1.2.1. Development of a suitable in vitro plant regeneration protocol for cotton:

1.2.1.1. Plant materials:

Commercial cotton (Gossypium hirsutum L.) varieties JK-1, LRA, LRK and non-commercial Coker 312 cv. (Gossypium hirsutum L.) were utilized as experimental plant materials for the present study.

1.2.1.2. Seed Sterilization:

Matured seeds of Coker 312, JK-1, LRA and LRK were surface sterilized by first treating with 70% ethanol for 1 min, then rinsed 2-3 times with sterile distilled water. This was followed by a 30 min treatment with 0.2% mercuric chloride (w/v) solution and detergent (2 drops of Tween-20 in 100ml water). Thereafter the seeds were washed 3-4 times aseptically and imbibed overnight in sterile distilled water.

1.2.1.3. Seed germination and cultivation of the seedlings:

Surface sterilized seeds without seed coats were germinated in MS (Murashige and Skoog, 1962) basal medium supplemented with 2% sucrose (w/v), pH 5.8. Seeds were incubated at 28±1°C in dark for 2 days and then transferred to the culture room for 5 days under 16/8h (day/night) photoperiod.

1.2.1.4. Induction and proliferation of callus:

Hypocotyl sections (3-5 mm length) of all the genotypes were excised aseptically from 7-days old germinated seedlings. The explants were placed on different callus induction media (CIM) to optimize the condition required for callus induction and proliferation. The optimal combinations of 2,4-D, NAA and kinetin (KT) were evaluated at varying concentrations in the growth media (Table 1.2.1) supplemented with MS basal salts, B5 vitamins (Gamborg et al., 1968), 3% glucose (w/v), 750mg/l MgCl2, 0.2% (w/v) phytagel (Sigma), pH 5.8. All cultures were maintained at 28±1°C under 16/8h (day/night) photoperiod. A friable primary calli masses with the colour ranging from yellowish-white to parrot-green were developed after 3 weeks of incubation. They were separated aseptically and subcultured onto fresh medium for further growth.
Table 1.2.1. Different media with varying concentrations of 2,4-D, NAA and kinetin (KT) used for optimization of callus induction in different cotton cultivars (Coker 312, JK-1, LRA and LRK):

<table>
<thead>
<tr>
<th>Callus induction medium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Growth regulators (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,4-D</td>
</tr>
<tr>
<td>MSB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.1</td>
</tr>
<tr>
<td>MSB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.1</td>
</tr>
<tr>
<td>MSB&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>MSB&lt;sub&gt;4&lt;/sub&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>All the media were supplemented with MS basal salts, B<sub>5</sub> vitamins, 3% glucose (w/v), 750mg/l MgCl<sub>2</sub>, 0.2%(w/v) phytagel, pH 5.8.

1.2.1.5. Development of embryogenic calli:

The primary calli masses emerged from hypocotyl sections of all the genotypes were spread uniformly as a monolayer on the surface of MST<sub>2</sub> medium (MS salts, B<sub>5</sub> vitamins, 3% glucose (w/v), 750mg/l MgCl<sub>2</sub>, 0.2% phytagel (w/v), pH 5.8) devoid of any plant growth regulators to initiate embryogenic calli formation. All the petriplates were kept at 28±1°C under 16/8h (day/night) photoperiod. After one month of culture, small areas of highly proliferating, friable, granular and yellowish-white embryogenic calli emerged from Coker 312 strain, whereas response to embryogenic calli formation obtained from JK-1 and LRA cultivars after 1½ months of incubation.

1.2.1.6. Differentiation of somatic embryos and plantlet regeneration:

The embryogenic calli masses emerged from Coker 312, JK-1 and LRA were selectively picked up and transferred to MST<sub>3</sub> medium (MS salts, B<sub>5</sub> vitamins, 3% maltose (w/v), 750mg/l MgCl<sub>2</sub>, 0.2% phytagel (w/v), pH 5.8 along with extra nitrate source i.e. 1.93gm/l KNO<sub>3</sub>) for the development of somatic embryos. The cultures were incubated at 28±1°C under 16/8h (day/night) photoperiod. Thereafter, the embryogenic calli were repeatedly subcultured onto fresh MST<sub>3</sub> medium every 2 weeks interval to promote formation of somatic embryos quite regularly. Almost after 2 months of incubation, plantlet regeneration took place from somatic embryos of Coker 312, while...
LRA variety responded to plantlet regeneration after maintenance of 3 months of culture. The non-regenerated somatic embryos were subcultured again in fresh MST₃ medium for further plantlet formation.

1.2.1.7. Growth and rooting of the regenerated plantlets:

The regenerated plantlets (2-3 cm long shoots with true leaves) of Coker 312 and LRA were separated aseptically and transferred to MSG medium (½MS salts, B₅ vitamins, 0.1mg/l GA₃, 0.05mg/l IAA, 3% glucose (w/v), 2gm/l active charcoal, 750mg/l MgCl₂, 0.2% phytage1 (w/v), pH 5.8) in sterile culture glass jars to promote elongation and rooting. All cultures were incubated at 28±1°C under 16/8h (day/night) photoperiod for 2-3 weeks.

1.2.1.8. Hardening and acclimatization of regenerated plants in greenhouse:

The elongated plantlets (5-7 cm. long shoots with 4-6 leaves) of both genotypes containing well developed roots were placed into small cups filled with sterilized 1:1 mixture of sand: soil and kept in a growth chamber under 60% relative humidity. The plants were then gradually hardened in the growth chamber under 16/8h (day/night) photoperiod at 28±1°C. After 2-3 weeks of incubation, they were transferred into large pots covered with transparent polyethylene bags which were intermittently removed for gradual adaptation of plants with outer environment. Finally, all the regenerated plants were grown in the greenhouse for maturation.

1.2.2. Experimental design and statistical analysis:

The experiments were treated as a randomized block design (RBD), consisting of an arrangement of four cotton cultivars, four types of different media and three replications. The data were represented in percentage. In order to perform the analysis of variance (ANOVA), the data were transformed into its corresponding angular values following Fisher and Yates table. Statistical analyses were performed by the SAS programme systems for windows, version 8.2 (SAS Inst., Inc. 2001, Cary, NC).

Standard error of the mean was calculated to measure the precision of the estimated mean. This was obtained by taking the standard deviation of the sample and dividing it by the square root of the number of observations.
Table 1.2.2. Composition of basic media used in the present study:

<table>
<thead>
<tr>
<th>Composition</th>
<th>MS (mg/l)$^1$</th>
<th>Bs (mg/l)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrient components:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>1900</td>
<td>2500</td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>1650</td>
<td>134</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>170</td>
<td>150</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>370</td>
<td>250</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>440</td>
<td>150</td>
</tr>
<tr>
<td><strong>Micronutrient components:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>27.85</td>
<td>27.85</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>37.25</td>
<td>37.25</td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>22.3</td>
<td>10</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>8.6</td>
<td>2</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>6.2</td>
<td>3</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>0.75</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>CoCl$_2$.6H$_2$O</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Organic components:</strong></td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.1</td>
<td>10.0</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>


1.2.3. Molecular cassette construction and *Agrobacterium* transformation:

1.2.3.1. Bacterial strains:

*Escherichia coli*:

*E. coli* strain DH10B (Gibco BRL) was used as the host for cloning experiments, while pUC18 (New England Biolabs) was used as the cloning vector. For growth of *E. coli* harbouring desired plasmids, LB or LA medium was used supplemented with 100μg/ml of ampicillin (Sigma Chemical Co. USA).
Materials and Methods

Table 1.2.3. Composition of the media used for *E. coli* culture:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>10gm/l tryptone, 5gm/l yeast extract, 10gm/l NaCl, pH 7.2</td>
</tr>
<tr>
<td>LA</td>
<td>LB supplemented with 1.2% agar.</td>
</tr>
</tbody>
</table>

*Agrobacterium tumefaciens*:  

*Agrobacterium tumefaciens* strain LBA4404 (Hoekama *et al.*, 1983) and the binary vector pCAMBIA 1300 harbouring the hygromycin phosphotransferase gene (*hptII*) as the selection marker, driven by a CaMV35S promoter and terminated by the CaMV35S polyA sequence were used to carry out plant transformation experiments. The LBA4404 *Agrobacterium* strain was cultured in AB minimal medium (Chilton *et al.*, 1974) containing 20mg/l of rifampicin (Duchefa Biochem.) and incubated in 28°C with shaking at 150 rpm. Culture of *Agrobacterium* strain was maintained in YEP medium solidified with 1.2% agar in presence of corresponding antibiotic.

Table 1.2.4. Composition of the media used for *Agrobacterium* culture:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>YEP</td>
<td>10gm/l Bactopeptone, 5gm/l NaCl, 10gm/l Yeast extract and 1.2% agar.</td>
</tr>
<tr>
<td>AB</td>
<td>5gm/l glucose, 5ml AB salt &amp; 5ml AB buffer for 100ml medium.</td>
</tr>
</tbody>
</table>

Composition of the AB salt (20X):

- Ammonium Chloride (NH₄Cl) : 20gm/l
- Magnesium Sulphate (MgSO₄·7H₂O) : 6gm/l
- Potassium Chloride (KCl) : 3gm/l
- Calcium Chloride (CaCl₂) : 200mg/l
- Ferrous Sulphate (FeSO₄·7H₂O) : 50mg/l

pH adjusted to 7.0 before autoclaving.

Composition of AB buffer (20X):

- Di-potassium hydrogen orthophosphate (K₂HPO₄) : 60gm/l
- Sodium dihydrogen orthophosphate (NaH₂PO₄·2H₂O) : 26gm/l
1.2.3.2. Construction of 2X3SS-gusA-nos chimeric gene expression cassette:

A constitutive 2X35S promoter element was developed in the laboratory as a HindIII/BamHI fragment. This promoter element was then fused at the 5'-end of the gusA reporter gene, present as a BamHI/SacI fragment and cloned in pUC18 vector. Thereafter the gusA gene along with the 2X35S promoter was retrieved from pUC18 as a HindIII/SacI fragment and ligated into the T-DNA region of modified pCAMBIA 1300 vector containing the nos terminator at SacI/EcoRI site of its multiple cloning sites to generate the plant transformation vector pCAM/BREF/GUS. The chimeric gene expression cassette 2X35S-gusA-nos thus developed in pCAMBIA 1300 vector as HindIII/EcoRI fragment was used for plant transformation experiments.

Transformation of the plasmid DNA into competent E. coli host cells, competent cell preparation, isolation of plasmid DNA by alkaline lysis method, restriction digestion, LMP gel elution, ligation, i.e. all the required steps for cloning procedure were carried out following the standard protocol (Sambrook et al., 1989).

1.2.3.3. Preparation of competent Agrobacterium cell:

Initially 5ml of strata culture of the Agrobacterium strain LBA4404 was made in the YEP broth containing 20mg/l of rifampicin. About 2ml of the strata culture was inoculated into a fresh 50ml of YEP medium supplemented with appropriate antibiotic and allowed to grow upto OD$_{600}$ - 0.6 in a 28°C incubator shaking at 150 rpm. The culture was then chilled in ice and centrifuged at 5000 rpm for 6 min. The soup was decanted and the cell pellet was resuspended in 1ml of ice-chilled 20mM CaCl$_2$. Finally, about 200µl of the resuspended competent cells were aliquoted into fresh eppendorf tubes and stored at -70°C freezer.

1.2.3.4. Transfer of plant transformation vector pCAM/BREF/GUS into Agrobacterium strain LBA4404 by transformation:

The direct DNA transformation method based on An et al., (1988) was followed for transferring pCAM/BREF/GUS into the Agrobacterium LBA4404 strain. For this, about 6µg DNA of pCAM/BREF/GUS was layered on the top of a frozen 200µl aliquot of competent Agrobacterium cell and gently flicked for mixing. The mixture was
incubated in 37°C water bath for 5 min and 1ml of YEP broth was then added to the 
mixture and set for shaking at 150 rpm in a 28°C incubator for 6h. Thereafter, it was 
centrifuged at 5000 rpm for 5 min. The supernatant was decanted leaving 100μl of the 
broth in which the cell pellet was resuspended and spread onto solidified YEP plates 
supplemented with 20mg/l rifampicin and 50mg/l kanamycin (Sigma Chemical Co. 
USA) and incubated at 28°C for 3 days.

**1.2.3.5. Mini plasmid isolation from *Agrobacterium* culture:**

The bacterial colonies of *Agrobacterium* strain LBA4404 : pCAM/BREF/GUS 
appeared after transformation were inoculated in 3ml of YEP medium containing 
appropriate antibiotics and allowed to grow overnight in a 28°C incubator shaking at 
150 rpm. The culture was centrifuged in an eppendorf tube at 5000 rpm for 6 min and 
the supernatant was completely drained out. The cell pellet was then resuspended in 
200μl of solution I (50mM glucose, 25mM Tris-HCl [pH-8.0] and 10mM EDTA [pH-
8.0]) containing 4mg/ml of lysozyme and incubated at 37°C for 30 min. About 400μl of 
freshly prepared solution II (0.2N NaOH and 1% SDS) was then added into it with 
gentle mixing and kept for 15 min at room temperature. This was followed by adding 
300μl of solution III (5M potassium acetate and glacial acetic acid), mixed well and 
incubated in ice for 30 min. Thereafter, it was centrifuged at 10,000 rpm for 10 min and 
the supernatant was collected. Equal volume of phenol:chloroform was added into it 
and subsequently treated with chloroform-isoamyl alcohol mixture. Finally, the DNA 
was precipitated with the addition of one-tenth volume of sodium acetate and double 
volume of chilled ethanol. The DNA pellet was then washed with 70% alcohol, dried 
and resuspended in 10:1 TE (Tris-EDTA). The plasmid DNA was checked in 0.8% 
agarose gel.

**1.2.3.6. Confirmation for the presence of plasmid:**

Presence of the pCAM/BREF/GUS was checked by digesting the DNA of 
different colonies with *HindIII/EcoRI* restriction endonucleases that dropped the full-
length gene cassette. Thereafter the digested DNA fragments were transferred onto 
Hybond-N nylon membrane (Amersham Pharmecia Biotech) by overnight capillary 
transfer. The membrane was crosslinked in UV cross linker (Amersham Life Science)
and subsequently hybridised with a gene specific probe to identify the positive colonies harbouring the construct of interest. The prehybridisation and hybridization were carried out according to the standard protocol (Sambrook et al., 1989). Finally, the autoradiogram was developed and different colonies which signaled for positive hybridization confirmed to carry the desired plasmid (pCAM/BREF/GUS) were selected for plant transformation experiments.

1.2.3.7. Development of ternary transformation system containing a constitutive \( \text{virG} \) mutant (\( \text{virGN54D} \)):

A ternary transformation system was developed in order to make use of the constitutive expression of VirGN54D. For this, the mutant \( \text{virG} \) gene (\( \text{virGN54D} \)) was cloned as \( \text{SacI}/\text{HindIII} \) fragment from pRAL6308 (Scheeren-Groot et al., 1994) into plasmid pBBR1MCS (Kovach et al., 1994). The plasmid pBBR1MCS containing \( \text{virGN54D} \) was then transferred by transformation into LBA4404 : pCAM/BREF/GUS to generate ternary transformation system LBA4404 : pCAM/BREF/GUS : \( \text{virGN54D} \). The plasmids pRAL6308 and pBBR1MCS were provided by Dr. Johan Memelink, Netherlands.

1.2.4. Sensitivity of the hypocotyl explants to hygromycin:

Hygromycin was used in the present study as the selection marker for plant transformation experiments. Sensitivity of cotton tissues in culture to hygromycin was determined. Hypocotyl explants of Coker 312 were placed on MSBi medium containing different concentration levels (2, 4, 6, 8 and 10 mg/l) of hygromycin. Each treatment containing twenty hypocotyl cut sections was performed in triplicate.

1.2.5. Use of ternary transformation system for \textit{Agrobacterium} mediated plant transformation:

1.2.5.1. \textit{Agrobacterium} mediated transformation of tobacco leaf discs using binary and ternary transformation systems:

The selected bacterial colonies of \textit{Agrobacterium tumefaciens} strains LBA4404 : pCAM/BREF/GUS and LBA4404 : pCAM/BREF/GUS : \( \text{virGN54D} \) were representing the binary and ternary transformation systems respectively. They were
separately grown in liquid AB medium (Chilton et al., 1974) upto OD$_{600}$ - 0.8 in presence of appropriate antibiotics as bacterial selection drug. The cells were then centrifuged, washed with 10mM MgSO$_4$ and resuspended in liquid infection medium [MS salts, MS vitamins, casamino acids (500mg/l), sucrose (68.5mg/l), glucose (36mg/l), pH-5.2]. Fresh leaf discs of one month old tobacco plants (*Nicotiana tabacum*) were inoculated in liquid infection medium and kept for 15 min. Thereafter the leaf discs were soaked on sterile filter paper to remove the excess bacteria and transferred to co-cultivation medium [MS salts, MS vitamins, IAA (8mg/l), kinetin (2.5mg/l), 3% sucrose (w/v), 1% glucose (w/v), pH 5.2] and incubated in dark at 25°C for 3 days. After co-cultivation, the leaf-discs were transferred to regeneration medium [MS salts, MS vitamins, IAA (8mg/l), kinetin (2.5mg/l), 250mg/l of cefotaxime, sucrose 3% (w/v), pH 5.8] and subcultured onto fresh medium every 2 weeks interval. Selection of the emerging plantlets was carried out with hygromycin (25mg/l). Transformation frequency was calculated in both the cases of binary and ternary transformation systems on the basis of total number of Hyg$^R$ plantlets survived against total number of green plantlets regenerated.

**1.2.5.2. *Agrobacterium* mediated transformation in cotton using ternary transformation system:**

Transformation of hypocotyl sections of cotton was carried out following *Agrobacterium* mediated transformation principle essentially that of Umbeck et al., (1987) with some modifications. Hypocotyl cut sections (3-5 mm length) from 7-days old germinated seedlings of Coker 312 were precultured on MSB$_1$ medium and kept at 28±1°C for 24h under 16/8h (day/night) photoperiod.

For conducting *Agrobacterium* mediated transformation, bacterial culture containing ternary vector system (LBA4404 : pCAM/BREF/GUS : virG54D) was allowed to grow upto OD$_{600}$ - 0.8 at 28°C in AB minimal medium supplemented with rifampicin (20mg/l), chloramphenicol (75mg/l) and kanamycin (50mg/l). The cell culture was then centrifuged at 6000 rpm for 5 min. The cell pellete was subsequently washed with 10mM MgSO$_4$, resuspended in liquid infection medium (LIM) [MS salts, B$_5$ vitamins, 3% glucose (w/v), pH 5.2] and kept for about one hour. The precultured
hypocotyl explants of Coker 312 were immersed into the *Agrobacterium* suspension and kept there for 10-15 min with mild shaking at room temperature. The excessive bacteria present on the surface of explants were then blotted dry on sterile filter paper aseptically and finally transferred to the co-cultivation medium (MSB\textsubscript{1}-Co) and incubated in dark at 25°C for 2 days. Following co-cultivation phase of *Agrobacterium* infection, the explants were soaked on sterile filter paper to get rid of excess bacteria and transferred to MSB\textsubscript{1} medium containing 250mg/l of cefotaxime (MSB\textsubscript{1}-Cf) to control further *Agrobacterium* growth. After a week, the infected hypocotyl explants were transferred to MSB\textsubscript{1} medium supplemented with 250mg/l of cefotaxime and 8mg/l of hygromycin B (Sigma Chemical Co.) (MSB\textsubscript{1}-Cf-Hy) for selection of the transformed calli masses. All cultures were maintained for 4 weeks at 28±1°C under 16/8h (day/night) photoperiod. The process was continued through recurrent selection by subculturing the calli masses onto fresh medium supplemented each time with 8mg/l of hygromycin.

### 1.2.5.3. Development of embryogenic calli from selected primary calli masses:

The selected primary calli masses were spread evenly as a monolayer onto hormone-free MST\textsubscript{2} medium to initiate embryogenic calli formation. The cultures were grown for at least 1½ month at 28±1°C under 16/8h (day/night) photoperiod. Small areas of highly proliferating, friable and yellowish-white granular embryogenic calli emerged from Coker 312 were selectively scooped out from the necrotic regions of calli masses.

### 1.2.5.4. Regeneration of the presumptive transformants:

The surviving hygromycin resistant embryogenic calli lines were transferred to MST\textsubscript{3} medium and incubated at 28±1°C under 16/8h (day/night) photoperiod. They were maintained in this growth medium by subculturing every 15 days interval until somatic embryos were developed. After one month of culture, the somatic embryos were again transferred to fresh MST\textsubscript{3} medium for their maturation and germination. Plantlet regeneration took place from the somatic embryos of Coker 312 after another 1½ month of culture, while the non-regenerated embryos were subcultured again in fresh medium for further plantlet formation.
1.2.5.5. Growth and rooting of the regenerated plantlets:

The bunch of HygR plantlets (2-3 cm long shoots with true leaves) coming out from the embryogenic clusters were separated individually and transferred to MSG medium in sterile culture glass jars to promote elongation and rooting. The cultures were incubated at 28±1°C under 16/8h (day/night) photoperiod for 2-3 weeks. The elongated plantlets with well-developed roots were then gradually hardened in the growth chamber under 60% relative humidity. After 2-3 weeks, they were finally transferred to large pots filled with soil and grown to maturity in the greenhouse.

Table 1.2.5. Composition of the media used for genetic transformation in cotton:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSB₁</td>
<td>MS salts, B₅ vitamins, 0.1mg/l 2,4-D, 0.5mg/l kinetin, 3% glucose (w/v), 750mg/l MgCl₂, 0.2% phytagel (w/v), pH 5.8</td>
</tr>
<tr>
<td>LIM</td>
<td>MS salts, B₅ vitamins, 3% glucose (w/v), pH 5.2</td>
</tr>
<tr>
<td>MSB₁-Co</td>
<td>MS salts, B₅ vitamins, 0.1mg/l 2,4-D, 0.5mg/l kinetin, 3% glucose (w/v), 750mg/l MgCl₂, 0.2% phytagel (w/v), pH 5.2</td>
</tr>
<tr>
<td>MSB₁-Cf</td>
<td>MS salts, B₅ vitamins, 0.1mg/l 2,4-D, 0.5mg/l kinetin, 3% glucose (w/v), 250mg/l cefotaxime, 750mg/l MgCl₂, 0.2% phytagel (w/v), pH 5.8</td>
</tr>
<tr>
<td>MSB₁-Cf-Hy</td>
<td>MS salts, B₅ vitamins, 0.1mg/l 2,4-D, 0.5mg/l kinetin, 3% glucose (w/v), 250mg/l cefotaxime, 8mg/l hygromycin, 750mg/l MgCl₂, 0.2% phytagel (w/v), pH 5.8</td>
</tr>
<tr>
<td>MST₂</td>
<td>MS salts, B₅ vitamins, 3% glucose (w/v), 750mg/l MgCl₂, 0.2% phytagel (w/v), pH 5.8</td>
</tr>
<tr>
<td>MST₃</td>
<td>MS salts, B₅ vitamins, 3% glucose (w/v), 1.93gm/l KNO₃, 750mg/l MgCl₂, 0.2% phytagel (w/v), pH 5.8</td>
</tr>
<tr>
<td>MSG</td>
<td>½ MS salts, B₅ vitamins, 0.1mg/l GA₃, 0.05mg/l IAA, 3% glucose (w/v), 2gm/l active charcoal, 750mg/l MgCl₂, 0.2% phytagel (w/v), pH 5.8</td>
</tr>
</tbody>
</table>
1.2.6. Analysis of the transformants:

1.2.6.1. PCR analysis of the presumptive transformants:

Genomic DNA was isolated from fresh leaves of the putative transformants selected on the basis of hygromycin resistance and the untransformed control plants following the CTAB method (Doyle and Doyle, 1996). Initial detection for the presence of hygromycin resistance gene (*hptII*) was carried out using PCR reaction with the help of *hptII* gene specific primers. The forward primer 5'- CTA TTT CTT TGC CCT CGG ACG AGT GCT GGG -3' and the reverse primer 5'- ATG AAA AAG CCT GAA CTC ACC GCG ACG TCT-3' were used for the PCR reaction. The DNA of untransformed plant and the binary vector pCAM/BREF/GUS were used as negative and positive controls respectively. PCR reaction was carried out in a reaction volume of 25μl containing 100ng of genomic DNA that served as the template, 1X PCR reaction buffer, 100μM of each dNTPs, 0.4μM of each primer along with 0.625U of Taq DNA polymerase (Roche Molecular Biochemicals). PCR was performed in a thermal cycler (Gene Amp PCR System 9600, Perkin-Elmer) following the amplification profile: initial heat denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30s, 60°C for 30s and 72°C for 1 min, with a final extension step of 72°C for 7 min. After PCR, the resulting samples were subjected to electrophoresis in a 1% agarose gel.

1.2.6.2. Histochemical localisation of gus gene expression:

Histochemical detection of GUS activity in different parts of the HygR plants was performed as described by Jefferson *et al.*, (1987) with the substrate 5-bromo-4-chloro-3-indoyl β-D-glucuronic acid (X-Gluc). GUS staining solution was prepared by taking 2mM X-Gluc (Sigma Chemical Co. USA) from a 20mM stock made in dimethylformamide, 50mM Na-Phosphate buffer, pH 7.0 and 0.001% Tween-20. GUS assay was carried out with the hygromycin resistant embryogenic calli masses. GUS expression was also monitored in the transverse sections of leaves, stems, petioles and other tissue parts of HygR plants. Different tissue sections were incubated in GUS staining solution overnight at 37°C and then washed several times with acetic acid and ethanol mixture [45% : 55% (v/v)] until the green colour of chlorophyll disappeared.
1.2.6.3. Southern blot analysis of transformed cotton lines expressing gus gene:

Southern blot analysis was carried out using approximately 10μg of genomic DNA isolated from the individual primary transformant lines expressing gus gene and the untransformed control plants. Genomic DNA was digested with EcoRI restriction endonuclease and separated electrophoretically on 0.8% agarose gel (USB, Amersham Life Science). The digested DNA fragments from agarose gel were thereafter transferred onto Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) by using a vacuum blotter (Bio-Rad). The blot was then soaked in 6XSSC, air dried and cross-linked using UV cross linker (Amersham Life Science). A BamHI/EcoRV cleaved 584 bp fragment from the coding region of gus gene was radiolabelled with α-[32P]-dCTP using random prime labelling system rediprime™ II (Amersham Life Science) and used as hybridization probe. Hybridization was carried out in Church buffer [0.25M Na-Phosphate buffer, pH-7.2; 1mM EDTA; 7% SDS and 1% BSA (w/v)] at 65°C for 16-18h. Following hybridization, the membrane was washed successively in 2XSSC/1%SDS, 1XSSC/1%SDS, 0.5XSSC/1%SDS, 0.2XSSC/1%SDS and 0.1XSSC/1%SDS for 10-15 min each under high stringency at 65°C. Autoradiographic exposcer was allowed at -70°C by keeping the radiolabelled membrane on Kodak X-ray film in a cassette with intensifying screen for the required time period. The autoradiogram was then developed.

1.2.6.4. Extraction of plant protein and fluorometric quantitation of GUS activity:

Total protein was isolated from about 150mg of fresh young leaves of the transgenic cotton plants and the untransformed control plants. The leaves of the individual plants were homogenized separately by using liquid nitrogen and suspended in 1ml of protein extraction buffer (50mM Tris, pH 8.0; 100mM NaCl; 5mM EDTA; 0.15% Triton X-100 and 10mM PMSF). After thawing, the suspension was collected in fresh eppendorf tubes, placed in ice and subjected to sonication (3 bursts of 10 sec each) by an ultrasonic cell disruptor (Microson). The mixture was then centrifuged at 15000g for 15 min at 4°C and the supernatant was collected. Total protein content of each sample was estimated by the method of Bradford (1976).
Quantitative estimation of GUS activity in different primary transgenic lines as well as untransformed control plants was carried out by using a Spectrofluorometer according to Jefferson et al., (1987).

Solution A: About 5μg of protein sample extracted from each of the individual plants present in 50μl volume (volume made up with 0.25M Tris-HCl, pH 8.0).

Solution B: 50μl of assay buffer (50mM Na-Phosphate buffer, pH 7.0; 10mM β-ME; 10mM EDTA; 0.1% Sodium Lauryl Sarcosine; 0.1% Triton X-100 and 1mM MUG (Sigma).

50μl of each (A) and (B) solutions were taken together, mixed well and incubated at 30°C for 30 min. In all the cases, a standard control containing 50μl of 0.25M Tris-HCl (pH 8.0) and 50μl of assay buffer was used to provide an autozero reading. The reaction was stopped with the addition of 900μl of 0.2M Na₂CO₃ solution and the fluorescence was measured in a Spectrofluorometer-LS45 (Perkin-Elmer) with excitation at 365nm and emission at 455nm. The instrument was calibrated for each reading with a known standard. Three independent measurements were recorded for each plant line.

1.2.6.5. Inheritance pattern of the transferred gus gene in T₁ generation:

Self-pollinated seeds of different T₀ cotton lines transformed with the gus gene were germinated in MS basal medium supplemented with 30mg/l of hygromycin. Segregation for hygromycin resistance was estimated by counting the number of hygromycin resistant and sensitive plantlets. Chi square tests were carried out to determine the fitness of the segregation ratio for hptII gene amongst T₁ progenies. Expression of GUS activity was monitored in different tissue sections of hygromycin resistant T₁ plants by histochemical staining with X-Gluc (Jefferson et al., 1987) to check inheritance pattern of the transferred gus gene in T₁ generation.

1.2.6.6. Southern blot analysis of the T₁ transgenic lines:

Southern blot analysis was carried out using approximately 10μg of genomic DNA isolated from the selected T₁ transgenic lines and the untransformed control plants to check the integration pattern of the transferred gus gene in T₁ generation. Genomic DNA was digested with EcoRI restriction endonuclease and resolved on 0.8% agarose
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gel. Southern blot analysis was then conducted following the method as mentioned earlier (section 1.2.6.3).

1.2.7. Transfer of crylAc gene of Bacillus thuringiensis in cotton in order to generate insect resistant transgenic lines:

1.2.7.1. Development of the chimeric crylAc gene construct for plant expression:

A reconstructed active part of the crylAc gene (1854bp) using plant preferred codons along with five extra codons [four of the codons constituted the endoplasmic reticulum signal sequence Lys-Asp-Glu-Leu (KDEL), while the fifth codon constituted the stop codon] at the 3' end was generated. The synthetic crylAc gene was cloned in pUC18 vector at the KpnI/SalI site. Additionally, a dicot specific transit peptide (TP), present as BamHI/KpnI fragment was then frame ligated at the 5' end of the gene, while the nos terminator, present as SalI/EcoRI fragment was fused with the gene at the 3' end. Thereafter the reconstructed crylAc gene along with the TP and the nos terminator was ligated into the T-DNA region of pCAMBIA1300 vector at BamHI/EcoRI sites. Finally, a constitutive 2X35S promoter element, present as a HindIII/BamHI fragment was joined at the 5’ end of the TP to generate the chimeric gene expression cassette 2X35S-TP-crylAc-nos as a HindIII/EcoRI fragment. The plant transformation vector pCAM/BREF/Cry1Ac thus developed was then transferred by transformation into the Agrobacterium strain LBA4404/virGN54D (van der Fits et al., 2000) to form a ternary transformation system. The bacterial colonies containing pCAM/BREF/Cry1Ac were chosen using antibiotic selection as well as radioactive hybridization and finally used for transformation experiments in cotton.

1.2.7.2. Plant material:

Transformation experiments were carried out in Coker 312 strain. Hypocotyl sections (3-5 mm length) excised from 7-days old germinated seedlings of Coker 312 were used as explants for Agrobacterium mediated transformation.

1.2.7.3. Agrobacterium mediated transformation of cotton explants for insect resistant plant generation:

Hypocotyl cut sections were infected with Agrobacterium strain LBA4404/virGN54D harbouring pCAM/BREF/Cry1Ac. The transformation protocol
already optimized was adopted for the purpose. After co-cultivation, the transformed calli masses were selected in presence of 8mg/l hygromycin and differentiated to form embryogenic calli which were converted subsequently into somatic embryos. The HygR plantlets were germinated from the transformed somatic embryos and finally grown to maturity in the greenhouse, as described earlier. The putative transformants were then subjected to molecular analysis.

1.2.8. Analysis of the transformants:

1.2.8.1. PCR analysis of the putative transformants:

PCR analysis was carried out to detect the presence of hygromycin resistance gene (hptII) in each of the presumptive transformants with the help of hptII gene specific primers. The forward primer 5'- CTA TTT CTT TGC CCT CGG ACG AGT GCT GGG -3' and the reverse primer 5'-ATG AAA AAG CCT GAA CTC ACC GCG ACG TCT-3' were used for the PCR reaction. The DNA of untransformed plant and the binary vector pCAM/BREF/Cry1Ac were served as negative and positive controls respectively. Thermal profile maintained for PCR reaction was the same as mentioned earlier (section 1.2.6.1).

1.2.8.2. Insect feeding assay:

Insect feeding assay was conducted using the first instar larvae of cotton bollworm (Helicoverpa armigera) under laboratory condition. Fresh young leaves of hygromycin resistant plants of same age were subjected to insect feeding assay. To keep the leaves fresh and free from infection during the period of experiment, stalk of the leaves were embedded into 2% agar containing 200mg/l cefotaxim and 0.1% bavistin in petriplates. The leaf surfaces were separated from agar layer by Whatman filter paper. In each petriplate, four insect larvae were placed on a fresh leaf collected from each of the individual transformant lines. Insect feeding assay was carried out in three replicates and leaves of the untransformed plants were used as negative control. The petriplates were incubated in dark at 28°C in a growth chamber under 70% relative humidity. Growth of the larvae was monitored closely. Mortality and body weight of the insect larvae were recorded after 5 days of feeding assay.
Considering the natural death of the feeding larvae, the percentage of insect mortality was determined using Abbott's formula:

\[
\% \text{ of mortality} = \frac{(X-Y)}{X} \times 100
\]

(where, \( X \) =\% of survivability in the control line where no toxin is present and \( Y \) = \% of survivability in the transgenic line).

1.2.8.3. PCR analysis for the presence of \( crylAc \) gene:

PCR analysis was carried out in selected promising insect resistant plant lines to detect the presence of \( crylAc \) gene. For this, the forward primer 5'- ACC AGA TCA TGG CCT CTC CAG TT -3' and the reverse primer 5'- CTC GAA TCT GTC AAT GAT CAC TC -3' were designed to amplify 857 bp coding region of the reconstructed \( crylAc \) gene. The DNA of untransformed plant and the binary vector pCAM/BREF/CrylAc were used as negative and positive controls respectively. PCR reaction was performed following the amplification programme: initial heat denaturation at 94\(^\circ\)C for 3 min, followed by 30 cycles of 94\(^\circ\)C for 30s, 58\(^\circ\)C for 40s and 72\(^\circ\)C for 1 min, with a final extension step of 72\(^\circ\)C for 7 min.

1.2.8.4. Southern blot analysis of \( crylAc \) transformed plant lines:

Southern blot analysis was carried out using approximately 10\( \mu \)g of genomic DNA isolated from the individual primary transformant lines showing the presence of \( crylAc \) gene and the untransformed control plants. Genomic DNA was digested with EcoRI restriction enzyme and separated electrophoretically on 0.8% agarose gel (USB, Amersham Life Science). The digested DNA fragments from agarose gel were thereafter transferred onto Hybond-N\(^+\) nylon membrane (Amersham Pharmacia Biotech). A BamHI/SacI cleaved 506 bp fragment from the coding region of reconstructed \( crylAc \) gene was radiolabelled with \( \alpha\)-\([32P]\)-dCTP using random prime labelling system rediprime\(^TM\) II (Amersham Life Science) and used as hybridization probe. Southern blot analysis was then conducted following the method as already described earlier (section 1.2.6.3).

1.2.8.5. Northern blot analysis:

Total RNA was isolated from fresh leaves of the transgenic cotton plants and the untransformed control plants according to the hot borax method (Wan et al., 1994).
About 25μg of RNA from each plant line was electrophoresed on 1.2% denaturing agarose gel. The gel was then set to deformatyation and blotted onto Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) by using a vacuum blotter apparatus (Bio-Rad). The probe was the same as used in Southern blot analysis (section 1.2.7.4). Northern hybridization was carried out in a solution containing 20% (w/v) dextran sulphate in deionised formamide; 14% SDS (w/v); 0.25M Na-Phosphate buffer, pH 7.2; 4mM EDTA; 0.25(N) NaCl and denatured salmon sperm DNA (100μg/ml) at 42°C for 16-18h. Following hybridization, the membrane was washed and exposed to Kodak X-ray film at -70°C to develop the autoradiogram.

1.2.8.6. Western blot analysis:

Total protein was isolated from about 200mg of fresh young leaves of the transgenic plants and the untransformed control plants. The leaf tissues were crushed in liquid nitrogen and extracted with protein isolation buffer (50mM Na₂CO₃, pH 10.5; 100mM NaCl; 2.68mM KCl; 10mM EDTA; 0.15% Triton X-100; 10mM PMSF and 0.1% PVP). Keeping in ice, the mixture was homogenized by using a homogenizer and then subjected to sonication (3 bursts of 10 sec each). The suspension was incubated for 30 min at 20°C. After this, the pH of the solution was adjusted to 7.5-8 with 0.5 (N) HCl. The mixture was then centrifuged at 15,000g for 15 min at 4°C and the supernatant was collected. Total protein content was estimated by the method of Bradford (1976).

About 50μg of total protein from each plant sample was fractionated in 10% SDS-PAGE and then transferred onto PVDF membrane (Boehringer Mannheim) by using the trans-blot semidyrid transfer cell apparatus (Bio-Rad). The membrane was blocked with 5% (w/v) non-fat milk (Sigma) in 1XPBS for overnight at 4°C and then immunoprobied with Cry1Ac polyclonal antibody raised in rabbit at 1:5000 (v/v) dilution in PBST [1XPBS and 0.1% (v/v) Tween-20] for 2h at room temperature. The blot was washed with PBST for 4-5 times with fresh changes of wash buffer after every 15 min interval and finally incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG as the secondary antibody (Amersham Biosciences) at 1:50,000 (v/v) dilution in PBST for 1h. The blot was then washed extensively with PBST for 1h in the same way as before with fresh changes. Thereafter the membrane was soaked with enhanced chemiluminescence (ECL) detection solution (Amersham Biosciences)
according to the supplier's instruction and exposed to X-Ray film. The levels of expressed Cry1Ac protein generated by different transformants were estimated quantitatively with the help of a densitometer (Kodak Digital Science ID Image Analysis Software: 9/97-IB5430103 win version 2.0.1; New York) with respect to known amount of the His-tag purified Cry1Ac toxin protein.

1.2.8.7. Inheritance pattern of the cry1Ac gene in T1 generation:

Self-pollinated seeds of selected T0 transgenic lines transformed with the cry1Ac gene were allowed to germinate in MS basal medium supplemented with 30mg/l of hygromycin. Segregation for hygromycin resistance was estimated by counting the number of hygromycin resistant and sensitive plantlets. Chi square tests were performed to determine the fitness of the segregation ratio for hptII gene amongst T1 progenies. PCR analysis was conducted on HygR T1 plants with the help of gene specific primers to check the presence of cry1Ac gene in T1 generation. Thermal profile and primers used for PCR amplification were the same as mentioned earlier (section 1.2.8.3).

1.2.8.8. Insect feeding assay on the leaves of T1 transgenic lines:

Insect feeding assay was carried out using the first instar larvae of cotton bollworm (Helicoverpa armigera) on the leaves of different T1 transgenic lines. The experimental design for feeding assay was the same as described in section 1.2.8.2.