Development of Transgenic Cowpea Overexpressing *Btcry1Ac* and *Btcry1Ab*

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by

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CERTIFICATE

It is certified that the work described in this thesis, entitled "**Development of Transgenic Cowpea overexpressing *Btcry1Ac* and *Btcry1Ab***", done by Ms. Souvika Bakshi 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, 2011

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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, 2011 Souvika Bakshi

Dedicated to my beloved parents

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Date: *Souvika Bakshi*

CONTENTS

ABSTRACT

Cowpea (*Vigna unguiculata* L. Walp) is an important grain and fodder legume widely cultivated in Africa, India, Middle East and South America. Insect pest infestation, prevalently perennial damage of cowpea pods by pod borers, *Maruca vitrata* and *Heliothis armigera* is most severe. Breeders lack resistance background for incorporation to cultivated varieties to control the diseases. It has been demonstrated that the introduction and expresssion of crystalline toxin genes (*cry*) derived from *Bacillus thuringiensis* (Bt) through transgenic approaches are effective mechanisms for protecting crops against insect infestations. In cowpea, absence of an efficient system for shoot multiplication and plant regeneration amenable to *Agrobacterium*-mediated transformation, mechanisms to increase T-DNA transfer to regenerating cells and strategy for efficient selection of transformed shoots have been identified as major bottlenecks for implementing transgenic approaches for genetic improvement. This investigation was carried out to find out the role of seedling preconditioning with cytokinin on shoot proliferation efficiency of cotyledonary node explants. A significantly higher shoot proliferation (7.1 shoots per explants), and mean shoot length (2.6 cm) were obtained with cotyledonary node explants derived from seedlings preconditioned in 10 µM TDZ for 4 days. The preconditioned explants were employed for efficient regeneration of transgenic cowpea plants. Bt*cry1Ac* and *cry1Ab* overexpression constructs were prepared and mobilized to *Agrobacterium tumefaciens vir* helper strain EHA105.

Method for efficient recovery of transgenic cowpea plants using an improved kanamycin selection regime was established that showed 46.1% increase in efficiency as compared to the existing transformation methods. *Agrobacterium*-cocultivated cotyledonary node explants were selected on medium containing 150 mg/l kanamycin for 20 days and surviving explants were shifted to kanamycin-free media supplemented with reduced dosage of BAP (2.5 µM) that resulted in profuse proliferation of kanamycin-resistant shoots with 63.6% increase in shoot length within 15 days of culture. Transgenic cowpea plants with introduced *cry1Ac* were recovered that showed presence, integration, expression and inheritance of transgene.

The role of sonication and vacuum infiltration on increase in T-DNA transfer efficiency to cotyledonary node explants were investigated. A combination of 20 s sonication followed by 5 min vacuum infiltration treatments were found to significantly

enhance T-DNA transfer to target regenerating cells. Fertile transgenic cowpea plants expressing *cry1Ac* were generated through sonication- and vacuum infiltration-assisted *Agrobacterium* mediated transformation of cotyledonary node explants. The presence, integration and expression of *nptII* and *cry1Ac* genes in transgenic plants were confirmed by polymerase chain reaction (PCR), genomic Southern and qualitative reverse transcription (RT)-PCR analysis. Western blot hybridization and Enzyme Linked Immunosorbant assay (ELISA) detected the accumulation of Cry1Ac protein in transgenic plants. The *cry1Ac* gene was transmitted in a Mendelian fashion.

The potential of newer explants based on sliced cotyledonary node on regeneration and transformation in cowpea was investigated. The split cotyledonary node explants allowed access of large number of regenerating cells to *Agrobacterium*-mediated transformation. Stable transgenic plants expressing *cry1Ab* were recovered using split cotyledoary node explants with an average transformation efficiency of 5.01%. The presence and expression of *cry1Ab* in transgenic plants were confirmed by PCR, and qualitative RT-PCR and ELISA respectively.

A positive selection system based on use of *phosphomannose isomerase* gene (*pmi/manA*) of *E. coli* and mannose as carbon source was established for efficient regeneration of transgenic cowpea plants. The selection scheme was determined on the basis of judicious choice of mannose and sucrose combination that allowed initial proliferation of shoot buds followed by their starvation. A combination of 20 g /l of mannose and 5 g/l sucrose was found optimal for selection of transformed shoots. The presence and expression of *pmi* in transgenic cowpea plants were confirmed by PCR, and RT-PCR and chlorophenol red assay respectively.

This research has demonstrated efficient recovery of cowpea transgenic plants through improved regeneration, T-DNA delivery system and selection approaches. Transgenic cowpea plants expressing *cry1Ac* and *cry1Ab* were generated which would facilitate their evaluation to test the impact of expression of Cry1Ac and Cry1Ab for resistance against target insects.

ABBREVIATIONS

ANOVA	Analysis of variance
B5	Gamborg's medium (1968)
BAP	6-benzylaminopurine
Bt	*Bacillus thuringiensis*
CAMBIA	Centre for Application of Molecular Biology to International Agriculture
CaMV	Cauliflower Mosaic Virus
CMPS	Cestrum yellow leaf curling virus constitutive promoter
CPR	Chlorophenol red
CTAB	Cetyltrimethyl ammonium bromide
DIG	Digoxygenin
DMRT	Duncan's multiple-range test
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
gus	*β-1-4 glucuronidase*
HCl	Hydrochloric acid
hpt	*hygromycin phosphotransferase*
IBA	Indole-3- butyric acid
LB	Luria-Bretani
LCM	Liquid co-cultivation media
MS	Murashige and Skoog's medium (1962)

MSB_5	MS medium supplemented with B5 vitamins
NaOH	Sodium hydroxide
NBT/BCIP	Nitro blue tetrazolium/ bromo chloro indolyl phosphate
nptII	*neomycin phosphotransferase* II
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pmi	*phosphomannose isomerase*
PPFD	Photosynthetic photon flux density
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-Polymerase chain reaction
SAAT	Sonication-assisted *Agrobacterium*-medited transformation
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
SISM	Shoot induction and selection medium
SRM	Shoot regeneration medium
SSC	Sodium chloride and sodium citrate buffer
SV	Sonication followed by vacuum infiltration treatment
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween-20
T-DNA	Tranferred-DNA
TDZ	N-phenyl-1,2,3-thidiazol-5-yl-urea
Ti-plasmid	Tumer inducing plasmid
vir	*virulence* gene

UNITS

µg	microgram
µl	microliter
µM	micromolar
^{0} C	degree celsius
bp	base, basepair
cm	centimeter
g	gram
g/l	gram per liter
kb	kilo base, kilo basepair
kDa	kilodalton
kHz	kilohertz
mg	milligram
mg/l	milligram per liter
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mm Hg	millimeters of mercury
pH	negative log of H^{+} ion
psi	pounds per square inch
rpm	Revolution per minute
s	second
v/v	volume/volume (concentration)
w/v	weight/volume (Concentration)

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Chapter 1

Introduction

1.1 Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is one of the most important food legume crop. The seeds contain about 25% proteins and constitute an important source of quality nourishment for the rural and urban poor in semiarid tropics. Cowpea leaves are used as vegetables and the haulms which contain over 15% protein forms an invaluable source of high quality fodder for livestock (Omo-Ikerodah et al. 2009). It is widely cultivated in the semi-arid tropics covering Asia, Africa, and South America. Cowpea is well adapted to high temperatures, drought and poor soils as compared to other crop species (Kolawale et al. 2000; Sanginga et al. 2000). In spite of its importance, cowpea faces numerous production constraints. Among the major constraints, insect pests cause significant reduction in yield. The pod borers, major lepidopteran insects perennially damage cowpea pods on fields. The legume pod borer, *Maruca vitrata* (Lepidoptera: Crambidae) and *Heliothis armigera* are key cowpea pests. Their larvae feed on the tender parts of the stem, peduncles, flower buds, flowers and pods (Singh and Jackai 1988). Severe infestations may often result yield loss by 90% (Murdock et al. 2001).

Currently, insecticide sprays are the only viable control technology available, but this approach has been compromised with the emergence of resistant biotypes due to indiscriminate use of multiple classes of insecticides (Ekesi 1999). Therefore, development of cowpea cultivars with insect resistance can form the backbone for integrated management of these legume pod borers (Malick Ba et al. 2009). Advances have been made in the various ramifications of cowpea breeding during the last few decades to ameliorate the insect pest problem. Conventional breeding has been exploited to both increase the genetic diversity of the cowpea as well as to improve the selection of desirable characteristics. While some sources of insect resistance have been reported in wild cowpea relatives (*Vigna* spp.) as well as other non-*Vigna* legumes such as African yam bean

(*Sphenostylis stenocarpa*), none of these can inter-cross with cowpea via conventional breeding (Machuka 2002). Several crosses of cultivated lines with wild cowpea cultivars such as 'Tvu 946', identified to be moderately resistant to *M. vitrata*, had produced offsprings with unacceptable agronomic characters and inadequate level of resistance as compared with the wild parent (Singh 1995). It also faces formidable challenges in incorporating all of the desirable traits into individual cultivars with acceptable grain quality and adaptation to an array of farming systems.

It is known that insect pests, especially lepidopterans, can be controlled by *Bacillus thuringiensis* (*Bt*) – an ubiquitous, soil-dwelling, spore-forming bacterium – when applied topically on crops as spore formulations. Incidentally, *Bt* sprays are often washed away by rain, degrade under solar ultra violet radiation and are not optimally targeted against certain insect pests that live within plant tissues. Therefore, the application of biotechnological tools and technologies hold great promise in overcoming the constraints in cowpea production by expressing candidate genes for insect resistance. Incorporation of *Btcry* genes encoding *Bt* crystal proteins in legumes (Sharma et al. 2006; Dufourmantel et al. 2005; Sanyal et al. 2005) has resulted in built-in protection against target lepidopteran insects through the conceptual framework of genetic transformation. Artificial diet bioassays with Cry1Ab (LC_{50} 0.207 ppm) has been shown that Cry1Ab is most potent against second instar larvae of *Maruca vitrata* followed by Cry1Ac (Srinivasan 2008). Therefore, the overexpression of Bt*cry1Ab* and Bt*cry1Ac* in transgenic cowpea is most promising for development of a durable and enhanced resistance against target insects.

Gene transfer to both *Agrobacterium* and direct DNA delivery has been attempted in cowpea, however, the procedure is still far from routine for large scale recovery of transgenics with desirable traits. Development of an efficient plant regeneration system amenable to genetic transformation is the bottleneck in developing transgenics in cowpea, a

highly recalcitrant grain and forage legume. Seed derived explants such as cotyledons (Muthukumar et al. 1996), mature embryos (Popelka et al. 2006), cotyledonary nodes (Chaudhury et al. 2007; Solleti et al. 2008a) and shoot apices (Ivo et al. 2008) have been used to regenerate transgenic cowpea plants albeit with a low transformation frequency. In most of the instances, the shoots formed as a result of proliferation of pre-existing meristems. It is expected that the efficiency of genetic transformation will be significantly improved if the proportion of meristematic cells in cotyledonary node explants is increased and/or if the number of shoots regenerated from the existing meristems is increased. Addition of BAP during seed germination has shown to improve regeneration from cotyledonary node explants in cowpea (Chaudhury et al. 2007; Solleti et al. 2008a, b; Raveendar et al. 2009). However, the effect of TDZ, a highly potent synthetic cytokinin, on seed preconditioning has not been evaluated for its influence of plant regeneration in cowpea. Therefore, to develop efficient plant regeneration system amenable to genetic transformation of cowpea, the effect of the cytokinin dose and duration of seed preconditioning was examined on efficiency of multiple shoot induction and recovery of plants from different cowpea seedling explants.

Conventional negative selection system such as neomycin phosphotransferase II (*npt*II) gene, conferring resistance to aminoglycoside antibiotics, kanamycin and geneticin, and herbicide resistance genes, *bar* which encodes for phosphinothricin acetyltransferase and confers resistance to herbicides l-phosphinotricin, and *hpt* gene that confers resistance to hygromycin have been used to select and recover transformed shoots in cowpea (Choudhury et al. 2007; Solleti et al. 2008a, b; Popelka et al. 2006; Muthukumar et al. 1996). However, the use of antibiotic resistance markers to select transformed plants has generated widespread public concern (Aragao and Brasileiro 2002; Bakshi 2003; Ramessar et al. 2007). Beside the lack of public acceptance of transgenic plants, which is mainly based

on concerns about health implications of consuming genetically modified food containing antibiotic resistance genes, also regulatory authorities ensure for the avoidance of such genes. In this context, positive selection system based on genes that confer selective metabolic and growth advantage to transformed plant cells enable efficient selection and recovery of transformed plants (Joersbo and Okkels 1996). Genes such as phosphomannose isomerase (*pmi/manA*) of *E. coli* (Miles and Guest 1984) code for the enzyme that can metabolize alternate source of sugar i.e., mannose, and therefore, the incorporation of *pmi* gene provides the transformed cell the ability to metabolize mannose that is not usually metabolized by target plants. The mannose based selection system has been successfully used for regeneration of transgenics in wide array of crop plants (Stoykova and Stoeva-Popova 2011). Evaluation of the efficacy of positive selection based on mannose in cowpea is expected to establish efficient as well as environmentally safe method to recover transgenics in cowpea.

The present study was undertaken with the objectives to establish an efficient plant regeneration system amenable to *Agrobacterium* mediated transformation, investigate the role of sonication and vacuum infiltration in enhancing T-DNA transfer, and formulate a mannose based positive selection system in cowpea. The established transformation systems were adopted for introduction and expression of the candidate genes, *Btcry1Ac* and *Btcry1Ab* in cowpea in order to develop durable resistance against target insects, *Maruca vitrata* and *Heliothis armigera*.

1.2 Objectives

The present investigation is carried out with the broad objectives to establish an efficient plant regeneration system from seedlings explants amenable to *Agrobacterium* mediated transformation, prepare *Btcry1Ab* and *Btcry1Ac* overexpression constructs, enhance T-DNA transfer through sonication and vacuum infiltration and generate stable transgenic plants overexpressing *Btcry1Ab* and *Btcry1Ac* through a robust selection system, and perform molecular analysis for expression of the candidate gene(s). The objectives are outlined as

- Studies on role of seedling preconditioning on plant regeneration efficiency in cowpea
- Preparation of binary constructs for overexpression of *Btcry1Ac* and *Btcry1Ab*
- Establishment of an improved selection system and recovery of transgenic cowpea expressing *cry1Ac*
- Investigation on sonication and vacuum infiltration assisted *Agrobacterium*-mediated transformation in cowpea
- Establishment of split cotyledonary node based plant regeneration system and recovery of transgenic cowpea overexpressing *cry1Ab*
- Development of a mannose based positive selection system for the recovery of transgenic cowpea

Chapter 2

Literature review

2.1 Cowpea: A protein-rich grain legume

Cowpea (*Vigna unguiculata* L. Walp) is an important grain legume as well as fodder crop widely cultivated in the semi arid tropics covering Asia, Africa, Southern Europe, Central and South America (Timko and Singh 2008). Cowpea has superior nutritional attributes containing 24-26% leucine, lysine and methionine (Bressani 1985). It is also valuable sources of dietary fiber as well as vitamins and minerals including folate, thiamin and riboflavin (Phillips et al. 2003). Dry cowpea grains are the principal product for human consumption of the plant, but leaves, fresh peas and fresh green pods are also consumed as a fresh vegetable. The crop is also used for green manure and fodder (Ehler and Hall 1997). This crop is adaptable to harsh environments and withstands extreme temperatures, water limiting conditions and poor soil fertility (Shimelis and Shiringani 2010). Due to adaptation versatility, ability to fix atmospheric nitrogen and considerable level of seed protein, minerals and vitamin contents cowpea emerge as viable and attractive crop in low input farming systems.

Cowpea is predominantly a self fertilizing crop. The primitive and wild relatives of *V. unguiculata* are reportedly found in Southern Africa. The Limpopo Province in the Republic of South Africa was suggested to be the centre of diversity due to the presence of most primitive wild botanical varieties (Ng and Marachel 1985). West Africa is a major center of diversity of domesticated cowpea when India appears to be a secondary center of diversity since significant genetic variability occurs on the subcontinent (Ehler and Hall 1997). Cowpea (2n = 2x = 22) with genome size 620 Mb belongs to the genus *Vigna* Savi. (subgenus *Vigna* sect. Catiang) in the Phaseoleae group (Das et al. 2008) and the cultivated cowpeas have been divided into five groups (Unguiculata, Melanophthalmus, Sesquipedialis, Biflora and Textilis) based on pod and seed characteristics (Fang et al. 2007).

2.2 Cowpea production and its constraints

Cowpea is most popular and widely cultivated among recourse-poor farmers in Africa, Asia and Central America. Cultivation of cowpea in these farming systems supplement and diversify the starch nutrition prevalent in these regions to improve the health and livelihoods of millions of the poor people particularly in the developing countries. The nutritional quality of its protein is limited by the presence of antinutrients as well as an inherent resistance to digestion of the major globulins.

Further, its production is limited by major abiotic stresses including photoperiod sensitivity, drought, heat, salinity and mineral toxicities. Insect pest infestations contribute to major yield penalty to cowpea (Jackai and Daoust 1986). Every part of the cowpea plant has an adapted pest species that can cause substantial damage and among these the flower and pod pests cause major damage to yield. A detailed study by Karungi et al. (2000), has found established that among all the biotic constraints, legume pod borers cause a significant production loss in field (Fig. 2.1).

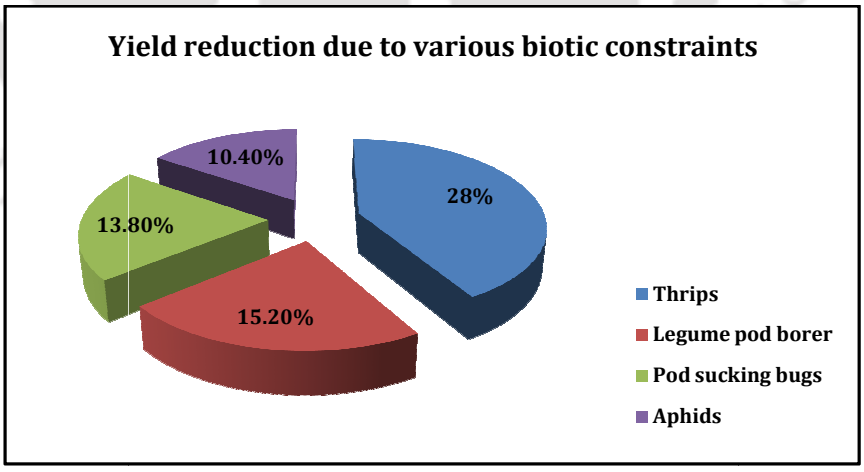


Fig. 2.1 Percentage of cowpea yield reduction due to various biotic constraints (Karungi et al. 2000)

Among the pod borers, lepidopteran insects *Maruca vitrata* and *Heliothis armigera* perennially damage cowpea pods on fields, the tender parts of the stem, peduncles, flower buds and flowers (Singh and Jackai 1988). Severe infestations may reduce yield by 90% (Murdock et al. 2001).

2.3 Strategies to address production constraint due to insect infestation

Various control strategies have been used for the management of insects of cowpea. These include culture controls, natural and biological control and use of insecticides.

2.3.1 Cultural control

Insect pest problems on cowpea can be reduced by the use of practices which involve ecological manipulations. These include crop diversification (inter cropping), alteration of planting dates, crop rotation, trap cropping, soil amendments and weed control. However, the most common control strategies are manipulation of insect environments, use of intercropping, alteration of planting dates and plant density.

2.3.2 Natural and biological control

Cowpea field pests can also be regulated to some extent by adverse weather conditions and natural enemies (Ogenga-Latigo 1988; Ekesi et al. 1998). Unfortunately, weather conditions cannot be manipulated, and rain is a density-independent mortality factor that cannot be relied upon as a strategy in pest management. Strategic use of natural agents must, be based on the manipulation of biotic mortality factors, particularly insect predators and parasitoids in biological control programs (Adipala et al. 2000).

2.3.3 Use of insecticides

Insecticide application on cowpea to control the major field pests through foliar sprays is most common. Although, foliar sprays are very effective against most insect pests of cowpea but they are very expensive and the residual chemicals are harmful to human health and the environment (Luck et al. 1977; Jackai 1983; Jackai and Adalla 1997). Vulnerability to the

emergence of tolerant strains of pest or pathogen is probably the most severe; chemical methods are also often insufficiently selective and very wasteful. Therefore, additional control through the development of insect resistance cowpea cultivars seems more economic, effective and safe compared with using insecticides and it can form the backbone for integrated management of the pod borers (Malick Ba et al. 2009).

2.3.4 Genetic improvement for insect resistance by conventional breeding

Conventional breeding methods have been exploited to both increase the genetic diversity of the cowpea and improve the selection of desirable characteristics. Most cowpea breeders employ backcross, pedigree, or bulk breeding methods to handle segregating populations because cowpea is a self-pollinating species. Due to the evolutionary 'bottleneck', insufficient genetic variability may exist in the species for pest resistance and to support long term crop improvement efforts. Wild cowpeas possess extensive genetic variability and valuable traits that are not available in cultivated germplasm (Ng and Padulosi 1988).

Developing cultivars with sustainable resistance to insects is a key objective of many breeding programs worldwide. While some sources of insect resistance have been reported in wild cowpea relatives (*Vigna* spp.) as well as other non-*Vigna* legumes such as African yam bean (*Sphenostylis stenocarpa*), none of these can inter-cross with cowpea via conventional breeding approaches (Machuka 2002). Despite the extensive germplasm screening, effective sources of resistance to *Maruca vitrata* and pod sucking bugs have not been identified among cultivated varieties of cowpea. Repeated crosses of cultivated lines with wild cowpea cultivars such as 'Tvu 946', identified to be moderately resistant to *M. vitrata*, had produced offsprings with unacceptable agronomic characters and inadequate level of resistance as compared with the wild parent (Singh 1995). Furthermore, breeders lack an array of well-adapted insect resistant parental lines that could be recombined to

generate cultivars with multiple resistances. It also faces formidable challenges in incorporating all of the desirable traits into individual cultivars with acceptable grain quality and adaptation to an array of farming systems.

2.4 Genetic engineering for insect resistance

Genetic engineering of crops may complement conventional breeding program to develop resistant cultivars. It offers many advantages, not just widening the potential pool of useful genes but also permitting the introduction of a number of different desirable genes at a single event and of reducing the time needed to introgress introduced characters into an elite genetic background. Since the first reports of transgenic plants appeared in 1984 (Horsch et al. 1984) there has been enormous progress directed at using transgenic technology for the practical ends of crop improvement. Protection of crops from insect pests was quickly seized upon as a major goal of plant genetic engineering (Fig. 2.2).

All plants possess a certain degree of resistance to insects, and so only a limited range of herbivores are able to feed on each individual species. This inherent resistance is based on various defense mechanisms, including a wide range of noxious secondary metabolites produced by the plant (Schuler et al. 1998). Individual plants within one genus, or even one species, vary in their level of insect resistance. At present, insect-resistance transgenes, whether of plant, bacterial or other origin, can be introduced into plants to increase the level of insect resistance, a technology that has dramatically extended the scope of resistance genes available to plant breeders.

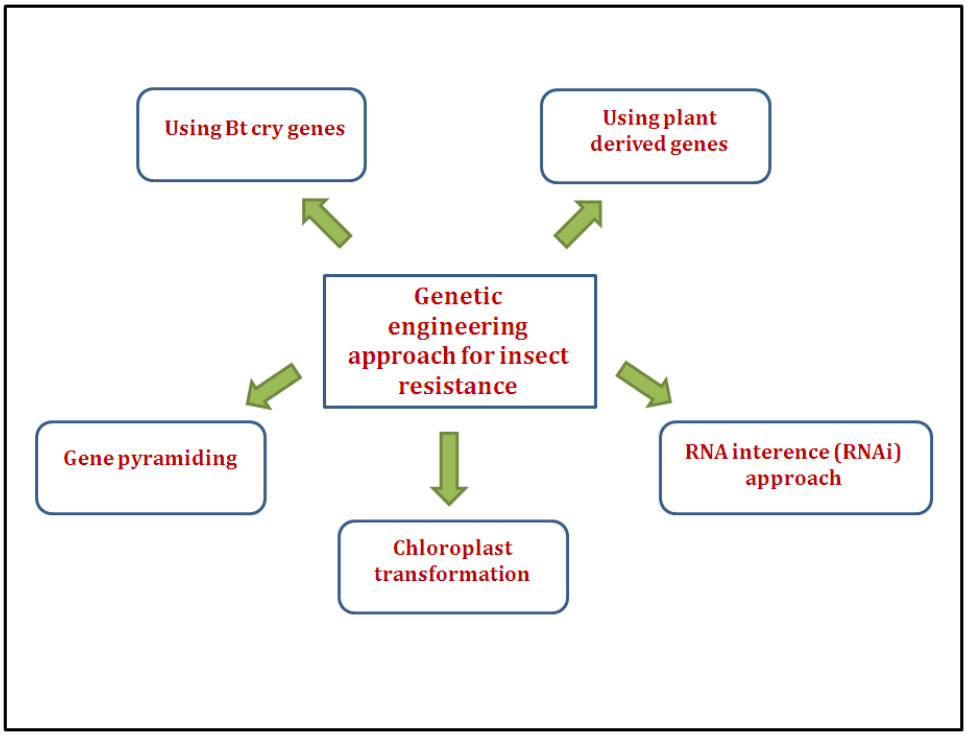


Fig. 2.2 Genetic engineering approaches to control insects

2.4.1 Plants derived genes conferring insect resistance

Several classes of genes have the potential to provide genetically-engineered resistances to insects in crop plants. Among the plant-derived genes, protease inhibitors, α-amylase inhibitors, plant lectins have been widely used in genetic engineering of legumes for insect resistance.

2.4.1.1 Protease inhibitor

Protease inhibitor gene expression has been detected in leaves of several species following wounding, suggesting their role in protecting plants from insect attack and microbial infection. After the identification of protease inhibitor as a valuable trait suitable for developing insect-resistant transgenic plants, there was intense interest to identify the protease inhibitor gene from different plant species. Protease inhibitor gene has been

identified and cloned from a wide array of plant sources, including alfalfa (Gurel et al. 1995), tomato (Lee et al. 1986), potato (Cleveland et al. 1987; Sanchez-Serrano et al. 1986), maize (Rohrmeier et al. 1993), mustard (Ceci et al. 1995), poplar (Hollick et al. 1993), tobacco (Atkinson et al. 1993), rice (Abe et al. 1987), sweet potato (Yeh et al. 1997), soybean (Song et al. 1991), amaranthus (Rodriguez et al. 1999), cowpea (Lee et al.1986) and barley (Odani et al. 1983). Although the ubiquity of occurrence of protease inhibitors in plants initially obscured their function in protecting the plant from insect attack, abundant evidence now exists for the defensive role of these proteins (Gatehouse et al. 1991, 1992). Much of the present research is focused on expressing protease inhibitors in transgenic plants. These protease inhibitor genes have advantages over genes encoding for complex pathways *i.e.* by transferring single defensive gene from one plant species to another and expressing them from their own wound inducible or constitutive promoters thereby imparting resistance against insect pests (Boulter 1993). Considering the high complexity of protease/inhibitor interactions in host pest systems and the diversity of proteolytic enzymes used by pests and pathogens to hydrolyze dietary proteins or to cleave peptide bonds in more specific processes (Graham et al. 1997), the choice of an appropriate proteinase inhibitor (PI) or set of PIs represents a primary determinant in the success or failure of any pest control strategy relying on protease inhibition. Engineering of plant protease inhibitors into a variety of crops can protect the plant from targeted insect-pest infestation (Hilder et al. 1987; Xu et al. 1996; Girard et al. 1998; Lee et al. 1999; Lawrence et al. 2001; Lawrence and Koundal 2001).

2.4.1.2 Plant lectins

Lectins are another important plant protein that play an important role in the plant's defense against insect pests, and have been found to be toxic to viruses, bacteria, fungi, insects and higher animals. Lectins are proteins with atleast one non-catalytic domain that

can reversibly bind to specific carbohydrates, either simple monosaccharides or more complex glycans (Peumans and Van Damme 1995). The classical plant lectins are often found at high concentrations in certain plant tissues (e.g. seeds, bark, bulbs). Although the exact function of these proteins has yet to be elucidated, the high concentrations and their source tissues suggest a role as storage proteins. Furthermore, lectins can also serve as defense molecules against insect herbivores and pathogens (Michiels et al. 2010).

Till date, a broad spectrum of plant lectins has been tested on several insect species. The snowdrop lectin (GNA) has previously been shown to be toxic to Homoptera (Rahbe et al. 1995; Powell et al. 1995, 1998), Lepidoptera (Fitches et al. 1997), and Coleoptera (Gatehouse et al. 1996; Elden 2000). Snowdrop lectin (2%) inhibited feeding and reduced the weight of spotted pod borer, *Maruca vitrata* larvae (Machuka et al. 1999) and tomato moth (*Lacanobia oleracea*) (Fitches et al. 1997). The biological effects of plant lectins from field bean (*Phaseolus vulgaris*), pigeonpea (*Cajanus cajan*), chickpea (*Cicer arietinum*), and garlic (*Allium sativum*) along with snowdrop (*Galanthus nivalis*) lectin on the growth and development of *H. armigera* so as to identify the candidate genes for deployment through transgenic plants to control this pest (Arora et al. 2007). 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).

2.4.1.3 α-amylase inhibitors

Cowpea seeds are rich in protein, carbohydrate and lipid and therefore suffer extensive predation by bruchids (weevils) and other pests. The larvae of the weevil burrow into the seed pods and seeds and the insects usually continue to multiply during seed storage. α-

amylase inhibitors are attractive candidates for the control of seed weevils as these insects are highly dependent on starch as an energy source.

The enzyme, α-amylases plays a key role in carbohydrate metabolism of microorganisms, plants and animals. α-amylases (α-(1-4)-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a family of endoamylases catalyzing the cleavage of α-(1-4) glycosidic bonds in starch and related carbohydrates with retention of α-anomeric configuration in the products. Different plant α-amylase inhibitors exhibit different specificities against α-amylases from diverse sources. Determination of specificity of inhibition is the important first step towards the discovery of an inhibitor that could be useful for generating insect-resistant transgenic plants. α-amylase inhibitors conveniently classified by their tertiary structure into six classes: lectin-like, knottin-like, cereal-type, Kunitz-like, c-purothionin-like and thaumatin-like (Richardson 1990).

The first practical demonstration involving α-amylase inhibitors used α-AI1, which specifically inhibits the α-amylases of the three Old World bruchids; the pea weevil *Bruchus pisorum*, the cowpea weevil and the azuki bean weevil. 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*. Similarly, azuki bean plants expressing α-AI1 were completely resistant to the azuki bean weevil (Ishimoto et al. 1996). Transfer of bean αAI-1 gene into chickpea, mungbean and cowpea (Sarmah et al. 2004; Sonia et al. 2007; Solleti et al. 2008) in a seed specific manner resulted in accumulation of αAI-1 in the seeds conferred complete protection from bruchid beetles.

2.4.2 Potential Bt cry genes to target insect

Bacillus thuringiensis (Bt), a wide spread soil bacterium, produces insecticidal proteins called Bt toxins. There are many Bt strains that produce characteristic sets of toxins, each

with its own activity spectrum that targets larvae of specific insect species. Bt endotoxins have been used as biological control reagents since the 1920's, but use of specific Bt toxins has increased dramatically since 1996 with the introduction of these candidate Bt genes in crops through conceptual framework of genetic engineering. These transgenic plants were significantly toxic to the target insects while there was no direct effect on non-target species (Qaim and Zilberman 2003).

Bt toxins are also called Cry toxins because they exist as crystals inside the bacterium. Full length Cry toxins are inactive until cleaved to generate their active form in the insect midgut (Lemaux, 2009). The cry genes code for proteins with arrange of molecular masses from 50 to 140 kDa. Upon ingestion by the susceptible target, the protoxins are solubilized and proteolytically processed to release the toxic fragment (Babendreier et al. 2005) (Fig. 2.3). During proteolytic activation, peptides are removed from both amino-and carboxyl-terminal ends of the protoxin. For the 130-to 140-kDa protoxins, the carboxyl-terminal proteolytic activation removes half of the molecule, resulting in an active toxin fragment of 60 to 70 kDa (Bravo 1997). Binding of activated forms of Cry toxins to receptors in the midgut is generally believed to be essential for toxicity. According to one model (Jim´enez-Ju´arez et al. 2007; Sober´on et al. 2007), after binding to midgut receptors, activated toxins form oligomers that create pores in midgut membranes, causing contents to leak, ultimately killing the larvae. The precision of Bt proteins for certain insects and their lack of effects in mammals are due to the specificity of receptor binding (Lemaux 2009).

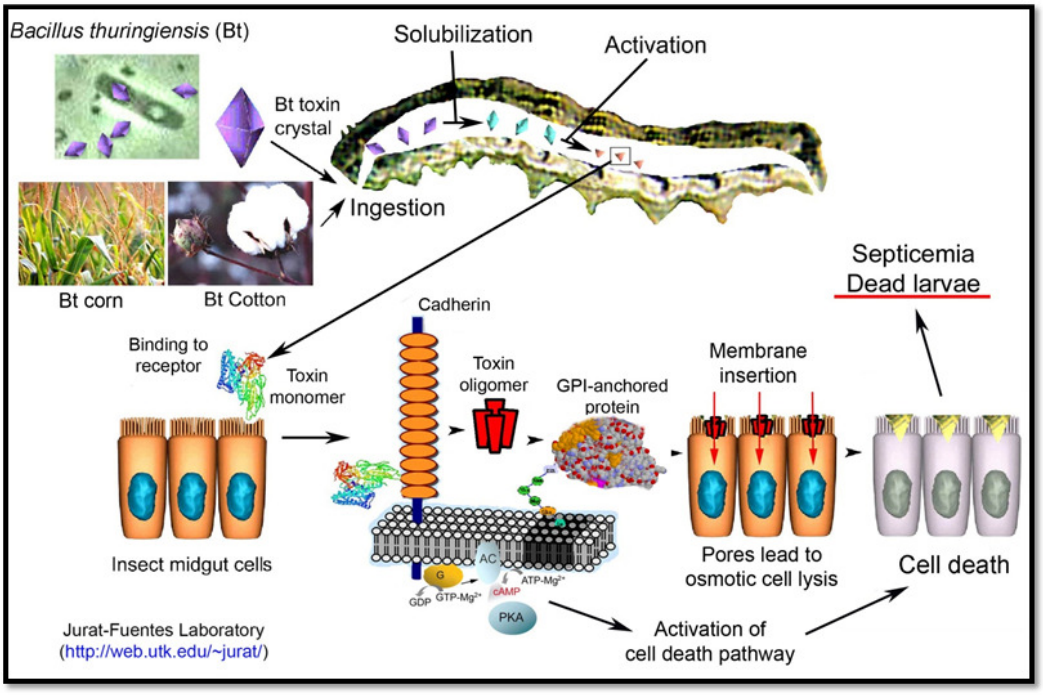


Fig. 2.3 Mode of action of Bt crystalline proteins (http://web.ulk.edu/~jurat)

The remarkable variety of known Cry proteins is the result of a continuing international effort to isolate and characterize new strains of *B. thuringiensis* with the hope of finding toxins with novel properties particularly suited for the control of agronomically or medically important pests. Thousands of strains have been screened and there are currently143 unique Cry toxins, according to the *B. thuringiensis* Toxin Nomenclature webpage (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). The three-dimensional structures of Cry toxins have provided considerable insight into the mechanism of toxin function and have helped to explain differences in toxin specificity. To date, seven structures have been solved by X-ray crystallography: Cry1Aa, Cry1Ac, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, and Cry4Ba (Lemaux 2009). These toxins show considerable

differences in their amino acid sequences and insect specificity but, remarkably, they all have highly similar three domain structures.

Domain I was first described in Cry3Aa by Li et al. (1991). It consists of an alpha-helical bundle in which six helices surround a central helix. Each of the outer helices is amphipathic in nature; polar or charged residues are generally solvent exposed and hydrophobic residues, typically aromatic in nature, project towards the central helix. These structural properties led to the hypothesis that domain I was the major determinant of pore formation in Cry toxins (Pigott and Ellar 2007).

Domain II is formed by three anti parallel β-sheets packed together to form a β-prism with pseudo three-fold symmetry. Structurally, domain II is the most variable of the toxin domains (Boonserm et al. 2005). Due to this variability, domain II is believed to be an important determinant of toxin specificity.

Domain III forms a β-sandwich. In this arrangement, two antiparallel β-sheets pack together with a "jelly roll" topology. Both sheets are composed of five strands, with the outer sheet facing the solvent and the inner sheet packing against domain II. Domain III shows less structural variability than domain II, and the main differences are found in the lengths, orientations, and sequences of the loops. The importance of these differences is particularly evident with Cry1Aa and Cry1Ac, where a loop extension in Cry1Ac creates a unique N-acetyl galactosamine (GalNAc) binding pocket implicated in receptor binding (Pigott and Ellar 2007).

Cry toxin binding to insect midgut epithelial receptors is an important determinant of specificity. Cry toxin receptors have since been reported, of which the best characterized are the aminopeptidase N (APN) receptors and the cadherin-like receptors identified in lepidopterans. In nematodes, glycolipids are believed to be an important class of Cry toxin

receptors (Griffitts et al. 2005). Other putative receptors include alkaline phosphatases (ALPs), a 270-kDa glycoconjugate, and a 252 kDa protein (Pigott and Ellar 2007).

Several teams working in the domain of plant transgenes is decided to make use of the insecticidal potential of *Bt* to generate genetically modified plants expressing δ-endotoxin genes. A first decisive step in this direction was taken in 1987, with the production of tobacco plants transformed with the *Btcry1Ab* gene (Vaeck et al. 1987) a gene whose product is active against the European corn borer (ECB), one of the main pest attacking maize in the US and Europe. The young ECB caterpillars burrow into the apical bud and then penetrate into the interior of the stem, creating a network of holes in the soft tissue. Thus, the insect rapidly finds shelter from classical insecticides and the damage it causes is not immediately apparent. A promising approach to control this type of pests was to create genetically engineered plants, expressing a cry Bt transgene in the tissues that are prone to the insect attack, in order to neutralize it before causing major damage.

However, despite the use of strong promoters, toxin production in plants was initially too weak for effective agricultural use (Koziel et al. 1993). Unlike plant genes, Bt genes have a high A+T content (66%), which is a suboptimal codon usage for plants, and potentially leads to missplicing or premature termination of transcription (Dela Riva and Adang 1996). The coding sequence of *cry* genes has been modified (without modifying the encoded peptide sequence) to ensure optimal codon usage for plants, and this allowed toxin production in plants to be increased by two orders of magnitude (Perlak et al. 1991). This strategy has been successfully used in many plants: cotton, rice and maize have been transformed with modified *cry1* genes and potato has been transformed with a modified *cry3A* gene.

2.4.3 Gene pyramiding

As insect resistance is a highly complex multigenic trait, generally single gene transformations result in insufficient and/or narrow spectrum insect resistance. In addition, there is always a possibility for reversal of resistance because of appearance of resistant strains of pathogens (Muthurajan and Balasubramanian 2010). Till date, many of the candidate genes, that have been used in genetic transformation of crops, are either too specific or are only mildly effective against the target insect pests. Some insect species are also insensitive to some of these genes. Therefore, to convert transgenics into an effective weapon in pest control, e.g., by delaying the evolution of insect populations resistant to the target genes, it is important to pyramid genes with different modes of action in the same plant. Hence, genetic engineering of crop plants with (i) a combination of genes encoding/controlling interdependent or synergistic sub-components of insect-resistance to realize effective resistance against a particular insect and (ii) a combination of genes conferring resistance against wide-spectrum insect resistance would be more logical. A well planned genetic engineering strategy involving a well balanced expression of transgenes with different modes of action would ensure enhanced and durable resistance against different target insects at a time.

2.4.4 Chloroplast transformation

The genetic transformation in plants generally concentrates on the nuclear expression of foreign genes. Researchers have developed another important landmark in methods of transformation, enhancing the ease and efficiency of plastid transformation globally. Chloroplast transformation besides providing high foreign protein expression also ensures maternal transmission of the foreign gene and therefore avoiding the spread of transgene through pollen. Boynton et al. (1988) reported the first successful chloroplast transformation in *Chlamydomonas reinhardtii* using gene gun and biolistic technology,

followed by introduction of spectinomycin resistance into tobacco (Svab et al. 1990). Stable chloroplast transformation depends on the integration of the foreign DNA into the chloroplast genome by homologous recombination and therefore must be flanked by sequences homologous to the chloroplast genome (Staub and Maliga 1992). Recent advancements in the plastid transformation systems in Arabidopsis (Sikdar et al. 1998), potato (Sidorov et al. 1999), and rice (Khan and Maliga 1999) come as a viable way forward, not only in the modification of a number of economically important crop plants, but also for a number of reasons such as high levels of protein expression, simultaneous expression of several genes as a polycistronic unit, and in the elimination of positional effects and gene silencing (Sharmah et al. 2005). Plastid expression of foreign genes also aims at eliminating environmental risks that arise due to the gene flow via pollen to other plants.

2.4.5 RNA interference (RNAi) approach

Application of RNAi technology is emerging as a novel approach to confer resistance to herbivorous insects in crop plants. Transgenic plants could be protected from the herbivorous insects by engineering them to express double-stranded RNAs (dsRNAs) directed against vital insect genes (Gordon and Waterhouse 2007).The key to the success of these approach is (i) identification of a suitable insect target and (ii) dsRNA delivery, which includes *in planta* expression of dsRNA and delivery of suitable amounts of intact dsRNA for uptake by the insects. Experimental validation of this strategy however, has now been studied in Arabidopsis and corn. Mao et al. (2007) identified a cytochrome P450 gene (CYP6AE14) from cotton bollworm (*Helicoverpa armigera*) and dsRNA specific to CYP6AE14 was expressed in Arabidopsis. Similarly, transgenic corn plants expressing western corn root worm (WCR) dsRNAs show a significant reduction in WCR feeding damage, suggesting that the RNAi pathway can be exploited to control insect pests by expressing a dsRNA.

2.5 Current status of transgenic research in cowpea and scope for developing insect-pest resistance

Recent progress in genetic transformation of cowpea offers opportunities to engineer with candidate genes for insect pest resistance. Although, considerable progress has been made, through conventional genetics and breeding, on cowpea improvement over the decades with respect to resistance to most of these stress factors (Singh 2007), the problem of insect pests still remains largely unresolved. Hence genetic engineering approaches stand out as the only effective alternative means of transferring genes that confer desirable agronomic traits more specifically to address insect infestation in cowpea.

In the past two decades, considerable success has been achieved in cowpea transformation (Table 2.1). Genetic transformation protocols have been developed for cowpea widening possibilities of introduction of candidate genes for crop improvement. To date, only three reports are available on generation of stable transgenic plants in cowpea and all reports are based on *Agrobacterium* mediated transformation. Muthukumar et al. (1996) recovered four hygromycin-resistant transformed plants, from mature de-embryonated cotyledons, of which one showed stable integration of *hpt* gene. However, the established plant failed to produce viable seeds. Popelka et al. (2006) generated transgenic plants from longitudinally bisected embryonic axes, attached with cotyledons but devoid of shoot and root apices, on phosphinothricin selection at a frequency of 0.001–0.003% in 5–8 months. Chaudhury et al. (2007) reported recovery of transgenic cowpea from cotyledonary nodes under kanamycin selection at a frequency of 0.76% in 5–6 months. However, Ikea et al. (2003) explored direct DNA delivery through particle bombardment in cowpea transformation. However, molecular evidence of stable transformation and inheritance of the transgenes to progeny was not provided. Employing the biolistic method of gene transfer, Ivo et al. (2008) reported generation of transgenic cowpea plants at an efficiency of 0.9% from bombarded embryonic axes that showed inheritance of transgenes.

Solleti et al. (2008a) demonstrated remarkable increase in the transformation efficiency of cowpea (1.64-1.67%) through constitutive expression of additional virulence genes in the LBA4404 *Agrobacterium* strain coupled to a regime of geneticin selection, experiencing effective selection than phosphinothricin (Popelka et al. 2006) and kanamycin (Chaudhury et al. 2007) respectively. The heterologous expression of the common bean *α-amylase inhibitor* 1 gene in the transgenic cowpea seeds (Solleti et al. 2008b) conferring resistance to storage pests, *Callosobruchus maculatus* and *C. chinensis* was the first report on the expression of any candidate gene in cowpea. These recent successes in cowpea genetic transformation have therefore pave way for the introduction of more agronomic important genes to cowpea for enhancing its genetic diversity and consequently for complementation of existing breeding programs.

2.6 Factors for improving cowpea transformation

Although many different techniques have been tested for gene delivery to plant cells, two major methods, *Agrobacterium*-mediated and particle bombardment, have been extensively employed for genetic transformation of cowpea. However, the overall transformation efficiency in these protocols was much low, most likely due to (i) inefficient T-DNA delivery from *Agrobacterium tumefaciens* to the regenerating cells of embryo-derived explants; (ii) inefficient selection of proliferating transgenic cells; (iii) difficulty with regeneration of shoots from transformed cells under the growth regulator and tissue culture regime followed (Fig. 2.4). Hence, enhancement of transformation efficiency is expected from the optimization of early gene transfer steps, use of efficient selection scheme, and tissue culture regime for maximal growth of transformants (Solleti et al. 2008a).

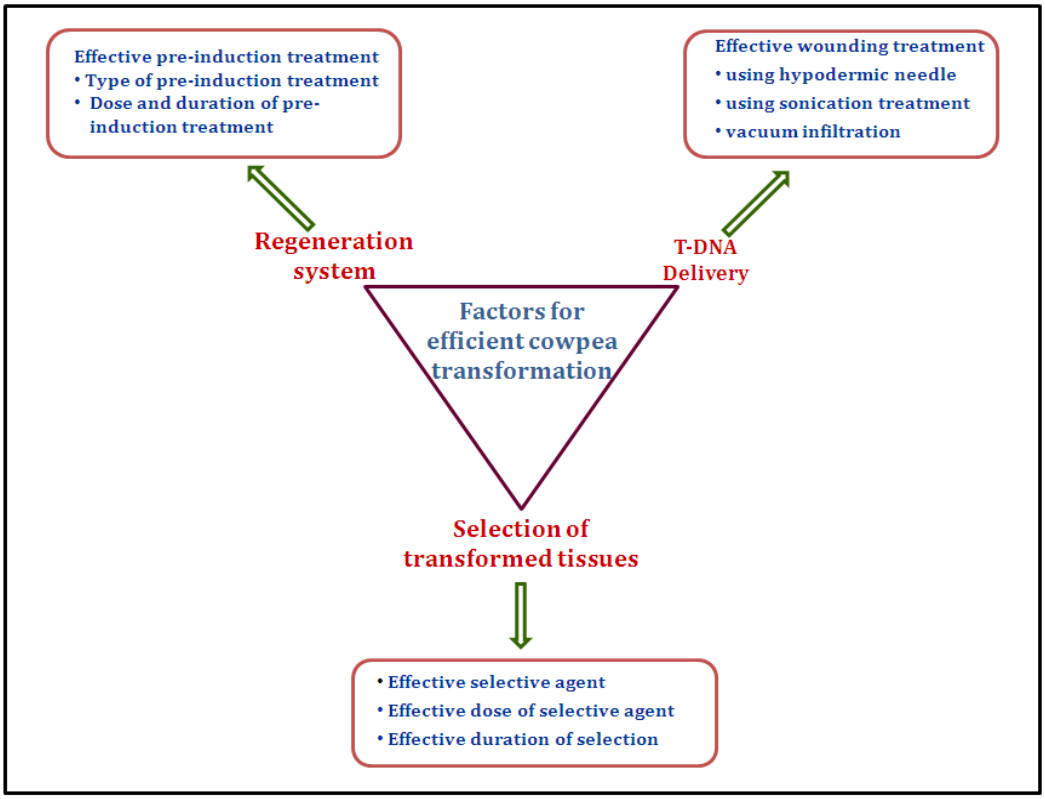


Fig. 2.4 Factors for improving cowpea transformation

The frequency of recovery of transgenic plants has been very low as the efficiency of the multiple shoot induction for genetic transformation is inadequate. Plant regeneration in cowpea has been achieved from different explants, cotyledon (Brar et al. 1999; Pellegrineschi 1997), shoot tip (Kartha et al. 1981; Brar et al. 1997; Mao et al. 2006; Aasim et al. 2009), cotyledonary node (Chaudhury et al. 2007; Solleti et al. 2008a; Raveendar et al. 2009), leaf (Muthukumar et al. 1996; Prem Anand et al. 2000; Ramakrishnan et al. 2005) and hypocotyl (Pellegrineschi 1997). Among them, only seedling explants, cotyledons (Muthukumar et al. 1996), mature embryos (Popelka et al. 2006) and cotyledonary nodes (Chaudhury et al. 2007; Solleti et al. 2008a, b) have been most preferred for *Agrobacterium*-mediated transformation of cowpea as T-DNA delivery to axillary meristem followed by regeneration via adventitious bud formation minimizes the risks of chimeras and

somaclonal variation (Tzfira et al. 1997). Yet, the efficiency of recovery of transgenic plants using seedling explants is inadequate. It is expected that the efficiency of genetic transformation will be improved if the proportion of meristematic cells in cotyledonary node explants is increased or the number of shoots regenerated from the existing meristems is enhanced. An efficient and rapid regeneration protocol need to be established that would allow formation of more shoots capable of elongation and regeneration to plantlets in a short period.

Plant transformation via *Agrobacterium* can be limited by both host specificity and the inability of *Agrobacterium* to reach the proper cells in the target tissue. Therefore, a robust *Agrobacterium*-based transformation technology is needed that overcomes these barriers and enhances DNA transfer in recalcitrant grain legumes. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) is a relatively new method for introducing *Agrobacterium* into the target cell. Plant cells have a hard and thick cell wall and the SAAT treatment produces a large number of small and uniform wounds across the tissue, allowing *Agrobacterium* easy access into the target plant cells or tissue. It allows the *Agrobacterium* to travel deeper and more completely throughout the tissue than normal cocultivation will permit (Trick and Finer 1997; Santare´m et al. 1998; Tang et al. 2001; Liu et al. 2005), thus enhancing the bacteria colonization and infection of the tissue. SAAT has been shown to provide efficient delivery of T-DNA to cells in a number of plants (Santare´m et al. 1998; Tang et al. 2001; Zaragoza et al. 2004; Beranova´ et al. 2008), especially those that are typically more recalcitrant to *Agrobacterium*-mediated transformation (Trick and Finer 1997).

Plant transformation technologies rely on the use of selectable marker genes, which are co-introduced with the gene of interest to select the transformation events. Selectable marker genes enable transgenic cells expressing the marker gene to survive in the presence

of the appropriate selective agent. For the transformation of cowpea, the most common selection systems based on the use of the neomycin phosphotransferase II (*npt*II) gene, conferring resistance to aminoglycoside antibiotics, such as kanamycin (Choudhury et al. 2007) and geneticin (Solleti et al. 2008a, b) or of the *bar* gene, which encodes for phosphinothricin acetyltransferase and confers resistance to herbicides l-phosphinotricin (Popelka et al. 2006), glyphosinate and bialaphos, or of the *hpt* gene that confers resistance to hygromycin (Muthukumar et al. 1996). However, the use of such markers for selecting transformed plants has continued to generate widespread public concerns of potential harmful effects to the environment and human health (Qiao et al. 2010). Therefore, alternative methods for selection have been developed that avoid the use of antibiotic resistant genes.

Till date, there are several approaches have been followed. One of these methods involve the use of the positive selection system that enables identification and selection of genetically modified cells without using the antibiotic resistance genes, which are based on enabling transformed plant cells to metabolize compounds that are usually not metabolized by plants (Joersbo and Okkels 1996). Genes such as phosphomannose isomerase (*pmi/manA*) isolated from *E. coli* (Miles and Guest 1984) code for the enzyme that can metabolize different source of sugar i.e., mannose, and therefore, the incorporation of *pmi* gene provides the transformed cell the ability to metabolize mannose that is not usually metabolized by target plants. The hexose sugar mannose is taken up by plants and phosphorylated by hexokinase to mannose-6-phosphate, which is not further utilizable. The accumulation of mannose-6-phosphate leads to a block in glycolysis by inhibition of phosphoglucose-isomerase (Goldsworthy and Street 1965), resulting in severe growth inhibition. In addition, synthesis of mannose-6-phosphate depletes cells of orthophosphate that is required for ATP production. The PMI catalyzes the reversible isomerization of

mannose-6-phosphate to fructose-6-phosphate, which serves as precursor for the glycolytic pathway. Plant cells transformed with the *manA* gene are able to utilize mannose as a carbon source and grow either in the presence of or with the addition of only small amounts of other carbon sources such as glucose or sucrose. This selection system has been successfully used for regeneration of transgenic sugar beet (Joersbo et al. 1998), cassava (Zhang and Puonti-Kaerlas 2000), maize (Negrotto et al. 2000; Wright et al. 2001), Arabidopsis (Todd and Tague 2001), wheat (Wright et al. 2001), tobacco and potato (Kunze et al. 2001), pepper (Kim et al. 2002), sweet orange (Boscariol et al. 2003), pearl millet (O'Kennedy et al. 2004), tomato (Sigareva et al. 2004), papaya (Zhu et al. 2005), bentgrass (Fu et al. 2005), apple (Degenhardt et al. 2006), onion (Aswath et al. 2006), almond (Ramesh et al. 2006), cucumber (He et al. 2006), cabbage (Min et al. 2007), sugarcane (Jain et al. 2007), flax (Lamblin et al. 2007), sorghum (Gurel et al. 2009) and chickpea (Patil et al. 2009). However, the pmi/Man system has not been tested previously in cowpea.

2.7 Concluding remarks and Future perspectives

Plant biotechnology continues the trend of improving crops by offering new ideas and techniques applicable to agriculture. The newly acquired ability to transfer genes into existing crop varieties improves the efficiency of production and increases the utility of agricultural crops (Sharmah et al. 2005). However, the insect-resistant transgenic plants were among the first products of plant biotechnology to reach the marketplace. Recent commercial releases of genetically engineered crops have included transgenic corn, cotton and potato, which express *Bacillus thuringiensis* (*Bt*) toxins (Maagd et al. 1999). However, one of the ecological risks of releasing transgenic *Bt*-plants, would be the unforeseen effects of the toxin on organisms that are not pests of the crop itself – especially if those organisms are predators and parasites of pests and therefore of benefit to agriculture. Furthermore, concern about insects rapidly becoming resistant to *Bt* toxins as a consequence of the

release of transgenic *Bt*-plants has received a great deal of attention (Maagd et al. 1999). Hence, it is feasible to increase the levels of resistance in transgenic plants and also employ different genes for managing development of resistance to Bt in insect populations.

Several strategies have been proposed to prevent or delay the rapid development of resistance to insect resistance transgenic plants, mostly the transgenic *Bt*-plants. One of the most plausible strategies is the use of temporal or spatial refuges. Rotation of *Bt*-crops with non-transgenic plants would slow down development of resistance, particularly if resistance is not stable in the insect population. With spatial refuges, part of a field is set aside for non-transgenic plants. This allows *Bt*-resistant insects that have survived on the transgenic plants to mate with non-selected, sensitive insects from the non-transgenic plants, and prevents the rise of a population that is homozygous for a recessive or semi-dominant resistance allele. A refinement of the spatial refuge strategy is the refuge/high dose-combination, which entomologists consider to be the most promising. In this strategy, refuges of non-transgenic plants are combined with transgenic plants that express *Bt* at a high level which should be enough to kill insects that are heterozygous for a recessive or semi-dominant resistance allele. This strategy is currently part of the resistance management plans that are imposed by the US Environmental Protection Agency on the companies selling *Bt*-cotton and *Bt*-maize (Maagd et al. 1999). Therefore, the large-scale commercial cultivation of these crops necessitates stringent implementation of resistance management strategies as also evaluation of the ecological, economic and social concerns (Kaur 2004). For that, the long-term effectiveness of the transgenic approach will need to be established. A combination of the new technology with existing technologies or treatments might provide the most effective and durable basis for future control of these important plant pathogens.

Chapter 3

Studies on role of seedling preconditioning on plant regeneration efficiency in cowpea

3.1 Introduction

Cowpea (*Vigna unguiculata* L. Walp) is an important grain and fodder legume of tropics and subtropics (Timko et al. 2007). The seeds are invaluable source of high-quality dietary protein for millions of local populace. There is currently significant interest in creating novel plants for insect pest and virus resistance in order to curb substantial yield loss due to these pathogen infestations (Solleti et al. 2008a). Cultivated cowpea however has a narrow genetic base and barriers in crossing with distant wild species allowing novel traits (Gomathinayagam et al. 1998; Fang et al. 2007). Therefore, gene transfer technology offers an alternative for the introduction of insect pest and virus resistance genes to potentially address the constraints in production. However, success in genetic manipulation in cowpea relies on the establishment of a highly efficient plant regeneration system for the production of transgenic plants from seedling explants by *Agrobacterium*-mediated transformation.

Regeneration of plants via organogenesis from cotyledonary node explants presents an attractive target for *Agrobacterium*-mediated transformation as T-DNA delivery to axillary meristem followed by regeneration of shoots via adventitious bud formation without involvement of a *de novo* regeneration pathway minimizes the risks of chimeras and somaclonal variation (Tzfira et al. 1997). In cowpea, there are only a few reports demonstrating the possibility of recovering transgenic plants through *Agrobacterium* mediated transformation of cotyledonary node explants (Chaudhury et al. 2007; Solleti et al. 2008a, b). From these experiments, it appears that shoot proliferation from transformed cotyledonary node explants is largely inefficient due to the formation of few shoots per explants, mostly stunted in nature and therefore incapable of elongating to shoots amenable for root induction (Solleti et al. 2008b). Nevertheless, the efficiency of transformed shoot recovery could be improved if the proportion of meristematic cells in explants is increased

or the number of shoots regenerated from the existing meristems is enhanced (Zhihui et al. 2009). Among the factors which could potentially facilitate increase in proportion of meristematic cells and shoot proliferation is the use of cytokinin especially thidiazuron (TDZ) in the induction phase of regeneration. TDZ (N-phenyl-1,2,3-thidiazol-5-yl-urea) is a non-purine cytokinin compound that has been established as a potent growth regulator of morphogenetic responses in many plant species (Jahan et al. 2011). Preconditioning of source of explants (seedlings) with high dose of cytokinin has been reported to improve subsequent regeneration efficiency in various plants (Gurel et al. 2011; Jahan et al. 2011) including grain legumes, mungbean (Amutha et al. 2006), common bean (Dang and Wei 2009) and pea (Zhihui et al. 2009). However, no systematic study has been conducted concerning the influence of seedling preconditioning on shoot proliferation and its effect on transgenic plant recovery.

In the present investigation, the effect of seedling preconditioning with cytokinin (type and dose) and duration of preconditioning on shoot regeneration and elongation, and efficiency of transgenic plant recovery were investigated.

3.2 Materials and methods

3.2.1 Pretreatment of seedlings and shoot multiplication

Seeds of commercially important cowpea cultivar, Pusa Komal were obtained from Indian Agricultural Research Institute, New Delhi, India. The seeds were surface sterilized with 0.2% (w/v) mercuric chloride for 5 min followed by rinsing 4-5 times with sterilized deionized water. The seeds were inoculated in MSB_5 medium [Murashige and Skoog (1962) salts and Gamborg B5 vitamins (1968)] or MSB_5 medium containing different concentrations (0, 5, 10 and 20 μM) of TDZ or BAP for 2-6 days (Fig. 3.1). Seeds cultured in hormone free media were considered as control.

In the first set of experiment, cotyledonary node explants (5-6 mm) excised from control seedlings (untreated) were cultured in three different regeneration medium, MSB_5 medium containing i) 5 µM BAP [M1], ii) BAP (5 µM) and kinetin (0.1 µM) [M2], and iii) BAP (5 µM) and kinetin (0.5 µM) [M3] for shoot multiplication. In the second set of experiment, both the effect of cytokinin (TDZ and BAP) preconditioning at different dose (0, 5, 10 and 20 µM) and for different duration (2-6 days) was tested. Cotyledonary node explants excised from pretreated seedlings were cultured onto three different regeneration media (M1, M2, M3) for shoot multiplication (Fig. 3.1). In both sets of experiments, after 2 weeks, shoots were excised from the explants and transferred to fresh medium for elongation. The mother explants were recultured on fresh medium after every 2 weeks for four consecutive times.

MSB_5 medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Hi-Media, Mumbai, India) was used throughout this study. The pH of the medium was adjusted to 5.8 with 1 N NaOH or HCl prior to autoclaving at 121^0C at 15 psi for 15 min. All the cultures were maintained at 25 ± 2^0 C under 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} s^1 provided by 40 W cool white fluorescent lamps (Philips, India).

3.2.2 Rooting and acclimatization

Regenerated shoots (3-4 cm) were transferred to MS basal medium for rooting. After 2 weeks of culture, the rooted plantlets were washed thoroughly in running tap water and then transferred to plastic pots containing sterilized soil and vermiculite (1:1). For acclimatization, pots were covered with transparent plastic bags to avoid dessication. The plastic bags were gradually removed in 2 weeks, plants were maintained in the greenhouse. After 4 weeks, plants were transferred to pots containing soil:compost (1:1) and grown to maturity.

3.2.3 Data collection and statistical analysis

Shoot multiplication data was recorded after every passage of 2 weeks and shoot elongation data was recorded after two weeks of culture of solitary shoots. The experiments were designed as four-factorial: the type of cytokinin (BAP and TDZ), dose of cytokinin (0, 5, 10 and 20 µM), duration of exposure (2-6 days) during seedling pretreatment and the type of medium (M1, M2 and M3) used for shoot multiplication to select the best media for maximum shoot multiplication and elongation. The effect of subculture passages was also examined with optimal concentration of TDZ (10 µM). The shoot multiplication and elongation were evaluated by the same manner described before. All experiments were set up in a completely randomized design and repeated thrice with a minimum of twenty replicates employed for each treatment. The data were subjected to analysis of variance (ANOVA) to detect significant difference between means. Significant differences were compared manually using Duncan's multiple range test at P=0.05.

3.2.4 *Agrobacterium* strain, binary plasmid and bacterial culture

A. tumefaciens strain EHA105 harboring the binary vector pCAMBIA 2301 (Fig. 3.2) was used for transformation. The T-DNA of pCAMBIA2301 includes neomycin phosphotransferase gene (*npt*II) and β-glucuronidase gene (*gus*) interrupted by catalase intron, both driven by the cauliflower mosaic virus (CaMV) 35S promoter. A single colony of the *A. tumefaciens* was inoculated into 25 ml of liquid AB minimal medium (Chilton et al. 1974) with 5 mg/l of rifampicin and 25 mg/l kanamycin and grown overnight at 28^{0}C at 180 rpm until the O.D. of the culture reached to 0.8 at 600 nm. For infection, the cells were collected by centrifuging at 5,000 rpm for 5 min and the pellet was resuspended in liquid cocultivation medium (LCM). LCM is defined as MSB_5 medium containing 1 µM BAP, 3% sucrose, with pH adjusted to 5.5 and supplemented with 100 µM acetosyringone.

3.2.5 Optimization of transformation amenable regeneration procedure

Thirty to forty explants each derived from seedlings pretreated with different concentration (0, 5, 10 and 20 µM) of TDZ or BAP for varied duration (2, 4 and 6 days), were injured with a hypodermic needle, and inoculated with the bacterial suspension for 30 min by occasional shaking. The explants were then blotted dry on sterile filter paper. After infection, they were cocultivated in petridishes lined with filter paper moistened with LCM supplemented with 100 µM acetosyringone, for 3 days in dark condition at 22^0C. A number of crucial parameters associated with preconditioning of source seedlings and regeneration media were evaluated to examine their effect on transformation efficiency and recovery of transformed shoots in cowpea. The parameters included the type and dose of cytokinin, the duration for seedling preconditioning and the regeneration medium used during selection of transformed shoots. Each of these experiments was repeated thrice on independent days, keeping two replicates per experiment.

Following cocultivation, the explants were washed with sterile distilled water containing 100 mg/l cefotaxime, blotted dry, and transferred to three kinds of regeneration medium (M1, M2 and M3) differring in dose of BAP and kinetin and their combinations in shoot multiplication medium. All of the three regeneration media contained 150 mg/l kanamycin and 500 mg/l cefotaxime. Both regeneration controls (no cocultivation, no selection) and transformation controls (no cocultivation but selection on kanamycin) were used for all the experiments and at all cycles of selection. The cultures were transferred to fresh selection medium at an interval of 5, 7 and 8 days. After 20 days of culture on selection, the proliferating kanamycin resistant shoots (>1.5 cm) were transferred to rooting medium.

In order to evaluate the rooting efficiency of kanamycin resistant shoots, they (1.5-2 cm) were transferred to MS basal medium supplemented with different concentrations of IBA (0, 0.5, 1.5, 2.0, 2.5 and 5 µM) for root induction. All the root induction media contained

500 mg/l cefotaxime. The kanamycin resistant shoots cultured on MS medium containing 500 mg/l cefotaxime were treated as control. Well-rooted transformed plantlets were washed thoroughly in running tap water and acclimatized and maintained in greenhouse as per the procedure described earlier.

3.2.6 Histochemical GUS assay

Histochemical GUS staining was performed to verify GUS activity in transgenic cowpea (Jefferson 1987). Transient expression was examined after 3-days of cocultivation (Solleti et al. 2008a). Transient expression of GUS was scored on a per explant basis by estimating the number of blue foci visible on the axillary region of each cotyledonary node explant. The blue foci were discrete areas of cells with GUS activity. Stable gus expression was detected in various plant parts following the described histochemical procedure.

The efficiency of transient transformation was calculated as number of explants showing GUS expression at regenerating site and stable transformation as the number of PCR (polymerase chain reaction) positive shoots obtained on kanamycin selection per inoculated explants.

3.2.7 Molecular analysis

Molecular characterization of the transformants was carried out by PCR, Southern hybridization, and RT-PCR analyses for confirmation of the presence, integration, expression and inheritance of the introduced genes. Genomic DNA was isolated from the young leaves of T_0 putative transformants and T_1 transgenic plants using the modified CTAB method (Solleti et al. 2008a). PCR amplification was carried out with gene specific primers for *npt*II and *gus* using genomic DNA from putative transformed plants, non-transformed control plants (negative control) and pCAMBIA2301 (positive control) as templates. The 540 bp region of *npt*II and 580 bp coding region of *gus* were amplified using respective 20 mers (*npt*II Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAG GCTATTCGGCTA) and 18 mers

(*gus* Fw: CTGTGGGCATTCAGTCTG; Rv: ACGCTGACATCACCATTG)–oligonucleotide primers. The amplification reaction was carried out under the following conditions: 94^{0}C for 5 min (1 cycle), 94^{0}C for 1 min (denaturation), 58^{0}C for 1 min (annealing), 72^{0}C for 1 min (extension) for 35 cycles followed by the final extension at 72^{0}C for 7 min (1 cycle). PCR was performed using ~100 ng of purified genomic DNA and Taq DNA polymerase (Genei, Bangalore, India) according to manufacturer's instruction. The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

Randomly selected PCR-positive T_0 transgenic cowpea plants were further analyzed by Southern hybridization for the integration of the *npt*II gene. 10 µg samples of genomic DNA from non-transformed control and transgenic plants were digested with *Eco*RI. The digested samples were fractioned on a 0.8% agarose gel and transferred to Zeta- Probe membrane (Bio-Rad, USA). The blot was hybridized with DIG-labeled 540 bp PCR product, corresponding to the coding region of *npt*II gene. The probe labeling and Southern hybridization were performed using the nonradioactive DIG Labeling and Detection system (Roche, Germany) following supplier's instructions. Pre-hybridization and hybridization were carried out using high hybridization buffer containing 5XSSC, 1% blocking solution, 0.1% (w/v) N-lauroyl sarcosine and 0.02% (w/v) sodium dodecyl sulfate. Washing and detection were performed according to the instruction of the DIG labeling and detection system (Roche Diagnostics, Mannheim, Germany).

For RT-PCR analysis, total RNA was isolated from the PCR-positive transgenic T_0 plants using Trizol Reagent (Invitrogen, USA) from 100 ng of leaf tissue according to the manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.5% denaturing agarose gel (Sambrook et al. 1989). RT-PCR was conducted using First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions.

PCR of the coding sequences of *npt*II gene in the cDNA was carried out using respective primers as described earlier. The house keeping gene *actin* was used as a control to indicate the amount of starting RNA. The sequences were designed from the *Jatropha curcas* actin gene (Accession no: HM044307.1). The primers were 5′-ATGAGCTTCGAGTTGCAC-3′ and 5′-ACCATCACCAGAATCCAG-3′ which gave a 200 bp product with cDNA. The PCR products were electrophoresed on 1.0% agarose gels, detected by ethidium bromide staining, and photographed through the Bio-Rad gel documentation instrument.

The leaves of T_1 transgenic plants generated from eight independent transformation events were analyzed for the presence of *npt*II and *gus* genes using PCR, as described earlier. Segregation patterns were analyzed with the Chi-square test ($\chi 2$) as described by Solleti et al. (2008b).

3.3 Results and discussion

The effect of cytokinin preconditioning of seedlings on shoot regeneration capacity and recovery of transgenic plant from cotyledonary node explants in cowpea were investigated using two different cytokinins at different doses in the induction phase of regeneration for different interval of time. A substantial amount of data were obtained by testing a total of 54 different treatments and generating different types of data; mean number of shoots per explant and mean shoot length. Comparisons of different types of seedling preconditioning treatments used during germination and different shoot multiplication medium compositions were made irrespective of the other parameters tested.

3.3.1 Comparison of different cytokinins with varying dose in seedling preconditioning on shoot regeneration

Cowpea seeds germinated on all the tested media within 2 days. Seeds cultured on MSB_5 medium germinated normally (Fig. 3.3 b). However, the seeds cultured on MSB_5 media supplemented with either TDZ or BAP (0, 5, 10 and 20 µM) germinated with thickened seedlings, stunted and enlarged cotyledons, swelled cotyledonary nodes, and thick and

short roots (Fig. 3.3c-e). Exposure to higher dose of TDZ or BAP caused increased swelling of hypocotyls (Fig. 3.3d, e). Prolonged exposure (6 days) to high dose of TDZ or BAP increased the length of the seedlings and induced the formation of secondary roots. No callus was formed on intact seedlings.

When eight different germination media were compared for shoot regeneration capacity, the medium containing no cytokinins was found the least effective (Fig. 3.4). The explants excised from seedlings preconditioned on medium supplemented with either TDZ or BAP showed significant increase in multiple number of shoots irrespective of the shoot multiplication medium used (Fig. 3.4a-f). Seedling preconditioning in either TDZ or BAP enhanced mean shoot number by 2 to 4 fold. Nevertheless, TDZ as compared to equimolar dose of BAP exerted 1.5 to 2 fold increase in mean shoot number. Among the different dose of TDZ and BAP tested for seedling preconditioning, maximum mean number of shoots (7.1 shoots) and also maximum shoot length (2.6 cm) were induced from explants, derived from the seedlings preconditioned with 10 μM TDZ (Fig. 3.4c, 3.5c). A further increase in TDZ dose (20 μM) resulted in reduction in mean shoot number and shoot length (Fig. 3.4, 3.5). Moreover, the shoots appeared as a cluster of leafy structures which failed to develop further. On the other hand, seedlings preconditioned with 20 μM BAP induced the lowest number of shoots (Fig. 3.4). Consequently, seedlings preconditioned with 10 μM TDZ was used for all experiments for optimal multiple shoot induction.

3.3.2 Comparison of different duration of seedling preconditioning on shoot regeneration

When three different seedling preconditioning durations were compared, for shoot regeneration capacity, it was observed that 4 days preconditioning induced maximum mean shoot number developing shoots irrespective of cytokinin type used for preconditioning and shoot multiplication medium tested (Fig. 3.4, 3.5c, d). Longer duration of seed preconditioning (6 days) in either TDZ or BAP showed no significant improvement in mean

number of shoot (Fig. 3.4e, f). Therefore, seed preconditioning for 4 days in 10 μM TDZ was considered as optimal for induction of multiple shoots and used in all subsequent regeneration studies.

3.3.3 Comparison of different regeneration media on shoot regeneration

The shoot proliferation was observed at the axils of the cotyledonary node explants (Fig. 3.6a) while their basal end formed non-friable green callus (Fig. 3.6b). With respect to the regeneration capacities of the explants of preconditioned seedlings cultured on three different regeneration media, the medium containing no cytokinin was found the least productive in multiple shoot formation. In terms of both mean number of shoots per explant and frequency of explants developing shoots, it was observed that the medium which contained a combination of 5 μM BAP and 0.5 μM kinetin, produced highest mean number of shoots per explants (7.1 shoots) (Fig. 3.4c; 3.6d) as compared to medium with either 5 μM BAP or a combination of 5 μM BAP and 0.1 μM kinetin. The mother explants recultured on the fresh medium induced formation of new shoots (Table 3.1; Fig. 3.6c), which could be further elongated on culturing isolated shoots on fresh regeneration medium. The mother explants induced new shoots on reculturing for two consecutive times (Table 3.1). However, further reculture of the explants failed to induce shoots. The shoots excised and elongated during successive subculture on the fresh media, formed well developed roots on MS basal medium containing within 2 weeks (Fig. 3.6e).

Following transfer to soil, plantlets were successfully hardened with 94% survival. The regenerated plants were apparently morphologically normal and flowered normally and set seeds (Fig. 3.6f). The entire procedure from seed germination to establishment of plants under greenhouse conditions took approximately 7 weeks.

In this study, it was observed that cowpea explants from seedlings (pretreated in medium with 10 μM TDZ) were significantly more productive for shoot formation, 1.5 to 2.0

fold higher than seedlings pretreated in medium with equimolar dose of BAP and 2 to 4 fold higher than untreated seedlings. Our results are in agreement with previous report of higher competition in diverse cultivars of recalcitrant rose to form adventitious shoots in the result of preculturing on the medium containing TDZ instead of BAP (Kucharska and Orlikowska 2009). These results were consistent with the findings in recalcitrant sugarbeet (Gurel et al 2011; Zhang et al. 2001) wherein the petiole explants taken from donor plants pretreated with BAP were the most prolific in regenerating shoots whereas those explants excised from non-pretreated seedlings (i.e., seeds were germinated on hormone-free medium) produced no shoots at all. Sorvari et al. (1993) reported the increase in regeneration capacity by conditioning of donor cultures of strawberry on media containing BAP and IBA in comparison to the media without growth regulators. However, contrasting observation on considerably better results of preculturing the *Castanea sativa* donor shoots in equimolar dose of BAP were recorded as compared to preculturing in TDZ (San-Jose et al. 2001). The histological documentation presented by the authors indicated that explants precultured on BAP medium have in their shoot buds more meristematic cells from which shoot primordia were formed. However, TDZ plays an important role in adventitious regeneration and has been recommended for use in the induction stage for many plants, including roses (Kucharska and Orlikowska 2009; Ibrahim and Debergh 2001; Dubois and De Vries 1997; Rosu et al. 1995), blueberry (Meng et al. 2004), pear (Lane et al. 1998), apple (Sriskandarajah and Goodwin 1998), gerbera (Orlikowska et al. 1999), safflower (Orlikowska and Dyer 1993) and blackberry (Swartz et al. 1990).

In accordance with this theory, we suggest that the cowpea cotyledonary node explants obtained from seedlings germinated in medium with TDZ and then further grown on medium containing BAP might have gained a kind of competence at an earlier stage of development, so that their cells can be more readily directed into shoot induction. The

beneficial effect of TDZ was not only recorded significant in the first regeneration passage but once induced activity for organogenesis was found consistent in the subsequent passages upto 3 passages (Table 3.1).

3.3.4 Effect of seedling preconditioning on frequency of transformation

All the explants showed GUS activity following 3 days of co-cultivation with EHA105 harboring pCAMBIA2301, predominantly in the regenerating sites (Fig. 3.7b) which was not observed in untransformed control explants (Fig. 3.7a). The effect of cytokinin type, dose and duration of exposure during seedling preconditioning on transformation rate was compared on the basis of both transient transformation efficiency of explants and generation of kanamycin resistant transformed shoots. Explants of TDZ preconditioned seedlings showed significantly higher transient transformation efficiency as compared to that of explants of BAP preconditioned seedlings (Fig. 3.8). Among all the preconditioning treatments tested, explants from seedlings preconditioned in 10 µM TDZ for 4 days showed maximum transient transformation efficiency (Fig. 3.8a).

The frequency of explants regenerating shoots on kanamycin selection in *Agrobacterium*-inoculated explants was significantly higher (38.9% than BAP) in explants of seedlings preconditioned in 10 µM TDZ for 4 days and cultured on medium containing a combination of 5 µM BAP and 0.5 µM kinetin (Table 3.3). Almost all (92%) transformed shoots were phenotypically normal and elongated and rooted earlier (6 weeks) than those obtained with explants from seedlings in absence of preconditioning, on kanamycin free MS medium containing IBA (Fig 3.7e). Among different concentration of IBA tested for root induction from kanamycin resistant transformed shoots, 2.5 µM IBA induced maximum rooting efficiency (96.4%). Furthermore, the maxium number of roots from a single shoot (4.8) and average root length (6.4) was obtained when the kanamycin resistant shoots were cultured on 2.5 µM IBA containing rooting medium (Table 3.2).

3.3.5 Molecular analyses of transformants

The presence of the *npt*II and *gus* genes in putatively transformed T_0 plants was confirmed by PCR detection of the expected 540 bp and 580 bp amplification products, respectively (Fig. 3.9 a,b). A strong, uniform and stable *gus* expression was detected in leaf and flower of PCR-positive T_0 plants and no endogenous *gus* expression was detected in the tissues of control plants (Fig. 3.7g-j).

5 PCR-positive T_0 transformed plants (lines 1–5) were randomly selected for Southern blot analysis to confirm the integration of *npt*II gene. Hybridizations of DIG-labeled *npt*II probe to total genomic DNA digested with *Eco*RI were expected to identify DNA fragments unique to individual integration events greater than 2.1 kb (Fig. 3.9c). As shown in Fig. 3.9c, Southern blot analysis using the *npt*II gene as a probe revealed that all the 5 randomly selected T_0 transgenic plants were found positive for *npt*II gene integration and furthermore, they showed differential integration events, confirming that these plants were derived from independent transformation events (Fig. 3.9c, lanes 1, 2, 3, 4 and 5). The T_0 transgenic plants exhibited simple hybridization patterns that ranged from single integration event to 3 loci and, in general, most fragments were greater than 2.1 kb (Fig. 3.9c). No amplification was detected in the control untransformed plants (Fig. 3.9c, lane C).

Semi-quantitative RT-PCR analysis was performed with six PCR positive T_0 transgenic lines as well as with untransformed control plants. Expression of *npt*II and *gus* gene was confirmed by the presence of a band at the expected size of 540 bp and 580 bp in the PCR products of transgenic lines, while untransformed control plants showed no amplification (Fig. 3.9d). Furthermore, the amplification of the *npt*II sequence from plant cDNA templates in RT-PCR ruled out the possibility of *Agrobacterium* contamination. Both transformed and untransformed plants showed expression of reference gene *actin* as

indicated from amplification of 200 bp (Fig. 3.9d). There were no significant differences in the levels of gene expression.

Based on the GUS histochemical assay of kanamycin resistant shoots, 95.7 % of the shoots expressed GUS (Fig. 3.10), thus demonstrating high transformation efficiency. The transformation rate was significantly increased when donor seedlings were preconditioned (in medium with 10 μM TDZ for 4 days), from 0.6% to 2.1% as compared to untreated donor seedlings (Table 3.3).

The seeds from T_0 generation were advanced to T_1 generation and the T_1 transgenic lines generated from 8 independent transformation events were analyzed for the segregation pattern of *npt*II by PCR analysis. Presence of the expected 540 bp amplified product corresponding to *npt*II in T_1 transgenic lines confirmed the inheritance of *npt*II gene. The segregation pattern of these selected transgenic events showed typical 3:1 Mendelian ratio as expected for single dominant gene inheritance (Table 3.4).

3.5 Conclusion

This is the first report on the efficiency of *Agrobacterium*-mediated genetic transformation of cowpea using TDZ as a growth regulator in the preconditioning of donor seedlings. Although it is rather difficult to compare the transformation efficiency obtained in this study with those reported by others, the high efficiency of TDZ-induced regeneration in both *in vitro* control culture and *Agrobacterium*-mediated genetic transformation further confirmed the effectiveness of donor seedling preconditioning. It has been reported that TDZ-induced regeneration is a manifestation of a metabolic cascade that includes an initial signaling event, accumulation, and transport of endogenous plant signals such as auxin and melatonin, a system of secondary messengers, and a concurrent stress response (Jones et al. 2007). Moreover, cytokinin-stimulation of cell division increases the numbers of cells entering the S/G2 phase of the cell cycle, thus promoting higher expression of homologous

recombination proteins such as Rad51 (Chen et al. 1997). Furthermore, cells undergoing active division are more likely to be targeted by a stable T-DNA integration event. An absolute requirement for the S-phase for transfer and/or T-DNA integration has been previously demonstrated in *P. hybrida* (Villemont et al. 1997; Thirukkumaran et al. 2009). An important role of active cell division in plant transformation is also supported by higher transformation efficiency observed in maize cells expressing a modified version of the viral replication-associated protein (RepA) that stimulates cell division (Gordon-Kamm et al. 2002). It can thus be hypothesized that the effects of TDZ on plant transformation are based on its combined influence on homologous recombination activity and cell division. However, the biochemical mechanisms underlying TDZ induced regeneration in plant cells have not been clearly elucidated (Jones et al. 2007).

Cowpea is known to be a recalcitrant grain legume with respect to genetic manipulation in *in vitro*, and the enhanced regeneration and transformation protocol described in this report is expected to contribute to the efforts of biotechnological improvement of cowpea by incorporation of candidate genes for biotic and abiotic stress tolerance, since the establishment of an efficient and reproducible regeneration protocol is a precondition for routine generation of transgenic plants with desirable traits in recalcitrant grain legumes.

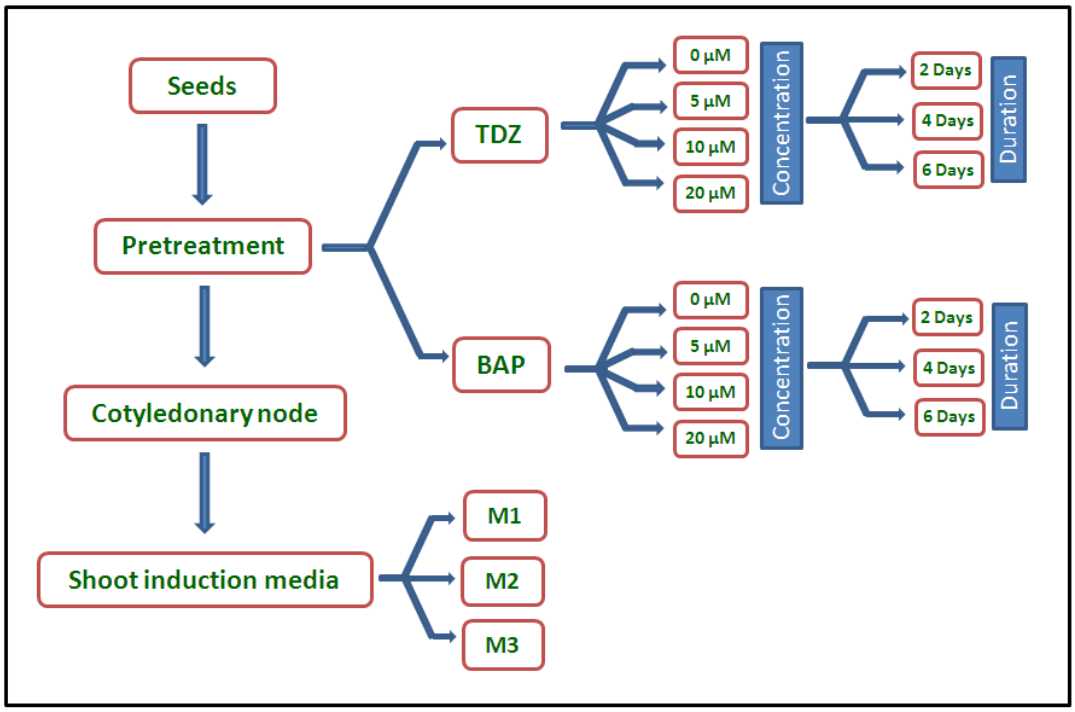


Fig. 3.1 Experimental design scheme of cowpea regeneration from pretreated cotyledonary node explant

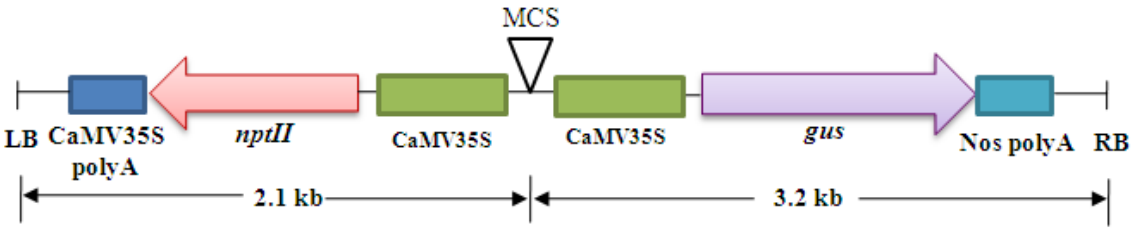


Fig. 3.2 T-DNA region (5.3 kb) of binary vector pCAMBIA2301. Abbreviations: RB, right border; LB, left border; 35S, CaMV 35S promoter or terminator; NOS, nopaline synthase terminator; intron-*gus,* intron interrupted *β-glucuronidase; nptII, neomycin phosphotransferase.*

Fig. 3.3 Cowpea seed germination. Cowpea seeds (a); Seedling germinated on MSB_5 medium (b) or MSB_5 medium containing 5 µM TDZ (c) or 10 µM TDZ (d) or 20 µM TDZ (e). Bar represents 5 mm (a); 1 cm (b); 1 cm (c); 1 cm (d) and 1 cm (e).

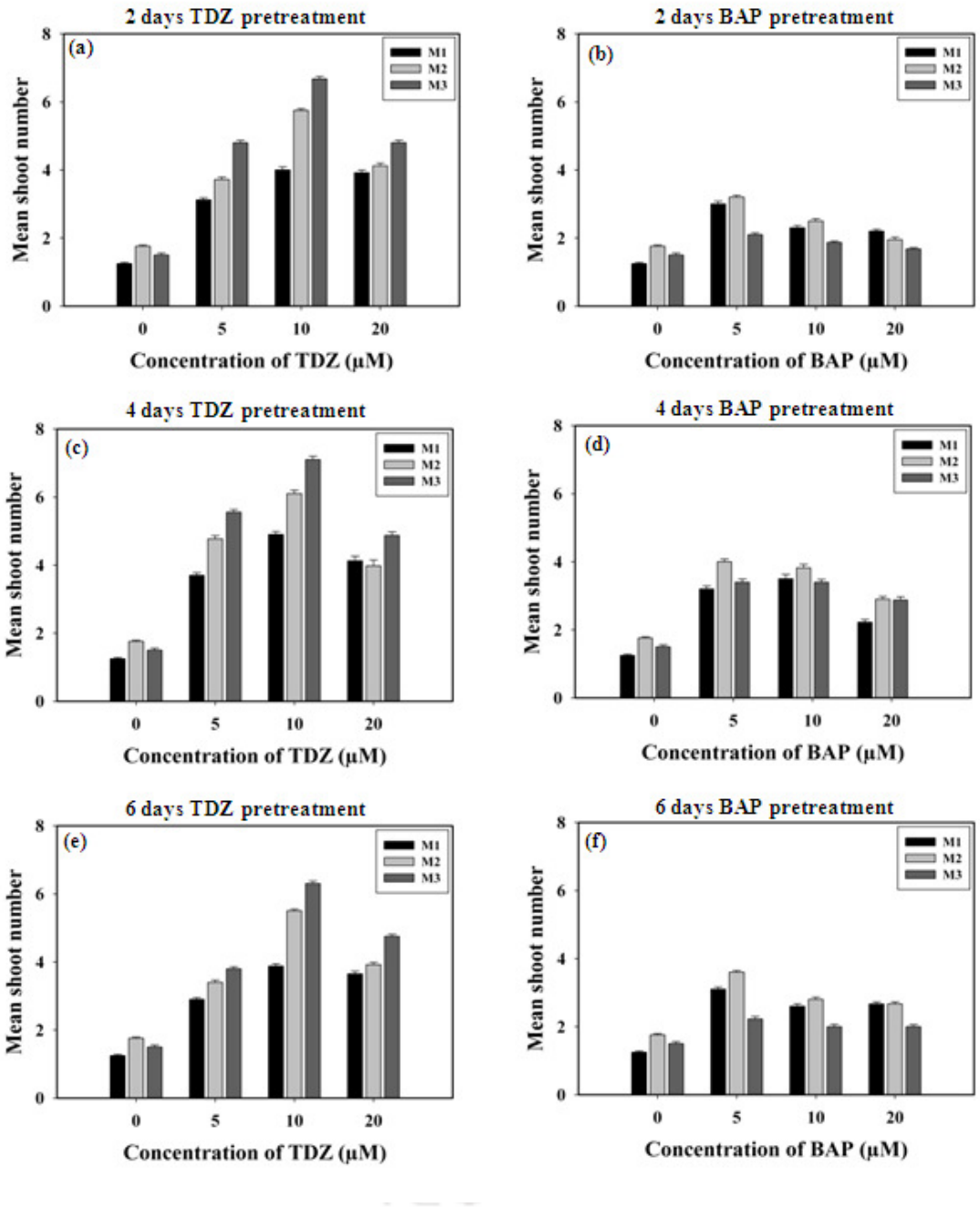


Fig. 3.4 Effect of seed pretreatment in the presence of different concentrations (5, 10, 20 µM) of TDZ and BAP, and the duration of pretreatment (2, 4, 6 days) on multiple shoot induction from cotyledonary node explants of cowpea (along X-axis: Concentration of TDZ or BAP in µM, along Y-axis: Mean shoot number).

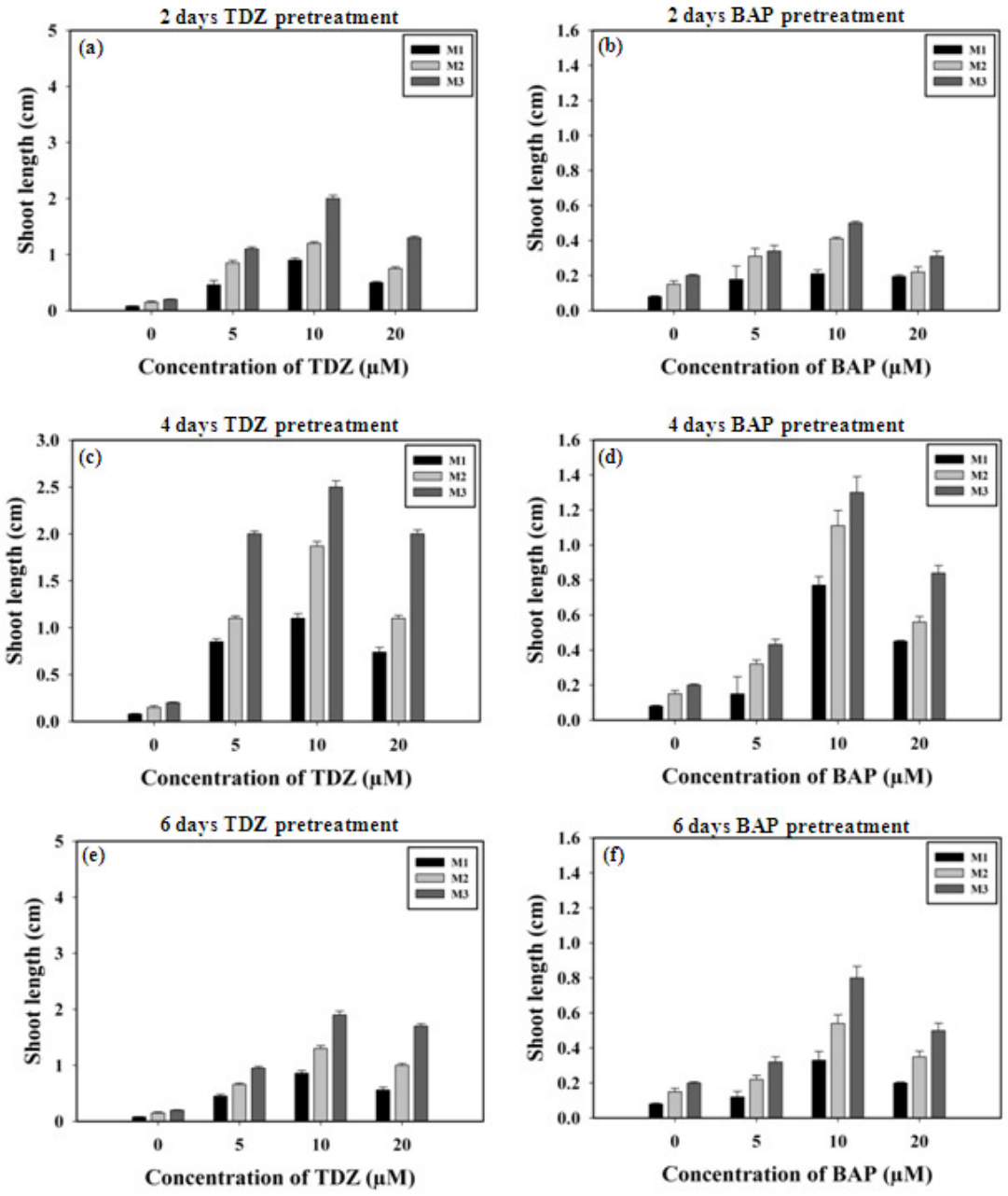


Fig. 3.5 Effect of seed pretreatment in the presence of different concentrations (5, 10, 20 µM) of TDZ and BAP, and the duration of pretreatment (2, 4, 6 days) on shoot length from cotyledonary node explants of cowpea (along X-axis: Concentration of TDZ or BAP in µM, along Y-axis: Shoot length).

Fig. 3.6 Multiple shoot induction and plant regeneration from cotyledonary node explants of cowpea (*Vigna unguiculata* L. Walp) cv. Pusa Komal. (a) Explant at the time of culture (Bar=1 mm). (b) Shoot induction from axils of explant within 1 week (Bar=7 mm). (c) Shoot proliferation from mother explants in subsequent reculture (Bar=5 mm). (d) Proliferation of multiple shoots within 4 weeks of culture (Bar=1 cm). (e) A rooted shoot after 2 weeks of culture (Bar=1 cm), (f) Plant acclimatized in greenhouse (Bar=10 cm).

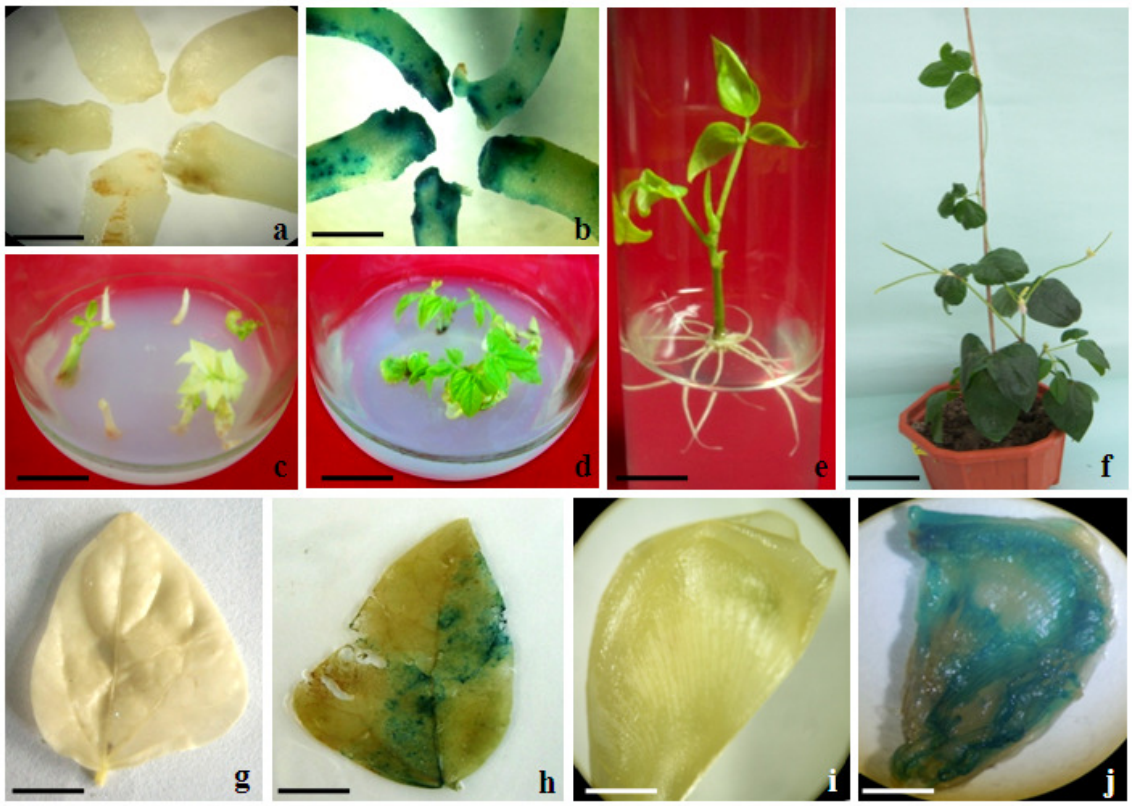


Fig. 3.7 Transient and stable gus expression and regeneration of transgenic plants. (a and b) Transient GUS expression, non-transformed (control) explants not showing GUS activity (a), cotyledonary node explants showing transient GUS activity after 3 days of co-cultivation (b). (c and d) Selection of transformed explants, (c) Untransformed explants were bleached on selection media; (d) Shoots were proliferated from transformed explants when cultured on selection media. (e) *In vitro* rooting of elongated transformed shoot, (f) Acclimatized plant maintained in transgenic greenhouse, (g) Non-transformed leaf, (h) Transformed leaf, (i) Non-transformed flower, (j) Transformed flower.

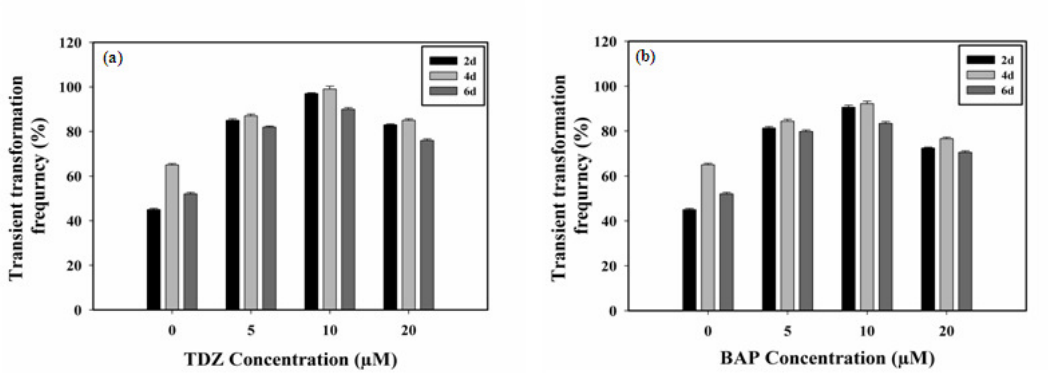


Fig. 3.8 Effect of pretreatment on transient transformation frequency. (a) Effect of different dose and duration of TDZ pretreatment on transient transformation frequency; (b) Effect of different dose and duration of BAP pretreatment on transient transformation frequency.

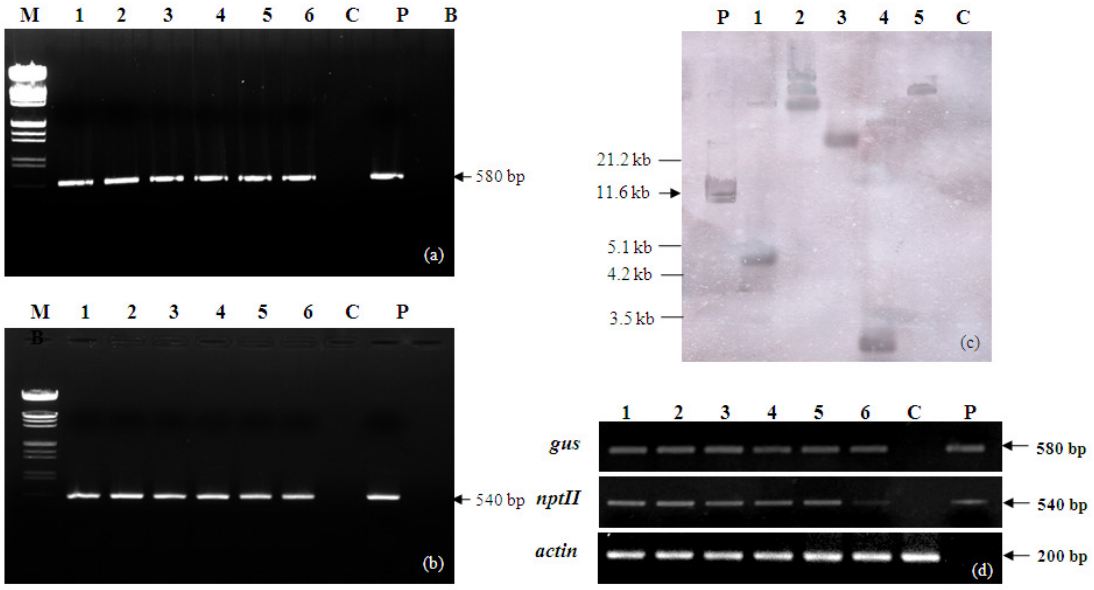


Fig. 3.9 Molecular analysis of T_0 transgenic plants. (a) PCR amplification of the 580 bp fragment of the *gus* gene, (b) PCR amplification of the 540 bp fragment of *npt*II gene. Lane M λDNA/*Eco*RI + *Hind*III marker, lane P pCAMBIA2301 plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lane B blank, lanes 1–6 DNA from independently transformed plants. (c) Southern blot hybridization analysis of junction fragments of five randomly selected PCR-positive T_0 lines. The plasmid and genomic DNA were digested with *Eco*RI, and hybridized with nptII probe. Lanes 1–5 genomic DNA from four T_0 lines, lane C genomic DNA from untransformed plant, lane P pCAMBIA2301/*Eco*RI. (d) RT-PCR analysis of *gus, nptII,* and reference gene *actin*, Lane M λDNA/*Eco*RI + *Hind*III marker, lane P pCAMBIA2301 plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lanes 1–6 T_0 transgenic plants

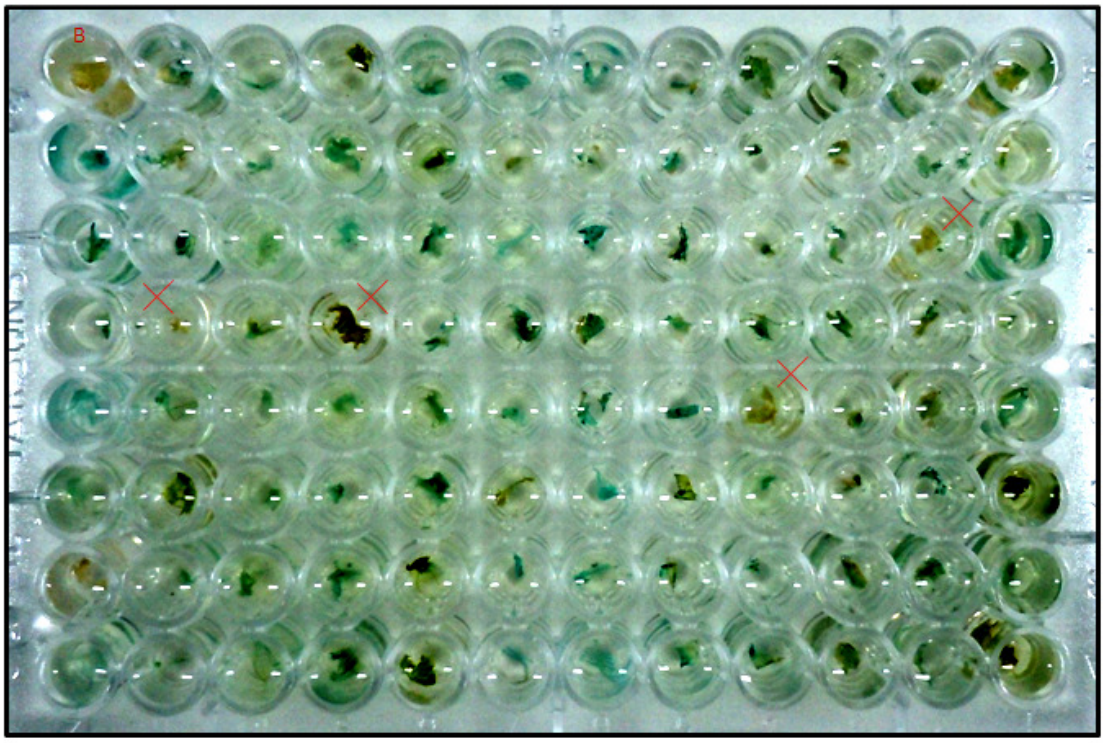


Fig. 3.10 Transient GUS expression in leaves from randomly selected 95 kanamycin resistance shoots; 91 shows blue staining and 4 lines (X) failed to show any blue staining. Line (B) signifies no *gus* expression in untransformed control leaf.

Table 3.1 Evaluation of morphogenetic potential of shoot cultures of cotyledonary nodes for four subculture passages on shoot multiplication medium[x]

Subculture passage	Mean number of shoots / explant	Shoot length increment (cm)
First	7.1 ± 0.54	0.94 ± 0.02
Second	6.0 ± 0.42	0.82 ± 0.04
Third	4.7 ± 0.76	0.41 ± 0.03
Fourth	1.8 ± 0.33	0.33 ± 0.03

[x]Shoot cultures were subcultured on MSB_5 medium containing MSB_5 medium supplemented with 5 µM BAP and 0.5 µM kinetin.

Values represent means ± SE

Means followed by the same letters are not significantly different by the Duncan's multiple range test at p=0.05

Table 3.2 Effect of different concentrations of IBA on root induction from kanamycin resistant transformed shoots[x] of cowpea after 2 weeks of culture

IBA (µM)	Rooting (%)	Mean number of roots (per shoot)	Average root length (cm)
0 (Control)	0	0	0
0.5	10.5 ± 1.1	1.2 ± 0.5	0.7 ± 0.07
1.5	30 ± 1.3	2.3 ± 0.43	2.5 ± 0.32
2.0	55 ± 1.3	2.8 ± 0.55	3.4 ± 0.92
2.5	96.4 ± 1.5	4.8 ± 0.73	6.4 ± 1.1
5.0	50 ± 1.2	3.1 ± 0.66	3.8 ± 0.42

[x]Kanamycin resistant transformed shoots were cultured on MS medium containing 500 mg/l cefotaxime and different concentrations of IBA.

Each treatment comprised of 20 kanamycin resistant shoots in three replicates.

Values represent means ± SE

Means followed by the same letters are not significantly different by the Duncan's multiple range test at p=0.05

Table 3.3 Comparative profile of transgenic plants of cowpea

Experimental condition	No of explants co-cultivated	Percentage of explants expressing GUS at regenerating sites[b] (%)	Frequency of explants regenerating shoots on kanamycin-containing media[c] (%)	Stable transformation efficiency[d] (%)
C	100	9.7	11.3	0.61
	95	10.5	15.3	0.59
Total[e]/ Average[f]	**195[d]**	**30.7[e]**	**13.3[e]**	**0.6[e]**
1	110	32.4	45.3	1.57
	105	35.5	39.2	1.69
Total[d]/ Average[e]	**215[d]**	**33.95[e]**	**42.25[e]**	**1.63[e]**
2	120	39.2	55.2	1.98
	125	38.8	62.2	2.23
Total[d]/ Average[e]	**245[d]**	**39[e]**	**58.7[e]**	**2.1[e]**

[a] Number of explants showing GUS expression per number of explants co-cultivated X 100

[b] Number of explants showing GUS expression at the regenerating sites per number of explants co-cultivated X 100

[c] Regeneration medium containing 150 mg/l kanamycin

[d] Number of T_0 plants PCR-positive for *nptII* divided by the total number of explants co-cultivated

C Transformation studies without pretreated seedlings

1 Transformation studies using BAP pretreated seedlings

2 Transformation studies using TDZ pretreated seedlings

Table 3.4 Segregation of *npt*II gene in T_1 progeny of transgenic cowpea plants

T_0 plants	Number of T_1 plants tested for *npt*II			χ^2 value	Expected Ratio
	Total	*npt*II +ve	*npt*II -ve		
L1	32	25	7	0.167	3:1
L2	29	22	7	0.011	3:1
L3	25	19	6	0.013	3:1
L4	24	20	4	0.889	3:1
L5	22	18	4	0.545	3:1
L6	32	23	9	0.167	3:1
L7	26	20	6	0.054	3:1
L8	34	32	2	0.008	15:1

Chapter 4

Preparation of binary constructs for overexpression of *Btcry1Ac* and *Btcry1Ab*

4.1 Introduction

Cowpea is one of the most important grain legumes in the tropics including Africa, which accounts for 64 percent of the world production (Mbene et al. 2000). Its global annual production stands at 7.6 million tonnes. In spite of the significance of cowpea as a food crop to millions of people on the continent, grain yields today remain low averaging 0.3 tonnes/ha due to several biotic and abiotic factors. Among these constraints are the pod borers, major lepidopteran insects which perennially damage cowpea pods on fields. The legume pod borer *Maruca vitrata* (Lepidoptera: Crambidae) is one of the key cowpea pest species apart from *Heliothis armigera*, as the larvae feed on the tender parts of the stem, peduncles, flower buds, flowers and pods (Singh and Jackai 1988), severe infestations may reduce yield by 90% (Murdock et al. 2001).

It is known that insect pests, especially lepidopterans, can be controlled by *Bacillus thuringiensis* (*Bt*) – an ubiquitous, soil-dwelling, spore-forming bacterium – when applied topically on crops as spore formulations. Unfortunately, *Bt* sprays are often washed away by rain, degrade under solar ultra violet radiation and are not optimally targeted against certain insect pests that live within plant tissues. The limitations associated with the use of conventional methods in effectively dealing with cowpea's insect pest problem makes the application of biotechnological procedures for overcoming the constraints to cowpea production particularly promising. Transgenic approach envisages incorporation of candidate genes in cowpea that encode expression of *Bt* crystal proteins, thus providing to the plant built-in protection against lepidopteran insects such as *Maruca* pod borer through the conceptual framework of genetic transformation.

Artificial diet bioassays at International Institute of Tropical Agriculture have shown that Cry1Ab, Cry1C, and CryIIA proteins are toxic to *Maruca* pod borer (Machuka 2002). The efficacy of different Cry proteins against second instar larvae of legume pod borer showed

that the toxin Cry1Ab was the most potent toxin (LC_{50} 0.207 ppm) followed by Cry1Ac (Srinivasan 2008). Overexpression of Bt*cry1Ab* in transgenic pigeon pea (Sharma et al. 2006) and soybean (Dufourmantel et al. 2005) has conferred complete protection against pod borer, *Helicoverpa armigera* and velvetbean caterpillar (*Anticarsia gemmatalis*) respectively. Expression of truncated native Bt *cry1Ac* gene in chickpea has shown effective resistance in transgenic plants to the major pod borer insects (Sanyal et al. 2005). However, development of transgenic crops being a long lasting and high investment option a major threat for the long lasting effect of such crops is the potentiality of the insects to develop resistance against such crops. Therefore, it is envisaged introduction of *Btcry1Ac* and *Btcry1Ab* in transgenic plants could be a potentially more viable strategy for controlling *Helicoverpa armigera* and *Maruca vitrata.*

The most efficient system for gene transfer into plants is *Agrobacterium*-mediated transformation that exploits the natural ability of the soil microorganism to transform wide range of plant species (Uranbey et al. 2005). The candidate gene(s) to be transferred *via* bacterial Ti plasmid are cloned between the borders of its T-DNA region. The majority of binary vectors harbor an origin of replication from a broad host range plasmid that ensures replication in both *Escherichia coli* (cloning procedure) and *Agrobacterium* (transformation of plants).

In order to drive efficient transgene expression in transgenic cowpea, it is essential to use a promoter that regulates gene expression to sufficient levels in tissues of interest. Although several reports described inducible promoters or tissue specific promoters that confer high levels of expression of *Btcry* genes in transgenic plants, the majority of crop plant constructions for insect-pest or disease resistance employ a constitutive promoter from cauliflower mosaic virus (CaMV). The CaMV promoter is preferred above other

potential promoters because it is a more powerful promoter compared to others and is not greatly influenced by environmental conditions or tissue types.

Here, in the present work, the overexpression constructs of *Btcry1Ac* and *Btcry1Ab* are prepared for their subsequent use in developing transgenic cowpea for resistance to pod borers.

4.2 Materials and methods

4.2.1 Cloning of *Btcry1Ac* in a plant binary vector

The standard binary vector pCAMBIA2301 (CAMBIA, Australia) was used to sub clone synthetic Bt*cry1Ac* gene expression cassette between the T-DNA borders of the binary vector. 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 expression cassette of Bt*cry1Ac* is under control of (CaMV) 2X35S promoter and Nos terminator, released as a 2.94 kb *Eco*RI/ *Hind*III fragment from 1AcPRD vector (Valderrama et al. 2007).

The plasmids, pCAMBIA2301 and p1AcPRD were isolated and digested with *Eco*RI/*Hind*III restriction enzyme. Digested p1AcPRD sample was separated in 0.8% agarose gel and the resulting fragment carrying the expression cassette was eluted using GenElute™ Gel Extraction Kit (Sigma-Aldrich), and the *Eco*RI/*Hind*III digested pCAMBIA2301 vector was also purified using the GenElute™ Gel Extraction Kit (Sigma-Aldrich). T4 DNA ligase (NEB enzyme) was used to ligate the *Btcry1Ac* gene expression cassette to linearized pCAMBIA2301 and the ligated product was transformed to *E. coli* DH5α. The recombinant clones were confirmed by Polymerase chain reaction using 24 mers (*Btcry1Ac* Fw: CCCAGAAGTTGAAGTACTTGGTGG; Rv: CCGATATT GAAGGGTCTTCTGTAC). The amplification reaction was carried out under the following conditions: 94^0C for 5 min (1 cycle), 94^0C for 1 min (denaturation), 58^0C for 1 min (annealing), 72^0C for 1 min (extension)

for 35 cycles followed by the final extension at 72^{0}C for 7 min (1 cycle). PCR was performed using ~100-200 pg of purified plasmid DNA and Taq DNA polymerase (Genei, Bangalore, India) according to manufacturer's instruction. The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989). Furthermore, the clones are additionally confirmed by restriction digestion with *Eco*RI/*Hind*III. The resulting Bt*cry1Ac* over expression construct was designated as pSouv:cry1Ac (Fig. 4.1). Standard molecular biology techniques were used for all DNA manipulations (Sambrook et al. 1989).

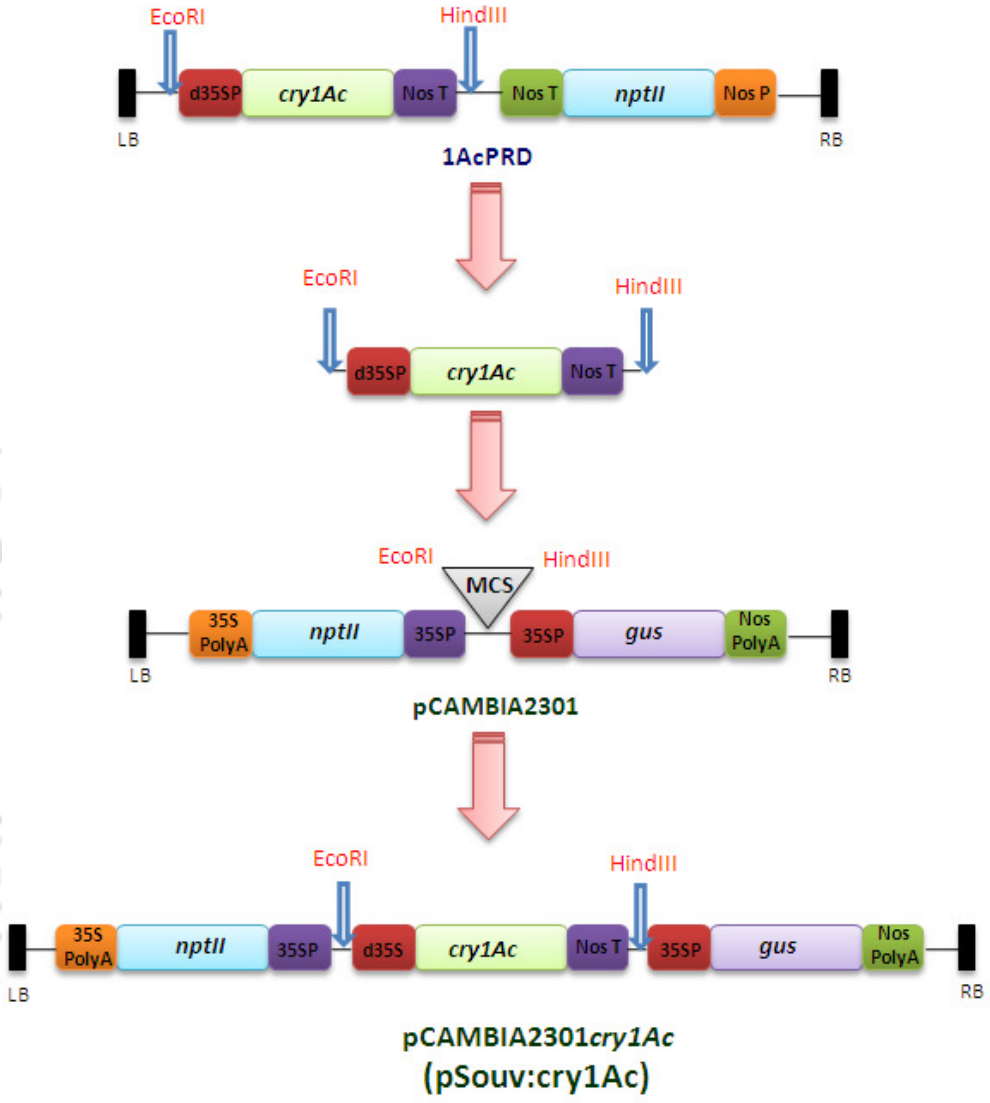


Fig. 4.1 Cloning strategy of *Btcry1Ac* in T-DNA overexpression vector pCAMBIA2301

4.2.2 Mobilization of pSouv:cry1Ac plant binary construct to *Agrobacterium tumefaciens*

The construct pSouv:cry1Ac was mobilized to *A. tumefaciens* hypervirulent strain EHA105 (Hood et al. 1993) by triparental mating (Bevan 1984) and the integrity of region of *cry1Ac, nptII* and *gus* within the *Agrobacterium* cells was confirmed through colony PCR using respective *Btcry1Ac, npt*II and *gus* gene specific oligonucleotide primers. The amplification reaction was carried out following conditions described in the previous section. The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

4.2.3 Cloning of *Btcry1Ab* in a plant binary vector

The full length plant codon optimized *Bt cry1Ab* ORF (1.86 kb) was amplified from the plasmid pUBB using primers corresponding full length *Btcry1Ab* with restriction sites *Xho*I and *Kpn*I added at its 5' and 3' nucleotide ends respectively. The amplified *cry1Ab* fragment was cloned in the intermediate vector pRT101 in between a 35S promoter and 35S terminator sequence.

The *Btcry1Ab* expression cassette (35S:*Btcry1Ab:*polyA) was amplified from pRT101*cry1Ab* using M13 universal primers 16 mers (*M13* Fw: GTAAAACGACGGCCAG; Rv: CAGGAAACAGCTATGAC). The amplification reaction was carried out under the following conditions: 94^0C for 5 min (1 cycle), 94^0C for 1 min (denaturation), 47^0C for 1 min (annealing), 72^0C for 1 min (extension) for 35 cycles followed by the final extension at 72^0C for 7 min (1 cycle). The amplified fragment was PCR purified and subsequently digested with *Pst*I. The *Pst*I digested fragment was ligated into the *Pst*I site of plant binary vector pCAMBIA2301 resulting in pCAMBIA2301*cry1Ab,* designated as pSouv:cry1Ab (Fig. 4.2). The recombinant clones were confirmed by polymerase chain reaction for the presence of *cry1Ab* gene using 21 bp full length gene specific primers (5'-CCCAGAAGTTGAAGTACTTGG-3' and 5'-CCGATATTGAAGGGTCTTCTG-3'). The amplification reaction was carried out

under the following conditions: 94^{0}C for 5 min (1 cycle), 94^{0}C for 1 min (denaturation), 60^{0}C for 1 min (annealing), 72^{0}C for 1 min (extension) for 35 cycles followed by the final extension at 72^{0}C for 7 min (1 cycle). Furthermore, the clones are additionally confirmed by restriction digestion with *Pst*I.

4.2.4 Mobilization of pSouv:cry1Ab plant binary construct to *Agrobacterium tumefaciens*

The construct pSouv:cry1Ab was mobilized to *A. tumefacience* hypervirulent strain EHA105 (Hood et al. 1993) by triparental mating (Bevan 1984) and the integrity of region of *cry1Ab, nptII* and *gus* within the *Agrobacterium* cells was confirmed through colony PCR using respective *Btcry1Ab, npt*II and *gus* gene specific oligonucleotide primers. pSouv:cry1Ab was used as a positive control. The amplification reaction was carried out following conditions described in the previous section. The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

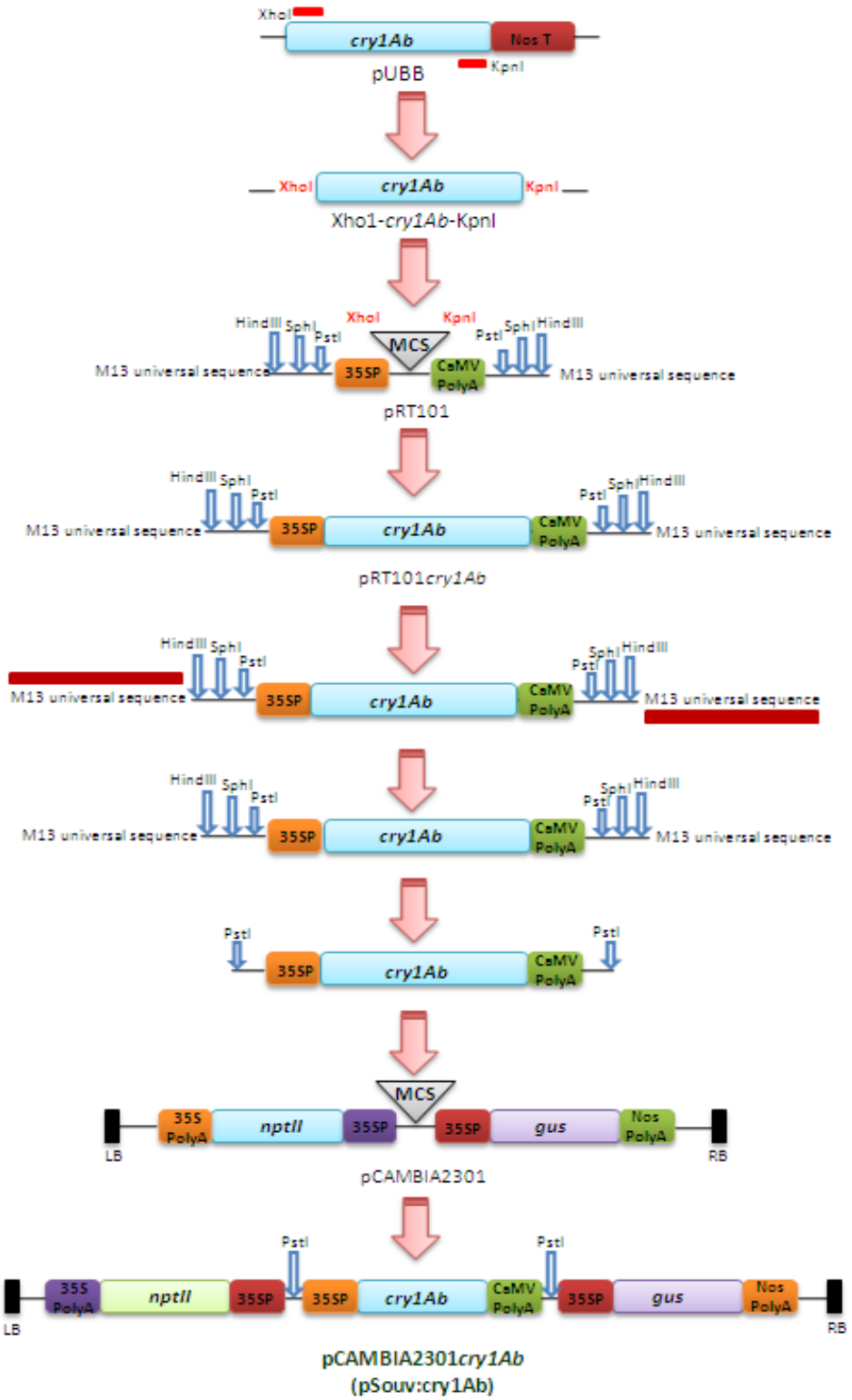


Fig. 4.2 Cloning strategy of *Btcry1Ab* in T-DNA overexpression vector pCAMBIA2301

4.3 Results and discussion

4.3.1 Confirmation of *Btcry1Ac* in plant binary construct

The plant binary vector pCAMBIA2301 was digested with *Eco*RI/ *Hind*III and 11.6 kb linearised plasmid was used as a vector for the cloning (Fig. 4.3A). Similarly 1AcPRD was digested with *Eco*RI/*Hind*III and 2.9 kb of Btcry1Ac expression cassette was released which was used as an insert for the cloning it in pCAMBIA2301 (Fig. 4.3B). After ligation, the recombinant clones were confirmed by PCR using gene specific primers for cry1Ac. The internal fragment of 1.0 kb was amplified which confirmed the presence of *cry1Ac* gene in all the recombinant clones (Fig. 4.3C). The pSouv:cry1Ac plasmid was isolated from recombinant clones and additionally confirmed by restriction digestion. The expression cassette (2.9 kb) was released from vector backbone (11.6 kb) upon digestion with *Eco*RI/*Hind*III (Fig. 4.3D).

4.3.2 Confirmation of *Btcry1Ab* in plant binary construct

The 1.86 kb fragment of *Btcry1Ab* was amplified from pUBB plasmid (Fig. 4.4A) and cloned in TA cloning vector, pGEMTeasy. The ligated clones were confirmed by restriction digestion where the 1.86 kb *cry1Ab* fragment was released from 3 kb vector backbone upon digestion with *Xho*I/ *Kpn*I (Fig. 4.4B). However, the 1.86 kb *Xho*I/ *Kpn*I digested product (Fig. 4.5A) was cloned in *Xho*I/*Kpn*I digested intermediate vector pRT101 (Fig. 4.5B). The ligated clones were confirmed by PCR as well as restriction digestion. The amplification of 1.86 kb was confirming the presence of *cry1Ab* gene in ligated clones (Fig. 4.5C). The release of 1.86 kb fragment of *cry1Ab* gene from the vector backbone (3.3 kb) upon digestion with *Xho*I/*Kpn*I (Fig. 4.5D) was further confirmed the ligated clones.

The 2.5 kb of expression cassette of *cry1Ab* (35S:*Btcry1Ab:*polyA) was amplified from the recombinant clone pRT101cry1Ab (Fig. 4.6A). The amplified fragment was PCR eluted and digested with *Pst*I (Fig. 4.6B) and further cloned in plant binary vector

pCAMBIA2301, linearized with *Pst*I (Fig. 4.6C). The recombinant clones were confirmed by colony PCR using gene specific *cry1Ab* primer which amplified 1.86 kb fragments from the ligated clones (Fig. 4.6D). The clones were further confirmed by restriction digestion with *Pst*I. 2.5 kb fragment of the *cry1Ab* expression cassette was successfully released from 11.6 kb of vector backbone of pCAMBIA2301 (Fig. 4.6E).

4.3.3 Confirmation of *Agrobacterium tumefaciens* transconjugants

Both the plant binary constructs pSouv:cry1Ac and pSouv:cry1Ab were successfully mobilized to *A. tumefacience* hypervirulent strain EHA105 and mobilization was confirmed by colony PCR. The amplification of 540 bp, 580 bp and 1 kb fragments from EHA105pSouv:cry1Ac clones were confirming the presence of *nptII, gus* and *cry1Ac* genes respectively (Fig. 4.7). Similarly, the amplification of 540 bp, 580 bp and 1.86 kb fragments from EHA105pSouv:cry1Ab clones were confirming the presence of *nptII, gus* and *cry1Ab* genes respectively (Fig. 4.18).

4.4 Conclusions

In conclusion, the binary overexpression constructs for *Btcry1Ac* and *Btcry1Ab* were prepared. The restriction digestion pattern and PCR confirmed the cloning. The constructs were mobilized into *A. tumefacience* vir helper strain EHA105 and the transconjugants were confirmed by PCR. The strains could be used for routine transformation of cowpea for introduction and expression of cry1Ac and cry1Ab for developing transgenic plants for insect resistance.

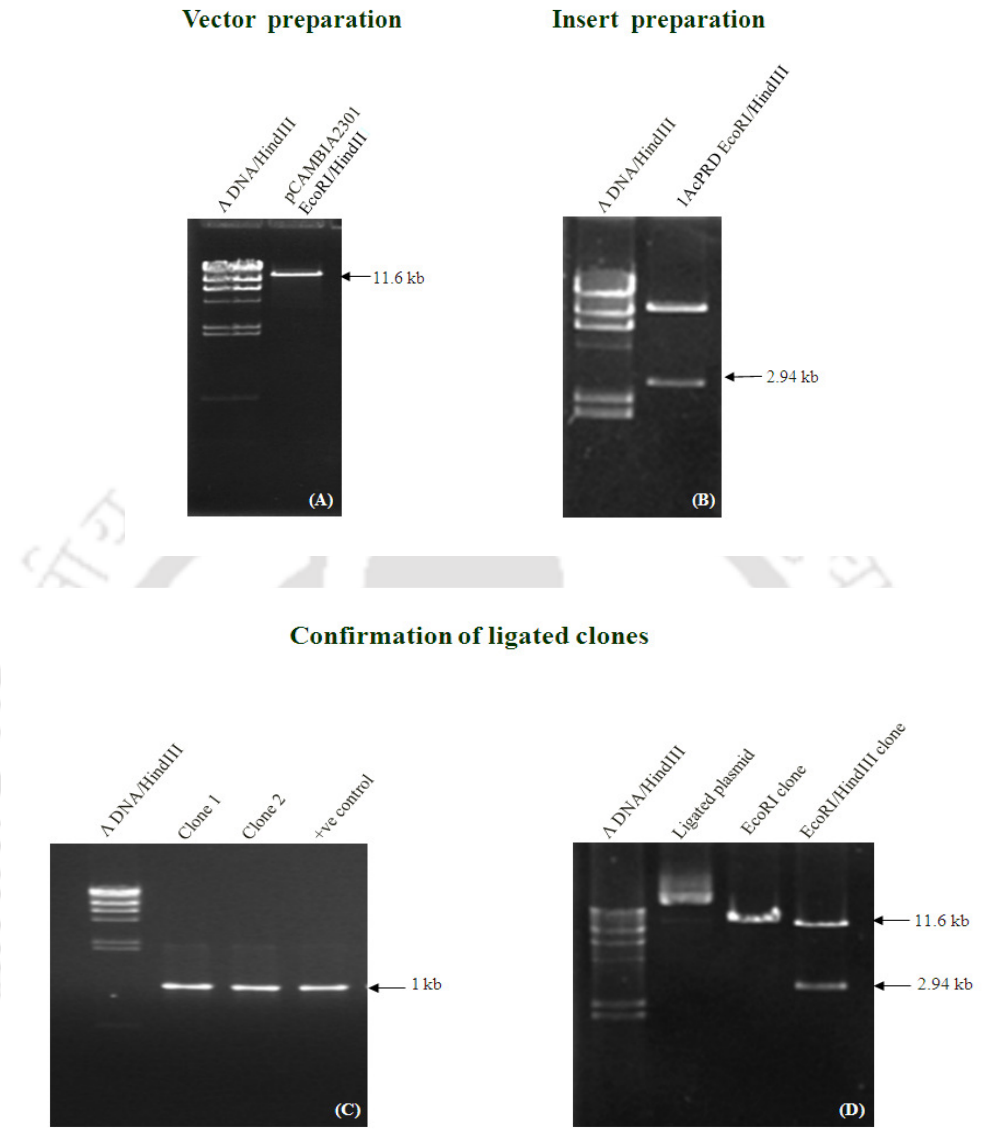


Fig. 4.3 Cloning of *Btcry1Ac* in pCAMBIA2301. (A) 11.6 kb linearised pCAMBIA2301 upon digestion with *Eco*RI/*Hind*III. (B) 2.94 kb Promoter-*Btcry1Ac*-Terminator casette was released from 1AcPRD upon digestion with *Eco*RI/*Hind*III. (C, D) Confirmation of pCAMBIA2301cry1Ac clones. (C) 1kb *cry1Ac* gene segment was amplified from ligated clones. (D) Confirmation of clones by restriction digestion. L1: λ DNA/*Hind*III marker; L2: Ligated plasmids; L3: 14.5 kb linearised plasmid from ligated clones upon digestion with *Eco*RI.

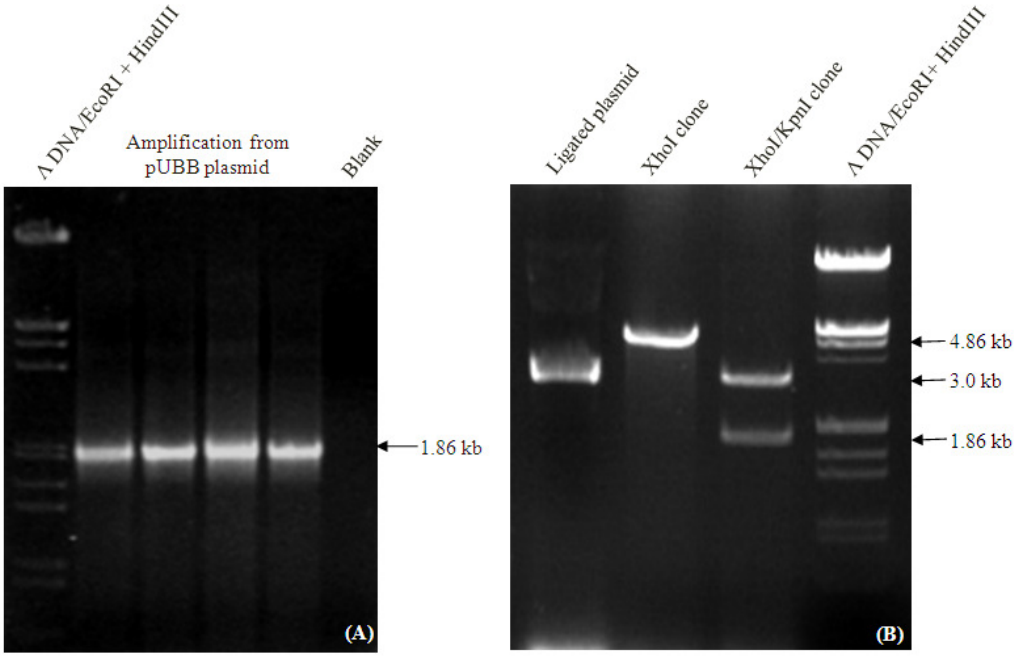


Fig. 4.4 (A) 1.86 kb full length *cry1Ab* PCR amplified product from pUBB; (C) Confimation of clones containing 1.86 kb *Xho*I-*cry1Ab*-*Kpn*I PCR product in pGEMT-Easy vector: L1: ligated plasmid; L2: 4.8 kb of *Xho*I digested linearised fragment; L3: 1.86 kb *cry1Ab* fragment was released upon digestion with *Xho*I and *Kpn*I

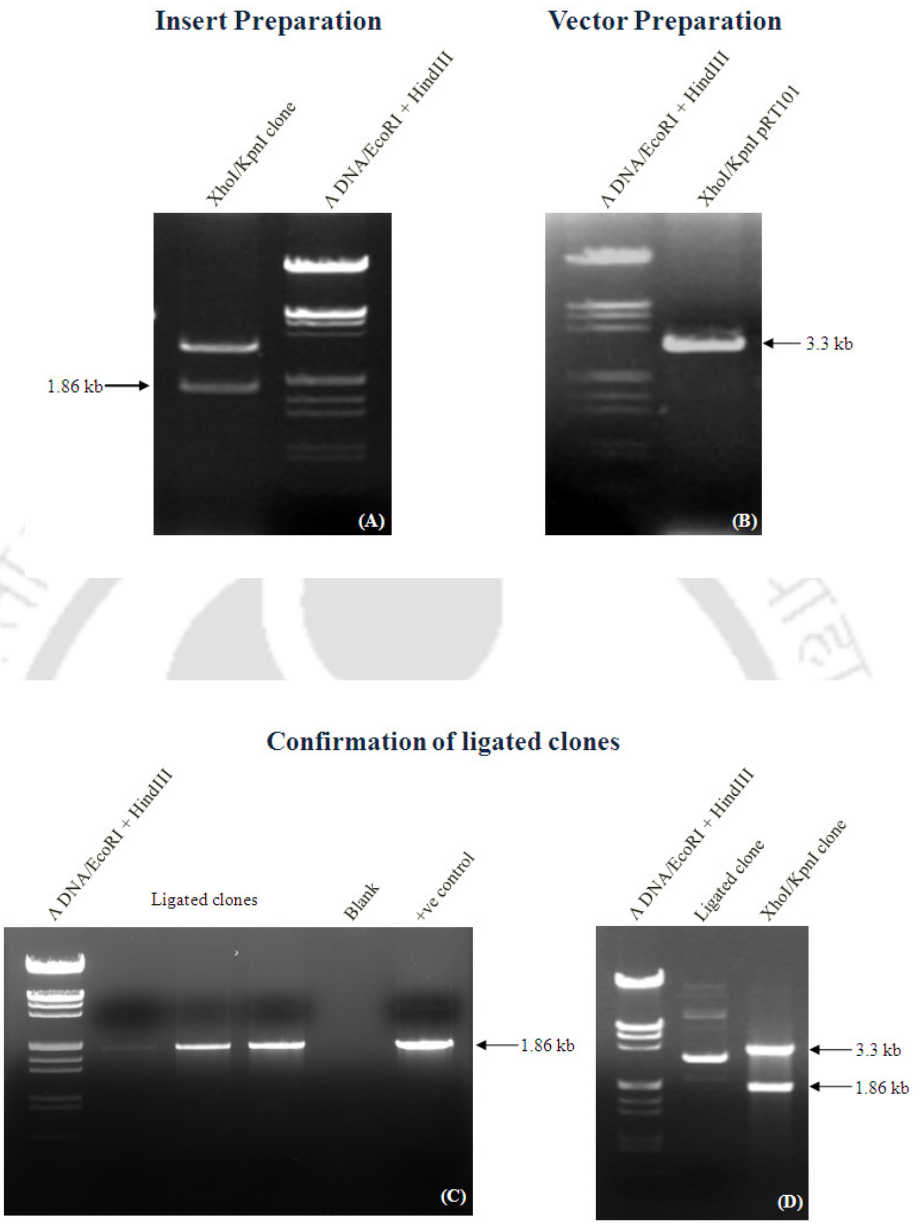


Fig. 4.5 Cloning of *Btcry1Ab* in pRT101. (A) 1.86 kb *Btcry1Ab* was released from pGEMT vector upon digestion with *Xho*I/*Kpn*I. (B) 3.3 kb linearised pRT101 upon digestion with *Xho*I/*Kpn*I. (C, D) Confirmation of pRT101*cry1Ab* clones. (C) 1.86 kb *cry1Ab* gene segment was amplified from ligated clones. (D) Confirmation of clones by restriction digestion. L1: λ DNA/*Eco*RI + *Hind*III marker; L2: Ligated plasmids; L3: 1.86 kb linearzed fragment released upon digestion with *Xho*I/*Kpn*I

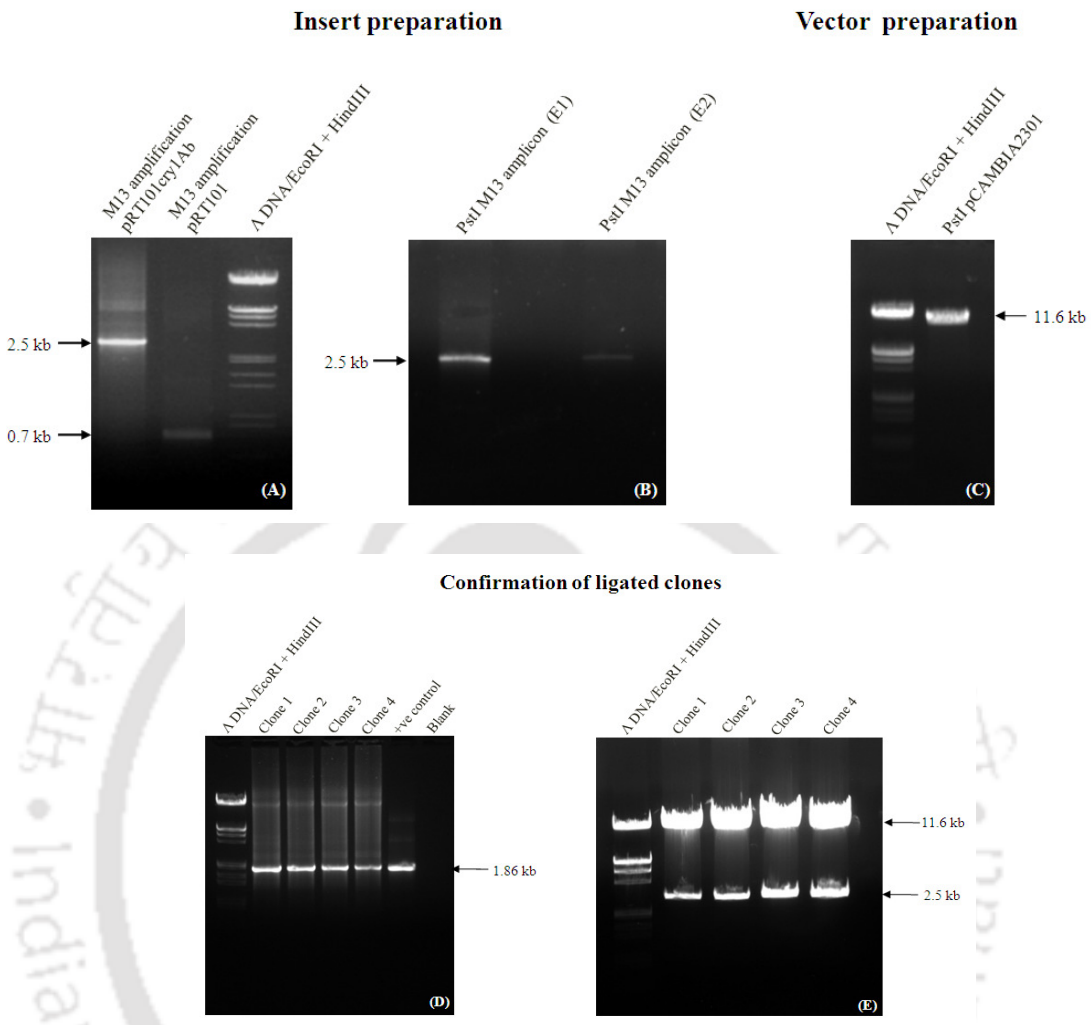


Fig. 4.6 Cloning of *Btcry1Ab* in pCAMBIA2301. (A) *Btcry1Ab* expression cassette was amplified from pRT101*cry1Ab* with M13 primer. L1: 2.5 kb of 35S promoter-c*ry1Ab*-polyA was amplified from pRT101*cry1Ab* using M13 universal primer, L2: 700bp of 35S-polyA fragment was amplified from pRT101 using M13 primer, L3: λ DNA/ *Eco*RI + *Hind*III marker. (B) 2.5kb fragment of 35SP-*cry1Ab*-polyA upon digestion with *Pst*I. L1: 1st elution; L3: 2nd elution. (C) 11.6 kb of linearised pCAMBIA2301 upon digestion with *Pst*I. (D, E) Confirmation of pCAMBIA2301c*ry1Ab* clones. (A) 1.86 kb *cry1Ab* gene segment was amplified from ligated clones. (B) Confirmation of clones by restriction digestion. L1: λ DNA/ *Eco*RI + *Hind*III marker; L2-L5: 1.86 kb linearised fragment released upon digestion with *Pst*I

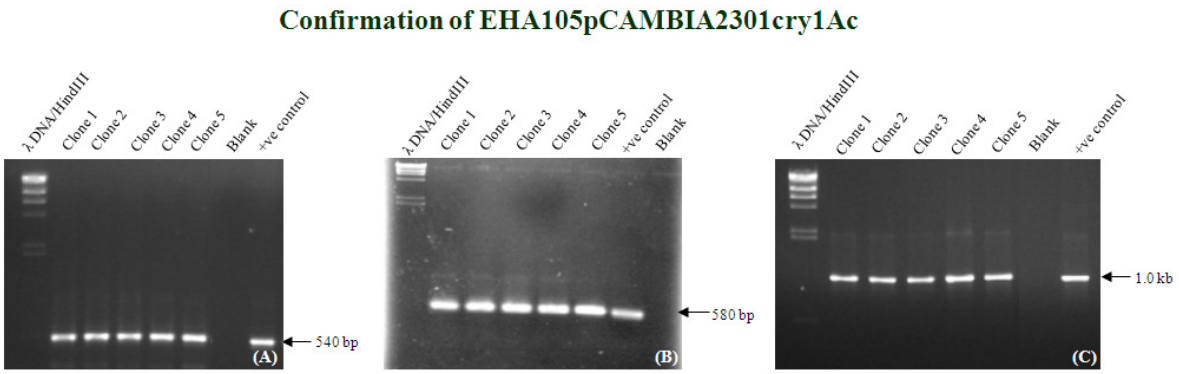


Fig. 4.7 Confirmation of *Agrobacterium* mobilisation of pCAMBIA2301cry1Ab in EHA105. (A) 540bp of *nptII* gene segment was amplified, (B) 580 bp of *gus* gene segment was amplified, (C) 1kb of *cry1Ac* gene segment was amplified from mobilised clones

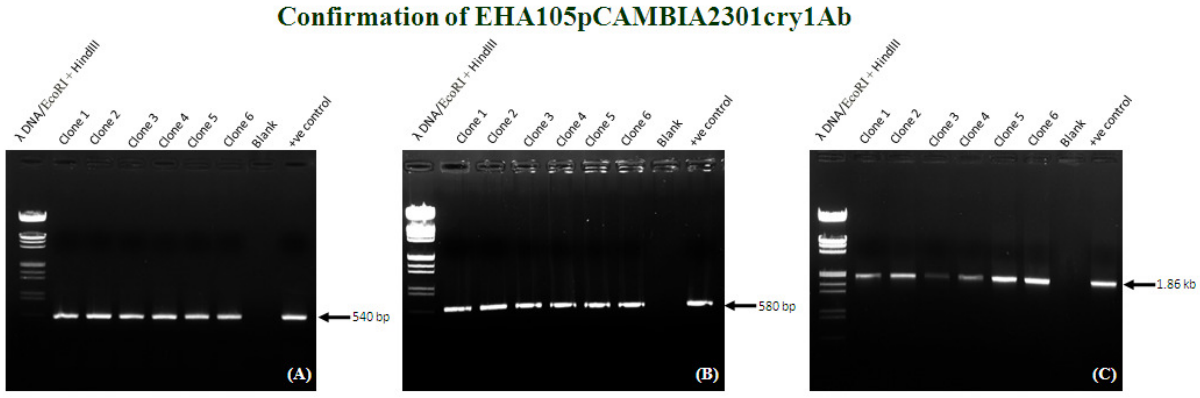


Fig. 4.8 Confirmation of *Agrobacterium* mobilisation of pCAMBIA2301*cry1Ab* in EHA105. (A) 540bp of *nptII* gene segment was amplified, (B) 580bp of *gus* gene segment was amplified, (C) 1.86 kb of *cry1Ab* gene segment was amplified from mobilized clones

Chapter 5

Establishment of an improved selection system and recovery of transgenic cowpea expressing *BtCry1Ac*

5.1 Introduction

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important grain and fodder legumes widely grown in tropical countries. The production of cowpea is affected by both biotic and abiotic stresses (Solleti et al. 2008a). Among biotic stresses, the pod borers, *Maruca vitrata* and *Heliothis armigera* cause extensive damage to pods, tender parts of stem, peduncles, flower buds and flowers of cowpea fields (Singh and Jackai 1988). Severe infestations accounts for as high as 90% reduction in yield (Murdock et al. 2001). Although conventional breeding methods have enhanced yield and quality of cowpea, low levels of insect resistance in cowpea germplasm and failures in interspecific crosses have limited the success in breeding for protection (Machuka 2002). Genetic engineering of cowpea with entomocidal genes for insect resistance offers a directed approach for protection against insect infestation (Zaidi et al. 2005). Genetic transformation of cowpea has been reported mainly through *Agrobacterium*-mediated transformation of cotyledons (Muthukumar et al. 1996) and cotyledonary node explants (Solleti et al. 2008a; Popelka et al. 2006; Chaudhury et al. 2007) with an exception of DNA delivery to shoot apices by biolistic (Ivo et al. 2008). Regardless of the availability of alternative plant transformation tool (Ivo et al. 2008), *Agrobacterium* is the most preferred method of gene delivery for its simplicity, cost-effectiveness and frequent single copy gene integration into the cowpea genome (Solleti et al. 2008b). In cowpea, cotyledonary node explants have been successfully used for regeneration of transgenic plants due to their competence for prolific shoot regeneration and T-DNA delivery (Solleti et al. 2008a). We have recently reported the development of transgenic cowpea expressing bean α-amylase inhibitor gene for storage pest resistance by using *Agrobacterium*-mediated transformation of cotyledonary node explants (Solleti et al. 2008b). Nevertheless, the efficiency of stable transformation in cowpea is far less for

production of numerous independent transformants with desirable genes to allow the selection of those with the appropriate level of gene expression.

One of the major bottlenecks in *Agrobacterium*-mediated transformation of grain legumes in general, and cowpea in particular is its high recalcitrance. The limitation in recovery of transformed cowpeas is neither the T-DNA transfer to the host plant cells nor plant regeneration from transformed cells, but the growth retardation of putative transformed shoots on selection medium and subsequent poor rooting collectively pose serious technical difficulties and restrict progress in cowpea biotechnology (Solleti et al. 2008b). Hence, there is a need to study the basis of cowpea recalcitrance to *Agrobacterium*-mediated transformation in view of the impediments in efficient recovery of stable transgenic plants. Exploring the basis of plant recalcitrance to *Agrobacterium*-mediated transformation would be useful to improve the transformation efficiency of recalcitrant grain legumes. Such attempt has so for not been made in any of the recalcitrant grain legumes to the best of our knowledge. Hence, we analyzed the basis of cowpea plant recalcitrance to *Agrobacterium*-mediated transformation in the current investigation by assessing various selection regimes based on kanamycin. Our results showed that judicious choice of selection dose and effectiveness of selection regime applied to the transformed tissues hold the keys to efficient recovery of transgenic cowpea plants. Several transgenic lines expressing *cry1Ac* were recovered that showed presence, integration, expression and inheritance of transgene in subsequent generation.

5.2 Materials and methods

5.2.1 Plant material and culture initiation

The mature seeds of cowpea cultivar Pusa Komal (IARI, New Delhi, India) were used for all the experiments in the present study. Seeds were surface-sterilized (Solleti et al. 2008a) and cultured on MSB_5 medium [MS salts (Murashige and Skoog, 1962) + B5 vitamins (Gamborg

et al. 1968)], 3% sucrose (w/v), 0.8% agar agar (w/v) and 10 µM TDZ. The cultures were incubated at 25±2^{0}C under 16-h photoperiod regime provided by cool white fluorescent lamps (36 µmolm^{-2}s^{-1}). Cotyledonary node explants (5–6 mm) were excised from 4-d-old seedlings as described previously (Solleti et al. 2008a).

5.2.2 Plasmid construct, bacterial strain and transformation

The *A. tumefaciens* strain EHA105 harbouring binary vector pSouv:cry1Ac (Fig. 5.1) (Contruct preparation was described in Chapter 4) were maintained on solid YEP medium (An et al. 1988) supplemented with 10 mg/l rifampicin and 50 mg/l kanamycin. The bacterial suspension was prepared and explants were infected and cocultivated as per the protocol described earlier (Chapter 3; Section 3.2.5). Following co-cultivation, the explants were washed three to four times with LCM and blotted dry on sterile filter paper and subjected to selection.

5.2.3 Selection regimes and regeneration of transgenic plants

In order to establish an efficient selection system to effectively select transformed shoots without suppressing shoot induction and elongation processes, the cocultivated explants were cultured onto initial multiple shoot induction and selection medium, P1 (MSB_5 medium containing 5 µM BAP and 0.5 µM kinetin supplemented with 150 mg/l kanamycin and 500 mg/l cefotaxime) for 20 days with 3 rounds of subculture at an interval of 5, 7 and 8 days respectively (Fig. 5.2). Following first round of selection, the survived explants were subjected to four different selection regimes (S1, S2, S3 and S4), and degree of tissue necrosis and increase in shoot length were determined. In S1, the explants were subjected to continuous high selection pressure (150 mg/l kanamycin) by culturing onto P1 medium for 15 days and in S2, the selection pressure was gradually reduced by culturing onto selection medium containing 125 mg/l kanamycin for 10 days followed by culturing onto kanamycin free medium for 10 days (Fig. 5.2). In S3, removal of selection pressure and

reduction in BAP dosage were implemented by culturing explants on kanamycin free medium containing 2.5 µM BAP for 15 days, and in S4, explants were cultured onto selection media containing 150 mg/l kanamycin and 2.5 µM BAP.

The efficiency of selection regimes was calculated by determining the number of plants, PCR-positive for Bt*cry1Ac* and *npt*II per the number of shoots recovered on selection.

Elongated putative transformed shoots (>2 cm) were transferred to rooting medium (MS + 2.5 µM IBA) devoid of any antibiotics for root induction. Rooted putative transformed plants were transferred to pots containing sterile soil:compost (1:1) and were acclimatized in greenhouse containment for 3 weeks.

5.2.4 Evaluation of transgenic plants

Molecular characterization of the T_0 transformants was carried out by GUS histochemical analysis, PCR, Southern hybridization, RT-PCR for confirmation of the presence, integration, expression and inheritance of the introduced genes.

5.2.4.1 Histochemical GUS assay

Transient and stable *gus* expression were detected in explants and various plant parts respectively, following the histochemical procedure as described by Jefferson (1987). Three days after cocultivation, the cotyledonary node explants and also the flower, anthers, pollens and pistils were incubated in GUS substrate solution (Solleti et al. 2008a) at 37^0C overnight. The tissues were bleached with 100% ethanol for few hours before examination under a stereomicroscope.

5.2.4.2 PCR analysis

Genomic DNA was isolated from young leaves of putative transformants in T_0 and T_1 using the modified CTAB method (Solleti et al. 2008a). PCR amplification was carried out with gene specific primers for *npt*II and *Btcry1Ac* using genomic DNA from putative transformed plants, non-transformed control plants (negative control) and pSouv:cry1Ac (positive

control) as templates. The 540 bp region of *npt*II and 1 kb coding region of *Btcry1Ac* were amplified by using respective oligonucleotide primers. The amplification reaction was carried out under following conditions: 94^0C for 1 min (1 cycle), 94^0C for 1 min (denaturation), 58^0C for 1 min (anneling), 72^0C for 1 min (extension) for 35 cycles followed by the final extension at 72^0C for 7 min (1 cycle). The PCR was performed by using ~100 ng of purified genomic DNA and Taq DNA polymerase (Genei, Bangalore, India) according to manufacturer's instruction. The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

5.2.4.3 Southern hybridization

Four randomly selected PCR-positive T_0 transgenic cowpea plants were further analyzed by Southern hybridization for the integration of the cry1Ac. 10 µg samples of genomic DNA from non-transformed control and transgenic plants were digested with *HindIII*. The digested samples were fractioned on a 0.8% agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, USA). The blot was hybridized with DIG-labeled 1 kb PCR product, corresponding to the coding region of *cry1Ac* gene. The probe labeling and Southern hybridization were performed using the nonradioactive DIG Labeling and Detection system (Roche, Germany) following supplier's instructions. Pre-hybridization and hybridization were carried out using the protocol described earlier (Chapter 3; Seection 3.2.7). The stable transformation efficiency was calculated on the basis of percentage of co-cultivated explants that developed to plants positive for *cry1Ac* by Southern hybridization.

5.2.4.4 RT-PCR analysis

Total RNA was isolated from the PCR-positive transgenic T_0 plants using Trizol Reagent (Invitrogen, USA) from 100 ng leaf tissue according to the manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.5% denaturing agarose gel (Sambrook et al. 1989). RT-PCR was carried out by using First Strand cDNA Synthesis Kit

(Fermentas, USA) according to the manufacturer's instructions. PCR of the coding sequences of *npt*II and *Btcry1Ac* genes in the cDNA was carried out by using respective primers as described earlier.

5.2.5 Segregation analysis

The leaves of T_1 transgenic plants generated from all the 11 T_0 transformants recovered through S3 selection regime were analyzed for the presence of *Btcry1Ac* gene by PCR, as described earlier. Segregation patterns were analyzed with the chi-square test (χ^2) as described by Solleti et al. (2008b).

5.2.6 Cry1Ac protein expression assay

To test for the presence of Cry1Ac protein, total soluble protein was isolated from leaves of T_1 transgenic lines using sample extraction buffer (DesiGen, India) according to the manufacturer's instructions. Rapid detection of Cry1Ac gene expression in the leaves of T_1 plants was carried out using immunodiagnostic Xpresstrips™ (DesiGen, India). DesiGen Xpresstrips™ are lateral flow devices which detect the Cry1Ac protein in extracts from plant samples. Each strip has a sample absorbing pad at one end and the extract moves up the pad into the clear window and reaches a second absorbant pad at the top of the strip. A line appears in the white window (control line), near the top absorbant pad, indicates that the assay is functional. The test line that appears near the sample pad indicates positive for Cry1Ac protein. Absence of test line signifies no expression of Cry1Ac.

5.2.7 Western hybridization

Proteins were extracted from about 1 g of young leaves of randomly chosen PCR positive T_1 transgenic lines using an extraction buffer containing 100 mM potassium phosphate buffer (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT). The protein concentration was determined by the method of Bradford (Bradford 1976). Thirty microgram of protein was fractionated on 12%

acrylamide gels with sodium dodecyl sulfate (SDS-PAGE) and blotted on to a PVDF membrane by electro transfer blotting unit. Blots were blocked for 2 h at room temperature in 5% bloking buffer (nonfat powdered milk in Tris-buffered saline with 0.1% Tween-20). Goat polyclonal antibodies (Amar Diagnostics, India) were used at 1/500 dilution in bloking buffer and incubated for overnight at 4^0C. The samples were washed three times in TBST (Tris-Buffered Saline Tween-20) for 5 min each. A secondary rabbit anti-goat antibody alkaline phosphatase conjugate (Amar Diagnostics, India) was then used for final detection, at a dilution of 1/1000. Blots were incubated for 40 min at 4^0C, washed five times for 5 min each with TBST followed by development in nitro blue tetrazolium/ bromo chloro indolyl phosphate (NBT/BCIP) substrate solution (Sigma, USA) for 15–20 min. The reaction was stopped by washing the membrane with distilled water.

5.2.8 Data analysis

Data were subjected to analysis of variance (ANOVA) and mean separation by Duncan's multiple-range test (DMRT) using single-factor completely randomized block design in order to study the effect of different selection regimes on recovery of stable transformants of cowpea. The four selection regime experiments consisted of 120 explants each. Each experiment was repeated at least three times.

5.3 Results and discussion

5.3.1 Transformation

Axillary meristems of the cotyledonary-node explants possess cells that are competent for regeneration and T-DNA delivery (Chandra and Pental 2003). Regeneration of shoots from cotyledonary node explants after *Agrobacterium* coculture is emerging as a rapid and relatively efficient method in a number of legume species including soybean (Hinchee et al. 1988; Purcell et al. 1996; Meurer et al. 1998; Donaldson et al. 2000), pea (Davies et al. 1993; Jordan et al. 1993; Bean et al. 1997), goat's rue (Colle´n and Jarl 1999), groundnut

(Venkatachalam et al. 1998), lentil (Mahmoudian et al. 2002a, b; Akcay et al. 2009), mungbean (Jaiwal et al. 2001; Sonia et al. 2007) and blackgram (Saini and Jaiwal 2007). In our experiments, cotyledonary node explants, prepared from 4-d-old seedlings germinated on MSB5 media containing 10 μM TDZ, were used to synchronize the multiple shoot bud induction with efficient T-DNA transfer process to regenerating cells. In the initial regeneration optimization experiments, explants derived from 4-d-old seedlings, germinated on MSB_5 media containing 10 μM TDZ were found competent for efficient multiple shoot induction and plant regeneration. Following cocultivation, all the cotyledonary node explants showed GUS activity predominantly in the regenerating sites indicating the competence of these explants to T-DNA transfer (Fig. 5.3a).

5.3.2 Sensitivity of explants and shoot elongation to different selection regimes

A judicious choice of selection level is an important criterion for the recovery of transformed shoots, because too high a level would be deleterious even to the transformed cells at initial stages of screening (Sharma and Anjaiah 2000). Preliminary dose response experiments with kanamycin using untransformed cowpea cotyledonary node explants showed the inhibition of shoot bud induction at 150 mg/l. Continuous exposure of these explants to 150 mg/l kanamycin resulted in growth retardation of kanamycin resistant shoots. Similar observation on growth retardation of transformed shoots on continuous high kanamycin selection pressure has been reported to produce transgenic plants at low frequencies (Popelka et al. 2006; Chaudhury et al. 2007). Inability of transformed shoots to elongate and form roots has been a major constraint in generating large number of transgenic cowpea via *Agrobacterium*-mediated transformation.

In order to establish an efficient selection regime, the cocultivated explants after first round of selection on P1 medium were subjected to continuous high selection pressure (S1) or gradual removal of selection pressure (S2) or removal of selection pressure

accompanied with reduction in BAP dosage (S3) or only reduction of BAP dosage (S4). Significant difference was observed in degree of necrosis of survived explants among the different selection regimes with highest degree of explant necrosis recorded in S1 followed by S2, S4 and S3 respectively (Fig. 5.4). In S1, the explants displayed proliferation of multiple shoot buds however, the shoots experienced growth retardation. The shoots were unable to elongate and this in turn significantly affected their rooting efficiency (Table 5.1). This may be attributed to the continuous exposure of explants to high selection pressure. Inhibition of root formation of transformed shoots selected on continuous high selection pressure has been reported in pea (Puonti-Kaerlas et al. 1990; Schroeder et al. 1993). Gradual decrease in kanamycin concentration in S2 from 125 to 0 mg/l induced 30.4% increase in shoot length as compared to continuous high selection pressure in S1 (Fig. 5.4). Gradually relieving selection had a great improvement on the development of transgenic shoots through adaptation of tissues to decreasing concentrations of selective agent in lentil (Gulati et al. 2002). Reducing the concentration of the selective agent relieved selection pressure on the explants and provided better elongation of the shoot buds into shoots. Removal of selection pressure accompanied by reduction in BAP dosage to 2.5 µM in S3 resulted in profuse proliferation with 63.6% increase in shoot length as compared to S1 (Fig. 5.4). The shoots were healthy and green apparently with no sign of bleaching. On transfer to rooting medium the shoots readily developed a good root system enabling successful transplantation to soil. Reduction in BAP dosage in kanamycin-free medium (S3) might have played a beneficial role in promoting elongation of kanamycin resistant shoots. A 57.5% increase in length of kanamycin resistant shoots was recorded in S4 as compared to S1. Among the four selection regimes tested, S3 induced maximum shoot elongation and proficient rooting (Table 5.1; Fig. 5.3b). The observation clearly indicated that the exposure of explants to appropriate duration of selection pressure was critical for elongation of

kanamycin resistant shoots in cowpea. Therefore, S3 selection regime was adapted for recovery of kanamycin resistant shoots in all subsequent experiments. The concentration and timing for the selection process are important factors that determine the efficiency of transformation (Tee et al. 2003). Application of short duration and timely selective pressures has resulted in rapid and efficient production of normal transgenic plants in plum (Padilla et al. 2003), apple (James et al. 1989) and recalcitrant turfgrass (Cao et al. 2006).

All the rooted plantlets transferred to soil survived, grew to maturity and produced seeds in transgenic greenhouse containment (Fig. 5.3c). The seeds were collected, sown in soil and T_1 plants were raised.

5.3.3 PCR analysis and efficiency of selection regimes

The PCR analysis detected the presence of the expected 540 bp and 1 kb amplified product corresponding to *nptII* and *cry1Ac* respectively (Fig. 5.5a, b) in kanamycin-resistant T_0 transformed plants. No amplification was detected in the control untransformed plants. PCR analysis was correlated with the efficiency of different selection regimes in recovering stable transformed plants (Table 5.1). In general, removal of kanamycin selection pressure accompanied with reduction in BAP dosage (S3) resulted in maximum numbers of selected plants (41.6%) transformed in nature as revealed by PCR analysis (Table 5.1). The selection efficiency of S3 regime was found significantly higher than other tested regimes (Table 5.1) and 3 fold higher than previous report on kanamycin based selection of cowpea transgenics (Chaudhury et al. 2007).

5.3.4 Stable *gus* expression, Southern hybridization and RT-PCR analysis

A strong, uniform and stable *gus* expression was detected in flower, anthers, pollens and pistils of PCR-positive T_0 plants and no endogenous *gus* expression was detected in the tissues of control plants (Fig. 5.3d-k).

Four PCR-positive T_0 transformed plants (lines 1–4) were randomly selected for Southern blot analysis to confirm the integration of *cry1Ac* gene. Hybridizations of DIG-labeled *cry1Ac* probe to total genomic DNA digested with *Hind*III were expected to identify DNA fragments unique to individual integration events greater than 5.0 kb (Fig. 5.5c). As shown in Fig. 5.5c, Southern blot analysis using the *cry1Ac* gene as a probe revealed that all the 4 randomly selected T_0 transgenic plants were found positive for *cry1*Ac gene integration and furthermore, they showed differential integration events, confirming that these plants were derived from independent transformation events (Fig. 5.5c, lanes 1, 2, 3 and 4). The T_0 transgenic plants exhibited simple hybridization patterns that ranged from single integration event to 3 loci and, in general, most fragments were greater than 5.0 kb (Fig. 5.5c). No amplification was detected in the control untransformed plants (Fig. 5.5c, lane C).

RT-PCR analysis showed the presence of transcripts of transgenes in different T_0 transformants. The amplification of a 0.54 kb fragment of *nptII* and 1 kb fragment of *cry1Ac* confirmed the accumulation of transcripts of both npt*II* and *cry1Ac* in T_0 transformants (Fig. 5.5d, e) indicating the absence of gene silencing events. Furthermore, the amplification of the transgenes sequences from plant cDNA templates in RT-PCR ruled out the possibility of *Agrobacterium* contamination.

The stable transformation efficiency was determined based on the number of T_0 plants PCR-positive for *Btcry1Ac* and *nptII* divided by the total number of explants co-cultivated. The stable transformation efficiency was evaluated as frequency of T_0 transgenics positive for *cry1Ac* by Southern hybridization. The stable transformation efficiency of different selection regimes varied from 1.43 to 2.44 with maximum stable transformation efficiency (2.44) recorded for selection regimes S3 (Table 5.1). The stable transformation efficiency of S3 was recorded 46.1% higher than our previous report (Solleti

et al. 2008a, b), 3.2 fold and 2.7 fold more efficient than other reports on cowpea transformation (Chaudhury et al. 2007; Ivo et al. 2008).

5.3.5 Segregation analysis

A total of 31 transgenic lines were obtained from four selection regimes. Segregation pattern of *cry1Ac* was analyzed on the basis of PCR analysis of *cry1Ac* in progeny plants of 11 T_0 transformants recovered from S3 selection regime. Presence of the expected 1 kb amplified product corresponding to *cry1Ac* in T_1 transgenic lines confirmed the inheritance of *cry1Ac* gene (Fig. 5.6). Segregation analysis showed a typical 3:1 segregation ratio for 9 line of T_0 lines suggesting a single independently segregating locus (Table 5.2). Two lines of the T_0 lines (C10 and C11) showed a 15:1 segregation ratio indicative of two loci segregating independently (Table 5.2).

5.3.6 Cry1Ac expression analysis

The randomly chosen PCR-positive T_1 transgenic lines were subjected to Cry1Ac protein expression analysis by rapid qualitative immunodiagnostic assay and Western hybridization. The clear appearance of test line in the DesiGen Xpresstrips™ confirmed the presence of Cry1Ac protein in T_1 transgenic lines (Fig. 5.7).

Expression of the Cry1Ac was tested by Western analyses on proteins extracted from T_1 transgenic lines generated from 4 independent transformation events. The polyclonal antibody detected an expected signal of 68 kDa protein corresponding to Cry1Ac protein in T_1 transgenic lines (Fig. 5.8). No signals for the presence of Cry1Ac were observed in extracts from non-transgenic plant controls (Fig. 5.8). The Western analysis confirmed the expression of *cry1Ac* in T_1 transgenic lines generated from 4 independent transformation events demonstrating the efficacy of this transformation system in cowpea. The total time from inoculation of the explants to a transgenic plant established in the greenhouse was approximately 2 months.

5.4 Conclusion

In any recalcitrant crop transformation through *Agrobacterium*-mediated approach, the major impediments in recovery of transgenic plants lie with either the inefficient T-DNA delivery to cells competent for regeneration or poor proliferation of the small minority of transformed cell populations or growth retardation of transformed shoots on selection medium (Solleti et al. 2008a). From our study, it is clear that an efficient selection regime based on a short duration and timely kanamycin selection pressure accompanied by reduction in BAP dosage in cowpea rapidly evokes its responses, leading to significant increase in stable transformation efficiency by as high as 48.7% (~50%) as compared to the previous reports in cowpea. The formulated selection regime allowed generation of a large number of phenotypically normal fertile transgenic plants expressing *Btcry1Ac*. The T_0 transformed plants reported here showed integration of *cry1Ac,* expression in RT-PCR. The *cry1Ac* was transmitted in a Mendelian fashion in transgenic plants. The rapid immunodiagnostic strip assay and Western hybridization studies confirmed the expression of Cry1Ac protein in T_1 transgenic lines. The transgenic cowpea plants overexperssing *cry1Ac* could represent a good opportunity to analyze the impact of *cry1Ac* for resistance to its target insects. Our protocol that addressed the key factor for recalcitrance of cowpea to *Agrobacterium*-mediated transformation should permit improvement in recovery of stable transgenic plants in other recalcitrant grain legumes.

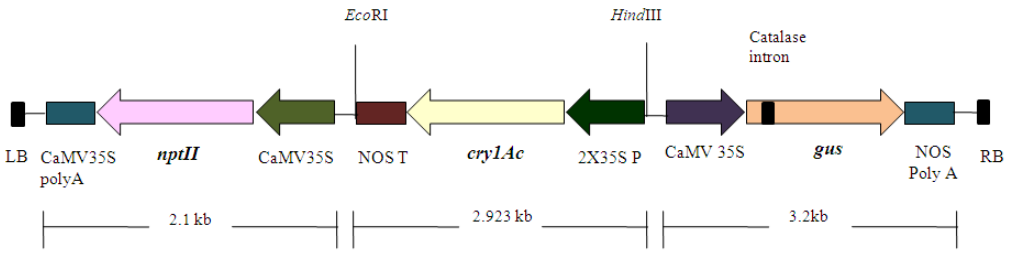


Fig. 5.1 Schematic construction of pSouv:cry1Ac (14.5 kb). The 2.9 kb (*Eco*RI-*Hind*III) fragment containing Bt*cry1Ac* under control of CaMV 2X35S promoter and NOS terminator was released from p1AcPRD and cloned at the *Eco*RI-*Hind*III sites of T-DNA of pCAMBIA2301. The resulting construct was designated as pSouv:cry1Ac. LB and RB: left border and right border of T-DNA region, NOS T: nos terminator, 2X35P: double 35S promoter, *npt*II: neomycin phosphotransferase II

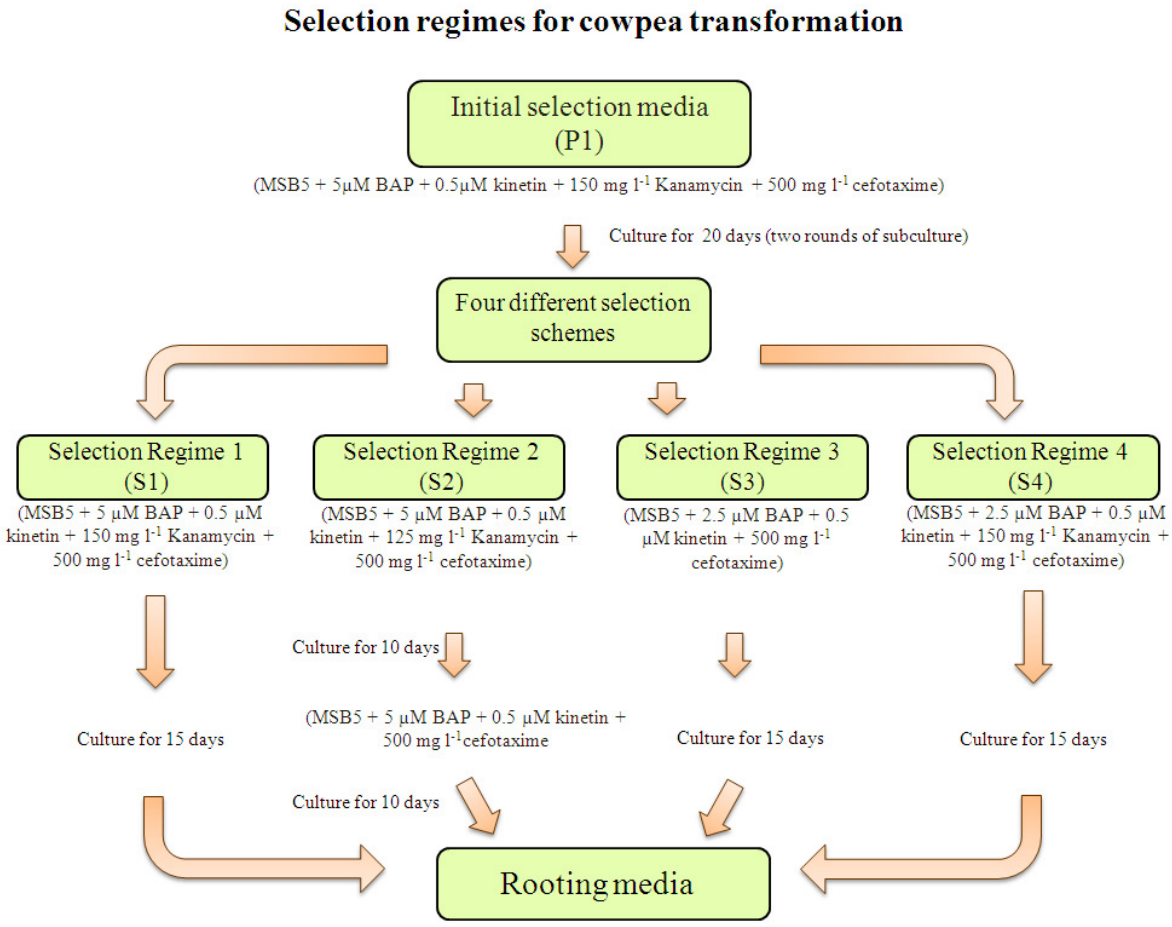


Fig. 5.2 Flow diagram showing the different selection regimes using kanamycin after 3-d coculture of explants with *A. tumefaciens* EHA105pSouv:cry1Ac. For media abbreviations, see text

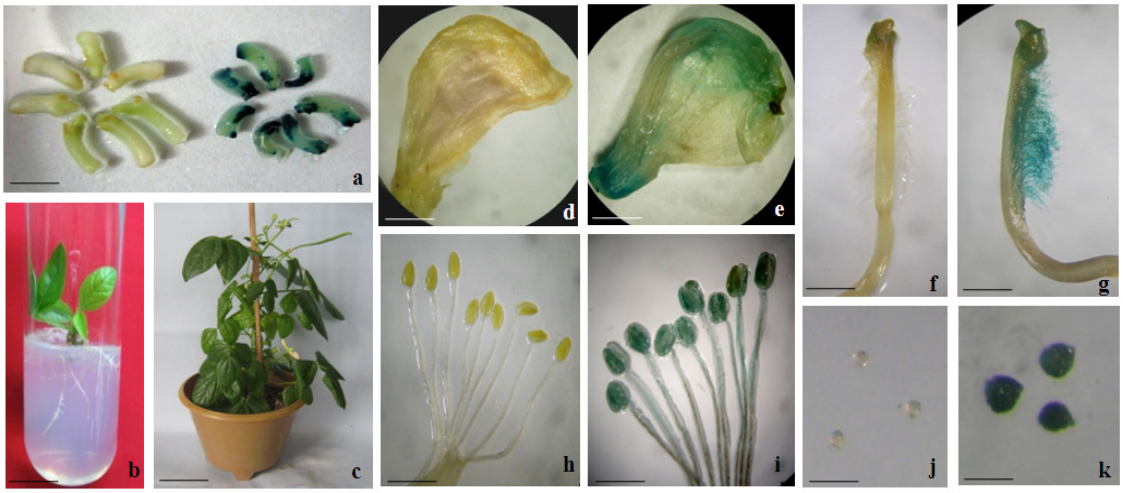


Fig. 5.3 Transient and stable *gus* expression and regeneration of transgenic plants. (a) cotyledonary node showing transient *gus* expression after 3 days of cocultivation (*Bar* 5 mm), (b) *In vitro* rooting of elongated transformed shoot (*Bar* 10 mm), (c) acclimatized plant maintained in transgenic green house (*Bar* 15 cm), (d) non-transformed control flower (*Bar* 7 mm), (e) transformed flower (*Bar* 7 mm), (f) control pistil (*Bar* 5 mm), (g) transformed pistil (*Bar* 8 mm), (h) control anthers (*Bar* 8 mm), (i) transformed anthers (*Bar* 8 mm), (j) control pollens (*Bar* 3 mm), (k) transformed pollens (*Bar* 3 mm)

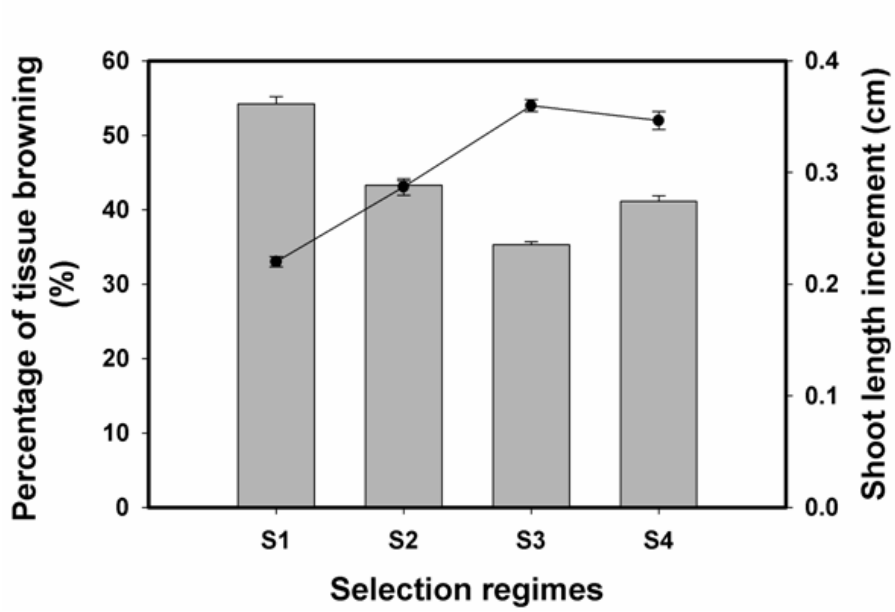


Fig. 5.4 Effect of different selection regimes on degree of tissue browning and increase in shoot length

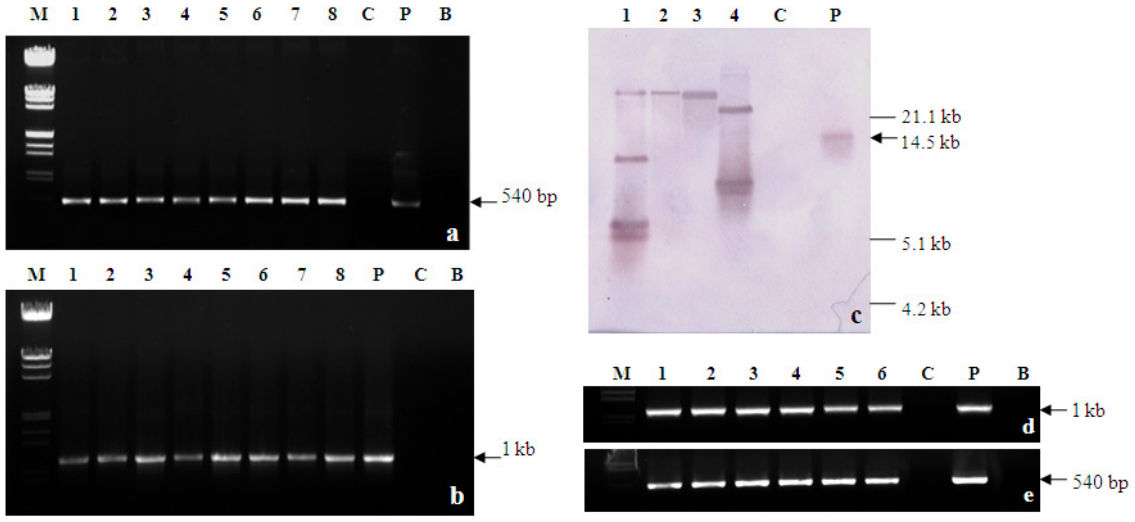


Fig. 5.5 Molecular analysis of transgenic plants. (a–d) analysis of T_0 transgenic plants, (a) PCR amplification of the 540 bp fragment of the *nptII* gene, (b) PCR amplification of the 1 kb fragment of the *cry1Ac* gene. Lane M molecular weight marker; lane P pSouv:cry1Ac plasmid DNA (positive control); lane C DNA from untransformed plant (negative control); lanes 1–8 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 *HindIII*, and hybridized with cry1Ac probe. Lanes 1–4 genomic DNA from four T_0 lines, lane C genomic DNA from untransformed plant, lane P *HindIII* digested pCAMBIA2301cry1Ac, (d) RT-PCR analysis of *nptII* gene (e) RT-PCR analysis of *cry1Ac* gene. Lane M molecular weight marker; lane C untransformed plant (negative control); lanes 1–6 T_0 transgenic plants.

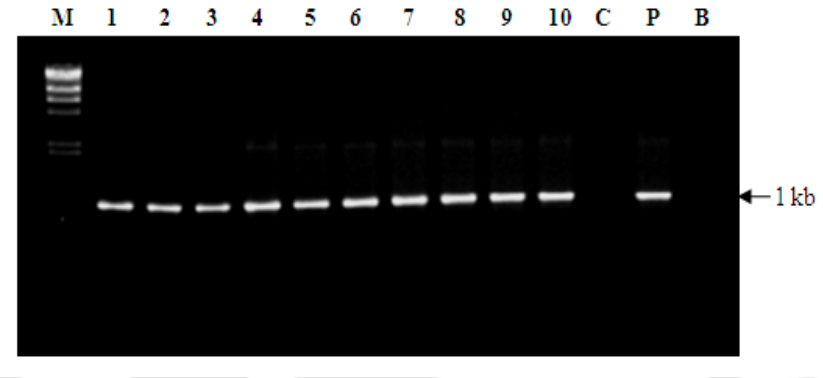


Fig. 5.6 PCR amplification of the 1 kb fragment of the *cry1Ac* gene of T_1 plants. Lane M molecular weight marker; lane P pSouv:cry1Ac plasmid DNA (positive control); lane C DNA from untransformed plant (negative control); lanes1–10: DNA fromT_1 transgenic plants

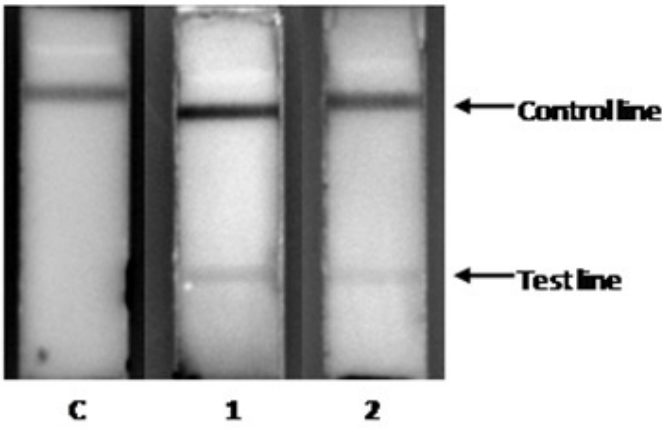


Fig. 5.7 Rapid immunodiagnostic test for detection of Cry1Ac in T_1 transgenic lines using DesiGen Xpresstrips™. C no test line appeared from control plants after lateral flow assay; (1-2) transgenic lines showed test line signifies the presence of Cry1Ac

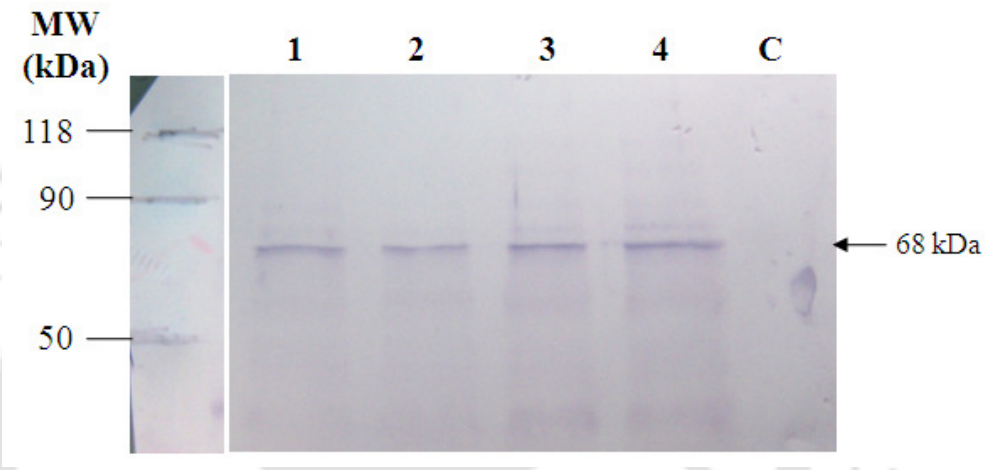


Fig. 5.8 Detection of Cry1Ac protein by Western blotting analysis in T_1 transgenic cowpea leaves. M: Protein molecular weight marker, lanes 1–4: *cry1Ac* transgenic lines (CT1A, CT1B, CT1C and CT1D) respectively, lane 5: non-transformed plant

Table 5.1 Effect of selection regimes on frequency of transformation in cowpea

Selection regime	Rooting efficiency (%)	Selection efficiency (%)	Stable transformation efficiency (%)
S1	31.5^{d}	32.4^{d}	1.43^{cd}
S2	42.5^{c}	33.3^{c}	1.55^{c}
S3	100^{a}	41.6^{a}	2.44^{a}
S4	81.5^{b}	34.5^{b}	2.00^{b}

Each value in the table is the average of the three replicates. Values sharing the same letters in each column are not significantly different from each other at *p=0.05.*

Selection efficiency (%) = No. of PCR-positive plants per no. of explants survived selection

Stable transformation efficiency (%) = Number of T_0 plants PCR-positive for *Btcry1Ac* and *nptII* divided by the total number of explants co-cultivated

Table 5.2 Segregation of *cry1Ac* gene in T_1 progeny of transgenic cowpea plants

T_0 plants	Number of T_1 plants tested for *cry1Ac*			χ^2 value	Expected Ratio
	Total	*cry1Ac*+ve	*cry1Ac*-ve		
C1	41	29	12	0.39	3:1
C2	27	18	8	0.47	3:1
C3	35	24	11	0.77	3:1
C4	33	23	10	0.12	3:1
C5	27	19	8	0.31	3:1
C6	38	26	12	0.88	3:1
C7	18	12	6	0.67	3:1
C8	45	31	14	0.89	3:1
C9	41	29	12	0.40	3:1
C10	32	29	3	0.28	15:1
C11	44	40	4	0.61	15:1

Chapter 6

Investigation on sonication and vacuum infiltration assisted *Agrobacterium*-mediated transformation in cowpea

6.1 Introduction

An efficient genetic transformation system could provide a valuable tool for functional genomics studies of cowpea. *Agrobacterium*-mediated transformation has been extensively applied to many crop plants including grain legumes, because this method offers several advantages such as the defined integration of transgenes, potentially low copy number, and preferential integration into transcriptional active regions of the chromosome (Koncz et al. 1989; Hiei et al. 1994). Till date, *Agrobacterium*-mediated transformation of cotyledonary explants has led to the generation of stable transgenic plants in cowpea (Muthukumar et al. 1996; Popelka et al. 2006; Chaudhury et al. 2007; Solleti et al. 2008a, b). Cotyledonary explants are preferred for *Agrobacterium*-mediated transformation of cowpea as T-DNA delivery to axillary meristem followed by regeneration via adventitious bud formation minimizes the risks of chimeras and somaclonal variation (Tzfira et al. 1997). However, cowpea transformation still remains inefficient and consequently, production of transgenic cowpea is far from being a routine procedure due to poor transformation efficiency and low numbers of regenerated transgenic plants. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) (Joersbo and Brunstedt 1992; Trick and Finer 1998; Santare'm et al. 1998) and vacuum infiltration (Charity et al. 2002; Park et al. 2005; Paz et al. 2006) methods have been reported to enhance the efficiency of *Agrobacterium*-mediated transformation of recalcitrant plant species. Exposure of the explants to short periods of sonication in the presence of *Agrobacterium* carrying desired T-DNA vector is thought to produce large numbers of micro wounds across the tissue which permits the *Agrobacterium* to penetrate deeper and more completely throughout the tissue as compared to the natural infection obtained during co-cultivation (Trick and Finer 1997; Santare'm et al. 1998; Tang et al. 2001; Liu et al. 2005), thus enhancing the bacterial colonization and infection of the tissue. Performed scanning electron and light microscopy observations revealed that

ultrasound treatment produces small and uniform fissures and channels throughout the plant tissue, which allows *Agrobacterium* access to internal plant tissue (Trick and Finer 1997). SAAT method has been successfully employed in improving transformation of a number of recalcitrant plants (Oliveira et al. 2009).

Agroinfiltration is an effective method in enabling the regenerating cells, often located a few cell layers beneath the surface of explants, rapid access to *Agrobacterium* and consequently increasing transient transgene expression in many recalcitrant plant species (Bechtold and Pelletier 1998; Tague and Mantis 2006). This method has been adapted for the successful transformation of number of recalcitrant plants (Subramanyam et al. 2011). Although the benefits of sonication and vacuum infiltration during *A. tumefaciens*-mediated transformation methods are evident, no effort has been made to apply these methods to cowpea. In order to improve the *Agrobacterium*- mediated transformation in cowpea for routine generation of transgenic plants with candidate genes, we investigated the effect of sonication and vacuum infiltration on *Agrobacterium*-mediated transformation of cowpea cotyledonary node explants. Stable transgenic cowpea plants expressing cry1Ac were recovered using both SAAT and vacuum infiltration, which showed presence, integration, expression and inheritance of transgenes.

6.2 Materials and methods

6.2.1 Plant material and explant preparation

The mature seeds of cowpea were surface sterilized and cultured on MSB_5 medium [MS salts (Murashige and Skoog 1962) + B5 vitamins (Gamborg et al. 1968)] supplemented with 3% sucrose (w/v), 0.8% agar agar (w/v) and 10 µM TDZ. Cotyledonary node explants (5–6 mm) were excised from 4-day-old seedlings and used for transformation experiments.

6.2.2 Binary plasmid, bacterial strain and culture conditions

The binary plasmid pSouv:cry1Ac (Fig. 6.1) was mobilized into *Agrobacterium tumefaciens* strain EHA105 and used for transformation experiments. The bacterial inoculums were prepared as described in Chapter 1 (Section 3.2.4) and was resuspended in liquid co-cultivation medium, LCM (MSB_5 medium containing 1 µM BAP, pH adjusted to 5.5) supplemented with 100 µM acetosyringone and used for inoculation.

6.2.3 Inoculation of explants with *A. tumefaciens*

For each experiment, 30–40 explants were subjected to wounding treatment either by mechanical injury with needle or by sonication, and inoculated in bacterial suspension by occasional shaking for 30 min or by vacuum infiltration. The explants inoculated in bacterial suspension without prior wounding treatment were considered as control. After inoculation in all cases, explants were blotted on a sterile filter paper to remove excess liquid and cocultivated for 3 days under dark condition at 22^0C, in petridishes lined with filter paper moistened with LCM supplemented with 100 µM acetosyringone. Following cocultivation, the explants were rinsed three to four times with LCM and blotted dry on sterile filter paper and placed onto initial multiple shoot induction and selection medium, SISM (MSB_5 medium containing 5.0 µM BAP and 0.5 µM kinetin supplemented with 150 mg/l kanamycin and 500 mg/l cefotaxime) for 20 days with three rounds of subculture at an interval of 5, 7 and 8 days, respectively.

6.2.4 Wounding and SAAT treatments

The cotyledonary node explants were wounded at axils by puncturing approximately 1.5 mm in depth with a sterile hypodermic needle (0.56 mm in diameter.) prior to inoculation with *Agrobacterium* cell suspension. For SAAT, the explants were immersed in 15 ml flat bottom glass culture tubes (Borosil, India) containing 6 ml of *Agrobacterium* cell suspension. The tubes were capped, placed in a float at the center of a bath-type sonicator

(Telsonic ultrasonic TPC-40, Switzerland) and then subjected to ultrasound at a frequency of 30 kHz. The treatments differed as to sonication duration (5, 10, 15, 20, 25, and 30 s). Following sonication, explants were removed from the tubes, placed on sterile filter paper surface to blot off excess bacteria and then transferred to co-cultivation medium.

For vacuum infiltration experiment, the explants with or without wounding and 20 s sonication treatments were placed in vacuum system consisted of a vacuum pump at 600 mm Hg (Rocker 400, Tarson, India) to which a desiccators was attached. Glass petri dishes containing explants immersed in *Agrobacterium* cell suspension were placed in the desiccator and vacuum was applied for different durations (2.5, 5, 10, 15 and 20 min).

The best treatments achieved in SAAT and vacuum infiltration experiments were combined to evaluate the effect of sonication followed by vacuum infiltration in contrast to the use of these methods alone.

In all experiments, the frequency of transient GUS expression was analyzed after 3 days of co-cultivation. The optimal wounding, sonication and vacuum infiltration treatments were determined as the levels that led to a perceived increase in GUS positive foci in explants at the site of regeneration without any perceived decrease in explant viability. Control treatments consisted of explants either uninoculated or inoculated with *Agrobacterium* without wounding, sonication and vacuum infiltration treatments.

6.2.5 Histochemical GUS assays

GUS activity was visualized using the histochemical assay (Jefferson 1987). Transient expression of GUS was scored on a per explant basis by estimating the number of blue foci visible on the axillary region of each cotyledonary node explant. The blue foci were the discrete areas of cells with GUS activity.

6.2.6 Shoot recovery

Following three rounds of kanamycin selection on SISM, the survived explants were transferred to SIEM [shoot induction and elongation medium (MSB_5 medium containing 5.0 µM BAP, 0.5 µM kinetin and 500 mg/l cefotaxime)] and cultured for 10 days for optimal elongation and selective regeneration of transformants. Elongated putative transformed shoots (> 1.5 cm) were transferred to rooting medium (MS + 2.5 µM IBA) devoid of any antibiotics for root induction. Rooted putative transformed plants were transferred to pots containing sterile soil:compost (1:1) and were acclimatized in greenhouse containment for 3 weeks.

6.2.7 Evaluation of transgenic plants

Molecular characterization of the transformants was carried out by PCR, Southern hybridization, GUS histochemical analysis of different plant tissues, RT-PCR, ELISA and Western blot hybridization analysis for confirmation of the presence, integration, expression and inheritance of the introduced genes.

6.2.7.1 Stable GUS assay

Stable gus expression was detected in various plant parts including flower, anthers, pollens and pistils following the histochemical procedure as described previously.

6.2.7.2 Screening of putative transformed plants using polymerase chain reaction (PCR)

Genomic DNA was isolated from the young leaves of T_0 putative transformants and T_1 transgenic plants using the modified CTAB method (Solleti et al. 2008a). PCR amplification was carried out with gene specific primers for *nptII* and *Btcry1Ac* using genomic DNA from putative transformed plants, non-transformed control plants (negative control) and pSouv:cry1Ac (positive control) as templates. The 540 bp region of *nptII* and 1 kb coding region of *Btcry1Ac* were amplified using respective gene specific oligonucleotide primers. The amplification reaction was carried out following the conditions described previously

(Chapter 5; section 5.2.4.2). The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

6.2.7.3 Southern hybridization

Randomly selected PCR-positive T_0 transgenic cowpea plants were further analyzed by Southern hybridization for the integration of the *cry1Ac*. 10 µg samples of genomic DNA from non-transformed control and transgenic plants were digested with *HindIII*. The digested samples were fractioned on a 0.8% agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, USA). The blot was hybridized with DIG-labeled 1 kb PCR product, corresponding to the coding region of *cry1Ac* gene. The probe labeling and Southern hybridization were performed using the nonradioactive DIG Labeling and Detection system (Roche, Germany) following supplier's instructions.

6.2.7.4 Qualitative reverse transcription (RT)-PCR analysis

Total RNA was isolated from the PCR-positive transgenic T_0 plants using Trizol Reagent Invitrogen, USA) from 100 ng of leaf tissue according to the manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.5% denaturing agarose gel (Sambrook et al. 1989). RT-PCR was carried out using First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. PCR of the coding sequences of *Btcry1Ac* gene in the cDNA was carried out using respective primers as described earlier.

6.2.7.5 Western blot hybridization

Proteins were extracted from 1 g of young leaves of T_0 transgenic plants and the protein concentration was determined by the method of Bradford (1976). 30 µg of protein was fractionated on 12% acrylamide gels with sodium dodecyl sulfate (SDS–PAGE) and blotted on to a PVDF membrane by electro transfer blotting unit. The bloting and the hybridization protocol was described earlier (Chapter 5; Section 5.2.7).

6.2.7.6 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to quantify the accumulated levels of Cry1Ac protein in T_0 transgenic plants using Desigen Quan T-ELISA-96 well plate kit (Desigen, Maharashtra, India) following manufacturer's protocol. Total protein was extracted using supplier's instruction. For the estimation of Cry1Ac, the 96-well titre plate was coated with 150 µl per well (1:1,000) of goat anti-Cry1Ac antibodies. The plate was then loaded with 100 µl samples and buffer was used in control wells. The plate was incubated at 37^0C for 1.5 h, followed by washing with wash buffer twice. After washing, the plate was incubated with alkaline phosphatase conjugated secondary antibodies at a dilution of 1:1,000 with 250 µl per well for 45 min at 37^0C. The plate was then washed with wash buffer twice and 250 µl of freshly prepared substrate (p-nitro phenyl phosphate, 1 mg/ml) was added per well. The plate was incubated at room temperature in the dark for 30 min and reaction was stopped and readings recorded at 405 nm in a micro plate reader (Tecan, Switzerland).

6.2.8 Segregation analysis

The leaves of T_1 transgenic plants generated from eight independent transformation events were analyzed for the presence of *nptII* and *Btcry1Ac* genes using PCR, as described earlier. Segregation patterns were analyzed with the Chi-square test ($\chi 2$) as described by Solleti et al. (2008b).

6.2.9 Data analysis

Data were subjected to analysis of variance (ANOVA) and mean separation by Duncan's multiple-range test (DMRT) using single-factor completely randomized block design in order to study the effect of different treatments on frequencies of transient expression. All experiments were performed at least three times with a minimum of 30–40 explants per treatment.

6.3 Results and discussion

6.3.1 Effect of wounding

Efficient *Agrobacterium*-mediated transformation requires optimal delivery of the T-DNA to regenerable cells of the explants. Wounding of explants allows *Agrobacterium* to better access plant cells as it stimulates the production of potent *vir* gene inducers, like phenolic substances and enhances the plant cell competence for transformation (Stachel et al. 1985; Shimoda et al. 1990; Bidney et al. 1992). Only plants with an appropriate wound response develop larger populations of wound adjacent competent cells for regeneration and transformation (Potrykus 1991). Although excessive wounding is probably detrimental to stable transformation, the frequency of gene transfer via *Agrobacterium*-mediated transformation in recalcitrant species can be significantly enhanced by inducing wounds in the target tissue (Bidney et al. 1992). In cowpea, infection of cotyledonary node explants with most effective supervirulent *A. tumefaciens* strain EHA105, in absence of injury treatment resulted in 85% transient GUS expression frequency (Solleti et al. 2008a). However, the accounted GUS foci were located mostly at the cotyledons detachment site of cotyledonary node explants, and not at the regenerating site. The low stable transformation efficiency, 1.64% in cowpea (Solleti et al. 2008a) could be attributed to poor conversion of transient transformation to stable transformation. Wounding of regenerating sites of the cotyledonary node explants of cowpea by a hypodermic needle and co-cultivation with *A. tumefaciens* resulted in more efficient transient expression especially on needle wounded explants, mainly in terms of the percentage of explants showing GUS foci at the regenerating sites as compared to unwounded explants infected with *A. tumefaciens* (Fig. 6.2). This clearly indicated that higher transient transformation of regenerating cells of meristematic tissue-based explants such as cotyledonary nodes, required an efficient wounding treatment. Wounding at the regenerating sites before co-cultivation allowed better bacterial

penetration into the regenerating cells of cotyledonary node explants, facilitating the accessibility of plant cells for *Agrobacterium* infection. Such mechanical wounding treatments greatly enhanced transformation efficiency in a number of plant species including recalcitrant grain legumes (Roome 1992; Rohini et al. 2005; Supartana et al. 2006; Saini and Jaiwal 2007).

6.3.2 Effect of sonication and vacuum infiltration

To identify more efficient methods to improve access of *Agrobacterium* and also to create an area of wounding to induce cotyledonary node cells and to produce phenolic compounds for *vir* gene induction in cowpea, we evaluated the effect of sonication and vacuum infiltration on *A. tumefaciens*-mediated transformation of cotyledonary node explants. These treatments have the potential to increase transformation efficiency by improving penetration of *Agrobacterium* cells into the cell layers beneath the epidermis of cotyledonary node region. This is an important criterion as regenerating cells of cotyledonary node explants are positioned a few cells layers beneath the surface at the axils in *Vigna* species including cowpea, mungbean and blackgram (Sahoo and Jaiwal 2009). A control experiment with explants without inoculation with *Agrobacterium* was designed to determine whether these treatments could be used without a negative effect on shoot regeneration from cotyledonary node explant. Sonication was very effective in increasing transient GUS expression frequency (Figs. 6.3, 6.4a). With the increase in sonication treatment time, the number of transiently transformed explants increased significantly with a maximum of 79% of the explants showing GUS foci at the regenerating sites when sonication treatment was prolonged to 20 s (Figs. 6.3, 6.4a). The number of GUS foci appeared to be quite variable among cotyledonary node explants. At lower sonication treatment time (10–20 s), the GUS foci were well defined, corresponding to probably one or a collection of small individual spots (Fig. 6.3a–f). With the increase in sonication treatment

time beyond 20 s, a diffuse GUS expression was presented all over the surface of the cotyledonary node explants, making the quantification of the number of foci difficult (Fig. 6.3g–h). Moreover, with increase in sonication treatment time to 30 s, the untransformed explants showed a decrease in their bud-forming capacity indicating that longer sonication treatment compromised viability of regenerating cells. SAAT has been used to enhance stable transformation of many recalcitrant plant species including soybean (Trick and Finer 1998), loblolly pine (Tang 2003), black locust (Zaragoza' et al. 2004), sweet potato (Wang et al. 2006), rice (Yookongkaew et al. 2007), *Chenopodium rubrum* (Flores Solı's et al. 2007), chickpea (Pathak and Hamzah 2008), flax (Beranova' et al. 2008) and *Theobroma cacao* (Silva et al. 2009).

We attempted various time intervals of vacuum infiltration of explants at 600 mmHg in an *Agrobacterium* suspension, and of the different time intervals tested, a 5 min vacuum infiltration resulted in a maximum of 93% transient transformation efficiency as accounted on the basis of number of explants showing GUS foci at the regenerating sites (Fig. 6.4b). Vacuum infiltration of cotyledon explants of *Pinus radiata* in an *Agrobacterium* suspension has allowed *Agrobacterium* to penetrate several layers deep through the sub-epidermal layer to mesophyll cells and vascular tissues (Charity et al. 2002), although the cells buried several layers deep, were not necessarily those that would induce shoots (Yeung et al. 1981). The vacuum infiltration of *Agrobacterium* has been successfully used to produce transgenic plants of model plant Arabidopsis (Clough and Bent 1998), and recalcitrant crop species including wheat (Cheng et al. 1997), mungbean (Jaiwal et al. 2001), pinus (Charity et al. 2002), cotton (Leelavathi et al. 2004), kidney bean (Liu et al. 2005), coffee (Canche-Moo et al. 2006), chickpea (Indurker et al. 2010) and banana (Subramanyam et al. 2011).

6.3.3 Combined treatment of sonication and vacuum infiltration

In order to evaluate the combined action of sonication and vacuum infiltration on transient transformation, the effect of 20 s sonication and 5 min vacuum infiltration was tested as compared to the two treatments separately. The combination of 20 s sonication followed by 5 min vacuum infiltration resulted in maximum frequency of cotyledonary node explants expressing GUS at the regenerating sites (Fig. 6.4c). Sonication coupled with vacuum infiltration has increased transient and stable transformation of radish (Park et al. 2005), kidney bean (Liu et al. 2005), citrus (Oliveira et al. 2009), Fraxinus pennsylvanica (Du and Pijut 2009), chickpea (Indurker et al. 2010) and banana (Subramanyam et al. 2011).

6.3.4 Production of transgenic cowpea plants carrying *cry1Ac* gene

Putative transformed plants were regenerated from cotyledonary node explants, which were subjected to a combination of 20 s sonication followed by 5 min vacuum infiltration prior to *Agrobacterium* co-cultivation, on kanamycin selection medium and established in greenhouse containment (Fig. 6.5a–g). A strong, uniform and stable *gus* expression was detected in flower, anthers, pollens and pistils of PCR-positive T_0 plants and no endogenous *gus* expression was detected in the tissues of control plants (Fig. 6.5h–o).

6.3.5 Analysis of transgenic cowpea plants

The detection of the expected 540 bp and 1 kb amplified products corresponding to *nptII* and *cry1Ac* in PCR analysis confirmed the presence of the transgenes in T_0 transformed plants (Fig. 6.6a, b). No amplification was detected in the control untransformed plants.

Four randomly selected PCR-positive T_0 transgenic cowpea plants were further screened by Southern analysis to confirm the integration of *cry1Ac* gene. Southern blot analyses of four T_0 transgenic plants are shown in Fig. 6c. Hybridizations of DIG-labeled cry1Ac probe to total genomic DNA digested with *HindIII* were expected to identify DNA fragments unique to individual integration events greater than 5.0 kb (Fig. 6.1). All the four

randomly selected T_0 transgenic plants were found positive for *cry1Ac* gene and furthermore, they showed differential integration events, confirming that these plants were derived from independent transformation events (Fig. 6.6c, lanes 1, 2, 3 and 4). The T_0 transgenic plants exhibited simple hybridization patterns that ranged from single integration event to three loci and, in general, most fragments were greater than 5.0 kb (Fig. 6.6c). A signal of size less than 5.0 kb was detected in lane 3 (Fig. 6.6c), suggesting the possibility of rearrangement of the T-DNA near the left border upon integration into the plant genome. No hybridization signal was detected in the untransformed plant (Fig. 6.6c, lane C).

The expression of the cry1Ac genes in leaves of T_0 transgenic plants was determined by RT-PCR analysis. RT-PCR showed the presence of expected transcripts of transgenes in different T_0 transgenic plants. The amplification of a 1 kb fragment of *cry1Ac* confirmed the accumulation of transcripts of cry1Ac in T_0 transgenic plants (Fig. 6.6d, e) indicating the absence of gene silencing events. Furthermore, the amplification of the *cry1Ac* sequence from plant cDNA templates in RT-PCR ruled out the possibility of *Agrobacterium* contamination.

The stable transformation efficiency was determined based on the number of T_0 plants PCR-positive for *Btcry1Ac* and *nptII* divided by the total number of explants co-cultivated. An average stable transformation efficiency of 3.09 was recorded (Table 6.1), which was significantly higher than the previously published report on *Agrobacterium*-mediated transformation of cowpea using extra copies of *vir* genes (Solleti et al. 2008a).

6.3.6 Cry1Ac expression analysis

The randomly chosen PCR-positive T_0 transgenic lines were subjected to Cry1Ac protein expression analysis by Western hybridization and ELISA. The expression of the Cry1Ac protein was analyzed in T_0 transgenic lines generated from four independent

transformation events by Western blot hybridization. A single band of 68 kDa corresponding to Cry1Ac toxin protein was detected immunologically in T_0 transgenic plants confirming stability of cry1Ac expression. Protein extracts of control nontransformed plants did not show the 68 kDa protein band (Fig. 6.7a).

The level of expression of Cry1Ac protein in transgenic lines ranged from 0.001 to 0.089% of the total leaf soluble protein (Fig. 6.7b). The results described above demonstrated that expression of the cry1Ac regulated by the double 35S-promoter led to the accumulation of Cry1Ac protein in transgenic plants.

6.3.7 Segregation analysis

The seeds from T_0 generation were advanced to T_1 generation and the T_1 transgenic lines generated from eight independent transformation events were analyzed for the segregation pattern of cry1Ac by PCR analysis. Presence of the expected 1 kb amplified product corresponding to *cry1Ac* in T_1 transgenic lines confirmed the inheritance of *cry1Ac* gene (Fig. 6.8). The segregation pattern of these selected transgenic events showed typical 3:1 Mendelian ratio as expected for single dominant gene inheritance (Table 6.2).

6.4 Conclusion

In conclusion, an improved *Agrobacterium*-mediated transformation system was developed for cowpea by employing sonication and vacuum infiltration was enhanced by 88.4% using SAAT in combination with vacuum infiltration as compared to simple *Agrobacterium*-mediated transformation. This is the first report on cowpea transformation using SAAT and vacuum infiltration. Furthermore, cowpea transgenics expressing cry1Ac is reported for the first time.

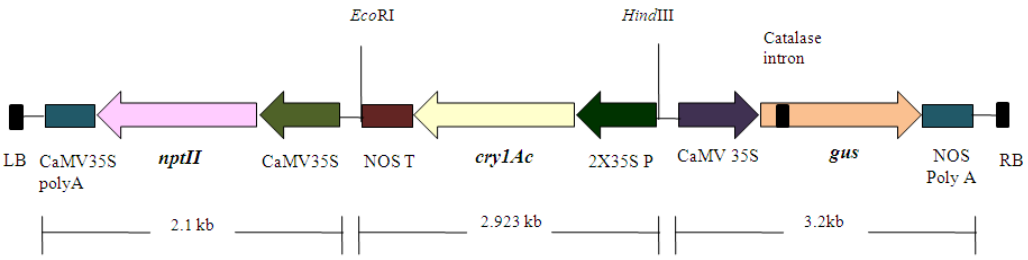


Fig. 6.1 Schematic construction of pSouv:cry1Ac (14.5 kb). The 2.9 kb (*Eco*RI-*Hind*III) fragment containing Bt*cry1Ac* under control of CaMV 2X35S promoter and NOS terminator was cloned at the *Eco*RI-*Hind*III sites of T-DNA of pCAMBIA2301. LB and RB: left border and right border of T-DNA region, NOS T: nos terminator, 2X35P: double 35S promoter, *npt*II: neomycin phosphotransferase II

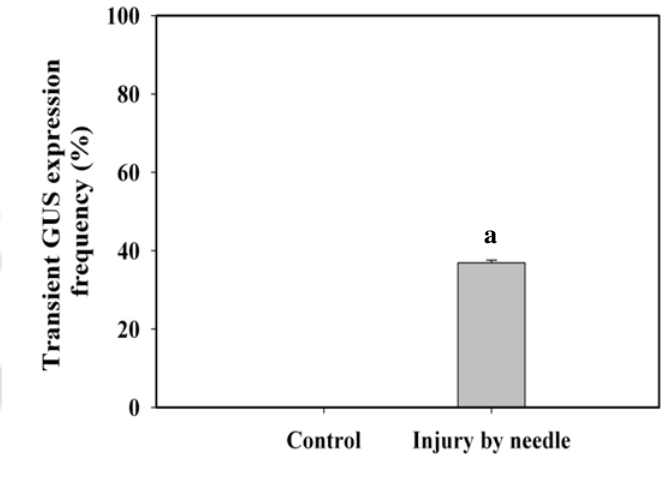


Fig. 6.2 Effect of mechanical injury by hypodermic needle on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. Control – injury is omitted in explants. The bars indicate ± standard errors. Means followed by the same letter are not statistically significant at $P < 0.05$.

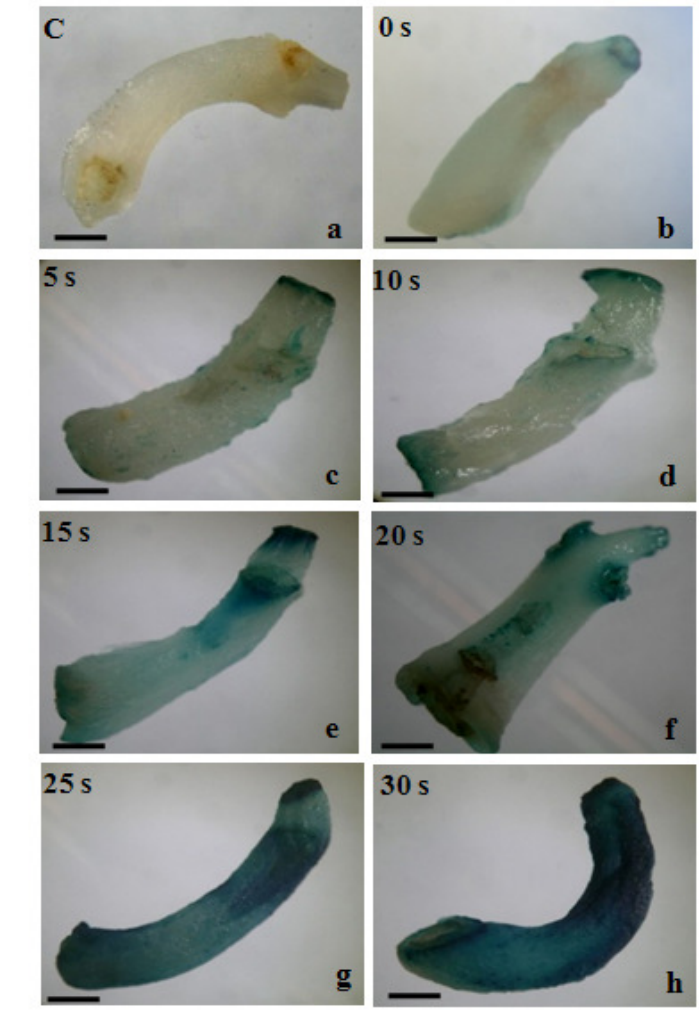


Fig. 6.3 Transient expression of GUS at the regenerating sites of sonication-treated cotyledonary node explants after 3-days of co-culture. (a) Control (untransformed). (b) *Agrobacterium*–treated explants (without sonication treatment). (c-h) sonication treated cotyledonary nodes (c: 5s, d: 10s, e: 15s, f: 20s, g: 25s and h: 30s). Bar (in all figures): 1mm

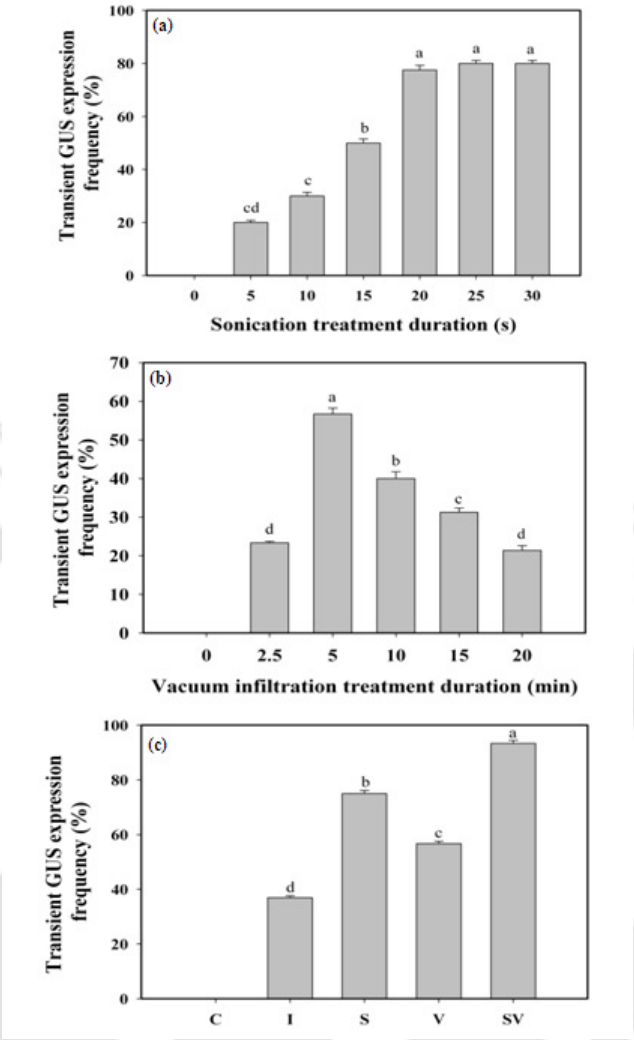


Fig. 6.4 (a) Effect of SAAT treatment duration and (b) vacuum infiltration treatment duration on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. (c) Effect of different wounding methods on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. C Without wounding. I Injury treatment by hypodermic needle. S 20 s sonication treatment. V Vacuum infiltration treatment for 5 min. SV 20 s sonication followed by vacuum infiltration treatment for 5 min. The bars indicate ± standard errors. Means followed by the same letter are not statistically significant at P>0.05

Fig. 6.5 Transient and stable gus expression and regeneration of transgenic plants. (a) cotyledonary node explants. Bar 2 mm. (b and c) Transient GUS expression, non-transformed (control) explants not showing GUS activity (b), cotyledonary node explants showing transient GUS activity after 3 days of co-cultivation (c). Bar 4 mm. d Shoot induction from axils of explant after 5-day culture on SISM. Bar 2 mm. (e) Proliferation of multiple shoots within 4 weeks of culture. Bar 10 mm. (f) In vitro rooting of elongated transformed shoot. Bar 12 mm. (g) Acclimatized plant maintained in transgenic green house. Bar 10 cm. h Non-transformed control flower. Bar 7 mm. (i) Transformed flower. Bar 7 mm. (j) Control anthers. Bar 8 mm. (k) Transformed anthers. Bar 8 mm. (l) Control pistil. Bar 8 mm. (m) Transformed pistil. Bar 8 mm. (n) Control pollens. Bar 3 mm. (o) Transformed pollens. Bar 3 mm

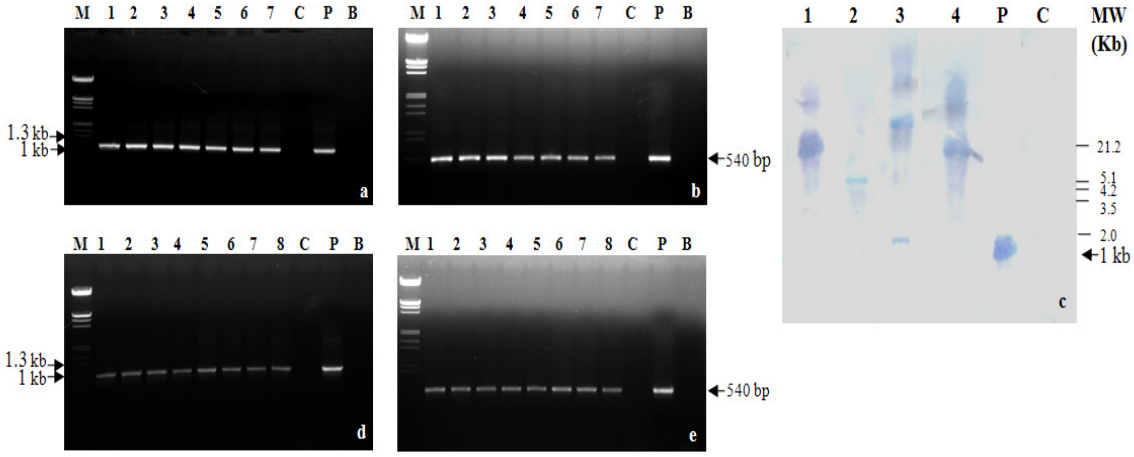


Fig. 6.6 Molecular analysis of T_0 transgenic plants. (a) PCR amplification of the 1 kb fragment of the *cry1Ac* gene, (b) PCR amplification of the 540 bp fragment of the *npt*II gene. Lane M λ DNA/*Eco*RI + *Hind*III marker, lane P pSouv:cry1Ac plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lane B blank, 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 HindIII, and hybridized with cry1Ac probe. Lanes 1–4 genomic DNA from four T_0 lines, lane C genomic DNA from untransformed plant, lane P *cry1Ac* PCR amplicon. (d) RT-PCR analysis of *cry1Ac* gene, (e) RT-PCR analysis of *npt*II gene. Lane M λ DNA/*Eco*RI + *Hind*III marker; lane C untransformed plant (negative control); lane B blank; lanes 1–8 T_0 transgenic plants

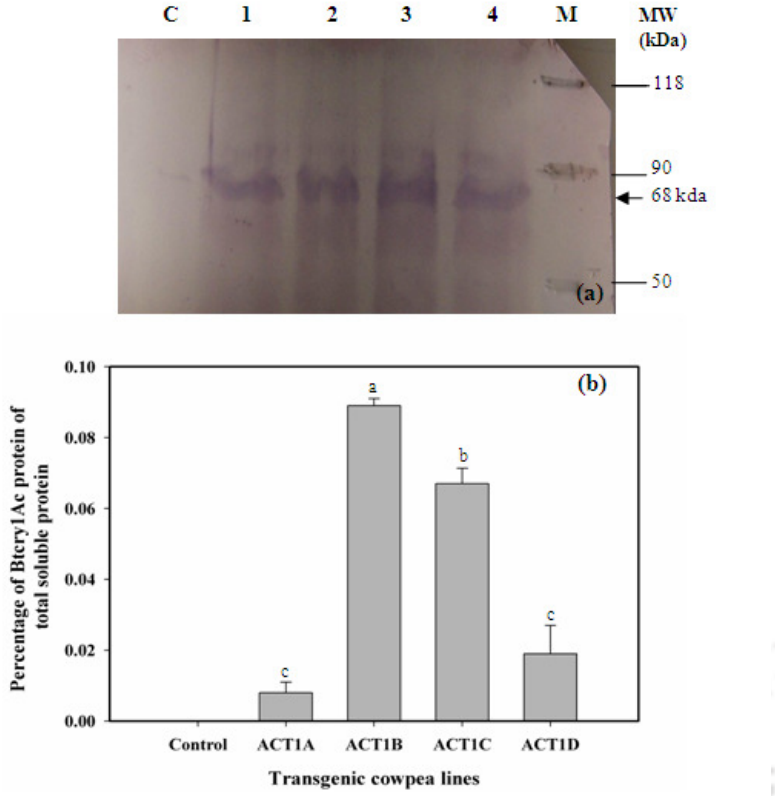


Fig. 6.7 (a) Detection of Cry1Ac protein by Western blotting analysis in transgenic cowpea leaves. M Protein molecular weight marker, lanes 1–4 cry1Ac transgenic lines (CT1A, CT1B, CT1C and CT1D), respectively, lane 5 non-transformed plant. (b) Expression level of BtCry1Ac protein in transgenic cowpea lines (CT1A, CT1B, CT1C and CT1D) from enzyme-linked immunesorbent assay (ELISA). Error bars represent ± standard error of the means. Means followed by the same letter are not statistically significant at P>0.05

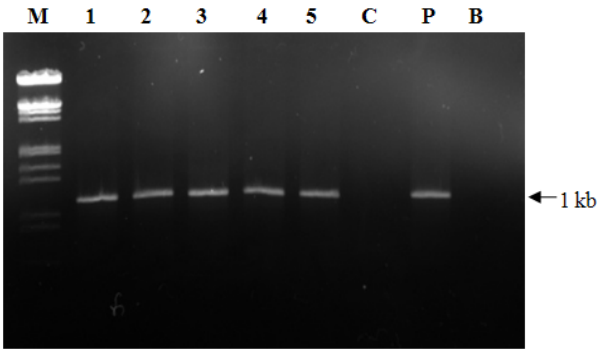


Fig. 6.8 PCR amplification of the 1 kb fragment of the *cry1Ac* gene of T_1 plants. Lane M λ DNA/*Eco*RI + *Hind*III marker, lane P pSouv:cry1Ac plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lane B blank, lanes 1–5 DNA from T_1 transgenic plants

Table 6.1 Summary of the *Agrobacterium*–mediated transformation of cowpea cotyledonary node explants subjected to 20 s sonication followed by vacuum infiltration for 5 min with *Agrobacterium tumefaciens* EHA105pSouv:cry1Ac

Exp. no.	No. of explants inoculated in *Agrobacterium* suspension	Transient transformation efficiency[a] (%)	No. of shoots recovered on selection medium	No. of plants positive for *cry1Ac* and *npt*II genes by PCR	Transformation efficiency[b] (%)
1	247	91.10	15	8	3.20
2	239	95.00	12	7	2.93
3	204	92.12	11	6	2.94
4	243	95.10	12	8	3.30
Total[c] / Average[d]	933 [c]	93.33[d]	50 [c]	29 [c]	3.09[d]

[a] Number of explants showing GUS foci at the regenerating sites per number of explants cocultivated with *Agrobacterium tumefaciens* EHA105pSouv:cry1Ac

[b] Number of plants PCR-positive for *cry1Ac* and *npt*II per number of explants co-cultivated

Table 6.2 Segregation of *cry1Ac* gene in T_1 progeny of transgenic cowpea plants

T_0 plants	Number of T_1 plants tested for *cry1Ac*[a]			χ^2 value	Expected Ratio
	Total	*cry1Ac*+ve	*cry1Ac*-ve		
C1	52	39	14	0.10	3:1
C2	35	24	11	0.77	3:1
C3	39	27	12	0.69	3:1
C4	55	40	15	0.15	3:1
C5	28	21	7	0.18	3:1
C6	43	30	13	0.56	3:1
C7	31	22	9	0.27	3:1
C8	44	40	4	0.61	15:1

[a] Presence of *cry1Ac* was analyzed by PCR

Chapter 7

Establishment of split cotyledonary node based plant regeneration system and recovery of transgenic cowpea overexpressing *Btcry1Ab*

7.1 Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is one of the important food legumes widely cultivated in the semi-arid tropics covering Asia, Africa, southern Europe, Central and South America (Singh 2002). Cowpea has excellent nutritional attributes containing 24–26% protein and well balanced essential amino acid composition with high amounts of leucine, lysine and methionine (Bressani 1985). However, cowpea production is limited by numerous constraints both biotic and abiotic of which several diseases, insect pests, nematodes, and parasitic weeds cause significant cowpea yield loss (Shimelis and Shiringani 2010). Losses due to insect pests alone can exceed 90 percent. Even in cases where modest levels of insect resistance have been developed in cowpea varieties against some of the insect pests, there is virtually none with demonstrable resistance against pod borer *Maruca vitrata,* a serious field pest of cowpea (Mignouna et al. 2010). The limitations associated with the use of conventional breeding methods in effectively dealing with cowpea's pest problem makes the biotechnological interventions most attractive by engineering the host with candidate gene, *cry1Ab* thus providing in built protection against *Maruca vitrata*.

For this purpose, a highly efficient cowpea transformation is required. The first cowpea transgenic plants were reported through *Agrobacterium*-mediated transformation of cotyledons (Muthukumar et al. 1996) followed by cotyledonary node explants (Popelka et al. 2006; Chaudhury et al. 2007; Solleti et al. 2008a, b; Bakshi et al. 2011). Alternative target tissues such as shoot apices have also been used for cowpea transformation through biolistic (Ivo et al. 2008). However, cotyledonary node explants still remain the target tissue of choice for cowpea transformation due to their competence for prolific shoot regeneration and T-DNA delivery (Bakshi et al. 2011). In comparison to DNA delivery to cowpea by particle bombardment, *Agrobacterium*-mediated transformation was more efficient and

preferred for its simplicity, cost-effectiveness and frequent single copy gene integration into the cowpea genome (Bakshi et al. 2011).

Although, cotyledonary explants are most preferred for *Agrobacterium*-mediated transformation of recalcitrant cowpea, the transformation efficiency has been reported to be in a range of 0.05-1.67%. In a more recent report, we described a transformation efficiency of 3.09%., averaged across four experiments starting with a total of 933 cotyledonary node explants (Bakshi et al. 2011). However, a number of different modifications were employed during the study including employment of sonication and vacuum infiltration in order to improve access of *Agrobacterium* to regenerating cells, located few cell layers beneath the epidermis of cotyledonary node region. Although these treatments consequently increased the number of cells transiently expressing transgenes, the method is still complex to generate numerous independent transgenic events with candidate genes to allow the selection of those with the appropriate level of gene expression. Furthermore, absence of a reproducible, simple and highly efficient transformation technology in cowpea will be a major impediment in developing of resources for functional genomics studies, such as T-DNA insertion mutants that require thousands of independent transgenic lines.

In the present study, we examined the potential of newer explants, split cotyledonary nodes and effect of dose and duration of preconditioning of source seedlings with cytokinin on multiple shoot induction in order to significantly improve the regeneration rate. Furthermore, the regeneration system was combined with a straightforward *Agrobacterium*-mediated transformation, for the first time in cowpea, in the pursuit of developing a robust, simple, rapid and highly efficient method for recovery of transgenic plants. The procedure yielded large numbers of independently transformed cowpea plants expressing *cry1Ab* gene with average transformation efficiency of 5.01%,

opening avenues for targeted expression of a number of desirable genes for varietal improvement as well as development of functional genomics resources in cowpea.

7.2 Materials and methods

7.2.1 Pretreatment of seedlings and explant preparation

Mature seeds of cowpea cultivar Pusa Komal (IARI, New Delhi) were surface-sterilized (Solleti et al. 2008a) and inoculated in MSB_5 medium containing different concentrations (0, 5, 10 and 20 µM) of TDZ or BAP for 2-6 days. Seeds cultured in hormone free media were considered as control. Whole cotyledonary nodes were excised from aseptic seedlings by detaching primary leaves and epicotyls leaving approximately 5-6 cm long hypocotyls intact (Fig. 7.1). The resulting cotyledonary nodes were sliced longitudinally through both the axils with sterile surgical blades to produce two split cotyledonary nodes (Fig. 7.1) and used for multiple shoot induction.

7.2.2 Multiple shoot induction and plant regeneration

The explants were cultured horizontally with their cut surface slightly embedded in the multiple shoot induction medium. In the first set of experiment, explants excised from control seedlings (untreated) were cultured in three different regeneration medium, MSB_5 medium containing i) 5 µM BAP [M1], ii) BAP (5 µM) and kinetin (0.1 µM) [M2], and iii) BAP (5 µM) and kinetin (0.5 µM) [M3] for shoot multiplication. In the second set of experiment, both the effect of cytokinin (TDZ and BAP) preconditioning at different dose (0, 5, 10 and 20 µM) and for different duration (2-6 days) was tested. Explants excised from pretreated seedlings were cultured onto three different regeneration media (M1, M2, M3) for shoot multiplication. In both sets of experiments, after 2 weeks, shoots were separated from the cultured explants and transferred individually to fresh medium for elongation.

MSB_5 medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Hi-Media, Mumbai, India) was used throughout this study, including rooting experiments. The

pH of the medium was adjusted to 5.8 with 1 N NaOH or HCl prior to autoclaving at 121^{0}C at 15 psi for 15 min. TDZ was filter sterilized and added to the medium after autoclaving and BAP was added prior to autoclaving.

Regeneration response was recorded based on the mean number of shoots per explant multiple shoot frequency. Shoot multiplication data was recorded after every passage of 2 weeks. The experiments were designed as four-factorial: the type of cytokinin (BAP and TDZ), dose of cytokinin (0, 5, 10 and 20 μM), duration of exposure (2-6 days) during seedling pretreatment and the type of medium (M1, M2 and M3) used for shoot multiplication to select the best media for maximum shoot multiplication. All experiments were set up in a completely randomized design and repeated thrice with a minimum of thirty replicates employed for each treatment. The data were subjected to analysis of variance (ANOVA) to detect significant difference between means. Significant differences were compared manually using Duncan's multiple range test at P=0.05.

Regenerated shoots (1.5-2.5 cm) were transferred to MS basal medium for rooting. After 2 weeks of culture, the rooted plantlets were washed thoroughly in running tap water and then transferred to plastic pots containing sterilized soil and vermiculite (1:1). For acclimatization, pots were covered with transparent plastic bags to avoid dessication. The plastic bags were gradually removed in 2 weeks, plants were maintained in the greenhouse, and the percentage of survival was recorded. After 4 weeks, plants were transferred to pots containing soil:compost (1:1) and grown to maturity. The percentage survival was recorded 15 days after transfer to greenhouse.

7.2.3 Establishment of selection system

Prior to transformation experiments, a threshold concentration of kanamycin for selection of putative transformed shoots was determined by culturing the explants on M3 medium supplemented with different concentrations of kanamycin (25, 50, 75, 100 and 125 mg/l).

Explants cultured on M3 medium in absence of any antibiotics served as control. The optimum selection pressure was determined based on the kanamycin dose that allowed initial proliferation of shoot buds, but bleached them afterwards. The observations were scored after 2 weeks on selection medium.

7.2.4 *Agrobacterium* strain, binary plasmid and transformation

A. tumefaciens strain EHA105 harboring the binary plasmid pSouv:cry1Ab (Bt*cry1Ab* expression cassette cloned in binary vector pCAMBIA2301) (Fig. 7.2) was used for transformation experiments. The T-DNA of pCAMBIA2301 includes neomycin phosphotransferase gene (*npt*II) and β-glucuronidase gene (*gus*) interrupted by catalase intron, both driven by the cauliflower mosaic virus (CaMV) 35S promoter. The bacterial suspension was prepared and explants were infected and co-cultivated as per the protocol described in Chapter 3; Section 3.2.5. The competence of explants derived from both untreated as well as treated (on 10 µM TDZ) seedlings for transformation was compared.

7.2.5 Shoot recovery

Following co-cultivation, the explants were washed four to five times with sterile double distilled water and blotted dry on sterile filter paper. The explants were cultured on M3 medium supplemented with 50 mg/l kanamycin and 500 mg/l cefotaxime for selective regeneration of transformants. Same levels of antibiotics were maintained during subsequent subcultures. Both regeneration controls (no cocultivation, no selection) and transformation controls (no cocultivation but selection on kanamycin) were used for all the experiments and at all cycles of selection. 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 greenhouse.

7.2.6 Histochemical GUS assay

Histochemical GUS staining was performed to verify GUS activity in transgenic cowpea (Jefferson 1987). Transient expression was examined after 3-days of cocultivation (Bakshi et al. 2011). Transient expression of GUS was scored on the basis of number of explants showing GUS expression. Stable *gus* expression was detected in various plant parts including flower, anthers, pollens and pistils following the described histochemical procedure. The efficiency of stable transformation was calculated as the number of PCR (polymerase chain reaction) positive shoots obtained on kanamycin selection per inoculated explants.

7.2.7 Evaluation of transgenic plants

Molecular characterization of the transformants was carried out by PCR, RT-PCR, rapid diagnostic assay and ELISA for confirmation of the presence, expression and inheritance of the introduced genes.

7.2.7.1 Screening of putative transformed plants using polymerase chain reaction (PCR)

Genomic DNA was isolated from young leaves of T_0 putative transformants using the modified CTAB method (Solleti et al. 2008a). PCR amplification was carried out with gene specific primers for *npt*II and *Btcry1Ab* using genomic DNA from putative transformed plants, non-transformed control plants (negative control) and pSouv:cry1Ab (positive control) as templates. The 540 bp region of *npt*II and 1.86 kb coding region of *Btcry1Ab* were amplified by using respective oligonucleotide primers. The amplification reaction was carried out under following conditions: 94^0C for 5 min (1 cycle), 94^0C for 1 min (denaturation), 58^0C for 1 min (anneling for *nptII*) and 60^0C for 2 min (annealing for *cry1Ab*), 72^0C for 1 min (extension) for 35 cycles followed by the final extension at 72^0C for 7 min (1 cycle). The PCR was performed by using ~100 ng of purified genomic DNA and Taq DNA polymerase (Genei, Bangalore, India) according to manufacturer's instruction. The

amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

7.2.7.2 Qualitative reverse transcription (RT)-PCR analysis

Total RNA was isolated from the PCR-positive transgenic T_0 plants using Trizol Reagent (Invitrogen, USA) from 100 ng of leaf tissue according to the manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.5% denaturing agarose gel (Sambrook et al. 1989). RT-PCR was carried out using First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. PCR of the coding sequences of *cry1Ab* gene in the cDNA was carried out using respective primers as described earlier. The house keeping gene *actin* was used as a control to indicate the amount of starting RNA. The PCR products were electrophoresed on 1.0% agarose gels, detected by ethidium bromide staining, and photographed through the Bio-Rad gel documentation instrument.

7.2.7.3 Cry1Ab Protein Expression Analysis

To test for the presence of Cry1Ab protein, total soluble protein was isolated from leaves of transgenic lines using sample extraction buffer (DesiGen, India) according to the manufacturer's instructions. Rapid detection of Cry1Ab gene expression in the leaves of transgenic plants was carried out using immunodiagnostic Xpresstrips™ (DesiGen, India). DesiGen Xpresstrips™ are lateral flow devices which detect the Cry1Ab protein in extracts from plant samples. The protocol is described previously in Chapter 5; Section 5.2.6. The test line that appears near the sample pad indicates positive for Cry1Ab protein. Absence of test line signifies no expression of Cry1Ab.

7.2.7.4 Enzyme Linked Immunosorbant assay (ELISA)

ELISA was performed to quantify the accumulated levels of Cry1Ab protein in T_0 transgenic plants using Desigen Quan T-ELISA-96 well plate kit (Desigen, Maharashtra, India)

following manufacturer's protocol. Total protein was extracted from 5 mg dry leaf powder using 500 µl of sample extraction buffer. The sample was chilled and spun at 8000 rpm for 15 min and 100 µl of supernatant was used for loading to anti-Cry1Ab pre-coated plate. For the estimation of Cry1Ab, the 96 well titre plate was coated with 150 µl per well (1:1000) of goat anti-Cry1Ab antibodies. The plate was then loaded with 100 µl samples and buffer was used in control wells. The plate was incubated at 37^0C for 1.5 h, followed by washing with wash buffer twice. After washing, the plate was incubated with alkaline phosphatase conjugated secondary antibodies at a dilution of 1:1000 with 250 µl per well for 45 min at 37^0C. The plate was then washed with wash buffer twice and 250 µl of freshly prepared substrate (p-nitro phenyl phosphate, 1 mg/ml) was added per well. The plate was incubated at room temperature in the dark for 30 min and reaction was stopped and readings recorded at 405 nm in a micro plate reader (Tecan, Switzerland).

7.2.8 Segregation analysis

The leaves of T_1 transgenic plants generated from twelve independent transformation events were analyzed for the presence of *cry1Ab* genes using PCR, as described earlier. Segregation patterns were analyzed with the Chi-square test ($\chi 2$) as described by Solleti et al. (2008b).

7.3 Results and discussion

Development of a high-throughput *Agrobacterium* mediated cowpea transformation technology is dependent on the establishment of a protocol for proficient multiple shoot differentiation, shoot development and whole plant regeneration from tissues competent for transformation. We evaluated the organogenic potential of split cotyledonary nodes of cowpea, derived from pre-treated seedlings, using shoot multiplication media with three different combinations of cytokinins.

7.3.1 Effect of seedling preconditioning on direct organogenesis and plant regeneration

Seeds cultured on MSB_5 medium germinated normally whereas those cultured on media supplemented with either TDZ or BAP (0, 5, 10 and 20 µM) germinated with thickened seedlings, stunted and enlarged cotyledons, swelled cotyledonary nodes, and thick and short roots. The explants from untreated seedlings showed least response in shoot regeneration media (Fig. 7.3) as compared to the explants from seedlings treated with either TDZ or BAP which showed significant increase in regeneration of shoots irrespective of the shoot multiplication medium used (Fig. 7.3a, c, e). Therefore, the effect of cytokinin dose and duration of seedling pretreatment on regeneration response of explants on different shoot multiplication medium were evaluated. Although preconditioning in either TDZ or BAP enhanced mean shoot number by 2.5-7.5 fold, TDZ at equimolar dose of BAP exerted 1.5 to 3 fold increase in mean shoot number. Among the different dose of TDZ and BAP tested for seedling preconditioning, maximum mean number of shoots (16.11 shoots) were induced from explants of seedlings pretreated with 10 µM TDZ (Fig. 7.3c). Further increase in TDZ concentration to 20 µM, reduced the number of shoots induced per explants. Moreover, shoots formed as a cluster, and it took a much longer time for the cluster to develop into individual shoots. Consequently, seedlings preconditioned with 10 µM TDZ was used for all experiments for optimal multiple shoot induction. The dose dependent positive effect of seed pretreatment in TDZ on shoot multiplication efficiency of split cotyledonary node explants in cowpea clearly reaffirmed the role of seedling preconditioning on shoot organogenesis of explants.

Among the different pretreatment durations, 4 days pretreatment in either TDZ or BAP induced higher mean number of shoots as compared to 2- or 6-days pretreatment (Fig. 7.3). Longer pretreatment duration (6 days) in either TDZ or BAP showed no significant

improvement in mean number of shoots (Fig. 7.3e, f). Four days pretreatment in 10 µM TDZ was found optimal for maximum shoot induction and therefore, used in all subsequent regeneration and transformation studies.

7.3.2 Effect of culture media on shoot proliferation

The frequency of the regeneration and mean number of shoots were affected by the size and type of the explants. In the present study, the effects of three different media supplements were investigated on shoot regeneration from split cotyledonary node explants. The results showed significant differences in shoot regeneration response for the various media supplements. Among them, medium containing a combination of 5 µM BAP and 0.5 µM kinetin induced significantly higher shoot proliferation (Fig. 7.3) as compared to medium with either 5 µM BAP or a combination of 5 µM BAP and 0.1 µM kinetin.

After 1 week on regeneration medium, callus began to appear at the bottom of shoot clusters in the medium (Fig. 7.4b). Therefore, explants needed to be transferred to fresh medium every week. Once shoot clusters formed, shoots needed to be separated and transferred to fresh medium in order to elongate, or shoots would deteriorate. The shoots excised and elongated during successive subculture on the fresh media, formed well developed roots on MS basal medium within 2 weeks (Fig. 7.4d). Following transfer to soil, plantlets were successfully hardened with 94% survival (Fig. 7.4e). The regenerated plants were apparently morphologically normal and flowered normally and set seeds. The entire procedure from seed germination to establishment of plants under greenhouse conditions took approximately 5 weeks.

7.3.3 Effect of kanamycin on shoot organogenesis from explants

The split cotyledonary nodes used as explants were susceptible to all the tested concentrations of kanamycin, whereas green shoots were formed in antibiotic-free regeneration medium. Nevertheless, the study showed significant difference among

different kanamycin treatments (Fig. 7.5; 7.6). The regeneration efficiency gradually declined with increase in kanamycin concentration in the medium (Fig. 7.5). However, a few explants that regenerated on medium with kanamycin concentration above 50 mg/l on reculturing on fresh media with same level of kanamycin resulted in inhibition of shoot proliferation from explants, and the appeared green shoots became chlorotic within 20 days of incubation (Fig. 7.6c-f; Fig. 7.7). Therefore, M3 media with 50 mg/l of kanamycin was used for selection of transformed shoots.

7.3.4 Recovery of transgenic plants

In the study to establish a selection regime it was observed that, after the first cycle of selection, the explants in the regeneration control cultures showed induction and proliferation of green shoot buds in all the explants (Fig. 7.8d) while those on the transformation control cultures showed formation of shoot buds in few explants (Fig. 7.8c, d). On further selection, the explants on transformation control cultures showed complete chlorosis of differentiated shoots (Fig. 7.8c). However, the frequency of shoot regeneration as well as the number of shoots per surviving explant decreased with each cycle of selection. To decrease the undue effects of the selection agent with continued exposure on the putative transformants the concentration of kanamycin in the third cycle of selection was decreased to 35 mg/l and a selection regime of 50–50–35 mg/l was followed.

The primary transformants obtained after the third cycle of selection from 4 sets of experiments. A total of 161putative transformed shoots that were 2–4 cm in length with 2–3 distinct nodes were transferred to rooting medium, where the shoots developed roots after 15–20 days in culture. The frequency of rooting of the primary transformants (82%) was lower compared with that of the regenerated shoots (97%). About 132 shoots showed rooting, but a very few rooted shoots failed to survive on acclimatization. The shoots that

survived on sterile soilrite (Fig. 7.8e) were transferred to soil in pots and left in the greenhouse.

Under the transformation and selection conditions described here, 153 kanamycin-resistant plantlets out of 975 infected explants were obtained (15.7%) after one round of selection; however, after 4 weeks of recurrent 3 rounds of selection, only 132 plantlets were selected (13.5%). No shoot emerged from control non-infected explants placed on selection medium. Although the transformation frequency determined as the percentage of kanamycin-resistant plant regenerated over the total infected explants is not a reliable measure of transformation efficiency, it provided an indirect measure of this; in this sense, putative-transformed plants of cowpea were generated at transformation rate of 13.5%. Almost all transformed shoots were phenotypically normal and rooted 6 weeks after co-cultivation on rooting media in absence of kanamycin and finally established in greenhouse containment (Fig. 7.8e).

A strong and uniform GUS expression was detected in flower, anthers, pollens and pistils of kanamycin resistant putative-transformed plants (Fig. 7.8f-j) confirming the presence and stable expression of the transgene. No endogenous GUS-like activity was detected in the tissues of uninfected control plants.

7.3.5 Molecular analysis of putative transformants

Molecular characterization of the T_0 transformants was carried out by GUS histochemical analysis, PCR, RT-PCR, rapid immunodiagnostic assay and ELISA for confirmation of the presence, integration and expression of the introduced genes.

All the explants showed GUS activity following 3 days of co-cultivation with EHA105 harboring pCAMBIA2301*cry1Ab*, predominantly in the regenerating sites (Fig. 7.8b) which was not observed in untransformed control explants (Fig. 7.8a).

The putative transformants obtained after the 3 cycles of selection were subjected to PCR with *npt*II and *cry*1Ab gene specific primers. PCR amplification of transformants with the *npt*II and *cry*1Ab gene specific primers showed DNA fragments of the expected size of 540 bp and 1.86 kb, respectively, confirming the presence of the introduced DNA in the transformed shoots (Fig. 7.9a, b). Both *npt*II and *cry*1Ab DNA fragments were not detected in the control (untransformed) plants.

It is not only essential to determine the presence of the transgene but also important to check the expression of the introduced gene. Transcript detection in leaves of PCR positive T_0 transgenic plants was done through RT-PCR analysis with *cry1Ab* gene specific primers, which showed the expected band size of 1.86 kb, in all 5 plants tested (Fig. 7.9c), confirming the accumulation of transgene transcripts. Furthermore, the amplification of the *cry1Ab* sequence from plant cDNA templates in RT-PCR ruled out the possibility of *Agrobacterium* contamination. Amplification was not observed in the control (untransformed) plants for either *npt*II or *cry1Ab* genes. Both transformed and untransformed plants showed expression of reference gene *actin* as indicated from amplification of 200 bp (Fig. 7.9c). There were no significant differences in the levels of gene expression.

The stable transformation efficiency was determined based on the number of T_0 plants PCR-positive for *cry1Ab* and *nptII* divided by the total number of explants co-cultivated. An average stable transformation efficiency of 5.01was recorded (Table 7.1).

The randomly chosen PCR-positive transgenic lines were subjected to Cry1Ab protein expression analysis by rapid qualitative immunodiagnostic assay and ELISA. The clear appearance of test line in the DesiGen Xpresstrips™ confirmed the presence of Cry1Ab protein in transgenic lines (Fig. 7.10a). The level of expression of Cry1Ab protein in transgenic lines ranged from 0.001% to 0.008% of total leaf soluble protein (Fig. 7.10b).

Such variation in the level of Cry1Ab protein is usually attributed to unpredictable levels of transgene expression as a consequence of position effects resulting from differences in the integration site.

The seeds from T_0 generation were advanced to T_1 generation and the T_1 transgenic lines generated from 12 independent transformation events were analyzed for the segregation pattern of *cry1Ab* by PCR analysis. Presence of the expected 1.86 kb amplified product corresponding to *cry1Ab* in T_1 transgenic lines confirmed the inheritance of *cry1Ab* gene. The segregation pattern of these selected transgenic events showed typical 3:1 Mendelian ratio as expected for single dominant gene inheritance (Table 7.2).

7.4 Conclusion

We report here the development of a simple, robust and highly efficient *Agrobacterium*-mediated transformation protocol for cowpea in which split cotyledonary nodes were used as the explants for the generation of transgenic plants. The plants were induced from split cotyledonary node explants of cowpea seedlings pretreated with TDZ. To our knowledge, this is the first report of reproducible production of transgenic cowpea plants using split cotyledonary node explants as the target for *Agrobacterium*-mediated transformation, and also the first report of *cry1Ab* expression in transgenic cowpea plants. Cowpea is known to be stubbornly recalcitrant to genetic transformation by *Agrobacterium* as evident from low stable transformation efficiency in published reports (Solleti et al. 2008b). One of the main bottlenecks in regenerating transformed plants through axillary shoot proliferation from most preferred target tissue, intact cotyledonary nodes was the limited accessibility of *Agrobacterium* to regenerating cells, located few cell layers beneath the epidermis of cotyledonary node region (Bakshi et al. 2011).

Only two types explants have been previously used in the development of *Agrobacterium* mediated transformation protocols for cowpea (Muthukumar et al. 1996;

Popelka et al. 2006; Chaudhury et al. 2007; Solleti et al. 2008a, b; Bakshi et al. 2011). Our success in obtaining transgenic cowpea using split cotyledonary node explants is probably due to the fact that by splitting them in halves, we increased the exposure of actively dividing plant cells to *A. tumefaciens*, facilitating infection and subsequent transfer of the T-DNA into the plant cell. It has been previously shown that host-cell division is required for successful *Agrobacterium* transformation (Binns and Thomashow 1988). We found that the mean shoot number could be dramatically increased by 2.1 fold respectively by longitudinally cutting cotyledonary node tissues as relative to uncut explants (Solleti et al. 2008a). Explants prepared by longitudinally cutting have been reported to enhance shoot multiplication in green bean (Franklin et al. 1991), sweet orange and citrange (Yu et al. 2002), sunflower (Hawezi et al. 2003), cotton (Hazra et al. 2002; Guru Prasad et al. 2011) and common bean (Dang and Wei 2009).

The high-throughput cowpea transformation system developed using split cotyledonary node as explants would accelerate cowpea genetic improvement program by transfer of candidate genes for both biotic and abiotic stress tolerance. The protocol would also lead to the possibility of the development of improved functional genomics tools for use in cowpea improvement by over-expression or silencing of target genes. In addition, with routine 5.01% transformation efficiencies, the development of tools such as T-DNA insertion populations in cowpea would now be a possibility. It is expected that with further optimisation, even higher efficiencies will be achieved opening up even more possibilities for transformation based resources in cowpea. Furthermore, the large number of transgenic cowpea plants expressing *cry1Ab* would pave way in verifying the efficacy of Cry1Ab proteins against cowpea insects.

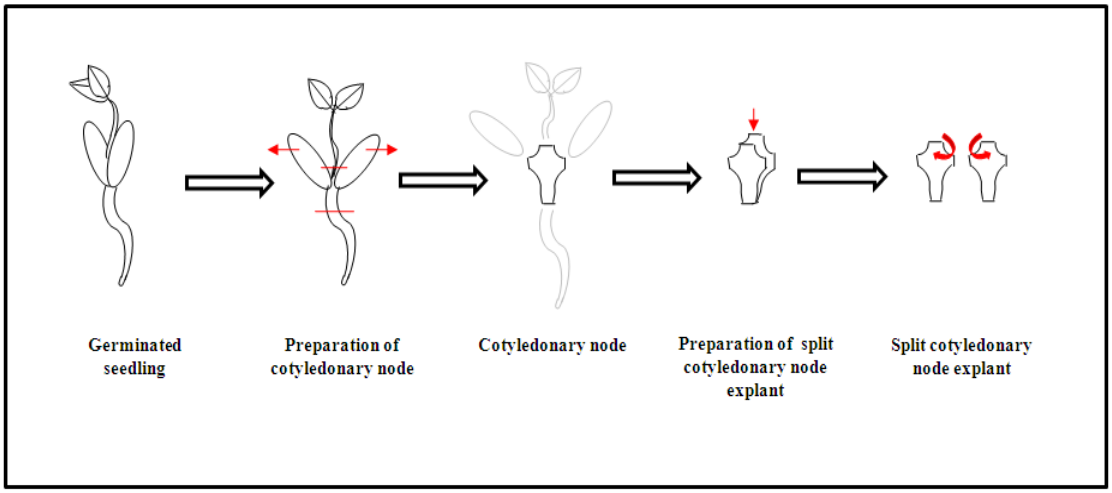


Fig. 7.1 Schematic representation of split cotyledonary node

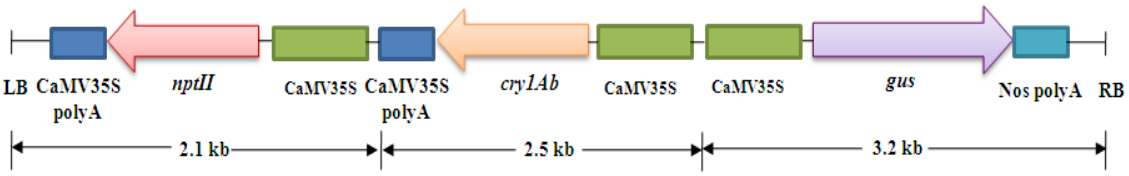


Fig. 7.2 T-DNA region (7.8 kb) of pSouv:cry1Ab. LB left border, RB right border, 35SP CaMV 35S promoter, CaMV35polyA CaMV35S terminator, NOS nopaline synthase terminator, *gus* intron interrupted β-glucuronidase, *npt*II neomycin phosphotransferase II. *cry1Ab Bacillus thuringiensis* crystalline protein toxin 1

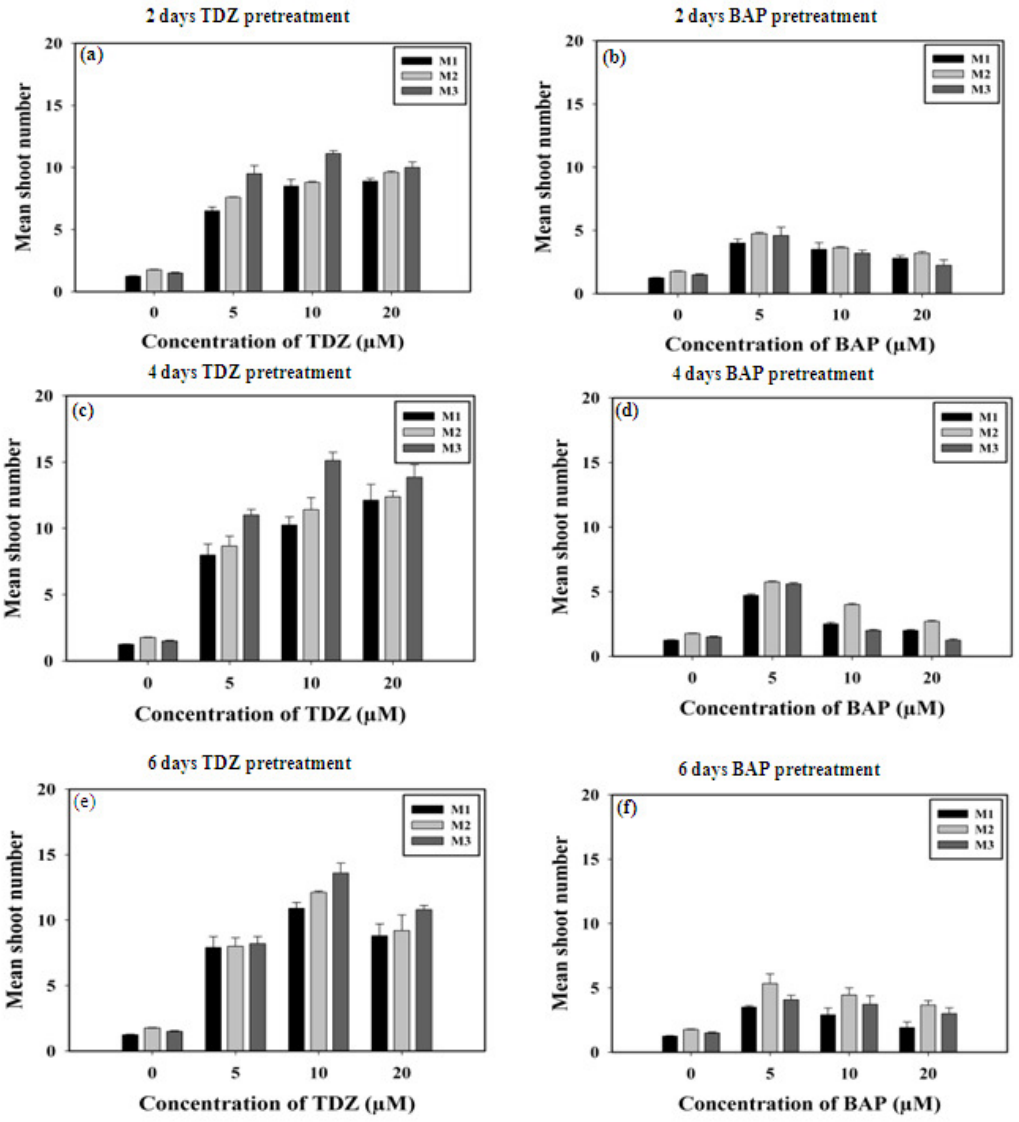


Fig. 7.3 Effect of seed pretreatment in the presence of different concentrations (5, 10, 20 µM) of TDZ and BAP, and the duration of pretreatment (2, 4, 6 days) on multiple shoot induction from split cotyledonary node explants of cowpea (along X-axis: Concentration of TDZ or BAP in µM, along Y-axis: Mean shoot number).

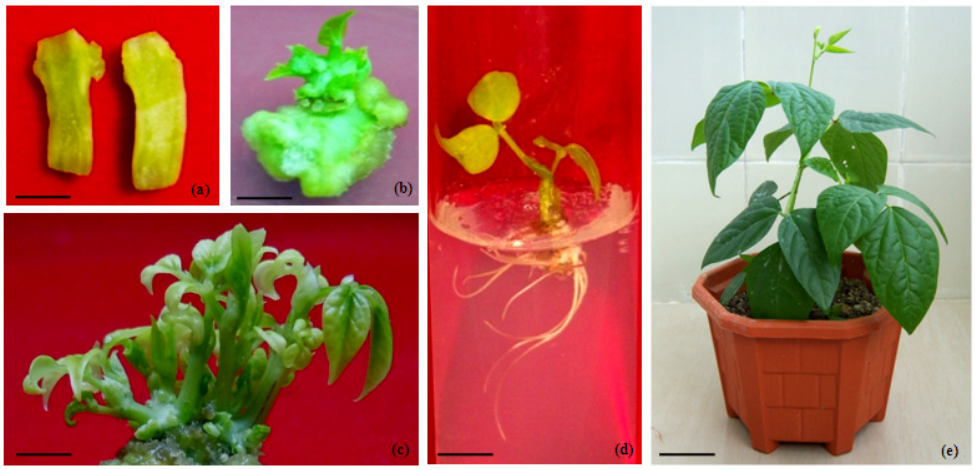


Fig. 7.4 Multiple shoot induction and plant regeneration from split cotyledonary node explants of cowpea (*Vigna unguiculata* L. Walp) cv. Pusa Komal. (a) 4-d old split cotyledonary node explant (Bar=3 mm); (b) Shoot induction from explant (Bar=3 mm); (c) Multiple shoot proliferation on regeneration media (Bar=1 cm); (d) *In vitro* rooting (Bar=1 cm); (e) Transgenic plant acclimatize into Greenhouse (Bar=10 cm)

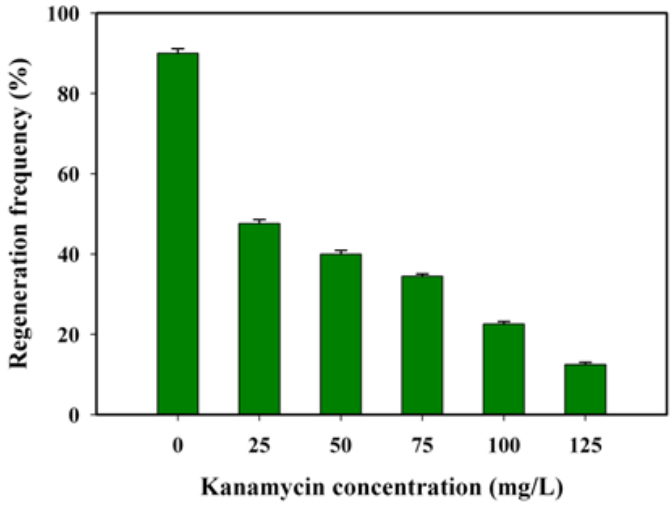


Fig. 7.5 Effect of various kanamycin concentration (0, 25, 50, 75, 100, 125 mg/l) on shoot regeneration rate

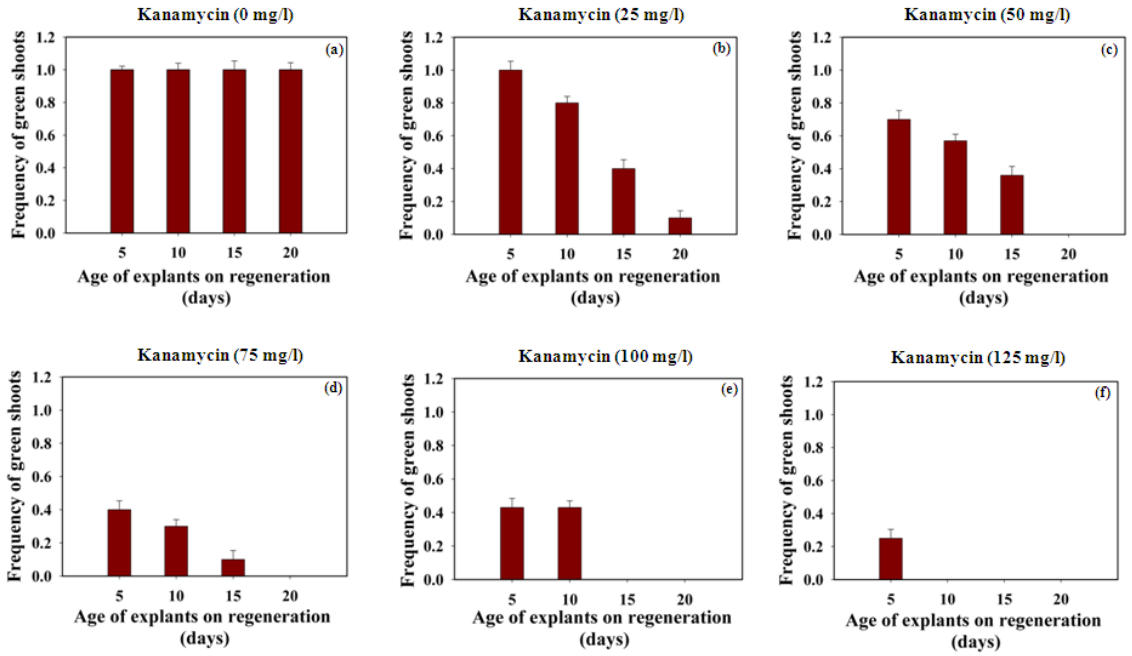


Fig. 7.6 Selection optimisation for split cotyledonary node explants on various concentration of kanamycin (0, 25, 50, 75, 100, 125 mg/l)

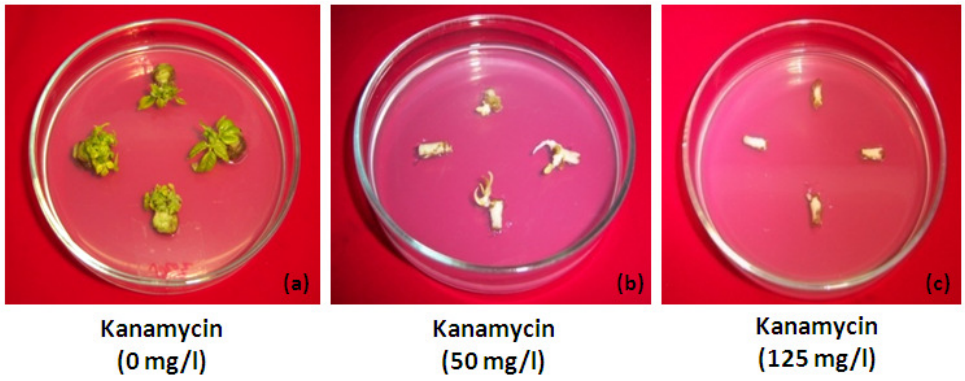


Fig. 7.7 Effect of kanamycin concentration on split cotyledonary node regeneration. (a) Green shoot proliferation on media devoid of kanamycin; (b) Responded explants turned bleached on media containing 50 mg/l kanamycin; (c) Explants were uncapable to regenerate on media containing 125 mg/l kanamycin. Data were taken after 15 days of culture.

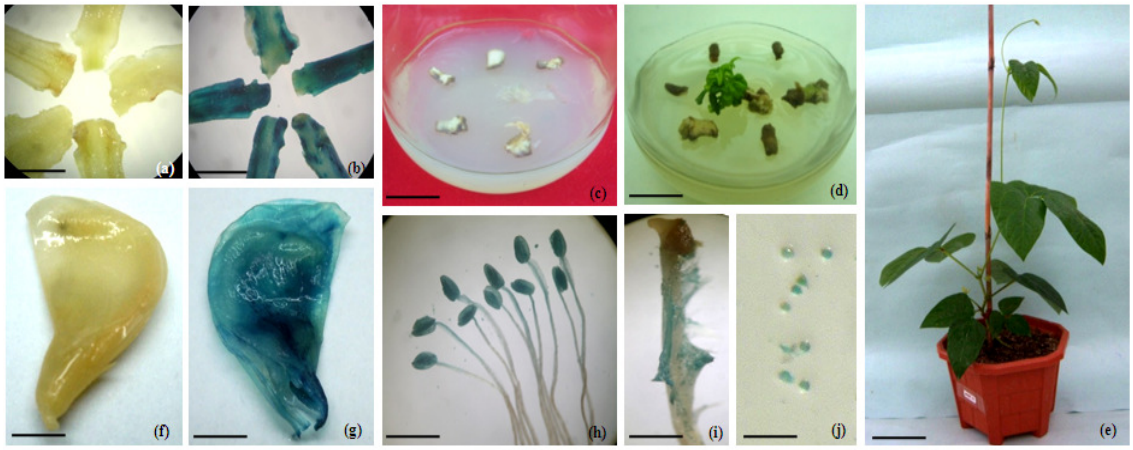


Fig. 7.8 Transient and stable *gus* expression and regeneration of transgenic plants. (a and b) transient GUS expression, non-transformed (control) explants are not showing GUS activity (a), cotyledonary node explants showing transient GUS activity after 3 days of cocultivation (b). Bar=4 mm; (c and d) Selection of transformed explants, (c) Untransformed explants were bleached on selection media; (d) Shoots were proliferated from transformed explants when cultured on selection media. Bar=2 cm; e acclimatized plant maintained in transgenic green house. Bar=10 cm; (f - j) Stable GUS expression in different part of flower of T_0 transgenic plants f non-transformed control flower. Bar=7 mm; g transformed flower. Bar=7 mm; h transformed anthers. Bar=8 mm; i transformed pistil. Bar=8 mm; j transformed pollens. Bar=3 mm

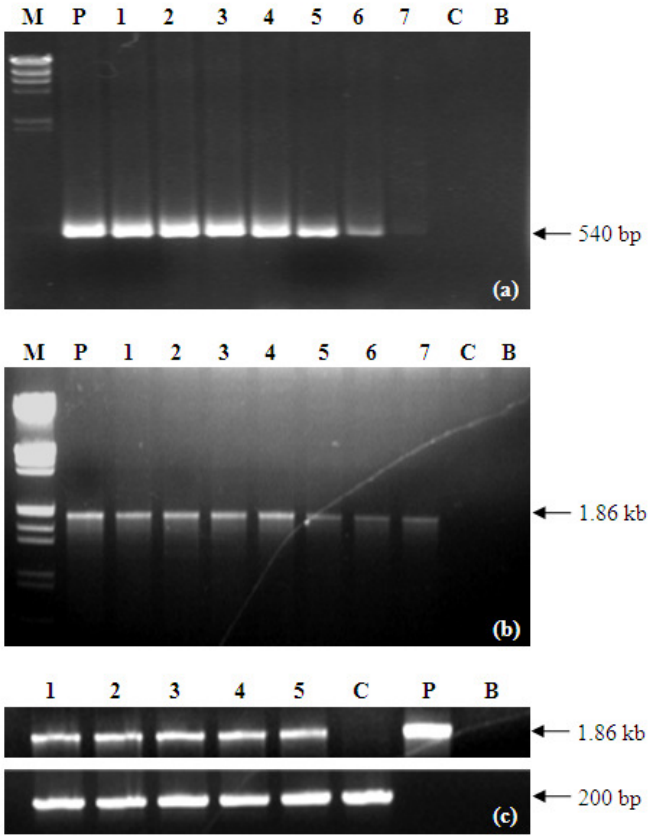
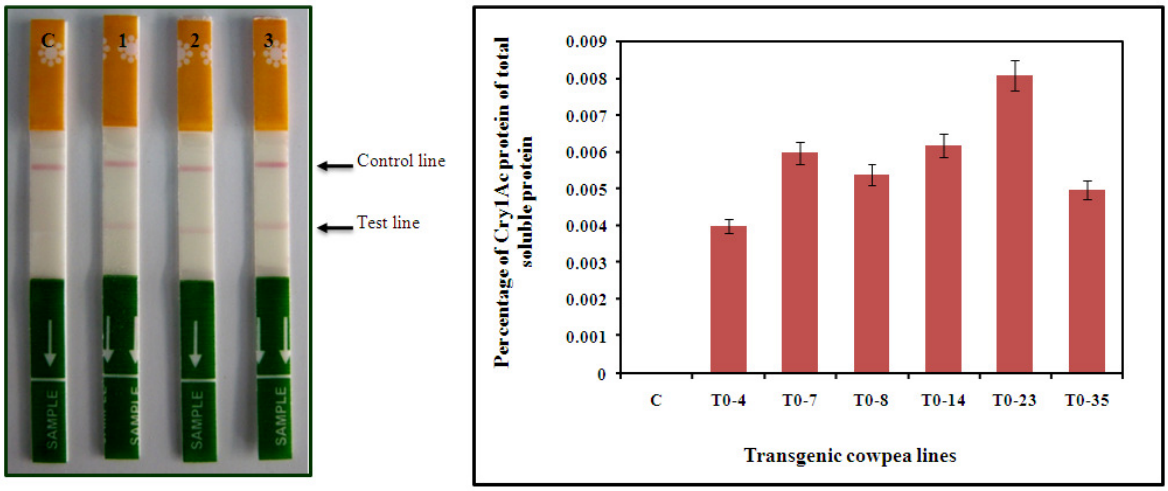


Fig. 7.9 Molecular analysis of T_0 transgenic plants. (A) PCR amplification of the 540 bp fragment of *npt*II gene. Lane M λDNA/*Hind*III marker, lane P pCAMBIA2301cry1Ab plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lane B blank, lanes 1–7 DNA from independently transformed plants. (B) PCR amplification of the 1.86 kb fragment of *cry1Ab* gene. Lane M λDNA/*Eco*RI + *Hind*III marker, lane P pCAMBIA2301cry1Ab plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lane B blank, lanes 1–7 DNA from independently transformed plants. (C) RT-PCR analysis of *nptII,* and reference gene *actin*, Lane M λDNA/*Eco*RI + *Hind*III marker, lane P pCAMBIA2301cry1Ab plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lanes 1–5 T_0 transgenic plants



(a) (b)

Fig. 7.10 Detection of Cry1Ab protein expression in transgenic lines. (a) Rapid immunodiagnostic test for detection of Cry1Ab in T_0 transgenic lines using DesiGen Xpresstrips™. C no test line appeared from control plants after lateral flow assay; (1-3) transgenic lines showed test line signifies the presence of Cry1Ab, (b) Expression level of BtCry1Ab protein in transgenic cowpea lines (T0-4, T0-7, T0-8, T0-14, T0- 23 and T0-35) from enzyme-linked immunesorbant assay (ELISA). Error bars represent ± standard error of the means

Table 7.1 Summary of the *Agrobacterium*–mediated transformation of cowpea split cotyledonary node explants

Exp. no.	No. of explants inoculated in *Agrobacterium* suspension	Transient transformation efficiency[a] (%)	No. of shoots recovered on selection medium	No. of plants positive for *cry1Ab* genes by PCR	Transformation efficiency[b] (%)
1	245	100	48	12	4.89
2	255	100	51	14	5.49
3	240	99	39	11	4.58
4	235	100	23	12	5.1
Total[c] / Average[d]	975[c]	99.75[d]	40.25[d]	12.25[d]	5.01[d]

[a] Number of explants showing GUS expression per number of explants cocultivated with *Agrobacterium tumefaciens* EHA105pSouv:cry1Ab

[b] Number of plants PCR-positive for *cry1Ab* and *npt*II per number of explants co-cultivated

Table 7.2 Segregation of *cry1Ab* gene in T_1 progeny of transgenic cowpea plants

T_0 plants	Number of T_1 plants tested for *cry1Ab*[a]			χ^2 value	Expected Ratio
	Total	*cry1Ab*+ve	*cry1Ab*-ve		
C-AB1	43	33	10	0.069	3:1
C-AB2	36	28	8	0.148	3:1
C-AB3	52	40	12	0.101	3:1
C-AB4	28	20	8	0.189	3:1
C-AB5	29	23	6	0.286	3:1
C-AB6	44	31	13	0.484	3:1
C-AB7	51	38	13	0.005	3:1
C-AB8	37	29	8	0.224	3:1
C-AB9	42	30	12	0.285	3:1
C-AB10	37	28	9	0.008	3:1
C-AB11	28	20	8	0.189	3:1
C-AB12	36	33	3	0.266	15:1

[a] Presence of *cry1Ab* was analyzed by PCR

Chapter 8

Development of a mannose based positive selection system for the recovery of transgenic cowpea

8.1 Introduction

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important food legumes which provide quality protein for the world's poorest and most food-insecure populace in the underdeveloped and developing nations of Sub Saharan Africa, Asia and Central America (Shimelis and Shiringani 2010). However, the production of cowpea is severely limited by biotic constraints which reduce the overall average grain yield losses by 0.37 ton per hecter (Waddington et al. 2010). Conventional breeding for improvement of cowpea for insect pest and disease resistance has met with limited success due to narrow genetic base and barriers in crossing with distant wild species (Fang et al. 2007). Development of reliable genetic transformation system for cowpea would form the basis to transfer genes for desirable traits and accelerate crop improvement program by complementing classical breeding approaches. Furthermore, the robust genetic transformation system would provide a valuable tool for functional genomics studies of cowpea. However, grain legumes in general and cowpea in particular are known to be highly recalcitrant to genetic manipulation in *in vitro* (Singh and Singh 2010). Absence of robust and efficient genetic transformation system in cowpea has remained as the bottleneck in targeted improvement for biotic and abiotic stress tolerance (Obembe 2008).

Possibility of recovering stable transgenic plants of cowpea has been reported through *Agrobacterium*-mediated transformation of cotyledonary explants (Muthukumar et al. 1996; Popelka et al. 2006; Chaudhury et al. 2007; Solleti et al. 2008a, b; Bakshi et al. 2011) and direct DNA delivery to shoot apices by particle bombardment (Ivo et al. 2008). Using *Agrobacterium*-mediated transformation of cotyledonary node explants, recently, we reported the development of transgenic cowpea expressing common bean α-amylase inhibitor (*α-ai1*) gene for storage pest resistance (Solleti et al. 2008b) and *Btcry1Ac* gene (Bakshi et al. 2011) towards insect resistance. For the transformation of cowpea, the

published protocols have utilized the use of the hygromycin phosphotransferase (*hpt*) gene, conferring resistance to aminocyclitol antibiotic, hygromycin (Muthukumar et al. 1996) or neomycin phosphotransferase II (*npt*II) gene, conferring resistance to aminoglycoside antibiotics, such as kanamycin (Chaudhury et al. 2007; Solleti et al. 2008b; Bakshi et al. 2011), and geneticin (Solleti et al. 2008a) or of the *bar* gene, which encodes for phosphinothricin acetyltransferase and confers resistance to herbicide l-phosphinotricin (Popelka et al. 2006) or the mutated *ahas* gene, which encodes for a mutated acetohydroxyacid synthase and confers resistance to herbicide imazapyr (Ivo et al. 2008).

Although promising results have been obtained, cowpea genetic transformation still remains far from routine due to the poor selection of transformed shoots on antibiotic- or herbicide-supplemented medium leading to regeneration of escapes, growth retardation of selected transformed shoots owing to long-time exposure to stringent selection and consequently low rooting efficiency of the transgenic plants (Bakshi et al. 2011). During such negative selection, the majority of the cells in the cultured tissue die. These dying cells may release toxic substances (such as phenolics), which in turn may impair regeneration of the transformed cells. In addition, dying cells may form a barrier between the medium and the transgenic cells, thereby preventing or slowing the uptake of essential nutrients (Joersbo and Okkels 1996). Furthermore, these few well-characterized selection systems for higher plants may not work efficiently with all species of interest. In addition, paucity of selection options can be a constraint for the stacking of multiple transgenes in a given plant line and consequently may be a barrier to a wider adoption of transformation technology as retransformation would preclude the use of the same marker gene (Hare and Chua 2002). Moreover, the use of such markers for selecting transformed plants has continued to generate widespread public concerns of potential harmful effects to the environment and human health (Qiao et al. 2010). These include the real or perceived threats of transfer of

herbicide- or antibiotic-resistant genes to weedy relatives or microorganisms. An additional consideration is that antibiotic and herbicide resistance genes in widely grown crops may pose real or perceived threats of transfer to weedy relatives or microorganisms. Although all evidence indicates that selection genes pose no health threat to human or animal consumers, there is a definite possibility of poor consumer acceptance of food products containing antibiotic-inactivating proteins (Zhu et al. 2005). Beside the lack of public acceptance of transgenic plants primarily due to the perceived health implications of consuming genetically modified food containing antibiotic resistance genes, the regulatory authorities demand for the avoidance of such genes. New European laws restrict the deliberate release of genetically modified plants transformed with marker genes conferring resistance to clinically important antibiotics (Zhu et al. 2005).

To circumvent problems associated with antibiotic- or herbicide-based selection systems, methods to either eliminate selection genes from the transformed plants or strategies to avoid selection of transformed cells with antibiotics and herbicides have been developed. Among them, methods based on "positive" selection are becoming increasingly popular. A "positive" selection regimen essentially incorporates a physiologically inert metabolite as the selection agent and a corresponding selectable marker gene that confers a metabolic advantage to transformed plant cells to metabolize compounds that are usually not metabolized by plants (Joersbo and Okkels 1996). One of these methods involves the use of mannose, a hexose sugar, as the carbon source in media because plants are not able to metabolize mannose as easily as other sugars (Lee and Matheson 1984). The mannose-positive selection system favours the regeneration and growth of the transgenic cells expressing phosphomannose isomerase (PMI) gene, *manA* while the growth of the non-transgenic cells is inhibited through carbohydrate starvation (positive selection). The utility of the *pmi* from *Escherichia coli* (Miles and Guest 1984) as a positive selectable marker in

conjunction with mannose for recovery of transgenic plants has been demonstrated in number of crops, sugar beet (Joersbo et al. 1998), cassava (Zhang and Puonti-Kaerlas 2000), maize (Wang et al. 2000; Wright et al. 2001), wheat (Wright et al. 2001), pepper (Kim et al. 2002), sweet orange (Boscariol et al. 2003), pearl millet (O'Kennedy et al. 2004), tomato (Sigareva et al. 2004), papaya (Zhu et al. 2005), onion (Aswath et al. 2006), almond (Ramesh et al. 2006), and cucumber (He et al. 2006), maize (Privalle 2002), rice (He et al. 2004), wheat (Wright et al. 2001), barley (Reed et al. 2001), pearl millet (O'Kennedy et al. 2004), pepper (Kim et al. 2002), tomato (Sigareva et al. 2004), apple (Degenhardt et al. 2006), cabbage (Min et al. 2007), sugarcane (Jain et al. 2007), flax (Lamblin et al. 2007), citrus (Ballester et al. 2008), sorghum (Gurel et al. 2009) and chickpea (Patil et al. 2009).

To our knowledge, the *pmi*/Man system has not been tested previously in cowpea. For each new crop, mannose selection system must be optimized for the production of transgenic plants efficiently. In this chapter, we report the development of an efficient system with mannose as a selectable agent for the *Agrobacterium*- mediated transformation of cowpea.

8.2 Materials and methods

8.2.1 Plant material

Cowpea seeds were surface sterilized (described in Chapter 3; Section 3.2.1) and cultured in plankton boxes (Tarson, Kolkota, India) containing MSB_5 medium [Murashige and Skoog (1962) salts and Gamborg B5 vitamins (1968)], 3% sucrose, and 0.8% (w/v) agar agar (HiMedia, Mumbai, India) containing 10 µM of TDZ. The cotyledonary nodes (5-6 mm) excised from 4 days old *in vitro* raised seedlings were used as explants for transformation and regeneration.

8.2.2 Binary plasmid, bacterial strain and culture conditions

The binary vector pNOV2819 (kindly provided by Syngenta, Switzerland) was used for cowpea transformation (Fig. 8.1). The T-DNA carried the *pmi* gene under the control of a 400-bp fragment of the *Cestrum* yellow leaf curling virus constitutive promoter (CMPS). It contained the essential promoter motifs plus a 62-bp fragment of the leader sequence downstream of the transcriptional start site (Stavolone et al. 2003). This promoter was shown to be highly active in vegetative and reproductive tissues of various mono- and dicotyledonous species (Stavolone et al. 2003). The pNOV2819 vector was mobilised by triparental mating into the disarmed *Agrobacterium tumefaciens* strain EHA105 and maintained on solid YEP medium (An et al. 1988) supplemented with 10 mg/l rifampicin and 100 mg/l spectinomycin. Prior to transformation, single bacterial colony was inoculated into 25 ml of liquid AB minimal medium (Chilton et al. 1974) with appropriate antibiotics and grown overnight at 28^{0}C on a rotary shaker at 180 rpm, until optical density at 600 nm reached to 0.8. The cells were collected by centrifuging at 5,000 rpm for 5 min, and then the pellet was resuspended in liquid co-cultivation medium, LCM (MSB_5 medium containing 1 µM BAP, pH adjusted to 5.5) supplemented with 100 µM acetosyringone prior to inoculation of the explants.

8.2.3 Explant sensitivity to the selection agent

Cotyledonary node explants were cultured on shoot regeneration medium (SRM) [MSB_5 medium containing 5 µM BAP and 0.5 µM kinetin containing 0.8% (w/v) agar-agar, pH 5.8] supplemented with various concentrations of mannose (0, 5, 10, 20, 30, 40 and 50 g/l) or sucrose (0, 2.5, 5, 7.5, 10, 20 and 30 g/l) to determine whether cowpea cotyledonary node explants could metabolize these sugars as the sole carbon source during shoot organogenesis. The experimental design was completely randomized with three replications, each consisting of 25 explants, with a total of 75 explants per treatment. After 4

weeks, the explants were scored for their regeneration response based on the percentage of explants forming shoots (regeneration efficiency) and percentage of necrotic explants.

8.2.4 Determination of mannose concentration for efficient selection

In order to find out an appropriate selection condition, the explants were cultured on SRM supplemented with various combinations of sucrose (S) and mannose (M), 2.5:0; 2.5:10; 2.5:20; 2.5:30; 5:0; 5:10; 5:20; 5:30; 7.5:0; 7.5:10; 7.5:20; 7.5:30. For each combination, 30 explants were used with three replicates per treatment. All cultures were incubated at 27°C with a 16-h photoperiod using cool white fluorescent tubes with an irradiance of 40 $\mu molm^{-2}s^{-1}$. After 4 weeks of culture, the explants were morphologically evaluated for their regeneration response based on the percentage of explants forming shoots (regeneration efficiency). A mannose dose-response curve was plotted from these completely randomized design treatments and data were analyzed.

8.2.5 Transformation and regeneration

The transformation procedure described in a recently published protocol by our laboratory (Bakshi et al. 2011) was used with few modifications. The explants were subjected to a combination of 20 s sonication followed by 5 min vacuum infiltration prior to inoculation in bacterial suspension for 30 min with occasional shaking. The explants were blotted on a sterile filter paper to remove excess liquid and cocultivated for 3 days under dark condition at 22^0C, in petridishes lined with filter paper moistened with LCM supplemented with 100 µM acetosyringone. Following cocultivation, the explants were rinsed three to four times with LCM and blotted dry on sterile filter paper and placed onto multiple shoot induction and selection medium, (SISM) [SRM containing 20 g mannose and 5 g sucrose, supplemented with 500 mg/l cefotaxime] for 42 days with two rounds of subculture at an interval of 14 days each. After 6 weeks of culture on selection medium, the surviving shoots were transferred to the rooting medium (MS + 2.5 µM IBA), containing 30 g/l sucrose

supplemented with 500 mg/l cefotaxime. Rooted putative transformed plants were transferred to pots containing sterile soil:compost (1:1) and acclimatized in greenhouse containment for 3 weeks.

8.2.6 Evaluation of transgenic plants

Molecular characterization of the transformants was carried out by PCR, Southern hybridization, RT-PCR analysis, and chlorophenol red (CPR) assay for confirmation of the presence, integration, expression and inheritance of the introduced *pmi* gene.

8.2.6.1 Screening of putative transformed plants using polymerase chain reaction

Genomic DNA was isolated from young leaves of putative T_0 putative transformants and T_1 transgenic plants using the modified CTAB method (Solleti et al. 2008a). PCR amplification was carried out with gene specific primers for *pmi* using genomic DNA from putative transformed plants, non-transformed control plants (negative control) and pNOV2819 (positive control) as templates. The 1.2 kb *pmi* coding region was amplified by using respective 30 mers (Fw: 5'-GCACTCGAGCTCTTACAGCTTGTTGTAAAC-3'; Rv: 5'-GCACTCGAGCATGCAAAAACTCATTAAACTCAG-3') oligonucleotide primers. The amplification reaction was carried out under following conditions: 94^0C for 1 min (1 cycle), 94^0C for 1 min (denaturation), 62^0C for 1 min (annealing), 72^0C for 1.5 min (extension) for 35 cycles followed by the final extension at 72^0C for 7 min (1 cycle). The PCR was performed by using ~100 ng of purified genomic DNA and Taq DNA polymerase (Genei, Bangalore, India) according to manufacturer's instruction. The amplified products were resolved on a 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

8.2.6.2 Southern hybridization

Randomly selected PCR-positive T_0 transgenic cowpea plants were further analyzed by Southern hybridization for the integration of the *pmi* gene. Ten µg samples of genomic DNA from non-transformed control and transgenic plants were digested with *HindIII*. The

digested samples were fractioned on a 0.8% agarose gel and transferred to Zeta- Probe membrane (Bio-Rad, USA). The blot was hybridized with DIG-labeled 1.2 kb PCR product, corresponding to the coding region of *pmi* gene. The probe labeling and Southern hybridization were performed using the nonradioactive DIG Labeling and Detection system (Roche, Germany) following supplier's instructions. Pre-hybridization and hybridization were carried out using high hybridization buffer containing 5XSSC, 1% blocking solution, 0.1% (w/v) N-lauroyl sarcosine and 0.02% (w/v) sodium dodecyl sulfate. Washing and detection were performed according to the instruction of the DIG labeling and detection system (Roche Diagnostics, Mannheim, Germany).

8.2.6.3 Qualitative reverse transcription (RT)-PCR analysis for pmi gene expression

Total RNA was isolated from the PCR-positive transgenic T_0 plants using Trizol Reagent (Invitrogen, USA) from 100 ng of leaf tissue according to the manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.5% denaturing agarose gel (Sambrook et al. 1989). RT-PCR was carried out using First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. PCR was performed with cDNA as template using primers specific to the coding sequences of pmi gene as described earlier. To check the integrity of cDNA and normalise the amount of cDNA template in each PCR reaction, a PCR product (200 bp) corresponding to the *β-actin* gene (internal standard) was amplified with *Jatropha curcas* actin (Accession no: HM044307.1) primers (Fw: 5′-ATGAGCTTCGAGTTGCAC-3′) and (Rv: 5′-ACCATCACCAGAATCCAG-3′). Amplified products were resolved on a 1% agarose gel.

8.2.6.4 Chlorophenol red assay

A chlorophenol red (CPR) assay was performed to visually verify PMI activity in transgenic plants (Wright et al. 2001). Leaf segments of PCR-positive transgenic T_0 plants were placed in separate wells of a multiwall plate containing MS liquid medium supplemented with 15

g/l mannose (control), pH 6.0, and 50 mg/l chlorophenol red and incubated for 24 hours in dark at 27^0C. Red or purple indicated no enzyme activity whereas yellow or orange indicated PMI activity due to acidification of medium by the mannose-resistant leaf tissue.

8.2.7 Inheritance of mannose resistance in the progeny of cowpea transformants

The leaves of T_1 transgenic plants (progeny of greenhouse-grown primary transformants, T_0 generation) generated from eight independent transformation events were analyzed for the presence of *pmi* gene using PCR, as described earlier. Segregation patterns were analyzed with the Chi-square test ($\chi 2$) as described by Solleti et al. (2008b).

8.3 Results and discussion

8.3.1 Effects of mannose on shoot formation from cotyledonary node explants

The use of the *pmi*/mannose selection system in genetic transformation is based on its ability to inhibit shoot organogenesis from non-transformed explants cultured on medium using mannose as a carbon source. In cowpea, cotyledonary node explants have been most preferred for transformation and regeneration of transgenic plants due to their competence for prolific shoot regeneration and T-DNA delivery (Bakshi et al. 2011). Efficient shoot proliferation is induced from cowpea cotyledonary node explants cultured in media containing salts, vitamins, growth regulators and sucrose as a carbon source (Solleti et al. 2008a, b; Bakshi et al. 2011). In order to recover transgenic plants of cowpea using mannose selection system, it is imperative to evaluate the sensitivity of cowpea explants to mannose for determining selective concentration as some leguminous plants such as soybean have been reported to have PMI activity (Lee and Matheson 1984). The effect of various mannose concentrations (0-50 g/l) on shoot formation from non transformed control explants in absence of sucrose was studied (Fig. 8.2a). The explants showed regeneration in the media containing mannose upto 10 g/l (Fig. 8.2a) which indicated that non transformed cowpea cotyledonary node explants has little basal level of PMI activity.

The percentage of explants forming shoots decreased with further increase in mannose concentration (Fig. 8.2a). The media containing 20 g/l mannose allowed regeneration of explants till 14 days in culture albeit with reduced regeneration frequency. However, the shoot growth in the regenerated explants in media containing mannose 20 g/l or above was completely retarded within 28 days of culture (Fig. 8.2b). Mannose at concentrations of 30-50 g/l resulted in no regeneration of explants. Complete suppression of shoot regeneration potential of cowpea cotyledonary node explants within 28 days in media containing 20 g/l mannose indicated the duration of exposure to minimum inhibitory concentration is crucial for determining mannose selection system.

The regeneration response explants gradually declined with decrease in concentration of sucrose in culture media. Explants remained healthy in media containing 2.5 g/l sucrose, however low shoot regeneration frequency was observed (Fig. 8.2c). Furthermore, no further shoot growth was observed from the regenerated explants at sucrose concentration upto 5 g/l (Fig. 8.2d).

8.3.2 Establishment of selection pressure by mannose

In order to determine the optimal mannose concentration for selection of putative transformed shoots, effect of the combinations of sucrose and mannose on growth and regeneration of control non transformed explants was analyzed from the completely randomized design treatments. The sucrose was supplemented with mannose-containing medium in order to alleviate the toxic effect of mannose-6-phosphate. A dose-response curve was designed for cowpea cotyledonary node explants for testing increasing mannose concentration (0 to 30 g/l) and decreasing sucrose content (30 to 0 g/l) (Fig. 8.3; 8.4). The results showed that mannose inhibited shoot regeneration from explants in a dose-dependent manner up to 30 g/l, a concentration at which the regeneration was completely suppressed, even when the medium was supplemented with sucrose (Fig. 8.4). Increase of

sucrose concentration (upto 7.5 g/l) in the media containing higher content of mannose (20 and 30 g/l) could not alleviate the inhibitory or starvation effect of high mannose dose (Fig. 8.4). Nevertheless, increase of sucrose concentration (to 5 and 7.5 g/l) in media containing 10 g/l mannose (that allowed shoot proliferation from explants till 28 days of culture) slightly enhanced the shoot proliferation ability of explants indicating recovery from starvation effect in presence of higher sucrose concentration (Fig. 8.4). Addition of sucrose to the mannose containing medium has been shown to modulate the inhibitory effect of mannose on shoot formation and allowed efficient recovery of transgenic shoots in many plants (Joersbo et al. 1998; Negrotto et al. 2000; Zhang et al. 2000; He et al. 2004). Based on our results, a combination of 5 g/l sucrose and 20 g/l mannose (mannose selection medium) was chosen for selection in order to suppress the emergence of escape shoots from cotyledonary node explants.

8.3.3 Genetic transformation of cowpea with *pmi* gene

Four independent *Agrobacterium*-mediated transformation experiments were performed with pNOV2819 vector using cotyledonary nodes as explants. Initial selection of transformed shoots was carried out on SISM medium that contained 20 g/l mannose and 5 g/l sucrose. After 2 weeks on the selection medium, the transformed explants showed the sign of regeneration with emergence of shoot buds whereas the untransformed explants remained starved (Fig. 8.5a, b). The regenerating explants were subcultured onto SISM medium every 14 days. After 4 weeks on the selection medium (with two rounds of subculture), the individual shoots were isolated and transferred to shoot elongation medium devoid of mannose where they elongated within 2 week of culture. The elongated shoots formed roots in MS supplemented with 2.5 µM IBA medium within 2 week of culture (Fig 8.5c). Independently regenerated putative cowpea transformed plants on mannose

selection with robust root systems were successfully established in the greenhouse (Fig. 8.5d).

The optimal concentration of mannose required for efficient selection of cowpea (20 g/l) was about eight times higher than that used for sugar beet (Joersbo et al. 1998) and almond (Ramesh et al. 2006), twice that for cucumber (He et al. 2006), maize (Negrotto et al. 2000), four times that for rapeseed (Wallbraun et al. 2009), similar to that for chickpea (Patil et al. 2009) and one quarter less that for rice (He et al. 2004). This indicates that cowpea is less sensitive than other plants to the toxic effects of mannose. However, this difference could be attributed to the inherent PMI activity which may differ between plants and target tissues used for transformation. Chickpea has also been reported to have tolerance to higher concentration of mannose (Patil et al. 2009). Chiang and Kiang (1988) have reported insensitiveness of soybean to the inhibitory effects of mannose due to high PMI activity rendering ineffectiveness of PMI/Man selection system for use in transgenics in soybean.

8.3.4 Molecular analysis of transgenic cowpea plants

Out of a total of 970 explants used for transformation in 4 batches, 137 mannose-resistant shoots were obtained (Table 8.1). Preliminary screening of putative transgenic plants was performed by PCR. The detection of the expected 1.2 kb amplified product corresponding to *pmi* in PCR analysis confirmed the presence of the *pmi* in T_0 transformed plants (Fig. 8.9a). No amplification was detected in the control untransformed plants. The stable transformation efficiency was determined based on the number of T_0 plants PCR-positive for *pmi* divided by the total number of explants co-cultivated. An average stable transformation efficiency of 3.6 was recorded (Table 8.1) which was significantly higher than the previously published reports on *Agrobacterium*-mediated transformation of

cowpea in our lab using negative selection system based on *npt* as selection system (Solleti et al. 2008a, b; Bakshi et al. 2011).

In order to verify integration of *pmi*, 5 randomly selected PCR-positive T_0 lines were analyzed by Southern hybridization. The *pmi* gene was detected in transgenic plants but not in the non-transgenic control plant (Fig. 8.9b). Hybridizations of DIG-labeled *pmi* probe to total genomic DNA digested with *Hind*III were expected to identify DNA fragments unique to individual integration events greater than 2 kb (Fig. 8.1). All the 5 randomly selected T_0 lines identified through the PCR screening were confirmed for stable genomic integration of the *pmi*, and none of the transgenic lines showed loss of the transgene under the mannose selection regimen. The transformed plants exhibited differential integration events, confirming that these plants were derived from independent transformation events (Fig. 8.9b, lanes 1, 2, 3, 4 and 5). The pattern of integration appeared to be simple with most of the events containing only one copy of the *pmi* gene and a few containing multiple copies (Fig. 8.9b).

The expression of the *pmi* gene in leaves of T_0 transgenic plants was determined by RT-PCR analysis. RT-PCR showed the presence of expected transcripts of transgenes in different T_0 transgenic plants and confirmed their transgenic nature. The results presented in these sections are of different independent transformation events and do not have any direct correlation. The amplification of a 1.2 kb fragment of *pmi* confirmed the accumulation of transcripts of *pmi* in T_0 transgenic plants (Fig. 8.6c) indicating the absence of gene silencing events. Furthermore, the amplification of the *pmi* sequence from plant cDNA templates in RT-PCR ruled out the possibility of *Agrobacterium* contamination. Both transformed and untransformed plants showed expression of reference gene, a*ctin* as indicated from amplification of 200 bp (Fig. 8.6d). There were no significant differences in the levels of gene expression.

8.3.5 Chlorophenol red assay

The PMI activity in transgenic plants was evaluated by cholorophenol red assay. 22 plants, including untransformed control plants were tested independently. The addition of chlorophenol red resulted in the appearance of a red color in the medium (Fig. 8.7). A few leaves from each plant were taken for evaluation. Incubation of transgenic leaf tissues in the medium containing cholorophenol red (pH 6.0) gradually changed the color from an original purple to yellow or orange. The color change due to progressive acidification of growth medium is indicative of active metabolic status of tissue expressing transgenic PMI (Fig. 8.7). Leaves of untransformed plant incubated in chlorophenol red medium did not show medium acidification, leaving the red color of the medium unchanged. The cholorophenol red assay has been shown to be valuable for rapid visual screening of PMI positive transgenic plants (Lucca et al. 2001; Jain et al. 2006; Patil et al. 2009; Dutt et al. 2010).

8.3.6 Segregation analysis

The seeds from T_0 lines were advanced to T_1 generation and the T_1 transgenic lines generated from 6 independent transformation events were analyzed for the segregation pattern of *pmi* by PCR analysis. Presence of the expected 1.2 kb amplified product corresponding to *pmi* in T_1 transgenic lines confirmed the inheritance of *pmi* gene. The segregation pattern of these selected transgenic events showed typical 3:1 Mendelian ratio as expected for single dominant gene inheritance (Table 8.2).

8.4 Conclusion

In conclusion, we demonstrate here for the first time, that “positive” selection based on mannose is compatible with cowpea transformation. Early identification of transgenic events by PCR (indicating presence of the *pmi* gene) followed by their confirmation by Southern hybridization (for integration and copy number evaluation), RT-PCR (for studying

expression of the *pmi* gene), or the chlorophenol red assay for the PMI enzyme showed the robustness of the *pmi/*mannose based positive selection for the efficient recovery of stable transgenics in cowpea. The procedure would enable introduction of candidate genes for biotic and abiotic stress tolerance and recovery of transgenic cowpea through environmentally safe positive selection system.

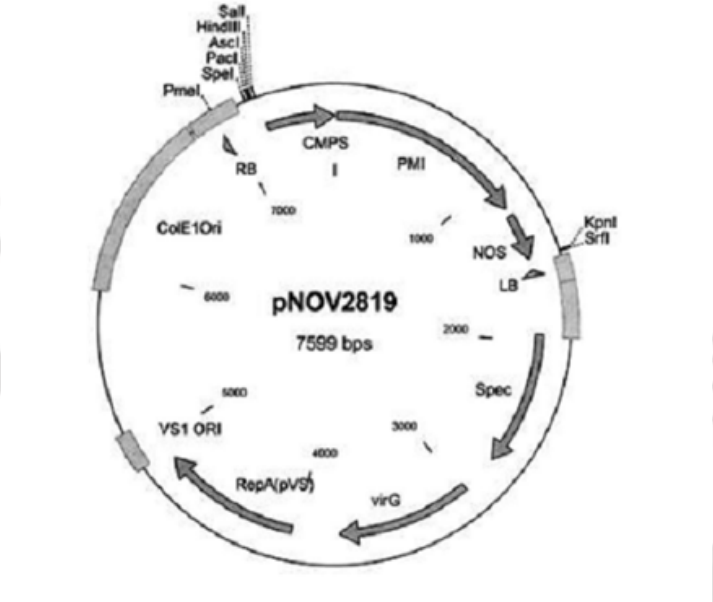


Fig. 8.1 Plasmid map of pNOV2819 (kindly provided by Syngenta). The *pmi* gene is under the constitutive CMPS promoter (cestrium yellow leaf curling virus promter short version). LB-left border sequence, RB-right border sequence, *pmi*-phosphomannose isomerase gene

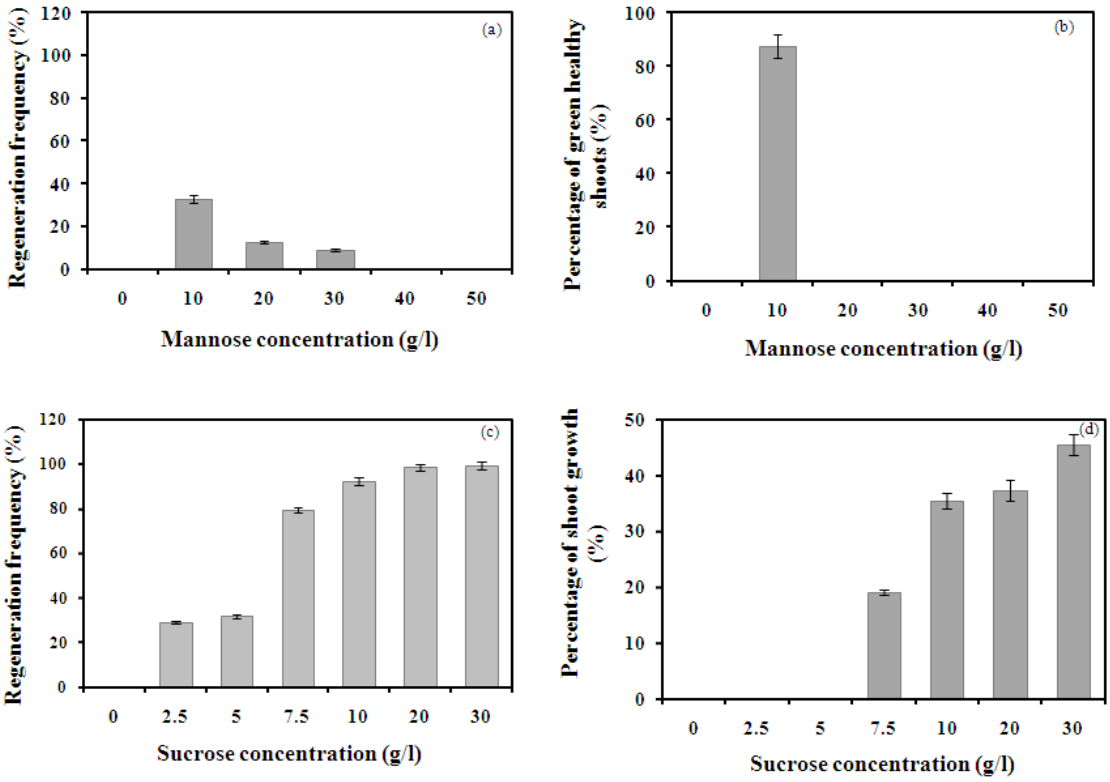


Fig. 8.2 Effect of mannose and sucrose on shoot formation from cowpea cotyledonary node explant. (a) Effect of different mannose concentration (0, 10, 20, 30, 40 and 50 g/l)on shoot regeneration frequency, (b) Percentage of regenerated shoots remained green on medium containing different mannose concentration after 4 weeks, (c) Effect of different sucrose concentration (0, 2.5, 5, 7.5, 10, 20 and 30 g/l) on shoot regeneration frequency, (d) Percentage of shoot growth on medium containing different sucrose concentration after 4 weeks.

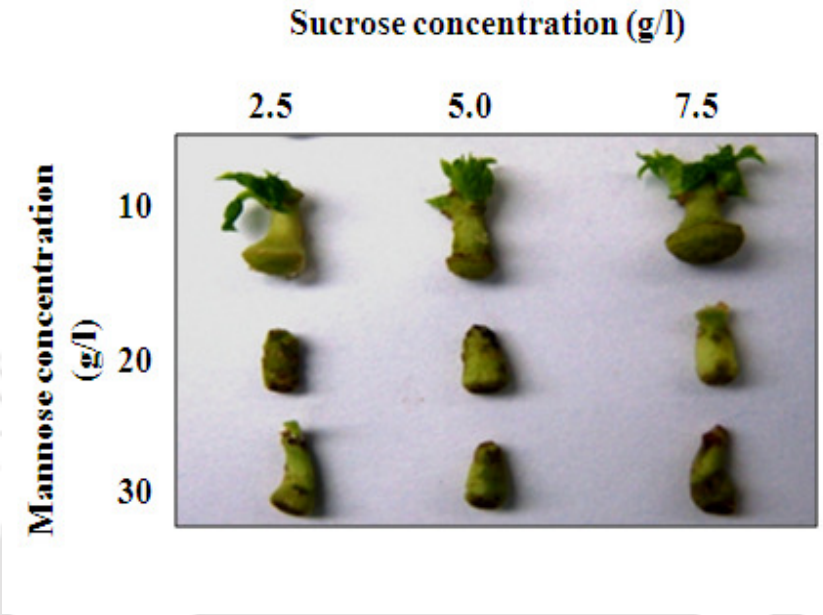


Fig 8.3 Effect of sucrose and mannose combination on regeneration of shoots from cotyledonary node explants after 2 weeks

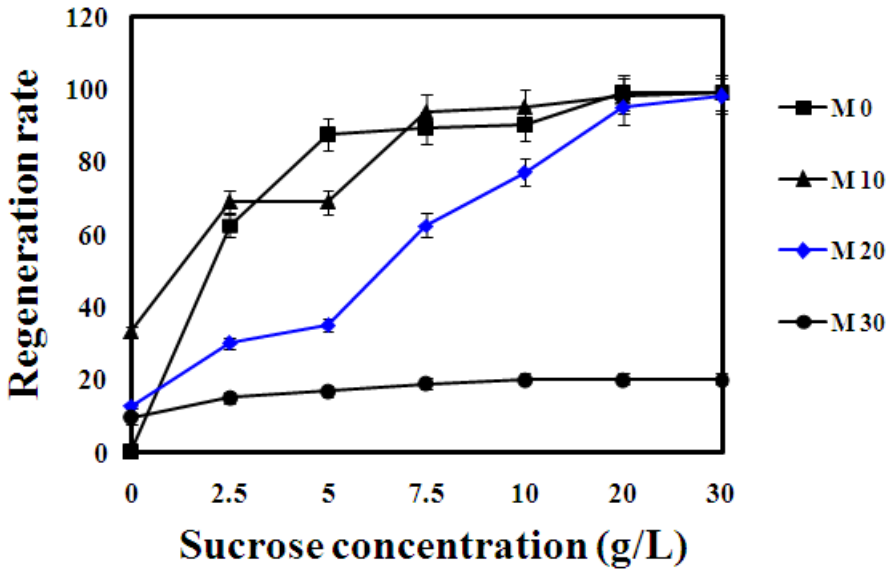


Fig 8.4 Dose response curve of mannose and sucrose concentration for regeneration rate (%)

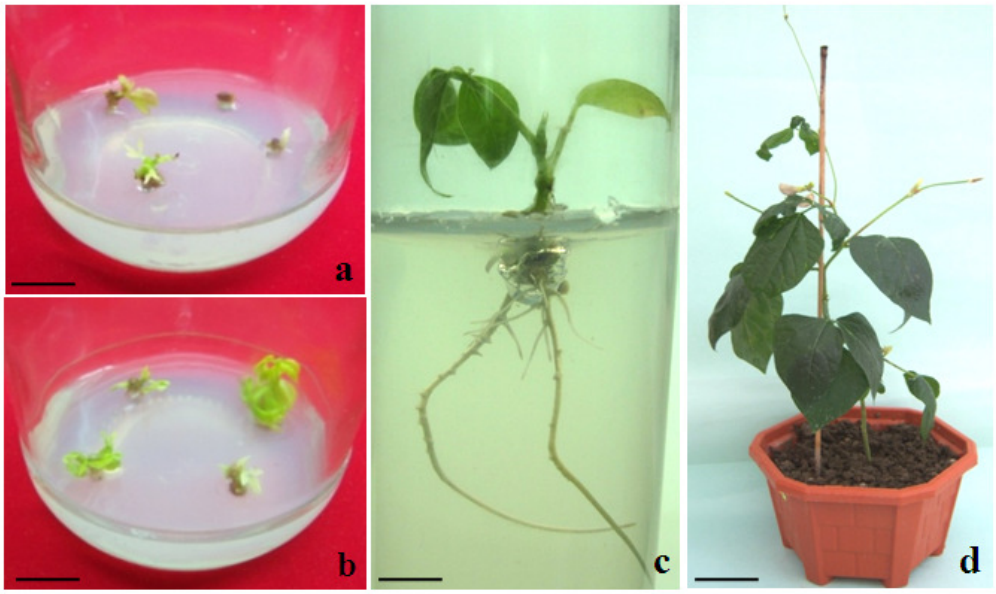


Fig. 8.5 Developmental stages of transformed cowpea using pNOV2819. (a) Control explants cultured on selection media supplemented with 20g/L mannose and 5g/L sucrose, (b) Selection of transformed explants on selection media, (c) *In vitro* rooting of selected shoot, (d) Acclimatized plants maintained in greenhouse.

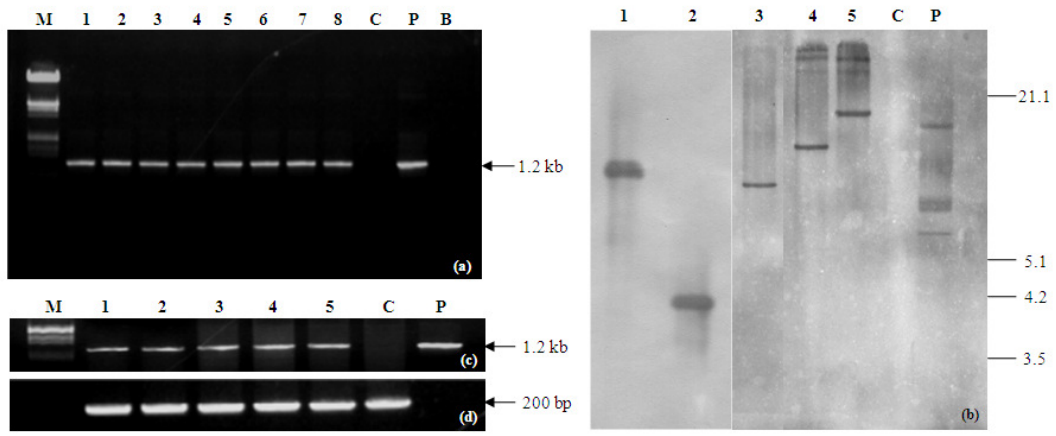


Fig. 8.6 Molecular analysis of T_0 transgenic plants. (a) PCR amplification of the 1.2 kb fragment of the *pmi* gene, Lane M λDNA/EcoRI + HindIII marker, lane P pNOV2819 plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lane B blank, lanes 1–8 DNA from independently transformed plants. (b) Southern blot hybridization analysis of junction fragments of five randomly selected PCR-positive T_0 lines. The plasmid and genomic DNA were digested with *HindIII*, and hybridized with *pmi* PCR product as a probe. Lanes 1–5 genomic DNA from four T_0 lines, lane C genomic DNA from untransformed plant, lane P pNOV2819 plasmid. (c) RT-PCR analysis of *pmi* gene and reference gene *actin*, Lane M λDNA/EcoRI + HindIII marker, lane P pNOV2819 plasmid DNA (positive control), lane C DNA from untransformed plant (negative control), lanes 1–5 T_0 transgenic plants

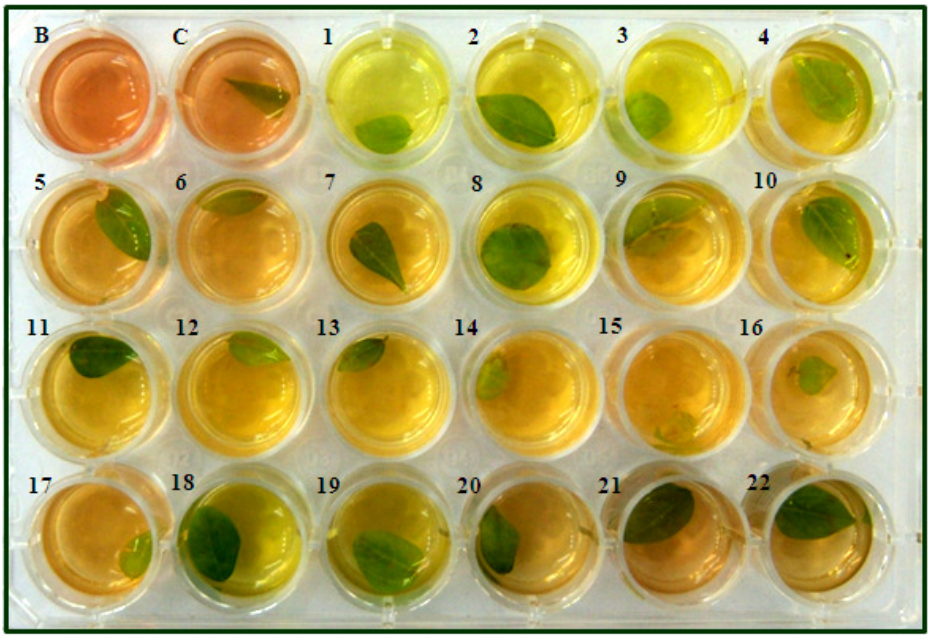


Fig. 8.7 Chlorophenol red assay of leaves from 22 transformed lines and untransformed control plants. Tissue that is able to utilize Mannose due to PMI activity acidifies the medium, which is indicated by a color change from red to orange or yellow. B: Blank; C: control (Leaves from untransformed plants)

Table 8.1 Effect of mannose based selection on regeneration of cowpea, following transformation with *Agrobacterium tumefacience* EHA105pNOV2819 carrying *pmi* gene

Exp. no.	No. of explants inoculated in *Agrobacterium* suspension	No. of shoots recovered on mannose selection medium	No. of plants positive for *pmi* by PCR	Stable transformation efficiency[a] (%)
1	280	42	10	3.57
2	210	31	8	3.80
3	230	36	8	3.48
4	250	28	9	3.6
Total[b]/ Average[c]	970[b]	137[b]	35[b]	3.6[c]

[a] Number of T_0 plants PCR-positive for *pmi* divided by the total number of explants co-cultivated

[b] Total

[c] Average

Table 8.2 Segregation of *pmi* gene in T_1 progeny of transgenic cowpea plants

T_0 plants	Number of T_1 plants tested for *pmi*			χ^2 value	Expected Ratio
	Total	*pmic*+ve	*pmi*-ve		
P1	43	30	13	0.63	3:1
P2	54	39	15	0.22	3:1
P3	26	17	9	1.28	3:1
P4	55	40	15	0.15	3:1
P5	60	44	16	0.08	3:1
P6	52	41	11	0.41	3:1

Chapter 9

Concluding remarks

9.1 Significance and salient features of the study

Cowpea (*Vigna unguiculata* L.Walp) is an important grain legume widely grown in tropical and subtropical regions of Indian subcontinent. Their seeds provide a major source of cheap and high quality dietary protein (Sahoo et al. 2008). The major constraints in its production are its prominent susceptibility to pod borer insects (Ehlers and Hall, 1997). Among insects, pod borers (*Maruca vitrata* and *Heliothis armigera*) cause massive damage to the pods in field (Eicher et al. 2006).

There are only few reports are 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; Solleti et al. 2008a, b). However, the overall transformation efficiency in thesis 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 selection regime followed. A critical step in the development f 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.

In the present study, the overall efficiency of *Agrobacterium*-mediated cowpea transformation was significantly increased by adapting to an improved cotyledonary node based regeneration system and efficient selection scheme. Transgenic plants overexpressing insecticidal genes, *Btcry1Ac* and *Btcry1Ab* were generated for the first time in cowpea. Furthermore, a novel positive section system based on mannose was established for the first time in cowpea. The transgenic cowpea plants overexpressing *Btcry1Ac* and *Btcry1Ab* offers promise of protection of cowpea from insect infestation. The developed transformation system would enable transfer of a wide range of candidate genes for both biotic and abiotic tolerance genes in cowpea which in turn speed up the process of cowpea improvement program.

The salient features of the present study are summarized below:

- An efficient and prolific shoot regeneration system using cotyledonary node explants of cowpea was developed by inducing efficient seed pretreatment with TDZ.
- *Bacillus thuringiensis cry1Ac* (*Btcry1Ac*) and *Btcry1Ab* overexpression constructs were developed and the constructs were mobilised to hypervirulent *Agrobacterium* strain EHA105.
- Highly efficient cowpea stable transformation system was developed with a 46.1% increase in efficiency as compared to the existing transformation methods by employing an improved kanamycin selection regime. Fertile transgenic plants expressing *Btcry1Ac* were generated.
- *Agrobacterium*-mediated transformation of cowpea was improved by employing sonication in combination with vacuum infiltration. This is the first report on cowpea transformation using SAAT and vacuum infiltration.
- An efficient shoot regeneration system was developed using split cotyledonary node explant and the system was adapted to *Agrobacterium*-mediated transformation and transgenic plants overexpressing *Btcry1Ab* were generated.
- A novel and efficient mannose based positive selection system was established for cowpea transformation.

9.2 Future prospects

Genetically engineering inherent crop resistance to insect pests offers the potential of a user-friendly, environment-friendly and consumer-friendly method of crop protection to meet the demands of sustainable agriculture. Despite its economic and social importance in the developing world, cowpea remains to a large extent an under exploited crop. Among the major goals of cowpea breeding and improvement programs is the stacking of desirable agronomic traits, such a disease and pest resistance and resistance to abiotic stress. Work to date has concentrated on the

introduction of genes for expression of common bean *α-amylase inhibitor* and *Bacillus thuringiensis* (Bt) toxins in order to control infestations by insect-pest.

However, cowpea is susceptible to several other insect pests and they bring about great losses in yield. The plant derived genes which can be transferred to cowpea to achieve insect pest resistance include lectins, chitinases 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 effectiveness and durability of disease and pest resistance are likely to be greater in engineered transgenic plants in which multiple resistance genes are introduced ("gene pyramiding").

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).

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. Furthermore, application of RNAi technology is emerging as a novel approach to confer resistance to herbivorous insects in crop plants. Transgenic plants could be protected from the herbivorous insects by engineering them to express double-stranded RNAs (dsRNAs) directed against vital insect genes (Gordon and Waterhouse 2007).

From our results, it is apparent that recalcitrance in cowpea is overcome for genetic transformation and trait improvement as evident by the stable expression and inheritance of the transgenes to the progeny. 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. It is to be hoped that the encouraging progress will be maintained and developed so as to make a significant contribution towards redressing the balance between world food productions and world food requirements in the coming century.

Chapter 10

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List of Publications

Journals:

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1. **Bakshi S**, Sadhukhan A, Mishra S and Sahoo S (2011) Improved Agrobacterium-mediated transformation of cowpea via sonication and vacuum infiltration. Plant Cell Reports. doi:10.1007/s00299-011-1133-8

2. Solleti S, **Bakshi S**, Purkayastha J, Panda SK and Sahoo L (2008) Transgenic cowpea (*Vigna unguiculata*) seeds expressing a bean α-amylase inhibitor 1 confer resistance to storage pests, bruchid beetles. Plant Cell Reports 27:1841-1850

3. Solleti SK, **Bakshi S**, and Sahoo L (2008). 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. Journal of Biotechnology 135: 97–104

Submitted/Under review

1. **Bakshi S** and Sahoo L (2011) Efficient recovery of transgenic cowpea expressing BtCry1Ac through improved selection system. Planta (Under review)

2. **Bakshi S**, Roy NK and Sahoo L (2011) Preconditioning of seedlings in thidiazuron improves adventitious shoot regeneration in recalcitrant grain legume-cowpea (*Vigna unguiculata* L. *Walp*). Plant Cell Tissue Organ Culture (Communicated)

3. **Bakshi S**, Saha B and Sahoo L (2011) Establishment of a mannose based positive selection system for the recovery of transgenic cowpea. Plant Cell Report (Communicated)

Conferences:

1. **Bakshi S** and Sahoo L (2011) Expression of *Btcry1Ac* in transgenic cowpea and genetic stability. National Symposium on PTC and Biotech & XXXII PTCA (I) meet on 4th -6 th Feb, Rajasthan, India

2. **Bakshi S**, Sadhukhan A, Roy NK, Kailasam S, Sahoo DP and Sahoo L (2011) Genetic engineering of cowpea with *Btcry1Ab* for resistance to Maruca pod borer. National Symposium on PTC and Biotech & XXXII PTCA (I) meet on 4th -6 th Feb, Rajasthan, India

3. Borah P, Kalita N, **Bakshi S**, Mishra S and Sahoo L (2011) Genetic transformation of mungbean with *cry1Ac* gene for insect resistance. National Symposium on PTC and Biotech & XXXII PTCA (I) meet on 4th -6 th Feb, Rajasthan, India
4. **Bakshi S** and Sahoo L (2010) Expression of *cry1Ac* gene in Indian cowpea cultivar Pusa Komal for resistance to pod borer. International Conference on Biotechnology: A Global Scenario held on 2nd-4th November, Warangal, India
5. Solleti SK, **Bakshi S,** Purkayastha J, Panda SK and Sahoo L (2008). Transgenic cowpea seeds expressing bean α-amylase inhibitor 1 conferred complete protection against storage pests, bruchid beetles. International Conference on Plant Biotechnology and Molecular Biology, Department of Biotechnology, Kakatiya University, Andhra Pradesh, August 15-17.
6. Solleti SK, **Bakshi S,** and Sahoo L (2008). Pronounced effect of synergistic and temporal application of two cytokinins, 6-benzyl aminopurine and kinetin on cyclic morphogenesis from shoot apex explants in cowpea: a morphological insight. International Conference on Plant Biotechnology and Molecular Biology, Department of Biotechnology, Kakatiya University, Andhra Pradesh, August 15-17.
7. Purakayastha J, Sugla T, **Bakshi S**, Solleti S and Sahoo L (2006). Rapid in-vitro plant regeneration from nodal explant of *Andrographis paniculata* Nees: a valuable medicinal plant. Abstract of "National Seminar on Biodiversity & Indigenous Knowledge System", Rajiv Gandhi University, Arunachal Pradesh, India.

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ORIGINAL PAPER

Improved *Agrobacterium*-mediated transformation of cowpea via sonication and vacuum infiltration

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Abstract An improved method of *Agrobacterium*-mediated transformation of cowpea was developed employing both sonication and vacuum infiltration treatments. 4 day-old cotyledonary nodes were used as explants for co-cultivation with *Agrobacterium tumefaciens* strain EHA105 harbouring the binary vector pSouv-cry1Ac. Among the different injury treatments, vacuum infiltration and their combination treatments tested, sonication for 20 s followed by vacuum infiltration for 5 min with *A. tumefaciens* resulted in highest transient GUS expression efficiency (93% explants expressing GUS at regenerating sites). After 3 days of co-cultivation, the explants were cultured in 150 mg/l kanamycin-containing selection medium and putative transformed plants were recovered. The presence, integration and expression of *nptII* and *cry1Ac* genes in T_0 transgenic plants were confirmed by polymerase chain reaction (PCR), genomic Southern and qualitative reverse transcription (RT)-PCR analysis. Western blot hybridization and enzyme-linked immunosorbent assay (ELISA) detected and demonstrated the accumulation of Cry1Ac protein in transgenic plants. The *cry1Ac* gene transmitted in a Mendelian fashion. The stable transformation efficiency increased by 88.4% using both sonication-assisted *Agrobacterium*-mediated transformation (SAAT) and vacuum infiltration than simple *Agrobacterium*-mediated transformation in cowpea.

Keywords *Agrobacterium tumefaciens* · *cry1Ac* · Cowpea · Sonication · Vacuum infiltration · Wounding

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Abbreviations

BAP	6-Benzylaminopurine
TDZ	Thidiazuron
GUS	*β*-Glucuronidase
nptII	Neomycin phosphotransferase II
SAAT	Sonication-assisted *Agrobacterium*-mediated transformation

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is widely cultivated in Africa, India, Middle East and, South America mostly for dry grain and fodder (Ehlers and Hall 1997; Timko et al. 2007), and is a major source of high-quality dietary protein for millions of local poor people (Singh 2002; Diouf and Hilu 2005; Xu et al. 2010). Cowpea production is seriously affected by a number of biotic and abiotic constraints, of which notably insect pests and viral diseases cause substantial yield loss worldwide (Solleti et al. 2008a). Despite its economic importance, progress in genetic improvement of cowpea for insect pest and disease resistance through conventional breeding is slow primarily due to narrow genetic base and barriers in crossing with distant wild species (Gomathinayagam et al. 1998; Fang et al. 2007). Consequently, the transfer of insect pest and virus resistance genes by genetic transformation could potentially aid plant breeders in overcoming these constraints and accelerate the development of resistant cultivars for breeding programs. Furthermore, efficient genetic transformation system would provide a valuable tool for functional genomics studies of cowpea. *Agrobacterium*-mediated transformation has been extensively applied to many crop plants including grain legumes, because this method offers several advantages such as the defined

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integration of transgenes, potentially low copy number, and preferential integration into transcriptional active regions of the chromosome (Koncz et al. 1989; Hiei et al. 1994). *Agrobacterium*-mediated transformation of cotyledonary explants has led to the generation of stable transgenic plants in cowpea (Muthukumar et al. 1996; Popelka et al. 2006; Chaudhury et al. 2007; Solleti et al. 2008a, b). Cotyledonary explants are preferred for *Agrobacterium*-mediated transformation of cowpea as T-DNA delivery to axillary meristem followed by regeneration via adventitious bud formation minimizes the risks of chimeras and somaclonal variation (Tzfira et al. 1997). However, cowpea transformation still remains inefficient and consequently, production of transgenic cowpea is far from being a routine procedure due to poor transformation efficiency and low numbers of regenerated transgenic plants. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) (Joersbo and Brunstedt 1992; Trick and Finer 1998; Santare′m et al. 1998) and vacuum infiltration (Charity et al. 2002; Park et al. 2005; Paz et al. 2006) methods have been reported to enhance the efficiency of *Agrobacterium*-mediated transformation of recalcitrant plant species. Exposure of the explants to short periods of sonication in the presence of *Agrobacterium* carrying desired T-DNA vector is thought to produce large numbers of micro wounds across the tissue which permits the *Agrobacterium* to penetrate deeper and more completely throughout the tissue as compared to the natural infection obtained during co-cultivation (Trick and Finer 1997; Santare′m et al. 1998; Tang et al. 2001; Liu et al. 2005), thus enhancing the bacterial colonization and infection of the tissue. Performed scanning electron and light microscopy observations revealed that ultrasound treatment produces small and uniform fissures and channels throughout the plant tissue, which allows *Agrobacterium* access to internal plant tissue (Trick and Finer 1997). SAAT method has been successfully employed in improving transformation of a number of recalcitrant plants (Oliveira et al. 2009).

Agroinfiltration is an effective method in enabling the regenerating cells, often located a few cell layers beneath the surface of explants, rapid access to *Agrobacterium* and consequently increasing transient transgene expression in many recalcitrant plant species (Bechtold and Pelletier 1998; Tague and Mantis 2006). This method has been adapted for the successful transformation of number of recalcitrant plants (Subramanyam et al. 2011).

Although the benefits of sonication and vacuum infiltration during *A. tumefaciens*-mediated transformation methods are evident, no effort has been made to apply these methods to cowpea. In order to improve the *Agrobacterium*-mediated transformation in cowpea for routine generation of transgenic plants with candidate genes, we investigated the effect of sonication and vacuum infiltration on *Agrobacterium*-mediated transformation of cowpea cotyledonary node explants. Stable transgenic cowpea plants expressing *cry1Ac* were recovered using both SAAT and vacuum infiltration, which showed presence, integration, expression and inheritance of transgenes.

Materials and methods

Plant material and explant preparation

The mature seeds of cowpea cultivar Pusa Komal (IARI, New Delhi) were surface sterilized with 70% ethanol (v/v) for 30 s followed by 0.2% of mercuric chloride (w/v) for 5 min. The sterilized seeds were rinsed 5 times with sterile water and cultured on MSB5 medium [MS salts (Murashige and Skoog 1962) + B_5 vitamins (Gamborg et al. 1968)] supplemented with 3% sucrose (w/v), 0.8% agar agar (w/v) and 10 μM TDZ. The cultures were incubated at 26 ± 2°C under 16 h-photoperiod regime provided by cool white fluorescent lamps (36 μmol m^{-2} s^{-1}). Cotyledonary node explants (5–6 mm) were excised from 4-day-old seedlings by removing both the cotyledons, and decapitating epicotyls as close as possible and hypocotyls 3 mm below the nodal region, and used for transformation experiments.

Binary plasmid, bacterial strain and culture conditions

The binary plasmid pSouv:cry1Ac (Bt*cry1Ac* expression cassette cloned in binary vector pCAMBIA2301) (Fig. 1) was mobilized into the disarmed hypervirulent *Agrobacterium tumefaciens* strain EHA105 and used for transformation experiments. The T-DNA of pCAMBIA2301 includes neomycin phosphotransferase gene (*nptII*) and *β*-glucuronidase gene (*gus*) interrupted by catalase intron, both driven by the cauliflower mosaic virus (CaMV) 35S promoter. The *A. tumefaciens* strain harboring pSouv:cry1Ac was maintained on solid YEP medium (An et al. 1988) supplemented with 10 mg/l of rifampicin, and 50 mg/l of kanamycin. Single bacterial colony was inoculated into 25 ml of liquid AB minimal medium (Chilton et al. 1974) with appropriate antibiotics and grown overnight at 28°C on a rotary shaker at 180 rpm, until optical density at 600 nm reached to 0.8. The cells were collected by centrifuging at 5,000 rpm for 5 min, and then the pellet was resuspended in liquid co-cultivation medium, LCM (MSB5 medium containing 1 μM BAP, pH adjusted to 5.5) supplemented with 100 μM acetosyringone and used for inoculation.

Inoculation of explants with *A. tumefaciens*

For each experiment, 30–40 explants were subjected to wounding treatment either by mechanical injury with

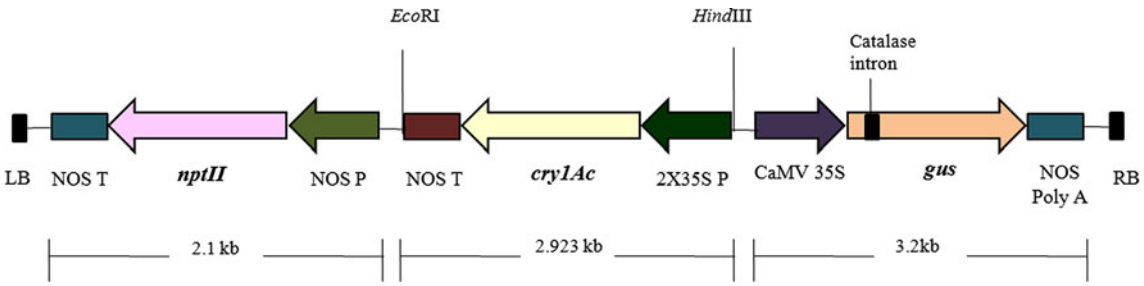

Fig. 1 Schematic construction of pSouv:cry1Ac (14.5 kb). The 2.9 kb (*Eco*RI–*Hin*dIII) fragment containing Bt*cry1Ac* under the control of CaMV 2X35S promoter and NOS terminator was cloned at the *Eco*RI–*Hin*dIII sites of T-DNA of pCAMBIA2301. *LB* and *RB* left border and right border of T-DNA region, *NOS T* nos terminator, *2X35SP* double 35S promoter, *npt*II neomycin phosphotransferase II

needle or by sonication, and inoculated in bacterial suspension by occasional shaking for 30 min or by vacuum infiltration. The explants inoculated in bacterial suspension without prior wounding treatment were considered as control. After inoculation in all cases, explants were blotted on a sterile filter paper to remove excess liquid and co-cultivated for 3 days under dark condition at 22°C, in petri dishes lined with filter paper moistened with LCM supplemented with 100 µM acetosyringone. Following co-cultivation, the explants were rinsed three to four times with LCM and blotted dry on sterile filter paper and placed onto initial multiple shoot induction and selection medium, SISM (MSB5 medium containing 5.0 µM BAP and 0.5 µM kinetin supplemented with 150 mg/l kanamycin and 500 mg/l cefotaxime) for 20 days with three rounds of subculture at an interval of 5, 7 and 8 days, respectively.

Wounding and SAAT treatments

The cotyledonary node explants were wounded at axils by puncturing approximately 1.5 mm in depth with a sterile hypodermic needle (0.56 mm in diameter.) prior to inoculation with *Agrobacterium* cell suspension.

For SAAT, the explants were immersed in 15 ml flat bottom glass culture tubes (Borosil, India) containing 6 ml of *Agrobacterium* cell suspension. The tubes were capped, placed in a float at the center of a bath-type sonicator (Telsonic ultrasonic TPC-40, Switzerland) and then subjected to ultrasound at a frequency of 30 kHz. The treatments differed as to sonication duration (5, 10, 15, 20, 25, and 30 s). Following sonication, explants were removed from the tubes, placed on sterile filter paper surface to blot off excess bacteria and then transferred to co-cultivation medium.

For vacuum infiltration experiment, the explants with or without wounding and 20 s sonication treatments were placed in vacuum system consisted of a vacuum pump at 600 mm Hg (Rocker 400, Tarson, India) to which a desiccator was attached. Glass petri dishes containing explants immersed in *Agrobacterium* cell suspension were placed in the desiccator and vacuum was applied for different durations (2.5, 5, 10, 15 and 20 min).

The best treatments achieved in SAAT and vacuum infiltration experiments were combined to evaluate the effect of sonication followed by vacuum infiltration in contrast to the use of these methods alone.

In all experiments, the frequency of transient GUS expression was analyzed after 3 days of co-cultivation. The optimal wounding, sonication and vacuum infiltration treatments were determined as the levels that led to a perceived increase in GUS positive foci in explants at the site of regeneration without any perceived decrease in explant viability. Control treatments consisted of explants either uninoculated or inoculated with *Agrobacterium* without wounding, sonication and vacuum infiltration treatments.

Histochemical GUS assays

GUS activity was visualized using the histochemical assay (Jefferson 1987). Transient expression was examined after 3 days of co-cultivation (Solleti et al. 2008a). The explants were bleached with 100% ethanol for 24 h prior to examination under a stereomicroscope. Transient expression of GUS was scored on a per explant basis by estimating the number of blue foci visible on the axillary region of each cotyledonary node explant. The blue foci were the discrete areas of cells with GUS activity.

Shoot recovery

Following three rounds of kanamycin selection on SISM, the survived explants were transferred to SIEM [shoot induction and elongation medium (MSB medium containing 5.0 µM BAP, 0.5 µM kinetin and 500 mg/l cefotaxime)] and cultured for 10 days for optimal elongation and selective regeneration of transformants. Elongated putative transformed shoots (>1.5 cm) were transferred to rooting medium (MS + 2.5 µM IBA) devoid of any antibiotics for root induction. Rooted putative transformed plants were

transferred to pots containing sterile soil:compost (1:1) and were acclimatized in greenhouse containment for 3 weeks.

Evaluation of transgenic plants

Molecular characterization of the transformants was carried out by PCR, Southern hybridization, GUS histochemical analysis of different plant tissues, RT-PCR, ELISA and Western blot hybridization analysis for confirmation of the presence, integration, expression and inheritance of the introduced genes.

Stable GUS assay

Stable *gus* expression was detected in various plant parts including flower, anthers, pollens and pistils following the histochemical procedure as described previously.

Screening of putative transformed plants using polymerase chain reaction (PCR)

Genomic DNA was isolated from the young leaves of T_0 putative transformants and T_1 transgenic plants using the modified CTAB method (Solleti et al. 2008a). PCR amplification was carried out with gene specific primers for *npt*II and *Btcry1Ac* using genomic DNA from putative transformed plants, non-transformed control plants (negative control) and pSouv:cry1Ac (positive control) as templates. The 540 bp region of *npt*II and 1 kb coding region of *Btcry1Ac* were amplified using respective 20 mers (*npt*II Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAG GCTATTCGGCTA) and 24 mers (*Btcry1Ac* Fw: CCCAG AAGTTGAAGTACTTGGTGG; Rv: CCGATATTGAAG GGTCTTCTGTAC) oligonucleotide primers. The amplification reaction was carried out under the following conditions: 94°C for 5 min (1 cycle), 94°C for 1 min (denaturation), 58°C for 1 min (annealing), 72°C for 1 min (extension) for 35 cycles followed by the final extension at 72°C for 7 min (1 cycle). PCR was performed using ~100 ng of purified genomic DNA and Taq DNA polymerase (Genei, Bangalore, India) according to manufacturer's instruction. The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (Sambrook et al. 1989).

Southern hybridization

Randomly selected PCR-positive T_0 transgenic cowpea plants were further analyzed by Southern hybridization for the integration of the *cry1Ac*. 10 µg samples of genomic DNA from non-transformed control and transgenic plants were digested with *Hin*dIII. The digested samples were fractioned on a 0.8% agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, USA). The blot was hybridized with DIG-labeled 1 kb PCR product, corresponding to the coding region of *cry1Ac* gene. The probe labeling and Southern hybridization were performed using the non-radioactive DIG Labeling and Detection system (Roche, Germany) following supplier's instructions. Pre-hybridization and hybridization were carried out using high hybridization buffer containing 5XSSC, 1% blocking solution, 0.1% (w/v) *N*-lauroyl sarcosine and 0.02% (w/v) sodium dodecyl sulfate. Washing and detection were performed according to the instruction of the DIG labeling and detection system (Roche Diagnostics, Mannheim, Germany).

Qualitative reverse transcription (RT)-PCR analysis

Total RNA was isolated from the PCR-positive transgenic T_0 plants using Trizol Reagent (Invitrogen, USA) from 100 ng of leaf tissue according to the manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.5% denaturing agarose gel (Sambrook et al. 1989). RT-PCR was carried out using First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. PCR of the coding sequences of *Btcry1Ac* gene in the cDNA was carried out using respective primers as described earlier.

Western blot hybridization

Proteins were extracted from 1 g of young leaves of T_0 transgenic plants using an extraction buffer containing 100 mM potassium phosphate buffer (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT). The protein concentration was determined by the method of Bradford (1976). 30 µg of protein was fractionated on 12% acrylamide gels with sodium dodecyl sulfate (SDS–PAGE) and blotted on to a PVDF membrane by electro transfer blotting unit. Blots were blocked for 2 h at room temperature in 5% blocking buffer (non-fat powdered milk in Tris-buffered saline with 0.1% Tween-20). Goat polyclonal antibodies (Amar Diagnostics, India) were used at 1/500 dilution in blocking buffer and incubated for overnight at 4°C. The samples were washed three times in TBST (tris-buffered saline tween-20) for 5 min each. A secondary rabbit anti-goat antibody alkaline phosphatase conjugate (Amar Diagnostics, India) was then used for final detection, at a dilution of 1/1,000. Blots were incubated for 40 min at 4°C, washed 5 times for 5 min each with TBST followed by development in nitro blue tetrazolium/bromo chloro indolyl phosphate (NBT/BCIP) substrate solution (Sigma, USA) for 15–20 min. The reaction was stopped by washing the membrane with distilled water.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to quantify the accumulated levels of Cry1Ac protein in T_0 transgenic plants using Desigen Quan T-ELISA-96 well plate kit (Desigen, Maharashtra, India) following manufacturer's protocol. Total protein was extracted from 5 mg of dry leaf powder using 500 µl of sample extraction buffer. The sample was chilled and spun at 8,000 rpm for 15 min and 100 µl of supernatant was used for loading to anti-Cry1Ac pre-coated plate. For the estimation of Cry1Ac, the 96-well titre plate was coated with 150 µl per well (1:1,000) of goat anti-Cry1Ac antibodies. The plate was then loaded with 100 µl samples and buffer was used in control wells. The plate was incubated at 37°C for 1.5 h, followed by washing with wash buffer twice. After washing, the plate was incubated with alkaline phosphatase conjugated secondary antibodies at a dilution of 1:1,000 with 250 µl per well for 45 min at 37°C. The plate was then washed with wash buffer twice and 250 µl of freshly prepared substrate (*p*-nitro phenyl phosphate, 1 mg/ml) was added per well. The plate was incubated at room temperature in the dark for 30 min and reaction was stopped and readings recorded at 405 nm in a micro plate reader (Tecan, Switzerland).

Segregation analysis

The leaves of T_1 transgenic plants generated from eight independent transformation events were analyzed for the presence of *npt*II and *Btcry1Ac* genes using PCR, as described earlier. Segregation patterns were analyzed with the Chi-square test (χ^2) as described by Solleti et al. (2008b).

Data analysis

Data were subjected to analysis of variance (ANOVA) and mean separation by Duncan's multiple-range test (DMRT) using single-factor completely randomized block design in order to study the effect of different treatments on frequencies of transient expression. All experiments were performed at least three times with a minimum of 30–40 explants per treatment.

Results and discussion

Effect of wounding

Efficient *Agrobacterium*-mediated transformation requires optimal delivery of the T-DNA to regenerable cells of the explants. Wounding of explants allows *Agrobacterium* to better access plant cells as it stimulates the production of potent *vir* gene inducers, like phenolic substances and enhances the plant cell competence for transformation (Stachel et al. 1985; Shimoda et al. 1990; Bidney et al. 1992). Only plants with an appropriate wound response develop larger populations of wound adjacent competent cells for regeneration and transformation (Potrykus 1991). Although excessive wounding is probably detrimental to stable transformation, the frequency of gene transfer via *Agrobacterium*-mediated transformation in recalcitrant species can be significantly enhanced by inducing wounds in the target tissue (Bidney et al. 1992). In cowpea, infection of cotyledonary node explants with most effective supervirulent *A. tumefaciens* strain EHA105, in absence of injury treatment resulted in 85% transient GUS expression frequency (Solleti et al. 2008a). However, the accounted GUS foci were located mostly at the cotyledons detachment site of cotyledonary node explants, and not at the regenerating site. The low stable transformation efficiency, 1.64% in cowpea (Solleti et al. 2008a) could be attributed to poor conversion of transient transformation to stable transformation. Wounding of regenerating sites of the cotyledonary node explants of cowpea by a hypodermic needle and co-cultivation with *A. tumefaciens* resulted in more efficient transient expression especially on needle-wounded explants, mainly in terms of the percentage of explants showing GUS foci at the regenerating sites as compared to unwounded explants infected with *A. tumefaciens* (Fig. 2). This clearly indicated that higher transient transformation of regenerating cells of meristematic tissue-based explants such as cotyledonary nodes, required an efficient wounding treatment. Wounding at the regenerating sites before co-cultivation allowed better bacterial penetration into the regenerating cells of cotyledonary node explants, facilitating the accessibility of plant cells for *Agrobacterium* infection. Such mechanical wounding treatments greatly enhanced transformation efficiency in a

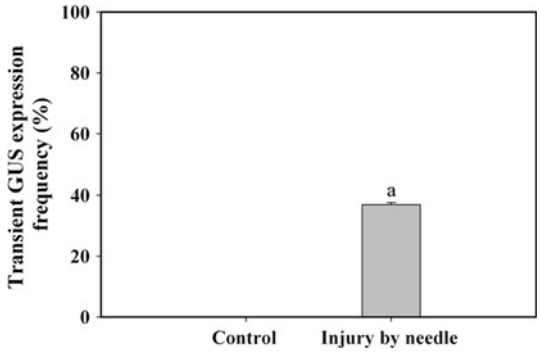

Fig. 2 Effect of mechanical injury by hypodermic needle on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. Control–injury is omitted in explants. The *bars* indicate ± standard errors. Means followed by the same letter are not statistically significant at $P < 0.05$

number of plant species including recalcitrant grain legumes (Roome 1992; Rohini et al. 2005; Supartana et al. 2006; Saini and Jaiwal 2007).

Effect of sonication and vacuum infiltration

To identify more efficient methods to improve access of *Agrobacterium* and also to create an area of wounding to induce cotyledonary node cells and to produce phenolic compounds for *vir* gene induction in cowpea, we evaluated the effect of sonication and vacuum infiltration on *A. tumefaciens*-mediated transformation of cotyledonary node explants. These treatments have the potential to increase transformation efficiency by improving penetration of *Agrobacterium* cells into the cell layers beneath the epidermis of cotyledonary node region. This is an important criterion as regenerating cells of cotyledonary node explants are positioned a few cells layers beneath the surface at the axils in *Vigna* species including cowpea, mungbean and blackgram (Sahoo and Jaiwal 2009). A control experiment with explants without inoculation with *Agrobacterium* was designed to determine whether these treatments could be used without a negative effect on shoot regeneration from cotyledonary node explant. Sonication was very effective in increasing transient GUS expression frequency (Figs. 3, 4a). With the increase in sonication treatment time, the number of transiently transformed explants increased significantly with a maximum of 79% of the explants showing GUS foci at the regenerating sites when sonication treatment was prolonged to 20 s (Figs. 3, 4a). The number of GUS foci appeared to be quite variable among cotyledonary node explants (data not shown). At lower sonication treatment time (10–20 s), the GUS foci were well defined, corresponding to probably one or a collection of small individual spots (Fig. 3a–f). With the increase in sonication treatment time beyond 20 s, a diffuse GUS expression was presented all over the surface of the cotyledonary node explants, making the quantification of the number of foci difficult (Fig. 3g–h). Moreover, with increase in sonication treatment time to 30 s, the untransformed explants showed a decrease in their bud-forming capacity indicating that longer sonication treatment compromised viability of regenerating cells (data not shown). SAAT has been used to enhance stable transformation of many recalcitrant plant species including soybean (Trick and Finer 1998), loblolly pine (Tang 2003), black locust (Zaragoza' et al. 2004), sweetpotato (Wang et al. 2006), rice (Yookongkaew et al. 2007), *Chenopodium rubrum* (Flores Solı́s et al. 2007), chickpea (Pathak and Hamzah 2008), flax (Beranova′ et al. 2008) and *Theobroma cacao* (Silva et al. 2009).

We attempted various time intervals of vacuum infiltration of explants at 600 mmHg in an *Agrobacterium* suspension, and of the different time intervals tested, a

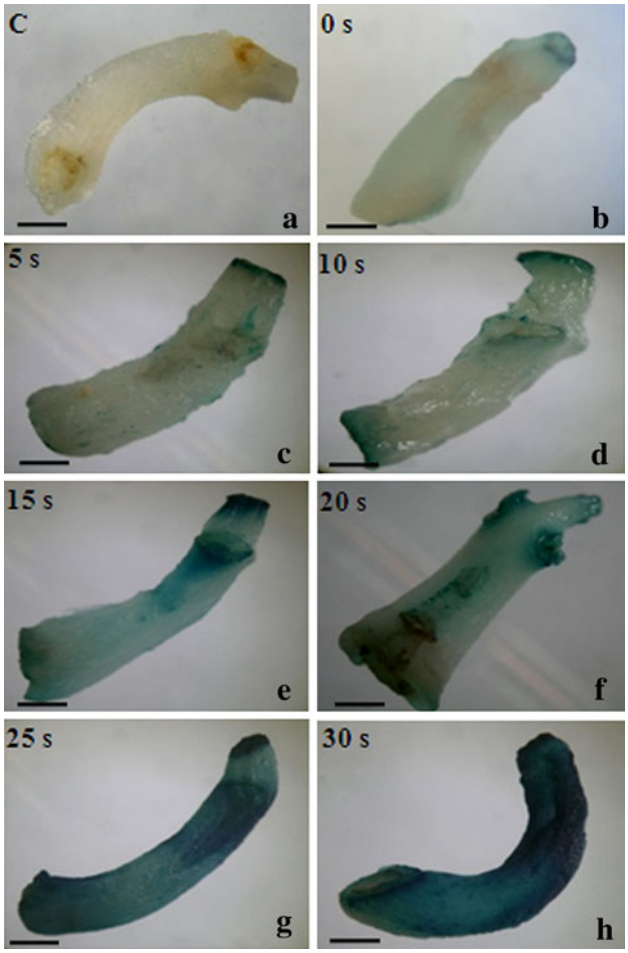

Fig. 3 Transient expression of GUS at the regenerating sites of sonication-treated cotyledonary node explants after 3 days of co-culture. **a** Control (untransformed). **b** *Agrobacterium*-treated explants (without sonication treatment). **c–h** sonication-treated cotyledonary nodes (**c** 5 s, **d** 10 s, **e** 15 s, **f** 20 s, **g** 25 s and **h** 30 s). *Bar* (in all figures) 1 mm

5 min vacuum infiltration resulted in a maximum of 93% transient transformation efficiency as accounted on the basis of number of explants showing GUS foci at the regenerating sites (Fig. 4b). Vacuum infiltration of cotyledon explants of *Pinus radiata* in an *Agrobacterium* suspension has allowed *Agrobacterium* to penetrate several layers deep through the sub-epidermal layer to mesophyll cells and vascular tissues (Charity et al. 2002), although the cells buried several layers deep, were not necessarily those that would induce shoots (Yeung et al. 1981). The vacuum infiltration of *Agrobacterium* has been successfully used to produce transgenic plants of model plant *Arabidopsis* (Clough and Bent 1998), and recalcitrant crop species including wheat (Cheng et al. 1997), mungbean (Jaiwal et al. 2001), pinus (Charity et al. 2002), cotton (Leelavathi et al. 2004), kidney bean (Liu et al. 2005), coffee (Canche-Moo et al. 2006), chickpea (Indurker et al. 2010) and banana (Subramanyam et al. 2011).

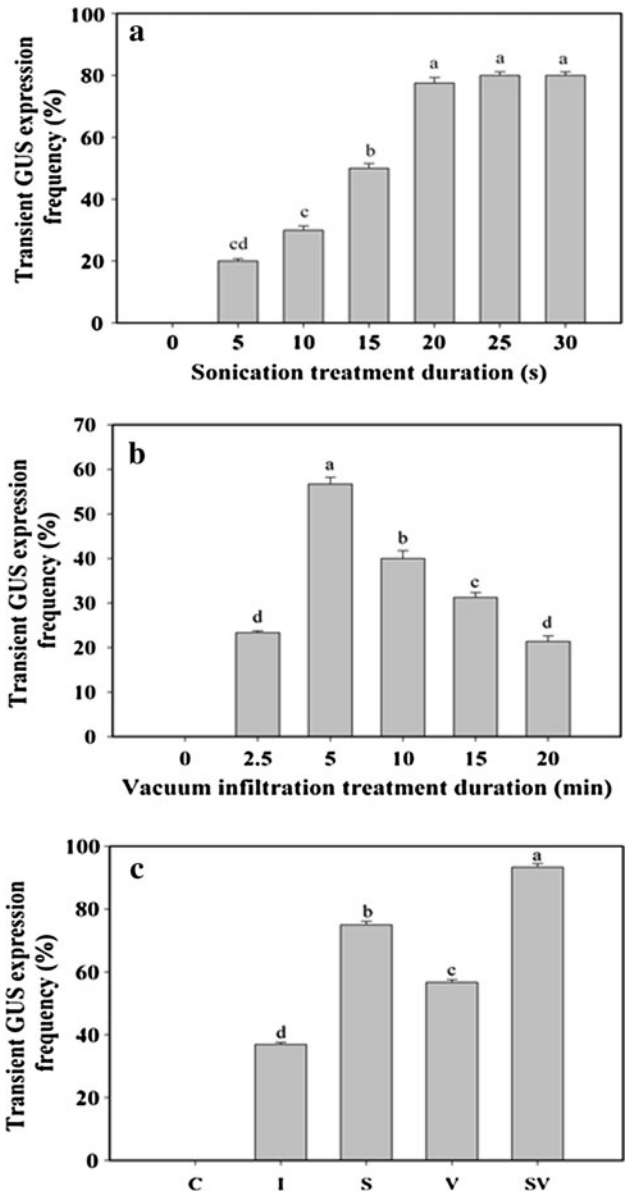

Fig. 4 **a** Effect of SAAT treatment duration and **b** vacuum infiltration treatment duration on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. **c** Effect of different wounding methods on transient transformation of cowpea cotyledonary nodes as evaluated with GUS assay. *C* Without wounding. *I* Injury treatment by hypodermic needle. *S* 20 s sonication treatment. *V* Vacuum infiltration treatment for 5 min. *SV* 20 s sonication followed by vacuum infiltration treatment for 5 min. The *bars* indicate ± standard errors. Means followed by the same letter are not statistically significant at $P < 0.05$

Combined treatment of sonication and vacuum infiltration

In order to evaluate the combined action of sonication and vacuum infiltration on transient transformation, the effect of 20 s sonication and 5 min vacuum infiltration was tested as compared to the two treatments separately. The combination of 20 s sonication followed by 5 min vacuum infiltration resulted in maximum frequency of cotyledonary node explants expressing GUS at the regenerating sites (Fig. 4c). Sonication coupled with vacuum infiltration has increased transient and stable transformation of radish (Park et al. 2005), kidney bean (Liu et al. 2005), citrus (Oliveira et al. 2009), *Fraxinus pennsylvanica* (Du and Pijut 2009), chickpea (Indurker et al. 2010) and banana (Subramanyam et al. 2011).

Production of transgenic cowpea plants carrying *cry1Ac* gene

Putative transformed plants were regenerated from cotyledonary node explants, which were subjected to a combination of 20 s sonication followed by 5 min vacuum infiltration prior to *Agrobacterium* co-cultivation, on kanamycin selection medium and established in greenhouse containment (Fig. 5a–g). A strong, uniform and stable *gus* expression was detected in flower, anthers, pollens and pistils of PCR-positive T_0 plants and no endogenous *gus* expression was detected in the tissues of control plants (Fig. 5h–o).

Analysis of transgenic cowpea plants

The detection of the expected 540 bp and 1 kb amplified products corresponding to *nptII* and *cry1Ac* in PCR analysis confirmed the presence of the transgenes in T_0 transformed plants (Fig. 6a, b). No amplification was detected in the control untransformed plants.

Four randomly selected PCR-positive T_0 transgenic cowpea plants were further screened by Southern analysis to confirm the integration of *cry1Ac* gene. Southern blot analyses of four T_0 transgenic plants are shown in Fig. 6c. Hybridizations of DIG-labeled cry1Ac probe to total genomic DNA digested with *Hind*III were expected to identify DNA fragments unique to individual integration events greater than 5.0 kb (Fig. 1). All the four randomly selected T_0 transgenic plants were found positive for *cry1Ac* gene and furthermore, they showed differential integration events, confirming that these plants were derived from independent transformation events (Fig. 6c, *lanes 1, 2, 3* and *4*). The T_0 transgenic plants exhibited simple hybridization patterns that ranged from single integration event to three loci and, in general, most fragments were greater than 5.0 kb (Fig. 6c). A signal of size less than 5.0 kb was detected in *lane 3* (Fig. 6c), suggesting the possibility of rearrangement of the T-DNA near the left border upon integration into the plant genome. No hybridization signal was detected in the untransformed plant (Fig. 6c, *lane C*).

Fig. 5 Transient and stable *gus* expression and regeneration of transgenic plants. **a** cotyledonary node explants. *Bar* 2 mm. (**b** and **c**) Transient GUS expression, non-transformed (control) explants not showing GUS activity (**b**), cotyledonary node explants showing transient GUS activity after 3 days of co-cultivation (**c**). *Bar* 4 mm. **d** Shoot induction from axils of explant after 5-day culture on SISM. *Bar* 2 mm. **e** Proliferation of multiple shoots within 4 weeks of culture. *Bar* 10 mm. **f** In vitro rooting of elongated transformed shoot. *Bar* 12 mm. **g** Acclimatized plant maintained in transgenic green house. *Bar* 10 cm. **h** Non-transformed control flower. *Bar* 7 mm. **i** Transformed flower. *Bar* 7 mm. **j** Control anthers. *Bar* 8 mm. **k** Transformed anthers. *Bar* 8 mm. **l** Control pistil. *Bar* 8 mm. **m** Transformed pistil. *Bar* 8 mm. **n** Control pollens. *Bar* 3 mm. **o** Transformed pollens. *Bar* 3 mm

The expression of the *cry1Ac* genes in leaves of T_0 transgenic plants was determined by RT-PCR analysis. RT-PCR showed the presence of expected transcripts of transgenes in different T_0 transgenic plants. The amplification of a 1 kb fragment of *cry1Ac* confirmed the accumulation of transcripts of *cry1Ac* in T_0 transgenic plants (Fig. 6d, e) indicating the absence of gene silencing events. Furthermore, the amplification of the *cry1Ac* sequence from plant cDNA templates in RT-PCR ruled out the possibility of *Agrobacterium* contamination.

The stable transformation efficiency was determined based on the number of T_0 plants PCR-positive for Bt*cry1Ac* and *npt*II divided by the total number of explants co-cultivated. An average stable transformation efficiency of 3.09 was recorded (Table 1), which was significantly higher than the previously published report on *Agrobacterium*-mediated transformation of cowpea using extra copies of *vir* genes (Solleti et al. 2008a).

Cry1Ac expression analysis

The randomly chosen PCR-positive T_0 transgenic lines were subjected to Cry1Ac protein expression analysis by Western hybridization and ELISA. The expression of the Cry1Ac protein was analyzed in T_0 transgenic lines generated from four independent transformation events by Western blot hybridization. A single band of 68 kDa corresponding to Cry1Ac toxin protein was detected

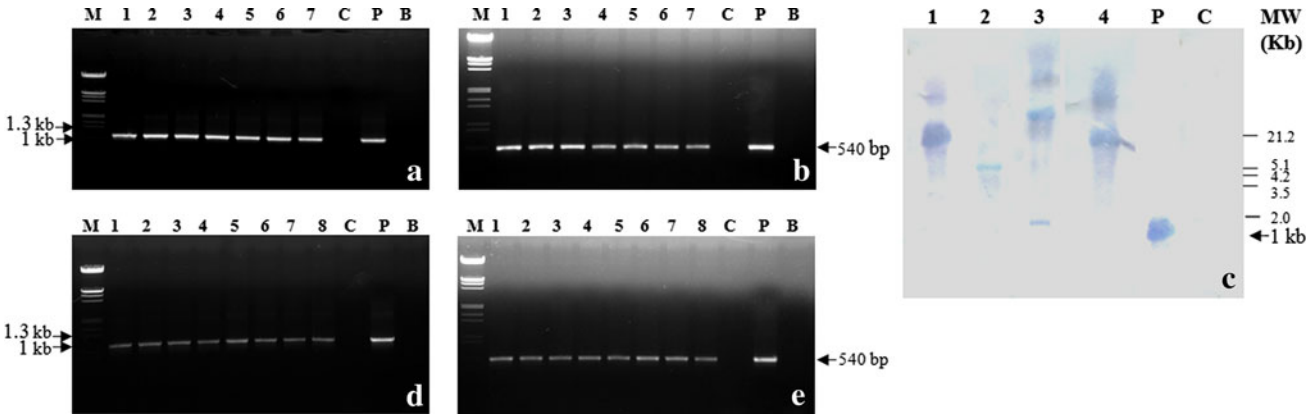

Fig. 6 Molecular analysis of T_0 transgenic plants. **a** PCR amplification of the 1 kb fragment of the *cry1Ac* gene, **b** PCR amplification of the 540 bp fragment of the *nptII* gene. *Lane M* λ DNA/*Eco*RI + *Hin*dIII marker, *lane P* pSouv:cry1Ac plasmid DNA (positive control), *lane C* DNA from untransformed plant (negative control), *lane B* blank, *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 *Hin*dIII, and hybridized with cry1Ac probe. *Lanes 1–4* genomic DNA from four T_0 lines, *lane C* genomic DNA from untransformed plant, *lane P* cry1Ac PCR amplicon. **d** RT-PCR analysis of *cry1Ac* gene, **e** RT-PCR analysis of *nptII* gene. *Lane M* λ DNA/*Eco*RI + *Hin*dIII marker; *lane C* untransformed plant (negative control); *lane B* blank; *lanes 1–8* T_0 transgenic plants

Table 1 Summary of the *Agrobacterium*–mediated transformation of cowpea cotyledonary node explants subjected to 20 s sonication followed by vacuum infiltration for 5 min with *Agrobacterium tumefaciens* EHA105pSouv:cry1Ac

Exp. no.	No. of explants inoculated in *Agrobacterium* suspension	Transient transformation efficiency[a] (%)	No. of shoots recovered on selection medium	No. of plants positive for *cry1Ac* and *npt*II genes by PCR	Transformation efficiency[b] (%)
1	247	91.10	15	8	3.20
2	239	95.00	12	7	2.93
3	204	92.12	11	6	2.94
4	243	95.10	12	8	3.30
Total[c]/average[d]	933[c]	93.33[d]	50[c]	29[c]	3.09[d]

[a] Number of explants showing GUS foci at the regenerating sites per number of explants co-cultivated with *Agrobacterium tumefaciens* EHA105pSouv:cry1Ac

[b] Number of plants PCR-positive for *cry1Ac* and *npt*II per number of explants co-cultivated

[c] Total

[d] Average response

immunologically in T_0 transgenic plants confirming stability of *cry1Ac* expression. Protein extracts of control non-transformed plants did not show the 68 kDa protein band (Fig. 7a).

The level of expression of Cry1Ac protein in transgenic lines ranged from 0.001 to 0.089% of the total leaf soluble protein (Fig. 7b). The results described above demonstrated that expression of the *cry1Ac* regulated by the double 35S-promoter led to the accumulation of Cry1Ac protein in transgenic plants.

Segregation analysis

The seeds from T_0 generation were advanced to T_1 generation and the T_1 transgenic lines generated from eight independent transformation events were analyzed for the segregation pattern of *cry1Ac* by PCR analysis. Presence of the expected 1 kb amplified product corresponding to *cry1Ac* in T_1 transgenic lines confirmed the inheritance of *cry1Ac* gene (Fig. 8). The segregation pattern of these selected transgenic events showed typical 3:1 Mendelian ratio as expected for single dominant gene inheritance (Table 2).

In conclusion, an improved *Agrobacterium*-mediated transformation system was developed for cowpea by employing sonication and vacuum infiltration was enhanced by 88.4% using SAAT in combination with vacuum infiltration as compared to simple *Agrobacterium*-mediated transformation. This is the first report on cowpea transformation using SAAT and vacuum infiltration.

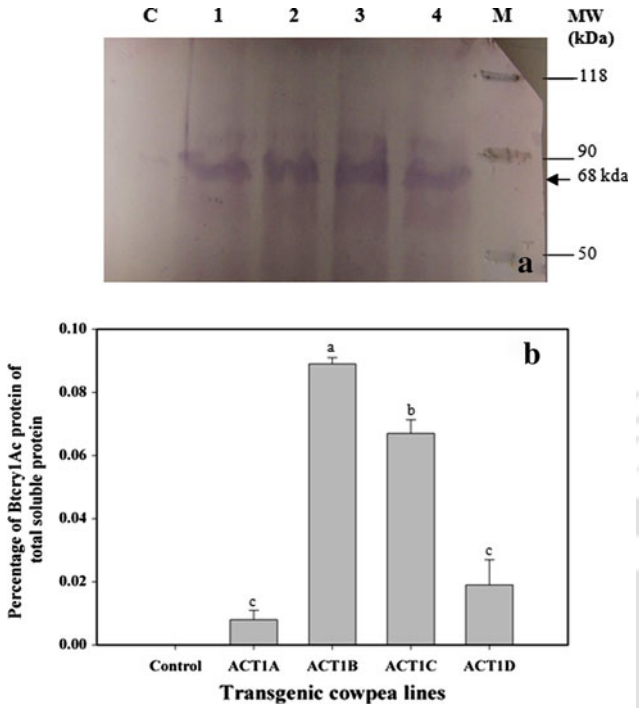

Fig. 7 **a** Detection of Cry1Ac protein by Western blotting analysis in transgenic cowpea leaves. *M* Protein molecular weight marker, *lanes 1–4 cry1Ac* transgenic lines (CT1A, CT1B, CT1C and CT1D), respectively, *lane 5* non-transformed plant. **b** Expression level of BtCry1Ac protein in transgenic cowpea lines (CT1A, CT1B, CT1C and CT1D) from enzyme-linked immunesorbent assay (ELISA). *Error bars* represent ± standard error of the means. Means followed by the same letter are not statistically significant at $P < 0.05$

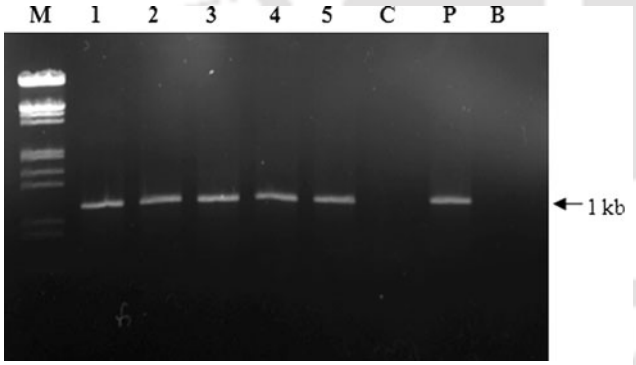

Fig. 8 PCR amplification of the 1 kb fragment of the *cry1Ac* gene of T_1 plants. *Lane M* λ DNA/*Eco*RI + *Hin*dIII marker, *lane P* pSouv:cry1Ac plasmid DNA (positive control), *lane C* DNA from untransformed plant (negative control), *lane B* blank, *lanes 1–5* DNA from T_1 transgenic plants

Furthermore, cowpea transgenics expressing *cry1Ac* is reported for the first time.

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Table 2 Segregation of *cry1Ac* gene in T_1 progeny of transgenic cowpea plants

T_0 plants	Number of T_1 plants tested for *cry1Ac*[a]			χ^2 value	Expected ratio
	Total	*cry1Ac* +ve	*cry1Ac* −ve		
C1	52	39	14	0.10	3:1
C2	35	24	11	0.77	3:1
C3	39	27	12	0.69	3:1
C4	55	40	15	0.15	3:1
C5	28	21	7	0.18	3:1
C6	43	30	13	0.56	3:1
C7	31	22	9	0.27	3:1
C8	44	40	4	0.61	15:1

[a] Presence of *cry1Ac* was analyzed by PCR

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GENETIC TRANSFORMATION AND HYBRIDIZATION

Transgenic cowpea (*Vigna unguiculata*) seeds expressing a bean α-amylase inhibitor 1 confer resistance to storage pests, bruchid beetles

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Abstract Cowpea is one of the important grain legumes. Storage pests, *Callosobruchus maculatus* and *C. chinensis* cause severe damage to the cowpea seeds during storage. We employ a highly efficient *Agrobacterium*-mediated cowpea transformation method for introduction of the bean (*Phaseolus vulgaris*) α-amylase inhibitor-1 (*αAI-1*) gene into a commercially important Indian cowpea cultivar, Pusa Komal and generated fertile transgenic plants. The use of constitutive expression of additional *vir* genes in resident pSB1 vector in *Agrobacterium* strain LBA4404, thiol compounds during cocultivation and a geneticin based selection system resulted in twofold increase in stable transformation frequency. Expression of *αAI-1* gene under bean phytohemagglutinin promoter results 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.

Keywords Bean α-amylase inhibitor 1 (αAI-1) · *Callosobruchus* spp. · Geneticin selection · Storage pest resistance · Transgenic cowpea

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Introduction

Cowpea (*Vigna unguiculata* L. Walp) is a grain legume widely cultivated in tropical countries of the world (Sahoo et al. 2003), which provides a major source of cheap and high-quality dietary proteins supply (Langyintuo et al. 2003). Bruchids are a major threat to stored cowpea grains (Singh et al. 2000), and infestations by the most prominent species, *Callosobruchus maculatus* and *C. chinensis* are responsible for grain losses estimated at 20–60% (Abrol 1999; Tarver et al. 2007). 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. 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; 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. 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.

We report introduction of the common bean αAI-1 gene to cowpea through an improved *Agrobacterium tumefaciens*-mediated transformation method using additional virulence genes for efficient T-DNA delivery to regenerating cells, thiol compounds during cocultivation, exerting synergistic action of two different cytokinins for promotion of regeneration of proliferating transformed cells, and adapting a geneticin-based selection system for rapid selection of transgenic shoots. We 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.

Materials and methods

Plant material and explant preparation

Seeds of a commercially grown cultivar of cowpea in India cv. Pusa Komal were used in the present study. Seeds were surface sterilized (Sahoo et al. 2000) 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-day-old in vitro raised seedlings by removing both the cotyledons, and epicotyls and hypocotyls approximately 1 and 3 mm, respectively, from above and below the nodal region.

Vector construction and *Agrobacterium* strain

The standard binary vector pCAMBIA2301 (CAMBIA, Australia) was used to subclone an α-amylase inhibitor 1 cDNA (accession J01261) flanked by seed-specific 5′ and 3′ control regions of the bean phytohemagglutinin gene by ligating a *Hin*dIII fragment from pTA3. The pTA3 contains an α-amylase inhibitor 1 cDNA derived from the bean cultivar Tendergreen (Altabella and Chrispeels 1990). 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 construct pSiva (Fig. 1) was mobilized to *A. tumefaciens* strain LBA4404 (Hoekema et al. 1983), carrying pSB1 by triparental mating (Bevan 1984). The pCAMBIA vectors lack super-virulent genes and do not give high transformation frequencies in recalcitrant grain legumes. Plasmid SB1 lacks T-DNA but has all the virulence genes present in pTOK 233 (Hiei et al. 1994) and contains a tetracycline resistance gene as selection marker. 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.

Plant transformation

Single colony of the bacterial strain was inoculated in 25 ml of liquid AB minimal medium (Chilton et al. 1974) with appropriate antibiotics, and grown overnight at 28°C until OD_{600} reached to 0.8. The cells were collected by centrifuging at 5,000 rpm for 5 min and the pellet was re-suspended in liquid cocultivation medium, LCM (MSB medium containing 1 μM BAP, 1 mM dithiothreitol and

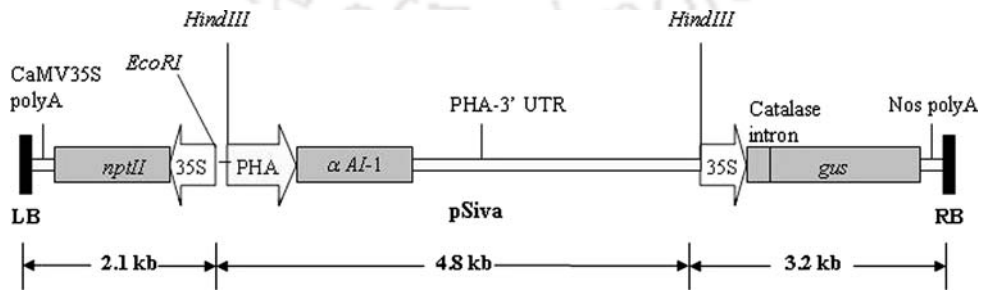

Fig. 1 T-DNA region (10.1 kb) of pSiva. *RB* right border, *LB* left border, *35S* CaMV 35S promoter, *CaMV35polyA* CaMV35S terminator, *NOS* nopaline synthase terminator, *intron-gus* intron interrupted β-glucuronidase, *npt*II neomycin phosphotransferase. *PHA* phytohemagglutinin promoter, *αAI-1* α-amylase inhibitor-1, *PHA-3′ UTR* phytohemagglutinin terminator. Also highlighted are the positions of unique *Eco*RI restriction site, and the distance between *Eco*RI site and LB (2.1 kb)

8.3 mM L-Cysteine, pH adjusted to 5.5) supplemented with 100 μM acetosyringone for inoculation. The 3-day-old cotyledonary node explants were inoculated in bacterial suspension for 30 min with occasional shaking. The explants were then blotted on sterile filter paper and co-cultivated in petri dishes lined with filter paper moistened with LCM supplemented with 100 μM acetosyringone for 3 days under 16 h photoperiod at 22°C.

Following co-cultivation, the explants were washed three to four times with LCM and blotted dry on sterile filter paper. The explants were cultured 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. The cultures were transferred after 1 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. 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.

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-day-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°C. 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.

Polymerase chain reaction analysis and Southern hybridization

Genomic DNA was isolated from both non-transformed and putative transformed plants by CTAB method (Roger and Bendich 1988). The 0.54 kb of *npt*II and 0.57 kb of *αAI-1* coding regions in putative T_0 transformants and their progenies were amplified by using the following primer combinations: *npt*II gene: forward 5′-CC ACC ATG ATA TTC GGCAAC-3′ and reverse 5′-GTGGAGAGGCTATT CGGCTA-3′, and *αAI-1* gene: forward 5′-TCATGGCTT CCTCCAAGTTAC-3′ and reverse 5′-GGACGATGTTG-GAACGTTCA G-3′. The amplification reaction was carried out with initial denaturation at 94°C for 1 min and followed by 38 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and final extension for 7 min at 72°C for detection of both *npt*II and *αAI-1* gene amplification. Amplified DNA fragments were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining (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.

Ten microgram samples of genomic DNA from non-transformed control and progeny of four independent transgenic cowpea lines were digested with *Eco*RI, 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). The probe consisted of the coding region of the *αAI-1* gene isolated as a PCR fragment using primers used for genomic PCR. The probe was labeled and Southern hybridization was performed using the non-radioactive DIG Labeling and Detection system (Roche, Germany) following supplier's instructions.

Prehybridization and hybridization were carried out using high-SDS 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. Washing and detection were performed according to the instruction of the DIG labeling and Detection System (Roche Diagnostics, Mannheim, Germany).

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.

RT-PCR

Total RNA was isolated from the PCR-positive transgenic T_0 embryo lines 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 RNA, oligo(dT) primer, and the M-MuLV Reverse Transcriptase, according manufacturers' instructions. These templates were used for the amplification of the *αAI-1* gene transcripts using the same primer sets as those used in genomic PCR.

α-Amylase inhibitory activity

Total seed protein from non transgenic and transgenic lines was extracted into 2 ml of extraction buffer (10 mM Tris–HCl pH 7.5, 10 mM β-mercaptoethanol, 0.1 M NaCl, 0.2 mM PMSF). Hundred milligram of seeds from each line was ground to fine powder in a cold mortar pestle. Extract was centrifuged at 15,000 rpm for 10 min, and the supernatant was used. Protein concentration was estimated by the method of Bradford (1976).

One microgram 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 2–10 µg) of total soluble protein from mature seeds of non transgenic or transgenic cowpea for 60 min at 37°C. Hundred microliters 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 min. 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; mean and SD were determined. A standard curve was constructed from a range of maltose concentrations. 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.

Insect bioassays

A 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). For bioassays, seeds were conditioned at 28 ± 1°C and 70% relative humidity for a period of 1 week before use. 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 1 week after they laid eggs and the percentage of mortality was calculated. The seeds were maintained for a further 5 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.

Results

Production of transgenic cowpea plants carrying αAI-1 gene

All the cotyledonary node explants showed GUS activity after cocultivation with *A. tumefaciens* LBA4404/pSB1 harboring pSiva, predominantly in the regenerable sites (Fig. 2a) 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. A total of 358 explants, in three different experiments, produced 35 shoots on geneticin selection medium (Table 1). 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. 2b; Table 1). 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 2 months. While endogenous *gus* expression was not detected in the tissues of control plants, 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 (Fig. 2c–h). The *gus* expression in germinated T_1 seedlings clearly demonstrated inheritance and expression of the transgene in the progeny (Fig. 2i, j). We determined the stable transformation efficiency based on the percentage of T_0 plants that showed stable *gus* expression in T_1 seedlings. A total of six independently derived transgenic plants were obtained from a total of 358 explants, giving an average transformation frequency of 1.67%.

Molecular analysis of transgenics

PCR analysis detected the presence of the expected 540 and 570 bp fragments (Fig. 3a, b) in geneticin-resistant T_0 transgenic plants, that showed stable *gus* expression in flowers demonstrating the efficiency of geneticin based selection scheme in elimination of escapes. No amplification was detected in the control untransformed plants.

Progenies of independent T_0 transgenic lines were screened by Southern analysis to confirm the integration of *αAI-1* gene. Southern blot analyses of progeny plants of

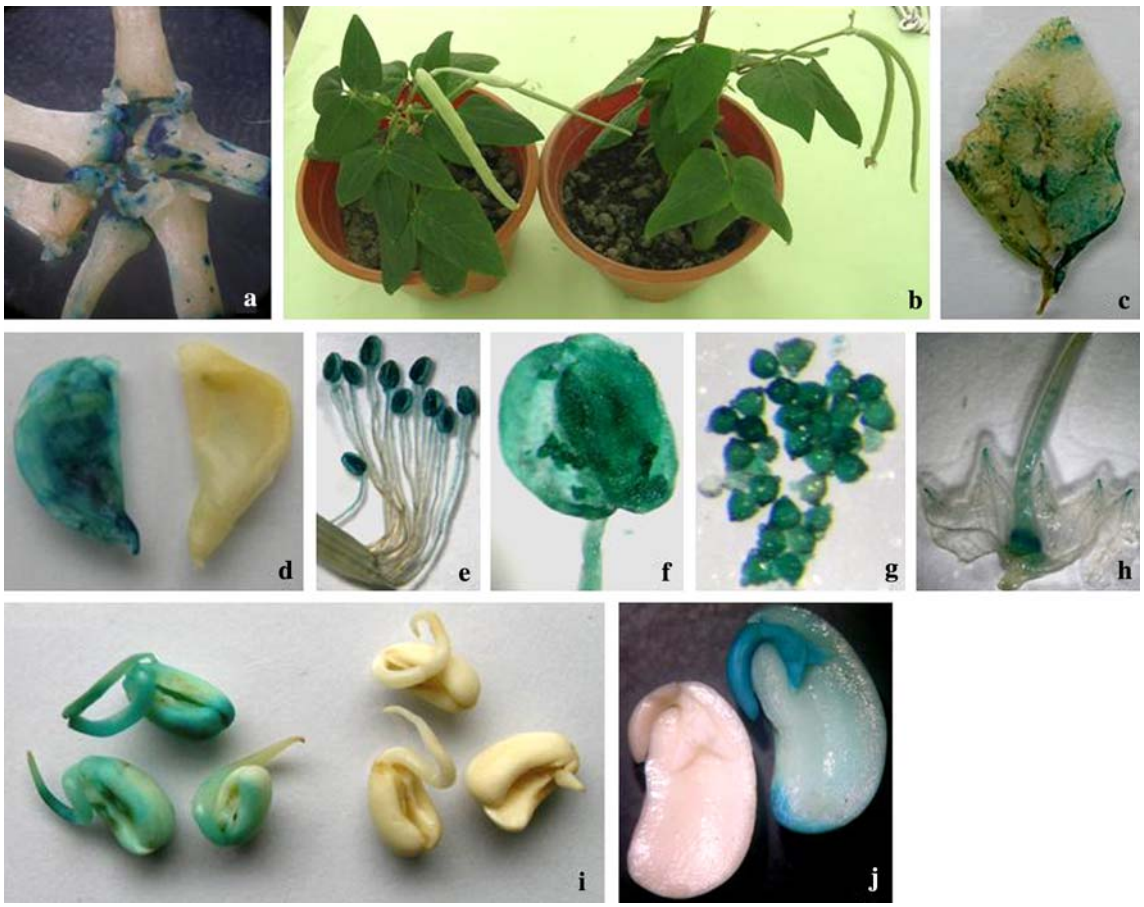

Fig. 2 Transient and stable GUS activity. **a** Cotyledonary node explants showing transient GUS activity after 3 days of cocultivation with *A. tumefaciens* LBA4404/pSB1pSiva. **b** Geneticin resistant T_0 transgenic plants established in green house, **c–j** Stable GUS activity in geneticin-resistant 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*)

Table 1 Summary of the transformation of 3-day-old cotyledonary node explants of *Vigna unguiculata* cv. Pusa Komal cocultivated with *Agrobacterium tumefaciens* strain LBA4404/pSB1 harboring 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 1 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

four independent T_0 transgenic lines are shown in Fig. 3c. 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. 1). All the four randomly selected T_1 transgenic lines were positive for *αAI-1* gene which showed differential integration events, confirming that these plants were derived from independent transformation

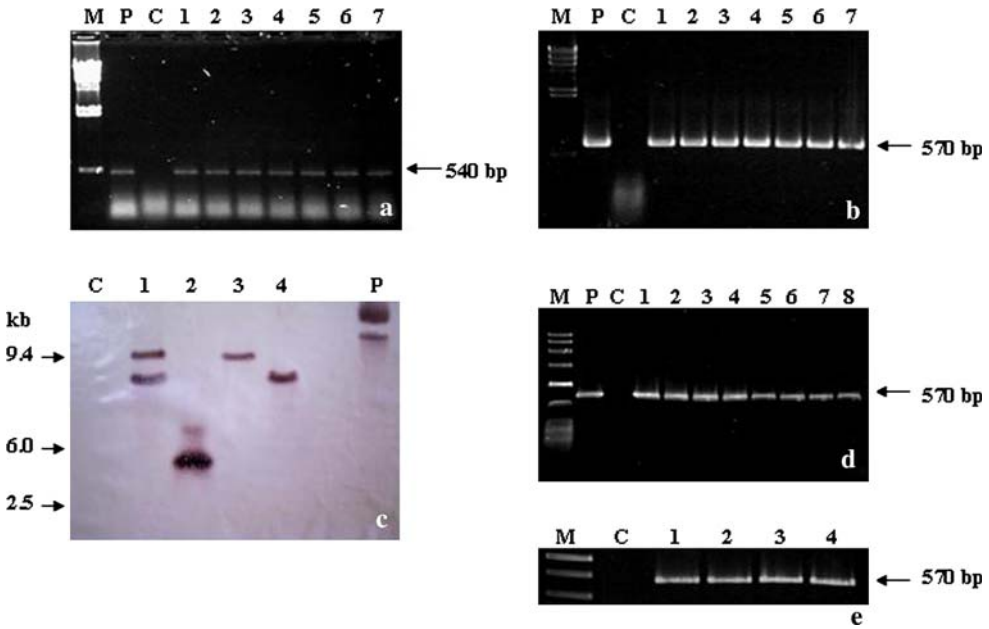

Fig. 3 Molecular analysis of transgenic plants. **a–c** Analysis of T_0 transgenic plants, **a** PCR amplification of the 540-bp fragment of the *npt*II gene, **b** PCR amplification of the 570-bp fragment of the *αAI-1* gene. *Lane M* molecular weight marker; *lane P* pSiva plasmid DNA (*positive control*); *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

events (Fig. 3c, lanes 1, 2, 3 and 4). The T_1 transgenic lines exhibited simple hybridization patterns that ranged from single integration events (e.g. lines, 2, 3 and 4) to two loci (e.g. line 1) and, in general, most fragments were greater than 8.0 kb (Fig. 3c). A signal of size less than 8.0 kb was detected in line 2 (Fig. 3c), suggesting 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 (Fig. 3c, lane C). The result was consistent with the PCR analysis of T_0 plants and stable *gus* expression analysis in their seedlings.

Inheritance of αAI-1 gene in transgenic plants

All T_0 plants produced viable T_1 seed. The segregation of *αAI-1* gene was analyzed in T_1 plants from four independent T_0 transgenic lines. PCR analysis confirmed the presence of 570 bp of coding region of *αAI-1* gene in tested progeny lines, a representative gel of which is shown in Fig. 3d. 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 2). 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 2). Detection of strong GUS activity in germinated T_1 seedlings indicated the inheritance and stable expression of the *gus* gene in the progeny.

Expression of αAI-1 gene in transgenic plants

RT-PCR analysis showed that the seeds of transgenic lines contained the transcripts of *αAI-1* gene (Fig. 3e),

Table 2 Segregation of *αAI1* transgene in T_1 plants of *Vigna unguiculata* cv. Pusa Komal

T_0 line	No. of plants tested	Positive (*αAI1*)	Negative (*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

confirming the expression of the *αAI-1*gene in seeds. The result verified the functional expression of the *αAI-1* gene in the transgenic seeds.

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. The percentage of inhibition was 37 in line-1, 36.6 in line-2, 31.9 in line-3 and 32.9 in line-4 (Fig. 4). No α-amylase inhibitory activity was detected in extracts from seeds of untransformed control cowpea.

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. 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

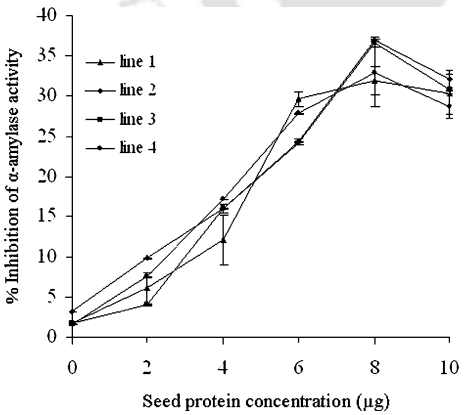

Fig. 4 Inhibitory activity of seed extracts from transgenic cowpea against porcine pancreatic α-amylase in vitro. Seed protein extracts from transgenic cowpea lines 1, 2, 3 and 4 were incubated with porcine α-amylase as described in "Materials and methods". The amount of reducing sugars released from 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. Inhibition (%) against porcine α-amylase. *Error bar* represents the SD ± values of mean of triplicate data

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 eight 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 3).

Discussion

We report successful recovery of transgenic cowpea plants expressing bean α-amylase inhibitor 1 in seeds. 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 co-cultivating 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 role of extra copies of *vir* genes for optimal transformation of this highly recalcitrant *Vigna* species was evident from the fact that 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 LBA4404pSiva clearly indicates the beneficial role of extra copies of *vir* genes in enhancing the 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 of many crops, particularly monocot species (Park et al. 2000; Wu et al. 2007).

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 acting as inhibitors of plant defense response mechanisms, making this approach useful in recovering transgenic plants at a high frequency. Cocultivation in presence of thiol compounds have been described beneficial in recovering transgenic plants in soybean (Olhoft

Table 3 *Callosobruchus maculatus* and *C. chinensis* development in T_1 seeds of control and two transgenic cowpea plants

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

et al. 2001). Moreover, co-cultivation of explants in the presence of mixtures of the thiol compounds combined with geneticin selection resulted in enhanced recovery of transgenic plants to an average of 1.67%. Transformation efficiencies obtained for thiol compounds treated cotyledonary node explants using PPT selection in cowpea were 0.001–0.003% (Popelka et al. 2006).

Selection strategy plays 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. Incomplete selection of transformed cells by kanamycin (Chaudhury et al. 2007), and generation of high percentage of 'escapes' and chimeric plants upon phosphinothricin selection (Popelka et al. 2006) have resulted in lower stable transformation efficiency in cowpea. Previous report on cowpea transformation (Popelka et al. 2006) observed that 150 mg/l geneticin inhibited the development of control untransformed shoots of cultivar Sasaque. In our study, the low concentration (45 mg/l) of geneticin had a much quicker and pronounced effect on bleaching of shoots compared to other antibiotics and further, necrosis of explants was completely absent in geneticin-containing medium (data not shown). The selection pressure imparted by the geneticin throughout the shoot regeneration period was very effective in early identification of putative transgenic shoots. Combining these two novel improvements resulted in a synergistic increase in the regeneration of transformed plants, without nontransformed 'escapes', in a relatively short period of time in culture.

Employing extra copies of *vir* genes and geneticin-based selection scheme, we recovered fertile transgenic plants at a frequency of 1.67%, a twofold higher than kanamycin selection based recovery of transgenics in cowpea (Chaudhury et al. 2007). Recovery of fertile transgenic plants in our case took an approximately 2 months which was significantly less than previous reports in cowpea, i.e., 5–8 months (Popelka et al. 2006) and 5–6 months (Chaudhury et al. 2007). The presence, integration and expression of *αAI-1* gene were demonstrated in T_1 transgenic plants. The *αAI-1* gene was transmitted in a Mendelian fashion. RT-PCR analysis verified the functional expression of the *αAI-1* gene in the transgenic seeds and accumulation of α amylase inhibitor protein in a seed specific manner by bean phytohemagglutinin promoter. 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.

The αAI-1 protein was active in inhibiting porcine pancreatic α-amylase in vitro, and inhibited the emergence of bruchid beetles from the transgenic cowpeas in insect bioassays and the results were in accordance with RT-PCR analysis demonstrating the functionality of the *αAI-1* transcripts. In all the lines tested, the maximum percentage of inhibition was observed with 8 µg of the total seed proteins. The percentage 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. All the four lines studied showed percentage of inhibition in a range 32.0–37.0 and as predicted the transgenic cowpea expressing *αAI-1* gene in their seeds reduced fecundity of *C. maculatus*, the major storage pest of cowpea by over 97%, and the seeds were also resistant to *C. chinensis* as predicted. The number, weight and longevity of the newly emerged adults were significantly reduced when the insects were reared on seeds of both transgenic lines tested.

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.

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Additional virulence genes in conjunction with efficient selection scheme, and compatible culture regime enhance recovery of stable transgenic plants in cowpea via *Agrobacterium tumefaciens*-mediated transformation

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Geneticin selection

ABSTRACT

A critical step in the development of robust *Agrobacterium tumefaciens*-mediated transformation system in recalcitrant grain legume, cowpea is the establishment of optimal conditions for efficient T-DNA delivery into target tissue and recovery of transgenic plants. A dramatic increase in efficiency of T-DNA delivery was achieved 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 1 week 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. The protocol developed for cultivar Pusa Komal will facilitate the transfer of desirable traits into cowpea.

1. Introduction

Cowpea (*Vigna unguiculata* L. Walp) is an important grain legume crop widely cultivated by the resource poor farmers of extended Sub-Saharan Africa and Asia for its protein rich seeds, fresh peas and fresh green pods (Sahoo et al., 2003). However, its production is limited by a number of biotic and abiotic stresses (Singh et al., 2000). Among biotic stresses, the post-flowering insect pests such as pod borers (*Maruca vitrata*), pod-sucking bugs (*Clavigralla* spp.) and cowpea weevil *Callosobruchus maculates* cause severe grain yield loss. Attempts for genetic improvement of cowpea by conventional breeding have not been successful (Singh et al., 2000), making these approaches unlikely to provide an immediate feasible solution.

Genetic transformation provides novel opportunities for transferring candidate genes to elite cultivars of cowpea to address the constraints in yield (Sahoo and Jaiwal, 2008). However, genetic manipulation of cowpea is limited due to the absence of a transformation amenable plant regeneration system, efficient method for selection, and recovery of viable and fertile transgenic plants from transformed sectors at a reasonable high frequency (Sahoo et al., 2003). Several cultivars and breeding lines of cowpea have been evaluated for their regeneration potential (Mao et al., 2006). To date, only three reports are available on generation of stable transgenic plants in cowpea and all reports are based on *Agrobacterium*-mediated transformation. Muthukumar et al. (1996) recovered four hygromycin-resistant transformed plants, from mature de-embryonated cotyledons, of which one showed stable integration of *hpt* gene. However, the established plant failed to produce viable seeds. Popelka et al. (2006) recovered transgenic plants from longitudinally bisected embryonic axes, attached with cotyledons but devoid of shoot and root apices, on phosphinothricin selection at a frequency of 0.001–0.003% in 5–8 months. Chaudhury et al. (2007) recovered transgenic cowpea from cotyledonary nodes under kanamycin selection at a frequency of 0.76% in 5–6 months. However, the overall transformation efficiency in these protocols was much low, most likely due to (i) inefficient T-DNA delivery from *Agrobacterium tumefaciens* to the regenerating cells of embryo-derived explants; (ii) inefficient selection of proliferating transgenic cells and regenerated shoots by phosphinothricin and kanamycin; (iii) difficulty with regeneration of shoots from transformed cells under the growth regulator and tissue culture regime followed. Hence, enhancement of transformation efficiency is expected from the optimization of early gene transfer steps, use of efficient selection scheme, and tissue culture regime for maximal growth of transformants.

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We developed a highly efficient *A. tumefaciens*-mediated transformation protocol for production of fertile transgenic cowpea from cotyledonary nodes within 2 months at a much higher frequency of 1.64% by using additional virulence genes for efficient T-DNA delivery to regenerating cells, employing synergistic action of two different cytokinins for promotion of regeneration of proliferating transformed cells, and adapting a geneticin-based selection system for rapid selection of transgenic shoots with a very low escape frequency.

2. Materials and methods

2.1. 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.

2.2. 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 and 3 mm respectively from above and below the nodal region (Fig. 2a). 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) 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 were excised after 4 weeks of culture and transferred to MS medium supplemented with 2.5 μM IBA for rooting.

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 °C for 20 min. Cultures were maintained at 25 ± 2 °C under a 16-h photoperiod provided by cool white fluorescent lights (35 μmol m^{-2} s^{-1}). 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. Plantlets were then transferred to pots containing soil:compost (1:1), grown to maturity and the seeds were collected.

2.3. Establishment of selection system

The explants were cultured on SIM containing different concentrations of three aminoglycoside antibiotics, i.e., kanamycin (50–150 mg/l), geneticin (15–75 mg/l) or paromomycin (25–125 mg/l) for determination of their threshold concentration for the selection of transformed shoots. Explants cultured on SIM in absence of any antibiotics served as control. The selection scheme, i.e., choice of antibiotic and its optimal concentration, for the selection of transformed shoots were based on those that allowed initial proliferation of shoot buds, but bleached them afterwards. The observations were scored after 3–4 weeks on selection media.

2.4. Agrobacterium strains and transformation vector

Four *A. 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 were compared for their efficiency through early detection of transient transformation events in explants. 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. 1). The binary vector was mobilized to each of four *A. tumefaciens* strains by triparental mating (Bevan, 1984). 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 pTOK 233 (Hiei et al., 1994) and contains a tetracycline resistance gene as selection marker. 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.

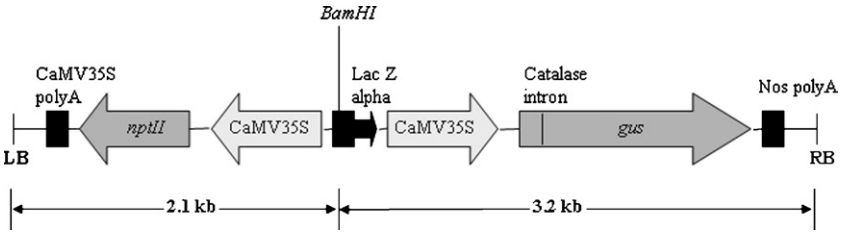

Fig. 1. T-DNA region (5.3 kb) of binary vector pCAMBIA2301. Abbreviations: RB, right border; LB, left border; 35S, CaMV 35S promoter or terminator; NOS, nopaline synthase terminator; intron-*gus*, intron interrupted β-glucuronidase; *npt*II, neomycin phosphotransferase. Also highlighted are the positions of unique BamHI restriction site, and the distance between BamHI site and LB (2.1 kb).

2.5. Bacterial inoculation and co-cultivation

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 °C. 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 co-cultivation medium, LCM (MSB medium containing 1 μM BAP, pH adjusted to 5.5) supplemented with 100 μM acetosyringone for inoculation. The 3-day-old cotyledonary node explants were inoculated in bacterial suspension for 30 min with occasional shaking. The explants were then blotted on sterile filter paper and co-cultivated in petri dishes lined with filter paper moistened with LCM supplemented with 100 μM acetosyringone for 3 days under 16 h photoperiod at 22 °C.

2.6. Selection and regeneration of transformants

Following co-cultivation, the explants were washed three to four times with LCM 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 greenhouse. The T_0 seeds were collected and sown in soil to raise the T_1 plants. Seeds were collected from T_1 plants.

2.7. Histochemical analysis of transient and stable gus expression

Histochemical GUS assays (Jefferson, 1987) were used to assess transient and stable expression of the *gus* gene. Transient *gus* expression in explants was scored after 3 days co-cultivation with different *A. tumefaciens* strains. Stable *gus* expression in geneticin-resistant T_0 transgenic plants was checked by immersing the leaves, flowers, perianth, ovules, stigma, stamen and pollen grains in GUS substrate solution for 24 h at 37 °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.

2.8. 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 (Rogers 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′-CC ACC ATG ATA TTC GGCAAC-3′ and reverse 5′-GTGGAGAGGCTATTCGGCTA-3′, and *gus* gene: forward 5′-TAACCTTCAC CCGGTTGCCAGAGG-3′ and reverse 5′-CCTTAACTAAGC CGGAATCCATCG-3′. The amplification reaction was carried out with initial denaturation at 94 °C for 1 min and followed by 38 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min, and final extension for 7 min at 72 °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.

2.9. 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 BamHI. The distance of the left border from the BamHI site in the T-DNA was indicated in Fig. 1. 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 BamHI, 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).

2.10. 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 T0 transgenic plants, and non-transformed control plant 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 RNA, oligo(dT) primer, and the M-MuLV Reverse Transcriptase, according manufacturers' instructions. 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.

2.11. Analysis of transgene inheritance

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

3. Results and discussion

3.1. Establishment of an efficient plant regeneration system

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 1). 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 1). BAP alone or in combination with other plant growth regulators has been reported to favorably induce multiple shoot induction from diverse explants of cowpea (Pellegrineschi, 1997; Mao et al., 2006). However, the mean shoot length declined with increase in BAP concentrations (Table 1) 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

Table 1
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 2
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.

often been reported to stimulate shoot proliferation while inhibiting shoot elongation (Figueiredo et al., 2001). 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 as GA_3 containing medium induced non-uniform elongation and moreover, the elongated shoots were found thin and lanky, unsuitable for rooting (Table 2). 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 3; Fig. 2b) 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. The 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. Among the eight commercially important cultivars compared for their shoot regeneration potential (data not shown), Pusa Komal produced maximum shoots (9.1 shoots/explant) in 94% of the cultures. Formation of roots was observed in 100% of shoots transferred to MS medium containing 2.5 μM IBA (Fig. 2c). 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. 2d). The plants were apparently normal and showed no variation with respect to their morphology and growth characteristics.

3.2. Selection

The three aminoglycoside antibiotics exerted varied effects on the regeneration of the explants. Kanamycin up to 100 mg/l permitted regeneration of healthy and green shoots while visually impaired growth at 150 mg/l (Table 4). Higher concentrations of

Table 3
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]

Mean followed by different alphabets in the same column differ significantly ($P < 0.05$) by Newman–Keul's multiple range test.
[a] Kinetin was supplemented to MSB medium containing 5.0 μM BAP after varying interval of culture.

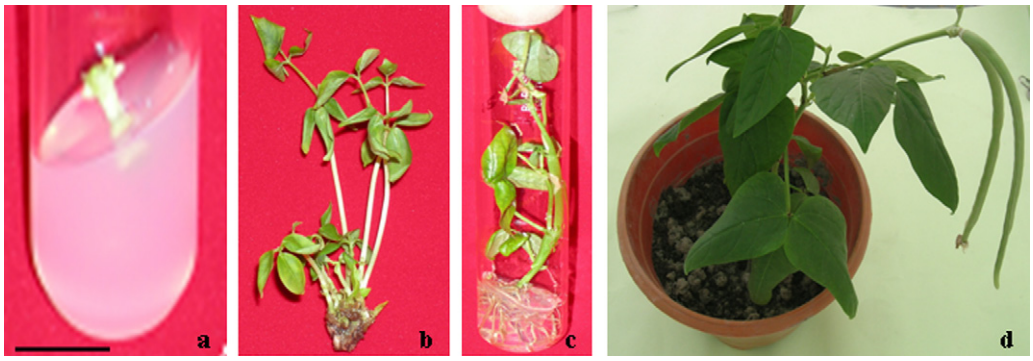

Fig. 2. (a–d) *In vitro* multiple shoot proliferation and plant regeneration of *Vigna unguiculata* cv. Pusa-Komal. (a) Cotyledonary node excised from 3-day-old seedlings raised on MSB medium containing 10 μM BAP. Bar represents 5 cm. (b) Direct multiple shoot regeneration from cotyledonary node explant 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.

kanamycin were too toxic to allow host cells to survive long enough for *Agrobacterium* infection to occur, and to undergo division thereafter. The cotyledonary node explants showed high degree of resistance to kanamycin (Popelka et al., 2006), which prolonged the duration for selection of transformants in cowpea (Chaudhury et al., 2007). Paromomycin up to 100 mg/l allowed shoot regeneration while drastically reducing the regeneration frequency. Both kanamycin and paromomycin induced necrosis of the explants to a varying degree interfering with the regeneration process (Table 4). 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 4) without any symptoms of necrosis in cultured explants. Efficient demarcation between transformants and non-transformants at low concentration of geneticin (45 mg/l) with negligible interference on regeneration of genuine transformed sectors, presented a quick and effective scheme for selection of transformants in our study, making the genetic modification of cowpea a commercially viable endeavor.

3.3. Effect of additional virulent genes

Among the five *A. tumefaciens* strains tested, EHA105 was most effective with higher transient transformation efficiency (76%) followed by LBA4404 (64%), AGL1 (61%) and GV2260 (23%). However, 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. 3). The constitutive expression of extra copies of *vir* genes in moderately virulent strain LBA4404 lead to 56% increase in transformation efficiency as compared to their absence, and 31% increase as compared to hypervirulent strain EHA105, which clearly demonstrate the role of extra copies of *vir* genes in enhancing the transformation efficiency in cowpea. 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).

Table 4
Effect of different aminoglycoside antibiotics on regeneration of 3-day-old cotyledonary node explants

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]

Explants cultured on MSB medium supplemented with 5.0 μM BAP for 1 week followed by 3 weeks of culture on MSB medium supplemented with 5.0 μM BAP and 0.5 μM kinetin.

3.4. Regeneration of transgenic plants

All the explants showed GUS activity after co-cultivation with LBA4404/pSB1 harboring pCAMBIA2301, predominantly in the regenerating sites (Fig. 4a). In contrast, only 80% of the explants showed GUS activity following 2 days of co-cultivation with EHA105 (Chaudhury et al., 2007). 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

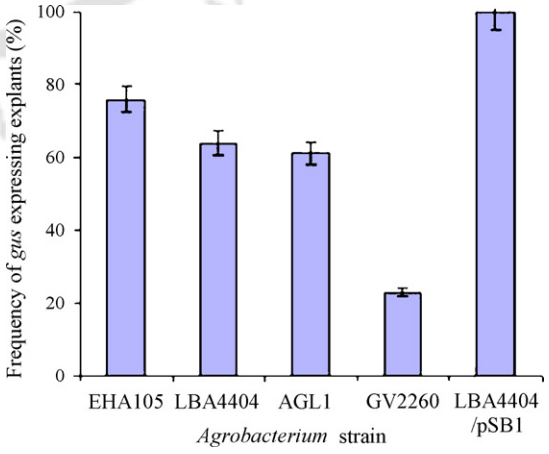

Fig. 3. Effects of *Agrobacterium* strains and additional *vir* genes (EHA105, LBA4404, AGL1, GV2260 and LBA4404/pSB1) on the transformation efficiency of cotyledonary node explants cowpea 3 days after co-cultivation. Three replicates for each treatment with at least 30 explants per replicate were used. Vertical bars represent the standard error.

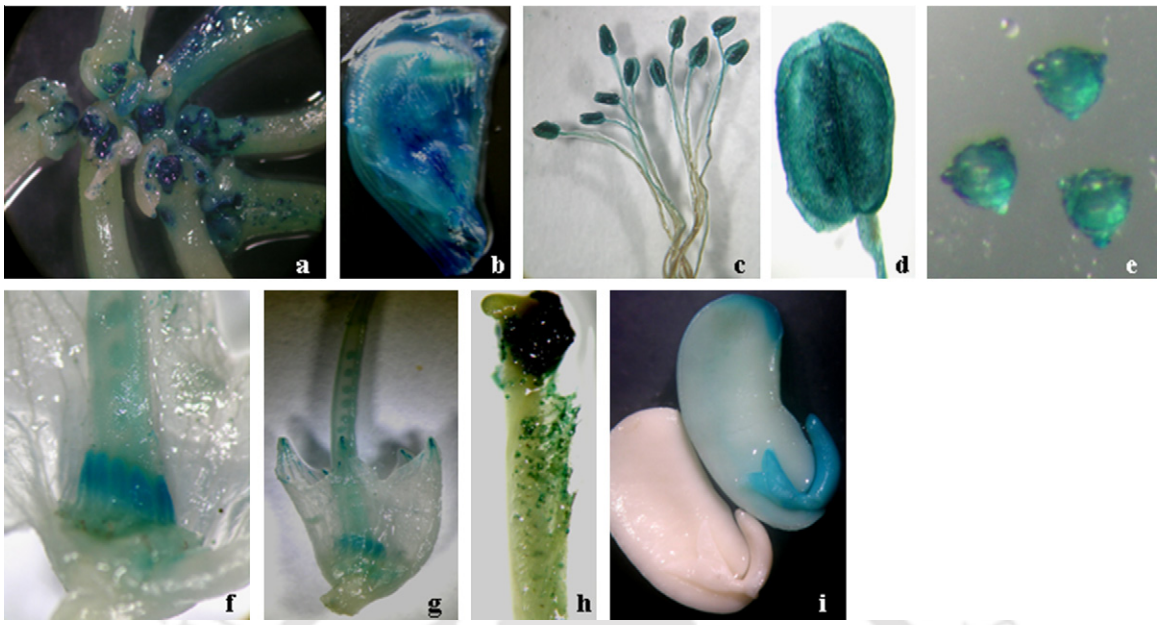

Fig. 4. (a–i) Transient and stable GUS activity. (a) Cotyledonary node explant showing transient GUS activity after 3 days of co-cultivation with *A. tumefaciens* LBA4404/pSB1pCAMBIA2301. (b–h) Stable GUS activity in geneticin-resistant T0 transgenic plants, (b) flower buds, (c) anthers, (d) anther, (e) pollens, (f) perianth, (g) ovules, and (h) stigma; (i) germinating cotyledon with attached embryo from non-transformed (bottom) and transformed (top) T1 plant.

different experiments, produced 46 shoots on geneticin selection medium (Table 5), out of which 29 shoots formed roots (63%). All the rooted plantlets produced seeds in greenhouse (Table 5). A strong, uniform and stable *gus* expression was observed in leaves, developing stamens, anthers, pollen grains, perianth, ovules and stigma of all the geneticin resistant T_0 plants while endogenous *gus* expression was absent in the tissues of control plants (Fig. 4b–h). The *gus* expression in T_1 seedlings clearly demonstrated inheritance and expression of the transgene in the progeny (Fig. 4i). The stable transformation efficiency was determined based on the percentage of T_0 plants that showed stable integration of transgene. The use of extra copies *vir* genes, compatible culture regime and geneticin based selection scheme dramatically enhanced stable transformation efficiency in cowpea to an average of 1.64%.

3.5. Molecular analysis of transgenics

PCR analysis detected the presence of the expected 540 and 240 bp fragments corresponding to *npt*II and *gus* genes respectively (Fig. 5a and b), in 25 out of the 29 geneticin-resistant transgenic plants demonstrating the efficiency of geneticin based selection scheme in elimination of escapes (Table 5). 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. Southern hybridization analysis revealed signals corresponding to junction fragments, having a defined portion of the T-DNA (BamHI site to close to the left border, i.e., 2.1 kb), and portions of plant DNA (Fig. 5c). The four independent transformed plants showed simple integration patterns with one to three copies of the 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. 5d) confirming the expression of the transgenes in the transgenic plants. The result verified the

Table 5
Summary of the transformation of 3-day-old cotyledonary node explants of *Vigna unguiculata* cv. Pusa-Komal co-cultivated with *Agrobacterium tumefaciens* strain LBA4404/pSB1 harboring a binary vector pCAMBIA2301

Explant 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 1 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.

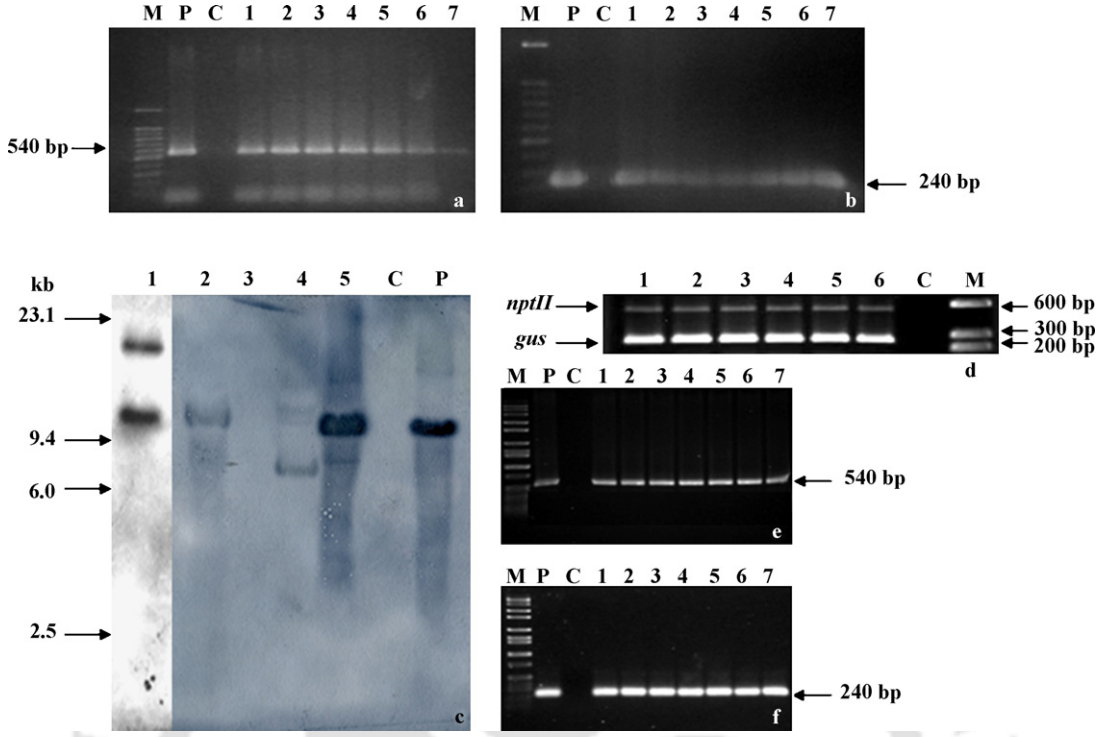

Fig. 5. (a–f) Molecular analysis of transgenic plants. (a–d) Analysis of geneticin-resistant T_0 transgenic plants, (a) PCR amplification of the 540-bp fragment of the *npt*II gene, (b) PCR amplification of the 240-bp fragment of the *gus* gene. Lane M: molecular weight marker; Lane P: pCAMBIA2301 plasmid 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 T_0 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 T_0 lines, lane C: genomic DNA from untransformed plant, lane P: plasmid DNA (pCAMBIA2301).

Table 6
Segregation of *npt*II and *gus* genes in T_1 transgenic plants of *Vigna unguiculata* cv. Pusa-Komal

T_1 Transgenic line	No. of plants tested	Positive (*npt*II)	Negative (*npt*II)	Ratio	χ^2	Positive (*gus*)	Negative (*gus*)	Ratio	χ^2
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

functional expression of the *npt*II and *gus* genes in the transgenic plants.

3.6. 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. 5e and f). A segregation ratio of 3:1 for both *npt*II and *gus* genes was observed in three lines whereas one line showed a segregation pattern of 15:1 for both genes (Table 6).

4. Conclusion

We demonstrated a dramatic increase in T-DNA delivery leading to 100% transient transformation frequency 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 1 week of culture, and 3 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 2 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.

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