CHAPTER 2
Materials and Methods

Note: Most of the basic molecular biology protocols such as plasmid isolation, restriction analysis, ligation etc., have been adapted from the standard book "Molecular Cloning: A Laboratory Manual" (Sambrook et al., 1989; Sambrook and Russell, 2001).

2.1 Similarity searches and retrieval of other information using Cyanobase database

Cyanobase Kazusa genome resource database (http://dev-genome.annotation.jp/cyano base) was used to find out the cyanobacterial homologues of BicA transporter using Cyanobase ortholog table (http://genome.microbedb.jp/cyanobase/GCA_000019485.1/ genes/SYNPCC7002_A2371) and information of their gene/protein sequences, length, conserved domains etc., was obtained by InterProScan table of the database. The presence of BicA transporter was checked in other cyanobacterial species using BLASTP tool of Cyanobase similarity search online server (http://genome.microbedb.jp/blast/ blast_search/cyanobase/GCA_000317635.1/genes).

2.2 PSI blast and retrieval of various protein sequences

BicA protein sequence of *Synechococcus* PCC 7002 was retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/protein) and was used as a query sequence to perform PSI blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) search, which was performed in two different ways so that maximum possible hits could be obtained. Firstly, blast was performed against all the possible organisms excluding cyanobacteria with 1000 target sequences, which were screened manually to select proteins, which performed other functions different from transporting bicarbonate ions like the query protein BicA.

Secondly blast was performed independently against domain archaea, eubacteria and also independently against each eubacterial phyla with 1000 target sequences, out of which again only those types of proteins were selected which were performing different functions. In few cases, the hits obtained were again used as the query sequence and then successive blast searches were performed to explore the maximum possible BicA related proteins. After performing these repeated blast searches, an array of proteins were obtained from all the possible organisms excluding cyanobacteria, showing identity to the query sequence but performing diverse functions other than transport of bicarbonate

ions. A total of 43 proteins were selected for further analysis and all the protein sequences were retrieved from NCBI database.

2.3 Conserved domain (CD) analysis

The conserved domain search was performed for all the 43 protein sequences using NCBI CD (Marchler-Bauer et al., 2015) search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The BicA protein was again analyzed to look for maximum possible conserved domains using HHpred online server (https://toolkit.tuebingen.mpg.de/hhpred) which is based on Hidden Markov Model (HMM) and can detect homologues even with <20% sequence identity (Söding, 2005).

2.4 Phylogenetic tree construction

All the 43 protein sequences were subjected to multiple sequence alignment (MSA) by clustal W and MSA was used to find out the best fit amino acid substitution model (Nei and Kumar, 2002). Best model (LG model) (Le and Gascuel, 2008) with lowest BIC (Bayesian Information Criterion) score was used to build a phylogenetic tree by Maximum Likelihood (ML) method with 1000 bootstrap values using MEGA version 6.0 "Molecular Evolutionary Genetics Analysis" (Tamura et al., 2013).

2.5 Information retrieval for inner chloroplast located proteins of *Arabidopsis thaliana*

The AT_CHLORO database (http://at-chloro.prabi.fr/at_chloro/) was used to manually acquire all the relevant information about the chloroplast proteome of *A. thaliana* (Ferro et al., 2010). A total, 1856 proteins have been annotated in this database and majorly categorized as per their sub-plastidial localization i.e., envelope, stroma, thylakoid and grana/stroma-lamellae proteins.

The AT_CHLORO database maintains information for all the proteins that have been identified from *A. thaliana* chloroplast. Information provided by the database includes accession numbers, sub-plastidial localization, analytical coordinates of all peptides, curated and predicted localization and functions of all the proteins. This database is also linked to other websites named as TAIR, AtProteome, PPDB, Aramemnon, POGs, SUBA.

2.6 Prediction of transit peptides

For prediction of transit peptides, ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) and TargetP 1.1 servers (http://www.cbs.dtu.dk/services/TargetP/) were used. Both the software are available online, in which protein sequence to be analyzed was added as a query. ChloroP (Emanuelsson et al., 1999) is a neural network (NN) based program and it specifically predicts the chloroplast transit peptide (cTP). The results obtained confirm the presence or absence of cTP, and the cTP length, which indicates towards the corresponding cleavage site (CS), determined by NN output score and CS score, respectively. The score is calculated from an automatic algorithm called MEME. TargetP (Emanuelsson et al., 2000) is also an NN based program and it predicts cTP, mitochondrial targeting peptide (mTP) and secretory pathway signal peptide (SP). The confirmation of protein localization is based on the NN score. Results obtained also include the predicted presequence length (TPlen) and reliability class (RC) values, which inversely indicate the reliability of prediction.

2.7 Prediction of TM helices and orientation of N- and C-termini

TMHMM and TOPCONS were used for prediction of TM protein topology by using protein sequences as query. To estimate the number of TM helices in the proteins under study, TMHMM server, v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001) was used while TOPCONS server (http://topcons.cbr.su.se/) (Bernsel et al., 2009) determined both the number of the TM helices and the orientation of the N- and C-termini of proteins. Although both the programs are hidden markov model (HMM) based, but TOPCONS is more advanced and efficient because it provides the results based on the individual topologies predicted using five different software, namely SCAMPI-single, SCAMPI- multi, OCTOPUS, PRO-TMHMM and PRODIV-TMHMM.

2.8 Codon usage and rare codon analysis

The *bicA* gene was analysed to find the differences in codon usage preference with respect to the genome of organisms that were used as expression hosts; namely *Arabidopsis* and *Nicotiana* species. The codon adaptation values were determined using a web server CAIcal (http://genomes.urv.es/CAIcal) (Puigbò et al., 2008) while an online available tool named Graphical Codon Usage Analyzer (GCUA) (Fuhrmann et al., 2004) was used to determine relative adaptiveness (http://gcua.schoedl.de/seqoverall_v2.html)

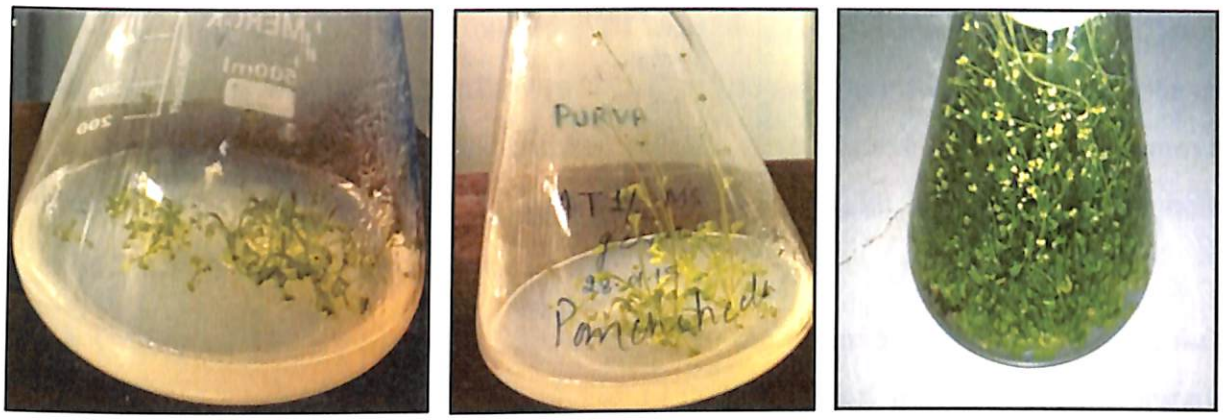
and low frequency codons (http://gcua.schoedl.de/sequential_v2.html). GCUA software takes into account the codon usage table of respective organisms, which is available in codon usage database (http://www.kazusa.or.jp/codon/) and then provides the results in a graphical format.

2.9 Standardizing growth conditions for *A. thaliana* (https://abrc.osu.edu/seed-handling) (Rivero et al., 2014)

The *A. thaliana* (ecotype Columbia) plants were grown in the lab using two different methods. In the first method, plants were grown in Murashige and Skoog (MS) basal medium under aseptic conditions. Seeds (Lehle seeds, USA) were surface sterilized in 0.1% mercuric chloride ($HgCl_2$) solution for approximately 30 seconds followed by 4-5 washes with autoclaved Milli-Q water. Each successive wash was carried out for about 3-5 minutes so that excess of $HgCl_2$ is removed to prevent toxic effects on the seeds. The exposure of the seeds to $HgCl_2$ beyond optimal concentration and/or time may reduce their germination potential. After the final wash, seeds were inoculated on the medium and allowed to germinate (Figure 2-1).

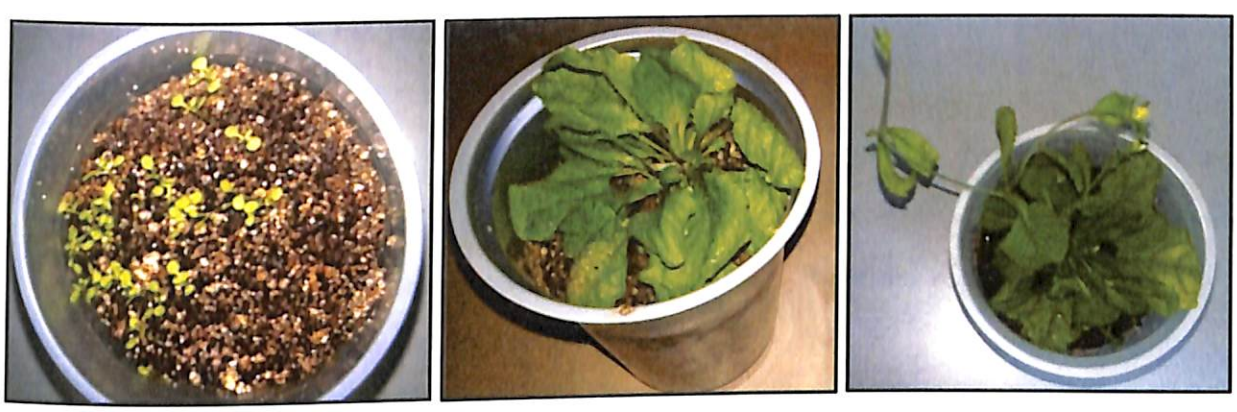
In the second method commercially purchased soilrite mixture (perlite, peat moss and vermiculite) was used, which was procured from Keltech Energies Limited (http://www.keltechenergies.com/horticulture-products.html). Firstly, soilrite mixture was filled in plastic pots or disposable plastic glasses with small holes at the base to allow bottom watering of plants. Soilrite was watered until appropriately wet and then approximately 5-10 seeds were inoculated into each pot or plastic glass (Figure 2-2).

The *A. thaliana* seeds need to undergo stratification i.e., exposure to low temperature in order for them to break the dormancy and germinate. Upon inoculation on the appropriate medium, the containers (flask, pots and plastic glasses) were covered with aluminium foil to maintain dark and humid conditions and then kept in cold room at 4°C for three days. After the completion of cold treatment, the plants were shifted to plant growth chamber under controlled growth conditions of 16 hrs light and 8 hrs dark at 22°C temperature and 75% humidity.



(A) (B) (C)

Figure 2-1: Various growth stages of *A. thaliana* plantlets grown on MS basal medium: (A) Emergence of leaflets (10-15 days old), (B) Initial flowering stage (25-30 days old) and (C) Late flowering stage showing multiple buds (6-7 weeks old)



(A) (B) (C)

Figure 2-2: Various growth stages of *A. thaliana* plantlets grown in soilrite mixture: (A) Emergence of leaflets (7-10 days old), (B) Rosette leaves stage (20-25 days old) and (C) Flowering stage (4-5 weeks old)

2.10 Genomic DNA isolation from plant leaves using DNeasy Plant Mini Kit (Qiagen)

Genomic DNA was isolated from *Arabidopsis thaliana*, *Nicotiana tabacum* and *Nicotiana benthamiana* leaves and was further used as the template for various types of PCR reactions.

The detailed protocol is as follows:

1) An amount of 100 mg of 6-8 weeks old plant leaves were collected and ground using liquid nitrogen in a pre-chilled mortar-pestle. Before the sample thawed, the finely powdered sample was immediately transferred into a microcentrifuge tube.

2) A volume of 400 µL of buffer AP1 and 4 µL of RNase A solution (stock concentration was 100 mg/mL) was added to the sample powder and proper invert mixing was done. In some cases, tissue clumps were observed which were removed by vigorous vortexing or pipetting. For proper lysis of cells, samples were incubated at 65°C in a water bath for 10-12 min.

3) A volume of 130 µL of buffer P3 was added to the lysate and incubated on ice for 5 min and this mixture was centrifuged at 14,000 rpm for 5 min at room temperature (RT).

4) After centrifugation, supernatant obtained was added into the QIAshredder spin column, which was placed in a 2 mL collection tube and centrifuged at 14,000 rpm for 2 min. The flow through was carefully collected in a 2 mL microcentrifuge tube without disturbing the pellet (if any) and 1.5 volume of buffer AW1 was added and mixed by pipetting.

5) The 650 µL mixture was added into the DNeasy Mini Spin Column, which was placed in a 2 mL collection tube and centrifuged at 8,000 rpm for 1 min (in case the total volume of the mixture was more than 650 µL, this step was repeated using the same column) and flow through was discarded.

6) The spin column was placed in a new collection tube followed by addition of 500 µL buffer AW2 and centrifuged at 8,000 rpm for 1 min. After centrifugation, flow through was discarded.

7) Again, 500 µL buffer AW2 was added to the column and centrifuged at 14,000 rpm for 2 min. The column was then carefully removed from the collection tube and transferred to a 1.5 mL microcentrifuge tube.

8) A volume of 50 µL nuclease free water (NFW*) was added to the column for elution and incubated for 5-10 min at RT and then centrifuged at 8,000 rpm for 1 min.

9) The eluted genomic DNA was analyzed on 0.8% agarose gel (Figure 2-3) and the remaining sample was stored at -20°C.

Note: * - To prepare NFW, autoclaved Milli-Q water was filtered through 0.22 µm syringe filter (Merck Millipore) and stored at -20°C in 1 mL aliquots.

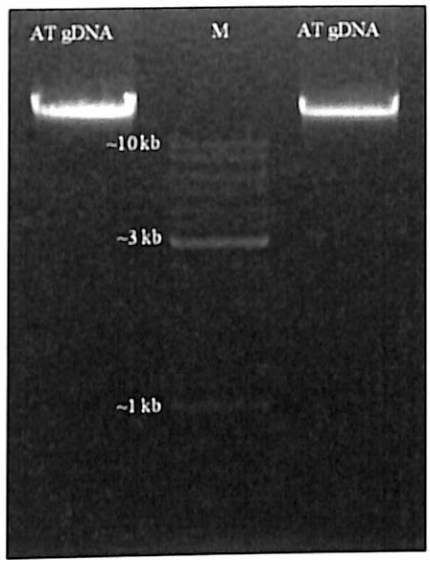


Figure 2-3: The agarose gel electrophoresis image for the genomic DNA isolated from *A. thaliana* leaves; Lanes 1 and 3: Genomic DNA, Lane 2: Gene Ruler DNA Ladder Mix (SM0331)

2.11 Plasmid DNA isolation by alkaline lysis method

Plasmid DNA isolation was done by minipreparation method (Bimboim and Doly, 1979) and the detailed protocol is as follows:

1) A single bacterial colony (obtained from transformed colonies or a streak culture from glycerol stock) was inoculated in 5 mL of Luria-Bertani broth (LB) medium with appropriate antibiotic* and allowed to grow at 37°C for 12-14 hrs at 220 rpm in an orbital shaker (Mac or Genetix). To collect the bacterial cells, properly grown culture (~3 mL) was centrifuged at 13,000 rpm for 1 min and the supernatant was discarded (tubes were kept on tissue paper in an inverted position to remove any remnants of supernatant).
2) The pellet was resuspended in 100 µL of chilled solution I [50 mM Glucose, 25 mM Tris-HCl (pH 8), and 10 mM EDTA (pH 8)] by vortexing (until pellet was completely dissolved).
3) A volume of 200 µL of freshly prepared solution II (1% SDS and 0.2 N NaOH) was added to the above solution and mixed by inverting (4-5 times) and then the sample was incubated at RT for 5 min.
4) After incubation, 150 µL of chilled solution III [3 M sodium acetate (pH 5.5)] was added and the sample was mixed by inverting the tubes (4-5 times) and incubated on ice for 15-20 min.

5) The sample was centrifuged at 10,000 rpm for 20 min at 4°C and a clear supernatant was collected in a fresh tube without taking any part of the pellet. DNase-free RNase A solution was heat treated at 100°C for 10 min and then again kept on ice for few min. This RNase solution was added to the supernatant collected in a final concentration of 20 µg/mL then mixed and incubated in water bath at 37°C for 1 hr.

6) After incubation, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) mixture was added to the sample and vigorous invert mixing of tubes was done, after which sample starts appear milky white in colour. The sample was then centrifuged at 10,000 rpm for 10 min at 4°C.

7) After centrifugation, the upper aqueous phase was carefully collected and transferred to a fresh tube. An equal volume of chilled absolute isopropanol was added into it and sample was incubated at -20°C for 15-30 min.

8) After incubation, sample was centrifuged at 10,000 rpm for 30 min at 4°C, the supernatant was discarded, and the pellet was washed with 70% ethanol by centrifugation at 10,000 rpm, for 10 min at 4°C.

9) The final pellet was air dried by inverting the tubes on a tissue paper for 20-30 min at RT (after proper drying there should not be any ethanol drops in the tube). Dried pellet was dissolved in an appropriate volume of NFW (30-40 µL).

10) The plasmid DNA was analyzed on 0.8% agarose gel and the remaining sample was stored at -20°C.

Note: * For *pGEM®-T*, *pCold-IV* and *pET-15b* vectors ampicillin (100 µg/mL) was used while for *pRI101-AN DNA*, *pBI101* and *pCAMBIA-1302* vectors kanamycin (50 µg/mL) was used.

2.12 Preparation of genetic constructs

2.12.1 Primer designing and PCR amplification

Throughout the study, various DNA sequences were amplified using specific primer sets and while designing primers, appropriate restriction enzyme sites were incorporated in both forward and reverse primer. Primers were designed manually by following standard guidelines (Lorenz, 2012) and their properties were checked using specific software *viz.* DNAMAN (Lynnon Biosoft), Oligoanalyzer 3.1-Integrated DNA Technologies (http://eu.idtdna.com/calc/analyzer) and NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All these software analyzed the presence of hairpin

structure, primer dimer formation, primer self-dimer formation and primer nonspecific binding for the template and the necessary modifications were done in the primer sequences to resolve these issues. Primer melting temperature (T_m) and percent GC content were calculated using Promega BioMath calculators (http://www.promega.com/a/apps/biomath/index.html?calc=tm) at default settings of Tm for oligos. Details of primers used are mentioned in Table 2-1.

Table 2-1: List of primers used throughout the course of this study for the amplification of various transit peptide sequences, genetic constructs for transformation and analyses of transformants

Sr. No.	Primer name (amplicon-RE-primer orientation)	Primer sequence (5' to 3')	Primer length (bases)	Tm (°C)
1.	TICTP-*NdeI*-F	GCGTCCCATATGGCTGTTCCATTTCTA	27	61.0
2.	TICTP-*SacI*-R	ATAGAGCTCGCGGAGGAGACGTAG	24	62.0
3.	MEXTP-*NdeI*-F	CGCCATATGGAAGGTAAAGCCATC	24	58.0
4.	MEXTP-*SacI*-R	ATAGAGCTCAGAGACAGCACCACC	24	60.0
5.	TICTP-*bicA-BglII*-F	GGTAAAGATCTATGGCTGTTCCATTTC	27	56.0
6.	TICTP-*bicA-SpeI*-R	TAGATTGACTAGTACCCATCTCTGAAC	27	56.0
7.	MEXTP-*bicA-BglII*-F	GGTAAAGATCTATGGAAGGTAAAGCC	26	56.0
8.	MEXTP-*bicA-SpeI*-R	TAGATTGACTAGTACCCATCTCTGAAC	27	56.0
9.	*bicA* gene-*SacI* -F	GAAAAATGCGAGCTCATGCAGATAAC	26	58.0
10.	*bicA* gene -*XbaI*-R	CGCAGATCTCTAGATTAACCCATCTC	26	57.0
11.	*bicA* gene internal-F	GTGTTTGTCGCTAATGTGCTC	21	55.0
12.	*bicA* gene internal-R	ATCAAAGACGAGGGCATCAC	20	55.0
13.	*mgfp5* gene-F	GGTAGATGGATCCAGTAAAGGAGAAG	26	56.0
14.	*mgfp5* gene-R	GGTGGTGGAGCTCTTTGTATAGTTC	25	58.0
15.	*mgfp5* gene internal-F	CAGGATCGAGCTTAAGGGAATC	22	55.0
16.	*mgfp5* gene internal-R	GGTAATGGTTGTCTGGTAAAAGG	23	54.0

Note: Primer name denotes firstly the name of amplicon followed by name of the restriction enzyme (if present) whose recognition site is being incorporated in the primer sequence. 'F' and 'R' represent forward and reverse primer respectively.

The synthesis of the primers was outsourced to Eurofins scientific company, Jaipur and they provided lyophilized primers, which were diluted in the $1/10^{th}$ strength of TE buffer [10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8)]. A final working solution of primers was prepared in NFW to obtain 10 pM/µL concentration. The PCR reaction mixture (For 50 µL reaction volume) composition is as follows:

- Buffer A with $MgCl_2$ (10X) - 1X (with 1.5 mM $MgCl_2$)
- Primers (10 pM/ µL) - 30 pM
- dNTPs (10 mM) - 200 µM
- Taq DNA Polymerase (3 Units/uL) - 1 to 2 Units
- Template - 10 to 50 ng

Final required volume was made-up using NFW. The template used for amplification of transit peptides (TICTP and MEXTP) was *A. thaliana* genomic DNA, and for amplification of fusion constructs (TICTP-*bicA* and MEXTP-*bicA*) was their respective *pCold-IV* positive clone's plasmid DNA (detailed results are given in chapter 3; section 3.9.1). As mentioned in Table 2-2, PCR conditions were standardized for all the amplicons under study using Taq DNA polymerase (Bangalore Genei) and the reactions were carried out in Veriti™ Thermal Cycler machine (Applied Biosystems). PCR products were analyzed on 0.8% to 1.5% of agarose gel, depending on the expected size of amplicons.

Table 2-2: PCR conditions for various amplifications carried out throughout the study

Sr. No.	Name of amplicon	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
1.	TICTP	94°C, 3 min	94°C, 1 min	58.5°C, 30 sec	72°C, 30 sec	72°C, 3 min	35
2.	MEXTP	94°C, 3 min	94°C, 1 min	59.5°C, 30 sec	72°C, 30 sec	72°C, 3 min	35
3.	TICTP-*bicA*	94°C, 3 min	94°C, 1 min	58.9°C, 1 min	72°C, 1:30 min	72°C, 3 min	30
4.	MEXTP-*bicA*	94°C, 3 min	94°C, 1 min	58.7°C, 1 min	72°C, 1:30 min	72°C, 3 min	30
5.	*bicA* gene	94°C, 3 min	94°C, 1 min	60°C, 1 min	72°C, 1:30 min	72°C, 3 min	30

Note: For confirmation of transgene/s in recombinant samples, PCR reactions were performed to amplify the above mentioned and few other sequences *viz. bicA* and *mgfp5* genes (full length and partial amplicons) using various genomic DNA (isolated from transformed *N. tabacum* and *N. benthamiana* plants), cDNA and plasmid DNA samples (details are mentioned in chapter 3).

2.12.2 Agarose gel electrophoresis

This technique was used to visualize various DNA sequences (PCR products, bacterial plasmids, plant genomic DNA, plant RNA samples, restriction enzyme digested products, recombinant clones etc.) on an agarose gel and to obtain a rough estimate about the concentration and size of DNA which was further confirmed by sequencing. The apparatus used for running the gel was submerged horizontal agarose slab gel (Bio-Rad) and generally, it was used at optimal voltage and time duration as per the DNA sample type.

A required amount of low electroendosmosis (EEO) agarose powder was dissolved in 1X Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic acid, and 1 mM EDTA) by boiling in the microwave. After boiling, melted agarose was allowed to cool at 50°C and then 1 µg/mL Ethidium Bromide (EtBr) concentration was added (stock concentration was 10 mg/mL). This was mixed by swirling the flask and then immediately poured into the pre-fixed casting tray and the comb was placed to prepare the wells in agarose after solidification. It took 25-45 min for proper solidification of agarose (time depends on the percent of agarose used). After solidification, the comb was carefully removed and the tray was put in the electrophoresis tank filled with an appropriate amount of 1X TAE buffer. DNA samples were mixed with a tracking dye and loaded into the wells and then the gel was run at 80 volts/cm and DNA was visualized under 302-312 nm range of UV light in a gel documentation system (Molecular Imager® Gel Doc™ XR - Bio-Rad).

2.12.3 Digestion of PCR amplicons and plasmid DNA using appropriate restriction enzymes

Throughout the study, various sticky end clonings were done, for which the inserts and vectors were digested using their respective restriction endonuclease enzymes (NEB) as detailed in Table 2-3. For confirmation of positive clones, various digestion reactions were performed using one or two enzymes (when using two enzymes in single digestion reaction their buffer compatibility was checked). The digestion reactions were carried out at 37°C in a circulating water bath for an optimal time duration as recommended by the manufacturer (time varied from 1 hr to overnight/12 hrs).

The reaction mixture composition was as follows:

- DNA - 1µg
- Buffer (10X) - 1X
- Restriction enzyme A - 1 unit
- Restriction enzyme B - 1 unit
- BSA (if needed) (10X) - 1X

Finally, required volume was made-up using NFW. After completion of digestion reaction, DNA products were analyzed on agarose gel and stored at -20°C.

Table 2-3: Details of all the restriction enzymes (RE) used in the study

Sr. No.	Name of RE	Recognition sequence of RE
1.	*SacI*	$GAGCT^{v}C$ $C_{\wedge}TCGAG$
2.	*XbaI*	$T^{v}CTAGA$ $AGATC_{\wedge}T$
3.	*NdeI*	$CA^{v}TATG$ $GTAT_{\wedge}AC$
4.	*BamHI*	$G^{v}GATCC$ $CCTAG_{\wedge}G$
5.	*XhoI*	$C^{v}TCGAG$ $GAGCT_{\wedge}C$
6.	*SpeI*	$A^{v}CTAGT$ $TGATC_{\wedge}A$
7.	*BglII*	$A^{v}GATCT$ $TCTAG_{\wedge}A$

2.12.4 Gel elution of PCR amplicons and digested DNA fragments using QIAquick® Gel Extraction Kit (Qiagen)

Gel elution was performed either to purify various PCR amplicons and digested DNA fragments or to separate the desired DNA fragment from a mixture of digested DNA samples. The detailed protocol is as follows:

Note: All the centrifugation steps were carried out at 13,000 rpm for 1 min at RT.

1) The desired DNA fragment was analyzed on the agarose gel and the specific band of desired DNA was quickly visualized under UV light (here UV exposure should be minimized to prevent mutations by the formation of pyrimidine dimers in DNA) and then immediately excised with the help of a sterile scalpel.

2) Excised piece of agarose gel was weighed after putting it into a pre-weighed microcentrifuge tube and thrice the gel volume QG buffer was added (for 100 mg gel, 300 µL QG buffer was used).

3) Sample was incubated at 50°C until the agarose melted properly (10-15 min) and once a clear solution was obtained, isopropanol was added equal to gel volume, (isopropanol addition is critical for <500 bp and >4 kb length DNA) and mixed thoroughly.

4) A volume of 750 µL of sample was added into the QIA quick spin column placed in 2 mL collection tube and centrifugation was done. Flow through was discarded and this step was repeated with remaining sample.

5) A volume of 500 µL of QG buffer was added into the column and centrifugation was done and flow through was discarded.

6) To the column, 750 µL of PE buffer was added and incubated at RT for 5 min followed by centrifugation and the flow through was discarded. To get rid of any remnants of PE buffer, the empty column was again centrifuged.

7) The column was removed from the collection tube and transferred to a 1.5 mL microcentrifuge tube. For elution of DNA, an appropriate volume of NFW was carefully added in the centre of the column and incubated at RT for 5-10 min.

8) After incubation, column was placed in microcentrifuge tube and centrifuged to allow the DNA to get collected in the tube.

9) Eluted DNA was analyzed on agarose gel and the remaining sample was stored at -20°C.

2.12.5 Ligation of genes of interest into cloning and plant expression vectors

Digested and purified gene sequences and vectors were ligated together using T4 DNA ligase enzyme (Thermo Fisher Scientific or Promega) and ligation reactions were performed overnight at 4°C or 16°C (as recommended by the manufacturer). To set up the ligation reaction standard molar ratio of 3:1 (insert: vector) was used.

In all the reactions 100 ng of the vector was taken and the insert concentration was determined using the following formula:

$$\text{Concentration of insert} = \frac{\text{(Insert/vector) molar ratio } \mathbf{X} \text{ (size of insert/size of vector)}}{\text{Concentration of vector}}$$

The reaction mixture composition is as follows:

- T4 DNA ligase (5 Weiss units/μL) - 1 μL
- 10X ligation buffer - 1X
- Insert - Variable
- Vector - 100 ng

Finally, required volume was made-up using NFW. Upon completion of incubation for ligation reaction, the ligated products were immediately transformed into competent cells of *E. coli.* DH5α, which were prepared as described in section 2.12.7)

2.12.6 Cloning strategy and selection of multiple vector systems

The ultimate objective of the study was to express the *bicA* gene into a plant system, for which it had to be cloned in a plant expression vector. However, for proper subcellular targeting of the protein, transit peptide sequences were fused upstream of the gene. The genetic elements were first ligated and cloned in cloning vectors for ease of handling. Subsequently, intermediate clonings and PCR amplifications were also done to incorporate appropriate restriction enzyme sites in the fusion gene sequences, and they were used for further cloning into plant expression vectors. In the complete process (Figure 2-4 and Table 2-4), several vectors and inserts were used to prepare optimal constructs to be expressed in a plant system.

Note: All the recombinant vector maps shown in Figure 2-5 and 2-7 were generated using software SnapGene® 1.5.3 and SnapGene® Viewer 4.0.5 (from GSL Biotech; available at snapgene.com)

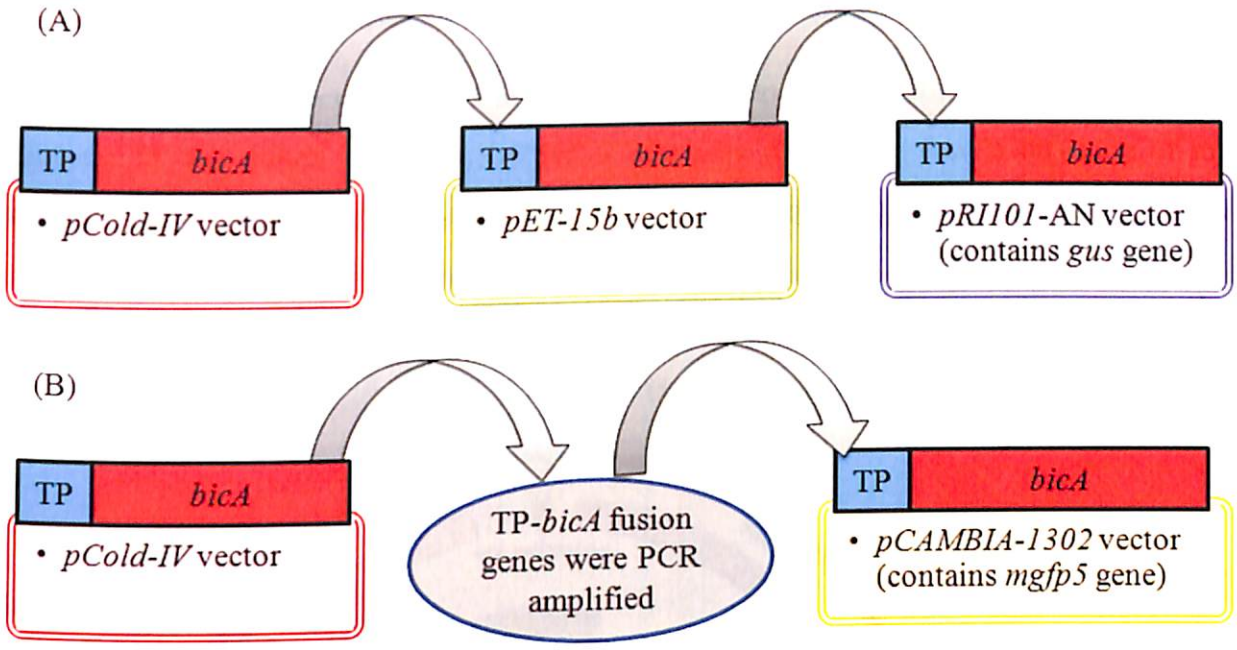


Figure 2-4: A schematic representation of the complete cloning strategy of *bicA* gene to generate fusion constructs for genetic transformation of model C_3 plants

2.12.6.1 Amplification of transit peptide sequences from A. thaliana genome

The transit peptide sequences were PCR amplified from *A. thaliana* genomic DNA using sequence specific primers (Figure 2-5) (details are mentioned in chapter 3).

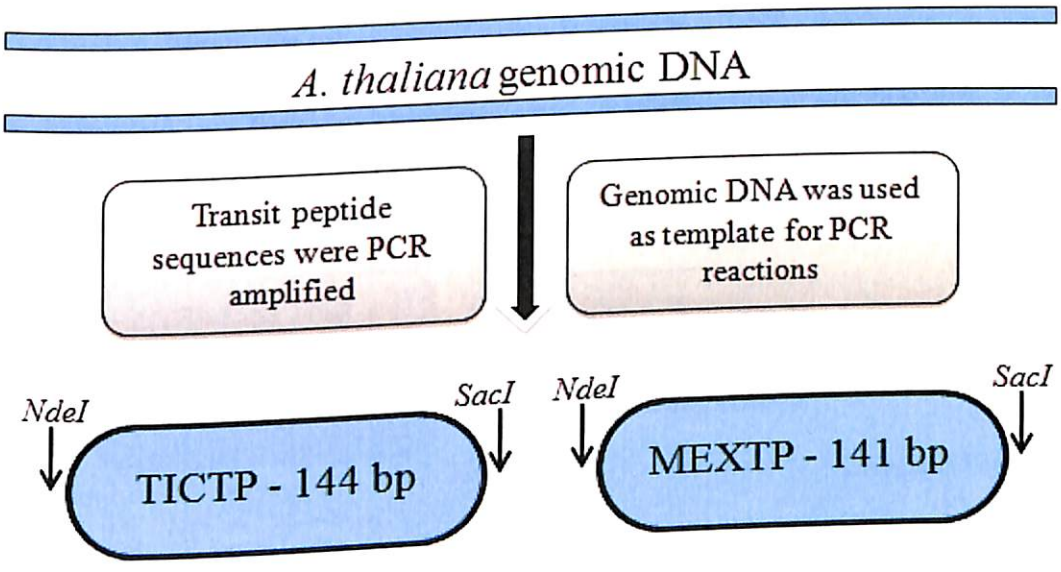


Figure 2-5: Schematic representation of the amplification of transit peptides (indicated by blue color)

2.12.6.2 Splicing of bicA gene segment from pGEM®-T clone

The *bicA* gene was procured in the form of *pGEM®-T* clone from Prof. G. Dean Price, ANU, Canberra. It was taken out from *pGEM®-T* clone by performing double digestion of the recombinant plasmid with *SacI* and *XbaI* restriction enzymes (Figure 2-6) (details are mentioned in chapter 3).

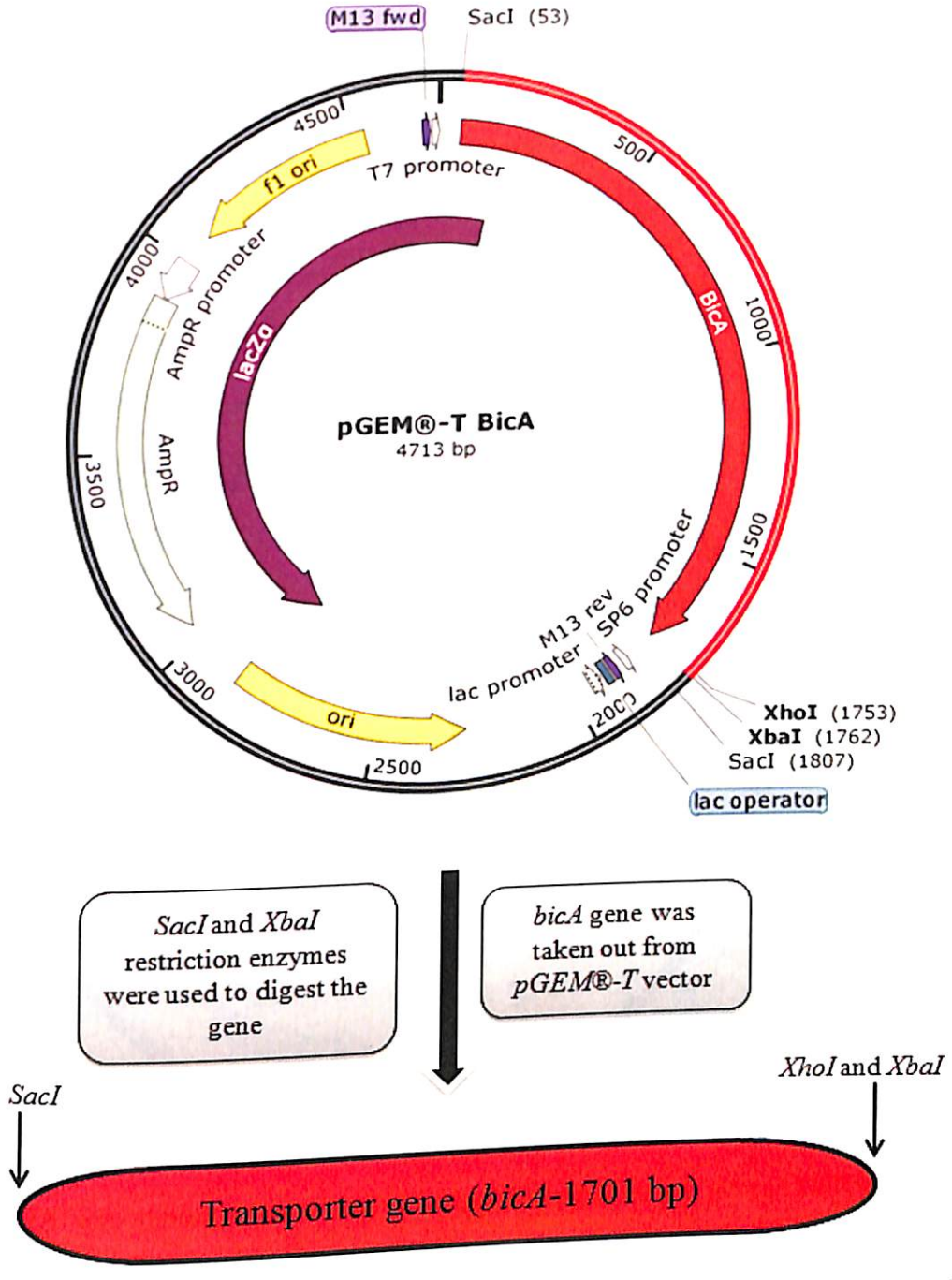


Figure 2-6: Schematic representation of the splicing of *bicA* gene from *pGEM®-T* clone (indicated by red color)

The detailed cloning strategy and recombinant vector maps are shown in Figure 2-7A-E and the elaborated results of each cloning are discussed in chapter 3.

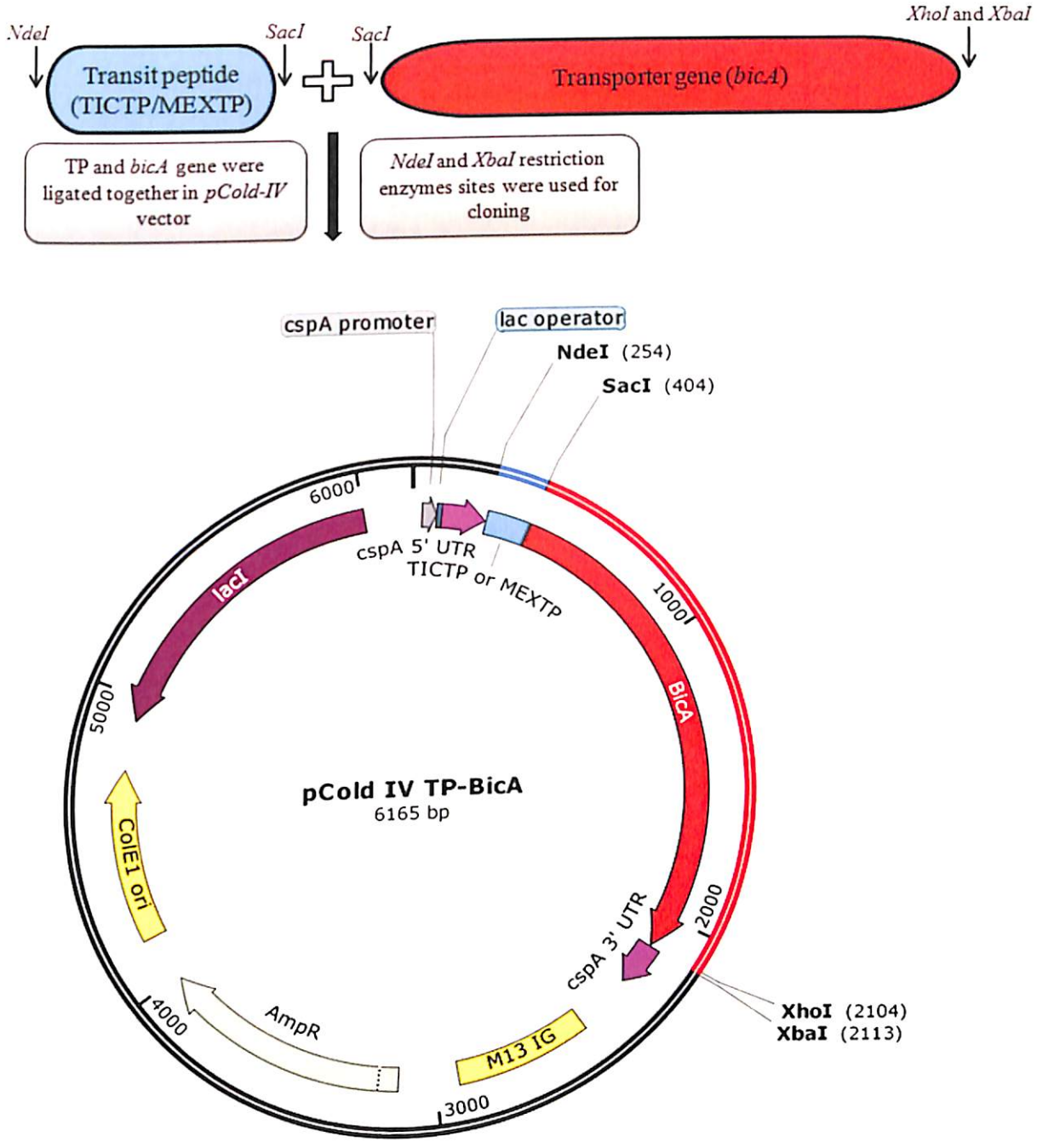
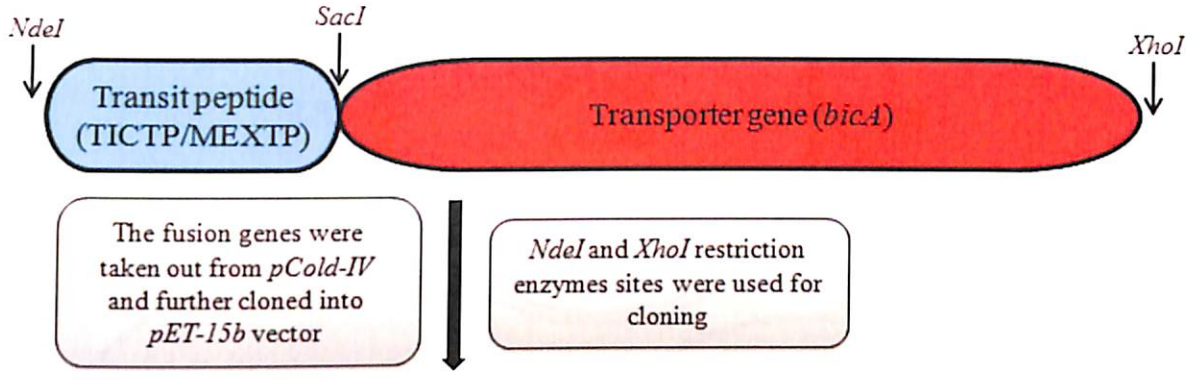


Figure 2-7A: Recombinant vector map showing cloning of fusion genes (TICTP/MEXTP + *bicA* gene) into *pCold-IV* vector



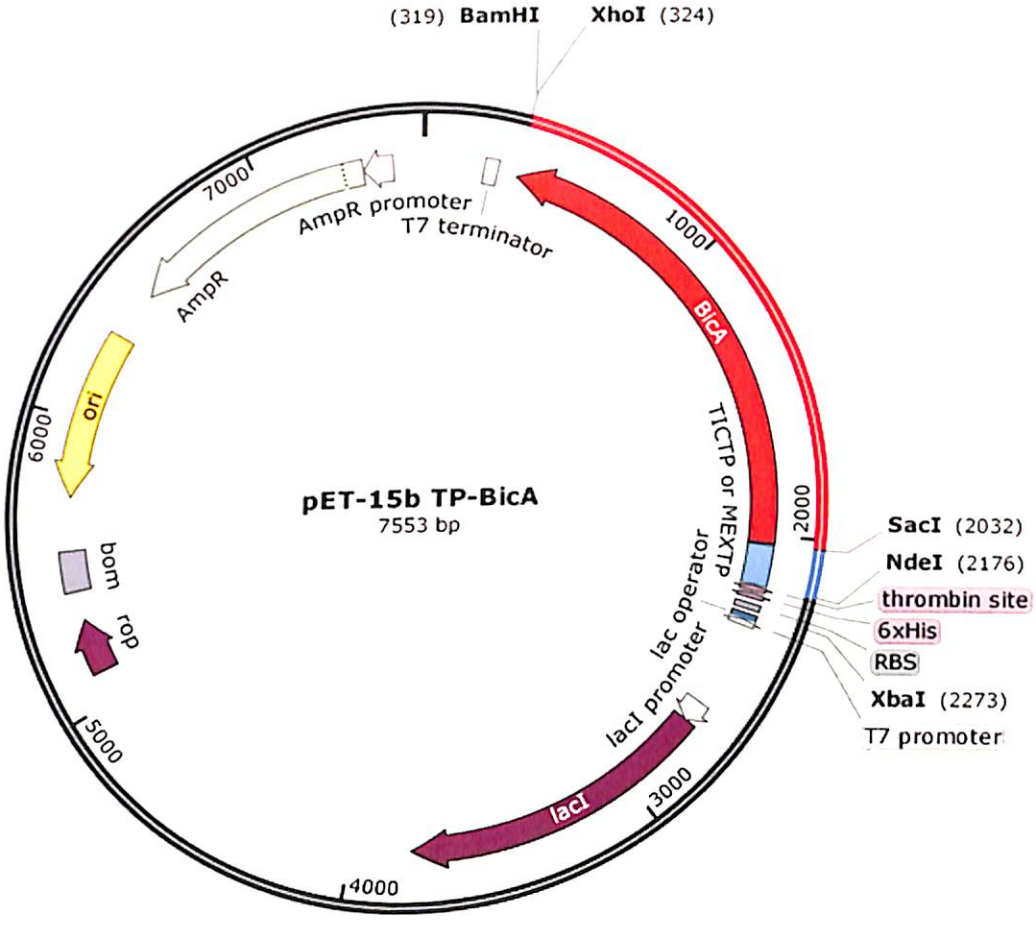


Figure 2-7B: Recombinant vector map showing cloning of fusion genes (TICTP/MEXTP + *bicA* gene) into *pET-15b* vector

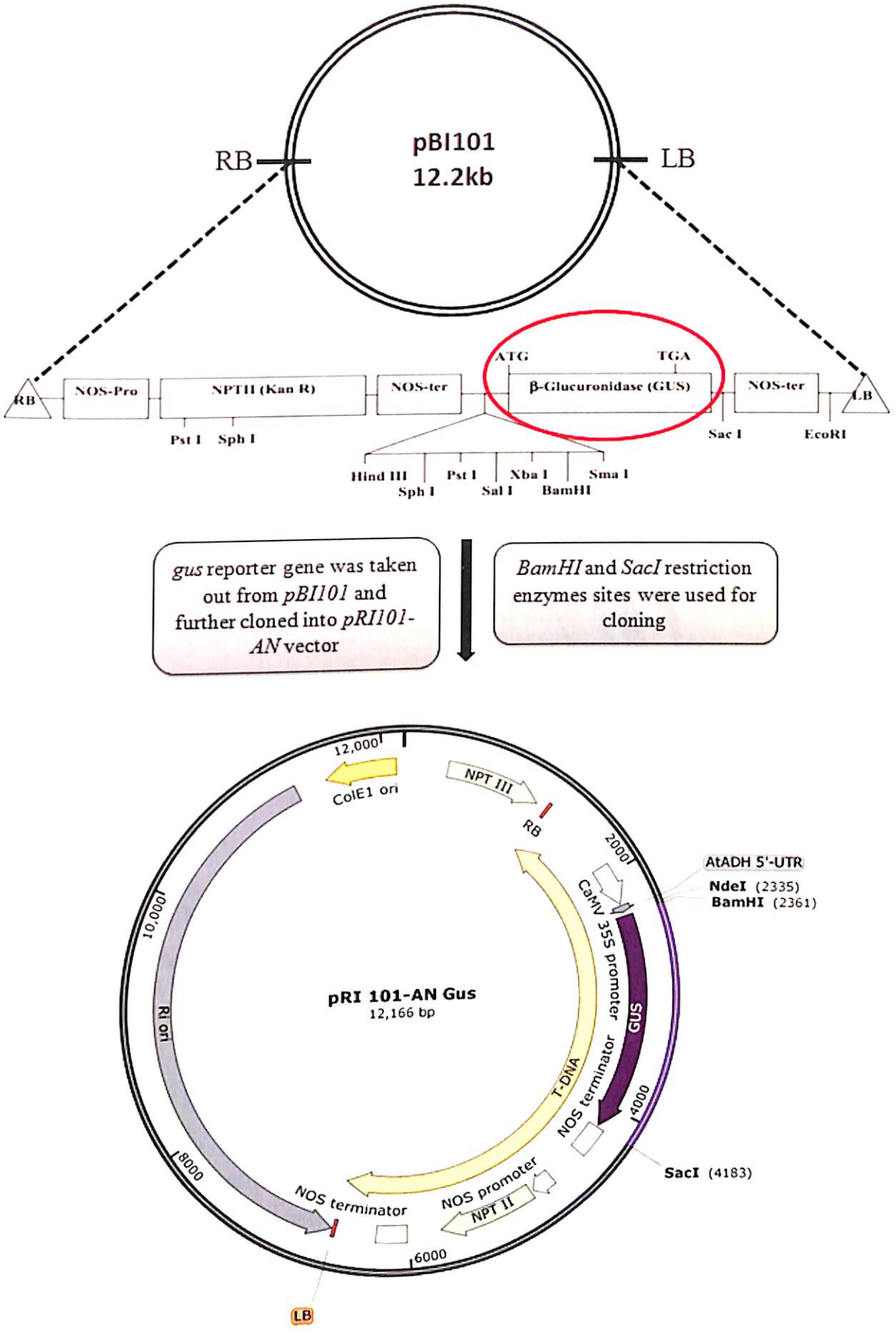


Figure 2-7C : Recombinant vector map showing cloning of *gus* gene into *pRI 101-AN* vector

Note: The circular vector map of *pBI101* was hypothetically drawn.

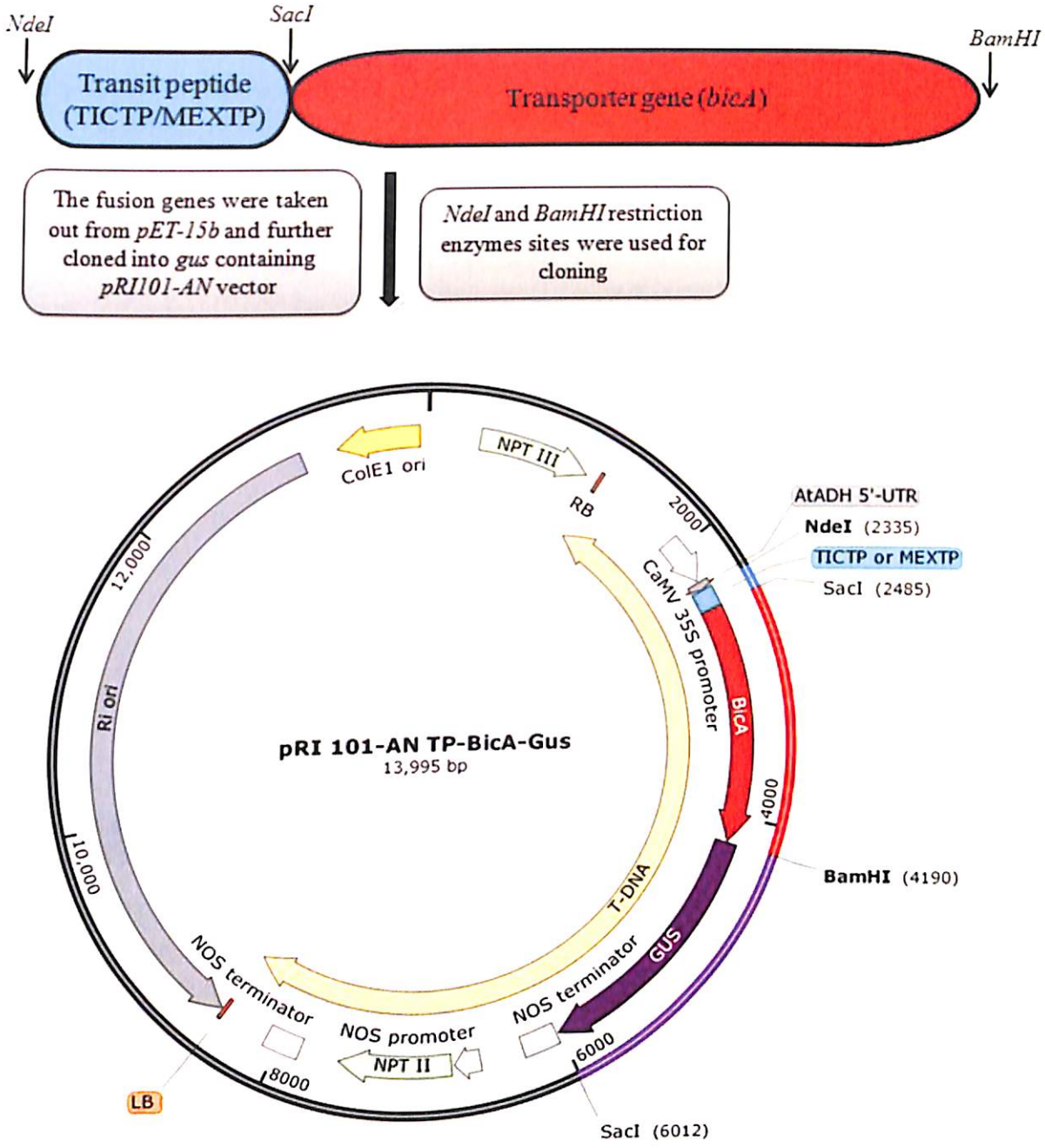


Figure 2-7D: Recombinant vector map showing cloning of fusion genes (TICTP/MEXTP + *bicA* gene) upstream of the *gus* gene into *pRI101-AN* vector

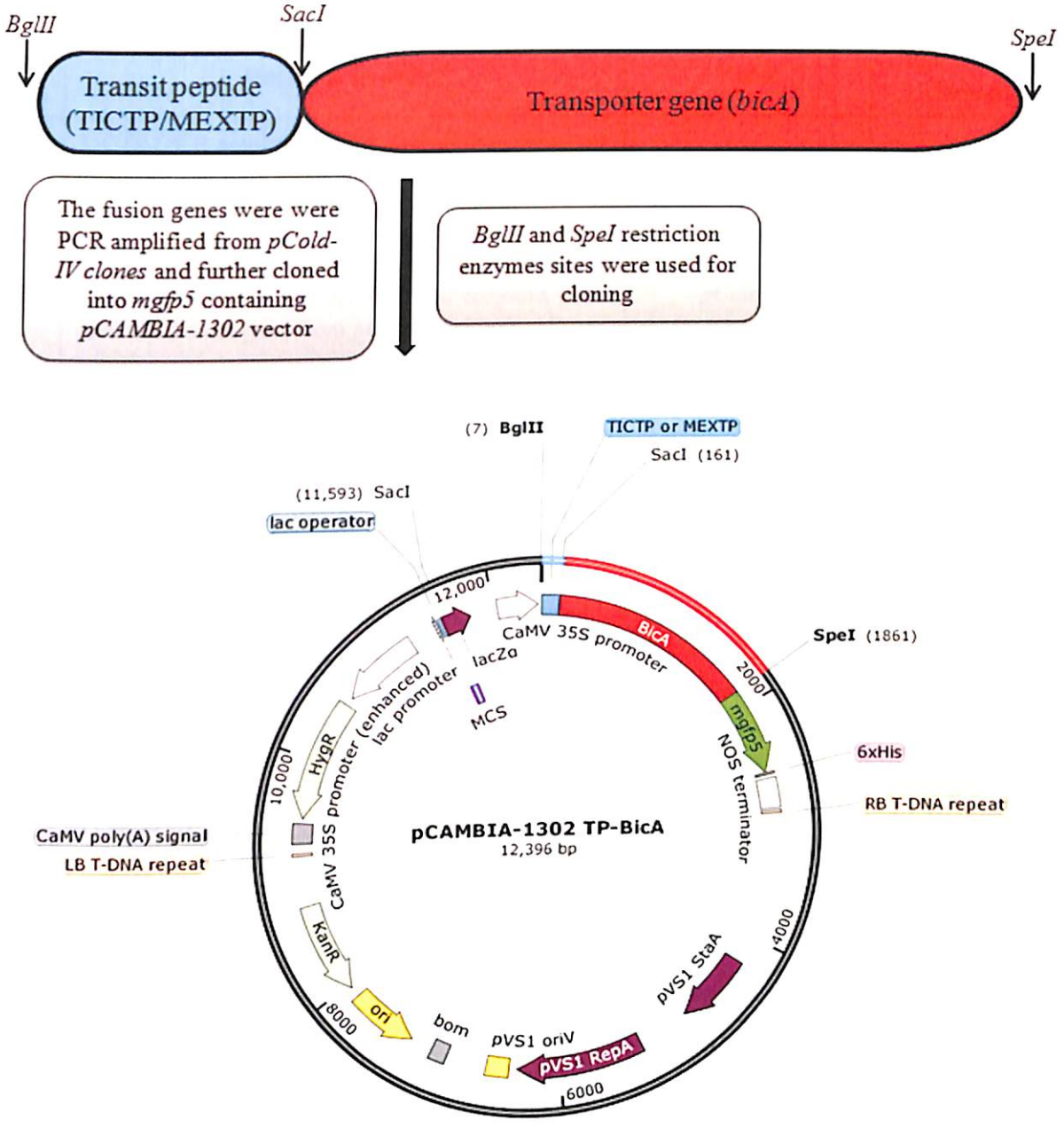


Figure 2-7E: Recombinant vector map showing cloning of fusion genes (TICTP/MEXTP + *bicA* gene) upstream of the *mgfp5* gene into *pCAMBIA-1302* vector

Table 2-4: Details of all the inserts, their sources, cloning vectors, plant expression vectors and restriction enzymes used for clonings

Sr. No.	Insert and its source	Vector	REs used in cloning
1.	TICTP (PCR amplified using *A. thaliana* genome as template) and *bicA* (*SacI* and *XbaI* enzymes were used to take out gene from *pGEM® T* clone)	*pCold-IV*	*NdeI* and *XbaI*
2.	MEXTP (PCR amplified using *A. thaliana* genome as template) and *bicA* (*SacI* and *XbaI* enzymes were used to take out gene from *pGEM® T* clone)	*pCold-IV*	*NdeI* and *XbaI*
3.	TICTP-*bicA* (*NdeI* and *XhoI** enzymes were used to take out gene from *pCold-IV* clone)	*pET-15b*	*NdeI* and *XhoI*
4.	MEXTP-*bicA* (*NdeI* and *XhoI** enzymes were used to take out gene from *pCold-IV* clone)	*pET-15b*	*NdeI* and *XhoI*
5.	*gus* reporter gene (*BamHI* and *SacI* enzymes were used to take out the gene from *pBI-101* vector)	*pRI101-AN DNA*	*BamHI* and *SacI*
6.	TICTP-*bicA* (*NdeI* and *BamHI* enzymes were used to take out gene from *pET-15b* clone)	*pRI101-AN DNA* containing *gus* gene	*NdeI* and *BamHI*
7.	MEXTP-*bicA* (*NdeI* and *BamHI* enzymes were used to take out gene from *pET-15b* clone)	*pRI101-AN DNA* containing *gus* gene	*NdeI* and *BamHI*
8.	TICTP-*bicA* (PCR amplified using *pCold-IV* clone as template)	*pCAMBIA-1302* containing *mgfp5* gene	*BglII* and *SpeI*
9.	MEXTP-*bicA* (PCR amplified using *pCold-IV* clone as template)	*pCAMBIA-1302* containing *mgfp5* gene	*BglII* and *SpeI*

Note: * - *XhoI* enzyme site was already present at the end of *bicA* gene sequence, upstream of the *XbaI* enzyme site

2.12.7 Preparation of competent cells of various bacterial hosts

In order to make the bacterial host cells more transformable, they were made competent by either treatment with some high concentration ionic buffer ($CaCl_2$) or by providing electric current to generate chemically competent and electrocompetent cells, respectively. These treatments modify the cell wall in such a way that the cell can easily take up the DNA upon transformation. To increase the transformation efficiency, all the steps were carried out at 4°C while preparing competent cells. Before starting the experiment, all the reagents, oak ridge centrifuge tubes, tip boxes, microcentrifuge tubes etc., were kept in cold room. Pellet dislodging step was done very gently and carefully using cut tips to maintain the cell viability.

Note: The positively charged calcium ions help attract negatively charged phosphate groups of DNA, which leads to the easy entry of recombinant vector DNA into chemically competent host cells. Electrocompetent cells are prepared by washing the cells several times with appropriate buffer to remove any salts present in the medium, because the salts may interfere while performing electroporation. By doing so, electric current is conducted entirely through the cells and not through the medium. Electric current will create temporary pores in the cell membrane, which allow DNA to enter inside the competent host cells.

2.12.7.1 Preparation of chemically competent cells of E. coli DH5α

1) Primary culture was prepared by inoculating a single colony of *E. coli* DH5α cells in 5 mL of LB broth medium and it was grown at 37°C for 12-14 hrs in an orbital shaker at shaking speed of 220 rpm.
2) A volume equal to 1% of primary culture was used to inoculate secondary culture (25 mL) which was grown in same conditions till OD at 650 nm wavelength reached 0.3-0.4 (it took 3-4 hrs). Once the optimal bacterial growth was obtained, cells were centrifuged at 6,000 rpm for 10 min at 4°C.
3) After centrifugation, the supernatant was discarded and the pellet was resuspended in 100 mM of $CaCl_2$ solution (8.33 mL) by dislodging the pellet using pipetting.
4) The suspended cells were centrifuged at same conditions (as mentioned in step 2) and pellet obtained was again resuspended in 100 mM of $CaCl_2$ solution (1.33 mL), and finally, 100% glycerol (200 µL) was added to the suspension and mixed properly.
5) Cells were aliquoted in a volume of 300 µL and transferred to sterile microcentrifuge tubes.
6) Tubes were frozen in liquid nitrogen and immediately stored in a deep freezer at -80°C.

Note: The $CaCl_2$ solution was either filter sterilized through 0.22 µm syringe filter or autoclaved before use.

2.12.7.2 Preparation of chemically competent cells of Agrobacterium tumefaciens strain GV3101

1) Primary culture was prepared by inoculating a single colony of *Agrobacterium* cells in 5 mL of LB broth medium containing rifampicin (50 µg/mL) and gentamicin (30 µg/mL) antibiotics and was grown at 28°C for 36-48 hrs at 220 rpm in an orbital shaker.

2) A volume equal to 1% of primary culture was used to prepare secondary culture (50 mL) which was grown in same conditions in antibiotics containing medium till OD at 600 nm wavelength reached 0.3-0.4 (it took 8-10 hrs). Once the optimal bacterial growth is obtained, cells were centrifuged at 4500 rpm for 10 min at 4°C.

3) After centrifugation, the supernatant was discarded and the pellet was resuspended in 20 mM of $CaCl_2$ solution (5 mL).

4) The suspended cells were centrifuged at same conditions (as mentioned in step 2) for 5 min and pellet obtained was again resuspended in 20 mM of $CaCl_2$ solution (1 mL).

5) Cells were aliquoted in a volume of 200 µL into sterile microcentrifuge tubes. Tubes were frozen in liquid nitrogen and immediately stored in a deep freezer at -80°C.

Note:

- The $CaCl_2$ solution was either filter sterilized through 0.22 µm syringe filter or autoclaved before use.
- *Agrobacterium tumefaciens* strain GV3101 harbors Ti plasmid pMP90, which codes for gentamicin resistant gene and its chromosomal background contains rifampicin resistance gene, therefore both these antibiotics were added into the culture medium while preparing competent cells.

2.12.7.3 Preparation of electrocompetent cells of Agrobacterium tumefaciens strain GV3101

A bacterial pellet was obtained (50 mL initial culture was taken) as mentioned in the previous protocol and resuspended in 1 mM of HEPES buffer (this was filter sterilized through 0.22 µm syringe filter before use) pH 7 (10 mL). The suspended cells were centrifuged and pellet obtained was again resuspended in 1 mM of HEPES buffer containing 10% glycerol in it. This step was repeated twice and cells were resuspended first in 2 mL and then in 200 µL of buffer (1 mM of HEPES buffer+10% glycerol). Cells were aliquoted in a volume of 100 µL, transferred to sterile microcentrifuge tubes, and immediately stored in a deep freezer at -80°C.

2.12.8 Transformation of chemically competent *E. coli* DH5α cells with recombinant plasmids

1) Competent cells were taken out from the deep freezer and incubated on ice for 20-30 min or until completely thawed.

2) After thawing, 300 µL of cells were equally distributed into three microcentrifuge tubes. In one of the microcentrifuge tubes ligation mixture was added and mixed properly while the remaining two were used as positive and negative controls for transformation.

3) Cells were again incubated on ice for 15-20 min and then a heat shock was given for 90 sec by keeping the tubes in water bath at 42°C temperature. After heat shock, cells were immediately transferred to ice (this sudden transition from low to high and again from high to low temperatures creates several temporary pores on bacterial surface to facilitate easy entry of DNA).

4) A volume of 800 µL of LB broth medium (without any antibiotic) was added to the cells which helped them recover after heat shock and incubated at 37°C for 45 min in an orbital shaker with slow speed.

5) Bacterial cells were collected by centrifugation at 3000 rpm, for 5 min at RT.

6) A volume of 750 µL of supernatant was discarded while remaining 50 µL was used to resuspend the bacterial pellet. Tubes with the ligation mixture and the negative control were used to spread plate the bacteria on antibiotic containing Luria Agar medium while for positive control only Luria Agar was used.

7) Plates were incubated at 37°C for 12-14 hrs and a variable number of transformed colonies were observed on the test plate. While there were no colonies on negative control plate, a bacterial lawn was observed on the positive control plate.

Note: Antibiotics chosen for selection of transformed colonies were decided according to the vector used. Test plate and negative control plate contained either ampicillin or kanamycin while positive control plate did not contain any antibiotic in it.

2.12.8.1 Screening of transformed colonies

Master plate was prepared using transformed colonies and was allowed to grow in optimal conditions. For further experiments, plasmid isolation was done using bacterial colonies grown on master plate. The presence of gene in recombinant plasmid was

checked by performing colony PCR, restriction analysis and DNA sequencing. Positive clones were further streaked on a Luria Agar medium with appropriate antibiotic to obtain single colony, which was used to prepare glycerol stock.

1) Colony PCR: In a 1.5 mL microcentrifuge tube 30 µL of NFW was taken. A small part of the bacterial colony was picked up from master plate using a sterile toothpick or pipette tip and properly resuspended in NFW. Sample lysis was done at 100°C for 4 min followed by incubation on ice for 4 min. The sample was centrifuged at 12,000 rpm for 4 min at RT. The supernatant (3-5 µL) was used as PCR template. PCR reaction was carried out using gene specific primers and products were checked on agarose gel for the presence of the desired gene.

2) Restriction analysis: Recombinant plasmid samples were digested with specific restriction enzymes and digested samples were analyzed on an agarose gel and compared with control samples. A significant difference in band sizes (according to the size of the gene) or the number of bands obtained, allowed us to determine the presence/absence of the gene in transformed samples.

3) DNA sequencing: Once the presence of gene was checked through restriction analysis, positive clones were sent for DNA sequencing. The results were analyzed by NCBI blast, in which the gene sequence (from NCBI database) was aligned with the sequence obtained after performing DNA sequencing.

2.12.8.2 Glycerol stocks preparation

A single bacterial colony was inoculated in 5 mL of LB broth medium and allowed to grow for 12 hrs. A volume of 800 µL of this bacterial culture was mixed with 200 µL of 100% sterile glycerol in properly labelled cryovials and immediately transferred to a deep freezer at -80°C for long term storage.

2.12.9 Transformation of chemically competent *Agrobacterium* cells with recombinant plasmids

1) Competent cells were taken out from the deep freezer and incubated on ice for 20-30 min or until completely thawed.

2) After thawing, 200 µL of cells were equally distributed into three microcentrifuge tubes. In one of the microcentrifuge tubes, recombinant vector DNA was added and

mixed properly while the remaining two were used as positive and negative controls for transformation.

3) Cells were again incubated on ice for 15-20 min and then all the tubes were frozen in liquid nitrogen for 5 min and then immediately heat shock was given at 37°C for 5 min. After the heat shock, tubes were immediately put on ice and incubated for 5 min.

4) A volume of 800 µL of LB broth medium (without any antibiotic) was added for recovery of the cells and tubes were incubated at 28°C for 3-4 hrs in an orbital shaker with slow speed.

5) After incubation, bacterial cells were collected by centrifugation, spread plated on the selection agent containing medium, and allowed to grow at 28°C. It took approximately 48 hrs to obtain bacterial colonies, which were screened by colony PCR and from positive colonies, glycerol stocks were prepared and stored in a deep freezer at -80°C.

Note: Antibiotics chosen for selection of transformed colonies were decided according to the vector and *Agrobacterium* strain used. Test plate and negative control plate contained gentamicin, rifampicin and kanamycin while positive control plate contained only gentamicin and rifampicin.

2.12.10 Transformation of electrocompetent *Agrobacterium* cells with recombinant plasmids

1) Competent cells were taken out from the deep freezer and incubated on ice for 20-30 min or until completely thawed.
2) After thawing, 100 µL of cells were equally distributed into three microcentrifuge tubes. In one of the the microcentrifuge tubes recombinant plasmid DNA was added and mixed properly while the remaining two were used as positive and negative controls for transformation.
3) Cells were again incubated on ice for 15-20 min and at the same time, three electro-cuvettes (0.1 cm) were put on ice to make them chilled.
4) After 20 min, cells were transferred from microcentrifuge tubes to chilled electro-cuvettes which were still kept on the ice. To perform electroporation, electric pulses were delivered to the cells, using Micropulser™ (Electroporation apparatus- Bio-Rad).

5) For *Agrobacterium* electroporation, a preset default program "Agr" was used, in which five pulses (each for 5 msec) at 2.20 kV were given to the sample. Electro-cuvettes were kept on ice while performing electroporation.

6) Once the pulses had been delivered, immediately 800 µL LB broth medium was added into the cuvettes, mixed properly and all the content was transferred to 1.5 mL microcentrifuge tubes, followed by incubation at 28°C for 3-4 hrs in an orbital shaker with slow speed.

7) After incubation, bacterial cells were collected by centrifugation, spread plated on the selection agent containing medium, and allowed to grow at 28°C. It took approximately 48 hrs to obtain bacterial colonies, which were screened by colony PCR and from positive colonies, glycerol stocks were prepared and stored in a deep freezer at -80°C.

2.13 Transformation of *N. tabacum* by particle bombardment method (Ucki et al., 2013)

This method allows direct entry of recombinant plasmid DNA into *N. tabacum* leaves using Biolistic® PDS-1000/He Particle Delivery System (Bio-Rad). This method can be used to transform bacterial, animal, fungal and plant cells etc. Pressurized helium gas is used to accelerate gold-coated DNA particles which facilitate DNA entry inside the specific cell type due to high velocity and transform the explant with the gene of interest. This method is useful for both stable and transient transformation studies. Here transient study was the main objective and that was analyzed by determining the expression level of *gus* reporter gene in the transformed leaves. This experiment was carried out at NABI, Mohali, Punjab.

2.13.1 Preparation and purification of recombinant plasmid constructs using Plasmid Mini Kit (Qiagen)

1) A single bacterial colony (harboring recombinant *pRI101* constructs) (obtained from transformed colonies or a streak from glycerol stock) was inoculated in 5 mL of LB broth medium with kanamycin antibiotic and allowed to grow at 37°C for 12-14 hrs at 220 rpm in an orbital shaker.

2) The bacterial cells were collected by centrifugation at 6,000g, for 15 min at 4°C.

3) Pellet was resuspended in 0.3 mL of buffer P1 containing RNase A by vortexing until completely dissolved.

4) A volume of 0.3 mL of buffer P2 was added into it, mixed properly by inverting the tubes 4-6 times and incubated at RT for 5 min.

5) A volume of 0.3 mL of chilled buffer P3 was added and immediately mixed vigorously by inverting the tubes 4-6 times and incubated on ice for 5 min.

6) Samples were then centrifuged at ~18,000*g* at 4°C for 10 min and a clear supernatant was collected in 2 mL microcentrifuge tube, which would be used in further steps.

7) A Qiagen-tip 20 was equilibrated with 1 mL buffer QBT and the column was allowed to get empty by gravity flow.

8) Once the column drained completely, the previously collected supernatant was applied and allowed to enter inside the column resin by gravity flow.

9) Qiagen-tip 20 was then washed twice with 2 mL buffer QC. DNA was eluted in 0.8 mL buffer QF and the eluate was collected in 1.5 mL microcentrifuge tube.

10) A volume of 0.56 mL (7 times the DNA volume) of isopropanol (stored at RT) was added to the eluted DNA, mixed and centrifuged at ≥15,000*g* at 4°C for 30 min and the supernatant was carefully decanted.

11) The DNA pellet was washed with 1 mL of 70% ethanol and centrifuged at 15,000*g* at 4°C for 10 min. The supernatant was carefully decanted without disturbing the pellet.

12) The final pellet was air dried by inverting the tubes on a tissue paper for 20-30 min at RT and dried pellet was then dissolved in an appropriate volume of NFW (30-40 µL).

13) DNA concentration was determined by taking the absorbance at 260 nm wavelength in a U.V. spectrophotometer using following formula:

[Double stranded DNA concentration = Absorbance $_{260}$ **x** dilution factor **x** 50 µg/ mL]

2.13.2 Preparation of plant material for transformation by particle bombardment

N. tabacum plants were grown on MS basal medium under aseptic conditions (Figure 2-8). First, seeds (Lehle seeds, USA) were surface sterilized, then inoculated on the MS basal medium and allowed to germinate at 25-28°C temperature in a plant tissue culture

room under 16 hrs light and 8 hrs dark period. It took 4-6 weeks to obtain appropriate sized leaves (1.5 to 4 cm^2). Leaves were excised from the *in-vitro* raised young plants and cut in a way so that the midrib was removed and the final explant size was not smaller than 1.5 cm X 1.5 cm. Explants were transferred to MS basal medium and incubated for 12 hrs at 22°C. These pre-incubated explants were further used to perform particle bombardment.

(A) (B) (C)

Figure 2-8: Various growth stages of *N. tabacum* plantlets grown on MS basal medium: (A) 10-20 days old, (B) 20-30 days old and (C) 5-6 weeks old

2.13.3 Preparation of gold particle (microcarriers) suspension and coating onto DNA

Two constructs (TICTP-*bicA-gus-pRI101* and MEXTP-*bicA-gus-pRI101*) were used for this experiment and for each one of them triplicates were transformed, and therefore the below described method is applicable for six bombardments.

1) Gold particles (1 µm size) were weighed (3 mg) into 1.5 mL sterile microcentrifuge tube and baked overnight at 192°C in a hot air oven.
2) For washing of gold particles, 1 mL of 70% ethanol was added into the above tube and vortexed for 5 min followed by incubation at RT for 15 min so that gold particles can settle down.
3) After the incubation, a short spin was given for 5 sec and supernatant was discarded without disturbing the gold particles.
4) For washing of gold particles, 1 mL of autoclaved Milli-Q water was added into the above tube, vortexed for 1 min and then allowed to settle at RT. A short spin was given for 5 sec and supernatant was discarded without disturbing the gold particles.

5) The above step was repeated three times and the final pellet was dissolved in 50 µL of sterile 50% glycerol. It was mixed properly by vortexing for 5 min and then it was divided into two tubes. Now each tube has 3 mg/50 µL or 60 mg/mL concentration of gold.

6) Gold particles tended to form clumps therefore vortexing was done before every step so that each preparation should have even distribution of gold particles.

7) In both the tubes, the following items were added in the order given below:

- 5 µL plasmid DNA (0.5 µg/µL)
- 25 µL sterile calcium chloride (2.5 M stock solution)
- 10 µL filter sterilized spermidine (0.1 M stock)

8) While adding all these components the tubes were continuously stirred at low speed and then vortexing was done for 3 min and allowed to settle down at RT for 5 min.

9) After the incubation, a short spin was given for 2-5 sec and supernatant was discarded.

10) A volume of 70 µL of 70% ethanol is added to the pellet (should not disturb or dissolve the pellet) and a short spin was given for 2 sec and liquid was removed by pipette.

11) A volume of 70 µL of 100% ethanol is added to it, a short spin was given for 2 sec and liquid was removed by pipette.

12) Finally, the pellet was dissolved in 24 µL of 100% ethanol by gentle tapping and continuous vortexing for 2-3 sec was done.

13) A volume of 8 µL of above suspension (gold particles coated with DNA) was spread onto the macrocarrier.

2.13.4 Particle bombardment

DNA coated macrocarriers were taken and fixed on the macrocarrier holder and were air dried for 3-5 min. The particle bombardment apparatus was assembled and the leaf (to be transformed) containing plates were kept inside the apparatus chamber of Biolistic® PDS-1000/He Particle Delivery System (Bio-Rad). The bombardment was performed at 1100 lbs/cm^3 pressure using 1100 psi rupture disk (Bio-Rad). Once the bombardment

was finished, the explants were incubated onto the same medium for 48 hrs under 16 hrs light and 8 hrs dark period at 25°C in a plant growth chamber.

2.13.5 GUS fluorometric assay (Jefferson et al., 1987)

This assay was performed using leaves bombarded with recombinant plasmid keeping the non-bombarded leaves as a negative control.

1) About 10-15 mg leaves were crushed in liquid nitrogen using mortar-pestle, resuspended in 150 µL of extraction buffer [50 mM sodium phosphate buffer (pH 7), 10 mM DTT, 1 mM EDTA (pH 8), 0.1% SDS, 0.1% triton-X) and all the content was transferred to microcentrifuge tubes. Hereafter all the steps were carried out on the ice.

2) Debris were pelleted by centrifugation at 13,000 rpm for 20 min at 4°C and the supernatant was collected in a microcentrifuge tube.

3) A volume of 90 µL of the supernatant was mixed with 10 µL of assay buffer [50 mM sodium phosphate buffer (pH 7), 10 mM DTT, 1 mM EDTA (pH 8), 0.1% SDS, 0.1% triton-X, 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG)] and incubated at 55°C for 20 min.

4) After incubation, 20 µL of methanol was added to inhibit intrinsic GUS activity and the further reaction was carried out at 37°C for 2 hrs.

5) Thereafter incubation reaction was stopped by addition of 900 µL of 0.2 M sodium carbonate solution followed by proper mixing of the contents.

6) A volume of 200 µL sample was used to detect the relative fluorescence of 4-methylumbelliferone (MU) using spectrofluorometer (Thermo scientific-Fluoroskan Ascent). MU is the fluorogenic reaction product, which is formed by the action of GUS enzyme on the substrate MUG. The excitation and emission wavelengths for MU are 365 nm and 455 nm, respectively.

7) While taking reading in spectrofluorometer, extraction buffer was taken as blank along with control (non-transformed), the test sample (transformed) and MU standards. Blank sample's reading was subtracted from all the remaining readings.

8) MU standard curve was prepared (Figure 2-9). From the curve, value of 1 Relative Fluorescent Unit (RFU) was calculated and a formula was derived to calculate specific GUS activity.

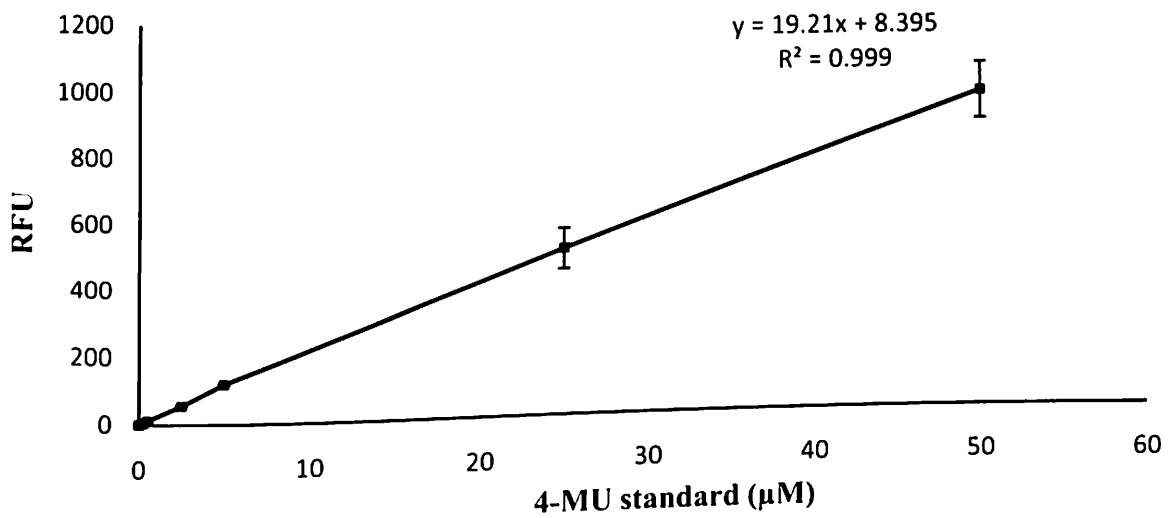


Figure 2-9: Standard curve for the relative fluorescence of 4-methylumbelliferone (MU standards were prepared from 0-50 µM concentration). The vertical bar indicates standard deviation for each MU standard prepared in triplicates.

The formula derived is as follows:

Number of nanomoles of MU/mg of protein/minute = 0.07 X/Y

Here X = RFU value of sample, Y = Total protein concentration of sample

9) Total soluble protein concentration was determined in the leaf extract supernatant by Bradford assay (Bradford, 1976). A volume of 100 µL of sample was mixed with 100 µL of Bradford reagent and in the same way Bovine Serum Albumin (BSA) standard samples were prepared with variable concentrations.

10) All the samples were added to a 96 well microtiter plate and incubated in dark at 37°C for 30 min at low shaking speed. Absorbance was recorded at 595 nm wavelength in an ELISA plate reader (STAT FAX 2100).

11) BSA standard curve was prepared from which total protein concentration was determined for control and test samples. The values obtained were used to calculate specific GUS activity.

12) Specific GUS activity was calculated by estimating the concentration of MU in the test as well as in control samples using the above mentioned formula and a final graph was plotted against each sample under study.

2.14 Transformation of *A. thaliana* and *N. tabacum* by *Agrobacterium* mediated co-culture method

Plant transformation was performed by *Agrobacterium* mediated co-culture method (Gallois and Marinho, 1995; van der Graaff and Hooykaas, 1998). The detailed methodology followed is mentioned below:

1) Standardization of growth conditions - For *A. thaliana* plants, growth conditions have already been described in section 2.9. *N. tabacum* plants were grown (Figure 2-10) in a mixture of 1:0.5:3 ratios of vermiculite, clay soil and sandy soil and plants were maintained at 25-28°C temperature in a plant tissue culture room under 16 hrs light and 8 hrs dark period.

(A) (B) (C)

Figure 2-10: Various growth stages of *N. tabacum* plantlets grown in soil mixture: (A) 20-25 days old, (B) 5-6 weeks old and (C) 7-8 weeks old

2) Explant (leaves or callus) preparation- (i) Young and juvenile leaves of pot grown plants (*A. thaliana and N. tabacum*) were taken from 6-8 weeks old plant and washed with detergent followed by treatment with 0.1% mercuric chloride solution for one min. After this, explants were properly washed four times with autoclaved Milli-Q water and cuts were made on all the four sides of explants using a sterile surgical blade. (ii) *In-vitro* raised callus tissue (*N. tabacum*) was used as an explant for agroinfection.

3) *Agrobacterium* growth- For the preparation of primary culture, *Agrobacterium* (harboring recombinant *pRI101* constructs) was grown in LB broth medium containing appropriate antibiotics at 28°C for about 48 hrs, which was then used to inoculate secondary culture. The secondary culture was grown until an optimal OD of 0.6-0.8 was achieved when observed at 600 nm wavelength and this culture was used for the further experiment.

4) Agroinfection- Once the optimal OD of *Agrobacterium* culture was achieved, freshly prepared explants were immersed in the culture containing acetosyringone (20 µM) for a duration of 10-20 min at 28°C, so that agroinfection could take place.

5) Co-cultivation- Immediately after agroinfection, explants were dried on sterilized filter paper, then transferred to co-cultivation medium [MS medium+appropriate plant growth hormone (PGR*) + acetosyringone], and thereafter maintained in this medium for two days in dark conditions at 22°C.

6) Regeneration- After two days of co-cultivation explants were washed with MS liquid medium containing cefotaxime (500 µg/mL) to kill the excess *Agrobacterium* and then dried on sterile filter paper. After washing, explants were transferred to regeneration medium [MS medium + PGR* + kanamycin (50 µg/mL) + cefotaxime (250 µg/mL)] containing selection agent for further growth under 16 hrs light and 8 hrs dark period at 22°C. Subculturing of callus was performed every 12-15 days.

Note: *PGRs used were variable as per the final objective of the experiment. In the case of callus induction 2,4-D (4 mg/L) was used while for multiple shoot regeneration BAP (2 mg/L) and (NAA 0.2 mg/L) was used.

2.14.1 GUS histochemical assay

This assay was performed using transformed callus keeping untransformed callus as a negative control. A small piece of callus tissue was incubated in GUS assay staining solution [2 M X-gluc, 20 mM sodium phosphate buffer (pH 7), 1 mM $K_3Fe(CN)_6$, 0.1% triton-X, 10 mM EDTA (pH 8)] at 37°C for 1 hr to 24 hrs. In this reaction, a chromogenic compound 5-bromo-4-chloro-3-indolyl β-D glucuronide (X-Gluc), which is a component of staining solution, was used as a substrate that is oxidized by GUS enzyme resulting in the development of blue colored precipitate. Blue coloured callus

was subsequently analyzed under the compound microscope (Olympus-bright field) (this protocol was adapted from Vitha, 2012)

2.15 Transformation of *A. thaliana* by floral dip method

In order to generate stable transgenic plants, transformation of *A. thaliana* by floral dip method (Clough and Bent, 1998; Zhang et al., 2006) using *A. tumefaciens* strain GV3101 was performed. The detailed protocol is as follows:

1) *A. thaliana* plants were grown in the lab under standard conditions as described in section 2.9. When primary bolts developed in around 6-8 weeks, they were cut from the top end to generate multiple side branches and allowed to generate large number of floral buds.

2) Once the plants reached the flowering stage (8-10 weeks), further experiment was carried out. Immediately before the experiment, enough watering of the plants was done and *Agrobacterium* (harboring recombinant *pRI101* constructs) culture was prepared.

3) For the preparation of primary culture, *Agrobacterium* was grown in LB broth medium containing appropriate antibiotics at 28°C for about 48 hrs, which was then used to inoculate secondary culture. The secondary culture was grown overnight until an optimal OD of ~1.5 was achieved when observed at 600 nm wavelength, and this culture was used for further experiment.

4) *Agrobacterium* culture was centrifuged at 4500 rpm for 10 min at RT to pellet down the bacterial cells. Collected cells were resuspended in equal volume of 5% (wt/v) freshly prepared sucrose solution and then 0.02% (v/v) Silwet L-77 (Lehle seeds, USA) was added into it and mixed properly by pipetting and swirling the container.

5) The floral inflorescences of the *A. thaliana* plants were submerged into the above solution for 10-20 seconds. Plant pot was tilted at a particular angle so that floral buds were properly dipped into the solution (plants with short stems were dipped into the solution with the help of sterile forceps)

6) Successful transformation was indicated by a thin film of liquid seen on the buds. Transformed plant pots were completely covered in a plastic bag or aluminium foil to maintain the appropriate humidity and kept under dark condition for 12 hrs at 22°C.

7) After 12 hrs, the plastic cover was removed and plants were kept under normal growth conditions. To increase the transformation efficiency, floral dip procedure was repeated after 3-5 days using the same plants.

8) Transformed plants were grown for another 3-4 weeks until the siliques started drying. Thereafter T_1 seeds were collected, surface sterilized and germinated on MS medium containing kanamycin (50 mg/mL) for selection of transformed plantlets.

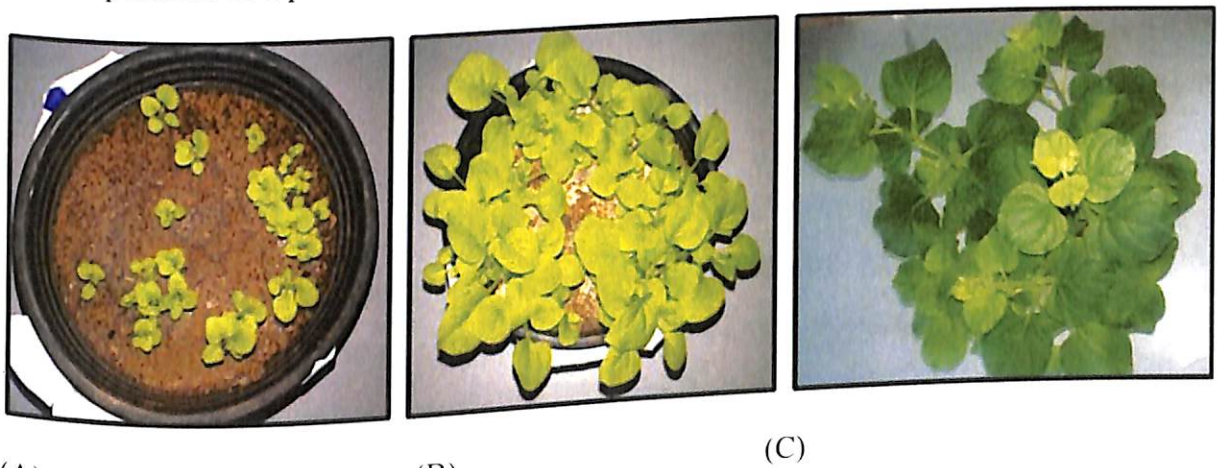
9) When resistant plantlets reached 2-4 leaf stage (this took around 2-3 weeks), they were transferred to soilrite mix in plastic pots (this does not contain antibiotic). These plants were termed as T_1 plants.

10) T_1 plants were grown for 6-8 weeks until the siliques started drying. Thereafter T_2 seeds were collected and again screened on MS medium containing kanamycin and resistant plantlets were transferred to soilrite mix in plastic pots. These plants were termed as T_2 plants.

2.16 Transformation of *N. benthamiana* by *Agrobacterium* mediated agro-infiltration method

N. benthamiana plants were transformed by agroinfiltration method (Ma et al., 2012; Leuzinger et al., 2013) to study transgene expression at DNA, mRNA and protein level and to determine intracellular localization of the expressed proteins.

1) *N. benthamiana* plants were grown (Figure 2-11) in a mixture of 1:0.5:3 ratios of vermiculite, clay soil and sandy soil and plants were maintained at 25-28°C temperature in a plant tissue culture room under 16 hrs light and 8 hrs dark period.



(A) (B) (C)

Figure 2-11: Various growth stages of *N. benthamiana* plantlets grown in soil mixture: (A) 20-25 days old, (B) 6-7 weeks old and (C) 8-9 weeks old

2) For the preparation of primary culture, *Agrobacterium* (harboring recombinant *pCAMBIA* constructs) was grown in LB broth medium containing appropriate antibiotics at 28°C for about 48 hrs which was then used to inoculate secondary culture (contains 20 µM acetosyringone). The secondary culture was grown until an optimal OD of 0.4 was achieved when observed at 600 nm wavelength and this culture was used for agroinfiltration.

3) *Agrobacterium* cells were harvested by centrifugation at 4,500 rpm for 15 minutes and the pellet obtained was resuspended in the equal volume of resuspension solution [10 mM $MgCl_2$ and 10 mM MES-K (pH 5.6) and 100 µM acetosyringone].

4) This solution was kept at RT for 2-3 hrs and used to infiltrate the leaves of *N. benthamiana* plants. Here the leaves of 6-8 weeks grown plants were used and agroinfiltration was performed using a needleless 5 mL or 2 mL sterile syringe.

5) In order to facilitate the entry of *Agrobacterium* cells into the plant, the syringe was pressed on the ventral side of the leaves. Post infiltration, plants were again kept under normal growth conditions. After 4 days of incubation plant leaves were excised and used for further experiments.

2.16.1 Genomic DNA isolation using DNeasy Plant Mini Kit (Qiagen)

Genomic DNA was isolated from *N. benthamiana* leaves four days post agroinfiltration. The methodology followed has already been described in section 2.10.

2.16.2 Total RNA isolation using RNeasy Plant Mini Kit (Qiagen)

Total RNA was isolated from *N. benthamiana* leaves four days post agroinfiltration. All the solutions required for RNA work were prepared in RNase-free water (0.1% DEPC was allowed to dissolve in water by continuous stirring for 10-12 hrs at RT followed by autoclaving) and microcentrifuge tubes, PCR tubes, micropipette tips etc. were soaked overnight in DEPC water and then autoclaved to remove traces of DEPC. DEPC is known to inactivate RNases from plasticware, glassware and solutions. RNaseZAP (Sigma) solution was used to clean the working area and pipettes and gloves which were used throughout the isolation procedure. To check RNA quality, samples were analyzed on 1% agarose gel as described in section 2.12.2. The running buffer i.e., 1X TAE was freshly prepared in RNase-free water and electrophoresis apparatus was cleaned with

0.5% SDS and then rinsed with DEPC water and finally wiped with 70% ethanol to remove RNase contamination. The detailed protocol of RNA isolation is as follows:

1) 100 mg plant leaves were weighed and grounded using liquid nitrogen in pre-chilled mortar-pestle. Finely powdered sample was immediately transferred into a microcentrifuge tube before it was thawed.

2) A volume of 450 µL of buffer RLT (with 1% v/v β-mercaptoethanol added before use) was added and the sample was vigorously vortexed. The lysed sample was added into the QIAshredder spin column, which was placed in a 2 mL collection tube and centrifuged at 14,000 rpm for 2 min. The flow through (supernatant) was collected in a 2 mL microcentrifuge tube.

3) A 0.5 volume 100% ethanol (Merck) was added to the supernatant and mixed by pipetting. The mixture was further added into the RNeasy Mini spin column, which is placed in a 2 mL collection tube and centrifuged at 10,000 rpm for 15 sec. The flow through was discarded.

4) A volume of 700 µL of buffer RW1 was added to the above column and it was incubated at RT for 5 min and then centrifuged at 10,000 rpm for 15 sec. The flow through was discarded.

5) A volume of 500 µL of buffer RPE was added to the above column and centrifuged at 10,000 rpm for 15 sec. The flow through was discarded. Again, 500 µL of buffer RPE was added to the above column and centrifuged at 10,000 rpm for 2 min. (to dry the membrane, the empty column was again centrifuged for 1 min)

6) To elute out the RNA from column, 30 µL RNase-free water was added to the column which is placed in 1.5 mL microcentrifuge tube and incubated for 5-10 min at RT and then centrifuged at 10,000 rpm for 1 min.

7) The eluted RNA sample was analyzed on 1% agarose gel and the remaining sample was stored at -80°C.

2.16.2.1 cDNA synthesis using QuantiTect® Reverse Transcription kit (Qiagen)

1) RNA samples were thawed on ice and as mentioned below, genomic DNA contamination (if any) was removed by treating the RNA samples with gDNA wipeout buffer provided in the kit.
    - gDNA wipeout buffer, 7X - 2 µL
    - Template RNA - 1 µg to 2 µg
    - RNase-free water - variable
2) The above reaction mixture was incubated at 42°C for 2-5 min and then immediately placed on ice.
3) Once the genomic DNA elimination reaction was completed, the same reaction mixture was further used for the synthesis of cDNA.

The composition of reverse transcription reaction mixture is as follows:

- Quantiscript reverse transcriptase - 1 µL
- Quantiscript RT buffer, 5X - 4 µL
- RT primer mix - 1 µL
- Template RNA (whole mixture from genomic DNA elimination reaction) - 14 µL

4) The above reaction mixture was incubated at 42°C for 15-30 min for the synthesis of cDNA and then at 95°C for 3 min to inactivate the Quantiscript reverse transcriptase.
5) The cDNA samples were stored at -20°C.

Note: All the reaction mixtures were prepared on ice and incubations were carried out in PCR machine.

2.16.3 Diagnostic PCR using genomic DNA and cDNA as template

Genomic DNA of transformed plants was used to amplify *bicA* and *mgfp5* using gene specific primers for full-length amplicon while cDNA was used to perform PCR using primers specific to the internal region of the gene for partial amplicon (primer and other details are already mentioned in Table 2-1 and section 2.12.1).

2.16.4 Total protein extraction using G-Biosciences kit

Total protein was isolated from *N. benthamiana* leaves after four days of agroinfiltration using Total Protein Extraction kit (TPE™) (G-Biosciences). The detailed protocol of protein extraction is as follows:

1) 100 mg plant leaves were weighed and grounded using liquid nitrogen in pre-chilled mortar-pestle until finely powdered sample was obtained.

2) A volume of 200 µL-250 µL of TPE Buffer-I containing 5 µL protease inhibitor cocktail (Sigma) was added to the powdered sample and again proper grinding was done to obtain homogenous lysate, which was transferred into a microcentrifuge tube.

3) A volume of 40 µL of warm TPE Buffer-II was added into the tube and the lysate was vortexed for 30 sec and then incubated at 100°C for 30 sec. This step of vortexing and heating was repeated until a clear solution was observed. Lastly, the samples were again incubated at 100°C for additional 10 min.

4) Samples were centrifuged at 15,000g for 10 min at 4°C to remove cellular debris and protein extract was collected in the form of the supernatant.

5) The protein samples were stored at -20°C.

Note: Minor modifications were done in the composition of TPE Buffer-I i.e., the addition of 5% PVPP and 2 mM DTT. A higher volume (120 µL of TPE Buffer-II was used for every 1 mL of TPE buffer-I) of TPE-II buffer was used because it contains SDS, which enhances the yield of membrane proteins in the total protein extract.

2.16.4.1 Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

This technique is used to separate proteins on the basis of their molecular weights. SDS provides almost uniform negative charge to the proteins which allow them to migrate only according to their size. The polyacrylamide gel (Table 2-5) is made up of two gels i.e., upper stacking gel (5% acrylamide) with larger pore size and lower resolving gel (12% acrylamide) with smaller pore size.

Table 2-5: The composition of polyacrylamide gel

Sr. No.	12% resolving gel (5 ml)	5% stacking gel (2.5 ml)
1.	Water - 1.65 mL	Water - 1.7 mL
2.	30% acrylamide mixture - 2 mL	30% acrylamide mixture - 415 µL
3.	1.5 M Tris (pH 8.8) - 1.25 mL	0.5 M Tris (pH 6.8) - 315 µL
4.	10% SDS - 50 µL	10% SDS - 25 µL
5.	10% ammonium per sulfate (APS)	10% ammonium per sulfate (APS) - 25 µL
6.	N, N, N', N'-Tetramethylethylenediamine (TEMED) - 5 µL	N, N, N', N'-Tetramethylethylenediamine (TEMED) - 2.5 µL

Sample preparation for SDS-PAGE: 10 µL of protein samples (approximately 50-100 ug) were mixed with equal volume of 2X SDS gel loading buffer [100 mM Tris-Cl (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue (BPB)] and incubated at 100°C for 10 min to denature the proteins.

Electrophoretic run: Electrophoresis was carried out in vertical electrophoresis apparatus (Bio-Rad) at 80-100 volts/cm for 3 hrs using 1X tris-glycine electrophoresis buffer (0.125 mM tris base, 0.96 M glycine and 0.5% SDS).

Staining and destaining of the gel: Once the run is over, the gel was stained with Coomassie Brilliant Blue (CBB) R- 250 staining solution [0.1% CBB R-250 and 5:4:1 (methanol: water: glacial acetic acid)] for 3-4 hrs. After proper staining, gel was incubated in the destaining solution (30% methanol and 10% acetic acid) for 10-12 hrs. Gel images were captured by gel documentation system (Molecular Imager® Gel Doc™ XR - Bio-Rad) under white light.

2.16.5 Western blot analysis

Proteins samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (0.45 µm) by wet transfer method using western transfer equipment (Bio-Rad). The detailed procedure is as follows:

1) Once the electrophoretic run was over, the desired area of the gel was cut and equilibrated in transfer buffer (45 mM tris base, 39 mM glycine and 20% methanol) for 10-15 min.
2) At the same time sponges, filter pads and PVDF membranes were also equilibrated in the transfer buffer. PVDF membrane was first activated by incubating in 100% methanol for 10 min and then incubated in the transfer buffer.
3) Transfer sandwich was assembled using equilibrated sponges, filter pads, gel and membrane membrane. In transfer cassette, the gel was placed towards cathode while membrane was placed towards the anode and this was sandwiched between sponges and filter pads in such a way that air bubbles should not form and gel and membrane should be in tight contact.
4) The cassette was placed in transfer tank in which transfer buffer was filled and the cooling pack was placed and then transfer was done at 50 volts/cm for 2.5 hrs in a cold room to avoid generation of excess heat.

5) After the transfer process was carried out for 2.5 hrs, the sandwich was disassembled and the membrane was taken out and stained with the poncaeuS solution (0.1% w/v poncaeuS in 5% v/v acetic acid) to observe protein bands. The poncaeuS solution was subsequently washed off with autoclaved Milli-Q water.

6) The membrane was incubated with blocking buffer [(5% skimmed milk powder prepared in Tris Buffer Saline containing Tween-20 (TBST- 50 mM tris base and 50 mM NaCl; pH 7.5 and then 0.05% tween-20 was added into this)] for 4-5 hrs at RT on slow shaking (10 rpm).

7) The membrane was rinsed with TBS for 7 min (two times) and then once with TBST for 5 min. Once the blocking buffer was washed off properly, the membrane was incubated in primary antibody solution i.e., an anti-GFP primary antibody raised in rabbit (GFP tag polyclonal antibody; Thermo Fisher Scientific; A-11122 with 1:2000 dilution) or anti-Tic110 primary antibody raised in rabbit (polyclonal antibody; Agrisera; AS08 293 with 1:1000 dilution). This incubation was done for 12-15 hrs in cold room with moderate shaking (20-25 rpm).

8) Once the incubation was over, the membrane was washed as mentioned in step 7 and then incubated with secondary antibody solution i.e., horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody raised in goat (Goat anti-rabbit IgG - HRP; Merck; 621140380011730 with 1:5000 dilution). This incubation was done for 2 hrs at RT with moderate shaking (20-25 rpm).

9) Once the incubation was over, the membrane was washed as mentioned in step 7 and then incubated with substrate solution i.e., 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) Liquid Substrate System for Membranes for 5-10 min or until the blue coloured bands start appearing. The image was then captured by gel documentation system.

Note: All the antibody dilutions were prepared in blocking buffer. Chilled buffers were used throughout the procedure.

2.16.6 Protoplast isolation from agroinfiltrated leaves

Agroinfiltrated leaves were chopped into approximately 0.5 cm pieces using a sterile scalpel blade and transferred into plastic petriplate containing 3 ml washing solution-1 [0.5 M mannitol, 4 mM MES-K (pH 5.5) and 20 mM KCl]. Thereafter gentle mixing of leaves was done by hand and the WS1 solution was replaced with freshly prepared enzyme solution [1.5% cellulase R-10, 0.4% macerozyme R-10, 0.4 M mannitol, 20 mM KCl, 20 mM MES (pH 5.5), 0.1% BSA and 10 mM $CaCl_2$. This mixture was incubated in a shaker at 25-28°C temperature with low speed shaking at 60-80 rpm speed in dark for 4-5 hrs. After the incubation period, the whole mixture was transferred into a falcon tube containing an equal volume of washing solution -2 [154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES (pH 5.7)] and a gentle mixing was done. In order to remove leaf debris, the complete mixture was passed through nylon mesh filter of 80 µM, which was followed by centrifugation at 50g for 10 minutes to settle down the protoplast. Protoplast pellet was washed 2-3 times using WS2 and finally dissolved in 1 mL of MMG solution [0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES (pH 5.7) (Jabnoune et al., 2015; Chamani and Tahami, 2016)

2.16.6.1 Confocal microscopy analysis

Protoplasts samples (2-3 µL) were mounted with Antifade Mounting Medium (2-3 µL) vectashield (Vector laboratories) and were imaged by LEICA TCS SP5 confocal laser scanning microscope under 63X oil immersion. The wavelength of 488 nm was used for excitation of both GFP and chlorophyll. Emission of GFP was recorded at 499-550 nm and chlorophyll autofluorescence was recorded at 630-735 nm.