Synopsis of the thesis on

The Role of Tomato Type II Metacaspase (s) during Plant Development

To be submitted to

The Maharaja Sayajirao University of Baroda

For the degree of
Doctor of Philosophy in Botany

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Introduction

Programmed cell death (PCD) is a key process responsible for the removal of unwanted assembly and shape remodeling during plant development. But, the molecular mechanisms behind this is poorly understood [1]. Bioinformatics analysis revealed the presence of structural analogues of animal caspases, the metacaspases which are considered as the potential cell death regulators in plants [2].

Metacaspases are divided into type I and type II groups based on their sequence similarity and domain structure. Type I metacaspases contain a proline rich pro-domain and a zinc-finger motif in the N-terminus region similar to the pro-domain found in animal caspases and Type II metacaspases don't have the pro-domain, but contains a longer linker region between p10 and p20 subunit [3]. Type I metacaspases can be found in all lineages between algae to flowering plants, protozoa and fungi, whereas type II metacaspases are found only in plants and algae [2].

Identification of metacaspase genes encouraged consideration of their potential involvement in cell death events in fungi, protozoa, and plants. Studies in yeast, *Saccharomyces cerevisiae* shown that the only metacaspase present in displays caspase-like proteolytic activity that is activated when yeast cells are stimulated by H_2O_2 to undergo cell death [4]. Subsequently, characterization of plant metacaspases was carried out in Norway spruce, where suppression of type II metacaspase mcII-Pa causes inhibition of terminal cell differentiation and PCD in embryo suspensor cells results into developmental arrest of early embryogenesis [5].

In *Arabidopsis thaliana*, type-II metacaspase AtMC9 plays important role during postmortem clearance of xylem elements [6]. Type-II AtMC8 is involved in the regulation of PCD induced by oxidative stress [7], while type-II AtMC4 is a positive regulator of biotic and abiotic stress-induced PCD [8]. Whereas, *Arabidopsis* type I AtMC1 is shown to act as positive regulator of HR cell death and its near relative AtMC2 is antagonist of AtMC1; their manipulation eliminates the hypersensitive cell death response (HR) activated by plant intracellular immune receptors [9]. In *Capsicum annuum* metacaspase 9 (CaMC9) plays a role in pathogen-induced cell death [10]. TaMCA4, a novel wheat metacaspase gene functions in PCD induced by a fungal pathogen [11]. In *Litchi chinensis*, a type II metacaspase LcMCII-1 was shown to play an important role as a positive regulator of ROS dependent senescence [12]. A tomato metacaspase, LeMCA1 was shown to up regulated during PCD in *Botrytis cinerea* infected leaves [13]. *In silico* study of metacaspases has revealed that there are eight metacaspases in tomato and potato, respectively in comparison with nine in Arabidopsis [14, 21, and 22].

Using bioinformatics analysis we have identified eight metacaspases in tomato (*Solanum lycopersicum* L.). Our phylogenetic analysis shows that two of type II metacaspases were found very close to *Arabidopsis* AtMC4 and AtMC9 therefore named as SolycMC4 and SolycMC9. The expression patterns of these two genes were analyzed in young seedlings as well as in vegetative and reproductive tissues of 60 days old mature plants. The expression analysis shows that SolycMC4 was constitutively expressed in seedlings as well as in various developmental stages of leaves, stem, flowers and fruits. However, expression of SolycMC9 was detected only in some developmental stages of leaves, flowers and fruits. We also observed strong expression of SolycMC9 in xylem layer of stem. We found that amongst all the tomato metacaspases SolycMC4 was found to be most expressed during development, therefore we aim to

characterize the role of SolycMC4 using various molecular genetics and biochemical approaches in plants.

Aim and Objectives

Aim of proposed work is to understand the role of tomato type II metacaspases in plant development using molecular and biochemical approaches.
The objectives of proposed work are:

1. Spatial and temporal expression analysis of type II Metacaspase in tomato.
2. Characterization of type II Metacaspase in tomato by gene silencing.
3. Biochemical characterization of type II Metacaspase.
4. Subcellular localization of type II Metacaspase.

Results

Identification of Type II metacaspases

In total eight metacaspase genes were identified in tomato genome from ITAG2.4 based annotated database using Arabidopsis metacaspase genes as query sequences [23]. Two of the eight tomato metacaspases, Solyc09g098150.3.1 and Solyc10g087300.1.1 were identified as type II metacaspases. The multiple sequence alignment and phylogenetic analysis showed that Solyc09g098150.3.1 and Solyc10g087300.1.1 were found close homologues of AtMC4 and AtMC9 in Arabidopsis; therefore, named as SolycMC4 and SolycMC9, respectively. The lengths of SolycMC4 and SolycMC9 coding sequences were found to be 3064 and 1928 bp encoding polypeptides of 416 and 320 amino acids length, respectively. Isoelectric points of SolycMC4 and SolycMC9 was on the acidic pH with pI values 4.84 and 5.77, respectively. SolycMC4 and SolycMC9 genes were located on chromosome 9 and 10, respectively. SolycMC4 consist of two exons separated by one intron, while SolycMC9 had three exons interrupted by two introns.

In order to understand the transcriptional regulation of type II metacaspases during development, potential cis-elements in their promoter sequences were analyzed. Several light responsive elements such as Box-4 and TCT-motif were identified in the promoter regions. We have also identified some additional cis-acting elements associated with light stress include G-box, AE-box, GT1 and chs-CMA1a.

Expression analysis of SolycMC4 using qRT-PCR

qRT-PCR was performed to analyze the expression of identified candidate metacaspases during plant development. For expression analysis various developing tissues like; root, hypocotyl and cotyledons from 15 dag old seedlings and various vegetative and reproductive tissues from 60 dag old mature tomato plants were used. Immature leaves, young leaves, mature leaves and senescent leaves; outer layer of stem (cortex and phloem), inner layer of stem (xylem); abscission zone of flowers; eight developmental stages of flowers (Developing flower bud 1, Developing flower bud 2, Developing flower bud 3, Immature flower 1, Immature flower 2, Immature flower 3, Mature flower, Senescent flower); eight developmental stages of fruits (Immature fruit 1, Immature fruit 2, Immature fruit 3; Mature green fruit, Mature breaker fruit, Mature turning fruit, Mature pink fruit, Mature red fruit) were taken for RNA extraction and

cDNA synthesis. qRT-PCR expression analysis showed that SolycMC4 was strongly expressed in the roots, hypocotyl and cotyledons of the seedlings. In mature tomato plants, its expression was also observed in all stages of developing leaves, stem, and roots. The expression was also observed in phloem and xylem tissues of stem. During reproductive development, SolycMC4 was strongly expressed in all stages of developing flowers and fruits. As compare expression of SolycMC9 was specifically detected in late stage of unopened flower to early stages of open flowers. In developing fruits, its expression was found mainly in late stage of developing fruits. Also, expression level of SolycMC9 was relatively higher in early stages of developing leaves and in xylem layer of stem. In short, the expression analysis showed that SolycMC4 gene was consistently and predominantly expressed in all developing tissues of tomato plants. On the basis of expression analysis, SolycMC4 was selected for further characterization in tomato.

Spatiotemporal expression analysis using PromSolycMC4::GUS lines

The spatiotemporal expression pattern of SolycMC4 during development was analyzed using PromSolycMC4::GUS histochemical assays. For generation of promoter::GUS lines, 870 bp sequences upstream of SolycMC4 initiation codon was PCR amplified and first cloned into the entry vector pDONR207 (Invitrogen, USA) then recombined into the Promoter-GUS destination vector pMDC163 [24] The positive clones were transferred into *Agrobacterium* cells followed by their transformation in tomato. The GUS histochemical analysis showed that SolycMC4 expression was observed in most tissues of 4 weeks old young tomato plants. The expression was observed in leaves, petioles, stem and primary and lateral roots. The expression was also seen in the meristematic regions of stem and roots. The expression was also consistently observed in the vascular tissues of roots, leaves and stem. Transverse sections of the stem showed promoter expression in epidermis, cortex, interfascicular fibers and in developing vascular bundles and pith of the stem. Within the cortex, expression was observed in chlorenchyma, collenchyma and parenchyma tissues. The expression was also seen in phloem, cambium and xylem tissues of the vascular system. Also, promoter expression was observed to be induced by wounding and oxidative stress (H_2O_2) indicating that SolycMC4 also play a role in plant defense responses.

Generation of amiRNA-SolycMC4 expressing gene silencing lines

To gain an insight into the functions of SolycMC4, 35S::amiRNA-SolycMC4 gene construct was generated and transformed into tomato cv. MicroTom to mediate suppression of its mRNA using endogenous RISC mediated post-transcriptional gene silencing mechanism in plants. Expression of amiRNA precursors under the control of a strong, constitutive promoter in transgenic plants leads to an accumulation of mature amiRNAs which guide the RISC to cleave their target genes. WMD3 artificial microRNA designer tool was used to design amiRNA sequences based on the input gene sequences of SolycMC4 [25] Overlapping PCR was carried out in two steps to generate a miRNA precursor in which an endogenous miRNA of MIR319 and complementary strand present in pRS300 vector was replaced with an amiRNA sequence of the candidate SolycMC4 [26]. The chimeric sequence was first cloned in entry vector pDONR207 followed by in plant specific vector pMDC32 by Gateway cloning [24]. Further the recombinant vector carrying 35S::amiRNA-SolycMC4 was transformed to *Agrobacterium tumefaciens* strain GV3101pMP90 by electroporation followed by screening of transformed cells by colony PCR. The PCR verified Agrobacterium cells carrying 35S::amiRNA-SolycMC4

construct was co-cultivated with isolated cotyledons from 10 dag old *in vitro* grown tomato seedlings for genetic transformation as procedure described [15]. A total of four independently transformed 35S::amiRNA-SolycMC4 T1 lines were selected on MS media supplemented with 20 mg/l Hygromycin. These lines were PCR verified for the presence of amiRNA-SolycMC4 construct insert.

qRT-PCR analysis was performed to verify the level of silencing of SolycMC4 in 35S::amiRNA-SolycMC4 expressing lines. The expression analysis showed that transcript of SolycMC4 was significantly suppressed in amiRNA lines. Confirmed lines were grown *in vitro* and in controlled growth room for further characterization.

Phenotypic characterization of amiRNA-SolycMC4 expressing lines

To determine the developmental role of SolycMC4, amiRNA-SolycMC4 expressing lines were grown in both *in vitro* and in pots under controlled growth room conditions for further phenotypic characterization. When grown in *in vitro*, amiRNA lines (T1, T2, T3 and T4; after which T1 and T4 selected for detailed study) initially produced more vigorous growth than the control plants. Their subsequent growth was reduced as compared with control resulting in a healthy dark green dwarf phenotype of the plants. The length of amiRNA plants was approximately 38.6 ± 8.1 mm, while control was 63.6 ±7.02 mm in length after 60 days of *in vitro* culture. The difference in length was about 39.4% shorter for the amiRNA line plants. The internode length of stem of these plants was also compared. We observed that average internode length of amiRNA lines (3.4 ± 1.1mm) was 34.7 % reduced than the control plants (5.2 ±1.3 mm). The diameter at base of main stem was also measured. The results showed that stem diameter of amiRNA lines (7.04 ± 0.64mm) were thicker than those of the control (6.11 ± 0.63 mm). This increase in stem diameter was approximately 15.2% more in amiRNA lines than the control. Similar growth patterns of amiRNA-SolycMC4 plants were also observed when grown in the pots.

To further evaluate the increase in the diameter of stem, a series of paraffin embedded stem sections was prepared and observed under a Zeiss discovery V.12 stereo microscope and Zeiss axioplan 2 microscope and images were captured using an axiocam camera. We observed the significantly increased cell number and cell size in the parenchyma of cortex of amiRNA plants. However, there was no such obvious difference observed in vascular tissues and pith. This increase in thickness of stem was appeared to be resulted from both increased in cell number and cell size in cortical layer of amiRNA lines indicating that SolycMC4 may have some role in the stem development.

The morphology of leaves was also altered, as they appeared to be vigorous in amiRNA-SolycMC4 lines. We observed that leaves in amiRNA lines were highly pigmented and thick in texture than the control plants. These alterations in leaf morphology prompted us to investigate further into the anatomical details of leaves. Thin parafilm embedded sections of mature leaves were made from both control and amiRNA lines and observed under axioplan 2 microscope. We observed that in comparison with the control, photosynthetic mesophyll cells were more in number and bigger in size in the leaves of amiRNA lines. However, we observed no difference in other cell layers of the leaves. Like stem, the increase in size of leaves was appeared to be

resulted from increased number and size of cells in amiRNA plants, reflecting the overall increased thickness of their leaves.

We observed that when grown in pots, amiRNA-SolycMC4 lines showed delayed leaf yellowing than the control plants. This indicates that rate of chlorophylls degradation during senescence of leaves was delayed in amiRNA lines. To evaluate whether senescence programs are affected in these plants, two known senescence associated genes, SGR1 (Stay Green protein1) and SAG12 (Senescence Associated Genes 12) were used as markers to analyze the onset of senescence. SAG12 encodes the senescence specific cysteine protease; it is upregulated as per the progression of leaf age and also as leaf senescence progresses [16]. SAG12 is commonly used senescence marker as it was specifically induced during early senescence. SGR1 is highly conserved in plants and its knockout resulted into stay green phenotype. SGR1 is associated with thylakoid membrane of chloroplasts and silencing of this gene leads to dark green coloration. Silencing of LeSGR1 is found to inhibit chlorophyll degradation in tomato [17]. Also, SGR1 gene is negative regulator of ROS production and chloroplast stability. Expression levels of SGR1 and SAG12 genes during different developmental stages of leaves of control and amiRNA lines were 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 program 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 indicate that SolycMC4 function in modulating senescence and act upstream or modulate pathways upstream of the senescence.

Autophagy can contribute to the development of plants. It is a proteolytic process that can eliminate defective proteins and organelles from the cells and have both pro-survival and pro-death roles [27]. In plants, autophagy is implicated to have roles in xylem development, embryogenesis and senescence [28]. As shown previously, Metacaspase MC-Pa II acts upstream of autophagy and along with autophagy required for PCD of suspensor during embryogenesis in spruce [18]. A similar activation of autophagy pathway downstream of metacaspase was also reported during tracheary elements development in Arabidopsis [18]. To examine whether silencing of SolycMC4 also affected autophagy, expression of three well known autophagy marker genes, ATG5, ATG8 and TOR1 (Target of Rapamycin) was analyzed in amiRNA-SolycMC4 lines. Both ATG5 and ATG8 are known to be positive regulators of autophagy, whereas TOR is its negative regulator [19]. qRT-PCR analysis showed that expression of both ATG5 and ATG8 increased significantly in amiRNA lines. However, as expected, expression of TOR1 was reduced in the amiRNA lines. This indicates that silencing of SolycMC4 resulted in increased autophagy in amiRNA-SolycMC4 plants.

Biochemical characterization of type II Metacaspase

Morphological characterizations showed that amiRNA expressing lines appeared highly pigmented and have stay green phenotype. To investigate this further, we measured the anthocyanin and chlorophyll contents in the leaves of amiRNA-SolycMC4 plants. Consistent with the phenotype results, total chlorophyll and anthocyanin levels of amiRNA lines was significantly higher than the control plants. We observed that both anthocyanin and chlorophyll

content was approximately two-fold increase in amiRNA lines. This higher chlorophyll content could be due to a greater number of chloroplasts or larger size of the chloroplasts in cells of amiRNA-SolycMC4 lines.

In order to understand whether silencing of SolycMC4 in tomato resulted in alteration in ROS contents in plants, we also examined H_2O_2 content in plants using DAB (3,3'-diaminobenzidine) histochemical staining. The H_2O_2 content in leaves was also measured spectrophotometrically. Our results showed significantly reduced H_2O_2 content in amiRNA expressing lines suggesting that the silencing of SolycMC4 may have resulted in reduced ROS activity in plants.

Plant cells possess ROS scavenging enzyme system to counterbalance excessive H_2O_2 production, which includes antioxidant enzymes such as catalase (CAT), peroxidase (PXD), ascorbate peroxidase (APX) and superoxide dismutase (SOD). The antioxidant enzymes activity was measured biochemically in amiRNA expressing lines and control plants. We observed 23% increase in SOD activity, whereas activity of CAT, PXD and APX was also dramatically higher with approximately 200, 400 and 200-fold increased activity, respectively, in silencing lines than control plants.

qRT-PCR of CAT, SOD, APX and Guaiacol peroxidase (GP) genes in amiRNA-SolycMC4 lines and control was also analysed to quantify the expression pattern of these REDOX regulator genes. Consistent with the enzymatic activity, expression analysis also showed that APX, CAT, PXD and SOD genes were upregulated in silencing lines.

Subcellular localization of SolycMC4

To localize the SolycMC4::GFP fusion protein in subcellular compartment, SolycMC4 coding sequence was PCR amplified and cloned into pDONR207 and subsequently recombined into pGWB5 [29] for generation of C::terminus-GFP fusion construct. For subcellular localization, the fusion protein was transiently expressed in the protoplast isolated from cell suspension culture of tomato cv. Microtom and visualization using Zeiss LSM510 laser scanning confocal microscope. Transiently transformed protoplast shows that SolycMC4::GFP fusion proteins were localized in cytoplasmic compartment of cells. Protein prediction localization with WoLF PSORT tool also predicts that SolycMC4 is localized to the cytoplasm.

Conclusion

Amongst all metacaspases, SolycMC4 was found to be most expressed during development in tomato. Therefore, we aimed to characterize the roles of SolycMC4 in plant development using various molecular and biochemical based approaches. The spatial and temporal expression analysis using PromSolycMC4::GUS lines shows that the SolycMC4 was expressed in most of the developmental tissues including leaves, stem and roots. The expression was also seen in the cortex and vascular tissues of stem and mesophyll cells of leaves. For functional characterization, we have generated several independent 35S::amiRNA-SolycMC4 expressing lines, when grown in soil shows more vigorous and stay-green phenotype. Also, the stem of amiRNA expressing plants was thicker and slightly shorter in length providing the plant a very robust and healthy phenotype compared to its control. In addition to these, leaves of silencing lines shows dark coloration due to higher accumulation of chlorophyll and anthocyanin

pigments in the plants. We further observed that silencing of SolycMC4 resulted in increases antioxidant enzyme activity and delayed leaf senescence. The expression of senescence associated marker genes, SGR1 and SAG12 was found to be down regulated, where expression of autophagy marker genes, ATG5 and ATG8 increased in amiRNA lines. This show that the onset of senescence, a cell death program was delayed which resulted in vigorous and dark green growth of plants. This also indicates that SolycMC4 act upstream or modulates pathways upstream of the senescence and autophagy. Other possible explanation for such growth phenotype would be that gene silencing of SolycMC4 triggered more biogenesis or recycling chloroplast due to altered ROS signaling. There is another possibility that silencing of SolycMC4 leads to less sensitive of plants to some specific proteolytic process which altered the senescence and autophagy pathways resulted in overall vigorous growth of the plants. How SoylcMC4 influence plant development and crosstalk and regulate these pathways needs to be further investigated.

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