

STUDIES ON GENETIC TRANSFORMATION IN COTTON (*Gossypium hirsutum* L.)

Thesis submitted to the
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Degree of
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in
GENETICS AND PLANT BREEDING

By
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IN COTTON (*Gossypium hirsutum* L.)**

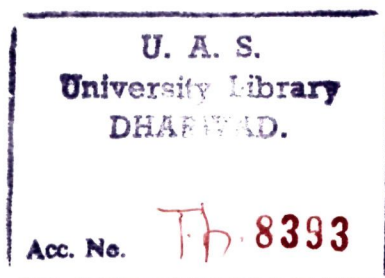
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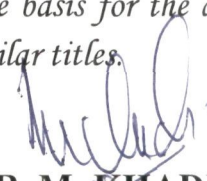


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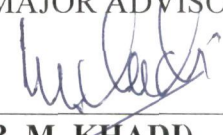
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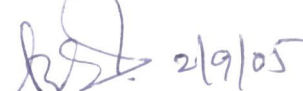

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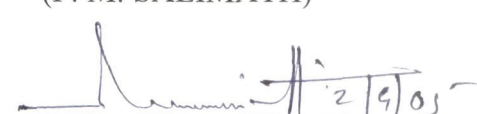
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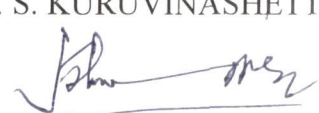
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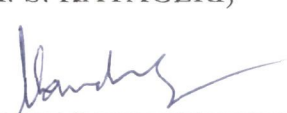
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Introduction

I . INTRODUCTION

Cotton is an important economic and fiber crop, grown in more than 60 countries in the world. This crop is of great commercial importance as it sustains livelihood of a large number of rural people through cultivation, picking and a large work force employed in both small and large-scale textile and other industrial units. Over 180 million people are associated with the fiber industries that produce 20 to 30 billion dollars worth of raw cotton. It is one of the most important cash crops in India, which plays a significant role in national economy. India has the distinction of having the largest cotton area of 8.9 m ha in the world across ten major states in India , with a production of 2.32 m bales. Thus the productivity is estimated to be 476 kg lint/ha (Anon, 2005). India is the third largest global cotton producer next to USA and China. Karnataka produces 9 lakh bales of cotton lint from an area of 0.533 m ha with a productivity of 287 kg/ha (Anon, 2005). The projected requirement by 2050 A.D. is placed around 282 lakh bales of cotton (Narayanan *et. al.*, 1997). The most significant constraint in cotton cultivation is the insect pests, which cause substantial yield losses. About 162 species of insects are known to attack cotton crop at various stages of growth, 15 of which are important. Several studies have shown that American bollworm, *Helicoverpa armigera* (Hubner) is the most prevalent and damaging pest of cotton. Other lepidopteron pests like pink bollworm, *Pectinophora gossypiella* (Saunders) and spotted bollworm *Earias vitella* (F.) also cause extensive damage. *H.armigera* being polyphagous insects, they are present throughout the year in the field. Pest problems in cotton are rampant and have caused social calamity, predominantly suicide of farmers in India. Bt cotton gives at least some relief to farmers as it reduces losses due to bollworms and thus ensures reduction in the cost of plant protection. It is reported that, in India, an estimated 0.509 billion-dollar worth of pesticides are used in cotton bollworm control alone. Instead the latest practice is to use seed as a carrier of plant protection either by external seed treatment or by breeding for pest tolerance.

Insect pest management by chemical pesticides has brought about considerable protection to crop yields over the past five decades. Unfortunately, extensive and very often indiscriminate use of chemical pesticides has resulted in environmental degradation, adverse effects on human health and other organisms and eradication of beneficial insects. Certain morphological features can serve as a hindrance to the easy multiplication of pests. Genetic control of desirable features has been established. Some of which provide a strong defense, while others may help in minimizing the multiplication of pests. The conflicting plant features desired for resistance to bollworms and jassids makes it difficult to obtain pest resistant genotypes in cotton. Increasing the density of trichomes will increase the tolerance to jassids (Tidke and Sane, 1962; Yadav *et. al.*, 1967) but make the plants more susceptible to bollworms as it attracts female moths for egg laying (Kadapa *et. al.*, 1983). There being no source of resistance available against bollworms, the breeder has to develop genotypes that are early with synchronous flowering and fruiting to mitigate the losses due to the pest by escape mechanism. Some morphological characters *viz.*, frego bract, coloured plant body, nectorilessness, thickness of bolls and bio-chemical factors are said to confer tolerance to boll worms, but attempts to obtain pest resistant genotypes of cotton by conventional breeding methods have not been successful because of incompatibility associated with pest resistant wild species. Genetically engineered resistance has been actively investigated in recent years as an alternative for genes from wild relatives.

Tools of molecular biology and genetic engineering have provided human kind with unprecedented power to manipulate and develop novel crop genotypes toward a safe and sustainable agriculture in the 21st century. As one enters the new millennium with the objective of achieving higher and stable crop productivity, it is imperative to apply safe and environment friendly strategies. Insect pest management in an eco-friendly manner is no longer a dream. Tools of molecular biology and genetic engineering can facilitate

harnessing and deployment of these molecules in crop plants in a safe and sustainable fashion.

Engineering of plant species to acquire novel traits involves the production of foreign genes into plant genome and expression of these transgenes to the desirable extent. It encompasses the techniques to transfer the genes to plant chromosomes and regulate their expression by employing suitable promoters. Various methods have been devised to introduce foreign genes into plants. Among the various transformation methods available *Agrobacterium tumefaciens* mediated transformation is the most popular, especially for the dicots.

The development of a method to obtain transformants, which is independent of problems inherent to tissue culture procedures, has been the ultimate goal of plant breeders. The alternative method, which involves *Agrobacterium*, but does not require tissue-culture steps, is *in-planta transformation*.

Bacillus thuringiensis (Bt) is a gram positive, aerobic, sporulating bacterium, which synthesises crystalline proteins that are highly insecticidal at very low concentrations. These proteins called δ -endotoxins are highly toxic to lepidopteron, dipteran and coleopteran insects. With the advent of genetic engineering techniques, it is now possible to transfer useful genes from other species / organisms. Genes responsible for these crystalline proteins (Cry) have been identified and cloned to transfer into plants. These proteins are harmless to human beings, other vertebrates and also to non-target insects.

Agrobacterium tumefaciens a gram negative soil dwelling micro-organism that causes crown gall disease in many species of dicotyledonous plants with its disarmed T_i plasmid can be used as a vector to transfer genes into plant cells without altering their capacity to regenerate whole plants *in vitro*. Among the methods available for gene

transformation, *Agrobacterium* mediated gene transfer is the most reliable especially in dicots. Most protocols involve a tissue culture step. But tissue culture response is highly genotype dependent and Indian cultivars of cotton are shy for regeneration. Apart from genotype dependent response, the other problems that circumvent somatic embryo genesis are prolonged culture period, low frequency of embryo maturation, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets and lack of shoot elongation. So genotype independent shoot apex plant regeneration method was originated for cotton (Gould and Smith, 1988). Use of *Agrobacterium* as a vector is technically simple and gene transfers are often low copy, permanent and heritable. Therefore *Agrobacterium* mediated transformation procedures are those that often minimize or eliminate the tissue culture component would be advantageous under such circumstances. With these points in view, present investigations were carried out with the following objectives:

- To study the *Agrobacterium* mediated transformation efficiency through meristem culture .
- To study the transformation possibilities through Pollen Tube Pathway (PTP) .
- To transfer genes encoding Cry proteins to *Gossypium hirsutum* genotypes.

Review of Literature

II. REVIEW OF LITERATURE

Plant genetic engineering involves techniques and approaches to introduce foreign genes into plants. This requires availability of suitable procedures to regenerate plant species of interest *in vitro*. Advances made in tissue culture, have led to the development of techniques to regenerate a wide range of plant species (Birch 1997). Plant transformation is a key methodology that has fostered diverse forms of scientific inquiry and technology development. Insect pests are the major scourge of agriculture. Resistance to these pests may be successfully engineered by exploiting insecticidal proteins. In the present study, an attempt has been made to regenerate transgenic cotton plants conferring resistance to bollworms. Transformation methods that employ *in vitro* techniques can be labour intensive and are plagued by low success rates, excessive somoclonal variation and chimeras. Attempts were also made to obtain the transformed plants through *in planta* transformation methods. Relevant literature on these aspects is reviewed in this chapter.

The development of efficient transformation techniques has catalyzed rapid progress in plant genetic engineering. Among the various methods developed to introduce foreign genes into plants, *Agrobacterium* mediated transformation, electroporation and biolistics have potential application. Mechanical transformation methods such as microinjection, sonication are also adopted.

2.1 *Agrobacterium tumefaciens* – nature's smallest genetic engineer

Although no naturally occurring plasmids are known in higher plants, bacterial plasmid, the T_i plasmid of *Agrobacterium tumefaciens* is of great importance. *A. tumefaciens* is a soil micro-organism that causes crown gall disease in many species of dicotyledonous plants. Crown gall occurs when the bacteria invade through the wound and in the stem allows *A.tumefaciens* bacteria to invade the plant and cause cancerous proliferation of cells. The ability to cause crown gall disease is associated with the

oncogenes present on tumour inducing(T_i) in the bacterium. Remarkably T_i-plasmid after infection, part of the T_i- plasmid is integrated into the plant chromosomal DNA. Engineered T_i-plasmid is now used to transport desired genes into the plant cells. All that is necessary would be to insert the new genes into the T-DNA and then the bacterium could do the hard work of integrating them into the plant chromosomal DNA. The main problem is of course that a unique restriction site is an impossibility with a plasmid 200kb in size. Novel strategies have to be developed for inserting new DNA into the plasmid. Two general strategies, currently being used are, The binary vector strategy and The co integration strategy. The former is based on the observation that the T-DNA does not need to be physically attached to the rest of the T_i-plasmid. A two plasmid system, with the T-DNA on a relatively small molecule, and the rest of the plasmid in normal form, is just as effective as transforming plant cells. In fact some strains of *A.tumefaciens*, and related *Agrobacteria*, have natural binary plasmid systems. The T-DNA plasmid is small enough to have a unique restriction site and to be manipulated using standard techniques. While the latter strategy uses an entirely new plasmid, based on PBR-322 or a similar *E.coli* vector but carrying a small portion of the T-DNA. The homology between the new molecule and the T_i-plasmid means that if both are present in the same *A.tumefaciens* cell, recombination can integrate the PBR-plasmid, into the T -DNA region. The gene to be cloned is therefore inserted into a unique restriction site. On the small PBR plasmid, introduced into *A.tumefaciens* cells carrying a T_i-plasmid, and the natural recombination process left to integrate the new gene into the T-DNA. Infection of the plant leads to insertion of the new gene, along with the rest of the T-DNA into the plant chromosomes.

If *A.tumefaciens* bacteria that contain an engineered T_i-plasmid are introduced into a plant in the natural way, by infection of a wound in the stem, then only the cells in the resulting crown gall will possess the cloned gene. This is of little value to the

biotechnologist. Instead a way of introducing the new gene into every cell in the plant is needed.

A number of disarmed Ti cloning vectors are now available, a typical example being the binary vector PBIN-19. Other disarmed strain of *Agrobacterium* are PLBA 4404 (Hoekema *et al.*, 1983), PGV 3850 (Zambrysky *et al.*, 1983), pEHA 101 (Hood *et al.*, 1986). The left and right T-DNA borders present in this vector flank a copy of the lac z gene containing a number of cloning sites, and a kanamycin resistance gene that functions after integration of the vector sequences into the plant chromosome. The initial manipulation that result in insertion of the gene to be cloned into pBIN 19 are carried out in *E.Coli*, the correct recombinant molecule then being transferred to *A.tumefaciens* and hence into the plant. Transformed plant cells are selected by planting onto agar medium containing kanamycin.

Although many different techniques have been tested for gene delivery in to plant cells, *Agrobacterium* mediated transformation has been extensively employed. The first transgenic plants of *Nicotiana tabaccum* were produced via *Agrobacterium* mediated transformation (Horsch *et al.*, 1984). With this success many crop plants were transformed via *Agrobacterium*. This is the most simple method now available for transferring genes into intact plant tissue with host range being its limitation. But it is now possible with the addition of acetosyringone.

2.1.1 *Agrobacterium* mediated *in vitro* transformation

Gynheung (1985) has reported that tobacco calli were transformed at levels upto 50per cent by co-cultivation of tobacco cultured cells with *Agrobacterium tumefaciens* harbouring the binary transfer DNA vector, PGA-472, containing a Kanamycin resistance marker. Transformation frequency was dependent on the physiological state of the tobacco cells, the nature of agrobacterium strain and, less so on the expression of vir genes of the

tumour inducing plasmid. Maximum transformation frequency was obtained with exponentially growing plant cells, suggesting that rapid growth of plant cells is an essential factor for efficient transformation of higher plants.

Firoozabady *et al.* (1987) have efficiently transformed and regenerated cotton cotyledon tissues. Transformation was confirmed by opine production, kanamycin resistance, immunoassay and DNA blot hybridization. This process for producing transgenic cotton plants facilitates transfer of genes of economic importance to cotton.

Chee *et al.* (1989) have confirmed that about 0.01 per cent of the total seeds infected with *Agrobacterium tumefaciens* harbouring npt-II gene have shown transformation in case of germinating seeds of *Glycine max*.

Potrykus, (1990) has reported that the shoot apical meristem of plants generates the whole green part of the plant body, including the flowers, thus it is a very important tissue in the developmental biology of plant. In particular, the meristem is a tissue with a very high regeneration potential. Gene transfer to meristem cells could therefore, overcome many of the regeneration problems due to difficult tissue culture systems in many important crop species.

Medford (1992) has reported that the meristem is often confused with the complete shoot apex, which also contains the leaf primordia and the young leaves. Furthermore, the meristem as a tissue may represent a complicated pattern of cells. Each of these cells may differ physiologically due to its unique position in the meristem.

Dillen *et al.* (1997) indicated that temperature plays an important role in transformation with *A. tumefaciens*. They observed the best transformation efficiency at 22^o C in both *phaseolus acutifolius* callus and tobacco leaves, irrespective of the type of helper plasmids. Although 25^o C is commonly employed for *in vitro* co-cultivation

experiments, they showed that a lower temperature (19-22⁰C) is more optimal because *in planta* tumour formation occurs more frequently at 22⁰ C.

Mahalaxmi and Khurana (1997) have studied the age and physiological status of the plant and reported that the meristematic tissues, explants from the young plants and cells undergoing dedifferentiation are the choicest material for *Agrobacterium* mediated transformation. The age and physiological status of the plant plays an important role during plant-microbial interactions. Usually, 3-4 days old seedlings were used for agro-infection in maize, wheat and other cereals than dry dissected seeds with exposed apical meristems. Immature embryos of maize were differentially susceptible to *Agrobacterium*; the best stage corresponded to 12-22 days after pollination when the two leaf initials were formed, indicating a specific window of competence in the host plant. It was also observed that leaves were more competent than roots, scutellum or seed remnants in maize, rice and in wheat and barley.

Cervera *et al.* (1998) and Cao *et al.* (1998) have suggested that low efficiency of transformation was due to an insufficient length of co-cultivation, however they have also reported that it was more difficult to eliminate *Agrobacterium* after longer periods of co-cultivation. Further they have also reported that the 5 day culture period resulted in over growth of *Agrobacterium* and a subsequent decrease in the regeneration frequency of transformed shoots, although 5 day co-cultivation was the most effective for increasing the frequency of transient GUS-expression in citrange explants. Therefore the period of co-cultivation should be optimized.

Gould and Cedeno, (1998) have presented a protocol for rapid genotype independent transformation and regeneration of cotton (*Gossypium* spp.) from shoots isolated from germinating seedlings. They have inoculated the isolated shoots with a super virulent strain of *Agrobacterium tumefaciens*, subjected them to a mild antibiotic selection

and directly regenerated as shoots *in vitro*. They have reported that by this method the shoots do not dedifferentiate and mutation rates are low. Rooted shoots can be obtained within 6-10 weeks of isolation and inoculation depending on the cotton cultivar.

Hemphill *et al.* (1998) have reported a rapid clonal propagation system developed to regenerate mature (*G. hirsutum* L.) plants from pre-existing meristems from *in vitro* grown tissues. Shoot apices, lateral node and cotyledonary, nodes were co-cultivated with *Agrobacterium tumefaciens* (Plus PBI 121) and then regenerated (Plus kanamycin) to a two leaf stage by this *in vitro* culture system(s). They further report that, this procedure has generated 121 developing kanamycin selected shoots and 40 mature viable plants, which produced viable T₁ seeds. Shoot elongation in the presence of kanamycin (37 to 50 to 75 µg/ml) represented the first screening procedure to identify putative transgenic plants. They have also measured, β-glucuronidase (GUS-activity) fluorometrically in leaf homogenates of some but not all kanamycin selected T₀ plants. Also, GUS-activity was localized histochemically in the pollen, pollen sacs and or pollen tubes from many of these putative transgenic T₀ plants. The expression of GUS activity in T₀ pollen grains suggested that the transgene was germline stable. More-over, some plants which are negative for GUS-activity in leaf assays produced GUS-positive pollen, suggesting, in part, that these T₀ plants possessed a chimeric growth pattern and/or demonstrated transgene variability for GUS expression. Transgenic T₁ seeds derived from each T₀ boll were tentatively identified by a second kanamycin-selection procedure. Seeds (T₀ progeny) within each boll and different bolls of the same T₀ plant showed different seed germination patterns which also suggest that these T₀ plants were chimeric. Mature T₁ plants expressed GUS-activity in pollen grains which suggested that the transgene was inherited by progeny from parent. Ultimately, the incorporation of the GUS transgene into both T₀ and its corresponding inheritance in T₁ plants was substantiated by southern blot analysis. Collectively, their results demonstrated that the selectable marker (npt-II) and

reporter (uid.A) genes were incorporated directly into different cotton cultivars (Paymaster HS-26, Stoneville 7A and CA 3076) by a new procedure that offers substantial improvements (simplicity, flexibility and efficiency) over existing methodologies.

Moralejo *et al.* (1998) have described a procedure for genetic transformation of *Eucalyptus globulus* (Labill) and they have studied the influence of explant pre-cultivation and reported that when seedlings were pre-cultivated for 4-6 days, the level of GUS transient expression was significantly greater than that of control (i.e. without pre-culture) and that the seedlings pre-cultured for 6 days seemed to be more suitable for stable integration of transgenes. They further reported that the improvement of DNA uptake could be due to stimulation of cell division by the hormones in the pre-cultivation medium, since mitotic cells would be more susceptible to *Agrobacterium* or would have a higher level of transcription. However, they also opine that the physiological status optimal for DNA uptake (transient expression) is not necessarily optimal for integration of foreign DNA in the host genome. Infact, division of cells would not be sufficient for transformation, and integration may primarily depend on the availability of DNA repair enzymes. These proteins, although not yet identified are thought to play a key role in the last steps of T-DNA integration (Tinland 1996).

Wang *et al.* (1998) have constructed a plant expression vector, pBINLK, carrying two insecticidal genes: pea lectin gene and soybean Kunitz trypsin inhibitor gene and successfully transferred into upland cotton cultivars, via *Agrobacterium* mediated transformation, Hypocotyl segments from aseptic seedlings were used as transgene recipients. After co-cultivation, kanamycin resistant calli were screened, and somatic embryos and regenerated plants were obtained using various media. Transgenic cotton plants with two insecticidal genes were confirmed by ELISA, PCR and southern analysis. Bioassays demonstrated that the transgenic plants had significant resistance to larvae of cotton bollworm.

Donaldson and Simmonds (2000) have reported the overall rate of *in vitro* transformation for co-cultural explants placed on selection media ranged from 27-92 per cent depending on the cultivar. However transformation was predominantly confined to non-regenerable hypocotyl callus or other non-regenerable tissue, in regeneration, competent tissue of the cotyledonary node or in differentiated tissue was rare.

Satyavathi *et al.* (2002) have given a protocol for consistent production of transgenic cotton plants in three Indian varieties established utilizing *Agrobacterium* mediated transformation. Shoot tip explants were transformed by co-cultivation with *Agrobacterium tumefaciens* strain LBA 4404. The strain harbors a binary vector pBAL₂ carrying the reporter gene β -glucuronidase intron (GUS-INT) and the marker gene-neomycin phosphotransferase (NPT-II). Regeneration potential of explants or different hormones was studied in detail. Among the different combinations of BAP and NAA tested, 0.1 mg per ml of BAP and NAA in the medium influenced efficient regeneration of shoots by organogenesis. Shoot bud proliferation and elongation was achieved in 3-4 weeks time on medium supplemented with GA₃. The putatively transformed shoots were harvested and placed for rooting on medium containing IBA and 75 mg/l kanamycin. Transgenic plants were recovered in 12-16 weeks from the time of gene transfer to establishment in pots. Molecular analysis of the field established plant lets was carried to confirm the transgenic nature. The presence of *gus* and *npt- II* genes in the transgenic plants was verified by histochemical GUS assay and polymerase chain reaction (PCR) analysis, respectively. Integration of T-DNA into the genome of putative transgenics was further confirmed by southern blot analysis. A total of 70-75 transgenic plants were raised in pots. Progeny analysis of these plants showed a classical mendelian pattern of inheritance.

2.1.1(a) Elimination of *Agrobacterium tumefaciens*

One of the major problems encountered during plant tissue transformation with *A. tumefaciens*, is the effective elimination of the *Agrobacterium* after transfer of genetic information has taken place. If *A. tumefaciens* is not eliminated, it will overgrow the tissue and affect the further growth of explants. Antibiotics are used for this purpose, but the levels of antibiotics needed to kill the organism may have deleterious effects on the regeneration. So the level of antibiotic requirement is to be standardized for individual crops.

Although many antibiotics have been described for the effective elimination of *Agrobacterium* and cefotaxime have minimal toxicity on most of the tissues and efficiently eliminate *Agrobacterium* cells (Pollock et.al.,1983). However this antibiotic showed plant hormone like activity on plant tissues, especially when the plant tissues were grown in a medium containing low concentration of carbenicillin or cefotaxime.

2.1.2 Improvement of efficiency of *Agrobacterium* mediated transformation

The frequency of *Agrobacterium* mediated transformation is very low. Recently it has been investigated that the efficiency of transformation can be enhanced by supplementing with other methods such as microprojectile bombardment, preculture of the explants, ultrasound mediated transformation, sonication assisted *Agrobacterium* transformation (SAAT) techniques, influence of macerating enzymes etc.

Bidney *et al.* (1992) have reported that bombardment of plant tissues with microprojectiles is an effective method of wounding to promote *Agrobacterium* mediated transformation. Tobacco cultivar Xanthi leaves and sunflower apical meristems were subjected to microprojectile bombardment prior to application of *Agrobacterium tumefaciens* strains containing genes within the T-DNA encoding GUS or NPT-II. Stable

kanamycin resistant tobacco transformants were obtained using *npt-II* construct from particle / plasmid, particle wounded / *Agrobacterium* treated or scalpel-wounded / *Agrobacterium* treated potato leaves. Those leaves bombarded with particles suspended in TE buffer prior to *Agrobacterium* treatment produced atleast 100 times more kanamycin resistant colonies than leaves treated by the standard particle gun transformation protocol. In addition, large sectors of GUS expression, indicative of meristem cell-transformation, were observed in plants recovered from sunflower apical explants only when the meristems were wounded first by particle bombardment prior to *Agrobacterium* treatment. Similar results in two different tissue types suggest that, particles may be used as a wounding mechanism to enhance *Agrobacterium* transformation frequencies, and *Agrobacterium* mediation of stable transformation is more efficient than the analogous particle/ plasmid protocol.

Bombardment of the plant tissues with micro-projectiles promoted *Agrobacterium* mediated transformation in tobacco (Bidney *et al.*, 1992) and *Eucalyptus* (Moralejo *et al.*, 1998).

Preculturing of the explants on the regenerating medium prior to inoculation of *Agrobacterium* increased the frequency of transformants in pigeonpea to 62 per cent from cotyledonary node and 27 per cent from shoot tip (Geetha *et al.*, 1999) 28.8 per cent from callus and 1.2 per cent from shoots regenerated (Lawrence and Koundal, 2001), cowpea (Muthukumar *et al.*, 1996), chickpea (Kar *et al.*, 1996).

Weber *et al.* (2000) demonstrated that the concentration of enzyme plays an important role for the transformation efficiency. Although no influence on transient expression was observed. Pectinase and cellulase showed beneficial effects on stable GUS-expression at 0.05 and 0.1 per cent respectively, while macerozyme proved to be inefficient in all concentrations tested. They have reported enhancement of the frequency

of explants producing shoots with GUS expression applying cellulases of different origin. Substantial progress was achieved by applying a combination of cellulase and pectinase at optimum levels which held to a mean frequency of 5.7 per cent of explants carrying shoots expressing GUS uniformly and upto 13.6 per cent in one repetition, while no such shoots were observed in the non treated control. In addition, the percentage of explants regenerating shoots chimeric for GUS expression was elevated from 3.6 per cent in the control to 9.1 per cent in the explants treated with combination of cellulase and pectinase. The treatment of explants with ultra sound (SAAT, Sonication assisted Agrobacterium mediated transformation) was effective on the level of transient EFP expression as indicated by the mean number of spots per explant.

Ultrasound mediated transformation and sonication assisted Agrobacterium transformation (SAAT) techniques were introduced to enhance the efficiency of gene transfer in soybean (Trick and Finer, 1998; Santarem *et al.*, 1998), cowpea, water spruce, wheat, maize (Trick and Finer, 1997) and *Eucalyptus grandis* x *E.urophylla* hybrids (Gonzalez *et al.*, 2002). The application of ultrasound to plant tissues induces acoustic cavitation, which generates microscopic wounds, which favours exposure of internal plant tissue to *Agrobacterium* (Trick and Finer, 1997) and thus increases level of transient expression of T-DNA. The average transformation rate was 17.64 per cent in seeds of *Eucalyptus* hybrid with sonication and that of control was 9.16 per cent (Gonzalez *et al.*, 2002). Ishida *et al.* (1996) reported enhanced transformation in *Zea mays*, when modified vectors expressing *viz. genes* constitutively were used.

Likewise there are different techniques to enhance the efficiency of *Agrobacterium* mediated transformation, one can adopt appropriate technique, which is simple, economically feasible, less laborious, time saving and beneficial for a given crop species.

2.1.3 Hardening

Acclimatization or habituation of *in vitro* grown plantlets to the external condition is a gradual process. It is recommended that removal of nutrient media from the plantlets to be hardened, pre-conditioning to low relative humidity, high light intensity and high temperature can ensure higher survival during transfer of plantlets to natural conditions. The gradual removal of sugar is known to stimulate photosynthetic ability.

A mixture of Soil: Sand: Peat in 2:1:1 ratio can be used for successful hardening of plants. Sand provide better aeration, while soil gives good anchorage, nutrients and water holding capacity and peat along with soil adds to these properties.

Iradparah and Khosh (1989) acclimatised *in vitro* rooted plants in pasteurised 1/3 loam soil, 1/3 sand and 1/3 peat moss (v/v) medium for two weeks and then transplanted them to pots with the same mix in green house.

2.2 *Agrobacterium* mediated *in planta* transformation methods

Numerous gene transfer methods have been developed in the past two decades for a wide range of plant species (Christou, 1995; Birch, 1997). Most of these methods are tissue culture based, requiring regeneration of whole plants from transformed cells. The utility of the techniques greatly depends on the establishment of tissue culture procedures in the species (Birch, 1997).

Developing tissue culture independent genetic transformation systems is of great interest because such systems would avoid constraints imposed by genotype specificity in transformation and regeneration and eliminate tissue-culture induced genetic variation. In addition, transgenic plants would be produced inexpensively and rapidly. A few of the important *in planta* transformation techniques have been reviewed below.

Feldman and Marks (1987) have reported utility of germline transformation, which allows avoidance of tissue-culture and regeneration in the vacuum infiltration, seed transformation, in planta transformation and floral dip methods. With all of these methods, selection with antibiotic or herbicide is not applied to the *Agrobacterium* treated T₀ plant. Instead progeny seed are harvested and selection is applied to the resultant seedlings as they germinate.

Raful and Riazuddin (1992) have reported transformation protocol in *ex-vitro* conditions using wild strain of *Agrobacterium* and have done the screening through tumour formation in chickpea.

Bechtold *et al.* (2000) have shown that transformation events occurring prior to or early in floral development of the T₀ plant would be expected to give rise to identically transformed male and female gametophytes, which upon self fertilization could produce significant number of T₁ plants that are homozygous for the T-DNA insertion. The rarity of total absence of such lines indicates that the relevant transformation events usually occur during germline development after divergence of male and female gametophyte cell lineages, or possibly soon after fertilization of the T₁ embryo separate transformants from a single plant carry independent T-DNA insertions even in methods that apply *Agrobacterium* to T₀ seed early in the growth of the plant, again suggesting that transformation occurs late in floral development.

Bondt *et al.* (1994) have studied the factors influencing transfer of an intron-containing β -glucuronidase gene to apple leaf explants during early steps of an *Agrobacterium tumefaciens* mediated transformation procedure. The gene transfer procedure was evaluated by counting the number GUS expressing leaf zones immediately after co-cultivation, as well as by counting the number of GUS expressing calli developing on the explants after 6 weeks of post cultivation in the presence of 50 mg/l kanamycin. Of

three different tested disarmed *Agrobacterium tumefaciens* strains, EHA 101 (pEHA101) was the most effective for apple transformation. Cocultivation of leaf explants with *Agrobacterium tumefaciens* on a medium with a high cytokinin level was more conducive to gene transfer than cocultivation on media with high auxin concentrations. Pre-cultivation of leaf explants, prior to co-cultivation slightly increased the number of β -glucuronidase expressing zones measured immediately after co-cultivation, but it drastically decreased the number of transformed calli appearing on the explants 6 weeks after infection. Other factors examined were : *Agrobacterium* cell-density during infection, bacterial growth phase, nature of the carbon-source, explant age, and explant genotype.

Reproductive inflorescences were clipped off in *Arabidopsis*; *Agrobacterium* was applied to the center of the plant rosette, new inflorescence formed a few days later were again removed, *Agrobacterium* was reapplied and plants were then allowed to develop and set seed. Cheng *et al.* (1998) thus succeeded in generating transformed lines by using the inflorescence. Plants obtained from these seed were confirmed as transgenic through PCR.

Clough and Bent (1998) suggested that the success of floral dip-method, in which flowering plants are simply dipped into a solution of *Agrobacterium* can be attributed to the cellular targets which are present on the exterior of the plant. They further reported that the transformants are often derived at high frequency as high as 4 per cent of all T₁ seed.

Plants were vacuum infiltrated in a suspension of *Agrobacterium* carrying the PGK 35 tagging vector. The efficiency of transformation increased with addition of the surfactant silwet L-77 (0.005per cent v/v) to the agrobacterium suspension (Richardson *et al.*, 1998).

Desfeux *et al.* (2000) have reported that the floral-dip method for *Agrobacterium* mediated transformation of *Arabidopsis* allows efficient plant transformation without the

need of tissue culture. In manual out crossing experiments, application of *Agrobacterium tumefaciens* to pollen-donor plants did not produce any transformed progeny, whereas, application of *Agrobacterium* to pollen recipient plants yielded transformants at a rate of 0.48 per cent. Their results suggested that the ovules are the sites of productive transformation in the floral-dip method and further suggested that *Agrobacterium* must be delivered to the interior of the developing gynoecium prior to locule closure if efficient transformation is to be achieved.

Rohini and Rao (2000) have produced transgenic peanut plants by a tissue-culture independent *Agrobacterium tumefaciens* mediated transformation procedure. Embryo axes of mature seeds with one cotyledon excised were incubated on Murashige and Skoog (MS) gelled medium for 2 days, then pricked randomly with a sterile needle and infected by immersion in a suspension of *Agrobacterium* in Winan's AB medium that was added with wounded tobacco leaf extract. *Agrobacterium* strain LBA 4404 harboring the binary vector pKIWI 105 that carries the genes for β -glucuronidase (GUS) and neomycin phosphotransferase (NPT-II) was used for transformation. Following a 16 hour infection, 18 hour decontamination with cefotaxime and germination and growth on soilrite for 16 days under growth room conditions, the seedlings were transferred to greenhouse. They have reported about 3.3 per cent of the seedlings to be GUS positive as determined by histochemical assay and by PCR-analysis. Further they have done the molecular characterization of primary transformants as well as T₁ and T₂ generation plants and shown that the method ensured insertion, expression and inheritance of foreign genes in peanut.

In *Medicago truncatula*, there was an improvement in transformation efficiency by *Agrobacterium* vacuum infiltration. Transformation rate ranged from 2.9 to 7.6 per cent of all seeds tested (Trieu *et al.*, 2000).

Vigorous plants with first siliques appearing and starting to form secondary floral stems were used for infiltration treatments in *Arabidopsis*. The treated plants were immediately planted in small individual pots. Seeds were collected from two plants and grown on selection media for selection of transformants. GUS-expression was seen in root, meristem, anther, pollen and immature embryos (Reddy *et al.*, 2002)

2.3 *cry* GENES

Major crops such as rice, cotton and pigeon pea are devastated by lepidopteron pests on regular basis, which necessitates use of large amounts of pesticides to manage the pests. An effective and safe strategy for insect pest management is to deploy insecticidal protein genes in transgenic crops. Since the first *cryIAC* Bt cotton variety was commercialized there have been numerous advancements for insect control with transgenic technology. Since the insects are developing resistance against the commercially available transgenic cotton with *cryIAC* gene, it necessitates the development of new transgenics with other genes encoding bt-toxins to delay the development of resistance.

Armes *et al.* (1996) have reported the resistance to insecticides in Indian cotton ecosystem. Further there are expectations that change in the pest complex due to single toxin protein.

Adamczyk and Gore (2004) have investigated the efficacy of transgenic cotton genotypes containing *cryIAC*, *cryIF* and *cryIAC* stacked with *cryIF* against beet armyworms and fall army worm in the laboratory bioassays and small experimental field plots and observed that cotton containing *cryIF* was more toxic than cotton containing only *cryIAC*.

Dogre *et al.* (2004), have developed an insect resistant cotton with cry1Aa₃ gene and their insect bioassay studies have revealed encouraging results with reference to gene expression studies using ELISA.

2.4 IDENTIFICATION OF TRANSFORMANTS

2.4.1 Selectable marker gene

A selectable marker gene is added to the gene construct in order to identify cells or tissues that have successfully integrated the transgene. This is necessary because, achieving incorporation and expression of transgenes in plant cells is an event, occurring in just a few percent of the targeted tissues or cells. Selectable marker genes encode proteins that provide resistance to agents that are non-toxic to plants, such as an antibiotics or herbicides. A suitable marker gene allows the preferential growth of transformed cells in the presence of the corresponding selective agent. Many antibiotics and herbicide genes are used as selectable markers. Selection efficiency depends on the size and developmental state of the plant cells, regeneration response and concentration of the selective agent.

Kanamycin resistance is the most widely used selection for higher plant transformation. The gene *npt-II* conferring resistance to the aminoglycoside antibiotics, such as kanamycin, was first established as useful dominant selectable marker for higher plants in 1983 (Bevans *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983). Since then its usefulness has been demonstrated with a broad group of plants (Steinbiss and Davidson, 1989). Kanamycin resistance is conferred by transgenic expression of neomycin phosphotransferase, the product of the *npt-II* gene from the bacterial transposon Tn₅. The enzyme neomycin phosphotransferase transfers a phosphate from ATP to the aminoglycoside and thereby inactivates it.

The neomycin phosphotransferase type-II (*npt-II*) gene has been used in transformation of more plant species than any other selectable marker. The original coding

sequence of npt-II was from bacterial transposon, Tn₅ Npt₅-II provides resistance to certain aminoglycoside antibiotics such as kanamycin, paramomycin and geneticin, but kanamycin is the most widely used. NPT-II coding sequence has been fused to constitutively regulated promoters such as nopaline synthase (nos) and cauliflower mosaic virus (CaMV) 35 S regulatory sequence (Fraley *et al.*, 1983) and later cloned into plant transformation and expression vectors by many researchers (An *et al.*, 1985, Bevan, 1984, Klee and Rogers, 1987).

Kanamycin has proven to be the most widely applicable selective agent but the concentration is species specific. Species like *Lycopersicum esculentum* (Fillatti *et al.*, 1987) *Brassica napus* (Moloney *et al.*, 1989) are selected at low concentration of kanamycin (15-100 mg/l) whereas *Beta vulgaris* needs relatively higher concentration of kanamycin (400 mg/l).

Neomycin phospho transferase-II, which is known to confer kanamycin resistance and a gene of bacterial origin of β -glucuronidase, which is commonly used as a reporter gene have been transferred to tobacco by leaf disc transformation using strain LBA 4404 of *A. tumefaciens*, which carries a binary vector (Oktem *et al.*, 1994).

Ming Cheng *et al.* (1996) have reported that selection on kanamycin (150 mg/l) facilitated the screening of regenerants and resulted in a relatively smaller population of putative transformants for further evaluation. Further indicated that all GUS-positive shoots rooted in rooting medium containing 150 mg/l kanamycin and the frequency of transformed fertile plants was 0.2 - 0.3 per cent of the explants inoculated.

They have further reported that a major obstacle to cotton transformation has been the optimization of methods for using a selectable marker such as kanamycin resistance. In a system such as tobacco, transformed tissue at any stage of development can be selected on high levels of kanamycin (up to 1000 mg/l.) while in cotton such levels are toxic.

De Neve *et al.* (1997) have reported that host factors are required for T-DNA integration. These authors have employed the root explant transformation procedure, which involves the treatment of sterilized *Arabidopsis* root section for transformed calli induced by transfer of a T-DNA carrying a selectable marker such as kanamycin resistance. Stable transformation was measured as the stable integration and somatic inheritance of neomycin phosphotransferase gene present on the engineered T-DNA. They further report that the protocol has an inherently high rate of variation from one explant to another.

Hemphill *et al.* (1998) have excised shoot apices from 1 day-old seedlings and demonstrated three different phenotypes during the kanamycin selection procedure. The 'green' phenotype showed early kanamycin resistance the leaves and stems were green and the shoots were cut/trimmed while growing throughout the selection phase. Following kanamycin selection, the green phenotypic shoots were placed on Ms3AC medium and then transferred to soil. The remaining shoots showed different degrees of stress. Some showed "mottled green" sectors on their cotyledonary leaves and their green primary leaves showed white vascular tissue and leaf edges. The non responsive apices ("tan" phenotype elongated to approximately 2 cm and eventually became necrotic and died. When the 'mottled green' phenotypes were transferred from kanamycin selection medium 1 to kanamycin selection medium 2, these shoots developed green leaves from the growing apical meristem. These shoots were routinely cut/trimmed to keep them under continual antibiotic selection pressure; many died at the higher kanamycin level. However, several of the mottled green phenotypes were transferred to soil and tested positive for histochemical localization of gus-activity.

Momtaz, *et al.* (1998) following co-cultivation with *A.tumefaciens*, callus induction media containing 500 mg/l carbencillin and 25 mg/l kanamycin allowed transformed tissues to form kanamycin resistant micro calli at wound sites after 12-15

days. No callus from control tissue initiated at this level of kanamycin. At the same time one to two kanamycin resistant calli per hypocotyl segment were observed. Further they have tested a total of 499 putative transformed calli for *npt II* production. On lower levels of kanamycin (15 mg/l), some non transformed calli proliferated very slowly but eventually turned brown and died. The percentage of kanamycin resistant and *npt-II* positive calli was higher when higher levels of kanamycin (25 or 35 mg/l) were used for selection.

Kanamycin selection has been proven to be the most widely applicable selective agent but the concentration is species specific. Species like *brassica napus* (Moloney *et al.*, 1989) and *lycopersicum esculentum* (Fillatti *et al.*, 1987) are selected at low concentration of kanamycin (15-100 mg/l), where as *Beta-vulgaris* and cotton (*Gossypium* spp) needs relatively higher concentration of kanamycin (400 mg/l).

Zapata *et al* (1999), reported that transgenic cotton (*Gossypium hirsutum* L.) plants of Texas cultivar CUBOHPRIS were obtained using *Agrobacterium* mediated transformation coupled with the use of shoot apex explants. After inoculation with Agro strain LBA 4404 containing the PBI-121 plasmid, regeneration of primary plants was carried out in a medium containing kanamycin (100 mg/l). Progeny obtained by selfing were germinated in the green house and selected for expression of the T-DNA marker gene encoding npt-II by painting kanamycin 2 per cent on the leaves. Plants that survived leaf painting were analysed by DNA blots. Evidence for integration of the (GUS) was observed in two successive generations from the regenerants (T₀). The transformed plants appeared to have more than one copy of the TDNA. The use of shoot apex circumvents the problem of genotype independent regeneration of cotton.

Zapata *et al.* (1999) have tested the kanamycin resistant plants for the presence of the GUS gene by DNA blot analysis. Hybridization of undigested DNA indicated that the

introduced T-DNA was integrated into high molecular weight DNA. Digestion of genomic DNA with ECOR-I produced three hybridizing bands that were different in mobility from that of the digestion with Hind-III, which again indicates genomic integration of the T-DNA. Furthermore, since the T-DNA of the PBI-121 has single ECOR-I and Hind-III sites. The presence of these hybridizing fragments suggests three independent insertions of the transforming T-DNA into the genome of this plant.

Seeds (T_2) from T_1 plant were grown, and a DNA blot analysis of some of the progeny was carried out. All these plants had the same hybridization pattern of DNA as in the T_1 plant. The fact that kanamycin resistant plants could be recovered and that most of the plants tested from resistant plants were GUS positive in the DNA blot, strongly suggested that the stable integration of the npt-II gene and gus gene.

Chengbin Xiang *et al.* (1999) have carried out the selection for transformants resistant to kanamycin. They have collected the seeds in bulk from plants vacuum infiltrated with *Agrobacterium* harboring PB 1101.2 gshl. Dry seeds were germinated either directly in soil (3000 seeds/flat) or on 150 mm agar plates (3000 seeds/plate). The medium in the plates was half-strength MS. Salts and B₅ vitamins solidified with 2.0 g/l phytogel, supplemented with 50 mg/l kanamycin. Seeds germinated *in solium* were kept in growth chamber. Plants grown at higher light intensities seemed to be more kanamycin resistant. Seeds germinated on plates and in soil received a 2 day cold treatment at 4° C to promote uniform germination. For *in solium* selection, seedlings at the stage of their first pair of true leaves were sprayed with 0.1per cent Triton X-100 solution with kanamycin concentrations ranging from 100 to 500 mg/l using a spray bottle. After spraying, the seedlings were covered for 16 h with a plastic dome to prevent excessive dehydration due to application of the Triton X-100. The kanamycin spray was applied daily in sufficient quantity to just wet the surface of the leaves. The plants were for 2 days with 100 mg/l kanamycin, followed by with 200 mg/l kanamycin, followed by 1 day with 500 mg/l kan.

There was a slight amount of burn on the edges of leaves following the final spray treatment.

Although selectable marker genes, that confer resistance to antibiotics or herbicides, are generally incorporated along with the gene of interest in a transformation process, so as to allow the recognition of the transformed cells from the background of untransformed ones, subsequent to the generation of transgenic plants, the presence of these marker genes becomes no more of practical utility and thus arguably a matter of public euphoria, speculating the risk they can pose to the environment and health. Since the recurrent transformations to pyramid desirable genes are practically difficult with the same selectable markers used, novel strategies are evolved to essentially eliminate these marker genes or to modulate their spatial / temporal expression. Use of environmentally safe selectable markers and transplastomic plants also serves the purpose.

2.4.1.1 Safer marker genes

The green fluorescent protein (*gfp*) gene is an ideal selectable marker and reporter for gene expression analysis and plant transformation. In nature GFP is made by the jellyfish *Aequorea victoria*, and has been used as a convenient marker in heterologous systems. The first example of its expression in graminaceous plant was reported by Sheen *et al.* (1995) where bright GFP expression was detected in maize mesophyll protoplasts. These results have since been extended and applied to other cereals and grasses (Pang *et al.*, 1996).

In the traditional selection system, using antibiotics or herbicides, the transgenic cells convert the selective agent to a detoxified compound that may still exert a negative influence on plant cells. Further, the release of toxic metabolites by dying adjacent cells may also inhibit the growth of transformed cells. In contrast, a new set of markers known as positive selection markers are being developed, which can overcome some of the limitations encountered by the traditional selection system.

Joersbo and Okkels (1996) have reported a selection system, which uses the β -glucuronidase gene from *E.coli* as a selectable gene and a glucuronide derivative of the cytokinins, benzyladenine as a selective agent, Benzyladenine N-3 glucuronide is inactive as cytokinin but upon hydrolysis by GUS becomes active cytokinin, stimulating growth and regeneration of cells *in vitro*. The gus gene served as both selectable as well as screenable marker. The efficiency of the transformation was reported to be two-fold higher than with kanamycin.

Likewise the other selection systems, which originated from the concept of favouring the transgenic cells while starving rather than killing the non transgenic cells, is mannose selection system, where in *Pmi* gene (Phosphomannose isomerase) is used as selectable gene and mannose as a selective agent. The authors have observed that the use of mannose selection in sugar beet resulted in a tenfold increase in transformation frequency when compared to traditional kanamycin selection.

Daniell *et al.* (2001) engineered chloroplast genome without the use of antibiotic selection. The Betaine aldehyde dehydrogenase (BADH) gene from Spinach was used as a selectable marker. The selection process involves the conversion of the toxic BA by the chloroplast BADH enzyme to a nontoxic glycine betaine, which also serves as an osmoprotectant for enhancing drought and stress tolerance in plants.

Komari *et al.* (1996) have reported that one of the strategies to produce transgenic plants free from selectable marker is that the marker and gene of interest are placed on two separate T-DNAs in a single plasmid or on separate plasmids, which are contained in one or more agrostrains. The transgene and marker gene are thus inserted at the loci, which should recombine at reasonably high frequencies; the gene of interest can be segregated from the selectable marker gene in the next generation. Using this technique, transgenic plants free of a selectable marker gene were developed in rice, *Brassica napus* and *Nicotiana tabacum*.

Over the past decade, several approaches have been developed either for excising marker genes from transgenic plants or their modulation and spatial / temporal expression. Further alternative environmentally safe selectable markers or containment of the transgenes through transplastomic plants have given a new dimension (Pawan *et al.*, 2002).

2.4.2 Polymerase Chain Reaction

Kary Mullis invented the polymerase chain reaction (PCR) in 1985 (Mullis K.B., 1990). One of the major applications of this outstanding invention is to know the presence of a transgene. The polymerase chain reaction results in the selective amplification of a transgene, if an appropriate primer is provided. Any region of transgene can be chosen, so long as the sequences at the borders of the region are known. The border sequences must be known because in order to carry out a PCR, two short oligonucleotides, must hybridize to the DNA molecule, one to each strand of the double helix. These oligonucleotides, which act as primers for the DNA synthesis reactions, delimit the region that will be amplified.

Amplification is usually carried out by the DNA polymerase-I enzyme from *Thermus aquaticus*, which lives in hot springs, and many of its enzymes, including Taq polymerase, are thermostable, meaning that they are resistant to denaturation by heat treatment. Thermostability of Taq polymerase is an essential requirement in PCR methodology along with dNTPs, MgCl₂, reaction buffer, sterile water.

To begin a PCR amplification, the enzyme is added to the primed template DNA and incubated so that it synthesises new complementary strands. The reaction mixture is then heated to 94° C so that the newly synthesised strands detach from the template, and then cooled, enabling more primers to hybridize at their respective positions, including positions on the newly synthesised strands. Taq polymerase, which unlike most types of

DNA polymerase is not inactivated by the heat treatment, now carries out a second round of DNA synthesis. The cycle of denaturation hybridization – synthesis is repeated, usually 25-30 times, resulting in eventual synthesis of several hundred million copies of the amplified DNA fragment.

At the end of a PCR, a sample of the reaction mixture is usually analysed by agarose gel electrophoresis, sufficient DNA having been produced for the amplified fragment to be visible as a discrete band after staining with ethidium bromide. This may by itself provide useful information about the presence of transgene that has been amplified.

Gould *et al.* (1998) have subjected Maize DNA from six F₁ of C₅₆ and from one F₂ of C₁ to PCR amplification of 250 bp fragment within the GUS coding region. In a separate amplification, primers for a 1000 bp fragment within the NOS/NPT II gene were used. Amplification of the expected fragments were achieved from the DNA of four C₅₆ F₁ as well as from the DNA of an F₂ of the C₁ family. These results indicated the presence of both GUS and NPT-II in the progeny of two original transformants.

Manoharan *et al.* (1998) have used to demonstrate the presence of T-DNA in the transgenic plants. Two specific primers derived from npt-II gene sequences were used to detect a 0.7 kb fragment. The amplified DNA samples were electrophoresed on 0.8 per cent agarose gel. 0.7 kb fragment co-running with the amplified product from PBI-121 could be detected from the transgenic plants but not from non transformed plant.

Zhang, *et al.* (1998) have reported that the resistance to the bollworm was observed in the R₂-progeny derived by selfing the five R₁ plants, which was evident from the high mortality rate of bollworm. On the basis of resistance analysis some resistant plants were sampled for the presence of the Bt-transgene with PCR. The amplified band on the Bt-transgene appeared in the samples of R₁ plants S 545 and S 591. The PCR products were

subjected to southern blot analysis with the labelled transgene as probe. All the amplified bands could hybridize with the probe, proved that the PCR bands were not amplified from contaminating DNA, but from the Bt-transgene, thus establishing that transgene was heritable through selfing.

The putatively transformed plantlets were subjected to PCR analysis to detect the presence of uid A and npt-II genes. In the transformants with GUS-activity, an expected fragment of 500 nucleotide uid A was amplified. An additional confirmation was also done using npt-II primers and PCR analysis revealed amplification of a 700 base-pair fragment. No amplification was found in non transgenic plants, pigeonpea (Geetha *et al.*, 1999).

For the detection of the *npt-II* coding sequence in the progeny, genomic DNA was subjected to PCR using the following primers and conditions: forward 5'TCATCTCACTTGCTC CTG-3' and reverse 5'AGC CAACGCTATGTCCTG-3' primers at a concentration of 50pM were used to amplify a 363-bp region (position 1860-2222 of Tn₅) of the npt-II gene (pHP 23 served as a positive control) with 0.5 µg genomic DNA of each sample, 2 mM MgCl₂, and 2 units of a (native) Taq Polymerase (and the recommended buffer) from Gibco in a 50 µl assay. Cycling conditions were – denaturation, 1 min at 94° C; annealing, 1 min at 50° C, elongation 2 min at 72° C; annealing, 1 min at 50° C, elongation 2 min at 72° C (30 cycles) and a final elongation step of 10 min. For detection of 363 bp fragment DNA was separated on 1per cent agarose gels. All the 4 plants indeed showed the presence of the expected 363 bp fragment, which is part of the coding region of the npt-II gene. In one of the putatively transformed lines, an additional fragment of approximately 2.5 kbp was amplified, probably due to re-arrangement of two or more incomplete copies (Krishnamurthy *et al.*, 2000).

Prasad *et al.* (2000) have carried out PCR analysis of the *npt-II* gene for initial screening of putative transformants of *Brassica juncea* for the presence of *npt-II* gene. About 85 per cent of the putative transformants gave the 0.7 kb full length *npt-II* gene. These results clearly indicated the successful integration of the gene cassette in these putative transformed lines of *Brassica juncea*.

Weir *et al.* (2001) have performed PCR in 10 μ l of reaction solution using 25-40 ng of wheat genomic DNA 100 μ M each dNTP, 10 pmol of each primer and 1 unit of Taq DNA polymerase. The cycling program was 1 cycle of 95° C for 5 min, 35 cycles of 95° C for 30s, 56° C for 30s and 72° C for 2 min. Primers to amplify the *npt* gene were 5'-AAAAGCCTG AACTCACCG-3' and 5'-TCGTCCATCACAGTTTGCC-3'. The PCR products were visualized by running the completed reaction in a one per cent agarose gel containing ethidium bromide and then photographed. PCR analysis of lines transformed with PBGxI and selected with hygromycin B showed that *npt* gene was present in callus lines showing different intensities of GFP expression, with the production of a well defined product of 250 bp.

PCR amplification of the npt-II gene using specific primers was carried out to check the presence of transgene in the plant genome. Plant DNA was isolated from immature leaves by cetyl trimethyl ammonium bromide (CTAB) method as described by Peterson *et al.* (1993) DNA concentration was estimated spectrophotometrically. The *npt-II* specific primer sequences (5'-3') GAG GCT ATT CGG CTA TGA CTG and ATC GGG AGG GGC, GAT ACC GTA were used. Each PCR reaction was performed in 25 μ l (total volume) of reaction mixture consisting of 10 x reaction buffer, 150 ng DNA, 200 mM dNTPs, 25 mM MgCl₂, 100 ng of each primer DNA and 1 U of Taq DNA polymerase. The PCR reaction buffer consisted of 10 mM Tris HCl (pH 8.3), 25 mM KCl and 0.01 per cent gelatin. PCR was carried out in a thermal cycler under the following conditions, 94° C for 3 min as preheating, then 35 cycles of 94° C denaturing for 30 S, 55° C annealing for

45 S, 72° C synthesis for 1 min and 7 min at 72° C as final extension. Amplified DNA fragments were electrophoresed on 1.0per cent agarose gel and detected by ethidium bromide staining and photographed under ultraviolet light (Sathyavathi *et al.*, 2002).

2.4.3 Expression Analysis

Smith *et al.* (1990) have reported that, tomato plants transformed with a chimeric polygalacturonase (PG) gene to produce a truncated PG transcript constitutively were found inhibiting the endogenous PG gene expression during ripening, resulting in a substantial reduction in PG mRNA and enzyme accumulation. The expression of the truncated gene in ripe fruit was substantially lower than its expression in green fruit. Inhibition of expression of artificially introduced genes has been demonstrated by transient expression of antisense RNA constructs in plant protoplasts and by stable expression in transgenic plants.

Five transgenics showed the expected 1kb amplified fragment in RT-PCR, indicating transcriptionally active *Cry1Ab* gene in the plant genome. The 2.2 kb mRNA transcript obtained after splicing of a 0.19kb intron from the Bt-gene further supported RT-PCR results (Chakraborty *et al.*, 2002).

2.4.4 Dot Blot Technique

This immunoblotting assay is a reliable technique used to detect the presence of a given sequence of DNA or RNA in the non-fractionated DNA. Where in sample DNAs from several DNAs can be tested in a single test run. Antigene may be applied directly to nitrocellulose filter papers as a dot to give a simple and a reliable assay with advantages over conventional immunoassays. Sometimes they are more sensitive than conventional ELISA because the dot permits the colour reaction to be viewed against a white background. The antigen is applied as a discrete spot and any remaining binding sites are

blocked using a solution on non cross reacting protein. The dot is then incubated with the putative antibody containing solution and washed. Antibody binding is detected with a second species specific antibody conjugated to horseradish peroxidase. It is a simple and reliable assay, feasible for screening a large number of samples, but precise information of integrated gene in genome is possible only by southern blotting.

The technique has been used in tobacco to confirm the transfer of glucanase chitinase gene (Dinesh, 1998).

2.4.5 Southern Hybridization

Southern blotting, developed by Southern (1975) is an extremely powerful tool for analyzing gene structure. To perform a southern blot, genomic DNA is cut with one or several restriction enzymes and the resultant fragments are separated by size on an agarose gel. The gel is then overlaid with a sheet of nitrocellulose filter or nylon membrane and a flow of buffer is set up through the gel toward the nitrocellulose filter, where they bind. Thus a replica of the DNA in the gel is created on the nitrocellulose membrane. A labelled probe, specific for the gene under study, is then allowed to hybridize to the DNA molecules bound on the filter. The labelled probe will hybridize to the specific molecule containing a complementary sequence. Autoradiography of the nitrocellulose filter will result in a pattern of bands indicating the number and size of the DNA fragments complementary to the probe. This technique was used to demonstrate the presence of the gene in question in transgenics, where detection of DNA fragments which are complementary to given DNA is critical. This is the common method to confirm the stable integration of DNA in the genome.

Umbeck *et al.* (1987) have analysed the DNA from transformed plants for the presence of *npt-II* gene by Southern hybridization. The results demonstrated that the expected 1kb *Ava-I/Hind-III* fragment corresponding to the region encoding *npt-II* in a

plant 1427 at approximately one copy per genome, consistent with the observation of enzyme activity in this plant. When probed with PBR-322 sequences, plant 1427 did not exhibit the expected 2.1 kb fragment or any other hybridizing fragment indicating that the portions of the modified T-DNA region are missing in this plant. They have reported that the results are not unusual because the analysis of the previously transformed plant species have shown that a significant fraction of recombinant plants contain partial or rearranged T-DNA inserts. Further they report that various screening procedures, such as Southern analysis or gene expression assays can be used to identify transformants with intact inserts. (Southern blot)

Wang *et al.* (1998) have carried out the southern hybridization with *uid A* probe. In the double restriction digest the hybridization signal was present as the 2.9 kb fragment of GUS expression cassette, in the transferred DNA, in five of the plants analysed. In two cases there were bands of different sizes possibly due to rearrangements or blocked restriction of one of the restriction enzymes.

When *ECOR-I* was the only restriction enzyme used, the hybridized fragments were as expected of varying size, depending on the location of the inserted DNA in the genome. One plant had more than a single band, which is indicative of multiple insertions.

Zapata *et al.* (1999) have subjected some of the plants surviving the leaf-test for kanamycin resistance to DNA blot analysis to test for the presence of *gus* gene. Hybridization of undigested DNA indicated that the introduced T-DNA was integrated into high molecular weight DNA. Digestion of genomic DNA with *ECOR-I* produced three hybridizing bands that were different in mobility from that of the digestion with *Hind-III*, which again indicates genomic integration of the T-DNA. Furthermore, since the T-DNA of *PBI 121* has single *ECOR-I* and *Hind-III* sites, the presence of these hybridizing fragments suggests three independent insertions of the transforming T-DNA into the genome of this plant.

Seeds (T₂) from a T₁ plant were grown, and a DNA blot analysis of some of the progeny was carried out. All these plants had the same hybridization pattern of DNA as in the T₁ plant. The fact that kanamycin-resistant plants could be recovered, and that most of the plants tested from resistant plants were *gus* positive in the DNA blot, strongly suggested the stable integration of the *npt-II* gene and the *gus* gene.

Geetha *et al.* (1999) have carried out southern hybridization to confirm the T-DNA integration in transgenic pigeon pea. A 0.7 kb PCR fragment of *npt-II* gene was used as probe. DNA from transgenic and untransformed plants was digested with *Hind-III*, which cuts only one site located between the *uidA* and *npt-II* genes in the T-DNA insert of PBI 121. $\alpha^{32}\text{P}$ labeled *npt-II* DNA probe hybridized to *Hind-III* digested nuclear DNA of transgenic plants and plasmid DNA double digested with *pst-I* revealed a strong hybridizing signals to the *npt-II* probe at about 1.9 kb, used as positive control. No hybridization could be detected for untransformed plants. The same hybridization pattern observed for transgenic plants indicate that the regenerants have originated from the same explant.

In order to ensure the insertion of the *codA* gene into the *Brassica* genome and determine the number of the integration sites (or loci) in each independent transgenic line, the genomic DNA of the transgenic plants that were *npt-II* positive were digested with *ECOR-I* and hybridized with 2.1 kb *codA* probe. With the exception of four of the *npt-II* positive putative transformants, the rest showed the presence of one to three bands that hybridized with the probe, suggesting the integration of the *codA* gene at one to three independent positions in the genome. Moreover, the position of the *codA* gene insertion varied among the transgenic lines. Further, it is known that *Agrobacterium* – mediated transformation of explants often results in the insertion of multiple of the transgene (Hobbs *et al.*, 1993). Results of the southern analysis showed that majority of the transformants contained more than one insert. Two of the independent transformed lines,

which showed the *codA* gene at a single insertion locus were multiplied and self pollinated in-order to obtain a homozygous population of these transformants (Prasad *et al.*, 2000).

The integration of the *npt-II* gene was analysed by southern blot (T_0). Genomic DNA was isolated from non-transformed plants and putative transformants according to the protocol of Rogers and Bendich (1985) with modifications. For Southern blotting the DNA was digested with Hind-III, separated on 0.8per cent agarose gels, transferred to Hybond N + nylon membrane (Amersham) and hybridized with a 0.85 kb *npt-II* – *Hind-III* fragment excised from pHp 23. After hybridization, the filter was exposed to X-ray film at -70° C for 5 days. Genomic analysis of 4 randomly selected plants from different transormation experiments, confirmed the integration of the T-DNA. The number of hybridization signals indicated integration of multiple copies of T-DNA (Krishnamurthy *et al.*, 2000).

Southern hybridization was carried out on PCR positive hygromycin resistant callus lines showing different levels of GFP expression, to test the relationship between intensity of GFP expression and the copy number of *gfp* gene. Digestion of callus DNA with *pst-I* and *ECOR-I* was expected to yield a 0.8 kb insert. Samples analysed showed that 0.8 kb sequence was present in two transformed callus lines with different intensities of green fluorescence. To check the number of insertions of the *gfp* gene in transformed calli, the DNA from four calli showing different levels of GFP expression was digested with *pstI* and hybridized with the radioactively labeled 0.8 kb *gfp* fragment. Sample 1 from callus line with GFP expression scored at level sorted at level 3, had three insertions of *gfp* gene with bands at 1.4, 5.6 and 11 kb, indicating that multiple copy insertion of the *gfp* gene had occurred. There was no apparent relation between the intensity of GFP fluorescence and transgene copy number in the callus tissue (Weir *et al.*, 2001).

2.4.6 Northern Hybridization

Northern hybridization is a technique used to detect transcription of DNA sequence that is used as a probe. It is useful as an adjunct to cDNA cloning because the size of specific mRNA can be compared with the size of cloned cDNAs, revealing whether the cloned cDNA is full length. In addition, this simple procedure can indicate which tissues or cell types express a particular gene or the factors that regulate its expression. This technique has been used to know the expression of cowpea protease inhibitor at mRNA level in transgenic pigeonpea (Lawrence and Koundal, 2001).

Bhattacharya *et al.* (2002) used southern confirmed plants for northern blotting of Bt [*CryIA(b)*] transformants in cabbage and reported differences in the level of transgene expression.

2.4.7 Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme immunoassays combine the specificity of antibodies with the sensitivity of simple spectrophotometric enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme that also possess a high turn over number. ELISA is relatively cheap to operate and lacks radiological hazards. It can be used for assaying antigen by either a competitive or a double antibody method and for assaying a specific antibody by an indirect method. All these methods require the preparation of calibration curve during the assay.

When eight of the transgenic plants were tested for NPT-II protein using the ELISA assay, the amount of protein ranged from 16 to 50 ng per mg of total soluble protein. The same plants also contained protein of the expected molecular weight in the immunoblot analysis. All the kanamycin resistant, octopine positive calli were positive for NPT-II protein, and they contained protein of the expected molecular weight (Firoozabady *et al.*, 1987).

NPT-II was detected and quantified in cotton extracts by an ELISA assay. When eight of the transgenic plants were tested for NPT-II protein using the ELISA assay, the amount of protein ranged from 16 to 50 ng per mg total soluble protein. The same plants also contained protein to the expected molecular weight in the immunoblot analysis. All the kanamycin resistant, Octopine positive calli were positive for NPT-II protein, and they contained protein of the expected molecular weight (Firoozabady, *et al.*, 1987).

The presence of NPT-II protein in transformed embryogenic callus of cotton was detected by a sandwich immunoassay with an NPT-II ELISA kit (5¹-3¹ Inc. Boulder CO, USA). Transformed cell-extract, non transformed cell-extract as control, and pure NPT-II protein concentrate as standard, were added to the wells. Callus extracts were made by grinding each tissue in 3-4 ml cold 0.25M Tris HCl, pH 7.8, containing 1mM phenyl methyl sulphonyl fluoride per gram of callus tissue. Extracts were centrifuged (7500 g, 4°C, 30 min) and 200 µl of diluted extract were loaded in the micro well ELISA plate. The NPT-II levels were determined and expressed as described in kit manual (Momtaz *et al.*, 1998).

Mandaokar *et al.* (2000) have transferred, a synthetic *CryI*Ac gene coding for an insecticidal crystal protein (ICP) of *Bacillus thuringiensis* by co-cultivating cotyledonary explants with *Agrobacterium tumefaciens*. Transformant plants resistant to kanamycin were regenerated. Hybridization experiments demonstrated gene integration and gene copy number in transgenic plants. Double antibody sandwich ELISA analysis revealed high levels of Bt ICP expression in the leaves and fruits against the larvae of tomato fruit borer (*Helicoverpa armigera*). Limited field trial of the transgenic plants (T₁-generation) confirmed the high levels of insect protection.

Further, they have also carried out double antibody sandwich ELISA analysis using the polyclonal rabbit antibodies specific for CRY1 AC protein to detect the

levels of toxin expression in the transgenic plants. Toxin levels ranged from 0.04 to 0.41 per cent of total soluble protein. The maximum level of *Cry1AC* expression reported here (0.41% of the total soluble protein) is among the highest reported in the literature (Schuler *et al.*, 1998). Various plant species transformed with synthetic Bt-genes under the control of constitutive promoters such as CaMV35s exhibited a wide range of levels of foreign protein expression. For example, out of total soluble protein 0.1 per cent of *Cry3A* in potato (Adang *et al.*, 1993), 0.03 per cent of *Cry1Ab* in tobacco (Perlak *et al.*, 1991), 0.05 per cent of *Cry1Ab* in rice (Fujimoto *et al.*, 1993), 0.4 per cent of *Cry1Ab* in maize (Koziel *et al.*, 1993) and 0.4 per cent of *Cry1Ac* in canola were observed. Recently, Cheng *et al.* (1998) reported extraordinary levels of *Cry1Ac* expression (3% of total soluble protein) in transgenic rice, the maximum level of Bt gene expression obtained to date, by nuclear transformation, McBride *et al.* (1995) demonstrated an alternative to the usage of synthetic Bt genes by engineering a native *Cry1Ac* into tobacco chloroplasts, yielding transgenic plants with 3-5 per cent of *CRY1Ac* protein. In another recent report dealing with transplastomic tobacco. Kota *et al.* (1999) have expressed *CRY2Aa* protein upto 2-3 per cent of total soluble protein.

2.4.8 Insect Bioassay

Transgenic tomato plants expressing a cry protein were shown to control tobacco hornworm (*Manducta Sexta*) larvae, showing essentially no feeding damage (Fischhoff *et al.*, 1987) under laboratory conditions. The same plants exhibited some activity against three other lepidopteran insects tobacco budworm (*Heliothis Zea*) and tomato pinkworm (*Keiferia lycopersicella*).

The surviving GUS positive plants were tested for resistance to bollworm, with Simian 3 and Zhongmianosuo 12 as control. Five R₁ plants were verified to be highly toxic to the insect, four from Simian 3 + Bt/GUS with bollworm mortality levels at

91.6per cent, 93.8per cent, 92.3per cent, 85.7per cent and one from Zhongmiansuo 12+Bt/GUS with 75.0per cent bollworm mortality. In the five plants, leaves were damaged slightly but bollworms damaged the boll surface of the transformed cotton plants only slightly. Bollworms bored into the center of the control plant bolls, causing severe damage.

Mandaokar *et al.* (2000) in their insect bioassay studies have reared *Helicoverpa armigera* in the laboratory on Semi-synthetic diet. Five second instar larvae were released on the leaf circles (2.5 cm diameter) of normal and seven transgenic tomato plants (T_0). Rapidly growing leaves of one month old tomato plants were selected for the bioassays. The leaf discs were placed on moist whatman (#1) filter paper discs in multi well tissue-culture plates (Greiner). Tomato fruits were sampled at full maturity and placed in Magenta boxes on moist filter paper. Larval mortality was recorded after seven days.

The results of the insect bioassays using second instar larvae of *H. armigera* revealed that the leaves and fruits of the seven transgenic plants (T_0 generation) were protected from the insect damage. Larval mortality was 100 per cent on the leaves of the plants except those of Bt_1 and Bt_5 , which was discernible after 48 hours. The larvae that could survive on the leaves of plants with partial protection, decreased in weight (50-92 %) relative to that of control larvae. Plants Bt_1 and BT_5 showed relatively lower levels of *Cry1AC* protein, which could be the reason for partial protection observed in these plant leaves.

The level of *Cry1Ab* in insects feeding on sources containing the *Cry1Ab* protein was uniformly low but varied with insect species as well as food source For the lipidopteran insects, *Ostrinia nubilalis* (Hubner), *Helicoverpa zea* (Boddie), and *Agrotis ipsilon* (Hufnagel), levels of *Cry1Ab* in larvae varied significantly with feeding treatment. After feeding larvae on artificial diets containing 20-100 ppm of *Cry1Ab* protein for 24

hours the level of protein in the larvae was about 57 to 142 times lower than the protein level in the diet for *O.nubilalis*, 20 to 34 times lower for *H.Zea*, and 10 to 14 times lower for *Agrotis ipsilon* (Graham *et al.*, 2001).

Henneberry *et al.* (2001) have compared larval mortality on different age cotton fruiting forms and determined the Bt susceptibility of different age PBW larvae. Infesting Bt bolls with PBW eggs that hatched within 24 h resulted in 92 per cent larval mortality after 48 hrs and 100 per cent mortality after 4 days or longer. There were no differences between cultivars in numbers of larval entrance holes into bolls. Larval mortality percentages decreased when older (second and third instar) larvae were placed on bolls compared with first instar larvae. Generally, days to pupation for both males and females were longer on Bt bolls compared with non-Bt cotton . 93 to 100 per cent of the progeny from pairs from second and third instar larvae that survived on non Bt bolls died when transferred to Bt bolls. There were no significant mortality differences for larvae feeding on Bt fruiting forms of different ages ranging from one-half grown flower buds to 40 day old immature green bolls.

Bhattacharya *et al.* (2002) have reported that Bt transformants, which showed detectable levels of mRNA expression for the transgene and stable presence of CRY protein in immunoblotting, were assayed for their tolerance to late, second instar larvae of *P.xylostella*. Larval mortality was recorded in order to test the influence of cry protein ingestion on larvae survival. Extent of leaf damage effected before cessation of larval feeding was measured to assess Bt mediated resistance. Bioassay on detached leaf discs showed significant larval mortality ranging from 51.84 to 74.06per cent. The highest larval mortality of 74.06per cent with a minimum leaf damage of 6.0per cent was obtained on transgenic BT₂. The damage on young leaves due to insect feeding was also significantly less on the transformed leaves compared to untransformed wild type plants.

The insect bioassays indicated effectiveness of the transgene against infestation by diamond backmoth *Plutella xylostella* larvae. The larvae showed different feeding patterns and growth on control and transgenic leaf discs (Chakraborty *et al.*, 2002).

2.4.9 Reporter gene

In all transformation protocols, invariably, many non-transformed plants (escapes) are noticed along with transgenic plants. The transformed plants can be identified by Southern blot analysis of the DNA isolated from them, recallusing assay, enzymatic assay, antibody assay and by detecting the selectable markers gene products. But these assays are laborious, time consuming and require radioactive substrate, antibodies, etc.

These drawbacks can be overcome by including independent reporter gene in the gene transfer cassette, whose presence can be easily visualized / measured. A reporter gene product should be stable, tolerate amino-terminal fusions, have simple and versatile assays and that it should not have intrinsic background activity in the organisms (Jefferson, 1987). β -glucuronide (*gus*) and luciferase (*lux*) are the widely used reporter genes.

Warkentin and McHughen *et al.* (1992) have studied the effect of co-culture on the proportion of lentil shoot apex explants with GUS expressions and per cent area with GUS expression per explant. As the duration of co-culture increased, the proportion of explants with GUS expression increased, although not significantly by ANOVA at the 0.05 level. Co-culture duration did not have a significant effect on the area with GUS expression per plant. Wounding by abrasion also did not significantly increase the proportion or area of tissue with GUS expression.

Leaf, stem and root tissues of 10 plants selected on kanamycin which were subjected to fluorometric GUS assays, eight plants showed GUS activity and these

measurements revealed that, two plants were, escapes, as there was no signal with *uidA* or *npt-II* probes. There was no significant difference in GUS-activity between leaves, stem or roots, but the highest activity was recorded in stem. This is, as might be expected (Hobbs *et al.*, 1993), variation between plants. Histochemical staining of stem cross sections indicated that not all cells showed the same GUS-activity; phloem tissue showed less activity than other vascular or ground tissue (Wang *et al.*, 1998).

Momtaz, *et al.* (1998) have detected the presence of NPT-II protein in transformed embryogenic callus by a Sandwich immunoassay with an NPT-II ELISA kit. Transformed cell extract, non transformed cell extract as control, and pure NPT-II protein concentrate as standard were added to the wells. Callus extracts were made by grinding each tissue in 3-4 ml cold 0.25 M Tris-HCl, pH 7.8 containing 1mM phenyl methyl sulfonyl fluoride per gram of callus tissue. Extracts were centrifuged and 200 ml of diluted extract were loaded in the micro well ELISA plate. The NPT-II levels were determined and expressed as in kit manual.

Zhang , *et al.* (1998) assayed the cotton root and leaf sections for GUS activity. Out of 3545 seedlings of Simian 3+Bt /GUS, 53 plants were GUS-positive and 21 plants survived. From 1360 seedlings of Zhogmiansuo 12+Bt/GUS, 17 were GUS positive and 13 plants survived. In total GUS-positive rate was 1.4 per cent.

Rohini and Rao (2000) have studied the expression and inheritance of the *uidA* gene in peanut. The susceptibility response of embryo-axes of peanut of *Agrobacterium* infection that was determined initially by scoring the transient GUS activity five days after infection, resulted in blue colour development. Deep blue sectors were seen occupying most part of the leaflet area. GUS activity was expressed in various organs and tissues of T₀, T₁ and T₂ plants. None of the control plants expressed GUS.

The expression of *uidA* gene assayed in the greenhouse established plants by Western blotting has shown a protein band at the expected position of 74 kDa in the total protein profile. This band was not detected by the antibody in the total proteins of uninfected plants.

2.5 TRANSGENIC PLANTS THAT CONFER INSECT RESISTANCE

Plants, by necessity, have evolved a fascinating array of structural and chemical defenses against insect attack. A variety of compounds are available that could be produced in transgenic plants with the goal of increasing insect resistance. The challenge for the future will be to use the limited genetic and financial resources that are available in the most efficient manner, so that plants with durable resistance can be developed at reasonable cost, and without jeopardizing other desirable agronomic characteristics of crops.

2.5.1 Choice of Defense Genes

The use of transgenic plants producing Bt-toxins has the advantage that these proteins are toxic to insects at very low levels. Plants can be obtained which have nearly complete resistance to pests without the necessity of producing large amounts of a foreign protein. Plant defense proteins such as proteinase inhibitors, amylase inhibitors, which interfere with insect metabolism and development. Lectins from plants in combination with Bt will prevent development of resistance to insects (Bowen *et al.*, 1998). Proteinase inhibitors may prove to be useful in combination with Bt-toxins as there appears to be a synergistic effect between these two types of defense compounds when consumed together by insects (Macintosh *et al.*, 1990). However Bt toxins are generally accepted as safer for users, wild life and consumers. The following table shows a chronology of the development of insect resistant transgenic plants.

Year	Plant	Gene/Protein	Result	Reference
1987	Tobacco	Bt-toxin	Low protein levels resistant to <i>Manduca sexta</i>	Vaeck <i>et al.</i> , 1987
1987	Tobacco	Cow Pea trypsin inhibitor	Resistant to <i>Heliothis virescens</i>	Hilder <i>et al.</i> , 1987
1989	Tobacco	Potato inhibitor-II Tomato inhibitor-II	Resistant to <i>Manduca sexta</i>	Johnson <i>et al.</i> , 1989
1990	Cotton	Modified Bt-toxin	High protein levels resistant to less sensitive insects	Perlack <i>et al.</i> , 1991
1990	Tobacco	Pea lectin	Resistant to <i>Heliothis virescens</i>	Boulter <i>et al.</i> , 1990
1993	Maize	Modified Bt-toxin	High protein levels resistant to <i>Ostrinia nubilalis</i>	Koziel <i>et al.</i> , 1993
1995	Pea	Bean α -amylase inhibitor	Resistant to <i>Bruchus pisorum</i>	Schroeder <i>et al.</i> , 1995
1995	Tobacco	Bt-toxin	Resistant to <i>Helicoverpa armigera</i>	Mc. Bride <i>et al.</i> , 1995
2003	cotton	Bt-toxin (<i>vip</i>)	Resistant to <i>Helicoverpa armigera</i>	Shotkoski <i>et al.</i> , 2003

The production of non protein insect defense compounds in transgenic crop plants is a novel strategy that may prove useful in the future. Understanding the enzymatic pathways involved in the production of compounds such as alkaloids and terpenes is increasing. Genes for more of the biosynthetic enzymes involved will certainly become available. As a result, it may be possible to alter existing biosynthetic pathways or to introduce new genes to divert the existing pathways into important crop plants. Alteration of biosynthetic pathways through genetic engineering has been successfully achieved in *Arabidopsis* a polygene which codes for the production of a series of enzymes, all under the direction of a single promoter (Von Bodman *et al.*, 1995).

Material and Methods

III. MATERIAL AND METHODS

A pre-requisite for transferring genes into plants is the availability of efficient transformation protocol. Although successful *Agrobacterium* mediated transformation has been reported earlier, the transformation efficiency was found to vary within and between plant species. It is therefore very important to identify conditions suitable to both high rates of transformation and genotype independent regeneration methods.

The details of the material used and the techniques adopted during the course of investigation are described in this chapter. The current investigations were carried out at Agriculture Research Station, Dharwad during 2001-2004.

3.1 EXPERIMENTAL MATERIAL

The experimental material consisted of two cotton genotypes namely, Sahana and BC-68-2 belonging to *G. hirsutum* were respectively obtained from Principal Scientist (cotton), Agriculture Research Station, Dharwad and Research Scientist (cotton), Navasari Agricultural University, Athwa Farm, Surat, Gujarat.

The disarmed and supervirulent *Agrobacterium tumefaciens* strain EHA 105 (Hood *et al.*, 1993), harbouring pBIN Ia₅, pCAMBIA AC, pBIN AR and pBIN Aa₃, that were used as vector systems for transformation were obtained from National Research Centre for Plant Biotechnology, IARI, New Delhi.

3.2 EXPERIMENTAL CONDITIONS

In vitro transformation studies were carried out under aseptic conditions in the laminar flow cabinet. Before using the laminar flow chamber, it was exposed to UV-light for 10-15 min. The working surface was sterilized by swabbing with 70 per cent alcohol. The walls of the hood were also sprayed with 70 per cent ethanol to ensure total sterile conditions. Any material being used from out side was flamed before and after use. Before

3.2.1 .Genotypes
Salient features of the genotypes used for transformation

Sl.No.	Characters	BC-68-2	Sahana
1	Plant height	120-140cm	120-150cm
2	Distinguishing morphological characters		
(a)	Habit	• 2-3 monopodial and other sympodial branches • Open growth habit	• 2-4 monopodial followed by multinodal sympodia
(b)	Foliage	• Leaves light green in colour • Hairy with generally 3 lobes and • Small to medium in size	• Medium hairy, nectariless, light green coloured medium sized leaves
(c)	Flower		
	i) Petal colour	Cream in colour	Cream to light yellow
	ii) Pollen colour (at anthesis)	Cream in colour	Cream
	iii) Petal spot	Absent	Absent
(d)	Bolls	• Medium to slightly big sized boll • Oval in shape • Pointed • 4 to 5 locules	• Round to oval shape • 3-5 loculed
(e)	Pigmentation	In 12 to 20 days old plant has fully pinkish red colour on the leaf edge near lamina juncture on first two leaves .	----
3	Maturity (Range in No. of days)		
	i) Sowing to 50% flowering	65 to 70	60 to 65
	ii) Sowing to boll opening	120 to 130	90 to 110
	iii) sowing to harvesting	180 to 190	150 to 160
4	Quality characters		
	i) Ginning outturn	33.4	39.0
	ii) Seed Index (g)	8.5	9.0
	iii) Lint Index.	4.3	5.2
5	Lint Characters		
	i) 2.5% Span length (mm)	28.3	26 to 27
	ii) Fibre fineness (mv)	3.9	4 to 4.5
	iii) Maturity co-efficient	0.85	0.85
	iv) Fibre strength (g/tex)	22.4	21 to 22
	v) UR.	48	48

starting the work, hands were also swabbed well with ethanol. The experiments were conducted under well defined conditions of the culture room maintained at $25\pm 2^{\circ}\text{C}$, uniform light (ca1000 lux) provided by fluorescent tubes (7200°K) over a light/dark cycles of 16/8 hour.

3.2.2 Glassware and chemicals

Glassware like culture tubes, conical flasks, petriplates, beakers, baby jars, measuring cylinders *etc.* were of borosil make. Glasswares were soaked in *labogel* overnight and washed in running tap water, thoroughly rinsed in distilled water and dried in hot air oven. The instruments like forceps, scalpels, *etc.* were also cleaned and dried.

All the chemicals and plant growth regulators were of analytical grade and were procured from standard chemical manufacturing companies.

Sterilization of the glassware

Cleaned glasswares were rinsed with double distilled water and dried in oven at 80°C and sealed with aluminum foil, petriplates, wrapped in filter paper, instruments like scalpel, forceps, blade holders wrapped in aluminium foil were autoclaved at 121°C , 15 lbs pressure for 15 mins. The glasswares were then transferred to sterile inoculation chambers.

3.2.3 Preparation of the stocks

The culture medium used for the study was MS-medium (Murashige and Skoog, 1962). The stock solutions were prepared by dissolving the chemicals on analytical grade in double distilled water and stored in reagent bottles. Stock solutions of auxins were prepared by initially dissolving in small volume of ethyl alcohol and the volume made up with double distilled water. Cytokinins were initially dissolved in 0.1N HCl and gibberlic

	Components	mg/l concentration
Macronutrients	NH ₄ NO ₃	1650.00
	KNO ₃	1900.00
	MgSO ₄ . 7H ₂ O	370.00
	KH ₂ PO ₄	170.00
	CaCl ₂ . 2H ₂ O	440.00
Micronutrients	FeSO ₄ . 7H ₂ O	27.80
	Na ₂ EDTA	60.00
	MnSO ₄ . 4H ₂ O	22.30
	ZnSO ₄ . 7H ₂ O	8.60
	H ₃ BO ₃	6.30
	KI	0.83
	Na ₂ MoO ₄ . 2H ₂ O	0.25
	CuSO ₄ . 5H ₂ O	0.025
	CoCl ₂ . 6H ₂ O	0.025
Organics	Thiamine HCl	10.00
	Pyridoxine HCl	1.00
	Nicotinic acid	1.00
	Glycine	10.00
	Myoinositol	100.00
	Biotin	0.50
Energy source	Sucrose	30000.00
Solidifying agent	Agar	8000.00

Separate stock solutions of Macrosalts, Microsalts-I, Microsalts-II, FeEDTA, CaCl₂.2H₂O and organics (except inositol) were prepared. They were as follows.

1. Macro salts	20x
KNO ₃	38.00g
NH ₄ NO ₃	33.00g
MgSO ₄ . 7H ₂ O	7.40g
KH ₂ PO ₄	3.40g
Volume made upto	500.00 ml
2. FeSO ₄ EDTA	25x
Na ₂ EDTA	1500.00 mg
FeSO ₄ , 7H ₂ O	695.00 mg
Volume made upto	500.00 ml
3. Micronutrients-I	50x

Na₂Fe EDTA was dissolved first and then FeSO₄.7H₂O was added

MnSO ₄ . H ₂ O	1115.00 mg
ZnSO ₄ . 7H ₂ O	430.00 mg
H ₃ BO ₃	315.00 mg
KI	41.50 mg
Na ₂ MoO ₄ . 2H ₂ O	12.50 mg
Volume made upto	500 ml
4. Micro nutrients-II	1000x
CuSO ₄ . 5H ₂ O	25.00 mg
CoCl ₂ . 6H ₂ O	25.00 mg
Volume made upto	100.00 ml

10 ml was taken from this and the final volume was made up to 500.00 ml.

5. CaCl ₂	20x
CaCl ₂ . 2H ₂ O	8800 mg
Volume made upto	250 ml

6. Organics	20x
Thiamine HCl	200 mg
Nicotinic acid	20 mg
Pyridoxine HCl	20 mg
Biotin	10 mg
Glycine	200 mg
Volume made upto	200.00 ml

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All stocks were kept in refrigerator (At 4°C)

Amount of stock solutions added to the media

Sl.No.	Stock solutions	Amount to be added ml/l
1.	Macro salts	25
2.	FeSO ₄ . EDTA	20
3.	Micro salts-I	10
4.	Micro salts-II	5
5.	CaCl ₂ . 2H ₂ O	10
6.	Organics (except inositol)	10

Inositol was weighed separately @ 100 mg/l and added during media preparation.

3.2.4 Preparation of culture media

Medium was composed from separate stocks (25x) of macronutrients, micronutrients (25x), Fe EDTA (25x) and organics (25x), which were stored in the refrigerator. Inositol, sucrose and agar were weighed and added in required amounts at the time of media preparation. Growth regulators were added from stock solutions of suitable strength. After mixing all the ingredients one by one, including sucrose (except agar), distilled water was used to make up the required volume. The pH of the medium was adjusted to 5.6-5.8 by using either 0.1N HCl or NaOH, with the help of digital pH meter.

Then the required amount of agar was added before autoclaving at 121°C and for 15 PSI for 20 min.

3.2.5 Preparation of media plates

After autoclaving was over, the media was taken into sterile laminar air flow. It was cooled to 50-60°C and then poured into the sterile petriplates. Before and after pouring, the conical flask containing the media was flamed in order to prevent contamination. The plates were kept open till the media was solidified, later closed and sealed thoroughly with Para film. The media plates were stored in culture rooms at 25±2°C. Inoculation of seeds or explants was done after 3-4 days ensuring that the containers are free from contamination.

3.2.6 Preparation of antibiotics

Kanamycin stock of 10 mg/ml was prepared in sterile double distilled water and was stored at 4°C. Cefotaxime stock of 100 mg/ml was also prepared in double distilled sterile water, filter sterilized and was stored at 4°C. Required amount of antibiotic from stock solutions was added following filter sterilization.

3.3 EXPLANT PREPARATION

Seeds were surface sterilized with 70 per cent (v/v) ethanol and followed by 0.2 per cent (w/v) aqueous mercuric chloride solution for 20 minutes and then rinsed five times with sterile distilled water. The seeds were cultured onto seed germination medium to germinate under light (1000 lux) and temperature (25±2°C), for 5-7 days. From these growing seedlings one cotyledon was removed by pushing down until it snapped off. Thus exposed shoot apices were isolated under the microscope from the apical region and were cultured on the agar solidified MS-medium containing 0.5 mg/l of zeatin (Fig.1).

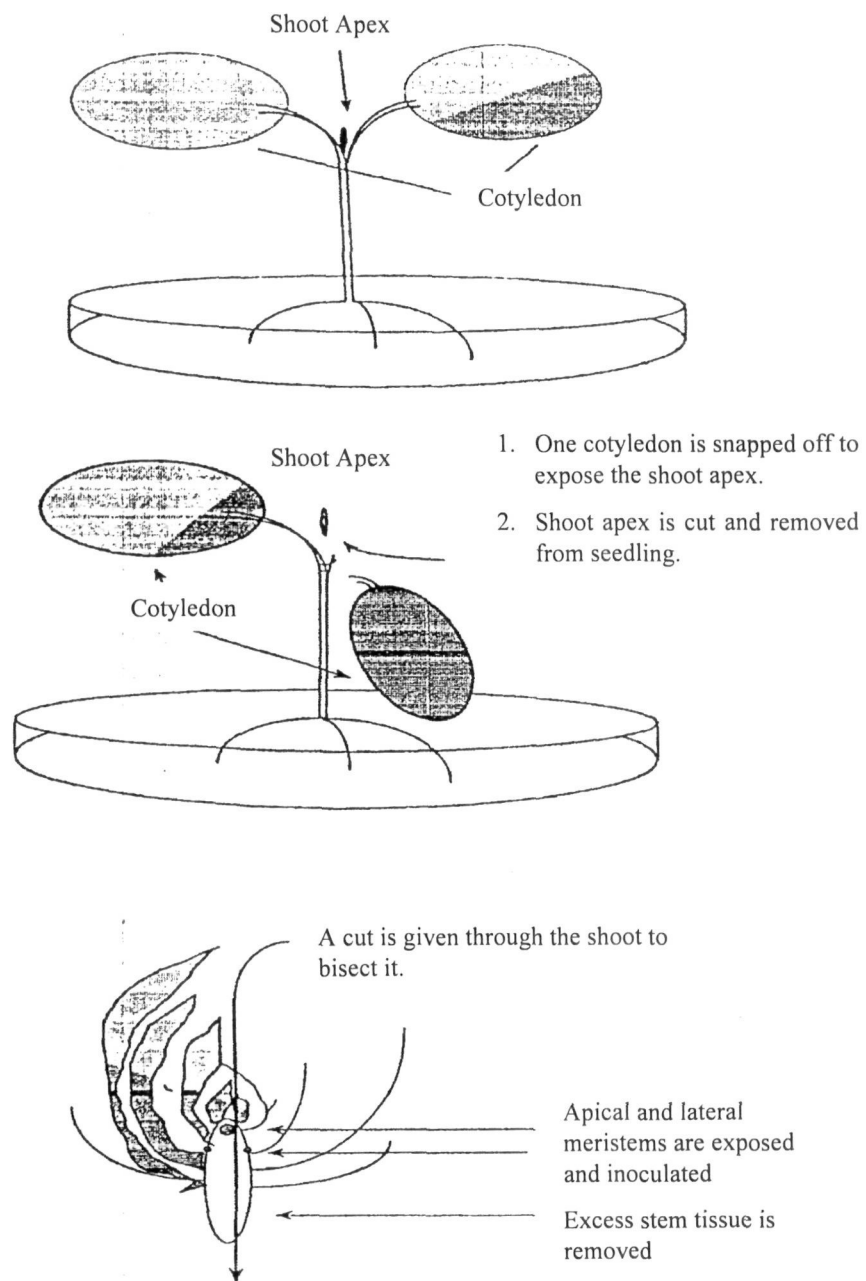


Fig. 1. Isolation of shoot apical meristem.

3.3.1 *Agrobacterium* strain and plasmid vector

The disarmed *Agrobacterium* strain EHA-105 harbouring binary vector pBIN1a₅, pBINAR, pBIN1Aa₃ and pCAMBIA, respectively carrying *Cry1Ia*₅, *Cry1F*, *Cry1Aa*₃ and *Cry1AC* gene linked to the CaMV35S promoter, OCS terminator II and *npt II* gene under the control of nopaline synthase (*nos*) promoter and terminator was used as a selectable marker (Figure 2).

3.3.2 Maintenance of *Agrobacterium*

The *Agrobacterium* EHA-105 containing all the four plasmids were maintained on solid Yeast Extract Mannitol Agar (YEM) medium(Appendix III) containing kanamycin @ 50µg/ml of YEM. Subculturing was done every month on fresh medium and incubated at 28°C temperature.

3.3.3 Preparation of starter culture

One loopful of *Agrobacterium* was picked using a sterile bacterial loop and transferred to 10 ml YEM liquid medium with 50 µg/ml of kanamycin which helps in applying selection pressure and allows only *Agrobacterium* to grow in the medium. Culture was grown at 28°C temperature for 24, 48 and 72 hrs as per the requirement of the experiment.

Working culture

From the starter culture, culture that read 0.2, 0.3, 0.6, 0.8 and 1.0 OD at 600 nm was used for infecting the explants.

pBIN1Aa3

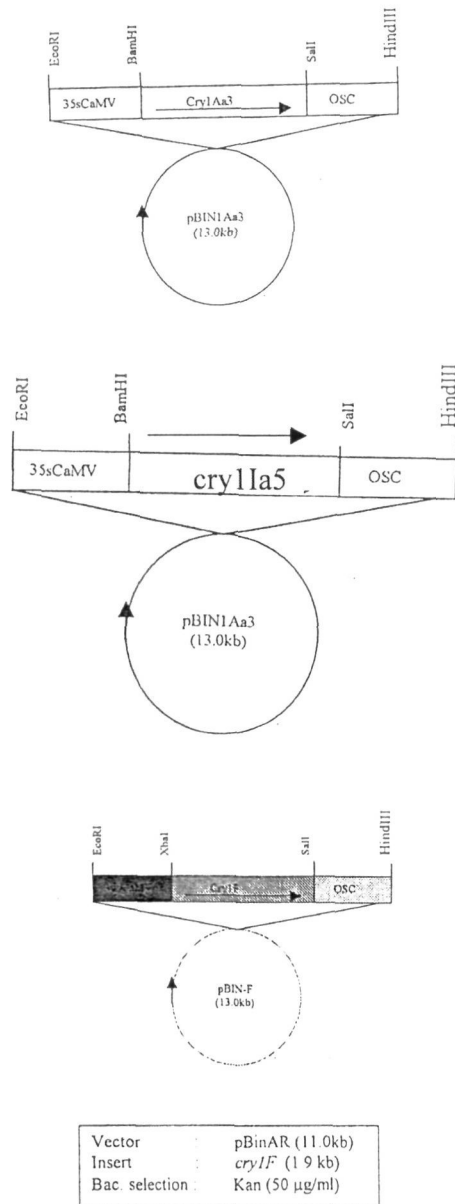


Fig. 2. *cry* constructs used in this investigation.

3.3.4 Cefotaxime sensitivity test

To find out the suitable concentration of cefotaxime to avoid bacterial contamination during morphogenesis and to know minimum level of cefotaxime which will completely eliminate the excess bacteria after co-cultivation, this test was conducted at 100, 200, 300, 400 and 500 mg/l cefotaxime along with control.

3.3.5 Kanamycin sensitivity

Initially, kanamycin sensitivity test was carried out to find out the minimum concentration of kanamycin required to kill normal, untransformed explants and to design the medium for selection of transformed plants. This was done by culturing the explants on optimised MS medium for regeneration (MS basal + BA @ 0.2 mg/l) of plants with the following levels of kanamycin added to it (0, 25, 50, 100 ,200 ,300 ,400 and 500 mg/l).

3.4 DETAILS OF THE EXPERIMENTS

The steps followed for transformation, viz., pre-culture, co-cultivation with *Agrobacterium*, selection using antibiotics, multiplication, rooting and hardening are described below.

3.4.1 Pre-culture

The shoot apical meristems isolated from 5-7 days old aseptically grown seedlings were incubated on MS-medium with zeatin @ 0.5 mg/l or BA @ 0.2 mg/l and incubated for 2 days before co-cultivating with *Agrobacterium*.

3.4.2 Co-cultivation

The pre-cultured explants were dipped in bacterial suspension. The optimization of bacterial suspension concentration (OD) needed was done by infecting the explants with

five inoculum densities (0.1, 0.2, 0.5 1.0 and 2.5 x 10 cells/ml) of bacterial suspension. In order to optimize bacteria to colonize the explants, they were immersed in *Agrobacterium* suspension for varying time intervals viz., 30 and 60 minutes. The standardization of co-cultivation period was done by incubating explants with *Agrobacterium* for different duration viz., 24, 48 and 72 hours. The explants were then transferred to the standardized regeneration medium containing BA @ 0.2 mg/l or zeatin @0.5 mg/l.

3.4.3 Culture

At the end of co-cultivation period the explants were washed with cefotaxime contained in liquid MS, dried and blotted and then transferred to MS + BA + Cefotaxime medium. Cefotaxime (ci) is effective in killing *Agrobacterium* at low concentration (300 mg/l), yet non toxic to plant tissue at higher concentrations. After the production of shootlets on this culture medium, the putative transformants showing regeneration were transferred to fresh medium with antibiotics for further growth.

3.4.4 Selection (MS + BA + Ci + kan)

The regenerated shoots were then transferred to MS + BA + Ci + kanamycin selection medium containing 50 mg/l, kanamycin with 50 mg/l cefotaxime for 7 days to 2 weeks. Subsequently selection pressure was increased, because the small shoot explants of cotton were found sensitive to kanamycin. The robust and healthy looking shoots were then transferred to medium containing 300 mg/l kanamycin. Subculturing was done at every 15 days interval on fresh medium to remove escapes.

Details of culture media used at different growth stages in cotton meristem transformation

Growth stage	Medium	Composition of the media	Duration
1. Seed germination	Germination Medium	Half MS with 2% sucrose and 0.8% agar	5-7 days
2. Shoot isolation and preculture	Shoot induction medium	MS + 0.2 mg/ benzyl adenine or 0.5 mg/l zeatin	1-2 days
3. Co-cultivation	Shoot induction medium	MS + 0.2 mg/L benzyl adenine or 0.5 mg/l zeatin	24-72 hrs
4. Reculture	Reculture medium	MS + Benzyl adenine @0.2 mg/l or 0.5 mg/l zeatin + 100 mg/l cefotaxime	1 week
5. Selection	Selection medium	MS + BA @ 0.2mg/l or zeatin @ 0.5mg/l+ 100 mg/l cefotaxime + 200 mg/l kanamycin	1-4 weeks
6. Rooting	Rooting medium	MS + 1 mg/l NAA + 0.5 mg/l IBA	2 weeks

Although varying periods of preculture, infection and cocultivation were attempted, kanamycin resistant shoots were obtained when preculture period was 48 hrs with an infection period of 30 min. and cocultivation duration of 48 hrs, hence this was adopted in large scale transformation studies.

3.4.5 Rooting (MS + NAA + IBA)

Surviving shoots were transferred to MS medium supplemented with 1 mg/l NAA and 0.5 mg/l IBA. Response to rooting was qualitatively graded based on number of primary and secondary roots and also on their length as good, better and profuse rooting.

3.4.6 Measurement of growth

In each of the replication of different treatments of an individual experiment, uniform explant tissues were inoculated and were incubated at $25\pm 2^{\circ}\text{C}$. Length of the shoot was recorded 30 days after inoculation to assess the growth and morphogenic responses such as shoot elongation, root development etc. Development of 2-3 nodes after 30 days of inoculation was considered as good response to shoot elongation.

Observations recorded

- 1) Number of explants inoculated / treatment
- 2) Number of explants responded / treatment

3.4.7 Hardening

Plantlets with a good root system were transferred to pots containing mixture of 2:1:1 of soil: sand: peat for hardening. The pots were moved to growth chamber under controlled conditions of temperature ($27\text{-}30^{\circ}\text{C}$), light intensity (Ca 2000 lux) and relative humidity (55-60%) for 2-3 weeks before keeping them in transgenic green house.

3.5 CONFIRMATION OF TRANSFORMATION

The plantlets, which survived on selection medium, were subjected to PCR analysis with *npt II* specific primers. Genomic DNA from putative transgenic plants, original cultivar and bacterial plasmid were isolated and purified as follows.

3.5.1 Isolation of plasmid DNA from *Agrobacterium* by alkali lysis method

- A single *Agrobacterium* colony was picked up aseptically using a sterile inoculation loop and was grown overnight in 10 ml YEM medium containing kanamycin (100 mg/l) in a flask by keeping on shaker at 150 rpm at $27\text{-}30^{\circ}\text{C}$ overnight.

- Overnight grown 1.5 ml culture was taken in 1.5 ml Eppendorf tubes and centrifuged at 12,000 rpm for 15 min.
- The cell pellet was collected by removing supernatant and the pellet was resuspended in 0.1 ml of solution I (Appendix -1), vortexed to disperse the pellet and kept on ice as soon as possible.
- After incubation 0.2 ml solution II was added to each tube and inverted gently 4-5 times and then placed on ice for 5 minutes.

For the above 0.15 ml of solution III (Appendix I) was added, mixed well and kept at -80°C for 20 minutes. This was melted for 3-5 minutes before centrifugation for 10-15 minutes at 12000 rpm and supernatant was transferred to a new tube.

0.5 ml of supernatant was mixed with phenol to form an emulsion, vortexed and centrifuged for 5 min at 2000 rpm. Supernatant was collected and poured to fresh 1.5 ml tubes.

To the supernatant 0.6 ml of CIA (chloroform: Isoamylalcohol 24:1) was added and centrifuged at 12000 rpm after mixing gently. Upper aqueous layer was carefully removed with 1 ml pipette as 0.2 ml aliquots.

The supernatant was treated with 0.2 ml sodium acetate and 0.6 ml isopropanol and stored at -80°C for 20 minutes or overnight in room temperature. The solution was centrifuged at 12000 rpm for 10 min and supernatant was discarded leaving behind the DNA pellet.

DNA Pellet was washed with 70 per cent ethanol @ 500 μl /tube, kept for 5 minutes at room temperature and centrifuged for 5 minutes at 12000 rpm and then 70 per cent ethanol was decanted and tubes were placed in sterile hood to dry. Then pellet was suspended in buffer and dissolved in water bath (37°C) for 20 min and stored at 4°C .

3.5.2 Isolation of genomic DNA by CTAB method

The DNA was extracted by following CTAB extraction method (Saghai-Marooof *et al.*, 1984) with required modifications.

1. One to two grams of fresh young leaf (not fully expanded and less than a week old) from shoot apex was harvested. The surface was cleaned with wet paper and towel. The sample was ground to fine powder using liquid nitrogen with prechilled pestle and mortar.
2. The powder was transferred into a sterile 2.0 ml eppendorf tube. Then added 1 ml of extraction buffer (0.35 M sorbitol, 100 μ M Tris and 5 μ M EDTA). Before using, 0.4 g/100 ml of sodium bisulphite, 1 g/100 ml of PVP and 9 g/100 ml of glucose was added in to the extraction buffer.
3. The solution was vortexed for 30-40 sec. centrifuged at 3000 rpm for 10 min.
4. Supernatant was discarded and to this 600 μ l lysis buffer, 1M Tris-HCl buffer(pH 8). 0.5 M ethylene diamine tetra acetic acid (EDTA) pH 8.0, 5M NaCl and 2 per cent (w/v)) Cetyl Trimethyl Ammonium Bromide (CTAB) and 50 μ l sarcosyl (5 %) was added.
5. The solution was vortexed for 30-40 sec.
6. The contents were incubated at 65° C in a water bath for 60 minutes with 2-3 times occasional shaking during incubation.
7. The tubes were kept for 10 min to allow them to return to room temperature.
8. Five hundred μ l of chloroform and Iso amyl alcohol (CIA) solution prepared in a ratio of 24:1, was added to the tubes.

9. The solution was vortexed for 40-60 sec and centrifuged at 8000 rpm for 10 min.
10. The aqueous supernatant solution was pipetted out and transferred to a fresh tube for DNA isolation. CIA extraction step was repeated.
11. Nucleic acids were precipitated by adding equal volume of ice cold isopropanol to the aqueous phase.
12. The solution was left for over night preservation at freezing temperature to enhance precipitation.
13. Solution was centrifuged at 8000 rpm for 5-10 minutes.
14. Supernatant was discarded (keeping pellet as it was).
15. The DNA pellet was washed with 70 per cent alcohol (400 μ l) for 30 minutes and centrifuged at 8000 rpm for 10 minutes, pellet was recovered.
16. The tubes were allowed to drain and dried at room temperature for 2-3 hours.
17. Then DNA was suspended in 500 to 700 μ l of high salt TE (Tris EDTA) buffer (10 mM Tris HCl + 1mM EDTA + 2M NaCl, pH 7.5) in sterile eppendorf tube and kept for 45 minutes at room temperature.
18. When DNA was fully suspended, 5 μ l of RNase (10 mg/ml) was added and incubated at 37°C for one hour or at room temperature overnight.
19. An equal volume of cold phenol : chloroform : isoamylalcohol (25:24:1) was added and mixed well (The molecular grade phenol buffered to pH 8 was used). The solution was centrifuged for five minutes at 14000 rpm.
20. Top layer was pipetted in to another eppendorf tube and an equal volume of chloroform : iso-amylalcohol (24:1) (v/v) solution was added and centrifuged for five minutes at 14000 rpm.

21. The top layer was pipetted in to new eppendorf tube and 0.5 volume of 7.5 mM ammonium acetate solution and two volumes of absolute ethanol was added, mixed thoroughly and centrifuged at 14000 rpm for 5 minutes.
22. DNA pellet was removed and washed twice with 250 μ l of 70 per cent ethanol. The supernatant solution was dispensed and the pellet was air dried for 10 minutes.
23. The DNA was dissolved in 200 to 500 μ l of TE (Tris-EDTA) buffer (10 mM Tris, 1 mM EDTA pH8).

3.5.3 Spectrophotometric assay of DNA concentration

Before carrying out of gene cloning or gene manipulative experiments, it is critical to know exactly how much DNA is present in the solution. DNA concentration can be accurately measured by ultraviolet absorbance spectrophotometry. The amount of uv-radiation at 260 nm absorbed by the solution of DNA is directly proportional to the amount of DNA present in the sample. Usually the absorbance is measured at wavelengths 260 and 280 nm. The reading at 260 nm allows the calculation of DNA concentration in the sample. An OD of 1 at 260 nm corresponds to 50 μ g of double standard DNA/ml of solution.

If the ratio of the absorbance at 260 nm and 280 nm($OD_{(260)}/OD_{(280)}$) is 1.8, then the sample of DNA is pure and the ratio less than 1.8 indicates that the preparation is contaminated either with phenol or proteins.

Procedure

- The spectrophotometer was first turned on for 20 minutes for the equipment to stabilize.
- 1 ml TE buffer was taken in quartz cuvette and placed in the reference slot as the reference sample and the equipment was auto zeroed.

- 10 µl of the sample DNA was taken in 990 µl of TE buffer (1x) in the other cuvette and mixed well. This was placed in the main reading slot and the absorbance at 260 nm and 280 nm wavelength was measured. 3 readings were taken and the average was calculated. Using the relationship, 1 OD at $A_{260} = 50 \mu\text{g/ml}$, the DNA concentration/ml and the concentration per µl was calculated. The ratio of A_{260}/A_{280} was also calculated to check the purity of the sample.

3.5.4 PCR amplification and analysis of amplified DNA fragments

The *npt-II* (Neomycin phosphotransferase II) gene confers resistance to kanamycin. This gene is present as a selection marker upstream of *CryIIa₅*, *CryIF* and *CryIAa₃* endotoxin genes. Presence of *npt-II* gene renders transformants be selected on the media containing kanamycin. Since both ‘Cry’ gene and *npt-II* are present with T-DNA borders, detection of either of them confirms the presence of both genes. Primers specific to *CryIIa₅*, *CryIF* and *CryIAa₃* genes and *npt-II* gene were employed to detect presence of transgene in putative transformants, nucleotide sequence of specific primers are as follows.

npt-II specific primers

Forward primer: 5¹ CAA TCG GCT GCT CTG ATG CCG3¹

Reverse primer: 5¹ AGG CGA TAG AAG GCG ATG CGC3¹

CryIF specific primers

Forward primer: 5¹ GGA GTG GGA GTG GCG TTT GGC CTG 3¹

Reverse primer: 5¹ CCA GTT TGT TGG AAG GCA ACT CCC 3¹

CryIAa₃ specific primer

Forward primer: 5¹ GGG ATG GCT AAC AAC CCA AAC 3¹

Reverse primer: 5¹ GGC AAA CTC TGG TCC AGA GAA ACC 3¹

CryI Ia5 Specific Primer

Forward Primer : 5¹ GGC AAG TTG AGA GGG CT GGT GAT 3¹

Reverse Primer : 5¹ TCC GGTA CCC AAT TCG CCC TA 3¹

3.5.5 Polymerase chain reaction

3.5.5.1 Requirements

Template DNA

50 ng template DNA of putative transformed plants and the control were used.

Primers, Taq polymerase and dNTPs

Cry1Ia₅, *Cry1F* and *Cry1Aa₃* specific primers were custom synthesized at M/S Bangalore Genei Pvt. Ltd. Bangalore. Taq DNA polymerase and 10x taq assay buffer and individual dNTPs were procured from M/S Bangalore Genei Pvt. Ltd., Bangalore.

Thermocycler

Mastercycler gradient^R of Eppendorf Ag, Barkhausenweg 1, 22339 Hamburg, Germany was used for cyclic amplification of DNA.

Gel documentation system: Herolab gel documentation system was used for gel documentation.

PCR reaction mix

The PCR reaction mixture comprises of 50 ng template DNA, 13.2 ng of primer. 2.5 mM of dNTPs, 0.33 µl (1 unit) of Taq DNA polymerase, 1x Taq assay buffer in a final volume of 25 µl.

3.5.5.2 Amplification conditions

The standardized amplification conditions were as follows.

For npt-II specific primer

Stage	Temperature (°C)	Duration (min)	Cycles
Denaturation	94	5	1
Denaturation	94	1	35
Annealing	55	1	
Primer extension	72	1	
Final extension	72	10	1
Hold	4	-	-

For CryIIa₅ specific primer

Stage	Step	Temperature (°C)	Duration (min)	No. of cycles
I	1. Initial denaturation	95	10	1
II	1. Denaturation	95	1	37 cycles
	2. Annealing	52	1	
	3. Extension	72	2	
III	1. Final extension	72	20	
	Hold	4	-	-
For CryIAa₃ specific primer				
I	1. Initial denaturation	94	5	1
II	1. Denaturation	94	1	37 cycles
	2. Annealing	50	1	
	3. Extension	72	2	
III	1. Final extension	72	20	1
	2. Hold	4		
For CryIF specific primer				
I	1. Initial denaturation	94	5	1
II	1. Denaturation	94	1	37 cycles
	2. Annealing	49	1	
	3. Extension	72	2	
III	1. Final extension	72	20	
	Hold	4	-	-

After the completion of required number of cycles of amplification the samples were stored at 4°C in a refrigerator and the contents were loaded on the agarose gel for electrophoresis.

3.5.6 Separation of PCR amplified products by agarose gel electrophoresis

Twenty five microlitres of PCR amplified products from each reaction along with 2 µl of loading dye (Bromophenol blue) was loaded on 1.2 per cent agarose gel. The electrophoresis was carried out using 1x TAE buffer (pH 8.0) (Appendix II). λDNA (EcoR-I and Hind III double digest) molecular weight marker was run in a separate lane. The DNA bands on gels were visualized under uv transilluminator and documented.

3.6 *In planta Agrobacterium* mediated transformation

Plant transformation is a key methodology that has fostered diverse forms of scientific inquiry and technology development. However, for many plant species, the generation of lines carrying stable heritable transformation events remains a technical challenge. Transformation can be labour intensive process that is plagued by low success rates, excessive somoclonal variation and the need for highly skilled practitioners. Germline transformation is the common feature that allows avoidance of tissue culture and regeneration. Transformation events occurring prior to or early in floral development of the T₀ plant would be expected to give rise to identically transformed male and female gametophytes, which upon self fertilization can produce a significant number of T₁ plants that are homozygous for T-DNA insertion (Desfeux *et al.*, 2000). Thus to derive stable transformation with high frequency, the following methodology was adopted.

The genotypes Sahana and BC-68-2 belonging to *Gossypium hirsutum* were planted at ARS, Dharwad farm on 3rd August 2003, in the transgenic green house.

3.6.1 Preparation of *agrobacterium* culture used for induction

Fortyeight hrs old *Agrobacterium* cultured on yeast extract mannitol agar (YEMA) medium containing kanamycin was used for the purpose. The bacteria were scraped from the culture plate and a slurry was made using 200 μ M acetosyringone.

In other set of experiment wherein the liquid *Agrobacterium* culture was used, 48 hrs old agroculture grown on YEMA media containing kanamycin was directly used for inoculation.

3.6.2 *In planta* transformation procedure

Flower buds, which would open the next day were chosen as pollen recipients and the entire corolla along with anthers were removed carefully without damaging the gynoecium, and the stigma of the flower. The stigmatic surface was treated with 5 per cent sucrose, boric acid at different concentrations ranging from 1-30 mg/l and combination of 5 per cent sucrose and 20 mg/l, of boric acid in independent experiments during evening hrs after the sunset. Pollen was dusted onto treated stigmatic surface on the following morning. Inoculated plants were tagged, labelled and grown in pest and disease free conditions. Seeds were harvested and sown in the next season for screening the transformants.

Different pollen tube pathway mediated transformation methods attempted to effect successful fertilization and boll setting

Method	Type of agrobacterial culture used	Time of emasculation and application of agrobacterial culture	Time of pollination
1.	Solid culture	Evening	On the same evening
2.	Liquid culture	Evening	On the same evening
3.	Solid culture	Evening	Next day morning
4.	Liquid culture	Evening	Next day morning

Under *in planta* transformation, four methods were attempted as seen in the table above. Method No. 4 involving liquid culture, pollinated during morning hours has yielded maximum boll set percentage. Therefore method 4 was employed for large scale *in planta* transformation studies.

3.6.3 Progeny test

Seeds set on the agroinoculated plants were harvested and sown under containment following recommended package of practice but under unprotected conditions. All the T₁ plants so raised were subjected to kanamycin screening. Further the kanamycin resistant plants in T₁ generation were selected and forwarded to T₂ generation. Again selection pressure was applied for kanamycin resistance among the plants of T₂ generation. Such of the seedlings which displayed a high level of resistance to kanamycin were subjected for PCR analysis and insect bioassay studies.

3.7 SCREENING OF THE TRANSFORMANTS

Kanamycin resistance encoded by the neomycin phosphotransferase-II gene (*nptII*) confers resistance to the aminoglycoside antibiotics, such as kanamycin. Kanamycin resistance is conferred by transgenic expression of neomycin phosphotransferase, the product of *npt-II* gene from the bacterial transposon Tn₅. The enzyme neomycin phosphotransferase transfers a phosphate from ATP to the aminoglycoside and thereby inactivates it. Hence kanamycin resistance was used as a criterion for screening the transformants.

3.7.1 Method I :*In vitro* kanamycin screening of the *in planta* transformed plants

Embryo axes exercised from the green bolls harvested from the *in planta* *Agrobacterium* treated plants were surface sterilized with 0.2 per cent mercuric chloride

and were germinated on half strength MS, supplemented with 200 mg/l kanamycin. The kanamycin resistant plants were potted and kept in growth chamber and transferred to pot house after hardening them for two weeks.

3.7.2 *Ex vitro* kanamycin screening methods

3.7.3 Method II

The seeds harvested from the *in planta* transformed plant were sown in the contained field and when the plants were around 90-100 days old, kanamycin spray was applied daily in sufficient quantity to just wet the surface of the leaves. The plants were sprayed for 2-3 days with 100, 200 and 300 mg/l kanamycin followed by 400 and 500 mg/l of kanamycin.

3.7.4 Method III *In solium* kanamycin screening

Cotton swab (200 mg weight) was completely saturated with different concentrations of kanamycin (*viz.*, 100, 200, 300, 400, 600, 800, 1000 and 1200 mg/l). Thus the cotton swab saturated with kanamycin weighing about 2.7 g was placed onto the surface of the primordial leaf preferably during early hours of morning. After 72-96 hrs, observation was made for mottled green sectors on leaves, burnt symptoms on margin of these leaves, while green plants bereft of such symptoms were screened and forwarded to T₂ generation.

For this purpose polythene bags of size 30 x 10 cm were filled with soil, sand and peat (2:1:1) and drenched with bavistin (0.2%) and dithane M-45 to avoid fungal contamination and other soil borne diseases and the seeds were sown. When the plants were about 30-35 days old, they were subjected to kanamycin screening by the method-III already cited earlier.

Such of the plants, showing kanamycin resistance were subjected to PCR, southern analysis and insect bioassay studies.

As method I and method III yielded promising results, they were employed for screening the large population of the *in planta* treated plants.

3.8 INSECT BIOASSAY STUDIES

For the insect bio-assay studies, larvae of *Helicoverpa armigera* (Hubner) was reared in the laboratory on artificial diet. The components of the artificial diet were as follows.

Component	Qty/l
Sorbic acid	1 g
Ascorbic acid	3.2 g
Methyl P hydroxy benzoate	2 g
Streptocyclin	0.4 g
Formaldehyde	1 ml
Agar	12.75 g
Chickpea seed	100 g
Wheat germ	8 g
Yeast	30 g

Five second instar larvae were released on the leaf bits of normal and transgenic cotton plants (T₂). Rapidly growing leaves of one month old cotton plants were selected for bioassays. The leaf bits were placed on the artificial diet in the multi cavity tissue-culture plates and larval mortality was recorded after 24 hrs, 48 hrs and 72 hrs.

In addition to the above method yet another novel method of insect bioassay was attempted.

Small glass vials of 15 ml were poured with 2 ml of two per cent agar-agar and were tightly plugged with sterile cotton. A leaf bit measuring about 10 sq mm was taken from the transformant to be tested and was placed onto the agar contained in a transparent glass vial. A second instar larva was placed with the help of a moistened camel hair brush carefully onto the leaf disc. The mouth of the vial was then sealed tightly with sterile cotton plug to avoid contamination, while it also facilitated aeration to both the larva and the leafbit. The vials were kept at 28°C temperature with 60 per cent RH and 14 hr. photoperiod (Plate 1).

Observations for mortality rate and extent of leaf damage were taken at 24 hrs, 48 hrs and 72 hrs interval. Observation on larval body weight were taken at the end of 72 hrs.

Thus the plants generated through different transformation methods, which were positive for different screening method viz., kanamycin spraying, kanamycin containing regeneration media, kanamycin cotton swab, insect bioassay, Bt-express sticks were finally subjected to PCR study, using plasmid DNA of the corresponding strain used as a positive control.

3.9 BT-EXPRESSION STRIPS

Bt-express sticks supplied by Innovative Biosciences, Nagpur, and Amarimmunodiagnosics, Hyderabad were used in this study. Plants that contain *Cry* gene express *Cry* protein residues in their tissues. These Bt-strips are lateral flow devices which protect the protein in extracts from plant samples. Each strip has a sample absorbing pad at one end indicated by the word sample and arrows. This end is to be inserted in the tissue extract. The extract moves up the pad into the clear window and reaches a second absorbant pad at the top of the strip. Leaf disc of 5 mm diameter or an embryo from matured seed was crushed in 500 µl of extraction buffer supplied by the manufacturer and was used as tissue extract for observing the Bt-expression. The strips work on the principle

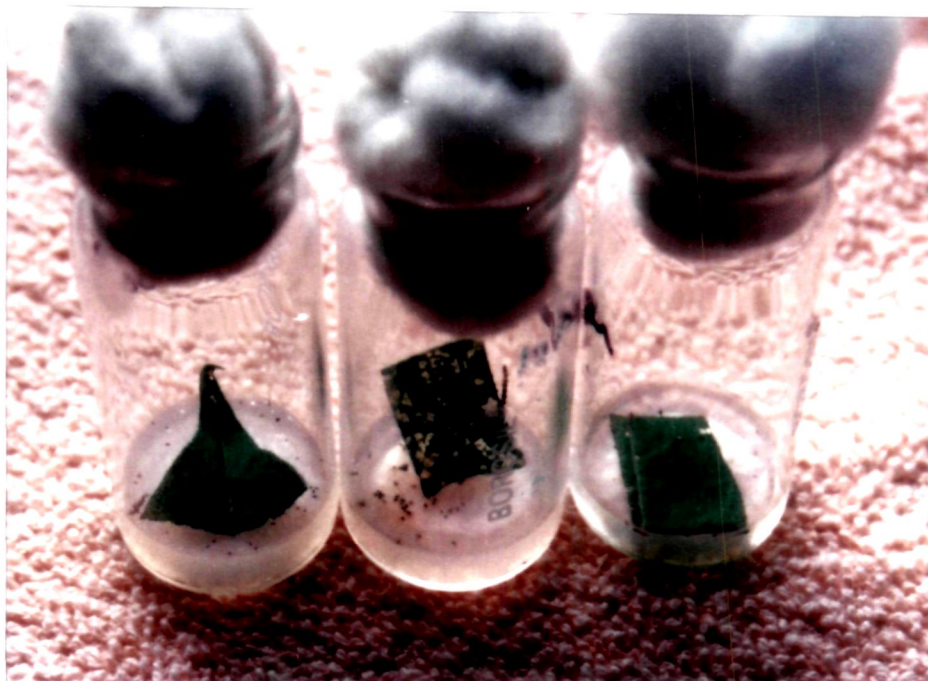


Plate 1. Method used for insect bioassay

of antigen-antibody specificity. When the specific protein was moving from the bottom to top of the strip, it will bind specifically to the antibody already impregnated on the strip showing a band within 3 min.

3.10 CALCULATION OF TRANSFORMATION FREQUENCY

3.10a *in vitro* transformation

out of the 1820 explants that were co-cultivated and survived only one was 'cry' positive in the T₁ generation, hence the transformation frequency was 0.0549 percent.

3.10b *in planta* transformation

A total of 5619 pollinations were carried out along with agroinoculating the female parent in T₀ generation and the total number of seeds harvested as a result of these pollinations was 22,861. Seven transformants were obtained, hence the transformation frequency was 0.03061 percent.

3.11 STATISTICAL ANALYSIS

The data obtained from the *in vitro* experiments that we were conducted in the laboratory, under uniform culture conditions were analysed under completely randomized design in a factorial format employing the following formula given by Gomez and Gomez (1983).

Further Z test for proportion was calculated as per Dixon and Massey (1957) employing the following formula,

$$Z = \frac{|P_1 - P_2|}{\sqrt{\hat{p}\hat{q} \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

Where P₁ = Proportion of I variable,

P₂ = Proportion of II variable

$$\hat{p} = \frac{n_1 p_1 + n_2 p_2}{n_1 + n_2}$$

$$\hat{q} = 1 - p$$

n = No. of observations

Paired t-test was employed as suggested by Steel and Torrie (1960)

$$Z = \frac{|\bar{d}|}{\sqrt{Sd^2/n}}$$

Where,

$$sd = \sqrt{\frac{\sum d^2}{n} - \left(\frac{\sum d}{n}\right)^2}$$

d = Final weight – Initial Weight

n = No. of observations

$\bar{d}$ = Mean difference in initial and final weight

The data obtained for a number of explants survived as a result of combined effect of cocultivation duration, inoculum densities of *Agrobacterium*, acetosyringone concentration were pooled and analysed as a factorial experiment to know the interaction effect between factors.

Experimental Results

IV. EXPERIMENTAL RESULTS

Numerous gene transfer methods have been developed in the past two decades for a wide range of plant species. Most of these methods are tissue culture based, requiring generation of whole plants from transformed cells. The utility of the techniques greatly depends on the establishment of tissue culture procedures in the species (Birch, 1997). Developing tissue culture independent genetic transformation system is of great interest because such systems would avoid constraints imposed by genotype specificity in transformation and regeneration and eliminate tissue culture induced genetic variation. In addition transgenic plants would be produced inexpensively and rapidly. Hence the current investigation was aimed to obtain transformants in cotton through both in *in vitro* and *in planta* methods. The results obtained are presented in this chapter.

4.1 SHOOT REGENERATION

Theoretically tissues of shoot apical meristems are suited for plant propagation and regeneration because these are programmed for shoot organogenesis. In practice, this method has a low incidence of somoclonal variation in regenerated plants (Murashige, 1974).

4.1.1 Standardization of appropriate hormonal concentration for regulating morphogenesis in explants

The composition of media used in the regeneration of shoot apical meristems with appropriate hormonal concentrations was studied and the response of explants to various levels of Benzyl adenine (BA) and Zeatin are presented in Table 1 and 2 respectively.

Among the various concentrations of BA tried along with the basal MS medium, 0.2 mg/l BA gave rise to maximum number of well developed shoots (93.5%), this was followed by 0.5 mg/l with 87 percent response. This morphogenetic response was obtained

Table 1. Response of cotton apical meristems to MS medium with various levels of BA on elongation of the shoot

Sl.No.	Media	No. of explants responded	Response (%)
1	MS basal	157.0 ^d	78.5
2	0.1mg/l BA	182.5 ^{ab}	91
3	0.2mg/l BA	187.0 ^a	93.5
4	0.3mg/l BA	174.0 ^b	87
5	0.4mg/l BA	171.5 ^{bc}	85.5
6	0.5mg/l BA	162.0 ^{cd}	81
	Mean	172.3	
	S.Em±	2.49	
	CD (1%)	8.20	

Note : A total of 200 explants were cultured under each treatment in two replications

Table 2. Response of cotton apical meristems to MS medium with various levels of zeatin on elongation of the shoot

Sl.No.	Media	No. of explants responded	Response (%)
1	0.01mg/l Zeatin	167.5 ^c	83.5
2	0.05mg/l Zeatin	173.0 ^{bc}	86.5
3	0.1mg/l Zeatin	178.5 ^{ab}	89
4	0.2mg/l Zeatin	181.0 ^{ab}	90.5
5	0.3mg/l Zeatin	181.5 ^a	90.5
6	0.5mg/l Zeatin	185.0 ^a	92.5
7	0.6mg/l Zeatin	185.0 ^a	92.5
	Mean	178.78	
	S.Em±	1.86	
	CD (1%)	6.12	

Note : A total of 200 explants were cultured under each treatment in two replications

within 15-20 days of culture. The response to shoot elongation was the least in basal MS medium (78.5%).

Further the morphogenetic response of the explants in the MS medium supplemented with various levels of zeatin was examined. Efficiency of shoot elongation of meristem in response to different concentrations of zeatin increased the response to shoot elongation. Zeatin at 0.3 and 0.6 mg/l gave better response which were statistically on par. However there was swollen appearance of the elongating shootlets at 0.6 mg/l. So, for further experimentation 0.5 mg/l of zeatin was used. In comparison with BA, zeatin served as a better growth regulator without any abnormalities in the growth and development of shootlets.

4.1.2 Rooting and plant recovery

Rooting of the shoots and plant recovery from apical meristems was direct and simple. More than 80 percent of the shoots could root spontaneously on half strength MS (Murashige and Skoog, 1962) medium. An experiment was conducted to select medium with optimum concentration of growth regulators for better root induction (Plate 2). The regenerated shoots were transferred to MS media supplemented with different auxins. The results indicated that out of two auxins (NAA and IBA) used, NAA at 1 mg/l of MS and IBA at 0.5 mg/l of MS media in combination gave good root induction. The details are furnished in Table 3. The plants thus recovered were then hardened (Plate 3).

4.2 *IN VITRO* TRANSFORMATION

4.2.1 Assessment of optimum factors affecting gene transfer efficiency during early transformation steps

4.2.1.1 Preculture

As the first step in transformation standardization of preculture duration was carried out. The sterile shoot apices were isolated and inoculated on MS-morphogenetic

Table 3. Effect of different levels of PGR supplemented MS medium on rooting of the regenerated shoots in cotton

Sl.No.	Media composition	Type of response
1	Half MS	+++
2	MS + 0.1mg/l NAA and 0.05mg/l IBA	++
3	MS + 0.2mg/l NAA and 0.1mg/l IBA	++
4	MS+1mg/l NAA and 0.5mg/l IBA	++++

++ = Good rooting
 +++ = Better rooting
 ++++ = Profuse rooting



A. Initiation of shoot bud
B. Shoot elongation and rooting

Plate 2. Regeneration of plants from shoot apical meristems of cotton



Plate 3. Hardening of *in vitro* grown plants

medium (MS+BA/zeatin) and precultured for different periods of time. Explants precultured for 24 or 48 hrs did not differ significantly with respect to their survival after cocultivation. However percent survival was reduced by 24 percent on direct cocultivation. Explants preincubated beyond 48 hours and without preculture turned brown at the cut end after cocultivation. So further preincubation was carried out for 48 hours. (Table 4).

4.2.1.2 Infection period

The precultured explants were treated with EHA105 (pBIN1a5) suspension by shaking for 30 min to 1 hour.

Initially as a first comparative experiment, the influence of duration of infection for 30 min and 60 min was studied. It was observed that percent survival of explants was more when the infection period was 30 min (53.8%), when compared to 60 min (42.4%). So for further experimentation, explants were infected with agrobacterium suspension culture for 30 min (Table 7a and 7b).

4.2.1.3 Duration of cocultivation

At the end of infection period, the explants were blotted dry by using sterile blotters and placed back on the same morphogenetic media for cocultivation for 24 to 72 hrs, cocultivation beyond 48 hrs resulted in overgrowth of the bacteria, resulting in softening and death of the tissue.

Although the effect of cocultivation for 24 hrs and 48 hrs was statistically on par (80% and 76% respectively), when the explants were cocultivated with *Agrobacterium* for less than 48 hrs, percent survival on antibiotic media was less, hence the optimum time period for cocultivation is determined to be 48 hrs (Table 5).

Table 4. Effect of preculture period on survival of cotton explants after cocultivation

Sl.No.	Duration (Hrs.) after preparation of explants	No. of explants alive	% Survival
1	Direct Cocultivation	30.5 ^b	60
2	24	42.5 ^a	84
3	48	43.0 ^a	86
4	72	44	88
	Mean	40	
	S.Em±	0.4769	
	CD (5%)	1.438	

Note : 50 explants were cultured in five replications under each treatment

Table 5. Effect of cocultivation period on the explant survivability in cotton

Sl.No.	Duration of cocultivation (Hrs.)	Response after fifteen days		Agrobacterium growth
		Alive	%Survival	
1	24	40 ^a	80	-
2	48	38 ^a	76	+
3	72	14 ^b	28	+++
	Mean	30.6		
	S.Em±	1.73		
	CD (1%)	5.70		

Note : A total of 50 explants were cultured under each treatment in two replications

- complete inhibition of Agrobacterial growth
- + slight appearance of Agrobacterial growth
- +++ prominent growth of *Agrobacterium*

To facilitate standardization of the other basic elements of the culture medium, particularly the concentration of acetosyringone and inoculum densities were determined in the subsequent experiments.

4.2.1.4 Acetosyringone concentration

When acetosyringone was excluded from the inoculation and cocultivation media, no Cry protein expression was seen. Increasing the acetosyringone concentration to 200 mM resulted in an increased number of explants, which showed Cry protein expression as indicated by Bt expression kit. There was 92 percent survival of explants when acetosyringone @ 200 mM was used. When the acetosyringone concentration was increased to 400 mM, the explants turned brown and only 44 percent of the cultured explants survived. Two of the explants showed kanamycin resistance when 200 mM acetosyringone was used and only one shootlet showed positive reaction for Bt-expression sticks. Hence acetosyringone @ 200 mM was used in subsequent experiments (Table 6).

4.2.1.5 Effect of inoculum densities of *Agrobacterium*

Optimum concentration of *Agrobacterium* culture is important because, though it may not really influence the frequency of transformation, it will surely influence the growth of explants after cocultivation. Because higher O.D leads to excess growth of *Agrobacterium*, and the elimination of it from explants becomes difficult. The explants were cocultivated in different concentration of bacterial suspension (O.D. measured at 600nm) at which the initial cell density of 0.5×10^8 cells/ml, wherein the explants survived without yellowing and death. Survival of explants was maximum (47) [Table 7(a)], when 0.2 O.D bacterial culture was used for cocultivation. Although survival of explants was less (38) when bacterial culture of 0.6 O.D was used, a single kanamycin resistant shoot was obtained at this inoculum density (0.5×10^8 cells/ml). Hence this was used for further experimentation (Table 7a).

Table 6. Effect of acetosyringone on transformation of cotton

Acetosyringone concentration (mM)	Number survived	Percent survival	No. of Kan ^r shoots	No. of shoots positive for Bt expression sticks
0	47 ^a	94	-	-
200	46 ^a	92	2	1
400	22 ^b	44	-	-
Mean	38.33			
S.Em±	0.504			
CD (5%)	1.497			
CV	3.48			

Note : 50 explants were cultured under each treatment in seven replications

Table 7(a). Interaction effect of cocultivation duration, inoculum densities and acetosyringone concentration on survival of explants for 30 min Infection Period .

O.D. of bacterial suspension (c)	24 hrs cocultivation duration (a ₁)		$\bar{X}$ (a×c)	48 hrs cocultivation duration (a ₂)		$\bar{X}$ (a×c)	Interaction (b×c)	
	Acetosyringone concentration (b)			Acetosyringone concentration (b)			$\bar{X}(b_0)$	$\bar{X}(b_1)$
	O (b ₀)	200 mM (b ₁)		O (b ₀)	200 mM (b ₁)			
0.2 (c ₁)	47	46	46.5	44	42	43	45.5	44
0.3 (c ₂)	42	40	41.0	40	37	38.5	41.0	38.5
0.6 (c ₃)	38	35	36.5	34	32 (1*)	33	36.0	33.5
0.8 (c ₄)	27	20	23.5	20	10	20	23.5	15.0
1.0 (c ₅)	10	4	7	8	2	5	9.0	3.0
Mean	32.8 (a×b) interaction	29.0	30.9 _(A)	29.2 (a×b)	24.6	26.9 _(A)	31.0	26.8
% survival of explants	65.6	58	61.8	58.4	49.2	53.8	62	53.6

Note : 50 explants were used for culture under each treatment
* 1 Kan' shoot obtained

4.2.1.6 Interaction effect of cocultivation duration, inoculum densities and acetosyringone concentration on survival of explants and early steps of transformation

Factorial analysis of the interaction effects between different factors revealed that there was significant interaction when the infection period was 30 min. However the interaction effects among cocultivation duration, bacterial inoculum density and acetosyringone concentration was not significant when the infection period was increased to 60 min.

Highest survival of explants resulted from the effect of interaction between 24 hrs cocultivation duration and 0.2 O.D of bacterial suspension (46.5). The interaction effect of acetosyringone concentration and bacterial inoculum density was also significant. This interaction effect was maximum on survival of explants when the bacterial culture with 0.2 O.D without acetosyringone was used. Lowest survival of explants was recorded when 1.0 O.D of bacterial suspension was used with 200 mM acetosyringone. However a single kanamycin resistant shoot was obtained when the explants were cocultivated with 0.6 O.D bacterial suspension for a period of 30 min. Hence this treatment was followed for a large scale transformation studies. As far as higher order interaction among cocultivation duration bacterial concentration and acetosyringone concentration was considered, no significant interaction was noticed (Tables 7a and 7b).

4.2.2 Inhibition of growth of *A. tumefaciens*

The cultured explants need to survive and develop healthy shoot and root system without being contaminated by overgrowth of bacteria. Hence the cefotaxime levels from 0 to 500 mg/l was tested for elimination of bacteria without any adverse effect on plantlet growth. All the five cefotaxime levels differed significantly with respect to inhibition of agrobacterial growth. Growth of *A. tumefaciens* was inhibited even at 300 mg/l, but started

Table 7(b). Interaction effect of cocultivation duration, inoculum densities and acetosyringone concentration on survival of explants for 60 min Infection Period

O.D. of bacterial suspension (c)	24 hrs cocultivation duration (a ₁)		$\bar{X}$ (a×c)	48 hrs cocultivation duration (a ₂)		$\bar{X}$ (a×c)	Interaction (b×c)	
	Acetosyringone concentration (b)			Acetosyringone concentration (b)			$\bar{X}(b_0)$	$\bar{X}(b_1)$
	O (b ₀)	200 mM (b ₁)		O (b ₀)	200 mM (b ₁)			
0.2 (c ₁)	42	40	41	38	34	36	40	37
0.3 (c ₂)	36	32	34	32	28	30	34	30
0.6 (c ₃)	34	31	32.5	31	29	30	32.5	30
0.8 (c ₄)	10	8	9	8	6	7	9	7
1.0 (c ₅)	4	3	3.5	4	2	3	4	4.5
Mean	25.2 (a×b) interaction	22.8	24.0	22.6 (a×b)	19.8	21.2	23.9	21.7
% survival of explants	50.4	45.6	48	45.2	39.6	42.4	47.8	43.4

Source	S.E.	C.D.(5%)	C.D.(1%)
a	0.317	0.939	1.284
b	0.317	0.939	1.284
c	0.502	1.485	2.03
a×b	0.449	NS	NS
a×c	0.710	2.100	NS
b×c	0.710	NS	NS
a×b×c	1.004	NS	NS

Note : 50 explants were used for culture under each treatment

reappearing at the rate of nine percent with the advance in culture period. Cefotaxime @ 400 mg/l was added to the culture medium routinely in the experiments. As and when there was reappearance of growth of agrobacterium, the explants were washed with cefotaxime contained in liquid MS, blot dried and subcultured on (MS+Zeatin+C_i) medium (Table 8).

4.2.3 Determination of kanamycin sensitivity

Kanamycin was used as a selective agent to pick up the transformed plants *in vitro*. In order to avoid the possibility of selecting the untransformed plants on medium containing Kanamycin, the intrinsic kanamycin resistance of cotton shoots was determined.

In this experiment various levels of kanamycin were used at increasing concentrations. kanamycin at 50 mg/l was the lowest concentration used. The next levels of concentration of kanamycin were 100, 200, 300, 400 and 500 mg/l. kanamycin concentration at 200mg/l and higher levels resulted in immediate death of explants (Table 9). This conclusion was drawn by observing browning of the explants placed on the medium containing 200mg/l of kanamycin after two weeks of incubation (plates 4, 5A and 5B).

4.2.3.1 Selection of the putative transformants

Out of 1820 successfully cocultivated and regenerated plants established *in vitro*, 65 of them were shifted to green house and only 13 of them showed kanamycin resistance (0.714%) (Table-10). Analysis of these primary transformants for the presence and expression of Cry-genes revealed that only one plant (0.0549%) was positive for PCR and southern (Table 11).

Table 8. Sensitivity of *Agrobacterium tumefaciens* to various levels of cefotaxime on the cotton shoot apical meristems

Sl.No.	MS + BA + Ci (mg/l)	Reappearance of agrobacterium in explants	% Reappearance Response	Growth of Agrobacterium
1	0	50 ^a	100	++
2	100	34 ^b	68	++
3	200	21 ^c	42	++
4	300	4.5 ^d	9	+-
5	400	2.5 ^e	5	--
6	500	0 ^f	0	--
	Mean	18.6		
	S.Em±	0.45		
	CD (5%)	0.94		
	CD (1%)	1.47		

Note : 50 explants were cultured under each treatment in two replications

Table 9. Effect of different concentrations of kanamycin on the explants of cotton

Sl.No.	Treatment Kan. mg/l	Explants after two weeks
1	Control	+
2	50	+
3	100	+
4	200	-
5	300	-
6	400	-

+ = Survival

- = Death

Table 10. Large Scale *in vitro* transformation studies using *cry 1 Ia5* gene in cotton

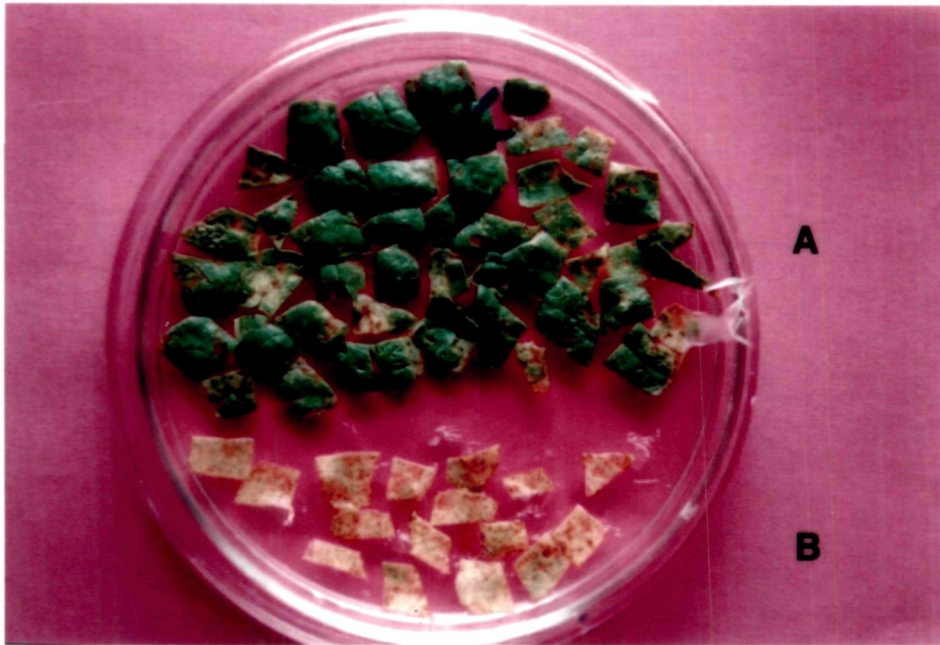
Sl.No.	Genotype	No. of explants cocultivated	Number of explants died ¹	Number of explants survived*	Number of plantlets transferred to growth chamber	No. of plants transferred to green house	No. of Kan ^r . plants
1	Sahana	2300	498	1802	65	63	11
2	BC-68-2	500	482*	18	13	2	2
	Total	2800	980	1820	78	65	13

¹ : Lost due to media contamination 3 months after cocultivation

* : Seeds of BC-68-2 were infected by endophytic bacteria, hence there was poor survival

Table 11. Gene integration studies in T₀ (putative) transgenic plants

Cultivar	No. of well established plant	No. of plants Kan ^r	PCR positive	Southern positive	Transformation frequency
Sahana	1802	11	1	1	0.0549%
BC-68-2	18	2	-	-	
Total	1820	13	1	1	



- A. Kanamycin resistant
B. Untransformed kanamycin treated plants

Plate 4. Leaf discs of transformed cotton plants



Plate 5a. *In vitro* response of transformed and untransformed cotton plants to kanamycin



Plate 5b. Later stages of the growth of the transformed plants

4.2.4 Confirmation of gene integration

PCR Amplification:

To look for the presence of the transgenes, amplification of *npt-II* gene was done through PCR.

The genomic DNA from the putatively transformed plants as well as the control plants and plasmid DNA from the *Agrobacterium* was isolated. Plasmid DNA served as a positive control. DNA from control untransformed plant was used as a negative control. The primer specific for the *npt-II* gene was used in the PCR analysis. The PCR products were electrophoresed on a 1.2% agarose gel using 1kb ladder as the marker (Plates 6, 7 and 8). An approximately 700 base-pair band of the *npt-II* gene was obtained. The untransformed control plants naturally did not produce any bands.

Although eleven plants belonging to Sahana cultivar were resistant to kanamycin, only one was PCR positive. Neither of the two kanamycin resistant BC 68-2 plants were PCR positive (Table-11).

4.2.5 Expression studies

4.2.5.1 Bt expression sticks

The Bt expression sticks designed to recognize the Cry protein in seed and leaf tissue extracts were used to study the expression of Cry proteins. As per the instruction of the manufacturers these are known to impart cross reactivity without causing false positive results. Hence, Cry1Ac sticks were used to detect the expression of the Cry1 Ia5 carrying cotton transformants. The details are furnished in Table 12. Although five plants showed positive reaction for Bt-expression sticks, four of them showed chimeric expression and the T₁ progeny of only one plant showed consistently positive response for Bt-expression (Table 12).

Table 12 . *Cry1 I_{a5}* gene expression studies using Bt-expression sticks in cotton

No. of <i>in vitro</i> Kan ^r plants	No. of Bt. stick positive plants
11	5

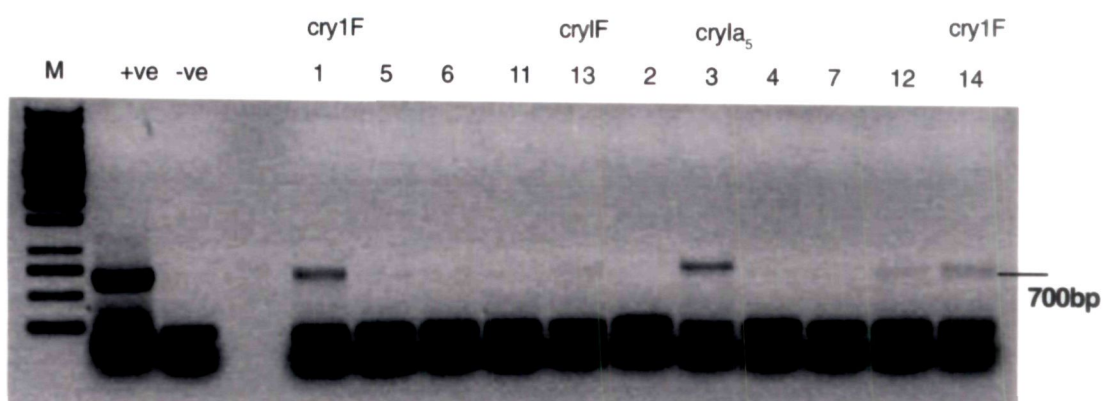


Plate 6. *nptII* amplifications of the transformed plants M;
1Kb Marker lane, 1, 3, 12 and 14 lanes show positive amplification;
+ plasmid DNA, - Untransformed plant

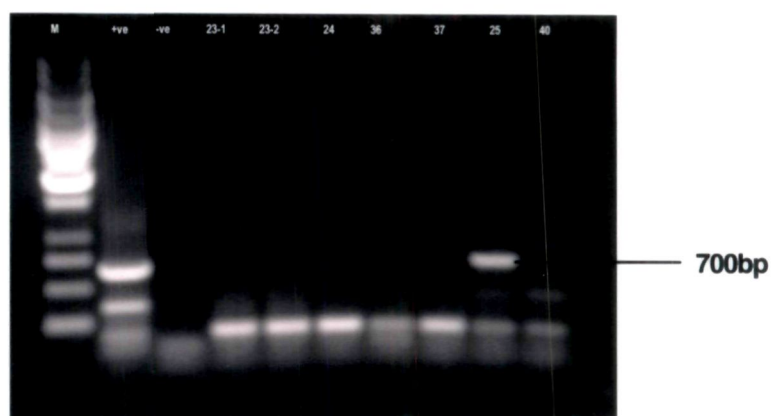


Plate 7. *nptII* amplifications of the *cry1Aa₃* transformed plants
M; 1Kb Marker lane, Lane 25 positive amplification,
+ plasmid DNA, - Untransformed plant



Plate 8. *nptII* amplifications of the *cry1Ia₅* transformed plants
M; 1Kb Marker lane, Lane 28, 30 show positive amplification,
+ plasmid DNA, - Untransformed plant

4.3 IN PLANTA AGROBACTERIUM MEDIATED TRANSFORMATION

There are different methods available for *in planta* transformation. These are advantageous over *in vitro* transformation methods as they are bereft of inherent problems associated with tissue cultural procedures. With a view to harness these benefits, *in planta* cotton transformation was attempted in this study.

4.3.1 Identification of efficient methods of pollen tube pathway transformation in which effective fertilization occurs

Forty eight hours old *Agrobacterium* solid and liquid cultures with 200 mM acetosyringone were used to identify a suitable method, which facilitates effective fertilization leading to good boll set. Out of the four methods attempted to see the efficiency of PTP transformation, method 4 yielded significantly higher percent of bollset (28.5%), followed by method 3 (21.0%) and method 1 yielded the least boll set percent (3.25%). The details are furnished in Table 13.

4.3.2 Effect of application of sucrose to stigmatic surface on the boll set

Various methods attempted to achieve successful boll setting with direct application of agrobacterial culture to stigmatic surfaces resulted in very poor boll setting (6.7%). 93.3 percent of the pollinations were abortive, the size of the bolls was also extremely small and they were shed 3-4 days after fertilization, so the stigmatic surface was treated with 5 percent sucrose solution, which resulted in significantly higher boll set percent (23.5%). The results are presented in Table 14.

4.3.3 Sole effect of boron and combined effect of application of boron and sucrose solution to stigmatic surface on the boll set

Five different concentrations of boron viz., 1, 5, 10, 20 and 30 mg/l of boric acid were applied to the stigmatic surface prior to application of *Agrobacterium*. DMRT

Table 13. Effect of type of *Agrobacterium* culture and time of pollination on pollen tube pathway transformation in cotton

Method	Type of <i>Agrobacterium</i> culture used	Time of emasculation and application of <i>Agrobacterium</i>	Time of pollination	No. of bolls set	boll Set (%)
1	Solid culture	Evening	On the same evening	13.0 ^d	3.25
2	Liquid culture	Evening	On the same evening	22.5 ^c	5.00
3	Solid culture	Evening	Next day morning	84.0 ^b	21.0
4	Liquid culture	Evening	Next day morning	114 ^a	28.5
		Mean		58.37	
		S.Em±		1.38	
		CD (1%)		4.53	

Note : 400 pollinations were carried out in two replications

Table 14. Effect of application of sucrose to stigmatic surface on the boll set in cotton

Sl.No.	Treatment	No. of pollinations carried out	No. of bolls set	Success of pollination (%)
1	Without Sucrose	400	27	6.7
2	With 5% Sucrose	400	94*	23.5

* Significant at 1 per cent based on z-test for proportion

analysis revealed that application of 20 mg/l of boric acid resulted in significantly higher success of pollination (24%), followed by 30 mg/l, which yielded 20.5 per cent boll set. Least pollination success was observed (7.5%) when 1 mg/l of boric acid was used (Table 15).

Further the combined effect of boron and sucrose was investigated in another set of independent experiments. Treatment of stigma with 5 per cent sucrose along with 20 mg/l boric acid resulted in further improvement in bollset (32.5%). The results are presented in Table 16. Further in the large scale *PTP* mediated transformation studies, this strategy was adopted.

4.3.4 Details of the pollen tube pathway mediated transformation studies

A total of 5619 flowers were treated, out of which 1858 flowers were treated with agrobacterial culture harbouring pCAMBIA AC, 2068 with *CryI Ia5*, 804 with *CryI Aa3* and 889 with *CryI F* gene constructs. The boll set ranged from 23.1 to 29.9 per cent. This low rate of boll set was due to combined effect of abortive pollinations and insect damage. Out of the total of 22,861 seeds harvested, only 6954 seeds germinated and it accounts to barely 30.4 per cent (Table 17).

4.3.5(a) Response of cotton for kanamycin under field conditions

Out of 25 plants that were treated with various concentrations of kanamycin an average of 15 plants (60%) which were treated with kanamycin @ 100 mg/l, showed single minute chlorotic spec on III leaf. With the increase in kanamycin concentration to 200 mg/l, there was a slight increase in size of the chlorotic patch and 19 plants, which accounts for 72 per cent of the treated plants showed such a symptom. Further on increasing the kanamycin concentration from 300-1200 mg/l, the response manifested as mottled green symptoms on leaf was statistically on par, ranging from 92-96 per cent.

Table 15. Effect of application of boron to stigmatic surface on the boll set in cotton

Sl.No.	Treatment with boric acid (mg/l)	No. of bolls set	boll set (%)
1	1	15 ^d	7.5
2	5	18 ^{cd}	9
3	10	23.5 ^c	11
4	20	48 ^a	24
5	30	40.5 ^b	20.5
	Mean	29.0	
	S.Em±	1.44	
	CD (1%)	4.74	

Note : 200 pollinations were carried out under each treatment in two replications

Table 16. Combined effect of application of boron and sucrose to stigmatic surface on the boll set of cotton

Sl.No.	Treatment	No. of pollinations carried out	No. of bolls set	Success of pollination (%)
1	5% Sucrose + 20mg/l Boric acid	200	63	32.5

Table 17. Large scale pollen tube pathway transformation studies in cotton

Sl.No.	Gene construct	No. of pollination	No. of abortive pollination	No. of bolls dropped due to insect damage	No. of bolls formed	Percent boll formation	Total No. of seeds harvested (T ₁)	No. of plants established	Germination(%)
1	<i>P C_{AMBIA} Ac</i>	1858	880	421	557	29.9	6684	2531	37
2	<i>CryI I_{a5}</i>	2068	1063	526	479	23.1	8622	2174	26.2
3	<i>CryI A_{a3}</i>	804	458	118	228	28.3	4104	1265	30
4	<i>CryIF</i>	889	492	194	203	22.8	3451	984	28
	Total	5619	2893	1259	1467	26.10	22861	6954	30.4

However there was absolutely not much difference between the response of plants that were treated with kanamycin @ 200 and 300 mg/l. At the highest concentration of kanamycin of 1200 mg/l, there was severe mottling on leaves (Table 18 and Plates 9 and 10 and 11).

4.3.5 (b) In *solium* selection for cotton transformants resistant to kanamycin

In order to assess the response of cotton plants to various concentrations of kanamycin in the field, cotton swab saturated with various concentrations of kanamycin viz., 100, 200, 300, 400, 600, 800 and 1200 mg/l was placed on the primordial leaf of the plant to be screened and observation was taken on the fifth day of treatment. Bt positive plants showed kanamycin resistance by remaining green despite the application of kanamycin @ 300 mg/l (Plate 12). The untransformed plants, which could not resist the kanamycin showed mottled green symptoms on the leaf surface (Plate 10). However the transformed plants, which were treated with kanamycin @ 1200 mg/l did not show absolute kanamycin resistance, nevertheless a spec of mottled green colour was noticed on the surface of leaf (Plate 13).

4.3.6 Details of *in vitro* kanamycin screening of the *in planta* transformed plants

About 2358 immature seeds were harvested from T₁ plants that showed kanamycin resistance in the field, their embryo-axes were exercised and subjected to *in vitro* kanamycin resistance and four of them developed into healthy seedlings and were maintained in growth chamber and later transferred to green house and among them only one was found to be PCR positive (Table 19).

4.3.7 Kanamycin screening results

In T₀ generation, no selection pressure for kanamycin resistance was imposed. All the 22861 seeds harvested from the bolls set on T₀ plants were sown in the field under

Table 18. Response of cotton for kanamycin under field conditions

Treatments* (mg l ⁻¹ Kan)	R ₁	R ₂	R ₃	R ₄	$\bar{X}$	% Response	Remarks
Control (0)	0	0	0	0	0	0	Chlorotic symptoms are not noticed
100	14	16	18	14	15.5 ^c	60	Single minute chlorotic spec on III leaf
200	17	18	17	19	17.75 ^b	72	Very small chlorotic spec on III leaf
300	24	22	23	24	23.25 ^a	92	Chlorotic patch on III leaf
400	23	24	22	24	23.25 ^a	92	Chlorotic patch on III & IV leaf
600	24	24	22	24	23.5 ^a	96	mottled green leaves
800	24	22	24	25	23.75 ^a	96	mottled green leaves
1200	25	24	25	22	24.00 ^a	96	severe mottling on leaves
S.Em±					0.6099		
L.S.D.					1.794		

Note : * 25 plants were subjected to kanamycin treatment

Table 19. *In vitro* Kanamycin screening of the *in planta* transformed cotton plants

No. of embryoaxes subjected for kanamycin screening	No. of kan ^r plantlets	No. of healthy plantlets transferred to growth chamber	No. of PCR +ve plants
2358	14	4	1



**Plate 9. Response of untransformed cotton plant to
kanamycin @ 200 mg/l**



**Plate 10. Response of untransformed cotton plant to
kanamycin @ 300 mg/l**



Plate 11. Response of untransformed cotton plant to kanamycin @ 1200 mg/l



Plate 12. Response of untransformed cotton plant to kanamycin @ 300 mg/l



Plate 13. Transformed cotton plant showing mottled green speck to kanamycin @ 1200 mg/l

containment. Only 6954 seeds, which were perfectly germinated and developed into well established plants were grown under unprotected conditions with protective irrigation (Table 17). When the seedlings were 90 days old, they were subjected to kanamycin screening. Around 521 plants were found to exhibit kanamycin resistance. Out of 521 plants, 189 plants carried *CryI AC* and 162 plants carried *CryI Ia5* genes. 94 plants carried *CryI Aa3* and 76 plants were with *CryI F* genes. From these 521 plants 5629 healthy seeds were harvested and were forwarded to T₂ green house. Out of these only 1199 seeds germinated and it accounts for only 21.06 per cent germination (Table 20).

4.3.8 Summary of PTP transformation in cotton

All the 1199 seedlings which exhibited resistance to kanamycin were further analysed using both PCR and Southern blot analysis. The overall transformation frequency was 0.58 per cent. From the gene integration study it was clear that out of the seven transformants obtained from *in planta* method. Four plants carried *CryI Ia5* genes, one transformant had *CryI Aa3* gene and two plants were transformed with *CryIF* gene (Table 21).

4.3.9 Insect bioassay studies

4.3.9.1 Larval mortality of *Helicoverpa armigera* in leaf bit bioassay

Young T₂ plants of about 60 days old were subjected to expression studies of Cry proteins using insect bioassay. The plants which were shown to be positive for PCR were assessed for their tolerance to second instar larvae of *Helicoverpa armigera*. Larval mortality was recorded in order to test the influence of Cry protein ingestion on larvae. Survival bioassay on detached leaf bits showed significant larval mortality ranging from 81-90 per cent (Table 21). The highest larval mortality of 90 percent was obtained on transgenics BT₂, BT₄ and BT₆. The damage of young leaves due to larval feeding was

Table 20. Screening of T₁ generation cotton plants with kanamycin resistance

Sl.No	Gene construct	No. of T ₁ plants treated with kanamycin	No. of T ₁ plants found to be kanamycin resistant	No. of healthy seeds harvested	No. of plants established	Germination (%)
1	<i>PCAMBIA Ac</i>	2531	189	2042	411	20.1
2	<i>CryI I_{a5}</i>	2174	162	1971	385	19.5
3	<i>CryI A_{a3}</i>	1265	94	733	186	25.3
4	<i>CryIF</i>	984	76	946	217	22.9
	Total	6954	521	5692	1199	21.06

Table 21. Frequency of T₂ generation cotton transformants obtained by pollen tube pathway transformation in cotton

Sl.No.	Gene construct	No. of plants established	No. of Kan ^r plant	No. of PCR +ve plants
1	<i>P C_{AMBIA} Ac</i>	411	-	-
2	<i>CryI I_{a5}</i>	385	38	4
3	<i>CryI A_{a3}</i>	186	21	1
4	<i>CryIF</i>	217	29	2
	Total	1199	88	7

restricted to shot hole size compared to the wild type, on which there was voracious feeding (Plates 14A and 14B).

4.3.9.2 Effect of cry proteins in larval body weight

Observations made on the growth and development of larvae fed on the transgenic leaves were assessed based on their body weight. There was drastic reduction in body weight of the larvae fed on the transgenic leaf bits. The larvae fed on transgenic leafbits were severely stunted in growth, when compared to larvae fed on the wild type of leaf. This was reflected in terms of increment or decrement in body weight of the larvae fed respectively on non transformed negative control and the transgenic plants. There was significant increase in the final body weight of the larvae fed on negative control (111.55%). Where as the larvae fed on leaf bits of transgenic plants showed significantly decreased body weight (56.36%). The results are furnished in Tables 22(a) ,22(b) and 23.

Table 22. (a) Larval mortality of *Helicoverpa armigera* in leaf bit bioassay

Sl.No.	Sample	Genotype / gene construct	No. of insects used for bioassay	Larval mortality after 72 hrs	Corrected larval mortality %	Nature of damage
1	Positive control	RCH-2Bt	25	24	95	Shot hole
2	Negative control	DCH-32	25	3	0	Voraciously fed
3	Plant-1 BT ₁	Cry1 I _{a5}	25	22	86	Shot holes
4	Plant-2 BT ₂	Cry1 A _{a3}	25	23	90	Shot holes
5	Plant-3 BT ₃	Cry1 I _{a5}	25	21	81	Shot holes
6	Plant-4 BT ₄	Cry1 F	25	23	90	Shot holes
7	Plant-5 BT ₅	Cry1 I _{a5}	25	22	86	Shot holes
8	Plant-6 BT ₆	Cry1 F	25	23	90	Shot holes
9	Plant-7 BT ₇	Cry1 I _{a5}	25	22	86	Shot holes
10	Plant-8 BT ₈	Cry1 I _{a5}	25	22	86	Shot holes

$$\text{Corrected Larval Mortality} = \frac{\text{percent mortality in transgenics} - \text{percent mortality in control}}{100 - \text{percent mortality in control}} \times 100$$

Table 22 (b) Change in *Helicoverpa* larval body weight consequent to feeding on leaves of non Bt and Bt transgenics

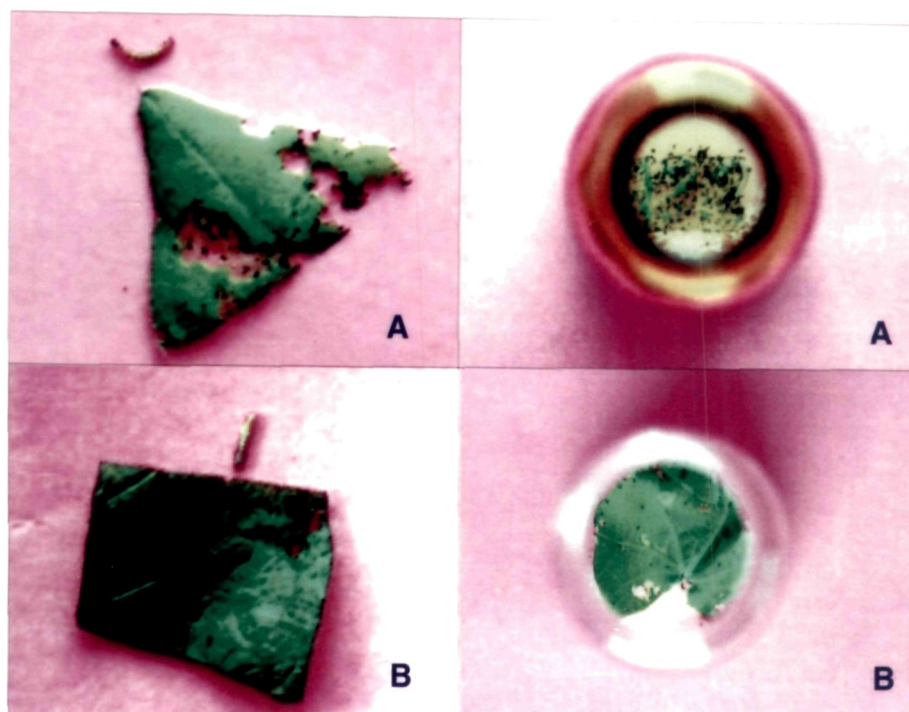
Repli- cation	Initial larval weight before feeding (in mg)	Final larval wt. 72 hrs after feeding (Bt -ve control)	Final larval wt. 72 hrs after feeding (Bt +ve)	% increment in larval body wt. of - ve control	% decrement in larval body wt. of Bt +ve
1	2.8	5.3	1.22	89.2	-56.0
2	2.4	4.9	1.17	104.1	-51.25
3	2.7	6.3	1.02	133.3	-62.22
4	2.9	6.4	1.18	120.6	-59.31
5	2.7	5.8	1.32	114.8	-51.11
6	2.3	4.9	1.12	113.04	-51.30
7	2.9	6.7	1.08	131.03	-62.75
8	3.2	6.9	1.28	115.62	-60.0
9	2.8	5.4	1.21	92.8	-56.78
10	2.4	5.1	1.02	112.5	-57.50
11	2.3	4.8	1.01	108.6	-56.08
12	2.9	6.1	0.98	110.3	-66.20
13	2.1	4.2	0.92	100.0	-56.19
14	3.4	6.8	2.02	100.0	-40.58
15	2.9	6.6	1.21	127.5	-58.27
Total	40.7	86.2	17.76	1673.39	-845.54
$\bar{X}$	2.71	5.74	1.18	111.55	-56.36

Table 23. Change in mean body weight of larvae subjected to insect bioassay studies

Sl.No.	Details of treatment	Weight in mg*	Percent increment or decrement in larval body weight
1	Initial larval weight (Before feeding)	2.71	+111.55
2	Final larval weight (negative control) (72 hrs. after feeding)	5.74**	
3	Final larval weight (Bt positive) (72 hrs. after feeding)	1.18**	-56.36

* Mean body weight of 15 larvae

** Significant at 1 percent based on paired t-test



A. Untransformed

B. Transformed

Plate 14. Damage of transformed and untransformed leaf bits by II instar larvae of *Helicoverpa armigera*

Discussion

V. DISCUSSION

Transformation of crop plants with desired genes is the focus of many plant genetic engineering programmes and several techniques to introduce foreign genes into plants have been developed. Recent advances in recombinant DNA technology combined with classical plant breeding techniques may prove useful to revolutionize plant improvement. To the delight of a plant breeder, there is no barrier for flow of genes across biological systems. Gene transfer techniques serve as powerful tools for targeting and characterizing potentially useful plant genes and gene regulatory sequences required for tailored expression of introduced genes in transgenic plants.

Plant genetic transformation was initiated and developed during the eighties. Convergence of constant progress in protocols for regeneration from tissue culture and molecular techniques leading to well expressed marker genes after transfer in plant cells and the diversification of DNA- delivery methods have served as an important tool for genetic improvement of agronomically important crop species.

A basic requirement for a successful gene transfer system for producing transgenic plants is the availability of a target tissue made up of a large number of regenerable cells that are accessible to the gene transfer treatment and that will retain the capacity for regeneration (Birch, 1997). There are several techniques for genetic manipulation, however certain procedures are specific to some crop species. Stable integration and expression of foreign genes in plants essential for crop improvement. In spite of several methods developed for transformation of plants, those that use *Agrobacterium tumefaciens* are still preferred. *Agrobacterium* mediated transformation is an excellent system for introducing genes into dicot plants, the integration of DNA in this method is relatively a precise process and transgene is stable in these cases.

Although cotton has been transformed by *Agrobacterium* mediated systems and plants have been subsequently regenerated (Firoozabady *et al.*, 1987). Commercially important cultivars have proven very difficult to regenerate due to the inability to generate embryogenic cell lines. Consequently *Agrobacterium* mediated transformation of cotton has been limited to those specific cultivars that can be regenerated by tissue culture. To circumvent difficulties with cultivar dependent regeneration, shoot apical meristem culture, which is genotype independent, was originated for cotton (Gould *et al.*, 1998). The shoot apical meristem has been widely used for cotton transformation and represents the only type of explant, which allowed recovery of stable transformants so far (Weber *et al.*, 2000) from any genotype. The protocol adopted in the current study uses transformation competent cells in the shoot apex of germinating seedlings for *Agrobacterium* mediated transformation and for plant driven regeneration. Mature seed is used because it is readily available and easily germinated when needed. Isolation and inoculation of shoots is followed by regeneration of normal fertile plants that flower and set viable seed.

Developing tissue culture independent genetic transformation systems is of great interest because such systems would avoid constraints imposed by genotype specificity in transformation and regeneration and eliminate tissue culture induced genetic variation.

Early stages of the revolution that transformed *Arabidopsis* transformation were carried out by Feldman and Marks (1987). They applied *Agrobacterium* to *Arabidopsis* seeds, grew plants to maturity in the absence of any selection, then collected progeny seeds and germinated them on antibiotic containing media to identify transformed plants. Although the procedure was difficult to reproduce consistently, successful rounds produced transformants at a high enough and rate that thousands of transformed lines were produced in a matter of few years. Other laboratories later succeeded in generating transformed *Arabidopsis* lines by “clip ‘n squirt” method (Chang *et al.*, 1994).

Reproductive inflorescences were clipped off, *Agrobacterium* was applied to the center of plant rosette, plants were then allowed to set seed.

Bechtold *et al.* (2000) and Desfeux *et al.* (2000) have obtained transformants by crossing *Agro* inoculated pollen recipients with non inoculated pollen donors. The *Agrobacterium* mediated floral dipping method developed for model plant *Arabidopsis thaliana* has greatly impacted biological research of that species. Use of pollen-tube pathway to deliver foreign DNA into embryo-sacs was first reported in cotton (Zhou *et al.*, 1983). Most initial reports used total genomic DNA for transformation. In view of the above, current investigation was aimed at developing transgenic cotton using both *in vitro* and *in planta* transformation protocols and the findings are discussed under the following heads.

5.1 *IN VITRO* TRANSFORMATION

5.1.1 Factors influencing transformation of cotton shoot apical meristems

Hormonal balance is a key factor in regulating morphogenesis in explants. The positive effect of growth hormones has already been observed on cotton shoot apical meristems (Gould and Cedeno 1998). Inclusion of cytokinin in the culture medium is to promote shoot growth. In this investigation low concentration of cytokinin was found to be more effective than high concentration for shoot induction. Among thirteen concentrations of growth regulators tried, BA@ 0.2 mg/l and zeatin at 0.5 mg/l were found to be optimum for inducing shoots. This hormonal concentration produced 93.5 and 92.5 per cent shoot induction respectively. Similar observations were made by Satyavathi *et al.* (2002) but reported cent per cent shoot induction. However, more vigorous and healthier shoots were obtained by the use of zeatin. Nevertheless the shoots induced by benzyl adanine in the medium were swollen in appearance initially but produced normal shoots subsequently.

Gould *et al.* (1998) have obtained rooting in the shoots by using MS. Media containing three per cent activated charcoal. They have also reported the use of rootone, a rooting hormone powder to induce rooting in non-rooted shoots. Another alternative suggested by them is to graft the shoot onto a germinated seedling of the same variety. In our study profuse rooting was obtained with the use of MS-media containing 1 mg/l of NAA together with 0.5 mg/l IBA.

Transformation is a complex multi-event process. Number of factors are known to affect transformation frequency viz., precultivation of explants, co cultivation period, inoculum density of the bacterium, Use of phenolic compounds like acetosyringone, post co cultivation wash with bactericide are considered critical. Use of precultured shoots in the inoculation step is to ensure activation of cell division in apical meristematic tissues. The *in vitro* transformants obtained in this study were from the explants precultured for 48 hrs before cocultivation with *Agrobacterium tumefaciens*. The results show that the explants precultured were more suitable for integration of transgenes as they would provide a higher probability for transformed cells to initiate division. Gould *et al.* (1998) also opine that presence of actively dividing cells (transformation competent cells) in the shoot apex are important in *Agrobacterium* mediated transformation.

The positive effect on expression of cry proteins in a medium containing zeatin @ 0.5 mg/l has also been observed. Cytokinins are supposed to improve uptake of DNA due to a stimulation of cell division in the pre cultivation medium, and mitotic cells are more susceptible to *Agrobacterium*. These observations are in agreement with Moralejo *et al.* (1998). However, the physiological status optimal for DNA uptake is not necessarily optimal for integration of foreign DNA in the host genome. Tinland (1996) opines that infact division of cells would not be sufficient for transformation and integration may primarily depend on availability of DNA repair enzymes. These proteins, although not yet identified are thought to play a key role in the last steps of T-DNA integration. According

to Moralejo (1998), DNA uptake has often been suggested to be cell-cycle dependent, whereas, DNA integration may primarily rely on the availability of DNA repair enzymes.

The periods of cocultivation differ according to plant species in the literature. Longer periods of cocultivation seem to be effective for efficient transfer of the T₁ plasmid to plant cells. Cao *et al.* (1998) suggested that low efficiency of transformation in earlier work on apples was due to insufficient length of cocultivation. Prolonged period of cocultivation from 2 to 4 days increased GUS expression by almost 100 fold. In the current study, the optimum time period for cocultivation was determined to be 48 hrs. Cocultivation beyond this period, resulted in softening, browning and death of the explants and the survival was very poor. Among different days to cocultivation based on resistant shoot production on media containing hygromycin, Muthukumar *et al.* (1996) have observed overgrowth of bacterium beyond three days of cocultivation. Cervera *et al.* (1998) reported that the 5-day culture period resulted in over growth of *Agrobacterium* and a subsequent decrease in regeneration frequency of the transformed shoots. Therefore in our study, the effects of varying periods of cocultivation was studied and found that 48 hrs was optimum.

Titer of bacterium is another factor that is known to affect transformation frequency. Minimum effective titer of bacteria (0.5×10^8 cells/ml) is very important for inoculation. High titers usually result in overgrowth of bacteria. Washing the explants after cocultivation and also later with cefotaxime @ 300 mg/l contained in liquid MS at definite intervals was essential to check the growth of *Agrobacterium*, which otherwise usually developed on the explants. These findings are in agreement with those of Momtaz *et al.* (1998) who also maintained a low titer of bacteria for inoculation.

Certain phenolic compounds produced by dicots are known to stimulate transformation. Acetosyringone, a phenolic compound, is known to increase virulence of

Agrobacterium. Cotton shoot apical meristems do not release phenolic compounds or compounds or other signal molecules in sufficient quantities so as to elicit *vir* gene induction. With the inclusion of acetosyringone, it is likely that signal molecules are sufficiently released to elicit *vir* gene induction. When acetosyringone was excluded from the inoculation and cocultivation media, no cry protein expression was seen. The putative transformants, which were kanamycin resistant, were all treated with 200 mM of acetosyringone. When this concentration was increased to 400 mM, the explants turned brown, indicating harmful effect on TDNA transfer. This may reflect the observation that while acetonyringone is effective at low concentration in inducing TDNA transfer, it may act as bacteriostatic at higher concentration.

Bondt *et al.* (1994) have reported that low cytokinin concentration in the media resulted in higher *gus A* expressing zones per leaf than for the media with high auxin content. Our findings are also in line with this fact and the transformant obtained was grown in media with 0.2 mg/l of BA. Lower concentrations of cytokinins in the culture media seem to stimulate cellular division and active mitotic cells are supposed to be more susceptible to *Agrobacterium*. In contrast, however Lu *et al.* (1991) have found that for carnation, high auxin levels in the co cultivation medium resulted in high transformation efficiencies compared with media with high cytokinin. In this investigation, hormonal manipulation was kept to a minimum to permit the native developmental program in the shoot apex to control plant regeneration. This observation is also in agreement with Schroeder *et al.* (1993) who has also reported that presence of growth regulators in culture medium enhanced transformation frequency and absence of which resulted in sporadic recovery of transgenic plants.

Although cefotaxime is non toxic to plant tissues, but at higher concentrations it inhibits the growth of the plant. In this study, cefotamxime at 300 mg/l was found to control *Agrobacterium* effectively. This finding is in agreement with Arundati (1999),

Geetha *et al.* (1999) and Lawrence and Koundal (2001), who observed effective inhibition of *Agrobacterium* at 300 mg/l in all the explants. But Manoharan *et al.* (1998), have found that cefotaxime @ 400 mg/l was optimum effective control of *Agrobacterium*.

Prior to starting gene transfer experiments the shoot apical meristems were tested for *in vitro* kanamycin sensitivity. Isolated shoot apical meristems were inoculated on MS medium with different concentrations of kanamycin and 200 mg/l was found to be lethal to all explants of cotton as this concentration resulted in browning and complete death of explants two weeks after incubation. However, Momtaz *et al.* (1998) chose 25 mg/l of kanamycin for selection of transformed callus from cotyledons and hypocotyle tissues. In a system such as tobacco, transformed tissue at any stage of development can be selected on high level of kanamycin (up to 1000 mg/l), while in cotton such levels were found to be toxic.

One of the major disadvantages of obtaining plants through organogenesis is the involvement of more than one cell in the shoot initiation process. Similar observation was made by Vasil (1985). When organogenesis is used for transformation and subsequent transgenic plant regeneration, the risk of recovering chimeric plants exists. In this study although five plants showed positive test for Bt expression strip test, but when a similar test was performed taking leaf samples from different branches only one was PCR positive for all the branches tested. This was confirmed by progeny test. These observations were in conformity with Kar *et al.* (1996).

The presence of kanamycin in the rooting media was inhibitory for root formation. Rooting was achieved on kanamycin free medium. Giovanna *et al.* (1992) made similar observation and the use of effective kanamycin selection, concentration, timing and absence of kanamycin in the root development phase were crucial in screening of chickpea transformants. However, Draper *et al.* (1988) feel that roots being more sensitive to

antibiotics, the ability to root on selection medium containing high levels of selective agent is by itself a strong indication of transformed nature of plantlets. While escapes failed to root, but the transformant did root in kanamycin containing rooting medium. According to Geetha *et al.* (1998), the efficient uptake of kanamycin by shoots during rooting stage might provide more effective selection of positive transformants. During rooting stage, shoots which apparently escaped previous selection eventually died within 2 weeks. Putatively transformed shoots that survived on selection medium produced roots within 3 weeks.

5.2 SCREENING AND CONFIRMATION OF TRANSFORMANTS

As the preliminary experiments showed that kanamycin at 200 mg/l was lethal to non transformed plants. This concentration was used to screen the putative transformants *in vitro*. Two types of shoots developed on the selection medium, shoots that were green and actively growing and those that bleached and died. Small shoot explants of cotton are sensitive to kanamycin. In the meristem based method, if the meristem is killed the procedure fails. One of the current problems in the shoot apex method is that the promoters used to drive *npt-II* may not be efficiently expressed in meristem. Under such circumstances, the selection pressure used must be low. Then the escapes and the transformed shoots are not killed. This is the reason, why there is a wide gap between the number of kanamycin resistant shoots and PCR positive transformants. Gould and Cedeno (1998) also opined that the use of a permissive selection protocol is important in the practice of this method because plant regeneration is dependent on vitality of the meristem. Further they also explain that the promoter used with the selectable resistance gene such as *npt II*, must be active in the meristem for selection to work, as one would normally expect. If cells in the meristem die, the organization inherent in the meristem is destroyed and regeneration from meristem is abolished. The CaMv 35S promoter is not active in plant meristem or shoot apex and the transformation events in this region do not

provide protection to this vital region. The amount of selection tolerated also depends on the size and preparation of shoot explant, which is skill dependent and highly individualistic. Although earlier workers have recommended 10-50 mg/l of kanamycin for the selection passage, we have used 50 mg/l for initial screening and during the subsequent subcultures, this level was increased to 200 mg/l to avoid too many escapes. Ramana *et al.* (1996) have also used 50 mg/l of kanamycin in the selection medium.

Despite the popular belief that meristematic tissues of plants cannot be used in *Agrobacterium* mediated transformation, there is no direct evidence to support this view. To date, meristem based methods have been successfully used in Petunia (Ulian *et al.*, 1988), Pea (Hussey *et al.*, 1989) and sunflower (Schrammeijer *et al.*, 1990). Despite the recalcitrance of the *Gossypium* genus, shoot apical meristem has widely been used for cotton transformation. However regenerants from cocultured shoot apices with *A. tumefaciens* carry the transgenes mainly in chimeric fashion since the transformation events giving rise to shoots occur in pre-existing multicellular meristems as *de novo* formation of shoots from single transformed cell, is less probable. In this investigation, although, five transformants were obtained (as confirmed by Bt expression strips) in R₀ generation, out of 13 kanamycin resistant *in vitro* regenerated and well established plants, five were confirmed for expression (Bt strip) but only one was positive for PCR in R₁ regeneration, which reflects the fact that the transgenic progeny will not be chimeric and will reflect the transformation event that was present in the germline.

Presence of *npt-II* specific band in the *in vitro* transformant, positive control and absence in negative control and untransformed plant confirmed the presence of transgene in the tested plant. The transformation frequency was 0.0549 percent. This low frequency of transformation was probably due to problems in sampling tissues from chimeric transformants obtained through organogenesis, which involves more than one cell in the shoot initiation process. Kar *et al.* (1996) also opine that in an organogenetic system, there

is a risk of losing events of transformation due to chimera.. It was observed that repeated application of selection pressure during shoot formation largely inhibits shoot formation by eliminating untransformed cells. Repeated selection in rapidly multiplying shoots is thought to eliminate untransformed tissues. Lowe *et al.* (1995) adopted a similar strategy to enrich transgenic sectors and eliminate chimeras. However even during the last stages of selection, there were four escapes and they were eliminated by progeny tests.

5.3 IN PLANTA TRANSFORMATION

The choice of pollen tube pathway transformation stems from the underlying assumption that at fertilization the egg cell accepts the donation of entire genome from the sperm cell and it might thus be appropriate stage to deliver transgenes. This concept was demonstrated by the successful transformation method developed for *Arabidopsis*. Based on this principle, *Agrobacterium* harbouring the plasmids containing donor DNA are applied directly on to the previously treated stigma (with 5% sucrose and 40 mg/l boric acid) allowing for the formation of pollen tubes extending from the style into the ovule.

In nature, when pollen grain falls on stigma it germinates and pollen tube grows through stylar tissues and ultimately arrives at the ovary. It is also known that the pollen tube enters ovule at the micropyle, when the degenerated nucellus cells form a column to facilitate passage of the pollen tube. Exogenous DNA can pass through the column, and enter the embryonic sac. This is indeed an intelligent method of introducing the exogenous DNA directly into a germline, which is sure to be inherited. Although use of pollen tube pathway to deliver foreign DNA into embryosacs first reported in cotton (Zhou *et al.*, 1983) was encouraging, the overall quality of the data was very weak. Most results did not demonstrate integration of the transgene into the plant genome (Potrykus, 1990).

In the present case all the experiments were performed in the late evening on flowers that were emasculated during the previous evening and agroculture was applied to

the exposed stigma, which was previously treated with 5 per cent sucrose and 20mg/l boric acid. Time of pollination and type of agroculture was varied in four sets of independent experiments. In methods 1 and 2 when pollination was carried out during evening hours, irrespective of the nature of agroculture, whether solid or liquid, there was very poor boll set (3-5%). Since the pollen was not treated with pollen germination medium and also, as the stigma receptivity was poor during evening hours in cotton, leading to abortive pollination, nevertheless pollination during morning hours resulted in successful fertilization leading to boll set of 21 to 28.5 per cent.

Another reason for poor boll set could be due to contamination by *Agrobacterium*. Like any other infections agent, *Agrobacterium* is alien to plant system, infecting the ovules leading to boll shedding.

Boll set was significantly higher (28.5%) when the plants were infected with liquid agroculture when compared to the boll set resulting from the use of solid agroculture. This difference can perhaps be attributed to the fact that the bacterial load is more in solid culture because of higher inoculum density resulting in decreased cell viability. These observations appear to give credence to the hypothesis that each plant cell binds to a finite number of bacteria (Gutlitz *et al.*, 1987). Beyond this threshold, it appears that cell viability may be compromised resulting in lower proportion of boll set. Moreover, from the broth bacteria can move with the pollen tube path more easily than from agar medium and reach the embry sac to effect fertilization. This indicates that the precise guidance of the pollen tube to the embry sac is critical to the successful sexual reproduction in flowering plants.

It is necessary to investigate the exact cause for poor boll set. Using different inoculum densities of *Agrobacterium* to treat the stigmatic surface so as to avoid heavy bacterial load could be a promising proposition, which may improve transformation

frequency. In a preliminary investigation, a number of factors that might facilitate *in situ* pollen germination were tried.

With the treatment of stigmatic surface by 5 per cent sucrose solution, pollination success dramatically improved was up to 23.5 per cent. Tetsuya *et al.* (1998) have also reported that sucrose concentration of 5 per cent appeared to be optimum for pollen tube growth. Reddy *et al.* (2004) have also observed that sucrose level was more crucial for the pollen penetration. Further there was marginal increase in rate of boll set by addition of boron, Tetsuya *et al.* (1998) have also achieved improvement in the growth of pollen tubes by increasing the amount of boric acid from 0.5 to 10 mg/l. Encouraged by the results of sucrose and boric acid inclusion in the treatment, combined effect of both were tried in subsequent experiments and improvement in the boll set was obvious.

5.4 IN SOLIUM SELECTION FOR COTTON TRANSFORMANTS AGAINST KANAMYCIN

Application of kanamycin (400mg/l) through sterile cotton swab to the primordial leaf of the plant under screening makes kanamycin as useful as the herbicide resistance genes in providing a truly culture free transformation technique of cotton. It bypasses the problems associated with sterile techniques and those that result when moving plants from a culture to soil environment. This selection method also decreases the time involved in the screening process (five days). But for such novel, quick and economic method of screening, it would be virtually impossible to screen thousands of plants generated through PTP-mediated transformation. Our experience with this procedure has showed that this technique requires only one tenth and one twentieth the quantity required for spray based technique respectively.

Application of the right concentration of kanamycin is very crucial in any technique. There are two ways that a selection could fail. If the concentration were very

high, the hard earned valuable transformants could be lost. On the other hand if the concentration of kanamycin was low the untransformed plants could be misconstrued to be positive. It was found that kanamycin concentration of 400 mg/l produced clear mottled green symptoms on the third leaf, although cotton swab soaked with kanamycin was kept on the primordial leaf. The chlorotic patches were at the 5th day of treatment on 2nd or 3rd leaf, while the primordial leaf showed the same symptoms after two weeks. This could be due to the fact that the youngest leaf is photosynthetically very active and it can resist any kind of selection pressure (chlorosis in this case) upto a threshold period and only at the end of this threshold period these symptoms were manifested. However it was apparent from the fact that there were no clear cut symptoms between 300 and 400 mg/l concentration of kanamycin, so either of these treatments could be used in detecting the transformed plants. About 92 per cent of the plants were killed at 300 and 400 mg/l kanamycin, when the same concentration was sprayed on positive control, no such symptoms were seen. However at 800, 1000 and 1200 mg/l, even the positive control showed a just a spec of chlorosis. Kanamycin an aminoglycoside antibiotic inhibits protein synthesis by binding to ribosomes. At higher concentration kanamycin is likely to inhibit the protein synthesis even in the transformant having *npt-II gene*. Application of right concentration of kanamycin of kanamycin is thus critical.

Transformation frequencies

Transformation is a rare event. Our attempts of pollen tube path transformation with no tissue culture approaches is a very useful alternative other methodologies available for cotton transformation. Out of the seven transformants selected through PTP-mediated method, six were obtained by treating the stigmatic surface with liquid culture. Four of them had a transformation frequency of 1.03 and two transformants carrying cry1F gene had a transformation frequency of 0.92. Nevertheless the transformation frequency of the transformant obtained through solid culture was 0.53. These results indicate that although the plants respond both to solid and liquid cultures, transformation frequency was more

when stigma was covered with liquid culture. This appeared to be due to free mobility of the bacteria here compared to application of bacteria in agar medium. As there was poor germination of seeds recovered in T_1 and T_2 generation, segregation pattern of the transgene could not be analysed.

Although there are reports of PTP transformation procedure such as ovarian injection, use of transfected pollen, vacuum infiltration, necessity to find a simple, quick and cost effective method is always there. PTP method, in which solutions containing *Agrobacterium tumefaciens* were applied directly to the stigmatic surface, which had been previously treated with boron and sucrose, before pollination may turn out to be a method of choice. Huang *et al.* (1999) opine that the possible mechanism involved here is the adhesion of *Agrobacterium* to the elongated pollen tube, where by it is carried on to the ovum, the zygote itself or the developing embryo.

Bhojwani and Bhatnagar (1974) have suggested that addition of definite amount of sugar limits the diffusion of water in to pollen and thus prevents pollen tube bursting. Of the many sugars tested for their ability to promote pollen germination and tube growth, sucrose had the best effect on pollen germination and pollen tube growth. They have also reported that among inorganic substances boron in the form of boric acid has the most dramatic effect on pollen germination and pollen tube growth. In the current investigation, perhaps this very fact aided in increase in successful pollination leading to enhancement of boll set up to 32.5 per cent, when the stigmatic surface was treated with 5 per cent sucrose and boric acid @ 20 mg/l.

5.5 INSECT BIOASSAY

Insect bioassay was carried out with the available *in vitro* and *in planta* generated cotton transformants using larvae of cotton bollworm. Seventy-two hours after infestation, the transgenic plants showed notable resistance to larvae compared to non-transformed

plants. Larval mortality in positive control was 95 per cent and that of the transgenics ranged from 80 to 90 per cent. On the contrary, the larvae survived and grew well in the negative control. It is not only the larval mortality and growth inhibition that is important to be considered, but also the nature and extent of damage, resulting from larval feeding has a serious bearing on the conclusion drawn. There was voracious feeding in the negative control and the damage increased with the progress in duration of feeding. Only the veins and vein lets were left unfed by the larva within 72 hrs. Nevertheless the leaf bits of Bt positive plants showed only the pin hole size shot holes. Deterrence to feeding was noticed with the advance in duration of feeding and the damage was static. This feeding inhibition was accompanied by decrease in larval weight. These results are in accordance with the studies conducted by Chakraborty *et al.* (2002), where larvae showed different feeding patterns and growth in control and transgenic leaf discs of cabbage.

5.6 *cry* GENES

A new dimension has been added in the crop improvement by developing the transgenic plant for insect resistance using a wide range of *cry* genes. But, the insects are developing resistance against commercially available transgenic cotton with *cryIAC* gene, keeping this fact in view, attempts have also been made to develop transgenic cotton with *cryIAa₃*, *cryIF* and *cryIIa₅* genes. In the current investigation *cryIF* and *cryIAa₃* genes exhibited higher toxicity towards the larvae of *Helicoverpa armigera* (90 % larval mortality) , compared to *cryIIa₅* ,which exhibited an average of 81% larval mortality. Adamczyk and Gore (2004) have also observed that cotton containing *cryIF* was more toxic than cotton containing only *cryIAC*. Further the insect bioassay studies carried out by Dongre *et al.* (2004) have also revealed encouraging results with insect resistant cotton with *cryIAa₃* gene.

FUTURE AREAS OF RESEARCH

Based on the results obtained in the present study the following points need to be considered as thrust areas of research:

- Optimisation of the factors influencing the efficiency and frequency of transformation to enable production of a large number of primary events.
- Although shoot apical meristem has been widely used for cotton transformation, efficiency is low and the regenerants are likely to be chimeric. Attempts should be made to increase the efficiency of transformation by:
 1. Injuring the explants by needle pricks, prior to inoculation .
 2. Vacuum infiltration during cocultivation period.
 3. By enhancing the activity of vir-genes, by exposing the explants to virulence induction medium.
- Although *in solium* selection for cotton transformants resistant to kanamycin was helpful in screening a large number of *in planta* transformed plants, there was a lot of discrepancy between the number of kanamycin resistant and PCR positive plants. This gap must be bridged by further standardization by varying concentration, time and method of application, *etc.*
- Research need to be focussed to investigate the causes for poor boll set using different inoculum densities of *Agrobacterium* used during the course of PTP transformation.

Summary

VI. SUMMARY

In the current investigation, successful attempts were made to obtain bollworm resistant transgenic cotton plants through *in vitro* as well as *in planta* *Agrobacterium* mediated transformation protocols. For *in vitro* transformation, the protocol adopted uses transformation competent cells in the shoot apex of germinating seedling for *Agrobacterium* mediated transformation and for plant driven regeneration. As hormonal balance is a key factor in regulating morphogenesis in explants, effect of various hormones on shoot regeneration and rooting was studied. Low concentration of cytokinin @ 0.2 mg/l of BA or 0.5 mg/l of zeatin was found effective for shoot induction. Profuse rooting of the shoots was obtained with the use of MS medium containing 1 mg/l of NAA together with 0.5 mg/l of IBA.

Further, the factors influencing transformation of cotton shoot apical meristems such as the influence of explant pre-cultivation, co-cultivation duration, bacterial inoculum density, acetosyringone concentration and hormonal composition and the interaction effect among these factors were also studied. Use of pre-cultured shoots in the inoculation step is to ensure activation of cell division in the apical meristematic tissues and it was found to be optimum at 48 hours. Attempts to standardize co-cultivation duration revealed that co-cultivation for 48 hours resulted in development of kanamycin resistant shoot and co-cultivation beyond this period resulted in softening, browning and death of the explants. A low titer of bacteria (0.5×10^8 cells/ml) was found to be important for cocultivation, while the high titers resulted in overgrowth of *Agrobacterium* on explant tissues.

Acetosyringone, a phenolic compound known to induce virulence of *Agrobacterium* was found to be effective @ 200 mM, beyond which it was bacteriostatic. In the present study, hormonal manipulation was kept to a minimum (BA @ 0.2 mg/l or

zeatin @ 0.5 mg/l) to permit the native developmental program in the shoot apex to control plant regeneration, allowing regeneration to be plant driven and therefore genotype independent. The interaction effect among various factors indicated that the explants pre-cultured for 48 hours, infected with agrobacterial suspension having with 0.5×10^8 cells/ml (i.e. 0.6 OD) for 30 min along with acetosyringone @ 200 mM for 48 hrs yielded the transformants under *in vitro* conditions. Analysis of the putative transformants for the presence and expression of *cry* gene revealed that the transformation efficiency was 0.274 per cent. As four of those transformants showed chimeric expression, transformation efficiency reduced to 0.0549.

Although use of pollen tube pathway to deliver foreign DNA into embryosacs first reported by Zhou *et al.* (1983) in cotton was encouraging the overall quality of the data was very weak. The study did not demonstrate integration of the transgene into the plant genome. Therefore our attempts to modify this method yielded fruitful results. Out of 5619 flowers, where the stigmatic surface was treated with 5 per cent sucrose along with 20 mg/l boric acid prior to application of *Agrobacterium* resulted in a boll set of 32.5 per cent and 22,861 seeds were harvested. Out of these only 6954 seeds germinated. *In solium* selection for these cotton transformants resistant to kanamycin resulted in 521 kanamycin resistant plants in T₁ generation. From these plants, 5692 healthy seeds were harvested and were forwarded to T₂ generation. Out of these only 1199 seeds germinated. All these 1199 seedlings were further subjected to stringent *in solium* kanamycin screening in the transgenic greenhouse. From the gene integration study it was clear that only seven were PCR positive and hence the transformation efficiency of PTP mediated transformation was 0.3062 per cent.

Although *in solium* selection for kanamycin resistant cotton transformants is easy, highly economic method for screening, thousands of plants generated through PTP mediated transformation. There was a lot of discrepancy between the number of

kanamycin resistant and PCR positive plants, so this gap must be bridged by further standardization of screening methods with kanamycin in terms of concentrations, time method and mode of application *etc.*

Insect bioassay was carried out with the available *in vitro* and *in planta* generated cotton transformants using the laboratory reared *Helicoverpa armigera*. Larvae. Seventy two hours after infestation, the transgenic plants showed notable resistance to larvae compared to wild plants. Larval mortality ranged from 80 to 90 per cent, while the larval mortality was zero in negative control. It was also noticed that the larval body weight was decreased by 56.36 per cent in the larvae fed on leaf bits of transgenic plants. Nevertheless there was a significant increase in the final body weight of the larvae fed on negative control (111.55%). The nature and extent of damage resulting from larval feeding also has a serious bearing on the conclusion drawn. There was voracious feeding in the negative control and the damage increased with the progress in duration of feeding. Only the veins and vein lets were left unfed by the larvae within 72 hrs. On the contrary, the leaf bits of Bt positive plants showed only limited feeding resulting in pinhole sized shot holes. Deterrence to feeding was also observed.

In conclusion it can be said that it is necessary to optimise the factors influencing the efficiency of transformation under *in vitro* conditions. While attempts of the current PTP transformation methods with no tissue culture approaches have been a tremendous source of alternate methodologies available for transformation of cotton. This successful attempt to obtain transgenic cotton up to T₃ generation along with simpler procedural steps, inexpensiveness has inspired a renewal of efforts to adopt these methods to the development of transformation of cotton.

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VII . REFERENCES

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Appendices

APPENDIX I

Extraction solutions

- Sol I: Stocks**
1. 1M glucose (stored at 4°C)
 2. 0.5 M EDTA
 3. 1M Tris-HCl (pH 8.0)

Working solutions: 5 ml of 1M glucose + 2ml of 0.5M EDTA + 2.5 ml of 1M Tris-HCl were combined and 5 mg/ml lysozyme was added to solution I before use.

- Sol II: Stock solutions**
1. 10 N NaOH
 2. 10% SDS

Working solution: 0.8 ml 10 N NaOH + 4 ml 10% SDS + 35.2 ml of sterile distilled water.

Sol III: Stock solution (stored at 4°C)

1.5 M Potassium acetate

Working solution: 60 ml of 5M potassium acetate was mixed with 28.5 ml of glacial acetic acid and 11.5 ml of sterile distilled water. pH of the final solution was adjusted to 4.8-5.3 using glacial acetic acid.

Sol IV: 3M sodium acetate

200 µl sodium acetate and 600 µl Isopropanol were added in sequence.

Loading dye (6x)

0.25% Bromophenol blue

40% (w/v) sucrose in water

Stored at 4°C

TAE tris Acetate (Sambrook *et al.*, 1989) 50x

Tris Base	242gm
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Distilled water	1000 ml

APPENDIX III**MSY medium (Mannitol salts yeast extract medium)**

Mannitol	-	10g
Yeast extract	-	1g
$\text{KH}_2\text{PO}_4(2\%)$	-	10 ml
$\text{K}_2\text{HPO}_4(2\%)$	-	10 ml
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1M)	-	0.8 ml
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1M)	-	0.4 ml
Agar	-	16 gm
Water	-	1000 ml

DNA extraction buffer (Edwards *et al.*, 1991)

Tris HCl (pH 7.5)	-	200 mM
NaCl	-	250 mM
EDTA	-	25 mM
SDS (w/v)	-	0.5%