MATERIALS AND METHODS

3.1 Identification of metacaspase gene family in tomato genome

Protein sequences of *A. thaliana* metacaspase genes acquired from TAIR were used to identify *Solanum lycopersicum* L. (tomato) metacaspase genes (https://www.arabidopsis.org/) (Berardini et al., 2015). Known protein sequences of *Arabidopsis* metacaspases were BLAST searched in SOL Genomics Network (SGN; https://solgenomics.net/) against the tomato genome {Fernandez-Pozo et al., 2015; The tomato genome consortium, 2012}.

3.1.1 Gene structure analysis and protein properties

Gene Infinity tool was used to forecast the theoretical molecular weight (Mw) and isoelectric point (pI) of identified candidate proteins. (http://www.geneinfinity.org/sms/sms proteiniep.html). The genomic sequences of putative metacaspase genes were found using the SGN database's gene search. (https://solgenomics.net/search/locus) and their exon-intron structures were identified using Gene Structure Display Server tool (GSDS; Hu et al. 2015). The SGN database was used to find the distribution of these putative genes on tomato chromosomes.

3.1.2 Multiple sequence alignment and Phylogenetic analysis

Peptide and nucleotide sequences of *Arabidopsis* and tomato metacaspase were downloaded from TAIR (https://www.arabidopsis.org/) and SGN (https://solgenomics.net/) database, respectively. ClustalW was used to create multiple sequence alignments of tomato candidate proteins and *Arabidopsis* proteins. (www.clustal.org/) (Larkin et al., 2007). The phylogenetic tree was generated using MEGAX (Kumar et al., 2018).

3.2 Analysis of Cis-regulatory elements in promoter sequences of metacaspases

The tomato SGN database was used to obtain 1 kb genomic sequences upstream of each candidate metacaspase gene's initiation codon (https://solgenomics.net). The putative cis-regulatory elements were identified using PlantCARE tool (Lescot et al., 2002). TB tool was used for visual representation of the data (Chen et al., 2020).

3.3 Plant materials and gene expression analyses

The seeds of *Solanum lycopersicum* L. (cultivar 'MicroTom') were surface sterilized and germinated in tissue culture boxes on 1/2 strength Murashige and Skoog (MS) medium

(Murashige and Skoog, 1962) supplemented with 1% sucrose and 1% plant agar, and incubated in a culture room at 25±1°C under 16h light and 8h dark and at 70±10% relative humidity. For gene expression analysis, root (Rt), hypocotyl (hy), and cotyledon (ct) samples were taken from 15 days after germination (dag) old seedlings and different vegetative and reproductive tissue and organ samples from 60 dag old mature plants. Various stages of leaf such as immature leaf (IL), young leaf (YL), mature leaf (ML) and senescing leaf (SL), phloem tissue from stem (Ph), xylem tissue from stem (Xy), abscission zone (AZ) of flower pedicel, eight stages of developing flowers {flower bud 1 (Fl1), flower bud 2 (Fl2); flower bud 3 (Fl3), immature flower 1 (Fl4), Immature flower 2 (Fl5), Immature flower 3 (Fl6); Mature flower (Fl7); Senescent flower (Fl8)}and eight different stages developing fruits {Immature fruit 1 (Fr1), Immature fruit 2 (Fr2), Immature fruit 3 (Fr3), Mature green fruit (Fr4), Mature breaker fruit (Fr5), Mature turning fruit (Fr6), Mature pink fruit (Fr7), Mature red fruit (Fr8)} were collected for isolation of RNA samples.

3.4 RNA isolation

RNAqueous total RNA isolation kit (Ambion, USA) was used to isolate total RNA from various tomato plant tissues following the method used was as mentioned in the manufacturar's protocol. The method used was as For RNA isolation, 0.1 g of plant tissue was isolated and stored in liquid nitrogen. samples were grinded with micropestles in microcentrifuge tubes in liquid nitrogen. In each tube, 8 volumes of lysis/ binding buffer were added, followed by 1 volume of plant RNA isolation Aid (Ambion, USA). Micropestle was then again used to homogenize the plant tissues in the respective tubes. To remove insoluble plant debris, the tubes were spin at 15,000g. The supernatants were re-circulated in fresh tubes. Then, in each tube, equal volume of 70% ethanol was added and gently mixed. The solution was centrifuged at 15,000g for 1 min. The collection tubes were reused and flowthrough discarded. Wash solution 1 was added to the column and centrifuged for 1 min until the wash solution passed through. The collection tube was reused for successive washings. 500 µl of Wash solution 2/3 was added and centrifuged again. The column was centrifuged for 30 seconds at 15,000g. Finally, by adding 50 µl of pre-heated elution buffer, the RNA was eluted.

3.5 Removal of DNA contaminant from RNA samples

DNA contamination was removed from RNA samples using the TURBO DNA-free TM Kit (Ambion, USA) by performed as follows; In each RNA samples, 0.1 volume of 10x DNase buffer and 1 ul of DNase I enzyme and the samples were incubated for 30 min at 37 °C for

DNA digestion. After that, DNase inactivation reagent (0.1 volume) was added and reaction was terminated. The samples were gently mixed and incubated for 2 minutes at room temperature. After spinning the samples at 10,000 x g for 2 min at room temperature, the supernatant was collected in fresh microcentrifuge tubes. Using agarose gel electrophoresis, the integrity of total RNA was determined. Then, using a Implen Nanophotometer, quality of RNA samples was measured.

3.6 First Strand cDNA synthesis

The first strand of cDNA was synthesised from RNA samples using a reverse transcription reaction with the PrimeScriptTM 1st strand cDNA synthesis kit (Takara, Japan) according to the manufacturer's instructions. 0.5 µg of RNA template was taken for synthesis of cDNA.

3.7 Gene expression analysis using semi-quantitative PCR

Expression of the candidate metacaspase genes was analyzed by semi-quantitative RT-PCR using metacaspase primers and cDNA templates synthesized from different plant samples as described in section 3.6. The semi-quantitative RT-PCR reaction was carried out using the Veriti thermal cycler (Applied Biosystems, USA). SolycAPT1 was used as a reference gene. Each reaction was performed in 10 µl volume and consist of EmeraldAmp® GT PCR Master Mix (Takara, Japan), 10µmol of each primer, 1µl cDNA template and sterile dH_2O. The PCR steps used were: 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 30-35 cycles. Metacaspase-specific and control gene specific primers were designed by primer3 tool (Untergasser et al., 2012). The sequence of primers are showed in table 4.

3.8 Expression analysis using Quantitative RT-PCR (qRT-PCR)

The cDNAs generated as shown in material and methods were used for the quantitative RT-PCR expression analysis. The PCR reaction was performed on ABI QuantStudio 12K Flex qPCR detection system using the SYBR® premix Ex TaqTM (Tli RNaseH Plus; Takara, Japan) kit, which contains SYBR premix Ex Taq and ROX reference dye (Applied Biosystems, USA). 1x Takara SYBR Green master mix, ROX, 10 mol of each primer, 1µl cDNA template, and sterile H2O were used in each reaction, which had a total volume of 10 µl. The following were the qRT-PCR steps that were carried out: Step 1: 95°C for 15 seconds; step 2: 55°C for 30 seconds; 40-45 cycles and step 3: 72°C for 30 seconds. Gene transcript specific primers were designed using vector NTI.

3.9 Cloning of promoter and gene silencing constructs

3.9.1 Cloning of Promoter of SolycMC4

Cloning of SolycMC4 (Solyc09g098150.2.1) 870 bp upstream promoter region was performed using Gateway cloning method (Karimi et al., 2007; Invitrogen, USA). Promoter fragments of 870 bp upstream of the initiation codon of SolycMC4 were obtained by PCR using Gateway-compatible primers (table 5). The PCR amplified promoter fragments were cloned into the pDONR207 vector (Invitrogen, Abingdon, UK; http://www.invitrogen.com) and subsequently, expression clone was generated using destination vector pMDC163 (Curtis and Grossniklaus, 2003; figure 4). Maps of this vector is shown in figure 4 and designed using Snap Gene (v 4.2) software (http://www.snapgene.com).

3.9.2 Cloning of amiRNA-SolycMC4

3.9.2.1 Designing of SolycMC4 specific amiRNA (amiRNA-SolycMC4)

Using the WMD3 (Web MicroRNA Designer V3) tool, a SolycMC4 (Solyc09g098150.2.1) gene-specific artificial microRNA (amiRNA) for gene silencing was designed (Schwab et al., 2006). The input sequence was the sequence of the selected gene SolycMC4, with the minimum acceptable included target set to one and zero off target. The first amiRNA sequences was selected from the output. primers sequences for the cloning of amiRNA were then designed based on the amiRNA sequence 5'-TAGCTACATCCTTTTGTGTAT-3'.

Primers for cloning of amiRNA-SolycMC4 for silencing of SolycMC4 were:

I miR-s: GATTACACAAAAGGATGTAGCTTTCTCTCTTTTGTATTCC

II miR-a: GAAAGCTACATCCTTTTGTGTAATCAAAGAGAATCAATGA

III miR*s: GAAAACTACATCCTTATGTGTATTCACAGGTCGTGATATG

IV miR*a: GAATACACATAAGGATGTAGTTTTCTACATATATATTCCT

By site directed mutagenesis, the four primers sequences (I to IV) were used to generate amiRNA into an endogenous miRNA precursor miR319a. The miR319a precursor sequence, which is a part of plasmid pRS300 and employed as a template. (figure 5).

3.9.2.2 Overlaping PCR for amiRNA-SolycMC4 construct generation

The amiRNA-SolycMC4 is generated by overlapping PCRs as per the protocol described previously (Schwab et al., 2006). In first round of PCR, amplicons a, b and c were generated

and their amplification strategy is shown in the table 6. PCR consist 5µl of 10x PCR buffer, 5µl of dNTPs, 2ul each of primers A and B, 2µl of pRS300 plasmid and 0.5µl of phusion DNA polymerase (ThermoFischer, USA). These DNA fragments a, b and c were finally fused in amplicon (d). Primer A and B were used which based on plasmid pRS300 (table 5); (figure 6). PCR was performed at 98°C for 1min followed by 35 cycles of 98°C for 15 sec, 55°C for 30 sec, 72°C for 30 sec and 7 min final extension at 72°C. PCR products a, b, and c, were gel eluted according to the manufacturer's instructions, QIAquick Gel Extraction Kit (Qiagen, USA). The concentration of three eluted fragments (a, b, and c) was determined by using the Nano photometer (lmplen GmbH, Germany).

For next d-PCR, Reaction mixture composed of 5µl 10x buffer, 5µl dNTPs, 2µl each of primer A and B, 0.5µl each of amplicon a, b and c and 0.5µl of Phusion DNA polymerase in total reaction of 50µl. The PCR 98°C for 1 min followed by 35 cycles of 98°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and 7 min for final extension at 72°C. Then the final PCR product (d) was also gel eluted as method used earlier. Finally, the PCR amplicon (d) which contained the amiRNA-SolycMC4 cloned in entry vector pDONR207 through BP reaction.

3.9.3 BP and LR reaction

For promSolycMC4, PCR product of 870 bp was cloned into pDONR207 using BP reaction, using BP Clonase II Enzyme mix to construct entry clone promSolyMC4-pDONR207 (Invitrogen, USA). The BP reaction consists of 3µl of PCR product (d), 1µl of vector pDONR207 and 1µl of BP Clonase II in a total volume of 5µl. The reaction mixture was incubated at 25°C for 1 h after that Proteinase K (0.5 µl) was added and reaction was terminated. The entry clone promSolycMC4-pDONR207 was transformed into *E. coli* strain "Top 10" and then cloned into expression vector pMDC163 to construct promSolycMC4-pMDC163 clone by LR reaction (Invitrogen, USA). Reaction mixture contained 1.5µl of entry clone, 1µl of destination vector pMDC163, 1µl of LR Clonase II enzyme, and TE buffer was added to a final volume of 5µl and incubated overnight at 25 °C. The reaction was terminated by adding Proteinase K (0.5 µl). Further, LR reaction was transformed in to *E. coli* Top 10 cells. The selection for the expression clone was carried out on LB medium supplemented with 50mg/l Kanamycin and confirmed by PCR using gene specific primers (Table 5). The PCR validated clones was finally transformed to *Agrobacterium tumefaciens* strain GV3101pMP90G^{R}.

For amiRNA-SolycMC4, the PCR product (d) was utilized to clone amiRNA-SolyccMC4 into entry vector pDONR207 using BP reaction to construct entry clone amiRNA-SolyMC4-pDONR207 (Invitrogen, USA). The BP reaction used was 3µl of PCR product (d), 1µl of pDONR207 and 1µl of BP Clonase II enzyme in a total volume of 5µl. The reaction mixture was incubated at 25°C for 1 h. The entry clone amiRNA-SolycMC4-pDONR207 was subsequent transformed into *E. coli* strain "TOP10" and subsequently cloned into destination vector pMDC32 to construct 35S::amiRNA-SolycMC4-pMDC32 clone (Curtis & Grossniklaus, 2003). The entry clone amiRNA-SolyMC4-pDONR207 was recombined with pMDC32 by LR reaction (Invitrogen, USA). Further, LR reaction was transformed in to *E. coli* TOP10 cells as methods described in section 3.9.4. The selection for the expression clone was carried out on LB medium supplemented with 50mg/l Kanamycin and confirmed by PCR using primers A and B (Table 5). The PCR genotyped clones were finally transformed to *Agrobacterium tumefaciens* strain GV3101pMP90G^{R}.

3.9.4 *E. coli* transformation

E. coli strain TOP10 cells (Invitrogen, USA) were transformed to select the both recombinant entry and expression clones. Tubes containing the TOP10 competent cells (50 µl) were kept on ice for 30 min with 2 µl of the BP or LR reactions. Heat shock was done by incubating the tubes at 42°C for 2 min before cooling it for 5 min on ice. 700 µl of sterile LB media was added, and incubated for 1 h at 37°C with 200 rpm agitation. The cells were collected by centrifuging for 3 min at 3000 rpm, then resuspending the pellet in sterile LB broth and spreading the bacterial suspension on a selection plate supplemented with 10 mg/ml gentamycin for entry clones and 50 mg/l kanamycin for expression clones. The selection plates were incubated at 37°C overnight. Few transformed colonies from each gene constructs were plated on antibiotic selection for overnight growth. Colony PCR was performed for genotyping of positive transformed colonies. The various gene specific and vectors specific primers were used to verify the above constructs by genotyping PCRs. Details of primers are mentioned in table 5.

3.9.5 Plasmid Isolation

The SolycMC4 promoter and amiRNA-SolycMC4 constructs carrying plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen, USA) according to the manufacturer's manuals. Purified entry clones and expression clones carrying the SolycMC4 promoter::GUS and

amiRNA-SolycMC4 constructs were validated by PCR also sequenced (Eurofins Genomics, India).

3.9.6 *Agrobacterium* transformation

For PromSolycMC4, electroporation method was used to transform the promSolycMC4 expression clone obtained following the LR reaction into *Agrobacterium tumefaciens* strain GV3101 (helper plasmid $pMP90^{GR}$). Electrocompetent agrobacterium cells were thawed and 0.5µl of expression clone was added and incubated on ice for 30 min. After that, mixture of cells were transferred to electroporation cuvette (BioRad). Electroporation was done using Bio-Rad Gene Pulser apparatus at 25mF with 2.5KV and pulse resistance to $200^{1/2}$ for 5 to 7 sec. Immediately, 750 µl LB media was added and the cells were transferred to 1.5 µl microcentrifuge tube. Then, the tube was incubated at 28°C for 3 h. The cells were centrifuged at 3000 rpm for 5 min. Pellet was spread on YEP plate supplemented with kanamycin 50mg/l and rifampicin 30mg/l and plate was incubated for 3 days at 28°C for selection of transformed colonies.

For amiRNA-SolycMC4 also, electroporation method was used to transform the amiRNA-SolycMC4 expression clone obtained following the LR reaction into *Agrobacterium tumefaciens* strain GV3101 (helper plasmid $pMP90^{GR}$).

3.9.7 Agrobacterium-mediated genetic transformation of tomato

For SolycMC4 promoter lines, tomato seeds (cv. Sangenta) were rinsed in 70% ethanol for 1 minute before being surface sterilized for 20-30 minutes with 0.8 percent sodium hypochlorite. The seeds were then washed with sterile distilled water. The sterilized seeds were grown in the dark for 3-5 days on 1/2 strength MS medium supplemented with 10g/l Sucrose and 1% plant agar before being grown in a culture room at 22 °C with a 16:8 h light: dark photoperiod. Agrobacterium-mediated genetic transformation was performed on seedlings that were 7-10 days old and had their first pair of leaves. Explants of the cotyledon and hypocotyl were isolated in liquid MS medium supplemented with 3% sucrose, dry blotted with sterile filter paper, and transferred to MS media supplemented with 3% Sucrose, 2 mg/l BA, and 20 mg/l acetosyringone in culture plates and pre-incubated in the dark for two days. Agrobacterium cells expressing the promSolycMC4 expression clone were first cultivated in 5 ml of YEP medium containing Kanamycin (50mg/l) and Rifampicin (30mg/l) overnight at 28 °C to prepare them for co-cultivation. On the next day, the overnight developed starter culture was incubated in YEP medium without antibiotics on an orbital shaker until bacterial growth

reached an OD of 0.3 to 0.5 at 600 nm. After that, the Agrobacterial cells were spined down at 3000g for 10 min. The pellet is then resuspended in an equal volume of MS liquid medium containing 30 gm of sucrose per litre and 20 mg of acetosyringone per litre, and diluted 10 to 20 times, depending on the OD of the Agrobacterial cells. After that, the Agrobacterial cells were poured on the pre-incubated explants. After 30 min, the agrobacterium cells were removed from the plates and explants were incubated in the dark for two days. Explants were cleaned with sterile water, dry blotted and transferred to fresh MS medium supplemented with 2 mg/l BA, 25 mg/l hygromycin, 250 mg/l timentin after two days of co-cultivation. For shoot bud regeneration, the explants were then sub cultured to fresh medium every 15 days. Explants were switched to shoot elongation medium comprising MS medium supplemented with 3 percent sucrose, 0.1 mg/l BA, 0.04 mg/l IAA, 25 mg/l hygromycin, 250 mg/l timentin after regeneration of early shoot buds with 2-3 little leaves. 2-4 cm in length of shoots were isolated and transferred to root induction medium containing MS medium supplemented with 3% sucrose, 0.1 mg/l IAA, 25 mg/l hygromycin and 250 mg/l timentin. The pH of all used media was adjusted to 5.8.

For SolycMC4 silencing lines, tomato seeds (cv. MicroTom) were rinsed for 1 min in 70% ethanol and then surface sterilized with 0.8 percent sodium hypochlorite for 30 min. The seeds were then rinsed with sterile water three times. Sterilized seeds were grown on 1/2 strength MS medium supplemented with 10g/l sucrose and 1% plant agar in the dark for 3-5 days before being cultured in a growth chamber at 22 °C temperature and 16::8 h of light and dark photoperiod. About 7-10 days old germinated seedlings with first pair of leaves were selected for Agrobacterium mediated genetic transformation. Cotyledon and hypocotyl explants were isolated in liquid MS medium supplemented with 3% Sucrose and dry blotted with sterile filter paper and transferred to MS media supplemented with 3% sucrose, 2 mg/l zeatin, and 20 mg/l acetosyringone in culture plates and pre-incubated in the dark for two days. To prepare the Agrobacterium cells for co-cultivation, Agrobacterium cells carrying the 35S::amiRNA-SolycMC4 expression clone were first cultured in 5 ml of YEP medium containing Kanamycin (50mg/l) and Rifampicin (30mg/l) overnight at 28 °C. Next day, overnight grown starter culture was inoculated in YEP medium without antibiotics on orbital shaker until the bacterial growth reached an OD of 0.3 to 0.5 at 600 nm. The Agrobacterial suspension was then centrifuged for 10 min at 3000g. The pellet is then resuspended in an equal volume of MS liquid medium containing 30g/l sucrose and 20 mg/l acetosyringone and diluted 10 to 20 times, depending on the OD of the Agrobacterial cells. The Agrobacterial suspension was then poured over the pre-

incubated explants. The suspension was withdrawn from the plates after 30 min and incubated in the dark for two days. After two days of co-cultivation, explants were washed with sterile water, dry blotted and transferred to MS media contained 2 mg/l zeatin, 25 mg/l hygromycin, 250 mg/l carbenicillin, and 250 mg/l cefotaxime. Explants were then subcultured periodically to fresh medium every 15 days for shoot bud regeneration. After that young shoot buds were moved to shoot elongation medium containing MS medium supplemented with 3% Sucrose, 0.1 mg/l Zeatin, 0.04 mg/l IAA, 25 mg/l hygromycin, 250 mg/l carbenicillin and 250 mg/l cefotaxime. About 2-3 cm long shoots were taken and transferred to rooting medium containing MS medium supplemented with 3% sucrose, 0.1 mg/l IAA, 25 mg/l hygromycin, 250 mg/l carbenicillin and 250 mg/l cefotaxime. The pH of all used media was adjusted to 5.8.

3.10 Genotyping of SolycMC4 promoter::GUS and 35s::amiRNA-SolycMC4

Genomic DNA of the putative transformed SolycMC4 promoter::GUS and 35s::amiRNA-SolycMC4 were extracted and PCR verified for integration of respective gene constructs in plants. 35s::amiRNA-SolycMC4 lines were verified by primer A and B, whereas SolycMC4 promoter: GUS lines were PCR genotyped by SolycMC4promoter-attB1 and SolycMC4promoter-attB2 primers. Other pairs of primers used for genotyping of all these lines were specific for CaMV 35S promoter and NosT. For SolycMC4 promoter::GUS lines, PCR reaction mixture consisted of 5µl 2x PrimeSTAR GXL polymerase 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 5 sec, 55°C for 10 sec, 68°C for 30 sec. For 35s::amiRNA-SolycMC4, PCR reaction mixture 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. The PCR products were run on agarose gel and visualized using UVP Gel-documentation-system (UVP, LLC).

3.11 Expression analysis using PromSolycMC4::GUS lines

Tomato SolycMC4 promoter::GUS lines were screened on MS media containing 25 mg/l hygromycin and PCR genotyped. Leaves, other plant parts and stem sections of tomato SolycMC4promoter::GUS lines were immersed and incubated overnight in the GUS staining solution (100 mM sodium phosphate, pH 7.0 and 1 mM 5-bromo-4-chloro-3-indolyl b-D-glucuronic acid) at 37˚C for GUS staining. Tissue samples were cleared in 70% ethanol solution and mounted in 70% glycerol solution on glass slides. Samples were observed under

a Zeiss discovery V.12 stereo microscope and Zeiss axioplan 2 microscope and images were captured using axiocam camera.

3.12 Expression analysis of SolycMC4 silencing lines

Expression of SolycMC4 in amiRNA-SolycMC4 lines were analyzed by semi-quantitative RT-PCR. Total RNA isolation and cDNA were synthesized as in section 3.6 and 3.8, respectively. PCR was performed using metacaspase-specific primers and cDNAs synthesized were used as templates. Primers SolycAPT1, SolycUBQ5 and SolycEF1α were taken as internal control. Details of primers used are listed in table 4. For expression analysis of amiRNA-SolycMC4 lines, cDNA synthesized in section 3.6 were used as templates and qRT-PCR was performed as in section 3.8. qRT-PCR was done using gene-specific primers listed in table 4.

3.13 Phenotypic analysis and plant growth measurments

To determine the plant length, stem diameter, internode and leaf length, 30 days old *in vitro* grown 10 plantlets from each of control and amiRNA-SolycMC4 lines were selected. The measurements were taken in cm and mm.

3.14 Anatomical analysis

The plant samples from both control and amiRNA-SolycMC4 lines were prepared and sectioned as methods previously described (Singh et al., 2005). Stem and Leaf sections (50–80 um) were prepared using a Leica Microtome, stained with 0.05% Toluidine blue O (SRL) solution for 1–2 min, rinsed and mounted on glass slides in 50% glycerol solution, covered with thin coverslips and observed in an Axioplan 2 microscope (Zeiss). Images were taken using an Axiocam camera (Zeiss).

3.15 Detection of Hydrogen peroxide (H_2O_2) accumulation

In situ accumulation of H_2O_2 in the leaves of amiRNA-SolycMC4 lines and control was analysed by histochemical method with 3,3'-diaminobenzidine (DAB) (Snyrychová et al. 2009). In addition, spectrophotometric measurements of H_2O_2 in the leaf discs of control, amiRNA-SolycMC4 was performed using earlier described method (Junglee et al., 2014).

3.16 Antioxidant enzymes assays

Antioxidant enzymes activity of Catalase, Superoxide dismutase, Ascorbate peroxidase and Guaiacol peroxidase was measured in samples prepared from young, mature and senescent leaves of amicRNA-SolycMC4 lines and the control.

3.16.1 Enzyme extraction

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

3.16.2 Catalase (CAT) assay

CAT activity was evaluated by spectrophotometry to track the decline in A240 at room temperature, as previously reported, with some adjustments (Hadwan, 2018). A total of 1.9 ml of 0.05 M phosphate buffer and 0.1 ml of enzyme extract were used in the procedure. The reaction was started by adding 1 ml of fresh H_2O_2 solution (39 mM). The decrease in absorbance at 240 nm was used to record the use of H_2O_2 at 30 sec intervals for 3 min. The amount of H_2O_2 decomposed per min per mg protein was used to define one unit of CAT activity. H_2O_2 has an extinction coefficient of 0.0394 m/M/cm.

3.16.3 Superoxide dismutase (SOD) assay

The activity of SOD was measured as previously described (Porra, 2002). 1.5 ml of 0.1 M Tris HCl buffer, 0.6 mM EDTA, and 1 ml of 6 mM freshly prepared pyrogallol made up the reaction mixture. 0.1 ml enzyme extract was used to start the process. For 3 min, change in absorbance at 420 nm at 30 second intervals. Blank was prepared without the use of enzymes. A unit of SOD activity was defined as the amount of enzyme that inhibited the auto-oxidation of pyrogallol by 50% when compared to the blank samples.

3.16.4 Ascorbate peroxidase (APX) assay

With some modification, APX activity was monitored as previously described (Nakano & Asada, 1981). 1 ml phosphate buffer, 0.8 ml 0.5 mM ascorbic acid, and 0.2 ml enzyme extract made up the reaction mixture. 1 ml of fresh H_2O_2 solution was used to start the reaction (39 mM). At 265 nm, the decrease in absorbance was measured every 30 sec for 3 min. The activity of APX is measured in nmoles of monodehydroascorbate produced in min^{-1} mg^{-1}. Monodehydroascorbate has a value of 2.8 m/M/cm for its extinction coefficient.

3.16.5 Guaiacol peroxidase assay

As previously described, GP activity was measured (Egley et al., 1983). 3 ml 0.01 M potassium phosphate buffer, 0.05 ml 20 mM guaiacol, and 0.1 ml enzyme extract made up the reaction

mixture. The reaction was started with 0.03 ml fresh 12.3 mM H_2O_2 solution and change in absorbance was recorded. The rate of production of dehydrogenated guaiacol per min per mg was used to determine one unit of activity. The Extinction coefficient was 6.39 m/M/cm. Blank sample was used without the addition of H_2O_2 solution.

3.16.6 Estimation of total chlorophyll

The contents of chlorophyll in both amiRNA-SolycMC4 lines and control was determined by the procedure previously described (Porra, 2002). 500 mg of leaf tissue was homogenised in 2 ml of 80 % (w/v) cold acetone and centrifuged at 3500g for 5 min. The supernatant was kept and absorbance measurements were taken at 663 and 646 nm and 80 % (w/v) cold acetone was used as a blank. Each sample's absorbance was subtracted from the absorbance of the blank.

Chlorophyll content was calculated using following formula:

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

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

3.16.7 Anthocyanin Measurement

The anthocyanin content was measured as per the method described previously (Rabino & Mancinelli, 1986). 200 mg of leaf tissues from amiRNA-SolycMC4 lines and control were crushed using a mortar pestle in 1 ml of methanol (95 ml): HCl (5 ml) mixture. Up to 5 ml of reaction mixture was prepared and incubated overnight. At 657 nm, the absorbance was measured.

Anthocyanin was measured using the formula:

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

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

3.17 Expression analysis of Senescence marker genes

Two well-known senescence maker genes, stay green 1 (SGR1) and senescence-associated gene 12 (SAG12), were used to examine their expression patterns by qRT-PCR in amiRNA-SolycMC4 lines and control plants. Total RNA was extracted from mature and senescent leaves of these plants as described in section 3.4, and cDNA was synthesized as described in section 3.6. To normalize the expression analysis, SolycAPT1 was used as control. The primer sequences are listed in table 4.

3.18 Expression analysis of Programmed Cell death (PCD) marker genes

For the qRT-PCR expression analysis of PCD marker genes, Total RNA from leaf tissues of plants was isolated using RNAqueous Total RNA Isolation Kit (Ambion) and cDNA synthesized by reverse transcription reaction using PrimeScript^{TM} 1st strand cDNA synthesis kit (Takara) as per manufacturer's recommendations. qRT-PCR was done using gene transcripts specific primers and analyses was done by relative expression and comparative 2_ΔΔCt methods (Livak & Schmittgen, 2001). SolycUBQ5, SolycEF1a and SolycAPT1 were used as internal control to normalize the expression.

3.19 Expression analysis of Chloroplast associated marker genes

qRT-PCR was used to examine the expression of chloroplast marker genes, RBCL, PETB, and RPOA in both control and amiRNA-SolycMC4 lines. As described previously, RNA was extracted from leaf samples and cDNA was prepared. Reference gene SolycAPT1 was used to normalize the expression. Primer sequences used are mentioned in table 4.

3.20 Mitochondrial Complex-I activity

Mitochondrial complex-I activity was determined in amiRNA-SolycMC4 lines and control using modified method (Oliveira et al., 2013). 200 g leaf tissue was isolated and immediately ground in liquid nitrogen to powder using a mortar pestle and directly homogenized in 0.5 ml 10 mM Tris buffer at 4˚C. Samples were passed to the freeze-thaw cycle 3 times and centrifuged at 3000 rpm for 5 min at 4˚C. After removing the supernatant reaction mixture was prepared. Assay mixture contains 100 mM potassium phosphate buffer (pH 7.5), 1mg/ml BSA, 0.1 M Na-azide, 0.1 M NADH and deacyl ubiquinone. After incubation for 3 min activity was measured spectrophotometrically at 340 nm.

3.21 Subcellular localization of SolycMC4::GFP fusion protein

Subcellular localization prediction of SolycMC4 was carried out using *in silico* prediction tools, such as Plant-mPloc (Chou & Shen, 2010) and wolf-pSORT (Horton et al., 2007). For cloning of SolycMC4::GFP fusion construct, the coding sequence of SolycMC4 gene was amplified using cDNA as template and gene-specific primers. The amplified product was subcloned into a pDONR207 vector (Invitrogen, http://www.invitrogen.com) subsequently cloned into destination vector pGWB5 (Nakagawa et al., 2007) and placed the SolycMC4::GFP cassette is under the transcription control of 35S CaMV promoter. This fusion construct was transiently expressed into tomato protoplasts to localize the SolycMC4::GFP fused protein in subcellular compartment.

The isolation of tomato protoplasts was performed as previously described (Miao & Jiang, 2007). Protoplasts were isolated from the cell suspension culture of tomato originated from leaf callus of cv "MicroTom". Suspension culture was incubated with 50 ml enzyme solution at 25°C for 5 h with gentle agitation. After incubation, the protoplast suspension was filtered through a mesh, overlaid on a 30% sucrose solution, and centrifuged to harvest protoplasts. The protoplasts were washed with W5 solution and finally suspended in W5 solution at a density of 1×10^6 protoplasts/ml. 35S::SolycMC4::GFP-fusion construct (≈100-150 µg) was transformed into protoplasts using a PEG-mediated method. To allow expression of the fusion construct, protoplasts were incubated in the dark at 22°C for 24 h (Mathur & Koncz, 1998). The cells were observed in Zeiss LSM 710 confocal microscope (Carl Zeiss, Inc., Germany). Images were taken by Zen Black software.

3.22 Statistical analysis

Data were subjected to statistical analysis for comparison using software for Student's t-Test. Differences between samples were considered significant if the probability of the difference arising from random sampling errors was <0.05, expressed as *P < 0.05. The Tukey's Honest Significant Difference (HSD) test was used for multiple comparisons of means. Statistical significance was set at $\alpha = 0.05$. Biochemical assays were performed in triplicates (n = 3). All statistical analyses were performed using Graph pad prism 8.0 and relative expression analysis was analysed as previously described method (Livak & Schmittgen, 2001).

Table 4: Gene specific primers used for semi-quantitative RT-PCR and qRT-PCR analysis

Gene	Gene ID/ Accession No.	Primer Sequence
SolycMC4	Solyc09g098150.2.1	Sense: GCCTTCTCGAGTAGCTGTTGA Antisense: TCACATGATGGAGCATGGTT
SolycMC9	Solyc10g081300.2.1	Sense: TGGTTGCAACTATGAAAATACACC Antisense: GGCATAATTGGACTCCCTGA
SolycMC1	Solyc01g088710.2.1	Sense: AGGCCTCTTCCTTACGGAGT Antisense: TTTCCAGACACCAGAACGGG
SolycMC1-L1	Solyc03g094160.2.1	Sense: TCTGCCATTCGCAGTTCTGA Antisense: TGCTGTCAATTGTGGCTCCT
SolycMC1-L2	Solyc05g052130.2.1	Sense: CGCTAAGCTTCACGCCATCA Antisense: GCCTCTCCTCCACTTGTTCC
SolycMC3	Solyc01g105300.2.1	Sense: TAAGGCCTTTACCCCCTGGA Antisense: TCGCATCCGATGATCTTCCC
SolycMC3-L1	Solyc01g105310.2.1	Sense: TGAAACTCAGGAGCCCCAAC Antisense: CTCGGGCTGGAGTTGGATAC
SolycMC3-L2	Solyc01g105310.2.1	Sense: ACCACTGCTTCCAGGTGTTAC Antisense: TTAGCACCAGATGGGGGTCT
SolycUBQ5	Solyc12g098940.2.1	Sense: TAAGCTCGCTGTCCTCCAGT Antisense: GGTTAGCCATGAAGGTTCCA
SolycEF1α	Solyc06g009970.2.1	Sense: TTGAGGCTCTCGACCAGATT Antisense: GTCAAACCAGTAGGGCCAAA
SolycAPT1	Solyc08g079020.2.1	Sense: AGTTGCCGGAGCTAGAAACA Antisense: ACGTGGATCTTGACCTTTGC
SolycAPX	Solyc06g005160.2.1	Sense: CTTCACGGAGCTTTTGAGTGG Antisense: ATTTCTCAACAAGGGGACGGA
SolycSOD	Solyc01g067740.2.1	Sense: GAGCTGTTGTTGTTCATGCTGA

		Antisense: CCCTGGAGGCCAATAATACCA
SolycCAT	Solyc04g082460.2.1	Sense: TCGGATGATAAGCTTCTCCAAACT Antisense: TGAGCACACTTGGGAGCATT
SolycPXD	Solyc05g046000.2.1	Sense: TGGCAAAAAGGAGAGGGCTT Antisense: GGTCGATCCATGAGACATAGCT
SolycATG8	Solyc02g080590.2.1	Sense: TTCTCTGATGTCTGCAATTTATGAGG Antisense: TTCCAAGCTCAACGAACCCA
SolycATG5	Solyc10g076580.1.1	Sense: GTTGACAATAGTGGAATCAACTCCA Antisense: TCATCCACCCTTTCGCCAAA
SolycTOR	Solyc01g106770.2.1	Sense: AGGCCTTTGTTCATGACCCA Antisense: TCACAACAGGAGGCACATGT
SolycTBN1	Solyc02g078910.2.1	Sense: GCCGGTGAAACTTTATCAGATGA Antisense: TGTTGAGAAGCTCCAAAAACGT
SolycPIRIN	Solyc09g098160.2.1	Sense: AGGTTTGTTCTGATAGGAGGGC Antisense: TCTTGATAAGCCTGCATAATCTCAC
SolycSBT3	Solyc01g087850.2.1	Sense: TGTGGAGTTCTTGGGAGCTT Antisense: AGGTTTGTTCTGATAGGAGGGC
SolycRCR3	Solyc09g020110.2.1	Sense: TGAAGCTGGATATGTACCATCAGA Antisense: TCGCCTGAGCATACCAACTC
SolycPR1a	Solyc01g106620.2.1	Sense: GTGCTAGGGTCAGGTGCAAC Antisense: TCAAAAGCCGGTTGATTTTCAAGA
SolycMLO1	Solyc04g049090.2.1	Sense: AGCAGACCAACCACACCATT Antisense: CTGCAGGATCTCTCCCTCCT

SolycSAG12	Solyc02g076910.3.1	Sense: GCTACGGAAGGGCTACACCAAC Antisense: TCATCCTCTCCTTCGACATCACAGT
SolycSGR1	Solyc12g056480.3.1	Sense: AGCATCTTCACTATCTTCCACCA Antisense: CCCTTGACATGGCTGTGGTA
SolycRBCL	Solyc00g500063.1.1	Sense: ACAGAGACTAAAGCAAGTGT Antisense: GGAGTTACTCGGAATGCTG
SolycPETB	Solyc00g500073.1.1	Sense: AATAGGATCACCTTTGGTCG Antisense: AGTAAGAAGCGGCAATACAA
SolycND1	Solyc00g500142.1.1	Sense: TATGTTAAGTCTGGTCGCTC Antisense: GCGAAGATATGGCAAACAAA

Table 5: Primers used for cloning of various gene constructs

Gene	Primer Sequence
amiRNA-SolycMC4	I miR-s: GATTACACAAAAGGATGTAGCTTTCTCTCTTTTGTATTCC II miR-a: GAAAGCTACATCCTTTTGTGTAATCAAAGAGAATCAATGA III miR*s: GAAAACTACATCCTTATGTGTATTCACAGGTCGTGATATG IV miR*a: GAATACACATAAGGATGTAGTTTTCTACATATATATTCCT
PromSolycMC4: GUS	Sense: GGGGACAAGTTTGTACAAAAAAGCAGGCTTACTTAACAACCAATCTTGG Antisense: GGGGACCACTTTGTACAAGAAAGCTGGGTATGTCTTCCTCAGCAAAT
35S::SolycMC4:: GFP	Sense: GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCGAAAAAAGCAGTATTAATT Antisense: GGGGACCACTTTGTACAAGAAAGCTGGGTAACAAACAAAAGGAGCATC
Primer A Primer B	Sense: CTGCAAGGCGATTAAGTTGGGTAAC Antisense: GCGGATAACAATTTCACACAGGAAACAG
Primer A attB1 Primer B attB2	Sense: GGGGACAAGTTTGTACAAAAAAGCAGGCTTACTGCAAGGCGATTAAGTT Antisense: GGGGACCACTTTGTACAAGAAAGCTGGGTAGCGGATAACAATTTCACA
35S promoter NosT	Sense: GTAAGGGATGACGCACAATCC Antisense: GGACTCTAATCATAAAAACCC

Table 6: PCR strategy for generating amiRNA-SolycMC4

PCR product	Forward oligo	Reverse oligo	Template
a	A	IV	pRS300
b	III	II	pRS300
c	1	B	pRS300
d	A	B	a + b + c

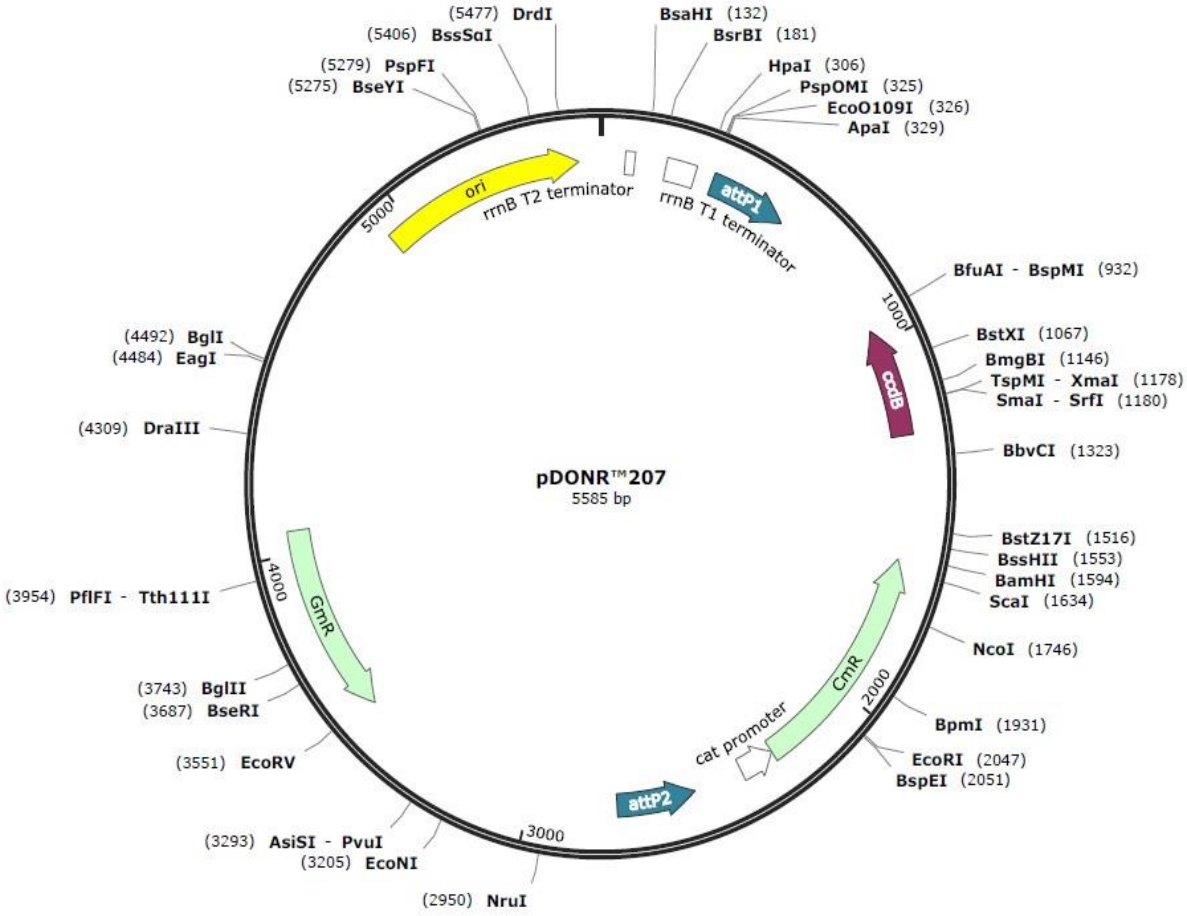

Figure 2: Map of entry vector pDONR207. ori; origin of replication site, ccdB gene, attP1 and attP2; recombination sites for BP reaction, CmR gene; confers resistance to chloramphenicol, GmR; a gentamycin resistance marker, cat promoter, rrnB T1 and rrnB T2 transcription terminators.

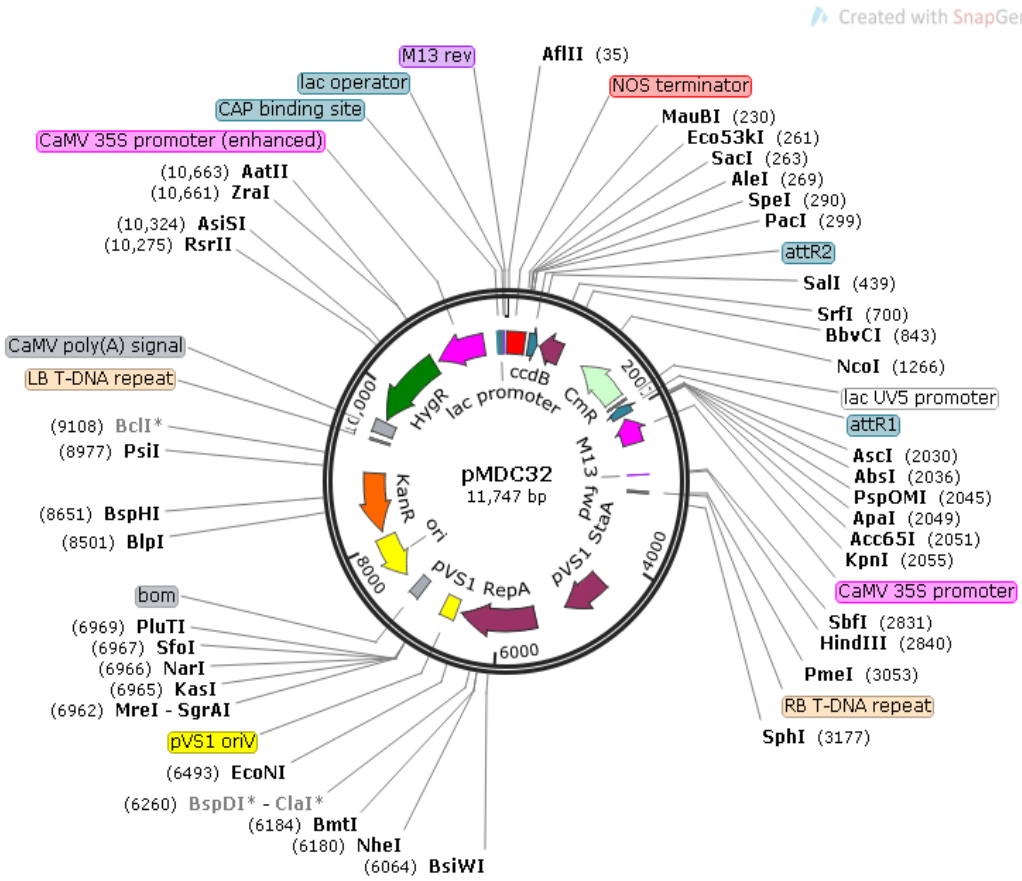

Figure 3: Map of destination vector pMDC32. ori; origin of replication site, pVS1 oriV; origin of replication for the Pseudomonas plasmid pSV, ccdB gene, CmR gene; confers resistance to chloramphenicol, HygR gene; hygromycin resistance marker, KanR; confers resistance to kanamycin, attR1 and attR2; recombination sites for LR reaction, CaMV35S promoter; strong constitutive promoter from cauliflower mosaic virus, NOS terminator; nopaline synthase terminator, LB and RB t-DNA repeat; left and right border from nopaline C58 T-DNA.

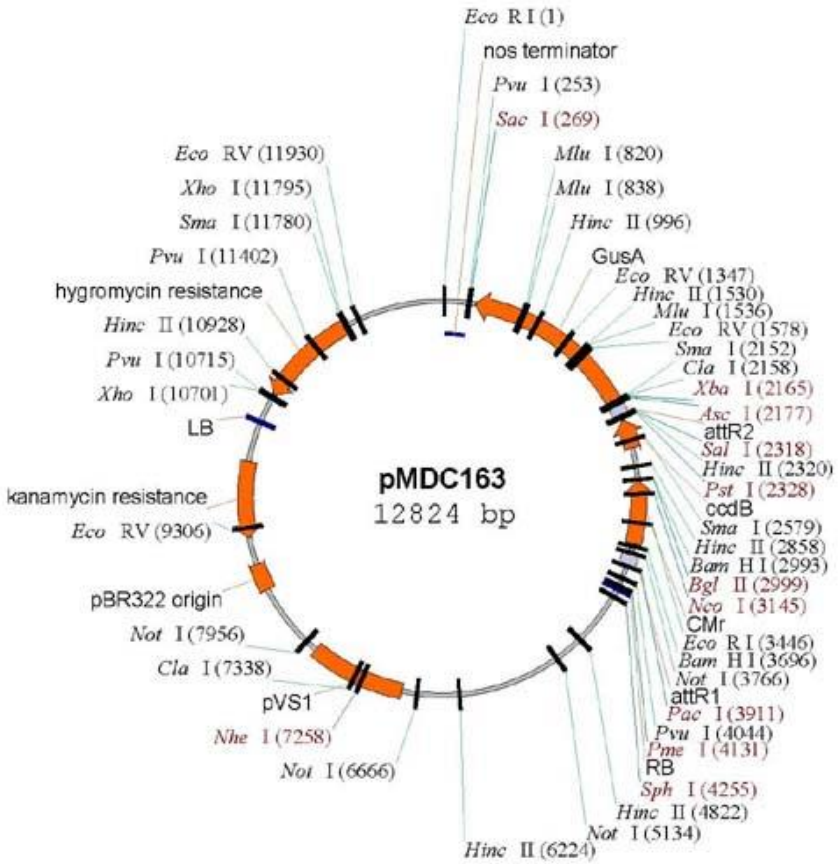

Figure 4: Map of destination vector pMDC163. ori; origin of replication site, pVS1 oriV; origin of replication for the Pseudomonas plasmid pSV, ccdB gene, CmR gene; confers resistance to chloramphenicol, HygR gene; hygromycin resistance marker, KanR; confers resistance to kanamycin, attR1 and attR2; recombination sites LR reaction, CaMV35S promoter; strong constitutive promoter from cauliflower mosaic virus, NOS terminator; nopaline synthase terminator, LB and RB t-DNA repeat; left and right border from nopaline C58 T-DNA.

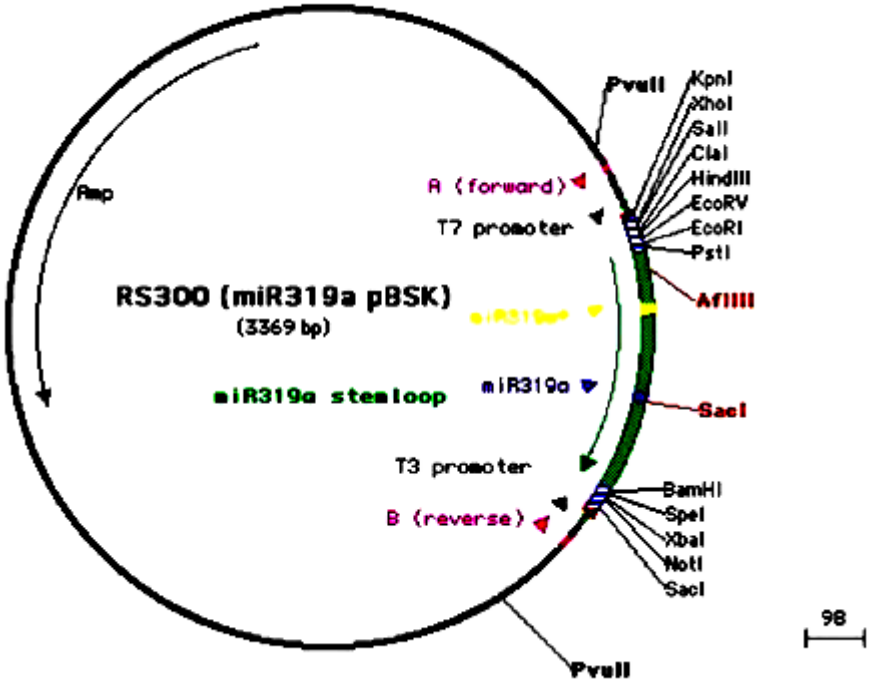

Figure 5: Map of plasmid pRS300 having precursor miR319a; site for A and B primers (http://wmd3.weigelworld.org)

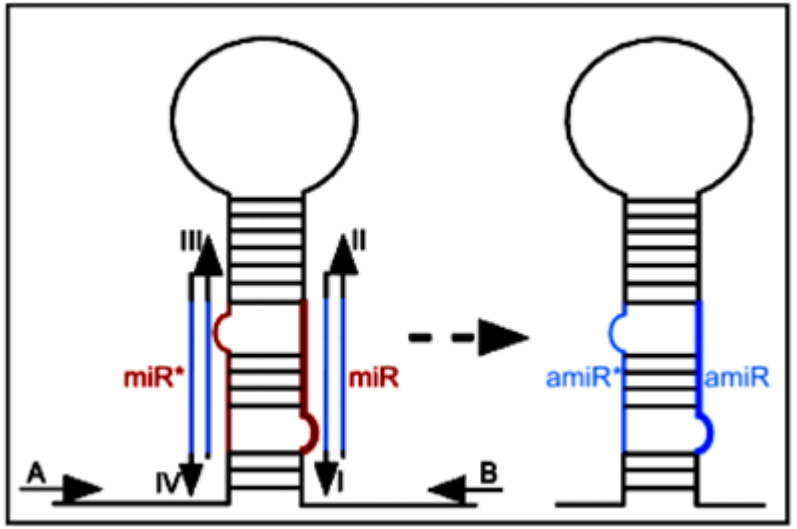

Figure 6: Cloning strategy of amiRNA (http://wmd3.weigelworld.org). I: microRNA forward; II: microRNA reverse; III: microRNA*forward; IV: microRNA* reverse.