DEVELOPMENT AND ANALYSIS OF TRANSGENIC CHICKPEA FOR RESISTANT TO *Helicoverpa armigera (Hubner*

Thesis submitted in partial fulfillment of the requirement for the degree of **Master of Science (Agriculture)**
to the Tamil Nadu Agricultural University, Coimbatore-641 003

By

Mohammed Mubarak, Y

(ID No. 07-612-005)

INTERNATIONAL CROPS RESEARCH INSTITUTE FOR THE SEMI-ARID TROPICS, PATANCHERU, ANDHRA PRADESH

&

CENTRE FOR PLANT BREEDING AND GENETICS
TAMIL NADU AGRICULTURAL UNIVERSITY
COIMBATORE-641 003

2009

CERTIFICATE

This is to certify that the thesis entitled **"Development and analysis of transgenic chickpea for resistant to *Helicoverpa armigera (Hubner)*"** submitted in part fulfillment of the requirements for the degree of **Master of Science (Agriculture)** to the Tamil Nadu Agricultural University, Coimbatore, is a record of ***bonafide*** research work carried out by **Mohammed Mubarak, Y.** ID No. 07-612-005, under my supervision and guidance and that no part of this thesis has been submitted for the award of any degree, fellowship or other similar prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

Place: Coimbatore
Dated:

(Dr. N. NADARAJAN)
Chairman

Approved by

Chairman : **(Dr. N. NADARAJAN)**

Co-chariman : **(Dr. KIRAN. K. SHARMA)**

Member : **(Dr. S. RAJESWARI)**

(Dr. G. ASOKAN)

External Examiner
Date:

CONTENTS

Acknowledgement

ACKNOWLEDGEMENT

Talents bloom in compatible climate; efforts capture enviable acclaim in unexpected moments, Odds are prone to over-shadow a sensitive soul in an insensitive age. At some point of time, I thought, I was diffident in my venture. However, as time rolled on, to my delight, I found myself transforming into an entirely different boy, capable of capturing the heights. I have come to believe the incredible. If with profound satisfaction, I could present this piece of investigation work in its present form, I owe a great deal to several of those who showered unstinted support on me throughout. I need no second thought to place on record resplendent letters, the overwhelming patronage granted to me by these noble souls but for which I would have been only groping in dark.

*In the array of names, sparkle an immaculate personality, **Dr.N.Nadarajan**, Professor and Head, Department of pulses, CPBG, TNAU, Chairman, whose stupendous capacity, elegance and gracious disposition left indelible impressions in my mind. He stands out as a beacon light having brought about a sea change in the quality of my approach and perspective, planting the spirit of enthusiasm like the permeating fragrance of blooming flower. In unequivocal terms, I express my intense indebtedness and heartfelt gratitude to him.*

*I have been very much fortunate to have the guidance and patronage of two prominent **Dr.S.Rajeswary**, Associate Professor, CPBG, TNAU and **Dr.G.Asokan**, Professor, Department of Entomology, TNAU, as the members of Advisory committee. Their erudition and expertise have been of great advantage in my pursuit. I have no reservation in acknowledging the valuable advice rendered by them at all stages.*

I remember with gratitude that Dr.T.Ravindran, Director, Centre for Plant Molecular Biology, has been so kind to me and highly helpful.

*I record my gratitude and thanks to acknowledge **Dr.K.K.Sharam, Internationally recruited Scientist**, International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Prades, Co-Chairman for the guidance, timely admonition, suggestions and constant encouragement throughout the research work that was carried out in ICRISAT.*

I place my sincere thanks to International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) for providing fellowship during my stay for research.

I gratefully thank Dr.Pooja Bhatnagar and Dr.D.Srinivasa Rreddy, Genetic Transformation Laboratory, ICRISAT, for their valuable guidance in carrying out my research.

I will be failing in my duty if I don't record here the invaluable help rendered by Dr.Babu, Associate Professor, Dr.P.Shanmugasundram Professor and PG Coordinator, CPBG, and Dr.M.Yassin, Professor, Department of Agronomy, TNAU I take this opportunity to thank them all.

In my endeavor, I had the special privilege to enjoy the voluntary help and guidance in abundance from various people. I would gladly make a special mention about the affectionate help offered by Pandari, Lakshmi, Mannama, Lakshminarayanan ansd Yosuf during my thesis work at ICRISAT.

My special thanks go to my ever-caring seniors, Dr.M.Kiruthika, Visiting Scientis, ICRISAT, without her utmost cooperation, perhaps, I would not have been so successful in my endeavor.

How can I wink my eyes over the spirit, enthusiasm and earnestness of my dear labmates, Venkatesh, Gaurav, Kalyani, Mayank, Prasanthi, Madurima, Sowmini, Kiruthika and Ranadeer. My heart throbs to thank them for the earnest encouragement and assuring support in my undertaking.

Jotting customary thanks is not enough for the care and support I enjoyed from my loving father, Mr.M.Yakeen, mother, Mrs.Y.Noorjahan and brother, Musthak, So, I would like to keep my feelings towards them close to my heart. Thanks are also to my friends, Ram, Shoba, Muthu Selva and Govindraj for all their timely help in the course of my study.

I humbly submit my work at the feet of Almighty, whose grace and blessings all through has helped in completing my work successfully.

The sprawling, complex and sylvan surroundings at TNAU have been a source of inspiration to me. It several to assuage the agonies of my heart at times of despair. My heart is with it, for I liked it; I loved it;

(MOHAMMED MUBARAK Y.)

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

LIST OF ANNEXURES

Annexure No.	Title
1	Media composition used for regeneration of in vitro chickpea plants
2	Composition of Yeast broth Medium (YEB)
3	Murashige and Skoog's media stock solution
4	Amon's nutrient solution
5	Isolation of plant genomic DNA
6	Buffers used for Southern blot
7	Preparation of hybridization buffer
8	Primary wash buffer
9	Secondary wash buffer

ABBREVATIONS

%	Percentage
µg	microgramme
µl	microlitre
M	molar
bp	base pair(s)
kb	kilo base pair
d	Days
ha	hectares
DNase	Deoxyribonuclese
dNTP	deoxynucleotide triphosphate
min	minute(s)
mM	millimolar
wk	week
mRNA	messenger ribonucleic acid
cDNA	complementary deoxyribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
LB	Luria-Bertani Broth
ng	nanogramme(s)
O.D$_{600}$	Optical density at 600 nm
IBA	Indole-3-butryic acid
GA_3	Gibberellic acid
2-iP	2-isopentenyl adenine
TDZ	Thidiazuron
MS medium	Murashige and Skoog medium
Bt	*Bacillus thuringiensis*
CaMV 35S	Califlower Mosaic Virus 35S promoter

Abstract

ABSTRACT

DEVELEOPMENT AND ANALYSIS OF TRANSGENIC CHICKPEA FOR RESISTANCE TO *HELICOVERPA ARMIGERA*

By

MOHAMMED MUBRAK, Y.

Degree : **MASTERS IN PLANT BREEDING AND GENETICS**

Chairman : **Dr. N. Natarajan, Ph. D.,**
Professor and Head
Department of Pulses
Centre for Plant Breeding and Genetics
Tamil Nadu Agricultural University
Coimbatore 641 003, Tamil Nadu, India.

2009

Chickpea (*Cicer arietinum* L.), an important grain legume which is adversely affected by the lepidopteran pest *Helicoverpa armigera* or the legume pod borer which can cause a substantial reduction in grain productivity and crop loss. The use of genetically engineered crops expressing the lepidopteran-specific Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) is an effective method to control this polyphagous pest. A reproducible method of *Agrobacterium*-mediated transformation would help in generating chickpea transgenics with enhanced resistance to insect pests. Axillary meristem explants from the in vitro germinated seedlings of chickpea cultivar C 235 were co-cultivated with *Agrobacterium tumefaciens* harbouring the binary plasmid pPZP200-cry1Acleg under the control of the constitutive 35SCaMV promoter. Tissue culture medium (MS) with 4 µM TDZ, 10 µM 2-iP and 2 µM kinetin induced a maximum of 70 shoots from a single cotyledonary explant after 2 weeks of culturing at an overall frequency of 88.3 %. The induced multiple shoots when cultured on MS medium with

5 μM 2-iP, 2 μM kinetin and 3 μM GA_3 showed elongation in 9% of the shoots from 88.3 % of regenerating explant. Subsequent culturing of the 111 elongated shoots on liquid MS medium with 5 μM IBA resulted in 48 well-established putative T_0 transformants with 4% and 31% regeneration and transformation efficiency, respectively. Molecular analysis of the putative transformants by PCR revealed the presence of the *cry1Acleg* gene in 17 T_0 plants. Southern analysis of the PCR products of the putative transformants confirmed integration of the transgene in the genome. RT-PCR analysis of randomly selected transgenic plants revealed the expression of the functional *cry1Acleg* gene at transcript level. Currently, 48 plants are being advanced to T_1 generation prior to being evaluated in insect bioassays.

Introduction

CHAPTER I

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an annual, self-pollinated legume crop belonging to the family Fabaceae. It is generally cultivated as a rainfed crop in the semi-arid regions with an annual output of 9.3 million metric t on an area of 11 million ha with the average productivity of 797 Kg/ha worldwide. India produces 5.97 million t of chickpea with 782 kg / ha productivity on 7 million ha contributing to about 70 % of the world's production (FAO, 2007), thus occupying the position of 2nd most significant grain legume in terms of cultivable area and production (Agri stat, 2004; Kumar and Kumar, 2005; FAO, 2007). A major portion of India's total production is contributed by desi type (80 %) and slightest by kabuli type (20 %) (Muehlbauer and Singh, 1987; Malhotra et al., 1987).

The production of chickpea has been reduced considerably for the past 2 to 3 decades mostly due to biotic and abiotic stress factors. On the global basis, annual yield loss due to these factors was estimated to be 11.2 million t, wherein 4.8 million t is being contributed by biotic factors alone (Ryan, 1997, www.icrisat.org). Amongst the biotic factors, the notorious pod borer *Helicoverpa armigera* causes a severe pod damage of up to 90% accounting for about 10-33 % per cent yield failure, resulting in annual losses of over US $325 million (ICRISAT, 1992; Yadav et al., 2006). Often, the extent of losses caused by this pest has led to the total failure of the crop (Jayaraj, 1990). This serious threat has been ascribed to frequent and fast changes occurring in cropping pattern of agroecosystem and the polyphagous and cosmopolitan feeding nature of *H. armigera*.

So far, the use of insecticides has been the major approach for controlling this pest in different crops (John et al., 2000). Despite such a high proportion of pesticide usage, the problem is ever increasing, since the pest has acquired resistance to almost all kinds of insecticides to varying degrees (Mehrotra, 1990). This has necessitated the use

of target-specific compounds with low persistence, and an increased emphasis on integrated pest management. But these strategies have not offered higher level of returns as the pesticides. Several breeding approaches have been made to evolve resistant chickpea lines through wide hybridization and host plant resistance. Although, the levels of resistance in the available germplasm have been found to be low to moderate (Lateef and Sachan, 1990; Sharma et al., 2001) and the problem with the conventional breeding involving wild species is that, most of the *Helicoverpa* resistant lines are highly susceptible to wilt and blight and also the resistance is broken in the due course by some other race of the same pest (Clement et al., 1993). This has again necessitated a look at additional technologies to provide adequate crop protection for sustainable food and feed production in future. Currently, biotechnology and genetic engineering of crop plants for insect resistance represents an attractive opportunity to reduce the insect damage and thereby minimize the use of chemical pesticides (Kumar and Sharma, 1994).

Over the past one-decade, spectacular successes have been achieved in developing insect-resistant plants, which culminated in commercial release of transgenic crops in 1996. Global area of transgenic crops has increased 40 fold from 1.7 million hectares in 1996 to nearly 1100 million hectares in 2005 (James, 2003). Transgenic plants with genes encoding for toxin proteins from the bacterium *Bacillus thuringiensis (Bt)* have been found to be quite efficient in reducing insect damage (Sharma and Ortiz, 2000). A variety of genes encoding for different classes of insecticidal proteins such as protease inhibitors (Hilder et al., 1987), lectins (Boulter et al., 1990), amylase inhibitors (Ignachimuthu and Prakash, 2006), chitinase (Ding et al., 1998) and δ-endotoxins (Indurker et al., 2007), of *Bt* are being tested for insect control. Amongst these, the insecticidal crystal proteins of *Bt* assumed significance due to their potency, insect specificity, and lack of toxicity against mammals and other organisms. The gene had

been introduced in several important crops like cotton, potato and maize with a fine level of expression (Peferoen, 1997).

In the ongoing efforts, several workers have attempted to develop transgenic chickpeas with different genes by utilizing different genetic transformation techniques. Due to a lack of proper evaluation to measure the inherent resistance offered to the target pests in subsequent generations when compared with non-transgenic chickpea a gap has been so far existed to deploy the transgenic chickpea for commercial purpose. In this regard, ICRISAT has involved in standardizing various screening techniques to identify the host plant resistance to insects, and develop protocols for in vitro regeneration, and molecular characterization of transgenic chickpea. A reliable plant regeneration and transformation protocol is a prerequisite for efficient application of the genetic transformation strategies to generate transgenic chickpea habouring insect resistance. A rapid, reproducible and efficient regeneration method was reported earlier for chickpea using single cotyledon with half embryonal axis as explants (Jayanand et al., 2003; Anwar et al., 2008; Sharma et al., 2007) which will offer a resourceful way for in vitro exploitation of chickpea.

The prokaryotic origin *cry1Ac* gene was optimized for the GC content to maximize the codon usage for its expression in a eukaryotic system (legume plants) by, eliminating the termination signals (AATAAA or any continuous 5 bases with only A/T) and modifying a fragment of 500 bp size between 200 and 700 bp, thus forming a modified *cry1Acleg* gene, which was used for plant transformation studies. Thus, to find appropriate solutions for reducing the yield loss due to *Helicoverpa armigera* and to enhance the efficiency of generating transgenic chickpea for insect resistance in the semi-arid ecosystems, this research was carried out with the following objectives:

1. Generation of transgenic events of chickpea expressing the modified *Bt* *cry1Acleg* gene through *Agrobacterium*-mediated genetic transformation.
2. Molecular analysis of the putative transgenic chickpea plants expressing the insecticidal gene.

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

2.1. Chickpea

Chickpea (*Cicer arietinum* L.) is an important grain legume, which has worldwide acceptance as a major source of protein for human as well as animal consumption. It plays a significant role in the nutrition of the rural and urban poor in the developing world. It is a good source of carbohydrate (48.2-67.6 %), protein (12.4-31.5 %), fat (6 %) and nutritionally important minerals (Geervani and Umadevi, 1989). Among the legumes it is the best hypocholesteremic agent, followed by black gram and green gram (Soni et al., 1982).

2.2. Limitations on chickpea production

The global production of chickpea is around 9.3 million metric t annually, covering and area of 11 million ha with the productivity of around 782 kg/ha. Despite significant gains occurred in world pulse production during the past two decades with an annual growth rate of 1.9% (Sharma et al., 2006), Chickpea production has been stagnated due to the susceptibility nature of the crop towards various biotic and abiotic stresses. Among these the yield loss due to insect damage is estimated to be around 10 to 33 % annually (Yadav *et al.*, 2006) which is caused by varoius insects like pod borer (*Helicoverpa armigera*), cutworms *(Agrotis* sp.), lesser armyworms *(Spodoptera exigua),* groundnut aphid (*Aphis craccivora*), pea aphid (*Acyrthsosiphon pisum*), cowpea bean seed beetle (*Callosobruchus maculatus*), and adzuki bean seed beetle (*Callosobruchus chinensis*).

2.2.1. Severity of *Helicoverpa armigera* damage on chickpea

Helicoverpa armigera or the legume pod borer is one of the most important insect pests in the world due to its mobility, high polyphagy, short generation duration, and high

reproductive rate (Filt, 1989; Sharma, 2005). The infestation starts on chickpea usually a fortnight after germination and becomes serious just after the initiation of flower bud coupled with cloudy and humid weather. The young larvae feeds on all green parts and defoliate young chickpea crops, where as the large larvae cut round holes in the pod wall and devour the seed inside. The yield loss due to this pest alone accounts for 21 % of the total damage by the insects (Kambrekar et al., 2003).

Currently, the application of chemical spray is the most common method of controlling this pest in chickpea (Shanower et al., 1998; Sharma et al., 2007). Inspite of these chemical sprays having environmental concerns that creates human health problems (Pray et al., 2002; Qaim et al., 2008), the pod borer also have developed resistance to almost all the insecticides used for its control (Forrester et al., 1993; Kranthi et al., 2002). These problems have paved a way to inbuilt resistance in chickpea against this pest. This can be achieved by two different approaches. The first possible way is through the conventional breeding approach through selection and hybridization of resistant lines from the gene pool and the next approach is the production of genetically modified chickpea, expressing genes for insect resistance.

2.3. Approaches for generating resistance to insects in chickpea

2.3.1. Breeding approaches

Genetic improvement of chickpea has continued since domestication of the crop. However the major advances through breeding process appear to be confined to recent times as systematic research works started only in 1966, when the All India Coordinated pulse improvement project (AICPIP) was initiated. The opportunities were created to infuse new variability in breeding programmes through hybridization and exchange, which started giving rich dividend in terms of new varieties of wider adaptability coupled with resistance to key stresses. In addition, screening of more than 14,800 germplasm accessions against the pod borer at ICRISAT has resulted in

identification of one accession, ICC 506, with fairly tolerance to pod borer (Chopra, 2001). As the screening of cultivated genotypes has not identified any inherent resistance so far (Sharma and Ortiz, 2000), breeders are turning to wild annual *Cicer* species as a possible source of desired traits. Unfortunately, inter-specific hybridization has been largely unsuccessful (Ahmad et al., 1988) because the wild species have not responded well to introgression through conventional breeding techniques for yield improvement (Van Rheenen et al., 1993).

2.3.2. Genetic engineering for crop improvement

The ability to isolate and manipulate single genes through recombinant DNA technology (Watson et al., 1987), together with the ability to insert specific genes into a chosen variety (Chilton, 1983), has opened a new era to overcome the problems that are encountered on crop production. Significant progress has been made over the past two decades in introducing foreign genes into plants, and this has provided opportunities to modify crops to increase yield, impart resistance to biotic and abiotc stresses and improve nutritional quality (Sharma et al., 2002). A variety of genes encoding for different classes of insecticidal proteins such as protease inhibitors (Hilder et al., 1987), lectins (Boulter et al., 1990), amylase inhibitors (Ignachimuthu and Prakash, 2006), chitinase (Ding et al., 1998) and δ-endotoxins (Indurker et al., 2007), of *Bacillus thuringiensis* (*Bt*) are being tested for insect control. Among these, transgenic plants with genes encoding for toxin proteins from *Bt* have been found to be quite efficient in reducing insect damage (Sharma and Ortiz, 2000).

2.3.2.1. *cry* genes from Bacillus thuringiensis showing resistance against Helicoverpa armigera

Bt is a gram positive, aerobic and an endospore-forming bacterium recognized by its parasporal body (known as crystal synthesized during sporulation) that is proteinaceous in nature and possesses insecticidal properties. Hydrophobic bonds and

disulphide bridges tightly pack these insecticidal proteins. The most common shape is a bi-pyramidal structure. *B. thuringiensis* was discovered from diseased silkworm (*Bombyx mori*) larvae by Ishiwata, (1901). It was re-isolated, in a diseased Mediterranean flour moth population (Berliner, 1915) and designated as *Bt*. Further research by Steinhaus, (1951) on *Bt* led to renewed interest in using it as a biopesticide, as a result of which, more potent products such as Sporeine, Thuricide and Dipel were introduced. The use of conventional *Bt* insecticides, however, was found to have limitations like narrow specificity, short shelf life, low potency, lack of systemic activity, and the presence of viable spores (Lambert and Pferoen, 1992). Some of these problems have now been overcome by various approaches that utilize the tools of molecular biology and genetic engineering.

Bt strains can be characterized by a number of techniques including serotyping, crystal serology, crystal morphology, protein profiles, peptide mapping and insecticidal activity. The most useful scheme of classification of Bt toxins is based primarily on analyzing the homology of toxin gene sequences and their spectrum of insecticidal activity. Hofte and Whiteley, (1989) have classified 42 *Bt* genes into 14 distinct types and grouped them into four major classes. Those are, *cryI* (specific to lepidopteran), *cryII* (specific to lepidopteran and diptera), *cryIII* (specific to coleoptera) and *cryIV* (specific to diptera). Feitelson, (1992) added two new major classes, *cryV* (specific to lepidoptera and coleopteran) and *cryVI* groups. Crickmore et al. (1998) have introduced a systematic nomenclature for classifying the *cry* genes and their protein products. Most *cry* genes retain the name as signed by Hofte and Whiteley, (1989) with a substitution of Arabic for Roman numerals (eg; *cry1Aa)* to accommodate the newly discovered genes. So far more than 150 Cry toxins have been cloned and tested for their toxicity on various insect species.

Bt-based genes are usually plasmid borne (Gonzalez et al., 1981, Gonzalez and Carlton, 1984) and also chromosomally located (Cralson and Kolsto, 1993). The genes encoding the δ -endotoxins of *Bacillus thuringiensis* are located on plasmids ranging in size from 11.3 kb to 1944 kb (Ward and Ellar, 1983; Gonzalez and Carton, 1984; Hoflack et al., 1997; Rolle et al., 2005). Bt toxin protein has three domains (Chona and Kalpan, 1990; Convents et al., 1990), the domain I is required for toxicity (Chen et al., 1995) and domain II is important for specificity (Dean et al., 1996; Smedley and Ellar, 1996) and domain III, near the carboxyl end, was not defined, is speculated that it may have a role in the processing of protoxin and channel forming function (Wolfersberger et al., 1996; Schwartz et al., 1997). However, experiments involving reciprocal exchange of domain segments between toxins has produced evidences suggesting that in a number of cases, domain III may also be a determinant of insect specificity/receptor binding (deMaagd et al., 1996).

The major benefits of using the Bt-toxin genes for the genetic transformation of crops include economic, environmental friendly and qualitative aspects. In addition to the reduced input by the farmer, the transgenic plant provides an effective control of boring insects that are difficult to reach with sprays at all the stages of insect development.

2.3.2.2. Mode of action of Cry protein

The mode of action of Cry proteins has been reviewed by Schnepf et al. (1998) and is summarized in the following stages: 1) ingestion of sporulated *Bt* with insecticidal crystal protein (ICP) by an insect larva, 2) solubilisation of the crystalline ICP in the midgut, 3) activation of ICP by midgut proteases, 4) binding of the activated ICP to specific receptors in the midgut cell membranes and, 5) insertion of the toxin in the cell membrane and formation of pores and channels in the insect gut cell membrane, followed by destruction of the epithelial cells (Cooksey, 1971; Norris, 1971). The main conditions that lead to activation of *Bt* protein in the insect midgut are as follows:

2.3.2.2.1. pH of the insect midgut

For most of the *cry* toxins, the midgut pH must be strongly alkaline (pH >9.5) for dissolution of the crystals (Bradley et al., 1995), while some of the coleopteran-specific toxins function at a much lower pH (Koller et al., 1992; Bauer, 1995). Rate and extent of crystal solubilisation influence the toxicity levels in different hosts, and pH may influence the effectiveness and specificity of some toxins (Bradley et al., 1995). The lepidopteran and dipteran midguts are highly alkaline, whereas the coleopteran midgets are neutral to acidic. It ha been postulated that expression of a truncated (pre-solubilised) form of *Bt* gene in transgenic plants removes the need for the initial gut barrier of solubilisation and, therefore, may imply a higher risk of toxicity in both target and non-target organisms (Hilbeck, 2002; Stotzky, 2002).

2.3.2.2.2. Mid-gut structure

Cry toxins pass through the peritrophic membrane and bind reversibly to receptors on the brush border membrane of the midgut cells. Final, irreversible binding is linked to insertion of part of the toxin (domain I) into the midgut membrane (Jurat-Fuentes and Adang, 2001). There is a positive correlation between the toxin activity and ability to bind brush boarder membrane vesicles (BBMV) (Gill et al., 1992), and the toxicity is correlated with the receptor number rather than receptor affinity (Van Rie et al., 1989). A large portion of the molecule (domain I) inserts into the membrane, forming low selective ion channels (Knowels and Dow, 1993; Luo et al., 1999; Miranda et al., 2001). The formation of toxin-induced pores in the columnar cells of the membrane allows rapid fluxes of ions. The pores are K+ selective (Sacchi et al., 1986), permeable to cations (Wolfersberger, 1989), permeable to anions (Hendrickx et al., 1989), or permeable to solutes such as sucrose, irrespective of the charge (Schwartz et al., 1991). Carroll and Ellar, (1993) observed that midgut permeability in the presence of Cry 1Ac was altered for cations, anions, neutral solutes and water. Knowles and Dow, (1993)

suggested that Bt toxins lead to cessation of K+ pump that results in the swelling of columnar cells and osmotic lysis. The disruption of gut integrity leads to death of the insect through starvation or septicaemia. These pores possess both selective (only K+ passes through) and nonselective (Na+ and anions pass through) properties depending on the pH (Schwartz et al., 1993). The lepidopteran insect midgut is alkaline and the pores probably permit K+ leakage. Formation of this cation selective channel destroys the membrane potentials (English and Slatin, 1992), thus resulting in midgut necrosis, degeneration of peritrophic membrane and epithelium and ultimately bacterial septicemia, which occurs after larval death due to toxins (Sneh and Schuster, 1981; Salama and Sharaby, 1985). Channels lead to osmotic swelling, cell lysis, damage to the mid-gut haemocoel barrier and leading ultimately to the death of the host (Federici and Bauer, 1998).

Other factors like feeding stimulants are also known to greatly enhance the performance of *Bt* toxins since the most susceptible insects cease to feed after consumption of *Bt* toxin-containing food (Bauer, 1995).

2.3.2.3. Transgenic plants with Bt crystal protein genes

Although the GM approach to using the cry genes to obtain pest resistance in plants is conceptually simple, it does provide an object lesson in the detailed molecular biology that may be required to achieve high levels of expression of a bacterial gene in a transgenic plant. This goes beyond the obvious requirements of plant promoter and terminator sequences to regulate transcription. The first attempts to express Cry1A and Cry3A proteins under the control of the CaMV 35S or Agrobacterium T-DNA promoters resulted in very low levels of expression in tobacco, tomato and potato plants. It was realized that the prokaryotic gene sequence itself would need to be extensively modified in order to obtain high levels of stable expression. For efficient expression of *Bt* gene, the gene should be first converted from AT-rich (typical of bacteria) to GC-rich (typical of

higher plants). Most changes are made to the third codon thereby minimising changes in the amino acid sequence and increasing the expression of Bt toxin by 10 to 100-fold (Perlak et al., 1991). The design criteria for the synthetic genes has often included sequence changes targeted at potential mRNA instability elements (Perlak et al., 1990, 1991, 1993; Sutton et al., 1992; Adang et al., 1993; va der Salm et al., 1994).

Since the first report on the introduction of *Bt*-derived cry genes into tobacco (Barton et al., 1987) and tomato (Fischoff et al., 1987; Vaeck et al., 1987), there has been a rapid increase in the transformation of other crop plants to achieve resistance against insect pests. At least ten different genes encoding different *Bt* toxins, viz., *cry1Aa, cry1Ab, cry1Ac, cry1Ba, cry1Ca, cry1H, cry2Aa, cry3A, cry6A and cry9C* have been engineered into different crop plants (Schuler et al., 1998). Cotton plants with *cry1Ab* (Benedict et al., 1996), *cry1Ac* and *cry2Ab* (Adamzacky et al., 2001a,b) against *H. armigera* and *H. viriscens*, corn transgenics with *cry1Ab* against *H. zea* (Lynch et al., 1999), *O. nubilalis* (Burkness et al., 2001) and rice plants with *cry1Ab* and *cry1Ac* for résistance to yellow stem borer and stripped stem borer (Cheng et al., 1998), hybrid rice plants with *cry1Ac* and *cry1Ab* together for leaf folder and yellow stem borer (Tu et al., 2000), rice plants with *cry1Ac* for stem borer (Nayak et al., 1997) tobacco plants with *cry1Ab* and *cry1Ac* against *S. exgua, M. sexata* and *H. viriscens* (Van der Salm et al., 1994), soybean with *cry1Ac* and *cry1Ab* independently for bean moth larvae (Parrot et al., 1994; Stewart et al., 1996) and chickpea with *cry1Ac* (Kar et al., 1997, Sanyal et al., 2005) for resistance to *H. armigera* were produced. All these transgenics showed resistance to the respective pests. These results show that *Bt* gene is an efficient insecticidal gene that can be deployed for producing transgenic chickpea plants for pest resistance with the availability of suitable tissue culture amenable protocol.

2.3.2.4. Regeneration and transformation in chickpea

2.3.2.4.1. Chickpea regeneration

Modern biotechnology, including tissue culture, genetic engineering, and genetic transformation techniques, has provided new opportunities to enhance the germplasm of the plants (Sharma and Ortiz, 2000). A reliable shoot regeneration protocol is a prerequisite for efficient application of genetic transformation strategies, Several regeneration protocols involving somatic embryogenesis and shoot organogenesis in chickpea have been reported with varying success (Rao and Chopra, 1987, 1989; Riazuddin et al., 1988; Rao, 1990, 1991; Dineshkumar et al., 1994; Sonia et al., 2002). Considerable work has been done on the induction of somatic embryogenesis from mature (Rao and Chopra, 1989) and immature leaflets (Barna and Wakhlu, 1993), mature

(Suhasini et al., 1994) and immature embryo axes (Sagare et al., 1993), or cell suspension cultures (Prakash et al., 1994). However, the recovery frequency of plants has been very low which has limited genetic transformation studies. Regeneration of shoot buds from various explants has also been reported to produce shoots, either directly (Shri and Davis, 1992; Kar et al., 1996; Sharma and Amla, 1998; Subhadra et al., 1998) or indirectly through a callus phase (Khan and Ghosh, 1984; Prakash et al., 1992; Barna and Wakhlu, 1994). However, to date effective chickpea regeneration has been possible only through the use of explants based on cotyledonary nodes or shoot apices derived from seedling explants (Sonia et al., 2002). In most of the instances, the shoot were formed as a result of proliferation of pre-existing meristems, marking these systems inefficient for transformation studies. Such systems have been used for genetically transforming chickpea (Fontana et al., 1993; Kar et al., 1997; Krishnamurthy et al., 2000) but the success has been very low and often the protocols are not reproducible in different laboratories. A comprehensive protocol for successful transplantation of the in vitro-

produced plants using axillary meristem from the cotyledonary nodes has been reported by Jayanand et al, (2003) with a maximum of 90 % rooting frequency.

2.3.2.4.2. Chickpea transformation for insect resistance

Senthil et al. (2004), reported 5.1 per cent transformation frequency in chickpea. Southern blot analysis and histochemical and leaf painting assays demonstrated integration and expression of the transgenes I the initial transformants, and two generations of progeny. Indraneel et al. (2005), standardized the protocol for *Agrobacterium*-mediated gene transfer in chickpea from cotyledonary nodes explants production of transgenic chickpea plants with *cry1Ac* gene driven by CaMV 35S promoter and *npt*II gene for Kanamhycin resistance. The regeneration and transformation frequency has been recorded as 1.12 %. Shivani et al. (2005) developed the transgenic chickpea by introducing *cry1Ac* gene through particle bombardment method using epicotyl explants. These transgenic plants showed moderate protection and mortality for *Heliothis armigera* and *Spodoptera litura* larvae as compared to control plants with the transformation frequency of 18 per cent.

Sarmah et al. (2006), developed transgenic plants using a *Bt cry1Ac* gene, the progeny did not confer resistance to pod borer. He reconstructed the *Bt* toxin genes (*cry1Ac* and *cry2Aa*) for expression in green tissues (using *Arabidopsis* SSU gene promoter and a tobacco SSU gene terminator) and inserted them into twin binary cassettes for transformation. Western blot analysis of 6 independent T_0 plants confirmed expression of the *cry2Aa* gene in 5 out of 6 plants. These results suggest that genetic engineering of crops is an effective method for the production on pod borer resistant chickpea plants.

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The present study was carried out with the aim of generating transgenic chickpea plants with enhanced resistance to *Helicoverpa armigera* that ultimately could increase the yield potential of the crop. All these studies were conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Patancheru, Andhra Pradesh. Details of materials and methods used in the study are as follows:

3.1. Development of *Helicoverpa armigera* resistant transgenic chickpea plants

3.1.1. *Agrobacterium* strain and plasmid construct used

The genetic transformation in chcikpea was carried out by using the *Agrobacterium* strain C-58 harboring the binary vector pPZP 200 carrying *Bt cry1Acleg* gene (pPZP200-*cry1Ac-leg*) (Figure 1) driven by a dual *CaMV35S* promoter and *Nos* terminator without any reporter gene.

3.1.2. Genetic transformation and regeneration of chickpea

3.1.2.1. Plant material

A widely grown chickpea cultivar C 235 (desi type) obtained from ICRISAT, India was used because of its good ability for transformation. The methodology for in vitro regeneration of chickpea used in this study was reported earlier from ICRISAT (Jayanad et al., 2003; Sharma et al., 2006).

3.1.2.2. Seed sterilization

Mature seeds were surface-sterilized with 70 % (v/v) ethanol for 1 min followed by 0.1% (w/v) mercuric chloride ($HgCl_2$) treatment for 10 min on a rotary shaker at 150 rpm. Thereafter, the seeds ware rinsed 5 times with sterile distilled water to remove the residues of $HgCl_2$ prior to overnight soaking. The soaked seeds were again washed with

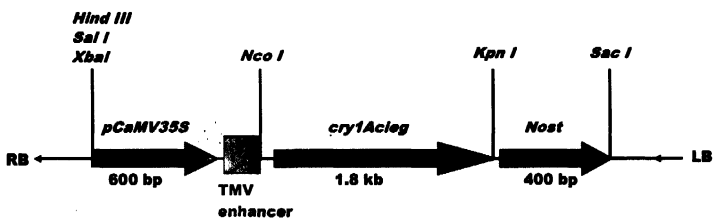

Figure 1. Linear map of the binary plasmid pPZP200 carrying the *cry1Acleg* gene used for genetic transformation of chickpea var. C235 by *Agrobacterium*-mediated transformation.

distilled water, removed the seed coat and kept for germination the Shoot Induction Medium containing MS salts (Murashige and Skoog, 1962), organics and 3% sucrose (SIM; Annexure 1).

3.1.2.3. Preparation of *Agrobacterium* inoculum harboring pPZP 200-*cry1Acleg*

A single colony of *Agrobacterium tumefaciens* was inoculated into 25 ml of YEB medium (Annexure 2) containing 100 µM streptomycin sulphate and incubated at 28 °C on a shaker at 100 rpm for 16-18 h. The culture at late log phase (when the absorbance at A_{600} at 0.6) was used for co-cultivation. Five ml of the culture was centrifuged at 5,000 rpm for 10 min and the pellet was dissolved in 20 ml of half strength MS liquid medium (Annexure 3). This suspension was stored at 4°C for 2 h and used for co-cultivation.

3.1.2.4. Explant preparation

Axillary meristems (AM) from the cotyledonary nodes of the in vitro germinated seedlings were excised and used as explants for genetic transformation and regeneration. The de-coated chickpea seeds were germinated on SIM and the seedlings were allowed to grow for 5-7 d until the axillary buds had grown significantly. The axillary meristem explant (AME) was prepared by removing the axillary bud from the cotyledonary node. The hypocotyle and epicotyle regions were also removed by giving two cuts at the base of the buds thus resulting in two axillary meristem explants (AME) per seedling. The explants were then cultured on SIM for shoot bud induction through direct shoot organogenesis for 7-9 d until multiple shoots were produced. The tips of growing shoots meristems were excised and used as explants for plant transformation.

3.1.2.5. Co-cultivation with *Agrobacterium* harboring pPZP 200-*cry1Acleg*

The AME was dipped into the *Agrobacterium* culture harboring pPZP 200-*cry1Acleg* for 1 to 2 s and cultured on plain MS medium (with no growth regulators). After 48 h, the infected explants were sub-cultured on hormone-free MS medium

containing 250 mg/l cefotaxime to terminate the growth of the *Agr* *...terium* cells. Repetitive sub-culturing on MS medium containing 250-mg/l cefotaxi... was continued for 8-10 d until the growth of *Agrobacterium* cells were absolutely t...minated and clusters of multiple shoot buds started emerging.

3.1.2.6. Elongation of multiple shoots

The multiple shoots that were initiated on MS medium con... ...ng 250 mg/l cefotaxime were sub-cultured on the shoot elongation media (SEM I; M... ...5 µM 2ip + + 2 µM kinetin) (Annexure I) for 10-11 d or till the shoots attains the hei...ht of 3-4 cm. Multiple shoot clusters were placed in shoot elongation media II (SEM ...: MS + 3 µM GA_3) (Annexure I) for further elongation, until they reached a height of ... cm.

3.1.2.7. Initiation and proliferation of roots

Optimization of rooting from the elongated shoots occurred in ... phases, *viz.*, phase 1, phase 2 and phase 3. Dark green and healthy shoots that were ...bout 5 cm or more in length were cultured in culture tubes (25X200 mm) containing filter paper bridges immersed in liquid root induction medium (RIM; MS + 5 µ... IBA) for the induction of adventitious roots in phase 1. The shoots devoid of roots ...phase 1 were carried to phase 2 where the shoot length should be at least a minimum of ... cm. Such shoots were briefly dipped in filter-sterilized solution of 100 mM IB... and placed on filter paper bridges in culture tubes containing hormone free liquid MS ...RIM). Shoots, which did not root even after two or three subcultures on RIM, we... carried to the hydroponic system that was generally used for hardening during th... ...nsplantation process. Quarter-strength Arnon's solution (Annexure 4) was filled in a ... ...n Magenta-jar and the shoots were suspended with support such that 1 cm of the s...oot base was immersed in the solution that contained 3 µM IBA. The medium was ch...ged every 4-5 d until the root primordia appeared. The rooted shoots were transferred ... the hormone-free Arnon's solution for further growth and hardening. Subsequent... ...fter sufficient

root growth, the plants were transferred to 20 cm diameter pots contain g potting mix, comprising sand and black farm soil (3:2) along with 10 % organic matter

3.1.3. Hardening and acclimatization of regenerated chickpea plants

Well-rooted regenerated plantlets were removed gently from the magenta jars and the roots were washed under tap water to remove the media attached to the roots. The roots were then dipped in the diluted Thiram (0.3 %) solution and transferred to 8 cm diameter pots containing autoclaved coarse sand and black soil (3:2) mixed with 10% organic matter for hardening. The plantlets were covered with polythene bags to minimize the loss of water through transpiration and for maintaining high humidity conditions. These pots were placed at 26 °C temperature at a relative humidity of 40% in a growth chamber for 8-10 d. The plants were carefully transferred to 20 cm diameter pots filled with sterilized sand and black soil mix (3:2) with 10% organic matter and transferred to the contained greenhouse to expose completely to the natural environment under controlled conditions. After their transfer to the greenhouse, small holes were made on the sides of the polythene bag. After 3 d, top portion of the polythene bag was removed and eventually after 7 to 8 d the whole bag was removed. This helped the plant to harden and withstands the sudden change in the atmosphere. These plants were allowed to grow till maturity in glasshouse.

3.1.4. Molecular characterization of stable chickpea transformants with *cry1Acleg* gene

Molecular analysis of putative *cry1Acleg* chickpea transgenics was carried out to determine the integration of transgene and to evaluate the transgene expression.

3.1.4.1. Isolation of plasmid DNA

Single colony of bacteria harbouring the recombinant plasmid pPZP200 *cry1Acleg* was taken from the plate and inoculated in 5 ml LB broth (10 g/l tryptone, 10

g/L NaCl, 5 g/L yeast extract, pH 7.2) containing spectinomycin sulphate (100 mg/L) and grown overnight at 37 °C on a shaker (200 rpm). The plasmid DNA was isolated by Wizard® Plus Miniprep DNA Purification Systems (Catalog # A1700; Promega, Madison, USA). The solution was centrifuged for 10 min at 14000 rpm, the supernatant was transferred to fresh tubes to which 3 to 5 µl of RNase (10 mg/ml) was added, to remove RNA, and incubated at 37 °C for 30 min. Equal volumes of phenol: chloroform (1:1) were added, mixed thoroughly and centrifuged at 12,000 rpm for 15 min to remove the proteins from the DNA mixture. Further, to the aqueous phase, equal volume of chloroform was added and the sample was centrifuged at 12,000 rpm for 10 min. The top aqueous phase was collected into fresh tubes to which 0.8 volume of isopropanol was added to precipitate nucleic acids and stored at -20 °C for 30 min. The sample was centrifuged for 10 min at 10,000 rpm at 4 °C, the pellet was washed with 70 % ethanol, air-dried and the plasmid DNA pellet was finally dissolved in 50 µl of 1 X TE.

3.1.4.2. Isolation, purification and quantification of plant DNA

3.1.4.2.1. CTAB-based extraction method (Porebski et al., 1997)

Genomic DNA was isolated from chickpea leaves of glasshouse grown putative transformants. Leaf tissue (500 mg) was ground in liquid nitrogen with a mortar and pestle. To the powdered tissue, 8 ml of CTAB extraction buffer (Annexure 5) was added and transferred to 30 ml polypropylene tubes. The contents were mixed well by inverting the tubes, 3 to 5 times and incubated at 65 °C for 45 min. To the incubated mixture, 8 ml of chloroform: octanol solution (24:1 ratio) was added and centrifuged at 6,000 rpm for 20 min. The supernatant was collected and the above step was repeated. To the supernatant, ½ the volume of 5 M NaCl and double the volume of 95 % ethanol was added to precipitate the DNA. Thus precipitated DNA was incubated at -20 °C for 20 min. The DNA pellet was collected by centrifuging at 10,000 rpm for 10 min. The pellet was washed with ice-cold 70 % ethanol, air-dried and dissolved in 500 µl of 1X TE

(Annexure 7). To this pellet, 10 µl of RNase (10 mg/ml) was added to degrade RNA by incubating at 37 °C. After 30 min, 3 µl of proteinase K was added to degrade the protein and incubated at 37 °C in water bath for 30 min. Later equal volumes of phenol:chloroform (1:1) was added to the DNA solution. The vial consisting of the DNA solution was inverted slowly for three to four times and centrifuged for 10 to 15 min at 14,000 rpm. To the clear supernatant 0.1 volume of 3 M sodium acetate and equal volume of 100 % ethanol were added and incubated at -80 °C. Samples were centrifuged at 14,000 rpm and the pellet was washed in 70 % ethanol, air dried and then dissolved in 1X TE.

3.1.4.2.2. Purification and quantification of genomic DNA

To 800 µl of isolated DNA, 1 ml of DEAE-cellulose suspension was added and mixed gently for 3 min to keep the DEAE-cellulose suspended, thereby maximizing its interaction with DNA. Centrifuge for 30 s at 3,000 rpm to sediment the DEAE-cellulose to which the DNA has been bound. The supernatant was carefully removed and the DEAE-cellulose was resuspended in 1-2 ml of wash medium to eliminate proteins, polysaccharides and secondary metabolites that are not bound to DEAE-cellulose. The suspension was centrifuged for 30 s at 2,627 g and the supernatant was removed. This washing step was repeated at least once followed by the addition of 1 ml of elution medium to the DEAE-cellulose pellet and mixed gently to elute the DNA. A brief centrifugation followed by the collection of supernatant was done prior to the addition of 300 µl of elution medium to DEAE-cellulose, mixed, centrifuged and pooled the supernatants. Isopropanol (0.8 volumes) was added to the supernatant, mixed and centrifuged at 7,100 g for 10 min at room temperature. The pellet was washed with 70% ethanol and the pellet was air-drie before dissolving in 100 µl of 1X TE buffer. The purified DNA was quantified at A260/A280 by using UV spectroscopy.

3.1.4.3. PCR (Polymerase Chain Reaction) analysis of putative chickpea transgenics

PCR analysis of the genomic DNA isolated from the putative transgenics was carried out to amplify a 1.8 kb *cry1Acleg* gene fragment by using primers 5'AACCDCCATGGATGGACAACAACCCAA3' and 5'AACCGGTACCTTCAGCCTCGAGTGTTGC3'. The genomic DNA from the untransformed plants was used as negative control while the plasmid DNA of pPZP *200 cry1Acleg)* from *Agrobacterium* was used as positive control. PCR reaction was performed with 25 µl of total reaction mixture, containing 200 ng of genomic DNA, 2.5 µl of 10 X PCR buffer (10X PCR buffer: 200 mM Tris HCl, 500 mM KCl), 1 µl of 50mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 10 µM of each primer and 0.25 µl of 1.25 units of Taq DNA polymerase (Invitrogen). The total volume was made up to 25 µl with sterile distilled water. Amplification reactions were carried out by using eppendorf gradient thermal cycler under the following conditions: Initial denaturation at 95 °C for 5 min, Denaturation at 95 °C for 1 min, Annealing at 60 °C for 1 min, Extension at 72 °C for 2 min and final Extension at 72°C for 10 min for 38 cycles. PCR products were separated by electrophoresis on 0.8 % agarose gels containing 0.5 µg ethidium bromide, for two hours at 50 V using 1X TAE as running buffer. The resolved bands were visualized using a UV transilluminator.

3.1.4.4. Nucleic acid blotting and detection of PCR amplicons (Southern blotting)

To verify the fidelity of the PCR amplicons, the PCR fragments resolved on the agarose gels (0.8%) were transferred to Hybond+nylon membranes and hybridized at 55 ^{0}C overnight with with *cry1Acleg* fragment labeled with the non-radioactive Alk Phos direct system ^{TM} (Amersham).

3.1.4.4.1. Transfer of DNA onto a positively charged nylon membrane

The agarose gel, resolving the PCR products was washed with MilliQ water,

incubated in depurination solution for 15-20 min and rinsed with ste[illegible] MilliQ water twice. The resolved DNA in the gel was denatured by incubating i[illegible] solution (Annexure 6) for 30-45 min and washed with MilliQ water for 10 min tw[illegible]. Finally the gel was neutralized in the neutralization buffer (Annexure 6) for 15 min [illegible] DNA on the gel was transferred to positively charged Nylon membrane (Bio-Dyne, Pal[illegible] Systems) by capillary transfer in 10x SSC buffer (Annexure 6) method as d[illegible]ribed by Sambrook *et al.* (1989). After 20 h transfer, the membrane was rinsed in 2x [illegible]ri[illegible] and UV crosslinked in an automated UV crosslinker (Stratgene). Membra[illegible] [illegible]alibrated by incubating the in a pre-hybridization solution (Annexure 7) for 2 h [illegible]ridization bottles at 55 °C and hybridized with non-radioactive DIG-labeled probe

3.1.4.4.2. Probe labelling and hybridization

The probe was prepared with non-radioactive labeling (An[illegible] labeling and detection system, Amersham) by excising the PCR amplified 1.8 [illegible] fragment of *cry1Acleg* from 0.8 % agarose gel and purified using Biogene gel extra[illegible] kit. About 16 µl of gel-eluted DNA (comprising 100-200 ng) was boiled in water [illegible] for 10 min, snap chilled in ice and mixed with 4 µl of DIG-High prime mix (Gmb[illegible] ling DIG high prime kit, ROCHE Diagnostics). The reaction mix was in[illegible] 7 °C for overnight. The reaction was boiled for 10 min in a boiling water-b[illegible] filled in ice and directly used for hybridization. The denatured probe was [illegible] 4 ml of hybridization buffer and used for hybridizing the pre-hybridized m[illegible] for 20 h at 55 °C.

3.1.4.4.3. Post Hybridization stringency washes

After overnight hybridization of the probe and membrane, they [illegible] subjected to stringency washes with primary wash buffer and secondary wash buffer (Annexure 8 and 9). The primary wash was done at 55 °C where the blot was transferred into a tray and washed for two times for 10 min each. After primary wash the m[illegible]mbra[illegible] was washed

thrice with secondary wash buffer at room temperature for 5 min.

3. 1.4.4.4. Signal generation and detection

For signal detection through non-radioactive method, CDP-sta[illegible] (Amersham Biosciences, UK) was used which is a chemi-luminiscent detection substrate utilizing the probe bound alkaline phosphatase protein. The alkaline phosphatase enzyme reacts with the added substrate, CDP-Star[IM] and emits photons in the form of signal that can be identified on an X-ray film. The blot is placed on the glass plate where the CDP*(Alkphos) substrate was spread. After 5 min of reaction with substrate, the membrane was wrapped in the saran wrap and fixed in the X-ray cassette. This X-ray film was exposed in the dark room for 30 min. For signal detection the X-ray film was removed from the cassette and placed for 60 to 120 sec in a tray containing the X-ray Kodak GBX developer. Later the film was rinsed with water for 2 sec. The film then fixed with Kodak GBX fixer and then incubated. After 60 to 120 sec the film was rinsed with water for 2 min followed by air drying.

3.1.4.5. RNA extraction and RT-PCR

Total RNA was extracted from leaves of transgenic and non-transgenic by the TRIzol protocol, according to the directions of the manufacturer (Ambion Inc. USA). RNA was quantified by UV spectrophotometry at 260 and 280 nm (A260 /A280 = 1.0 = A260 = 40 µg RNA/ml) and quality was assessed by electrophoresis in 1.5 % non-denaturing agarose gels stained with ethidium bromide (Sigma Chemical Company®). DNA was removed from total RNA extracts by treatment with RNase-free DNase I (Ambion Inc. USA). The gene-specific amplification of *cry1Aclcg* by reverse-transcription polymerase chain reaction (RT-PCR) was performed from the total RNA extracted from transformed and non-transformed plants using two-step RT-PCR kit (Protoscrip®, BioLabs Inc.). The cDNA

was synthesized by using ~1 µg of poly $(A)^+$ RNA primed with 1 µl 50 µM Oligo $dT_{23}VN$ and 2 µl 10 mM of dNTP mix at 55 °C. The full length gene specific primer was used to amplify the complete *cry1Aclegl* gene fragment from the synthezied cDNA through PCR as described in 3.1.4.3.

Experimental Results

CHAPTER IV

EXPERIMENTAL RESULTS

The present study was undertaken with an aim of generating transgenic chickpea by introducing the *Bt cry1Acleg* gene into cultivar, C-235 for developing resistant to pod borer *Helicoverpa armigera*. The results of the experiments are detailed in this chapter.

4.1. Development of transgenic chickpea with *cry1Acleg* gene

4.1.1. Genetic transformation and regeneration of chickpea cultivar C-235 by using axillary meristem explants (AME) derived from *in vitro*-germinated seedlings

Transgenic chickpea plants were developed through *Agrobacterium*-mediated gene transformation method. The AME explants were co-cultivated with *Agrobacterium* strain C 58 harboring the binary plasmid pPZP200-*cry1Acleg*. The recovery of complete plants was obtained through direct *in vitro* somatic organogenesis pathway.

The de-coated seeds of chickpea cultivar C 235 were cultured on shoot induction media (SIM), containing 4 µM TDZ, 10 µM 2iP and 2 µM kinetin. The mean percentage of germination response for this cultivar, in 8 batches was recorded after 6-8 days as 95 (Table1; Plate 1).

4.1.1.1. Induction of multiple shoots from axillary meristem explants

Explants consisting of single cotyledon with half embryonal axis obtained from 6 day old seedlings on SIM, was found to be good for producing optimal shoot/adventitious buds. Embryonal axis attached to cotyledon showed significant swelling and exhibited initiation of shoot induction within duration of 6-8 d. Regeneration of multiple shoots from all over the surface of the swollen embryonal axis was observed by 11-15 d. The morphogenic responses of the cultured explants were recorded at the end of the second week. About 88.3 % of cotyledonary explants produced

Table 1. Germination response of chickpea cultivar C 235 cultured in vitro on shoot induction medium.

Batches	Mean percentage of germination
1	90.0
2	93.3
3	100.0
4	93.3
5	93.3
6	93.3
7	100.0
8	100.0
Average germination	**95**

Table 2. Multiple shoot regeneration and elongation from the axillary meristem explants of chcikpea*.

Batches	No. of explants cultured	No. of explants showing multiple shoot induction	**No. of shoots induced per explant	Percentage explants with multiple shoots	**No. of shoots elongating per explant*
1	21	13	30	61.9	0.6
2	21	14	35	66.0	1.3
3	21	19	31	90.5	2.1
4	21	20	52	95.2	1.2
5	21	20	57	95.2	3.1
6	21	21	70	100.0	2.6
7	21	21	41	100.0	0.8
8	21	21	40	100.0	2.2
Overall Mean				**88.3**	

*Results were recorded at the end of 2 wk for shoot regeneration and at the end of 5 wk for shoot elongation.

**The values are mean of three replications.

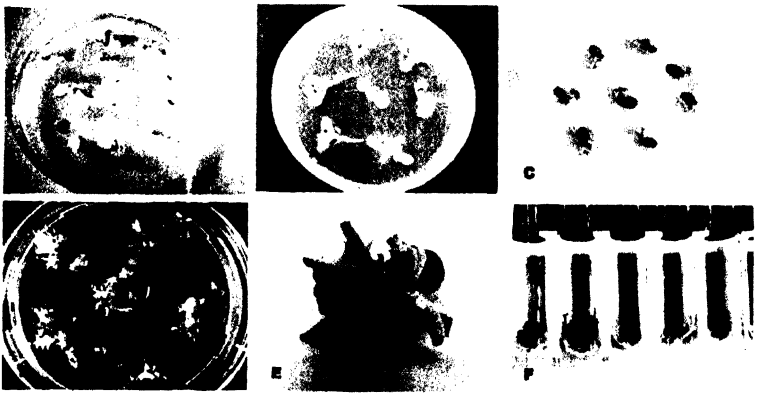

Plate 1. Regeneration of multiple shoots from co-cultivated axillary meristem explant of chickpea var. C235 with *Agrobacterium tumefaciens* strain C58 harbouring the binary plasmid pPZP200-*cry1Acleg*.

A) De-coated seeds after 14 h of imbibition; B) In vitro germinated chickpea seedlings after 6 d of culturing on SIM; C) Swollen embroynal axis attached to the cotyledon explant; D) 2-wk-old culture of embryonal axis with single cotyledon showing the emergence of a multiple adventitious shoot buds; E) Axillary meristem explant infected with *A. tumefaciens* after 15 d of cocultivation; F) Putatively transformed shoots after 7 d on SEM I.

multiple shoots with 50-70 shoots arising from each explant within a time period of 15 d (Table 2; Plate 1).

4.1.1.2. Co-cultivation of axillary meristem explants with *Agrobacterium* harboring pPZP 200-*cry1Ac-leg*

Axillary meristem explants were infected with *Agrobacterium* harboring pPZP 200-*cry1Acleg* and co-cultivated on hormone free MS medium. After 48 h of co-cultivation, the explants were subcultured on hormone free MS medium containing 250-mg/l cefotaxim that eliminates the growth of *Agrobacterium* cells.

4.1.1.3. Elongation of regenerated shoots

The induced multiple shoots/adventitious buds were excised from the bunch after 12-15 days and cultured on the shoot elongation media (SEM I) consisting of MS medium supplemented with 5 µM iP and 2 µM kinetin. Since the elongation frequency of the induced multiple shoots was brought down by prolonged culture of the explants on the TDZ-containing medium, 12-15 d old explants were transferred to SEM I which was devoid of TDZ. Elongation of 0.1-3 shoots per explant, in the initial stages was obtained (Table 2). The development of adventious multiple shoots to a height of 2 to 3 cm were obtained after 10-12 d in SEM I. Explants that were not elongated was regularly sub-cultured at an interval of 10-15 d on SEM I until it reached upto 2-3cm. After 2 wk the elongated shoots were cultured on another elongation medium (SEM II) containing GA_3 until shoots developed to a height of 5-6 cm (Plate 2).

4.1.1.4. Development of strong root system

Elongated shoots showed the differentiation and development of roots on root induction media (RIM, MS + 5 µM IBA). The roots developed in the first week were relatively weak and requires over a period of 3 wk for further development. Elongated shoots that were unsuccessful to produce adventitious roots in RIM were subjected to a

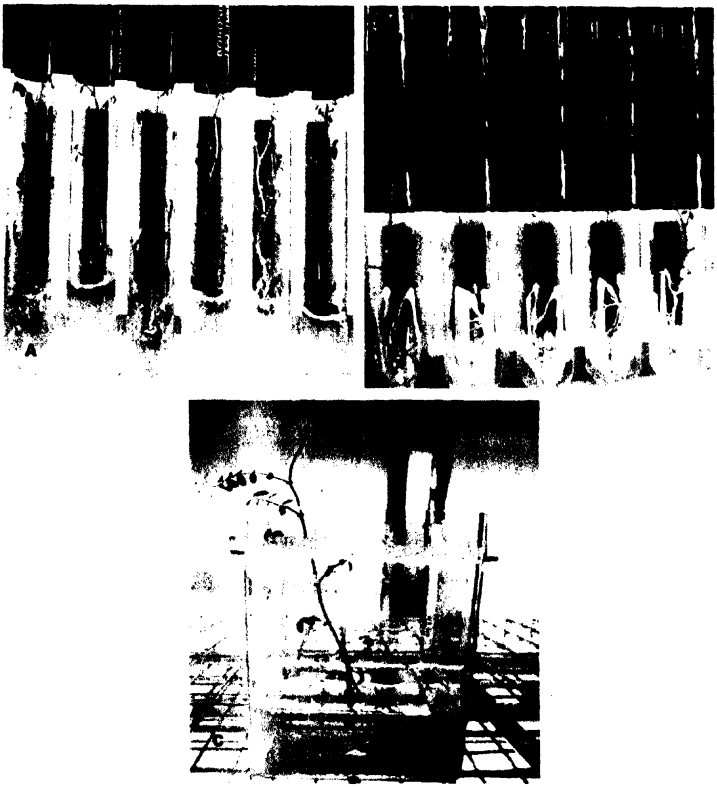

Plate 2. Elongation and rooting of the in vitro transformed and regenerated shoots of chickpea var. C235 plants following transformation with *cry1Acleg gene.*

A) Multiple shoots on SEM II after 15 d; B) Rooting of elongated shoots on filter paper bridges in liquid medium containing 3 μM IBA after 10 d; C) Hardening of the rooted plant in static culture containing 1/4-strength Arnon's nutrient solution.

10 sec pulse treatment with 100 mM IBA. This led to root induction within 4 d and subsequently resulted in the development of strong root system within 10-12 d in liquid MS basal medium (Plate 2). Necrosis of the entire shoots occurred when they were subjected to prolonged exposure (>5-10 min) to IBA.

Root initiation was observed within 4-7 d in about 20 % of the elongated shoots. By the end of 10 d another 30 % shoots generated roots. The remaining 10 % developed roots in the third week. The lateral roots appeared within 2-3 wk. About 50 -60 % of elongated shoots transferred to the hydrophonic system (1/4 th Arnon solution + 3 µM IBA) for root induction illustrated well-developed adventitious root establishment of 5 % elongated shoots (Table 3, Palte 2).

4.1.1.5. Establishment of in vitro raised chickpea plantlets in glasshouse

Plantlets with shoot length of 5-6 cm survived well when transferred to pots containing autoclaved coarse sand and black soil (3:2) mixed with 10 % organic matter for hardening in glasshouse. The transplanted plants exhibited recovery and normal growth in 60–65 d to produce morphologically normal flowers and pods that contains viable seeds (Plate 3). A total of 48 (1-48) putative transgenic plants (4 % regeneration efficiency) were generated in the present study (Table 3). These putative transformants were further molecular characterized to study the integration and expression of the introduced *cry1Acleg* gene in the chickpea genome.

4.2. Molecular characterization of transgenic chickpea plants for *cry1Acleg* gene

4.2.1. Detection of *cry1Acleg* gene in genome of independently transformed T_0 transgenic lines

The presence and integration of *cry1Acleg* gene in the putative T_0 transgenic lines was ascertained by PCR amplification using gene specific primers and Southern hybridization respectively. PCR amplification results obtained showed that 17

Table 3. Frequencies of shoot regeneration and transformation of transgenic plants of chickpea var. C 235 with *cry1Acleg* gene.

S. No.	Stages of regeneration	No.	Percent compared at various stages (Sl. No. 1-4) of regeneration / transformation			
			1	2	3	4
1	Axillary meristem explants used for co-cultivation	1195				
2	Elongated shoots	111	9			
3	Rooted shoots	65	5	59		
4	Putative transformants	48	4	43	74	
5	PCR positive events	17	1	15	26	35

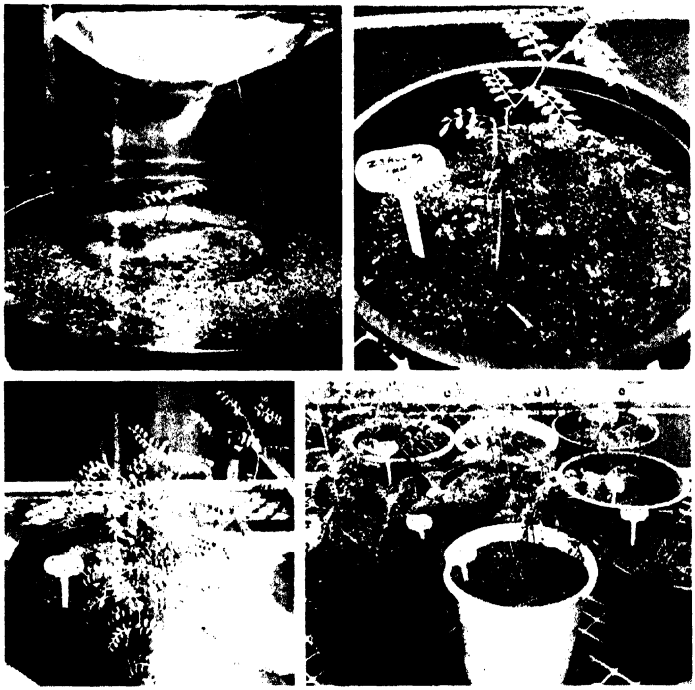

Plate 3. Hardening and establishment of the putative chickpea transformants harbouring the *cry1Acleg* gene.

A) and B) Plantlets transferred to the pots and covered with polythene bags initially for maintaining the humidity; C) and D) in vitro transformed chickpea plants after 2 months in the containment greenhouse showing flowering and pod set.

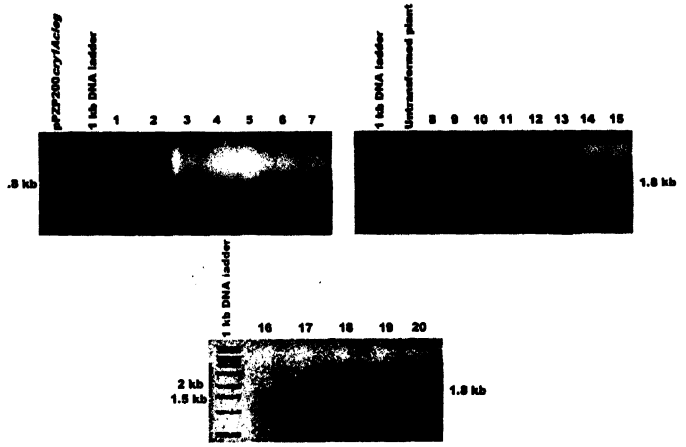

Plate 4a. PCR amplification of the putative chickpea transformants in T_0 generation containing the 1.8 kb *cry1Acleg* gene fragment.

50 ng of DNA was extracted from the leaves of the transgenic events. (1-20) – Following amplification, the PCR products were resolved on 0.8 % agarose gel along with 1 kb DNA ladder. Amplification from the untransformed in vitro generated chickpea plant and plasmid pPZP200- *cry1Acleg* was used as controls for identifying the *cry1Acleg* gene integration in transformed plants.

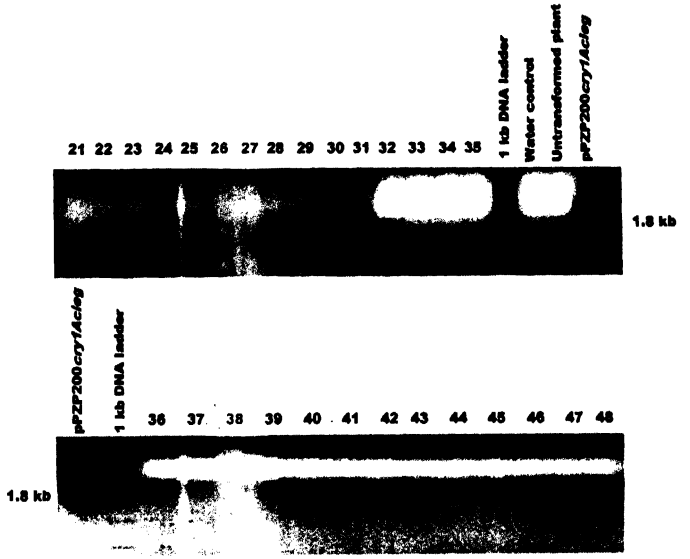

Plate 4b. PCR amplification of the putative chickpea transformants in T_0 generation containing the 1.8 kb *cry1Acleg* gene fragment.

50 ng of DNA was extracted from the leaves of the transgenic events (21-48) - the PCR products were resolved on 0.8 % agarose gel along with 1 kb DNA ladder and water control. Amplification from the untransformed in vitro generated chickpea plant and plasmid pPZP200- *cry1Acleg* was used as controls for identifying the *cry1Acleg* gene integration in transformed plants. The products stained with EtBr and visualized by UV-illuminator.

(3, 4, 5, 7, 13, 16, 18, 22, 24, 25, 26, 30, 31, 32, 39, 41 and 47) out of 48 putative T_0 transformed plants produced an amplified fragment of 1.8 kb, the expected size of PCR product, with *cry1Acleg* specific primers. As expected, no amplification was observed when genomic DNA from untransformed control plants was used as template (Plate 4a; 4b). A summary of the transformation experiments and molecular characterization of regenerated plants is presented in Table 3.

To study the stable integration of transgene in the chromosome that was obtained through independent transformation events, the experimental results of few representative transformed plants that showed amplification for 1.8 kb *cry1Acleg* gene (13, 16, 18, 22, 24, 25 and 26) were analyzed further by Southern blot hybridization. Southern blotting was performed on the PCR product that was resolved, blotted and probed with non-radioactive Alkphos Direct®–labelled 1.8 kb PCR amplified *cry1Acleg* DNA fragment. Southern hybridization results showed identical pattern of hybridization signals except for 22, thus confirming the integration of transgene in the plant genome (Plate 5). Variations in the intensity of hybridization bands were also observed. Based on these observations, the transformation frequency was 31.25 % in the present investigation. The 17 T_0 plants harbouring the *cry1Acleg* transgene were fertile and set normal seed.

4.2.2. RT-PCR analyses of *cry1Acleg* gene in T_0 transgenic chickpea lines

Expression of introduced gene at transcript level was analyzed through RT-PCR from randomly selected PCR positive T_0 plants (16, 18, 22, 24, 25, 26 and 30). The expected 1.8 kb amplification band corresponding to the *cry1Acleg* gene was detected (Plate 6) on 16, 26 and 30.

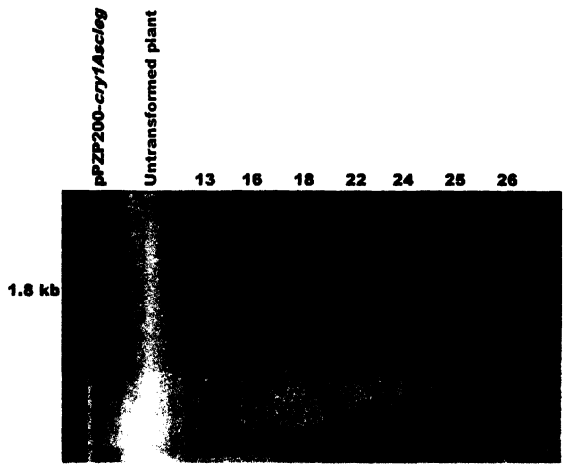

Plate 5. Southern blot hybridization of the *cry1Acleg* gene in PCR products of putative chickpea transformants in the T_0 generation.

The blot was probed with a 1.8 kb PCR-amplified *cry1Acleg* gene fragment. (13, 16, 18, 22, 24, 25, 26) - PCR product of the positive transformants. Amplification from untransformed plant and plasmid pPZP200-*cry1Acleg* was used as controls for identifying the *cry1Acleg* gene integration in transformed plants.

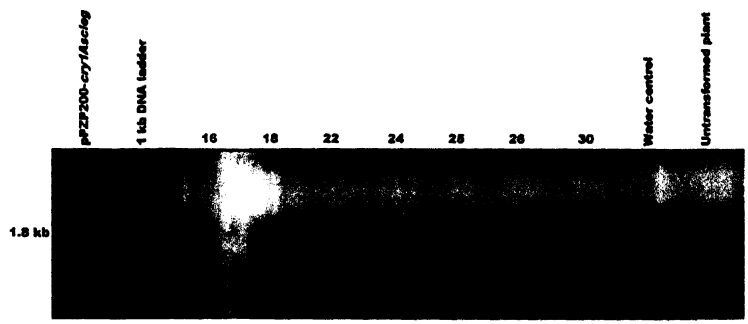

Plate 6. RT-PCR amplification of the 1.8 kb *cry1Acleg* gene fragment by using the gene-specific primers from chickpea transformants in the T_0 generation.

0.5 µg of the total RNA was extracted from leaves of the transformed plants. (16, 18, 22, 24, 25, 30) – the RT-PCR products were resolved on 0.8 % agarose gel along with 1 kb DNA ladder and water control, stained with EtBr and visualized by UV-illuminator. Amplification from untransformed *in vitro* generated chickpea plant and plasmid pPZP200- *cry1Acleg* was used as controls for identifying the expression of *cry1Acleg* gene in transformed plants.

Discussion

CHAPTER V

DISCUSSION

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is one of the vital insect pests due to its mobility, high polyphagy, short generation duration, and high reproductive rate (Fitt, 1989; Sharma, 2005). Currently, the application of chemical spray insecticides is the most common method of controlling this pest on crops, including cotton (King, 1994; Durairay et al., 2005) and chickpea (Shanower et al., 1998; Sharma et al., 2007). This pest is known to develop resistance to almost all the insecticides used for its control (Forrester et al., 1993; Kranthi et al., 2002). These chemical sprays are also of environmental concern and are responsible for human health problems (Pray et al., 2002; Qaim et al., 2008). Thus, alternative control methods are increasingly being employed. The use of genetically engineered crops (GECs) that express insecticidal genes such as those derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) provide a powerful option to control the Lepidopteran pests (Shelton et al., 2002). This technology, for example, is applied to protect cotton plants from damage by the bollworm by expressing *B. thuringiensis cry1Ac* gene. Thus, the expression of *cry* genes is an option to protect a crop like chickpea from damage by this notorious pest (Romeis et al., 2004). Chickpea plants that express either Cry1Ac or Cry2Aa, or both proteins are currently under development and could become commercially available in the future (Sanyal et al., 2005; McPhee et al., 2007).

Developments in molecular genetics of chickpea for the expression of genes for crop improvement require efficient genetic transformation methods. Hence, in the present study, the development of a simple, rapid and a high frequency transformation system in chickpea *through Agrobacterium*-mediated transformation has been shown, to benefit further prospects in this area of research. The development of high frequency plant regeneration protocol using a de novo re-generable source such as axillary meristem

explant from a suitable genotype like C235 which is susceptible to *H. armigera* (Patil et al., 2007) but is highly amenable to in vitro regeneration (Jayanand et al., 2003; Sharma et al., 2007) as employed in the present study, will be helpful in genetic modification of this important legume.

Regeneration of shoots from explants in chickpea can be accomplished indirectly via a callus phase leading to development of somatic embryos (Khan and Ghosh, 1984; Prakash et al., 1992; Barna and Wakhlu, 1994). However, the recovery of mature somatic embryos is significantly low which makes it an unproductive system for any genetic transformation studies (Jayanand et al., 2003). Hence, the direct method of regeneration through pre-formed meristems has been valuable in the development of chickpea transgenic plants. Several reports are available on the proliferation of multiple shoots from pre-existing meristems in the cotyledonary nodes, shoot tips and epicotyls (Shri and Davis, 1992; Kar et al., 1996; Subhadra et al., 1988). However, the recovery of a reasonable frequency of transformation from the shoots emanating from the pre-formed meristems is very low (Jayanand et al., 2003). At ICRISAT, it has been observed that the axillary meristems present in the axils of the in vitro germinated seedlings of chickpea play a significant role in initiation of adventitious shoot bud through induced morphogenesis from the target cells (Sharma et al., 1991: Jayanand et al., 2003; Anwar et al., 2008). Hence, in the present study, the axillary meristem explant was used for generating in vitro chickpea plants for genetic transformation of *cry1Acleg* gene.

Explants consisting of single cotyledon with half embryonal axis obtained from in vitro germinated seedlings on MS medium supplemented with 4 μM TDZ, 2 μM kinetin and 10 μM 2-iP was found to be good for producing multiple shoots/adventitious buds. The cytokinin, TDZ (Thidiazuron) has been shown to have high potential for shoot induction in chickpea (Malik and Saxena, 1992; Barna and Wakhlu, 1993; Huetteman and Preece, 1993; Murthy et al., 1996; Rizvi and Singh, 2000; Jayanand et al., 2003;

Senthil et al., 2004; Tewari- Singh et al., 2004; Kumar et al., 2005) by regulating the endogenous growth regulators (Malik and Saxena, 1992). Jayanand et al. (2003) reported the use of other cytokinins such as 2-iP and kinetin with an optimal concentration of 4 μM TDZ to support rapid shoot multiplication in chickpea. In the present investigation, these growth regulators were also found to improve the frequency of multiple shoot initiation which was as high as 50 to 60%. As gibberellins (GA_3) promotes the elongation of shoots (Jayanand et al., 2003), the regenerated shoots were transferred to shoot elongation medium containing 3 μM GA_3 which showed on an average 3 shoots elongating per explant.

Numerous reports are available on the use of grafting for rooting of transgenic plants since root development was considered as the foremost obstacle on in vitro regeneration of chickpea (Krishnamurthy et al., 2000, Sarmah et al., 2004, Senthil et al., 2004, Sanyal et al., 2005, Chakraborti et al., 2006). While the technique enclosed considerable deviations on success rates on establishment of plants in soil (Dinesh kumar et al., 1994; Polisetty et al., 1996) and time consuming, a high frequency rooting of about 50-60 % was obtained in this study when the elongated shoots were immersed on paper bridges in liquid MS medium with 9.4 mM KNO_3, 2 % sucrose and 5 μM IBA for 2 weeks, which was consistent with the earlier report of Jayanand et al. (2003). About 65 plantlets with strong root system were transferred to the hardening phase within 10d of the root primordial being observed. A total of 48 putative transgenic plants (4% regeneration efficiency) were generated in the present study.

PCR analysis of the putative T_0 transformed plants showed that 17 out of 48 produced the expected size of 1.8 kb *cry1Acleg* gene amplified fragment. The experimental results of few representative transformed plants showed the integration and expression of *cry1Acleg* gene when analyzed by Southern blotting and RT-PCR analysis respectively.

The present study reports a high frequency *Agrobacterium*-mediated transformation in chickpea using axillary meristem explants. This will help in advancement of molecular genetics of chickpea in expressing genes for crop improvement in future.

Summary

CHAPTER VI

SUMMARY

The present study was undertaken with an aim of generating chickpea trangenics expressing *cry1Acleg* gene providing scope for improving resistance to *Helicoverpa armigera*. Summary of the key findings are as follows:

1. Transgenic chickpea plants were developed through *Agrobacterium*-mediated transformation method.
2. The de-coated seeds of chickpea cultivar C 235 were cultured on shoot induction media (SIM) containing 4 μM TDZ, 10 μM 2-iP and 2 μM kinetin. The mean germination response of this cultivar was 95%.
3. About 88.3 % of the cotyledonary explants produced multiple shoots with 50-70 shoots arising from each explant.
4. The axillary meristem explants containing multiple shoots were co-cultivated with *Agrobacterium tumefaciens* strain C 58 harbouring the binary plasmid pPZP200-*cry1Acleg*.
5. The transformed multiple shoots cultured on shoot elongation media (SEM) containing 5 μM 2-iP, 2 μM kinetin and 3 μM GA_3 showed about 3 elongated shoots per explant.
6. About 60 % of elongated shoots showed the differentiation and development of roots on root induction media (RIM) containing 5 μM IBA.
7. A total of 48 putative transgenic plants (4% regeneration efficiency) were generated.
8. The transformation frequency was recorded as 31.25 % through PCR analysis showing the presence of *cry1Acleg* gene in 17 putative T_0 transgenic events. Southern blotting of the randomly selected PCR products and RT-PCR analysis revealed the integration and expression of *cry1Acleg* gene in the genome, respectively.

References

REFERENCES

*Ahmad, G., A.E. Slinkard and G.J. Scoles. 1988. Investigations into the barrier(s) to site-specific hybridization between *Cicer arietinum* L. and eight other annual *Cicer* species. **Plant Breed., 100:**193-198.

Anwar, F., P. Sharmila and P. Pardha Saradhi. 2008. An optimal protocol for in vitro regeneration, efficient rooting and stable transplantation of chickpea. **Physiol. Mol. Biol. Plants, 14:** 329-335.

Barna, K.S. and A.K. Wakhlu. 1993. Somatic embryogenesis and plant regeneration from callus cultures of chickpea (*Cicer arietinum L.*). **Plant Cell Rep., 12:** 521- 524.

Barna, K.S. and A.K. Wakhlu. 1994. Whole plant regeneration from *Cicer arietinum* from callus cultures *via* organogenesis. **Plant Cell Rep., 13:** 510-513.

Chakraborthi, D., A. Sarkar and S. Das. 2006. Efficient and rapid in vitro plant regeneration system for Indian cultivars of chickpea (*Cicer arietinum* L.). **Plant Cell Tiss. Org. Cult., 86:** 117-123.

*Chilton, M.D., M.H, Drummon, D.J, Merlo, D. Sciaky, A.L. Montoya, M.P. Gordon and E.W. Nester. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. **Cell, 11:** 263-271.

Chopra, V.L. 2001. **Chickpea In:** Breeing field crops. Oxford and IBH publishing Co. pvt. Ltd. New Delhi. pp. 305-334.

Clement, S.C., N. El-Din, S. El-Din, S. Weigand and S.S. Lateef. 1993. Research achievements in plant resistancce to insect pest of cool season food

legumes. **Euphytica, 70:** 41-50.

Dineshkumar, V., P.B. Dirti, J.K.S. Sachan and V.L. Chopra. 1994. Plant regeneration *via* somatic embryogenesis in chickpea (*Cicer arietinum* L). **Plant cell Rep., 13:** 468-472.

Ding, X., B. Hopalakrishnan, L.B. Johnson, F.F. White, X. Wang, T.D. Morgan, K.J. Kramer and S. Muthukrishnan. 1998. Insect resistance of transgenic tobacco expressing an insect chitinase gene. **Transgenic Res., 7:** 77-84.

Durairay, C., G.V. Subbaratnam, T.V.K. Singh and T.G. Shanower. 2005. ***Helicoverpa* in India: spatial and temporal dynamics and managment options In:** *Heliothis/Helicoverpa* management: emerging trends and strategies for future research. H.C. Sharma (ed.), IBH Publishing Co., New Delhi, India, pp. 91-117.

Fitt, G.P. 1989. The ecology of *Heliothis* species in relation to agroecosystems. **Annu. Rev. Entomol., 34:**17-52.

Food and Agriculture Organization, (FAO). (2007) Food Outlook.

Forrester, N.W., M. Cahill, L. Bird and J.K. Layland. 1993. Management of pyrethoid and endosulfan resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. **Bull. Entomol. Res. Suppl. Ser., 1:** 1-132.

Geervani, P. and T. Umadevi. 1989. Effect of maturation of nutrient composition of selected vegetable legumes. **J. Sci. Food Agric., 46:** 243–248.

Gill, S.S., E.A. Cowels and P.V. Pietrantonio. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. **Annu. Rev. Entomol., 37:** 615-636.

*Hilder, V.A., A.M.R. Gatehouse, S.E. Sheerman, R.F. Barker and D. Boulter.

1987. A novel mechanism of insect resistnce engineered into tobacco. **Nature, 300:** 160-163.

Huetteman, C.A. and J.E. Preece. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. **Plant Cell Tiss. Org. Cult., 33:** 105-119.

ICRISAT 1992. The medium term plan. International Crops Research Institute for the Semi-Arid Tropics. Patancheru, Andhrapradesh, India.

Ignacimuthu, S. and S. Prakash. 2006. *Agrobacterium*-mediated transformation of chickpea with α-amylase inhibitor gene for insect resistance. **J. Biosci., 31:** 339–345.

Indurker, S., H.S. Misra and S. Eapen. 2007. Genetic transformation of chickpea (*Cicer arietinum* L.) with insecticidal crystal protein gene using particle gun bombardment. **Plant Cell Rep., 26:** 755-763.

James, C. 2002. Global review of commercialized transgenic crops: 2002. International service for the Acquisition of Agri-biotech Applications, Ithaca, New York. http://www.isaaa.org/publications/briefs_27.htm.

Jayanand, B., G. Sudarsanam and K.K. Sharma. 2003. An efficient protocol for the regeneration of whole plants of chickpea (*Cicer arietinum* L.) by using axillary meristem explants derived from in vitro-germinated seedlings. **In Vitro Cell. Dev. Biol. Plant, 39:** 171–179

Gatehouse, J.A. and A.M.R Gatehouse. 2000. **Genetic Engineering of plants for insect resistance In:** Biological and Biotechnological control of insect pests. CRC Press Lewis, pp. 211-241.

Kar, S., T.M. Johnson, P. Nayak and S.K. Sen. 1996. Efficient transgenic plant regeneration through *Agrobacterium* mediated transformation of Chickpea (*Cicer arietinum*. L). **Plant Cell Rep., 16:** 32-37.

Khan, S.K. and P.D. Ghose. 1984. Plantlet regeneration from cotyledon nodes of chickpea. **Int. Chickpea Newsl., 11:** 22-24.

King, A.B.S. 1994. ***Heliothis/Helicoverpa* (Lepidoptera: Noctuidae) In:** Insect pests of cotton. G.A. Matthews and J.P. Tunstall (ed.), CAB International, Wallingford, Oxon, United Kingdom, pp. 39-106.

Kranthi, K.R., D.R. Jadhav, S. Kranthi, R.R. Wanjari, S.S. Ali, and D.A. Russell. 2002. Insecticide resistance in five major insect pests of cotton in India. **Crop Prot., 21**:449-460.

Krishnamurthy, K.V., K. Suhasini, A.P. Sagare, M. Meixner, A. de Kathen, T. Pickardt, O. Schieder and A. Kathen de. 2000. *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) embryo axes. **Plant Cell Rep., 19:** 235-240.

Kumar, P.A. and R.P. Sharam. 1994. Genetic engineering of insect resistant crop plants with *Bacillus thuringiensis* crystal protein genes. **J. Plant Biochem. Biotechnol., 3:** 3-5.

Lateef, S.S. and J.J. Schan. 1990. **Host plant resistance to *H. armigera* (Hub.) in different agro- ecological conditions pp 181-190 In:** Chickpea in nineties. Proceedings of 2nd National work shop on Chickpea 4-8 Desi 1989. ICRISAT Patancheru India.

Malhotra, R.S., R.P.S. Pundir and A.E. Slinkard. 1987. **Genetic resources of chickpea In:** M.C. Saxena and K.B. Singh (ed.), The Chickpea. C.A.B. International Cambrian News Ltd, Aberystwyth, UK, pp. 67-81.

Malik, K.A and P.K. Saxena. 1992. Thidiazuron induces high frequency shoots regeneration in intact seedlings of pea (*Pisum sativum*), Chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*). **Aust. J. Plant Physiol., 19:**

731-740.

Mehrotra, K.N. 1990. Pyrithroid resistance in insect pest management India experience. **Pesticide Res. J., 2:** 44-45.

Muehlbauer, F.J. and K.B. Singh. 1987. **Genetics of chickpea In:** The Chickpea. M.C. Saxena and K.B. Singh (eds.), CAB International, Wallingford, Oxon, OX10 8DE, UK. pp. 99-125.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. **Physiol. Plant., 15:** 473-497.

Murthy, B.N.S., J. Victor, R.P. Singh, R.A. Fletcher and P.K. Saxena. 1996. *In vitro* regeneration of chickpea (*Cicer arietinum* L.): stimulation of direct organogenesis and somatic embryogenesis by thidiazuron. **Plant Growth Regul., 19:** 233-240.

Peferoen, M. 1997. Progress and prospects for field use of *Bt* genes in crops. **Trends Biotechnol., 15:** 173-177.

Polisetty, R., P. Patil, J.J. Deveshwar, S. Khetarpal and R. Chandra. 1996. Rooting and establishment of in vitro tip explants of chickpea (*Cicer arietinum* L.). **Ind. J. Exp. Biol., 34:** 806-809.

Prakash, S., J.B. Chaudhury, R.K. Jain and V.K. Choudhury. 1992. Factors affecting plant regeneration in chickpea, *Cicer arietinum* L. **Ind. J. Exp. Biol., 30:** 1149-1153.

Pray, C.E., J. Huang, R. Hu, and S. Rozelle. 2002. Five years of *Bt* cotton in China-the benefits continue. **Plant J., 31:** 423-430.

Qaim, M., C.E. Pray and D. Zilberman. 2008. **Economic and social considerations in the adoption of *Bt* crops In:** Integration of

insect-resistant genetically modified crops within IPM programs. J. Romeis, A.M. Shelton and G.G. Kennedy (ed.), Springer, Dordrecht, The Netherlands, pp. 329-356.

Rizvi, S.M.H. and R.P. Singh. 2000. *In-vitro* plant regeneration from immature leaflet-derived callus cultures of *Cicer arietinum* L. *via* organogenesis. **Plant Cell Biotechnol. Mol. Biol., 1:** 109-114

Romeis, J., H.C. Sharma, K.K. Sharma, S. Das and B.K. Sarmah. 2004. The potential of transgenic chickpeas for pest control and possible effects on non-target arthropods. **Crop Prot., 23:** 923-938.

Ryan, J.G. 1997. **A global perspective on pigeonpea and chickpea sustainable production systems: present status and future potential In:** Recent advantages in pulses research. Indian Society of Pulses Research and Development, Asthana AN, Ali M (eds) IIPR, Kanpur, pp. 1–31.

Sanyal, I., A.K. Singh, M.A. Kaushik and D.V. Amla. 2005. *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis Cry1Ac* gene for resistance against pod borer insect *Helicoverpa armigera*. **Plant Sci., 168:** 1135-1146.

Sarmah, B.K., A. Moore, W. Tate, L. Molving, R.L. Morton, D.P. Ress, P. Chiaiese, M.J. Chrispeels, L.M. Tabe and T.J.V. Higgins. 2004. Transgenic chickpea seeds expressing high level of a bene α-amylase inhibitor. **Mol. Breed., 14:** 73-82.

Sarmah, B.K., S. Acharjee, A. Moore, J. Carlos and T.J.V Higgins. 2006. **Development of transgenic chickpeas using Twin T-DNA Binary vectors Harbouring *Bt* Endotoxinn Cenes to confer resistance against pod borer *Helicoverpa armigera* In.** Annual Pulse Net work meeting 2-4

February 2006. Indo-Swiss collaboration in Biotechnology. ICRISAT Patancheru Andrapradesh. Abstracts, pp.13.

Senthil, G., B. Williamson, R.D. Dinkins and G. Ramsay. 2004. An efficient transformation system for chickpea (*Cicer arietinum* L.) **Plant cell Rep., 23:** 297-303.

Shanower, T.G., T.G. Kelley and S.E. Cowgill. 1998. **Development of effective and environmentally sound strategies to control *Helicoverpa armigera* in pigeon pea and chickpea production systems In:** Tropical entomology. R.K. saini (ed.), Proceedings of the 3rd International Conference on Tropical Entomology. ICIPE Science press, Nairobi, Kenya. pp. 239-260.

Sharama, H.C., J.H. Crouch, K.K. Sharam, N. Seetharama and C.T. Hash. 2002. Application of biotechnology for crop improvement: prospects and constraints. **Plant Sci., 163:** 381-395.

Sharma, H.C. 2005. *Heliothis/Helicoverpa* management: emerging trends and strategies for future research. IBH Publishing Co., (ed.). New Delhi, India.

Sharma, H.C., C.L.L. Gowda, P.C. Stevenson, T.J. Ridsdill-Smith, S.L. Clement, G.V. Ranga Rao, J. Romeis, M. Miles and M. Bouhssini. 2007. **Host plant resistance and insect pest management in chickpea In:** Chickpea breeding and management. S.S. Yadav, R.R. Redden, W. Chen, and B. Sharma (ed.), CAB International, Wallingford, United Kingdom, pp. 520-537.

Sharma, H.C., K.K. Sharma, N. Seetharama and R. Ortiz. 2001. Genetic transformation of crop plants risks and opportunities for the rural poor. **Curr. Sci., 80:** 1495-1508.

Sharma, K.K., S.S. Bhojwani and T.A. Thorpe. 1991. Role of the cotyledonary tissue in the in vitro differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. **Plant Cell Tiss. Organ Cult., 24:** 55-59.

Sharma, K.K, and R. Ortiz. 2000. Program for the application of genetic transformation for crop improvement in the semi-arid tropics. **In Vitro Cell. Dev. Biol. Plant, 36:** 83–92

Sharma, K.K., P.B. Mathur and B. Jayanand. 2006. **Chickpea (*Cicer arietinum* L.) In:** Methods in Molecular Biology, Human press Inc., Totowa, Nj Ken Wang (ed). pp. 313-324.

Shelton, A.M., J.Z. Zhao and R.T. Roush. 2002. Economic, ecological, food safety, and social consequences of the deployment of *Bt* transgenic plants. **Annu. Rev. Entomol., 47:** 845-881.

Shri, P.V and T.M. Davis. 1992. Zeatin-induced shoot regeneration from immature chickpra (*Cicer arietinum* L.) cotyledons. **Plant Cell Tiss. Org. Cult., 28:** 45-51.

Soni, G.L., M. George and R. Singh. 1982. Role of common Indian pulses as hypocholesterolemic agents. **Indian J. Nutr. Diebet., 19:** 184–189.

Subhadra, R., K. Vashishth, J.B. Chowdhury, M. Singh, P.K. Sareen. 1998. Multiple shoots from cotyledonary node explants of non-nodulating genotype (ICC435M) of chickpea, *Cicer arietinum* L. **Ind. J. Exp. Biol. 36:** 1276-1279.

Tewari-Singh, N., J. Sen, H. Kiesecker, V.S. Reddy, H. J. Jacobsen and S. G. Mukherjee. 2004. Use of herbicide or lysine plus threonine for non-antibotic selection of transgenic chickpea. **Plant Cell Rep., 22:** 576-583.

Van Rheenen, H.A., R.P.S. Pundir and J.H. Miranda. 1993. How to accelerate the genetic improvement of a recalcitrant crop species such as chickpea. **Curr. Sci., 65:** 414-417.

Yadav, S.S., J. Kumar, S.K. Yadav, S. Singh, V.S. Yadav, N.C. Turner and R. Redden. 2006. Evaluation of *Helicoverpa* and drought resistance in desi and kabuli chickpea. **Plant Genet. Res., 4:** 198-203.

* Originals not seen

Annexure

ANNEXURE 1

Media composition used for regeneration of in vitro chickpea plants

Culture media	MS + Hormones (µM /L)					Difco-Bacto agar (%)	pH
	TDZ (µM)	2iP (µM)	Kinetin (µM)	GA_3 (µM)	IBA (µM)		
Shoot induction medium (SIM)	4	10	2	...	...	0.8	5.2
Shoot elongation medium (SEM – 1)	...	5	2	....	....	.08	5.5
Shoot elongation medium (SEM – 2)	...	5	2	3	...	.08	5.5
Root induction medium (RIM)	MS liquid with 9.4 mM KNO_3 + 1.5% sucrose				5 µM	....	6.0
Root proliferation media (RPM)	Amon's nutrient solution				.....	....	6.5

ANNEXURE 2

Composition of Yeast broth Medium (YEB) (per Litre)

Compound	Weight (g)
Bacto peptone	5
Yeast Extract	1
Beaf Extract	5
Sucrose	5
Megnesium sulphate heptahydrate ($MsSO_4 . 7H_2O$)	0.5
Agar	15
pH	7

ANNEXURE 3

MS Medium stock solution (per Liter)

MS Organic salts / L distilled water	**Quantity**
Nicotinic acid	50 mg
Pyridoxine HCl	50 mg
Thiamine HCl	10 mg
Glycine	200 mg
MS Minors / L distilled water	
Potassium Iodide (KI)	83 mg
Boric acid (H_3BO_3)	620 mg
Magnese sulphate ($MnSO_4$)	2.23 mg
Zinc sulphate ($ZnSO_4.7H_2O$)	860 mg
Sodium molybdate ($Na_2MoO_4.2H_2O$)	25 mg
Copper sulphate ($CuSO_4.5H_2O$)	2.5 mg
Cobalt chloride ($CoCl_2.6H_2O$)	2.5 mg
MS inorganic salts / 400 ml distilled water	
Ammonium nitrate (NH_4NO_3)	66 mg
Potassium nitrate (KNO_3)	38 mg
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	14.8 mg
Potassium ortho phosphate (KH_2PO_3)	6.8 mg
Calcium chloride ($CaCl_2$)	17.6 mg
Myoinositol	2.5 g / 250 ml distilled water

Murashige and Skoog's media (MS) (per Liter)

Compound	**Volume**
Ammonium nitrate (NH_4NO_3)	10 ml
Potassium nitrate (KNO_3)	20 ml
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	10 ml
Potassium ortho phosphate (KH_2PO_3)	10 ml
Calcium chloride ($CaCl_2$)	10 ml
MS Minor	10 ml
Fe EDTA	10 ml
MS Organics	10 ml
Myoinositol	10 ml

Sucrose – 30 g
Agar – 0.8 %
pH- 5.8

ANNEXURE 4

Arnon`s nutrient solution (Arnon, 1938)

Stock No.	Compound	Quantity (mg/L)	Stock solution (g/L)
1	Potassium ortho phosphate (KH_2PO_3)	122	12.2
	Potassium chloride (KCL)	155	15.5
	Magnesium sulphatre heptahydrate ($MnSO_4.\ H_2O$)	250	25
2	Calcium chloride ($CaCl_2.\ 2H_2O$) /	215	21.5
	Calcium sulphate ($CaSO_4.\ 2H_2O$)	250	25
3	Manganese sulphate heptahydrate ($MnSO_4.\ H_2O$)	1	1
	Zinc sulphate ($ZnSO_4.\&H_2O$)	0.25	0.25
	Copper sulphate ($CuSO_4.5H_2O$)	0.25	0.25
	Boric acid (H_3BO_3)	0.25	0.25
	Sodium Molybdate ($Na_2MoO_4.2H_2O$)	0.05	0.05
4	Ferric citrate ($FeC_6H_2O_7.5H_2O$) /	30	30
	Ferric Chloride ($FeCl_3$) /	15	15
	(NaFe EDTA)	59	59

ANNEXURE 5

Isolation of plant genomic DNA

Extraction buffer	Concentration of stocks	Stock solutions used (for 100 ml)
Tris	1 M	20 ml
NaCl	5 M	56 ml
EDTA (pH 8.0)	100 Mm	40 ml
CTAB (HexadeCylTrimethyl Ammonium Bromide)	10 %	40 ml
β- mercaptoethanol	0.3%	300 µl
H_2O	-	40 ml

ANNEXURE 6

Buffers used for Southern blot

Buffers	Chemical compounds
Depurination buffer / L	250 mM HCl
Neutralization buffer / L	1.5 M NaCl
	0.5 M Tris
	pH
Denaturation Buffer / L	1.5 M NaCl
	0.5 M NaOH
20 X SSC / L	3M NaCl (Sodium chloride)
	Tri Sodium citrate dihydrate
	pH (HCl)

ANNEXURE 7

Preparation of hybridization buffer

Hybridization buffer (Alkaline phosphatase) – 25 ml

0.5 M NaCl – 0.73125 g

Blocking reagent – 1 g

For best results add the blocking reagent slowly to the buffer solution while stirring. Continue mixing at room temperature for 1-2 hours on a magnetic stirrer or roller mixer. This buffer can be used immediately or stored in suitable aliquots at -15 °C to -30 °C.

Note: Pre-heated buffer (55 °C) to be added to the blots.

ANNEXURE 8

Primary wash buffer

Chemicals	**Quantity (1L)**
2M Urea	120 g
0.1% (w/v) SDS	1 g
0.5 M Na phosphate pH 7.0 50 mM	100 ml
150 mM NaCl	8.7 g
1M $MgCl_2$- 1 mM	1 ml
Blocking reagent 0.2% (w/v)	2 g

- 0.5 M Na Phosphate can be made by using Sodium Dihydrogen Phosphate (monobasic, $NaH_2PO_4.xH_2O$) and adjust the pH to 7.0 with sodium hydroxide.
- The primary wash buffer can be kept for up to 1 week in a refrigerator at 2-8 °C. Avoid reheating.

ANNEXURE 9

Secondary wash buffer – 20x stock

Chemical	Quantity (g/1L)
1M Tris base	121
2M NaCl	112

Adjust pH to 10.0. Make up to 1 L with water. This can be kept for up to 4 months in a refrigerator at 2-8 °C.

Secondary wash buffer- working dilution

Dilute stock 1:20 and add 2 ml/L of 1M $MgCl_2$ to give a final concentration of 2 mM magnesium in the buffer. This buffer should not be stored.