RESULTS

4.1 Identification and In-silico analysis of tomato metacaspase

4.1.1 Identification and nomenclature of tomato metacaspases

In silico based approach was used to identify putative metacaspases (MCs) in the tomato (*Solanum lycopersicum* L.) genome. The putative metacaspases in tomato genome were identified using known gene and protein sequences of metacaspases from *A. thaliana* retrieved from TAIR (https://www.arabidopsis.org/; Berardini et al., 2015). These sequences were BLAST analyzed to the tomato genome v ITAG2.3 (https://solgenomics.net/; Goodstein et al. 2012) and a total of eight metacaspases were identified based on homology with *Arabidopsis* metacaspases. Gene ID of identified metacaspases were: Solyc01g088710.1.1, Solyc03g094160.1.1, Solyc05g052130.1.1, Solyc01g105320.1.1, Solyc01g105300.1.1, Solyc01g105310.1.1, Solyc10g081300.1.1, and Solyc09g098150.1.1. Amongst these, six of metacaspases were found to be like type I metacaspases and other two found to be similar to type II metacaspases. On the basis of sequence homology with *Arabidopsis* metacaspases, tomato metacaspases were designated as SolycMC1, SolycMC1-L1, SolycMC1-L2, SolycMC3, SolycMC3-L1 and SolycMC3-L2, SolycMC4 and SolycMC9. Interestingly, no close homologs of Arabidopsis AtMC5, AtMC6, AtMC7 and AtMC8 were identified in tomato. Thus, only two type II metacaspases, named SolycMC4 and SolycMC9, were present in tomato as compared to six type II metacaspases in Arabidopsis. Our analysis also showed that three tomato metacaspases, SolycMC3, SolycMC3-L1 and SolycMC3-L2 were tandemly duplicated. Our analysis also revealed that three tomato metacaspases, SolycMC3, SolycMC3-L1 and SolycMC3-L2 were tandem duplicated. Type I MCs from tomato found to have N-terminal zinc finger prodomain. This prodomain is absent in type II MCs. Like Arabidopsis, all the tomato metacaspases also contain a predicted caspase-like His-Cys proteolytic dyad domain. The length of coding sequences of this gene family ranged from 1548 bp (SolycMC3) to 6510 bp (SolycMC1). The encoded metacaspase peptides length ranged from 320 to 416 amino acids with their molecular weight in the ranged from 35.68 to 45.57 kD. Type I metacaspases, has its pI of wide pH range from 4.84 to 9.02 whereas Type II metacaspases have its pI of the acidic pH values with pI-4.84 for SolycMC4 and pI-5.71 for SolycMC9.

4.1.2 Phylogenetic analysis and conserved motifs of tomato metacaspases

The conserved domains and structural characteristics of tomato metacaspases were identified using the InterProScan database (figure 8; Blum et al., 2021). Phylogeny of identified tomato metacaspases was also analysed to show their relationship with *Arabidopsis* metacaspases (figure 7). The multiple sequence alignment revealed the presence of conserved His-Cys residue (figure 9) and prodomain of tomato metacaspases which was generated using MEGAX and Vector NTI tools (Kumar et al., 2018; Lu & Moriyama, 2004). Amongst the six types I metacaspases, only SolycMC1 and SolycMC1-L2 have both N-terminal zinc finger prodomain and LSD1 domain, whereas, SolycMC1-L1 has only LSD1 domain. These prodomain are absent in other type I and II Metacaspases. Like Arabidopsis, all the tomato metacaspases also contain a predicted caspase-like His-Cys proteolytic dyad domain known as peptidase-C14 domain. Multiple sequence alignment showed 55% to 70% sequence similarity between amino acid sequence of type II metacaspases of Arabidopsis and tomato, whereas 38% to 81% sequence similarity was found between members of type I metacaspases. Multiple sequence alignment also revealed presence of two Ca^{+2} binding sites in p20 domain along with His-Cys Domain.

4.1.3 Molecular properties and gene structure of tomato metacaspases

Molecular weight of tomato metacaspases varies from 39 to 46 kD in which type II metacaspase SolycMC4 has the highest molecular weight of 45.578 kD. Tomato metacaspases have ORF length of 312 to 416 amino acid sequences and 1548 to 6510 bp genomic sequences. Type I metacaspases are distributed on chromosome number 1, 3 and 5, while type II metacaspases are located on chromosome 9 and 10 (figure 11). The coding sequences of all the metacaspases are on the antisense strand except for the SolycMC1-L1 and SolycMC1-L2 genes which have their coding sequences on the sense strand (table 4).

4.1.4 Gene structure analysis

Gene structural analysis of tomato metacaspases showed that type I metacaspases contain five exons and four introns of variable lengths, whereas type II metacaspases, SolycMC4 have two exons and one intron, and SolycMC9 have three exons and two introns. Gene SolycMC1-L2 has both the TSS (transcriptional start site) and Poly A tail in its structure, whereas SolycMC9 does not contain these structures. The remaining six tomato metacaspases have poly A tails but do not have a TSS site in their structure (figure 12; Solovyev, 2007).

4.2 Identification of cis-regulatory elements (CREs) in promoter of tomato metacaspase genes

Putative CREs in 1000 bp long promoter sequences were identified using the Plant CARE to obtain more insight into the transcriptional control of candidate metacaspase genes. (Lescot et al., 2002). A total of 32 CREs were identified in promoter region of metacaspase genes. The conventional cis elements like CAAT and TATA boxes were found in all the promoters. Besides these, some identified CREs were specific to only few promoters of metacaspase genes and divided into different groups of CREs, ARE: element essential for the anaerobic induction; ABRE: involved in the abscisic acid responsiveness; Box-4; GA-motif; GT1; MRE; SP1, GATA, chs-CMA1a : involved in light responsiveness; ERE: ethylene responsive element; GC-motif: anoxia specific inducible; TCA: salicylic acid responsiveness; CAT box: meristem expression; P- Box: gibberellin responsive element; TGACG, CGTCA: MeJa responsiveness; TC rich repeats- involved in defence and stress. For ease of understanding, we divided all cis-regulatory elements in two groups, first are cis-regulatory elements related to growth and development and second related to hormone fluctuation and biotic and abiotic stress. All tomato metacaspases has Box-4 and ABRE cis-regulatory elements in their promoters which is involved in light and abscisic acid responsiveness, respectively (table 8; figure 10).

4.3 Expression analysis of tomato metacaspases during plant development

The relative expression patterns of metacaspase genes in various developmental tissues of tomato were analysed by qRT-PCR. Detailed analysis showed that all the metacaspase genes have differential expression patterns in both vegetative and reproductive tissues of tomato plants. Expression analysis showed that SolycMC1 (figure 13) was highly expressed in cotyledons, hypocotyl and roots of 15 days old seedlings. Whereas, SolycMC1-L1 (figure 14) was expressed higher in hypocotyl and roots than in cotyledons. While, expression of SolycMC1-L2 (figure 15) was higher in hypocotyl than cotyledons and roots. In leaves, expression of SolycMC1, SolycMC-L1 and SolycMC1-L2 was observed to gradually increase from immature to mature leaves. Amongst these three MCs, SolycMC1-L1 was highly expressed in developing leaves and its expression was found to be maximum in mature leaves and leaves undergoing senescence. In stem, expression of SolycMC1 was higher in xylem, while expression of SolycMC1-L1 and SolycMC1-L2 was higher in outer layer of stem, i.e., phloem. In reproductive tissues, expression of SolycMC1 was relatively higher in flower buds' stage 5 than other flowering stages. This shows that SolycMC1 is differentially expressed and has some role during early stages of flower development. In contrast, expression of SolycMC1-L1 and SolycMC1-L2 is found consistently throughout all stages of flowers. In fruits, expression of SolycMC1 and SolycMC1-L1 was more or less same throughout all stages of developing fruits, however SolycMC1-L2 was strongly expressed only in fruit stage 5.

Expression patterns of SolycMC3 (figure 16), SolycMC3-L1 (figure 17) and SolycMC3-L2 (figure 18) were also analyzed in different vegetative and reproductive tissues. In general, SolycMC3-L2 was found to be low expressing gene than the SolycMC3 and SolycMC3-L1. Relatively higher expression of these MCs was found in hypocotyl than in cotyledons and roots of seedlings. Expression of SolycMC3 and SolycMC3-L2 was also detected in the abscission zone of flower pedicels and in the leaves undergoing senescence. In addition, SolycMC3 was observed to be consistently expressed throughout all stages of flower. In fruits, its expression was relatively higher in early stages of developing fruits, i.e., in immature fruits (1 to 3 stages) and then gradually decreased from young fruits to mature fruits undergoing ripening. In the stem, SolycMC3 was evenly expressed in both xylem and phloem layers of stem. In contrast, SolycMC3-L1 expression was observed to be higher only in the outer layer of the stem. In flowers, a higher expression of SolycMC3-L1 was detected from the early stage of opened flowers (Flower Stage 4) to the fully opened mature flowers. Higher expression of SolycMC3-L1 was also observed in fruits undergoing ripening. However, expression of SolycMC3-L2 was found to be undetectable in many of these tissues. Nevertheless, its expression was found to be detectable in hypocotyl, stem and in the abscission zone of flower pedicels.

Expression of type II metacaspases SolycMC4 and SolycMC9 was also analyzed in various vegetative and reproductive tissues of tomato. Expression of SolycMC4 (figure 19) was found to be more or less constitutively expressed in seedlings and in all stages of developing leaves. In reproductive tissues, expression of SolycMC4 was uniformly observed in all stages of developing flowers and fruits. However, its expression was relatively higher in flower buds' stage 4 and 5 and in developing fruits stage 5 and 6. Expression of SolycMC4 was also observed in xylem and phloem layers of the stem. Expression of SolycMC9 (figure 20) was specifically detected in later stages of unopened flowers to early stages of open flowers. In fruits, expression of SolycMC9 was mainly observed in the late stage of developing fruits. Expression of SolycMC9 was also relatively

higher in early stages of developing leaves and in the xylem layer of the stem. On the basis of constitutively strong expression pattern in most of the vegetative tissues SolycMC4 was selected for functional analysis.

4.4 Functional analysis of tomato SolycMC4 gene

4.4.1 Spatiotemporal expression analysis of SolycMC4 using PromSolycMC4::GUS lines

The spatiotemporal expression of SolycMC4 was analysed using PromSolycMC4::GUS reporter lines. Three independent transformed PromSolycMC4::GUS lines T1.1, T1.2 and T1.3 in tomato were generated. Out of these three, PromSolycMC4::GUS T1.2 was selected for detailed expression analysis. The expression of PromSolycMC4::GUS line was observed in most tissues of 4 weeks old young plants. Expression was observed in leaves, stem, primary and lateral roots. Expression was also detected in the meristematic regions of both stem and roots and the vascular tissues of roots, leaves and stem. Transverse section of the stem also showed expression in epidermis, cortex (chlorenchyma, collenchyma and parenchyma), interfascicular fibre region, vascular bundles, and pith of the stem (figure 21). Most interestingly, PromSolycMC4::GUS expression was induced by wounding of leaves indicating that SolyMC4 may have a role in stress responses.

Figure 21: Expression analysis of SolycMC4 using PromSolycMC4::GUS line. 870bp long SolycMC4 promoter sequence was cloned to drive the expression of the β-glucuronidase (GUS) ORF in transgenic tomato plants. Expression was analyzed in stem (A), cross section of the stem (B), leaflets (C middle part and D tip part) and roots (E-G), Leaf (H) and wounded part of leaflets (I, J) was 3-week-old plants. Scale bars: 200 µm in A, 30 µm in B.

4.4.2 Silencing of SolycMC4 by amiRNA-SolycMC4 in tomato

4.4.2.1 Cloning of amiRNA-SolycMC4

A SolycMC4 specific amiRNA was designed for posttranscriptional silencing of SolycMC4 (figure 22). SolycMC4 gene specific amiRNA was generated using overlapping PCRs. Generated amiRNA-SolycMC4 was first cloned in pDONR207 then transformed into *E. coli* to generate entry clone. PCR with the appropriate primers was used to validate the isolated entry clones and subsequently sequence

verified. To construct the expression clones, the entry clone was transferred in the pMDC32 expression vector and transformed into *E. coli*. After that, the PCR verified expression clone was transformed into the *Agrobacterium tumefaciens* strain GV3101pMP90, for genetic transformation of tomato explants. Resultant *A. tumefaciens* carrying the amiRNA-SolycMC4 construct under control of constitutive CaMV 35S promoter was transformed into tomato to develop 35S::amiRNA-SolycMC4 expressing gene silencing lines. On hygromycin selection plates, we were able to successfully generate several independent putatively transformed shoot buds. After a few weeks of growth, the transformed shoot buds grew to be 1-2 cm long shoots. These shoots were rooted and genotyped for the presence of transgene using genotyping primers A and B by PCR. Four positively transformed lines were further verified for SolycMC4 gene transcript level by qRT-PCR analysis. Our results show that the level of expression of SolycMC4 was reduced 96 to 98 percent in 35S::amiRNA-SolycMC4 lines relative to control plants.

4.4.2.2 Phenotypic characterization of 35S::amiRNA-SolycMC4 expressing tomato lines

To phenotypically characterize the 35S::amiRNA-SolycMC4 expressing T1 and T4 lines were grown *in vitro* and in potted soil in controlled growth condition in the growth room. When grown on MS agar in tissue culture, T1 and T4 silencing lines showed increased stem diameter compared to the control plants. After 30 days of *in vitro* growth, the diameter of T1 and T4 lines was 7.0 ± 0.6 mm and 7.1 ± 0.6 mm, respectively, compared to 6.1 ± 0.6 mm, in the control plants. An increase of about 15% in diameter of the stem was observed in these lines. Other growth parameters such as length of plants, leaf length and internode length were also undertaken for phenotypic characterization. We observed distinct morphological alterations in these growth parameters in amiRNA lines than in the control plants. Both T1 and T4 lines showed reduced plant length, 38.6 ± 8.1 mm and 39.1 ± 6.1 mm, respectively, compared to 63.0 ± 7.0 mm length in the control. The length of stem internode and leaves was also decreased in the silencing lines, internode length and leaf length of 30 days old T1 plants were 3.1 ± 1.1 mm and 30.2 ± 3.5 mm

Figure 22: A. Target site of amiRNA used for silencing of SolycMC4 gene. Truncation site was shown at point. Black box represents exon region and black line shows intron region. White box indicates UTR regions. B. Genotyping of amiRNA-SolycMC4 lines using construct specific A and B primers. C. Semi-quantitative RT-PCR analysis of amiRNA-SolycMC4 expressing lines: a. SolycMC4 gene transcript specific PCR b. Internal control SolycEF1α. D. qRT-PCR analysis of SolycMC4 expression levels in amiRNA-SolycMC4 silencing lines and control. Expression levels was normalized using the SolycAPT1 gene as internal control. Data represent the mean +/− SE of three biological replicates.

and in T4 plants 3.2 ± 0.9 mm and 31.1 ± 3.4 mm, respectively, compared with 5.2 ± 1.0 mm and 37.2 ± 3.3 mm in the control (figure 26). Approximately 60-62 % reduced length of plants and 81-83 % decreased length of leaves was observed in the amiRNA lines than the control. We also observed that the roots of *in vitro* grown amiRNA lines were thicker and produced fewer laterals than the roots in the control. Also, *in vitro* rooting of isolated 2-3 cm long shoots of amiRNA lines was delayed for at least 2-3 days than the control shoots. Rooted plantlets of 35S::amiRNA-SolycMC4 lines were transferred to pots and grown in controlled growth conditions, where they showed similar reduced stem and leaf growth, as well as fewer flowers and fruits (figure 23 and 24). In comparison to the control, the fruit was relatively smaller and produced no seeds. Because of the seedless nature we could not propagate these lines using seeds in pots, therefore amiRNA lines were maintained and sub cultured in tissue culture and majority of the subsequent investigations were undertaken on *in vitro* produced plants. The seedless phenotype of amiRNA lines is currently being investigated and is not included in the thesis.

A

Figure 23: Growth of 35S::amiRNA-SolycMC4 silencing line T1 and control plantlets cultured in vitro on MS medium scale bar- 1 cm.

B

Figure 24: Growth of 35S::amiRNA-SolycMC4 silencing line T1 and control plants in potted soil grown under controlled growth room scale bar- 1 cm.

4.4.2.3 Altered anatomy of stem in amiRNA-SolycMC4 lines

Anatomical analysis of the stem of amiRNA lines showed thicker cortical layer as compared to the control. The transverse section of 30 days old stems of amiRNA lines showed a greater number of cortical cells, as well as a larger size of the cells, resulting in an 82 % increase in thickness of

cortex over the stem of control. The cortex of T1 and T4 lines showed 2.94 ± 0.2 mm and 2.8 ± 0.2 mm thickness, respectively, compared to 1.6 ± 0.2 mm of control. Other tissues like phloem, xylem, inter fascicular-fibre region (IFR) and pith also showed some variation in thickness (figure 25).

The leaf morphology of plants of amiRNA lines was also altered, making the leaflets leathery. We also found that the venation patterns in the leaves of amiRNA lines were more complex than in control, with many closed thick vasculature loops tightly linked with the major midvein of the leaflets. The number of closed venation loops ranged from three to eight in leaflets depending on their location in a leaf, and only amiRNA lines showed such a thick vascular loop. Cross section was also analyzed to further explore anatomical details of the leaflets. The amiRNA lines showed thicker leaflets with larger and increased number of mesophyll cells and vascular system as compared to the control.

These observations indicate that the increase in diameter of stem and thickness of leaflets of amiRNA lines was largely due to the increased number and size of cortical and mesophyll cells, respectively (figure 25).

1.4.2.6 Altered pigment content in amiRNA-SolycMC4 plants

An additional phenotype of amiRNA-SolycMC4 lines was an intensified green colouration of leaves and stems of plants. To examine this phenotype, we analyzed the pigment content of amiRNA-SolycMC4 lines. We observed that both chlorophyll and anthocyanin pigment content of leaves of amiRNA lines were higher compared with leaves of the control. Chlorophyll-a, chlorophyll-b and total chlorophyll were assayed almost 150 times higher in the leaves of amiRNA lines which is approximately 6.0 mg g-1FW higher than the control. Total chlorophyll content in the leaves of T1 and T4 lines was 8.8 ± 1.1 mg g^{-1}FW and 8.9 ± 1.7 mg g-1FW, respectively compared to 3.4 ± 1.1 mg g^{-1}FW of chlorophyll in the control leaves. Other than this, anthocyanin pigment content was also almost 87% higher in the plants of amiRNA lines. We found 32 ± 0.4 mg g^{-1}FW and 32.1 ± 0.5 mg g^{-1}FW of anthocyanin in the leaves of T1 and T4 lines compared to 28 ± 0.1 mg g^{-1}FW of anthocyanin in control (figure 27).

4.4.2.6 Biochemical characterization of amiRNA-SolycMC4 silencing lines

4.4.2.6.1 Reactive Oxygen Species (ROS) analysis of amiRNA-SolycMC4 plants

To further characterize the 35S::amiRNA-SolycMC4 expressing lines, ROS generation activity using histochemical staining of leaves and roots with 3–3-diaminobenzidine tetrahydrochloride (DAB) was performed and samples were observed under Zeiss discovery V12 stereo microscope. When samples were observed after DAB staining, leaves and root tissues of control showed darker coloration as compared to amiRNA T1 and T4 lines, indicating that plants of amiRNA lines generated less ROS than the control.

To verify the difference in the ROS content of amiRNA lines and the control, H_2O_2 measurement in the leaves was analyzed spectrophotometrically using the standard procedure described previously (Junglee et al., 2014). The analysis showed a significantly reduced level of H_2O_2 in amiRNA lines. We measured about 3.15 ± 0.6 $mg^{-1}FW$ and 3.42 ± 1.2 $mg^{-1}FW$ of H_2O_2 in the leaves of amiRNA-SolycMC4 T1 and T4 lines, respectively compared to 10.0 ± 0.9 $mg^{-1}FW$ of H_2O_2 in the control leaves (figure 28).

4.4.2.6.2 Antioxidant enzyme activity in amiRNA-SolycMC4 plants

As we observed reduced ROS contents in the amiRNA lines, the activities of antioxidant enzymes were also assayed in amiRNA expressing T1 and T4 lines as per previously described methods (Junglee et al., 2014). Plant cell possesses ROS scavenging enzyme system to regulate cellular ROS molecules (H_2O_2, O^{-}, O^{2-}, etc.), which includes antioxidant enzymes such as catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11) and superoxide dismutase (SOD; EC 1.15.1.1). We found increased activities of CAT, POD, APX and SOD in amiRNA expressing lines. The activity of CAT was observed to be 472 ± 65 $Umg^{-1}min^{-1}$ and 468 ± 85 Umg^{-1} min^{-1}, respectively in T1 and T4 lines compared to 149.3 ± 118 Umg^{-1} min^{-1} in control leaves which was approximately 200 times higher in amiRNA expressing lines.

Figure 28: A-C. Detection of H2O2 production by DAB histochemical analysis in the leaf and root of amiRNA-SolycMC4 line and control scale bar – 50 µm and 200 µm D. The quantification of H_2O_2 in leaves of amiRNA-SolycMC4 lines and control. Error bar represents SE with three biological replicates. Significance is shown as *P < 0.05; **P < 0.01 according to the Tukey's test.

Similarly, POD activity was 3.16 ± 0.1 U µmole and 3.23 ± 0.5 U µmole in T1 and T4 lines, respectively compared to 0.58 ± 0.2 U µmole in control which was about 400-fold increased amiRNA expressing lines. Furthermore, APX activity was 2.87 ± 0.11 $Umg^{-1}min^{-1}$ and 2.79 ± 0.1 $Umg^{-1}min^{-1}$ in T1 and T2 lines respectively, compared to 0.9 ± 0.08 $Umg^{-1}min^{-1}$ in control which was approximately 200-fold higher than the control. Approximately 23% increase in activity of SOD was also observed in T1 and T4 lines. SOD activity was 0.21 ± 0.01 $Ugm^{-1}min^{-1}$ and 0.23 ± 0.01 $Ugm^{-1}min^{-1}$, respectively, in T1 and T4 lines compared to activity of 0.17 ± 0.01 $Ugm^{-1}min^{-1}$ in control plants (figure 29).

qRT-PCR analysis was also performed to confirm the expression patterns of ROS scavenging genes, such as APX, CAT, POD, and SOD in amiRNA lines. The expression levels of APX, CAT, POD, and SOD genes was also significantly higher in amiRNA lines compared to the control (figure 30), suggesting that the altered growth responses of amiRNA lines could also be attributed by ROS imbalance and altered antioxidant enzyme activity in the plants.

4.4.2.7 Role of SolycMC4 in delaying senescence

As we observed the dark green leaves and higher chlorophyll contents in amiRNA-SolycMC4 lines. We further investigated the senescence pattern of these leaves in amiRNA lines and observed that yellowing of leaves was highly suppressed in these plants than the control (figure 31). Comparison of leaf senescence pattern in amiRNA-SolycMC4 and control showed that the older leaves of amiRNA lines remained dark green with little visible yellowing at the margin which was frequently found completely yellow in the control plants. It was also noticed that the leaves of

Figure 30 Expression analysis of ROS scavenging genes, APX1, Catalase, Cu/Zn SOD and PXD27 in amiRNA-SolycMC4 lines and control plants. Error bar represents SE with three biological replicates. Significance is shown as *P < 0.05; **P < 0.01according to the Tukey's test.

amiRNA lines were found less brittle. These alterations in the developmental senescence pattern in the leaves of amiRNA-SolycMC4 plants prompted us to analyse expression patterns of known senescence marker genes. The expression of leaf senescence marker genes, Senescence Associated Genes 12 (SAG12) and Stay Green proteins (SGR1) were analysed in different developmental stages of leaves. SAG12 encodes the senescence-specific cysteine protease; it is upregulated as per the progression of leaf age and also as leaf senescence progresses (Zentgraf et al., 2004). SAG12 is commonly used as a late-stage senescence marker. Stay Green proteins (SGR1) which is an early senescence marker shows a highly conserved role in plants and its absence results in a stay-green phenotype (Hörtensteiner, 2009b). Silencing of LeSGR1 was found to inhibit chlorophyll degradation in tomato (Z. L. Hu et al., 2011). Our qRT-PCR analysis showed significantly decreased expression of SAG12 in mature and senescent leaves of amiRNA lines as compared with the control (figure 31). Expression of SGR1 was also suppressed in leaves of amiRNA plants indicating delayed senescence program in amiRNA-SolycMC4 lines (figure 31). As shown in figure 31, the expression of SAG12 was 97-98 % reduced in the mature and senescent leaves, while expression of SGR1 decreased by 90% in mature leaves of amiRNA lines compared with the leaves of control.

The change in the expression pattern of SAG genes also prompted us to investigate the expression pattern of autophagy marker genes ATG5, ATG8 and TOR1 in amiRNA lines as leaf senescence also involves upregulation of autophagy. Knockout of autophagy gene found to have accelerated senescence phenotype in *Arabidopsis* showing the importance of autophagy in senescence. qRT-PCR analysis of autophagy-related (ATG) genes in the leaves of amiRNA-SolycMC4 lines showed upregulated expression of ATG5 and ATG8 genes, whereas expression of TOR1 gene was downregulated in amiRNA lines than the control. The downregulation of TOR1, a negative regulator of autophagy, further validated our finding that the onset of senescence program is delayed in the plants of amiRNA lines. As autophagy is involved in cellular content and nutrition recycling during senescence, the time of expression of these ATG genes could be a key regulation for autophagy induction in senescing leaves. The enhanced expression of ATGs in the plants of amiRNA lines suggests greater cellular and nutritional recycling, which could be a reason for delayed leaf senescence in silencing lines. These results indicate that some of the phenotypes observed in silencing lines could be due to the altered senescence pathway.

4.4.2.8 Role of chloroplast and mitochondria in delayed senescence of amiRNA-SolycMC4 lines

As plants of amiRNA-SolycMC4 lines showed delayed senescence, higher chlorophyll and anthocyanin pigments and increased antioxidant enzyme activity. We also analysed the functions of mitochondria and chloroplast in these lines in order to examine whether these alterations observed in amiRNA lines were due to impairments of chloroplast and mitochondria. To validate this, we analysed the cell organelle DNA content and also the expression patterns of several chloroplast and mitochondrial marker genes in amiRNA lines. Chloroplast encoded genes *RbcL, RpoA* and *PetB* were used as markers for chloroplast, while 26srRNA and ND1 were used as markers for mitochondria. We noticed no distinguishable difference in mitochondrial and chloroplast DNA content in amiRNA lines compared with control plants (figure 31). However, the expression analysis of both the mitochondrial and chloroplast encoded marker genes showed a noticeable decrease in the expression of PetB (97%) and ND1 (98%) genes of chloroplasts and mitochondria, respectively in amiRNA lines than the control. In contrast, expression of RbcL which encodes a subunit of Rubisco was increased in the leaves of amiRNA lines. Both the PetB and ND1 genes are involved in energy metabolism. Mitochondrial ND1 is part of complex-I in electron transport chain (ETC) and its decreased expression indicates increased ROS production in the cell. This prompted us to further investigate the complex-I activity in amiRNA lines. The enzyme activity of mitochondrial complex--I activity of amiRNA lines showed a 50% decreased activity compared with the control. The amiRNA-SolycMC4 T1 and T4 lines shows 0.92 ± 0.03 Umg-1min-1 and 0.93 ± 0.02 Umg-1min-1, respectively activity compared with 2.0 ±0.18 Umg-1min-1 in control plants (figure 31), further suggesting that functions of mitochondria also altered due to the silencing of the SolycMC4 in plants.

4.4.2.9 Expression pattern of cell death marker genes in amiRNA-SolycMC4 lines

We demonstrated that silencing of SolyMC4 resulted in dark pigmented dwarf plants which also show delayed senescence process indicating altered regulation of cell death nucleases and proteases in plants. To validate this expression pattern of known nucleases and proteases and related cell death marker genes. For example, Subtilase 3 (SBT3), Required for Cladosporium Resistance-3 (RCR3; Papain-like Cysteine Protease), MLO-like Protein1 (MLO1), Pathogenesis-Related gene 1a (PR1a), Tomato Bifunctional Nuclease (TBN1) and Pirin1 (PRN1) was analyzed in amiRNA-SolycMC4 lines and compared with their expression in control plants. An increased

expression of the SBT3 gene was observed in the SolycMC4 silencing line, in contrast, expression of RCR3 was down regulated in the amiRNA-SolycMC4 lines. Both SBT3 and RCR3 genes are serine and cysteine proteases, respectively. We also analyzed the expression of MLO1 and PR1a, both involved in plant defence and pathogenesis, respectively. Both MLO1 and PR1 are proteases, MLO1 is involved in the modulation of pathogen defence and leaf cell death, whereas PR1a is an extracellular pathogenesis-related protein. In the amiRNA-SolycMC4 plants, expression of MLO1 was upregulated, however PR1a was downregulated as compared with control plants showing possibly perturbed roles due to the silencing of SolycMC4 in amiRNA-SolycMC4 plants (figure 32).

4.4.4 Subcellular localization of SolycMC4::GFP fusion protein

Protein localization prediction using Plant-mPloc (Chou and Shen, 2010) and wolf-pSORT tools (Horton et al., 2007) suggested that SolycMC4 is localized in the cytoplasm. For analysis, AtMC4 was used as a control as it has been shown to be localized in the cytoplasm (Watanabe and Lam, 2011). To explore the SolyMC4 subcellular localization, a 35S::SolycMC4::GFP C-Terminus fusion construct was generated, transiently expressed in tomato protoplasts and visualized using Zeiss LSM700 confocal microscopy. The green fluorescence of the SolycMC4::GFP fusion protein was detected in the cytoplasmic compartment of the tobacco protoplast suggesting that SolycMC4 is a cytoplasmic protein (figure 33).

Figure 33: Subcellular localization of SolycMC4::GFP fusion protein in the protoplast of tomato showing SolycMC4 localization in cytoplasm scale bar= 10 µm.

Table 7 Molecular properties of tomato metacaspases

Type	Gene ID	Gene Name	bp	AA	PI	Mol wt. kD	Locus
I	Solyc01G088710.2	SolycMC1	6510	368	6.49	39.789	75,230,243 - 75,236,204 (83.31 centisomes) on Chromosome 1
	Solyc03G094160.2	SolycMC1-L1	2914	361	6.3	39.412	49,509,087 - 49,512,000 (76.35 centisomes) on Chromosome 3
	Solyc05G052130.2	SolycMC1-L2	2176	362	8.69	40.284	61,588,518 - 61,590,693 (94.72 centisomes) on Chromosome 5
	Solyc01G105300.2	SolycMC3	1548	361	8.82	40.458	85,277,359 - 85,278,906 (94.43 centisomes) on Chromosome 1
	Solyc01G105310.2	SolycMC3-L1	1549	362	7.97	40.314	85,282,764 - 85,284,312 (94.44 centisomes) on Chromosome 1
	Solyc01G105320.2	SolycMC3-L2	1812	355	9.02	40.424	85,287,892 - 85,289,703 (94.45 centisomes) on Chromosome 1
II	Solyc09G098150.2	SolycMC4	2621	416	4.84	45.578	67,309,895- 67,312,515 (99.48 centisomes) on Chromosome 9
	Solyc10G081300.1	SolycMC9	1928	320	5.71	35.684	61,728,461- 61,730,388 (95.21 centisomes) on Chromosome 10

Table 8: Identified CREs in promoters of tomato metacaspase genes

Gene	cis-element (growth and development related)	cis-element (stress and hormone related)
SolycMC1	Box -4; GA-motif; GT1; MRE; SP1	ARE[5]; ERE
SolycMC1-L1	Box -4; GA-motif; GT1; GATA; G-box[2]	ABRE[3]; GC- motif
SolycMC1-L2	Box -4; G-box; TCCC; TCT	ABRE[3]; ARE; TCA
SolycMC3	AF1; Box -4; G-box; GATA; TCT; CAT box	ABRE
SolycMC3-L1	Box -4; G-box; GT1; MRE; TCCC; TCT; ATCT	ARE; ABRE;
SolycMC3-L2	Box -4; ATCT; CAT box; TCT	ARE; ABRE; P-box; TCA; TGACG
SolycMC4	Box -4[2]; AE; TCT	ABRE; CCAAT box; CGTCA; TGACG; O2-site
SolycMC9	3-AF1; ATC-motif; Box -4[2]; G-box; GT1; chs-CMA1a; TCT motif	ABRE[2]; ABRE3a[2]; ABRE4[2]; ARE[2]; TC rich repeats

DISCUSSION

Caspases are important regulators of PCD in animals. Similar caspase like proteases, such as metacaspases, were also discovered in plants, fungi, and other organisms. Although caspases and metacaspases are structurally similar, functionally they are found to be different (Uren et al., 2000). In plants, initially the function of metacaspases was investigated in spruce and *Arabidopsis* to characterize their roles in developmental PCD (Bozhkov et al., 2005; Suarez et al., 2004; Uren et al., 2000; Vercammen et al., 2004; Watanabe & Lam, 2004). Subsequently, identification of this important family of cysteine proteases also performed in other plant species, such as poplar, rubber, litchi, wheat, pepper, potato, rice and tomato (Bollhöner et al., 2018; Dubey et al., 2019; Kim et al., 2013; Liu et al., 2016a, 2016b; Piszczek et al., 2012; Wang & Zhang, 2014; Zhang et al., 2013).

Tomato is one of the important vegetable crops and also a genetic model system (Giovannoni 2001). The genome of tomato is sequences and encodes for 34,384 predicted protein-coding genes (Su et al., 2021). Tomato genome encodes for eight MC genes (Liu et al., 2016; Dubey et al., 2019). Despite of identification and their importance, function of none of the tomato MC genes have been characterized. Functional characterization of these genes could increase our understanding of this important family of proteases in plants. Also, these genes could be targeted to improve various crop traits such as seedless-ness, delayed senescence, male sterility and disease resistance in tomatoes. With these targets in view, in present study, we characterized the role SolycMC4 during development of tomato. Using *in silico* based approaches we have identified MCs in the tomato and phylogenetically compared with MCs from *Arabidopsis*. Our results showed that as compared with *Arabidopsis* which has 6 type-II MCs, the tomato genome possesses only two type II MCs which were found to close homologs of AtMC4 and AtMC9; therefore, named these two gene as SolycMC4 and SolycMC9, respectively.

Cis-regulatory elements, which act as molecular switches, are fundamental in the onset of transcription. Therefore, to understand the transcriptional regulation, promoter sequences of SolycMCs were isolated and various cis-regulatory elements related to growth and development were identified. These cis-regulatory elements could be important in controlling SolycMC genes expression during development. To explore the involvement of SolycMCs in the development, we examined the expression of all eight SolycMCs genes in various tissues at different developmental stages of plants. We found nearly all SolycMCs showed differential

expression patterns in different developing tissues. These results suggested that SolycMC genes are actively involved in developmental regulation of plants. SolycMC4 found to be expressed during most the developmental stages in tomato, which was also observed in PromSolycMC4::GUS lines. Expression was found in most regions of the plant, including the leaf, root, and stem in PromSolycMC4::GUS lines. Expression of SolycMC4 was observed in most of anatomical tissues, such as cortex and vascular tissues of stem and leaves and also induced by wounding. Similarly, *Arabidopsis* AtMCP1b was found to be upregulated in vasculature after wound responses and pathogens (Castillo-Olamendi et al., 2008), Also strong GUS expression was observed in *Arabidopsis* in seedlings and tracheary elements (Tsiatsiani et al., 2013; Watanabe & Lam, 2011). In case of *Populus* sp. expression of metacaspase was also observed in vascular tissues (Bollhöner et al., 2018).

Due to presence of only two type II MC genes in tomato, the silencing of SolycMC4 decreases the chances of gene redundancy in context to illustrate a functional role in tomato compared with *Arabidopsis*. Although we found highly conserved gene structure and conserved amino acid sequences between SolycMC4 and AtMC4, it cannot be predicted that the tomato SolycMC4 will have the same function as its counterpart in *Arabidopsis*. Amongst all tomato MC genes, expression of SolycMC4 was most abundantly found in the developing vegetative tissues indicating that SolycMC4 is one of the most important candidate MCs in tomato. We observed that SolycMC4 also has a similar expression pattern in tomato as its counterpart AtMC4 has in *Arabidopsis* (Watanabe & Lam, 2011a).

Subcellular localization of SolycMC4::GFP fusion protein shows that SolycMC4 was found to be localized in the cytoplasmic compartment of the cell. Similar to our observation its closest homolog AtMC4 was also localized in the cytoplasm (Watanabe & Lam, 2011). Another type-II MC, MC-IIPa in *P. abies* was localized at the nuclear periphery and in the perinuclear cytoplasm (Bozhkov et al., 2005). Whereas LcMCII-1 from *Litchi chinensis* was identified as type-II MC and was predominantly found in the cytoplasm (Wang et al., 2016). These data support that most of the type-II MC proteins seem to have cytoplasmic localization.

Artificial microRNA-based silencing is a reliable method to generate stable knockdown lines in plants (Schwab et al., 2006). We used a similar approach to silence SolycMC4 post transcriptionally in tomato. Our phenotyping results shows that plants due to silencing of SolyMC4 produce various developmental changes in plants which include vigorously growing dark green leaves and shorter and thicker stems, resulting in the reduced length of the plants.

Similarly leaves and roots of amiRNA-SolycMC4 lines were also leathery and thicker compared with control. We further characterized these observations anatomically and noticed a greater number of larger cells in the cortical region of the stem. There appear to be a link between more expansions of cells as we found similar observation in leaf anatomy. We could not find any possible explanation for this yet; further investigations are needed to understand the enlargement of the cortical cells exhibited by amiRNA-SolycMC4 lines.

A further consequence of silencing of SolycMC4 is a high accumulation of chlorophyll as well as anthocyanin pigments in the plants of amiRNA-SolycMC4 lines producing visual darker plant growth phenotype. As these pigments are part of chloroplast – a photosynthesizing machinery of plant, we further validated the expression of various marker genes for chloroplast and mitochondria in the amiRNA-SolycMC4 lines. *RbcL* is a widely used marker of chloroplast and its increased expression is supported by the proof of high chlorophyll biosynthesis in amiRNA lines. Surprisingly, the expression of *PetB* and *ND1* which are energy metabolism-associated markers of chloroplast and mitochondria, respectively, was decreased significantly compared with the control. Plants increases its photosynthetic efficiency by regulating the light-harvesting complexes there by managing balance of excitation energy where ND1 is involved (Pesaresi et al., 2009).

In both, chloroplast and mitochondria excessive ROS production is due to over reduction of the electron transport system (Davidson & Schiestl, 2001). Therefore, we further verified the ROS level in the amiRNA-SolycMC4 lines using DAB staining methods and found that there was an increase in ROS production. Cellular ROS production greatly dependent on balance between ROS producing and antioxidant pathways (Mittler et al., 2004). We found a significantly higher activity of antioxidant enzymes, SOD, CAT, APX, and GPX in amiRNA-SolycMC4 plants compared with the control; the same was also confirmed by analyzing their expression by qRT-PCR. An increased antioxidant genes expression and enzyme activity indicate induction of the ROS maintenance system. In plants, ROS plays important role as a signal molecules during development, programmed cell death and abiotic and biotic stresses (Mittler et al., 2004; Torres & Dangl, 2005). ROS could also play the role as a signalling molecule, not for short, but for long distances at the cellular level (Mittler et al., 2004). It has also been reported that antioxidant enzymes function as redox regulators and mediate metabolism that modulates programmed cell death and plant acclimation (Foyer & Halliwell, 1976; Foyer & Noctor, 2005; Takahashi & Asada, 1988). Currently ROS-scavenging enzymes

are shown to be involved in signalling besides their roles in ROS management and cellular protection (Laxa et al., 2019)

As amiRNA-SolycMC4 lines showed almost 10-fold decreased expression of *PetB* and *ND1* marker genes and Complex-I activity was inhibited, implying that mitochondrial dysfunction leads to an over-reduction of the ubiquinone pool and enhanced ROS generation. A retrograde ROS signal was predicted to be triggered by changes in ROS levels produced by the disruption of complex-I. (Rhoads & Subbaiah, 2007). These changes shows that organelle-based retrograde signalling is involved in mediating ROS signals between ROS-producing organelles and the nucleus. (Pogson et al., 2008). In the case of amiRNA-SolycMC4 line later one seems to be more convincing.

Type-II MCs act upstream of autophagy. In Norway spruce, MC-IIPa and autophagy were shown to require for PCD of embryonic suspensor cells (Minina et al., 2013). Similarly, *Arabidopsis* AtMC9 was found to regulate autophagy during the development of xylem elements (Escamez et al., 2016). Also in *Arabidopsis*, autophagy mutants are shown to have accelerated senescence (Ishida et al., 2014). We found increased expression of *ATG5* and *ATG8* marker genes in amiRNA-SolycMC4 lines which is further supported by reduced expression of the *TOR1* gene, a negative regulator of Autophagy in *Arabidopsis* (Y. Liu & Bassham, 2012). Together these data supported our finding that silencing of SolycMC4 delays leaf senescence in tomato. It is known that ATGs actively participate in the cellular nutrient recycling (Michaeli et al., 2016) and their increased expression indicates increased recycling and maintenance in amiRNA-SolycMC4 plants which is in support of its vigorous leaf growth.

Leaf chlorosis is an indication of leaf senescence. We observed that leaves in amiRNA-SolycMC4 lines showed different senescence patterns as their leaves remained dark green produce very little visible yellowing compared to the control. The possible explanations for this delayed senescence phenotype are: it is likely that the amiRNA-SolycMC4 lines possess high chlorophyll content thus its degradation process may take longer or silencing of SolycMC4 results in the alteration of expression of senescence-associated genes. To verify these two possibilities, we checked the expression of the senescent marker gene *SAG12*, and the result indicated an almost 10-folds decreased expression of *SAG12* in mature leaves as well as in senescent leaves of amiRNA-SolycMC4 lines as compared with control. This clearly indicate delayed or suppressed leaf senescence program as expression of SAG12 was suppressed in amiRNA-SolycMC4 lines. Another study supported this finding as knockout of

MC type-II, LcMCII-1 delays ROS-dependent leaf senescence in *Litchi chinensis* (Wang et al., 2016). Further evidence for this observation is that Stay green 1 (*SGR1*) which regulates degradation of chlorophyll and chlorophyll-binding proteins in senescence (Hörtensteiner, 2009) was also found to decreased by 10-folds and it supports dark green phenotype and delayed senescence of tomato amiRNA-SolycMC4 lines.

In the present study, we characterized the SolycMC4 gene in tomato and clearly show that it plays important role in the development plasticity and involved in ROS maintenance and leaf senescence. However further investigations are needed to understand the mechanism by SolycMC4 is involved in such processes. Moreover, SolycMC4 possible targets could still be identified and characterized. Further investigations are needed to precisely know the role of this important member of tomato metacaspase gene family during plant development and PCD.

Figure 33: Pictorial representation of role of SolycMC4 gene during plant development

Figure 34: Model representation of role of SolycMC4 gene during plant development