GENETIC ENGINEERING OF COWPEA FOR STORAGE PEST RESISTANCE

A thesis submitted to Indian Institute of Technology Guwahati for the award of degree of

Doctor of Philosophy in Biotechnology

by

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STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the guidance of Dr. Lingaraj Sahoo.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

September, 2008. Siva Kumar Solleti

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CERTIFICATE

It is certified that the work described in this thesis, entitled **"*Genetic engineering of cowpea for storage pest resistance*"**, done by Mr. Siva Kumar Solleti for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

September, 2008.

Dr. Lingaraj Sahoo
Associate Professor
(Thesis Supervisor)

Dedicated to my beloved, late grand parents

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ABSTRACT

Cowpea (*Vigna unguiculata* L. Walp) is an important grain legume widely consumed by 120 million people. Cowpea seeds and fresh peas are a rich source of protein, certain minerals and vitamins. However, cowpea seeds are highly susceptible to storage pests, bruchid species causing massive damage to the stored seeds and seriously limiting its yield potential. Success through conventional breeding methods for developing resistant varieties is limited due to the natural genetic barriers in cowpea germplasm. Consequently, the transfer of insect pest resistance genes by genetic engineering could potentially aid plant breeders in overcoming these constraints.

A critical step in the development of robust *Agrobacterium tumefaciens*-mediated transformation system in recalcitrant grain legume is the establishment of optimal conditions for efficient T-DNA delivery into target tissue and recovery of transgenic plants. We report a dramatic increase in efficiency of T-DNA delivery by constitutive expression of additional *vir* genes in resident pSB1 vector in *Agrobacterium* strain LBA4404. A geneticin based selection system permitted rapid and efficient identification of transgenic shoots without interfering with their regeneration, and eliminated the bulk of escapes. Supplementation of 0.5 µM kinetin to medium containing 5.0 µM benzyl aminopurine after 1week of culture followed by 3 weeks of culture were found critical for optimal multiplication and elongation of transformed shoots from cotyledonary node explants. Combining these three developments, we recovered fertile transgenic plants at a frequency of 1.64%, significantly higher than previous reports. The presence, integration, expression and inheritance of transgenes were confirmed by molecular analysis.

We employed the developed *Agrobacterium*-mediated cowpea transformation method for introduction of the bean (*Phaseolus vulgaris*) α-amylase inhibitor-1 (α*AI-1*) gene into a commercially important Indian cowpea cultivar, Pusa Komal and generated fertile transgenic plants at a frequency of 1.67%. The presence, integration, expression and inheritance of α*AI-1*gene was confirmed by molecular analysis. Expression of α*AI-1* gene under bean phytohemagglutinin promoter resulted in accumulation of αAI-1 in transgenic seeds. The transgenic protein was active as an inhibitor of porcine α-amylase *in vitro*. Transgenic cowpeas expressing αAI-1 strongly inhibited the development of *C. maculatus* and *C. chinensis* in insect bioassays.

Key words: transgenic cowpea, storage pest resistance, additional virulence genes, geneticin selection.

ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid.
ABA	Abscisic acid
AdS	Adenine sulfate
ANOVA	Analysis of variance
AS	Acetosyringone (3,5-dimethoxy-4-hydroxy Acetophenone)
B5	Gamborg's medium (1968)
BA or BAP	6-benzylaminopurine
CAMBIA	Center for Application of Molecular Biology to International Agriculture
CaMV	Cauliflower Mosaic Virus
CDNA	Complimentary DNA
CTAB	Cetyltrimethylammonium bromide
C-terminal	Carboxyl (COOH)-terminal
DIG	digoxygenin
DNA	deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization of the United Nations
GA3	Gibberellic acid
GFP	green fluorescent protein
GUS	β-1,4-Glucuronidase
HCl	Hydrochloric acid
HPT	Hygromycin Phosphotransferase
hpt or *hph*	hygromycin phosphotransferase gene
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IBA	Indole-3-butyric acid
IITA	International Institute of Tropical Agriculture
Kin	Kinetin or (6-furfuryl amino purine)
LB	Luria-Bretani
LUC	luciferase
mRNA	messenger RNA
MS	Murashige and Skoog's medium (1962)
MSB5	MS medium supplemented with B5 vitamins
NAA	α-naphthalene acetic acid

NAA	α-Napthalene acetic acid
NaCl	Sodium chloride
NaPO4	Sodium phosphate buffer
Nos poly-(A)	Nopaline Synthase Poly (adenylation)
*npt*II	neomycin phosphotransferase II gene
NPTII	Neomycin Phosphotransferase II
OD	Optical density
ORF	open reading frame
PCR	Polymerase Chain Reaction
PEG	polyethylene glycole
PIG	particle inflow gun
PMI	phosphomannose isomerase
PVP	Polyvinylpyrrolidone
RNA	ribonucleic acid
RT	Room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SD	Standard Deviation
SDS	sodium dodecyl sulphate
SSC	Sodium chloride and sodium citrate buffer
TBE	Tris borate-EDTA buffer
T-DNA	transferred DNA
TDZ	Thidiazuron (1-phenyl-3-(1,2,3-thidiazol-5yl) urea
Ti plasmid	Tumor-Inducing Plasmid
T-Pilus	Transfer Pilus
UV	Ultraviolet (light)
vir gene	virulence Gene
X-gluc	5-bromo-4-chloro-3-indolyl-β-d-glucuronide
$\chi 2$	chi-square test
YEP	Yeast extract peptone
Zeatin	6-(4-hydroxy-3-methyl-2-butenylamino) purine

UNITS

° C	Degrees Celsius
Cm	centimeter
µg	Microgram
µl	Micro liter
µM	Micro Mole
bp	base pair
kb	kilo base, kilo base pair
kDa	kilo Dalton
mg	Milligram
min	minute/sec
mg/l	Milligrams per liter
mM	Micromolar
pH	negative log of of H^+ ion
psi	pounds per square inch
rpm	Revolustions per minute
v/v	Volume/Volume (concentration)
w/v	Weight/Volume (Concentration)

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Chapter 1

Introduction

INTRODUCTION

1.1 Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is an important staple food legume widely grown in Africa, Latin America, Southeast Asia and southwestern regions of North America, with a world annual production of about 3.9 million metric tons (FAOSTAT 2006). The seeds, fresh peas and green pods provide a major source of cheap and high-quality dietary protein supply to the resource poor populace (Langyintuo et al. 2003). One advantage of cowpeas compared to many other legumes is that the leaves and green pods can be eaten before crop maturity. This provides an important food source before cowpea is harvested – acting as a food bridge during the 'hunger gap' between harvests. Farmers in the dry savanna use cowpea haulms as a nutritious fodder for their livestock. The plants are tolerant to poor and dry soil conditions with considerable adaptability to high temperature and drought. Under conditions of intensive cultivation, cowpea is a crop with possibly highest yield potential in *Vigna* group (Latunde–Dada, 1990).

Cowpea is highly susceptible to many viral diseases, such as cowpea aphid borne mosaic virus, and it is the host for a wide range of insect pests, notably flower bud thrips, pod borers, leaf beetles, pod suckers, aphids and leaf hoppers, which seriously limits the realization of its yield potential (Singh et al. 2000; Aliyu, 2007; Taiwo et al. 2007). Bruchids are a major threat to stored cowpea grains (Singh et al 2000), and infestations by *Callosobruchus maculatus* and *C. chinensis* are responsible for grain losses estimated at 20–60% (Abrol, 1999; Tarver et al. 2007). Durable and adequate levels of resistance to the bruchids are lacking in the primary gene pool, but are available in distant wild species, which present barriers for gene transfer through conventional breeding procedures (Singh et al. 2000), limiting these approaches untenable in transferring resistance to cultivated cowpea (Fatokun et al. 2002). In addition, limited genetic diversity in cowpea breeding programs is of special concern because cowpea appears to have lower inherent genetic diversity than other cultivated crops as a result of a hypothesized single domestication event (Fang et al. 2007). Consequently, the transfer of insect pest resistance genes by genetic engineering has the potential to address the breeding constraints (Machuka, 2000).

Artificial diet bioassays carried out on cowpea weevils indicate that these insects can be controlled by α-amylase inhibitors of common bean (*Phaseolus vulgaris*) (Ishimoto et al. 1999; Matchuka, 2002). Introduction of a gene encoding an α-amylase inhibitor 1 (αAI-1) of common bean has conferred resistance to several bruchid beetles when expressed in seeds of transgenic peas (Shade et al. 1994; Schroeder et al. 1995; Morton et al. 2000), azuki beans (Ishimoto et al. 1996) and chickpeas (Sarmah et al. 2004; Ignacimuthu and Prakash, 2006).

Therefore, bean αAI-1 gene has been considered to be a strong candidate for conferring resistance to *Callosobruchus* spp. in cowpea. However, absence of a robust plant regeneration system amenable to routine genetic transformation methods, and lack of efficient selection scheme for recovery of viable and fertile transgenic plants from transformed explants, at a reasonable high frequency have proved to be major stumbling blocks in adapting the two previously published protocols (Popelka et al. 2006; Chaudhury et al. 2007) for transferring candidate genes to cowpea.

1.2 Objectives

This thesis has evolved around the research performed in cowpea for development of 1) a robust transformation amenable regeneration system from cotyledonary node explants, 2) an efficient *Agrobacterium*-mediated transformation method, 3) preparation of a plant binary construct harbouring common bean α-amylase inhibitor 1 (αAI-1) under bean phytohemagglutinin promoter, 4) overexpression αAI-1 in cowpea cv. Pusa Komal, and 5) molecular analysis of transgenic plants and insect pest bioassay of transgenic seeds for bruchid resistance. Chronologically, the research began by developing a regeneration system from cotyledonary node, and geneticn-based selection system followed by optimization of critical parameters for enhanced *Agrobacterium*-mediated transformation of cowpea. Along with the development of the transformation method, the αAI-1 gene was introduced and transgenic plants were generated. Molecular analysis was performed to confirm the presence, integration, expression and inheritance of the transgene(s). The resistance of transgenic seeds to storage pests, *C. maculatus* and *C. chinensis* was demonstrated by insect pest bioassay.

Chapter 2

Literature review

REVIEW OF LITERATURE

2.1 Plant Genetic Engineering

Plant genetic engineering has opened new avenues to modify crops, and to provide novel solutions to address regional constraints in productivity (Estruch et al. 1997). The process offers plant breeders to access an infinitely wide array of novel genes and traits across the organisms, which can be introduced through a single event into high-yielding and locally-adapted cultivars through the conceptual framework and technical approaches of plant tissue culture and genetic transformation. Dramatic progress has been made over the past two decades in manipulating genes from diverse and exotic sources, and incorporating them into crop plants to confer resistance to insect pests and diseases, tolerance to diverse abiotic stresses, improved post-harvest quality; enhanced nutrient uptake and nutritional quality; increased photosynthetic rate, sugar, and starch production; improved understanding of gene function and metabolic pathways; and production of drugs and vaccines in crop plants.

The technology has enabled the control of the timing, tissue-specificity, and expression level of transferred genes for their optimal function. The powerful combination of genetic engineering and conventional breeding programs permits useful traits encoded by transgenes to be introduced into commercial crops within an economically viable time frame, saving precious time required for introgression of desired trait from the wild relatives by conventional practices. Moreover, with the establishment and expansion of genomics programs, a much broader range of genes with potential for crop improvement are being identified and, in some cases, tailored and/or re-designed for further enhancement of their properties within specific crops. This has further intensified the interest in developing efficient plant transformation technologies to be able to concurrently test and capture the value of these genes. In many species, the development of rapid, highly efficient, and routine transformation systems is still in progress and thus represents a bottleneck in the development of stable high yielding transgenic plants. Plant regeneration system amenable to gene delivery methods has remained as an important pre-requisite for efficient transformation and recovery of fertile transgenic plants in a given plant species.

2.1.1 Plant regeneration

The key to successful regeneration of transgenic plants lies in combining the competence of plant cells for stable integration of the foreign DNA into the genome and their ability to regenerate to fertile plant. The very basis of plant regeneration in *in vitro* is the recognition that somatic plant cells are totipotent and can be stimulated to regenerate into whole plants via

organogenesis or somatic embryogenesis, provided they are given the appropriate hormonal and nutritional conditions (Skoog and Miller, 1957).

The unique ability of isolated plant cells to regenerate into whole plant means that there is a wide range of potential target cell types for transformation (Walden and Wingender, 1995). Cell division can be induced using plant growth regulators to generate callus from almost any plant tissue. Friable callus can be used to generate suspension cultures-one route to generating protoplasts. Explants – either organs or cell types are also used as a source of protoplasts, which can be prepared either by mechanical or enzymatic removal of the cell wall. Regeneration of plants from protoplasts requires the hormonal induction of callus formation, followed by organogenesis or somatic embryogenesis that generally requires defined media. Trivially, every plant species possesses regeneration potential from single cells or from small cell groups. Regeneration can take place, in principle, through two main routes which are often, but not always clearly distinct. Organogenesis, usually achieved by optimizing the cytokinins/auxin balance in the medium, is the direct differentiation of buds and organs from callus or explants through the concerted meristematic activity of a group of cells. Somatic embyrogenesis, often induced by treatment with potent auxins, is the formation of non-zygotic embryo-like structures from the somatic tissues such as embryos, microspores or leaves (Chandra and Pental, 2003).

2.1.2 Plant regeneration in Asiatic *Vigna* species

Plant regeneration in *Vigna* species, like in the large-seeded grain legumes can occur through three pathways namely de novo organogenesis, somatic embryogenesis or through proliferation of shoot meristems from areas surrounding a shoot bud (See Jaiwal and Singh, 2003). Among the three modes of regeneration as target cells for transformation, meristematic areas of cotyledonary nodes are the most preferred explant source as in *Vigna mungo* (Ignacimuthu et al. 1997; Sen and Guha-Mukherjee, 1998; Avenido and Hattori, 1999; Franklin and Ignacimuthu, 2000; Saini et al. 2003), *Vigna radiata* (Mathews, 1987; Gulati and Jaiwal, 1994; Avenido and Hattori, 2001; Avenido et al. 2001; Kumar et al. 2003; Amutha et al. 2006; Mundhara and Rashid, 2006; Vijayan et al. 2006), and *Vigna unguiculata* (Chaudhury et al. 2007; Solleti et al. 2008).

Direct organogenesis in *Vigna radiata* has been achieved from preexisting meristems of different seedling explants such as shoot-tips (Bajaj and Dhanju, 1979; Goel et al. 1983; Singh et al. 1985; Mathews, 1987; Gulati and Jaiwal, 1992), cotyledons (Mathews, 1987; Gulati and Jaiwal, 1994; Patel et al. 1991), cotyledon attached to embryonic axis (Das and Pal, 2004), and non meristemic explants such as primary leaf petiole with lamina and hypocotyls (Mahalakshmi et al. 2006). The type and size of explants, physiological status of

explant, age of donor seedlings, and orientation of explants on medium and genotype influenced the shoot regeneration in *Vigna radita* (Gulati and Jaiwal, 1990, 1992, 1994; Mahalakshmi et al. 2006). Among different cytokinins, BAP was found most effective for multiple shoot regeneration. However, the concentraton of BAP varied with the type of explant and genotype used (Gulati and Jaiwal, 1994; Vijayan et al. 2006). A combination of IAA and BAP induced high frequency regeneration from immature cotyledons of *Vigna radita* wherein the regeneration of shoot buds was obtained not only from the proximal end as reported earlier from mature cotyledons (Gulati and Jaiwal, 1990) but from the entire surface of the immature cotyledons isolated from embryos one week prior to harvest (Tivarekar and Eapen, 2001). Chandra and Pal (1995) reported differential regeneration responses from the two cotyledons in *Vigna radiata* i.e., one that is firmly attached to embryonal axis and the other that is easily detachable from germinating embryonal axis. The regeneration from cotyledon and cotyledonary node is highly site specific. Shoots developed from the adaxial side at proximal ends of cotyledons and from the nodal regions in cotyledonary nodes (Gulati and Jaiwal, 1992, 1994; Chandra and Pal, 1995; Avenido and Hattori, 1999). Thidiazuron, a non-purine cytokinin has been shown to exhibit stronger effects than other conventionally used cytokinins in a wide range of species of *V. radiata* (Murthy et al. 1998). The ability of TDZ in induction of shoot regeneration from mature cotyledons (Sen and Guha-Mukherjee, 1998), immature seeds (Tivarekar and Eapen, 2001), cotyledonary node and hypocotyls (Kumar et al. 2003; Amutha et al. 2006), and seedling (Mundhra and Rashid, 2006) has been reported. Continuous exposure of cotyledon explants to TDZ at high concentration resulted in complete loss of regeneration ability of explants whereas low TDZ concentration, brief exposure period (Kumar et al. 2003) and successive subculture in basal medium or basal medium supplemented with reduced TDZ levels (Amutha et al. 2006) were found to be optimal for shoot regeneration in mungbean. Histological observations revealed that in cotyledon cultures, shoots originated from epidermal and subepidermal cells present on the basal adaxial region of the petiolar residue (Mendoza et al. 1993). In cotyledonary nodes, regeneration of shoots occurred directly from epidermal and subepidermal cells without callus formation at the node (Gulati and Jaiwal, 1994). Both axillary and adventitious shoot formation from cotyledonary node explant was reported (Amutha et al. 2006). Therefore, cotyledonary nodes are considered promising explants for genetic transformation because of easy accessibility of *Agrobacterium* to regenerable surface cells (Gulati and Jaiwal, 1994 and Amutha et al. 2006).

In vitro plant regeneration in *V. mungo* has been reported via shoot organogenesis and somatic embryogenesis from various seedling explants. Among various explants, plant regeneration from cotyledonary node is dependable and reproducible. Multiple shoot

induction was influenced by the number and size of cotyledons attached to the cotyledonary node, type and concentration of cytokinin used, presence or absence of shoot tip, and genotype. The cotyledonary node explants with or without cotyledons developed multiple shoots from axillary meristems in blackgram (Saini et al. 2003; Sen and Mukherjee, 1998). The explant with both the cotyledons was found to be more efficient than those with one or no cotyledon. Complete removal of both the cotyledons delayed the shoot induction and shoot regeneration response (Saini et al. 2003). The morphology of leaves was also dependent on initial explants (presence /absence of cotyledon). In the presence of cotyledons, the axillary shoots produced from node were healthy and possessed trifoliate leaves whereas in the absence of cotyledons, the shoots were weak and possessed a simple leaf (Franklin and Ignacimuthu, 2000). The younger node gave a better shoot regeneration response than older explants (Saini et al. 2003). Excised mature cotyledons produced multiple shoot buds via *de novo* meristem formation in sub-epidermal cell layers on medium supplemented with low TDZ concentration (Sen and Mukherjee, 1998). TDZ induced shoots were incapable of elongation and therefore, required subculturing to basal medium with reduced hormones for proper elongation of regenerated shoots. Seed derived mature cotyledons and embryonic axis explants produced multiple shoot initials in blackgram (Ignacimuthu and Franklin, 1999). The duration of exposure of the explants to cytokinins was critical for the initial shoot induction. Cotyledons showed much higher regeneration response than embryonic axes. On cotyledon explant, shoots developed from preexisting meristem while on embryonal axes, shoot appeared to develop *de novo* from sub-epidermal parenchyma cell (Ignacimuthu and Franklin, 1999). Efficient multiple shoot induction and plant regeneration was reported from shoot apices of blackgram (Saini and Jaiwal, 2005). Preconditioning of the explant with BAP was effective in increasing the number of shoots by 3-fold over those without preconditioning treatment. A combination of two cytokinins was found more effective than either of the cytokinins in induction and proliferation of shoot buds from shoot tip (Agnihotri et al. 2001).

Avenido and Hattori (1999) observed induction of multiple adventitious shoots at the basipetal end (hypocotyls) of the cotyledonary explants in *V. angularis*, which is in contrast to all epigeal species (*V. radiata* and *V. mungo*) where multiple shoots developed from the node. In *V. angularis*, cotyledonary radicle junction possessed a pair of unique clamp-like connecting tissue termed as cotyledon flap (Endo and Ohashi, 1997), as a result of which the cotyledons do not abscise from the node. Development of adventitious shoots from the epicotyl explants derived from *in vitro* etiolated seedlings (Sato, 1995) and epicotyl derived from seedling explants (Takaheshi et al. 1998). The direct organogenesis method (Sato, 1995) was efficiently used in conjunction with *Agrobacterium tumefaciens* to develop transgenics in *V. angularis* (Ishimoto et al. 1996).

2.1.3 Gene delivery systems

Several methods have been developed for introducing genes from diverse organisms, encoding for desirable traits into plant cells (Potrykus, 1990, 1991). However, the production of transgenic plants relies on the stable introduction of foreign DNA into the genome of plant cells capable of regenerating to intact plants and the subsequent expression of the introduced genes. Successful transformation of plants demands the following criteria be met.

- Target tissues competent for propagation or regeneration
- An efficient DNA delivery method
- Agents to select the transformed tissues and recover fertile transgenic plants at a reasonable frequency
- A simple, efficient, reproducible, genotype-independent and cost-effective process
- A tight timeframe in culture to avoid somaclonal variation and possible sterility

During the past two decades, numerous methods have been developed to deliver exogenous DNA into plant cells (Potrykus, 1990, 1991). The most widely used DNA delivery systems which have potential practical applications include those based on the natural gene transfer mechanism of the gram negative soil bacterium *Agrobacterium*, microprojectile bombardment with DNA or biolistics and direct DNA transfer into isolated protoplasts by electroporation or chemical treatment. While these procedures differ in the way in which DNA is delivered into plant cells (De Block, 1993), they all require the regenerable cultured cells or tissues as recipients of foreign DNA. *Agrobacterium tumefaciens* and *A. rhizogenes* are the most extensively exploited gene transfer agents for generating transgenic plants in a wide variety of plant species (Hooykaas, 1989).

2.1.3.1 *Agrobacterium*-mediated gene transfer

The unique ability of *Agrobacterium* to precisely transfer defined DNA sequences to plant cells forms the basis of gene transfer mediated by *Agrobacterium* (Veluthambi et al. 2003). During infection, *Agrobacterium* introduces its T-DNA into compatible host plant cells which stably integrates into the plant genome via highly evolved molecular mechanisms (Walkerpeach and Velten, 1994). The plant's molecular machinery and nutritional resources are then utilized to produce unique amino acid derivatives, opines, that provide wild-type *Agrobacteria* with a novel food source, and plant type phytohormones that leads to the imbalance of auxins and cytokinins in the infected cells. The result of this genetic transformation by *Agrobacterium tumefaciens* is the generation of crown gall tumors on infected plant tissues (Gelvin, 2003). This tumor-inducing capability is due to the presence of auxin and cytokinins biosynthesis genes on a large Ti (tumor-inducing) plasmid in virulent strains of *Agrobacterium* (Tinland, 1996). Likewise, Ri (root-inducing) megaplasmids are

found in virulent strains of *Agrobacterium rhizogenes*, the causative agent of 'hairy root' disease i.e., formation of proliferative multibranched adventitious roots at the site of infection (Chilton et al. 1982). The Ri-plasmid shares large functional homologies with the Ti plasmid and appears to have evolved from a common ancestor (Sinkar et al. 1987).

The fact that foreign genes of agronomic value inserted between the T-DNA borders are also integrated into recipient plant genomes has led to the development of wide range of Ti or Ri based plant transformation vectors. Removal of oncogenes from the T-DNA of wild type strains of *Agrobacterium* results in disarmed Ti or Ri plasmids, which are then used to introduce foreign genes into plant cells. Regardless of the *Agrobacterium* species classification, as a genus, *Agrobacterium* can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species (Anderson and Moore, 1979; DeCleene and DeLey, 1976, porter, 1991; Van Wordragen and Dons, 1992) and gymnosperms (Levee et al. 1999; Loopstra et al. 1990; McAfee et al. 1993; Morris and Morris, 1990; Stomp et al. 1990; Wenck et al. 1999; Yibrah et al. 1996). In addition, *Agrobacterium* can transform fungi, including yeasts (Bundock et al. 1995, Bundock and Hooykaas, 1996; Piers et al. 1996), ascomycetes (Abuodeh et al. 2000; Groot et al. 1998) and basidiomycetes (Groot et al. 1998). Recently, *Agrobacterium* was reported to transfer DNA to human cells (Kunik et al. 2001).

The underlying molecular mechanisms of T-DNA transfer to plant cells have been extensively reviewed (Gelvin, 2003). Three genetic elements, *Agrobacterium* chromosomal virulence genes (*chv*), T-DNA delimited by a right border and a left border and Ti plasmid virulence genes (*vir*) constitute the T-DNA transfer machinery. During the course of infection, *Agrobacterium* processes a region of DNA, (the T-transferred DNA), from a resident Ti-(tumor inducing) plasmid and transfers T-DNA and several virulence (Vir) proteins to plant cells. These Vir proteins include VirD2 (an endonuclease that is involved in T-DNA processing and that is covalently attached to the 5′ end of the processed single-stranded T-strand), VirE2 (a sequence-independent single-stranded DNA binding protein that probably coats T-strands) and VirF (Gelvin, 2003). It has been proposed that VirD2, VirE2 and the T-strand form a complex, the T-complex, which is the probable molecular form that is transported through the plant cytoplasm into the nucleus (Howard and Citovsky, 1990). The site of assembly of this complex (within the bacterium or within the plant cell) remains somewhat controversial (Gelvin, 2000). The mechanisms governing the transfer of 'T-complex' via the conjugation channel and the roles of plant and *Agrobacterium* proteins in T-DNA integration are being intensely studied (Veluthambi et al. 2003).

Initially, vectors used for *Agrobacterium*-mediated plant transformation were deletion derivatives of Ti plasmids, termed as 'co-integrative vectors'. The gene(s) of agronomic

interest to be introduced in the T-DNA of disarmed Ti plasmid, are cloned into an intermediate vector (which is small and easily manipulated *in vitro*) and subsequently cloned between the T-DNA borders of disarmed Ti plasmid by the process of homologous recombination within the bacterium (Walkerpeach and Velten, 1994). In the co-integrative vector types, the T-DNA and *vir* functions reside in the same replicon and as a part of Ti plasmid. However, the problem encountered with co-integrative vectors is their size, which makes their manipulation difficult. Subsequent research has not demonstrated any specific advantage of retaining the T DNA and *vir* functions on a single replicon, except plasmid stability in *Agrobacterium*.

Binary vectors have increasingly been adopted, primarily because of their ease of manipulation (Walden and Wingender, 1995). The binary vector system consists of two autonomously replicating plasmids within *A. tumefaciens*, a shuttle (or more commonly, binary) vector, which is a small plasmid containing T DNA, which is easy to manipulate in *Escherichia coli* and capable of replication both in *E. coli* and *Agrobacterium*, and a 'helper' Ti plasmid (disarmed Ti plasmid lacking the T DNA) that provides the *vir* functions to facilitate transfer into plant cells. Disarmed helper Ti plasmids have been engineered by removing the oncogenic genes while still providing the necessary *vir* functions required for T DNA transfer to the host plant cell. Several disarmed helper Ti plasmids are in common usage, which includes pAL4404, pGV3850, pEHA101, pTiBo542ΔT, pMOG101, pEHA105 etc. Binary vectors obviate the need for *in vivo* recombination and these relatively small size vectors are preferred due to the ease of both *in vivo* and *in vitro* DNA manipulation and their higher transformation efficiencies. Since, binary plasmids exist as separate replicons, copy number is not strictly tied to that of the Ti plasmid.

Since the binary vector such as pBIN19 (which offer one selectable marker) constructed by Bevan in 1984, many modifications have been made in these vectors to expand the range of their utility and to improve their transformation efficiency. The construction of the superbinary vector pTOK233 (Hiei et al. 1994) by cloning the *vir*B, *vir*G and *vir*C genes of pTiBo542 in pGA472 has led to the successful transformation of recalcitrant plant species such as rice (Hiei et al. 1994; Aldemita and Hodges, 1996; Mohanty et al. 1999; Kumaria and Rajam, 2001; Dong et al. 1996), maize (Ishida et al. 1996), sorghum (Zhao et al. 2000), onion (Zheng et al. 2001) and mungbean (Jaiwal et al. 2001). pBIN20, the improved version of pBIN19, with many additional single restriction sites in the MCS was reported (Hennegan and Danna, 1998). A new series of pPZP vectors have been developed which are small in size and stable in *Agrobacterium* (Hajdukiewicz et al. 1994). The pPZP vector backbone was used to construct the pCAMBIA series of vectors with a combination of selectable marker and reporter genes. The pCAMBIA vectors are very widely used for plant transformation. Plant

expression vectors of pRT100 series (Topfer et al. 1993) permit construction of gene cassettes with CaMV 35S promoter and its polyA signal. These cassettes can be excised and placed in the MCS of binary vectors.

A typical binary vector system comprising an octopine type *vir* helper strain such as LBA4404 (Hoekema et al. 1983) that harbours the disarmed Ach5 Ti plasmid and a binary vector such as pBin19 (Bevan, 1984) is very commonly used for plant transformation. The available range of *vir* helper strains has been expanded with the nopaline-type MP90 (Koncz and Schell, 1986) and the L, L-succinamopine-type EHA101 (Hood et al. 1986). The bacterial kanamycin resistance gene in EHA101 was deleted to develop the *vir* helper strain EHA105 (Hood et al. 1993). EHA101 and EHA105, by virtue of harbouring the 'supervirulent' *vir* genes, exhibit broader host-range and higher transformation efficiency. Many recalcitrant plants such as rice (Rashid et al. 1996), wheat (Cheng et al. 1997) and barley (Tingay et al. 1997) have been transformed using EHA101 and EHA105. A new *vir* helper strain pTiChry5 has been constructed from an *Agrobacterium* strain virulent on soybean (Torisky et al. 1997).

2.1.3.2 Gene delivery by Biolistics

Biolistics (Finer et al. 1991) is the delivery of microprojectiles, usually of tungsten or gold coated with DNA and propelled into the target cells by acceleration. The acceleration can be provided by gun powder, by gases, such as helium or CO2, or by an electric discharge. This method can introduce DNA into virtually any tissue from any cultivar and the success depends critically upon the ability of target tissue to proliferate and give rise to a fertile plant. There are basically three systems for particle bombardment with various modifications. Electric discharge particle acceleration device ACCEL^{TM} utilizes an instrument to accelerate DNA-coated gold particles with any desired velocity by varying the input voltage. The biolistic PDS 1000 He device is powered by a burst of helium gas that accelerates the macrocarrier upon which; DNA-coated gold particles (microprojectiles) are uniformly bombarded over target cells. Minor alterations in the standard biolistic protocol have yielded tremendous improvements (Quecini et al. 2006; Indurker et al. 2007; Kamble et al. 2003; Singh et al. 2004; Lacorte et al. 1997; Rech et al. 2008; Strömvik et al. 1999; Li et al. 2005; Aragao et al. 1997; Chiera et al. 2004; Ivo et al. 2008; Bonfim et al. 2007; Magbanua et al. 2000) These include:

- Proper pre-culture of the explant material
- Use of baffling screens
- Use of small size microprojectiles
- Subjecting the tissue to an osmotic pretreatment by either partial drying in a laminar flow hood or culturing in a medium containing an osmotic agent

The advantages of particle bombardment system are: (1) virtually any type of meristematic totipotent cells, tissues, organs and monocots that are not readily amenable to *Agrobacterium*-infection, can be transformed, (2) DNA may be transferred without using specialized vectors, (3) The introduction of multiple DNA fragments/plasmids can be accomplished by co-bombardment, thus eliminating the necessity of constructing a single large plasmid containing multiple transforming sequences, (4) False positives resulting from reporter gene expression in *Agrobacterium* are avoided, (5) Transformation protocols are applicable to plants, which lack good regeneration systems, (6) Organelle transformation is achieved only by particle bombardment and (7) Application in transient gene expression studies in differentiated tissues (Klein et al. 1992).

Though the biolistic gene delivery system has been successfully used to develop transgenics, molecular analysis of plants obtained by biolistic transformation generally reveals a complex pattern of transgene integration, for example, high copy number and rearrangements of transgene(s), thus causing gene silencing or genomic rearrangements. In addition, delivery of long fragment DNA is challenging because breaks can occur in the delivered DNA. Although the fate of introduced DNA is not clear, ligation of the transgenic DNA fragments before integration is proposed to account for the observation of arrays of transgenic DNA integrated at the same site into the plant genome (Pawlowski and Somers, 1998). This can result in the reduction of transgene expression by co-suppression. Some of the pitfalls of biolistics are circumvented by the delivery of fragment DNAs or by using the 'agrolistic' approach (Hansen et al. 1997). This novel method permits generation of a simple insert even after biolistic or protoplast transformation. Many recent improvements have been made to overcome problems relating to gene silencing and genetic integrity.

2.1.3.3 *In planta* transformation

There is considerable interest in developing plant transformation methods that obviates the tissue culture steps, eliminates somaclonal variation and relies on simple protocols. These methods are called *in planta* transformation because transgenes are generally delivered into intact plants in the form of naked DNA or from *Agrobacterium*. The stage of plant development selected is variable but for many species it is around the time of zygote formation. This choice stems from the underlying assumption that at fertilization the egg cell accepts the donation of an entire genome from the sperm cell and it might thus be the appropriate stage to integrate transgenes. This concept is exemplified by the successful transformation method developed for *Arabidopsis* wherein flowers were infiltrated or dipped into an *Agrobacterium* suspension and subsequently some of the harvested seeds were transgenic (Bechtold et al. 1993; Clough and Bent, 1998). Although the overall efficiency is

low, the sheer number of seeds recovered for screening and the ease of the method makes it an extremely attractive alternative. In a similar approach, cotton transformants were recovered following injection of DNA into the axil placenta about a day after self-pollination (Zhou et al. 1983). Similarly, a mixture of DNA and pollen was either applied to receptive stigmatic surfaces or DNA was injected directly into rice floral tillers (Landgridge et al. 1992), or soybean seeds were imbibed with DNA (Trick et al. 1997). These procedures, intriguing as they are, are impractical at present because of their low reproducibility.

Direct access to the male gamete is an option that can be achieved by biolistic delivery of DNA into tobacco pollen in culture. Subsequent pollination with the bombarded pollen led to the recovery of transgenic plants (Touraev et al. 1997). Similarly, this method could most certainly be applied to other crops (Stauffer et al. 1991). However, this apparently clear strategy might, in fact, be complicated by a variety of factors including the presence of nucleases and methylases in pollen (Oakeley et al. 1997), pollen survival and polyploidy (Leitch and Bennett, 1997). In another approach, meristematic cells located in the apical dome of nodal buds have also been considered ideal recipients for transgenes because growth and development occur in this area. Furthermore, some cell layers of the apex will eventually contribute to the germline and are thus transmitted to the sexual offspring. DNA and lipofectin injection followed by electroporation into pea apical meristems allowed the recovery of transgenic offspring (Trick et al. 1997). Excised apical meristems from embryonic axes and shoot tips (Park et al. 1996) have also been used as targets for transformation by particle bombardment or by *Agrobacterium* inoculation. After DNA delivery, these tissues are induced to form multiple shoots prior to whole plant regeneration. This method is, however, very labor-intensive because each regenerated plant must be screened for transgenic sectors that will potentially contribute to the germline. In addition, this method requires some tissue culture steps. Shoot apices are far less efficient as the target for transformation compared with embryogenic tissues, with the additional risk of obtaining chimeric plants (Park et al. 1996). However, the efficiency can be improved by multiplication of shoot meristems before DNA delivery (Livingstone and Birch, 1999; Santarem and Finer, 1999; Aragão et al. 2000; Gulati et al. 2002; Aragão et al. 2002).

2.1.3.4 Alternative methods of transformation

There is a perpetual quest to find more efficacious and economical methods for plant transformation. Some of these are described here, but are not often used because of their impracticability at present. Plants can be recovered after DNA delivery using silicone carbide whiskers (Thompson et al. 1995) but the use of these fibers requires caution because of their potential carcinogenic effects. The electroporation method (Lurquin, 1997) is not often used

because of its low reproducibility. Microinjecting DNA into zygotes (Leduc et al. 1996) is an alternative method that could eventually lead to recovery of transgenic plants. Uptake of naked DNA by plant cells can also be facilitated by laser microbeam (Hoffman, 1996). However, these options, which are still at the early stages of development, can be tedious and require sophisticated equipment.

2.1.4. Marker genes for selection of transgenic plants

Irrespective of the transformation methods followed, only a small proportion of the cells of the treated explants, exposed to the transformation process are stably transformed (Miki and McHugh, 2004). Therefore, all transformation systems for creating transgenic plants require separate processes for identifying or selecting those cells that have integrated the DNA into the appropriate plant genome (nuclear or plastid) and for regenerating or recovering fully developed plants from the transformed cell. Further, efficient processes are required to follow early gene transfer and monitor the genetic transformation events in plant species. The incorporation of marker genes along with genes conferring desired traits in plants enables the selection of transformed plant cells and tissues, and permits the early detection of transformation events.

2.1.4.1. Selectable marker genes

Selectable marker genes have been pivotal to the development of plant transformation technologies because the marker genes allow identification of the cells that are expressing the cloned DNA and to monitor and select for the transformed progeny. As only a very small proportion of cells are transformed in most experiments, the chances of recovering transgenic lines without selection are usually low. Hence, incorporation of a selectable marker gene along with the gene(s) of interest in the transformation vector construct confers a selectable advantage to the recipient cells expressing the introduced gene and encourages their preferential growth. Since the selectable marker gene is expected to function in a range of cell types, it is usually constructed as a chimeric gene using regulatory sequences that ensure constitutive expression throughout the plant. The selectable marker gene is usually co-transformed with a gene of interest. The selectable marker genes, that allow the recognition of the transformed cells from a background of untransformed cells, code for a product that effectively detoxify or evade the selective agent by enzymatic modification or expression of an altered target. These genes are dominant, usually of microbial origin, and placed under the control of strong, constitutive, eukaryotic promoters, often of viral origin (Birch, 1997).

Approximately 25 marker genes, mostly conferring resistance to antibiotics or herbicides, have been successfully used for plant transformation (Scutt et al. 2002). The most widely used

selectable marker gene is neomycin phosphotransferase-II (*npt*II), which confers resistance to the aminoglycoside antibiotics such as kanamycin sulfate and its analogues paromomycin and geneticin (G418) (Bevan et al. 1983). Hygromycin phosphotransferase (*hpt*) gene that confers resistance by specifically phosphorylating hygromycin B (Van den Elzen et al. 1985) has also been successfully employed for efficient recovery of transgenic plants (Sanyal et al. 2005; Krishnamurthy et al. 2000; Kelemu et al. 2005; Chiera et al. 2004; Orczyk et al. 2000; Lawrence and Koundal, 2001; Kumar et al. 2008; De Clercq et al. 2002; Magbanua et al. 2000; Bottinger et al. 2001; Czihal et al. 1999; Yamada et al. 2001; Jaiwal et al. 2001; Ignacimuthu, 2000; Polowick et al. 2000; Sharma and Anjaiah, 2000. The *bar* (bialaphos resistance) gene which confers resistance to the herbicide BASTA® (Murakami *et al*., 1986; Thompson et al. 1987) and *pat* (phosphinothricin acetyltransferase) gene for phosphinothricin (Strauch et al. 1988) have provided stringent selection of transgenic plants (Miki and McHugh, 2004). Phosphinothricin or PPT is an analogue of glutamate and competitively inhibits glutamine synthetase (GS), an enzyme that plays a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants (Skokut et al. 1978). It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation and photorespiration. The inhibition of glutamine synthetase by PPT causes rapid accumulation of ammonia and eventual cell death (Beriault et al. 1999). Two PPT derivatives have been successfully used as selective agents for transformation (Singh et al. 2004; Muruganantham et al. 2007; Krishnamurthy et al. 2000; Bean et al. 1997; Schroeder et al. 1995; Richter et al. 2007; Orczyk and Orczyk, 2000; Olhoft et al. 2003; Li et al. 2000). Bialaphos, a natural tri-peptide compound consisting of PPT and two alanine residues (Murakami et al. 1986; Thompson et al. 1987), is an antibiotic produced by *Streptomyces hygroscopicus* as an extracellular product (for review, see Thompson and Seto, 1995). Glufosinate, on the other hand, a synthetic commercial formulation that is the ammonium salt of PPT, is an active ingredient of the herbicide BASTA® (Aventis, France). Similarly the *EPSPS* gene, encodes for 5-enolpyruvyl-shikimate-3-phosphate synthase, confers tolerance to the herbicide glyphosate. The herbicide-based selection of transformants has been successful in a wide array of crops (Sahoo et al. 2001). Expression of the hygromycin B transferase gene has also been reported to confer tolerance to the herbicide glyphosate (Penaloza-Vazquez et al. 1995).

Many plants are naturally resistant to particular selective agents, and an effective antibiotic/herbicide for a particular species and further for an explant can only be determined empirically. Thus it is important to design an experiment to test the frequency of “escapes” in any particular explant and for each selective condition. Careful evaluation of the concentration of the selective agents, that inhibits growth in a large proportion of particular

explants but allows the “escape” of a few percent, is essential prior to any transformation experiment. Further a “kill curve” that determines the lowest concentration of selective agent that will kill 100% of explants facilitates the right concentration to be chosen. In addition, the application of selective agents can be applied in gradual increments (step-wise selection) or in lethal concentrations soon after transformation. Application of selective agent immediately after transformation or after a short recovery period is an important consideration for successful recovery of transgenics (Sharma and Anjaiah, 2000). All these approaches have been successful in the transformation of a number of species and a variety of explants. Prior to transformation, it is thus essential to screen different selective agents, and to find out their optimal concentration that preferentially permit growth and development of the transformed cells without hindering or impairing the normal regeneration process. There is a concern that the transformation efficiencies are suboptimal with toxic substrates because dying untransformed cells may inhibit transformed cells from proliferating by secreting inhibitors or preventing transport of essential nutrients to the living transformed cells (Hardrup et al. 1998a).

Mannose-6-phosphate isomerase (MPI) and xylose-isomerase genes facilitate the identification and selection of transformed cells without injuries or death of the non-transformed population of cells (negative selection). Mannose-6-phosphate isomerase (MPI) encoded by *manA*, converts the unusable carbon source mannose-6P to fructose-6P. Thus, transformants containing *manA* can grow on mannose as a sole carbon source. This selection has a positive mode of action that encourages the growth of transformed tissues rather than just permitting it. The MPI marker is extremely effective for the selection of transformed sugar beet (Joersbo et al. 1998), cassava (Zhang et al. 2000) and maize (Wang et al. 2000). Plant cells from species such as tobacco, potato and tomato cannot use d-xylose as a sole carbon source. The enzyme xylose isomerase catalyzes the isomerization of xylose to d-xylulose, which can then be used as a carbon source. The efficiency of selection of *xylA* genes, coding for xylose isomerase from *Streptomyces rubiginosus* (Haldrup et al. 1998a) and *Thermoanaerobacterium thermosulfurogenes* (Haldrup et al., 1998b), have been much greater than for the *npt*II gene and the regeneration of shoots was significantly faster. The enzyme from *S. rubiginosus* posed no biosafety issues as it is used in the food industry and considered safe (Haldrup et al. 1998a). You et al. (2003) have developed the *pflp* gene from sweet pepper as a new generation of selectable marker for orchid transformation.

Organogenesis *in vitro* occurs in three phases: the acquisition of competence, determination of organ formation governed by phytohormone balance and morphogenesis (Sugiyama, 1999). For shoot formation in culture, high cytokinin:auxin ratios are required. The *ipt* gene of T-DNA of *A. tumefaciens* Ti plasmids, codes for isopentyl transferase (IPT) enzyme that

catalyzes the synthesis of isopentyl-adenosine-5-monophosphate, the first step in cytokinin biosynthesis. The conditions promote and enhance regeneration of shoots endogenously thus providing a novel positive selection strategy. Moreover, the effectiveness of the *npt*II gene as a selectable marker was enhanced when it was co-transformed with the 35S-*ipt* gene construct (Endo et al. 2001). The *Arabidopsis* genome codes for a family of IPT genes which catalyze similar reactions and generate the same phenotype when expressed in transgenic plants (Takei et al. 2001; Sun et al. 2003). They may be effective substitutes for the *A. tumefaciens ipt* gene. The difficulty with this system is that all of the regenerated shoots have abnormal morphologies resulting from the high endogenous cytokinin levels which include the loss of apical dominance and lack of roots (i.e. the shooty phenotype). The use of a β-estradiol-inducible, artificial promoter system to restrict expression of the *ipt* gene during the selection phase appeared to eliminate these morphological abnormalities in regenerated tobacco shoots and plantlets (Kunkel et al. 1999). The reported high frequency of escapes are assumed to result from cytokinins produced in the transformed cells that migrate to non-transformed cells and induce shoot formation (Zuo et al. 2002a); however, this assumption is uncertain (Kunkel et al. 1999).

2.1.4.2. Reporter genes

A number of reporter genes have been used as convenient markers to visualize gene expression and protein localization in a wide range of plant species. For every transformation methods, transient expression experiments are generally performed with a reporter gene. These transient expression assays with reporter/scorable marker are a preliminary step used to identify conditions that will allow efficient DNA delivery. With so many variables and no evidence of combinations that are broadly applicable across plant species, this simple test provides some guidance for answering several initial questions, by providing the most convenient way of measuring both the quantitative (i.e. the level of gene expression) and qualitative (i.e. localization of gene expression) expression of introduced DNA into the target cells. As the conversion rate from transient expression to stable integration in the plant genome is low and estimated to be, 1% to 9% for DNA (Finer et al. 2001), it is worthwhile achieving a high frequency of transiently expressing cells following DNA delivery.

Each gene has specific, inherent characteristics that define both its limitations and the applications for which it will be useful. The *Escherichia coli gus* reporter gene (also referred to as *uidA*), which encodes ß-glucuronidase (GUS), has been extensively used in plants (for review see Martin et al. 1992). The ß-glucuronidase assay invented by Jefferson is very sensitive, and it is possible to obtain both qualitative (histochemical) and quantitative (fluorometric) data (Jefferson et al. 1987). This reporter gene is routinely used for promoter

analysis and to study protein targeting; it is also an invaluable tool when used to follow gene transfer and monitor the genetic transformation of plant species. However, the histochemical GUS assay is destructive for tissue and therefore not suitable for direct visual selection of transformed plants.

The firefly luciferase (*luc*) and green fluorescent protein (*gfp*) genes allow the non-toxic detection of tissues expressing the transgenes. The formation of Luciferase can be monitored *in vivo* but requires an exogenous substrate (luciferin) and emits light at low intensity (Ow et al. 1986). The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was reported to function as a sensitive reporter of *in vivo* gene expression (Chalfie et al. 1994; Heim et al. 1994). This gene emits bright fluorescence upon excitation with ultraviolet (excitation max=395 nm) or blue light (excitation max=475 nm). The formation of the fluorescent chromophore requires no exogenous substrates or cofactors and is easily visible. GFP is stable and is only denatured under extreme conditions. So far, only molecular oxygen has been identified as a possible cofactor for chromophore formation (Heim et al. 1994). These properties make GFP an ideal nondestructive marker for plant use. It can thus be used as a means to visualize the fate of transformed cells over time and rapidly test the influence of various factors through the successive steps of the transformation protocol. The selective subculture of transient *luc* or *gfp* expressing plant cells has led to the successful recovery of transgenic plants. However, the process carries additional complication of being need of a much precised regeneration system from the transformed region. The wild-type GFP has been used for expression studies in plants in various transformation systems or virus-based delivery systems (Haseloff and Siemering, 1998). However, to achieve efficient expression of GFP in plants, a cryptic intron sequence has to be removed from the coding sequence (Haseloff et al. 1997). Mutants of GFP with increased stability and enhanced fluorescence have been isolated (i.e. Davis and Vierstra, 1998; Haseloff and Siemering, 1998; Stewart, 2001).

2.1.5. Genetic transformation of Asiatic *Vigna* species

Production of *Vigna* species is severely limited by a number of biotic and abiotic stresses. Genetic improvement of these bean species through conventional breeding relies on the utilization of domestic cultivars and related genera as a source of genes. Genes conferring resistance to biotic and abiotic stresses have been found in many wild or related species (Sahoo and Jaiwal, 2008). However, the major impediments in using the wild cultivars as a source of resistance are the cross-incompatibility with the cultivated ones, and the linkage drag of undesirable traits (Watanasit and Pichitporn, 1996). The resistance in cultivated gene pool in subgenus *Ceratotropis* is rare when compared to the wild species (Tomooka et al.

2002). Genetic barriers existing in the native *Vigna* species further prevent the transfer of useful traits to the related species (Palmer et al. 2002).

Enormous potential for genetic manipulation of these species lies in increasing resistance to diseases, pests and abiotic stress, and qualitative change in their seed composition. Beyond crop improvement, the ability to engineer *Vigna* species holds a powerful and informative means for studying gene function and the regulation of physiological and developmental processes. However, development of efficient and routine genetic transformation systems in grain legumes and *Vigna* species in particular has posed a formidable challenge in the generation of stable transgenic plants. Advances in tissue culture, combined with improvements in transformation technology, have resulted in increased transformation efficiencies in many grain legumes (Somers et al. 2003). However, *Vigna* species have still remained recalcitrant to tissue culture and are stubbornly resistant to the overtures of genetic engineering (Chandra and Pental, 2003). Gene transfer to these species demands development of robust plant regeneration systems from tissues amenable to routine genetic transformation methods, efficient selection system for recovery of viable and fertile transgenic plants from transformed explants at a reasonable frequency and a timeframe in culture to avoid somaclonal variation and possible sterility (Sahoo et al. 2001). *Agrobacterium tumefaciens* mediated gene transfer approach has been employed for successful generation of transgenic plants in *Vigna* species. A summary of the key transformation events is presented in Table 1. However, severe host-genotype specificity, lack of wounding response, the choice of suitable explant source potentially competent for both regeneration and *Agrobacterium* infection have so far limited the optimal success through *Agrobacterium*-mediated transformation.

Direct organogenesis pathway from cotyledon, cotyledonary nodes and primary leaf explants has been adapted for *Agrobacterium*-mediated transformation of *V. radiata*. The axillary meristems at the junction of the cotyledon and cotyledonary node explants contain cells most responsive for regeneration through multiple shoot induction and hence offers suitable target for gene delivery. Pal et al. (1991) recovered primary transformants from cotyledons of *V. radiata* cvs. B1 and T44, and confirmed through dot blot assay that approximately 4-5% of the shoots produced on the kanamycin selection were transformed. However, large number of escapes on kanamycin selection, lack of evidence for stabilization and inheritance of transgenes ruled out the applicability of their transformation system. Transgenic calli were generated from primary leaf explants of *V. radiata* that exhibited β-glucuronidase activity, and integration of *npt*II gene (Phogat et al. 1999). However, the calli failed to regenerate to plants under tested culture conditions. Transformed hairy roots were developed at a high frequency from cotyledon and hypocotyls explants of *V. radiata* (Jaiwal et al. 1998). Stable transgenic plants were generated from cotyledonary nodes of *Vigna radiata* cv. K-851 by

employing a co-integrate vector pTOK233, that contained a duplicated *virG* region (Jaiwal et al. 2001). The plants exhibited stable GUS expression reproductive parts and seeds of T_0 plants. The authors reported recovery of transgenic calli from primary leaves and hypocotyls explants at a frequency of up to 50% and demonstrated integration of transgenes. Mahalakshmi et al. (2006) recovered transgenic plants of *Vigna radiata* cv. K-851 from primary leaf explants on hygromycin selection and confirmed the stable integration and inheritance of *hpt* gene. However, absence of prolific regeneration from primary leaf explants and generation of escapes on hygromycin containing medium prevented the use of these explants for routine introduction of candidate genes. Sonia et al. (2007) generated fertile transgenic plants from cotyledonary node explants of *V. radiata* that carried *bar* gene and *Phaseolus vulgaris* α-amylase inhibitor-1 (αAI-1) gene. Preculture and wounding of the explants, presence of acetosyringone and PPT-based selection in the protocol resulted in enhancing transformation frequency to 1.51%. Bhargava and Smigocki, (1994) reported 4-fold increase in the level of transient *gus* expression in the germinated embryos of *V. aconitifolia*, *V. mungo* and *V. radiata* after two to three times bombardment of DNA-coated particles.

Karthikeyan et al. (1996) obtained transformed calli from primary leaves segments of *V. mungo* which showed stable integration of *npt*II gene. The gene expression was demonstrated by neomycin phosphotransferase assay. Saini et al. (2002) demonstrated the competence of seedling epicotyl explants of *V. mungo* for *Agrobacterium* mediated transformation. Saini et al. (2003) generated fertile transgenic plants of *V. mungo* from cotyledonary node explants through *A. tumefaciens*-mediated transformation at a frequency of 1%. Mechanical wounding of the explants prior to inoculation with *Agrobacterium*, time lag in regeneration due to removal of the cotyledons from explants, and a second round of selection on kanamycin at the rooting stage were found to be critical for transformation. The low efficiency of transformation of meristematic cells in the axil of the cotyledonary node regions was attributed to the presence of limited number of regenerable cells whose capacity for regeneration was short-lived and inefficient T-DNA delivery to the regenerable cells (Saini and Jaiwal, 2005). An *Agrobacterium* mediated transformation of shoot apex explants was developed to overcome these limitations (Saini and Jaiwal, 2005). A significant increase in production of transgenic plants was observed from an average of 1% to 6.5% when shoot apices were preconditioned with BAP, and wounded prior to their inoculation with *A. tumefaciens*. Shoot apex is preferred because of the high regeneration potential of the pre-existing meristems, which require minimal tissue culture manipulations for rapid and direct multiple shoot induction in a less genotype independent fashion. Various factors involving *Agrobacterium*-mediated transformation of *V. mungo* cotyledonary node explants were

optimized (Saini and Jaiwal, 2007). Preculture and wounding of explants, manipulations in inoculation and co-cultivation conditions were found to play a significant role in influencing tissue competence, *Agrobacterium* virulence and *Agrobacterium*-tissue compatibility for leading to a high stable transformation efficiency, 4.31 %. Bhomkar et al. (2008) overexpressed the glyoxalase I (gly I) gene of *Brassica juncea* (Veena et al. 1999) under a novel constitutive Cestrum yellow leaf curling viral (CmYLCV) promoter (Stavalone et al. 2003) in transgenic *V. mungo* that survived salt stress (up to 100 mM) and set seed. The authors achieved a transformation frequency of 2.25% by employing cotyledonary node explants for *Agrobacterium*-mediated transformation.

Reproducible transformation in azuki bean (*V. angularis*) was reported using *A. tumefaciens*-mediated gene transfer to epicotyl explants (Yamada et al. 2001). The combined action of acetosyringone and high concentration of BA in co-cultivation medium played important roles in improving transformation efficiency in epicotyl explants of azuki bean. The authors observed the effect of *Agrobacterium* strain on transformation efficiency. Ishimoto et al. (1996) generated fertile adzulkibean with bean α-amylase inhibitor gene by employing *Agrobacterium*-mediated transformation of epicotyl explants. The transgenic seeds expressing *αAI* gene driven by the promoter of phytohemagglutinin resulted in high levels of αAI in the seeds which conferred complete protection from bruchid development on the seeds. The D6-fatty-acid desaturase gene isolated from *Mortierella alpina* 1S-4 was introduced and constitutively expressed in *V. angularis* by *Agrobacterium*-mediated transformation (Chen et al. 2005). The genetic transformation procedure was performed using a modified version of the method of Yamada et al. (2001). The γ-linolenic acid (GLA) accumulated at levels of up to 0.44 and 1.26% of the total fatty acids in the leaves and seeds, respectively, of a transgenic *V. angularis* line and at 0.69% in the leaves of its progeny. These findings reveal the modification of the fatty-acid biosynthetic pathway as a possible approach to produce specific polyunsaturated fatty acids in grain legume. Yamada et al. (2005) isolated cDNAs encoding for two isoforms of α-amylase inhibitor i.e., αAI-Pa1 and αAI-Pa2 from tepary bean (*Phaseolus acutifolius*) and expressed these cDNAs in *Vigna angularis*, which revealed that active forms of the inhibitor proteins accumulated in seeds and exhibited specificities for insect α-amylases identical to those of the native proteins purified from tepary bean seeds. Seeds transgenic for ai-Pa1 thus inhibited the larval α-amylase activity of *C. chinensis* but not that of *Z. subfasciatus*, whereas ai-Pa2 transgenic seeds inhibited the larval α-amylases of both of these insects. Seed extracts of both types of transgenic plant inhibited the larval α-amylases of the cereal storage pests *T. molitor* and *T. confusum* but had no effect on porcine pancreatic α-amylase. The inhibitory specificities of the transgenic azuki bean seeds thus further confirmed that ai-Pa1 and ai-Pa2 encode αAI-Pa1 and αAI-Pa2, respectively. The

active form of αAI-Pa1 in transgenic azuki bean was a single polypeptide with a size similar to that expected for the full-length encoded protein. These results suggested that αAI-Pa1 defining a new type of bean αAI, structurally related to lectins and not activated by proteolytic processing.

2.2. Regeneration and genetic transformation of cowpea

A considerable progress has been made in plant regeneration and genetic transformation of cowpea (*V. unguiculata*) despite the fact that the species is notoriously recalcitrant to *in vitro* genetic manipulation (Ramakrishnan et al. 2005a). The recalcitrance towards *in vitro* regeneration has been a major constraint in transgenic cowpea production, required for both the validation and exploitation of data generated by molecular tools e. g. gene over-expression, gene suppression, promoter analysis and gene tagging (Solleti et al. 2008).

2.2.1. *In vitro* plant regeneration in cowpea

During the last two decades, several investigations have been made in a wide range of cowpea cultivars and breeding lines to explore their shoot regeneration potential. The regeneration systems based on direct or indirect shoot organogenesis and somatic embryogenesis have been adapted in cowpea (Sahoo et al. 2003).

Pandey and Bansal, (1989) reported plantlet formation in cowpea from callus cultures initiated from leaf explants. The regenerated plants showed among the normal diploid chromosome number with an occurrence of about 1% aneuploid ones. Muthukumar et al. (1995) regenerated shoots from callus induced from primary leaf explants including the intact petiole in cowpea. Plants regenerated from callus, cell suspensions are known to exhibit somaclonal variations, which may be genetic or epigenetic in nature. Potentially useful somaclonal variants have been identified in many crop plants including legumes (Jaiwal and Gulati, 1995; Singh et al. 1998; Sonia et al. 1998). The potential of somaclonal variations need to be harnessed for possible extension cowpea gene pool.

Somatic embryogenesis offers a means for propagating essentially limitless number of discrete propagules and also has potential for efficient production of non-chimeric transgenic plants. Kulothungan et al. (1995) reported establishment of suspension cultures and somatic embryo formation from callus induced from seedling leaf explants of cowpea. Plantlets were obtained from cotyledonary stage embryos. Induction of somatic embryos in cowpea has been reported from suspension cultures of calli derived from seedling leaf explants (Ganapathi and Anand, 1998). Maximum somatic embryos were obtained when calli were transferred to liquid medium supplemented with in 2-, 4-dichlorophenoxy-aceticacid. The characterization of the stages and processes of somatic embryo development and screening of explant sources

other than young leaves for somatic embryos induction in solid and liquid suspension cultures need thorough investigation. The basal medium developed for embryo development by Pellegrineschi et al. (1997) could form a starting point for formulating media for growth of somatic embryos *in vitro*. Growth medium supplements that enhanced embryo development included addition of sucrose, casein hydrolysate, and any one of three commonly used cytokins, namely zeatin, benzyl amino purine (BAP), and kinetin, for enhancing embryo maturation. Prem Anand et al. (2000) stressed the importance of organic supplements along with 2,4-D for the induction of embryogenic calli. Ramakrishnan et al. (2005a) reported an efficient protocol for plantlet regeneration from the cell suspension cultures from primary leaf-derived embryogenic calli of cowpea. The sequence of events in the functional body pattern formation during the somatic embryo development was described in cowpea suspension cultures (Ramakrishnan et al. 2005b).

The establishment and maintenance of embryogenic cultures as well as recovery of plants can be an extremely labor intensive and lengthy process that has the added risk of encountering morphological abnormalities and sterility among regenerants. In contrast, multiple shoot formation via organogenesis is simpler once a suitable explant has been identified. Various laboratories have independently reported successful regeneration of cowpea by direct organogenesis from a variety of explants. Kartha et al. (1981) described a method for cowpea regeneration from shoot apical meristem at low level of exogenously supplied cytokinin. The authors attributed the presence endogenous hormones in cowpea meristems to be adequate to effect plant regeneration. Multiple shoot induction from mature de-embryonated cotyledons with intact proximal end was reported albeit with low frequency in cowpea (Muthukumar et al. 1996). Addition of putrescine to high cytokinin medium enhanced the shoot forming response of axenic hypocotyls and cotyledons excised from green immature pods in five different genotypes of cowpea (Pellegrineschi, 1997). Fertile cowpea plants have been regenerated successfully using nodal thin cell layer (TCL) explants (Le et al. 2002). The TCL, approximately eight cells thick, was obtained by cutting twice over each cotyledonary node, followed by regeneration on MS media containing either zeatin and indole butyric acid (IBA) or BAP and IBA. Brar et al. (1999a) reported a regeneration system from cotyledon that was applicable to 17 US commercial cowpea cultivars and breeding lines. The regeneration capacity was reported to be significantly influenced by the BA concentration in the initiation stage, culture duration, and genotype used. The inclusion of silver nitrate, an ethylene inhibitor in shoot induction medium has been shown to improve regeneration from cotyledon of cowpea (Brar et al. 1999b). Popelka et al. (2006) reported multiple shoot formation from longitudinally bisected cotyledonary node explants in different BAP, and TDZ containing medium. The addition of TDZ resulted in formation of deformed shoots which could not be

elongated beyond the bud stage on transferring to medium containing low concentration of TDZ. An *in vitro* regeneration system from shoot apices was reported in cowpea cv. Blackeye, known for high yield potential as well as early flowering and maturation (Mao et al. 2006). Chaudhury et al. (2007) reported the multiple shoot induction and plant regeneration in Indian cowpea cultivar, V-585 from cotyledonary node explants without cotyledons.

These studies have, however, indicated that cowpea *in vitro* regeneration responses are genotype dependent requiring the optimization of tissue culture condition for each individual genotype. Furthermore, regeneration response was dependent on the type of explant used (Brar et al. 1999a), basal salt compositions, plant growth regulators (PGRs) and sucrose levels (Pellegrineschi, 1997; Popelka et al. 2006). Since nutritional requirements of different explants are different, a systematic optimization of media components and culture conditions for each individual explant type may overcome variable response in *in vitro* regeneration (Pellegrineschi, 1997; Popelka et al. 2006).

2.2.2. Genetic transformation of cowpea

Absence of efficient and routine genetic transformation system in cowpea poses a formidable challenge in the incorporation of candidate genes for biotic and abiotic stress tolerance. Although genetic transformation of cowpea has been difficult and challenging till date, considerable progress has been made in regeneration of stable transgenic plants through both *Agrobacterium* as well as biolistic mediated method (Solleti et al. 2008).

Garcia et al. (1986, 1987) were the first to investigate whether cowpea was susceptible to *Agrobacterium tumefaciens* infection and demonstrated that foreign genes could be stably introduced and expressed in cowpea cells. The authors recovered kanamycin-resistant calli from leaf discs of cowpea on infection with *Agrobacterium* (Garcia et al. 1986) and subsequently reported introduction of a full-length cDNA copy of the of cowpea mosaic virus, under the control of the 35S CaMV promoter or the nopaline synthase promoter into leaf-discs in order to study the interaction between cowpea mosaic virus and the natural host (Garcia et al. 1987). Northern blot analysis revealed the expression of the transgene in transformed calli confirming its stable integration. However, no plants could be regenerated from kanamycin-resistant calli. Stably transformed calli of cowpea was recovered from mature embryos, cotyledonary node buds, epicotyls, and apical meristems upon cocultivation with *A. tumefaciens* (Perkins et al. 1987; Filippone, 1990) using hypervirulent strain 6044. Penza et al. (1991) used sliced mature cowpea embryos as a target for genetic transformation by *A. tumefaciens* and were able to regenerate putatively transgenic plants. However, genetic evidence of transgene integration was not presented. Transgenic hairy roots were produced

from leaf, epicotyl, and hypocotyl explants of cowpea on co-cultivation with *A. rhizogenes* harbouring soybean cell wall protein gene (SbPRP1) promoter-GUS construct (Suzuki et al. 1993). The authors showed localization of SbPRP1 in actively growing roots (apical and elongating regions) during seedling growth. Nagl and Ehemann (1994) reported the transient and stable GUS expression in the calli obtained from transformed protoplasts of cowpea ssp. *susquipedalis*.

The first production of transgenic cowpea plants was reported by Muthukumar et al. (1996). These authors generated hygromycin resistant primary transformed shoots from mature de-embryonated cotyledons cocultivated with *A. tumefaciens* and confirmed the stable integration of transgene. The hygromycin-resistant shoots grew to maturity and set seed. Nevertheless, none of the seeds germinated and no evidence of transgene transmission to the progeny was obtained. The group suggested further studies to produce viable seeds for analysis of inheritance of the transgene. Kononowicz et al. (1997) reported the production of chimeric plants of cowpea from shoot apices of young seedlings as well as from embryonic axis and cotyledonary segments of immature seeds, under kanamycin selection, using both *A. tumefaciens* and particle bombardment method for gene transfer. Sahoo et al. (2000) reported the recovery of chimeric transformed plants of cowpea under kanamycin selection through *Agrobacterium*-mediated transformation of shoot apices. An extra wounding treatment in shoot apices prior to cocultivation was found necessary for optimal gene delivery to shoot apices. Popelka et al. (2006) obtained transgenic plants of cowpea through *Agrobacterium*-mediated transformation of longitudinally bisected embryonic axes attached to the cotyledons. Addition of thiol compounds during infection and co-culture, prolonged co-culture, application of a delayed selection in the protocol resulted in recovery of transgenics with a frequency of transformation of 0.15%. Transgenic lines transmitted the transgene (*bar* gene) to their progenies following Mendelian inheritance (Popelka et al. 2006). Chaudhury et al. (2007) used cotyledonary node explants to generate fertile transgenic plants with an efficiency of 0.76%. Transgenic plants were recovered on kanamycin selection and they inherited the transgenes in Mendelian fashion in the first generation. However, both the transformation systems (Popelka et al. 2006; Chaudhury et al. 2007) are time consuming and present low frequency of germ line transformation. Solleti et al. (2008) reported a dramatic increase in efficiency of T-DNA delivery to cotyledonary node explants of cowpea by constitutive expression of additional *vir* genes in resident pSB1 vector in *Agrobacterium* strain LBA4404. The authors employed a geneticin based selection system for rapid and efficient identification of transgenic shoots, and synergistic use of BAP and kinetin for optimal multiplication and elongation of transformed shoots. Stable transgenic plants transmitting transgenes to

progenies were obtained with a frequency of 1.64% which was significantly higher than previous reports on cowpea transformation.

The transient expression of reporter genes in cowpea seedlings following the electroporation of zygotic embryos with plasmid DNA harbouring the chimeric gus gene has been demonstrated (Penza et al. 1992; Akella and Lurquin, 1993). Delivery of linearised DNA following electroporation of seedling tissues was demonstrated in cowpea with remarkably high transient GUS expression in intact hypocotyls and epicotyls (Dillen et al. 1995). However, no stably transformed cowpea plants have been obtained through electroporation mediated transformation. Ikea et al. (2003) were able to generate transgenic cowpea plants after particle bombardment of embryonic axes. However, the transgenes were transmitted to only a small proportion of the progeny and no evidence for stable integration was presented. The authors presumed an unstable transgene integration, which could not be fixed in a homozygous line. In addition, the tissue culture protocol used was time consuming, involving several treatments and medium transfers of the bombarded embryos, prior to achieving putative transgenic plantlets. Ivo et al. (2008) demonstrated a novel system of exploiting the biolistic process to generate stable transgenic cowpea plants by combining the use of the herbicide imazapyr to select transformed meristematic cells after physical introduction of the mutated *ahas* gene (coding for a mutated acetohydroxyacid synthase, under control of the ahas 5' regulatory sequence) and a simple regeneration system from shoot apices. The authors generated transgenic plants with a frequency of 0.90%. The progenies (first and second generations) of all self-fertilized transgenic lines revealed the presence of the transgenes (*gus and ahas*) cosegregated in a Mendelian fashion (Ivo et al. 2008).

2.3. Storage pest resistance

The seeds of starchy grain legumes are an important staple food and source of dietary protein in developing countries. In spite of their superior nutritional value, the yields of grain legumes are diminishing as a result of predation by insects and storage pests which cause considerable crop losses. Particularly devastating is the pre- and post-harvest damage done by larvae of different species of bruchids, coleopteran insects that belong to the family *Bruchidae*. Many bruchids are storage pests and the understanding of the bruchid life cycle provides with the cause for such an extensive crop losses (Talekar, 1988). The bruchid adults emerge from the seeds and mate within an hour after emergence. Subsequently, they lay 80-100 eggs, depositing on seeds. The eggs are glued to the outside of the seed and are clearly visible as small white dots. Soon after hatching the larvae burrow into the seed through the seed coat and the seed pod (if the egg was laid on the pod) creating a small, dark entry hole inside the seed, where it continues to feed consuming cotyledon contents and grow until it pupates. Just

before the larva pupates, it eats a circular hole, leaving only a thin layer or window, of the intact seed coat. Development of eggs to adults takes 22-30 days depending on the species and conditions. Within a 30-day life cycle and a reproductive rate of 80 eggs for female, the population of bruchids mushroom when the seeds are stored for several months, even if the initial infestation is moderate (Wolfson et al. 1991).

The members of bruchid species include, cowpea weevil *Callosobruchus maculates* (F.), azuki bean weevil *Callosobruchus chinensis* (L.), Mexican bean weevil *Zabrotes subfasciatus* (Boheman), bean weevil *Acanthoscelides obtectus* and the pea weevil (*Bruchus pisorum*), that cause extensive damage to the crop. Bean weevil (*Acanthoscelides obtectus*) and pea weevil (*Bruchus pisorum*) starts to attack the crop while they are still in the field and continues the attack in storage. On the other hand, cowpea weevil (*Callosobruchus maculates*), azuki bean weevil (*Callosobruchus chinensis*) and Mexican bean weevil (*Z. subfasciatus*) attacks the seeds only during storage (Howe and Currie, 1964; Fernandez and Talekar, 1990).

Control of these storage pests by fumigants or other insecticides is not practical because of the primitive nature of seed-storage facilities in most developing countries, and the rising costs of farm inputs due to the use of expensive insecticides make all-important chemicals virtually out of reach of the farmers (Fillippone, 1993).

Though screening of legume genetic resources for insect resistance has showed some promise, the successful breeding of insect resistance into commercial cultivars has proven difficult in many legume crops.

2.3.1. Seed defence

Plants have evolved certain degree of resistance mechanisms to the insects and storage pests through the production of defense compounds most of which are accumulated in the seeds, as the seeds are the main targets for the insect predation. Like most plants, legumes rely on a suite of defences for protection against insect pests. Legume seeds are often protected by an accumulation of anti-nutritional compounds that remain until germination (Edwards and Singh, 2006). The seed tissues may accumulate, constitutively or after induction, a wide array of aproteic or proteic defensive compounds like plant secondary metabolites (PSMs), lectins, ribosome-inactivating proteins (RIPs) of types 1 and 2, inhibitors of proteolytic enzymes and glycohydrolases, vicilins (Ryan, 1990; Bowles, 1990; Chrispeels and Raikhel, 1991; Barbieri et al. 1993; Peumans and Van Damme, 1995 and Koiwa et al. 1997) and other proteins like arcelins (Osborn et al. 1988), chitinases (Herget et al. 1990 and Cohen, 1993), canatoxin (Carlini et al. 1997) and modified forms of storage proteins (Macedo et al. 1993 and Sales et al. 2000) that are involved in complex defense mechanisms. Considering the vast numbers of

plant and insect species, it is not hard to imagine the complexity and the diversity of specific defense mechanisms associated with different plant-insect systems.

The use of cheaper vegetal protein is progressively substituting protein of animal source as food and feed, therefore, human and cattle are increasingly exposed to plant defense proteins in edible crops. Some of these proteins are relatively thermostable and are only partially inactivated by heat during cooking (Carlini and Udedibie, 1997). The major defensive chemicals that have been characterized in various plant insect systems include inhibitors of insect digestive enzymes and lectins.

2.3.1.1 Proteinase inhibitors

Inhibitors of insect digestive enzymes are the most extensively studied group of anti-insect chemicals. There are two major kinds of digestive enzymes in insect digestive systems: proteases and amylases (Terra et al. 1996). Inhibitors of both proteases and amylases are widely distributed in plant tissues, especially in seeds (Zavala et al. 2004). Digestive enzyme inhibitors as a defense mechanism were first discovered by Ryan and his associate (Green and Ryan, 1972). They found that the expression of protein proteinase inhibitors was rapidly induced in potato and tomato leaves in response to insect attacks, and the induction was mediated by a systemic signal, which could also be activated by mechanical wounding. Since then, the roles of digestive enzyme inhibitors in plant defense have been extensively studied (reviewed by Ryan, 1990; Ussuf et al. 2001; Rawlings et al. 2005).

A large number of naturally occurring proteinase inhibitors have been described in animals, plants and microorganisms and have been extensively studied in order to elucidate their structural and functional properties (Bode and Huber, 1992; Hibbetts et al. 1999). They are classified according to the type(s) of enzyme they inhibit (Bode and Huber, 2000). In plants, different roles for proteinase inhibitors have been suggested, including their action as storage proteins, as regulators of endogenous proteolytic activity (Ryan, 1990), as participants in many developmental processes, including programmed cell death (Solomon et al. 1999), and as components associated with the resistance of plants against insects and pathogens (Lu et al. 1998; Pernas et al. 1999). They may be synthesized constitutively during normal development or may be induced in response to insect and pathogen attacks (Ryan and Pearce, 1998).

Plant serine proteinase inhibitors fall into a number of structurally distinct subfamilies based on their amino acid sequences (Bode and Huber, 1992). Bowman–Birk type inhibitors are small polypeptides (8 kDa), typically found in legume seeds. They are double-headed, binding simultaneous and independently to two separate proteinase molecules, such as trypsin and chymotrypsin (Bode and Huber, 1992). Kunitz-type inhibitors are 20 kDa proteins, usually made of one polypeptide chain or two disulphide linked chains, with an arginine

residue in their single reactive site. In general, these inhibitory proteins behave as pseudo-substrates, with the amino acid at position P1 of the inhibitor determining the specificity for the enzyme, either trypsin or chymotrypsin (Bode and Huber, 1992). In spite of differences in primary structure and topology, the reaction center structure and mechanism of action are well preserved among serine proteinase inhibitors. Some plant serine proteinase inhibitors are bifunctional molecules, being able to inhibit trypsins as well as α-amylase (Strobl et al. 1995).

In plants, members of the cystatin or cysteine proteinase inhibitors are known as phytocystatins (5-87 kDa) and show characteristics found in cystatins subfamilies I and II (Turk et al. 1997; Arai et al. 1998). Phytocystatins have been identified in a variety of monocot and dicot species, such as maize, rice, potato, soybean and apple (Abe et al. 1991, 1996; Kondo et al. 1990; Botella et al. 1996; Gruden et al. 1997; Ryan et al. 1998). Several cysteine proteinase inhibitors have been identified and their primary and tertiary structures were determined (Kondo et al. 1990; Nagata et al. 2000; Wu and Haard, 2000; Kouzuma et al. 2000; Kuroda et al. 2001). Some of them show homology with serine proteinase inhibitors, such as the potato tuber cysteine proteinase inhibitor that belong to the Kunitz-type trypsin inhibitor family and do not contain the conserved region (Gln-X-Val-Y-Gly) that characterizes the cystatin superfamily (Ishikawa et al. 1994; Hatano et al. 1998). The phytocystatins differ largely from animal cystatins (Kondo et al. 1991; Brown et al. 1997), by displaying high inhibitory activity toward insect gut proteinases (Bode and Huber, 1992; Koiwa et al. 1997) making them attractive as biological control agents of insect pests (Gatehouse and Gatehouse, 1998; Ussuf et al. 2001). Serine and cysteineproteinase inhibitors frequently have deleterious effects when fed to lepidoptera and coleoptera, respectively, which may include reduced fecundity, decreased weight, increased mortality and severe deformations (Murdock et al. 1988; Kuroda et al. 1996; Gruden et al. 1998; Elden, 2000). The potential of these inhibitors has already been demonstrated by diet incorporation assays or by *in vitro* inhibition studies of digestive proteinases (Carlini and Grossi-de-Sa, 2002).

2.3.1.2. α-Amylase inhibitors

α-Amylases (α-1,4-glucan-4-glucanohydrolases) are widespread hydrolytic enzymes found in microorganisms, animals and plants. They catalyze the initial hydrolyses of α-1,4-linked sugar polymers, such as starch and glycogen, into shorter oligosaccharides, an important step towards transforming sugar polymers into single units that can be assimilated by the organism. These widely distributed molecules are the most important digestive enzymes of many insects that feed exclusively on seed products during larval and/or adult life. When the action of the amylases is inhibited, nutrition of the organism is impaired causing shortness in energy. α-Amylase inhibitors occur in many plants as part of the natural defense mechanisms.

They are particularly abundant in cereals (Abe et al. 1993; Feng et al. 1996; Yamagata et al. 1998; Franco et al. 2000; Iulek et al. 2000) and legumes (Marshall and Lauda, 1975; Ishimoto et al. 1996; Grossi-de-Sa et al. 1997). Research into α-amylase inhibitors is relevant with respect to several aspects of human health, from diagnosis of pancreatic hyperamylasemia disorders to control of diabetes, obesity and hyperlipaemia (Bischoff et al. 1994) and nutritional and toxicological aspects of foods. In addition, amylase inhibitors are of great interest as potentially important tools of natural and engineered resistance against pests in transgenic plants (Chrispeels et al. 1998; Gatehouse and Gatehouse, 1998; Valencia et al. 2000).

The effect of the bean amylase inhibitors on the amylases of different organisms was well determined not only by enzymatic activity, but also in feeding assay experiments (Ishimoto and Kitamura, 1989; Ishimoto et al. 1996; Grossi-de-Sa et al. 1997). Complete resistance against bruchids, the pea weevil (*Bruchus pisorum*), the cowpea weevil (*C. maculatus*) and the azuki bean weevil (*Callosobruchus chinensis*), was found in transgenic pea and azuki bean seeds expressing the inhibitor, αAI-1, of the domesticated common bean *P. vulgaris* (Shade et al. 1994; Schroeder et al. 1995; Ishimoto et al. 1996; Chrispeels, 1996; Morton et al. 2000; Sarmah et al. 2004). The transgenic grains showed minimal effects on mammalian digestion system (Pusztai et al. 1999) suggesting that these proteins can be safely introduced into food plants.

In *Phaseolus* seeds, αAI is a member of a protein family that includes two other defense proteins, the hytohemagglutinin (PHA) and arcelins (Arc) (Chrispeels and Raikhel, 1991). The members of these plant defense proteins are encoded by tightly linked genes in the *P. vulgaris* genome and their deduced amino acid sequences are highly homologous (45–85% identical) (Chrispeels and Raikhel, 1991; Nodari et al. 1993; Mirkov et al. 1994). It is likely that these genes family originated from a common ancestral gene through both duplication and divergence (Osborn et al. 1986; Kornegay et al. 1993; Nodari et al. 1993). The proteins display insecticidal activities and protect seeds against different predators through different mechanisms. PHA is a lectin that binds to the glycans on the glycoproteins of the intestinal epithelium of animals and act as a mitogen. In contrast, arcelins are suggested to bind to the peritrophic membrane of the insect gut interfering with nutrient absorption and causing rupture of gut membranes (Paes et al. 2000). As mentioned, αAI inhibits the activity of some mammalian and insect α -amylases (Grossi-de-Sa et al. 1997).

Phaseolus genus contains at least four phenotypes of αAIs (αAI-1, αAI-2, αAI-3 and the null type) with alleles encoding the inhibitors being co-dominant and with the presence of inhibitors dominant in relation to its absence (Suzuki and Ishimoto, 1999). Of particular interest is the specificity of the two isoforms αAI-1 and αAI-2 towards different α-amylases.

αAI-1, found in most cultivated common bean varieties, inhibits mammalian α-amylases such as porcine pancreatic amylase (PPA) and the insect larval α-amylases of *C. chinensis*, *C. maculatus* and *B. pisorum*, but is not active against the α-amylase of the Mexican bean weevil (*Zabrotes subfasciatus*), which is an important storage pest of the common bean (Grossi-de-Sa and Chrispeels, 1997). The second variant, αAI-2, which shares 78% amino acid homology with αAI-1, is found in few wild accessions of common beans and specifically inhibits the *Z. subfasciatus* larval α-amylase, ZSA, (Ishimoto and Kitamura, 1992, 1993; Suzuki et al. 1993; Grossi-de-Sa and Chrispeels, 1997; Grossi-de-Sa et al. 1997). αAI-2 can also weakly (40%) inhibit the pea bruchid α-amylase, thus delaying the maturation of the larvae (Morton et al. 2000).

This inhibitor provides an excellent example of the coevolution of insect digestive enzymes and plant defense proteins. Different species of bruchids are found all over the world. For example, while the two main bruchids species of common bean, *Z. subfasciatus* and *Acanthoscelides obtectus*, have evolved in the Americas and are able to feed on all cultivated varieties of the common bean, the pea and cowpea weevil have evolved in the Old World and are not able to consume the common bean. They thrive on cowpeas, mung beans, and other Eastern Hemisphere legumes. The amylase inhibitor found in the common bean does not inhibit the amylase of the Mexican bean weevil, but completely inhibits the amylases of the pea and cowpea weevils (Grossi-de-Sa et al. 1997; Chrispeels et al. 1998), which suggest a co-evolution between insect and their food source. The toxic effect of αAI-1 toward bruchid pests is assumed to be caused by inhibition of digestive amylases. Its failure, however, to affect *Z. subfasciatus* has two possible explanations: either the amylase of the insect is not inhibited by αAI-1, or the insect has an intestinal serine proteinase that is able to digest the inhibitor (Ishimoto et al. 1996; Silva et al. 2001a). Recent studies implicated the presence of the inhibitor in the insect's diet with the induction of new amylase activities and inhibition of the constitutive larval *Z. subfasciatus* α-amylase by αAI-1 when starch granules were used as substrate (Silva et al. 1999, 2001b). αAIs are synthesized as an initial translation product that is co-translationally and post-translationally modified to yield a holoprotein consisting of two glycoproteins, α and β. These form a heterotetrameric structure, $\alpha_2\beta_2$, that can form a very tight complex with two molecules of α-amylase (Kasahara et al. 1996). Several α-amylases and α-amylase/inhibitor complexes have been crystallized and their threedimensional structures determined (Matsuura et al. 1984; Bompard-Gilles et al. 1996; Strobl et al. 1997, 1998).

2.3.1.3. Lectins

Lectins are a very diverse group of proteins of non-immune origin that possess at least one non-catalytic domain that reversibly bind to specific mono- or oligosaccharides and are widely distributed in microbial, plant, and animal tissues (Komath et al. 2006). Lectins are usually considered a complex and heterogeneous group of proteins, due to the differences in molecular structure, biochemical properties, and carbohydrate-binding specificity. Plant lectins are classified into four major families (Van Damme et al. 1998), namely, the legume lectins, the chitin-binding lectins composed of hevein domains, the type 2 ribosome-inactivating proteins (RIP), and the monocot mannose-binding lectins, that comprises the majority of all currently known plant lectins. As a group, lectins from different sources show very few if any similarities. Seeds, particularly those of the Leguminoseae, are rich sources of lectins. Typical lectins from different plant sources exhibit a considerable degree of structural similarity. Amino acid sequence homologies exist among the lectins from *Canavalia ensiformis* (concanavalin A) and from other leguminous seeds, despite differences in their carbohydrate-binding specificities (Carrington et al. 1972; Foriers et al. 1977; Rouge et al. 1987, Van Damme et al. 2000).

A role of lectins in the plant defense mechanisms has been proposed (Hirsch, 1999) (Chrispeels and Raikhel, 1991; Peumans and Van Damme, 1995; Gatehouse et al. 1995). Some lectins are highly toxic when ingested by mammals, surviving gastrointestinal passage, and inducing a variety of systemic effects. Among them are the lectins from the common bean, soybean, wheat germ (Pusztai et al. 1986, 1993; Bardocz et al. 1995; Kordas et al. 2000), and concanavalin A, the lectin present in the jack bean (Udedibie and Carlini, 1998). Various plant lectins have shown entomotoxic effects when fed to insects from Coleoptera, Homoptera, and Lepidoptera orders. One of the most widely studied, and perhaps one of the most promising chemicals for aphid control, is snowdrop lectin. The particular interest in this protein is based on the fact that it acts on sap-sucking insect pests that are not targeted by the known *Bt*-toxins (Rahbe et al. 1995; Sauvion et al. 1996; Powell et al. 1993, 1995a, 1995b, 1998; Powell, 2001). Transgenic plants that express snowdrop lectins (GNA) offer partial resistance to homopteran pests, as has been observed in tobacco (Hilder et al. 1995; Yuan et al. 2001), potato (Down et al. 1996; Gatehouse et al. 1996), rice (Rao et al. 1998; Foissac et al. 2000; Sun et al. 2002), and wheat (Stoger et al. 1999). In addition to effects on sap-sucking pests, insecticidal effects of snowdrop lectin were also found to lepidopteran pests (Fitches et al. 1997; Gatehouse et al. 1997; Irvine and Mirkov, 1997; Legaspi et al. 1997; Fitches and Gatehouse, 1998; Setamou et al. 2003) and a coleopteran herbivore (Nutt et al. 1999). Genes coding for entomotoxic lectins like Man-specific snowdrop lectin (*Galanthus nivalis agglutinin*; GNA), wheat germ Lectins (WGA), Man-specific lectin from garlic (*Allium*

sativum) leaves (ASA-L), Concanavalin-A, pea lectin (P-lec) have been introduced into different crops rendering the transgenic plants less susceptible to the attack of insects (Rao et al. 1998; Shukle and Murdock, 1983; Sharma et al. 2000; Gatehouse et al. 1995; Powell et al. 1995; Saha et al. 2006a; Cao et al. 1999; Boulter et al. 1990).

It has been demonstrated that some lectins bind to the brush-border membrane of the insect's intestinal epithelial cells or, in the case of chitin-binding lectins, to the peritrophic membrane (Chrispeels and Raikhel, 1991; Peumans and Van Damme, 1995). Other possible toxic effect includes binding of lectins to glycosylated digestive enzymes. However, the precise mechanism of action of lectins in insects is still unknown. However, the applicability of lectins for engineering insect resistant plants limited due to their toxicity to mammals and/or non-target insects. Because of the wide spread presence of lectins in the plant kingdom, this class of proteins should be screened more consistently for an entomotoxic activity not associated to these potentially harmful effects, which can be explored for the construction of insect resistant transgenic plants.

The other group of defense molecules, arcelins, belongs to the bean lectin-like family which includes the two types of phytohemagglutin subunits (PHA-L and PHA-E) (Chrispeels and Raikhel, 1991). To date, seven different allelic variants (designated Arc-1–7) of arcelin proteins have been isolated from *phaseolus* genus, with molecular weights ranging from 27 to 42 kDa (Osborn et al. 1986; Lioi and Bollini, 1989; Santino et al. 1991 and Acosta Gallegos et al. 1998) and each arcelin variant is composed of several polypeptides presumably encoded by a family of different genes (Hartweck et al. 1991). Arcelins acts by binding to the peritrophic membrane of the insect gut and interfere with nutrient absorption and causing rupture of gut membranes (Paes et al. 2000). Of all arcelins characterized, Arc-5 and Arc-1 are the ones conferring highest resistance levels to the bean bruchid pest *Z. subfasciatus* (Cardona et al. 1990 and Fory et al. 1996).

2.3.2 Conventional breeding for resistance to insect pests in legumes

Though screening of legume genetic resources for insect pest resistance has showed some promise, successful breeding of insect pest resistance into commercial cultivars has proven difficult in many legume crops. Despite having the most successful legume screening program for identifying pest resistance, the soybean program in the USA released only four insect-resistant soybean cultivars in their first 26 years and the adoption of these has been limited because of issues of agronomic inferiority (Boethel, 1999). In tropical bean systems, there have been some successes in developing cultivars resistant to single pests, but multiple insect and disease resistant varieties are desperately needed to be commercially viable (Cardona and Kornegay, 1999). In cool season legumes, improvements in insect resistance

have rarely been greater than incremental (Muehlbauer, 1996; Clement et al. 1994); perhaps the greatest success has been aphid resistance in cultivated narrow-leafed lupins in Australia (Cowling, 1999). In pasture legumes, there has been more success. Alfalfa/lucerne breeders have had the greatest success in developing insect resistance, with the successful development of resistance against three aphid species, leafhoppers, and the alfalfa weevil (Manglitz and Sorensen, 1999). There have also been some successes with annual pasture legumes, where breeders have exploited wild germplasm to breed resistance to bluegreen aphid, *Acyrthosiphon kondoi* Shinji, into a number of cultivars (Berlandier et al. 2000; Nair et al. 2003).

However, there has been very little progress in improving insect resistance in many legume crops primarily due to the limited genetic resources available. Effective resistance in beans to the Mexican bean weevil, *Zabrotes subfasciatus*, controlled by a single, dominant gene was located in a small collection of wild lines by van Schoonhoven et al. (1983). Identifying effective resistance to *Helicoverpa* spp. in chickpea germplasm collections also has met with limited success (Srivastava and Srivastava, 1989), but recent efforts have identified promising levels of resistance in accessions of the wild relatives *Cicer bijugum* Rech. and *C. reticulatum* Ladiz. (Sharma et al. 2001). Collections of wild accessions of crop species or their non-domesticated relatives may provide the answer to the problems posed by the limited genetic resources in legume crops (Clement, 2002). Other recent examples include resistance to pod fly and pod wasp in wild relatives of pigeonpea (Sharma et al. 2003), resistance to aphids in wild *Vicea* spp. (Holt and Birch, 1984), resistance to *Apion godmani* Wagner in landraces of *Phaseolus vulgaris* L. (Garza et al. 2001), and volatiles deterring the brown pod bug, *Clavigralla tomentosicollis* Stal., in wild *Vigna* spp. (Koona et al. 2003).

For some wild relatives, such as *C. bijugum*, success relies on effective embryo rescue technology. Achieving pest resistance without reducing agronomic quality has been a second problem as in many instances the resistance traits are under polygenic control or have low dominance, resulting in many undesirable qualities being introduced along with the desired trait during the breeding process. Such problems have plagued breeding of resistance for bean weevil (*Sitona lineatus* (L.) in beans, and for Mexican bean beetles and leafhoppers in soybeans (Boethel, 1999). A modified recurrent selection procedure has been adopted to successfully overcome this problem and improve resistance and tolerance to leafhoppers in common beans (Kornegay and Cardona, 1990). It is particularly difficult to breed for pest resistance when the mechanism of resistance in itself reduces crop quality. This can be true for many physical resistance mechanisms including pod thickness (e.g. pea weevil resistance), and for chemical resistance when the compounds involved are also toxic to mammals (e.g. alkaloids in lupins). Finally, in many cases the failures have been implicated due to the

absence of establishment of proper links between researchers identifying resistance mechanisms and the breeders that would introduce these mechanisms into commercial lines.

2.3.3. Transgenic grain legumes for storage pest resistance

While conventional breeding will continue to have a valuable role in providing insect resistant cultivars in many legume crop species, it appears that in some systems there will continue to be barriers to achieving insect resistance through this approach. Fortunately, there are exciting opportunities arising from our growing understanding on candidate genes from plants encoding for insect pest resistance, and transfer these genes across the sexual barrier to susceptible grain legumes through conceptual framework of genetic transformation. The approach promises novel strategies to achieve enhanced storage pest resistance in seeds of transgenic grain legumes within a short time frame.

The common bean contains a family of plant defense proteins that includes phytohemagglutinin (PHA), arcelin and α-amylase inhibitor (αAI) (Chrispeels and Raikhel, 1991). The genes for these three proteins are encoded at a single locus in the *P. vulgaris* genome (Nodari et al. 1993) and it is likely that these homologous genes have arisen by duplication of an ancestral gene. The three proteins have quite different modes of action: PHA is a lectin that binds to the glycans on the intestinal mucosa of mammals and acts as a mitogen, arcelin binds to the peritrophic membrane of insect larvae and may interfere with nutrient absorption, and αAI inhibits the activity of certain mammalian and insect α-amylases but not that of plant enzymes. Of particular interest is the specificity of these inhibitors to the α-amylases of different insect species. Because it is an anti-nutritional factor for humans, the biochemical properties of bean αAI have been studied in many laboratories since 1945 (Bowman, 1945). It is a relatively heat-stable protein that exists as a heterotetramer and forms a very tight complex with two molecules of porcine pancreatic α-amylase (PPA). Complex formation is pH dependent and occurs maximally at the acidic pH (around pH 5.0) of the intestinal track of mammals and coleopteran larvae.

Recent studies show that bean αAI exists in at least two allelic variants. The isoform found in cultivated beans (Moreno and Chrispeels, 1989) is now called αAI-1; a second variant, αAI-2, is found in some wild accessions of the common bean that contain arcelin as the major storage protein instead of phaseolin. Of particular interest is that the two isoforms differ in their specificity toward α-amylases. Thus, αAI-1 inhibits PPA as well as the amylases of the cowpea weevil and the azuki bean weevil, but it does not inhibit the amylase of the Mexican bean weevil; αAI-2, on the other hand, does not inhibit the three amylases mentioned above that are inhibited by αAI-1, but it does inhibit the Mexican bean weevil amylase (Ishimoto and Chrispeels, 1996; Suzuki et al. 1993). Furthermore, neither inhibitor inhibits the α-

amylase of the bean weevil, *Acanthoscelides obtectus*. Both proteins have been purified and their cDNAs have been cloned. The derived amino acid sequences share 78% amino acid identity. The related species *Phaseolus acutifolius* has an immunologically related α-amylase inhibitor of the same molecular weight that has again a different specificity, but its cDNA has not yet been isolated.

The fact that αAI can act as a plant defense protein has been demonstrated by feeding experiments in which artificial seeds were created out of flour of azuki beans or cowpeas to which varying amounts of purified bean αAI-1 had been added. The presence of the inhibitor at levels above 0.3% inhibited the development of the larvae of the azuki bean weevil and the cowpea weevil. Azuki bean weevil larvae were much more sensitive to the inhibitor than cowpea weevil larvae. Because strong seed specific promoters can give levels of expression for seed proteins in the 0.5 to 1.0% range, it follows that genetically engineered legume plants that express this inhibitor in their seeds should be resistant to these bruchid larvae. Transfer of common bean α-amylase inhibitor 1 gene (αAI-1) to pea (Shade et al. 1994; Schroeder et al. 1995; Morton et al. 2000) conferred complete resistance to pea weevil, *Bruchus pisorum*, in addition to three *Callosobruchus* species *C. chinensis*, *C. maculatus*, and *C. analis*. Incorporation and expression of *αAI-1* in azuki bean resulted in complete resistance to the azukibean weevil (Ishimoto et al. 1996). Transfer of bean αAI-1 gene into a desi type of chickpea in a seed specific manner resulted in accumulation of αAI-1 in the seeds up to 4.2% of total seed protein (Sarmah et al. 2004). The transgenic protein was active as an inhibitor of porcine α-amylase *in vitro*. Transgenic chickpeas expressing α-AI1 strongly inhibited the development of *C. maculatus* and *C. chinensis* in insect bioassays. Ignacimuthu and Prakash (2006) reported introduction of *αAI-1* into chickpea cultivar, K850 that resulted in a significant reduction in the survival rate of bruchid weevil *C. maculatus* reared on transgenic chickpea seeds in bioassay. Sonia et al. (2007) reported introduction of *αAI-1* to mungbean cultivar, Pusa-105 and showed stable integration of *αAI-1* into T_0 transgenic plants.

2.3.4 Genetic improvement of cowpea for storage pest resistance

Among the storage pests, cowpea weevil, *Callosobruchus maculatus* (F.) and azukibean weevil, *C. chinensis* cause serious cowpea grain losses in storage (Singh et al. 2000), that damage the unprotected seeds within 4-6 months (Seck et al. 1991), rendering the cowpea grains unfit for consumption or sell as they are riddled with bruchid holes. Under the adverse uncontrolled conditions the insect pests may cause up to 100% yield losses.

The most economical and environmentally friendly way of controlling these insect pests would be through host-plant resistance. To this end, a wide range of accessions from the cowpea germplasm collection as well as those of its wild and weedy cross-compatible

relatives was screened in order to identify those with genes for resistance to the pests that cause massive damage to cowpea production. None of the tested cultivated cowpea lines and their cross-compatible wild relatives showed the desired high level of resistance to these pests (Fatokun, 2002). Several accessions of some *Vigna* species, such as those belonging to *V. vexillata*, *V. davyi*, *V. oblongifolia*, and *V. luteola*, were also screened, among others, for resistance to insect pests of cowpea. The results showed that some accessions of *V. vexillata* and *V. oblongifolia* have good levels of resistance to the insect pests that devastate cowpea.

The various accessions of *V. vexillata* showed high levels of resistance to pod sucking bugs, flower thrips, *Maruca vitrata*, bruchid, and *Striga gesnerioides* among others. Possession of these traits makes interspecies crosses between it and cowpea very attractive and worth pursuing. Hence, crosses were initiated between cowpea and *V. vexillata* with the aim of transferring the genes conferring resistance to insect pests from the latter to cowpea. While making these crosses (*V. vexillata* × cowpea) it was observed that some pods were retained for up to seven days or even more when cowpea is the pollen parent. However, in the reciprocal crosses pods were not retained as emasculated flowers drop within one day following cross pollination (Fatokun, 2002). On the other hand, the pods that were retained by pollinating *V. vexillata* with cowpea developed slowly as the seeds contained therein. By the time these pods attained their maximum size, they only approximated the size attained by four-day-old pods resulting from selfing. In all the crosses, flowers were emasculated and pollinated a day before anthesis. This was to ensure that pollen tubes reached the ovule in order to release the male (sperm) nuclei in time for fertilization to take place. No viable interspecific hybrid seed was obtained from any of the several hundreds of crosses made, thus suggesting a strong cross incompatibility between the two species, *V. vexillata* and *V. unguiculata* (Fatokun, 2002).

Efforts have also been underway to identify plant genes that affect bruchid beetles development in cowpea. The majority of artificial seed bioassays that involved the use of α-amylase inhibitors have shown their potential to protect cowpea seeds against infestation from bruchid beetles (Ishimoto et al. 1999). Plans are underway to introduce the common bean *αAI-1* gene into agronomically important but bruchid susceptible cowpea lines once the transformation system becomes routine.

Chapter 3

DEVELOPMENT OF AN EFFICIENT PLANT REGENERATION SYSTEM

Increasing attention is being focused on genetic engineering as a viable option for overcoming limitations in cowpea improvement. Availability of efficient *in vitro* regeneration system is the primary requirement for genetic transformation of most plants. Cowpea has been regenerated via direct or indirect shoot organogenesis and somatic embryogenesis (Sahoo et al. 2003). However, recovery of cowpea transgenics through these regeneration systems have remained difficult primarily due to failure in combining the competence of plant cells for stable DNA integration, and their regeneration to fertile plant. Furthermore, studies indicated that cowpea *in vitro* regeneration responses are genotype dependent, requiring the optimization of tissue culture condition, type of explant used, medium composition and plant growth regulators, for each individual genotype (Brar et al. 1999a; Pellegrineschi 1997; Popelka et al. 2006). Hammatt et al. (1986) suggested that the common recalcitrance of large seed legumes to *in vitro* regeneration could result from their long history of inbreeding and selection for high performance genotypes, leading to reduced genetic variability in modern varieties.

Therefore, development of an efficient regeneration system amenable to genetic transformation demands selection of appropriate explant competent for transformation, and systematic optimization of media components and plant growth regulators, and culture conditions for the selected explant. Genetic transformation using seedling explants has been suggested as a suitable alternative for improvement of highly recalcitrant *Vigna* species (Avenido and Hattori, 1999) as the freshly injured tissue provided by young seedlings are in an active state of cell division, and thus, the direct access of these freshly wounded tissue to *Agrobacteriim tumefaciens* is vital in the success of the *Agrobacterium*-mediated transformation (Sahoo et al. 2003).

In legumes, most investigations on plant regeneration were carried out with cotyledonary node explants derived from *in vitro* germinated seedlings. Cotyledonary nodes from mature seeds have been most responsive for the induction of multiple shoots via organogenesis, the process by which a cell or a group of cells differentiates to form organs, may occur directly from the explant (Parrott et al. 1992). In contrast to other explant sources, cotyledonary node explants have shown intrinsic organogenic competence in a variety of legume species (Distabanjong and Geneve, 1997) and high frequency multiple shoot formation have been reported for various legume species including pea (Jackson and Hobbs, 1990; Mallick and Rashid, 1989), dry bean (Franklin et al. 1991; Mohamed et al. 1992a, 1992b), soybean (Kothari et al. 1991; Wright et al. 1986), pigeonpea (Prakash et al. 1994) and chickpea (Polisetty et al. 1997). Moreover, such explants have the advantage of being easily and quickly available throughout the year. Microbial contamination of such explants has never been a serious problem.

Meristematic explants are considered amenable to *Agrobacterium*-mediated transformation as the proliferation from pre-existing meristems concomitant to T-DNA delivery results in more number of transformed cells that could be regenerated to fertile plants. Such regeneration systems have advantages over *de novo* organogenesis as the shoot organogenesis from these explants is direct; therefore the changes like somaclonal variation are very low (Parrott et al. 1992). Since the shoot regeneration in cotyledonary node explants occurs via adventitious proliferation from the axillary meristems at the junction of the cotyledon and the embryo axes whose cells are competent for regeneration and hence could be useful targets for gene delivery. Apart from the production of a maximum number of shoots per explant, the regeneration site was completely exposed and easily accessible to the *Agrobacterium* infection. Since the infectivity of *Agrobacterium* needs wound response, and most of the cotyledonary node explants are responsive to cytokinins, especially BAP and thidiazuron (TDZ), explants and media regimes that allow regeneration after wounding have to be optimized for transformation.

Employing a cotyledonary node regeneration system for *Agrobacterium*-mediated gene transfer, transgenic plants were successfully regenerated in pea (Davies et al. 1993; Jordan and Hobbs, 1993), white clover (Voisey et al. 1994), soybean (Hinchee et al. 1988; Di et al. 1996; Meurer et al. 1998; Zhang et al. 1999; Donaldson and Simmonds, 2000), mung bean (Kumari et al. 2001; Sonia et al. 2007), blackgram (Saini et al. 2002; Saini and Jaiwal, 2002; Bhomkar et al. 2008).

The present study was undertaken with the objective to develop an efficient plant regeneration system from seedlings explants, amenable to *Agrobacterium*-mediated transformation.

3.1 MATERIALS AND METHODS

3.1.1 Plant materials

Seeds of eight cultivars, i.e., Pusa Komal, Pusa Safed, Pusa Sampada, Rambha, V-16, V-240, V-130 and V-585 of cowpea, obtained from Indian Agricultural Research Institute, New Delhi, India, were tested for shoot regeneration response while the commercially important cultivar, Pusa Komal was used for detailed studies.

3.1.2 Explants preparation and plant regeneration

Healthy and uniform seeds were rinsed with 70% alcohol for 1 min, surface-sterilized with 0.2% aqueous solution of $HgCl_2$ (w/v) for 5 min. The seeds were subsequently rinsed five times with sterile distilled water and cultured on MSB medium [MS salts (Murashige and Skoog, 1962) +B5 vitamins (Gamborg et al. 1968)] supplemented with 10 µM BAP. The cotyledonary node explants (5-6 mm) were excised from 3 days-old *in vitro* raised seedlings

by removing both the cotyledons, and excising both epicotyls and hypocotyls approximately 1 mm and 3 mm respectively from above and below the nodal region (Fig. 1a).

Explants were cultured on MSB medium supplemented with various cytokinins [benzylaminopurine (BAP), kinetin and thidiazuron (TDZ)] individually at different concentrations (1.0, 2.5, 5.0 and 7.5 µM) in culture tubes (25×100 mm) for multiple shoot induction. After 2 weeks, the regenerated explants were subcultured on fresh medium of the same composition for 2 weeks. The shoot clusters developed on BAP supplemented media showed stunted shoot growth. Therefore, after 4 weeks, the cultures induced on MSB medium containing 5 µM BAP were transferred to basal media supplemented with kinetin and gibberellic acid (GA_3) individually at different concentrations (0.1, 0.5 and 1.0 µM) for 2 weeks for shoot elongation.

The synergistic effect of kinetin on proliferation and elongation of shoots from the regenerating explants was examined by supplementing kinetin (0.1, 0.5 and 1.0 µM) to SIM, shoot induction media (MSB medium containing 5.0 µM BAP) after varying interval of culture (0, 1 and 2 weeks). The mother explants were repeatedly subcultured on SIM, after harvesting elongated shoots, for further induction of shoots.

Elongated shoots (2-3 cm) were excised from proliferating shoot cultures after 4 weeks of culture and transferred to MS medium supplemented with 2.5 µM IBA for rooting.

3.1.3 Culture conditions

The culture media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar agar. The pH of the media was adjusted to 5.8 prior to autoclaving at 121^0 C for 20 min. Cultures were maintained at 25 ± 2^0 C under a 16-h photoperiod provided by cool white fluorescent lights (35 µmol m^{-2} s^{-1}).

For each treatment, 20 cultures were raised and each experiment was performed at least thrice. Visual observations of the cultures were taken periodically, and the effect of the treatments was quantified on the basis of the percentage of cultures showing regeneration and number of regenerants per culture. The data pertaining to the number of shoots per culture were subjected to analysis of variance and significant treatment differences selected by Newman-Keuls multiple range test (Brunning and Kintz, 1977).

3.1.4 Transplantation

Well-rooted plantlets were washed thoroughly in running tap water before being transplanted in plastic pots containing sterilized soil and vermiculite (1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture and transferred to the greenhouse for acclimatization at 28^0C day, 20^0C night temperatures, 16 h day-length and

70% relative humidity. The plants in pots were irrigated alternatively with Hoagland's solution and water. Plantlets were then transferred to the pots containing soil:compost (1:1), grown to maturity and the seeds were collected.

3.2 RESULTS AND DISCUSSION

3.2.1 Multiple shoot induction

Cotyledonary node explants cultured on MSB medium produced an average of 1.9 shoots in 98% of the cultures. The frequency of shoot multiplication and shoot number were significantly higher on BAP-supplemented media followed by kinetin and TDZ (Table 2). BAP at 5.0 µM induced maximum number of shoots (6.9) in 94% of the explants within 2 weeks of culture whereas the response was minimal at 7.5 µM TDZ with regeneration being restricted to stunted bud formation (Table 2). BAP alone or in combination with other plant growth regulators has been reported to favorably induce multiple shoot induction from diverse explants of cowpea (Muthukumar et al. 1995; Pellegrineschi, 1997; Brar et al. 1999; Popelka et al. 2006; Mao et al. 2006). However, the mean shoot length declined with increase in BAP concentrations (Table 2) and furthermore, elongation was very slow even after 4 weeks of culture. Lowering the concentration of BAP during subsequent subculture was not favorable for shoot elongation. The BAP have often been reported to stimulate shoot proliferation while inhibiting shoot elongation (Brassard et al. 1996; Figueiredo et al. 2001).

Like BAP, the effects of other cytokinins (TDZ and kinetin) on frequency of shoot regeneration were also examined. Addition of TDZ to the basal medium also induced a variable amount of callus at the base of the explants followed by induction of adventitious shoot buds from the explant. One interesting observation was made in the case of explants cultured on TDZ-supplemented media; with the increase in TDZ concentrations, there was an apparent decrease in frequency of shoot regeneration with drastic reduction in emergence of shoots in the responding explant with regeneration response restricted to stunted bud formation. TDZ at 1.0 µM induced a maximum of 0.9 shoots per explant in 74% of the cultures. Albeit TDZ found to have a higher cytokinin activity or a different action than other cytokinins during differentiation in different legumes, including pea, chickpea, lentil (Malik and Saxena, 1992c Malik and Saxena, 1992c), peanut (Gill and Saxena, 1992), white clover (Beattie and Garrett, 1995), tepary bean (Dillen et al. 1995) and goat's rue (Collen and Jarl, 1999), no such induction was observed in cowpea. This may be attributed to non-responsive genetic makeup or internal hormone concentration of the cotyledonary node tissue of cowpea towards TDZ.

Both BAP and Kinetin favored multiple shoot development; however, frequency of shoot multiplication and average number of shoots were less on kinetin containing medium (Table

2) clearly demonstrating that the type and concentration of the cytokinin used had a profound effect on frequency of shoot multiplication and number of shoots induced. However, the mean shoot length on kinetin was significantly higher than BAP, signifying the role of kinetin in shoot elongation.

The developmental behavior of cotyledonary node explants from seeds germinated in the presence of a high concentration of BAP is known to have definite advantages on shoot multiplication. Presumably, pre-cultivation on BAP activates and/or induces proliferation of pre-existing competent cells in the tissue (Veltcheva et al. 2005). The beneficial effect of pre-conditioning of seeds in BAP on shoot regeneration efficiency from cotyledonary explants has been reported in soybean (Thorne et al. 1995), pigeonpea (Shiv Prakash et al. 1994) and common bean species (Santalla et al. 1998). The modification of the physiological state of the initial explants is one of the approaches that may alter *in vitro* regenerative capacity (Malik and Saxena, 1991; Santalla et al. 1998; Cruz de Caravalho et al. 2000). This could be achieved by pre cultivation of the explants in various plant growth regulators for determination of organogenesis and embryogenesis in some of the legumes like bean. However, the physiological and molecular mechanism underlying cytokinins pre-cultivation in regeneration is still not properly known. However, since a specific physiological state is required for cells to be competent for regeneration, preconditioning might help in achieving the competence.

3.1.2 Shoot elongation

Incorporation of 0.5 µM of kinetin to MSB media enhanced the shoot elongation by three-fold in 86% of cultures within 2 weeks while higher concentrations proved ineffective whereas GA_3 containing medium induced non-uniform elongation and moreover, the elongated shoots were found thin and lanky, unsuitable for rooting (Table 3). However, the interval at which kinetin (0.5 µM) was supplemented to SIM was critical for obtaining optimal response. The maximum number of shoots with optimal shoot length was obtained when explants were cultured initially for 1 week on SIM, followed by 3 weeks on SIM supplemented 0.5 µM kinetin (SMM) (Table 4; Fig. 1b) suggesting that the time of exposure to the combination of these two cytokinins was critical for accelerated regeneration response from cotyledonary node explants of cowpea.

3.1.3 Cyclic organogenesis

The BAP-habituated cultures tended to produce shoots continuously on the subculture medium and therefore, after harvesting elongated shoots, the mother explants were repeatedly subcultured on MSB media supplemented with 5.0 µM BAP and 0.5 µM kinetin for 3 weeks for cyclic induction of shoots. These mother explants continuously produced shoots during

successive subculture without losing their shoot forming potential. An average of 36–38 shoots was produced from each explant in four consecutive harvests. Repeated transfer of meristematic explants on media containing cytokinins was found to cause rejuvenation of explants tissue, activation, conditioning of meristems and retention of their morphogenetic potential (Shekhawat et al. 1993; Naik et al. 2000; Vengadesan et al. 2002; Gopi et al. 2006). Continuous proliferation of BAP habituated culture of cotyledonary nodes during successive subcultures indicates its persistence in cowpea tissues.

The regeneration protocol described here has the basis for both adventitious and a non-adventitious system. The ability of proliferating meristematic/organogenic tissue to be maintained in a cyclic manner confers adventitious characteristics. However, the callus-like tissue that has developed upon prolonged culture on the regeneration medium itself is differentiated and produces small shoot clumps leaving small meristems, which are most probably the source of the formation of new buds in the next multiplication cycle, giving it non-adventitious characteristics.

Among the eight commercially important cultivars compared for their shoot regeneration potential, Pusa Komal produced maximum shoots (9.1 shoots/explant) in 94% of the cultures.

3.1.4 Rooting and acclimatization

Formation of roots was observed in 100% of shoots transferred to MS medium containing 2.5 µM IBA (Fig. 1c). Popelka et al. (2006) grafted rootless shoots onto cowpea seedlings in order to overcome the difficulties in rooting. Plantlets with developed roots were successfully established in soil (95%), and were eventually grown to maturity with normal seed set in greenhouse (Fig. 1d, e). The plants were apparently normal and showed no variation with respect to their morphology and growth characteristics.

The present study established a simple, efficient, rapid and direct plant regeneration through multiple shoot proliferation from cotyledonary node explants, precultured on BAP containing medium, on simple medium containing BAP and kinetin as growth regulators, and applicable to large number of genotypes.

Chapter 4

ESTABLISHMENT OF EFFICIENT SELECTION SYSTEM

Plant transformation is a rare process, and during transformation only a small proportion of the cells, of the target explant are transformed by the transformation vector and subsequently become stably transformed (Klee et al. 1987). As only a very small proportion of cells are transformed in most experiments, the chances of recovering transgenic lines without selection are usually low. Therefore, genetic manipulation requires a selection mechanism by which genetically modified cells are selectively favored to grow and divide in a background of untransformed cells. Different selection strategies have been proposed and successfully applied in plant transformation (Wilmink and Dons, 1993). This is usually accomplished by coupling or co-introducing the gene(s) of interest along with genes conferring resistance to antibiotics or herbicides. Thus, the selectable marker gene system confers a selectable advantage to those cells expressing the introduced gene. Incorporation of appropriate selective agents such as antibiotics or herbicides in the medium allow only transgenic cells to divide.

The marker genes, that allow the recognition of the transformed cells from a background of untransformed cells, code for a product that effectively detoxify or evade the selective agent by enzymatic modification or expression of an altered target. These genes are dominant, usually of microbial origin, and placed under the control of strong, constitutive, eukaryotic promoters, often of viral origin for ensuring its expression throughout the plant (Birch, 1997). The usefulness of a particular resistance marker depends on the characteristics of the selection agent, the resistant gene and the plant material. A selection agent that inhibits the growth of untransformed cells but does not have adverse effects on the transformed cells is preferred in plant transformation. The sensitivity of the plant cells to the selection agent depends upon the genotype, the explant type, the developmental stage, and the tissue culture conditions. However, selection mechanisms are not always 100% efficient. As a result, regenerated plants are not composed of only transgenic cells but contain either, mixed transgenic and untransformed cells or only untransformed cells. The first type of plants is called ‘chimeras’ and the second ‘escapes’.

The most exclusively exploited selectable marker gene is neomycin phosphotransferase-II (*npt*II) isolated from transposon Tn5 of *E. coli* K12 that confers resistance to the aminoglycoside antibiotics such as kanamycin sulfate and its analogues paromomycin and geneticin (G418) (Bevan et al. 1983) by inactivating them by phosphorylating the 3'OH of its 6-deoxy-6aminoglucose-1-alpha sugar residue. *E. coli* hygromycin phosphotransferase (*hpt*) gene, which confers resistance by specifically phosphorylating hygromycin B (van den Elzen et al. 1985) has also been successfully employed as a selectable marker in transformation of legume crops like *Cajanus cajan* (Kumar et al. 2004), *Glycine max* (Olhoft et al. 2003), *Vigna angularis* (Khalafalla et al. 2005), *Arachis hypogea* (Joshi et al. 2005), *Pisum sativum* (Lulsdorf et al. 1991), *Medicago sativa* (D’ Malluin et al. 1990) and (Finer et al. 1991). The

bar gene that confers tolerance to herbicide has been successfully used to select and recover transgenic plants in various grain legumes (Senthil et al. 2004; Aragao et al. 2002; Richter et al. 2007; Grant et al. 2003; Sonia et al. 2007; Krejci et al. 2007; Popelka et al. 2006, Muruganantham et al. 2007). There have been no reports of adverse effects of either NPTII or the *npt*II gene on humans, animals or the environment (Flavell et al. 1992; US-FDA, 1998 and European Federation of Biotechnology, 2001).

Many plants are naturally resistant to particular selective agents and an effective antibiotic/ herbicide for a particular species and further for explants can only be determined empirically. Thus it is important to design an experiment to test the frequency of "escapes" in any particular explant and for each selective condition. Careful evaluation of the concentration of the selective agent, that inhibit the growth in a large proportion of particular explants but allow the "escape" of a few percent, are essential prior to any transformation experiment. In addition, the application of selective agents can be applied in gradual increments (step-wise selection) or in lethal concentrations soon after transformation. Inclusion of selective agent in the plant culture medium, immediately after transformation or after a short recovery period is also crucial in recovering transformed plants (Sharma and Anjaiah, 2000). All these approaches have been used successfully in the transformation of a number of legume species in a variety of explants.

Prior to transformation, it is thus essential to screen different selective agents to find out their optimal concentration that would preferentially permit growth and development of the transformed cells without hindering or impairing the normal regeneration process.

The present study was undertaken with the objective to evaluate the efficacy of various aminoglycoside antibiotics, (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983), paromomycin (Guerche et al. 1987) and geneticin (Potrykus et al. 1985) on shoot regeneration from cotyledonary node explants of cowpea. The basal level of resistance/tolerance of explants to these selective agents was investigated. Thus preliminary experiments were set up to determine the lowest level of antibiotic that would suppress shoot organogenesis from cotyledonary node explants.

4.1 MATERIALS AND METHODS

4.1.1 Effect of kanamycin, paromomycin and geneticin on shoot regeneration

In order to determine the threshold concentration of these antibiotics for the selection of transformed shoots, the explants were cultured on shoot induction medium containing different concentrations of kanamycin (50, 75, 100, 125 and 150 mg/l), geneticin (15, 30, 45, 60, 75 mg/l) or paromomycin (25, 50, 75, 100, 125 mg/l).

All the culture conditions were maintained as similar to that of stated elsewhere. The same concentrations of antibiotics were incorporated during subsequent subculture in respective media, at an interval of 2 weeks. Control experiments were maintained by culturing the explants on shoot induction medium in absence of any antibiotics.

The selection scheme i.e., choice of aminoglycoside antibiotic and its optimal concentration, for the selection of transformed shoots was based on those that allowed initial proliferation of shoot buds followed by their bleaching without necrosis effect on the explants. The observations were scored after 3–4 weeks on selection media. For each treatment, 40 cultures were raised and each experiment was performed atleast thrice.

4.2 RESULTS AND DISCUSSION

4.2.2 Effect of kanamycin, geneticin, paromomycin on shoot regeneration

The effect of three aminoglycoside antibiotics kanamycin parmomycin and geneticin on the growth and morphogenic potential of untransformed cowpea cotyledonary node cultures were compared. Although the mode of action of *npt*II is highly specific and the antibiotics tested were all aminoglycosides, they exerted varied effects on the regeneration of the explants. All the three selective agents tested for their sensitivity, showed their toxicity differed at varied concentration range and so also their mode of action.

The regeneration remained unaffected in the presence of kanamycin upto 100 mg/l, which permitted emergence of healthy and green shoots with a survival frequency of 81%. However, the average number of shoots reduced gradually with the increase in kanamycin concentration (Table 5). With further increase in kanamycin concentration to 125 mg/l, the shoot regeneration was reduced to 64%, and necrosis was observed in the explants. Shoot growth was visually impaired at 150 mg/l, and the shoots remained stunted and bleached with a higher degree of necrosis in the explants. The higher concentrations of kanamycin were too toxic for the survival of the cotyledonary node cells as evident from the degree of necrosis observed, suggesting that higher kanamycin concentration may not allow the host cells to survive long enough for *Agrobacterium* infection to occur, and to undergo division thereafter. The host-cell division is required for successful *Agrobacterium*-mediated transformation and regeneration of transformants (Binns and Thomashow, 1988). The cotyledonary node explants exhibited high degree of resistance to kanamycin (Popelka et al. 2006), and needed a prolonged duration of selection for recovery of transformants in cowpea (Chaudhury et al. 2007).

The survival of untransformed explants on regeneration medium remained undeterred in the presence of paromomycin up to a concentration of 50 mg/l (Table 5). However, the frequency

of shoot regeneration decreased with increase in concentration of paromomycin. Shoot regeneration was permitted upto 100-125 mg/l while the regeneration frequency was drastically reduced. Moreover, early necrosis was observed in explants cultured in higher concentrations of paromomycin, which interfered with the regeneration process to a varying degree. These results substantiated the observation that early necrosis may be due to substances leached from the dying cells that probably had negative influence on regeneration. It is not completely understood how this effect is exerted, but it is likely that higher levels of antibiotic may interfere with photosynthesis, thereby reducing plantlet vigor (Wilmink and Dons 1993).

In contrast, a much lower concentration (45 mg/l) of geneticin was effective in selection as it permitted the initiation and proliferation of shoot buds, and thereafter completely bleached the regenerated shoots buds within a week (Table 5; Fig. 2), without any symptoms of necrosis in cultured explants. Therefore, low concentration of geneticin (45 mg/l) was chosen for selection and maintenance of putative transformed shoots in our study. Interestingly, the developmental stage of the initial explants and their subsequent regeneration were found concomitant to the activity of geneticin whereas kanamycin and paromomycin exerted a faster inhibitory activity and thus directed early necrosis. Geneticin selection has been successfully employed for the recovery of transgenic plants in papaya (Zhu et al. 2004), leek and garlic (Eady et al. 2005), sugar cane (Manickavasagam et al. 2004), oat (Cho et al. 2003), pine (Grace et al. 2005), safflower (Orlikowska et al. 1995) and *Acacia mangium* (Xie et al. 2002).

Selectable markers have been developed to permit preferential growth of engineered cells but these are only effective with fully dedifferentiated tissue. Attempts to effect selection with organized tissue have not met with much success with the notable exception of the shoot apical meristem-transformation of lupinus, cotton and soybean (Pigeaire et al. 1997; Zapata et al. 1999; Aragao et al. 2000). The major problem with organized tissue is that transformed cells permit proliferation of non-transformed tissue in their vicinity by effectively detoxifying the selective agent. This results in the creation of chimeric tissue, which subsequently give rise to both transformed and non-transformed plants, and the latter being the overwhelming majority (Christou, 1997). The judicious choice of selection levels for organized tissue is critical for facilitating the growth and division of transformed cells, as higher level would be deleterious even to the transformed cells at initial stages of screening (Sahoo et al. 2001).

Chapter 5

AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION OF COWPEA AND RECOVERY OF FERTILE TRANSGENIC PLANTS

Plant genetic transformation has become an important tool for functional genomics and complementation to conventional breeding programmes for crop improvement. *Agrobacterium tumefaciens* is the established method of choice for the genetic transformation of most plant species as it provides a reliable and well-documented means for introducing foreign DNA into plant cells. It is perceived to have several advantages over other methods of transformation (such as biolistics), including the ability to transfer large segments of DNA with minimal rearrangement, with fewer copies of inserted genes at higher efficiencies with lower cost and reduced gene silencing associated with integration of T-DNA (see reviews by Hansen and Wright 1999; Hiei et al. 1997; Gheysen et al. 1998; Shibata and Liu 2000). In addition, *Agrobacterium* transformation may facilitate the removal of plant selectable marker genes by segregation (Komari et al. 1996; Matthews et al. 2001; Miller et al. 2002). These are important considerations, particularly when creating genetically manipulated lines in crop species for field testing, when the presence of unnecessary DNA and transgene arrangement/copy number are scrutinized as part of the regulatory processes.

A. tumefaciens-mediated delivery of foreign genes into numerous plant species has been extensively described since Horsch et al. (1985) first demonstrated the utility of the system with subsequent report by other workers (see reviews in Gelvin 2003). However, *A. tumefaciens*-mediated foreign gene transfer in many recalcitrant plant species such as cowpea is laboratory dependent and far from routine (Barik et al. 2005; Chaudhury et al. 2006; Ignacimuthu and Prakash et al.2006; Liu et al. 2005; Hanafy et al. 2005; Saini et al.2007; Bhoomkar et al. 2008; see reviews by Eapen et al. 2008). Regeneration of fertile transgenic plants of cowpea by *A. tumefaciens*-mediated transformation has been reported using longitudinally bisected embryonic axes attached to the cotyledons (Popelka et al. 2006) and cotyledonary node system (Chaudhury et al. 2007). Addition of thiol compounds during infection and coculture, prolonged coculture, application of a delayed herbicide selection resulted in recovery of cowpea transgenics with a frequency of transformation of 0.15% (Popelka et al. 2006). Transgenic lines transmitted the transgene (*bar* gene) to their progenies following Mendelian inheritance (Popelka et al. 2006). Chaudhury et al. (2007) generated fertile transgenic cowpea plants on kanamycin selection with an efficiency of 0.76% and demonstrated the transgene inheritance in Mendelian fashion in the first generation. However, both the transformation systems (Popelka et al. 2006; Chaudhury et al. 2007) are time consuming, proved difficult to follow and moreover, present low frequency of germ line transformation.

A serious constraint to the genetic transformation of cowpea is low transformation efficiency. Part of the optimization process for efficient T-DNA delivery to the

regenerating tissues of grain legumes through *Agrobacterium*-mediated transformation involves matching broad host-range, disarmed strains with suitable binary vectors containing the T-DNA, additional *vir* genes, explant condition, use of chemical inducers, and cocultivation conditions, and testing these for their ability to transform the target cells of the desired host germplasm and successful generation of transgenic plants (Sahoo and Jaiwal, 2008). Optimization of these factors proved to be of considerable importance for the establishment of successful transformation system in the highly recalcitrant crops like cowpea, for which a routine transformation procedure is far from routine.

In the investigation reported in this chapter, we examined major variables influencing T-DNA delivery to cotyledonary node explants of cowpea and the regeneration of fertile plants. The goal of the investigation was to improve the transformation efficiency and to demonstrate the reproducibility of this transformation procedure for cowpea. Therefore, the influence of *Agrobacterium* strains, constitutive expression of *vir* genes, and different co-cultivation conditions on the efficiency of *Agrobacterium*-mediated gene transfer was assessed using the *gus* as reporter system. Secondly, the optimized transformation conditions were shown to facilitate the production of fertile transgenic cowpea plants in cultivar Pusa Komal. Transgene integration and inheritance in these transformants were examined.

5.1 MATERIALS AND METHODS

5.1.1 Plant material and explant preparation

Seeds of cowpea cv. Pusa Komal were surface-sterilized and cultured on MSB medium [MS salts (Murashige and Skoog, 1962) + B5 vitamins (Gamborg et al. 1968)] supplemented with 10 µM BAP for 3 days. Subsequently, the cotyledonary node explants were prepared from 3 days-old *in vitro* raised seedlings as described elsewhere, and soaked in liquid co-cultivation medium, LCM (MSB medium containing 1 µM BAP, pH adjusted to 5.5) in a sterile petridish to avoid de-hydration.

5.1.2 *Agrobacterium* strains and transformation vector

Four *Agrobacterium tumefaciens* strains, characterized by different chromosomal backgrounds and their respective tumour-inducing plasmids [LBA4404 (Hoekema et al. 1983), GV2260 (Deblaere et al. 1985), AGL1 (Lazo et al. 1991) and EHA105 (Hood et al. 1993)], all harboring a binary vector pCAMBIA2301 (http://www.cambia.org) were compared for their efficiency through early detection of transient transformation events in infected explants. The vector pCAMBIA2301 is a pPZP-based small binary vector (Hajdukiewicz et al. 1994) and the T-DNA of pCAMBIA2301 includes neomycin

phosphotransferase gene (*npt*II) and β-*glucuronidase* gene (*gus*) both driven by the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 3). The pCAMBIA vectors lack super-virulent genes and do not give high transformation frequencies in recalcitrant grain legumes. Therefore, the possible enhancing effect of additional *vir* genes on transformation was examined by separately mobilizing pCAMBIA2301 into *A. tumefaciens* strain LBA4404 carrying pSB1 by triparental mating, and the transformation efficiency of LBA4404pSB1 and hypervirulent strain EHA105 was compared. Plasmid SB1 lacks T-DNA but has all the virulence genes present in pTOK233 (Hiei et al. 1994) and contains a tetracycline resistance gene as selection marker.

5.1.3 Triparental mating

The binary vector was mobilized to each of four *A. tumefaciens* strains by triparental mating procedure (Bevan, 1984). The recipient *A. tumefaciens* strains were streaked on the YEP medium containing rifampicin (10 mg/l) and incubated at 28^0 C, two days prior to triparental mating. The donor strain DH5αpCAMBIA2301 and helper strain DH5αpRK2013 were streaked on LB medium (Sambrook et al. 1989) containing kanamycin (50 mg/l) and incubated at 37^0 C, one day prior to triparental mating. The plasmid pSB1 was mobilized from donor strain DH10BpSB1 to the *A. tumefaciens* strain LBA4404 following the same procedure, prior to the mobilization of pCAMBIA2301. Triparental mating was performed on the third day on YEP medium (An et al. 1988), and incubated at 28^0 C for 12-18 hrs. Serial dilution was performed and 100 µl from each dilution was plated on YEP medium containing rifampicin (10 mg/l) and kanamaycin (50 mg/l), and additionally 5 mg/l tetracycline (for selection of pSB1), for selection of transconjugants.

The plates were incubated at 28^0 for 12-16 hrs. Single colonies were analyzed by Colony PCR using primers specific to *npt*II gene (mentioned elsewhere in this chapter) to confirm the *Agrobacterium* transconjugants harboring the mobilized plasmid pCAMBIA2301. Plasmid DNA was isolated and restriction digestion was performed with *Bam*HI enzyme, and the plasmid DNA fragments were analysed to confirm the presence of mobilized vector in *A. tumefaciens* transconjugants.

All *A. tumefaciens* strains containing binary vector were maintained on solid YEP medium (An et al. 1988) supplemented with 10 mg/l of rifampicin, and 50 mg/l of kanamycin, and additionally 5 mg/l tetracycline was used to select pSB1.

5.1.4 Bacterial inoculation

Single colonies of the bacterial strains were transferred to 2 ml of liquid AB minimal medium (Chilton et al. 1974) with appropriate antibiotics, and grown overnight at 28^0C.

Then, 500 µl of *Agrobacterium* suspension were recultured in 25 ml of liquid AB minimal medium with respective antibiotics until OD_{600} reached to 0.8. The cells were collected by centrifuging at 5000 rpm for 5 min, and the pellet were re-suspended in liquid cocultivation medium, LCM (MSB medium containing 1 µM BAP, pH adjusted to 5.5) supplemented with 100 µM acetosyringone for inoculation.

5.1.5 Cocultivation

The 3-d-old cotyledonary node explants were gently stabbed three to four times using a sterile needle (26.5 G) at the nodal region and inoculated in bacterial suspension for 30 min with occasional shaking. The explants were then blotted on sterile filter paper and cocultivated in petri dishes lined with filter paper moistened with LCM (pH adjusted to 5.2, 5.5 and 5.8) containing different concentrations of acetosyringone (0, 50 and 100 µM) for 2-4 days under 16 hr photoperiod at different temperatures (20, 22, 24, 26 and 28^0 C).

5.1.6 Selection and regeneration of transformants

Following co-culture, the explants were washed three to four times with LCM (pH adjusted to 5.5) and blotted dry on sterile filter paper. The explants were cultured on SIM containing 45 mg/l geneticin and 500 mg/l cefotaxime for selective regeneration of transformants. Same levels of antibiotics were maintained during subsequent subcultures. The elongated shoots were rooted in MS medium supplemented with 2.5 µM IBA, and 500 mg/l cefotaxime. The putative transformed plants were established in soil:compost (1:1) and grown to maturity in transgenic greenhouse containment. The T_0 seeds were collected and sown in soil to raise the T_1 plants. Seeds were collected from T_1 plants.

5.1.7 Transient and stable *gus* expression analysis

Histochemical GUS assays (Jefferson, 1987) were used to assess transient expression of the *gus* gene in cotyledonary node explants after 2-, 3- and 4-d cocultivation with different *A. tumefaciens* strains under varied cocultivation conditions. Level of transient *gus* expression was assessed on a per explant basis by estimating the number of blue foci visible on the nodal region of each cotyledonary node.

Histochemical GUS assays were also used to assess stable expression of the *gus* gene in geneticin-resistant transgenic plants in the T_0 and T_1 generations. Leaves, flowers, perianth, ovules, stigma, stamen and pollen grains of T_0 transgenic plants and nontransformed control plants were immersed in GUS substrate solution consisting of 0.5 M NaPO4 (pH 7.0), 50 mM potassium ferricyanide, 50 mM potassium ferrocyanide, 10 mM EDTA (pH 7.0), 0.1% triton X-100 with 1 g/l of 5-bromo-4-chloro-3-indolyl-β-d-

glucuronide (X-gluc) (Biosynth AG, Staad, CH) for 24 h at 37^0 C. Following incubation, tissues were bleached with 100% ethanol, and examined under microscope. T_1 seedlings were also assayed for GUS activity after overnight imbibitions of seeds in distilled water.

5.1.8 Genomic DNA isolation and PCR analysis

Genomic DNA was extracted from young leaves of both non-transformed and putative transformed plants by the CTAB (cetyl trimethyl ammonium bromide) method (Roger and Bendich, 1988). Polymerase chain reaction (PCR) was performed to detect the presence of the *npt*II and *gus* genes in putative T_0 transformants and their progenies. The 0.54 kb of *npt*II and 0.25 kb of *gus* coding regions were amplified by using following primer combinations, *npt*II gene: forward (5′-CCACCATGATATTCGGCAAC-3′) and reverse (5′-GTGGAGAGGCTATTCGGCTA-3′), and *gus* gene: forward (5′-TAACCTTC ACCCGGTTGCCAGAGG-3′ and reverse (5′-CCTTAACTAAGCCGGAATCCAT CG -3′). The amplification reaction was carried out with initial denaturation at 94^0C for 1 min and followed by 38 cycles of denaturation at 94^0 C for 1 min, annealing at 58^0 C for 1 min and extension at 72^0 C for 1 min, and final extension for 7 min at 72^0 C for detection of both *npt*II and *gus* gene amplification. Amplified DNA fragments were analyzed by electrophoresis on 1% agarose gel, visualized by ethidium bromide staining (Sambrook et al. 1989) and photographed under ultraviolet light in gel documentation system. The PCR was carried out using approximately 100 ng of purified genomic DNA and Taq polymerase. DNA from non-transformed (control) plants was included in the experiments to discriminate from possible contamination.

5.1.9 Southern hybridization analysis

Southern hybridization analysis was carried out on four randomly chosen PCR positive T_0 plants to confirm the stable integration of *npt*II gene and to detect junction fragments of plant DNA and the T-DNA. Ten µg samples of genomic DNA from non-transformed control and transformed plants were digested with *Bam*HI. The distance of the left border from the *Bam*HI site in the T-DNA was indicated in Fig. 3. The digested samples were fractioned on a 0.8% agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA). Southern hybridization was performed using the non-radioactive DIG Labeling and Detection System (Roche, Germany) by probing with DIG-labelled coding regions of *npt*II (540 bp) following supplier's instructions. Genomic DNA isolated from independent transformation events is expected to generate unique patterns after digestion with *Bam*HI, and the number of signals generated upon hybridization with coding regions of *npt*II (540 bp), should reveal copy number of the integrated gene (*npt*II).

5.1.10 RNA isolation and RT-PCR

The presence of the *npt*II and *gus* transcripts was determined by reverse transcription polymerase chain reaction. Total RNA was isolated from fully expanded leaves of T_0 and T_1 transgenic plants, and non-transformed control plant using Rneasy Plant Mini Kit (Qiagen, USA) following manufacturers instructions. The first strand cDNA was synthesized using 5 µg of total RNA, and the First Strand cDNA Synthesis Kit (Fermentas, USA), with oligo(dT) primer, and the M-MuLV Reverse Transcriptase, according manufacturers' instructions. PCR amplification of the 0.54 kb of *npt*II and 0.25 kb of *gus* coding regions was performed using cDNA of the first reaction (10% of the volume), with primers specific to *npt*II and *gus* genes, as described earlier. These templates were used for the amplification of the *npt*II and *gus* gene transcripts using the same primer sets as those used in genomic PCR.

5.1.11 Analysis of transgene inheritance

The leaves of T_1 transgenic plants were analyzed for the presence of *npt*II and *gus* genes using polymerase chain reaction, as described earlier. Segregation patterns were analyzed with the chi-square test ($\chi 2$) against the expected Mendelian ratio of 3:1 for single locus insertion.

5.2 RESULTS AND DISCUSSION

5.2.1 Effect of *Agrobacterium* strains

We studied the influence of four commonly used disarmed strains of *A. tumefaciens* harboring pCAMBIA2301 on rate of T-DNA transfer, measured via the number of explants showing GUS expression under optimized cocultivation conditions presented in this chapter. Among the four *A. tumefaciens* strains tested, EHA105 was most effective with higher transient transformation efficiency (76%) followed by LBA4404 (64%), AGL1 (61%) and GV2260 (23%). The transformation efficiency of the strain EHA105 was significantly higher than that of LBA4404 (Fig. 4a). However, no significant difference was observed between LBA4404 and AGL1. It is already well established that virulence of the *Agrobacterium* strain against particular plant genotype (species) is a key factor of successful transformation (Hood et al. 1993). Virulence of *Agrobacterium* strains varies widely among plant hosts and is particularly important for the transformation of recalcitrant species (Han et al. 2000).

EHA105 is an L,L-succinamopine strain with a C58 chromosome background which contains as virulence helper plasmid pEHA105, originally derived from supervirulent pTiBo542 (Hood et al. 1986, 1993) whereas LBA4404 is an octopine strain with Ach5

chromosomal background carrying pAL4404 as virulence plasmid (Hoekema et al. 1983). EHA105 is generally recognized as being superior in facilitating gene transfer to plant cells in many plant species including leguminous species, soybean (Meurer et al. 1998; Donaldson and Simmonds, 2000), pea (Nadolska-Orczyk and Orczyk, 2000) and peanut (Egnin et al. 1998), and other crop species, apple (De Bondt et al. 1994), cabbage (Domínguez et al. 2004), blueberry (Cao et al. 1998), and wheat and barley (Guo et al. 1998). A previous assessment of different *Agrobacterium* strains (AGL0, AGL1 and LBA4404) in cowpea transformation found no difference in their transient transformation efficiency (Popelka et al. 2006).

5.2.2 Effect of pH

GUS expression was observed in the explants over the entire range of pH (5.2–5.8) tested. However, cocultivation medium set to pH 5.5 significantly enhanced the number of explants showing *gus* expression, and the number of GUS foci per explant. A further increase in pH of co-cultivation medium from 5.5 to 5.8 significantly decreased the frequency of *gus* expression (Fig. 4b). A comparatively low pH during co-cultivation has been described as an important factor influencing *Agrobacterium*-mediated gene transfer (AltMorbe et al. 1988). Several groups have reported that optimal induction of *vir* genes is attained when the pH is lower than those of commonly used plant tissue culture media (Stachel et al.1986; Vernade et al. 1988). Shrivastava et al. (2001) and Husnain et al. (1997) found a pH of 5.6 to be the most effective for transforming pigeonpea and chickpea respectively, pH 5.8 optimal for blackgram transformation (Saini et al. 2007), pH 5.5 (Meurer *et al*., 1998) and 5.4 (Dang and Wei, 2007) found optimal for transforming soybean. The stimulatory effect of low pH on transformation frequency may be due to the fact that an acidic pH induces the virulence genes of *Agrobacterium* (Stachel et al. 1985; AltMorbe et al. 1988).

5.2.3 Effect of acetosyringone

The presence of acetosyringone during cocultivation enhanced the frequency of transient transformation, and optimal transformation achieved with 100 µM acetosyringone (Fig. 4c). A further increase in its concentration resulted in necrosis of explants. This result suggested that maximal transformation was dependent on the presence of acetosyringone and its use at the appropriate pH. Alt-Moerbe et al. (1988) demonstrated that *vir* induction is strictly dependent on acidic pH and in the presence of acetosyringone. Clercq et al. (2002) showed that *vir* induction is optimal in the presence of acetosyringone at low pH (5.5).

The *vir* genes of the Ti plasmid as well as the *chv* genes on the bacterial chromosome are known to mediate the transfer of T-DNA from *A. tumefaciens* to plant cells and the steps preceding integration into the plant genome (for review, see Gelvin 2003). Transcription of these *vir* genes can be induced by plant specific phenolic compounds, such as acetosyringone (Stachel et al. 1985), and certain sugars act synergistically with these phenolic inducers (Cangelosi et al. 1990). The level of induction of this region determines the ability of the strain to transfer the T-DNA In plant species, where such compounds are not synthesized, addition of phenolic compounds such as acetosyringone during plant/bacteria interaction supports the gene transfer (Hiei et al. 1994).

Acetosyringone has been found to be a key component for successful *Agrobacterium*-mediated transformation of chickpea (Polowick et al. 2004), soybean (Torisky et al. 1997), mungbean (Jaiwal et al. 2001; Sonia et al. 2006), and blackgram (Saini et al. 2007, Bhomkar et al. 2008). Results from a wheat inflorescence transformation experiment showed that T-DNA cannot be transformed to the plant tissue without acetosyringone (Amoah et al. 2001). Transformation frequency was induced 2-fold with 100 µM acetosyringone in *Carrizo citrange* explants (Cervera et al. 1998).

5.2.4 Effect of cocultivation period and temperature

In the present study, extension of the cocultivation period from 2 to 3 day significantly enhanced the frequency of explants showing *gus* activity (Fig. 4d). Longer cocultivation periods frequently resulted in *Agrobacterium* overgrowth leading to its detrimental effect on regeneration potential of the explants. A short cocultivation period (2-3 days) has been reported optimal for T-DNA delivery in cowpea (Muthukumar et al. 1996), mungbean (Jaiwal et al. 2001; Sonia et al. 2007), blakgram (Saini et al. 2007, Bhomkar et al. 2008), pegionpea (Mohan and Krishnamurthy, 2003) and soybean (Li et al. 2004).

In the present investigation, results from three separate experiments showed consistent improvement in transformation efficiencies, when cocultivation was carried out at 22^0C, irrespective of the type of *Agrobacterium* strain used, and the transient GUS activity decreased at cocultivation temperatures above and below 22^0C (Fig. 4e). Our results on influence of cocultivation temperature on transient and stable T-DNA integration in cowpea were in line with the previous reports in diverse plant species (Dillen et al. 1997; Sales et al. 2001; Sunilkumar and Rathore, 2001; De Clercq et al. 2002; Grant et al. 2003). Cocultivation with *Agrobacterium* at lower temperature has been shown to improve *Agrobacterium*-mediated gene transfer to plant cells. Fullner et al. (1996) showed that low temperatures promoted pilus assembly leading to increased number of pili on the cell surface. It has been proposed that the increased transformation efficiency at low-

temperature may be due to better functioning of the VirB-VirD4 part of the T-DNA transfer machinery (Fullner and Nester 1996; Fullner et al. 1996). It can also be concluded that at temperatures above the optimum, a lack of *vir* induction is not the cause of reduced T-DNA transfer rather due to thermal instability of VirA (Jin et al. 1993). In particular for tropical legume species, for which regeneration is often performed at relatively high temperatures, lowering the temperature during *Agrobacterium* co-cultivation may prove beneficial.

For *in vitro* cocultivation experiments, 22^{o}C is a commonly employed temperature and literature reports mentioning higher temperatures abound. The temperatures assessed in this work pertain to the actual temperature of the co-cultivation medium. In many systems, the actual temperature inside the culture vessels is well above the temperature of the culture rack due to radiation heating from two lamps (a difference of two degree is not exceptional).

5.2.5 Effect of additional virulent genes

The presence of additional copies of *vir*G, *vir*C, *vir*B genes in resident pSB1 in LBA4404pCAMBIA2301 significantly increased the T-DNA delivery efficiency to 100% (Fig. 4f). The constitutive expression of extra copies of *vir* genes in moderately virulent strain LBA4404 significantly increased the T-DNA delivery efficiency to 100%. The constitutive expression of extra copies of *vir* genes in moderately virulent strain LBA4404 lead to 36% increase in transformation efficiency as compared to their absence, and 24% increase as compared to hypervirulent strain EHA105, which clearly demonstrated the role of extra copies of *vir* genes in enhancing the transformation efficiency in cowpea. The constitutive expression of *vir* genes in resident pSB1 vector dramatically increased the transient transformation frequency to 100% as opposed to only 80% in absence of extra copies of *vir* genes (Chaudhury et al. 2007). Optimal T-DNA delivery due to the presence of additional copies of *vir*G, *vir*C, *vir*B genes in resident pSB1 in LBA4404pCAMBIA2301 clearly indicates the beneficial role of extra copies of *vir* genes in enhancing the transient transformation efficiency of regenerating cells of cotyledonary node explants. Extra copies of *virG* (Hansen et al. 1994; Hiei et al. 1994) or constitutive mutant *virG* gene on a compatible plasmid (Van der Fits et al. 2000) have been reported to increase the efficiency of *Agrobacterium* mediated transformation for many crops, particularly monocot species (Park et al. 2000; Wu et al. 2008).

The pSB1plasmid carrying the virulence region encompassing genes *vir*C, *vir*B, and *vir*G when coupled with the disarmed octopine type *A. tumefaciens* strain, LBA4404 was more

consistent for efficient T-DNA delivery in cowpea, and similar results have been obtained in cereals, rice (Hoekema et al. 1983).

5.2.6 Selection and regeneration of transgenic plants

All the explants showed GUS activity after co-cultivation with LBA4404/pSB1 harboring pCAMBIA2301, predominantly in the regenerating sites (Fig. 5a). The selection pressure imparted by the geneticin was very effective in early identification of putative transgenic shoots as it permitted a significant amount of escapes to be discarded at the initial stage. A total of 486 explants, in three different experiments, inoculated with LBA4404/pSB1 harboring pCAMBIA2301 produced 46 green shoots on genetecin selection medium (Table 6) after 7-8 weeks of cocultivation, out of which 29 shoots formed roots (63%) within 3 weeks of transferring to rooting medium.

Geneticin provided an effective selection of transformed shoots as most of the un transformed shoot cultures bleached within 6 weeks on geneticin selection medium. The green shoot clusters were selected and transferred to fresh selection medium during subculture for further proliferation of the shoots. Two to three months after transplantation, all the rooted putative transgenic plants matured and produced flowers, and set seeds in the greenhouse (Table 6).

5.2.7 Stable GUS expression analysis

The transformed shoots recovered on selection medium showed a strong, uniform and stable GUS expression in in leaves, developing stamen, anthers, pollen grains, perianth, ovules and stigma of all the geneticin resistant T_0 plants, and endogenous GUS expression was absent in the tissues of control plants (Figs. 5b, c, d, e, f, g, h).

The GUS expression in germinated T_1 seedlings clearly demonstrated inheritance and expression of the transgene in the progeny (Fig. 5i). The stable transformation efficiency was determined based on the percentage of T_0 plants that showed stable integration of transgene.

5.2.8 Molecular analysis of transgenics

PCR analysis of the genomic DNA isolated from the putative transgenics detected the presence of the expected 540 bp and 240 bp fragments corresponding to *npt*II and *gus* genes respectively (Fig. 6a, b), in 25 out of the 29 geneticin-resistant transgenic plants demonstrating the efficiency of geneticin based selection scheme in elimination of escapes (Table 6). No amplification was detected in the control untransformed plants. The four plants tested negative in PCR analysis showed strong GUS expression in flowers

indicating that they were possibly germline transformants, thus stable transgenic lines could be recovered in the subsequent generations.

The integration of foreign DNA into the plant genome was further confirmed by Southern blot hybridization with the *npt*II specific probe. Genomic DNA was digested with *Bam*HI, which cleaves once in the T-DNA cassette. Consequently, this enzyme produces fragments characteristic of the integration site of T-DNA for the probe, and the numbers of bands can give an indication of the copy number of insertion. Southern hybridization analysis revealed signals corresponding to junction fragments, having a defined portion of the T-DNA (*Bam*HI site to close to the left border i.e., 2.1 kb), and portions of plant DNA. The pattern in Southern hybridizations using *npt*II probes clearly indicated that transgenes linked with T-DNA were randomly integrated into the cowpea genome (Fig. 6c). The four independent transformed plants showed simple integration patterns with one e.g. lines, J1 (lane 2), J3 (lane 4) to two e.g. line J7 (lane 1) and three e.g. line J6 (lane 5) transgene per genome (Fig. 5c). No hybridization signal was detected in the untransformed plant (Fig. 5c, lane C). The transgene copy number and the segregation ratios reported here were similar to those previous reports on cowpea (Popelka et al. 2006; Chaudhury et al. 2007).

RT-PCR analysis of the Southern positive plants showed that lines contained the transcripts of both *npt*II and *gus* genes (Fig. 6d) confirming the expression of the transgenes in the transgenic plants. The result verified the functional expression of the *npt*II and *gus* genes in the transgenic plants.

5.2.9 Transgene expression and segregation in the T_1 generation

Transgene expression and segregation were analyzed in the seeds of 4 randomly selected T_0 transgenic lines. Strong GUS activity in germinated T_1 seedlings indicated the inheritance and expression of the *gus* gene in the progeny. PCR analysis of the progeny of the each selected transgenic lines showed inheritance of *npt*II and *gus* genes in a Mendelian fashion (Fig. 6e, f). A segregation ratio of 3:1 for both *npt*II and *gus* genes was observed in three lines (J1, J3, J6) suggesting a single functional locus, where as the segregation ratio in progeny of line J7 appeared to be 15:1 rather than 3:1 for both genes suggesting segregation of two independent loci (Table 7).

The results described the development of an efficient and reproducible *Agrobacterium*-mediated genetic transformation system for the production of stable transgenic plants in cowpea. A dramatic increase in T-DNA delivery leading to 100% transient transformation frequency was demonstrated by constitutive expression of additional *vir* genes in resident pSB1 vector, for the first time in any grain legume. Incorporation of kinetin to medium containing BAP after one week of culture, and three weeks of exposure to the

combination of these two cytokinins were found critical for optimal multiplication and elongation of transformed shoots from cotyledonary node explants. The geneticin-based selection system allowed rapid and efficient identification of transgenic shoots. Incomplete selection by kanamycin (Chaudhury et al. 2007), and phosphinothricin (Popelka et al. 2006) has resulted in lower recovery of transgenics in cowpea. Combining the three components, extra copies of *vir* genes, geneticin-based selection scheme and compatible culture regime; we recovered transgenic plants of cowpea in cultivar Pusa Komal at a frequency of 1.64%, a two fold higher than previous report (Chaudhury et al. 2007). Further, the recovery of transgenic plants by our protocol took approximately two months as compared to 5-8 months (Popelka et al. 2006) and 5-6 months (Chaudhury et al. 2007) reported earlier. The presence, integration and expression of transgenes were demonstrated in T_0 transgenic plants, which transmitted the transgenes in a Mendellian fashion. T_1 progeny showed stable expression of transgenes. This method for efficient recovery of stable transgenics would envisage genetic improvement of cowpea for insect pest resistance.

Chapter 6

SEED SPECIFIC EXPRESSION OF A α-AI 1 GENE IN COWPEA FOR STORAGE PEST RESISTANCE

Cowpea is a key staple food in many developing countries, and it forms an integral part of the diet of about 120 million people around the world. It provides a major source of cheap and high-quality dietary proteins supply (Langyintuo et al. 2003) to resource poor populace. However, cowpea production suffers heavily due to insect and storage pest damage, both in the field as well as in the post-harvest storage of seeds. The seeds being rich in protein and carbohydrate, suffer from extensive predation by storage pests, bruchid beetles (Singh et al. 2000), and infestations by the most prominent species, cowpea weevil, *Callosobruchus maculatus* and azukibean weevil, *C. chinensis* are responsible for grain losses estimated at 20–60% (Abrol, 1999; Tarver et al. 2007). The larvae of the weevils burrow into the seedpods and seeds, and the insects usually continue to multiply by feeding on starchy cotyledons during storage (Wolfson et al. 1991). The damage causes extensive losses, especially if the seeds are stored for long periods causing dramatic degradation to dry seeds, obstructing the commercialization and human consumption and thus causing the severe crop loss (Seck et al. 1991).

None of the tested cultivated cowpea lines and their cross-compatible wild relatives has the desired level of resistance to bruchids. Some accessions of Vigna species, belonging to *V. vexillata* have shown high levels of resistance to bruchids, and other insect pests. However, interspecific crosses between *V. vexillata* and *V. unguiculata* resulted in nonviable seeds, making conventional breeding approach untenable in transferring resistance to cultivated cowpea (Fatokun et al. 2002). The alternative recourse is to transfer candidate genes to cultivated cowpea through genetic transformation for developing inherent resistance to bruchid beetles.

Many insecticidal proteins and molecules of plant origin such as lectins, α-amylase inhibitors and protease inhibitors can retard insect growth and development when ingested (Boulter 1993; Ussuf et al. 2001). The enzyme inhibitors impede digestion through their action on insect gut digestive α-amylases and proteinases, which play a key role in the digestion of plant starch and proteins. Artificial diet bioassays carried out on cowpea weevils and azuki bean weevils indicate that these insects can be controlled by α-amylase inhibitors of common bean (*Phaseolus vulgaris*) (Ishimoto et al. 1999; Matchuka, 2002). Different plant α-amylase inhibitors exhibit different specificities against α-amylases from diverse sources. The α-AIs have been purified and characterized from different accessions and varieties of the common bean *P. vulgaris* (Kasahara et al. 1996; Marshall and Lauda, 1975; Le Berre-Anton, 1997). The best-characterized isoform, known as *α-AI-1*, was cloned and identified as an α-amylase inhibitor homologous to phytohemagglutinin (PHA) (Moreno and Chrispeels, 1989). A second variant of *α-AI*, called *α-AI2*, is found in some wild accessions of the common bean (Grossi de Sa et al. 1997). These two allelic variants have different inhibition specificities.

The α-AI-1 inhibits PPA as well as the α-amylases of the *C. maculatus* and *C. chinensis*, but it does not inhibit the α-amylases of the *Z. subfasciatus* (ZSA). In contrast, α-AI2 does not inhibit the first three amylases mentioned above but it does inhibit ZSA (Grossi de Sa et al. 1997).

Introduction of a gene encoding an α-amylase inhibitor 1 (αAI-1) of common bean has conferred resistance to several bruchid beetles when expressed in seeds of transgenic peas (Shade et al. 1994; Schroeder et al. 1995; Morton et al. 2000; Sousa-majer et al. 2007), azuki beans (Ishimoto et al. 1996) and chickpeas (Sarmah et al. 2004; Ignacimuthu and Prakash, 2006). Therefore, bean αAI-1 gene has been considered to be a strong candidate for conferring resistance to *Callosobruchus* spp. in cowpea.

In this chapter, we report introduction of the common bean *αAI-1* gene to cowpea through the developed *Agrobacterium tumefaciens*-mediated transformation method, and demonstrate transmission of the transgenes to subsequent generations and high level expression of the common bean *αAI-1* in seeds of transgenic plants. The αAI-1 protein in transgenic seeds was active as an inhibitor against porcine α-amylase *in vitro*. The transgenic cowpea seeds were protected from *C. maculatus* and *C. chinensis* in insect bioassays.

6.1 MATERIALS AND METHODS

6.1.1 Construction of plant transformation vector carrying *α-AI-1* gene

The standard binary vector pCAMBIA2301 (CAMBIA, Australia) was used to sub clone α-amylase inhibitor 1 gene expression cassette between the T-DNA borders of the binary vector. The α-amylase inhibitor 1 cDNA (0.73 kb) derived from the bean cultivar tendergreen (Altabella and Chrispeels, 1990) (accession J01261), is flanked by 5'(PHA-L5', 0.53 kb) and 3'(PHA-L3', 3.6 kb) control regions of the bean phytohemagglutinin gene for seed-specific expression. The α-amylase inhibitor 1 expression cassette was kindly provided by Dr. T. J. V. Higgins (CSIRO Plant Industry, Australia) as 4.8 kb *Hin*dIII fragment in pTA3 (7.5 kb) plasmid.

The plasmids, pCAMBIA2301 and pTA3 were isolated and digested with *Hin*dIII restriction enzyme. Digested pTA3 sample was separated in 0.8% agarose gel and the resulting 4.8 kb *Hin*dIII fragment carrying the expression cassette was eluted using Quiaquick gel elution kit, and the *Hin*dIII digested pCAMBIA2301 vector was also purified using the Quiaquick gel elution kit. T_4 DNA ligase (MBI Fermentas) was used to ligate the α-AI1 gene expression cassette to linearized pCAMBIA2301 (Fig. 7). The T-DNA of pCAMBIA2301 includes neomycin phosphotransferase gene (*npt*II) and β-glucuronidase gene (*gus*) both driven by the cauliflower mosaic virus (CaMV) 35S promoter. The recombinant clones were confirmed by

restriction digestion. The resulting α-amylase inhibitor 1 over expression construct was designated as pSiva (Figure 8). Standard molecular biology techniques were used for all DNA manipulations (Sambrook et al. 1989).

6.1.2 *Agrobacterium* strain and mobilization

The construct pSiva was mobilized to *A. tumefaciens* strain LBA4404 (Hoekema et al. 1983), carrying pSB1 by triparental mating (Bevan, 1984) and their integrity within the *Agrobacterium* cells was confirmed through restriction digest analysis and colony PCR. The *A. tumefaciens* strain harboring pSiva was maintained on solid YEP medium (An et al. 1988) supplemented with 10 mg/l of rifampicin, and 50 mg/l of kanamycin, and additionally 5 mg/l tetracycline was used to select pSB1.

6.1.3 Plant material and explant preparation

The preparation of the 3 days old colyledonary node explants from cowpea cv. Pusa Komal, were described elsewhere in previous chapters.

6.1.4 Transformation, selection and plant regeneration

The optimized *Agrobacterium*-mediated genetic transformation protocol was used for the over-expression of the common bean α-amylase inhibitor 1 gene in cowpea. The innoculum preparation, *Agrobacterium* infection and cocultivation procedures were followed as described in chapter 3. However, the liquid cocultivation medium (LCM) used in the present study contained 1 mM dithiothreitol and 8.3 mM L-Cysteine along with the components described in the previous chapter (MSB medium containing 1 µM BAP, 1 mM DTT and 8.3 mM L-Cysteine, pH adjusted to 5.5).

Following cocultivation, the explants were washed three to four times with LCM and blotted dry on sterile filter paper. Selection was applied immediately after cocultivation, by culturing the explants on SISM, shoot induction and selection medium (MSB medium containing 5.0 µM BAP, 45 mg/l geneticin and 500 mg/l cefotaxime) for induction and selective regeneration of transformants. To allow faster penetration of geneticin throughout the explants and thus reduce competition from untransformed cells (Lulsdorf et al. 1991), 2-3 mm from hypocotyls end of all explants were excised immediately after the cocultivation period and the explants shifted to selection medium with the cut ends of the explants embedded in the medium. The cultures were transferred after one week to SIESM, shoot induction, elongation and selection medium (MSB medium containing 5.0 µM BAP, 0.5 µM kinetin, 45 mg/l geneticin and 500 mg/l cefotaxime) and cultured for 3 weeks for optimal induction, elongation and selective regeneration of transformants. Same levels of antibiotics were maintained during subsequent subcultures. Rooting and transplantation of the elongated green

shoots of 2-3 cm were performed as described in previous chapters. Putative transformed plants were grown to maturity in transgenic greenhouse containment and the T_0 seeds were collected and sown in soil to raise the T_1 plants. Seeds were collected from T_1 plants.

6.1.5 GUS assay

Histochemical GUS assays (Jefferson, 1987) were used to assess transient and stable expression of the *gus* gene. Transient *gus* expression in cotyledonary node explants was scored after 3-days of cocultivation. Stable *gus* expression in geneticin-resistant T_0 transgenic plants was detected by immersing the leaves, flowers, stamen, anthers, pollen grains and carpel in GUS substrate solution for 24 h at 37^0C. Following incubation, tissues were bleached with 100% ethanol, and examined under microscope. The T_1 seedlings and embryos were also assayed for GUS activity after overnight imbibitions of seeds in distilled water.

6.1.6 Molecular analysis of putative transformants

Total genomic DNA was extracted from fresh, young leaves (400-500 mg) of *in vitro* grown putative transformants (T_0) by the CTAB (cetyltrimethyl ammonium bromide) method (Rogers and Benedich, 1988) and the DNA estimation was done using UV spectrometer at 260/280 nm.

6.1.6.1 Polymerase chain reaction analysis

Putative transformants were screened by polymerase chain reaction (PCR) for the presence of *αAI-1* and *npt*II genes. The 0.54 kb coding region of *npt*II in putative T_0 transformants and their progenies were amplified by using 20-mer oligonucleotide prime combinations (forward 5′-CC ACCATGATATTCGGCAAC-3′ and reverse 5′-GTGGAGAGGCTATTCGGCTA-3′). The 0.57 kb of *αAI-1* coding regions in putative T_0 transformants and their progenies were amplified by using the following 21-mer primer combinations: *αAI-1* gene: forward (5′-TCATGGCTTCCAAGTTAC-3′ and reverse 5′-GGACGATGTTGGAACGTTCAG-3′). The amplification reaction was carried out using a thermal cycler (Perkin Elmer, USA) under the following conditions: initial denaturation at 94^0 C for 1 min and followed by 38 cycles of denaturation at 94^0 C for 1 min, annealing at 58^0 C for 1 min and extension at 72^0 C for 1 min, and final extension for 7 min at 72^0 C for detection of both *npt*II and *αAI-1* gene amplification.

The PCR amplifications were performed in 25 µl reaction mixture containing 1 U recombinant Taq DNA polymerase (Bangalore Genei, India) and 1 X Taq buffer (Bangalore Genei, India), 100 µM each dNTP, 100 pM of each primer and 100 ng of purified genomic DNA as template. Amplified DNA fragments were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining as per the standard protocol

(Sambrook et al. 1989). DNA from non-transformed (control) plants was used as a negative control while the pSiva vector was used as positive control.

6.1.6.2 Southern hybridization analysis

To determine the integration of the *αAI-1* gene in the transgenic lines, ten µg samples of genomic DNA from non-transformed control and progeny of four independent transgenic cowpea lines were digested with *Eco*RI enzyme, which cuts once within the T-DNA. The digested samples were fractioned on a 0.8% agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA) and fixed by UV crosslinking, as described in previous chapter. The blot was hybridized with 570-bp coding region of the *αAI-1* gene isolated as a PCR fragment using primers used for genomic PCR. The probe was labeled, column purified and Southern hybridization was performed using the non-radioactive DIG Labeling and Detection system (Roche, Germany) following supplier's instructions. DNA from non-transformed (control) plants was used as a negative control while the pSiva vector was used as positive control.

Prehybridization and hybridization were carried out at 65^0C using hybridization buffer containing 50% deionized formamide, 5X SSC, 50 mM sodium phosphate (pH 7.0), 2% blocking solution, 0.1% *N*-lauroylsarcosine and 7% SDS a hybridization oven. Post hybridization washing and detection were performed according to the instruction of the DIG labeling and Detection System (Roche Diagnostics, Mannheim, Germany).

6.1.6.3 Expression analysis of the *α-AI-1* gene in transgenic lines

Total RNA was isolated from the seeds of selected PCR-positive transgenic T_0 embryo lines and non-transformed control plants using Rneasy Plant Mini Kit (Qiagen, USA) following manufacturers instructions. RT-PCR was conducted using the First Strand cDNA Synthesis Kit (Fermentas, USA) to synthesize the first strand cDNA with 5 µg of total seed RNA, oligo(dT) primer, and the M-MuLV Reverse Transcriptase, according manufacturers' instructions. Subsequently, $1/10^{th}$ volume of the original cDNA was used for the amplification of the *αAI-1* gene using the same primer sets as those used in genomic PCR. These templates were used for the amplification of the *αAI-1* gene transcripts using the same primer sets as those used in genomic PCR.

6.1.7 Segregation analysis

The leaves of T_1 transgenic plants were analyzed for the presence of *αAI-1* gene using polymerase chain reaction, as described earlier. Segregation patterns were analyzed with the chi-square test ($\chi 2$) against the expected Mendelian ratio of 3:1 for single locus insertion.

6.1.8 Extraction of seed protein

Hundred mg of seeds from non transgenic and transgenic lines was ground to fine powder in a cold mortar pestle and the total seed protein was extracted into 2 ml of extraction buffer (10 mM tris-HCl pH 7.5, 10 mM ß-mercaptoethanol, 0.1 M NaCl, 0.2 mM PMSF). Extract was centrifuged at 15000 rpm for 10 min at 4^0 C, and the supernatant was used. Protein concentration was estimated by the method of Bradford (1976) using BSA as a standard.

6.1.9 α-amylase inhibitory activity

The α-Amylase activity was measured by a modification of the Bernfeld method (Bernfeld, 1955) as previously described by Morton et al. (2000). One µg of porcine pancreas α-amylase (Sigma A-6255) in 100 µl of succinate buffer (pH 5.6), (15 mM succinate, 20 mM $CaCl_2$, 0.5 M NaCl and 2 mg/ml BSA) was pre-incubated with a serial dilution (containing 2 to 10 µg) of total soluble protein from mature seeds of non transgenic or transgenic cowpea for 60 minutes at 37°C. Hundred µl of starch substrate solution [1% starch in phosphate buffer (pH 6.9), 40 mM potassium phosphate, 50 mM NaCl] was added and incubated at 37°C for further 20 minutes. The enzyme activity was monitored by adding 100 µl of dinitrosalicylic acid reagent (Bernfeld, 1955) to the reaction mix, and at the end of the reaction period the tubes were incubated in boiling water bath to develop the color. After 10 min of incubation, 600 µl of water was added to the samples to stop the reaction and the absorbance was measured at 530 nm.

Reactions were carried out in triplicate; means and standard deviation were determined. A range of maltose concentrations were used for constructing the standard curve. The α-amylase activity was measured by calculating the amount of maltose released (in µg) per minute. The initial rate of reaction was measured in samples containing protein from transformed and untransformed plants (*R*t and *R*ut, respectively). The percent inhibition was calculated [% inhibition =100(*R*ut - *R*t)/*R*ut] at each pH point.

6.1.10 Insect bioassays

Stock culture of *C. chinensis* was reared on green gram (*Vigna radiata* cv. K851) seeds at 28±1°C in glass jars covered with muslin cloth, and maintained on cowpea (cv. Pusa Komal) prior to bioassay. The stock culture of *C. maculatus* was reared and maintained on cowpea (cv. Pusa Komal) seeds. Both the stocks were maintained at 70% relative humidity.

For insect bioassays, before inoculation with the insects, seeds were conditioned at 28±1°C and 70% relative humidity for a period of one week. Ten seeds each from T_1 non-transgenic control cowpea plants and transgenic lines S-3 and S-4 were kept in glass vials covered with muslin cloth, and ten pairs each of newly emerged adults of *C. maculatus* and *C. chinensis*

from the stock cultures were introduced into glass vials. Vials were maintained at 28±1°C and 70% relative humidity.

Adults were removed one week after they laid eggs and the percentage of mortality was calculated. The seeds were maintained for a further five weeks and emerging F_1 adults were counted at regular intervals. The mean number of emerging adults per seed was determined for the non-transgenic control and the transgenic lines. The weights of the newly emerged adults were measured and the longevity of the adults was also recorded. All experiments were replicated three times.

6.2 RESULTS AND DISCUSSION

6.2.1 Preparation of the *αAI-1* gene construct

Restriction digestion of pSiva clearly indicated the ligation of *α*-amylase gene in pSiva. The plasmid pSiva isolated from *Agrobacterium* transconjugants, upon digestion with *Hind*III showed the presence of *α*-amylase inhibitor 1 gene cassette (4.8 kb) and 11.6 kb pCAMBIA2301 vector backbone (Fig. 7).

6.2.2 Production of transgenic cowpea plants carrying *αAI-1* gene

Following cocultivation with *Agrobacterium*, the regenerating explants were placed for selection on 45 mg geneticin, in SISM for 1 week followed by 3 weeks in SIESM. After the first 7 days explants have started to form de novo shoots in the cotyledonary-node region. In three different experiments, a total of 358 explants produced 35 shoots on geneticin selection medium (Table 8). After 4 weeks, the elongated shoots were transferred to rooting medium devoid of geneticin. All the rooted plantlets transferred to soil survived, grew to maturity and produced seeds in transgenic greenhouse containment (Fig. 9b; Table 8). The seeds were collected and sown in soil to raise the T_1 plants. The total time from inoculation of the explants to a plant in the greenhouse was approximately two months.

In the present study, the addition of L-cysteine and DTT to the liquid cocultivation medium (LCM) increased the T-DNA delivery and the production of transgenic shoots. In previous experiments, we found that explants treated without any thiol compounds exhibited increased T-DNA delivery but greater enzymatic browning on wounded surfaces, suggesting that multiple mechanisms may be involved in thiol-dependent increases in T-DNA transfer. Cocultivation of explants in the presence of mixtures of thiol compounds, DTT and L-cysteine increased the frequency of *Agrobacterium*-mediated transformation of regenerating cells of cotyledonary node explants, most likely by inhibiting the activity of plant pathogen- and wound-response enzymes, such as peroxidases (PODs) and polyphenol oxidases (PPOs) (Olhoft et al. 2001), making this approach useful in recovering transgenic plants at a high

frequency. Olhoft et al. (2001) have also reported the beneficial effect of thiol coumpounds during cocultivation in recovering transgenic plants in soybean.

Selection strategy has also played an important role in transformation efficiency and subsequent plant regeneration. The use of geneticin in conjuction with *npt*II gene based selection system allowed rapid and efficient identification of transgenic shoots, and eliminated the bulk of escapes without interfering with the regeneration capacity of the explants.

6.2.3 GUS assay

All the cotyledonary node explants showed GUS activity after cocultivation with *A. tumefaciens* LBA4404/pSB1 harboring pSiva, predominantly in the regenerable sites (Fig. 9a). Randomly selected transformed shoots derived from cotyledonary node were assayed for GUS activity. A strong, uniform and stable *gus* expression was observed in leaf, flower, stamens, anther, pollen grains, carpel, ovary, and ovules of all the geneticin resistant T_0 plants established in green house (Figs. 9c, d, e, f, g, h) whereas no endogenous *gus* expression was detected in the tissues of control plants. The *gus* expression in germinated T_1 seedlings clearly demonstrated inheritance and expression of the transgene in the progeny (Fig. 9i, j). We determined the stable transformation efficiency based on the percentage of T_0 plants that showed stable *gus* expression in T_1 seedlings. Detection of strong GUS activity in vegetative parts, flowers and germinated seedlings of transgenic plants clearly suggested the constitutive expression by CaMV35S promoter in cowpea.

6.2.4 Molecular analysis of transgenics

Molecular analysis of the transgenic plants was performed for detecting the presence, integration and expression of *αAI-1* gene. PCR analysis performed on the genomic DNA isolated form the transformants, detected the presence of the expected 540-bp and 570-bp fragments (Fig. 10a, b) in geneticin-resistant T_0 transgenic plants, that showed stable *gus* expression in flowers indicating the effective selection conferred by geneticin, and no amplification was detected in the control untransformed plants.

Progenies plants of 4 independent T_0 transgenic lines were screened by Southern analysis to confirm the integration of *αAI-1* gene in the genome of the transgenic plants (Fig. 10c.). Hybridizations of DIG-labeled *αAI-1* probe to total genomic DNA digested with *Eco*RI were expected to identify DNA fragments unique to individual integration events greater than 8.0 kb (Fig. 10c). Since there is a single restriction site of *Eco*RI in the T-DNA region of pSiva vector (Fig. 8), the presence of one hybridization band suggests a single transgene integration site while multiple bands suggest multiple integration loci.

All the four randomly selected T_1 transgenic lines were positive for *αAI-1* gene which showed hybridization bands of different sizes demonstrating differential integration and confirming that the plants were derived from independent transformation events (Fig. 10c, lanes 1, 2, 3 and 4) and that the transgene was stably integrated into the plant genome. The T_1 transgenic lines exhibited simple hybridization patterns with most of the transformants had single copy integration events in lines, 2, 3 and 4 (i.e derived from transgenic lines S1, S3, S4 respectively) to more than one hybridization band in line 1 (derived from transgenic line S6) which may indicate multiple transgene integrations and could reflect the approximate transgene copy number i.e two loci. In general, most fragments were greater than 8.0 kb in all the lines tested, except in line 2 where signal of size less than 8.0 kb was detected (Fig. 10c). This unexpected signal size could be due to the possibility of rearrangement of the T-DNA near the right border upon integration into the plant genome. No hybridization signal was detected in the untransformed plant used as a negative control (Fig. 10c, lane C), indicating the authentication of the results. The result was consistent with the PCR analysis of T_0 plants and stable *gus* expression analysis in their seedlings.

A total of 6 independently derived transgenic plants were obtained from a total of 358 explants cocultivated in the presence of mixtures of the thiol compounds followed by their culture on geneticin selection medium, giving an average transformation frequency of 1.67%. Transformation efficiencies obtained for thiol compunds treated cotyledonary node explants using phosphinothricin selection in cowpea were 0.001-0.003% (Popelka et al., 2006).

6.2.5 Expression of αAI-1 gene in transgenic plants

The RT-PCR analysis indicated that *αAI-1* gene was transcribed and accumulated in seeds of all the four transgenic lines tested (Fig. 10e). The amplification of a 570 bp DNA fragment derived from *αAI-1* gene mRNA is shown for transgenic lines (Fig. 10e). Because the *αAI-1* gene construct used in these experiments is regulated by flanking sequences from the seed-specific bean PHA (dlec2) gene, its expression would be restricted to the cotyledon and embryonic axis of the developing seed. No signal was obtained with cDNA from nontransgenic plants. These results were further verified with the functional expression of the *αAI-1* gene in the transgenic seeds.

6.2.6 Inheritance of αAI-1 gene in transgenic plants

Fertile transgenic plants were readily obtained after transferring the plants to the greenhouse. All T_0 plants produced viable T_1 seed after 6–9 weeks of growth in the greenhouse. Inheritance and segregation of the transgenes in the T_1 generation was investigated using a combined histochemical and molecular approach, allowing the estimation of the number of independent active loci in the T_0 lines. Four independent T_0 transgenic lines were chosen for

the analysis of the segregation of *αAI-1* gene in their T_1 progeny plants. PCR analysis confirmed the presence of 570 bp of coding region of *αAI-1* gene in tested progeny lines (representative gel of which is shown in Fig. 10d). The segregation ratios were derived from the *αAI-1* positive: *αAI-1* negative progeny plants, which showed the transgene inheritance in a Mendelian fashion (Table 9).

Chi-square analysis was performed on the segregation ratios obtained to determine goodness-of-fit for 3:1 and 15:1 ratios. A segregation ratio of 3:1 for *αAI-1* gene was observed in three lines suggesting a single functional locus, whereas one line showed a segregation pattern of 15:1 suggesting segregation of two independent loci (Table 9). Detection of strong GUS activity in germinated T_1 seedlings indicated the inheritance and stable expression of the *gus* gene in the progeny.

6.2.7 α-amylase inhibitory activity in transgenic seeds

We assayed the inhibitory activity of αAI-1 in T_0 transgenic seeds against porcine pancreas α-amylase enzyme *in vitro*. The inhibitory activity of seed extracts from the four transgenic cowpea lines against porcine pancreas α-amylase enzyme was compared. All the four lines showed % of inhibition in a range of 32.0-37.0. The % of inhibition was 37 in line-1, 36.6 in line-2, 31.9 in line-3 and 32.9 in line-4 (Fig. 11 a). No α-amylase inhibitory activity was detected in extracts from seeds of untransformed control cowpea. These results suggest the accumulation of α-amylase inhibitor protein in a seed specific manner by bean phytohemagglutinin promoter.

In all the lines tested, the maximum % of inhibition was observed with 8 μg of the total seed proteins. The % of inhibition was reduced with further increase in the seed protein concentration as opposed to the fact that α-amylase enzyme inhibition was directly proportional to the total seed protein. This indicated a saturation of enzyme inhibition was attended beyond 8 μg of the total seed proteins. All four transgenic lines tested showed similar level of α-amylase inhibition activity suggesting different transgene integration events had no adverse effect on transgene expression.

6.2.8 Insect bioassays

The bioassay was performed using *C. maculatus* and *C. chinensis* on T_1 seeds of transgenic lines S-3 and S-4 of cowpea. *C. maculatus* and *C. chinensis* reared on control seeds (untransformed) developed into adults (96% and 92 % respectively), and a mortality of only 4% and 8% was recorded respectively. There were clear reductions in the emergence of adult insects from the seeds of the both the transgenic lines compared with the untransformed control in all insect bioassays. Different transgenic lines showed the diversity of bruchid

resistance. The results of the bioassay were summarized in Table 10. These results showed the transgene was expressed in the transgenic cowpea and had high resistant to insect. The insects reared on transgenic seeds showed a high mortality rate which was found to be significantly higher than in the control (Table 3). The mean number of F_1 individuals that emerged and the weight of the newly emerged adults were also significantly reduced ($P < 0.05$) when the insects were reared on seeds of both transgenic lines (Table 3). In the case of *C. maculatus*, the mean number of adults emerged per seed was reduced from more than 8 on the untransformed to 1.3 and 0.9 on the transgenic lines 3 and 4 respectively (Table 3). In the *C. chinensis* bioassays, the mean number of emerged adults from untransformed seeds was 4.6, whilst adult emergence was negligible from the transgenic seeds. The adult longevity of the insects (both male and female) was also found to be significantly reduced in comparison to insects reared on untransformed seeds (Table 10; Fig. 11b). During its development in a nontransgenic seed, both the larva created large cavities in the cotyledons, causing a reduction of between 20 and 45% in the final seed weight and a concomitant loss in seed viability. In transgenic seeds, final weight loss ranged from 2 to 4.3 %, which is less than single seed weight variation.These results clearly demonstrate that the insect mortality and the reduced redundancy were the direct effects of inhibition of carbohydrate digestion of bruchid weevils by the expressed αAI-1 protein in the transgenic seeds. These results were in accordance with RT-PCR analysis demonstrating the functionality of the *αAI-1* transcripts.

In conclusion, we report successful recovery of transgenic cowpea plants expressing bean α-amylase inhibitor 1 in seeds of an elite variety Pusa Komal. The cultivar Pusa Komal was chosen for transformation as this variety is widely cultivated in India due to its high yield and short duration nature. The production of transgenic cowpea plants by *Agrobacterium*-mediated transformation of cotyledonary-node explants was significantly increased by using additional copies of *vir* genes, by cocultivating explants in the presence of mixtures of thiol compounds followed by selection on geneticin. The transgenic seeds strongly inhibited the development of two major storage pests of cowpea, *C. maculatus* and *C. chinensis*. To our knowledge, this is the first report of the successful recovery of transgenic plants expressing any candidate gene in cowpea.

The transgenic cowpea resistant to its storage pests would eliminate the need of post-harvest chemical pesticide applications. Pyramiding two insecticidal genes in cowpea that either target different sites in the insect or have different mode of inhibitory action would slow the rate of resistance development. Such a strategy would be effective by preventing rapid emergence of bruchid strains that are not affected by single inhibitor.

Chapter 7

CONCLUSIONS

7.1 SIGNIFICANCE AND SALIENT FEATURES OF THE PRESENT STUDY

Cowpea (*Vigna unguiculata* L. Walp) is an important grain legume widely grown in Africa, Latin America, Southeast Asia and southwestern regions of North America. Cowpea contributes immensely to human nutrition since the seeds and fresh peas are a rich source of protein, certain minerals and vitamins (Sahoo et al. 2003). Cowpea seeds are highly susceptible to storage pests, bruchid species causing massive damage to the stored seeds and seriously limiting its yield potential. Durable and adequate levels of resistance to the bruchids are lacking in the primary gene pool, but are available in distant wild species, which present barriers for gene transfer through conventional breeding techniques (Singh et al. 2000). In addition, limited genetic diversity in cowpea breeding programs is of special concern because cowpea appears to have lower inherent genetic diversity than other cultivated crops as a result of a hypothesized single domestication event (Fang et al. 2007). Consequently, the transfer of insect pest resistance genes by genetic engineering promises plant breeders in overcoming these constraints. However, transfer of candidate genes to cowpea for biotic stress resistance has not progressed primarily due to the absence of a robust genetic transformation system.

There are three reports published on generation of stable transgenic plants in cowpea, through *Agrobacterium*-mediated transformation (Muthukumar et al. 1996; Popelka et al. 2006; Chaudhury et al. 2007). However, the overall transformation efficiency in these protocols is much low, most likely due to (i) inefficient T-DNA delivery to the regenerating cells; (ii) inefficient selection of proliferating transgenic shoots; (iii) difficulty with regeneration of shoots from transformed cells under the growth regulator and tissue culture regime followed. A critical step in the development of robust *Agrobacterium tumefaciens*-mediated transformation system in recalcitrant grain legumes is the establishment of optimal conditions for efficient T-DNA delivery into target tissue and recovery of transgenic plants.

We employed an improved *Agrobacterium*-mediated cowpea transformation method (Solleti et al. 2008) for introduction of the bean (*Phaseolus vulgaris*) α-amylase inhibitor-1 (α*AI-1*) gene into a commercially important Indian cowpea cultivar, Pusa Komal and generated fertile transgenic plants. Expression of α*AI-1* gene under bean phytohemagglutinin promoter resulted in accumulation of αAI-1 in transgenic seeds. The transgenic protein was active as an inhibitor of porcine α-amylase *in vitro*. Transgenic cowpeas expressing αAI-1 strongly inhibited the development of *C. maculatus* and *C. chinensis* in insect bioassays (Solleti et al. 2008).

The salient features of the present study are summarized below:

- An efficient plant regeneration system was established for cowpea from the cotyledonary node explants.

- The preliminary studies on synergistic affect of the two cytokinins, BAP and kinetin on shoot proliferation showed that the time of exposure to the combination of these two cytokinins was critical for accelerated regeneration response from cotyledonary node explants of cowpea.
- Aminoglycoside antibiotics screening showed that geneticin at low concentration was highly efficient than kanamycin and paramomycin in distinguishing the cowpea transformants from that of non-transformants, and further, no interference of geneticin was observed on regeneration of transformed sectors.
- The choice of *Agrobacterium* strain, co-culture conditions, and constitutive expression of extra copies of *vir*G, *vir*C and *vir*B genes were found critical for efficient T-DNA delivery to cotyledonary node explants of recalcitrant cowpea.
- Transgenic cowpea seeds harboring bean α-amylase inhibitor 1 gene were successfully generated using our improved transformation protocol.
- The presence, integration and expression of the α-amylase inhibitor gene in the transgenic lines were confirmed by PCR, Southern hybridization and RT-PCR.
- The inhibitory activity of αAI-1 was assayed in T_0 transgenic seeds against porcine pancreas α-amylase enzyme confirming the functionality of the *αAI-1* transcripts.
- Transgenic cowpea expressing *αAI-1* gene in their seeds challenged against its storage pests *C. maculatus* and *C. chinensis* showed complete protection from these major storage pests infestation.

7.2 FUTURE PROSPECTS

Despite its economic and social importance in the developing world, cowpea remains to a large extent an underexploited crop. Among the major goals of cowpea breeding and improvement programs is the stacking of desirable agronomic traits, such as disease and pest resistance and resistance to abiotic stresses. Implementation of marker-assisted selection and breeding programs is severely limited by a paucity of trait-linked markers and a general lack of information on gene structure and organization. With a nuclear genome size estimated at ~620 Mb, the cowpea genome is an ideal target for reduced representation sequencing. Thus, relatively large genetic gains can likely be made with only modest investments in both applied plant breeding and molecular genetics. However, development of tractable gene transfer systems in cowpea, for its germplasm enrichment programme has been impeded as cowpea is not of major economic importance to the most technologically advanced countries in North America and Europe. This crop is mainly grown in tropical Africa, Asia, and Latin America

where technical expertise and infrastructure for biotechnology research are either lacking or poor.

Cowpea is susceptible to several insect pests and they bring about great losses in yield. The candidate genes which can be transferred to cowpea to achieve insect pest resistance include lectins, α-amylase inhibitors, chitinases, *Cry* genes from *Bacillus thuringiensis* and protease inhibitor genes (Dita et al. 2006). Cowpeas are also susceptible to diseases caused by bacteria, fungi, cowpea mosaic viruses and hence transfer of genes such as chitinase gene, stilbene synthase gene or antifungal protein genes for fungal resistance; coat protein gene, replicase gene or movement protein genes of viruses for viral resistance and T4 lysozyme gene for bacterial resistance may benefit the plant to combat damage by these biotic agents. Yields of edible cowpea seed are also severely reduced by infection of the roots by the parasitic angiosperm *Striga gesnerioides* and *Alectra volgetii* (Bashir and Haptom, 1996; Singh and Emechebe, 1997). The identification and cloning of resistance genes to these parasites would contribute significantly to the future improvement of cowpea germplasm. Several sources of resistance to nematodes were identified and the resistance in current cultivars is conferred by Rk gene locus (Fery and Dukes, 1980). This gene has been used extensively by breeders and it proven protective to many nematodes. But the emergence of root-knot nematode populations that can be damaging to cowpea carrying the Rk gene suggests new sources of resistance and broad-based resistance strategies are needed to ensure the continued effectiveness of resistance as a management tool.

Various abiotic stresses like drought, salinity, water-logging, mineral toxicities, temperature etc. also bring about a decline in yield (Singh et al. 2000) and transfer of genes involved in developing abiotic stress tolerance from other organisms to this tropical grain legume will improve its ability to withstand stress (Dita et al. 2006). Cowpea seed proteins are deficient in sulfur-containing amino acids and transfer of such genes from other plants such as sunflower or cereals will be beneficial in this respect. However, care should be taken to see that the introduced gene does not cause any allerginicity to humans and animals. RNAi technology can be fruitfully employed to improve the nutritional traits in cowpea for suppression of anti-nutritional chemicals/compounds. However, this technology is yet to be tried out in pulse crops. The effectiveness and durability of disease and pest resistance are likely to be greater in engineered transgenic plants in which multiple resistance genes are introduced (so-called “resistance gene pyramiding”). Such pyramiding is time consuming and often difficult to achieve through traditional breeding approaches due to interspecific barriers, but readily achievable through transgenic approaches.

Legume research, both fundamental and applied is undergoing a revolution in the field of genomics and functional genomics, ever since, legumes like *Medicago truncatula*

(www.noble.org, www.ncgr.org/research.mgi) (Bell et al. 2001; Tadege et al. 2005) and *Lotus japonicus* (Udvardi et al. 2005) were adopted internationally as model legumes. However, little attention has been paid to gene characterization and the development of resources in cowpea (Timko et al. 2007). Even though cowpea genome is one of the smallest among the legumes, it has received less attention for genome analysis (Paterson et al. 2006). Till date, approximately 1,000 cowpea ESTs have been deposited in public databases (NCBI dbEST: database of "Expressed Sequence Tags" (http://www.ncbi.nlm.nih.gov/dbEST/dbEST summary.html) and most of the genomic DNA sequence available relates to either rRNA coding and spacer regions or represents anonymous sequence exploited for RFLP mapping. Increasing our knowledge of the structure and composition of the cowpea genome will help in the interpretation of genome evolution and undoubtedly contribute substantially to efforts aimed at improvement of this crop.

Developing innovative biotechnologies for cowpea improvement requires not only an understanding of genome organization and complexity, but also of gene structure and function. In a large proportion of the sequences present in the databases for cowpea are sequences encoded by genes turned on in response to pathogen attack (e.g., acidic and basic chitinase, pathogenesis-related proteins, and various resistance gene analogs) or in response to abiotic stress such as drought and low temperature (e.g., dehydrin, acid phosphatases, and phospholipases, seed associated proteins and general metabolic enzymes. An approach would be the identification and cloning of these genes which eventually could be used to engineer cowpea to overcome these stresses. This is a much longer route to take but it might be worth the efforts because of the potential benefit. The identification and cloning of resistance genes would contribute significantly to the future improvement of cowpea germplasm.

Breeding insect resistant cowpeas would have a revolutionary impact on food availability and nutritional status in many regions. Genetic transformation of cowpea with entomocidal genes for insect resistance may show promise in protecting cowpea (Zaidi et al. 2005). The incorporation of gene encoding for α-amylase inhibitor in cowpea holds a great promise to protect the seeds from its storage pests. However, in order to be of practical use for the production of transgenic plants, α-amylase inhibitors should have appropriate specificity profiles. On the one hand, they should ideally be effective against the full range of potential predatory insects. However, they must not interfere with the action of endogenous α-amylases, which are of demonstrated importance, for example, in germination (Kadziola et al. 1998). They should also lack activity against the mammalian enzymes, although this is in general a lesser issue as cooking would denature any inhibitors before ingestion. In recent rat feeding experiments with transgenic peas containing αAI-1, it has been shown that there is no detrimental effect on weight gain, carbohydrate or nitrogen metabolism, or the growth of

internal organs when these peas were fed at 30% of the diet (Pusztai et al. 1999). The consumption of αAI-1 protein (marketed as a weight-loss aid in the United States in the 1980s) by humans (Carlson et al. 1983; Garrow et al. 1983) or rats (Savaiano et al. 1977) also has been shown to have no effect on their carbohydrate metabolism. Several factors probably contribute to the lack of inhibition in mammals i.e., the formation of the inhibitor–enzyme complex for this class of α-amylase inhibitors is pH-, time- and concentration-dependent (Franco et al. 2002). The inhibitor may be inactivated by gastric juices (Carlson et al. 1983), the pH optimum for inhibition is lower (pH 4.5-5.0) than the pH that prevails in the duodenum (pH 6-7) (Le Berre-Anton et al. 1997), and α-amylase is produced in vast excess in the human gut (Fogel et al. 1973). Moreover, the structural differences between the insect and mammalian α-amylases contribute to their specificity of interaction. Therefore, development of bruchid weevil-resistant cowpeas containing the bean α AI-1 protein should be safe for human consumption and animal feed.

Molecular markers should be used in cowpea breeding for a variety of purposes in the future. These purposes include primarily disease and pest resistance, but also introgression of exotic germplasm, tolerance to abiotic stresses, and improvement of quality traits such as nutritional quality. Development of PCR based markers helps in their wide spread use and focused efforts in cowpea genomics will have to be organized to develop SSR and SNP markers to exploit genomic resources both as a source of high-throughput markers and as tools of marker analysis and gene discovery.

Beyond disease and pest resistance, the ability to transform cowpea opens up the potential for manipulation of numerous other plant characteristics including seed protein composition and nutritional quality (Chopra et al. 1999) and abiotic stress tolerances (Vandemark, 1999). Each of these characteristics is being successfully manipulated in other crop species (Hilder and Boulter, 1999; Somerville and Somerville, 1999) where, well established protocols for transformation and regeneration already exist.

From our results, it is apparent that recalcitrance in cowpea is no longer a constraint for genetic transformation and trait improvement as evident by the stable expression and inheritance of the transgenes to the progeny. However, an improvement in existing cell and tissue culture systems to allow regeneration of stable transformed cowpea plants may further enhance the recovery of transgenic plants. With so many available advances and new breakthroughs in plant transformation technologies, it is precluded that cowpea's stubborn resistance to genetic engineering will soon be overcome. Thus, the application of biotechnological tools to cowpea improvement offers the promise of increased productivity by speeding the development of varieties that yield more, are more resistant to the above stresses, and are more economical and efficient to produce.

Chapter 8

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Figures

Fig. 1. (a-d) ***In vitro*** **multiple shoot proliferation and plant regeneration of** ***Vigna unguiculata*** **cv. Pusa-Komal.**

(a) Cotyledonary node excised from 3 days-old seedlings raised on MSB medium containing 10 µM BAP. Bar represents 5 mm.

(b) Direct multiple shoot regeneration from cotyledonary node explant on MSB medium containing 5 µM BAP for 1 week, followed by 3 weeks of culture on MSB + 5 µM BAP + 0.5 µM kinetin.

(c) Induction of roots from *in vitro* regenerated shoot cultured on MS basal medium supplemented with 2.5 µM IBA.

(d) Acclimatized plant in greenhouse.


a
b
c
d
e

Fig. 2. (a-e) Selectivity of non transformed 3-day old cotyledonary node explants to the aminoglycoside antibiotic geneticin.

Effect of various concentrations of geneticin [(a) 15 mg/l, (b) 30 mg/l, (c) 45 mg/l, (d) 60 mg/l, (e) 75 mg/l] on the growth and morphogenic potential of untransformed cowpea cotyledonary node cultures.

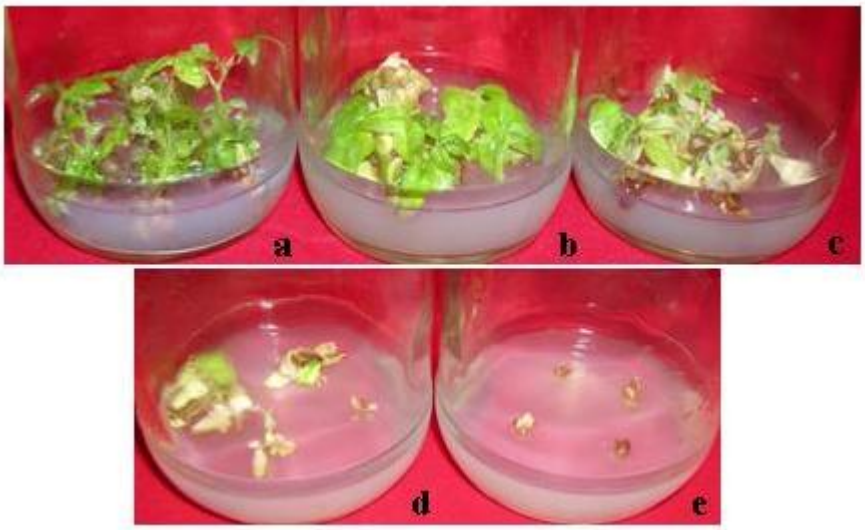


Fig. 3. T-DNA region (5.3 kb) of binary vector pCAMBIA2301.

Abbreviations: RB, right border; LB, left border; 35S, CaMV 35S promoter or terminator; NOS, nopaline synthase terminator; intron-*gus*, intron interrupted *β*-glucuronidase; *npt*II, neomycin phosphotransferase. Also highlighted are the positions of unique *BamHI* restriction site, and the distance between *BamHI* site and LB (2.1 kb).

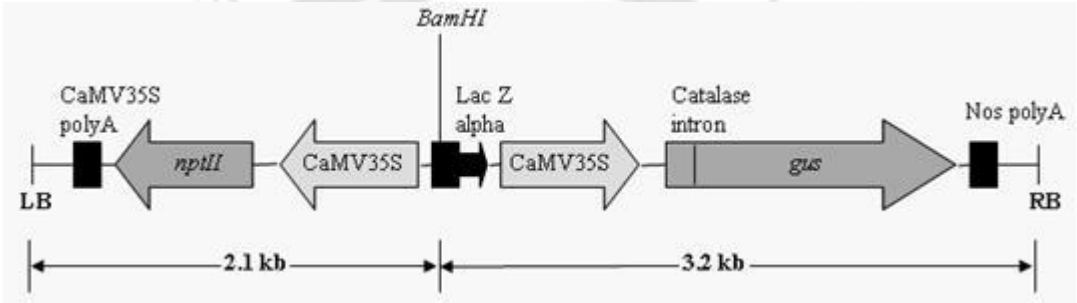
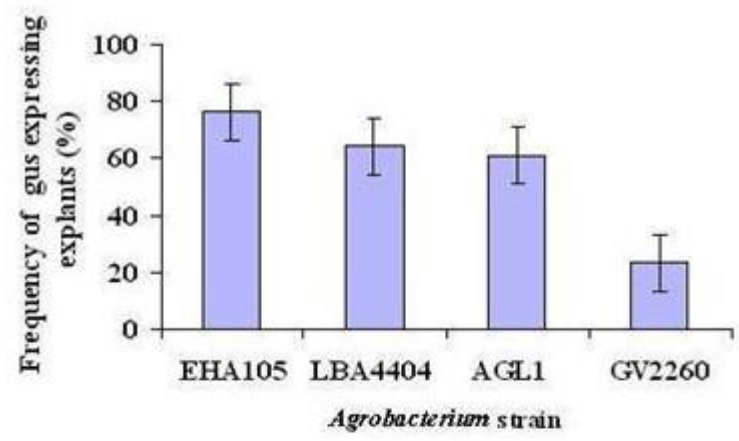

BamHI
CaMV35S
polyA
Lac Z
alpha
Catalase
intron
Nos polyA
nptII
CaMV35S
CaMV35S
gus
LB
RB
2.1 kb
3.2 kb

Fig. 4. (a-f) Optimization of various factors affecting the efficient T-DNA delivery to cotyledonary node explants of cowpea, 3-days after cocultivation through *Agrobacterium*-mediated transformation.

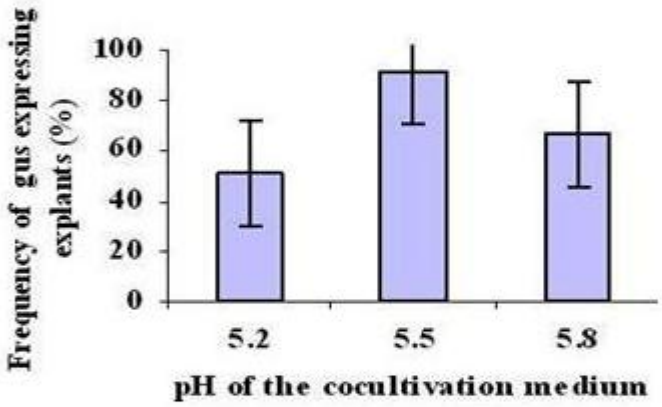
(a) Effects of *Agrobacterium* strains (EHA105, LBA4404, AGL1 and GV2260) on the transformation efficiency.

(b) Effects of pH (5.2–5.8) of the cocultivation medium.

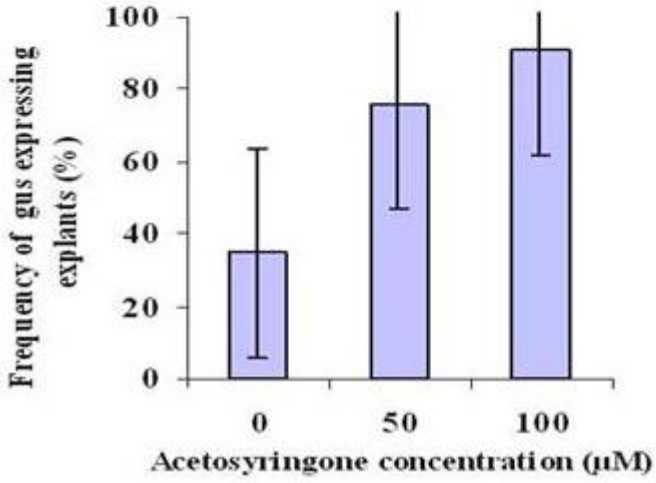
(c) Effects of the presence of various concentrations (0, 50, 100 µM) of acetosyringone during cocultivation.


Frequency of gus expressing explants (%)
100
80
60
40
20
0
EHA105
LBA4404
AGL1
GV2260
Agrobacterium strain

a


Frequency of gus expressing explants (%)
100
80
60
40
20
0
5.2
5.5
5.8
pH of the cocultivation medium

b


Frequency of gus expressing explants (%)
100
80
60
40
20
0
0
50
100
Acetosyringone concentration (µM)

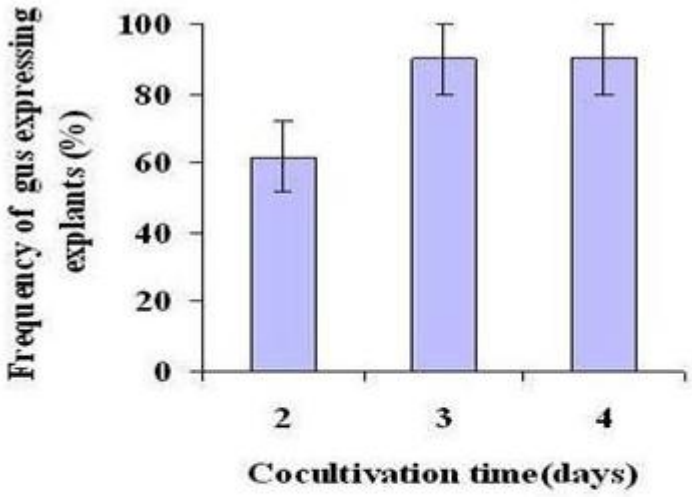
c

Fig. 4. (a-f) Optimization of various factors affecting the efficient T-DNA delivery to cotyledonary node explants of cowpea, 3-days after cocultivation through *Agrobacterium*-mediated transformation.

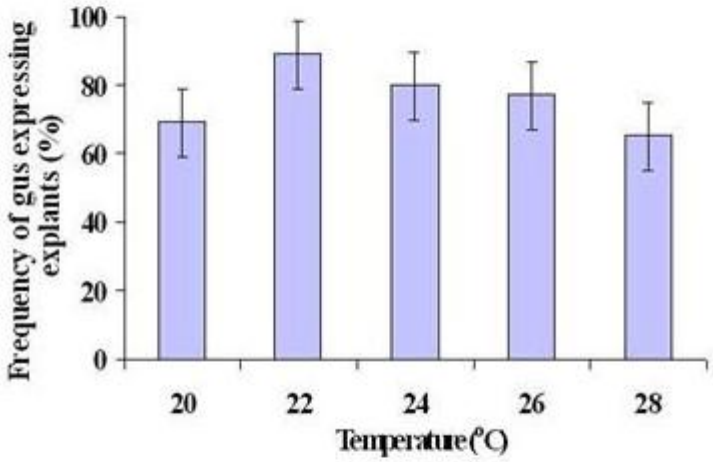
(d) Effect of cocultivation period (2-4 days) and

(e) Temperature (20, 22, 24, 26 and 28^0 C) during cocultivation.

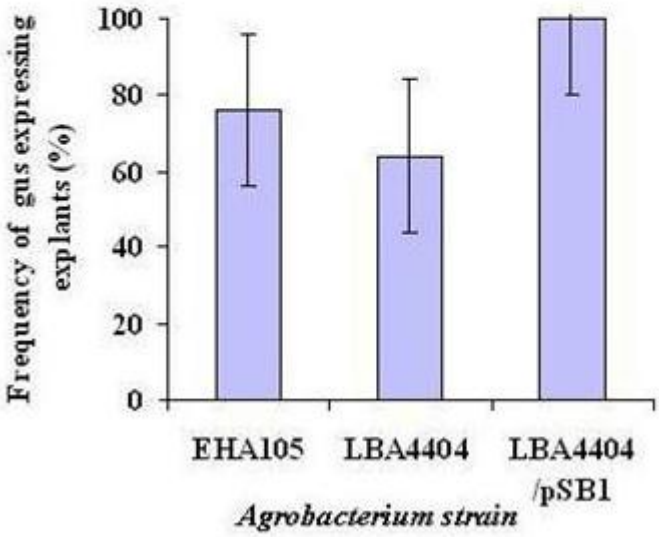
(f) Effect of additional *vir* genes in LBA4404/pSB1 on T-DNA delivery. Three replicates for each treatment with at least 30 explants per replicate were used. Vertical bars represent the standard error.


Frequency of gus expressing explants (%)
100
80
60
40
20
0
2
3
4
Cocultivation time(days)

d


Frequency of gus expressing explants (%)
100
80
60
40
20
0
20
22
24
26
28
Temperature(°C)

e


Frequency of gus expressing explants (%)
100
80
60
40
20
0
EHA105
LBA4404
LBA4404 /pSB1
Agrobacterium strain

f

Fig. 5. (a-i) Transient and stable GUS activity.

(a) Cotyledonary node explant showing transient GUS activity after 3 days of cocultivation with *A. tumefaciens* LBA4404/pSB1pCAMBIA2301.

(b-h) Stable GUS activity in geneticin-resistant T0 transgenic plants, (b) flower buds, (c) anthers, (d) anther, (e) pollens, (f) perianth, (g) ovules, and (h) stigma; (i) germinating cotyledon with attached embryo from non-transformed (bottom) and transformed (top) T1 plant.

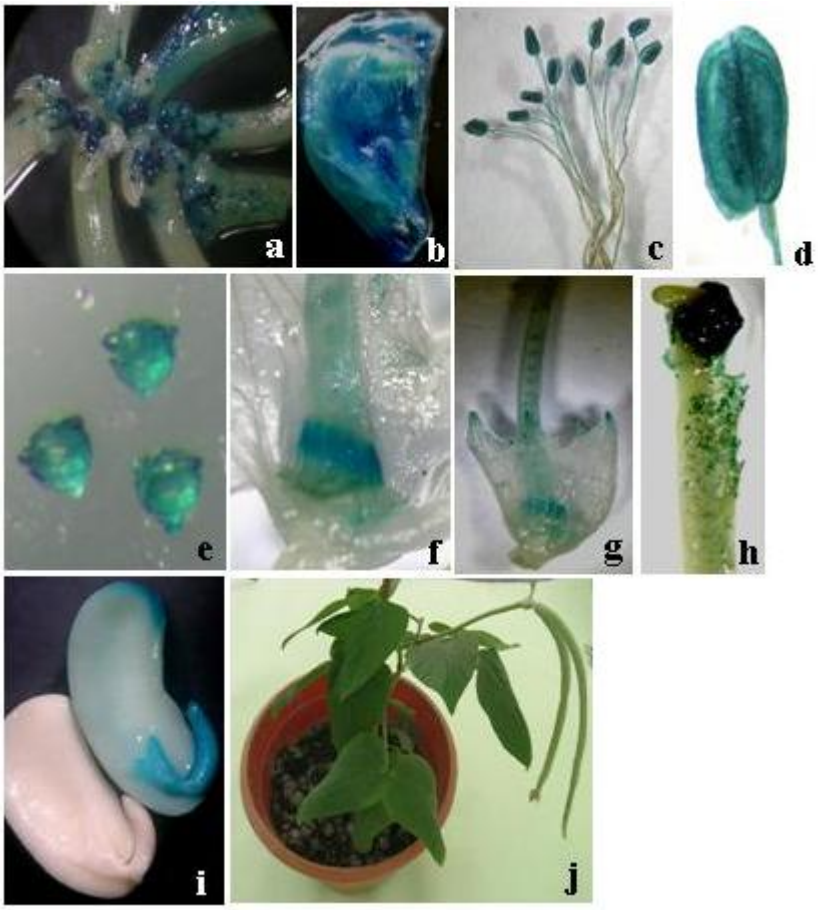


Fig. 6. (a-f) Molecular analysis of transgenic plants.

(a-d) Analysis of geneticin-resistant T0 transgenic plants.

(a) PCR amplification of the 540-bp fragment of the *npt*II gene, (b) PCR amplification of the 240-bp fragment of the *gus* gene. Lane M: Molecular weight marker; Lane P: pCAMBIA2301 plasmid DNA (positive control); Lane C: DNA from untransformed plant (negative control); Lanes 1–7: DNA from independently transformed plants.

(c) Southern blot hybridization analysis of junction fragments of four randomly selected PCR-positive T0 lines. The plasmid and genomic DNA were digested with *BamHI*, and hybridised with a *npt*II probe. Lane 1, 2, 4, 5 – genomic DNA from four T0 lines, lane C – genomic DNA from untransformed plant, lane P – plasmid DNA (pCAMBIA2301).

(d) RT-PCR analysis of the *npt*II and *gus* gene mRNA transcript levels in the T_0 transgenic plants. M, molecular weight marker; Lane C: untransformed plant (negative control); Lanes 1–6: T_0 transgenic plants.

(e) PCR amplification of the 540-bp fragment of the *npt*II gene of T_1 plants. Lane M: Molecular weight marker; Lane P: pCAMBIA2301 plasmid DNA (positive control); Lane C: DNA from untransformed plant (negative control); Lanes 1–7: DNA from T_1 transgenic plants.

(f) PCR amplification of the 240-bp fragment of the *gus* gene of T_1 plants. Lane M: Molecular weight marker; Lane P: pCAMBIA2301 plasmid DNA (positive control); Lane C: DNA from untransformed plant (negative control); Lanes 1–7: DNA from T_1 transgenic plants.

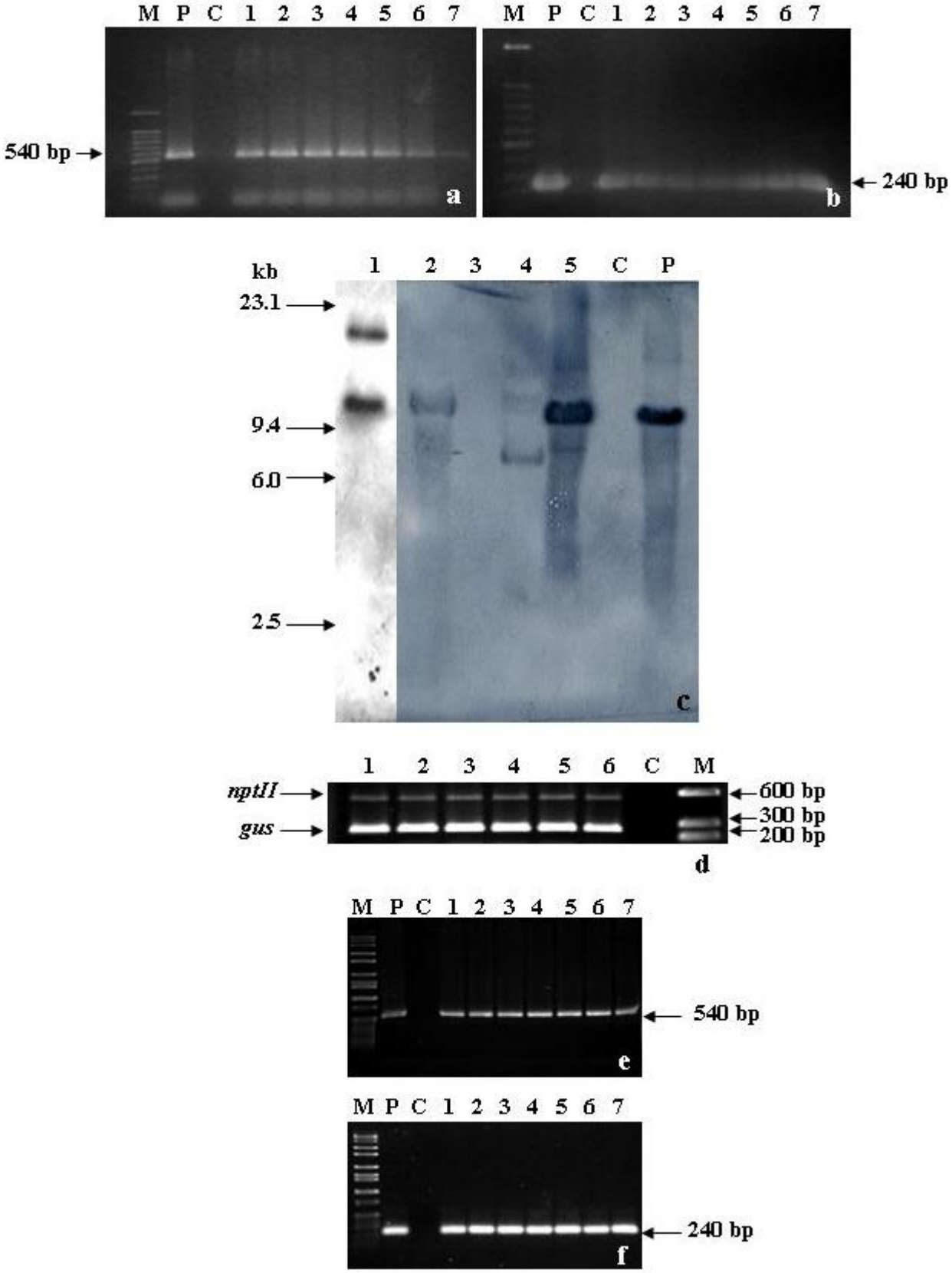

M P C 1 2 3 4 5 6 7
540 bp
a
M P C 1 2 3 4 5 6 7
240 bp
b
kb
1 2 3 4 5 C P
23.1
9.4
6.0
2.5
c
1 2 3 4 5 6 C M
nptII
gus
600 bp
300 bp
200 bp
d
M P C 1 2 3 4 5 6 7
540 bp
e
M P C 1 2 3 4 5 6 7
240 bp
f

Fig.7. (a-d) Construction of plant transformation vector carrying *α-AI-1* gene

(a) Digestion of plasmid, pTA(7.5 kb) with *Hin*dIII, resulted in release of a 4.8 kb α-amylase inhibitor 1 gene expression cassette from the vector backbone.

(b) Column eluted 4.8 kb α-amylase inhibitor 1 gene cassette.

(c) Digestion of pCAMBIA2301 (11.6 kb).

(d) The recombinant plasmid pSiva, upon digestion released the expected 4.8 kb *α-AI* cassette from the linear pCAMBIA2301 backbone.

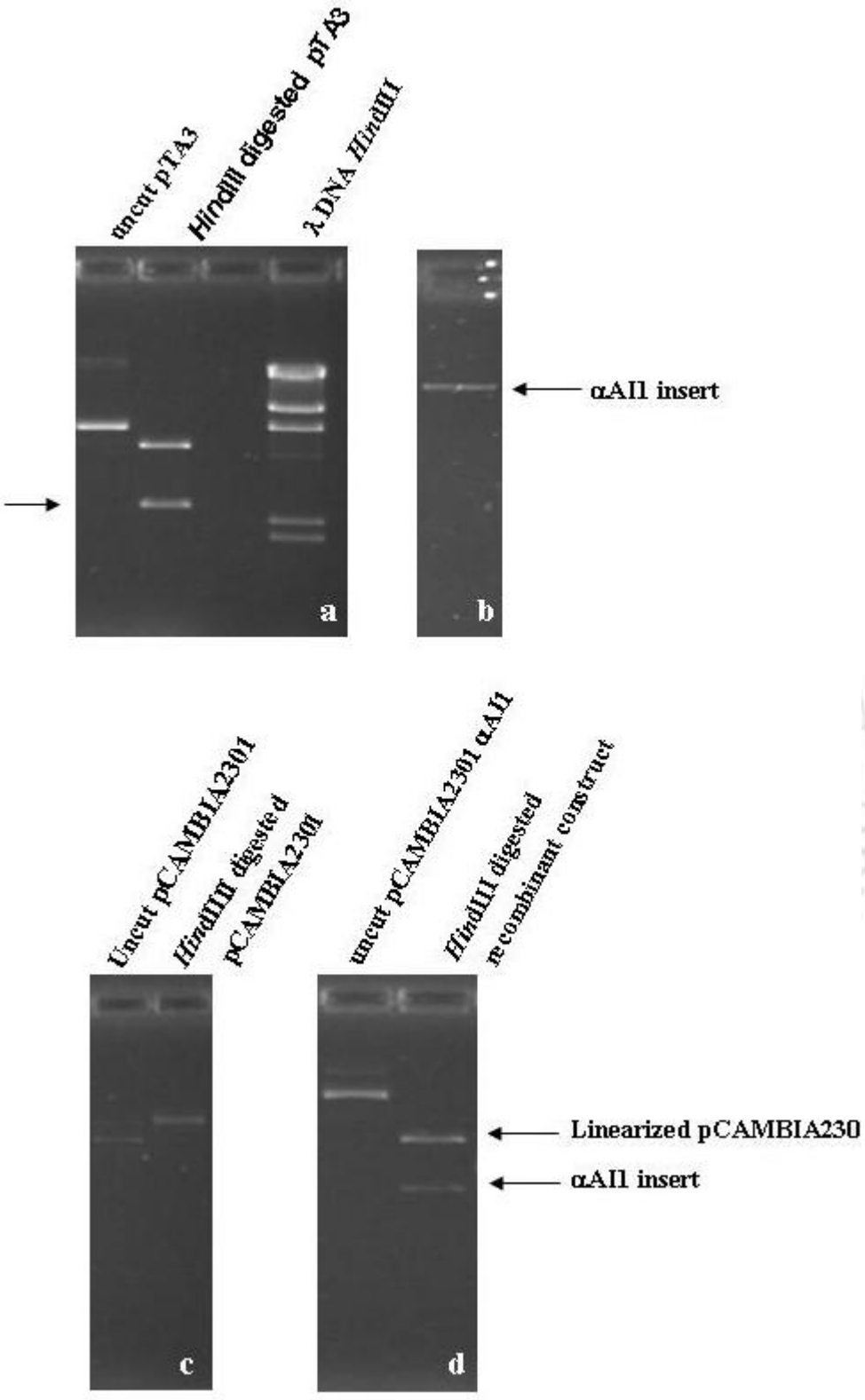

uncut pTA3
HindIII digested pTA3
λ DNA HindIII
αAI1 insert
a
b
Uncut pCAMBIA2301
HindIII digested pCAMBIA2301
uncut pCAMBIA2301 αAI1
HindIII digested recombinant construct
Linearized pCAMBIA230
αAI1 insert
c
d

Fig. 8. T-DNA region (10.1 kb) of pSiva.

Abbreviations: RB, right border; LB, left border; 35S, CaMV 35S promoter or terminator; NOS, nopaline synthase terminator; intron-*gus*, intron interrupted *β*-glucuronidase; *npt*II, neomycin phosphotransferase. PHA, phytohemagglutinin promoter; *αAI-1*, *α*-amylase inhibitor-1; PHA-3' UTR, phytohemagglutinin terminator. Also highlighted are the positions of unique *EcoR*I restriction site, and the distance between *EcoR*I site and LB (2.1 kb).

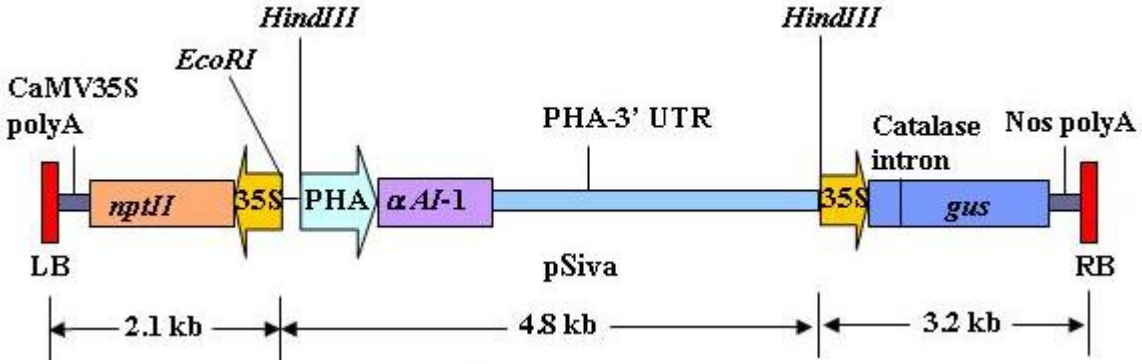


Fig. 9. (a–j) Transient and stable GUS activity.

(a) Cotyledonary node explants showing transient GUS activity after 3 days of cocultivation with *A. tumefaciens* LBA4404/pSB1pSiva.

(b) geneticin resistant T_0 transgenic plants established in green house.

(c-j) Stable GUS activity in geneticin-resistant T_0 transgenic plants, (c) leaf, (d) transformed flower (left) and non-transformed control flower (right), (e) stamens, (f) anther, (g) pollen grains, (h) carpel, perianth, ovary and ovules; (i) germinated T_1 transgenic seedlings (left) and non-transformed control T_1 seedlings (right); (j) germinating cotyledon with attached embryo from non-transformed control T_1 seedling (left) and transgenic T_1 seedling (right) .

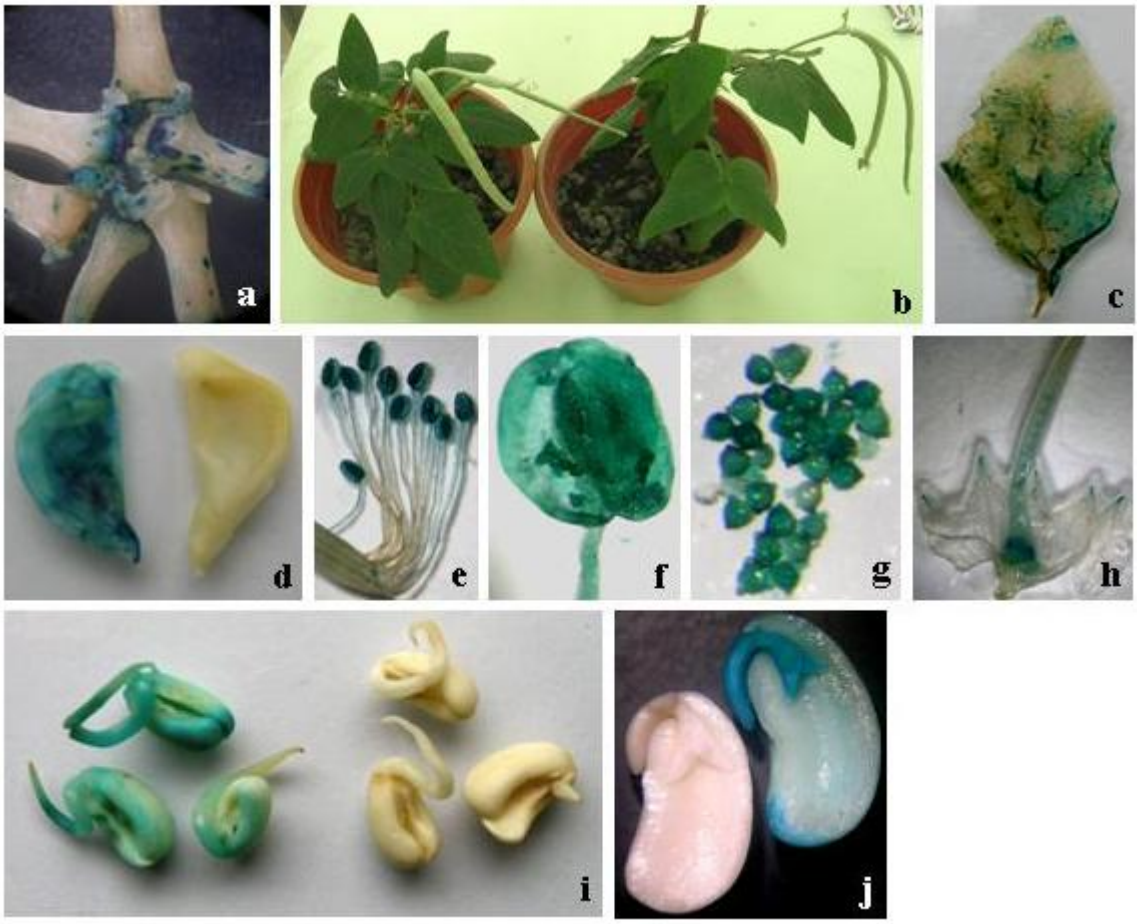


Fig. 10. (a-e) Molecular analysis of transgenic plants.

(a-c) Analysis of T_0 transgenic plants.

(a) PCR amplification of the 540-bp fragment of the *npt*II gene, (b) PCR amplification of the 570-bp fragment of the *αAI-1* gene. Lane M: Molecular weight marker; Lane P: pSiva plasmid DNA (positive control); Lane C: DNA from untransformed plant (negative control); Lanes 1–7: DNA from independently transformed plants.

(c) Southern blot hybridization analysis of junction fragments of four randomly selected PCR-positive T_0 lines. The plasmid and genomic DNA were digested with *Eco*RI, and hybridised with a *αAI-1* probe. Lane 1, 2, 3, 4 – genomic DNA from four T_0 lines, lane C – genomic DNA from untransformed plant, lane P – plasmid DNA (pSiva).

(d) PCR amplification of the 570-bp fragment of the *αAI-1* gene of T_1 plants. Lane M: Molecular weight marker; Lane P: pSiva plasmid DNA (positive control); Lane C: DNA from untransformed plant (negative control); Lanes 1–8: DNA from T_1 transgenic plants.

(e) RT-PCR analysis of the *αAI*-1 gene mRNA transcript level in the T_0 transgenic plants. M, molecular weight marker; Lane C: untransformed plant (negative control); Lanes 1–4: T_0 transgenic plants.

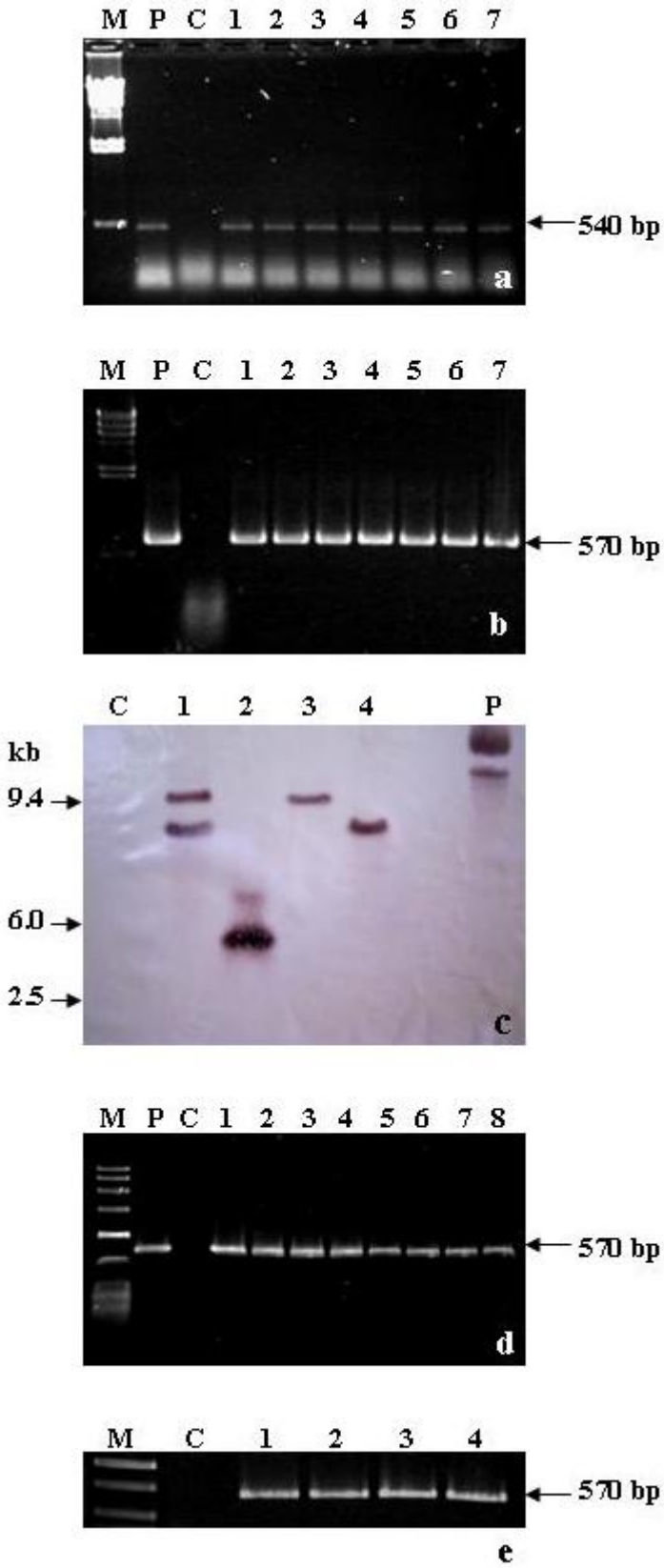
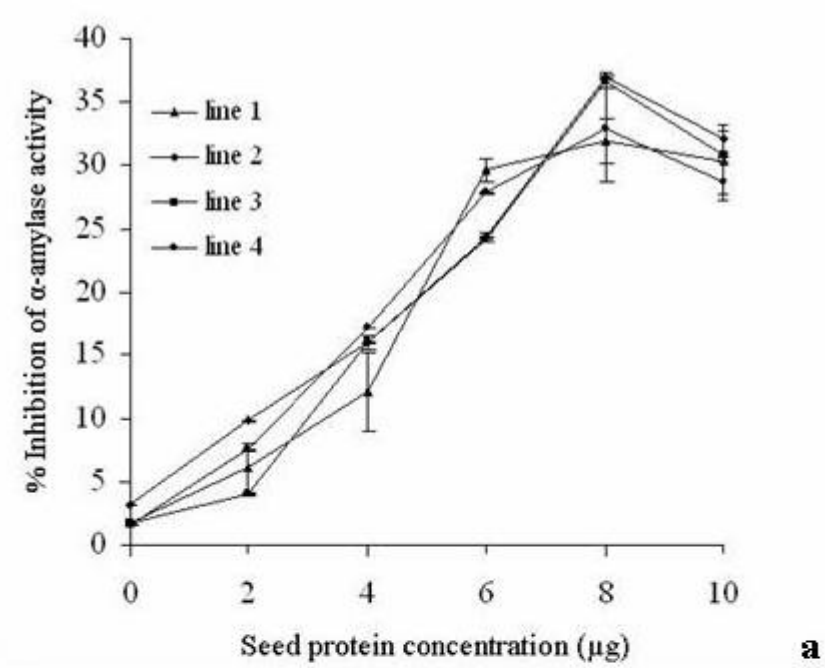

M P C 1 2 3 4 5 6 7
540 bp
a
M P C 1 2 3 4 5 6 7
570 bp
b
C 1 2 3 4 P
kb
9.4
6.0
2.5
c
M P C 1 2 3 4 5 6 7 8
570 bp
d
M C 1 2 3 4
570 bp
e

Fig. 11. Inhibitory activity of seed extracts from transgenic cowpea against porcine pancreatic α-amylase *in vitro*.

Seed protein extracts from transgenic cowpea lines 1, 2, 3 and 4 were incubated with porcine α–amylase as described in Materials and methods. The amount of reducing sugars released from the starch substrate (in maltose equivalents) was measured at A 530. The values shown are the means of three assays and the error bars reflect standard deviation. (a) Inhibition (%) against porcine α–amylase. Error bar represents the SD±values of mean of triplicate data.
(b) **Insect bioassay.** Resistance of transgenic cowpea seeds to cowpea weevil, *C. maculatus*. Transgenic cowpea seeds (left), control cowpea seeds (cv. Pusa Komal) (right).




भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

Tables

Table 1. Current status of genetic transformation of *Vigna* species.

Species & Cultivars	Explant and mode of regeneration	DNA Delivery Method	Vector/ Construct	Selection	Frequency of transformation	Transgenic plants and progeny analysis	Reference
Vigna radiata (B1 and T44)	Cotyledon, Shoot organogenesis	At LBA4404 GV2260	pBI121 (*uidA* & *npt*II) pGV2260	Kanamycin (300 mg/l)	Not reported	T_0 plants, Confirmed by dot blot assay	Pal *et al*., 1991
Vigna radiata (ML-5 and K-851)	Shoot regeneration from mature germinating embryos	PB	pBI221 (*gus* and *npt* II)	Kanamycin	Not reported	Putative transformed shoots rooted on kanamycin, No molecular analysis	Bhargava & Smigocki, 1994
Vigna radiata	Callus induction from primary leaf explants	At LBA4404	pKIWI105 (*uidA* & *npt*II)	Kanamycin	Not reported	Transgenic calli, Confirmed by Southern hybridization	Phogat *et. al.*, 1996
Vigna radiata (K-851)	Induction of roots and callus from hypocotyl and primary leaves	Ar LBA9402	----	Kanamycin	80-100%	Transgenic roots and calli	Jaiwal *et al*., 1998
Vigna radiata (K-851)	Shoot regeneration from cotyledonary nodes Callus regeneration from cotyledon and hypocotyl	At LBA4404 C58C1 EHA105 At EHA105	pTOK233 pIG121Hm pBin9Gus Int pBin9Gus Int	Kanamycin Kanamycin	0.9% 50%	T_0 plants confirmed by Southern analysis Transgenic calli confirmed by Southern analysis, failed to regenerate to shoots	Jaiwal *et al*., 2001
Vigna radiata (K-851)	Adventitious regeneration of shoots from primary leaves	At C58	pCAMBIA1301	Hygromycin	2% (mean)	Stable integration of the marker gene in the T0 transgenics and its inheritance in the T1 transgenics confirmed through molecular analysis	Mahalakshmi *et al*., 2006
Vigna radiata (Pusa 105)	Shoot regeneration from cotyledonary nodes	At EHA105	pKSB (*bar* and *αai 1*)	Phosphinothricin	1.51%	Integration *α*-ai 1 gene confirmed by Southern analysis. PCR analysis revealed inheritance of both the transgenes	Sonia *et al*., 2007

Species & Cultivars	Explant and mode of regeneration	DNA Delivery Method	Vector/Construct	Selection	Frequency of transformation	Transgenic plants and progeny analysis	Reference
V. angularis (cv. Beni-dainagon)	Adventitious shoot regeneration from epicotyl	At EHA105	pBI221 (*αAI-Pa1* or *αAI-Pa2*)	Kanamycin	Not reported	Genomic Southern analysis confirmed stable integration of transgene, seed extracts of azuki bean transgenic lines for ai-Pa1 or ai-Pa2 showed the same specificities as purified native aAI-Pa1 and aAI-Pa2, respectively,	Yamada et al. (2005)
V. mungo (cvs. T-9 & RV-19)	Shoot regeneration from mature germinating embryos	PB	pBI221 (*gus* and *npt* II)	Kanamycin	Not reported	Putative transformed shoots rooted on kanamycin, No molecular analysis	Bhargava & Smigocki, 1994
V. mungo (cvs. T-9 & RV-19)	Induction of callus from segments of primary leaves	At LBA4404 EHA105	pGA472 (*npt*II)	Kanamycin	23 % (LBA4404) 10% (EHA105)	Integration and expression of npt II in transformed calli confirmed by Southern analysis and neomycin phosphotransferase assay respectively	Karthikeyan et. al., 1996
V.mungo Pusa-2	Shoot regeneration from cotyledonary nodes	At LBA4404	pCAMBIA2301 (*npt*II & *gus*)	Kanamycin	1%	Stable integration & expression of transgenes in T_0, Inheritance to T_1 progeny demonstrated	Saini et al., 2003
V.mungo Pusa-2	Shoot regeneration from shoot apices	At EHA105	pCAMBIA2301 (*npt*II & *gus*)	Kanamycin	1-6.5 %	Stable integration & expression of transgenes in T_0, Inheritance to T_1 progeny demonstrated	Saini et al., 2005
V.mungo Pusa-2	Shoot regeneration from cotyledonary nodes	At EHA105	pCAMBIA2301 (*npt*II & *gus*)	Kanamycin	4.31%	Stable integration & expression of transgenes in T_0, Inheritance to T_1 progeny demonstrated	Saini et al., 2007
V.mungo LBG-20	Embryonal axis	At	pCAMBIA2301 (*npt*II and *gly* I)	Kanamycin	2.25%	Stable integration & expression (western blot) of *gly* I in T_1 is demonstrated. The T_1 transgenic lines were showing salt stress alleviation	
*V. angular*is (cv. Beni-dainagon)	Adventitious shoot regeneration from epicotyl	At LBA4404	pBinl9 (*npt*II, *gus* & *αai I*)	Kanamycin	Not reported	Presence of αAI in seeds of T_0 and T_1 plants confirmed by immunoblotting Transgenic seeds showed resistance to bruchids	Ishimoto et al., 1996

Species & Cultivars	Explant and mode of regeneration	DNA Delivery Method	Vector/Construct	Selection	Frequency of transformation	Transgenic plants and progeny analysis	Reference
V. angularis (cv. Beni-dainagon)	Adventitious shoot regeneration from epicotyl	At EHA105	pIG121 (*gus* &*npt*II) pSG65T (*gfp*, *npt*II & *gus*)	Kanamycin	2.0%	Transgenic plants Molecular analysis confirmed the stable integration & expression of foreign genes, GFP expression detected in progenies	Yamada et al., 2001
V. angularis (cv. Beni-dainagon)	Adventitious shoot regeneration from epicotyl	At EHA105	pSPB559 (*npt*II & Δ6-fatty-acid desaturase gene)	Kanamycin	Not reported	Genomic Southern analysis confirmed stable integration of transgene, RT-PCR indicated the transcription of D6-fatty-acid desaturase gene in transgenic lines, Fatty-acid compositions of the whole lipids extracted from the leaves and seeds of the T_0 transgenic plants & the leaves of their T_1 progenies were identified using GC-MS	Chen et al., 2005

Table 2. Effect of different cytokinins on shoot proliferation from cotyledonary node explants of *Vigna unguiculata* following culture on MSB medium for 4 weeks.

Cytokinin (µM)	% Regeneration	Mean number of shoots per explant	Mean shoot length (cm)
BAP			
1.0	98[a]	2.4[c]	1.6[c]
2.5	97[a]	3.7[b]	1.4[c]
5.0	94[a]	6.9[a]	1.1[d]
7.5	87[ab]	4.1[b]	0.8[d]
Kinetin			
1.0	93[a]	1.8[d]	3.1[a]
2.5	87[ab]	2.3[c]	2.4[b]
5.0	85[b]	2.9[c]	1.8[c]
7.5	84[b]	1.7[d]	1.3[cd]
TDZ			
1.0	74[c]	0.9[e]	0.8[d]
2.5	61[d]	0.8[e]	0.5[de]
5.0	45[e]	0.6[e]	0.3[e]
7.5	36[f]	0.4[e]	0.3[e]

Mean followed by different alphabets in the same column differ significantly ($P<0.05$) by Newman-Keul's multiple range test.

Table 3. Effect of gibberellic acid and kinetin on elongation of shoots from cotyledonary node cultures of *Vigna unguiculata* on MSB medium.

Cytokinin (µM)	Shoot elongation response (%)	Mean shoot length (cm)	Fold increase in shoot length
0	15^{f}	1.3^{c}	0.18^{e}
GA_3 (µM)			
0.1	28^{e}	1.6^{c}	0.45^{d}
0.5	36^{de}	2.1^{bc}	0.90^{c}
1.0	49^{d}	2.7^{b}	1.45^{b}
Kinetin			
0.1	64^{c}	2.4^{b}	1.18^{bc}
0.5	86^{a}	3.8^{a}	2.45^{a}
1.0	71^{b}	2.2^{b}	1.00^{c}

Mean followed by different alphabets in the same column differ significantly ($P<0.05$) by Newman-Keul's multiple range test.

Table 4. Effect of kinetin[a] on shoot proliferation and elongation from cotyledonary node explants of *Vigna unguiculata* cultured on MSB medium containing 5.0 µM BAP.

Kinetin (µM)	Interval of kinetin supplementation					
	0 week		1 week		2 weeks	
	Mean number of shoots/ explant	Mean shoot length (cm)	Mean number of shoots/ explant	Mean shoot length (cm)	Mean number of shoots/ explant	Mean shoot length (cm)
0.1	7.1^{bc}	1.9^{b}	7.5^{b}	2.1^{b}	7.0^{c}	1.7^{bc}
0.5	7.8^{b}	2.2^{b}	9.1^{a}	3.5^{a}	7.4^{b}	1.9^{b}
1.0	7.3^{b}	1.7^{bc}	7.8^{b}	1.9^{b}	7.1^{bc}	1.4^{c}

[a]Kinetin was supplemented to MSB medium containing 5.0 µM BAP after varying interval of culture.

Mean followed by different alphabets in the same column differ significantly ($P<0.05$) by Newman-Keul's multiple range test.

Table 5. Effect of different aminoglycoside antibiotics on regeneration of 3-days old cotyledonary node explants[a].

Antibiotic	Dosage (mg/l)	Regeneration (%)	Bleaching (%)	Necrosis (%)
Kanamycin	50	100[a]	0[e]	0[e]
	75	100[a]	0[e]	0[e]
	100	81[b]	0[e]	16[d]
	125	64[c]	18[cd]	29[c]
	150	47[d]	26[c]	68[a]
Geneticin	15	100[a]	11[d]	0[e]
	30	92[a]	64[b]	0[e]
	45	86[b]	96[a]	0[e]
	60	34[e]	98[a]	0[e]
	75	12[f]	98[a]	0[e]
Paromomycin	25	100[a]	0[e]	0[e]
	50	100[a]	0[e]	13[d]
	75	89[ab]	13[d]	31[c]
	100	62[c]	22[c]	48[b]
	125	49[d]	29[c]	61[a]

[a]Explants cultured on MSB medium supplemented with 5.0 μM BAP for one week followed by 3 weeks of culture on MSB medium supplemented with 5.0 μM BAP and 0.5 μM kinetin

Table 6. Summary of the transformation of 3-d old cotyledonary node explants of *Vigna unguiculata* cv. Pusa-Komal cocultivated with *Agrobacterium tumefaciens* strain LBA4404/pSB1 harbouring a binary vector pCAMBIA2301.

Exp. no.	No. of explants inoculated in *Agrobacterium* Suspension	No. of shoots recovered on selection medium[a]	No. of plants rooted[b]	No. of plants positive for *gus* and *npt*II genes by PCR	Selection efficiency[c] (%)	No. of plants positive for *npt*II by Southern hybridization	transformation efficiency[d] (%)
1	124	11	08	5	45	2	1.61
2	178	16	10	7	43	3	1.68
3	184	19	11	6	31	3	1.63
Total	486	46	29	18	39.6[e]	8	1.64[e]

[a]Explants cultured on selection medium for shoot regeneration: MSB+BAP (5.0 μM)+geneticin (45 mg/l)+cefotaxime (500 mg/l) for one week followed by 3 weeks of culture on MSB+BAP (5.0 μM)+kinetin (0.5 μM)+geneticin (45 mg/l)+cefotaxime (500 mg/l)

[b]Shoots rooted on MS+IBA (2.5 μM)+ cefotaxime (500 mg/l)

[c]Selection efficiency: Percentage of geneticin-resistant plants positive for *gus* and *npt*II genes by PCR

[d]Transformation efficiency: Percentage of initial explants that developed to plants positive for *npt*II by Southern hybridization

[e]Average

Table 7. Segregation of *npt*II and *gus* genes in T1 transgenic plants of *Vigna unguiculata* cv. Pusa-Komal.

T1 Transgenic line	No. of plants tested	+ve (*npt*II)	- ve (*npt*II)	Ratio	χ^2 value	+ve (*gus*)	- ve (*gus*)	Ratio	χ^2 value
J1	39	28	11	3:1	0.39	28	11	3:1	0.39
J3	43	30	13	3:1	0.43	30	13	3:1	0.43
J6	36	26	10	3:1	0.26	26	10	3:1	0.26
J7	41	37	4	15:1	0.35	37	4	15:1	0.35

Table 8. Summary of the transformation of 3-d old cotyledonary node explants of *Vigna unguiculata* cv. Pusa-Komal cocultivated with *Agrobacterium tumefaciens* strain LBA4404/pSB1 harbouring a binary vector pSiva.

Exp. no.	No. of explants inoculated in *Agrobacterium* suspension[a]	No. of plants rooted[b]	No. of plants positive for *αAI1* by Southern hybridization	Transformation efficiency[c] (%)
1	119	12	2	1.68
2	121	15	2	1.65
3	118	08	2	1.69
Total	358	35	6	1.67[d]

[a]Explants cultured on selection medium for shoot regeneration: MSB+BAP (5.0 μM) + geneticin (45 mg/l) + cefotaxime (500 mg/l) for one week followed by 3 weeks of culture on MSB+BAP (5.0 μM) + kinetin (0.5 μM) + geneticin (45 mg/l) + cefotaxime (500 mg/l)

[b]Shoots rooted on MS + IBA (2.5 μM)+ cefotaxime (500 mg/l)

[c]Transformation efficiency: Percentage of initial explants that developed to plants, positive for *αAI1* by Southern hybridization

[d]Average

Table 9. Segregation of *αAI1* transgene in T_1 plants of *Vigna unguiculata* cv. Pusa-Komal.

T_0 line	No. of plants tested	+ve (*αAI1*)	- ve (*αAI1*)	Ratio	χ^2 value
S1	38	27	11	3:1	0.34
S3	41	29	12	3:1	0.39
S4	34	24	10	3:1	0.26
S6	43	39	4	15:1	0.36

Table 10. *C. maculatus* and *C. chinensis* development in T_1 seeds of control and two transgenic cowpea lines.

Callosobrochus species	Control and transgenic cowpea lines	Mortality (%)	Mean no. of adult emergence	Mean F_1 adult insect weight (mg)	Adult longevity (days)
C. maculatus	Control	6.0±1.1[a]	8.0±1.1[d]	3.6±0.4[b]	128±8.9[d]
	S-3	71.2±2.8[bc]	1.3±0.3[b]	2.1±0.2[a]	32±5.2[b]
	S-4	64.6±3.1[b]	0.9±0.2[ab]	1.8±0.1[a]	28±3.7[b]
C. chinensis	Control	8.0±1.4[a]	4.6±1.6[c]	3.2±0.4[b]	109±7.5[c]
	S-3	82.3±2.3[c]	0.2±0.0[a]	1.9±0.3[a]	26±3.9[b]
	S-4	79.5±2.9[c]	0.3±0.0[a]	1.7±0.1[a]	19±3.1[a]

Numbers with same alphabet do not differ significantly

LIST OF PUBLICATIONS

1. In Refereed journals

1. **Solleti SK**, Bakshi S, and Sahoo L (2007). Additional virulence genes in conjunction with efficient selection scheme, and compatible culture regime enhance recovery of stable transgenic plants of cowpea via *Agrobacterium tumefaciens*-mediated transformation. J.Biotechnol. 135: 97-104
2. **Solleti SK**, Bakshi S, Purkayastha J, Panda SK and Sahoo L (2007). Transgenic cowpea (*Vigna unguiculata*) seeds expressing a bean α-amylase inhibitor 1 confer resistance to storage pests, bruchid beetles. Plant Cell Rep. (accepted)
3. Purkayastha J, Sugla T, Paul A, **Solleti SK** and Sahoo L (2008) Rapid and efficient *in vitro* plant regeneration from shoot apices – a method suitable for gene transfer by particle bombardment in *Jatropha curcas* L. Biol. Plant. (accepted)
4. Purkayastha J, Sugla T, Paul A, **Solleti SK** and Sahoo L (2008) Rapid *in vitro* multiplication and plant regeneration from nodal explants of *Andrographis paniculata*: A valuable medicinal plant. *In Vitro* Cell. Dev. Biol. Plant (accepted)
5. Sugla T, Purkayastha J, Singh SK, **Solleti SK** and Sahoo L (2007) Micropropgation of *Pongamia pinnata*, through enhanced axillary branching. *In Vitro* Cell. Dev. Biol. Plants 43(5): 409-414
6. Sahoo L, Mishra S, Purkayastha J, **Solleti SK** and Sugla T (2007) Genetic engineering of rice: Prospects for abiotic stress tolerance. J. Appl. Biosci. Biotechnol. 3(1): 1-28

2. In Conferences

1. **Solleti SK**, Bakshi S, Purukayastha J and Sahoo L (2008) Transgenic cowpea seeds expressing bean α-amylase inhibitor 1 conferred complete protection against storage pest, bruchid beetles. International conference on plant biotechnology and molecular biology, Kakatiya University, Warangal, India, 15-17th Aug 2008 (Received best poster presentation award)
2. **Solleti SK**, Bakshi S and Sahoo L (2008) Pronounced effect of synergistic and temporal application of two cytokinins, 6-benzy amino purine and kinetin on cyclic morphogenesis from shoot apex explants in cowpea: A morphological insight. International conference on plant biotechnology and molecular biology, Kakatiya University, Warangal, India, 15-17th Aug 2008

3. Sugla T, **Solleti SK** and Sahoo L (2008) Constitutive expression of virulence genes in conjunction with thiol compounds and geneticin selection enhanced recovery of transgenic plants in mungbean. International conference on plant biotechnology and molecular biology, Kakatiya University, Warangal, India, 15th -17th Aug 2008