MOLECULAR AND GENETIC ANALYSIS OF PUTATIVE TRANSFORMANTS IN PIGEONPEA

THESIS

Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
in partial fulfilment of the requirements
for the Degree of

MASTER OF SCIENCE
IN
AGRICULTURE
(AGRICULTURAL BIOTECHNOLOGY)

By

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Enrolment Number - NN-3168 **2017**

DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation in the thesis entitled **"MOLECULAR AND GENETIC ANALYSIS OF PUTATIVE TRANSFORMANTS IN PIGEONPEA"** or part thereof has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The source of materials used and all assistance received during the course of investigation have been duly acknowledged.

Place : Akola (Kartiki Dadasaheb Kadam)

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CERTIFICATE

This is to certify that thesis entitled **"MOLECULAR AND GENETIC ANALYSIS OF PUTATIVE TRANSFORMANTS IN PIGEONPEA"** submitted in partial fulfilment of the requirement for the degree of **"Master of Science in Agriculture (Agricultural Biotechnology)"** of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **Kartiki Dadasaheb Kadam** under my guidance and supervision.

The subject of the thesis has been approved by the Student's Advisory Committee.

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ACKNOWLEDGEMENT

Formal and dead words cannot carry the fragrance of emotions with them still they are the only available means of expressing emotions in such formal acknowledgement.

I consider myself to be fortunate for getting a golden opportunity to work under the dynamic and able guidance of Dr. A. A. Akhare, Assistant Professor, Biotechnology Centre, Department of Agriculture Botany, Dr. PDKV, Akola. My Vocabulary fails to express my deep and sincere feeling of gratitude. I take this opportunity to express my deep and sincere feeling gratitude to her for suggesting research topic, valuable guidance, counsel, sympathy, generous encouragement and constructive criticism till the successful completion of this.

I would also like to thank Dr. S. J. Gahukar, Professor and In-Charge Biotechnology Centre, Dr. PDKV, Akola for providing all the facilities required and for serving as co-chairman in my advisory committee and for his kind co-operation to carry out the research work.

My sincere gratitude is due to members of advisory committee Dr. S. J. Gahukar, Associate Professor and In-Charge Biotechnology Centre, Dr. PDKV, Akola Dr. M. P. Moharil, Assistant Professor, Biotechnology Center, Dr. PDKV, Akola, and Dr. R. M. Wadaskar, Department of Entomology, Dr. PDKV, Akola whose encouraging guidance, stimulating discussion and valuable suggestion have led my work.

I am highly obliged to Dr. R. S. Nandanwar, Professor and Head, Department of Agricultural Botany, Dr. PDKV, Akola, for providing necessary facilities during the course of present research work and valuable suggestions throughout the course.

I express my deep sense of gratitude to Dr. V. K. Kharche, Associate Dean, PGI, Dr. PDKV, Akola, for providing all the necessary facilities for degree programme and the present research work.

I express my deep sense of gratitude to Dr. P. V. Jadhav, Assistant Professor, Biotechnology Center, Dr. PDKV, Akola and Dr. D. R. Rathod, Assistant Professor, Biotechnology Center, Dr. PDKV, Akola for their kind support during the tenure.

I am also thankful Shri. R. R. Jirapure, Agriculture Assistant, Dr. Balwant Mundhe, Dr. Dpika Padole, for their valuable suggestion, encouraging guidance, stimulating discussion, constructive ideas and helping me a lot throughout my research. I also acknowledge my gratitude to Mr. Baban Nachone and Miss. Anjali Gawande supporting, helpful and friendly nature during the work.

I find no words to express my sincere thanks to my seniors Deepa Muske, Yashoda Ether, Madhuri Gavande, Shrikant, Anil Chunada, Ritesh Thakre, Dimpal Raut and Komal Shinde, for their meticulous help extended throughout the course of investigation. The word like thanks should not come in between friends, but it comes from the bottom of my heart for Anuja, Anita, Snehal, Manjiri and Pooja. My special thanks to Vishal, Haridas, Satish, Akshay and Manik my classmates, for their constant support, suggestion and most valuable comments, practical tips, good companionship and thanks for helping me during my whole research work.

The words are not worthy to mention the love and affectionate core of my beloved parents Mr. Dadasaheb Kadam, and Mrs. Alka Kadam and my brothers Dhanjay, Sanket and Pravin the “Beacon” of my life. They remain pillars of source and inspiration for me in stepping up educational career and molding of my life.

I am also thank to Mr. Nikhil B. Kathiwale (M/s. Nikhil Grafix, Akola) for timely and skilful typesetting and for neat binding of the manuscript.

I would like to express my sincere thanks to all authors whose literature has been cited in the thesis and other people also helped me directly and indirectly to accomplished this goal.

At the outset I am reminded of the saying “A person who is not grateful to his fellow cannot be grateful to God”. I therefore take this opportunity to express my heartful thanks and gratitude to all those people who have helped immensely in the successful completion of my research work.

Place : Akola (Kartiki Dadasaheb Kadam)

Date : / /2017 Enrollment No. NN-3168

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(C) List of Plates

(D) Abbreviations

%	:	per cent
°C	:	Degree Celsius
µl	:	Micro liter
Bp	:	Base pair
Cm	:	Centimeter
DNA	:	Deoxy ribonucleic acid
Dr. PDKV	:	Dr. Panjabrao Deshmukh Krishi Vidyapeeth
EDTA	:	Ethylene Diaminetetraacetic acid
et al.	:	et alia (And others)
etc.	:	Etcetera
Fig.	:	Figure
G	:	Gram
Ha	:	Hectare
i.e.	:	id est. (that is)
Kb	:	Kilo base
L	:	Litre
N	:	Normal
NaOH	:	Sodium Hydroxide
Ng	:	Nanogram
OD	:	Optical density
PCR	:	Polymerase chain reaction
pH	:	Hydrogen ion concentration
RNA	:	Ribonucleic acid
Rpm	:	Revolution per minute

(F) Thesis Abstract

a)	**Title of the thesis**	**:**	**MOLECULAR AND GENETIC ANALYSIS OF PUTATIVE TRANSFORMANTS IN PIGEONPEA**
b)	**Full name of student**	**:**	**Kartiki Dadasaheb Kadam**
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d)	**Degree to be awarded**	**:**	M.Sc. (Agriculture)
e)	**Year of award of degree**	**:**	2017
f)	**Major subject**	**:**	Agricultural Biotechnology
g)	**Total number of pages in the thesis**	**:**	69
h)	**Number of words in the abstract**	**:**	441
i)	**Signature of the student**	**:**	
j)	**Signature, name and address of forwarding authority**	**:**	

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ABSTRACT

The gram pod borer (*Helicoverpa armigera* Hubner) is the most serious insect pest of pigeonpea It is highly susceptible to the insecticidal proteins of *Bacillusthuringiensis* (*Bt*). A codon-optimized chimeric *Cry1Aabc* gene of *Bt* driven by a constitutive promoter was introduced in pigeonpea (PKV Tara). Molecular analyses were carried out by most of

worker in T_1 and T_2 generation.

From 7 plants of T_3 generation total 57 progenies were tested for PCR from which 47 plants were seen to be positive through PCR analysis. 38 plants of T_1 generation from DK+ material were tested for PCR analysis through gene specific primers from which 22 plants were seen to be positive.

Kamble in year 2014-15 attempted 130 embryo infections with *Agrobacterium* strain EHA 105 harboring *Cry1Aabc* and *NptII* gene out of which 73 were survived but only two plants were confirmed positive through PCR. T_1 and T_2 generation of these two plants namely A and B were sown. Three plants from each were selected for PCR confirmation. From two positive plants total 6 progenies from T_1 generation and 3 plants from T_2 generation were tested for PCR analysis all the 6 plants from T_1 generation were seen to be positive while from T_2 generation out 3 only two plants were positive. Sample population size of T_1 and T_2 plants was too less to study inheritance pattern.

In order to study Mendalian inheritance pattern in T_3 progeny PCR analysis for the amplification of the *cry1Aabc* gene and *NptII* gene was carried out. The plants from same parents plant was consider together for the inheritance study. For undertaking the Chi-square test for segregation the minimum population size of 6 was taken.

Calculated x^2 value is less than table value in all the plant progenies. Thus plants for the amplification of the *Cry1Aabc* gene and *NptII* gene and progeny of each clone shows 3:1 (presence: absence) segregation pattern.

For detached leaf bioassay 19 positive plants and one negative plant which were confirmed by DNA extraction and PCR amplification with *NptII* and *Cry1Aabc* gene specific primers were tested using leaf feeding assay with neonate larvae of *Helicoverpa armigera*. 20 putative transformants of pigeonpea were subjected to insect (*Helicoverpa armigera*) bioassay using neonates of *Helicoverpa armigera* and young trifoliate leaves and WT (non-transformed) PKV Tara was used as control.

Thus the result revealed that the level of Cry protein expressed in positive plants were able to reduce the leaf damage. Thus, it was concluded from above results that like positive plants selected for bioassay study having potential for providing tolerance against *Helicoverpa armigera*. The further confirmation in subsequent generation is required to come to more robust conclusion.

CHAPTER I

INTRODUCTION

1.1 Background information

1.1.1 introduction to pigeonpea

Pigeonpea [*Cajanus cajan*(L.) Mills.], is a short-lived perennial shrub, which is traditionally cultivated as an annual crop in the semi-arid tropics representing the majority of the developing nations. It is an important grain legume richer in proteins (21%) than cereal grains and is a source for a significant amount of dietary protein requirements (Gosh, 2014). Common names of pigeonpea are arhar, red gram, toovar, toor, and rohar, etc. Pigeonpea is member of family Leguminoceae (Fabaceae) under the division – Magnoliophyta, class – Magnoliopsida, order – Fabales and genus – *Cajanus.* It belong to monotypic genus and it is often cross pollinated crop with diploid chromosome no. 2n = 2x = 22.

Pigeonpea (*Cajanus cajan* (L.) Millsp) is protein-rich legumes of the semi-arid tropics grown throughout the tropical and subtropical regions of the world. In India its major area is lying between 14° and 28°N latitude, where the majority of the world's pigeonpea is produced.According to FAO statistics, worldwide pigeonpea was grown in about 4.23 million hectares with a production and productivity of 4.68 million tons and 751 kg/ha, respectively. India contributes in 66% in world pigeonpea production. The other major pigeonpea producing countries are Myanmar (17.09 percent), Malawi (6.15 percent), Kenya (4.36 percent), and United Republic of Tanzania (5.29 percent) as reported by Pal et.al, 2016. In India, it occupies an area of 3.81 million hectares with a production and a productivity of 3.07 million tons and 806 kg/ha, respectively. Pigeonpea is cultivated in more than 25 tropical and subtropical countries either as sole crop or mixed with cereals. Indian subcontinent, Eastern Africa and Central America in order are world's main three pigeonpea producing region. It accounts for about

11.8% of the total pulse area and 17% of total pulse production of the country. Maharashtra contributes in production of pigeonpea about 39.24 percent and the other major pigeonpea producing states are Karnataka (17.57 percent), Andhra Pradesh (10.94 percent), Uttar Pradesh (11.85percent), Madhya Pradesh (10.65 percent) and Gujrat (7.86). Total production of vidharbha region is 485kg/ha and 777kg/ha in year 2015-16 and 2016-17 respectively (General Statistical Information of Agril. Deptt.)

It is a rich source of protein and supplies a major share of the protein requirement of the vegetarian population of the country. It is mainly eaten in the form of split pulse as ‘dal’: Seeds of arhar are also rich in iron, iodine, essential amino acids like lycine, tyrocene, cystine and arginine. The outer covering of its seed together with part of the kernel, provides a valuable feed for milch cattle. The husk of pods and leaves obtained during threshing constitute a valuable cattle feed. Woody parts of the plant are used for fuel. It is a legume crop and, consequently, possesses valuable properties as restorer of nitrogen to the soil.

Red gram or pigeon pea ranks high amongst the grain legume of India, consumed by large population of country. Nutritionally they are two to three times richer in protein than cereal grains (Geetha, 1990). Because of its high protein content, pigeon pea forms a significant component of the diet of vegetarians. Pigeonpea contains approximately 19.2% protein and 57.3% carbohydrate in dried seed (Purseglove, 1988).

It provides excellent fodder and also made into hay and silage. It is also good source of fibers, vitamins and minerals but they are low in essential amino acid (sulphur containing) like metheonine and cystine (Kay, 1979).

Being a common food, pigeonpea is important in nutritional security of the poor, especially in the Indian sub-continent. Pigeonpea is being used as fuel. It is grown for subsistence and surplus is traded both within and outside the country. Despite being an important pulse, the

production of this crop has not undergone any significant improvement in the last three decades.

1.1.2 Helicoverpa armigera threats for pigeonpea

Production and productivity of pigeonpea are constrained by several diseases, including sterility mosaic, *Fusarium* wilt, *Phytophthora* blight, *Alternaria* blight and stem canker (Reddy et al. 1990) and by several pests. Pigeon pea suffers heavy losses in yield due to fungal, bacterial and viral diseases and insect pest like pod borer But one of the major pests attacking on crop is the legume pod borer *Helicoverpa armigera*. Over 150 species of insects damage pigeonpea (Shanower *et al*., 1999), of which the pod borer, *Helicoverpa armigera* (Hubner) is the most important pest. It causes an estimated annual loss of US$ 317 million in the semi-arid tropics (ICRISAT, 1992).

Among the various species of *Helicoverpa* found worldwide, three species namely *Helicoverpa armigera, Helicoverpa assulta* and *Helicoverpa peltigo* have been recorded on most important crops in India. *Helicoverpa armigera* is the most serious pest harboring over 181 plant species belonging to 45 families because of its high mobility, survival rate under adverse condition and capacity of complete several generation in year. *H. armigera* attack cause serious and extensive yield losses has been reported in some legumes from 28-40 ensuring economic loss up to 300 million dollars annually (Arshad Ali *et al.,* 2009).

Helicoverpa armigera has lead to series of consequences like, insecticide resistance, pest resurgence, outbreak of secondary pests, harmful residual effects, imbalances in natural ecosystem and higher production costs, which has been a concern in India and elsewhere. It is therefore necessary to develop more environment friendly approaches with minimum use of chemical pesticides. To overcome these problems, a radical technique such as fusion of protoplasts of wild and cultivated *Cajanus* species could be an attractive proposition to transfer agronomically useful traits such as pest and disease resistance to the cultivated varieties. The use

of molecular markers and the generation of a genetic map would also be desirable for marker assisted selection and the positional cloning of resistance genes in pigeon pea (Rao*et al*., 1992). The control of insect-pest *Helicoverpa armigera* is becoming a best alternative by transfer of insect resistant traits.The lack of an efficient high frequency plant regeneration system has deterred the production of transgenic plants in pigeon pea as it is considered as one of the recalcitrant crops and not amenable to tissue culture.

1.1.3 Transgenic pigeon pea

Many of the classical breeding methods are time consuming and labor-intensive, and their success is constrained by limited variability in the variable germplasm of different. This provided a strong impetus to develop newer technologies, which has provides access to novel genes from different sources. The advance in recombinant DNA technology has made it possible to clone the toxin genes and express in crop plants to confer resistance against insect pests (Bennet, 1994).

The biotechnological approaches such as genetic transformation using appropriate genes and DNA marker assisted selection have potential in mitigating these problems and eventually increasing the yield. Development of transgenics expressing insecticidal proteins in productive cultivars is one of the strategies followed in many crop species including pigeonpea. Use of cry genes from *Bacillus thuringiensis* have been found useful in controlling the pod borer when they are introduced into crop plants (Lisa and Raymond, 2007). *Cry1Ac* gene in BT cotton has phenomenally controlled pod borer pest all over the world including in India.

As pigeon pea is a self-pollinated plant with narrow genetic base, conventional breeding had no conspicuous effects on the genetic improvement. Thus, genetic transformation plays an important role in the incorporation of agronomically convenient characters. The development of transgenic plants is not only limited to the confinements of the laboratory, but also has a wider scope of application.

Any initiative to develop transgenic plants, particularly crop species, should be undertaken with a long-term goal rather than just the analysis of the initial transformants. Unfortunately, in case of pigeon pea, initial reports of transgenic research had no far reaching impacts (Gosh, 2014).Development of transgenics expressing insecticidal proteins in productive cultivars is one of the strategies followed in many crop species including pigeon pea. Use of cry genes from *Bacillus thuringiensis* have been found useful in controlling the pod borer when they are introduced into crop plants (Lisa and Raymond, 2007).

In-planta transformation is a tissue culture independent, quick and,efficient direct transformation system that produced large number of plants in very short time. Floral dip and vacuum infiltration methods are the two main *in-planta* transformation methods that were successfully used to transformed gene of interest in cereal crops, vegetables, oil seeds crops and many other plants. The transgenic plants produced through *inplanta* transformation show high transformation and regeneration frequency and need minimal labors and reagents requirements

1.1.4. Mode of action (*Cry1Aabc* construct gene)

Bacillusthuringiensis produces parasporal, proteinaceous, crystal inclusion bodies during sporulation. Upon ingestion, these are insecticidal to larvae of the order Lepidoptera and to both larvae and adults of a few Coleoptera. Once in the insect body, the crystal proteins are solubilised and the insect gut proteases convert the original pro-toxin into a combination of up to four smaller toxins. These hydrolyzed toxins bind to the insect's midgut cells at high-affinity, specific receptor binding sites where they interfere with the potassium-ion dependent, active amino acid symport mechanism. This disruption causes the formation of large pores that increase the water permeability of the cell membrane. A large uptake of water causes cell swelling and eventual rupture, disintegrating the midgut lining (Sharma *et al.,* 2008). Different toxins bind to different receptors in different insect species and with varying intensities: this explains species specificities. These genes have proved their field usefulness in cotton all

over the world. Numbers of methods are available including micro projectile bombardment electroporation, sonication, chemical method, Agrobacterium mediated transformation. Of this Agrobacterium transformation is more efficient as in results in integration of well defined DNA sequence, High expression of the introduced genes and preferential integration into actively transcribed region. Recently the novel in planta transformation technique was demonstrated in pigeonpea as an alternate to overcome recalcitrancy.

1.1.5. Inheritance study of transgenic

Agrobacterium-mediated transformation usually produces transgenic plants with a low copy number and the transgenes are transmitted to progeny according to Mendelian (Horsch *et al.,* 1984; Budar *et al.,*1986).

Transgenes are inherited sexually as a dominant trait (Christou *et al*., 1989; Misra, 1989; Pawlowski and Somers, 1996; Theuns *et al.*, 2002), with inheritance conforming to a 3:1 Mendelian ratio (Perret*et al.*, 2003; Yong *et al.*, 2006; Shrawat *et al.*, 2007).Many factors influence transgene expression and inheritance, including the transgene itself, the host genome and the interaction between them (Makarevitch*et al.*, 2003; Yin *etal.*, 2004; Shrawat *et al*., 2007).

1.2 Importance of study

Pigeon pea widely grown in the rain fed and dry land areas of India. Even though large acreage under these crops, total productivity remains near to the ground and has been stagnating for the last few decades. A number of biotic and abiotic stresses are severely affecting full realization of the yield potential of these crops.

A lepidopteron insect, legumes pod borer is most serious pest of pigeonpea. Its larvae attack the flowers pods of pigeonpea, resulting in substantial damage and yield losses over $300 million annually worldwide (Shanower *et al.,* 1990). This is despite use of over US$ 211 million worth of chemical pesticide to control this pest. For pest problem as complex and

intractable as *Helicoverpa armigera*, no signals strategy is useful in keeping population below economic threshold level. Although chemical control is one of the effective methods of controlling this pest, it has led to secondary pest problems, besides contamination of food and food products with insecticide residues. So, it makes more difficult to addresses these problems through chemical control.

Despite adaption several technologies, low productivity of pigeonpea lids the plant breeders to explore the feasibility of using alternative biotechnological approaches for the crop improvement series of consequences like insecticide resistance, pest resurgence, outbreak of secondary pest,harmful residual effect, imbalance in natural ecosystem and high production cost has been considered important for developing IPM strategies. Generally modified crops represent one of the most rapidly adopted technological innovation to have been commercialized in the history of agriculture (Sharma *et al.,* 2004). Stable introduction of transgene into genome of plant also offers strategies for over expression or suppressing endogenous gene. Thus, introducing new gene expression via transformation generates new phenotypic variation useful for investigating new function and crop improvement.

The control of insect pest *Helicoverpa armigera* by transfer of insect resistant traits is becoming a best alternative. The lack of efficient high frequency plant regeneration system has deterred the production of transgenic plants in pigeonpea as it is considered as one of the recalcitrant crops and not amenable to tissue culture. Development of transgenic plant is outcome of an integrated application of recombinant DNA technology, gene transfer methods and plant tissue culture techniques.

Molecular characterization of transgenic plants is essential, as the number of transgene copies influence the expression level and genetic stability of the transgene (Weng *et al.,* 2014). DNA is randomly inserted into the plant genome during *Agrobacterium* transformation procedures. This often leads to the generation of plants that can have multiple transgene copies integrated into one or more chromosomal locations. The number of

transgene copies in transgenic plants can influence the level of expression and the genetic stability of the target gene (Weng *et al.,* 2004). It is thus important to analyze primary transformants in order to determine the transgene copy number. A single or low copy transformation event confers stability over several generations of successive breeding. Due to variation that might exist between independent transgenic lines produced under identical conditions, it is imperative to also assess the mRNA expression levels of the transgene for each transgenic line as expression levels are dependent on insertion site and transgene copy numbers (Toplak *et al.,* 2004).

Southern and northern blot analyses are routinely used to determine copy number and expression levels in transgenic plants respectively. These procedures are however laborious, time-consuming, requires large amounts of plant material and may also involve the use of harmful radioisotopes.

Looking to the potential of pigeonpea transgenic in enhancing insect resistant variety this study was planned to carry analysis of putative transformants of T_1 and T_2 generation. Comparisons between parent and progeny performance should take into account the consequences of breeding. Usually, wherever feasible, researchers resort to selfing of the T_0 plants to obtain homozygous progenies. The resultant T_1 progenies are a complex mix of genotypes whose composition depends on the genetic constitution of the T_0 parents. Besides hemi- and homozygous transgenic, there will be non-transgenic plants in T_1 population further, transgenes inserted at multiple loci will segregate giving rise to transgenic with different dose of transgene. Determining exact genotypic constitution of individual plants will prove difficult because it is not easy to distinguish hemizygotes from homozygotes by Southern analysis. Again, real time PCR based techniques have been found suitable for such detection breeding behaviour of the plant species will have profound effect on progeny performance. If the species is cross-pollinated, inbreeding depression will manifest in selfed progenies. On the other hand, the physiological and non-heritable

somaclonal effects will disappear in progeny generations. If the transgene insertion disrupts a functional host gene, homozygotes may display altered phenotype corresponding to insertionally mutagenised locus. Transgenics that are chimeric may show abnortions, transgenics need to be generated in much larger numbers. Thus in asexually propagated plants evaluation of transgenics should be deferred to clonal generation.

1.3 Objectives

1) Confirmation of gene integration with gene specific markers of putative transformants of pigeon pea plants.
2) Insect bioassay of putative transformants of pigeon pea plants.
3) Genetic analysis of putative transformants of pigeonpea plants.

1.4 Hypothesis

The present investigation is undertaken with the assumption to maintain and confirm putative transformantsfor gene integration with gene specific markers**.** The putative transformants will be used to determine the behavioral response of *Helicoverpa armigera* through detached leaf bioassay in relationship with expression of Cry1Aabc protein transformant. The perfect transformant would contain single copy of transgene that would segregate as Mendalian trait, with uniform expression from one generation to next.

1.5 Scope and importance

The transfer of desirable genetic traits across species barriers offered potential promises to solve problems in managements of agricultural crops, provide new possibilities to improve human and animal health and provide new revenue stream for a farmer through contract production of pharmaceuticals and industrial crops (Anonymous, 2010). It has potential environmental benefits included reduce toxic pesticide use, improved control resulting in less management Furthermore new technologies promises increased yield due to minimizing losses.

In world, transgenic for various traits are available in crops like cotton for insect resistance, in soybean for glyphosate tolerant and in corn for resistance against lepidopteron pest, in Tomato for delayed ripening. In India cotton is the only crop in which commercial transgenic are available. Out off total area of cotton in year 2010, 90% of area was under Bt cotton, Bt gene has proved to be very effective in controlling cotton bollworm. The research for development of transgenic against pod borer in pigeonpea is under progress in many of research institute of private and public sectors like genetic transformation laboratory, ICRISAT, National center of plant biotechnology, IARI, New Delhi.

Vidarbha region is being a major producer of pigeonpea and losses due to pod borer are also reported. The most protocol reported in literature is genotype specific. Pigeonpea (*Cajanus cajan* (L) Millisp) is a protein rich grain legume of the semiarid tropics and more eco-friendly approach. Interspecific of agriculture has exacerbated the pest problems and farmers are responding by using more toxic pesticides more frequently. Plant transformation remains art that requires considerable training of the practitioner to develop the skill s needed to generate sufficient transgenic plants. In addition regeneration is often slow and the frequency of transformation (number of transformed plant generated from each explants) is often low.

The perfect transformant would contain single copy of transgene that would segregate as Mendalian trait, with uniform expression from one generation to next. Transformation technologies have been advanced to the point of commercialization of transgenic crops. The introduction of transgenic varieties in the market is multi step process that begins with registration of new variety followed by field trials and ultimately delivery of seed to the farmer.

CHAPTER II

REVIEW OF LITERATURE

Pigeonpea (*Cajanus cajan*) is a multipurpose legume crop grown by more than 25 tropical and sub-tropical countries, either as the sole crop or as a mixed crop with sorghum, pearl millet, maize or with short duration legumes such as groundnut. It is a perennial shrub that can survive for a period of 3-5 years but it is normally cultivated as an annual crop. In terms of global grain legume production, it is sixth after phaseolus beans, peas, chickpeas, broad beans and lentils. In Asia, pigeon pea is the third most important pulse crop which accounts for approximately 90% of world production. It occupies an important position in human diet as a protein source to about 20%-30% (Singh et al., 1984; Thu et al., 2003) and also is an abundant source of minerals and vitamins (Saxena et al., 2002). From natural resource management perspective, cultivation of pigeonpea improves the soil characteristics and fertility status (up to 200 kg N ha -1) ensuring better growth to succeeding crop by contributing about 40 kg/ha.

2.1. Molecular and Genetic analyses for insecticidal genes in transgenic plants

Molecular analyses were carried out by most of workers in the t_0 and t_1 stages to check the integration pattern and copy number of gene. PCR amplification of the marker gene or transgene is often taken as an indication of transgenic status of regenerants (Bhat, 2002). Vast differences in the level of expression of introduced genes have been observed among transgenic plants generated under identical conditions using the same DNA constructs. These have been generally attributed to copy number and position effect of the transgene (Bhat *et al*., 2002).

Kim *et al.* (1998) studied molecular and genetic analysis of transgenic rice plants expressing the maize ribosome-inactivating protein β-32 gene and the herbicide resistance *bar* gene. Segregation analysis of 15 R_3 plants revealed that transgene was stably transmitted to their progenies

and southern blot band patterns of R_1 progenies remained the same as the corresponding parents, suggesting that all the loci of multiple integration events are genetically linked. Also, in most of the lines, physical presence of the β-32 transgene cosegregated with the phosphinothricin-resistant phenotype, confirming that the transgene is behaving as a normal locus in the genome. However, some of R_1 seedlings that contained multiple copies of the transgene became sensitive to phosphinothricin, indicating that its expression was silenced.

XiongYing *et al.* (1998) reported transformation in rice plants expressing synthetic *cry1A(b)* and *cry1A(c)* genes highly toxic to striped stem borer and yellow stem borer. The plants were transformed with fully modified (plant codon optimized) versions of two synthetic *cryIA(b)* and *cryIA(c)* coding sequences from *Bacillus thuringiensis* as well as the *hph* and *gus* genes, coding for hygromycinphosphotransferase and β-glucuronidase .The integration, expression and inheritance of these genes were demonstrated in R_0 and R_1 generations by Southern, Northern, and Western analysis and by other techniques. Accumulation of high levels (up to 3% of soluble proteins) *of CryIA(b)* and *CryIA(c)* proteins was detected in R_0 plants. Bioassays with R_1 transgenic plants indicated that the transgenic plants were highly toxic to two major rice insect pests, striped stem borer.

Chakrabharty (2002) introduced *cry1Ab* gene in to cauliflower by *Agrobacterium* mediated transformation for the resistance against diamondback moth. Transformants were analyzed by PCR amplification by using gene specific primers and RT PCR and southern blotting. PCR with *nptII* and *cry1Ab* gene specific primers amplified 0.7 and 1.0 kb fragments respectively. Southern blotting revealed integration of transgene at one to three loci in independent transgenic plants and RT PCR demonstrating transcriptionally active *cry1Ab* gene in plant genome.

Dayal *et al.*(2003) studied shoot regeneration and genetic transformation in pigeon pea using leaf explants. Transformants were confirmed by PCR amplification of *uidA* and *nptII* and southern hybridization for the *nptII* gene, shoots were subjected to PCR analysis for the presence

of the *uidA* and *nptII* genes, over 90% of shoots showed positive amplification of the respective gene fragments. Fidelity of the amplified gene fragments was verified by subjecting the PCR gels to southern blot hybridization. RT-PCR amplification of cDNA from the putative transgenic plants showed positive amplification of the 1,200bp *uidA* gene fragment in all the selected transgenic lines.Southern blot analysis of these plants confirmed the transgenic nature of the selected shoots, where 50% of the transgenic lines showed single gene inserts and the rest contained two inserts.

Kumar *et al.* (2004) studied genetic transformation of pigeonpea with rice chitinase gene. Putative transformed pigeonpea plants were recovered with stringent selection pressure and confirmed using molecular techniques. Stable integration and expression of the chitinase gene has been confirmed in the T_0 and T_1 transgenics through molecular analysis. Molecular analysis was carried out to confirm the integration and expression of the transgene. Of the 17 (T_0) independently transformed plants, four that showed single copy transgene integration (P-1, P-2, P-29 and P-30) were advanced to the T_1 generation. In order to study any Mendelian inheritance pattern in the T_1 progeny, 25 seeds from each of the four T_0 plants were sown and a total of 83 T_1 plants were raised clone-wise in a glasshouse. PCR analysis was carried out on the 83 T_1 plants for the amplification of the *Rchit* gene and the progenies of each clone showed a 3: 1 segregation pattern.

Sharma *et al.*(2005) developed an efficient method to produce transgenic plants of pigeonpea by incorporating the *cry1Ab* gene of *Bacillus thuringiensis* through *Agrobacterium tumefaciens*-mediated genetic transformation. Over 75 independently transformed transgenic events of pigeonpea were produced and advanced to T_2 generation. Amongst the recovered primary putative transformation events, 60% showed positive gene integration based on initial polymerase chain reaction (PCR) screening. PCR analysis of the progenies from independent transformants followed gene inheritance in a Mendelian ratio and 65% of the transformants showed

the presence of single copy inserts of the introduced genes. Reverse transcription–polymerase chain reaction analysis showed that the transcripts of the introduced genes were normally transcribed and resulted in the expression of *Cry1Ab* protein in the tested T_2 generation plants. Interestingly, the content of *Cry1Ab* protein as a percent of total soluble protein varied in different tissues of the whole plant, showing the highest expression in flowers (0.1%) and least in the leaves (0.025%) as estimated by enzyme-linked immunosorbent assay.

Swathi *et al.* (2006) generated putative promoter tagged transgenic lines in *Arachis hypogaea* cv JL-24 using cotyledonary node (CN) as an explants and a promoterless *gus::npt*II bifunctional fusion gene mediated by *Agrobacterium* transformation. Over 141 putative T_0 plants by using the promoter less construct and transferred them to the field**.** Among these, 82 plants survived well in the green house and 5 plants corresponding to 3.54% showed stable integration of the fusion gene as evidenced by GUS, polymerase chain reaction (PCR) and Southern blot analyses. Twenty-four plants were positive for GUS showing either tissue-specific expression or blue spots in at least one plant part. The progeny of 15 T_0 plants indicated Mendelian inheritance pattern of segregation for single-copy integration. The tissue-specific GUS expression patterns were more or less similar in both T_0 and corresponding T_1 progeny plants.

Ahsan *et al.* (2007) reported *Agrobacterium* (LBA4404) mediated transformation, harbouring pIG121Hm-GS, which contained the glutathione synthetase gene under the control of the CaMV35S promoter. Optimal transformation frequency (20.7%) was observed with cotyledonary explants directly infected with a bacterial solution, followed by co-cultivation at 24ºC for 2 days. The presence of transgene, in putative transgenic plants, was confirmed by PCR and southern blot analysis

Keshamma *et al.* (2008) developed the transgenic plants of the groundnut (*Arachis hypogeae*) cv. TMV-2 expressing a chimeric Bt gene, cry1X, were generated using an *Agrobacterium tumefaciens* – mediated transformation system. A tissue culture-independent transformation method,

in planta which targets the *A. tumefaciens* to the apical meristem was used in this study. The protocol involves in planta inoculation of the embryo axes of the germinating seeds and allowing them to grow into seedlings ex vitro. PCR analysis indicated the putative transgenic nature of the T_1 generation plants. Enzyme Linked-Immuno Sorbent Assay (ELISA) was used to identify the high expressing plants. The appearance of the protein band in the quickstix confirmed the expression of the chimeric Bt toxin. Southern analysis of 10 high expressing plants confirmed the integration of the transgene.

Madappattuparambil *et al.*(2008) studied the stability of the transgenes was checked in three generations for integration, expression and efficacy against the two insects *H.armigera* and *Spodoptera litura*. Southern blot analysis of 10 high expressing plants confirmed the integration of the transgene. The presence and expression of synthetic cry gene and selectable marker *(nptII)* gene in regenerated plants were confirmed by PCR, southern and western blot analysis.

Rao *et al.* (2008) performed PCR on T_1 transformants for both *uidA* and *npt II* genes. Out of the 350 progeny analyzed, 48 plants amplified both transgenes to obtain confirmation onthe presence of the introduced genes in the plantsForty eight of the 350 T_1 plants amplified both transgenes.

Ashrani *et al.* (2011) introduce *cry1X* using in planta transformation and characterize putative transformants T_1 lines for molecular and biochemical characters by PCR analysis, ELISA and western blotting techniques. PCR analysis of ELISA positive plants with *nptII* specific primers revealed the *nptII* gene in 43 plants. Western blot analysis of transgenic chickpea using the antibody against the cry protein gave an indication that the plants were expressed as *cry* protein.

Ramu *et al.* (2011) studied the expression of synthetic *cry1AcF* gene in transgenic pigeonpea confers resistance against *Helicoverpa armigera*. PCR, Southern hybridization, RT-PCR and Western analysis

confirmed stable integration and expression of the *cry1AcF* gene in pigeon pea transgenics. When screened for efficacy of the transformants for resistance against *H. armigera*, the transgenics showed not only high mortality of the larva but also could also resist the damage caused by the larvae. Analysis for the stable integration, expression and efficacy of the transgenics resulted in the identification of four T_3 plants arising from two T1 backgrounds as highly promising.

Chetty *et al.* (2012) compared the transformation efficiency of tomato (*Solanum lycopersicum* L.) cv. Micro Tom with *Agrobacterium tumefaciens* strains AGL1, EHA105, GV3101, and MP90 harboring the plasmid pBI121 was compared. The presence of the *nptII* and/or *uidA* transgenes in regenerated T_0 plants was determined by PCR, southern blotting, and/or GUS histochemical analyses. In addition, a rapid and reliable duplex, qPCR TaqMan assay was standardized to estimate transgene copy number.

Geng *et al.* (2012) introduced a synthetic *cry8Ea1* gene in peanut (*Arachis hypogaea* L.) through *Agrobacterium rhizogenes*-mediated transformation in embryonic axes along 23 with cotyledons of peanut. Transcription and expression of gene were detected via RT-PCR and the GUS histochemical assay, respectively. RT-PCR assay showed that 59 % of 27 composite plants were positive and the positive composite plants carrying the synthetic *cry8Ea1* gene were transferred to pots. The damage degree of 63 % of the composite plants was D0. These plants grew well and were not damaged by *Holotrichia parallela* larvae.

Gowda (2013)studied transgenic pigeonpea plants expressing *cry1Ac* gene for the expression of *Cry1Ac* protein and resistance to pod borer *Helicoverpa armigera* The seeds obtained from homozygous transgenic plants (T_6 generation) from all five lines viz., *iabtppML43, iabtppML46, iabtppML49, iabtppAL52* and *iabtppAL55* in two genetic backgrounds Asha and Maruti were sown and T_7 generation plants were raised. The PCR analysis of T_7 generation plants validated the zygosity status as homozygous in all five transgenic lines. The PCR assay indicated

the amplification of 600 bp and 800 bp amplicon with gene specific primer pair. These transgenic plants were taken for further event characterization and bioefficacy studies.

Yogendra *et al*. (2013) performed plant transformation for stable expression of a transgene. However, some unintended transgene instability has occurred in the expression of 34 recombinant proteins. To study the genetic stability of a transgene, they transformed leaf explants of tobacco with them hepatitis B surface antigen (*HBsAg*) gene along with *npt-II* as an antibiotic selection marker using *Agrobacterium* mediated transformation. The presence of the *BsAg* gene in putative transformants was confirmed by polymerase chain reaction (PCR) analysis. The T_1 to T_4 generation seeds obtained from the transgenic tobacco plants were tested after germination in the presence of kanamycin and Mendelian segregation was confirmed. This phenotypic and molecular characterization strengthens the genetic stability and inheritance of the *HBsAg* gene in different generations of transgenic tobacco plants.

Gosh *et al*. (2014) studied genetic transformation of important pulses and described that transgenic plants needs successful bioassay evaluations to check the mortality levels of the desired insect on which resistance is being developed and also reported that in case of pigeon pea, initial reports of transgenic research had no far reaching impacts. Most of the studies conducted molecular analysis of the primary transformants, rather than proceeding further. Molecular analyses were carried out by most of the workers in the T_0 and T_1 stages to check the integration pattern and copy number.

Jain (2014) worked on development and characterization of pigeonpea for resistance against *Helicoverpa armigera* for that Bt pigeon pea variety Manak (H77-216) carrying *Cry1Ac* gene was used and molecular characterization of transgenic plant was done. Developing Bt pigeon pea for plasmid isolation was carried out and Bt pigeon pea was developed by using a novelty technique after that DNA was isolated and PCR analysis of putative transgenic pegionpea plant was carried out for that 324 progeny

were used out of 233 were randomly analyses to determine transgenic nature out of 233 plant 125 amplified *Cry1Ac* gene.

Kumar *et al*. (2016) carried out *In vitro* regeneration of pigeonpea through organogenesis byauxiliary buds as explants from the field grown plants of seven varieties and two hybrids under the influence of variable concentrations of two cytokinins; BAP and Kinetin. The elongated shoots were successfully rooted on MS medium containing different concentrations of auxins; IBA 1 mg/l induced maximum frequency of roots in all the cultivars. They did molecular confirmation using 12 SSR primers which was screened for both parents and regenerated plants concluded that 7 markers showed monomorphism/homozygous bands confirming that the regenerated plants were genuinelytrue-to-type to the mother plants.

Das *et al.* (2016) studied the expression of chimeric Bt gene, *cry1Aabc* gene in transgenic pigeonpea by PCR and alkaline phosphatase (ALP) chemistry baseddirect antigen coating (DAC) ELISA (qualitative ELISA) Horse Radish Peroxidase (HRP) chemistry based Double Antibody Sandwich ELISA (quantitative ELISA) were performed in all qualitative ELISA and PCR positive pigeonpea progenies for the quantiication of δ-endotoxin protein.

Insect bioassay

XiongYing *et al.* (1998) reported transformation in rice plants expressing synthetic *cry1A(b)* and *cry1A(c)* genes highly toxic to striped stem borer and yellow stem borer. Bioassays with R_1 transgenic plants indicated that the transgenic plants were highly toxic to two major rice insect pests, striped stem borer and yellow stem borer with mortalities of 97-100% within 5 days after infestation.

Gopalaswamy (2005) studied that the transgenic pigeonpea plant *cry1Ab* and soybean trypsin inhibitor genes were molecularly characterized for the presence of insecticidal genes and bioassay were conducted to their efficacy against the gram pod borer *Helicoverpa armigera* under the labrotory condition. They observed that the *cry1Ab* toxin level

present in the leaves of transgenic plant could not inhibit the feeding by the larvae.

Sharma *et al.*(2005) reported that the noctuidae *Helicoverpa armigera* is a major insect pest of chickpea chickpea *Cicer arietinum* L., pigeonpea *Cajanus cajan*L.Millsp., peanut *Arachis hypogaea* L. and cotton *Gossypium* spp.To overcome these problems, we standardized the detached leaf assay to screen for resistance to this pest in chickpea, pigeonpea, peanut, and cotton under uniform insect pressure under laboratory conditions. Terminal branch of chickpea, first fully expanded leaf of cotton, trifoliate of pigeonpea or quadrifoliate of peanut, embedded in 3% agar-agar in a plastic cup/jar of appropriate size (250-500-ml capacity) infested with 10-20 neonate larvae can be used to screen for resistance to *H. armigera.* The experiments can be terminated when the larvae have caused > 80% leaf damage in the susceptible check or when differences in leaf feeding between the resistant and susceptible checks are maximum. Detached leaf assay can be used as a rapid screening technique to evaluate germplasm, segregating breeding materials, and mapping populations for resistance to *H. armigera.*

Transgenic soybean carrying *cry1Ac* gene challenged with *H. armigera* larvae, the leaves of highest resistance found hardly eaten, on other hand larval growth fed on transgenic control plants seen evidently restrained, which represented less body, higher mortality and lower weight compared to non-transgenic where the leaves were completely eaten and had good growth (Dang Wei and Zhiming Wei, 2007).

Gaifullina *et al*. (2007) evaluated the effectiveness of detached leaf assay to assess the efficacy of transgenic pigeonpea (var; ICPL 88039 and ICPL 87) plants carrying *Bt cry1Ab* and *SBTI* genes for resistance to *H. armigera* and conclude that the levels of *Cry1Ab* or *SBTI* proteins in the transgenic pigeonpea plants were not sufficient to cause significant deterrent effects on leaf feeding, larval survival, and larval weight of *H. armigera*. However, detached leaf assay was found to be quite useful for evaluation of transgenic pigeonpea plants for resistance to *H. armigera.*

Transgenic soybean carrying *cry1Ac* gene challenged with *H. armigera* larvae, the leaves of highest resistance found hardly eaten, on other hand larval growth fed on transgenic control plants seen evidently restrained, which represented less body, higher mortality and lower weight compared to non-transgenic where the leaves were completely eaten and had good growth.

Adesoye *et al.* (2008) tested *Cry*1Ab transgenic cowpea of T1 and T3 generation resistance to *Marucavitrata* and found that transgenic plants significantly reduced larval survival and larval weight andinhibited *M. vitrata* feeding on cowpea leaves.

Gopalaswamy *et al.* (2008) evaluated transgenic pigeonpea plants of ICPL 88039 and ICPL 87 carrying *Bacillus thuringiensiscry1Ab* and soybean trypsin inhibitor (*SBTI)* genes were evaluated for resistance to *H. armigera* under field conditions, as well as in vitro bioassays using leaves, inflorescences, and pods. Lack of significant reduction in leaf feeding, larval survival, and larval weight indicated that the levels of *Cry1Ab* endotoxin or *SBTI* present in the transgenic pigeonpea plants were not sufficient. Infestation of transgenic plants with neonate larvae at flowering, supplemented with leaf or pod bioassays under laboratory conditions can be used effectively to evaluate transgenic pigeonpea for resistance to *H armigera.*

Keshamma *et al.* (2008) developed the transgenic plants of the groundnut (*Arachis hypogeae*) cv. TMV-2 expressing a chimeric Bt gene, *cry1X*, were generated using an *Agrobacterium tumefaciens* – mediated transformation system. Bioassay was carried against two major pests of the groundnut, *Helicoverpaarmigera* and *Spodoptera litura* revealed several T1 plants that perform well against both the larvae. This revealed that 22% of T1 plants harbor the transgene.

Ashrani *et al.* (2011) introduced *cry1X* in *chickpea* using in planta transformation and characterize putative transformants. The efficacy of the cry gene product was tested against pest of chickpea *H. armigera*.

The transgenic plants showed significantly higher tolerance to the pest and performed better when compared to the wild type.

Mehrotra *et al.* (2011) performed insect bioassay to demonstrate the entomocidal analysis and assessment of toxicity to *H. armigera* against transgenic chickpea plants co-expressing *cry1Ab* and *cry1Ac* genes. These transgenic plants reflected higher toxicity and protection to insects as compared to plant expressing single *cry1Ab* or *cry1Ac* gene at relatively higher levels. Transgenic chickpea plants harbouring both the genes have reflected significant mortality (95%) to second instar larvae of *H. armigera* at toxin levels of 15 ng mg-1 TSP while plants expressing *cry1Ac* gene could result similar toxicity at much higher levels of expression (22–25 ng mg-1TSP).

Krishna *et al.* (2011) investigated that *Agrobacterium* mediated genetic transformation was performed using embryonic axes explants of pigeonpea both pod borer resistant gene (*cry1Ac*) and plant selectable marker *neomycin phoshotransferasenptII* genes under the constitutive expression of cauliflower mosaic virus 35s promoter assembled in *Ppzp211* binary vector were used for the experiment. The plant obtained were subjected to multi and no choice test ton determined the behavioral responses and mortality through *helicoverpa armigera* bioassay on the leaf and relate their relationship with expression of *cry1Ac* protein which was found to be less in leaf as compared to the floral bud, anther, pod and seeds.

An insect mortality bioassay performed by Mehrotra *et al*., (2011) on transgenic chickpea plants with second instar larvae of *H. armigera* have indicated the effect of Cry toxin on insect larvae for their growth and overall health compared to larvae fed on non-transformed chickpea plants. They reported extensive feeding of plant tissues (90%) by the larvae for untransformed control plants and larvae were healthy, active and showed a normal developmental cycle. 100 % larval mortality reported after 4 days of incubation on plants expressing higher level (70–112 ng mg-1 soluble protein) of Cry1Ac protein and the plants showed high resistance to

the insect and suffered very little feeding damage to leaves. The plants expressing moderate level of crystal protein showed severely affected larval growth with impaired life cycle and early pupation.

Ramu *et al.* (2011) performed the insect bioassay to study expression of a chimeric *cry1AcF* gene in transgenic pigeon pea and its resistance towards *Helicoverpa armigera*. Bioefficacy of the plants against first and second instars *Helicoverpa* revealed significant variability in larval mortality and damage in both the pods and leaves. The mortality varied from 0% to 100% among the putative transformants. The effect of the *cry1AcF* gene was also seen on the larva as there was a considerable difference in the size of the larva that fed on the transgenics and wild type. The plants that showed high mortality exhibited les damage in leaves and pods. These experimental evidences gave clear evidence about the gene integration, expression and efficacy of *cry1AcF*.

Vemanna *et al.* (2013) carried out bioassay to examine the expression of the crystal protein in transgenic plants out using first instar larvae. The three transgenes were co-expressed in Arabidopsis as evidenced by gene expression, histochemical assay and insect bioassay. The damage of leaf and stem caused by the larvae was less in transgenics compared to the damage in wild-type plants. After 24–48 h, 80–100% mortality was recorded in the larvae fed with transgenic plants whereas larval growth was not inhibited and mortality was not observed in wild-type plants.

CHAPTER III

MATERIALS AND METHODS

The present investigation entitled “Molecular and genetic analysis of putative tranformants in pigeonpea” was carried out at Centre of Excellence in Plant Biotechnology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth Akola during Academic year 2016-17.

3.1 Material

Kalapad (2013) and Kamble (2014) generate Putative transformants resulted from *in-planta* inoculation of embryo axes of germinating seeds and floral dip with pBinAR-*cry*1*Aabc* mobilized into *Agrobacterium tumefaciens* strains EHA 105 are maintained in containment facilities in greenhouse condition at 28 - 30˚C temperature and 70-80% Relative Humidity and used for further study.

3.1.1 Chemicals and buffers

All the chemicals used in the present investigation were of analytical grade, molecular biology grade from SRL glasswares and plasticware used in various experiments were from Borosilicate, Riviera and tarson. They were thoroughly washed and sterilized as per standard procedure. All the plastic wares used in the molecular study were made up of polypropylene materials.

3.1.2 Equipments used

-20ºCrefrigerator	:	Vestfrost
Blender	:	Boss
Electrophoresis system	:	Tarsons
Gel documentation system	:	Bio-Rad
Micropipette	:	Bio-Rad
Microwave oven	:	LG
PCR machine	:	Applied biosystem

pH meter	:	ECIL
Refrigerated micro centrifuge	:	Eppendrof
Single pan digital balance	:	Sartorious
Water bath	:	Swastik Scientific

3.1.3 Solutions buffers reagents used

Solutions buffers reagents used to perform present study listed below

3.1.4 Plasmid DNA isolation

3.1.4.1 Reagents

1. Agrobacterium culture
2. Solution I
   - Gluose
   - Tris HCl
   - EDTA
3. Solution II
   - NaOH
   - SDS
4. Solution III
   - Pottasium acetate
   - Glacial acetic acid

3.1.4.2 Methodology for Isolation of plasmid DNA by Alkaline-lysis Method

1) 1.5 ml bacterial culture was taken and centrifuged at 12,000 rpm for 60 second. (Or 5000 rpm for 5 minutes).
2) 100 µl of solution I (ice cold) was added, vortexes and incubated on ice for 5 minutes.
3) 200 µl of solution II was added to microfuge tube and the content was mixed thoroughly by inverting and rolling the solution and incubated on

ice for 5 minutes. 100 µl of solution III (ice cold) was added to microfuge tube and incubated on ice for 4-6 minutes.

4) The samples were centrifuged at 12, 000 rpm for 10 minutes at 4°C temperature.

5) The supernatant was transferred to fresh microcentrifuge tube.

6) Equal volume of chilled ethanol or isopropanol was added in the supernatant mixed by inverting the tube 4-6 times. Incubated at -20°C for overnight

7) The tubes were again centrifuged was at 12, 000 rpm for 10 minutes at 4°C.

8) The supernatant was discarded and the pellet was dried at 35°C to remove traces of ethanol.

9) The 60 µl of TE buffer was added to dissolve the pellet and Agarose gel Electrophoresis was performed to check plasmid

3.1.5 DNA isolation from the leaves

3.1.5.1 Requirements

A. Extraction buffer (pH8.0)

- 5M Nacl (solution sterilized by autoclaving)
- 1M Tris Hcl
- 0.5M EDTA Na2

B. B marcaptoethanol (at the time of used)

C. Phenol: chloroform:isoamyl alcohol(25:24:1)

D. Chloroform : isoamyl alcohol(24:1)

E. 100% isopropanol(ice cold)

F. 70% ethanol (ice cold)

G. TE buffer (8.0)

- 10mM tris
- 1mM EDTA

3.1.5.2 Methodology for DNA Isolation using CTAB Method

1) 0.1gm of tissue sample was weighed and macerated in liquid nitrogen and was taken in a microcentrifuge tube.
2) 2 ml of pre-warmed CTAB buffer (65ºC) was added to tube and incubated for 1hour at 65ºC.
3) Centrifuge at 10000 rpm for 10 min at room temperature.
4) Supernatant was taken in another centrifuge tube. Equal volume of Chloroform: isoamly alcohol (24:1) was added to that tube.
5) The samples were centrifuged at 10000 rpm for 10 min at room temperature.
6) The aqueous phase was removed and taken in another microcentrifuge tube and equal volume of chilled isopropanol was added.
7) Incubated overnight at -20℃ or at -80 ˚ C for 1 hour. Centrifuged for 10,000 rpm for 10 minutes.
8) The supernatant was removed and washed with 70 % chilled ethanol twice
9) The pellet was air dried for 1 hour and dissolved in TE buffer or nuclease free water and stored in -20ºC.

3.1.6 DNA quantification

3.1.6.1 Requirements

- Agarose
- TBE buffer

3.1.6.2 Procedure

1) The DNA obtained by extraction was confirmed by running it on 0.8% agarose gel containing ethidium bromide @ 0.5 mg/ml) in a horizontal gel electrophoresis system.

2) And even DNA was quantified using nanophotometer at 260 nmThe purity of extracted genomic DNA was confirmed through its A_{260}/A_{280} (1:8) ratio having a concentration of 20 µg/ µg.l

3.1.7 Ribonuclease treatment

RNA was removed by giving RNase A treatment. RNase A (10mg/ml) was added to each DNA sample @ 100 µl/ml and the sample were incubated at 37ºCfor 1 hour in thermomixer.

3.1.8 PCR amplification

PCR amplification was carried out with Cry1Aabc and *NptII* gene specific primers synthesized from Eurofine Pvt.,Mumbai were listed below (Table 3.1).

Table 3.1. Lists of gene specific primers

Sr. No.	Primer name	Sequence (5'-3')	Direction
1	*nptII*	CAATCGGCTGCTCTGATGCCG	F
		AGGCGATAGAAGGCGATGCGC	R
2	*Cry1Aabc*	TTCTGCCCAAGGTATCGA	F
		CAGCCTGGAGTGTTGCA	R

3.1.8.1 Various components used for PCR are listed below

Master Mix for PCR reaction

Mix	**1 X**
10X *Taq* polymerase buffer with $MgCl_2$	2 µl
$MgCl_2$ (25 mM)	2 µl
dNTPs (10 mM)	0.25 µl

Forward Primer	1µl
Reverse Primer	1 µl
Taq polymerase, recombinant (5 U/µl)	0.2µl
Sterile distilled water	13.45 µl

3.1.8.2 PCR amplification

The isolated genomic DNA was amplified using cry1Aabc and *npt*-II specific primers. The plasmid DNA was used as control.

1. Prepare and label a1.5 ml microcentrifuge tube for PCR cocktail
2. Defrost all components of the cocktail at room temperature, except the Polymerase which has to keep at -20 at all time prior to use.
3. Prepare the PCR cocktail for 20 µl reaction volume by adding the components in order as listed in table (3.1)

3.1.8.2.1PCR amplification step for *Cry1Aabc* primers

PCR profile used for the amplification of Cry1Aabc gene is given below in Table 3.2.

Table 3.2. PCR amplification step for *Cry1Aabc* primers

Cycles	**Temperature**	**Time**	**Step**
Denaturation (One Cycle)	95 ^{0}C	5 min	Hot Start
Amplification program (34cycles)	94 ^{0}C	1 min	Denaturation
	54^{0}C	1 min	Annealing
	72 ^{0}C	2 min	Extension
Final Elongation (one cycle)	72 ^{0}C	10 min	Final Extension
Hold (Infinity)	4 ^{0}C	---	Hold

3.1.8.2.2The PCR amplification steps for *nptII* primers

PCR profile used for the amplification of *NptII* gene is given below in Table 3.3.

Table 3.3. PCR amplification steps for *NptII* primer

Cycles	Temperature	Time	Step
Denaturation (One Cycle)	95 ^{0}C	5 min	Hot Start
Amplification program (40 cycles)	94 ^{0}C	1 min	Denaturation
	62 ^{0}C	1 min	Annealing
	72 ^{0}C	1 min	Extension
Final Elongation (one cycle)	72 ^{0}C	10 min	Final Extension
Hold (Infinity)	4 ^{0}C	---	Hold

3.1.9PCR product determination on agarose gel Electrophoresis

The amplified product was resolved on Horizontal gel electrophoresis system by using the 1.5 % agarose in 1x TBE

1) Appropriate gel tray and combs were selected for the number of samples to be run (leaving an appropriate number of wells free for size standards). Gel –forming casettee were used for the gel preparation.
2) The level of comb was adjusted so it rests evenly with few mm of space between teeth and tray; that will allows wells to be form in the agarose.
3) Adequate volume of the electrophoresis buffer (TAE) was prepared to fill the tank and to prepare the gel.
4) Weighed agarose was placed in conical flask. To check PCR success, a 1.5 % agarose=1.5g agarose in 100ml 1x TBE buffer was used.
5) Weighted electrophoresis grade agarose added to a volume of electrophoresis buffer followed gently swirl.
6) The solution was heated in a microwave on maximum setting for approx 30 sec , flask was removed from the microwave and gently swirl and confirmed that all the agarose has been dissolved.
7) Gel was allowed to cool or hold under cold running water, until it is comfortable to touch the side of conical flask.

8) Appropriate volume of ethidium bromide was added to agarose gel to a final concentration of 0.5 µg EtBr/ ml of agarose gel. The solution to mix. EtBr binds to DNA and allows you to visualize the DNA under UV light.

9) Melted agarose was poured into the gel casting tray and left to set approx. 30 min, making sure that no bubbles are trapped underneath combs and all bubbles on agarose surface are removed before the gel sets.

10) Samples were prepared for gel electrophoresis by mixing the gel loading solutions with PCR product (3 µl gel loading solution and 5 µl PCR product).

11) The comb was carefully removed from the set.

12) Agarose gel was placed in electrophoresis tank containing 1x TBE buffer, making sure that the gel is totally immersed in buffer. The buffer should be just covering the surface of the gel.

13) Recommended volume of size standard ladder was loaded into the assigned lanes (typically, 0.1 µg of standard per millimeter lane width). Then, 5 µ of sample were loaded in to the subsequent wells taking care not to puncture well bottom. Gel was run for 30 min to 60 min at 80v.

14) Run the gel until the tracking dye was migrated to within ¾ th of the positive electrode end of the gel.

15) The gel to the imaging documentation system and digital image was captured.

3.1.10 Documentation

Gel was visualized and photographed with ethidium bromide by illumination with UV light using Bio-Rad gel documentation system and captured the image for further analysis

3.1.11 Rearing and maintenance of *Helicoverpa armigera*

3.1.11.1Artificial diet preparation

Requirements

The requirements for artificial diet for *Helicoverpa armigera* larvae (Reference Insecticide Resistance**:** Monitoring, Mechanisms and Management Manual by K. R. Kranthi) given in Table 3.4.

Table 3.4. Composition of artificial diet used for *H. armigera* rearing in laboratory conditions (for larvae)

Chickpea flour (Kabuli type)	160 g
Wheat germ	60 g
Sorbic acid	1.7 g
Dried yeast	53 g
Ascorbic acid	5.5 g
Methyl paraben	3.3 g
Auromycin	2.5 g
Formaldehyde	13.5 ml
Anti mould solution*	2 ml
Double distilled water ml	1200 ml

*The anti mould solution contains 5 % phosphoric acid and 45 % propionic acid in sterile water

Procedure

1. Masured quantities of chickpea flour, wheat germ, sorbic acid, ascorbic acid, methyl paraben and Aureomycin was taken into a large bowl. 500 ml of pre-boiled warm water, was added and mix well.
2. 53 gm active dried yeast was dissolve in 350 ml water and boiled for 5 min.
3. 16 gm agar was added to 350 ml water, disperses well and boiled for 5 min.

4. Yeast and agar solutions were mixed well and boiled for 5 min and added to the bowl containing other diet ingredients. The solution was mixed well using a blender.
5. 13.5 ml 10% formaldehyde was added in the solution and 2 ml anti-mould solution if necessary. Mix thoroughly using a bender.
6. The hot diet was transferred into soft plastic squeeze-bottles, close with lids having spouts trimmed to 1 cm and dispensed the diet into wells of multi-cell trays.
7. The trays were allowed to cool in laminar airflow under UV lamp for 2-3 hours to sterilize the diet surface.
8. The rearing trays were stored at 4-8ºC for a week.

3.1.11.2 Moth diet preparation

Requirements

Table 3.5. Moth diet preparation

Honey	5 g
Sucrose	5 g
Methyl paraben	0.2 g
Ascorbic acid	0.2 g
Sterile distilled water	90 ml

Procedure

1. 5 gm each of sucrose and honey was dissolved to 90 ml sterile water , boil for 5 minutes and simmer for a further 15 minutes.
3. Once the solution is cooled, 0.2 g each of ascorbic acid and methyl hydroxyl par benzoate was added in the solution. Mix well and store at 40C for 1- 2 weeks.
4. Sterile absorbent cotton wads was used to soak the solution and place them in the moth jars. Change the wads at least thrice a week.

3.1.11.3 Rearing of *Helicoverpa armigera*

Procedure

1. *H.armigera* larvae for laboratory bioassay experiment were maintained at Biotechnology Center, Dr.PDKV,Akola.
2. The *H.armigera* culture was maintained on semi synthetic diet.
3. The *H.armigera* neonates were reared in group on 200-250 in the petridish having 2-3 mm layer on artificial diet on the bottom.
4. After 5 days the larvae was transferred individually to twelve-cell well plates to avoid cannibalism.Each cell well will have sufficient diet to support larval develop until pupation.
5. The pupa was removed from cell wells, sterilized with 2% sodium hypochlorite solution for 2 min and kept in group of 20 in plastic jar containing soil rite.
6. 10 pairs of adult were released in inside ovipositor cage. Adults were provided with adult diet.
7. Muslin cloth was used for the female to lay egg.
8. After hatching of egg the larvae will be moved to artificial diet and the neonate larvae was used for bioassay.

3.1.12 Bioassay

Requirements

1) Agar agar
2) Helicoverpa armigera neonates
3) Tender pigeonpea leaves

Procedure

1. The bioassays performed in the laboratory at temp 27±2ºC and 65-75 RH with a photoperiod 12:12 (L: D. hrs).
2. The experiments carried out in 3% agar containing bottles

3. The solidified agar was used as substratum for holding pigeon pea leaf in slanting manner inside the bottles. 10 neonates *H. Armigera* larvae were released to the bottles on the pigeonpea leaves.
4. The observations were recorded after 5^{th} day of released larvae in bottles.
5. Weight of larvae was recorded after 5^{th} day
6. Leaf area damaged was estimated by scale
7. Mortality rate was calculated using formula.

3.2 Statistical analysis

The experiment was conducted in Completely Randomized Design (CRD) and data analyzed by PROC ANOVA.

Segregation analysis was done using the x^2 test in which observed values were compared to theoretical values corresponding to the integration of one or more copies of the transgene.

CHAPTER IV

RESULTS AND DISCUSSION

The present investigation was aimed to analyzed putative transformants of pigeonpea at molecular level. The research work was carried out at Center of Excellence in Plant Biotechnology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth Akola.The Result and discussion of investigation is presented in this chapter.

4.1 Maintenance of Putative transformed Plants

The introduction of a transgene into a recipient genome is a complex event depending on the transgene itself and the host genome. The PCR positive plants resulted from *in-planta* inoculation of embryo axes of germinating seeds and floral dip with pBinAR-*cry*1*Aabc* mobilized into *Agrobacterium tumefaciens* strains EHA 105 are maintained in containment facilities for further study of inheritance of transgene and the level of transgene expression.

The data for maintenance of the plants with its pedigree is given in below mentioned Table 4.1.

Table 4.1. Initiation of experiment: July 2013: Embryo Infection

Sr. No.	Activity	Month and Year	Remark	
1	Embryo Infection (T_0)(180 seeds attempted)	Aug. 2013	91 plants survived out of 180 infected seeds sown.	By Kalpad S.
2	Harvesting of seeds from embryo infected plants (T_0)	Feb14 – April 14	Seeds harvested plant wise and stored for further studies.	

3	Sowing of 54 seeds with single seed from one plant was carried out. (T_1)	March 2014	DNA was extracted from 1 to 54 embryo infected plants and confirmation through gene specific amplification in PCR (T_1)	Out of 54 two plants reported positive for *npt II* and *Cry1Aabc*.
4	Sowing of 37seeds with single seed from one plant was carried out. (T_1)	Feb 2015	DNA extraction from 55-91 embryo infected plants and confirmation through gene specific amplification in PCR (T_1)	Out of 37 ,Six plants reported positive for npt II and Cry 1 Aabc
5	Harvesting of seeds of positive plants (T_1) and its sowing.(T_2) (Total 8 plants)	Aug. 2015	10 seeds of each positive plant were sown. DNA was extracted from 5 plants form each row and confirmed using gene specific primers. The positive plants were maintained in greenhouse and harvested during March 2016 (T_2). The list of positive plants harvested is given in table 4.2.	By Kamble S.
6	Sowing of seeds of positive plants (T_3)	July 2016	10 seeds of each positive plant were sown as mentioned in table 4.2.	By Kadam K.
7	Testing of T_3 Plants	Jan. 2017	Three plants from each row of positive plant was selected for PCR confirmation and data of PCR amplification is presented in table 4.2	

4.1.1Testing of T_3 plants of Embryo Infection

Kalapad, 2013 attempted 180 embryo infections with *cry1Aabc* and *NptII*. Out of 180 infected seeds were grown from which 91 plants survived (T_0). Out of these 91 plants total 8 plants were positive. From these 8 positive plants one plant is lost due to damage and remaining 7 plants were harvested. From these 7 plants 10 seeds from each plant were sown and harvested which is mentioned in Table 4.2 and Plate 1 & 2.

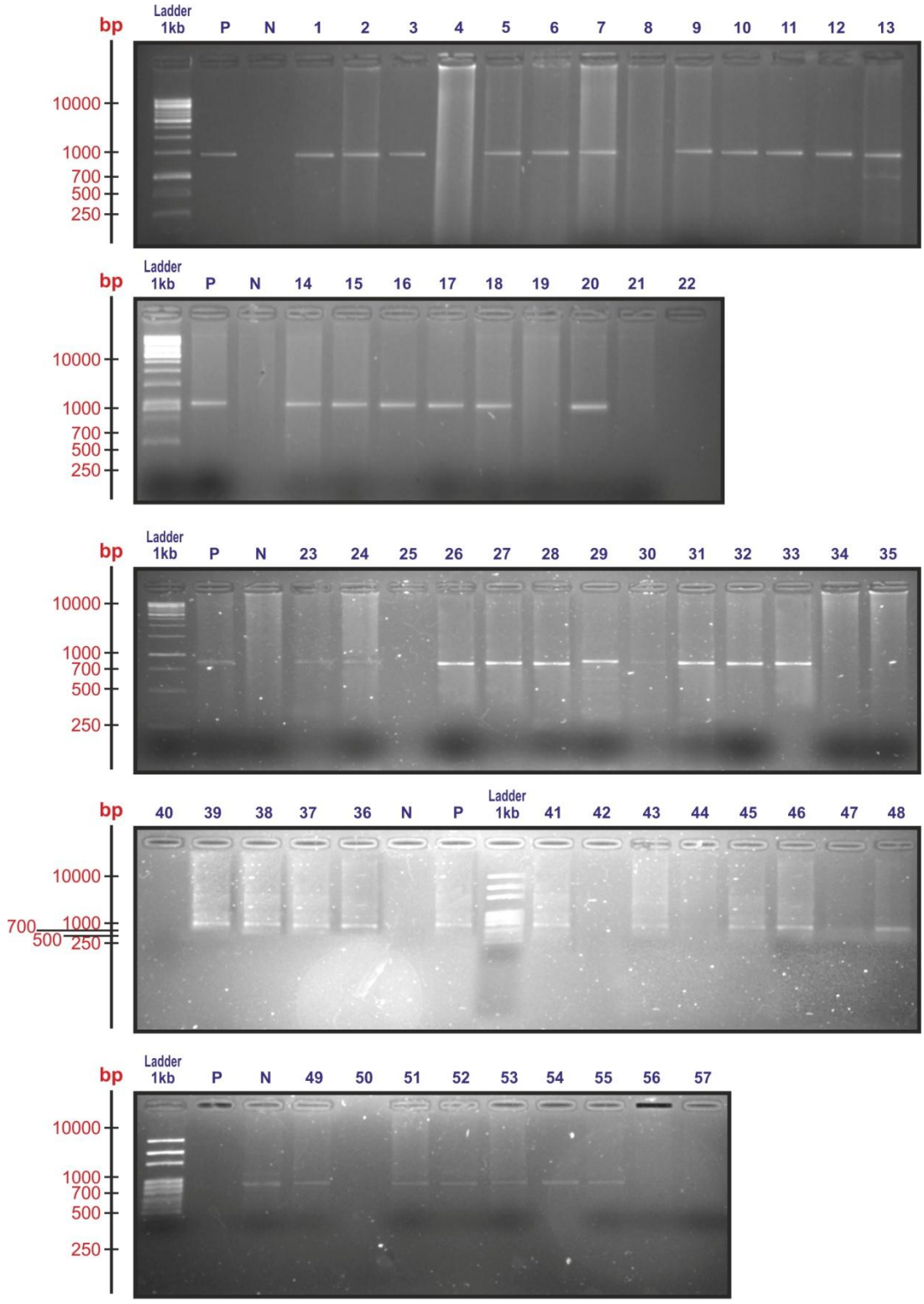


Plate 1. PCR result of *Cry1Aabc* (T_3 Embryo infections)

Ladder : 1 kb; **P :** Positive Control; **N :** Negative Control; **1 - 57 :** Plant Number

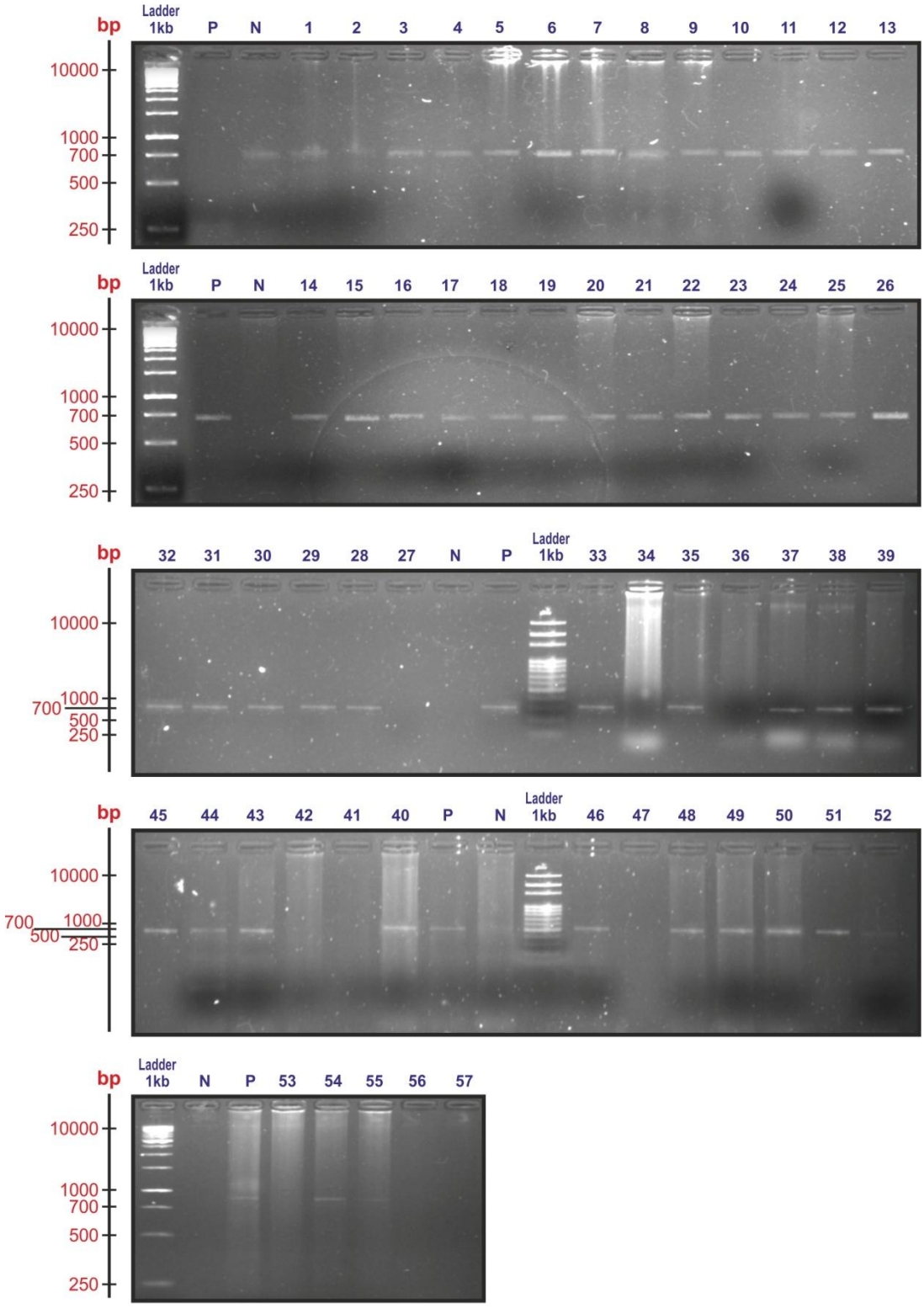


Plate 2. PCR result of *nptII* (T_3 Embryo infections)

Ladder : 1 kb; **P :** Positive Control; **N :** Negative Control; **1 - 57 :** Plant Number

Table 4.2. Analysis of T_2 and T_3 plants of year 2013

Plant No.	Progenies	Totalseedsown	Total seed harvest	Total number of plant selected for PCR	PCR positive p	PCR negative
Embryo infection T_3 2013-14						
1	1-1	10	96	3	3	0
	1-2	10	343	3	2	1
5	5-1	10	85	6	6	0
	5-2	10	123	6	4	2
	5-3	10	117	3	2	1
	5-4	10	85	3	3	0
	5-5	10	87	6	6	0
9	9-1	10	133	6	5	1
10	10-1	10	227	3	3	0
	10-2	10	193	3	2	1
11	11	10 10	290	3	2	1
19	19-1	10	91	3	2	1
	19-2	10	93	3	2	1
23	23-1	10	87	3	2	1
	23-2	10	105	3	3	0
Floral dip T_2 2014-15						
DK +1-38	DK +1-38	38	317	38	22	16

The PCR confirmation for integration of *Cry1Aabc* gene and *NptII* gene was carried out by extracting DNA from the three plants from each row of positive plant. Data of PCR amplification is presented in Table 4.2.

From 7 plants total 57 progenies were tested for PCR from which 47 plants were seen to be positive through PCR analysis. 38 plants from DK+ material were tested for PCR analysis through gene specific primers from which 22 plants were seen to be positive.

Kumar *et al.* (2004) carry out molecular analysis of the putative T_0 transformants by PCR and observed that out of a total of 40 transformants17 plants were found to be positive for the amplification of the 525-bp fragment of the *Rchit* gene by PCR.

Rao *et al.* (2008) performed PCR on T_1 transformants for both *uid* A and *npt* II genes. Out of the 350 progeny analyzed,48 plants amplified both transgenes in Pigeonpea

Das *et al.* (2016) carry out confirmation of *Cry1Aabc* gene in putative transgenic plants by PCR.PCR was performed in all progenies of pigeonpea derived from two T_0 events. In T_4 generation, 203 progenies derived from both the events were analysed of which 31 progenies have been identified as positive. These progenies were further advanced to T_5 generation. In T_5 generation, 2013 progenies derived from two T_0 events were analyzed and 84 progenies were identified as positive from both the events

4.1.2 Inheritance study of T_3

It is necessary to characterize the inheritance pattern of fusion gene in the T_1 and T_2 generation in order to understand the stability of foreign gene integration. In order to study Mendalian inheritance pattern in T_3 progeny PCR analysis for the amplification of the *cry1Aabc* gene and *NptII* gene was carried out. The plants from same parents plant was consider together for the inheritance study. For undertaking the Chi-square test for segregation the minimum population size of 6 was taken. Due to unavailability of probe and pure protein for particular Cry1Aabc gene the further analysis was not carried out so this particular study designed for the PCR analysis only. (Table 4.3)

Table 4.3. Segregation pattern of transgenic plants in T3 generation

Sr. No.	Plant Name	Total plant characterized by PCR	PCR analysis		3:1 segregation ratio	
			Positive	Negative	X^2	P
1	10-36	6	5	1	0.00	0.240
2	9-32	6	5	1	0.00	0.240
3	5-19	6	6	0	0.2	0.240
4	5-18	6	4	2	0.2	0.240
5	5-17	6	6	0	0.22	0.240
6	19-78	6	4	2	0.22	0.240
7	23	6	5	1	0.00	0.240
Df=1; P=0.005; x^2=3.841						

As observed in the Table 4.3 calculated x^2 value is less than table value in all the plant progenies. Thus plants for the amplification of the *cry1Aabc* gene and *NptII* gene and progeny of each clone shows 3:1 (presence: absence) segregation pattern (Table 4.2). However, based on the inheritance analysis, the selected lines indicated homozygosity. Sample population taken for the study is less so the prediction for the inheritance study is not foolproof.

Ramu et al. (2011) while working on expression of a synthetic *cry1AcF* gene in transgenic Pigeonpea confers resistance to Helicoverpa armigera carried out Segregation analysis with all the selected 25 lines and most of the plants followed the 3: 1 segregation pattern.

Thu *et al.* (2003) used four of six analysed lines have one transgene locus (segregation ratio 3:1), whereas two lines may have two loci (ratio 15:1).

Satyavathi *et al.*(2003) also tested Progeny from various independent transformations for the presence of the *nptII* gene by PCR analysis they observed segregation of the *nptII* transgene fit the predicted Mendelian ratio of 3:1 (presence: absence) of *nptII*.

Dayal *et al.*(2003) while working on shoot regeneration and genetic transformation of pigeonpea were observed that PCR amplification of the *uidA* gene in the T_1 generation of selected transformants showed that inheritance of the introduced genes segregated with a 3:1 Mendelian ratio for single copy integrations.

Swathi *et al.*(2006) carried out Chi-square test for segregation with minimum population size 12. However, they shown good fit for monogenic segregation suggesting single copy integration.

Table 4.4. Initiation of experiment: Dec. 2013: Floral Dip

Sr. No.	Activity	Month and Year	Remark	Progress
1	Floral Dip (T_0) (50+ flower buds attempted)	Dec 13 –Feb 2014	In all 03 attempts showed development of bud. Harvesting of pods developed out of floral dip was carried out. (T_0)	Seeds harvested plant wise (March 2014) and stored for further studies.
2	Sowing of harvested seed (T_1)	March April 2014	Single seed from each pod developed after floral dip was sown. DNA was extracted from plant grown through seed harvest of Floral dip.	One plant reported positive for npt II and Cry 1 Aabc.
3	Harvesting of seeds of positive plants (T_1)	January 2015	From one floral deep positive plant 38 healthy seeds were harvested.	In all 38 seeds were stored for further studies.
4	Sowing of seeds of positive plants (T_2) (A lot of 1-38 seeds was sown)	July 2016	In all 38 seeds were sown. Harvested from each plant from 1 to 38.	PCR confirmation of all 38 plants was carried out with gene specific marker.

4.1.3 Testing of T_2 floral dip putative transformants

Total 50 plants were attempted for floral dip transformation of Cry1Aabc gene. Out of this 3 attempts showed development of bud. Harvesting of pods developed out of floral dip was carried out. (T_0). These 3

plants were further confirmed through PCR. Out of three only one plant was seen to be positive. Total 38 seeds from one positive plant was sown and harvested from these 38 plants were confirmed through PCR by Kamble (T_1).Sowing of seeds of positive plants (T_2) (A lot of 1-38 seeds were sown) was carried out. PCR confirmations of all these 38 plants were carried out by using gene specific primer. Out of 38 plants 22 plants were shown to be positive (Plate 3 & 4).

Table 4.5. Initiation of experiment: July 2014 Embryo Infection

Sr. No.	Activity	Month and Year	Remark	
1	Embryo Infection (T_0) (130 seeds attempted)	August 2014	73 plants survived	Harvesting of seeds from embryo infected plants (T0)
3	Sowing of seeds (T_1) Single seed from one plant.	April 2015	Branch wise single seeds from all 73 plants were sown.	DNA extraction from 73 embryo infected plants and confirmation through gene specific amplification in PCR(T_1). Two plants reported positive
5	Harvesting of seeds of positive plants (T_1)	Feb-March 2016	Seeds of only one plant could be harvested.(Plant no. A)	
6	Sowing of seeds of positive plant (T_2)	July 2016	10 seeds of Plant no. A (T_2) harvested in March 2016 were sown.	
7	Re-sowing of seeds two positive plants from reserved seeds. (T_1)	July 2016	Plant No. A (T_1) and B (T_1) of Aug. 2014 infection were sown	Three seeds of plant no. A and eight seeds of plant no. B was sown.
8	Testing of T_1 and T_2 plants.	Jan. 2017	DNA was extracted from three plants form each A (T_2),A (T_1) and B (T_1)plants.	

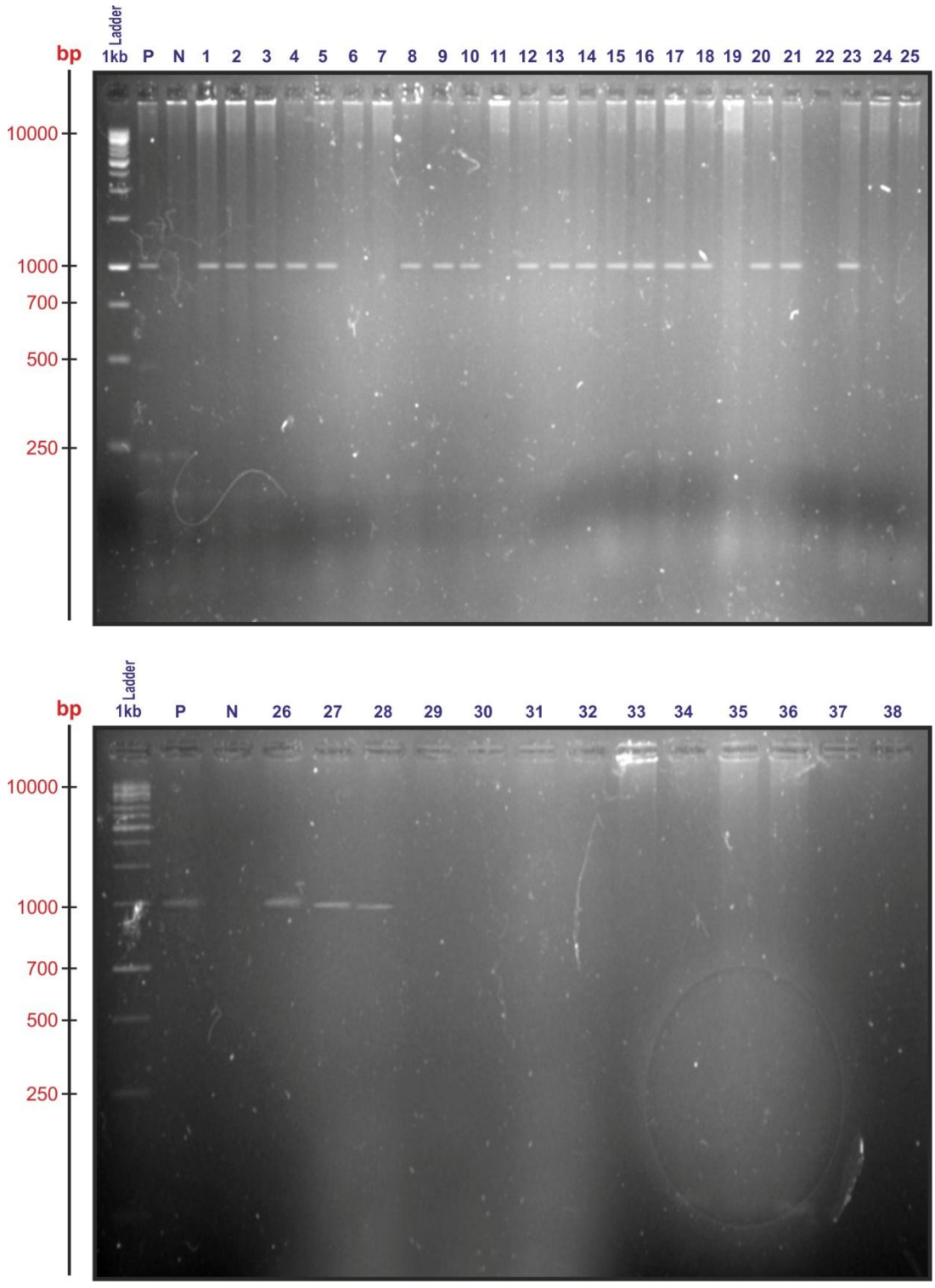
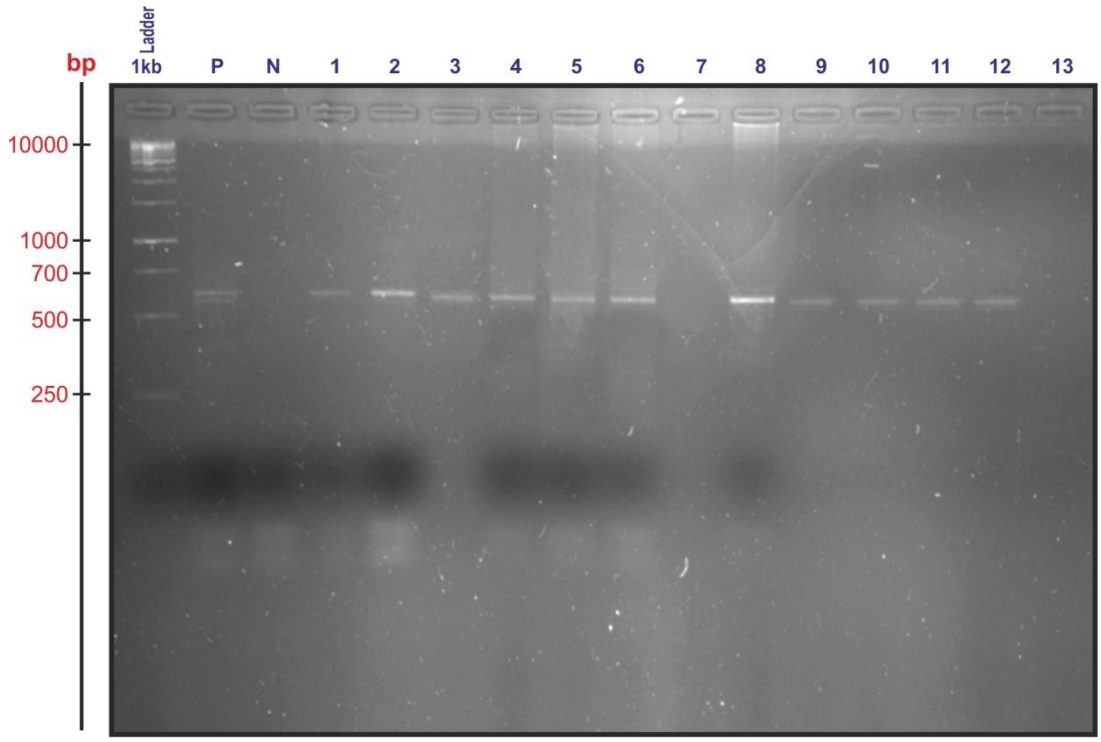


Plate 3. PCR result of *Cry1Aabc* (T_1 DK+ 1-38)

Ladder : 1 kb; **P :** Positive Control; **N :** Negative Control; **1 - 38 :** Plant Number



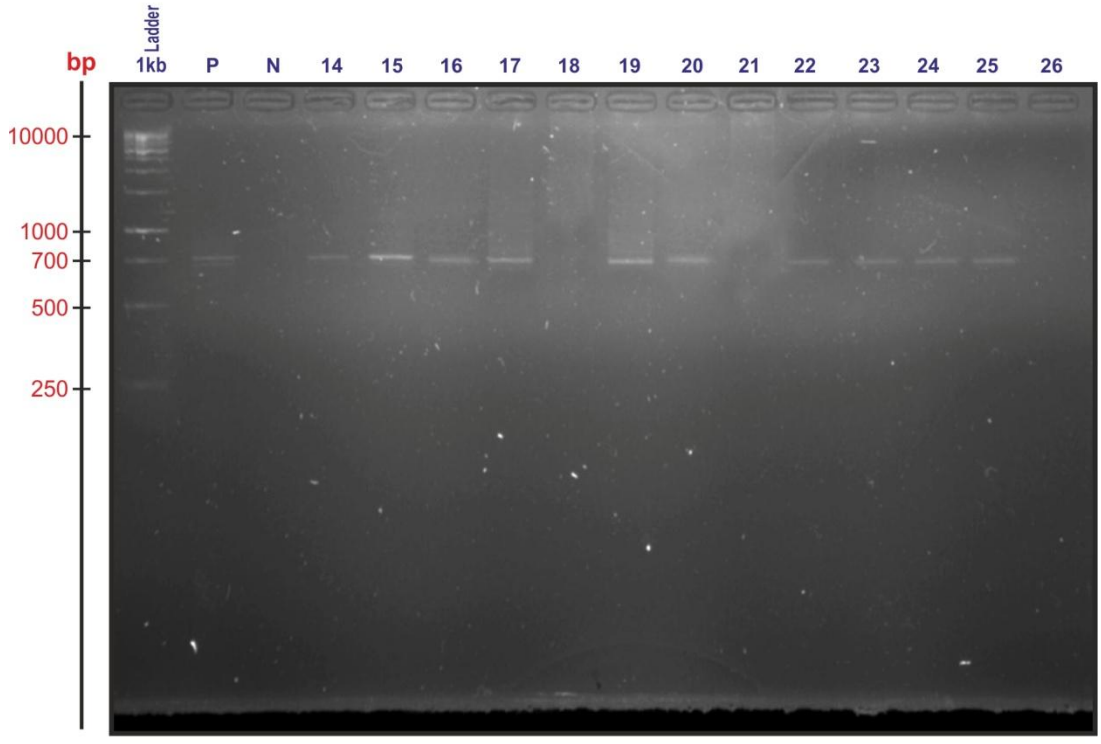


Plate 4. PCR result of *nptII* (T_1 DK+ 1-38)

Ladder : 1 kb; **P :** Positive Control; **N :** Negative Control; **1 - 26 :** Plant Number

Ramu *et al.* (2011) observed 22 primary transformants, 2640 T_1 plants were established in the green house.PCR analysis with *nptII* specific primers of the composite samples revealed the possibility of presence of the gene in 849 plants, These plants were further analysed for the expression and efficacy of the gene.

PCR analysis with *nptII* specific primers of 69 individual plant DNA samples revealed the presence of *N. phosphotransferase* gene (around 750 bp DNA fragment) in 43 individual plant DNA samples out of 69 verified by Ashrani *et al.* (2011) in chickpea.

4.1.4 Testing of T_1 and T_2 plantsJuly 2014 Embryo Infection

Kamble in year 2014-15 attempted 130 embryo infection with Agrobacterium strain EHA 105 harboring Cry1Aabc and NptII gene out of which 73 were survived but only two plants were confirmed positive through PCR. T_1 and T_2 generation of these two plants namely A and B were sown. DNA was extracted from three plants form each A (T_2), A (T_1) and B (T_1) plants and amplified by using Gene specific primers. PCR confirmation and data of PCR amplification is presented in Table 4.6.

Table 4.6. Initiation of experiment: July 2014 Floral Dip

Sr. No.	Activity	Month and Year	Remark	Progress
1	Floral Dip (T_0) (80 floral buds attempted)	Jan 2015	In all 13 attempts showed development of bud.	Harvesting of seeds from embryo infected plants and pods developed out of floral dip. (T_0)
3	Sowing of seeds (T_1)	April 2015	13 plants of FD were sown.DNA extraction from plant grown. (T_1) was carried out.	Two plants confirmed as positive. But seeds could not be harvested due to damage to plant.
5	Re-sowing of another remaining seed of same pod	July 2016	DNA extraction from plant grown. (T_1) was carried out.	Seed from each positive plants were harvested and stored for further studies.

4.1.6 Testing of T_1 floral dip 2014

80 floral buds attempted in year 2014-15 by Kamble out which in all 13 attempts showed development of bud. Out of 13 two plants were confirmed as PCR positive. But seeds could not be harvested due to damage to plant. Re sowing of the two plants were carried out. These two plants were confirmed through PCR by gene specific primers. Both the plants were showed positive result plate. (Table 4.7 and Plate 5 & 6)

Table 4.7. Analysis of T_1 and T_2 plants of year 2014

Plant No.	Progenies	Total seed sown	Total seed harvest	Total number of plant selected for PCR	PCR positive	PCR negative
Embryo infection T_1 and T_2 2013-14						
A	A (T_1)	3	120	3	3	0
	A(T_2)	10	335	3	2	1
B	B(T_1)	8	316	3	3	0
Floral dip T_2 2014-15						
9F D		1	13	1	1	0
5FD		1	13	1	1	0

From two positive plants total 6 progenies from T_1 generation and 3 plants from T_2 generation were tested for PCR analysis all the 6 plants from T_1 generation seen to be positive while from T_2 generation out 3 two plants were showed positive(Table 4.7).

Keshmma *et al.*(2008) established effective protocol to the extent of recovery of 17% of the T_1 generation plants as transformants.

In T_4 generation, 203 progenies derived from both the events were analysed of which 31 progenies have been identified as positive. These progenies were further advanced to T_5 generation. In T_5 generation, 2013 progenies derived from two T_0 events were analyzed and 84 progenies were identified as positive from both the events by das et al in pigeonpea (2016).

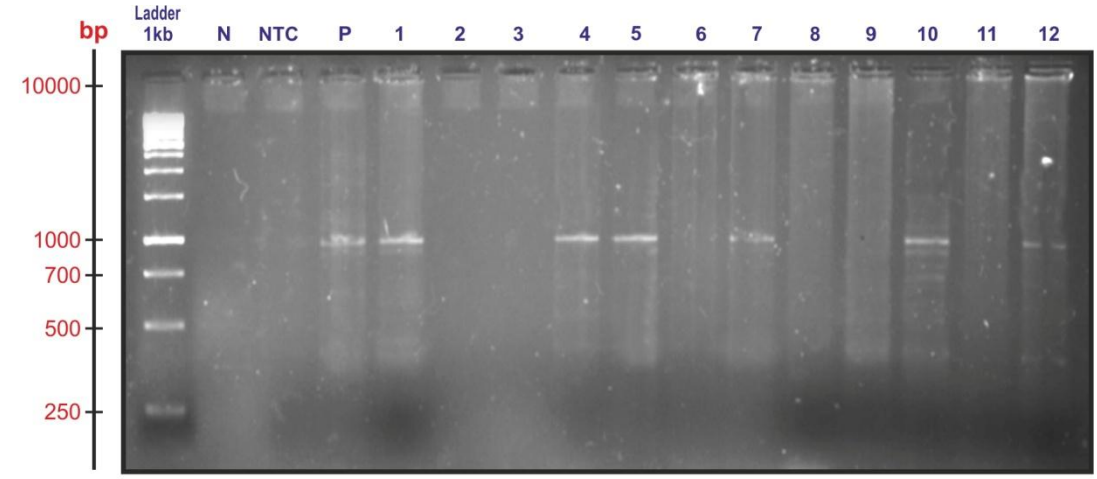
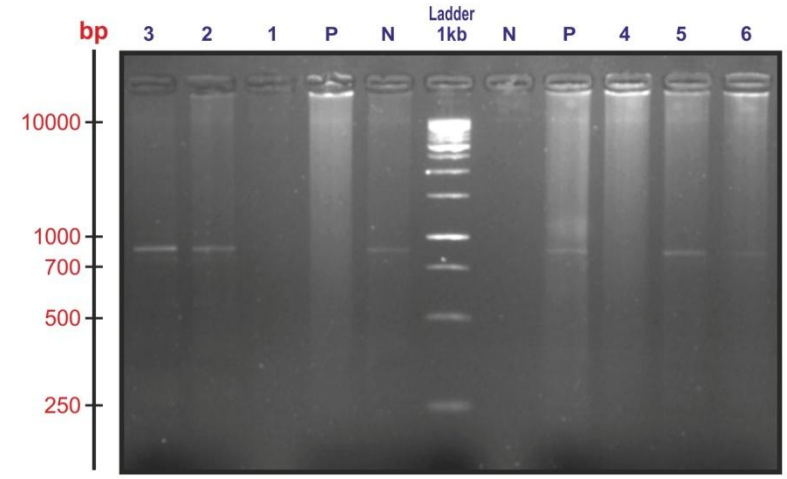


Plate 5. PCR result of *Cry1Aabc* (T_1 & T_2 2014)



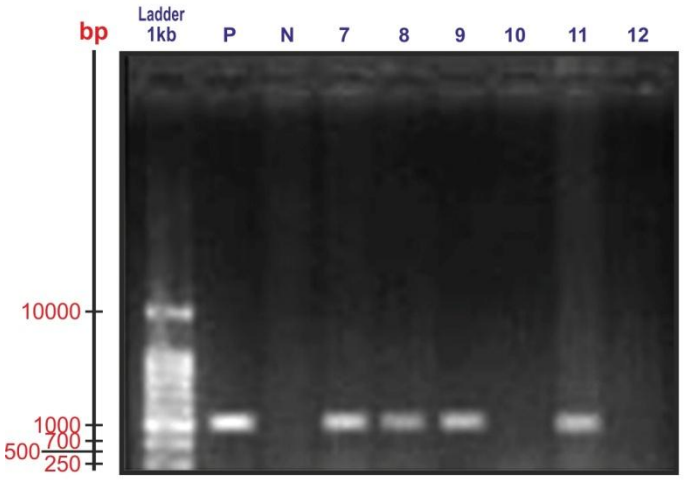


Plate 6. PCR result of *nptII* (T_1 & T_2 2014)

Ladder : 1 kb; **P :** Positive Control; **N :** Negative Control; **NTC :** Non Template Control;
1 - 12 : Plant Number

4.1.7Inheritance study of T_1 and T_3 plants of year 2014

Sample population size of T1 and T2 plants were too less to study inheritance pattern.

4.2 Insect bioassay

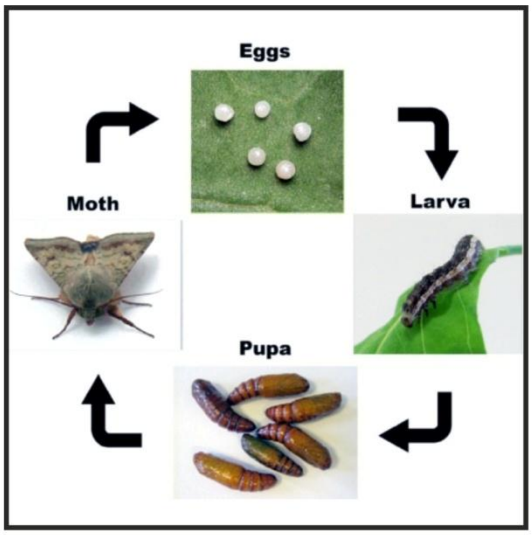
Bioassay was carried out to evaluate the potential of transformed pigeonpea plants. Leaves from normal (control PKVTARA) and transformed plants were used for insect feeding. 20 putative transformants of pigeonpea were subjected to insect (*Helicoverpa armigera*) bioassay using neonates of *Helicoverpa armigera* and young trifoliate leaves and WT (non-transformed) PKV Tara was used as control.

4.2.1Detached leaf bioassay

For detached leaf bioassay 20 plants were taken out of which 19 were PCR positive and one was PCR negative. These 20 plants were tested by using leaf feeding assays with neonate larvae of *Helicoverpa armigera.*

The solidified agar-agar was used as substratum for holding pigeonpea leaf in slanting manner inside the bottles. 10 neonates per replication were released on the pigeonpea leaves. Observation were recorded on average larval weight in mg, percent weight reduction as compared to the control, leaf area damage estimated by scale 1-10 , no. of larvae survive(Table 4.8 and Plate 8).

The detached leaf assay was carried out to test the effect of *cry1Aabc* gene in transformed plants to control the population of *H. armigera*. It was revealed that the positive plants had expressed cry protein in sufficient concentration to reduce the weight of larvae and leaf damage as shown in Table 4.8. It is recorded that the all positive plant resulted in larval weight reduction. The percent weight reductions in transgenic plants were observed were between 73%- 31%. One negative plant was resulted higher leaf damage and lower reduction in larval weight. Plant No. 17 showed highest weight reduction of 73.36%(Table 4.9 and Fig. 4.1).

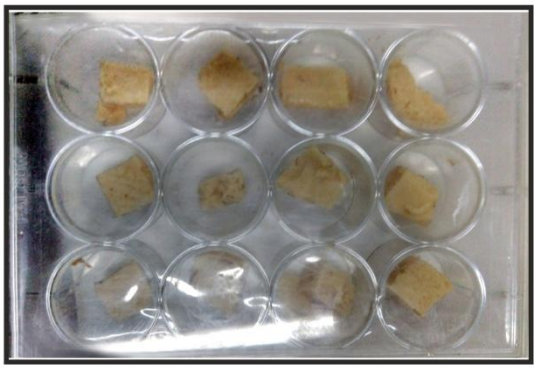


H. Armigera life cycle

H. armigera larvae on artificial diet

H. armigera pupa

Mating chamber



H. armigera neonates

Plate 7. Rearing of *Helicoverpa armigera*

Control

PCR negative plant

PCR positive plant

Plate 8. Bioassay result

Table 4.8. Observations recorded for insect bioassay by detached leaf method

Plant No	Average larval wt (mg)	% Weight reduction as compare to control	% survival rate	Leaf area damage rating 5DAI
Control	28.5	0.000	63.3	7.3
Negative	27.3	4.30	56.6	6.6
1(T_3)	13.4	52.8	40.0	4.3
2 (T_3)	12.8	56.6	40.0	4.3
3 FDA (T_2)	10.5	63.2	40.0	2.6
4(T_3)	10.3	63.9	36.6	2.3
5 (T_3)	14.6	48.8	50.0	4.6
6 (T_3)	12.5	56.2	30.0	4.0
7 (T_3)	11.7	59.0	30.0	3.3
8 (T_3)	7.8	72.2	36.6	2.6
9 (T_3)	11.7	59.0	36.6	4.3
10 (T_3)	13.4	53.0	30.0	3.6
11 (T_1)	8.8	69.2	33.3	3.0
12 (T_3)	15.8	44.6	23.3	3.3
13 (T_2)	12.0	58.0	40.0	4.3
14 (T_1)	10.3	63.9	30.0	2.6
15 (T_1)	11.5	59.7	23.3	3.0
16 (T_2)	9.9	65.3	33.3	3.0
17 (T_1)	8.0	72.0	33.3	3.0
18 (T_2)	13.0	54.4	36.6	4.6
19 (T_2)	13.6	52.3	23.3	2.3
C.D.	6.1	12.2	15.728	1.215
SE(m)	2.1	4.2	5.492	0.424
C.V.	28.1	14.3	7.766	19.365

It is observed that all positive plants showed lower leaf damaged as compare to the control i.e. PKVTARA after the interval of 5 days. Plant No. 4 and 19 shows lowest leaf damage i.e. (2.33), plant no 3 and 6 and 14 also showed less damage about 2.66, plant 11, 15, and 16 showed damage into scale 3. Plant no.6, 1, 2 10, 14, 5, 18 were showed leaf damage into scale 4-5 and one PCR negative plant showed higher leaf damage rating (6.6) (Table 4.8 and Fig. 4.1).

Similarly percent survival rate of larvae was also reduced as compared to the control plant. Plants No. 12, 15, 18 showed lowest survival rate of larvae 23.33%, plant 10, 11 shown survival rate of larvae 30%, plant 16, 17 and 11 recorded survival rate 33.33 %, plant 4, 8, 9 and 18 recorded 36.66%, plant 1, 2, 3 and 13 shown 40% plant 6 shown high survival rate of larvae i.e. 50% and negative plant recorded highest survival rate of larvae 56.66% (Table 4.8 and Fig. 4.1).

It observed that all positive plant showed lower average larval weight as compared to the control (non transformed). It ranges from 44.6 to 72.2. The PCR negative plant shows average larval weight nearly equal to the control. The plant 8 showed lowest larval weight i.e. 4.30.

Thus, the result revealed that the levels of cry protein expressed in positive plants are able to reduce the weight of larvae and reduce the leaf damage. Thus it was concluded from above results that positive plants selected for bioassay study having potential for providing tolerance against *helicoverpa armigera.*The further confirmation in sub sequent generation is required to come to more robust conclusion.

Simillar result were found by Das *et al.* (2016) work on expression of chimeric *Bt* gene, *Cry1Aabc* in transgenic pigeonpea (*cv*. Asha) confers resistance to gram pod borer (*Helicoverpaarmigera* Hubner

they reported that two pigeonpea lines IPCc1 and IPCc2 were subjected to insect (*Helicoverpa armigera*) bioassay using 5 days and 7 days old larvae and young trifoliate leaves and pods, respectively. WT (non-transformed) Asha was used as control. Larval mortality between the controls did not differ significantly. In leaf bioassay, there was significantly greater defoliation in control lines than in transgenic pigeonpea lines.

Gopalaswamy (2005) also reported that the transgenic pigeonpea plant carrying Bt *Cry1Ab* and soybean trypsin inhibitor genes were molecularly characterized for the presence of insecticidal genes and bioassay were conducted to test their efficacy against gram pod borer *H. armigera* under laboratory conditions. They observed that the *cry1Ab* toxin level present in the leaves of transgenic plant could not inhibit the feeding by larvae.

Gopalaswamy (2008) also reported that Introduction transgenic insect-resistant pigeonpea can be considered as one of the components for minimizing the losses due to *Helicoverpa armigera* (Hubner). Transgenic pigeonpea plants of ICPL 88039 and ICPL 87 carrying *Bacillus thuringiensiscry1Ab* and soybean trypsin inhibitor (SBTI) genes were evaluated for resistance to *H. armigera* under field conditions, as well as in vitro bioassays using leaves, inflorescences, and pods. Lack of significant reduction in leaf feeding, larval survival, and larval weight indicated that the levels of *Cry1Ab* endotoxin or *SBTI* present in the transgenic pigeonpea plants were not sufficient. Some plants though showed lower leaf damage, larval survival, and larval weight, owing to the inadequate levels of expression of the transgenes, resistance levels were not consistent. Infestation of transgenic plants with neonate larvae at flowering, supplemented with leaf or pod bioassays under laboratory conditions can be used effectively to evaluate transgenic pigeonpea for resistance to *H.*

armigera as the non transgenic plants of ICPL 88039 and ICPL 87 recorded, respectively and suffered significantly less damage among the transgenic lines tested than the non transgenic plants of larval weights as three day after infestation on was significantly lower compared to that on non transgenic plants.

Krishna *et al.* (2011) The transgenic plants were subjected to multi- and no choice tests to determine the behavioral responses and mortality through *Helicoverpa armigera* bioassays on the leaf and relate their relationship with the expression of *cry1Ac* protein which was found to be less in leaf as compared to the floral buds, anther, pod and seed.

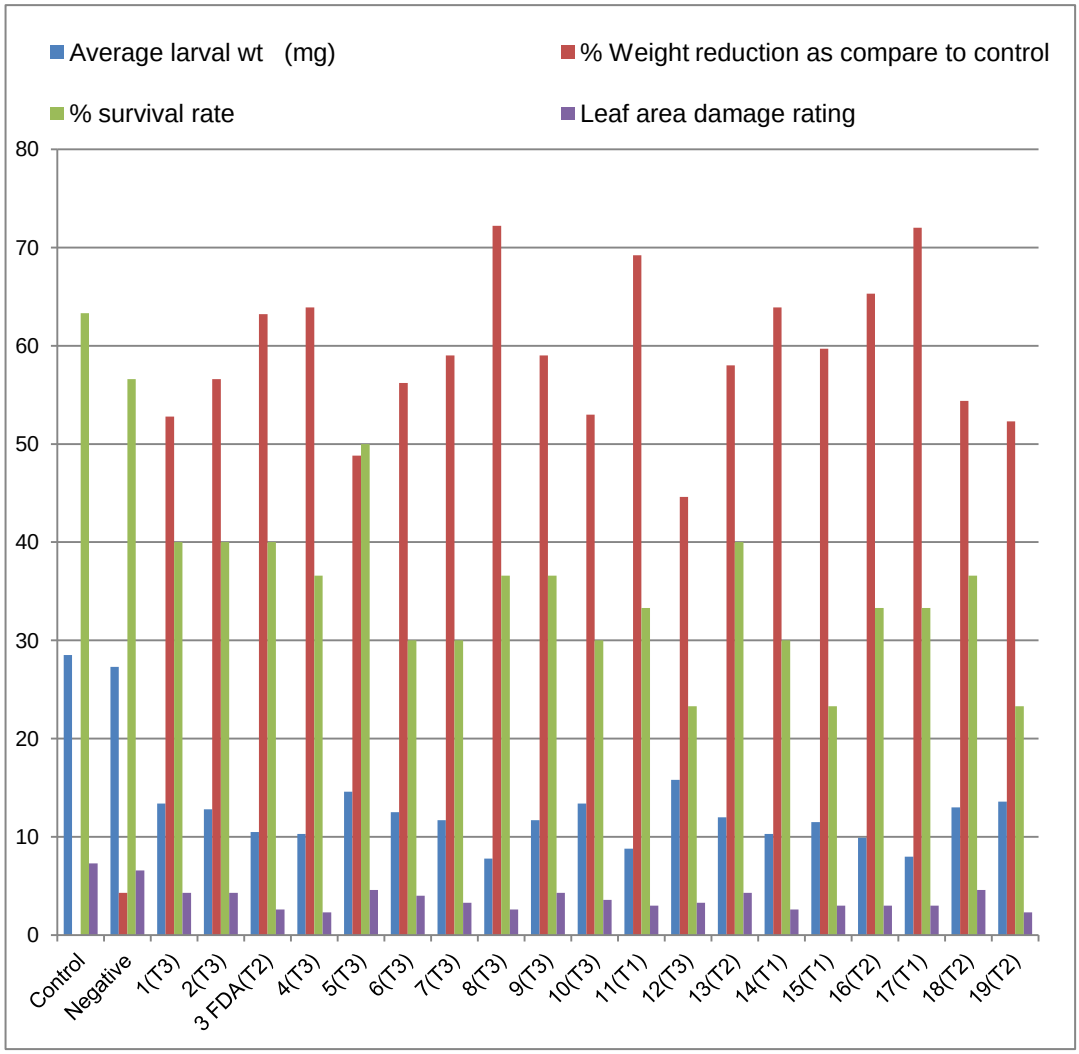


Fig. 1. Result of Detahed leaf insect bioassay

CHAPTER V

SUMMARY AND CONCLUSION

In present study molecular analysis of T_1, T_2 and T_3 generation of putative transformants in pigeonpea were carried out by PCR with gene specific primers. Molecular characterization of transgenic plants is essential, as the number of transgene copies influence the expression level and genetic stability of the transgene. DNA is randomly inserted into the plant genome during *Agrobacterium* transformation procedures. This often leads to the generation of plants that can have multiple transgene copies integrated into one or more chromosomal locations.

Stable introduction of transgene into genome of plant also offers strategies for over expression or suppressing endogenous gene. Thus, introducing new gene expression via transformation generates new phenotypic variation useful for investigating new function and crop improvement.

The introduction of a transgene into a recipient genome is a complex event depending on the transgene itself and the host genome. The PCR positive plants resulted from *in-planta* inoculation of embryo axes of germinating seeds and floral dip with pBinAR-*cry*1*Aabc* mobilized into *Agrobacterium tumefaciens* strains EHA 105 are maintained in containment facilities for further study of inheritance of transgene and the level of transgene expression.

From 7 plants of T3 generation total 57 progenies were tested for PCR from which 47 plants were seen to be positive through PCR analysis. 38 plants of T_1 generation from DK+ material were tested for PCR analysis through gene specific primers from which 22 plants were seen to be positive.

Kamble in year 2014-15 attempted 130 embryo infections with Agrobacterium strain EHA 105 harboring *Cry1Aabc* and *NptII* gene out of which 73 were survived but only two plants were confirmed positive through PCR. T_1 and T_2 generation of these two plants namely A and B were sown. Three plants from eachwere selected for PCR confirmation.From two positive plants total 6 progenies from T_1 generation and 3 plants from T_2 generation were tested for PCR analysis all the 6 plants from T_1 generation were seen to be positive while from T_2 generation out 3 only two plants were positive.Sample population size of T_1 and T_2 plants was too less to study inheritance pattern.

It is necessary to characterize the inheritance pattern of fusion gene in the T_1 and T_2 generation in order to understand the stability of foreign gene integration. In order to study Mendalian inheritance pattern in T_3 progeny PCR analysis for the amplification of the *cry1Aabc* gene and *NptII* gene was carried out. The plants from same parents plant was consider together for the inheritance study. For undertaking the Chi-square test for segregation the minimum population size of 6 was taken. Due to unavailability of probe and pure protein for particular Cry1Aabc gene the further analysis was not carried out so this particular study designed for the PCR analysis only.

Calculated x^2 value is less than table value in all the plant progenies. Thus plants for the amplification of the *cry1Aabc* gene and *NptII* gene and progeny of each clone shows 3:1 (presence: absence) segregation pattern. However, based on the inheritance analysis, the selected lines indicated homozygosity. Sample population taken for the study is less so the prediction for the inheritance study is not foolproof.

For detached leaf bioassay 19 positive plants and one negative plant which were confirmed by DNA extraction and PCR amplification with *NptII* and *Cry1Aabc* gene specific primers were tested using leaf feeding assay with neonate larvae of *Helicoverpa armigera*. 20 putative

transformants of pigeonpea were subjected to insect (*Helicoverpa armigera*) bioassay using neonates of *Helicoverpa armigera* and young trifoliate leaves and WT (non-transformed) PKV Tara was used as control.

Thus, the result revealed that the level of Cry protein expressed in positive plants were able to reduce the leaf damage. Thus, it was concluded from above results that like positive plants selected for bioassay study having potential for providing tolerance against *Helicoverpa armigera*. The further confirmation in subsequent generation is required to come to more robust conclusion.

CHAPTER VI

IMPLICATION

Molecular characterization of transgenic plants is essential, as the number of transgene copies influence the expression level and genetic stability of the transgene. DNA is randomly inserted into the plant genome during *Agrobacterium* transformation procedures. This often leads to the generation of plants that can have multiple transgene copies integrated into one or more chromosomal locations.

The perfect transformant would contain single copy of transgene that would segregate as Mendelian trait, with uniform expression from one generation to next. Transformation technologies have been advanced to the point of commercialization of transgenic crops. The introduction of transgenic varieties in the market is multi step process that begins with registration of new variety followed by field trials and ultimately delivery of seed to the farmer.

CHAPTER VII

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