SUMMARY

Metacaspases are cysteine proteases that belong to the C14 family, including caspases, orthocaspases, and paracaspases (Uren et al., 2000). In contrast to caspases, which are aspartate-specific, metacaspases are arginine/ lysine-specific proteases (Vercammen et al., 2004). Metacaspases are categorized into type I and II groups based on their domain organization and found in plants, algae, fungi, and protista. Type I metacaspases are composed of proline and glutamine-rich zinc finger-like pro domains (Vercammen et al., 2004). Metacaspases are found in several prokaryotic and eukaryotic organisms but not in animals. Metacaspase type II exist only in some green algae and land plants which is characterized by a longer linker and absence of pro-domain.

The function of very few metacaspases have been characterised in *A. thaliana* and Norway spruce leaving enough scope to further characterise the roles of some of the other interesting metacaspases in plants. Therefore, we aimed to characterise the role of tomato type II metacaspases in tomato plant development using molecular genetics and biochemical approaches.

Bioinformatics based genome wide analysis showed that tomato genome has eight metacaspases. 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 and named as SolycMC1, SolycMC1-L1, SolycMC1-L2, SolycMC3, SolycMC3-L1 and SolycMC3-L2, SolycMC4 and SolycMC9, respectively. Six of these were found to be like type I and other two found to be similar to type II metacaspases.

Like other metacaspases, tomato metacaspases also contain a predicted caspase-like His-Cys proteolytic dyad domain known as peptidase-C14 domain. Multiple sequence alignment analysis showed 55% to 70% similarity between amino acid sequences of type II metacaspases of Arabidopsis and tomato where as 38% to 81% amino acid sequence similarity between type I metacaspases. Multiple sequence alignment also showed presence of two Ca^{+2} binding sites in p20 domain along with His-Cys Domain. Two of six tomato type I metacaspases, viz., SolycMC1 and SolycMC1-L2 found to have N-terminal zinc finger prodomain and LSD1 domain. Whereas, SolycMC1-L1 has only LSD1 prodomain. These prodomains were absent in other three type I and two type II metacaspases.

Further, we have identified 32 different types of cis-regulatory elements in the 1kb upstream of promoter sequences to know better the transcriptional regulations of these genes in different conditions. General cis regulatory elements such as TATA and CAAT box were found in all promoters. However, some cis elements were found to be particularly to only a few metacaspase gene promoters and were divided into several groups, including as ARE: anaerobic induction essential element; ABRE: abscisic acid responsiveness; Box-4; GA-motif; GT1; MRE; SP1, GATA, chs-CMA1a: light responsiveness; ERE: ethylene responsive element; GC-motif: anoxia specific inducible; TCA: salicylic acid responsiveness Meristem expression is represented by the CAT box. TGACG, CGTCA: MeJa responsiveness; TC rich repeats- involved in defense and stress response; P- Box: gibberellin responsive element; TGACG, CGTCA: MeJa responsiveness; TC rich repeats- involved in defense and stress responsiveness.

Expression analysis of metacaspase genes in various developmental tissues of tomato such as the root, hypocotyl, cotyledons, stem, leaves, flowers, and fruits was analysed. Most of these metacaspase genes were found to differentially express during growth and development in plants. One of the type II metacaspase SolycMC4 showed very strong expression in almost all developing tissues therefore selected for functional analysis.

Spatiotemporal expression of SolycMC4 was also analyzed using PromSolycMC4::GUS reporter lines. GUS was observed in most of the tissues of 4-week-old tomato plantlets which includes leaves of all developmental stages, stem, primary and lateral roots. The meristematic areas of the stem and roots also showed GUS expression. GUS expression was also detected in vasculature tissues of the roots, leaves, and stem. Anatomy of stem showed expression in the epidermis, cortex (chlorenchyma, collenchyma, and parenchyma), interfascicular fibers, and developing vascular bundles, also in the pith.

For further characterization of SolycMC4, we designed and generated four independent constitutive expressing 35S::amiRNA-SolycMC4 gene silencing lines in tomato “MicroTom” using Gateway cloning technology. The location of designed amiRNA was targeted to second exon of SolycMC4 gene. The putatively transformed gene silencing lines were PCR genotyped and maintained in tissue culture for their multiplication. Expression analysis showed that transcript of SolycMC4 was significantly (98%) reduced in 35S::amiRNA-SolycMC4 lines as compared with control plants. The expression level of other SolycMC1-7 genes in amicRNA-SolycMC4 lines was verified and found mostly to be unchanged as compared to control plants.

Two of the selected lines when grown in vitro and in pots for detailed characterization produce more vigorous growth and higher pigmented stay green phenotype of plants. Phenotypic characterization of in vitro grown amiRNA-SolycMC4 plants showed reduced plant height by approximately 40% which almost 15 mm and increased diameter of stem by approximately 15% which almost 1 mm thicker stem. However, length of leaves was decreases by approximately 19% which accounted for almost 5 mm smaller leaves along with shorter internode length by approximately 35% which almost 1-2 mm shorter compared to control. As plants of silencing lines produce dark green phenotype, we also analyzed the pigment content in their leaves. We observed that both chlorophyll and anthocyanin pigments in these lines was higher compared to control plants. Chlorophyll-a, chlorophyll-b and total chlorophyll were assayed almost 150 times higher in the leaves of silencing lines which is approximately 6.0 mg g^{-1}FW more compared to control. Anthocyanin pigment was also almost 87% higher in the leaves of SolycMC4::amiRNA plants. We also observed that silencing of SolycMC4 results in reduced ROS production and more activity of some of the ROS scavenging enzymes. For ROS analysis we stained the leaves of silencing lines and control plants with 3,3, -diaminobenzidine (DAB). DAB histochemical analysis showed lower localized accumulation of brown precipitations indicating reduced ROS generation in the leaves of silencing lines. In addition, H_2O_2 contents in leaves of silencing lines was also analysed spectrophotometrically. Our results showed significantly reduced H_2O_2 content suggesting that due to the silencing of SolycMC4 in amiRNA-SolycMC4 expressing lines, there may have reduced ROS production in these lines. Also, higher chlorophyll contents and chloroplasts may have played important roles in ROS balancing inside the cell and tissues of silencing lines.

Plant cells possess ROS scavenging enzymes to counterbalance excessive ROS generation, which includes catalase (CAT), peroxidase (PXD), ascorbate peroxidase (APX) and superoxide dismutase (SOD). The antioxidant enzyme activities were also assayed in both SolycMC4 gene silencing lines and control. The CAT activity was observed to approximately 200 times higher in silencing lines, whereas SOD activity was 23% increase times in these plants. Activities of PXD and APX was also dramatically higher in silencing lines than control with 400- and 200-fold increase in their activity, respectively. qRT-PCR analysis of Catalase (CAT), Superoxide dismutase (SOD), Ascorbate peroxidase (APX) and Guaiacol peroxidase (GP) was also performed to quantify their expression in 35S::amiRNA-SolycMC4 lines and control. Expression analysis showed that APX, CAT, PXD and SOD genes were upregulated in silencing lines further supporting their higher enzymatic activities in these plants.

The expression patterns of several cell death, mitochondrial and chloroplast markers were also analyzed in order to verify whether chloroplast and mitochondria functions also altered due to the silencing of SolycMC4 in plants. As some of the phenotypes and biochemical changes such as dark green phenotype with higher chlorophyll contents and ROS imbalances observed in these plants could be the due to functional alterations in these cell organelles. Expression analysis of both the mitochondrial and chloroplast encoded marker genes in amiRNA lines showed a notable decrease in the expression of PetB (97%) and ND1 (98%) genes of plastids, respectively. The PetB and ND1 genes both have a role in energy metabolism. Mitochondrial ND1 is a component of the electron transport chain's (ETC) complex-I, and its decreasing expression signals that the cell is producing more ROS. To further validate this observation complex-I activity in amiRNA plants was investigated. The enzyme activity of mitochondrial complex-I was measured spectrophotometrically in the leaf samples and both silencing and control. We observed that the complex-I activity in the leaves of amiRNA lines was 50 percent lower than the control. In comparison to 2.00.18 Umg-1min-1 in control plants, the amiRNA-SolycMC4 T1 and T4 lines showed 0.92 0.03 Umg-1min-1 and 0.93 0.02 Umg-1min-1 activity, respectively.

To evaluate whether senescence programs are affected in amiRNA-SolycMC4 plants, two known senescence associated genes, SGR1 (Stay Green protein1s) and SAG12 (Senescence Associated Genes 12) were used as markers to analyze the onset of senescence. Expression levels of SGR1 and SAG12 genes during developmental stages of leaves of amiRNA lines and control was examined using qRT-PCR. Expression results showed that leaves of amiRNA lines have significant decreased expression of both SGR1 and SAG12 genes. Expression of SAG12 was reduced by 97.7% in mature and 98.3% in leaves undergoing senescence indicating that senescence in amiRNA-SolycMC4 line was delayed. Similarly, expression of SGR1 in senescent leaves of amiRNA plants was reduced by 84% than the control. These results suggest that SolycMC4 function in modulating senescence and is act upstream or modulate pathways upstream of the senescence.

To assess whether silencing of SolycMC4 leads to change in expression of other cell-death related marker genes, ATG5, ATG8 and TOR1 (Target of Rapamycin) was analyzed in amiRNA-SolycMC4 lines. Expression of these genes was compared between amiRNA lines and control. Results showed that expression of both ATG5 and ATG8 increased significantly in amiRNA line. As expected, expression of TOR1 was reduced in the amiRNA line. This indicate that down regulation of SolycMC4 resulted in increased expression of autophagy

genes in amiRNA-SolycMC4 plants. Expression patterns of known cell death and stress marker genes, such as 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 using qRT-PCR analysis. SolycMC4 is a cysteine protease, thus analyzing the effect of its silencing on the expression pattern of plant proteases, SBT3 and RCR3 genes, also both are serine and cysteine proteases, respectively. A 4-fold upregulated expression of SBT3 gene was observed in silencing line which indicates that there might be regulation of some compensatory mechanism due to loss of SolycMC4 function in plants. In contrast, RCR3 showed almost 6-fold down regulation in the amiRNA-SolycMC4 plants. We have also analyzed the expression of other proteases, MLO1 and PR1a, both involved in plant defense and pathogenesis, respectively. MLO1 is known to be involved in modulation of pathogen defense and leaf cell death, whereas PR1a is an extracellular pathogenesis related protein. In SolycMC4 gene silencing line, we observed 2-fold upregulated expression of MLO1, whereas expression of PR1a was downregulated by approximately 3-fold showing possibly perturbed pathogenesis and defense pathways. We have selected TBN1 nuclease gene as marker gene for nucleases and observed approximately 7.5-fold increased expression in silencing line compared to control which indicated that there might be higher nuclear degradation activity in the plants of SolycMC4 silencing lines. Contrary to this, Nuclease PIRIN1, an adjacent gene of SolycMC4 showed 2-fold decreased expression in silencing lines.

To visualize SolycMC4 subcellular localization, a 35S::SolycMC4::GFP C-Terminus fusion construct was designed and transiently expressed in tomato protoplasts. The SolycMC4::GFP fusion protein was localized to the cytoplasmic compartment of cell.

This study provides insights in to functional role of SolycMC4 in tomato. Our analysis shows that silencing of the SolycMC4 results in several developmental alterations which include stay green plant phenotype, ROS imbalances, activation of antioxidant enzymes and delayed the leaf senescence in plants.