# CHAPTER 3

# MATERIALS AND METHODS

# 3. Materials and Methods

## 3.1 Identification of polyamine biosynthetic genes in tomato genome

To identify the tomato polyamine biosynthetic genes, known protein sequences of *Arabidopsis* polyamine biosynthetic genes obtained from TAIR (https://www.arabidopsis.org/) (Berardini et al., 2015) were subjected to BLAST search against the tomato genome in the SOL Genomics Network (SGN; https://solgenomics.net/) (Fernandez-Pozo et al., 2014). On the basis of sequence homology with Arabidopsis we have identified several candidate polyamines biosynthetic genes in tomato. Protein sequences of all identified candidate genes were further validated by conserved domains searching using InterProScan database (http://www.ebi.ac.uk/interpro/scan.html) (Jones et al., 2014).

## 3.2 Protein properties and gene structure analysis

The theoretical molecular weight (Mw) and isoelectric point (pI) of identified candidate proteins were predicted by Gene Infinity tool (http://www.geneinfinity.org/sms/sms_proteiniep.html). The genomic sequences of candidate polyamine biosynthetic genes were obtained from the gene search of SGN database (https://solgenomics.net/search/locus) and their exon-intron structures were identified using Gene Structure Display Server tool (Hu et al., 2014). Distribution of these candidate genes on tomato chromosomes was identified from SGN database.

## 3.3 Multiple sequence alignment and phylogenetic analysis

The protein sequences of Arabidopsis and tomato polyamines biosynthetic genes were obtained from TAIR (https://www.arabidopsis.org/) and SGN (https://solgenomics.net/) database, respectively. Multiple sequence alignment of candidate proteins of tomato along with the Arabidopsis proteins was generated with ClustalW (www.clustal.org/) (Larkin et al., 2007). The phylogenetic tree was constructed using MEGA6 (Tamura et al., 2013).

## 3.4 Analysis of cis-regulatory elements in promoter sequences

The 1kb of genomic sequences upstream of 5'UTR of each candidate gene was obtained from tomato SGN (https://solgenomics.net). The putative cis-regulatory elements in the promoter regions were identified using PlantCARE database (Lescot et al., 2002).

## 3.5 Expression analysis of candidate polyamine biosynthetic genes in different tissues of tomato plants

### 3.5.1 Plant material

Seeds of tomato *Solanum lycopersicum* L. (cultivar 'MicroTom') were germinated on ½ Murashige and Skoog (MS) medium (Himedia; Murashige & Skoog 1962) and grown in a culture room at 25±1 °C under 16 h light and 8 h dark and approximately 70% relative humidity. For tissue-specific expression analysis Rt; roots, Hy; hypocotyl and Ct; cotyledons from 15 dag (days after germination) old seedlings and various vegetative and reproductive tissues from 60 dag old mature plants were isolated. Four stages of leaves IL; immature leaves, YL; young leaves, ML; mature leaves and SL; senescent leaves (leaves undergoing senescence), two layers of stem OL of St; outer layer of stem (cortical layer including phloem), IL of St; inner layer of stem (xylem layer), AZ; abscission zone of flower pedicels, eight developmental stages of flowers (FL1; developing flower bud stage 1, FL2; developing flower bud stage 2; FL3; developing flower bud stage 3, FL4; immature flower stage 1, FL5; immature flower stage 2, FL6; immature flower stage 3; FL7; mature flower; FL8; senescent flower) and eight different developmental stages of fruits (FR1; immature fruit stage 1, FR2; immature fruit stage 2, FR3; immature fruit stage 3, FR4; mature green fruit, FR5; mature breaker fruit, FR6; mature turning fruit, FR7; mature pink fruit, FR8; mature red fruit) were collected for RNA isolation.

### 3.5.2 RNA isolation

Total RNA from different tissues of plants was isolated using RNAqueous Total RNA Isolation Kit (Ambion, USA) following the method as described in the user manual. The protocol used was as follows:

For RNA isolation, 100 mg of plant tissues were excised and quickly frozen in liquid nitrogen before extraction or stored in deep freezer at -80 $^{o}$C for further use. Plant tissues were crushed using micropestle in a microcentrifuge tube containing liquid nitrogen. About 8 volume of lysis/binding buffer was added to the tube followed by addition of 1 volume of plant RNA isolation Aid (Ambion, USA). The plant tissues were then homogenized with the help of micropestles. The tubes were then centrifuged at 15,000×g to remove insoluble plant debris. The supernatants were transferred to new tubes. Then an equal volume of the 64% ethanol was added to each tube and mixed gently by inverting the tube several times. The lysate was

passed through a filter column/cartridge and then centrifuged at 15,000×g for 1 min. Flow through was discarded. The 700 µl of Wash solution 1 was added to the column and centrifuged for 1 min until the wash solution passed through the column. Flow through was discarded and collection tube was reused for subsequent washings. Again, 500 µl of Wash solution 2/3 was added and then centrifuged. This step was repeated twice. Finally, after discarding the flow through the column was centrifuged at 15,000×g for 30 seconds to remove the traces of wash solution. The RNA was eluted by adding 50 µl of preheated elution buffer.

### 3.5.3 DNase I treatment

Before proceeding for the further steps, trace of DNA contamination from the each isolated RNA sample was removed using TURBO DNA-free™ Kit (Ambion, USA) by the following steps:

1. 0.1 volume of 10x DNase buffer and 1 µl of DNase I enzyme were added to each RNA sample.
2. The samples were then incubated at 37 °C for 30 min for DNA digestion.
3. 0.1 volume of DNase inactivation reagent was added.
4. Samples were gently mixed and incubated at RT for 2 min.
5. Samples were then centrifuged at 10,000 x g for 2 min at RT and supernatant was transferred to new microcentrifuge tubes.

The integrity of total RNA was analysed by agarose gel electrophoresis. Then RNA was quantified using Nano Spectrophotometer (lmplen GmbH, Germany). For cDNA synthesis 0.5 µg DNA-free RNA was used.

### 3.5.4 First strand cDNA synthesis

Synthesis of first strand of cDNA from RNA samples was done by reverse transcription reaction using PrimeScript$^{TM}$ 1$^{st}$ strand cDNA synthesis kit (Takara, Japan) as per manufacturer's recommendations. Kit contains PrimeScript reverse transcriptase, 5X PrimeScript buffer, RNase inhibitor to prevent degradation of RNA template, dNTP mixture, Oligo dT primer and random 6 mers. The reaction was performed in two steps. Total reaction was set to 20 µl. First reaction mixture comprises of template RNA (0.5 µl g each), oligo dT primer and dNTPs were mixed with RNase free $dH_2O$ up to 10 µl. The reaction mixture was then incubated for 5 min at 65 °C and immediately cooled on ice. The second reaction mixture consists of template RNA primer mixture from the previous step, 5x PrimeScript buffer, RNase

inhibitor, PrimeScript RTase and RNase free $dH_2O$ upto 20 µl. The complete reaction mixture was then incubated in Veriti thermal cycler (Applied Biosystems, USA) at 42 °C for 60 min. and the amplification was inactivated at 70 °C for 15 min.

### 3.5.5 Expression analysis using semi-quantitative RT-PCR

Expression of the candidate genes in the different plant samples was analyzed by semi-quantitative RT-PCR using gene-specific primers and cDNA template synthesized in the section 3.5.4. The semi-quantitative RT-PCR expression was carried out with an Veriti thermal cycler. SolycUBQ5 (Solyc012g098940) was used as an internal control. Each reaction was performed in 10 µl (total volume) and consisted of EmeraldAmp® GT PCR Master Mix (Takara, Japan), 10 µmol of each primer, 1 µl cDNA template and sterile $dH_2O$. The PCR steps performed were as follows: step (1) 98 $^oC$, 2 min; step (2) 98 $^oC$, 15 sec; step (3) 55 $^oC$, 30 sec; step (4) 72 $^oC$, 45 sec x 40-45 cycles. Gene-specific and reference gene primers used were designed by Vector NTI (www.invitrogen.com/VectorNTI) and primer3 tool (Ye et al., 2012). The primers were listed in Table 3.1.

### 3.5.6 Expression analysis using quantitative RT-PCR (qRT-PCR)

For the quantitative RT-PCR expression analysis, cDNAs templates as synthesized in section 3.5.4 were used. Quantitative RT-PCR was performed by using SYBR® premix Ex Taq™ (Tli RNaseH Plus; Takara, Japan) kit which contains SYBR premix Ex Taq and ROX reference dye and the PCR reaction was carried out on ABI QuantStudio 12K Flex qPCR detection system (Applied Biosystems, USA). Each reaction was performed in 10 µl total volume and consisted of 1x Takara SYBR Green master mix, ROX, 10 µmol of each primer, 1 µl cDNA template and sterile $H_2O$. The qRT-PCR steps performed were as follows: step (1) 95 $^oC$, 15 sec; step (2) 55-60 $^oC$, 30 sec (3) 72 $^oC$, 30 sec x 40-45 cycles. Quantitative RT-PCR was performed using gene transcripts specific primers designed using vector NTI and primer3 tools and analysis was done by relative expression and comparative $2^{-\Delta\Delta Ct}$ methods (Livak & Schmittgen 2001). SolycUBQ5 (Solyc012g098940) and SolycAPT1 (Solyc08g079020) were used as references to normalize the expression. Primers used are listed in Table 3.1.

**Table 3.1: Primers used for semi-quantitative RT-PCR and qRT-PCR analysis**

| Gene Name | Gene ID | Primer Sequence |
|---|---|---|
| SolycADC1 | Solyc10g054440.1 | Forward: TAAAATGGCGGTCCAGCCTG<br>Reverse: CCCAAACTTGCCTTTCTCTCCA |
| SolycADC2 | Solyc01g110440.2 | Forward: TTGCTTTGGTCGCAAGAAAGC<br>Reverse: GCCAGAATGCTTTGTCCTGAGC |
| SolycODC1 | Solyc04g082030.1 | Forward: GAGATGCCCAATGGGTCCA<br>Reverse: GACTCACCGTCTCCGGCGTG |
| SolycODC2 | Solyc03g098300.2 | Forward: AATGGCACCCACAGTGCCAT<br>Reverse: TGAACCACTCCTGCATTACGCTT |
| SolycSAMDC1 | Solyc05g010420.1 | Forward: CTGAAGAAGTTGCTGTCCTCGATG<br>Reverse: TTCTGTGTTTTGTCGGGATTTCC |
| SolycSAMDC2 | Solyc1g010050.2 | Forward: GAACTCCTCGAGTCTCGGGTCA<br>Reverse: TCACAAGCAACATAATCAGCATGC |
| SolycSAMDC3 | Solyc02g089610.1 | Forward: GCTTACGTACCTCACATTCA<br>Antisense: TCTTCTCCATTTCCTCATCCT |
| SolycSAMDC4 | Solyc06g054460.1 | Forward: CCAGAATTGTTAAAGCTGTCG<br>Reverse: CCTATTTCCCGTCAACGTAA |
| SolycSAMDC5 | Solyc01g080380.3 | Forward: ACGAGAATTGCTAAAGCTGT<br>Reverse: TTCCGGCAAGATGTAAAAGT |
| SolycSPDS1 | Solyc05g005710.2 | Forward: GGAACTGAATTGCCAGTGAAGAGG<br>Reverse: GGGGGCTAATCTCAGAGAACCA |
| SolycSPDS2 | Solyc04g026030.2 | Forward: TGCCGCCAGATCTTCAAAGG<br>Reverse: CCCTCGGTAGAGCAGACCATGA |
| SolycSPDS3 | Solyc08g014310.2 | Forward: ACATGCTTTGCTCTACTGAG<br>Reverse: GTGTTGTAGAACTTGAGAGGT |
| SolycSPDS4 | Solyc06g053510.2 | Forward: TTGGTTACATGCTTTGCTCT<br>Reverse: AGAACTTCAAAGGTCCCTTG |
| SolycSPDS5 | Solyc06g053520.2 | Forward: TGTTATTTGCTCTACTGAGGG<br>Reverse: ACTTCAAAGGGGATTCAGATTT |
| SolycSPMS | Solyc03g007240.2 | Forward: TTATTGGGTTTCTTCTGTGCTCAAC<br>Reverse: TTGTAAAACTTGAGTTCCCGCTG |
| SolycACL5 | Solyc08g061970.2 | Forward: GGATGGGTTATGGCTTCTGA<br>Reverse: GTTTTGGCAACGGTCTTGTT |
| SolycACL5-Like1 | Solyc09g075900.2 | Forward: CAGGGCTGAGCTAGAGAGGA<br>Reverse: GCTGGTCCTGCCTGAGTAAC |
| SolycACL5-Like2 | Solyc07g041300.1 | Forward: CAACCATTGAAACTAGATGCTG<br>Reverse: AAAAGCAAGCATGAAAGCAG |
| SolycUBQ5 | Solyc012g098940 | Forward: TAAGCTCGCTGTCCTCCAGT |

| | | |
|---|---|---|
| | | Reverse: GGTTAGCCATGAAGGTTCCA |
| SolycAPT1 | Solyc08g079020 | Forward: AGTTGCCGGAGCTAGAAACA<br>Reverse: ACGTGGATCTTGACCTTTGC |
| NtUBQ5 | Nitab4.5_0003998 g0080.1 | Forward: TAAGCTCGCCGTCCTCCAGT<br>Reverse: GGTTAGCCATGAAAGTACCG |
| NtEF1- α | Nitab4.5_0000022 g0030.1 | Forward: TTGAGGCTCTCGACCAGATT<br>Reverse: GTCAAACCAGTAGGGCCAAA |
| NtAPT1 | Nitab4.5_0000312 g0400.1 | Forward: AGTTGCCGGAGCTAGAAACA<br>Reverse: ACGTGGATCTTGACCTTTGC |
| NtRPOA | Nitab4.5_0001564 g0040.1 | Forward: AGTGGAAGTGTGTTGAATCA<br>Reverse: CAAGTAAAGCTCTTCGCATC |
| NtRBCL | Nitab4.5_0006084 g0010.1 | Forward: ACAGAGACTAAAGCAAGTGT<br>Reverse: GGAGTTACTCGGAATGCTG |
| NtPETB | Nitab4.5_0003957 g0010.1 | Forward: AATAGGATCACCTTTGGTCG<br>Reverse: AGTAAGAAGCGGCAATACAA |

## 3.6 Expression analysis of tomato polyamine biosynthetic genes during stress and hormone treatments

### 3.6.1 Plant material

For stress treatments, *S. lycopersicum* variety "Money Maker" seedlings were grown *in vitro* as methods described in section 3.5.1. Seven day old seedlings were taken for inducing various stress treatments.

### 3.6.2 Stress treatments

We have induced different types of stresses such as cold, heat, UV-C, flood, drought, salt, mannitol, methyl viologen (MV), fumonisin B1 (FB1), rose bengal (RB), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) as given below:

**Cold stress**

The petriplates containing the seedling were kept at 4 °C in the refrigerator and then the samples were harvested for RNA isolation after 30 min. Plates containing seedlings at 25 °C were treated as control (Diao et al., 2016).

**Heat stress**

Petriplates containing seedlings were kept at 37 °C for 30 min for heat treatment and the samples were harvested after 30 min (Mainali et al., 2014). Control plates were kept at 25 °C.

**UV-C light stress**

For UV-C light stress, the plates containing seedlings were exposed to UV-C light for 15 min, and seedlings under normal growth conditions were taken as control (Kilian et al 2007).

**Drought stress**

The seedlings were taken out from the tissue culture boxes and exposed to a stream of air on a sterile petriplate for 15 min, and then the samples were harvested for RNA isolation (Kilian et al., 2007).

**Flood stress**

For flooding, the seedlings were taken out from the tissue culture boxes and kept in sterile water in a sterile petri plate for 15 min, and then the samples were harvested for RNA isolation (Kilian et al., 2007).

**Wounding stress**

To provide wounding stress we have just provided mechanical injury to the seedlings by piercing the seedling with a pointed needles tool. Then the RNA is isolated from the injured plant samples (Kilian et al., 2007).

**Salt stress**

For salt stress, the seedlings were transferred to a petriplate containing MS media supplemented with 150 mM NaCl salt. The petri plate was again incubated in the standard plant tissue culture growth condition. And then the samples were harvested at the time interval of 12 and 24 h. Seedlings grown on MS plates were used as control (Kilian et al., 2007).

**D-Mannitol**

To provide osmotic stress the seedlings were transferred to the petriplates containing MS media supplemented with 300 mM D-mannitol and further incubated in the standard plant tissue culture growth condition. The samples were harvested after 12 and 24 h time interval. Control samples were same as that of salt treatment (Kilian et al., 2007).

**Methyl Viologen, Rose Bengal, and Fumonisin B1 treatments**

Tomato seedlings grown in tissue culture boxes were transferred to the pertiplates containing MS medium supplemented with methyl viologen (10 µm) to generate oxidative stress, rose bengal (0.5 µm) and fumonisin B1 to a final concentration of 1 mM as a fungal toxin and the samples were harvested at the time interval of 12 and 24 h. Control seedlings were same as previously described for salt treatment.

**Salicylic (SA), jasmonic (JA) and abscisic acid (ABA) treatments**

To provide hormonal treatments, seedlings were transferred from the tissue culture boxes to the petriplates containing MS medium supplemented with SA, JA and ABA to a final concentration of 10 mM each, respectively, and kept in the standard plant tissue culture growth conditions for 3 h then the samples were harvested for RNA extraction. Seedlings grown on MS plates alone were taken as control. Each treatment was performed in triplicates. All the samples were immediately frozen in liquid nitrogen and used for RNA isolation.

### 3.6.3 RNA isolation

Total RNA from different control and treated tomato seedlings were isolated as methods described in section 3.5.2. For cDNA synthesis 0.5 µg RNA per sample was used.

### 3.6.4 First strand cDNA synthesis

First strand cDNAs from above isolate RNA samples were synthesized as the procedure mentioned in section 3.5.3.

### 3.6.5 Expression analysis using qRT-PCR

Quantitative real time expression analysis after each stress experiment was performed as in section 3.5.6. All the cDNAs were normalized with SolycAPT1 and SolycUBQ5 as internal control. The real-time PCR amplification values were quantified following comparative $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Data were expressed as the mean±SEM of three biological replicates. All the primers used are mentioned in Table 3.1.

## 3.7 Cloning of SolycACL5 (Solyc08g061970.2) gene for generation of gene silencing and overexpresser lines

Cloning of selected SolycACL5 (Solyc08g061970.2) gene was performed using Gateway cloning method (Hartley et al., 2000, Landy 1989). In Gateway cloning, a DNA fragment of interest is inserted into a donor vector by BP reaction, creating a entry clone. The DNA fragment is further transferred to a destination vector via LR reaction to create an expression clone. For cloning of selected gene SolycACL5 we used entry vector pDONR207 (Invitrogen, USA; Figure 3.1) and generation of expression clones we used pMDC32 ((Curtis and Grossniklaus 2003); Figure 3.2) as

a destination vector. Maps of selected vectors are shown in Figs 3.1 and 3.2 were designed using Snap Gene software (http://www.snapgene.com) (Adames et al., 2015)

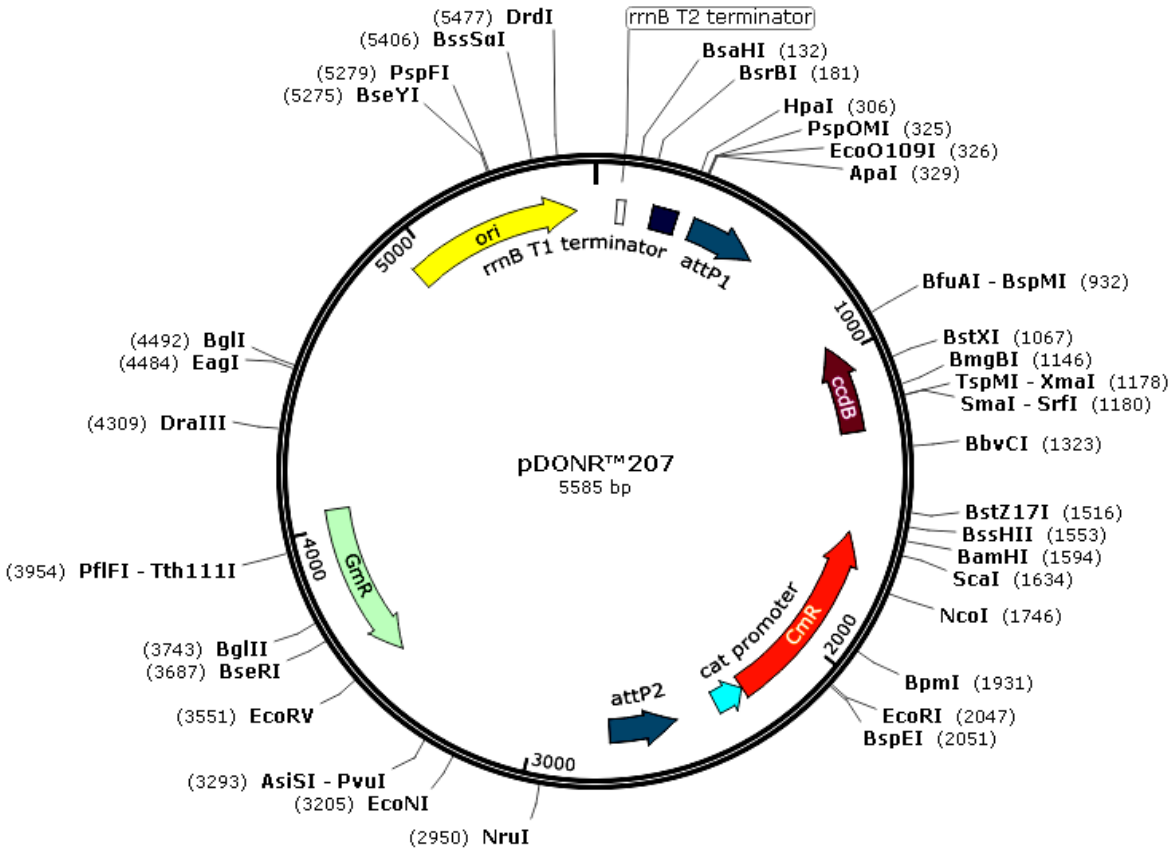


**Figure 3.1: Map of Gateway entry vector pDONR207**. i) ori; origin of replication site, ii) ccdB gene, iii) attP1 and attP2; recombination sites for the Gateway BP reaction, iv) CmR gene; confers resistance to chloramphenicol, v) GmR; a gentamycin resistance marker, vi) cat promoter, vii) rrnB T1 and rrnB T2 transcription terminators.

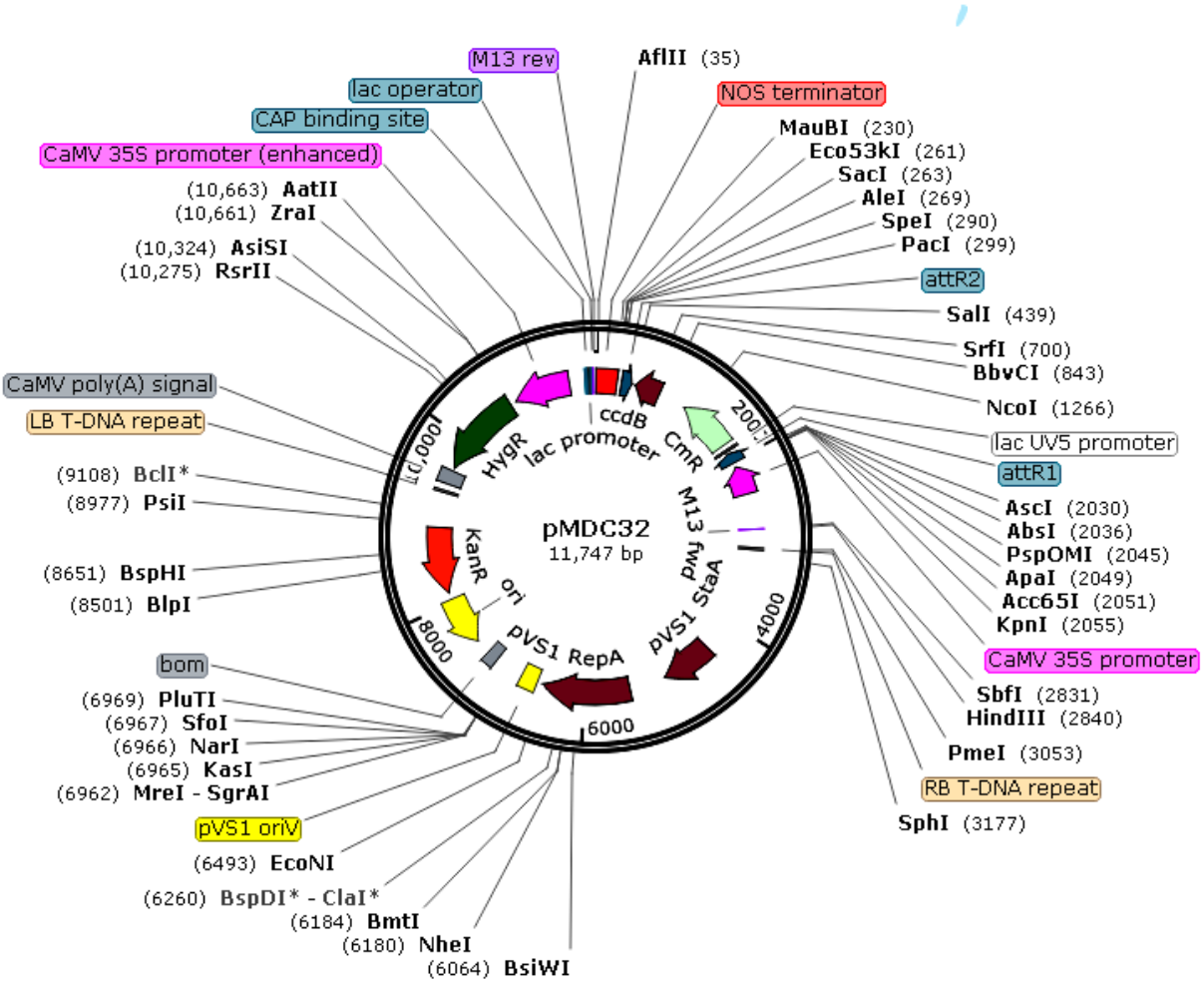


**Figure 3.2: Map of Gateway destination vector pMDC32.** i) ori; origin of replication site, ii) pVS1 oriV; origin of replication for the *Pseudomonas* plasmid pSV, iii) ccdB gene, iv) CmR gene; confers resistance to chloramphenicol, v) HygR gene; hygromycin resistance marker, vi) KanR; confers resistance to kanamycin, vii) attR1 and attR2; recombination sites for the Gateway LR reaction, viii) CaMV35S promoter; strong constitutive promoter from cauliflower mosaic virus, ix) NOS terminator; nopaline synthase terminator, x) LB and RB t-DNA repeat; left and right border from nopaline C58 T-DNA.

### 3.7.1 Gateway cloning technology

The basic principle of Gateway cloning technology (Hartley et al., 2000, Landy 1989) is based on the specific recombination that occurs between the infecting bacteriophage and the infected bacteria (*E. coli*). The specific recombination occurs between the attP site (242 bp) of the phage and attB site (25 bp) of *E. coli* in BP reaction, where the phage genome gets integrated into *E.coli* genome. The recombination reaction results in the generation of phage genome flanking at the two ends by attL (100 bp) and attR (168 bp) sites. The phage DNA gets excised from the *E. coli* genome by the recombination between the attL and attR sites during LR reaction which is a reverse recombination reaction. For the successful recombination, the reaction requires phage integrase (Int) and *E. coli* integration host factor (Ihf) called as BP clonase. And in LR reaction phage excision enzyme called excisionase (Xis) is needed in addition to Int and Ihf which are collectively known as LR clonase. The Gateway cloning method uses att sites and the Clonases for construction of recombinant DNA *in vitro*.

### 3.7.2 Cloning of artificial microRNA (amiRNA) for silencing of SolyACL5

#### 3.7.2.1 Designing of SolycACL5 specific amiRNA (amiRNA-SolycACL5)

SolycACL5 (Solyc08g061970.2) gene-specific artificial microRNA for the gene silencing was generated by using the WMD3 (Web MicroRNA Designer 3 tool; Ossowski et al., 2008). The sequence of selected gene SolycACL5 was used as the input sequence with the minimum accepted included target set at one and zero off target. From the output, the first amiRNA sequences were chosen. Then the oligonucleotide sequences were designed for the amiRNA based on the amiRNA sequences which are mentioned below.

Oligos for designing of amiRNA for silencing of SolycACL5

**I miR-s:** gaTCTTGTGTGTAAAAACCCCTAtctctctttgtattcc

**II miR-a:** TAGGGGTTTTTACACACAAGAtcaaagagaatcaatga

**III miR*s:** TAAGGGTTTTTACTCACAAGTtcacaggtcgtgatatg

**IV miR*a:** aACTTGTGAGTAAAAACCCTTAtctacatatatattcct

The four oligonucleotide sequences (I to IV) were used to engineer amiRNA into an endogenous miRNA precursor miR319a by site directed mutagenesis. Plasmid pRS300 was used as a template containing the miR319a precursor (Schwab et al., 2006) ( Figure 3.3).

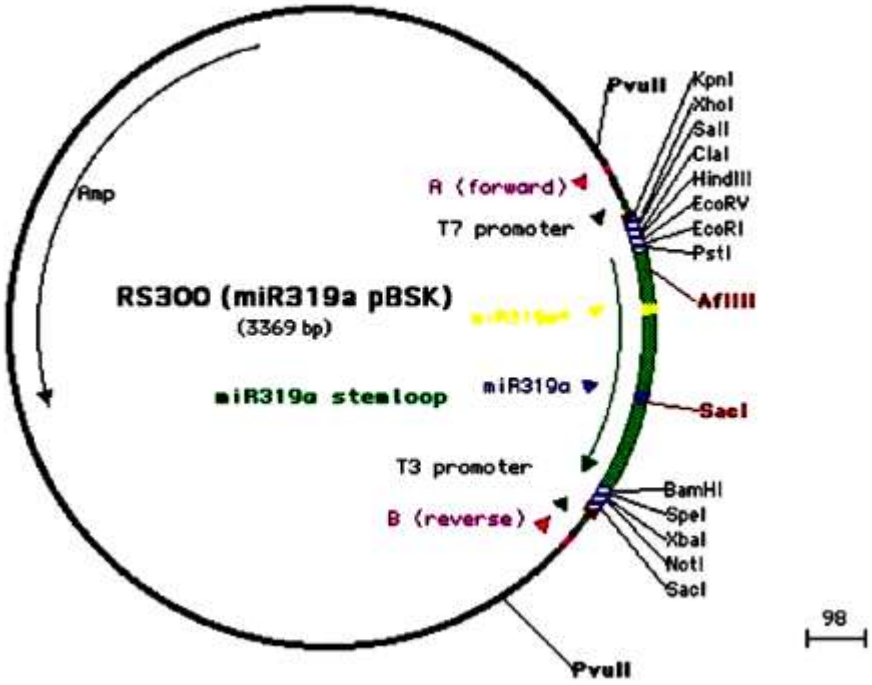


**Figure 3.3. Map of plasmid pRS300 showing the precursor miR319a and position of A and B oligonucleotides** (Schwab et al., 2006) (http://wmd3.weigelworld.org).

The amiRNA is generated by overlapping PCRs as per the protocol described previously (Schwab et al., 2006). A first round of PCR amplifies fragments (a) to (c), which are listed in the Table 3.2. Reaction mixture of first PCR contained 5µl of 10xPCR buffer, 5 µl of dNTPs, 2 µl each of primers A and B, 2 µl of pRS300 plasmid DNA and 0.5µl of high fidelity Phusion DNA polymerase (ThermoFisher, USA) in a total volume of 50 µl. These are subsequently fused in PCR (d). Oligonucleotides A and B are based on the template plasmid sequence (Table 3.3). Cloning strategy is shown in Figure 3.4.

The separate reaction mixture for the respective oligonucleotides (a), (b), and (c) was transferred in separate microcentrifuge tubes. PCR amplification was performed at 98 °C for 1 min followed by 25 cycles of 98 °C for 15 sec, 55 °C for 30 sec, 72 °C for 30 sec and 5 min final extension at 72 °C. The PCR products generated were separated by 1% agarose gel electrophoresis.

PCR purification and GEL elution was carried out first for the three PCR products a, b, and c. The DNA fragments were eluted as per the instruction is given by the manufacturer, QIAquick Gel Extraction Kit (Qiagen, USA). The sliced gels were dissolved by adding QG buffer 3 times the weight of the gel (100 mg equivalent to 100 µl). The gels were then incubated at 50 °C for 10 min and vortexed slightly after every 3 min interval to completely dissolve the sliced gel. The yellow color was observed after incubation, indicating that the gel was completely dissolved. Then isopropanol was added to the samples and transferred to the QIAquick column. The flow-through was discarded and any traces of agarose remaining were removed by the addition of 0.5 ml QC buffer. The collected samples were washed by the addition of PE buffer. Additional centrifugation was done for 1 min after discarding the flow through. The QIAquick column containing the DNA was placed in a sterile 1.5 ml Eppendorf tube and 30 µl EB buffer was added to elute the DNA. The eluted purified DNA were run on 1% agarose gel by loading 1:5 volume of loading dye to the purified DNA. The concentration of three respective fragments was determined by using the nanodrop spectrophotometer (lmplen GmbH, Germany).

The three amplified fragments, namely (a), (b), and (c) was fused by performing next PCR d. Reaction mixture consisted of 5 µl 10x buffer, 5 µl dNTPs, 2 µl each of primer A and B, 0.5 µl each of product a, b and c, 0.5 µl of Phusion DNA polymerase in total reaction of 50 µl. The PCR was set at 98 °C for 1 min followed

by 25 cycles of 98 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min and 5 min final extension at 72 °C. Then the PCR product d was eluted in 20 µl elution buffer as previously and validated by running 1% agarose gel electrophoresis. Finally, the PCR product (d) was cloned into entry vector pDONR207 through BP recombination reaction to generate amiRNA-SolyACL5-pDONR207 entry clone.

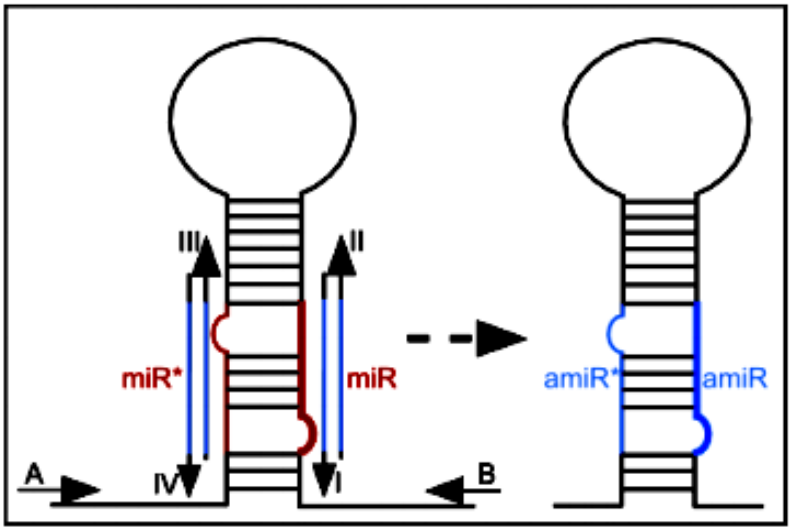


**Figure 3.4: Cloning strategy of amiRNA** (Schwab et al., 2006). I: microRNA forward; II: microRNA reverse; III: microRNA*forward; IV: microRNA* reverse.

**Table 3.2: PCR strategy for generating amiRNA-SolyACL5** (Schwab et al., 2006).

| | forward oligo | reverse oligo | template |
|---|---|---|---|
| (a) | A | IV | pRS300 |
| (b) | III | II | pRS300 |
| (c) | I | B | pRS300 |
| (d) | A | B | (a)+(b)+(c) |

#### 3.7.2.2 BP recombination reaction

To construct entry clone amiRNA-SolyACL5-pDONR207, the PCR product (d) was used for amiRNA-SolyACL5 cloning into entry vector pDONR207 through BP reaction using BP Clonase II Enzyme mix (Invitrogen, USA). The BP reaction mixture contained 3 µl of PCR product (d), 1 µl of entry vector pDONR207 and 1 µl of BP Clonase II enzyme in a total volume of 5 µl. The reaction mixture was mixed and incubated at 25 °C overnight. Proteinase K (0.5 µl) was added and incubated at 37 °C for 5 min to terminate the reaction. The entry clone amiRNA-SolyACL5-pDONR207 was used for subsequent transformation into *E.coli* and then cloning into destination vector pMDC32 (Curtis and Grossniklaus 2003).

#### 3.7.2.3 *E. coli* transformation

To select the recombinant entry clone *E.coli* strain TOP10 cells (Invitrogen, USA) were used for transformation. TOP10 competent cells (50 µl) mixed with 2 µl of the BP reaction was chilled on ice for 30 min. Heat shock was given by keeping the reaction mixture in a water bath at 42 °C for 2 min followed by chilling on ice for 5 min. To it, 700 µl of LB broth was added immediately and incubated at 37 °C for 1 hour agitating at 200 rpm. The cells were collected by spinning at 3000 rpm for 3 min, the pellet was resuspended in sterile LB broth, and the bacterial suspension was spread on gentamycin 10 mg/ml selection plate. The selection plate was incubated overnight at 37 °C. Some transformed colonies from each respective construct were streaked on a new plate containing the same selection media and were incubated overnight. Colony PCR was carried out for selection of transformed colonies. Primers attL1 and attL2 were used to validate the above construct and annealing temperature was set at 55 °C. Primer sequence listed in Table 3.3.

**Table 3.3: Primers used for PCR**

| Gene | Primer Sequence |
|---|---|
| attL1<br>attL2 | Forward- CTGGCAGTTCCCTACTCTCG<br>Reverse- TGTAACATCAGAGATTTTGAGACAC |
| attB1<br>attB2 | Forward- GGGGACAAGTTTGTACAAAAAAGCAGGCT<br>Reverse- GGGGACCACTTTGTACAAGAAAGCTGGGT |
| Primer A<br>Primer B | Forward- CTGCAAGGCGATTAAGTTGGGTAAC<br>Reverse- GCGGATAACAATTTCACACAGGAAACAG |
| SolycACL5-attB1<br><br>SolyACL5-attB2 | Forward-<br>GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGGGTAGTGAAGCTTTG<br>Reverse-<br>GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATTTCTAAATCCCAA |
| 35S promoter<br>NosT | Forward- GTAAGGGATGACGCACAATCC<br>Reverse- GGACTCTAATCATAAAAACCC |

### 3.7.2.4 Plasmid isolation

The positively transformed pDONR 207 plasmid carrying amiRNA-SolyACL5 gene construct were isolated using QIA prep Spin Miniprep kit (Qiagen, USA) as per the manufacturer instructions. One of the positively transformed colonies grown on the gentamycin selection media was picked and inoculated in 15 ml falcon tube containing 5 ml of sterile LB broth and 10 mg/L gentamycin. The cell suspension culture was incubated at 37 °C with agitation at 150 rpm overnight. The cells at log phase were harvested by centrifugation at 5000 rpm for 3 min. The pellet so collected was resuspended in 250 µl of P1 buffer and mixed thoroughly by pipetting. The same volume of P2 (lysis) buffer was added to the reaction mixture and mixed by inverting 4-6 times. Then the reaction mixture was centrifuged at 13000 rpm for 1 min. The supernatant was collected and loaded on Qiagen prep spin column. Centrifugation was done at 13000 rpm for 1 min. and flow through was discarded. 500 µl of PB buffer was added to the column and centrifuged same as above. Pellet so collected was washed by adding 750 µl PE buffer and again centrifuged as previously. Finally, the pellet was washed again to remove any residues. The spin column was placed in a fresh 1.5 µl

microcentrifuge tube and elution was done in 30 µl EB (elution buffer) and spin down at 13000 rpm for 1 min after standing for 1 min. Isolated entry clone was validated by PCR with the respective primers and also sequenced (Eurofins Genomics, India).

#### 3.7.2.5 LR recombination reaction

The entry clone amiRNA-SolyACL5-pDONR207 with L1 and L2 sites flanking the amiRNA-SolyACL5 gene construct were recombined with the destination vector pMDC32 having R1 and R2 sites using LR Clonase II Enzyme mix (Invitrogen, USA). Reaction mixture contained 1.5 µl of entry clone, 1 µl of destination vector pMDC32, 1 µl of LR Clonase II enzyme, and TE buffer was added to a final volume of 5 µl. Further, transformation in to *E.coli* Top 10 cells as methods mentioned in section 3.7.2.3. But, selection for the expression clone was carried out on Kanamycin 50 mg/l selection plate and confirmed by PCR using both primer pair attB1 and attB2 and primers A and B (Table 3.3). The PCR validated expression clone was further transformed to *Agrobacterium tumefaciens* strain GV3101pMP90G$^{R}$.

#### 3.7.2.6 *Agrobacterium* transformation

The amiRNA-SolycACL5 expression clone generated after LR reaction was transformed to A. *tumefaciens* strain GV3101 using electroporation method. *A. tumefaciens* stain GV3101 used is in C58 background also contain rifampicin resistant gene RIF in its nuclear DNA to facilitate selection. This strain also contains nopaline type Ti plasmid pmP90G$^{R}$. This helper plasmid has virulence genes (vir genes) and gentamycin resistance marker for efficient transgenic approach (Koncz et al 1994).

50 µl of *Agrobacterium* competent cells were thawed on ice in 1.5 µl micro centrifuge tubes. To it 0.5 µl of expression clone was added and incubated on ice for 30 min. Then a mixture of cells and vector carrying amiRNA-SolycACL5 gene construct were transferred to ice cold electroporation cuvette (Biorad) with tapping to avoid air bubbles. Electroporation was performed using Bio-Rad Gene Pulser apparatus at 25 mF with 2.5 Kv and pulse resistance to $200^{1/2}$ for 5-7 seconds. Immediately 750 µl LB broth was added to the cuvette and the cells were transferred to the new 1.5 µl microcentrifuge tube. Further, the tube was incubated at 28 °C for 3-4 hours in an orbital shaker. The cells were collected by spinning

down at 3000 rpm for 10 min. Pellet so obtained was spread on YEP selection media containing kanamycin 50 mg/l and rifampicin 30 mg/l and plate was incubated for 2-3 days at 28 °C for selection of transformed *Agrobacterium* colonies. Genotyping of all the transformed colonies was performed by PCR using primers A and B. Sequences of primers used is listed in Table 3.2.

**3.7.2.7 *Agrobacterium*-mediated genetic transformation of tomato**

*Agrobacterium* mediated genetic transformation of tomato was performed using method described earlier with some modifications (Park et al., 2003). Tomato seeds were sterilized by rinsing with 70% ethanol for 1 min followed by treating with 0.8% sodium hypochlorite for 20-30 min. Then the seeds were washed three times with autoclaved water. The sterilized seeds were grown on ½ MS medium supplemented with 10 g/l Sucrose and 1% plant agar and kept in dark for 3-5 days followed by their growth in culture room at 16 h light and 8 h dark. For *Agrobacterium* transformation, 7-10 dag old seedlings were used before the development of first pair of true leaves. Cotyledons were excised in MS + 3% Sucrose liquid medium. These explants were inoculated on MS medium supplemented with 3% Sucrose, 2 mg/l Zeatin and 20 mg/l acetosyringone. These explants were first incubated in dark for two days under tissue culture growth conditions. At the same time, *Agrobacterium* cells carrying amiRNA-SolycACL5 gene construct was grown in 5 ml of YEP medium containing kanamycin (50 mg/l) and rifampicin (30 mg/l). Next day the starter culture was again inoculated in YEP medium without antibiotics till the bacterial growth reaches OD 0.3 - 0.5 at 600 nm. Then the agrobacterial suspension was centrifuged at 3000 g for 10 min. The pellet is then resuspended in equal volume of MS liquid medium containing 30 g/l sucrose and further, it is diluted to 10 to 20 times depending on the OD of agrobacterial cells. To it, acetosyringone of 20 mg/l concentration is added and mixed. Then the bacterial suspension was poured over the preincubated explants. After 30 min the suspension was removed from the plates and incubated for 2 days in the dark. After 2 days of cocultivation the explants were transferred to fresh MS supplemented with 2 mg/l zeatin, 25 mg/l hygromycin, 250 mg/l carbenicillin and 250 mg/l cefotaxime. Then the explants were subcultured every 15 days. After regeneration of shoot buds with 2-3 small leaves the explants were transferred to shoot elongation medium containing MS + 3% Sucrose + 0.1 mg/l Zeatin + 0.04 mg/l IAA + 25 mg/l hygromycin +250 mg/l carbenicillin + 250 mg/l cefotaxime.

After the development of 2-4 cm long shoots, shoots were excised and transferred to root induction medium containing MS + 3% Sucrose + 0.1 mg/l IAA + 25 mg/l hygromycin + 250 mg/l carbenicillin + 250 mg/l cefotaxime. The pH of all media used was adjusted to 5.8.

### 3.7.3 Cloning of SolycACL5 gene for their overexpression in plants

#### 3.7.3.1 PCR amplification of SolycACL5 coding sequence for generation of attB1-SolycACL5-attB2 clone

The Gateway cloning method as described in section 3.7.1 was used for generation of overexpresser construct of SolycACL5 gene. PCR product attB1-SolycACL5-attB2 was generated from cDNA synthesized from tomato seedling using gene specific primers (5'-ATGGGTAGTGAAGCTTTG-3') and (5'-TCAATTTCTAAATCCCAA-3'), each containing respectively attB1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAT-3′) and attB2 (5′-GG GGACCACTTTGTACAAGAAAGCTGGGTC-3′) recombination sequences as adaptor sites at the 5' end. PCR reaction mixture was consisted of 2 µl of 5X phusion HF buffer, 0.2 µl of 10 mM dNTPs, 0.1 µl Phusion DNA polymerase, 50 ng of template cDNA, 0.1 µl each of gene specific primers SolycACL5-attB1 and SolyACL5-attB2 in a volume of 10 µl. PCR amplification was performed at 98 °C for 1 min followed by 35 cycles of 98 °C for 15 sec, 58 °C for 1 min, 72 °C for 2 min and 5 min final extension at 72 °C. The PCR product was electrophoresed on 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide and visualized under UV transillumination. The final PCR product attB1-SolycACL5-attB2 obtained containing attB1 and attB2 sites used for further cloning.

#### 3.7.3.2 BP Reaction

Gene coding sequence of SolycACL5 gene flanked by attB sites and pDONR207 vector were used for making entry clone, SolycACL5-pDONR207. The BP reaction was carried out as mentioned in section 3.7.2.2 and the entry clone generated were further transformed in to *E. coli* Top10 cells and plasmid purification were done as mentioned previously in sections 3.7.2.3 and 3.7.2.4, respectively.

#### 3.7.3.3 LR Reaction

SolyACL5-pDONR207 entry clone with L1 and L2 sites was recombined with the destination vector pMDC32 carrying R1 and R2 sites as mentioned above in

section 3.7.2.5. The further procedure was performed as in sections 3.7.2.3 and 3.7.2.4 for transformation. Selection of transformed colonies was done on 50 mg/l kanamycin. The SolyACL5 expression clone was further used for transforming *Agrobacterium* cells. Transformation of *Agrobacterium* cells was performed as in section 3.7.2.6.

**3.7.3.4 Genetic transformation of SolycACL5 overexpresser construct in tobacco**

Tobacco *Nicotiana tabacum* (cultivar Colombia) plants were transformed with SolycACL5 overexpresser construct **(**35S::SolycACL5) using method described previously with some modifications (Horsch et al., 1985)**.** A single colony of *Agrobacterium* carrying 35S::SolycACL5 gene construct was cultured in liquid YEP medium supplemented with 50 mg/l kanamycin and 50 mg/l rifampicin. Leaves of 4-8 weeks old *in vitro* cultured tobacco plants were used as explant for genetic transformation. Healthy and fully expanded leaves were taken and cut to about 1.0 cm square pieces. Leaf pieces were transferred into 50 ml falcon tube containing liquid regeneration medium (RM) containing MS medium + 3% sucrose + 2 mg/l BA +0.2 mg/l NAA. Agrobacteria suspension culture grown was centrifuged at 3000 g for 2 min to collect the cells. The pellet was resuspended in liquid RM and diluted to an OD of 0.5 to 0.8 at 600nm. Then the agrobacterial suspension was poured over the explants and incubated at 28 °C with shaking at 100 rpm for 30 min. The bacterial suspension was then poured off and explants were incubated at 25 °C in dark for 4 days for cocultivation. Following cocultivation, the explants were transferred to liquid RM medium supplemented with 300 mg/l timentin for 3 min and rinsed with liquid RM twice for 3 min. The explants were then blotted dry on sterile filter paper and transferred to the selection medium RM + 250 mg/l timentin + 25 mg/l hygromycin with the abaxial surface in contact with the medium. Explants were then cultured at 25°C in dark for 2 weeks followed by transfer to 16 h light and 8 h dark photoperiod. Then the explants were subcultured to fresh selection media at 2-3 weeks interval. After the regeneration of shoots, 2-4 cm long isolated shoots were transferred to rooting media containing MS medium supplemented with 3% sucrose, 25 mg/l hygromycin and 250 mg/l timentin.

## 3.8 Molecular and biochemical characterization of SolycACL5 overexpressing (35S::SolycACL5 OE) tobacco lines

### 3.8.1 Genotyping of putative SolycACL5 overexpressing tobacco lines

Genomic DNA of the putative overexpresser (OE) lines was extracted and integration of 35S::SolycACL5 gene construct was confirmed by PCR using gene specific primers SolycACL5-attB1 and SolycACL5-attB2, and other primers were specific for CaMV 35S promoter and NosT (Table 3.2). Each PCR reaction mixture was consisted of 5µl 2x EmraldAMP GT PCR Master mix, 0.1µl of each primer and 100ng DNA in a total volume of 10µl. PCR amplification was performed at 98°C for 1min followed by 35 cycles of 98 °C for 10 sec, 55 °C for 30 sec, 72 °C for 1.5 min (for SolycACL5) and 2 min for (CaMV 35S and NosT) and 5 min final extension at 72 °C. The PCR products were electrophoresed on 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide and visualized under UV transillumination.

### 3.8.2 Expression analysis of SolycACL5 overexpressing tobacco lines by semi-quantitative RT-PCR

Expression of SolycACL5 in different OE lines was analyzed by semi-quantitative RT PCR. RNA isolation and cDNA were synthesized as in section 3.5.2 and 3.5.3, respectively. PCR was performed using gene-specific primers and cDNAs synthesized as DNA templates. NtEF-1α (Nitab4.5_0000022g0030.1) was used as reference gene. Primers used are listed in Table 3.1.

### 3.8.3 Quantitative RT-PCR expression analysis of 35::SolycACL5 overexpressing tobacco lines

For detail expression analysis of SolycACL5 overexpressing tobacco lines, cDNA synthesized in section 3.8.3 were used and qRT-PCR was performed as in section 3.5.6. Quantitative RT-PCR was performed using gene-specific primers listed in Table 3.1.

### 3.8.4 Detection of Hydrogen peroxide ($H_2O_2$) accumulation

*In situ* accumulation of $H_2O_2$ in the leaves of control and overexpresser lines was detected by histochemical staining with 3,3'-diaminobenzidine (DAB) (Šnyrychová et al., 2009). In addition, $H_2O_2$ measurement in the leaf discs of

control and overexpressor lines was performed spectrophotometrically as method described previously (Junglee et al., 2014).

### 3.8.5 Antioxidant enzymes assays

Antioxidant enzymes activity such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GP) was measured from young, mature and senescent leaf samples of control and selected overexpresser lines.

#### 3.8.5.1 Enzyme extraction

For enzyme activity, leaf tissues (100 mg) were homogenized with extraction buffer containing 2.5 ml of 0.05 M phosphate buffer containing 1% PVP (polyvinyl pyrrolidone) and centrifuged at 15000 g for 30 minutes at 4 °C. The supernatant was collected and used for enzyme assays.

#### 3.8.5.2 Catalase assay (CAT)

CAT activity was determined by monitoring the decrease in $A_{240}$ at room temperature by spectrophotometry as mentioned previously with some modifications (Shin et al., 2012). The reaction mixture contained 1.9 ml of 0.05 M phosphate buffer and 0.1 ml of enzyme extract. The reaction was initiated by adding freshly prepared 1 ml of $H_2O_2$ (39 mM). Utilization of $H_2O_2$ at the interval of 30 seconds for 3 min was recorded by measuring the decrease in absorbance at 240 nm. One unit of CAT activity was measured as µmoles of $H_2O_2$ decomposed per min per mg protein. The extinction coefficient of $H_2O_2$ is 0.0394 $mM^{-1}cm^{-1}$.

#### 3.8.5.3 Superoxide dismutase assay (SOD)

SOD activity was determined as described previously (Marklund and Marklund 1974). The reaction mixture contained 1.5 ml of 0.1 M Tris HCl buffer, 0.6 mM EDTA and 1 ml of 6 mM freshly prepared pyrogallol. The reaction was initiated by adding 0.1 ml of enzyme extract. Change in absorbance at 420 nm at 30 seconds intervals was measured for 3 min. Blank was prepared without enzyme. A unit of SOD activity was measured as the amount of enzyme causing 50% inhibition of auto-oxidation of pyrogallol observed in the blank.

#### 3.8.5.4 Ascorbate peroxidase assay (APX)

APX activity was measured as per described previously (Nakano and Asada 1981) with some modifications. The reaction mixture contained 1 ml of 0.05 M phosphate buffer, 0.8 ml of 0.5 mM ascorbic acid and 0.2 ml of enzyme extract.

The reaction was initiated by adding 1ml of freshly prepared $H_2O_2$ solution (39 mM). The decrease in absorbance was measured at the 30-second interval for 3 min at 265 nm. One unit of APX activity is expressed as nmoles of monodehydroascorbate formed per min per mg. The extinction coefficient of monodehydroascorbate has a value of 2.8 $mM^{-1}cm^{-1}$.

#### 3.8.5.5 Guaiacol peroxidase assay (GP)

GP activity was measured as mentioned previously (Egley et al., 1983). The reaction mixture contained 3 ml of 0.01 M potassium phosphate buffer, 0.05 ml, 20 mM guaiacol and 0.1 ml enzyme extract. The reaction was initiated by adding 0.03 ml 12.3 mM freshly prepared $H_2O_2$. Change in absorbance was recorded. One unit of activity was measured as the rate of formation of dehydrogenated guaiacol per min per mg. Extinction coefficient used was 6.39 $mM^{-1}cm^{-1}$. Blank was run without $H_2O_2$.

### 3.8.6 Estimation of total chlorophyll

Chlorophyll content of both control and overexpresser plants was measured by using the procedure as described previously (Arnon 1949). Briefly, 500 mg of leaf tissue from the plants was homogenized in 2 ml of 80% (w/v) cold acetone and the homogenate was centrifuged at 3500g for 5 min. The supernatant was retained and the absorbance was recorded at 663 nm and 646 nm with only 80% (w/v) cold acetone as blank. The absorbance of the blank was subtracted from the absorbance of each sample.

Chlorophyll content was calculated using following formula:

$$\text{Total Chlorophyll } (\mu g/g \text{ FW}) = 0.0202\ A_{663} + 0.00802\ A_{645}$$

Where $A_{663}$ and $A_{645}$ are the absorbance at 663nm and 645nm, respectively.

### 3.8.7 Anthocyanin measurement

Anthocyanin content was measured as described in the previous protocol (Rabino and Mancinelli 1986). 200 mg of leaf tissues of both wild-type and overexpresser plants was crushed in 1 ml of methanol (95 ml): HCl (5 ml) mixture in a mortar pestle. Reaction mixture volume was made up to 5 ml and incubated overnight. Absorbance was measured at 657 nm.

Anthocyanin was measured using the formula:

$$\text{Anthocyanin content} = 0.25 * A_{657} * \text{extraction volume} * 1/\text{weight of tissue}$$

Where $A_{657}$ is the absorbance at 657 nm.

### 3.8.8 Malondialdehyde content

Malondialdehyde (MDA) content was measured according to the method described previously (Shin et al., 2012). 500 mg of leaves were homogenized in 0.5 ml of 0.1% (w/v) trichloroacetate (TCA) and centrifuged at 10,000 g for 10 min. Thereafter, 0.5 ml of the supernatant was mixed with 0.5 % (w/v) thiobarbituric acid (TBA). The mixture was incubated at 95° C for 30 min and quickly cooled on ice and centrifuged at 10,000 g for 5 min. The absorbance of the supernatant was recorded at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm.

MDA content was calculated as below:

$$\text{MDA Content (nmoles/gm fresh weight)} = A_{532} - A_{600} \times V \times 1000/155 \times W$$

Where V and W are the volume of extract and fresh weight of sample respectively and $A_{532}$ and $A_{600}$ is an absorbance at 532 nm and 600 nm, respectively. Extinction coefficient used was 155 $mM^{-1}cm^{-1}$.

### 3.8.9 Expression analysis of senescence marker genes

To analyze the expression pattern of senescence marker genes, we have selected two senescence-related genes namely Stay green 1 (SGR1) and Senescence-associated gene12 (SAG12). Expression pattern of both the genes was analyzed in both control and selected overexpresser lines using qRT-PCR. Samples were taken from mature and senescent leaves and RNA was isolated as in section 3.5.2 and cDNA was synthesized as in section 3.5.3. NtAPT1 was taken as a reference gene to normalize the expression. Sequences of primers are listed in Table 3.1.

### 3.8.10 Expression analysis of chloroplast associated marker genes

Expression pattern of chloroplast associated genes such as rbcL, petB, and rpoA was analyzed in both control and selected overexpresser lines using qRT-PCR. RNA was isolated and cDNA was synthesized from the leaf samples as described previously. NtAPT1 was used as internal control. Sequences of primers used are listed in Table 3.1.

### 3.8.11 Quantification of chloroplast DNA (cpDNA)

To analyze chloroplast DNA copy number, genomic DNA from mature leaf tissues of both control and overexpresser lines was extracted using CTAB method (Minas et al., 2011). For cpDNA copy number analysis, genes specific to chloproplast

such as rbcL, petB, and rpoA were used. qRT- PCR analysis was done using 100 ng of DNA as template and the steps performed were as follows: step (1) 95 $^{o}$C, 5 sec; step (2) 55 $^{o}$C, 30 sec (3) 72 $^{o}$C, 20 sec x 40 cycles. SolycAPT1 was used as reference gene and calculations were done by $2^{-}\Delta\Delta Ct$ methods (Livak and Schmittgen 2001). Primers sequences are listed in Table 3.1.

### 3.9 Statistical analysis

All experiments were repeated three times and subjected to statistical analysis. The statistical analysis was performed by unpaired t-test and one-way ANOVA. Data analysis and graphical representation were carried out using GraphPad Prism7 software (www.graphpad.com).