Studies on tissue culture and genetic transformation of pigeonpea [Cajanus cajan L. (Millsp.)]

A thesis submitted in
Partial fulfilment of the requirements for the degree of
Master of Technology in
Biotechnology

by
G.Krishnavenamma

SCHOOL OF BIOTECHNOLOGY
JAWAHARLAL NEHRU TECHNOLOGICAL UNIVERSITY
HYDERABAD, ANDHRA PRADESH
INDIA
1999

To

My Parents

CERTIFICATE

This is to certify that the dissertation entitled **"Studies on tissue culture and Genetic transformation of Pigeonpea [*Cajanus cajan* L. (Millsp.)]"** is submitted in partial fulfillment for the award of degree of Master of Technology in Biotechnology, to the Jawaharlal Nehru Technological University, is a bonafide work carried by **Mrs. G. Krishnavenamma**, under our guidance and supervision.

The results embodied in this dissertation have not been submitted to any other university or institution for the award of any degree or diploma.

Dr. Lakshmi Narasu
Head, Centre for Biotechnology
Jawaharlal Nehru Technological University
Hyderabad, India.

Dr. Kiran K. Sharma
Senior Scientist
Genetic Transformation Laboratory
ICRISAT, Patancheru, India

Acknowledgements

I express deep sense of gratitude to Dr. Kiran K. Sharma, Senior Scientist, Genetic Transformation Laboratory, ICRISAT, for his invaluable guidance, supervision and cooperation during the course of their study.

I deem it's a privilege in extending gratitude and heartfelt thanks to Dr. M. Lakshmi Narasu, Head, Department of Biotechnology, JNTU, Hyderabad for her valuable suggestions and support.

I am sincerely thankful to Dr. B. Diwakar, Senior Manager, Training and Fellowships Program, ICRISAT, for giving an opportunity to work in a prestigious institute.

I thank ICRISAT for providing the research facilities during the course of this study.

I am thankful to Dr. Anjaiah, Visiting Scientist, ICRISAT, for his guidance and suggestions in carrying out experiments.

I sincerely acknowledge the help rendered by technical staff of GTL, ICRISAT, Mr. Jaganmohan Reddy, Pandari, Bekkemma, and Maniamma.

I am thankful to Mr. Jayanand, Miss Sunitha, Miss Bhargavi, Mr. Ramchander of GTL and Miss Manjula for their affectionate care, where I was able to complete this course successfully.

I am highly indebted to my husband Dr. E. Sudhakar Reddy for his encouragement to successfully complete this project.

I am heartily thankful to my brother, G. S. M. Reddy, sister-in-law and sisters for their love and support.

Krishnavenamma

CONTENTS

Introduction

INTRODUCTION

High yielding plant varieties are usually prone to insect pests and diseases. About 50% of potential food production is lost due to this problem. Control of the insect pests have been mainly relied upon the use of synthetic chemical pesticides. Although, chemical insecticides have been valuable in the control of insect pests of crop plants, their use has posed certain problems. They tend to harm non-target organisms such as humans, domestic animals, beneficial insects that help in pollination, and control of harmful insects and wildlife. These pesticides are not easily degraded in the soil and tend to accumulate in the food chains, leading to amplification of their effects. Their residues not only remain in the crops, but also in air, water and soil. Continuous use of pesticides has led to the development of resistant strains of insect pests. Due to such complexities arising from the use of chemical insecticides the use of microorganisms as agents to control insect pests of crops has become very significant in recent years, as one of the promising alternatives (Aizawa, 1982).

A number of pathogenic microorganisms on insects, such as viruses, rickettsiae, mycoplasma, bacteria, fungi and protozoa have been recorded. Since microorganisms pathogenic to insects occur naturally, microbial insecticides are usually nontoxic to humans, domestic animals and plants. They are nonpathogenic to nontarget insects owing to their relatively high degree of specificity. The selective use of microorganisms towards target insects is advantageous in the conservation of ecosystem (Aizawa, 1982).

Some disadvantages in the use of pathogenic microorganisms for the control of insect pests are:

1. The immediate effectiveness is not seen owing to the incubation period of the microorganism in the body of insect.
2. The specificity or the narrow host range of microbial insecticide is disadvantageous in practical insect control.
3. The contact infection occurs only in the case of fungal infection while in the case of insects, they should necessarily ingest leaves coated with microbial insecticides before being infected.

However, during the past few years, bio-control agents like bio-pesticides and botanical pesticides (neem products) have been developed. Despite their remarkable safety and efficiency, they have not generated a good market because of low cost and longer persistence of synthetic chemical pesticides. Therefore, development of crop plants having inherent resistance mechanism against pests is the only permanent solution.

Advances in the genetic transformation of crop plants has facilitated the introduction of insecticidal genes not only across plants but also from lower organism like bacteria, viruses and fungi and expressing them in plant tissues in a controlled manner. The most widely used technique for the transfer of desired genes into crop plants is genetic transformation using *Agrobacterium tumefaciens*, a host pathogenic soil bacterium. Many plant species especially dicots have been successfully transformed using this method. However many monocots to which cereal crops belong, remain recalcitrant to "Agrobacterium" technique. Recently developed technique of plant transformation such as "particle gun or biolistic" have solved the major problem of incompatibility between the technique used and the crop plant transformed (Gatehouse, 1991).

The development of efficient regeneration system which is now available for some crop species and vigorously pursued in others will result in the production of novel plants. Combined with plant regeneration, the transformation of legumes with insect resistance genes will also aid in increasing yield to feed millions of poor people around the globe.

In model plants like tobacco, leaf discs are the most favoured explant source for *Agrobacterium*-mediated (Horsch et al. 1985) and direct DNA transfer using microprojectile bombardment (Tomes et al. 1990). Besides tobacco, which provides a model system for transfer and regeneration studies pigeonpea *(Cajanus cajan* (L.) Millsp.) is an important grain legume in South Asia, East Africa, and Latin America, providing dietary protein, fodder, firewood,and building materials, fixing of atmospheric nitrogen and plays an important role in the sustainability of semiarid agricultural system. The crop is attacked heavily by insects, the most important of which is *Helicoverpa armigera* (Lateef and Reed 1990, Shanower et al. 1994). This single pest causes yield losses of up to 100% in some years and locations, and worldwide losses to pigeonpea of more than $300 million per year (ICRISAT 1994). Farmers

increasingly rely on synthetic insecticides to manage this pest in pigeonpea. This has increased the risk of environmental contamination, the loss of biodiversity, and contributed to the development of insecticide resistant *Helicoverpa armigera* populations (Jadav and Armes, 1992 b). Identification and incorporation of resistance to *Helicoverpa armigera* in pigeonpea would facilitate the development of a more integrated and sustainable approach to managing insect pests in pigeonpea.

In an attempt to induce resistance to insect pests (*Helicoverpa armigera*) in pigeonpea, we carried out studies on Bt CrylA(b) gene from *Bacillus thuringiensis* with the following objectives:

a. Isolation of plasmid from *Agrobacterium tumefaciens*.
b. Plant regeneration of pigeonpea by organogenesis.
c. Transformation of axillary meristem of pigeonpea and leaf discs of tobacco with Bt gene through biolistic and *Agrobacterium*-mediated gene transfer.
d. Histologocal assay of Bt gene from recombinant cells and transformed tobacco plants.

Review of Literature

REVIEW OF LITERATURE

A. Economic importance of pigeonpea :

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an erect, woody, perennial shurb that is commonly grown as an annual. It is very important as a field crop in India, Eastren Africa, and in the Caribbean region, but it is also grown as a backyard or hedge crop in most countries in the tropics and subtropics. Its seed is harvested and eaten while greeen (mainly in Africa and the Caribbean) or when mature (mainly in India). The plants are also used for fodder, as fuel wood, for basket making, and in construction. The crop is well known as a soil improver, for its roots penetrate deep, its nodules (containing rhizobia) fix nitrogen, and the shed leaves add a considerable quantity of organic matter to the soil (Reed et al. 1989).

B. Constraints to pigeonpea productivity :

Pigeonpea is a host for many insects. Over 150 species have been reported to feed on it in India alone, and more will be found. Although many insects feed upon pigeonpea from the seedling stage, most of the economic damage is caused by pests that feed upon flowers and pods (Table 1). At the flowering stage, well grown, medium- and long-duration pigeonpea is generally too tall and dense to be treated effectively by insecticides applied manually. More attention is now being given to short-duration, sole crops of pigeonpea, which flower when about 1 m tall. Such crops can be conventionlly treated with insecticides. Short-duration, determinate genotypes with large, white seeds are particularly popular, but these are susceptible to insect damage and must be adequately protected (Reed et al. 1989). At ICRISAT, efforts have been made to identify sources of resistance to the major pests , particularly to *Helicoverpa armigera* and *Melanagromyza obtusa* and to incorporate these developed lines will produce greater yields in farmers' fields with no or minimal pesticide application.

C. Tissue culture and transformation of pigeonpea :

Attempts to obtain pest-resistant genotypes of pigeonpea by conventional breeding methods have not been successful because of limited genetic variation and sexual incompatibility with wild relatives (Nene et al. 1990). Genetic engineering approaches to

introduce genes coding for insecticidal proteins into pigeonpea may prove useful in obtaining pest resistant genotypes (Kumar et al. 1996). A prerequisite for the genetic transformation of a crop is the availability of a good protocol for in vitro plant regeneration system. Genetic improvement of this crop through biotechnological approaches has not been achieved so far mainly due to its recalcitrance in tissue culture. In vitro plant regeneration via organogenesis from leaf callus was successful in pigeonpea variety, ICPL161 (George et al. 1993). True breeding lines with dwarf stature and non-striped pods were isolated from the regenerants.

The techniques of plant tissue culture have also complemented many of the efforts of plant breeders by increasing the viability of wide-hybrids necessary to introgress useful genes from wild relatives of the crop into cultivated germplasm. The immense possibilities offered by the application of the techniques of tissue culture and protoplast fusion for genetic upgrading of economically important plants have been recently emphasised by Murashige (1978).

There have been several reports of plant regeneration via organogenesis in pigeonpea by using different explants. Rao and Narayanswamy (1975) reported regeneration of shoots from callus cultures of hypocotyls obtained from gamma-irradiated seeds on a differentiating medium. But they failed to regenerate unirradiated controls. Cultural conditions for regeneration from callus of leaves and cotyledons were defined by Kumar et al. (1983), with an emphasis on creating genetic diversity in this crop. They also reported production of 5 to 18 shoot-buds from excised cotyledons of pigeonpea when cultured on 2.25mg/L benzylaminopurine (BAP) containing medium. In a brief communication, Mehta and Mohan Ram (1980) reported formation of 5 to 35 shoot buds from the surface of the cotyledons of the seedlings raised in Gamborg's B5 (Gamborg et al. 1962) medium supplemented with BAP (2.25mg/L). They observed that when the cotyledons excised from water - soaked seeds were planted on B5 +BAP (2.25mg/L), only 3 to 5 shoot buds initiated (as against 5 to 35) from the surface. It appears that the presence of the embryonal axis stimulates the production of buds on the cotyledons.

Plant regeneration, which is the major limiting factor for transformation of pigeonpea has been reported (Shivaprakash et al. 1984) via multiple shoot formation from the cotyledonary node region of seedlings germinated on MS medium containing 2mg /L BAP

Within 2 weeks they observed a mass of multiple shoot-initials that formed at the axillary bud region of the cotyledonary node of the seedlings. It was also observed that the cotyledonary nodal explant (after removing the preexisting shoot initials on the surface layer), cultivated on 2mg./L BAP containing medium resulted in the formation of new shoot initials.

The influence of IAA and some IAA-aminoacid conjugates such as IAA-glycine, IAA-phenylalanine, IAA-alanine and IAA-aspartic acid at a concentration of 1.0 μM in combination with BAP on in vitro shoot regeneration from leaf discs of pigeonpea was investigated by George and Eapen (1994). However attempts to regenerate plants via somatic embryogenesis did not succeed although somatic embryos developed from immature cotyledon explants.

Efficient plant regeneration via somatic embryogenesis has been developed in pigeonpea by Sreenivasu et al. (1988). They observed cotyledon and leaf explants from 10-day-old seedlings to produce embryogenic callus and somatic embryos when cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 10 μM thidiazuron (TDZ). Subsequent withdrawal of TDZ induction medium resulted in the maturation and growth of the embryos into the plantlets on MS basal medium. High yields of protoplasts were obtained by (Sarangi et al. 1992) from leaves of asceptically grown plants and calli originated from different explants, in several cultivars of *Cajanus cajan*. However, no shoots or plants were obtained from these protoplasts

George and Eapen (1994) observed shoot regeneration from the distal end of cotyledons where whole cotyledons were cultured. No reports describing genetic transformation of pigeonpea are available, probably because an efficient, reproducible, high - frequency direct regeneration system is lacking. Mohan and Krishna Murthy (1998) reported for the first time de novo organogenesis from the distal half of cotyledon explants that lack pre-existing meristems in the pigeonpea genotypes T-15-15 and GAUT-82-90. The regeneration system may be applicable to *Agrobactarium* - mediated gene transfer as well as particle bombardment-mediated transformation.

The use of tissue culture procedure or in vitro culture was found to be highly mutagenic especially if the plant is regenerated from the callus induced from the explant (Maheshwari et al. 1995). This procedure has resulted in the possibility of getting true to type plants. Hence regenerated plants were found to be highly variable for many agronomic traits. The simple procedure has resulted in production of many useful variants which were superior to their parents. In vitro culture methods to facilitate introgression of insect resistance in crop has been made possible. However the exact mechanism by which the variability or somalonal variation (Larkin and Scowcroft,1981) is produced is not known, since many parameters influence the variability generated. Some of the changes observed in regenerated plants were lost in the progenies. Hence more studies are required on somaclonal variation technique to produce true to type plants having modified genetic traits. An attempt was made by Chintapalli et al. (1997) to exploit somaclonal variation for the varietal improvement of pigeonpea. Significant variation was observed for plant height, seed mass, and damage due to the insect pest *Helicoverpa armigera*.

The genetic transformation approach based on direct gene transfer overcomes the limitation of sexual incompatibility and therefore allows introgression of genes not possible by conventional means. If genetically engineered plants are to be used commercially, then the following requirements must be satisfied:

1) Introduction of the gene(s) of interest to all plant cells.
2) Stable maintainance of the new genetic information.
3) Transmission of the new gene to subsequent generations.
4) Expression of the cloned genes in the correct cells at the correct time.

Gene transfer refers to a process which moves a specific piece of DNA (usually a foreign gene ligated to a bacterial plasmid) into protoplasts or cells.If the gene transfer is efficient and the foreign gene is introduced into a sufficient number of cells, transient gene expression can be quantitatively measured (12-48 hours after gene transfer). Usually the level of protein encoded by the foreign gene is measured because it is simpler than measuring the level of mRNA to show the success of gene transfer. For study of transient gene expression, stable integretion of the introduced gene is not necessary but a reproducible and efficient gene transfer method is needed. Examples are PEG-mediated gene transfer electroporation, and the biolistic method (Jenes et al. 1993).

Genetic transformation refers to suitable integration of a foreign gene into the genome of explant regenerated from DNA treated protoplasts or intact cells. Integration can be demonstrated by the analysis of DNA,RNA and the production of new protein.

i. Transformation of protoplasts:-

Each single cell of a plant is potentially capable of developing into a whole plant This phenomenon is known as cell totipotence. Significant advances have been made in plant regeneration from protoplasts which has made possible to transfer foreign genes into protoplasts (Jenes et al. 1993). There are two major advantages, one is that the transfer of foreign gene is through the plasma membrane and the other is that all cells of a transgeneric plant regenerated from a protoplast will contain foreign genes of interest (nonchimeric).

Transformation of protoplasts can be achieved through various ways:-

a) Chemical agents such as polyethylene glycol (Negrutiu et al. 1987) that acts to increase the permeability of cell membranes.

b) Electroporation method, which is based on the use of short electrical pulses of high field strength to facilitate DNA uptake. Fromm et al. (1985) first reported gene transfer into maize protoplasts and Langridge et al. (1985) first reported the stable transformation of carrot protoplasts with DNA by eletroporation.

c) Protoplasts of many dicot and some monocot (nongraminaceous) species can be transformed using *A.tumefaciens* strain harboring foreign gene(s) of interest.

d) Sonication:-In this method the protoplasts are briefly exposed to ultrasound of specific frequency in the presence of a plasmid containing the desired gene (Zhang et al. 1991).

e) Microinjection is one of the most precise techniques for delivering macro molecules into protoplasts (Reich et al. 1986).

ii. Transformation of Intact cells and tissues:-

a) Using *Agrobacterium tumefaciens*, the bacterial agent of crown gall disease. Crown gall formation is the cosequence of the transfer, integration and expression of a specific segment of bacterial plamid DNA - called the T-DNA into the plant cell genome.Transformation using disarmed (nontumorigenic) *A. tumefaciens* plasmid vectors can

result in transgenic plants of normal phenotype which express the introduced gene(s). The methods for transformation of intact cells or tissues with *A. tumefaciens* have been developed using excised tissue of *Nicotiana* and *Petunia* sp. (Horsch et al. 1985; Rogers et al. 1986). But the monocots are not easily transformed by *Agrobacterium* (Raineri et al. 1990) due to the lack of evocation by *A. tumefaciens* of a wound response in these species (Potrykus, 1990).

b) Biolistic gene transfer: Biolistic gene transfer is a relatively new approach to plant transformation. The term "Biolistic" (biological ballistics) was coined to describe the nature of the delivery of foreign DNA into living cells or tissues through "Bombardment " with a biolistic device (a particle gun) (Klein et al. 1987). The process involves high-velocity acceleration of microprojectiles carrying foreign DNA, penetration of the cell wall and membranes by microprojectiles carrying foreign DNA, penetration of the cell wall and membranes by microprojectiles, and delivery of the DNA inside the cells. Some target tissues such as Embryogenic suspension culture (Fromm et al. 1990) and meristematic tissue (Mc Cabe et al. 1988) have proven to be transformable and able to give rise to transgenic plants. DNA has been reproducibly delivered into mitochondria in yeast (Johnston et al. 1988) & chloroplasts in chlamydomonas (Boynton et al. 1988) through biolistic process.

When the biolistic process is applied to plant tissues, plants regenerated from such tissues usually are chimeric in terms of introduced foreign genes due to random bombardment of a small number of cells in a multiple system. However, transformants can be sorted out and stabilised in their progenies, especially when selectable marker genes are used (Finer and Mc Mullen, 1990). Various successfully transformed plant species through particle bombardment technology are listed in table 1. Engineering of important agronomic crops such as soybean, cotton, maize, rice, etc has been restricted to a few non-commercial varieties when conventional methods are used. Particle bombardment technology allowed recovery of transgenic plants from many commercial cultivars.

D. Candidate genes used for conferring insect resistance in pigeonpea

The two types of insecticidal proteins which have been identified include bacterial toxins, notably from *Bacillus thuringiensis* (William et al. 1992), protease inhibitors, which are of plant origin (Richardson, 1977). By introduction of Bt gene from *Bacillus thuringiensis*, plants produce Bt toxin which is highly toxic to pod borer, *Helicoverpa armigera.* This endotoxin is also effective against dipteran, coleopteran and other lepidoptern insects. This endogeneuos production of Bt toxin does not harm the plants but confers on them the ability to prevent against insect attack (Urmila et al. 1988).

i). Bacillus thuringiensis :- *Bacillus thuringiensis* is an aerobic, gram positive, sporeforming bacterium found commonly in the environment. *B. thuringiensis* is a close relative of *B. cereus* with which at shares considerable DNA homology (Priest, 1981). Berliner proposed the name *B. thuringiensis* for the bacillus which he isolated from the diseased larvae of the mediterranian flour moth, *Anagosta kuhniella* in 1911. But in reality *B. thuringiensis* had earlier been discovered by Ishiwata in 1901 from silkworm, *Bombix mori* and named it sotto bacillus.

Bacillus thuringiensis can be readily recognised by the presence of the cytoplasmic parasporal body or crystal protein. The classification of *B. thuringiensis* is based on biochemical and serological criteria (de Barjac, 1982). The classification of *B. thuringiensis* has been done by the flagellar agglutination , which demonstrates the existance of flagellar antigen [H ag]. *B. thuringiensis* subspecies kurstaki, darmastadiensis, kyushuensis, israelensis are widely used in the control of insects.

a. **Insecticidal toxins produced by *B. thuringiensis*:-** *B. thuringiensis* produces several toxins such as alpha, beta, gamma exotoxins, delta-endotoxins. However, beta exotoxins and delta endotoxins are important from the viewpoints of microbial insecticides.

The beta-exotoxin is composed of adenine, ribose, glucose and alleric acid with a phosphate group. The beta-exotoxin is produced by some but not all strains of *B. thuringiensis*. The production is a strain specific property rather than a serotype property. The

exotoxin is known as thermostable exotoxin or flytoxin and is toxic not only to insects but also to mammals and plants. Usually one toxic crystal is formed in one sporangium and the shape is bipyramidal, although the size and shape of toxic crystals vary depending on the bacterial strains. Spindle shaped inclusions are frequently observed (Aizawa, 1982).

Delta-endotoxin is composed of a glycoprotein subunit. The proteinaceous toxin shows toxicity of endotoxin to a given insect varies among *B. thuringiernis* strains. Toxin genes are located on plasmids and there can be transmitted between two strains in a mixed culture (Aizawa, 1982). Since strains of *B. thuringiensis* frequently lose the ability to produce crystal proteins and rarely if even revert to crystal production, it seemed likely that the genes responsible might be located on plasmids (Debabov et al. 1977, Stahly et al. 1978). Since the crystal protein is synthesized during the early stages of sporulation, it has been suggested that sporulation-specific forms of RNA polymerase may be involved. Analysis of the promoler and mRNA initiation sites of the gene from subspecies kurstaki CHD-D supports this contention and shows that crystal protein synthesis is under tramuriptional control from a promoler that resembles the σ37 promoter of *B. subtilis* (Wong et al. 1983). A large number of distinct Bt toxin genes have now been cloned and referenced since the first one in 1985. In 1989, Hofte and Whiteley classified 42 Bt crystal protein genes, grouped into 4 major classes on amino acid sequence and host range.

Two important sources of heritable genetic variability that could influence insecticidal protein expression and efficacy against target lepidopteran insects are the effects from the BTK gene insertion position in the plant genome (positional effects), and effects from the tissue culture or plant regeneration process (somaclonal effects). The effect of foreign genes insertion position on plant physiology, morphology and growth is caused by the influence of the foreign DNA (for eg: CryIA base sequences plus selectable marker and promoter sequences) on the native gene(s) at or near the position of insertion in the plant genome and indirect effects caused by foreign gene expression and interactions with the plant genome. The tissue culture or plant regeneration effects are the result of the heritable genetic variability produced when whole plants are regenerated from somatic cells using plant tissue culture or regeneration techniques (Evans 1988, Stelly et al. 1989). These two sources of variability can independently effect any aspect of the plant that is under genetic control. Thus in a crop improvement program to develop insect resistant plants from transformed or

regenerated plants, it is critical to carefully screen a range of transformed and regenerated plants for the derived expression of agronomic and insect resistant characteristics (Altman et al. 1991, Benedict et al. 1993)

b) Mechaism of action of Bt toxins: The site of Bt toxin action is in the insect midgut, where it disrupts the cell membrane. In the bacterium, endotoxins are synthesized as large protein molecules and crystallized as parasporal inclusions. In susceptible insects, these inclusions dissolve in the midgut, releasing protoxins that are proteolytically converted into still smaller toxic polypeptides. There is extensive variation in the size and structure of the inclusion proteins athe intermediate protoxins and the active toxins that are presumed to relate to insect specifity (Mc Gaughey and Whalon 1992).

Following activation, these toxins bind with high affinity to receptors (glycoproteins) on the midgut epithelium. After binding, the toxins generate pores in the cell membrane, disturbing cellular osmatic balance and causing the cells to swell and lyse through a process that has been termed " Colloid-osmotic lysis "(Haider et al. 1987 : Knowles et al. 1987).

The ability to solubilize and activate inclusion proteins influences the susceptibility of insects to Bt toxin but the extent of that influence on host spectrum remains unresolved. In lepidoptera, binding affinity of individual toxins to receptor sites on the midgut membrane accounts for the sensitivity of different insect species to various toxins (Adang et al. 1991). However, binding site specificity may not be simple system in which each toxin binds to a unique receptor. There appears to be a high degree of heterogeneity among binding sites in some species, suggesting that some sites may bind a single toxin where as others may bind two or more toxins. Similarly specific toxins may bind to more than one site in some insect species (Mc Gaughey and Whalon 1992).

ii. Soybean trypsin inhibitor (SBTI)

Proteins that form complexes with proteases and inhibit their proteolytic activities are wide spread in nature. In additional to their role in regulating proteolytic activities they are important for protecting tissues that are particularly vulnerable to foreign proteases (Bowles et al.,1990). Several nonhomologous facilities of protease inhibition are recognised among the

animal, micro-organism and plant kingdom In plants atleast 8 and possibly 10 protease inhibitor families have been recognised (Ryan 1990).

The presence of inhibition of mammalian digestive proteases in seeds, particularly those of legumes, has been known since 1938, when Read and Haas reported that an aqueous extract of soybean flour inhibited the ability of trypsin to liquefy gelatin (Read & Haas,1938). The characterisation of these inhibitors as proteins was effectively demonstrated by the isolation of crystalline trypsin inhibitor are found specific for each of the 4 mechanistic aspartic and metallo proteases. By far the majority of the known protease inhibitors are specific for serine proteases.

The soybean trypsin inhibitor (SBTI) is a storage protein in soybean seeds that is regulated during embryo genesis.The first plant inhibitor to be well characterized was SBTI (Kunitz 1947).

a) Mechanism of action of protease inhibitors.: Most animals require proteolysis to degrade and use the component aminoacids of the protein they consume. The mechanism of enzyme inhibitors is usually through formation of a very strong complex containing a covalent bond between an active site residue and the inhibitor Interest in the effects of plant protease inhibitor on insects was aroused as early as 1947, when Mickel and Standish observed that larvae of certain pests were unable to develop normally on soybean products (Mickel and standish,1947). Lipke et al. (1954) subsequently studied the toxicity of soybean trypsin inhibitors on development *of Tribolium confusum* (flour beettle), common pest of stored grain. Although the SBTI gave negative results in their bioassay, the presence of specific inhibitor of Tribolium larval proteases was revealed, this inhibitor was later isolated and shown to inhibit completely the larval gut proteolysis of both *T.confusum* and *T.castaneum* (Birk et al. 1963), was inactive towards bovine trypsin and chymotypsin.

On the surface of each inhibitor molecule lies atleast one peptide bond called the reactive site, which specifically interacts with the active site of the enzyme (trypsin) in a substrate like manner. These interactions of the inhibitor with trypsin interfere with the normal degradation of the monitor peptide, which then abnormally activates the complex feedback mchanism that is loss of proteolytic activity, loss of appetite, starvation and

eventual death (Laskowski et al. 1988). In addition, these inhibitors cause hyper-production of digestive enzymes and enhance the loss of sulphur amino acids by the insects.

The recent results regarding the successful transform of Bt and SBTI genes into plants are listed in table-2.

One possible way to determine the toxicity levels of such insect control genes is by cloning these into plasmid vectors, containing powerful promoters which generate large amounts of mRNA complementary to cloned sequence of foreign DNA. Such genetically engineered constructs can be transferred to appropriate bacterial strains and the protein induced in these can be used for insect bioassay studies.

iii. Lectins:-

Lectins are carbohydrates-binding proteins found in many plant tissues, but are often present in relatively large amounts in seeds and other storage tissues. The ubiquitious occurrence of lectins in plants, animals and micro-organisms has been firmly established (Sharon et al. 1977). Lectins are invaluable tools in biological and medical research, in areas as diverse as bacterial typing and bone narrow transplantation. They are classified into a small number of specificity groups (mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, L-fucose, and N-acetylneuraminic acid) according to the monosaccharide that it is the most effective inhibitor of the agglutination of erychrocytes or precipitation of carbohydrate-containing polymers by the lectin. Although found primarily in seeds, lectins are also present in other plant tissues. In some plant families, such as the. Leguminoseae or the Gramineae. Lectins are present in many species, where as in others, such as the Euphorbiaecease, they have been found in a few species only. Usually, a particular source contains lectin (s) belonging to a single specificity found in the same plant (Pusztai, 1983)

Structually, 2 classes of legume lectins have been recognised ; (a) those comprised of either identical or nearly identical sub units of Mr 25,000 - 30,000 (one chain lectins); and (b) those made up of two different sub units, the light α chain and the heavy β chain (two-chain lectins). The first complete aminoacid sequence of a cereal (Gramineae) lectin, wheat germ agglutinin, has been established (Wright, 1984).

There is considerable support, but little solid evidence, for the belief that lectins function primarily as recognition molecules. This function may be expressed differently in different organisms and also in different organs or tissues of the organism.

In plants, two proposed function of lectins are currently attracting most attention:-

a) as mediators of symbiosis between plants and microorganisms;
b) in protection of plants against phyto pathyogens. At present the only lectin isolated from roots that can bind to a specific modulating strain of *Rhizobium* is trifolin of white clover (Sharon et al.,1986).

Lectins may be involved in the defense of plants against fungal, bacterial, and viral pathogens during germination and early growth of the seedlings this proposal is supported primarily by two lines of evidence:-

a) The binding of lectins to various fungi and their ability to inhibit fungal growth and germination and (Barkai-Golan et al. 1978)
b) The presence of lectins at the potential site of invasion by the infectious agents (Mishkind et al. 1992).

Interactions of lectins with fungal hyphae were first demonstrated by Mirelman et al., (1975), who found that wheat germ agglutinin (WGA), a lectin specific for chitin oligosaccharides, binds to hyphal tips and hyphal growth and spore germination of this chitin fungus. Lectins with sugar specificities different from those of WGA may function similarly, as natural inhibitors of the growth of fungi, the surfaces of which are covered by other ploysaccharides. Galun et al. (1976) examined the binding of WGA, as well as 5 other lectins with different sugar specificities, to 3 mycobionts isolated from lichens. From the binding characteristics they concluded that chitin is a mycobionts hyphal wall component, have suggested that chitins may be useful in studies of the chemical composition of hyphal wall surfaces.

An involvement of these proteins in resistance to insect attack was first put forward by Janzen et al.(1976), who showed that the lectin form *Phaseolus vulgaris* was toxic to developing larval of bruchid beetle *Callosobrochus maculatus*.

The potential of plant lectins as chemical defenses against insect pests were determined by Huesing et al. (1991) when they screened 17 plant lectins in an artificial seed system using the cowpea weevil, *Callosobruchus maculatus*, as a model insect. These lectins were classed into one of 2 groups lectins with specificity for N-acetylgalactosamine residues (Gal NAc), which included orange lectin and peanut agglutinin, and lectin with specificity for N-acetylglucosamine residues (GLc NAc), a constituent of chitin. Their results suggest that GLc NAc-specific plant lectin represent a class of biologically active proteins effective against the cowpea weevil. The GLc NAc-specific lectins studied were derived form wheat germ (WGA) rice (RL), tomato (TL), Jimson weed (DSA) and stinging nettle (UDA). Among the most acive lectins is WGA, the isolectins of which were found to be equally detrimental to the cowpea weevil. The GLc NAc-specifies lectins appear to be members of the chitin binding protein family. Lectins appear to members of the chitin binding protein family, which is characterised by stable proteins containing extensive disulfide cross linkages. Histological evidence suggests that WGA acts in the widgut to cause pathology. Good correlation exists between lectin dose, lession intensity, and impact on insect growth and sruvival. It appears that there exists in cowpea weevil and physiological/biochemical systems vulnerable to selected plant lectins. The genes coding for effective plant lectins could, in principle, serve as antibiosis factors to use in plant transformation to confer insect resistance (Edwin 1991).

Studies of Pustzai and co workers (Pustzai et al.,1979; King et al;1980 a,b) have shown that the toxic effects are consequent on the binding of the protein to glycoprotein receptor on the gut microvilli.

The efforts are concentrated on engineering and transforming the gene constructs into tobacco, typically used as a model system because of case of transformation. Subsequent selection and regeneration standardised technique will then be used to transform pigeonpea to develop resistance against the pod borers.

Materials and Methods

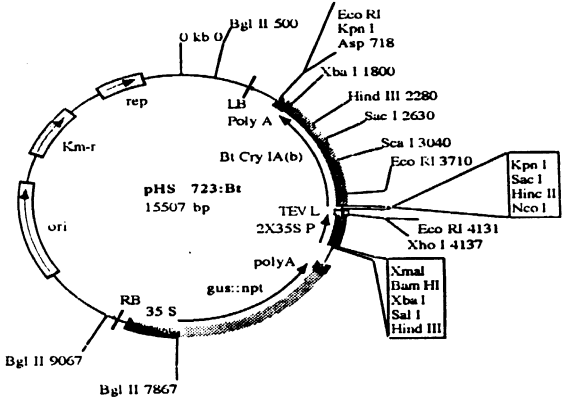
IV. MATERIALS AND METHODS

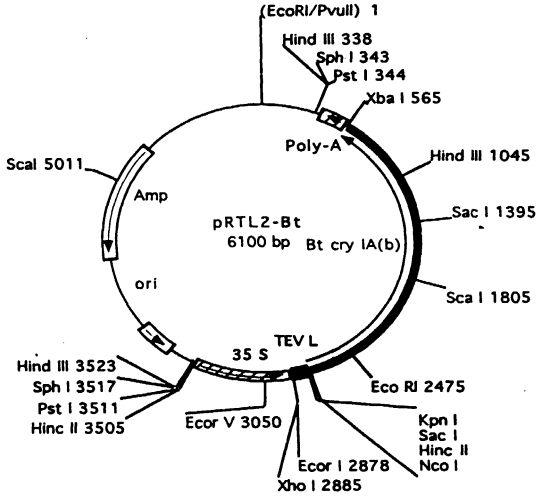
A. Plasmids used for transformation:

Agrobacterium tumefaciens strain C58 harbours a binary plasmid pHS 723 :Bt (Figure 1a) which was used for transformation studies. It was developed as abinary vector for use in *Agrobacterium tumifaciens* mediated gene transfer. It is 15507 bp in size with Bt CryIA(b) gene of 2000 bp. The Bt gene was cloned by K.K. Sharma into Sma I site of pHS 723 mcs from pRTL2 :Bt. (Figure 1b) It is driven by double 35S promoter and is also provided with termination sequences. The gus::npt fused gene driven by another 35S and provided polyadenylation sequences can be used as a reporter gene.

i. Isolation of PHS 723: Bt from *Agrobacterium tumefaciens*

The plasmid was isolated following the modifying form of protocol for small scale preparation of plasmid DNA (alkaline lysis) by Sambrook et al. (1989). A single bacterial colony was inoculated into 10ml of LB medium containing the appropriate antibiotic in a conical flask and inoculated overnight at 37°c on a gyrator shaker at 200 rpm. The culture was centrifuged at 4500 rpm for 10 min at 4°c. The supernatant was discarded, leaving the bacterial pellet as dry as possible. The pellet was resuspended in 400ml of ice-cold GTE (Glucose/Tris/EDTA) buffer by vigorous voltexing. 800ml of freshly prepared lysis buffer was added and mixed by inverting the tubes five times and the tubes were stored on ice for 5 min. 60 ml of a 5M potassium acetate solution was added with vigorous mixing prior to storing on ice for 5 min. The eppendorf tubes were then centrifuged at 12000 rpm for 10 min and the supernatant was transferred to fresh tubes. An equal volume of phenol .and chloroform (1:1) was added and voltexed for 2 min. The samples were then centrifuged for 2 min and the upper aqueous layer containing plasmid DNA was transferred to fresh tube. The DNA was precipitated by adding 0.8 volume of isopropanol. The contents were mixed by inverting the eppendorf tubes and stored at room temperature for 5 min. The


Bgl II 500
Eco RI
Kpn I
Asp 718
0 kb 0
Xba I 1800
rep
LB
Hind III 2280
Poly A
Sac I 2630
Km-r
Sca I 3040
Bt Cry IA(b)
Eco RI 3710
Kpn I
Sac I
Hinc II
Nco I
pHS 723:Bt
15507 bp
TEV L
ori
2X35S P
Eco RI 4131
Xho I 4137
polyA
Xmal
Bam HI
Xba I
Sal I
Hind III
gus::npt
RB
35 S
Bgl II 9067
Bgl II 7867


(EcoRI/PvuII) 1
Hind III 338
Sph I 343
Pst I 344
Xba I 565
Poly-A
Hind III 1045
Sac I 1395
Sca I 1805
Eco RI 2475
Kpn I
Sac I
Hinc II
Nco I
Xho I 2885
Ecor I 2878
Ecor V 3050
Hinc II 3505
Pst I 3511
Sph I 3517
Hind III 3523
35 S
TEV L
ori
Amp
ScaI 5011
pRTL2-Bt
6100 bp
Bt cry IA(b)

samples were then centrifuged at 12000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol. The ethanol was aspirated and the pellet was dissolved in 50 ml of TE (pH 8.0) containing DNase free pancreatic RNase

ii. Electrophoresis of PHS 723:Bt:-

The plasmid thus isolated was run in 0.9% gel containing ethidium bromide at 0.5μg/ml at 60 v, in 1X TAE electrophoresis buffer. The bands were examined by long wavelength UV illuminator.

iii.Restriction analysis of the plasmid pHS 723:Bt

pHS 723:Bt was restricted with several enzymes that have unique sites in the plasmid. A 20 μl of reaction was used in which 10 μl of DNA, 2 μl of buffer, 1 μl of restriction enzyme, 1 μl of Rnase and 6 μl of water was used. All the additions done on ice and the tubes were incubated for 3 hrs at 37 °c in hot water bath. The reaction stopped after electrophoresis with the addition of 0.5 M EDTA (PH 8.0). The fragments were then visualized after electrophoresis in 0.9% agarose run at 60 v in 1X TAE, by a UV transilluminator.

A. Transformation through *Agrobacterium tumefaciens* method:-

i.Preparation of inoculum for transformation through *Agrobacterium* :-

5ml of *Agrobacterium* culture harbouring pHS723:Bt plasmid was transferred into a 25 ml tube under aseptic conditions and centrifuged at 4000 rpm for 10 min at 4°c. The pellet was resuspended in 5 ml of sterile 1/2 MS and centrifuged for 10 min at same rpm and temperature. This step is repeated once again. The pellet dissolved in 25 ml of 1/2MS which was used for transformation.

ii. Sterilization of leaf discs of Tobacco :

The leaves of tobacco were surface sterilized by sequential treatments by dipping them in 70% ethonol for 30 sec and then wiping them with sterile tissue paper. These were

transferred to 15% clorox solution for 10 min. These were then washed in a series of sterile water for 3 times. Leaf discs were cut with a sterile leaf disc borer and placed on MS media supplemented with 2.25 mg/L of BAP and 0.1 mg/L of NAA.

iii. Sterilization of pigeonpea seeds:-

Pigeonpea seeds were rinsed in 75% ethanol for 60 seconds .The seeds were transferred to sterile container with 0.1% mercuric chloride solution and 0.1% Tween 20 for 8-9 minutes .The container was placed on a shaker (150-200rpm). The seeds were then washed thrice with sterile distilled water and finally soaked in sterilized water for four hours. The seed coat was removed from the imbibed seeds and the seeds were transferred to tubes containing MS+5mg/L BAP (MS5) medium for germination. After 10 days multiple shoots were removed from plants and were placed in the same medium.

iv. Cocultivation of pigeonpea multiple shoots with *Agrobacterium tumefaciens*:-

The axillary meristems from the seedlings were dipped in the inoculum of *Agrobacterium* at their based region form which the regeneration takes place. These multiple shoots were then placed on MS 5 media supplemented with BAP (5mg/L). After 2-3 days of cocultivation of explants with *Agrobacterium* these were transferred on to the same media containing cefatoxime (250mg/L). After induction of organogenisis for 2-3 weeks, they were transferred to the selection media supplemented with 50mg/L kanamycin for the specific growth of transformants.

V .Cocultivation of tobacco leaf discs with *Agrobacterium* :-

The leaf discs were dipped in *Agrobacterium* inoculum, this facilitates the adhesion of the bacteria to the cut ends of the leaves. Transfer the leaf discs to the same media with their abaxial side in contact with the media. The explant is cocultivated with bacterial suspension for 2-3 days after which the leaf discs were transferred to the media supplemented with 250 mg/L of cefatoxime. After induction for 2-3 weeks, the explants were transferred to

the selection media of the same composition but supplemented with 50mg/L kanamycin. This facilitates the growth of only those plants which were transformed, thus providing good selection.

C. Transformation through Biolistics :

i. Plasmid DNA used for Bombardment :-

The transformation strategy employs plasmids as vectors to transfer DNA. The vectors containing genes of agronomic interest were co-transformed with vectors containing reporter genes and selectable markers. The following vectors were used for bombardment .

a. PRT 99 GUS: Plasmid contains GUS, a reporter gene whose expression is controlled by strong 35S promoter and has poly A as the polyadenylation site. This bacterial gene uid A encoding.β-glucuronidase acts on a substrate X-Gluc, histoichemical stain 5 bromo-4 chloro-3indolyl D-glucuronide (Jefferson et al. 1986). Cleavage of this substrate leads to the production of a blue dye. The advanatage of histochemical staining is that individual cells expressing GUS can be visualized. The vector contains a selectable marker NPT II (Neomycin phosphotranferase) gene which is also controlled by 35S promoter and has a poly A termination site NPT II gene derived from the transposon Tn 5, which encodes the enzyme neomycin phosphotransferase which inactivates by phosphorylation a number of aminoglyside antibiotics such as kanamyein, neomycin, geneticin or paromomycin. kanamycin is generally used for the purpose.

b. pHS 723:Bt : Contains Cry 1A(b) as insect control gene. This gene from pRTL2:Bt was cloned as a pst I fragment into - sma1 site of pHS 723 multiple cloning site. The 35S promoter is derived from cauliflower mosaic virus and the polyadenylation signals are derived from nopaline synthase gene (NOS).

ii. Micro carrier preparetion :-

The procedure described was developed by Sanford et al. (1992). In a 1.5 ml eppendorf tube 60 mg of tungsten particles were weighed. 1ml of 70% alcohol was added to tungsten particles and vortexed for 3 to 5 seconds. The particles were incubated for 15 minutes and then given a spin for 5 seconds. Ethanol was removed and 1ml of sterilized water was added to the particle and vortexed for 1 minute. The particles were repelleted. This washing with sterilized water was repeated for 3 times. 1 ml of sterile 50% glycerol was added to the particle to get a final concentration of 60 mg/ml.

iii. Coating of DNA on to microcarriers :-

The particles were vortexed for 5 minutes on a platform vortexer to resuspend and disrupt agglomerated particles. 50 µl (3mg) of microcarriers were taken in a 1.5 ml microfuge tubes. 5 µl of DNA i.e. vector constructs (1mg/ml) was added to each aliquot of micro projectiles and mixed well with the pipetter. 50 µl of 2.5 M calcium chloride (filter sterilized) was added to DNA mixture followed by the addition of 20 µl of 0.1M spermidine. The whole mixture was vortexed for 2-3 minutes and then allowed to settle for 2 seconds and the liquid was removed. The particles were then washed with 140µl of 70% ethanol and subsequently with absolute ethanol. 48µl of 100% ethanol was added and vortexed at low speed for 2-3 seconds. From this 6µl was transferred to the centre of a sterile macrocarrier and desiccated immediately. The microprojectile with the DNA was placed in the biolistic gun. The stopping plate and tissue sample was inserted. The tissue was subjected to microprojectile bombardment using the particle accelerator, when the vacuum reaches the desired pressure(1350 psi).

D. Selection and regeneration of transformants :-

Twenty four hours after bombardment the tobacco leaf discs and the pigeonpea axillary meristem were evenly spaced in the petriplates containing 2.25mg/L BAP+ 0.1mg/L NAA (MS4) and 5mg/L BAP (MS5) media respectively. The cultures were then incubated in the light for 1 week. The culture room temperature was maintained between 26 ± 1°C. The regenerating tobacco explants were then transferred to MS basal medium containing 250mg/L cefotaxime and 50mg/L kanamycin. Similarly the regenerating pigeonpea culture were transferred to MS 5 medium containing 250mg/l cefotaxime and 50mg/L kanamycin. All culture were again incubated in light in a culture room maintained at 26 ± 1°C.

Three weeks later the concentration of the kanamycin in the media was raised. The tobacco culture was transferred to MS B containing 100mg/L kanamycin and the pigeonpea culture was transferred to MS 5 medium containing 100mg/L kamamycin.

E. GUS expression and analysis of putative transformants :

The GUS assay was done as described by Jefferson et al (1986). The tobacco leaf discs and pigeonpea axillary meristems were analyzed for transient expression of GUS gene after 24 hrs of bombardment. GUS assay was done 2 –3 weeks later for tobacco culture growing in the selection medium according to Jefferson et al (1986). The leaf discs were incubated in 100ml of 0.5mg/ml X-Gluc (5-Bromo 4-chloro 3-indolyl glucuronicacid). The solution was vacuum infiltrated into the disc for 5 min and then kept for overnight incubation in dark at 37°C. The GUS positive culture were transferred to MSB medium containing the appropriate antibiotic (figures 3 and 4).

Results and Discussion

RESULTS AND DISCUSSIONS

The isolated plasmid and the fragments separated by electrophoresis are shown in figure 1. pHS 723:Bt was digested singly with Xba I, Eco RI, and Hind III to check for 2.2 Kb fragment of CryIA(b) gene (figure 2). Several preparations made for pHS 723:Bt show the same pattern i.e., the plasmid cut singly with these enzymes and there was no fragment released but the size of the uncut plasmid was corresponded well with that of the expected size of pHS:723:Bt.

Studies on factors affecting biolistic transformation:-

Biological factors : An efficient transformation system should ensure production of transgenic plants, in quickest possible period. It is therefore very crucial to select actively growing meristernatic cells to serve as the target tissue for any transformation study. The target tissue selected should be competent enough to regenerate in vitro and produce fertile plants. Initial experiments in transformation used embryogenic callusses/cell suspensions (Vasil et al. 1992; Fromm et al. 1990) as target tissue. Regeneration from embryogenic cell suspensions, however, is limited to only a few genotypes. Immature embryos (Christon et al. 1991), microspores (Want and Lemaux,1994) and young embryogenic callus derived from immature embyos (Vasil et al. 1993) were successfully used for transformation in recent years.

In this study tobacco leaf discs and pigeonpea multiple shoots (axillary meristem) were used as the target tissue. These explants can help in efficient regeneration of plants from in vitro cultured tissue.

Physical parameters:- Design of the particle gun, the type size and amount of microprojectiles used, the velocity of microprojectiles, the amount of DNA per projectile and the number of bombardments determine the efficiency of the transformation process (Casas et al. 1995).

Model PDS-1000/ He system (Biorad) is generally used for bombardment. Helium gas used in this system is superior because it is a light, clean, inert and safe and expands much faster than other conventional bottled gases, imparting higher velocities to light weight. macroprojectiles (Sanford et al. 1991). The microprojectiles are accelerated in a wider macroprojectiles, and are subsequently dispersed much more widely and uniformly on impact against the stopping screen. The microprojectiles used were tungsten particles of 1.1μm diameter. Physical parameters studied using tobacco leaf discs and pigeonpea axillary meristem as the target tissue and the results obtained are discussed below:

The vaccum pump used to remove the overlying air so that the pressure comes down between 15-20 inches Hg. Increasing the vaccum results in tissue damage because of the residual water vapour pressure from the biological samples used as a target tissue. Also by removing most of the overlying gas the amount of decceleration of the microprojectiles is significantly reduced.

The helium pressure used and target distance at which the tissue is bombarded also influences transformation efficiency. Decreasing the target distance and increasing the Helium pressure results in higher transformation. In this study chamber vaccum was 20 inches Hg, 1350 psi helium pressure and target distance of 5cm

Regeneration of transformed tissues on selection medium:-

The tobacco leaf discs were bombarded by placing the tissue on standardised MS4 medium containing 2.25 mg/L BAP and 0.1 mg/L NAA. After 24 hrs of transformation swelling was seen on the surface of the leaf discs indicating the beginning of cell division and callus formation. After one week, when callusing was initiated in the induction medium the tobacco leaf disc calli were transferred to the medium containing a 50 mg/L Kanamycin. After one week the tobacco leaf disc calli were transferred to the regeneration medium i.e., MSB medium containing 50 mg/L kanamycin without any hormones. The regenertion medium has been used to suppress callusing and to promote organogenesis. Kanamycin limits the number of nontransformed cells that survive due to cross protection by the transformed cells (figure 7)

Pigeonpea axillary meristems were bombarded by placing the explants on MS5 medium containing 5mg/ L BAP. Incubation of the transformed tissue samples in MS5 medium for 2 weeks resulted in incubation of shoot buds from the explants. The axillary meristems with the proliferating shoot buds were transferred to MS5 medium containing 5mg/L of BAP and 50mg/L kanamycin. Less concentration of hormone was used in this medium to promote growth and kanamycin helped to select the transformed tissues. 40% of cultures showed formation of leaves from the shoot buds (figures.5, 6, and 8). In normal conditions, the pigeonpea cultures release large amounts of phenolic compounds in to the medium and pigment production is increased after bombardment, presumably as a consequence of the stress imposed on the tissue. The cultures were transferred to fresh MS2 medium containing 2mg/L of BAP and 50mg/L of kanamycin every ten days so as to enhance the growth of the transformants.

Fig. 1. Agarose gel electrophoresis of the plasmid pHS 723:Bt on 0.8% gel run at 60 v.
Lane 1.----------λ Hind III Marker
Lane 2 and 3.----------pHS 723:Bt

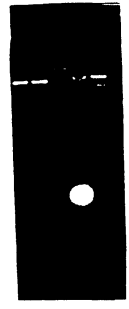


Fig. 2. Restriction analysis of the plasmid pHS 723:Bt separated on 0.9% agarose gel run at 60v.

Lane 1.--------Hind III digested pHS 723:Bt
Lane 2.--------EcoRI digested pHS 723:Bt
Lane 3.--------XbaI digested pHS 723:Bt
Lane 4.--------Uncut pHS 723:Bt
Lane 5.--------λ HindIII Marker

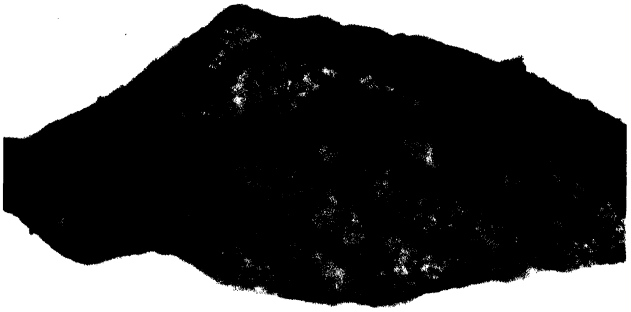


Fig 3. Histochemical expression of GUS in an explant of pigeonpea transformed with *Agrobacterium* with Bt gene.

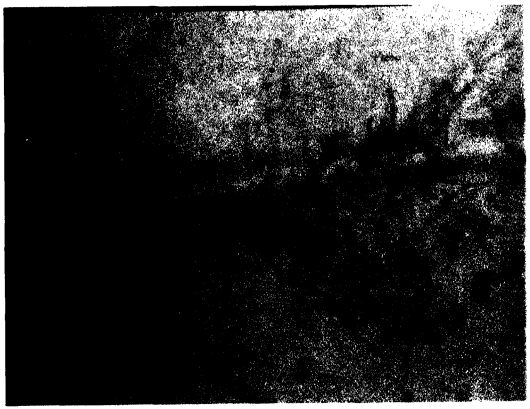


Fig. 4. Histochemical expression of GUS in an explant of pigeonpea transformed with biolistics with Bt gene.

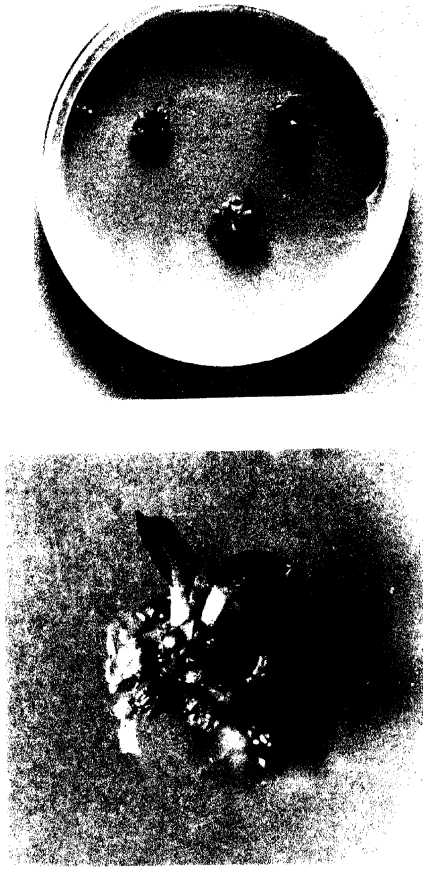


Fig. 5 and 6. Initiation of adventitious shoot buds from transformed axillary meristems of pigeonpea on shoot forming medium (5 mg/L BAP) containing kanamycin.

Fig. 7. Shoot formation on subcultured callusses, from transformed tobacco leaf disc, growing on medium containing kanamycin.

Fig. 8. Response of the putatively transformed axillary meristem of pigeonpea with Bt gene through *Agrobacterium* on MS media with 5mg/L BAP cotaining kanamycin

Conclusions

CONCLUSIONS

1. Transformation of the axillary meristem of pigeonpea with Bt CryIA(b) gene was done via *Agrobacterium tumefaciens based vectors*.
2. Axillary meristem of pigeonpea was also transformed via biolistics method. However, the efficiency of transformation was found to be low when compared with Agro-infection.
3. The putatively transformed explants with multiple shoots were assayed histochemically for the presence of GUS and thus tentatively suggesting the integration of the gene of interest.

Tables

Table 1. Insect pests attacking Pigeonpea

S.No	Name of the Pests	Part of the plant infected	Symptoms
1.	Nodule – damaging Fly	Nodules	Yellowing , indicating nitrogen deficiency and reduced growth
2.	Termites	Stems and roots	Wilting and holes in the stem.
3.	Jewel Beetles	Stem	A prominent gall around the stem
4.	Stem Weevils	Stem	Wilting , drying and stem breakage
5.	Stem Flies	Stem	Wilting and death
6.	Cow Bugs	Stem	Formation of corky calluses, wilting and reduced plant vigour
7.	Scale Insects	Stems and leaves	Secretions on stems and leaves
8.	Jassids	Leaves	Leaflets become cup shaped, yellow at the edges, red-brown sometimes with subsequent defo Defoliation and stunting
9.	Aphids	Leaves	Young leaves of seedlings become twisted and the heavy infestation can cause wilting
10.	Red Spider Mites	Leaves	Yellow or white spots on the upper surface of the leaflets, heavy infestation results partial defoliation
11.	Whiteflies	Leaves	White spots
12.	Leaf -cutter Bees	Leaves	Neat ,semi-circular portions are cut from the leaflets
13.	Grasshoppers and Locusts	Leaves	Defoliation, withering and death
14.	Leaf – damaging Weevils	Leaves	Adult weevils chew the leaflets , generally at the margins , causing a ragged effect
15.	Thrips	Flowers and pods	Shedding of buds and flowers
16.	Blister Beetles	Flowers and pods	Adult beetles feed on the flowers and greatly reduce the numbers of pods that are set.
17.	Bud Weevils	Flowers and pods	Larvae feed and pupate inside the flower buds , making them hollow
18.	Pod Weevils	Flowers and pods	Green seeds in the pods gets damaged, holes in the leaflets and flowers
19.	Pod sucking Bugs	Flowers and pods	The seeds become shrivelled with dark patches
20.	Pod Borers	Flowers and pods	Conspicuous holes in the pods, Loss of mesophyll tissue in the leaflets.
21.	Lablab Pod Borers	Flowers and pods	Bores in the buds, flowers and green pods

Table 2. Summary of recent results with Bt and SBTI genes incorporated into plants

S.No	Insecticidal compound	Target insect	Crop	References
1.	*Soybean Kunitz Trypsin Inhibitor*	*Manduca sexta*	Tomato, Tobacco, Potato	Shukle et al. (1983)
2.	*Soybean Kunitz Trypsin Inhibitor*	*Ostinia nubilalis*	Maize	Steffens et al (1978)
3.	*Soybean CII Trypsin Inhibitor*	*Spodoptera littocalis*	Maize, Rice, Cotton, Tobacco.	Jounanin et al (1990)
4.	Soybean Bowman-Birk Trypsin Inhibitor	*Tribolium castenum*	Cereal flours	Bier, Y. (1985)
5.	Synthetic CryIA(b)	*Manduca sexta*	Tobacco & tomato	Perlek et al (1991)
6.	Modifical CryiA(b)	*Heliothis zea*	Cotton	Perlek et al (1991)
7.	Cry III A	*Colarado potato*	Tobacco	Sutton et al (1992)
8.	Synthetic I A (b)	*Ostinia nubilalis* (European corn borer)	Corn	Koziel et al (1993)
9.	Synthetic Cry III A	*Leptinotorsa decemlineato*(Colarado Potato Beetle)	Potato	Perlak et al (1993)

References

REFERENCES

Aizawa, K. (1982). Microbial control of insect pests in Advances in Agricultural Microbiology, ed. Subbarao, N.S.,Published by Oxford and IBH., New Delhi. 397-413.

Armes, N.J., Jadav, D.R., and Kenneth, R.D. (1996). A survey of insecticide resistance in *Helicoverpa armigera* in the Indian subcontinent. Bulletin of Entomological Research, **86 :** 499-514.

Bailey, W.D., Zhao, G., Carter, L.M., Gould, F., Kennedy, G.G., and Roe, R.M. (1998). Feeding disruption bioassay for species and *Bacillus thuringiensis* resistance diagnosis for *Heliothis virescens* and *Helicoverpa zea* in cotton. Crop Protection, **17 :** 591-598.

Baruah, A.A.L.H., Ramesh, C. (1997). Bioefficacy of synthetic pyrethroids on the pod damage by pod borer, *Helicoverpa armigera* in pigeonpea. Legume Research, **20 :** 87-90.

Benedict, J.H., Sachs, E.S., Altman, D.W., Deaton, W.R., Kohel, R.J., Ring, D.R., and Berberich, S.A. (1996). Field performance of cottons expressing transgenic CryIA insecticidal proteins for resistance to *Heliothis virescens* and *Helicoverpa zea* Journal of Economic Entomology, **89 :** 230-238.

Bijaya, K.S., Nickolay, K., and Yuri Y.G. (1992). Isolation and culture of protoplasts of pigeonpea. Plant Cell Reports, **11 :** 462-465.

Bowles, d.j.,(1990). Defense related protein in higher plants. Annual Review of Biochemistry, **59 :** 873-907.

Casas, A., Karonowicz, A., Zahr, U., Zhang, L., Haan, T., Tomes, D.Bressan, R., and Hasegawa P. (1997). Approaches to the genetic transformation of Sorghum in Genetics, Biotechnology and Breeding of Maize and Sorghum ed. Tsaftaris, A.S,. Published by the Royal Society of Chemistry, 88-93.

Chaudhary, R.K., Gargav, V.P., and Parsai, S.K. (1993). Studies on the effect of the host plants on the developmental stages of gram pod borer *Helicoverpa armigera*, Legume Research, **16 :** 115-118.

Chaudhary, R.R.P and Sachan, R.B. (1997). Effect of differential protection against pod borer complex in early pigeonpea cv.Upas-120 in western plain zone of Uttar Pradesh. Legume Research, **20 :** 189-192.

Chintapalli, L.P., Moss, J.P., Sharma, K.K., and Bhalla, J.K. (1997). In vitro culture provides additional variation for pigeonpea crop improvement. In Vitro Cellular and Developmental Biology (P), **33** : 30-37.

Clarence, A.R. (1990). Protease inhibitors in plants. Annual Review of Phytopathology, **28** : 425-449.

Deml, R., Meise, T., and Dettner, K. (1999). Effects of *Bacillus thuringiensis* δ-endotoxins on food utilization, growth, and survival of selected phytophagous insects. Journal of Applied Entomology, **123** : 55-64.

Draper, T., and Scott, R., (1991). Gene transfer to plants. Plant Genetic Engineering, **1**. ed. Grierson, D. Blackie & Sons Ltd: London. 38-77.

Eapen, S., and George, L. (1993). Plant regeneration from leaf discs of peanut and pigeonpea. Plant cell Tissue and Organ culture, **35** : 223-227

Edwin, H.J., (1991). Lectins as plant chemical defenses against insects. Ph.D. Thesis, Purdue University.

Finer, J.J., and Vain, P. (1992). Development of the particle in flow gun for DNA delivery to plant cells. Plant Cell Report, **11** : 323-328.

Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., and Klein, T.M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Biotechology, **8** : 833-839.

Gatehouse, J.A., Hilder, V.a., and Gatehouse, A.M.R. (1991). Plant genetic engineering. 1 (ed) Grierson, D., Blackie and sons Ltd: London.

George, L., and Eapen, S. (1994). Organogenesis and embryogenesis from diverse explants in pegeonpea. Plant Cell Reports, **13** : 417-420.

Heckel, G.D., Gahan, J.L., Gould, F., Daly, C.J., and Trowell, S. (1997). Genetics of *Heliothis* and *Helicoverpa* resistanee to chemical insecticides and to *Bacillus thuringiensis*. Pesticide Science, **51** : 251-258.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtzr, Rogers,S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science, **27** : 1229-1231.

Iida, A., Seki, M., Kamad, M., Yamada, Y., and Morikawa, H. (1990). Gene delivery into cultured plant cells by DNA-coated gold particles accelerated by a pneumatic particle gun. Theoritical Applied Genetics, **80** : 813-816.

Jadhav,R.D., and Armes, J.N. (1996). Comparative states of insecticide resistance in the *Helicoverpa* and *Heliothis* species of south India. Bulltin of Entomological Research, **86** : 525-531.

Jenes, B., Moore, H., Jun, C., Zhang, W. and Wu, R. (1993). Techniques for Gene Transfer in Transgenic Plants, ed. Kung, S.D., Wu, R. Academic press, U.S.A. **1** : 125-144.

Klein, T.M., Wolf, E.D, and Sanford, J.C. (11987). High-Velocity microprojectiles for delivering nucleic acids into living cells. Nature, **327** : 70-73.

Kononowicz, A.K., Casas, T., Bressan, R.A., and Hasegawa, P.M. (1995). New vistas are opened for Sorghum improvement By Genetic transformation. African Crop Science Journal, **3** : 171-180.

Kunitz, M. (1947). rystalline soybean trypsin inhibitor 2. General properties. Journal of General Physiology, 30 : 291.

Larkin, P.J., and Scowcroft, W.R. (1981). Somaclonal variation-a novel source of variability from cell cultures for plant improvement. Theoritical Applied Genetics, 60 : 197.

Laskowski, M. Jr., and Kato, I (1980). Protein inhibitors of proteases. Annual Review of Biochemitry, 49 : 593.

Lis, H., and Sharon, N. (1986). Lectins as molecules and as tools. Annual Review of Biochemistry, **55** : 35-67.

Maheswari, N., Rajyalakshmi, K., Baweja, J. (1995). In vitro culture of wheat and genetic transformation-Retrospect and prospect. Critical Reviews in Plant Sciences, **14** : 149-178.

Mc Gaughey, W.H., Gould, F., and Gelernter, W. (1998). Bt Resistance management. Nature Biotechnology, **16** : 144-146.

Mehta, U., and Mohan Ram, H.Y. (1980). Regeneration of plantlets from the cotyledons of *cajanus cajan*. Indian Journal of Experimental Biology,. **18** : 800-802.

Murashige, T., in Frontiers of plant tissue culture, edited by T. A. Thrope IAPTC Publ., Calgary, (1978). 15.

Negrutiu, I., Shillito, R., Potrycus, I., Biasini, G., and Sala, F. (1987). Hybrid genes in the analysis of transformation conditions. Plant Molecular Biology, Plant Molecular Biology, **8** : 363-373.

Perlak, F.J., Fuchs, R.L., Dean, D.A., Sylvia L., McPherson, and Fischoff, A.D. (1991). Modification of the coding sequence enhances plant expression of insect control protein genes. Proceedings of National Academy of Science, **88** : 3324-3328.

Raineri, D.M., Bottino, P., Gordon, M.P.,and Nester, E.W. (1990). *Agrobacterium* -mediated transformation of Rice. Biotechnolog, **8** : 33-38.

Rathore, S.R., and Chand, L. (1997). In vitro transformation of pigeonpea genotypes by wild strains of *Agrobacterium tumefaciensis*. International Chickpea and Pigeonpea Newsletter, **4** : 38-39.

Reed W., Lateef, S.S., Sithanantham, S., and Pawar, C.S. (1989). Pigeonpea and Chickpea Insect Indentification Handbook, ICRISAT, INDIA.

Reed, W., and Lateef, S.S. (1990). Pest management in The Pigeonpea, ed. Nene, Y.L., Hall, S.D., and Sheila, V.K. ICRISAT. 349-374.

Reed, W., Lateef, S.S., Sithanantham, S. (1980). Pest management in low-input pigeonpea in Proceedings of the International workshop of Pigeopeas, ed. Nene, Y.L. ICRISAT.**1** : 99-117.

Reich, T.J., Iyer, V.N., and Miki, B.L. (1986). Efficient transformation of Alfalfa protoplasts by the intranuclear microinjection of Ti plasmids. Biotechnology, **4** : 1001-1004.

Richard, A.J.,Sean, M.B., and David, H. (1986). β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proceedings of National Academy of Science, **83** : 8447-8451.

Rivka, B.G., David, M., and Nathan, S. (1978). Studies on growth Inhibition by lectins of *Penicillia* and *Aspergilli*. Archieves of Microboilogy, **116** : 119-124.

Ryan,C.A. (1990). Protease inhibitors in plants. Annual Review of Phytopathology, **28** : 425-449.

Sautter, C., Waldner, H., Neuhaus-Url, G., Galli, A., Neuhaus, G., and Potrykus, I. (1991). High efficiency gene transfer using a novel approach for the acceleration of micro-projectiles. Biotechnology, **9** : 1080-1085.

Shanower, G.T., Yoshida, M., and John Peter, A. (1997). Survival, growth, fecundity and Behavior of *Helicoverpa armigera* on pigeonpea. and two wild cajanus species. Journal of Economic Entomology, **90** : 837-841.

Shiva Prakash, N., Pental, D and Bhalla-Sarin, N. (1994). Regeneration of Pigeonpea from Cotyledonary node via multiple shoot formation. Plant Cell Reports, **13** : 623-627.

Sims, R.S., and Sharon, Berberich, A. (1996). *Bacillus thuringiensis* CrylA protein levels in raw and processed seed of transgenic cotton. Journal of Economic Entomology, **89 :** 247-251.

Sreenivasu, K., Malik, S.K., Ananda Kumar, P. (1998). Plant regeneration via somatic embryogenesis in pigeonpea. Plant Cell Reports, **17 :** 294-297.

Takeuchi, Y., Dotson, M., and Keen, N.T. (1992). Plant transformation asimple particle bombardment device based on flowing helium. Plant Molecular Biology, **18 :** 835-839.

Urmila, V.K., Amonkar, S.V. (1988). Microbial control of *Heliothis armigera* (Hb) Indian Journal of Experimental Biology, **26 :** 703-707.

Vasil, V., Srivastava, V., Castillo, A.M., From, M.E., and Vasil, I.K.,(1993). Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Biotechnology, **11 :** 155301558.

Venugopal Rao, N., Rajasekhar, P., Venkatiah, M., and Rajasri, M. (1994). Estimation of insecticide resistance in *Helicoverpa armigera* in Andhra Pradesh. Indian Journal of Plant Protection, **20 :** 33-40.

William, H., Mc Gaughey., and Whalon, E.M. (1992). Managing insect resistance to *Bacillus thuringiensis* toxins. Science, **258 :** 1451-1455.

William, H., Mc Gaughey., Gould, F., and Gelernter, W. (1998). Bt Resistance management. Nature Biotechnology, **16 :** 144-146.

Xiongfei Ding, Gopalakrishnan, B., Johnson, B.L., White, F.F., Wang, X., Morgan, D.T., Kramer, J.K., and Muthukrishnan, S. (1998). Insect resistance of transgenic

Appendices

APPENDIX – A

1. LB (Luria Bertanii) medium
   Peptone(10g/L)
   NaCl (10g/L)
   Yeast Extract (5g/L) and adjust the pH to 7.0

2. GTE Buffer
   50 mM Glucose
   25 mM Tris.HCl (pH 8.0)
   10 mM EDTA (pH 8.0) di-sodium salt and autoclaved at 15 lb/sq in. in a liquid cyclefor 15 min and stored at 4°C

3. Lysis Buffer
   0.2 N NaOH (freshly diluted from a 10 N stock)
   1% SDS

4. Potassium acetate solution

5 M Potassium acetate solution	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

5. Rnase free of Dnase
   Pancreatic Rnase was dissolved in 10 mM Tris Cl (pH 7.5), 15 mM NaCl, at conc. of 10mg/ml and heated at 100°C for 15 minutes. Allowed to cool and placed at -20°C

6. TE (pH 8.5)
   0.1M Tris.HCl, 5mM EDTA (pH 8.0) di-sodium salt

7. TAE Buffer (50X):
   242 g Tris Base
   57.1 ml glacial acetic acid
   100 ml of 0.5 M EDTA di-sodium salt and make up the volume to one litre

APPENDIX – B

GUS HISTOCHEMISTRY

ASSAY MIXTURE
(for 5 ml)

1. Dissolve 5 mg. X – gluc in 50 µl Dimethyl formamide and add
2. 5 ml phosphate buffer (0.05 M, pH 7.0)
3. 1 mM potassium ferrocyanide (25 µl of 200 mM stock)
4. 1 mM potassium ferricyanide (25 µl of 200 mM stock)
5. 10 mM Na2 EDTA (50 µl of 1 M stock)
6. 0.1% Triton X 100 (100 µl of 1:10 diluted stock)
7. 0.2% Sodium azide (200 µl of 50 mg/ml stock)

MURASHIGE AND SKOOG (1962) MEDIUM

SALT	CONC. (mg/l)	STOCK (per l)	USE (per l)
MAJOR SALT (X 50)			
NH_4NO_3	1650	33.0 g/200ml	10 ml
KNO_3	1900	38.0 g/400ml	20 ml
KH_2PO_4	170	3.40 g/200ml	10 ml
$CaCl_2$	440	8.80 g/200ml	10 ml
$MgSO_4.7H_2O$	370	7.40 g/200ml	10 ml
MINOR SALTS (x 100)			
H_3BO_3	6.20	6.20 mg/l	)
KI	0.83	83.0 mg/l	)
$MnSO_4.4H_2O$	22.3	2230 mg/l	)
$ZnSO_4.7H_2O$	8.6	860 mg/l	) 5.0 ml
$Na_2MoO_4.2H_2O$	0.25	25 mg/l	)
$CuSO_4.5H_2O$	0.025	2.5 mg/l	)
$CoCl_2.6H_2O$	0.025	2.5 mg/l	)
IRON (X 100)			
$Na_2EDTA.2H_2O$	37.3	3.73 g/l	)
$FeSO_4.7H_2O$	27.8	2.78 g/l	) 10 ml
OR			
FE NA_2 EDTA	40	2.0 G/500 ml	10 ml
ORGANICS (X 100)			
GLYCINE	2.0	200 mg/l	) 10 ml
NICOTINIC ACID	0.5	50 mg/l	)
THIAMINE HCl	0.1	100 mg/l	)
PYRIDOXINE HCl	0.5	50 mg/l	)
m-Inositol	100	5.0 g/500 ml	10 ml