DEVELOPMENT OF TRANSGENIC CHICKPEA (*Cicer arietinum* L.) LINES RESISTANT TO *Helicoverpa armigera* (F.) (LEPIDOPTERA : NOCTUIDAE)

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Affectionately Dedicated

to

My Beloved Parents

DEPARTMENT GENETICS AND PLANT BREEDING
UNIVERSITY OF AGRICULTURAL SCIENCES, GKVK,
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CERTIFICATE

This is to certify that the thesis entitled **"Development of Transgenic Chickpea (*Cicer arietinum*L.)Lines Resistant to *Helicoverpa armigera* (F.) (Lepidoptera:Noctuidae)**" submitted by **Ms. ASHARANI,B.M. ID. NO. PAK 7187** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **GENETICS AND PLANT BREEDING** to the University of Agricultural Sciences, GKVK, Bangalore, is a record of research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other.

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THESIS ABSTRACT

Insect resistant transgenics provide an exciting option as they are likely to reduce the usage of insecticides for pest management and provide sources of resistance for deployment. Transgenic chickpea plants were generated through an *Agrobacterium* mediated, *in planta* transformation protocol by incorporating *cry1X* gene in to KAK-2 variety (strain EHA-105, harbouring the binary vector pBinAR). A tissue culture-independent method where in the apical meristem of germinating seedlings is inoculated and reared into single transformants. These putative T1 transgenics plants were then selected by adopting multiple evaluation strategies, such as PCR, ELISA and bioassays, for selection of plants for advancement.

The success rate of PCR is 15.6 per cent in T_1 generation was observed. The associational studies between Cry protein levels expressed in transgenic plants , mean per cent larval mortalities of *Helicoverpa armigera* and leaf damage caused by *Helicoverpa asmigera* was observed to be non-significant in KAK-2 variety. The western analysis of PCR-positive plants in T_1 generation revealed the presence of the *cry* protein in the transgenics.

From the detached leaf bioassays of PCR positive transformants, the per cent leaf damage caused by *Helicoverpa armigera* was significantly more in non-transgenic plants compared to transgenic plants in the KAK-2 variety in the tested generation. It is hoped that further advancement will help produce the *Helicoverpa armigera* tolerant and stable transgenic chickpea variety.

Signature of the Student **Signature of the Major Advisor**

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INTRODUCTION

I. INTRODUCTION

The world population is expected to increase from 6.5 billion at present to 7.5 billion by 2025. Most of this population lives in the rural areas of developing countries, where poverty, food and nutrition insecurities are likely to be the major problems.

One of the practical means of increasing crop production is to minimize the pest-associated losses. The input costs for the management of these pests were estimated during 2000 at $ 32 billion annually. As much as $30 billion of this is largely the expenses towards the pesticides. Massive application of pesticides results in health hazards leaves residues in the food, causing adverse effects on non-target organisms and the environment. Lastly, and more importantly, resistance development among the pests and the consequent pest emergence problem has led to a vicious insecticidal treadmill. As a result, alternatives to chemical control of insect pests have become an urgent necessity. This has necessitated a rigorous implementation of integrated pest management with a view to reduce both input levels and to increase the productivity through eco-friendly means.

Among the various options available is a novel delivery system of insecticidal toxin through crop plants *i.e.* transgenic crops with suitable insecticidal genes. A classic and highly successful example is the *Bt* cotton plants in India. Elsewhere in the world, *Bt* genes are extensively being used to develop insect resistant transgenic crops for the management of a wide variety of insect pests.

The single gene controlled δ-end toxins of *Bacillus thuringienesis,* widely labeled as *cry* genes or *Bt* toxins, were shown to be amenable for isolation and development of transgenic insect resistant crops (Hilder *et al.,* 1987). Thus a new avenue in the pest management of particularly Lepidopteran pests has been opened. The method is an elegant and perhaps the most effective delivery system for *Bt* toxins, that apparently provides a relatively long lasting and seed borne solution for the management of Lepidopteran pest (Tabaashnik *et al.,* 2003). The constitutive expression of the toxins makes the transgenic much more than the sprays of best insecticides. They remain active

throughout the season, they are not affected by the weather conditions, give effective control of even burrowing insects where insecticidal sprays are difficult to reach and also remain active and useful against all stages of insects. Because, the solution to the pest problem is packaged into seeds, the anticipated input for further management of the crop pests is greatly reduced thus saving the farmer from drudgery of pesticide use. Furthur, only the insects feeding on the crops are exposed to toxin and thus reduce the environmental pollution and become relatively safe to non-target organisms. Consequently, the technology of transgenics on deployment can now provide, long lasting, relatively easy, economical, and environmentally safe alternatives to many other options in pest management.

With the identification of crop, the development of the insect tolerant transgenic crops depends on the availability of an effective or potent gene, a transformation protocol, methodology for ascertaining the presence of the transgene in the transformation and a mechanism for evaluation of the gene expression.

cry genes from *Bacillus thuringienesis* are now widely being used for development of insect (particularly Lepidoptera) resistant transgenics. The directed desirable gene transfer from one organism to another and the subsequent stable integration and expression of a foreign gene into the genome is referred to as 'genetic transformation'. The transferred gene is known as 'trangenics'. The natural mechanism of *Agrobacterium* mediated gene transfer was the first successful plant transformation system, making the breakthrough in plant genetic engineering in 1987 (Hilder *et al.,* 1987).

The *cry* gene vary greatly in their ability to affect different groups of insects and in particular, *cry 1* genes are strongly Lepidoptera specific. The degree of specificity of these genes being high, and also due to enormous local variations in the activity pattern of these toxins (Raghunathe, 2002), a large number of gene sequences, that vary greatly in their activity patterns, have now been identified or synthesized (Tuli, *et al.,* 2000) and patented. Further, these genes have also been extensively engineered into many crops to combat a variety of crop pests including Lepidoptera, Diptera, and Coleoptera. One of the

greatest advantages seen in the engineering of these genes into crops is the avoidance of developing high levels of resistance to these same proteins when applied as sprays (Tabashink, *et al.*,2003). However, variable expression of native *Bt* genes has necessitated the use of codon modified and truncated versions of these genes to activate higher expression. When expressed in plants, these modified or synthetic genes have been shown to confer significant protection against insects in crops such as cotton, maize, rice, tomato, potato and soya.

Genetic transformation of almost all the major crop species is now feasible with the development of techniques ranging from the *A. tumefaciens* mediated transformation to electric discharge mediated particle acceleration procedure, However, not all techniques are easily adaptable for all the crops and the transformation of most crops depends on the ability to develop *in-vitro* culture techniques. Many recalcitrant crops that are not easily cultured under *in-vitro* conditions can now be effectively transformed using the *.A. tumefaciens* mediated *in-planta* transformation technique (Rohini, 2002).

Agrobacterium tumefaciens has been treated as nature's most effective plant 'genetic engineer'. It is the causative agent of 'crown gall' disease, characterized by the timorous growth of plant tissues in the stem and is an economically important disease of many plants (Smith and Townsend, 1907). It is suggested and later confirmed that the bacterium has the capacity to infect plants and transfer a segment of DNA (T-DNA) from its tumor inducing (Ti) plasmid into the plant cell that eventually results in converting the quiescent somatic cells to active cell dividers. The T-DNA then integrates into the plant nuclear genome where the gene on the T-DNA then integrates into the plant nuclear genome where the genes on the T-DNA are expressed.

Once a crop has been transformed using the right kind of gene, the presence of the gene can now be effectively verified by the PCR based molecular analysis technique. Further the southern hybridization analysis can provide insights into the number of copies of the gene that are inserted into the target plant or its progeny.

Further, if the gene is an anti-insect gene, standard procedure can be conveniently deployed for the assessment of the gene expressions, systematic molecular and bioassays through several generation would confirm the stable integration and expression of the gene. At the University of Agricultural Sciences, Bangalore, India, these procedures have been standardized both for molecular studies and bioassays (Kumar *et al., 1998).*

As the university is now equipped to handle all the required technical knowhow and the physical facilities to handle such a genetic engineering work, the study has been planned with chickpea, to perform the same using available and effective *cry* gene to combat several Lepidopteron pests that affect it under field conditions *Cicer arietinum* (L.) (Fabaceae), chickpea, is one of the 10 most important grain legumes of the world. Chickpea seeds contain high quality, easily digestible protein (25 %) and carbohydrates (20 %) making it an important source of protein for the vegetarians of the country and thus it is also called "Poor man's meat". Chickpea, either as grain or vegetable is widely used in Indian cuisines and an important source of vegetable protein. Not surprisingly, India stands first in both area and production of this crop. The demand for chickpea, worldwide, stands around 8.2 million tonnes per year and is expected to increase to around 11.1 Mt per year by 2010. Much of this increase in demand is expected to be met from increased production and expanding areas under the crop in India. The productivity of chickpea is extremely low at present at an average of just around 0.8 tonnes per ha. However, the crop is potentially capable of yielding up to 5 tonnes per ha with better management of pests and diseases.

Among several pests of the crop, the pod borer, *Helicoverpa armigera*, is the most important and is estimated to cause up to 40 % yield loss. In chickpea, the larvae of *H. armigera* begin to appear from the seedling stage itself, feeding on leaves and continue to damage the crop till harvest by feeding on pods. During the early stages, the crop suffers from heavy defoliation resulting in poor vegetative growth. This damage is quite crucial for the productivity of the crop since the yield is proportional to the vegetative growth. During the reproductive phase the crop suffers due to damage to flowers, pods and developing seeds. Much of the economic damage therefore occurs at this stage of the crop growth.

Although, an excellent management package is in place for combating *H. armigera*, it is rarely practiced by the farmers. High input costs in the form of seed and fertilizers coupled with uncertain monsoon makes it difficult for the dry land farmers to invest in the management of the insect pests. Therefore, it is important that investment free techniques be developed to manage this pest. Seed borne solutions thus provide the best option.

With the success of the *Bt* cotton, the potentialities of producing insect resistant, especially Lepidoptera resistant, transgenics has become a reality. The availability of local gene constructs has further enhanced the opportunity to develop the transgenics. But crops such as chickpea are recalcitrant and explants developed by various techniques are difficult to establish under field conditions. Thus the development of transgenics in chickpea is a real challenge. However, *Agrobacterium tumefaciens* mediated *in planta* transformation protocols are being developed for various recalcitrant crops such as groundnut, field bean, *etc.*

In this background, the present investigation has been planned to develop transgenic chickpea that can tolerate *Helicoverpa armigera* with the fallowing objectives.

1. Transformation of KAK- *2* variety of chickpea with *cry1X* using *in planta* transformation protocol and raising the T_0 plants
2. Molecular and biochemical characterization of putative transformants of T_1 lines of KAK-2
3. Evaluation of putative transformants of KAK-2 lines for their tolerance to *Helicoverpa armigera,* through *in vitro* bioassay
4. Selection of putative transformants for advancement to T_2 generation based on molecular, biochemical characteristics and insect tolerance

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Development of an insect tolerant transgenic plant basically revolves around the idea of selection of a suitable crop, target insect, a utility gene, a technology for gene transfer, evaluation of the putative transgenic against the target pest(s) and selection of plants for advancement. Therefore, the review of literature has been designed accordingly, to meet the demands of the work and is presented under various suitable headings.

2.1 The crop

Cicer arietinum(L.) (Leguminosae), chickpea, is one of the 10 most important grain legumes of the world. Chickpea seeds contain high quality easily digestible protein (25 %) and carbohydrates (20 %) making it an important source of protein for the vegetarians of the country and thus it is also called "Poor man's meat". The demand for chickpea, world wide, stands around 8.2 million tonnes per year and is expected to increase to around 11.1 Mt per year by 2010.

The productivity of chickpea is extremely low at present at an average of just around 0.8 tonnes per ha. However, the crop is potentially capable of yielding up to 5 tonnes per ha with better management of pests and diseases.

Among several pests of the crop, the pod borer, *Helicoverpa armigera*, is the most important and is estimated to cause up to 40 % yield losses. In chickpea, the larvae of *H. armigera* begin to appear from the seedling stage itself, feeding on leaves and continue to damage the crop till harvest by feeding on pods. During the early stages, the crop suffers from heavy defoliation resulting in poor vegetative growth. This damage is quite crucial for the productivity of the crop since the yield is proportional to the vegetative growth. During the reproductive phase the crop suffers due to damage to flowers, pods and developing seeds. Much of the economic damage therefore occurs at this stage of the crop growth.

Production of chickpea has remained constantly low because of its susceptibility to several pathogens and insect pests. Among the insect pests, bruchids cause substantial loss during storage (Singh *et al* 1994). The cowpea weevil *Callosobruchus maculatus*) and azuki bean weevil (*C. chinensis*) infest chickpea seeds heavily. Since seeds are used for consumption, use of chemicals to protect the seeds is not recommended. Hence, newer approaches such as genetic engineering and molecular breeding have been encouraged (Jaiwal *et. al., 2*001; Dayal *et. al.,* 2003; Grant *et. al.,* 2003; Saini *et. al.,* 2003)

2.1.1 The insect

Helicoverpa armigera is one of the most important insect pests of agricultural crops in the Asian tropics. This species is widely distributed throughout tropical and temperate Asia, Australia and the Pacific Islands (Feakin, 1973; Kranz *et al.,* 1977). It is a polyphagous pest recorded on more than 120 host plants and is known to cause severe damage to many crop plants.

The damage is done by larvae, which feed gregariously on leaves in early stages and later spread out to entire field causing extensive damage. It is also reported to feed on chickpea pods. Being polyphagous and voracious feeder it is the first in economic importance at the national level.

Helicoverpa armigera (Hubner) is one of the most important constraints to chickpea production worldwide, and has been estimated to cause a loss of $325 million annually in the semi-arid tropics (ICRISAT, 1992).

The noctuids *Helicoverpa armigera* and *Spodoptera litura* are serious pests of several crops such as cotton, pulses, and vegetables in Asia. These noctuids are reported to cause yield loss of more than US $2 billion in the semiarid tropics including India, despite application of insecticides costing $500 million annually (Ramesh, 2008)

Important insect pests of global importance viz., *Helicoverpa armigera* (Hubner), *Spodoptera litura* (Fabricius), *Plusia* spp. that attack crops such as groundnut, soybean, sunflower, cotton and tomato. (Vimala Devi, 2003)

2.1.2 Options for management of pests of chickpea.

Various options are available for the management of caterpillar pests of chickpea. *Helicoverpa armigera,* basically a defoliator is amenable for management through insecticide application. Various insecticides have been recommended for their management that can significantly reduce the damage due to this pest

Application of chemical insecticides only if the insect population crosses the economic threshold level (ETL), spraying of insecticides like endosulfan 35 EC @ 1000 ml/ha. Spray insecticides like deltamethrin 2.8 EC @ 750 ml/ha. Quinalphos 25 EC@ 1000ml/ha. In case of severe infestation Spray insecticides like polytrin C 44 EC @ 1000 ml/ha. Profenophos 50 EC @ 1500 ml/ha. Spark 36 EC at the rate of 1000 ml/ha. Chlorpyriphos 20 EC (200 ml) + acephate 100 ml/ha were effective in management of *Helicoverpa armigera.*

However, as pointed out earlier, these options involve additional crop protection expenditure to raise the crop. Much of the area being under rainfed conditions and also extensively cultivated by small and marginal farmers, pest management options entail increased economic risks that most farmers cannot afford. Therefore, seed borne solutions that can provide reasonably economical protection are the most desirable options. Unfortunately, sufficient variability in resistance against mandibulate defoliator pests is lacking in chickpea that makes the development of resistant cultivars a near impossible task. Secondly, conventional host-plant resistance to insects calls for manipulation of quantitative traits at several loci that slows down the progress and is difficult to achieve. So, conventional plant breeding techniques are unlikely to provide reasonable solutions for the management of these pests. Recent history of pest management technologies provide reliable and viable options that revolve around the recombinant DNA technology.

2.2 Genetic engineering of crop plants

Plant genetic transformation permits direct introduction of agronomically useful genes into important crops and offers a significant tool in breeding programs by producing novel and genetically diverse plant materials. The directed desirable gene transfer from one organism to another and the subsequent stable integration and expression of a foreign gene in the genome is referred to as 'Genetic Transformation'. The transferred gene is known as 'transgene' and the organisms that are developed after a successful gene transfer are known as 'transgenics' (Babaoglu *et al*., 2000)

Among the various r-DNA technologies, genetically modified plants expressing δ-endotoxin genes from *Bacillus thuringiensis* (*Bt*), protease inhibitors and plant lectins have been successfully developed, tested and demonstrated to be highly viable for pest management in different cropping systems during the last decade and a half (Gatehouse, 2008). Insect resistant crops have been one of the major successes of applying plant genetic engineering technology to agriculture. Most of the plant derived genes produce chronic rather than toxic effects and many insect pests are less or not sensitive to most of these factors. Therefore, the genes for δ-endotoxins are expected to provide better solutions. Considering the need for a seed borne solution, it is desirable that scientifically sound strategies be developed to deploy exotic and plant derived genes for minimizing the extent of losses caused by insect pests in different crops in general and chickpea in particular (Sharma *et al.,* 2000).

Advances in biotechnology have provided several unique opportunities that include access to various plant transformation techniques, novel and effective molecules, ability to change the levels of gene expression, capability to change the expression pattern of genes, and develop transgenics with different insecticidal genes.

With the advent of genetic transformation techniques based on recombinant DNA technology, it is now possible to insert foreign genes that confer resistance to insects into the plant genome (Bennett, 1994). To sustain the crop yield potential and to meet the growing demand for food, crop productivity needs to be increased. Recombinant DNA technology coupled with plant tissue culture has helped develop novel options for the

management of various kinds of biotic stresses including insect pests. These technologies, as of now, may not appear to greatly improve the potential yields, but would be of immense value in reducing the losses caused by biotic stresses, including insect pests.

Genes from bacteria such as *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus* have been the most successful group of genes identified for use in genetic transformation of crops for pest control on a commercial scale (Gill *et al.,* 1992; Charles *et al*., 1996). Transgenic plants rarely result in 100% control, but tend to retard insect growth and development (Estruch *et al*., 1997).

Genes coding for *Bt* δ-endotoxins have been deployed in a wide range of crop plants with considerable success (Sharma *et al*., 1999). The first *Bt* toxin gene was cloned in 1981 and the first transgenic plant was produced by mid-1980s. Since then, several crop species have been genetically engineered to produce *Bt* toxins to control the target insect pests. Genes conferring resistance to insects have been inserted into crop plants such as cotton, maize, potato, tobacco, rice, broccoli, lettuce, walnut, apple, alfalfa and soybean (Bennett, 1994; Federici, 1998; Griffiths, 1998). The first transgenic cotton crop was grown in 1994 and large-scale cultivation was taken up in 1996 in USA (McLaren, 1998).

Transgenic plants display considerable potential to benefit both developed and developing countries. Transgenic plants expressing insecticidal *Bt* proteins alone or in conjunction with proteins providing tolerance to herbicide are revolutionizing agriculture (Shelton *et al*., 2002). The use of such crops with input traits for pest management, primarily insects and herbicide resistance, has risen dramatically since their first introduction in the mid 1990s.

India, the largest cotton growing country in the world had 50000 ha of *Bt* cotton in 2002. In 2007, the *Bt* cotton area soared to 6.2 m ha grown by 3.8 million small and resource poor farmers (James, 2007). The spectacular growth in *Bt* cotton means that it has consistently delivered unprecedented benefits to farmers and to the nation. *Bt* cotton

has increased productivity by up to 50% while reducing the insecticide sprays by half, with environmental and health implications, besides increased income to cultivators.

Success achieved in cotton has served as an excellent model to emulate in many other crops such as rice, wheat, pulses and oilseeds that have the potential to make agriculture a viable profession for the peasants of India.

2.2.1 *Bacillus thuringiensis* toxin genes

Bacillus thuringiensis is a gram-positive soil bacterium, which produces proteinaceous crystalline inclusion bodies during sporulation. There are many subspecies and serotypes of *Bt* with a range of well characterized insecticidal proteins or *Bt* toxins. At present it has been estimated that over 60,000 isolates of *Bt* are being maintained in culture collections worldwide. Known *Bt* toxins kill insects belonging to the orders Lepidoptera, Coleoptera, Diptera (Hofte and Whiteley, 1989) and nematodes (Feitelson *et al.,* 1992) (Table 1). The host range of *Bt* has expanded considerably in recent years due to extensive screening programs. Insecticidal δ-endotoxins of *B. thuringiensis* (*Bt*) have acquired great significance in recent years because of their specificity to target insects, toxicity at very low concentrations and environment friendly nature (Kumar *et al*., 1998).

Primarily *Bt* toxins are classified based on homology of toxin gene sequences and the spectrum of insecticidal activity (Hofte and Whiteley, 1989). The nomenclature of Hofte and Whiteley (1989), failed to accommodate genes that were highly homologous to known genes but with a different insecticidal spectrum. Crickmore *et al.* (1998) have introduced a systematic nomenclature for classifying the *cry* genes and their products based on the homology of amino acids of full length gene products.

Eleven Lepidoptera specific δ-endotoxins have been reported towards *H. armigera,* an important polyphagous pest on cotton, chickpea, pigeonpea, tomato, sunflower, sorghum, *etc*. (Chakrabarti *et al*., 1998). However, mixtures of these toxins are expected to act synergistically. For example, a mixture of *cry1Ac* and Cry1F toxins will show synergistic effect and the EC_{50} of *cry1Ac* will be lowered by 13 times due to the presence of Cry1F. As a result, there are suggestions that *cry1Ac* and *cry1F* genes be

expressed together in transgenic crop plants for effective control of *H. armigera* and also as a durable resistance management strategy (Chakrabarti *et al*., 1998).

2.2.2 Mode of action of *Bt*

The crystalline protoxins are inactive until they are solubilized by the gut proteases (Tojo and Aizawa, 1983; Milne and Kaplan, 1993). The protoxins are activated in the midgut by trypsin like proteases to toxins at alkaline pH. In general, 500 amino acids from the C terminus of 130 kDa protoxins and 28 amino acids from the N terminus are cleaved leaving a 65 to 55 kDa protease resistant toxic active core comprising the N-terminal half of the protoxin (Hofte and Whiteley, 1989). The active toxin consists of three distinct structural domains. Domain I (7 α -helices) determines toxicity and pore formation. Domain II (3 ß–sheets) determines receptor binding and specificity whereas domain III (2 ß–sheets), is involved in receptor binding and protein processing (Schnepf *et al*., 1998). The active toxin binds to specific receptors located on the apical brush border membrane of the columnar cells in the midgut of target insect, the α -helices penetrate the membrane and lead to formation of pores (ion channels). The toxicity of *Bt* lies in the organization of α-helices derived from domain I. The toxin induced pores formed in the columnar cells allow rapid fluxes of ions leading to swelling of the cells and osmotic lysis. There is a positive correlation between toxin activity and ability to bind BBMV (Brush Border Membrane Vesicles) (Gill *et al*., 1992), and the toxicity is correlated with receptor number rather than receptor affinity (Rie *et al.*, 1989). The disruption of gut integrity leads to death of the insect through starvation or septicemia (Sneh and Schuster, 1981; Salama and Sharaby, 1985).

2.2.3 Transgenic crops with *Bt* genes

The concept of creating insect resistant plants began with identifying proteins with insecticidal properties. The soil microorganism, *Bacillus thuringiensis (Bt)* has proven to be a rich source for insecticidal protein genes. Known *Bt* strains contain a great diversity of δ-endotoxin encoding genes and have proven to be the source *par excellence* of insecticidal principles to be used in transgenic plants.

Table 1 : A broad classification of Cry toxins and the group of organisms susceptible to them

Sl. No.	Cry toxins	Active on
1	Cry 1, Cry 2, Cry 9	Lepidoptera
2	Cry 3, Cry 7, Cry 8, Cry18, Cry 23, Cry 43	Coleoptera
3	Cry 1B, Cry 11	Lepidoptera and Diptera
4	Cry 5, Cry 12, Cry 13 and Cry 14	Nematodes
5	Cry 2, Cry 4, Cry 10, Cry 11, Cry 16, Cry17, Cry 19, Cry 20, Cry 27	Mosquitoes

(Liao *et al*., 2002)

Table 2 : List of transgenic crop plants developed using various *cry* genes against insect pests

Crop group	Target crop	Toxin	Target pest/s	References
Commercial crops	Cotton	*cry1Ac*	*Helicoverpa zea* (Boddie) (Lep.) *Pectinophora gossypiella* (Saunders) (Lep.)	Bacheler and Mott, 1997
		cry1Ab	*H. virescens* (Fabr.) (Lep.) *H. zea*	Perlak *et al*., 1990
		cry 1Ac + cry 2Ab	*H. zea* *P. gossypiella* *Spodoptera exigua* (Hubner) (Lep.) *Spodoptera frugiperda* (J.E. Smith) *Pseudoplusia includens* (Walker) (Lep.)	Adamczyk *et al*., 2001 Chitkowski *et al*., 2003
		cry 1Ac+ CpTI	*H. zea*	Wu and Guo, 2005
	Tobacco	*cry1Aa*	*Manduca sexta* (L.) (Lep.)	Barton *et al*., 1987
		cry1Ab	*M. sexta*	Vaeck *et al*., 1987
		cry1Ab + CpTI	*M. sexta*	Perlak *et al*., 1991
		cry1Ab	*M. sexta*	Williams *et al*., 1993
		cry1Ac	*H. virescens, H. zea* *Spodoptera littoralis* (Boisduval) (Lep.)	McBride *et al*., 1995
		cry1C	*S. littoralis*	Strizhov *et al*., 1996
		cry2A	*Helicoverpa armigera* (Hubner)	Selvapandiyan *et al*., 1998
		cry2Aa	*H. virescens* *H. zea, Spodoptera exigua* (Hubner)	Kota *et al*.,1999
		Cry1Ia5	*H. armigera*	Selvapandian *et al*.,1998
Cereals	Corn	*cry1Ab*	*Ostrinia nubilalis* (Hubner) (Lep.)	Koziel *et al*., 1993
		cry9C	*O. nubilalis*	Jansen *et al*., 1997

Continued……

Crop group	Target crop	Toxin	Target pest/s	References
Cereals	Rice	*cry1Ab*	*Chilo suppressalis* Walker (Lep.)	Fujimoto *et al*., 1993
		cry1Ab and *cry1Ac*	*C. suppressalis*	Cheng *et al*., 1998
		cry1B	*C. suppressalis, Cnaphalocrosis medinalis* Guenee (Lep.)	Marfa *et al*., 2002
		cry1Ac, *cry2A* and GNA	*C. medinalis, Scirpophaga incertulas Nilaparvata lugens* Stal (Hom.)	Maqbool *et al*. 2001
		cry3A	*Dicladispa armigera* (Oliv.) (Col.) *Sitophilus oryzae* (L.) (Col.)	Jhonson *et al*., 1996
		cry2A	*S. incertulas and C. suppressalis*	Chen *et al*., 2005
		cry1Ab / cry1Ac	*S. incertulas C. suppressalis Sesamia inferens* Walker *C. medinalis*	Ye *et al.*, 2001
Pulses	Soybean	*cry1Ac*	*H. virescens, H. zea Pseudoplusia includens* Walker (Lep.) *Anticarsia gemmatalis* (Hubner) (Lep.)	Walker *et al.*, 2000
	Pigeonpea	*cry1E-C*	*Spodoptera litura*	Surekha *et al*., 2005
	Chickpea	*cry2Aa*	*H. armigera*	Sarmah and Deka 2004
		cry1Ac	*H. armigera*	Sanyal *et al*., 2003
Vegetables	Tomato	*cry1Ab*	*H. virescens*	Fischholff *et al*., 1987
		cry1Ac	*H. armigera*	Mandaokar *et al*., 2000
	Eggplant	*cry1Ab*	*Leucinodes orbonalis* Guenee (Lep.)	Kumar *et al*., 1998
		cry3A	*Leptinotarsa decemlineata* (Say) (Col.)	Jelenkovic *et al*., 1998

Continued……..

Crop group	Target crop	Toxin	Target pest/s	References
Vegetables	Cabbage	*cry1Ab*	*Plutella xylostella* (L.) (Lep.)	Bhattacharya *et al*., 2002
	Broccoli	*cry1C*	*P. xylostella*	Zhao *et al*., 2001
Sugars and Starches	Sugarcane	*cry1A(b)*	*Diatraea saccharalis* (F.)	Arencibia *et al.*, 1997
	Potato	*cry1Ab*	*Phthorimaea operculella* (Zeller) (Lep.)	Peferoen *et al*., 1992 Rico *et al*., 1998
		cry3Aa	*L. decemlineata*	Adang *et al*., 1993
		Cry9Aa2	*Phthorimaea operculella*	Gleave *et al.*, 1998
		Cry1Ia1 (cryV)	*Phthorimaea operculella* *Symmetrischema tangolias* (Gyen) (Lep.)	Lagnaoui *et al.*, 2000
		Cry1Ba / cry1Ia	*Leptinotarsa decemlineata* *Phthorimaea operculella*	Naimov *et al.*, 2003
Other crops	Canola	*cry1Ac*	*Thrichoplusia ni* (Hubner) (Lep.) *Spodoptera exigua, H. virescens, H. zea*	Stewart *et al*., 1996
	Alfalfa	*cry1C*	*S. littoralis* *Spodoptera exigua*	Strizhov *et al*., 1996

Lep = Lepidoptera; Col = Coleoptera; Hom = Homoptera

An elegant and the most effective delivery system for *Bt* toxins, is the transgenic plant. The major benefits of this system are economic, environmental, and qualitative. In addition to the reduced input costs to the farmer, the transgenic plants provide season-long protection independent of weather conditions, effective control of burrowing insects that are difficult to reach with conventional chemical sprays, and control at all of the stages of insect development. The *Bt* genes encoding δ-endotoxins were introduced into many crops to develop insect resistant transgenic plants.

Several *Bt* toxin genes have been inserted into crop plants to provide protection against several insect pests (Table 2). Many crops such as commercial crops, vegetables, cereals and forage crops are now being transformed to be protected against insect pests by *Bt* toxins (Shelton *et al*., 2002).

2.2.3.1 Transgenic crops with single *Bt* toxin

Transgenic plants containing *Bt* genes control pests more effectively than *Bt* formulations. So far, three species of *Bt* crops (cotton, maize and potato) have become commercially available world wide. In 2007, the estimated global area of *Bt* crops reached 20.3 m ha (James, 2007). Commercialization of *Bt* crops has significantly reduced the use of synthetic insecticides (Ferre and van Rie, 2002).

The first transgenic tobacco plants using *cry* genes were developed in 1987 (Vaeck *et al.,* 1987; Barton *et al.,* 1987; Fischoff *et al*. 1987; Carozzi *et al*.,1992; Ranjekar *et al.,* 2003). A significant breakthrough was made in 1990 by researchers at Monsanto (USA) who modified the *cry* genes, *cry1Ab* and *cry1Ac* for better expression in plant cells (Perlak *et al.,* 1990).

The tobacco plants engineered with truncated genes encoding *cry1Ac* and *cry1Ab* toxins were found to be resistant to the larvae of tobacco horn worm *Manduca sexta* (Kota *et al.,* 1999). Cotton cultivar Coker 312 was first transformed by using partially modified *cry1Ac* gene. The transformed plants showed total protection against *Trichoplusia ni*, *Spodoptera exigua* and *Heliothis zea*. The maximum level of toxin

protein in the plants was 0.1 per cent of the total soluble protein (Sharma and Anjaiah, 2000).

Selvapandian *et al.* (1998) transformed tobacco plants using *cry1Ia5* insecticidal toxin from an Indian *Bt* strain, that provided complete protection against *H. armigera*. The transgenic tobacco plants with the partially modified *cry1Ab* gene had a 10 fold higher level of insect control protein and plants with the fully modified *cry1Ab* had a 100 fold higher level of *cry1Ab* protein compared with the wild type gene and exhibited 100% larval mortality of tobacco horn worm (Perlak *et al.,* 1990). Tobacco and tomato plants expressing *cry1Ab* and *cry1Ac* genes have been developed to control lepidopteran insects (Salm *et al*., 1994). Synthetic *cry1C* gene in alfalfa and tobacco plants results in the production of 0.01- 0.2% of total soluble proteins as Cry1C toxin and provides 100% protection against the Egyptain cotton leaf worm (*S. littoralis*) and the beet army worm (*S. exigua*) (Strizhov *et al*., 1996).

Expression of modified genes *cry1Ac* in cotton and *cry3Aa* in potato conferred considerable protection against lepidopteran and coleopteran pests respectively (Ranjekar *et al.,* 2003). Successful control of pink bollworm (*P. gossypiella*) has been achieved through transgenic cotton using a truncated *cry1Ab* gene in transgenic cotton plants (Wilson *et al.,* 1992; Arencibia *et al.,* 1997).

Scientists at the Bose Institute (Kolkata) have introduced a modified *cry1Ac* gene in rice (IR 64) for resistance to yellow stem borer (Nayak *et al.,* 1997). A synthetic *cry1Ac* gene was introduced into rice lines (Pusa Basmathi 1, Karnal Local, and IR-64) exhibiting total protection against neonate larvae of yellow stem borer.

Rice cultivars (*indica* and *japonica* types) with truncated *cry1Ab* gene caused 100% mortality of the yellow stem *borer* (*S. incertulas*; Datta *et al.,* 1998). Transgenic sugarcane plants with *cry1Ab* showed significant larvicidal activity against neonate larvae of sugarcane borer (*Diatraea saccharalis* (Fabricius)).

'Jack', a transgenic line of soybean, *Glycine max* (L.), expressing a synthetic *cry1Ac* gene (Jack-Bt) showed 3 to 5 times less defoliation from corn earworm, *H. zea*

and eight to nine times less damage from Velvetbean caterpillar, *Anticarsia gemmatalis* (Hubner) (Walker *et al.,* 2000).

Transgenic broccoli with Synthetic *cry1C* was resistant to the cabbage looper (*T. ni*), and cabbage butterfly (*Pieris rapae* ; Selvapandian *et al.* 1998). Vegetable crops like brinjal and tomato were transformed by synthetic/modified *cry1Ab* and *cry1Ac* genes respectively to confer resistance to fruit borers (*L. orbonalis*) and *H. armigera* respectively (Kumar *et al*., 1998). The 'New Leaf' potatoes with *Bt* protein, Cry3A, are season-long resistant to Colorado potato beetle (Duncan *et al.,* 2002).

2.2.3.2 Two toxin / hybrid toxin *Bt* crops

Although no insect species resistant to *Bt* crops have been reported under natural conditions, the potential of insects to evolve resistance against *Bt* toxins is an inevitable threat to this technology. To meet this challenge, several strategies have been proposed to manage insect resistance, such as the high dose, refuge strategy, gene stacking and temporal or tissue specific expression of the toxin (Roush, 1998; Frutos, *et al*., 1999; Shelton *et al*., 2002). Among the strategies, only the high dose or refuge strategy has been used in developed countries such as the United States and Australia.

At the same time, small farmers in Asian countries could hardly devote their land to a refuge and more over, a high dose of a foreign protein could cause a phenotypic trade off resulting in a yield penalty (Datta *et al*., 2002). Hence, efforts are being made to develop two toxin *Bt* crops (other wise known as the pyramiding approach), since two toxin cultivars require smaller refuges to achieve successful resistance management and are expected to provide sustained long term protection as against the single gene transgenics (Cohen *et al*., 2000). The use of multiple toxin genes with different modes of action has been proposed so that cross resistance is likely to be a less serious problem. As a result, two cry genes for toxins with different receptors or a cry gene in combination with an altogether different unrelated toxin gene, are considered the ideal options (de Maagd *et al*., 1996; Frutos *et al*., 1999).

Further, hybrid toxins produced through inclusion of a domain from another toxin results in increased potency of a fused protein by the shift in receptor binding (Bosch *et al*., 1994). Alternate receptor ligand interaction may also be exploited to further broaden the host range of the *Bt* toxins (Sivasubramanian and Federici, 1994).

The dual-toxin Bollgard II genotype (*cry1Ac* + *cry2Ab*) was found highly effective against lepidopterous pests, *Helicoverpa zea*, *Pseudoplusia includens* (Walker) and *Spodoptera frugiperda* compared to Bollgard I (*cry1Ac*) and conventional cotton (Chitkowski *et al*., 2003).

Rice plants expressing *cry1Ab* and *cry1Ac* genes were highly toxic to striped stem borer (*Chilo suppressalis*) and yellow stem borer (*Scirpophaga incertulas*), with mortalities of 97 to 100% within 5 days after infestation (Nayak *et al.*, 1997).

Transgenic IR72 lines, TT9-3 and TT9-4, carrying a fused *Bt* gene (*cry1Ab* and *cry1Ac*) demonstrated that both the transgenic lines were highly resistant against natural infestation and artificial infestation of four lepidopteran species, *viz.*, striped stem borer, *Chilo suppressalis* (Walker), pink stem borer, *Sesamia inferens* (Walker), leaf folder, *Cnaphalocrocis medinalis* (Guenee) and green semilooper, *Naranga aenescens* Moore (Ye *et al.,* 2001). The elite Vietnamese rice (*Oryza sativa* L.) cultivars transformed with translationally fused *cry* genes (*cry1Ab-1B*) exhibit 100% mortality of the neonate larvae of yellow stem borer (YSB) within a week of infestation (Ho *et al.,* 2006).

Transgenic potato plants developed with a hybrid *Bt* gene *SN19* (domain I & III from *cry1Ba* and domain II from *cry1Ia*) was shown to be resistant against Colorado potato beetle, tuber moth and European corn borer. These are the first transgenic plants resistant to pests belonging to two different insect orders. In addition, the target receptor recognition of this hybrid protein is expected to be different from Cry proteins currently in use for these pests that makes it a useful tool for resistance management also (Naimov *et al.,* 2003).

Thus, introduction of *Bt* transgenic plants for commercial cultivation has launched a new era in Agriculture. The expression of very effective insecticidal proteins by plants

delivers a remarkable level of insect control unsurpassed by any other method of insect pest management. Therefore, current and novel *Bt* δ-endotoxins are fully expected to be part of the transgenic plant approach to combat pests in the future also.

Advantages of transgenic crops:

- Potential reduction in chemical insecticide use which are responsible for causing economic, environmental and health hazards (Shelton *et al*., 2002)
- Cost effective (Shelton *et al*., 2002)
- Ease in implementation
- In case of transgenic crops, no new practices need be learned for the basic use of the technology. The whole technology is 'all in the seed'. Therefore, the only challenge is to get the seed into the hands of farmers (Ranjekar *et al*., 2003).

However, transgenics are not a panacea for solving all pest problems. There are also some genuine or perceived concerns.

Limitations of transgenic plants are:

- Secondary pests are not controlled in the absence of sprays for the major pests
- Cost of producing and deployment of transgenics may be very high
- Proximity to sprayed fields will reduce the benefits of transgenics
- Insect migration may reduce the effectiveness of transgenics
- Development of resistance in insect populations may limit the usefulness of transgenics (Sharma *et al.,* 2000).

The story of *Bt* cotton in India is remarkable. With political will and farmer support in place, adoption is projected to continue increasing with *Bt* cotton plantings escalating from the current 66% to 80% or more (James, 2007). Coincidentally, new biotech products such as *Bt* eggplant, an important food and cash crop that can benefit up to 2 million small and resource-poor farmers, is in an advanced large scale field trials stage with expectations of approval in the near term.

2.3 Transformation studies

Plant transformation is now a core research tool in plant biology and a practical tool for transgenic plant development. There are many verified methods for stable introduction of novel genes into the nuclear genomes of diverse plant species. The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (DeBlock *et al*., 1984; Horsch, *et al*., 1984; Paszkowski, 1984) has been extended to many plant species in at least 35 families.

Successes include most major economic crops, vegetables, medicinal, fruit, tree and pasture plants. As a result, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation systems for many plant species that continues to evolve.

2.3.1 Transformation methods

Gene delivery systems involve the use of several techniques for transfer of isolated genetic materials into a viable host cell. At present, there are two classes of delivery systems: (a) Non-biological systems (which include chemical and physical methods) and (b) Biological systems (Table3). The desire for higher transformation efficiency has stimulated work on not only improving various existing methods but also in inventing novel methods (Table4).

2.3.2 Biological and practical requirements for transformation

2.3.2.1 Biological requirements

The essential requirements in a gene transfer system for production of transgenic plants are:

(a) Availability of a target tissue including cells competent for plant regeneration.

(b) A method to introduce DNA into those regenerative cells and

(c) A procedure to select and regenerate transformed plants at a satisfactory frequency.

2.3.2.2 Practical requirements

Beyond the biological requirements to achieve transformation and the technical requirements for verification of reproducible transformation, desired characteristics to be considered in evaluating alternative techniques or developing new ones for cultivar improvement include:

(1) High efficiency, economy, and reproducibility, to readily produce many independent transformants for testing.

(2) Safety to operators, avoiding procedures, or substances requiring cumbersome precautions to avoid a high hazard to operators (e.g. potential carcinogenicity of Silicone carbide whiskers).

(3) Technical simplicity, involving a minimum of demanding or inherently variable manipulations, such as protoplast production and regeneration.

(4) Minimum time in tissue culture, to reduce associated costs and avoid undesired somaclonal variation.

(5) Stable,uniform (non-chimeric) transformants for vegetatively propagated species, or fertile germline transformants for sexually propagated species.

(6) Simple integration patterns and low copy number of introduced genes, to minimize the probability of undesired gene disruption at insertion sites, or multicopy associated transgene silencing.

(7) Stable expression of introduced genes in the pattern expected from the chosen gene control sequences (DeBlock, 1993).

When tested against the above criteria, most published techniques for gene transfer into plant cells must be dismissed as either disproven or impractical for use in routine production of transgenic plants. As a result in many laboratories, virtually all the transformation work rely on Particle bombardment with DNA coated microprojectiles or *Agrobacterium* mediated transformation for gene transfer to produce transgenic plants from a range of plant species (Birch, 1997).

Table 3 : DNA delivery methods available to produce plant transformants

<table>
<tr><th colspan="2">Plant transformation</th></tr>
<tr><th>Non-biological based transformation
(Direct method)</th><th>Biological gene transfer
(Indirect method)</th></tr>
<tr><td>A) <u>DNA transfer in protoplasts</u>
1) Chemically stimulated DNA uptake by protoplast
2) Electroporation
3) Lipofection
4) Microinjection
5) Sonication</td><td rowspan="2">1) Agrobacterium mediated transformation
<u>Primarily two methods</u>
a) Co-cultivation with the explants tissue
b) In planta transformation
2) Transformation mediated by viral vector</td></tr>
<tr><td>B) <u>DNA transfer in plant tissues</u>
1) Particle bombardment / Biolistics
2) Silicon carbide fiber mediated gene transfer
3) Laser microbeam (UV) induced gene transfer</td></tr>
</table>

Birch, *et al*., 1997

Table 4 : Juxtaposition of gene delivery methods

Gene delivery method	Transformation efficiency	Range of transformable plant species	Tissue culture phase	Type of explant	Remarks
Electroporation	Low to high	Unrestricted	With and without tissue culture phase	Protoplasts, meristems or pollen grains	Fast, simple and inexpensive in contrast with biolistics
Lipofection	Low	Recoverable species from protoplast	With tissue culture phase	Protoplast	High efficiency with combination of PEG based method, simple and non-toxic
Microinjection	High	Recoverable species from protoplast	With tissue culture phase	Protoplast	Very slow, precise, single cell targeting possibility, requires high skill, the chimeric nature of transgenic plants and ability of whole chromosome transformation
Sonication	Low	Unrestricted	With and without tissue culture	Protoplast cells, tissues and seedlings	Effective to transfect by virus particles and able to increase the *Agrobacterium* based transformation efficiency
Particle bombardment	High	Unrestricted	With and without tissue culture phase	Intact tissue or microspores	Efficient for viral infection, complex integration patterns, without specialized vectors and backbone free integration

Continued……..

Gene delivery method	Transformation efficiency	Range of transformable plant species	Tissue culture phase	Type of explant	Remarks
Silicon carbide mediate transformation	Low to high	Unrestricted	With tissue culture	Variety of cell types	Rapid, inexpensive and easy to set up
Laser beam mediated transformation	Low	Unrestricted	With tissue culture phase	Variety of cell types	Rapid and simple
Agrobacterium mediated method	High and stable	Many species, specially dicotyledonous plants	With and without tissue culture method	Different intact cells, tissues or whole plant	Possibility of *Agroinfection*, combination with sonication and biolistic methods and transgene size up to 150 kb
Virus based method	High and transient	Virus host specific limitation	With tissue culture	In planta inoculation	Rapid, inducible expression and with mosaic status

Darbani *et al*., 2008

2.3.3 Non-biological based transformation

2.3.3.1 Particle bombardment / Biolistics

Particle bombardment was first described as a method for the production of transgenic plants in 1987 (Sanford *et al*., 1987) as an alternative to protoplast transformation and especially for transformation of more recalcitrant cereals. Unique advantages of this methodology compared to alternative propulsion technologies are discussed in terms of range of species and genotypes that have been engineered and the high transformation frequencies for major agronomic crops (McCabe and Christou, 1993). In plant research, the major applications of biolistics include transient gene expression studies, production of transgenic plants and inoculation of plants with viral pathogens (Southgate *et al*., 1995; Sanford, 2000; Taylor and Fauquet, 2002).

Gene constructs for biolistics can be in the form of circular or linear plasmids or a linear expression cassette. Embryogenic cell cultures are likely the best explants to use for biolistic transformation because they can be spread out as uniform targets of cells and have high recovery capacity (Kikkert *et al*., 2004). Rice transformation has also been successfully achieved via the bombardment of embryogenic calli (Li *et al*., 1993; Sivamani *et al*., 1996; Cao *et al*., 1992; Zhang *et al*., 1996), in which transformation efficiency has been raised to 50% (Li *et al*., 1993). Particle bombardment has emerged as a reproducible method for wheat transformation (DeBlock *et al*., 1997; Bliffeld *et al*., 1999) and the first stable transformation in a commercially important conifer species (*Picea glauca*) was achieved via embryogenic callus tissue as explant (Ellis *et al*., 1993).

Particle bombardment has some disadvantages. The transformation efficiency might be lower than with *Agrobacterium* mediated transformation and it is more costly, as well. Intracellular targets are random and DNA is not protected from damage. Many researchers have avoided particle bombardment method because of the high frequency of complex integration patterns and multiple copy insertions that could cause gene silencing and variation of transgene expression (Dai *et al*., 2001; Darbani *et al*., 2008).

2.3.4 Biological gene transfer

2.3.4.1 *Agrobacterium* mediated transformation

The natural ability of the soil bacteria, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenus,* to transform host plants has been exploited in development of transgenic plants. In the 1970s the prospect of using *A. tumefaciens* for the rational gene transfer of exogenous DNA into crops was revolutionary. Genetic transformation of plants was viewed as a prospect. In retrospect, *Agrobacterium* was the logical and natural transformation candidate to consider since it naturally transfers DNA (T-DNA) located on the tumor inducing (Ti) plasmid into the nucleus of plant cells and stably incorporated the DNA into the plant genome (Chilton *et al*., 1977). Now thirty years later, this method has been the most widely used and powerful technique for the production of transgenic plants. However, there still remain many challenges for genotype independent transformation of many economically important crop species, as well as forest species (Stanton, 2003; De la Riva *et al*., 1998).

Despite the development of other non-biological methods of plant transformation (Shillioto *et al*., 1985; Uchimiya *et al*., 1986; Sanford, 1988; Arenchibia *et al*., 1992, 1995), *Agrobacterium* mediated transformation remains popular and is among the most effective. This is especially true among most dicotyledonous plants, where *Agrobacterium* is naturally infectious. *Agrobacterium* mediated gene transfer into monocotyledonous plants was thought to be not possible. However, reproducible and efficient methodologies have been established for rice (Hiei *et al*., 1994), banana (May *et al*., 1995, corn (Ishida *et al*., 1996), wheat (Cheng *et al*., 1997), sugarcane (Arencibia *et al*., 1998), forage grasses such as Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) (Bettany *et al*., 2003). Among the commercially important conifers, hybrid larch was the first to be stably transformed via co-cultivation of embryogenic tissue with *A. tumefaciens* (Levee *et al*., 1997). Subsequently, this method was successfully applied to several species of spruce (Klimaszewska *et al*., 2001; Charity *et al*., 2005; Grant *et al*., 2004).

Methods relative to transformation targets can be classified into two categories: (a) those requiring tissue culture and (b) *in planta* methods.

In tissue culture systems for plant transformation , the most important requirement is a large number of regenerable cells that are accessible to the gene transfer treatment and that will retain the capacity for regeneration for the duration of the necessary target preparation, cell proliferation and selection treatments. A high multiplication ratio from a micropropagation system does not necessarily indicate a large number of regenerable cells accessible to gene transfer (Livingstone and Birch, 1995). Some time gene transfer into potentially regenerable cells may not allow recovery of transgenic plants if the capacity for efficient regeneration is short lived (Ross *et al*., 1995). Further, tissue culture based methods can lead to unwanted somaclonal variations such as alterations in cytosine methylation, induction of point mutations and various chromosomal aberrations (Phillips *et al*., 1994; Singh, 2003; Clough, 2004). On the other hand, realization of whole plant transformants has been a problem in a large number of crop species as these plants have proven to be highly recalcitrant *in vitro*. As a result, other strategies are being evolved wherein the tissue culture component is obviated in the procedure and these are known as *in planta* methods.

Plant genetic transformation is of particular benefit to molecular genetic studies, crop improvement and production of pharmaceutical materials. *Agrobacterium*-based methods are usually superior for many species including dicots and monocots. The others are typically not done on a routine basis. Biolistics is by far the most widely used direct transformation procedure both experimentally in research and commercially. So why have all these other methods emerged in the past 20 years if we already have efficient transformation techniques in *Agrobacterium* and biolistics? There are two reasons. First of all, there is hope that a more efficient and less expensive method would be developed. The second and most important reason is the biolitics and *Agrobacterium* are patented.

2.3.5 *In planta* transformation

Although successful plant regeneration methods have been developed, the technology has not provided regeneration in several other crops for use in transformation

protocols which is a serious limitation to the exploitation of gene transfer technology to its full potential. In the light of this major constraint, it becomes necessary to evolve transformation strategies that do not depend on tissue culture regeneration or those that substantially eliminate the intervening tissue culture steps. The plant transformation methods that exclude tissue culture steps, rely on simple protocols and required short time in order to obtain entire transformed individuals are known as *in planta* transformation methods. These methods are called *in planta* transformation because transgenes are generally delivered into intact plants in the form of naked DNA or via *Agrobacterium.*

In many cases *in planta* methods have targeted meristems or other tissues with the assumption that at fertilization, the egg cell accepts the donation of an entire genome from the sperm cell that will ultimately give rise to gametes (Chee and Slighton, 1995; Birch, 1997) and therefore is the right stage to integrate transgenes. For non tissue culture based approaches of *in planta* transformation, *Agrobacterium* co-cultivation or microprojectile bombardment have been directed to transform cells in or around the apical meristems (Chee and Slighton, 1995; Birch, 1997). Injection of naked DNA into ovaries has also been reported to produce transformed progeny (Zhou *et al.,* 1983a).

Arabidopsis thaliana was the first plant that saw successful *in planta* transformation. Early stages of success in *Arabidopsis* transformation came from the work of Feldmann and Marks (1987). Transformation rates greatly improved when Bechtold *et al.* (1993) inoculated plants that were at the flowering stage. At present, there are very few species that can be routinely transformed in the absence of a tissue culture based regeneration system. *Arabidopsis* can be transformed by several *in planta* methods including vacuum infiltration (Clough and Bent, 1998), transformation of germinating seeds (Feldmann and Marks, 1987) and floral dipping (Clough and Bent, 1998).

Other plants that were successfully subjected by vacuum infiltration include rapeseed, *Brassica campestris* and radish, *Raphanus sativus* (Ian and Hong, 2001; Desfeux *et al*., 2000). The labor intensive vacuum infiltration process was eliminated in

favor of simple dipping of developing floral tissues (Clough and Bent, 1998). Also, the results indicate that the floral spray method of *Agrobacterium* can achieve high rates of *in planta* transformation comparable to the vacuum infiltration and floral dip methods (Chung *et al*., 2000).

Utilizing naked DNA, cotton transformants were recovered following injection of DNA into the axil placenta about a day after self-pollination (Zhou *et al.,* 1983a). Similarly, a mixture of DNA and pollen was either applied to receptive stigmatic surfaces or DNA was injected directly into rice floral tillers, or soybean seeds were imbibed with DNA (Trick *et al.,* 1997, Langridge, 1992). These procedures, intriguing as they are, are impractical at present because of their low reproducibility.

Recent studies with *Agrobacterium* inoculation of germinating seeds of rice has provided transformation efficiencies higher than 40% (Supartana *et al.*, 2005), while providing 4.7 to 76% efficiency for the flower infiltration method and from 2.9 to 27.6% efficiency for the seedling infiltration method (Trieu *et al*., 2000).

Crop species that were successfully transformed by pricking the apical meristem of the differentiated embryo of the germinating seeds and then infecting with *Agrobacterium* include peanut, *Arachis hypogaea* L. (Rohini and Rao, 2000b & 2001), Sunflower, *Helianthus annuus* L. (Rao and Rohini, 1999), Safflower, *Carthamus tinctorius* L. (Rohini and Rao, 2000a), field bean, *Dolichos lablab* L. (Pavani, 2006), and cotton, *Gossypium* sp. (Keshamma *et al*., 2008). Maize, *Zea mays* L. was transformed by treating the silks with *Agrobacterium* and afterwards pollinated with the pollen of the same cultivar (Chumakov *et al*., 2006).

The above successes have in fact provided a great leverage for easy development of transgenic pants, as the methodology is simple, cost effective, does not call for high infrastructural requirement even to handle recalcitrant crops such as chickpea. Thus the technology of gene transfer for the development of recalcitrant crops has become a practical possibility for experimenting and producing viable transformants. However, the optimization of *Agrobacterium*-plant interaction is crucial for efficient transformation.

Many factors including type of explant are important and they must be suitable to allow the recovery of whole transgenic plants (De la Ravi *et al*., 1998; Opabode, 2006; Cheng, *et al*., 1997; Jones *et al*., 2005; Darbani *et al*., 2008).

2.4 Molecular analysis of transgenic plants

Development of transgenic plants is a routine exercise in plant molecular biology now-a-days. *Agrobacterium* mediated genetic transformation is the most preferred method of gene transfer because of advantages such as its simplicity, economy and generation of single copy transgenics to overcome transgene silencing (Gelvin, 2003). Polymerase chain reaction (PCR) is the most commonly used technique to screen putative transformants (Nain *et al*., 2005).

2.4.1 Polymerase chain reaction (PCR)

The presence of transgene in the transformants was analyzed by DNA amplification. For the PCR amplification, plant genomic DNA will be extracted by various extraction methods. PCR was used as the first proof for the analysis of the putative transformants.

PCR amplification with gene specific primers of 3 modified *cry9Aa2* genes was carried out and the results confirmed that the plants were transgenic (Gleave *et al*., 1998). Peanut cv. TMV-2 was transformed with tobacco chitinase gene. Integration, copy number and expression of transgene was confirmed by the PCR, Southern and Northern analysis. (Rohini and Rao, 2000b and 2001).

A synthetic *cry1Ab* gene coding for an insecticidal crystal protein of *Bacillus thuringiensis* (*Bt*) was transferred to cabbage cultivar 'Golden Acre'. Transformed plants resistant to kanamycin were regenerated and total genomic DNA was isolated. Presence of the *cry1Ab* sequence was initially detected through PCR analysis using *cry1Ab* specific primers (Bhattacharya *et al*., 2002).

A number of putative transgenic chickpea lines were developed using the reconstructed *Bt cry1Ac* genes for protection against pod borer *H. armigera*. The

molecular analysis was done to identify homozygotes with high levels of transgene expression. PCR analysis was done with T_0 and T_1 generation plants for confirmation of the presence of the gene and amplification of both *cry1Ac* and *nptII* genes were obtained. Southern blot analysis was carried out on PCR positives to determine the copy numbers of the genes (Sarmah and Deka, 2004). The presence of 1.1, 0.73 and 0.5kb PCR amplicons in the transformants but not in the non-transformed control indicated the transgenic status of the plants in Vietnamese rice for *cry1Ab/ cry1Ac*, *hph,* and *bar* genes respectively (Ho *et al*., 2006). Integration of T-DNA into maize genome was confirmed by PCR (the *nptII* and *gus* reporter genes) (Chumakov *et al*., 2006). The putative T_0 transgenic rice plants with cry2A gene were detected by PCR analysis (Chen *et al*., 2005).

2.4.2 Westernblot analysis:

Southern blot analysis confirmed the presence of viral coat protein genes in the plant genomes. Western blot analysis indicated that the CMV coat protein genes were expressed in transgenic spinach plants (Yang Y *et al*., 1997)

Tobacco plants (Nicotiana tabacum 'Xanthi') were transformed with a binary vector containing the coat protein gene of tomato mottle Begomovirus (ToMoV) modified by the deletion of 30 nucleotides in the 5' end. The transgene transcript was detected by northern blot analysis; however, the transgene product could not be detected by protein blot analysis using antisera reactive with ToMoV coat protein (Sinisterra et al, 1999).

Bau *et al.* (2002) reported a broad spectrum resistance to different geographic strains of *Papaya ringspot virus* in CP gene transgenic papaya. Molecular analysis was carried out by polymerage chain reaction, DAS-ELISA, Western blot, Northern blot.

Somatic embryos of *Glycine max* (L.) Merrill cultivar ‘Jack’ was co-transformed with coat protein (CP) gene of attenuated isolates of soybean mosaic virus (SMV) and hygromycin phosphotransferase (*hpt*) gene by means of microprojectile bombardment. These transformed embryogenic tissues were selected in hygromycin-containing liquid

medium and the presence of transgene transcript was confirmed by Northern blot analysis, and the transgene product was detected in two of them by Western blot analysis (Noriyuki Furutani *et al.,* 2005*)*

Fertile transgenic sweet pepper (*Capsicum annuum var. grossum*) plants were regenerated at relatively high rate from various explants that were cocultivated with *Agrobacterium tumefaciens* strain GV3111-SE harbouring a plasmid that contains the cucumber mosaic virus coat protein (CMV-CP) gene. Southern analysis of DNA isolated from putative transgenic plants revealed that 3 out of 5 R_1 plant lines reacted positively with the CMV-CP gene. Western blot analysis of CMV-CP containing R_1 plants showed that two of them accumulated significant levels of the foreign gene product while the other two expressed it only, to low levels. Thus, like many other dicotyledonous plant species, sweet pepper can be transformed by*A. tumefaciens* and regenerated into healthy, fertile plants that express foreign genes (Yu-Xian Zhu *et al.,1995)*

2.4.3 Enzyme Linked Immuno Sorbent Assay (ELISA)

The presence of the gene can be identified through PCR, but its expression level is identified and quantified by ascertaining the product of the gene using ELISA. The principle involves specific antigen-antibody reactions leading to the accumulation of the conjugate product that is identified at specific wavelength by an ELISA Reader (Sambrook *et al*., 1989). Quatification of *Bt* protein expressed in plants is very important, as part of post transformation analysis with *Bt* genes.

The use of monoclonal based technique for detection and quantification of Cry protein has been demonstrated in 1987 (Vaeck *et al*., 1987). Polyclonal-monoclonal antibody sandwich ELISA was adopted for detecting the presence of the *cry3A* protein in transgenic *indica* rice plants (Johnson *et al*., 1996). Quantification of the fused Cry1Ab/ *cry1Ac* produced in transgenic rice plants was performed using a double sandwich ELISA technique. A Cry1Ab/ *cry1Ac* plate kit (ENVIROLOGIX INC., Portland, Maine, USA) was used to determine nanogram quantities of the fused Cry1Ab/ *cry1Ac* per mg fresh leaf tissue (Ye *et al*., 2001).

Quantitative estimation of delta *cry1Ac* endotoxin expressed in transformed chickpea plantlets was performed using a double antibody sandwich ELISA (Adang *et al*., 1993). The quantitative levels of *cry1Ac* and the seasonal decline in expression differed significantly among the eight commercial Bollgard hybrids tested. Bashir *et al*. (2004) analyzed the Cry2A protein content of a highly insect resistant *cry2A* transgenic rice line. The Cry2A protein content in transgenic *indica* rice plants was determined by ENVIROLOGIX kits (Chen *et al*., 2005).

The *cry1Ac* protein expression was found to be variable among the hybrids and also between different plant parts. The leaves of *Bt* cotton plants were found to have the highest levels of *cry1Ac* expression followed by squares, bolls and flowers. The toxin expression in the boll-rind, square bud and ovary of flowers was clearly inadequate to confer full protection to the fruiting parts (Kranthi *et al*., 2005; Sanyal *et al*. (2005) reported that quantitative assay of *cry1Ac* protein in transgenic chickpea plants indicated maximum expression of *Bt*-toxin in leaves, pods followed by green portions of stem and minimum in roots.

2.5 Bioassay studies on transgenic plants

Insecticidal transgenic plants were assessed for insect resistance against target pest (s) by conducting insect bioassays under laboratory as well as under field conditions. Under laboratory conditions various plant parts were used in insect bioassays to assess the tolerance of the transformants. Under contained or field trials, whole insecticidal transgenic plants will be compared to their control counterparts to assess their tolerance level to insect pests and also the benefits that might potentially accrue to the farmers.

Insect feeding bioassay on transformed chickpea plants (T_0 and T_1) with larvae of pod borer, *H. armigera* showed high levels of toxicity to insects and protection of transgenic plants. Transformed chickpea plants expressing *cry1Ac* protein above 10 ng mg^{-1} soluble protein showed 80–85% protection and high mortality (>80%) of insects (Sanyal *et al*., 2005).

Field evaluation of soybean engineered with a synthetic *cry1Ac* transgene for resistance to *Helicoverpa zea* and *Anticarsia gemmatalis* was done by planting transgenic soybean (Jack-*Bt*) in the form of hill along with wild type. Each hill consists of six plants and infested with 140 larvae. Resistance was evaluated through visual estimates of per cent defoliation of the plants in a hill and estimates were made at 2 to 3 days intervals beginning 7-8days after initial infestation. Jack-*Bt* showed three to five times less defoliation from *H. zea* and eight to nine times less damage from *A. gemmatalis* (Walker *et al*., 2000).

Five neonate larvae of tobacco hornworm were placed on a leaf of a plant transformed with *cry1Ab*. The plants exhibited 100% mortality to tobacco hornworm and sustained no visible damage after 3 days (Perlak *et al*., 1991).

Detached leaf bioassays of transgenic alfalfa expressing synthetic *cry1C* gene were performed with *S. littoralis,* using ten neonate larvae placed on a moistened filter disc in petri dishes, produced 100% mortality of larvae 3 days after bioassay initiation (Strizhov *et al*., 1996). A detached leaf bioassay of *Bt- cry1Ia1* potato transgenic plants was performed against potato tuber moth (PTM), *P. operculella* by releasing ten neonate larvae into each petri dish and 99% mortality was observed (Lagnaoui *et al.,* 2000).

Sweet corn plants expressing *cry1Ab* gene were artificially infested with 30 *Ostrinia nubilalis* neonates per plant. Percentage of ears without larvae or damage was used to determine the percentage of marketable ears and obtained 100% marketable ears (Burkness, *et al*., 2001).

Bhattacharya *et al*. (2002) performed bioassay of insect resistant transgenic cabbage plants expressing a synthetic *cry1Ab* gene by releasing larvae of *P. xylostella.* The leaf discs (1.5 cm diameter) from young leaves were cut and placed in small petri dishes containing moistened filter paper. On each leaf disc, five late second instar (6-day old) larvae of *P. xylostella* were released and reared at room conditions. Bioassay on detached leaf discs showed significant larval mortality ranging from 51.84 to 74.06%.

Fall armyworm, *Spodoptera frugiperda* and beet armyworm, *S. exigua* were exposed as second instars to leaves, squares or bolls of Bollgard II for 6 days exhibited significantly greater mortality than larvae exposed to parts from plants of the Bollgard I variety and the conventional non-transgenic variety (Chitkowski *et al*., 2003).

Series of bioassays were performed on detached fully grown potato leaves stuck in water agar. For the Colorado potato beetle (CPB) bioassays, 10 neonate larvae were placed on the upper leaf surface. Transgenic potato leaves expressing *cry1Ba*/ *cry1Ia* showed complete resistance against larvae with 100% mortality and no visible damage in a leaf feeding assay after 4 days. For adult CPB, four newly emerged insects were placed on leaves and reared for up to 10 days. The transgenic leaves were completely undamaged and most of the Colorado potato beetles which were placed on transgenic potato leaves were still alive but not feeding but smaller in size than control beetles. Potato tuber moth bioassays were performed by placing 10 neonate larvae on the back surface of potato leaves. No live PTM larvae could be recovered from the transgenic potato plant leaves. In contrast to transgenic plant leaves, leaf infestation with PTM larvae resulted in extensive tunneling by four or five live larvae in control leaves after 4 days. For testing of European corn borer (ECB) resistance, two day old larvae were used. Larvae were allowed to feed on potato leaves for two days. ECB mortality was zero after 3 days in control leaves. In contrast, transgenic potato leaves remained healthy and caused 100% mortality of the ECB larvae (Naimov *et al*., 2003).

Five pieces of freshly cut stems (5-6 cm) with 12 first instar larvae of yellow stem borer were placed into a sealed glass bottle and incubated in the controlled environmental chamber for five days. The feeding assay of *cry2A* plants showed that all the yellow stem borer larvae in transgenic stem cuttings were killed within 5 days after infestation, whereas the larvae infesting the stem cuttings of Minghui 63 (control) grew normally and developed into second instar larvae (Chen *et al*., 2005).

Insects reared on transgenic seeds of rice showed a high mortality rate and reduction in weight, which was found to be significantly higher than in the control.

Bioassay studies proved that the transgenic seeds showed increased resistance to rice weevil *Sitophilus oryzae* than in controls (Ignacimuthu *et al*., 2006).

The T_0 and T_1 transgenic plants from the transgenic Vietnamese rice cultivars expressing *cry1Ab/ cry1Ac* at the maximum tillering stage were bioassayed for resistance to neonate larvae of yellow stem borer using the cut stem method. Three to five stems (including sheath) of 8cm length were collected at the booting stage. These were placed on a moistened filter paper disc in a 90 mm diameter petri dish and infested with six neonate larvae of yellow stem borer. The percentage larval mortality was based on dead and alive larvae 4 days after their release. The cut stem bioassay results showed that yellow stem borer neonate larval mortality after feeding for 4 days reached 100% in more than 95% of the tested Southern and Western positive T_0 plants, whereas the mortality was 0 to 16.6% in the non-transgenic control plants (Ho *et al*., 2006).

Detached leaf feeding bioassay tests were done on T_1 and T_2 generations of pigeon pea plants for insect resistance using the 1^{st} and 2^{nd} instar larvae of the pest *S. litura*. The highest mortality of the larvae found in the transgenic plants was 80% (four out of five larvae released). The larvae fed with leaves of transgenic plants were severely stunted in growth when compared to larvae fed with wild-type leaf (Surekha *et al*., 2005).

A perspective for pest management in Chickpea using transgenic approach

The fore going review has amply provided evidence to the effect that transgenic technology holds many options for pest management in various crops.

Considering chickpea, although various options are available for the management of its Helicoverpa pest, seed borne solutions for insect pest management are the most desirable options. Conventional plant breeding techniques are unlikely to provide reasonable solutions for the management of these pests and recombinant DNA technology provides an option to alleviate the problems of dry land chickpea farmers. Many *Bt* genes are available to meet this requirement. Quite a few constructs are also available in public domain or within the country. The technology to transfer these genes to many crops is in place. More importantly, studies have confirmed the possibility of

developing transgenics of even the recalcitrant chickpea using *Agrobacterium* mediated *in planta* transformation protocols. The procedures for evaluation of these transformants to select and advance the putative transformants to obtain stable transgenics are also available. On these counts, it would be worth making an attempt to produce insect tolerant /resistant transgenic chickpea. Therefore, the present study has been attempted to test this possibility using primarily *Helicoverpa* as the target insect to be tackled.

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The present investigation on development and evaluation studies on transgenic chickpea (Variety KAK-2) was carried out at the departments of Crop Physiology, Entomology and Genetics and plant breeding, UAS, GKVK, Bangalore.

3.1 Plant material:

Chickpea (*Cicer arietinum* L.) variety KAK-2, one of the leading varieties in Karnataka was used for transformation studies. The seeds were procured from the Zonal Agricultural Research Station, GKVK, University of Agricultural Sciences, Bangalore.

3.2 Bacterial strains and vectors

The A*grobacterium* strain EHA-105, harbouring the binary vector pBinAR with the gene of interest *cry1X*, was procured from Dr. P. Ananda Kumar, Principal Scientist, IARI, New Delhi. In this construct *neomycin phosphotransferase* (*nptII*) gene was used as the marker gene while 35s promoter of CaMv as promoter and *nos* as terminator sequences. This construct in EHA-105 was used to transform chickpea (Fig 1)

3.3 Isolation of Plasmid DNA from *Agrobacterium*

Materials:

Sterile microfuge tubes; Micropipeter and autoclaved microtips; Vortex mixer; Microfuge; Laminar flow; Inoculation needle; Bacterial culture plate; LB liquid medium

Procedure:

A single *Agrobacterium* colony was picked up aseptically using sterile inoculation needle and was inoculated in 3 ml of LB (Luria Broth) medium containing kanamycin 1.5 ml in a sterile culture tube.

↓

Incubated overnight at 28^0 C shaker.

↓

Overnight grown culture was transferred to eppendorf tube

↓

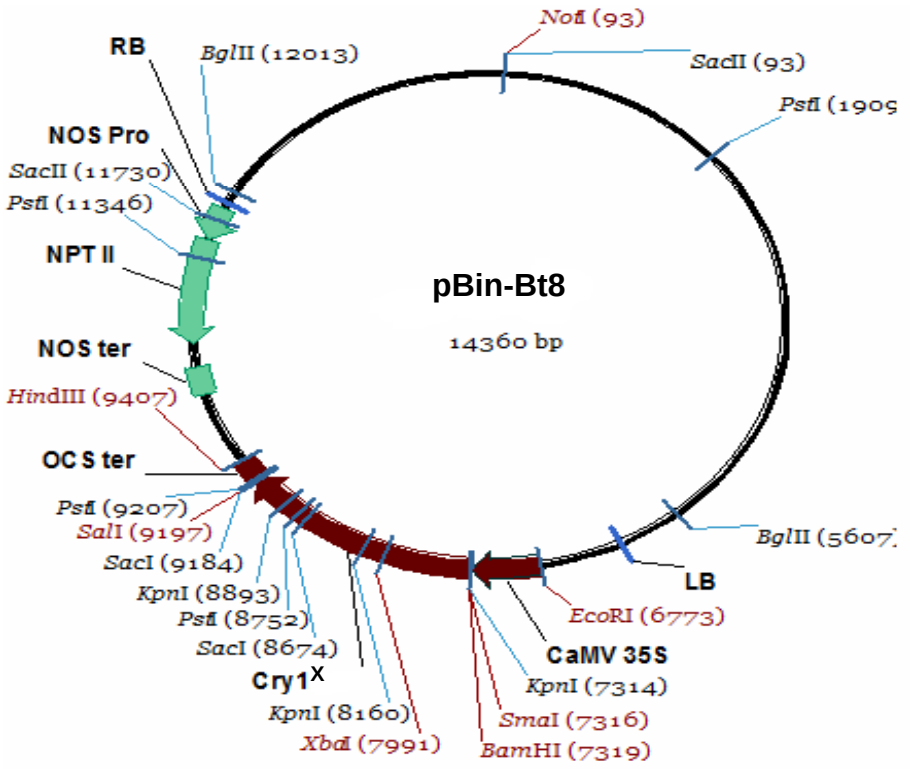

Fig. 1 : The genomic map of the plasmid pBin-Bt8 showing the insert and the restriction enzyme sites

Binary Vector	:	pBinAR (11.0kb)
Insert	:	*cry1X* (1.9 kb)
A. tumefaciens	:	EHA 105
Bacterial selection	:	Kanamycin (50 μg/ml)

Centrifuged at 10,000 rpm for 10 minutes at 4^0 C

↓

The supernatant was decanted and the cell pellet was resuspended in 200 µl of solution I

↓

Vortexed the tube for evenly dispersing the pellet into the solution.

↓

Add 2 µl of lysozyme enzyme to lyse the cell wall

↓

aqueous phase was aliquoted into fresh tube and added equal volume of phenol:chloroform:iso amylalcohol(25:24:1)

↓

200 µl of solution II and mixed gently by shaking and incubated at room temperature for 5 minutes

↓

Added 300 µl of solution III and inversed the tube and kept on ice for 5 minutes

↓

Centrifuge the tube at 10,000 rpm for 30 minutes

↓

Centrifuge the tube at 10,000 rpm for 10 minutes

↓

Collect the aqueous phase added equal volume of chloroform:isoamyl alcohol(24:1)

↓

Centrifuge the tube at 10,000 rpm for 10 minutes

↓

To the upper aqueous phase , added $1/10^{th}$ volume of 3M sodium acetate + 2 volume of ethanol

↓

The tube was stored overnight at - 70^0 C

↓

plasmid DNA was pelleted by centrifuging the tube at 10,000 rpm for 15 minutes

↓

Add 70% alcohol to the pellet and

↓

Again centrifuge the tube for 5 minutes at 10,000rpm, supernatant was aspirated off and

↓

The pellet was dried at 37^0 C for 30 minutes

↓

The pellet was suspended in 10-20 µl of TE and stored at 4^0 C

↓

2µl was checked by Agarose gel electrophoresis to confirm the presence of the plasmid.

3.4 Isolation and Identification of the Plasmid PBin-Bt8 In The *Agrobacterium*

The construct was ascertained primarily by culturing the labeled strain on Kanamycin loaded at the rate of 50µg/ml of agar or broth medium. Growth of the bacterium is an indirect indicator of the transgene in the bacterium. The plasmid DNA was then isolated following the protocol (Sachdev *et al.*,2003;Annexure-1). The isolated plasmid DNA was run on a 0.8 percent agarose gel to discern the presence of the transformed plasmids to confirm the existence of the *cry1X* gene construct in the *A. tumefaciens* culture of the strain EHA105.

3.5 *In planta* transformation of chickpea

Although many protocols can be employed for the transformation of plants, in the present study, *Agrobacterium* mediated *in planta* transformation protocol was employed due to the ease of the technique and the expertise available. Using these transformation protocols, kabuli chickpea variety KAK-2 was transformed with *cry1X* gene. The work was carried out at the Department of Crop Physiology, UAS, GKVK, in association with Dr. Rohini Sreevathsa, who has earlier done many similar transformations.

3.6 Preparation of *Agrobacterium* culture for the transformation

A single colony of *Agrobacterium* harboring recombinant binary vector was grown in LB medium containing 50 µg/ml Kanamycin overnight at 28 °C on a shaker. The bacterial cells were later resuspended in Winan's AB medium (pH 5.2) and grown for 18 hours at 28 ^{o}C on a shaker. Wounded tobacco leaf extract was kept overnight and after 18 hrs added to this suspension and incubated for 6 h on the shaker. This culture was used for transformation (Annexure-2)..

3.6.1.1 Preparation of seedlings for transformation.

Seeds of chickpea (cv. KAK-2) were soaked in water over night and later surface sterilized with 0.1% mercuric chloride for 5-7 minutes, followed by thorough rinsing with sterile water and germinated in Petri plates for a day.

3.6.1.2 Infection and recovery of transformants

The embryo axes were randomly pricked 4-5 times with a sterile sewing needle of 28 gauge and kept in the suspension of *Agrobacterium* in Winan's AB medium. The infection was carried out by gentle agitation at 28-30°C, for 1-2 hours and then these seedlings were blot-dried and washed thoroughly with 500 µg/ml of cefotaxime for 18 h and placed on autoclaved soilrite for further growth under aseptic conditions in capped bottles. After 5-6 days, the germ lings were transferred to soil rite in pots and the seedlings were allowed to grow under growth room conditions for at least 10 days and later they were transferred to green house. The growth chamber was maintained at 26-28°C under 14 hours photo period with fluorescent light of intensity 35 µmol $m^{-2}s^{-1}$. The plants thus raised were T0 plants which were chimeric. The seeds from T0 plants were sown to get T1 plants.

3.7 Molecular analysis of transformants

Transformants were subjected to molecular analysis to confirm the integration of transgene and its expression.

3.7.1 PCR analysis of transformants

The presence of transgenes in the transformants was analyzed by DNA amplification. For the PCR amplification, genomic DNA was extracted by rapid extraction protocol. PCR was used as the first proof for the analysis of the transformant.

3.7.2 Genomic DNA extraction by CTAB method

Cetyl trimethyl ammonium bromide is a detergent and is used along with other reagents to liberate nucleic acids from the plant cell. This is an efficient method for isolating plant genomic DNA from leaf tissues. The high molecular weight DNA obtained was purified by phenol- chloroform method to remove the proteins and other plant debris.

Materials

1. Extraction buffer – 4 % CTAB (composition as in annexure-6).
2. Chloroform: isoamylalcohol mix (24:1)
3. Isopropanol
4. 70 % alcohol
5. TBE buffer
6. Sterile double distilled water
7. Pestle and mortar
8. -70 °C Freezer
9. 1.5 ml Autoclaved eppendorf tube
10. Vortex mixer
11. Water bath
12. microfuge
13. Incubator
14. Micropipeter and autoclaved microtips
15. Leaf tissue from transformed and non transformed plants
16. Physical Balance.

Procedure

The leaf tissue was washed in water and the excess water was blotted with blotting paper and air dried briefly.

↓

2g leaf tissue was weighed from each transformed or control plant

↓

The leaf tissue was ground well to powder form using liquid nitrogen.

↓

Powdered leaf tissues were transferred to the sterilized microfuge tube

↓

Hot extraction buffer was added (CTAB) to each tube @ 750µl and 10 µl of β-mercapto ethanol

↓

The tubes were incubated at 65 °C in a water bath for 15-20 min with gentle invertion

↓

Equal volume of chloroform : isoamylalcohol (24:1) was added and mixed well by inverting the tubes

↓

The contents were centrifuged at 6000 rpm for 20 minutes at 10 °C

↓

The supernatant was taken and to this 600 µl of chloroform:isoamylalcohol (24:1) was added and mixed well and inverted.

↓

Centrifuged the tube at 6000 rpm for 20 minutes at 10 °C

↓

The supernatant was collected and equal volume of chilled Isopropanol was added and was kept overnight at -70 °C

↓

Centrifuged the tube at 6000 rpm for 20 minutes at 10 °C

↓

Decanted the supernatant and washed the pellets with 70 % alcohol

↓

Centrifuged the tube at 6000 rpm for 20 minutes at 10 °C

↓

Decanted the centrifuge and the pellet was air dried until alcohol smell disappeared

↓

Sterile water or TE buffer was added to the air dried pellet and stored at -20 °C (Sambrook *et al*., 1989).

3.7.3 Agarose gel electrophoresis of DNA

Electrophoresis involves the movement of charged molecules in an electric field. DNA molecules carry a net negative charge and therefore when placed in an electric field, they will migrate towards the positive pole. In a gel, the shape, size of the DNA fragment to be separated and the concentration of agarose used influence the migration rate of macromolecule. Smaller the DNA fragment, higher is the concentration of the agarose used in the gel to separate.

Agarose gel electrophoresis is not only used to resolve DNA fragments of different lengths but also to separate different forms of DNA, such as the super coiled or covalently closed circular DNA, the naked or relaxed DNA, and the linear DNA molecules. The compact DNA will migrate faster than the open circular forms. For a particular form of DNA, the migration rate in the gel is inversely proportional to the logarithm of the molecular weight of the DNA unit.

The size of the fragment is determined by electrophoresing simultaneously with DNA fragments of known size such as 1 Kb DNA ladder or *Hind* III lambda DNA digest. However, the rate of migration differs depending on the amount of nicking and super coiling. To confirm the correct size of DNA, the DNA should be linearized. The DNA is visualized by using the interacting dye, such as Ethidium bromide, which produces the orange fluorescence when exposed to UV-light at 260 nm.(Annexure-3)

Material

- TBE buffer (10X stock): Tris Base (54g /l): Boric acid (27.5 g/l): EDTA (0.5M, 10ml, pH 8.0)
- Loading dye (0.25 % Bromophenol blue + 40 % sucrose)
- 0.8% agarose gel (horizontal)
- DNA sample for PCR reaction
- Gel frames and comb (with teeth size 5mm and spaced at a distance of 3mm)
- Ethidium bromide stocks (10 mg/ml concentration)
- UV-transilluminator (260-280 nm)

Procedure:

The frame of gel-casting unit was cleaned and sealed with tape to form the mould

↓

The frame was placed on a flat platform to ensure a flat level base.

↓

The comb was then positioned to the open edge of the frame about 2mm above the surface

↓

Agarose powder was added to TBE buffer (1X) and was dissolved by melting at 100 °C. The solution was cooled to 50 °C and ethidium bromide was added to the gel to achieve a final concentration of 0.5 µg/ml. It was then poured into the gel frame and allowed to set. After setting, the gel was transferred to the gel tank such that the wells were towards the negative pole. The gel tank was filled with TBE buffer (1X) just enough to cover the surface of the gel.

↓

DNA samples were mixed with 2µl of loading dye, bromophenol blue and loaded into the wells of the submerged gel using a micropipette. 5 µl of 1Kb ladder was also mixed with loading buffer as a marker and loaded into one of the wells.

↓

The electrophoresis apparatus was connected to the power supply and electrophoresis was carried out at 50V for 3 hours or when the Bromophenol blue dye migrated to the end of the gel.

↓

It was then visualized on an UV- Transilluminator

Genomic DNA was checked by agarose gel (0.8 %) electrophoresis and then used for PCR amplification using the primers in an Eppendorf Master Cycler® at the optimal annealing temperature. Before that the annealing temperature of primers was standardized. Amplification was carried out in a total reaction volume of 20 µl with the following components:

Genomic DNA	:	100 ng
Taq assay buffer	:	10X
dNTPs	:	2mM
$MgCl_2$	:	2.5mM
Forward primer	:	10 picomoles /µL
Reverse primer	:	10 picomoles /µL
Taq polymerase	:	1 unit

PCR conditions*:*

The reaction mix was then used for amplification under the following conditions:

Hot start / Denaturation	:	94°C for 4 min
Denaturation	:	94 °C for 1 minute
Annealing	:	Depending on the primers
Extension	:	72 °C for 1 minute ,go to step 2 cycle
Final Extension	:	72 °C for 10 minutes

The resulting amplified product was resolved on agarose (0.8 %) gel to confirm the gene.

The *nptII* gene coupled with the target *cry* gene was being used as the marker for the transgene in the PBinAR plasmid. Therefore one method of indirectly confirming the presence of the transgene is to check for the marker gene. T1 generation, KAK-2 putative transformants were initially screened for the *nptII* (neomycine phosphotransferase-II) gene. The primers used for confirming the presence of *nptII* were:

nptII forward primer 5'GAG GCT ATT CGG CTA TGA CTG 3'

nptII reverse primer 5'ATC GCG AGG GGC GAT ACC GTA 3'

However, further confirmation of the target *cry1X* gene was attempted by PCR amplification using either 901 bp or 460 bp gene-specific primers (Annexure-4). The following were the primers used for the two different molecular sizes:

901bp primers for *cry1X*
'Forward 5'-AACCCAAACATCAACGAGTGC 3' and
'Reverse 5'-TTATGCTGTTCAAGATGTC 3'

460 bp primers
'Forward 5'-ATTCAGCGGGCCCGAGTTTACCTT 3' and
'Reverse 5'-CGGATGCGATGATGTTGTTGAA 3'

3.7.4 Enzyme Linked Immunosorbant Assay

The presence of the gene can be identified through PCR analysis; its expression is identified and quantified by ascertaining the product of the gene using Enzyme Linked Immuno Sorbent Assay (ELISA). The principle in ELISA involves specific antigen-antibody reaction linked to the accumulation of the conjugate product that is identified at specific wave length (Sambrook *et al*, 1989) observed in an ELISA reader. The 0.5 g leaf samples of putative tranformants in T_1 chickpea KAK-2 variety were collected and ground in 500 µL extraction buffer. This process calls for extreme caution to prevent sample to sample cross contamination. Brief spin was given to separate out the debris and the supernatant is expected to contain the protein. Commercially available ELISA

plates coated with antibodies raised against *cry* toxins were used for the purpose. However, the target gene, *cry1X* does not have any known monoclonal antibodies raised against it. Therefore, as the *cry1X* has some elements of *cry1Ac*, ELISA plates with antibodies for *cry1Ac* protein that are commercially available were used. Earlier experience has suggested that *cry1X* protein does respond to these antibodies (Keshavareddy, 2008), potentially determining the PCR positives. The wells of the ELISA plates were each added 50 µL of individual cleaned extracts of the putative tranformants along with some designated controls for check. The plates with the plant extracts were incubated at room temperature for one hour to undergo the antigen – antibody reaction. Unbound remaining extracts were then washed away and the bound antigens were detected by adding a substrate. The plate was incubated for 30 minutes and the reaction was stopped using stopping solution provided by Desigen^{TM}. This results in the development of yellow colour that is proportional to *cry* toxin concentration in the sample extracts (Sachdev *et al*., 2003). Darker the colour developed, higher will be the concentration of *cry* proteins in the samples. The colour was then read at 450 nm as optical density based on the absorbance in an ELISA reader. All materials used for the purpose of ELISA, including the plates were supplied by Desigen^{TM} as an exclusive *cry1Ac* protein based ELISA test kit (Annexure-5).

Although the immunosorbance of the *cry1X* could be detected using the ELISA with antibodies for *cry1Ac* protein, it was not clear whether the OD values reflect the true level of expression of the *cry1X* protein. Because it is also possible that the epitopes used for the detection of the transgene may not fully reflect the nature of the protein to respond to the antibodies. Antigen-antibody reactions, it is potentially possible may be dictated by factors independent of just the aminoacid sequences and extraneous factors such as nature of folding and the position of folding in the protein might affect the response pattern of the antibody to the antigen. Further as there is no guarantee that the expression patterns in terms of folding positions of the protein and consequently the reaction sites should remain static across plant species. Therefore, the observations valid for one plant species (e.g. Keshavareddy, 2008) need not remain the same for all other plants even if the source gene sequence remains identical. Nevertheless, putative chickpea

transformants did show significant variability in terms of OD values indicating a potential possibility for greater reliance on the use of the methodology for identification of true expression levels of the transformants. The observed OD values were converted to microgram per gram tissue on fresh weight basis using the standard OD values with 3.419 as equivalent to 100 ng pure *cry* protein after deducting the mean control OD value.

3.7.5 Western blot analysis to confirm *cry* protein gene

A western blot defined as the electrophoresis of the antigen followed by its subsequent transfer to nitrocellulose paper and incubation with specific antibody and then with labelled secondary antibody.

Procedure:

The protein samples were prepared and separated by SDS-PAGE. The length and width of the Resolving gel was measured.

↓

Once SDS-PAGE was completed, Whatmann no 1 filter paper was cut to the same dimension as that of the gel. Usually 12 such pieces were cut out and presoaked in the transfer buffer.

↓

The nitro cellulose membrane was also cut exactly to the same size as that of the gel and soaked in transfer buffer prior to use. The gel was soaked in the transfer buffer for equilibration.

↓

Preparation of the transfer stack: Six pieces of filter paper were stacked over one another followed by the nitrocellulose membrane, gel and over the gel another six pieces of filter paper were placed one over the other.

↓

Before placing the gel, suitable marks are made to indicate the position of the marker lane.

↓

Electroblotting should be done for 2 hrs at 50 volts and 45mA current.

↓

The membrane is kept at 4 °C overnight.

↓

The membrane is then placed in the blocking solution for 1Hr .(The blocking is done so that the area of the nitrocellulose where no protein has been transferred is occupied by milk powder, which in turn prevents non-specific binding of the antibody).

↓

After 1 hr blocking solution was poured out and washed the nitrocellulose membrane with PBST wash solution. 3 washes of 10 min each should be done.

↓

The nitrocellulose membrane was placed in the primary antibody solution and kept at room temperature on a rocker for 1 hr.

↓

The membrane was washed with PSBST wash solution. 3 washes of 10 min each was carried out, changing the wash solution after each wash.

↓

After washing, the membrane was placed in the secondary antibody solution for 1 hr at room temperature on a rocker.

↓

Later the membrane was washed with PSBT wash solution. 3 Washes of 10 min each was carried out, changing the wash solution after each wash.

↓

After washing, the membrane was placed in substrate solution which provides colour only where there is binding of the primary and secondary anti body. Usually colour develops within 30 min. The reaction was carried out in dark conditions.

↓

The reaction was stopped by rinsing the membrane with water and dried under dark conditions. The membrane should not be exposed to light as the bands fade out.

↓

A photograph was taken for permanent record.

3.8 Leaf Bioassay

The proof of efficacy of transgene expression depends on the performance of the transgenics against the target insects. As a result, attempts were made to assess the efficacy of transgenics against the target insect, *H. armigera.* Leaf material of all plants was subjected to biostress against larvae of *H. armigera.*

The plants which are positive in T1 generation were selected and bioassay against *H. armigera* followed this is the most common insect pest in chickpea crop in the recent past in the state of Karnataka. The methodology was adopted from Kumar *et al.,* (2000). Two young pinnate leaves were collected, washed with distilled water, and cleaned of all the debris. The leaf stalk was then plugged into wet cotton and transferred individually to 200 ml plastic containers. Each pinnate leaf represented one replication and at least 2 replications were maintained from each plant in all bioassays. Ten neonate larvae were then released in to each container and observations recorded at an interval of 24 h for at least four days from 48 h. Percent mortality of the released larvae and the extent of leaf damage were the two parameters recorded during the course of bioassays. The most tolerant plants identified were then selected for further advancement.

3.12 Statistical analyses

Means and standard deviations were worked out for all values depending on the need. The mean values of all the plant parameters were subjected to analysis of variance (Sokal and Rohlf, 1969).

Pearson's Correlation and regression analyses were done fallowing Snedecor and Cochran (1967). Scatter plot and frequency distribution graphs were necessary for representing the data and were plotted using MSExcel.

The per cent leaf damage caused by test insect were worked out and the values were angular transformed, then subjected to single factor analysis of variance (ANOVA) to compare the means.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Growth and yield potential of crop plants are significantly influenced by a number of biotic stresses. To meet the growing demand for food, there is a need to increase resistance among the crop plants to various biotic stresses. One of the viable approaches for crop improvement towards this end would be genetic engineering of crops to obtain cultivars that are tolerant towards biotic stresses. Advancement in tissue culture combined with improvement in gene transfer technology has played a vital role in this approach. In this study, efforts were made to develop transgenic chickpea over expressing *cry1X,* a synthetic gene through *Agrobacterium* mediated gene transfer technology. Results of this study are presented below under suitable headings.

4.1 Development of transgenic chickpea with *cry1X gene* for resistance against *Helicoverpa armigera*

4.1.1 Confirmation of presence of *cry1X* gene in *Agrobacterium* strain EHA-105

EHA-105 strain of *Agrobacterium tumefaciens* kindly procured from Dr. P. Anandakumar, IARI, New Delhi, has both the *cry1X* gene and the kanamycin resistance gene, *nptII*, in a single plasmid. *A. tumefaciens* was grown on Luria Broth agar medium provided with kanamycin and the colonies were obtained. The growth of the *A. tumefasciens* colonies in the kanamycin treated plate indirectly suggests the availability of the target *cry* gene. Thus the results indicated that the EHA colonies contained the required *cry* gene, so that the gene *cry1X* can be used for further transformation work. The colonies were further sub-cultured in Luria Broth medium. The *Agrobacterium* cells were lysed and the pBinAR plasmid carrying *nptII* marker gene and the specific *cry* gene were isolated and electrophoresed on an agarose gel (Plate 1). The plasmid digested with various restriction enzymes was run on an agarose gel to confirm the presence of the required pBinAR transformed plasmid with *cry1X* gene. Since the *A. tumefaciens* culture indicated the presence of the plasmid, an attempt was made to use the culture of *A. tumefaciens* for developing the transgenics using the *in planta* transformation method.

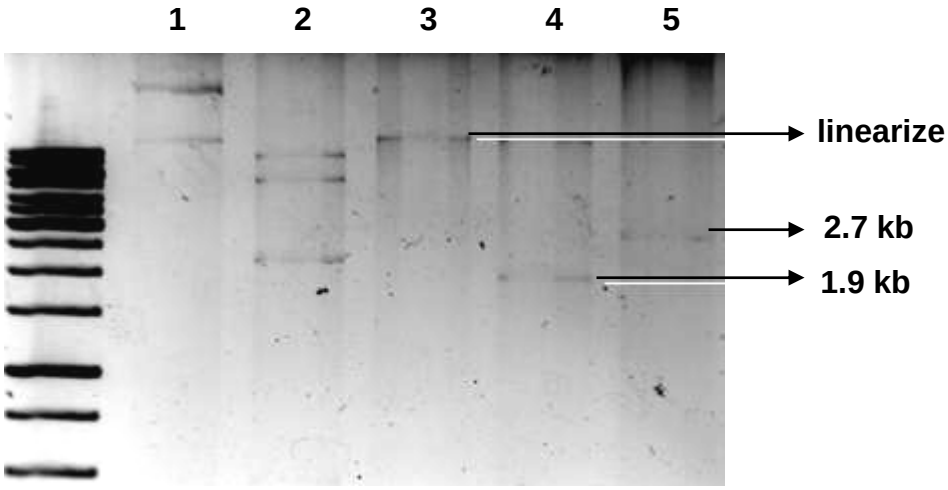

Plate 1 : Restriction analysis for confirmation of binary vector pBin- Bt8

Lane 1- uncut
Lane 2 - *Pst* (3) site in vector
Lane 3 – *Sma*I linearize
Lane 4 – *Bam*HI fragment released
Lane 5 – *EcoR* I-*Hin*dIII releases cassette

Two µg of plasmid DNA was restricted with Pst, Sma*I, *Bam*HI *Eco*R I-*Hin*dIII, *electrophoresed on 0.8% agarose gel

4.1.2 Transformation of chickpea kabuli variety KAK-2 with *cry1X* gene

As many as 60 pre-germinated seeds of KAK-2 were used for the transformation work. Of the total 60 seeds used only 44 viable plants could be obtained and an attempt was made to transform these seedlings by *in planta* transformation method. After 7-8 days, the seedlings were transferred to soilrite in glass bottles and the seedlings were allowed to grow under room conditions for 10 days and later they were transferred to pots and kept in green house. Under green house condition, the plants showed reasonably good growth and development. Plants in this generation represented the T_0 generation, which contained the putative chimeric transformed plants. All 44 plants yielded at least some seeds. These plants were labeled from to 44. An average of 24.09 ±16.60 seeds per plant with a range of 5 to 58 could be obtained from these plants. The seeds harvested from these plants were used for raising the T_1 generation plants. In all 1060 seeds were sown for the development of T1 generation plants.

4.2 Advancement of the transgenic KAK-2 chickpea lines with *cry1X* gene

4.2.1 T_1 generation KAK-2 chickpea plants.

4.2.1.1 Growth of plants

As many as 1060 seeds harvested from the 44 plants obtained in the T_0 generation of transformed KAK-2 chickpea variety were grown in the green house. Previous experiments had indicated that the chickpea crop is highly sensitive and germination tended to be extremely low during off season. In order to improve the chances of getting higher germination, all the 1060 T_1 seeds were germinated in a seed germinator by following the petriplate method. Germinated seedlings were initially grown on soilrite under controlled condition and later transferred to the pots. However, these efforts notwithstanding, only 275 plants were retrieved as germination of T_1 seeds remained quite poor.

These putative transformants with *cry1X* gene were grown under the green house condition with suitable labeling. Growth of all the plants was taken care to be uniform.

However, the growth under green house condition was observed to be variable and relatively poor compared to the control plants.

4.2.1.2 Plant characteristics

Plant transformation with an insecticidal gene may not necessarily help promote plant growth. It is possible that the gene might also affect the plant growth characteristics. Therefore, in order to understand the impact of the transgene if any on the plant traits, some of the growth and yield parameters were also measured and compared between the putative transformants and the control plants. This would also help identify the best of the plants in growth and yield characteristics among the transformed ones.

4.2.1.2.1 Plant Height

Plant height, an important agronomic parameter in chickpea, was recorded in 275 putative T_1 transformants and compared with non-transformed control (wild type) plants. Plant height was recorded at 10 DAS and 30 DAS and the values ranged from plant height at 10DAS range from 4cm to 14 cm in the putative transgenic plants with a mean 8.54(± 1.5) and in the wild types value ranged from 8cm to 16cm with a mean of 12.15(± 2.33). Plant height in 30 DAS as high as 57cm in the plant 30.16 to 57 cm in plant number 3.14 among the transformed plants with a mean of 39.52 (± 9.01) cm, whereas in the wild type plants the values ranged from 39 to 50 cm with a mean of 45.92 (± 3.30) cm (Table 5). The difference between the putative transformants and the wild type plants was found to be significant (30 DAS Student's 't' = 5.92 ; $p < 0.05$). Clearly the results suggested the transformants were not as exuberant as the wild type plants. (10 DAS Student's 't' = 5.5 ; $p < 0.05$)

4.2.1.2.2 No. of Primary and Secondary branches

An important yield attribute of annual species of Fabaceae family is the number of primary and secondary branches, since the number of flowers produced and the pod set is expected to be directly proportional to the branch number. Hence, the number of primary and secondary branches were counted among the putative transformants and

Table 5 : Mean height of Chickpea at 10 DAS and 30 DAS of T_1 generation transgenic plants carrying *cry1X* gene and non-transgenic plants of KAK-2 variety

		Plant Height (cm)			
	n	**10 DAS**		**30 DAS**	
		Range	**Mean ± SD**	**Range**	**Mean ± SD**
Transgenic plants	275	4-14	8.54 ± 1.5	22-57	39.52 ± 9.01
Non-transgenic plants	13	8-16	12.15 ± 2.33	39-50	45.92 ±3.3
't' value			5.5		5.92
'p' level			<0.05		<0.05

*'students 't' two sample, unequal variences.

compared with the wild type plants. The number of primary branches varied from 4 to 9 per plant with a mean of 5.35 (± 1.07) among the putative T_1 transformants. Among the wild type it ranged from 4 to 8 with a mean of 6.23 (± 1.30). The number of secondary branches also showed variation with the values ranging from a minimum of 7 in plant numbers 42.05, 4.35, and 4.07 to a maximum of 17 per plant in plants 4.01, 6.19 and 12.02 with a mean of 9.01 (± 1.69) among the transgenic plants. The values for the same parameter in wild type plants ranged from 8 to 15 per plant with a mean of 11.23 (± 2.12).

Student's t test revealed that the wild type plants had better primary ($t = 2.39$ $p < 0.05$) and secondary ($t = 3.6$ $p < 0.05$) branches compared to the putative transformants in T1 generation under greenhouse conditions (Table 6).

4.2.1.2.3 Pod characteristics

Pods are the economic plant parts in chickpea. The yield of the plant therefore should depend exclusively on the numbers of pods per plant. Among the transgenic plants the number of single seeded pods per plant ranged from a low of 6 per plant in plant numbers 32.01 and 42.03 to a high of 24 per plant in plant numbers 4.19 and 5.11with a mean of 14.4 (± 4.84) pods per plant. In wild type plants, however also had the identical range with a mean of 16.61 (± 6.17) single seeded pods per plant. In some of the plants, two seeded pods were also found, both among the putative transformants and also among the wild type. The number of two seeded pods per plant ranged from up to 4 among the transgenic plants with a mean of 0.42 (± 1.00) and from 0 to 2 among wild type plants with a mean of 0.53 (± 0.77) (Table 7).

Total number of pods per plant ranged from 6 per plant in plant numbers 6.20 to 26 in plant number 4.19 with a mean of 14.82 (± 4.88) among the transgenic plants and correspondingly a low of 6 to 24 pods per plant were observed in wild type plants with a mean of 17.15 (± 6.20) pods per plant. Similarly, total number of seeds per plants varied from 6 to 29 per plant with a mean of 15.25 (± 5.12) among T1 generation putative transformants and among the control plants the range was from 6 to 25 seeds per plant with a mean of 17.69 (± 6.32) (Table 8)

In the T0 generation plants were numbered from 1……n, from each of these plants seeds collected and plants were raised separately. These plants in the T1 generation were planted serially from 1 to n prefixing the plant number in the TO generation

As a result plants in the T0 are all numbered as 1 to n and in the T1 plants are numbered as 1.1, 1.2, 1.3……, n.n.

Although significant differences were observed between the wild type and transformed plants with respect to number of branches, the differences observed in respect of numbers of pods and seeds per plant were not found to be significant despite numerical superiority of the wild type plants

4.2.1.2.4 Interrelationships among the plant characteristics measured

Correlation coefficients of the different biometric parameters measured among themselves were computed and are presented in (Table 9) It was observed that most relationships were weak and only the pod numbers and the seeds per plant had the most significant relationships among themselves. Number of single seeded pods per plant was significantly correlated with both total number of pods per plant ($r = 0.979$; $p < 0.01$) and total number of seeds per plant ($r = 0.920$; $p < 0.01$). Number of double seeded pods per plant and total pods per plant ($r = 0.143$; $p < 0.05$) were also found correlated. The total number of pods was seen to be strongly correlated to total number of seeds per plant ($r = 0.981$; $p < 0.01$) as expected.

4.3 Molecular analysis of the protein

4.3.1 Analysis for the presence of protein using ELISA.

It was observed that the *cry1X* protein could be detected in 69 of the 275 plants by ELISA. Thus the results confirmed the expression of the transgene in some of the T_1 generation KAK-2 chickpea plants with *cry1X* gene. The *cry1X* protein detected by ELISA was calibrated and presented in microgram per gram tissue. Among these 69 plants, content of the toxin protein varied from 0.257 to 10.77 µg/g of plant tissue. The

Table 6 : Mean number of primary and secondary branches per plants observed in Chickpea T_1 generation transgenics carrying *cry1X* gene and non-transgenic plants of KAK-2 variety

		Branches per plant			
	n	Primary branches per plant		Secondary branches per plant	
		Range	Mean ± SD	Mean ± SD	Range
Transgenic plants	275	4-9	5.35± 1.07	7-17	9.01 ±1.69
Non-transgenic plants	13	4-8	6.23± 1.3	8-15	11.23 ±2.12
't' value			2.39		3.6
'p' level			<0.05		<0.05

*'students 't' two sample unequal variances

Table 7 : Mean number of single and double seeded pods per plant observed in Chickpea of T_1 generation transgenics carrying *cry1X* gene and non-transgenic plants of KAK-2 variety

		Number of pods			
	n	Single pods per plant		Double pods per plant	
		Range	Mean ± SD	Range	Mean ± SD
Transgenic plants	275	6-24	14.40± 4.84	0-4	0.42 ± 1.00
Non-transgenic plants	13	6-24	16.61± 6.17	0-2	0.53 ±0.77
't' value			0.5		1.27
'p' level			>0.05		>0.05

Students 't' two sample unequal variances .

Table 8 : Mean number of total pods and total seeds per plant observed in Chickpea of T_1 generation transgenics carrying *cry1X* gene and non-transgenic plants of KAK-2 variety

	n	Total number of pods per plant		Total number of seeds per plant	
		Range	Mean ± SD	Mean ± SD	Range
Transgenic plants	275	6-26	14.82± 4.88	6-29	15.26 ± 5.12
Non-transgenic plants	13	6-26	17.15± 6.20	6-25	17.69 ±6.32
't' value			1.33		1.36
'p' level			>0.05		>0.05

*'students 't' two sample, unequal variances

highest values were observed in the plant numbers 38.39, 6.08, 42.06 and 42.18 with 10.77, 10.67, 10.23 and10.13 µg of toxin protein /g of plant tissue, respectively. The lowest toxin protein content as measured by ELISA was observed in the plants numbering 12.09 and 12.12 with 0.257 and 0.262 µg of toxin protein /g of plant tissue, respectively(Plate 2). The means for toxic protein in transgenic plants (3.15 ± 3.09) was found to be significantly different from those of the wild types (0.16 ± 0.20) as compared by t test ($t = 3.46$; $p < 0.01$).

4.3.2 Molecular analysis by individual plant PCR

Plants that responded to ELISA were chosen for the identification of transgene among the putative transformants. Plant genomic DNA was extracted by CTAB method, a standard protocol that is commonly employed for DNA extraction. PCR analysis with *nptII* specific primers of 69 individual plant DNA samples revealed the presence of *nptII* gene (around 750 bp DNA fragment) in 43 individual plant DNA samples out of 69 verified (Plate 3).

However, further confirmation of the target *cry1X* gene was attempted by PCR amplification using either 901 bp or 460 bp gene-specific primers. (Plate 4 and 5)

4.3.3 Western blot analysis of transgenic chickpea harboring *cry1X* protein

Western blot analysis of transgenic chickpea using the antibody against the cry protein gave an indication that the plants expressed cry protein. Three plants were verified for the purpose and two of these clearly indicated the 20 KDa protein band depicting the expression of cry protein in transgenic plants (plate 6).

4.4 Laboratory bioassay of *cry1X* transgenic plants

The putative transformants, with *cry1X* gene, of T_1 generation grown in transgenic greenhouse facility were subjected to detached leaf bioassay by releasing ten neonate larvae of *H. armigera* on each leaf. Two such leaves were assayed for each plant. Observations were made on per cent larval mortality and per cent leaf damage. All the PCR positive plants of T_1 generation were screened for the purpose irrespective of

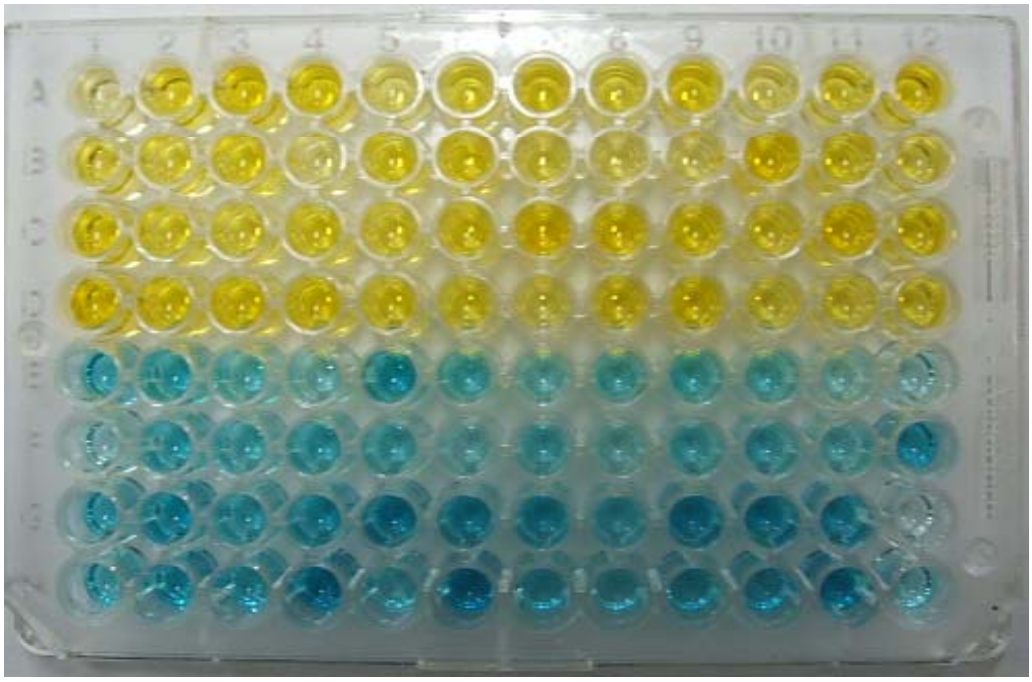

Plate 2 : ELISA plate showing cross reaction of cry protein with antibody

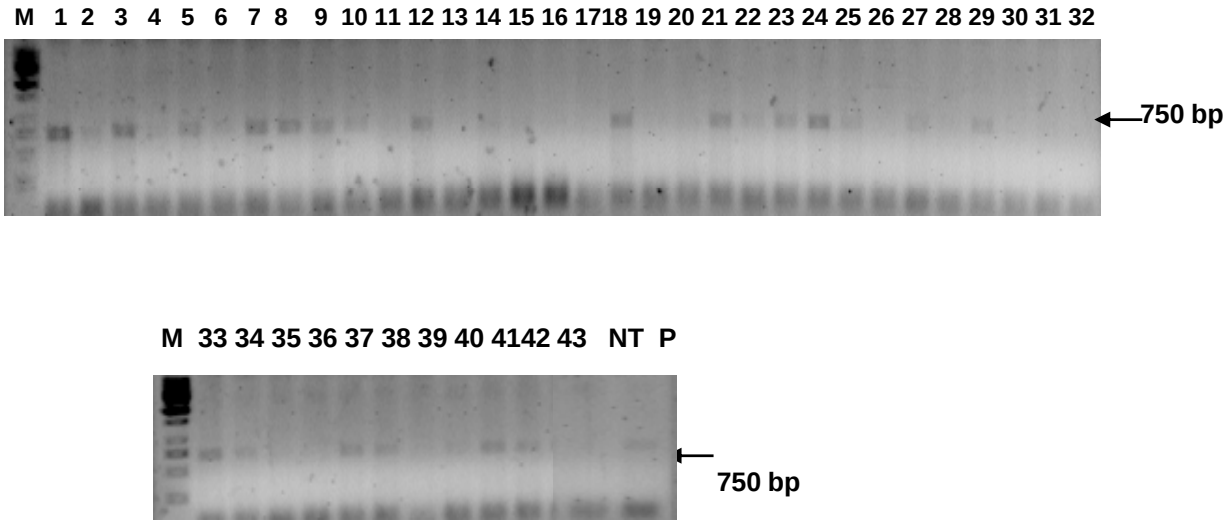

Plate 3 : PCR amplification of selected transgenic plants over expressing *cry1X* gene of T_1 generation of KAK-2 Chickpea with *nptII* 750bp primers Bands along the indicated arrow suggested positive samples

Lanes: M-1Kb ladder
P- positive control
1to 43- transgenic plants with *cry1X* gene
Nt- non- transgenic plant

Genomic DNA was extracted by CTAB method from selected plants leaf sample and PCR was carried out and the product was run on 0.8% agarose gel

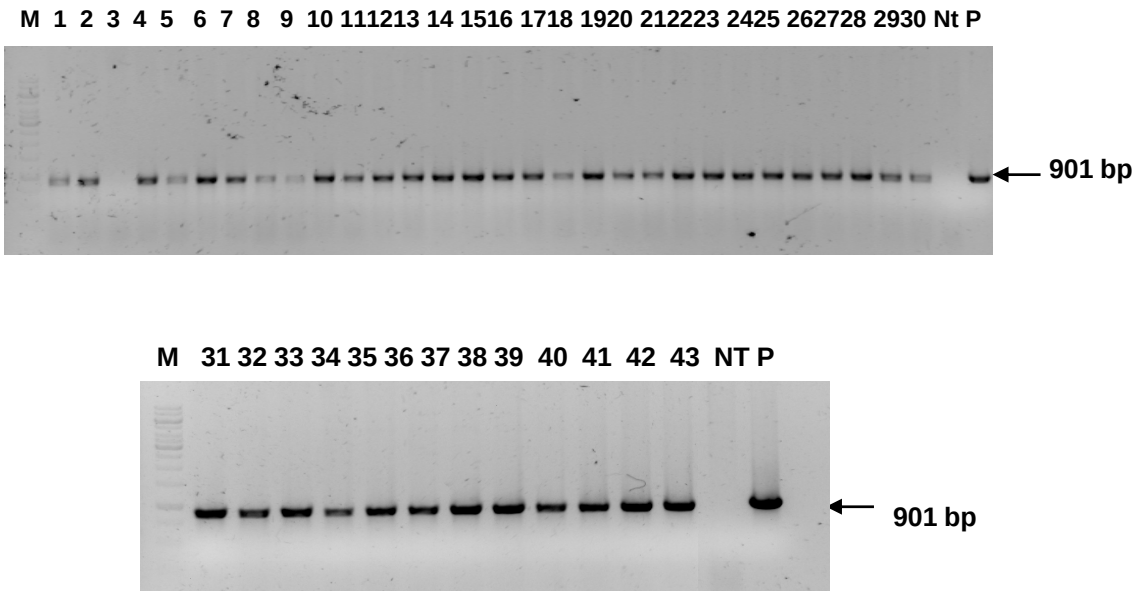

Plate 4 : PCR amplification of selected transgenic plants over expressing *cry1X* gene of T_1 generation KAK-2 Chickpea with *cry1X* gene specific 901bp primers. Bands along the indicated arrow suggested positive samples

Lanes: M-1Kb ladder
P- positive control
1to 43- transgenic plants with *cry1X* gene
Nt- non-transgenic plants

Genomic DNA was extracted by CTAB method from selected plants leaf sample and PCR was carried out and the product was run on 0.8% agarose gel

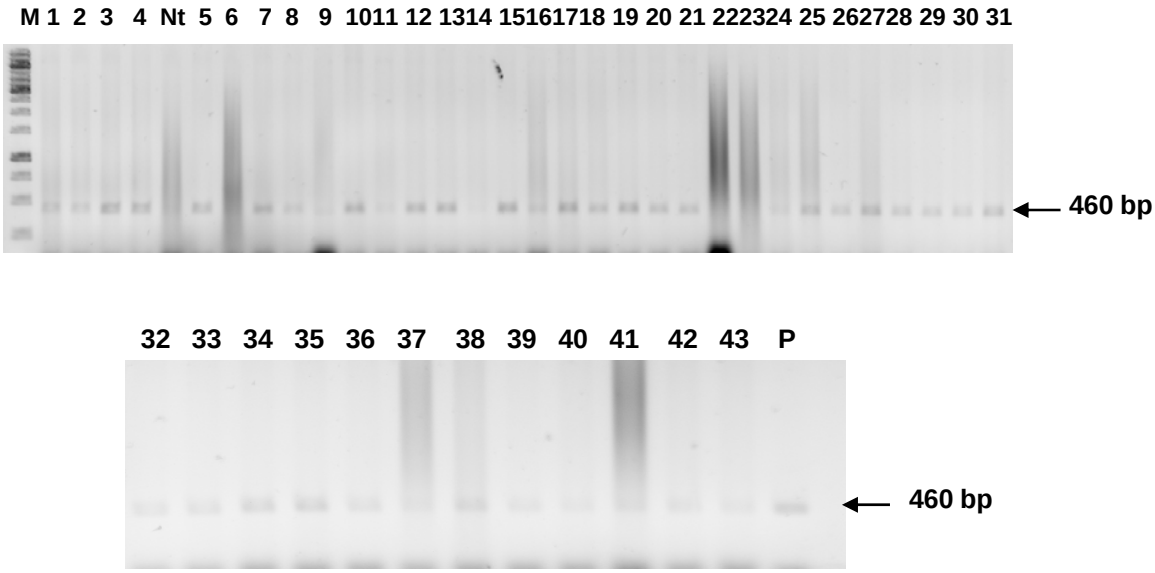

Plate 5: PCR amplification of selected transgenic plants over expressing *cry1X* gene of T_1 generation KAK-2 Chickpea with *cry1X* gene specific 460bp primers. Bands along the indicated arrow suggested positive samples

Lanes: M-1Kb ladder
P- positive control
1to 43- transgenic plants with *cry1X* gene
Nt – non-transgenic plant

Genomic DNA was extracted by CTAB method from selected plants leaf sample and PCR was carried out and the product was run on 0.8% agarose gel

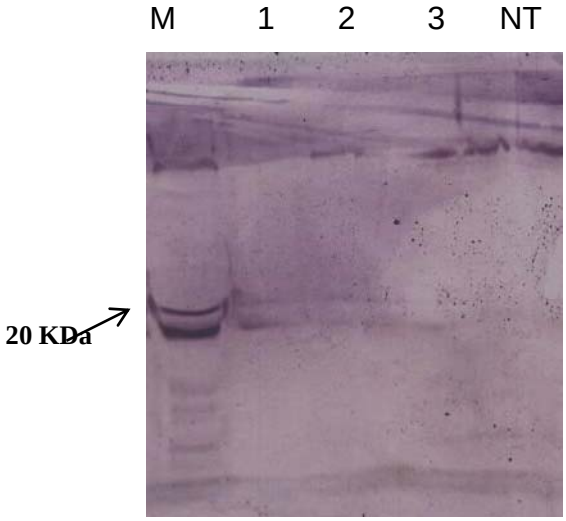

Plate 6 : Western blot analysis of *cry1X* gene in transgenics (T_1) of KAK- 2Chickpea

M- Marker 20KDa
1 to 3- transgenic plants
Nt – non- transgenic plants

the nature of insertion, integration and the expression of the transgene in the chimeric T_1 population (Plate 7).

4.4.1 Mean per cent larval mortality of *Helicoverpa armigera* in T1 generation *cry1X* gene incorporated KAK-2 chickpea plants

The extent of larval mortality of *H. armigera* observed on leaves of putative transformant plants with *cry1X* gene of KAK-2 chickpea variety in T1 generation varied from 0.0 to 100 per cent among the different plants studied. Highest mean of 100 per cent larval mortality was observer in the plants numbered 30.07, 37.03, 8.01, 12.02, 42.16, 5.12, 4.18, 38.3, 12.07, 38.42, 5.03, 12.05, 43.03, and 38.17. Further plants numbered 38.16, 38.31, 15.06, 38.11, 42.26, 39.15, 21.12, 43.05, 41.01, 42.19, 12.09 and 10.1 also recorded a mean mortality of more than 85 %. The average mean per cent larval mortality recorded in transgenic plants was 49.6 ± 25.59 (n =275) compared to that of 3.5 ± 4.73 in non-transgenic wild type plants (n = 13). The 't' test indicated significant differences between the transgenic and non-transgenic plants ($t = 12.94$; $p < 0.01$;). Thus the results demonstrate a high degree of variability in the insecticidal property of putative transgenics of T_1 generation plants generated by *in planta* method, besides some potentially highly insecticidal plants (Table 10).

4.4.2 Mean per cent leaf damage caused by *Helicoverpa armigera* in *cry1X* gene incorporated KAK-2 chickpea plants

The range of mean per cent leaf damage varied from 5.0 to 90.0 among different plants studied. The lowest mean per cent leaf damage of only 5.00 per cent was observed in the lines 15.03, 10.1, 39.15, 38.11, 43.03, 12.07, 38.16, 5.12, and 42.19. A few other plants, viz., 41.01, 38.42, 37.03, 30.70, 15.06 and recorded 10 or less percentage leaf damage. The average mean per cent leaf damage recorded in transgenic plants was 34.10 ± 20.53 (n = 275) compared to that of 92.7 ± 3.13 in non-transgenic plants (n = 13) and the difference between the two sets of plants was significant ($t = 20.36$; $p < 0.01$). It is possible that the plants with relatively high expression of the *cry1X* protein may still not bring about substantial mortality of the larvae, but may successfully reduce the damage

Plate 7 : Screening of transgenic KAK-2 T_1 generation plants for *Helicoverpa armigera* resistance. Insecticidal assay with neonate larvae reared for 4 days of *H. armigera* on leaves from transgenic plant with *cry1X* gene (left) and non-transgenic plant (right).

due to induced antibiosis. Therefore, associational study between the two traits may help identify the nature of activity of the toxin in the transgenics

4.4.3 Association between mean percent larval mortality of *Helicoverpa armigera* and mean percent leaf damage *in cry1X* plants

It is generally anticipated that more the mean percent larval mortality observed in a plant, less would be the mean percent leaf damage. This was checked by associating the two parameters for the putative *cry1X* transgenics in the T1 generation KAK-2 chickpea variety. A strong linear negative association between mean per cent larval mortality of *H. armigera* in a plant (x) and mean per cent leaf damage (y) ($r = -0.596$; $n = 288$; $p < 0.01$) was observed among the putative transgenic plants. This relation fallowed the equation, $y = -0.4134x + 54.59$. Therefore, plants with higher mean per cent larval mortality are ideal for further advancement due to their higher resistance against *H. armigera* for they also reduce the plant damage. The observed high correlation coefficient clearly suggests a straight forward relation between the toxicity and the corresponding leaf damage. As a result, potential antibiosis effect independent of toxicity does not seem to exist in *cry1X* transformed plants (FIG 3).

4.4.4 Relationship between the insect mortality and the response to ELISA among the transgenic plants

All putative transformants were subjected to both ELISA and detached leaf bioassay against *H. armigera*. However, only those that were positive to ELISA were further tested for the presence of the transgene by attempting to identify the coupled marker *nptII* gene in the putative transformants. But surprisingly, only 4 T1 plants recorded no larval mortality while 11s plant recorded 10 per cent or less mortality of the *H. armigera* larvae. Similarly, only 31 plants recorded less than 25 per cent larval mortality. As this was unexpected on the basis of the ELISA results, an attempt was made to associate the two parameters to understand the correspondence between the two parameters verified. In order to reduce the ambiguity, 25 per cent larval mortality was taken as the cut off value to consider a plant as potentially transformed as the mean mortality observed in any wild type plant was 22 per cent.

Table 9 : Association between the different biometric characteristics measured in the T1 generation of KAK2 chickpea transgenic plants

	Height at 10DAS	Height at 30DAS	Primary branches	secondary branches	# double pods	# single pods	#total pods	#total pods	% mortality
Height at 10DAS	1								
Height at 30DAS	0.062	1							
Primary branches	0.071	0.058	1						
secondary branches	0.034	-0.018	0.0025	1					
# double pods	-0.102	0.032	0.0070	0.057	1				
# single pods	-0.056	-0.071	0.0039	0.072	-0.064	1			
#total pods	-0.077	-0.064	0.0053	0.083	0.143	0.979	1		
#total pods	-0.093	-0.055	0.0064	0.091	0.333	0.920	0.981	1	
% mortality	-0.036	0.010	-0.0220	-0.027	-0.025	0.076	0.071	0.0624	1

NS: P>0.05, *p =0.05, ** p= 0.01

Contingency *Chi* square revealed that the two parameters are not congruent suggesting that it is possible that the expression of the toxin identified by ELISA as is being followed may not fully reflect the activity of the transgene *cry1X* in these putative transgenic plants. (λ=0.0034) (Table 12).

4.4.5 Mean per cent larval mortality and mean per cent leaf damage observed in *H. armigera* in *cry1X* gene incorporated KAK-2 T_1 generation chickpea plants selected for advancement

On the basis of the above results, as many as 43 plants of T_1 generation were selected for further advancement. Among the plants selected, the highest mean per cent larval mortality of 100 per cent was observed in the plants, 30.07, 37.03, 8.01, 12.02, 42.16, 5.12, 4.18, 38.3, 12.07, 38.42, 5.03, 12.05, 43.03 and 38.17. The lowest mean per cent larval mortality was observed in the plants numbered 14.01, 32.10 with 5 and 30 per cent mortality respectively. The average mean per cent larval mortality recorded in selected transgenic plants for further advancement was 80.59 ± 24.75 (n = 43) compared to that of 22.44 ± 17.52 in non-transgenic plants (n = 13) 96 h after bioassay initiation. The 't' test indicated significant difference between the T_1 generation transgenic plants selected for further advancement and the non-transgenic plants ($t = 15.95$; $p < 0.01$).

The extent of mean per cent leaf damage caused by *Helicoverpa armigera* varied from 5.0 to 62.50 among different plants selected for further advancement. The lowest mean per cent leaf damage of 5.0 was observed in the plants, 5.12, 38.16, 12.07, 43.03, 38.11, 39.15 and 15.03. The highest mean per cent leaf damage was observed in the plant numbered 38.39. The average mean per cent leaf damage recorded in selected transgenic plants for further advancement was 22.44 ± 17.52 (n = 43) compared to 92.7 ± 3.13 in non-transgenic plants (n = 13) and the difference was highly significant ($t = 20.71$; $p < 0.01$). Thus the plants selected were the superior most available among the putative transgenics both from the point of view of high mortality of *Helicoverpa armigera* larvae and in keeping the leaf damage to the minimum.(T.able 11)

4.4.6 Correlation between *cry1X* protein (µg/g of tissue) and mean per cent larval mortality of *Helicoverpa armigera* in T_1 generation KAK-2 chickpea plants

The range of toxic protein level was 0.0 to 10.8 microgram per gram tissue among transgenic T_1 generation KAK-2 chickpea plants. It is generally anticipated that, the larval mortality varies with the Cry protein level expressed. This was checked by associating the two parameters for the *cry1X* transgenics in T_1 generation. A non-significant association between *cry1X* protein in a plant (x) and mean per cent larval mortality (y) ($r = 0.0876$; $n = 288$; $p < 0.05$) was observed in these plants. However, plants with higher level of protein expression and high mean larval mortality were also observed. Nevertheless, the results are clearly counter intuitive and are the product of multiple consideration of both ELISA and mortality values in the selection of the plants for further advancement (FIG 2).

4.4.7 Relation between *cry1X* protein (µg/g of tissue) and mean per cent leaf damage caused by *Helicoverpa armigera* in total T_1 generation KAK-2 chickpea plants

As a corollary to the above relationship, one can anticipate similar results for associational studies between *cry1X* protein expression in a plant (x) and mean per cent leaf damage (y). This relation was observed to be negative ($r = -0.1928$; $n = 288$; $p < 0.05$). This association therefore clearly complements the above observation (FIG 2).

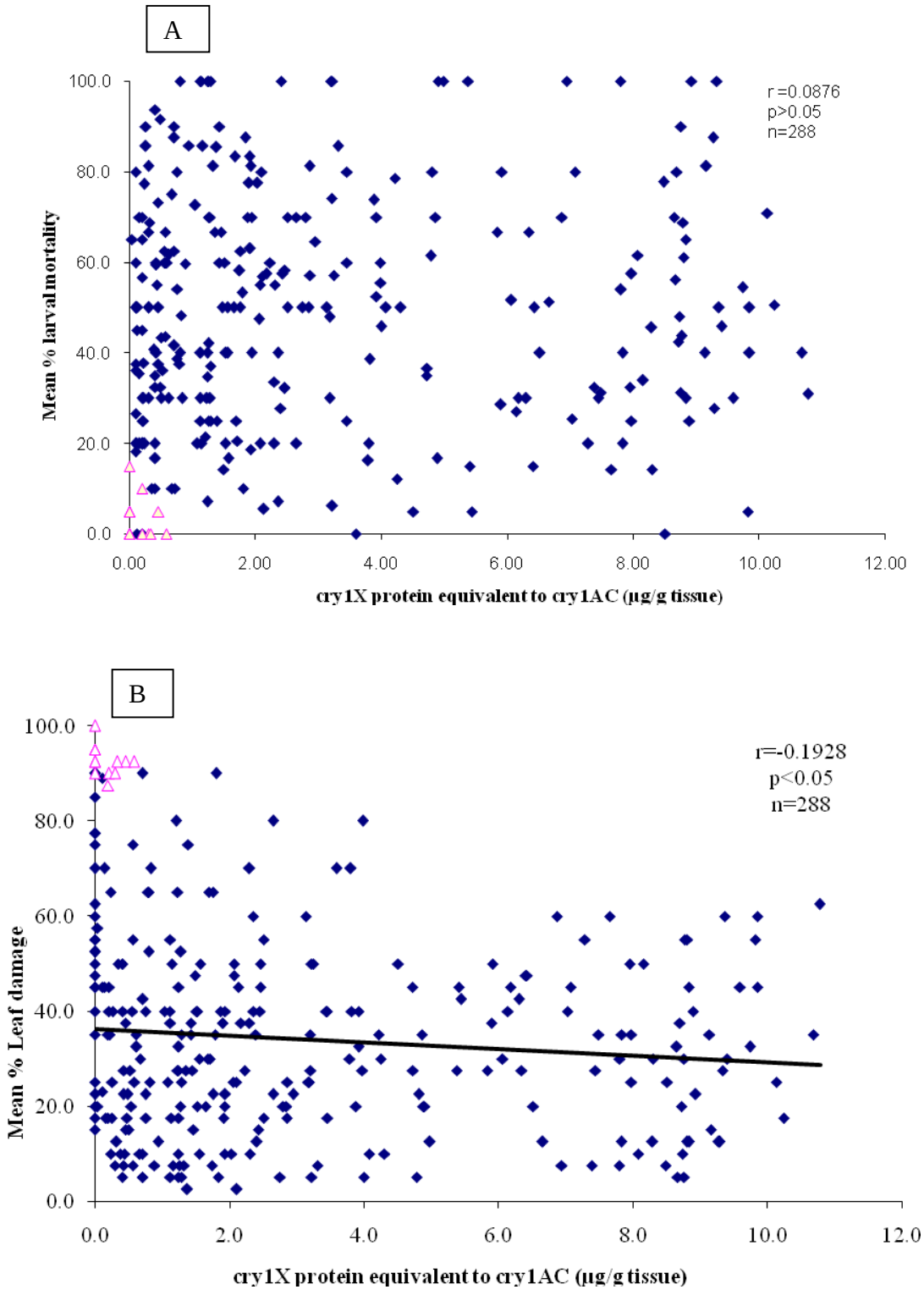

FIG. 2 : Relation between Cry1X protein expression (µg) and (A) larval mortality of *Helicoverpa armigera;* B) mean per cent leaf damage caused by *Helicoverpa armigera* in transgenic T_1 generation KAK-2 chickpea plants

Table 10 : Mean per cent larval mortality and mean per cent leaf damage observed in *Helicoverpa armigera* when tested against T_1 generation transgenic plants carrying *cry1X* gene and non-transgenic plants of KAK-2 chickpea variety

		Helicoverpa armigera			
	n	**% larval mortality**		**% leaf damage**	
		Range	**Mean ± SD**	**Range**	**Mean ± SD**
Transgenic plants	275	0-100 (0- 90.0)	49.6± 25.59 (44.77)	5-90.0 (12.9-71.57)	34.10 ± 20.53 (35.73)
Non-transgenic plants	13	0-15 (0-22.79)	3.5 ± 4.73 (10.78)	87.5-100 (69.3-90.0)	92.7 ± 3.13 (74.32)
't'value			12.94		20.36
'p' level			<0.01		<0.01

*'t' test: two samples assuming unequal variances Values in parentheses are arc sine transformed values.

Table 11 : Mean per cent larval mortality and mean per cent leaf damage observed in *Helicoverpa armigera* when tested against T_1 generation selected transgenic plants carrying *cry1X* gene and non-transgenic plants of KAK-2 variety

		Helicoverpa armigera			
	n	**% larval mortality**		**% leaf damage**	
		Range	**Mean ± SD**	**Range**	**Mean ± SD**
Transgenic plants	43	5-100 (0- 90.0)	80.59±24.75 (63.86)	5-62.5 (12.9-52.24)	22.44 ± 17.52 (28.28)
Non-transgenic plants	13	0-15 (0-22.79)	3.5 ± 4.73 (10.78)	87.5-100 (69.3-90.0)	92.7 ± 3.13 (74.32)
't' value			15.95		20.7144
'p' level			<0.01		<0.01

*'t' test: two samples assuming unequal variances Values in parentheses are arc sine transformed values.

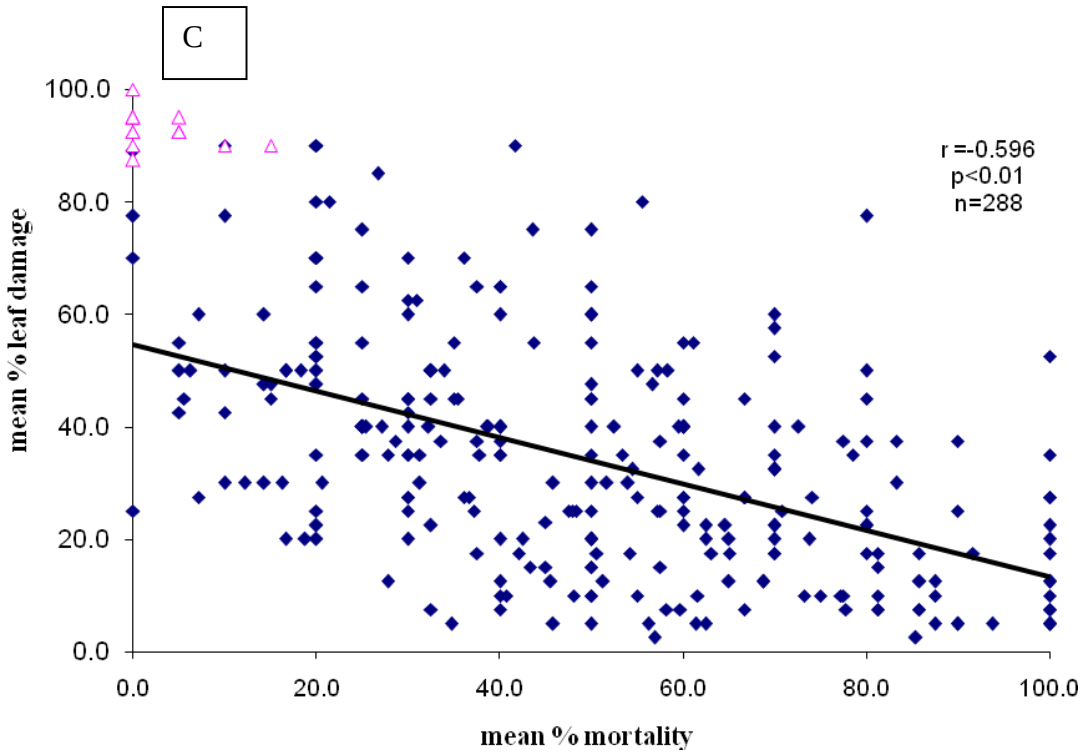

FIG. 3 : Relation between mean per cent larval mortality and mean per cent leaf damage of transgenic T_1 generation KAK-2 chickpea plants when tested against *Helicoverpa armigera* larvae

DISCUSSION

V. DISCUSSION

Plant biotechnology has become a source of agricultural innovation, providing new solutions to age old problems. The insect control area is one of its most active theaters of operation. The simple underlying reason for this interest is that worldwide crop damage inflicted by phytophagous insects is staggering despite the use of sophisticated crop protection measures, chiefly chemical pesticides. Over the past decade, the success in producing insect resistant crops through gene transfer has been impressive and the culmination of this process occurred in 1996 when the first generation of insecticidal plants genetically known as *Bt* plants, were introduced into the market. Insect resistant crops have been one of the major successes next to herbicide resistant crops in applying plant genetic engineering technology to agriculture. Benefits of the technology have now led to the widespread adoption in many crops. Cotton, maize and potato lead the chart, worldwide. However, most of these technological advancements in the development of insect resistant transgenic crops are largely limited to few selected cash crops of the world. Consideration of economic returns commensurate with the high cost of investment in transgenics has prompted corporate sector to enter this area in a big way, while the public sector is lagging behind. Due to economic considerations, the development of such transgenic crops has therefore, been limited to the crops that can easily and quickly pay back the investment made on the crop for improvement. As a result, marginalized crops or dry land crops have hardly attracted the attention of the corporate sectors. This technology would be of immense value to the farming community that depends on such marginalized or dry land crops, if adopted by the public sector.

Availability of suitable gene constructs, a technology of gene transfer to the desired crops and the methodology of evaluation through successive generations are in fact the major limitations at present for the development of these transgenics in dry land and marginalized crops (Ortiz *et al*., 2000). Keeping these limitations in mind, an attempt is made the public sector Sate Agricultural Universities in the area of transgenic crops for pest management. For the purpose, chickpea, a marginalized legume food crop, grown almost exclusively under dry land conditions by small and marginal farmers, but yet severely affected by *Helicoverpa armigera* the pod borer has been chosen. In

Karnataka, it is mainly grown under rain fed conditions during *Rabi* season. The studies on post transformation conducted up to the T_1 generation in KAK-2 kabuli chickpea plants , has helped clearly demonstrate the potency of the State Agricultural Universities or other public institutions in developing the desired transgenic varieties. The outcome of these studies is discussed here under.

The *cry* genes

The specificity of *Bt* Cry toxins towards target pest species is a major advantage in agriculture because effects on non-target insects and other organisms in the ecosystem are minimized. However, deployment of transgenic crops expressing a single specific *Bt* toxin can lead to problems in the field, where secondary pest species are not affected and can cause significant damage to the crop. Introduction of additional *Bt cry* genes or novel *Bt* genes into the crop can afford protection against a wider range of pests. Commercial cultivation of transgenic cotton containing two *Bt* genes began in 1999, in the USA Cotton plants expressing both *cry1Ac* and Cry2Ab proteins were more toxic to bollworm (*Helicoverpa zea*; target pest) than cotton expressing *cry1Ac* alone (Chitkowski *et al.,* 2003).

Similarly, many large *cry* genes particularly active against Lepidopteran pests with varying specificity and activity levels have been identified. A major limitation has been the specificity of the *Bt* toxins to certain groups of Lepidoptera. If several such receptor specific toxins are combined, the activity levels will be higher and more effective than the natural sequences that provide a one to one relationship of toxin to insect specific receptors. The structural similarity of all members of the family of three domain *Bt* toxins, and the separate roles of the domains in the processes of receptor binding and pore formation, suggested that combining domains from different proteins could generate active toxins with novel specificities.

The role of domain III of *Bacillus thuringiensis* δ-endotoxin *cry1Ac* is implicated in determining toxicity against *Heliothis virescens*. Hybrid toxins, containing domain III of *cry1Ac* with domains I and II of Cry1Ba, Cry1Ca, Cry1Da, Cry1Ea, and Cry1Fb,

respectively, were created. In this way Cry1Ca, Cry1Fb, and to a lesser extent Cry1Ba were made considerably more toxic (Rumyana Karlova, *et al.,*2004*).*

Cadherin protein has been identified as a putative receptor for *Bacillus thuringiensis* (Bt) *cry1Ac* toxin in *Helicoverpa armigera* and plays a key role in Bt insecticidal action. Production of a fragment from this *H. armigera cry1Ac* toxin-binding cadherin included the predicted toxin-binding region. Binding of *cry1Ac* toxin to this cadherin fragment facilitated the formation of a 250-kDa toxin oligomer. The cadherin fragment was evaluated for its effect on *cry1Ac* toxin-binding and toxicity by ligand blotting, binding assays, and bioassays. The results of ligand blotting and binding assays revealed that the binding of *cry1Ac* to *H. armigera* midgut epithelial cells was reduced under denaturing or native conditions *in vitro*. The addition of the cadherin fragment had no effect on Cry2Ab toxicity (Chenxi Liu[a], *et al.,*2009)

Considering this possibility, in order to improve the insecticidal property of the toxins, *cry1X* has been developed by Dr. P. Ananda Kumar, at IARI. The novel synthetic gene has elements of *cry1Ac* and *cry1F* toxins built into it. As a result, the potency and breadth of activity are expected to be much higher than any of the conventional natural toxins of *Bt*, against lepidopteran pests. Hence this *cry1X* gene was transformed, in the present study to explore the possibility of developing transgenic kabuli chickpea variety KAK-2 against *Helicoverpa armigera.*

Gene transfer, integration and expression

A variety of gene transfer methods are available for incorporation of foreign genes in to plants. Most of these methods however revolve around the development of explants using tissue culture as the primary mechanism. Tissue culture methods are not standardized for all plants in the first place and secondly, many crops are not easily amenable for tissue culturing. Such recalcitrant plants are also not amenable for gene transfer techniques that call for availability of tissue culture technique. As a result, alternative approaches for gene transformation is a necessity. Feldmann and Marks (1987) observed that transformed plant tissues could be obtained via *Agrobacterium*-mediated infection of the mesocotyl region of germinating *Arabidopsis* seeds. This

method largely referred to as *in planta* transformation method, has several advantages over the conventional tissue culture based methods. *In planta* transformation protocols have been developed for transformation in *Arabidopsis thaliana* (Feldmann and Marks, 1987), soybean (Trick *et al.,* 1997), groundnut (Rohini and Rao, 2000b & 2001), sunflower (Rao and Rohini, 1999), safflower (Rohini and Rao, 2000a), rice (Supartana *et al*., 2005), maize (Chumakov *et al*., 2006), field bean (Pavani, 2006) and cotton (Keshamma *et al*., 2008). The method has also been shown to work for a number of genes, encoding different kinds of proteins. Thus a similar method, with little modifications from Rohini and Rao (2000b and 2001) was employed in the present study to develop transformants. Similar tissue culture independent *Agrobacterium* mediated transformation protocols have been successfully employed for both recalcitrant and non recalcitrant species of plants (Rohini and Rao, 2000a and b; 2001).

An add factor to be noted is the simplicity of procedures after transformation with the *in planta* protocols. In the present study also, transformation protocol developed by Rohini and Rao (2000b & 2001) was employed for transformation of chickpea plants in the kabuli variety KAK-2 with *cry1X* gene. The putative transformants were successfully regenerated in green house conditions. T_0 transgenic plants showed good recovery and normal growth under green house conditions. Because of the chimeric nature of the T_0 transformed plants, T_1 generation plants were subjected to different as molecular analysis.

However, the *in planta* method will produce only the chimeric plants that show both transformed and untransformed vegetative and reproductive parts initially. As a result the success rates can vary greatly and are best measured in the T_1 stage of the crop rather than the T_0 generation. Little modifications can greatly improve the success rates as seen in the case of *Arabidopsis thaliana* seeds. A 12-hour exposure to *Agrobacterium* has for example can give raise to higher rates of success (Feldmann and Marks, 1987).

Yet, *in planta* methods are expected to give just around 10 percent success in transformation when measured in the T_1 generation. However the percent of actual transformants can be arrived at only by observing the PCR of individual plants of the positive which are selected by ELISA. Fairly good transformation efficiencies have been reported for *in planta* transformation in *Arabidopsis*. Efficiencies up to 40% with

Agrobacterium inoculation of germinating seeds of rice have also been reported (Supartana *et al.*, 2005).

Further, the PCR analysis of the putative transformants in subsequent generations, also the expression data, and the western analysis clearly demonstrate stable integration of the gene by *in planta* transformation technique.

The results were also confirmed by insect bioassays against *Helicoverpa armigera* of positive plants observed in the T_1 generations for the chickpea variety KAK-2. The *nptII* (neomycin Phosphotransferase II) gene confers resistance to Kanamycin. This *nptII* gene in the transgene is present as a selectable marker upstream of the insect resistant gene. By and large, it is anticipated that presence of this gene indicates the presence of *cry1X* gene also. Therefore, by amplifying the *nptII* gene, the presence of *nptII* gene and the intended transgene could be confirmed. Similar approaches were also followed by earlier workers for confirmation of the presence of transgene in the putative transformants (Rohini and Rao, 2000b & 2001), where molecular characterization of primary transformants as well as the representative method for selection uniform generation plants had shown that the method ensured insertion, and inheritance of foreign genes in KAK-2 chickpea variety.

Thus in essence the study confirms the utility of *in planta* method for insertion of foreign gene in to chickpea plants. Further, the gene integration was also demonstrated by the fact that T_1 generation plants showed PCR positives in kabuli chickpea variety KAK-2 during the course of advancement to further generations. This besides confirming the presence of *cry1X* gene, indicates the integration and inheritance of the gene in question.

Gene expression

While PCR studies with gene specific and *nptII* primers through successive generations indicate the integration of the gene, ELISA is expected to be crucial in understanding the *Bt* toxin expression pattern. Although, alternative methods do exist,

ELISA has been standardized for the Cry proteins and is expected to be the most ideal technique for quantification of the Cry protein levels in *Bt* transgenic plants.

However, due to non–availability of antiserum for *cry1X* proteins, the ELISA could not be conducted using the right target antiserum. While it is a straight forward situation to use the gene specific antiserum for ELISA for most natural genes, it is not clear whether the expression of the complex synthetic genes could also be followed in the same pattern. But several studies conducted earlier have used antibody sandwich ELISA, wherever the transgenes were of complex nature. For example, a double antibody sandwich ELISA was used for quantitative estimation of expression of fused Cry1Ab/ *cry1Ac* produced in transgenic rice plants (Ye *et al*., 2001). This however, could not be attempted in the present study, as a gene specific antisera was lacking for the *cry1X* gene. Since the *cry1X* gene has *cry1Ac* domain, an attempt was made to check on the possibility of using available *cry1Ac* antibodies for the ELISA. In the initial phases, it was observed that ELISA based on *cry1Ac* antibodies was useful, as was observed by Pavani (2006). As a result, in the present study also, ELISA was conducted based on the antiserum developed for *cry1Ac* protein, as *cry1Ac* protein antiserum was able to provide some indications of the protein levels for the *cry1X* protein being pursued.

Targeting *cry1Ac* commercial ELISA kits were developed using specific monoclonal antisera targeted specific epitopes of the complete *cry1Ac* gene. However, *cry1X* contains only specific domains of *cry1Ac*. As a consequence, the ELISA results presented in this study for *cry1X* have to be viewed cautiously to reduce the possibility of erroneous conclusions. The quantified protein levels were expressed as microgram per gram leaf fresh weight. The maximum Cry protein concentration of *cry1X* transgenic plants reached approximately 10.77 µg/g leaf fresh weight in T_1 generation KAK-2 chickpea plants. The associational studies between Cry protein levels expressed in transgenic plants and mean per cent larval mortality of *Helicoverpa armigera* were observed to be non-significant. Similar trend was also seen between Cry protein expression levels and mean per cent leaf damage caused by *Helicoverpa armigera* in the chickpea plants.

Further, concentrations of foreign protein usually show great differences among different independent transgenic plants despite identical construct (Mabqool *et al*., 2001; Ramesh *et al*., 2004). *Bt* protein level expressed in commercial *Bt* transgenic cultivars (cotton, maize and potato) is generally 1 to 11 µg/g leaf fresh weight, which is adequate to meet the requirement of the high-dose/refuge strategy (Cohen *et al*., 2000).

cry1X protein levels expressed in the transgenic chickpea plants as estimated in the present studies were considerably low compared to the above reports. But the veracity of these estimates needs to be further confirmed, due to two important reasons. First, it is not clear whether the current *cry1Ac* based immunoassay is good enough estimate. This was also substantiated by the fact that bioassay results did not match with ELISA values for *Helicoverpa armigera* . Basically, this would raise serious questions on the validity of the use of *cry1Ac* based immunoassay transformed with *cry1X* gene in chickpea. Similar mismatch between the immunoassay and the bioassay results were also known earlier. Murray *et al*., (1991), for example, observed the tomato plants with truncated *cry1Ab* gene to tolerate tobacco horn worm (*Manduca sexta*), tomato fruit borer (*Helicoverpa zea*) and tomato pinworm (*Keiferia lycopersicella* (Walshingham)). But in these plants, the *cry* protein was immunologically undetectable.

Consequent to the above, ELISA done on *cry1Ac* protein antiserum for the *cry1X* protein can only provide some indications of the protein levels but may not provide the true picture of the quantitative content of the *cry1X* protein. Therefore there is a need to develop specific antisera for *cry1X* protein and further standardization of ELISA protocols.

Selection of stable *cry1X* plants

A pertinent question to the success of development of transgenics would be the extent of homozygosity of the integrated transgene. It is necessary that a transgenic developed should be homozygous for the transgene such that the subsequent multiplication would not allow for segregation of the gene across progeny. Thus the homozygosity brings in the stability in the transgenics for the inserted gene. Stable transgenics are a necessity for extending every developed transgenic to the level of field-

testing or for transferring to the farmers' fields. This aspect can be best verified by estimating the success rates of the transgenics advanced through successive generations. Therefore, relative success rates on the basis of PCR positives in subsequent generations should provide us the idea of the homozygosity of the transgene in the plants under scrutiny. Considering this aspect, it is expected that on the basis of the PCR, the per cent success should show the tendency to increase in subsequent generations relative to T_1 generation in the chickpea variety KAK-2 for the transgene. PCR results showed success rates of 15.6 per cent in T1 generation chickpea for *nptII* in *cry1X* transformed plants.

Expression of the cryprotein gene was confirmed by isolating crude protein from the transgenic plants. Western blot analysis of the crude protein showed a band of 20 KDa proteins. Proteins from the control non-transgenic plant not showed any band. This confirms the expression of cry protein gene in the transgenic plants.

Insect bioassays under laboratory conditions

The detached leaf bioassays performed under laboratory conditions against neonate larvae of *Helicoverpa armigera* in T_1 generation of KAK-2 variety substantiated the fact that the identified transgenics do have activity against these larvae. The extent of larval mortality of *H. armigera* ranged from 0.0 to 100 per cent with average mean per cent larval mortality of 49.6 with an average mean leaf damage of 34.10 in T_1 generation KAK-2 putative transformants, where as the % larval mortality was 3.5 in the non-transgenic control plants with leaf damage reaching 92.7 per cent. Results demonstrated the potency of the *cry1X* gene in chickpea plants against the pod borer, *H. armigera*. The association between the mean per cent larval mortalities of *H. armigera,* and mean leaf damage caused by them in *cry1X* transformed plants were found to be significant. This result has a strong implication for the selection of the plants for further advancement and stabilization. Consequently it is desirable that careful selection be made of plants to be advanced, considering the mortality of *H. armigera*.

Biometric parameters of transgenic plants

Several biometric parameters recorded showed significant differences between transgenic and non-transgenic plants. Plant height, number of primary branches per plant and number of secondary branches per plant were found to be significantly different between the two groups of plants. The toxin production in transformants seems to have some kind of effect on the transgenic plants and cause them to be less vigorous than the non-transgenic plants for these parameters.

Since, the plants are in the T1 generation the major limitation is that the transgenic plants cannot be replicated. Such adverse effect on the transgenic plants needs to be addressed in subsequent generations.

Yield related parameters, the direct indicators of yield, did not vary significantly between transgenic and non- transgenic plants. The traits such as number of pods per plant, number of single seeded pods per plant, number of double seeded pods per plant, total number of pods per plant and total seeds per plant were not found to be significantly different, though plant height and number of branches per plant were found to be different between transgenic and non-transgenic plants.

The correlation co-efficient between different biometric parameters also suggests that there is no significant difference between biometric characteristics. This could be probably due to the timing of the recording of biometric observations (30 DAS) which was much before the bio-efficacy and bioassay tests were done. The pod characteristics were enumerated at harvest that might have contributed for plant recovery resulting in lack of differences between transgenic and non-transgenic plants. Mean larval mortality and mean damage did not correlate with any of the pod characteristics such as single seeded pods per plant, double seeded pods per plant, total pods per plant and total seeds per plant,

These preliminary results are in the T_1 generation, thus on the whole suggest that the transgenesis has not greatly affected the yield performance of the transgenics. Further studies should provide better data to reinforce these results.

Insect mortality levels and the significance of the defense achieved

The present study had highlighted many transgenic plants with high bioassay-efficacy. Hundred percent mortality of the larvae did show the effective expression in at least some of the transgenics. The lack of correlation between the mortality level and the ELISA remains a challenge to be tackled.

Why should the plants differ in expression when the transgene is same? ELISA currently used for this study was based on the *cry1Ac* toxin. The differences in homology between the *cry1Ac* and the *cry1X* used for plant transformation is only expected to be around 60 %. As a result, the contributing expression factor that could be detected using the antibodies for *cry1Ac* might not b equally well detected in the transgenics. However, some of the plants demonstrated both high mortality of the test insect and high detectable expression by the ELISA. As a result, the association between the two parameters may not have strong meaning and that the epitopes detected by the monoclonal antibodies in ELISA may not be directky involved in the nature of expression of the toxicity levels. Observed. Thus there appears to be a total disassociation between the two parameters. This aspect needs to be further carefully observed and the exact reason for the observed lack of association between these two parameters understood.

Conclusions

It is now firmly established that transgenics are bound to become the mainstay of the pest management technology in the years to come because of many advantages associated with their use. However, most of these efforts are limited to the crops that can potentially provide better market rewards for the investors. However, the role of insect pests in limiting the productivity of crops is of no less importance in the marginalized crops and Agro-ecosystems. Dry land crops are generally low input crops in the first place and next, due to vagaries of monsoon, risk associated with further investment can be very high and thus insect pests remain a potential perennial problem. As a result, seed borne solutions at meaningful cost prices would facilitate better performance of the crops against insect pests.

Crops such as chickpea, field bean, groundnut *etc.* thus deserve the attention in this regard for improving the productivity of dry land crops. Such an effort can only come from the public sector institutions. However, the infrastructural and investment needs for such research work in most public sector institutions such as State Agricultural Universities are largely insufficient. Keeping these limitations in mind, the present study was planned with a dry land crop, chickpea which is also recalcitrant for tissue culture, for modifications to incorporate insecticidal *cry* gene.

Study although is only an experimental attempt, substantially suggests the plausibility of developing efficient cry gene bearing transgenics that can provide good protection against *Helicoverpa armigera*, the podborer of chickpea. The study overwhelmingly demonstrated the following:

a. Good cry gene that can work against *Helicoverpa* is indigenously available.
b. *In planta* method can provide viable chickpea transformants.
c. It is possible to overexpress the *cry1X* gene in chickpea and overexpression of *cry1X* gene provides reasonably good resistance against *H. armigera.*

Further the study has also identified the following limitations,

a. ELISA methods have to be standardized for *cry1X* gene
b. More rigorous bioassays are required against *Helicoverpa*
c. The efforts should continue to develop stable transgenics

On the whole, these experimental efforts demonstrate significant success besides identifying the constraints to be addressed for further research efforts towards managing *Helicoverpa armigera* through transgenic approach in chickpea.

SUMMARY

VI. SUMMARY

Insect pests have major effects on agricultural productivity and food supply. Although the application of insecticides has helped minimize the impact of insect pests, chemical control entails economic, health and environmental costs. Therefore, the development of new strategies for insect pest control is critical for sustaining agricultural production and improving our environment and health. Insect resistant transgenics provide an exciting option as they are likely to a) reduce the usage of insecticides for pest management, b) provide sources of resistance for deployment, which are otherwise unavailable from natural plant sources, and c) help mitigate further investment on pest management over and above the basic requirements for raising a crop and this factor would be of unmatched importance in improving the productivity of dry lands, if deployed effectively against insect pests of dry land crops.

Keeping these factors in view, an attempt was made to explore the possibility of developing insect resistant chickpea overexpressing the synthetic *cry1X* gene against *Helicoverpa armigera.* In essence, the study aimed at understanding the development, evaluation and advancement of constitutively co-expressing *cry1X* gene in transgenic chickpea plant in the kabuli variety KAK-2, along with a kanamycin resistant marker gene, *nptII*. *cry1X* is a synthetic gene comprising the elements of *cry1Aa, cry1Ab, cry1Ac* and *cry1F*. All these genes are basically active against Lepidoptera and have varied efficacy against different taxa. The gene was synthesized by Dr. P. Ananda Kumar, Dept. of Biotechnology, IARI, New Delhi and kindly lent to the University of Agricultural Sciences, Bangalore for the work in the form of a binary vector, pBin-Bt8, cloned in to the strain EHA-105 of *Agrobacterium tumefaciens*.

The chickpea variety KAK-2 was tested for transformation with *cry1X* gene, primarily targeting against the chickpea podborer, *H. armigera*. Transgenic chickpea plants were generated by following an *Agrobacterium* mediated *in planta* transformation protocol for incorporating *cry1X* gene. Procedure involved co-cultivating differentiated embryo of the germinating seeds with *A. tumefasciens* carrying the intended transgene.

In T_0 generation 44 plants of KAK-2 could be obtained after transformation with *cry1X*. From these, 1060 seeds were obtained for sowing in T_1 generation, which represented the putative transgenics. As the T_0 plants are expected to be chimeric, it is only in the T_1 generation that the true trasformants can be identified.

These putative transgenics were then advanced to T1 generation and analysed adopting multiple evaluation strategies, such as PCR, ELISA, Western blotting and bioassays, for selection of plants for further advancement.

Individual PCR studies of selected plants were carried out with both gene specific and *nptII* primers. The PCR positives in subsequent generations provide us the idea of the homozygosity of the transgene in the plants under scrutiny. Per cent success of true transformants by PCR analysis showed about 15.63 per cent in the T1 generation.

ELISA was conducted based on the antiserum developed for *cry1Ac* protein. Protein levels were quantified based on standard OD values of the pure protein and expressed as microgram per gram leaf fresh weight. The maximum cry protein concentration of *cry1X* transgenic plants reached approximately 10.77 µg/g leaf fresh weight in T_1 generation KAK-2 plants. The associational studies between cry protein levels expressed in transgenic plants and mean per cent larval mortalities of *Helicoverpa* were observed to be non-significant. Similar trend was also seen between Cry protein expression levels and mean per cent leaf damage caused by *Helicoverpa.*

The western analysis of PCR-positive plants in T_1 generation in KAK-2 plants, revealed the presence of *cry1X* protein in the genome. Bioassays with *Helicoverpa* revealed an average mean per cent larval mortality of 49.6 and an average mean leaf damage of 34.10 in T_1 generation KAK-2 putative transformants, where as the corresponding figures for non-transgenic control plants were 3.5 and 92.7 per cent.

Under green house conditions, a study was also conducted to ascertain the agronomic parameters and efficacy against *H. armigera* in T_1 generations of putative transgenic KAK-2 variety. The results revealed that in KAK-2, transgenic and non-transgenic plants did not show much difference. There were significant differences in the

agronomic traits observed between the transgenic plants and wild types for plant height, number of branches per plant. Whereas non-significant differences were observed for the pod characters such as number of single seeded pods per plant, number of double seeded pods per plant, number of total pods per plant and number of total seeds per plant.

In summary, the study clearly demonstrated potentialities of developing insect resistant transgenics in chickpea Further, the study also was an example of symbiosis between, Biotechnologists and Entomologists that helped the progress of the work with a recalcitrant crop such as chickpea, a crop that is not easily amenable for tissue culture. Lastly, the current situation of the transgenic plants developed under this programme, despite being an experimental approach, still provides an opportunity for further test and advancement to reach the field utility level. This is because the bioassays under contained and forced feeding conditions may under estimate the true advantage to be gained under open field conditions.

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ANNEXURES

ANNEXURE-I

Composition of LB medium

- Bacto tryptone-10g/l
- Bacto yeast extract-5g/l
- Sodium chloride-10g/l
- pH adjusted to 7.0
- for solid medium Bacto agar @ 1.5% was included**.**

ANNEXURE-II

Composition of AB minimal medium

AB buffer (20X)-solution I

K_2HPO_4 – 60 g/l

NaH_2PO_4 – 20 g/l

pH should be 7.0

AB salt (20X)-solution II

NH_4Cl - 20g/l

$MgSO_4.7H_2O$ - 6g/l

KCl – 3g/l

$CaCl_2$ $2H_2O$ – 3g/l

$FeSO_4$. $7H_2O$ – 50mg/l

Prepare 0.5% glucose, add 1X concentration of the AB-buffer and AB salt and make up the required volume.

ANNEXURE-III

Methodology for gel electrophoresis to visualize the specific DNA fragments

Material

- TBE buffer (10X stock): Tris Base (108 g/l): Boric acid (55 g/l): EDTA (0.5M, 20ml, pH 8.0)
- Loading dye (0.25 % Bromophenol blue + 40% sucrose)
- 0.8% agarose gel (horizontal)
- DNA template (1µg) for PCR reaction
- Gel frames and comb
- Ethidium bromide stocks (10 mg/ml concentration)
- UV-transilluminator (260-280 nm)

Procedure:

- The frame of gel-casting unit was cleaned and sealed with tape to form the mould
- The frame was placed on a flat platform to ensure a flat level base.
- The comb was then positioned to the open edge of the frame about 2mm above the surface
- Agarose powder was added to TBE buffer (1X) and was dissolved by melting at 100^0 C. The solution was cooled to 50^0 C and ethidium bromide was added to the gel to achieve a final concentration of 0.5 µg/ml. It was then poured into the gel frame and allowed to set. After setting, the gel was transferred to the gel tank such that the wells were towards the negative pole. The gel tank was filled with TBE buffer (1X) just enough to cover the surface of the gel.
- DNA samples were mixed with 2µl of loading dye, bromophenol blue and loaded into the wells of the submerged gel using a micropipette. 5 µl of 1Kb ladder as a marker was also loaded into one of the wells.
- The electrophoresis apparatus was connected to the power supply and electrophoresis was carried out at 50V for 3 hours or when the Bromophenol blue dye migrated to the end of the gel.
- It was then visualized on a UV- Transilluminator (Sachdev *et al*; 2003)

ANNEXURE-IV

PCR amplification of the DNA

Genomic DNA - 100 ng

Taq assay buffer - 10X

dNTPs - 2mM

$MgCl_2$ - 2.5mM

Forward primer - 1µl

Reverse primer - 1 µl

Taq polymerase - 2 µl

Sterile water to makeup the volume.

ANNEXURE-V

Enzyme Linked Immunosorbent Assay for the cry genes

Materials

- Antibody coated plates
- Enzyme conjugate
- Substrate
- Wash buffer
- Positive control
- stop solution
- Extraction buffer(1X)
- microtiter plate reader.

Procedure

- Four leaf discs of groundnut plant were taken and ground well in a pestle and mortar with 500µl of extraction buffer
- A quali plate was taken and 50 µl enzyme conjugate was added to each well of the plate
- 50 µl of positive control was added to one well to compare the sample readings with it. 100 µl of extracted leaf sample was added to each well
- To compare the readings 100 µl of Monsanto leaf sample was also added. 100 µl of extraction buffer which acts as blank was added in one well and one well with plant extract from an untransformed plant as a control in one well.
- Plate was kept for incubation for two hours at ambient temperature.
- Sample extract was carefully removed and wells were washed with wash buffer, the plate was emptied and washing was repeated three times.
- Then 100 µl of substrate was added to each well.
- Plate was incubated for half an hour at ambient temperature.
- Then 100 µl of stop solution was added to each well and mixed thoroughly to turn the well contents yellow.

Plate was read in a plate reader at 450nm measurement wavelength and 620nm reference wavelength (Sachdev *et al*., 2003 and Sambrook *et al*., 1989).

ANNEXURE-VI

Chemical composition of working solutions:

A) 4% CTAB buffer (100ml):

CTAB – 4g
NaCl (1.4M) – 8.18g
EDTA – 4mL of 0.5M stock
Tris Cl – 20 mL of 1M stock
Sterile distilled water – 86mL

B) TE preparation for DNA dilution

Tris Cl -10 mM
EDTA -1mM
pH – 8

C) Loading Dye (100mL)

Bromophenol Blue – 0.25% (0.25g)
Sucrose – 40% (40g)
Sterile distilled water – 100mL

Note: dissolve sucrose completely in 80mL of sterile distilled water and then add dye and make up the volume.

D) Ethidium Bromide (1mL)

Ethidium bromide – 10mg
Sterile water -1mL

Note: wrap the eppendorf with Aluminum foil or black paper

E) TBE (Tris Borate EDTA) 10X (500mL)

Tris Cl – 54g
Boric acid – 27.5g
EDTA (0.5M) – 10mL

F) TAE (Tris Acetate EDTA) 50X (100mL)

Tris Cl – 24.4g
Acetic acid (CH_3 COOH) – 5.7 mL
EDTA (0.5M) – 10mL