3.1. Source of Materials and Growth Conditions

3.1.1. *Brassica* spp.

Seeds of *Brassica* species (Table 2.1) were purchased from National Seed Corporation (N.S.C), New Delhi and germinated in Herbal Garden and tissue culture lab at Jamia Hamdard, New Delhi, India. Experimental material was obtained from these seedlings and plants.

**Table 3.1: Brassica species used in the present study**

<table>
<thead>
<tr>
<th>Species</th>
<th>Variety /common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brassica rapa</td>
<td>R-O-18</td>
</tr>
<tr>
<td>Brassica rapa</td>
<td>Chinese Cabbage</td>
</tr>
<tr>
<td>Brassica juncea</td>
<td>Indian Mustard (Pusa Bold and Pusa Jaikisan)</td>
</tr>
<tr>
<td>Brassica oleracea</td>
<td>Botrytis (Cauliflower)</td>
</tr>
<tr>
<td>Brassica oleracea</td>
<td>Capitata (Cabbage)</td>
</tr>
<tr>
<td>Brassica oleracea</td>
<td>Brussels Sprout</td>
</tr>
<tr>
<td>Brassica carinata</td>
<td>Abyssinian Mustard</td>
</tr>
<tr>
<td>Brassica napus</td>
<td>Rape seed</td>
</tr>
</tbody>
</table>

3.1.2. *Arabidopsis thaliana*

For germination *Arabidopsis thaliana* (Col-0) seeds were sown in plastic pots filled with soilrite, vermiculite and perlite in 1:1:1 ratio and kept in plant growth chamber under controlled conditions of 22°C temperature, 65% relative humidity with 16 hrs light and 8 hrs dark cycle. Hoagland’s nutrient solution (Appendix II) was used for watering. Seedlings with at least four leaves were transferred to pots and irrigated regularly. Floral dip method for transformation was performed when the plants reached flowering stage.
3.2. Isolation of Genomic DNA

3.2.1. CTAB method

CTAB method of Doyle and Doyle (1990) was followed for isolation of genomic DNA from field grown Brassica species.

Two grams fresh weight young leaves was macerated in pre-warmed at 65 °C 10 ml CTAB buffer (0.2 % of β-mercaptoethanol added just before use) in autoclaved mortar pestle. The ground material was mixed thoroughly with additional 10ml of pre-warmed buffer. The mixture was incubated at 65°C for 1 hr, with mixing every 5 min. Equal volume of Chloroform: Isoamylalcohol (24:1) was added, mixed properly by gentle inversion and incubated at room temperature for 5 min. The samples were centrifuged at 8000 rpm for 20 min at 4°C and the aqueous phase collected in fresh tubes. 5 μl RNase (50 mg/ml) was added to the aqueous phase and incubated at 37°C for 1 hr. The samples were re-extracted with equal volume of Chloroform: Isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 10 min at 4 °C. The aqueous phase was transferred to fresh tube. Equal volume of chilled isopropanol was added and incubated at –20°C for 30 min to precipitate DNA. DNA was pelleted by centrifugation at 10,000 rpm at 4°C for 15 min. The supernatant was discarded and the DNA pellet washed with 80% ethanol. Pellets were air dried for 1-1½ hrs and dissolved in TE buffer.

3.2.2. Edward’s method

A quick method of DNA extraction for screening of in vitro grown transgenic lines was done according to Edwards et al (1991).

50-100 mg leaf tissue was taken in 1.5 ml eppendorf tube and pulverized with liquid nitrogen at room temperature. 400 μl extraction buffer (Appendix II) was added and incubated at 37°C for 30-45 min. Samples were centrifuged at 5000 rpm for 7 min. Supernatant was transferred into fresh tubes and equal volume of isopropanol was added to precipitate the DNA and incubated for 2 min at room temperature. DNA was pelleted out by centrifugation at 13,000 rpm for 5 min. Pellets were air dried and dissolved in 50 μl sterile H2O.
3.2.3. Isolation of plasmid DNA

Plasmid DNA from *E. coli* and *Agrobacterium tumefaciens* was isolated manually using alkaline-lysis method as well as column based method through commercially available plasmid DNA purification kit (Macherey-Nagel, Germany).

3.2.3.1. Alkaline-lysis method

A single colony of bacterial strain was inoculated into LB medium containing appropriate antibiotics and allowed to grow overnight at suitable temperature in an incubator shaker. 1 ml of culture was taken in a sterile 1.5 ml eppendorf tube and cells were harvested by centrifugation at 12,000 rpm for 1-2 min at room temperature and resuspended in 100 μl of solution I (Appendix II) through vortexing. 200 μl of freshly prepared solution II (Appendix II) was added to lyse the cells. Mixed gently by inverting the tube 5-6 times and stored on ice for 5 min. For renaturation of plasmid DNA, 150 μl of ice cold solution III (Appendix II) was added, mixed by inverting and stored on ice for 5 min. The cell debris was pelleted out by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was taken out in a sterile eppendorf tube and equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C and again supernatant was taken out in a fresh tube. Two volumes of chilled absolute ethanol was added and incubated at –20°C for half an hour. The plasmid DNA was pelleted out by centrifugation at 12,000 rpm for 10 min at 4°C. Pellet was washed with 1 ml 70 % ethanol. Air dried pellet was dissolved in 20-30 μl of TE buffer (pH 8) and stored at –20°C.

3.2.3.2. Column based method

Bacterial culture was taken into an eppendorf tube and cells were harvested by centrifugation at full speed for 1 min and this step was repeated to get appropriate amount of pellet. Pellet was resuspended into 250 μl buffer A1 supplied in the kit through vortexing. After complete resuspension of the cells 250 μl buffer A2 was added for cells lysis, mixed gently by inverting the tube 6-8 times and incubated at room temperature for 5 min or until lysate became clear. For neutralization of lysate 300 μl buffer A3 was added to that and mixed by inverting the tube 6-8 times. Lysate was centrifuged at 12,000 rpm for 5 min and clear supernatant was collected into the
NucleoSpin® plasmid column packed in 2 ml collection tube. Column was centrifuged at 12,000 rpm for 1 min for binding the plasmid DNA onto the column membrane. Supernatant was discarded and placed the column into the collection tube. Membrane was washed with 600 µl buffer A4 by centrifugation at 12,000 rpm for 1 min, discarded the flow-through and placed the column again into the empty collection tube. Membrane was dried by spinning the empty column at 12,000 rpm for 2 min. Column was placed in an eppendorf tube, 30-40 µl buffer AE was added and incubated at room temperature for 2 min. Plasmid DNA was eluted by centrifugation at 12,000 rpm for 2 min.

**3.3. Isolation of Total RNA**

Total RNA was extracted by using Trizol®/Tri-reagent® (Invitrogen/Sigma) according to manufacturer’s instruction. In order to avoid degradation of RNA, all glasswares were rinsed with DEPC (Diethyl Pyrocarbonate) treated water followed by autoclaving and the entire procedure was carried out by wearing gloves in nuclease free environment.

100 mg plant material was taken into 1.5 ml eppendorf tube, pulverized in liquid nitrogen, 1 ml of Tri-reagent was added and homogenized. Resultant slurry was incubated at room temperature for 5 min for complete dissociation of nucleoprotein complexes. Samples were centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was transferred to the fresh tube. 200 µl chloroform was added, mixed by vigorous shaking for 15 sec and allowed to stand at room temperature for 10 min. Centrifuged the sample at 13,000 rpm for 15 min at 4°C. Aqueous phase was transferred to fresh tube; 500µl of isopropanol was added and kept at -20°C for 30 min to precipitate RNA. RNA was pelleted out by centrifugation at 13,000 rpm for 15 min at 4°C. Supernatant was decanted and the pellet was washed with 80% ethanol. Air dried the pellet at room temperature and dissolved in 30-40 µl DEPC water.

**3.4. Qualitative and Quantitative Analysis of Nucleic Acid**

Estimation of quality and quantity of isolated DNA and RNA was done through spectrophotometer and agarose gel electrophoresis.
3.4.1. Spectrophotometric analysis

Concentration and purity of the genomic DNA and RNA was measured using spectrophotometer (Eppendorf, BioPhotometer plus) at 260 and 280 nm according to the formula:

$$C (\mu g/\mu l) = \frac{(OD_{260} \times df \times ec)}{1000}$$

Where $OD_{260}$ is the measured optical density at 260 nm, df: dilution factor, and ec: extinction coefficient. For double-stranded DNA, the absorbance of one OD at 260 nm (known as ec) is 50 μg/ml, and for single stranded RNA is 40 μg/ml. The purity was determined from $A_{260}/A_{280}$ ratio; 1.8 was considered to be pure DNA while 2.0 is pure RNA.

3.4.2. Agarose gel electrophoresis for DNA

Agarose gel electrophoresis was performed for quantitative as well as qualitative estimation of genomic DNA. 0.8% agarose was added in 1X TAE or 0.5X TBE buffer and dissolved by boiling in a microwave. Dissolved agarose mix was allowed to cool to 50-60°C and 0.05 μg/ml of Ethidium Bromide was added to the solution (Sambrook and Russell 2001). Gel was poured carefully into the clean casting tray by avoiding bubble formation. The comb was inserted immediately and allowed to polymerize for 15-20 min at room temperature. After polymerization comb was removed and the gel was kept in the gel running tank. 6X loading buffer was added to the DNA samples and loaded to the gel. Electrophoresis was performed at 5 V/cm in 1X TAE or 0.5X TBE running buffer. The gel was run until the bromophenol blue dye migrated approximately 2/3rd of the gel distance. DNA band was visualized under UV light in UV transilluminator (UVP, UK) at short wavelength (254 nm). Uncut λ DNA of 25-ng/μl concentrations was used as standard marker for quantitative assessment of the DNA. For size estimation of the resolved fragments, a 100bp, 1kb (Fermentas, USA) or HindIII and EcoRI restricted λ DNA were used.

3.4.3. Native agarose gel electrophoresis for RNA

Agarose gel electrophoresis was performed for estimation of RNA quality. To prevent degradation of RNA, electrophoresis apparatus (casting tray and comb) was soaked in DEPC treated water for at least 4-5 hrs and solutions were prepared in DEPC treated
water (Sambrook and Russell 2001). 1% agarose (w/v) was dissolved in 0.5X TBE buffer by boiling in a microwave. Ethidium bromide was added to a final concentration of 0.05μg/ml and gel was poured into RNase-free casting tray. Before loading, RNA samples were prepared; 2 μl (2-5μg) of RNA was mixed with an equal volume of loading buffer (Appendix II), denatured at 65°C for 5 min and immediately transferred on ice and loaded to the gel. Electrophoresis was performed at 10 V/cm for 30 min and gel was visualized under UV transilluminator (UVP, UK).

3.4.4. Denaturing agarose gel electrophoresis for RNA

RNA retains much of its secondary structure unless it is first denatured. The quantitative and qualitative analysis of RNA was also done through denaturing agarose gel electrophoresis in which formaldehyde was added to the gel for denaturation of the RNA.

1.2% (w/v) of agarose was dissolved in 42.5 ml of DEPC treated water through boiling in microwave. Solution was kept to cool down to 55-60°C, afterwards 5 ml pre-warmed 10X MOPS buffer and 2.5 ml formaldehyde (stock 37%) was added, mixed properly and poured into the casting tray. After solidification of the gel comb was removed and placed to equilibrate the gel into the gel tank containing running buffer (1X MOPS buffer, 2.2 M formaldehyde) for 30 min. Approximately 5 μg of RNA was taken in an eppendorf tube, 2 μl of 5X MOPS buffer, 3.3 μl of formaldehyde and 10 μl of formamide was added and incubated at 95°C for 10 min and chilled on ice. 2 μl of loading buffer was added to the samples and loaded to the gel. Electrophoresis was performed at 10 V/cm for at least 1hr or till the bromophenol blue migrated half the gel. The gel was stained with ethidium bromide (0.05 μg/ml) and visualized under UV (312 nm).

3.5. Computational Analysis

3.5.1. Identification of homologs

The homologous sequences corresponding to genes of interest namely REVOLUTA (At5g60690) and miR165a (At2g40805) were retrieved from The Arabidopsis Information and Resource (TAIR version 10). These were used as query to perform
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BLASTN search using default parameters (e value= 1E-10; max no. of hits= 50) in the Brassica genome gateway against nr, gss, EST and BAC database.

BAC sequences were aligned using global alignment tool AVID (Bray et al. 2003) against A. thaliana genome and visualized using VISTA (Couronne et al. 2003; Dubchak and Ryaboy 2006).

3.5.2. DOT plot analysis

Dotplot analysis was performed by using dottup under EMBOSS (European Molecular Biology Open Software Suite). The BAC sequence to be analysed were saved as “fasta” file and loaded on to “dottup” module of EMBOSS (Rice et al. 2000) as input file. Subsequently, the word size was specified and dotplot was created. The dotplot output was saved as .png or as postscript file for further analysis.

3.5.3. Gene prediction

BAC sequences with HSP were analyzed for gene content based on comparative analysis with other plant genomes using FGENESH+ (Salamov and Solovyev 2000; www.softberry.com). FGENESH+ employed Arabidopsis gene model as template. All predictions were based on default parameters. Predicted genes were validated by BLASTX, TBLASTX and TBLASTN against respective databases at NCBI.

3.5.4. Primer designing

Primers were designed in identified putative sequences by using online primer designing software; Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and net primer (http://www.premierbiosoft.com/netprimer). The primers were synthesised from MWG-Biotech (Munich, Germany).

3.5.5. Sequence analysis

Prior to sequence analysis, .abi files were exported and used to generate contig. For this, .abi files were exported as .seq files using Finch TV (Geospiza Inc. 2004-06 ver. 1.4.0); contigs were constructed using DNA program (ver. 2.2) employing default parameter (minimum match= 80%, minimum overlap= 40). Errors in sequences were corrected by manual comparison with chromatograms.
Pair-wise sequence alignment was performed with Blast2 (bl2seq) tool at NCBI using default parameters including expect threshold=10, wordsize=28, match, mismatch scores=1-2, and by applying a filter on low complexity region.

Multiple sequence alignment was performed with ClustalX version 2.0.7 (Larkin et al. 2007) using the following procedure. The sequences to be aligned were saved in a single fasta format files, and were loaded into the program. Any gap or spaces in the sequence were removed by using the command “reset all gaps before alignment” under “alignment parameter”. Output formats including clustal, GCG, MSF, PHYLIP and NEXUS were selected for further downstream analysis.

The Nexus formatted output files of ClustalX were used for Bayesian based clustering with BEAST (Drummond and Rambaut 2007). Initially, an .xml file was created using BEAUti v1.6.1 with default parameters (Hasegawa, Kishino and Yano model of DNA substitution; Strict clock with rate=1.0; Coalescent Tree:Constant Size; MCMC Chain length= 10000000). The .xml file was then employed for generating trees depicting clustering through BEAST v1.6.1, and a consensus Tree was obtained using Tree Annotator v1.6.1. FigTreev1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) was finally employed to visualize the clustering. Sliding window analysis of REV partial genomic clones from Brassica species and A. thaliana was performed using DnaSP 5.0 (Librado and Rozas 2009).

3.5.5.1. Prediction of stem-loop secondary structure

The secondary structures of pre-miRNAs were predicted using the web-based computational software MFOLD 3.2 (Zuker 2003) available at http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi using default values set as RNA sequence (linear), folding temperature (37°C) and ionic strength (1M NaCl).

3.6. Polymerase Chain Reaction (PCR)

PCR was performed to amplify genomic DNA fragments by using gene specific primer pairs for cloning, colony PCR for screening of transformed bacterial colonies and screening of transgene integration into the transgenic plants. Annealing temperature varied according to primer melting temperature, extension time was set as per expected amplicon size of the gene, (ca. 1 min/Kb). Annealing temperature (Tm)
of primers was calculated using the formula: \( T_m = 2^o \times (A+T) + 4^o \times (G+C) - 3 \text{ to } 5^o \text{C} \). Standard reaction mix for amplifications is given below and standardized cycling parameters are described in Table 2.2.

- Template (DNA) 50 ng
- 10 X buffer 1.5 µl
- 25mM MgCl\(_2\) 1.0 µl
- 10 mM dNTPs 0.5 µl
- 10 µM forward primer 1.0 µl
- 10 µM reverse primer 1.0 µl
- *Taq* DNA polymerase (1U/µl) 1.0 µl
- Sterile MQ water to 15 µl

### 3.6.1. Colony PCR

Colony PCR was done for the screening of putative recombinant clones by using gene specific primers. Putative colonies were picked up and resuspended in 50 µl of sterile water. Suspension was incubated at 100°C for 1 min to get cell lysate, which was used as template. The reaction components for colony PCR are as follows:

- Colony suspension (cell lysate) 1.0 µl
- 10 X *Taq* buffer 1.5 µl
- 25mM MgCl\(_2\) 1.0 µl
- 10 mM dNTPs 0.5 µl
- 10 µM forward primer 1.0 µl
- 10 µM reverse primer 1.0 µl
- Sterile MQ water 8.5 µl
- *Taq* DNA polymerase (1U/µl) 0.5 µl
- Total 15 µl

### 3.6.2. PCR for screening of transgenic lines

To check the integration of gene of interest (transgene) into putative transformants; PCR was done by using primer pairs specific for GUS (*uid A*) and hygromycin (*hpt II*). Genomic DNA was isolated from transgenic lines according to Edward’s protocol described in section 3.2.2 and used as template. The cycling parameter used for the amplification is given in the table 2.2.
Table 3.2: PCR programmes for amplification of specific genes

<table>
<thead>
<tr>
<th>Locus</th>
<th>Initial Denaturation (Temperature and Time)</th>
<th>Denaturation (Temperature and Time)</th>
<th>Annealing (Temperature and Time)</th>
<th>Extension (Temperature and Time)</th>
<th>Number of cycles</th>
<th>Final extension (72°C)</th>
<th>Hold Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR165a and REV (P)*</td>
<td>94°C 5 min</td>
<td>94°C 1 min</td>
<td>56°C 1 min 30 sec</td>
<td>72°C 1 min</td>
<td>32</td>
<td>10 min</td>
<td>4°C</td>
</tr>
<tr>
<td>REV (F)*</td>
<td>98°C 30 sec</td>
<td>98°C 10 sec</td>
<td>55°C 1 min</td>
<td>72°C 4 min</td>
<td>40</td>
<td>10 min</td>
<td>4°C</td>
</tr>
<tr>
<td>uidA (GUS)</td>
<td>94°C 4 min</td>
<td>94°C 1 min</td>
<td>54°C 1 min</td>
<td>72°C 30 sec</td>
<td>32</td>
<td>10 min</td>
<td>4°C</td>
</tr>
<tr>
<td>hprtII</td>
<td>94°C 4 min</td>
<td>94°C 1 min</td>
<td>54°C and 55°C 1 min</td>
<td>72°C 30 sec</td>
<td>32</td>
<td>10 min</td>
<td>4°C</td>
</tr>
</tbody>
</table>

*(P) – partial, (F) – Full length

3.7. Purification of PCR Products

The amplified DNA product was eluted from agarose gel using column based commercially available PCR clean-up gel extraction kit (Macherey-Nagel, Germany and Wizard™, Promega, USA) according to manufacturer’s protocol.

The amplified single DNA band was excised from the agarose gel with sharp and sterile scalpel and transferred to a pre-weighed 1.5 ml eppendorf tube. Double volume of NT or QG buffer was added and incubated at 50°C till the gel slice melted. In case of Promega’s kit, equal volume of isopropanol was added to the sample, mixed by inversion and incubated at 4°C for 2 min. The sample was transferred to the MinElute® or NucleoSpin® column, and centrifuged for 1 min at 13000 rpm. The flow-through was discarded and column was placed back in the same collection tube. The column was washed with 700 μl wash buffer NT3 or PE and centrifuged for 1 min at full speed. The column was then placed into 1.5 ml fresh microcentrifuge tube. Pre-warmed 10-15 μl of elution buffer was added to the center of the membrane, incubated at room temperature for 2 min and centrifuged for at maximum speed 2 min. The eluted product was quantified with known concentration of λ DNA marker through agarose gel electrophoresis (section 3.4.2).

3.8. Ligation into Cloning Vector (pGEM-T Easy®)

The eluted product was ligated into the pGEM-T Easy® vector (Promega, USA). Insert to vector ratio was maintained at 3:1 ratio and calculated by using the formula:
A standard ligation reaction is described below:

\[
\text{Insert conc.} = \frac{\text{Vector conc.} \times \text{Insert size}}{\text{Vector size}} + \frac{3}{1} \text{ (insert: vector)}
\]

2X Rapid Ligation Buffer 5.0 μl
pGEM-T Easy Vector (50ng/μl) 0.5 μl
PCR product (insert) XX μl
T4 DNA Ligase (3 Weiss units/μl) 1.0 μl
Sterile MQ water to 10 μl

After setting this reaction, ligation mix was incubated at 16°C for 8-16 hrs or at 25°C for 2 hrs.

3.9. Preparation of Competent Cells

3.9.1. Ultra competent of Escherichia coli (DH5α)

Ultra competent cells were prepared according to the protocol described by Inoue et al. (1990). E. coli strain DH5α was streaked on semi-solid LB plate and incubated at 37°C for 14-16 hrs. Isolated single colony was picked up and inoculated in 10 ml SOB medium (see Appendix II for composition), allowed to grow as starter culture at 37°C and 150 rpm shaking for overnight. 1ml of overnight grown starter culture was inoculated in 100 ml of SOB medium and the cells were grown till OD₆₀₀nm reached to 0.5. The culture flask was incubated on ice for 10 min subsequently the bacterial suspension was transferred to sterile centrifuge tubes, and cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C. The pellet was resuspended in 32 ml of ice cold Inoue buffer (Appendix II), incubated on ice for 10 min and centrifuged at 4000 rpm for 10 min at 4°C. The pellet obtained was gently resuspended in 8 ml Inoue buffer and repeated the above step. Finally, the pellet was resuspended in 4 ml ice cold Inoue buffer and 320 μl DMSO was added to it and incubated on ice for 10 min. The suspension was dispensed as 50μl aliquots in 1.5 ml eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80°C for future use.

3.9.2. Competent cells of Agrobacterium tumefaciens (GV3101)

Agrobacterium tumefaciens strain GV3101 was streaked on LB agar plate containing Gentamycin (25 mg/L) and Rifampicin (25 mg/L) and incubated at 28°C. A single
colony was picked up, inoculated in 5ml of YEM medium and kept at 28°C with vigorous shaking at 200 rpm. 2 ml of the grown culture was used as starter culture and used to inoculate in 50 ml YEM medium, incubated at 28°C with 150 rpm shaking until the culture had grown to an OD = 0.4 to 0.6 at 600nm. The culture was cooled on ice and transferred into 50 ml centrifuge tube. Cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C. The supernatant was discarded and pellet resuspended into 2ml of 50mM ice-cold CaCl₂ solution and centrifuged at 4500 rpm for 10 min. The supernatant was decanted-off and finally pellet was resuspended in 2 ml of pre-cooled 20mM CaCl₂ solution. The suspension was distributed as 100µl aliquots into 1.5 ml eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80°C.

3.10. Bacterial Transformation

3.10.1. E. coli transformation

Transformation of ultra competent cells of E. coli (DH5α) was done using heat shock method (Hanahan 1983). An aliquot of competent cells was thawed on ice and ligation mix or plasmid was added and mixed gently through pipetting. The mixture was incubated on ice for 30 min. Heat shock was given at 42°C for 2 min and immediately transferred to ice for 5 min. 800 µl of SOC medium (Appendix II) was added to the mix and the cells allowed to recover at 37°C for 1h in an incubator shaker. After 1 h incubation the cells were pelleted out by centrifugation at 4000 rpm for 4 min. The supernatant was decanted and pellet was resuspended in 100 µl SOC medium by gentle tapping or pipetting. The transformed cells were spread with the help of glass spreader on LB-agar plate containing appropriate antibiotics (Ampicillin 100 mg/L, or Kanamycin 50 mg/L). For blue-white screening, plating was carried out on LB agar medium containing X-gal (5-bromo, 4-chloro, 3-indoyl-β-galactopyranoside) and IPTG (Isopropyl thio-galactoside) at concentration of 10 mg/L and 0.1M respectively. Plate was incubated at 37°C for 14-16 hrs until isolated colonies appeared on the plate.
3.10.2. *Agrobacterium tumefaciens* transformation

Transformation of binary vector into the *A. tumefaciens* strain GV3101 harbouring plasmid pMP90 was done through freeze-thaw method (An et al. 1998). Competent cells of *A. tumefaciens* were thawed on ice and 1µg of plasmid DNA was added to it and mixed gently. The mixture were quick frozen in liquid nitrogen, followed by incubation at 37°C for 5 min and then kept on ice. 1ml of LB medium was added to the mix and incubated at 28°C for 3 hrs with gentle shaking in an incubator shaker. The suspension was centrifuged at 4000 rpm for 4 min. Supernatant was discarded and resuspended the cells in 100 µl LB medium. The cells were spread on LB agar plate containing Gentamycin (25 mg/L), Rifampicin (25 mg/L) and Kanamycin (50 mg/L) and incubated at 28°C for 36 to 48 hrs or till colonies appeared. The colonies were picked for screening the integration of gene into the *A. tumefaciens* through colony PCR (section 3.6.3).

3.11. Sequencing

Three to five positive clones of each species were taken for sequencing. Plasmid DNA was isolated according to the protocol given in section 3.2.5. Sequencing was done based on Sanger’s di-deoxy chain termination (Sanger et al. 1977) principle, using Big Dye™ terminator mix and either standard primers (T3, T7, SP6 M13- Fwd, M13- Rev) or custom primers by ABI 3700 sequencer at TCGA (Okhla, New Delhi).

Appropriate amount of plasmid DNA was mixed with 1.6 pmole primer, 2 µl Big Dye™ terminator v3.0 sequencing ready reaction mix, and sterile MQ water to give final volume of 10 µl. Sequencing was carried out as PCR based cycling reaction with the following parameters; 95°C for 5 min followed by 35 cycles of 30 sec at 95°C, 15 sec at 50°C and 4 min at 60°C.

3.12. Amplification and Cloning of Large Fragment DNA

Amplification of large fragment of DNA from *Brassica juncea* was performed by using long range Taq polymerase; Ex-Taq (Takara, Japan). 4.2 kb product of *REVOLUTA* was amplified from genomic DNA of *B. juncea*. Standardized reaction
for amplification is given below and cycling parameters for PCR are mentioned in Table 2.2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template (DNA)</td>
<td>75 ng</td>
</tr>
<tr>
<td>10 X buffer</td>
<td>5.0 μl</td>
</tr>
<tr>
<td>10 mM dNTPs mix</td>
<td>4.0 μl</td>
</tr>
<tr>
<td>10 μM primer Fwd</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>10 μM primer Rev</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Ex-Taq (5U/μl)</td>
<td>0.2 μl</td>
</tr>
<tr>
<td>Sterile MQ water to 50 μl</td>
<td></td>
</tr>
</tbody>
</table>

Amplified products resolved by agarose gel electrophoresis (section 3.4.2), gel purified the product according to protocol given in section 3.7. Purified product was cloned using T&A cloning kit (Real Biotech Corporation).

Ligation into TA cloning vector:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X ligation buffer A</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>10 X ligation buffer B</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>T&amp;A cloning vector</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>PCR product (REVOLUTA)</td>
<td>50 ng</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Sterile MQ water to 10 μl</td>
<td></td>
</tr>
</tbody>
</table>

Ligation mix was transformed into *E. coli* competent cells (section 3.10.1). Colonies were picked up randomly and screened through restriction digestion with suitable enzymes and finally through sequencing (section 3.11). For restriction digestion and sequencing plasmid DNA was isolated according to protocol given in section 3.2.5. Reaction for restriction digestion is as follows.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>5.0 μl</td>
</tr>
<tr>
<td>Sal I (10 U/μl)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Kpn I (10 U/μl)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>10X buffer</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Sterile MQ water to 20 μl</td>
<td></td>
</tr>
</tbody>
</table>
3.13. Expression Analysis

3.13.1. Small RNA Blotting

Small RNA Northern blot was performed for detection of microRNA in different tissues of *B. juncea* and *B. rapa*. To carry out this, total RNA was isolated from different tissues (Table 3) as per the protocol described in section 3.3. The quantitative and qualitative analysis of total RNA was done through spectrophotometer as well as gel electrophoresis (section 3.4.3, 3.4.4).

3.13.1.1. Denaturing PAGE

In order to avoid RNA degradation the electrophoresis apparatus, glass plates, combs and spacers were cleaned with detergent and rinsed thoroughly with autoclaved water followed by DEPC treated water. Glass plates were clamped together with binder clips. To prepare the gel, 20 ml of 15% Acrylamide : bisacrylamide, 1 ml 50X TBE and 21 g urea was taken in a clean flask and warmed up the solution briefly in microwave to dissolve the urea and volume was maintained upto 50 ml with DEPC treated water. Just before pouring the gel, 500µl freshly prepared 10% APS and 15µl TEMED was added to the solution. Gel was poured into the space between the two glass plates carefully by avoiding air bubble formation almost up to the top, and then immediately the desired comb was inserted. The gel was allowed to polymerize for 2-3 hrs. After polymerization the comb and spacer was removed carefully and gel casting assembly was loaded on the running apparatus. The tank was filled with 1X TBE buffer. Before running the gel wells were rinsed thoroughly with 1X TBE buffer by using a syringe and needle and the gel was pre-run at 400 V (40 mA) for 1 hr to achieve and maintain uniform temperature of ca. 55°C for proper denaturing conditions.

3.13.1.2. Sample preparation

50 µg of total RNA of each samples were taken in eppendorf tubes and equal volume of FLS (Appendix II) was added. Samples were denatured at 65°C for 20 min and quick chilled on ice.
3.13.1.3. Sample loading and electrophoresis

Immediately before loading the samples on to the warm gel, wells were rinsed again by using syringe and needle. Samples were loaded and allowed to come into the well completely by running the gel at 200V. Once the samples have migrated out of the well, gel was run at 400V until the bromophenol blue dye reached the bottom of the gel and xylene-cyanol was detected in the middle of the gel. Apparatus was dismantled and soaked the gel in 1X TBE for 10 min. Gel was cut into half; lower portion used for blottting as small RNA migrates between bromophenol blue and xylene cyanol. Upper portion was stained with EtBr for photography to be used as loading control.

3.13.1.4. Blotting and fixation

Semi-dry transfer of small RNA from gel to membrane was done using an electroblotter (Biometra, Germany) according to manufacturer’s instructions. Six pieces of 3 MM Whatman® blotting sheet was cut according to gel size and soaked in 1X TBE buffer for 3-5 min. Positively charged membrane ‘Nytran’ (Amersham, USA) pre-soaked in 1X TBE buffer was used for blotting. Blot was assembled in the order: 3 MM Whatman blotting sheet – membrane – Gel (upside down) – 3 MM Whatman blotting sheet. The transfer of small RNA to the membrane was carried out by using current flow 200 mA for 2 hrs. Blot was disassembled, placed the membrane on dry Whatman paper and cross-linked the RNA to the membrane by exposure to UV at 1200 µJ/cm² for 2 min (UVP, UK).

3.13.1.5. Probe labelling and purification

Single stranded oligo antisense to miR165a was end labelled with [γ-32P] ATP using T4 polynucleotide kinase (Fermentas, USA). The reaction of probe labelling is as follows:

- 10X T4 polynucleotide kinase buffer: 5.0 µl
- 10µM single stranded DNA oligos: 5.0 µl
- ATP [γ-32P] (370 MBq/ml): 5.0 µl
- T4 polynucleotide kinase (PNK; 10U/ µl): 1.0 µl
50 µl as final volume was maintained by adding RNase free water and mix was incubated at 37°C for 30 min. Reaction was stopped by incubating at 90°C for 5 min and quick chilled on ice.

Labelled probe was purified through Sephadex G-25 column which was prepared manually. Sephadex G-25 beads and Glass wool were soaked in 1X TE buffer for 3-4 hrs and autoclaved that. 0.5 ml decaped eppendorf tube was taken and created a tiny hole in the bottom of tube with the help of red hot needle. The bottom of the tube was packed with Glass wool with the help of packer and Sephadex G-25 beads were filled in glass wool packed tube, centrifuged at 4000 rpm for 1 min and flow through collected in the 1.5 ml collection tube. The step was repeated until 0.5 ml tube was packed with beads. Finally, G-25 column was transferred to a fresh 1.5 ml eppendorf tube, probe was added to the column and collected the purified probe by centrifugation at 4000 rpm for 2 min and used for hybridization.

3.13.1.6. Hybridization and detection

Cross-linked membrane was placed into a hybridization bottle and pre-hybridized. Buffer used for Pre-hybridization and hybridization was same and contained 7% SDS, 1M phosphate buffer and 0.5M EDTA (Appendix II). 15 ml pre-hybridization solution was added into hybridization bottle containing membrane and pre-hybridized for 2 hrs at 40°C in hybridization oven. After completion of pre-hybridization, buffer was replaced and 10 ml of fresh buffer was added containing purified probe and kept for hybridization at 40°C for overnight.

Next day, hybridization solution was removed and membrane was washed twice using wash buffer I (Appendix II) at the same temperature for 5-7 min. The washing time was dependent upon the radioactivity count of the membrane. Washing was continued until the counts become differential. The membrane was wrapped in a Clingfilm™ and exposed to X-rays film along with intensifying screens for 2 to 3 days at -80°C. Autoradiography was carried out as per standard protocol (Sambrook and Russell 2001).
3.13.2. DNase treatment of total RNA

DNA free RNA was prepared for RT-PCR, through DNase treatment as given below:

RNA 1.0μg
10X DNase Buffer 1.0 μl
RiboLock™ (1U/μl) 1.0 μl
DNase I (1U/μl) 1.0 μl
DEPC treated water to 10 μl

The reaction mixture was incubated at 37°C for 30 min. To inactive DNase 1 μl 0.5M EDTA was added and incubated at 65°C for 15 min. The samples were stored at -80°C till use.

3.13.3. Stem-loop RT-PCR (SLRT-PCR)

Transcript levels of mature miR165a were also evaluated by stem-loop RT-PCR (Gasic et al. 2007) and the primers were designed as per the protocol of Chen et al. (2005). The stem-loop RT primer was allowed to bind to the miRNA and reverse transcribed in a pulsed RT reaction using RevertAid™ H Minus cDNA synthesis kit (Fermentas, USA). Stem-loop RT primer and total RNA was mixed and incubated for 5 min at 65°C, chilled on ice for 5 min. After that, 2 μl 10mM dNTP, 4 μl 5X reaction buffer, 1 μl Ribolock and 1 μl reverse transcriptase (200U/μl) were added. The PCR conditions for the first strand were 16°C for 30 min, 50°C for 30 min and subsequently reaction was stopped by incubating at 85°C for 5 min. The RT reactions were performed as 94°C for 5 min for 1 cycle, followed by 94°C for 20 s, 60°C for 30 s and 72°C for 30 s up to 28 cycles. RT product was used as template to amplify the microRNA sequences through semi-quantitative RT-PCR by using microRNA specific forward primer and universal reverse primer (Appendix I). Amplification of 5S rRNA served both as internal control as well as to normalize the reaction. The amplified product was electrophoresed on 4% agarose gel and visualized after ethidium bromide staining (Sambrook and Russell 2001).
3.13.4. cDNA synthesis

First-strand cDNA was synthesized using RevertAid™ H Minus cDNA synthesis kit (Fermentas) from 1 µg of DNase treated total RNA according to the manufacturer’s instructions. 1 µl oligo (dT₁₈) primer was added to the RNA, mixed gently, incubated at 70°C for 5 min, and immediately placed on ice. Subsequently, the following components were added.

5X reverse transcription reaction Buffer 4.0 µl
Ribolock™ (1U/µl) 0.5 µl
10mM dNTP 2.0 µl
DEPC treated water 7.5 µl

Mixed gently and incubated at 37°C for 5 min. Finally, 1 µl M-MuLV Reverse Transcriptase H’ (RevertAid™ H Minus) was added, mixed and given a short spin to collect the droplets. The mixture was incubated at 42°C for 1 hr followed by heat inactivation of Reverse Transcriptase at 70°C for 10 min and stored at -20°C.

3.13.5. Semi-quantitative RT-PCR

Transcript level of genes was analyzed by using semi-quantitative RT-PCR. 2 µl from 1/10th dilution of prepared cDNA was used as template for subsequent second strand synthesis. The detailed description of reaction mix and cycling parameter for amplification is given below.

First strand cDNA (1/10 dilution) 2.0 µl
10 X Taq pol buffer 1.5 µl
25 mM MgCl₂ 1.0 µl
10 mM dNTPs 0.5 µl
10 µM primer (Fwd) 1.0 µl
10 µM primer (Rev) 1.0 µl
DEPC treated water 7.0 µl
Taq DNA polymerase (1U/µl) 1.0 µl
Total 15 µl
Cycling parameter for second strand synthesis is as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>4:00</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>1:00</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>56</td>
<td>1:00</td>
<td>26-28</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1:00</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>10:00</td>
<td>1</td>
</tr>
<tr>
<td>Storage</td>
<td>4</td>
<td>infinity</td>
<td></td>
</tr>
</tbody>
</table>

β-Actin was used as an internal control to normalize the amplification reaction.

### 3.13.6. Densitometric analysis of expression profile

The gel images were analyzed using Quantity-One 1-D software (Bio Rad, USA) to obtain the intensity values along with the standard error. The intensity values of transcript level of root was normalized against 5SrRNA (for *miR165a*) or actin (for *REV*); and the relative transcript levels of other tissues were then normalized against root. The statistical analysis of the densitometric data was done by one way ANOVA followed by Dunnett’s ’t’ test and expressed as mean ± SD.

### 3.14. Functional Characterization


The binary vector pCAMBIA 1304 was used for the construction of plant transformation vector.

The recombinant plasmid of *miR165a:pGEM-T Easy* was restricted with *EcoRI* to release the insert from cloning vector. To linearize and generate compatible ends for ligation, binary vector (pCAMBIA 1304) was also restricted with the same restriction enzyme (*EcoRI*). The common reaction for restriction of both is given below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>5.0</td>
</tr>
<tr>
<td>10X Restriction buffer</td>
<td>2.0</td>
</tr>
<tr>
<td><em>EcoRI</em> (10U/µl)</td>
<td>1.0</td>
</tr>
<tr>
<td>Sterile Milli-Q water</td>
<td>12</td>
</tr>
<tr>
<td>Total volume</td>
<td>20</td>
</tr>
</tbody>
</table>
The above reaction was incubated at 37ºC for overnight and next day reaction was terminated by heat inactivation at 65ºC for 10 min. Restricted product was electrophoresed at 0.8-1.0% agarose gel. The desired restricted fragment was eluted from gel according to protocol given in section 3.7. The restricted pCAMBIA 1304 was dephosphorylated using calf intestine alkaline phosphatase (CIAP) treatment.

Reaction of CIAP treatment is as follows:

\[
\begin{align*}
\text{Restricted plasmid} & \quad X \ \mu l \\
10X \text{ reaction Buffer} & \quad 3.0 \ \mu l \\
CIAP (1U/\mu l) & \quad 1.0 \ \mu l \\
\text{Sterile Milli-Q water} & \quad \text{to 30 } \mu l
\end{align*}
\]

The above reaction was incubated at 37ºC for 30 min and the reaction was stopped by heating at 85ºC for 15 min.

The eluted product was sub-cloned into CIAP treated binary vector pCAMBIA 1304 using ligation reaction as given below:

\[
\begin{align*}
\text{Restricted insert} & \quad X \ \mu l \\
\text{Restricted vector} & \quad Y \ \mu l \\
10X \text{ ligase Buffer} & \quad 1.0 \ \mu l \\
\text{T4 DNA Ligase (10 U/\mu l)} & \quad 1.0 \ \mu l \\
\text{Sterile Milli-Q water} & \quad \text{to 10 } \mu l
\end{align*}
\]

A 3:1 insert: Vector ratio was maintained and ligation mix was incubated at 16ºC for 8–16 hrs and was transformed into ultra competent cell of \textit{E. coli} (DH5α). The recombinant clones were screened through colony PCR followed by restriction digestion with appropriate enzyme. The orientation of ligated insert was checked by PCR using polyA tail reverse primer and \textit{miR}165\textit{a} specific forward primer. Confirmed clone with right orientation was transformed into \textit{Agrobacterium tumefaciens} strain GV3101 (section 3.10.2).
3.14.2. *Brassica* transformation

3.14.2.1. Sterilization of seed

Seeds of *Brassica juncea* var. Pusa Bold were surface sterilized by treating sequentially with 0.2% Cetrimide for 5 min, 0.5% Streptomycin sulphate for 5 min and mixture of 0.5% Streptomycine sulphate and 0.5% Bavistin in 1:1 ratio for 2 min. Seeds were washed with 0.1% HgCl₂ for 1 min and from 70% alcohol for 1 min. Finally, seeds were thoroughly rinsed thrice with sterile double distilled water to remove sterilants from the surface of seeds. Sterilised seeds were inoculated on Murashige and Skoog (MS) medium (Appendix II) for germination.

3.14.2.2. Source of explants

Hypocotyl segments (0.3-0.4 cm) from *in vitro* germinated 4-5-day-old seedling of *B. juncea* var. Pusa Bold were used as explants for transformation. One day before the co-cultivation the hypocotyl segments were inoculated on MS medium supplemented with BAP (1.0 mg/L) + 2,4-D (0.05 mg/L) + AgNO₃ (20µM). The pre-cultured hypocotyls were then co-cultivated with *A. tumefaciens* suspension harbouring gene of interest.

3.14.2.3. Co-cultivation

Single colony of *Agrobacterium tumefaciens* (GV3101) harbouring 35S::miR165a construct was inoculated in 50 ml LB medium (Appendix II) containing Gentamicin (25 mg/L), Rifampicin (25 mg/L) and Kanamycin (50 mg/L). The cells were grown at 28°C for 36 hrs until the culture had grown to an OD of 0.4 to 0.6 at 600 nm. The cells were then harvested by centrifugation at 5000 rpm for 10 min and resuspended into MS liquid medium. Pre-cultured hypocotyl segments were submerged into suspension for five min. Extra suspension from the surface of explants was blotted dry using sterile blotting paper. The hypocotyls were inoculated on MS medium supplemented with BAP (1.0 mg/L) + 2,4-D (0.05 mg/L) + AgNO₃ (20µM) and kept in dark for 36 hrs.
3.14.2.4. Selection and regeneration of putative transformants

After 36 hrs of co-cultivation thin film of Agrobacterium surrounding the explants was washed with liquid MS medium containing Carbenicillin, (250 mg/L), blotted dry on sterile filter paper and inoculated on regeneration medium containing Carbenicillin (250 mg/L).

Initially, till one week the explants were kept on regeneration medium containing only Carbenicillin. After one week, explants were transferred on regeneration medium containing selection antibiotic; Hygromycin (10 mg/L) as well as Carbenicillin (250 mg/L). The cultures were kept in culture room at 23 ± 2°C, 55% relative humidity and 8 hrs photoperiod. Hygromycin and Carbenicillin were removed from the medium after 8 weeks.

Shoot elongation and proliferation of cultures transformed with miR165a gene was obtained on MS medium (2% sucrose) supplemented with BAP (1.0 mg/L). Rooting of transformed lines was obtained in MS + IBA (1.0 mg/L).

3.14.3. Arabidopsis thaliana (Col-0) transformation

Floral dip transformation method was used for generation of transgenic lines of A. thaliana.

3.14.3.1. Preparation of bacterial suspension

A single colony of A. tumefaciens strain harbouring gene of interest in the binary vector was inoculate into 5-10 ml liquid LB medium containing appropriate antibiotics and incubated at 28°C in incubator shaker for growth. Cells were subcultured in 150 ml liquid LB with the appropriate antibiotics until cells had grown to an OD of ~0.8-1.0 at 600 nm. Cells were harvested by centrifugation at 5000 rpm for 15min at 4°C, and resuspended the cells in 5% freshly prepared sucrose solution to final OD$_{600}$ = ~ 0.6-0.8.

3.14.3.2. Floral dip method

To prepare transformation solution, 0.05% (v/v) Silwet L-77 was added to the bacterial suspension and mixed well with vigorous shaking. Siliques that have been formed in plants were removed before transformation. The transformation solution was poured into proper size beaker and the above-ground parts were dipped in
transformation solution for 20 to 30 sec with gentle agitation. Plants were covered by plastic bags for 16-24 hrs to maintain high humidity and kept in dark. Next day, covers were removed from the plants were allowed to grow in plant growth chamber. Plants were irrigated normally with Hoagland’s solution (Appendix II). Watering was stopped as seeds matured and after complete drying, seeds were harvested.

3.14.3.3. Selection of primary transformants of *A. thaliana*

1/2MS plates were prepared containing Carbenicillin to prevent bacterial growth and Hygromycin (10 mg/L) as selection antibiotic for positive transformants selection. Seeds of transformed plants were sterilized with the mix of 70% (v/v) ethanol and 0.05% Triton X-100 for 10 min. Subsequently, washed 5 times with sterile distilled water in laminar flow hood and vernalized for 3-4 days. Vernalized seeds were spread on autoclaved Whatman filter paper in laminar air flow and kept for drying for 10-15 min. Dry seeds were sprinkled on the plate of selection medium. Plates were sealed with parafilm and kept in the culture room condition till transformants could be distinguished as seedlings with healthy green cotyledons, true leaves and roots. Putative transformants were transplanted in the pots filled with soilrite, vermiculite and perlite in a 1:1:1 ratio placed in plant growth chamber and watered regularly with Hoagland’s solution till the maturation of seeds.

3.14.4. Screening of putative transformants

3.14.4.1. GUS assay

Histochemical analysis of transformed lines was done to determine the GUS expression according to Jefferson (1987). Tissues from putative transgenics were collected and immersed in GUS staining solution as given below:

- 2X Citrate-HCl buffer (pH 7.0) 1000 μl
- X-Gluc solution (10mg/1ml) 100 μl
- 0.1 M Potassium ferrocyanide 20 μl
- 0.1 M Potassium ferricyanide 20 μl
- 10 % (w/v) Triton X-100 10 μl
- Sterile Milli-Q water: 850 μl
- Total 2000 μl
Tissues dipped in GUS staining solution were incubated overnight at 37°C. After blue colour appeared, chlorophyll was removed by treating sequentially with 50%, 60% and finally 70% ethanol at 37°C and then photographed.

3.14.4.2. PCR screening of putative transgenic lines

After preliminary screening using GUS assay, DNA was isolated from putative transgenic lines according to Edward’s method (section 3.2.2). Integration of transgene into plants was verified by PCR using primer pairs specific for GUS (uid A) and Hygromycin (hpt II) (Table 2) according to protocol described in section 3.6.2.

3.14.4.3. Southern Blotting

In order to confirm the transgene integration and to determine the number of copies of transgene integrated, Southern blotting (Southern 1975) was performed. Genomic DNA was isolated from 100 mg fresh weight leaf material of in vitro grown transgenic plants as well as from non-transgenic plant to use as negative control using Edward’s method.

3.14.4.3.1. Restriction digestion and electrophoresis

15 µg genomic DNA from different transgenic lines and a non-transgenic plant was restricted with EcoRI in the reaction mix given below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>15 µg</td>
</tr>
<tr>
<td>10X restriction buffer</td>
<td>15 µl</td>
</tr>
<tr>
<td>EcoRI (10U/µl)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Sterile Milli-Q water</td>
<td>to 150 µl</td>
</tr>
</tbody>
</table>

Reaction mix was incubated at 37°C for overnight. Next day, reaction was stopped by incubating at 65°C for 15 min. Digested product was precipitated by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and 2.5 volume of ice cold absolute ethanol and incubated on ice for 30 min. The samples were centrifuged at 12,000 rpm for 20 min at 4°C. Pelleted DNA was washed with 70% ethanol (v/v), air dried and dissolved in 20-25 µl of water. Restricted DNA was fractionated on 0.8% (w/v) agarose gel in 1X TAE at 3-5 v/cm until the bromophenol blue migrated to 2/3rd distance of the gel. After completion of electrophoresis gel was stained in ethedium bromide and visualised under UV light and photograph was taken with fluorescent ruler.
3.14.4.3.2. Transfer of DNA on nylon membrane

DNA fragments from the gel were transferred onto nylon membrane (Hybond-N plus, Amersham) through capillary transfer method (Southern 1975). Before transfer, gel was treated with depurination solution (0.25M HCl) for 15 min, denaturation solution (0.5N NaOH, 1M NaCl) for 45 min and neutralization solution (1.5M Tris, pH 7.4 and 1.5M NaCl) for 45 min. Finally, the gel was washed thrice with sterile water to remove the solution from the gel surface. Meanwhile, a solid support larger than the gel was placed on the tray and transfer buffer; 20X SSC (Appendix II) was added to it. Two sheets of 3 MM chromatography paper (Whatman) were folded over the solid support and allowed to hang on both side of the support so that it would form a proper wick allowing efficient movement of transfer buffer. Three sheets of 3 MM Whatman paper and the nylon membrane were cut to the size of the gel. After neutralization, gel was placed upside down on the solid support with wet 3 MM paper wick. The nylon membrane was placed carefully on top of the gel such that one end extends just over the lines of slots at the top of the gel by avoiding trapping of air bubbles between the gel and the membrane. Three sheets of 3 MM paper equal to the gel size were placed on the nylon membrane. The stacks of blotting sheets of gel size were kept on top of the 3 MM papers. A glass plate was placed on the top of the stack over which weight of around 500 g was placed. The transfer of DNA from gel to membrane was allowed to proceed in capillary action manner for a minimum of 16 hrs. At the end of the transfer, the blotting sheets and the 3 MM paper layers were removed and the positions of the slots/wells on the membrane were marked with the help of HB pencil. The membrane was then removed and washed in 2X SSC buffer; subsequently, transferred DNA fragments were immobilized by UV crosslinking at 1200 µJ/cm² for 2 min (UVP, UK).

3.14.4.3.3. Pre-hybridyization

Membrane was kept in a hybridization bottle; 20 ml of Pre-hybridyization solution (Appendix II) was added and placed into rotating hybridization oven at 60°C for 2-3 hrs.
3.14.4.3.4. Probe labelling and purification

hptII gene was used for labelling. The hptII gene was amplified, gel eluted and quantified. Probe was labelled by using probe labelling kit (Roche, UK). Reaction of probe preparation is as follows:

Eluted hptII product 30 ng
Sterile MQ water to make 8 µl

Denatured in boiling water bath for 10 min, quick chilled on ice and given a short spin. After denaturing the probe following components were added.

High prime reaction mix 4 µl
dATP, dTTP, dGTP 1 µl each
α-P^{32}\text{-dCTP (50µci/mol)} 1-2 µl
Sterile Milli-Q water to 20 µl

Given a short spin to collect the components at the bottom of the tube, incubated at 37°C for 10 min. Reaction was terminated by heating at 65°C for 10 min and quick chilled on ice.

The labelled probe was purified using G-50 column supplied by GE-life sciences. Column was placed in an eppendorf tube after vortexing and centrifuged at 3000 rpm for 5 min. Flow through was discarded and placed the column in a fresh eppendorf tube. Prepared probe was added to the column, centrifuged at 3000 rpm for 1min and purified probe was collected into the tube and checked for count. Purified probe was denatured in boiling water bath for 5 min and quick chilled on ice.

3.14.4.3.5. Hybridization and detection

Prehybrydization solution was removed from the bottle and fresh solution along with denatured probe was added. The bottle was placed back in the oven for overnight at 60°C to for hybridization. Next day, membrane was washed with wash buffers. First wash was give with wash solution I (Appendix II) for 10 min at room temperature and second wash was stringent at 60°C with wash solution II (Appendix II) for 5 min and checked for the differential count. Membrane was taken out from the bottle, kept between the autoclaved bag/Saranwrap™ and exposed to X-ray film.
Autoradiography was carried out according to protocol given by Sambrook and Russell (2001).

3.15. Microscopy

The micromorphology of adaxial and abaxial surface of leaf was examined under FEI Quanta 200 FEG scanning electron microscope (SEM) at IIT, Delhi. Leaf samples were mounted directly on metal stubs using both side sticky tape (carbon tape) and observed the samples in low vacuum mode at different magnifications.