# INTRODUCTION

## 1.1 Plant development and PCD

Plant development process is regulated by the activity of apical meristems. Shoot apical meristem regulates the development of above-ground part of the plants, whereas root apical meristem gives rise to underground roots. Both the apical meristems consisted of pools of undifferentiated stem cells generating different lateral organs and tissues. Stem cells can renew themselves and support the growth throughout the life cycle of plants. Lateral meristem "vascular cambium" produces vascular tissues, phloem and xylem and is responsible for growth in girth of the mature stem and roots. There are two developmental phases of plants, first is the vegetative development where the plant produces stem, leaves and roots and later transition from vegetative to reproductive development promotes flower and embryo development. Both the development phases involve programmed cell death (PCD) that is needed for the normal development of the plants. PCD is a biological cellular activity seen in a majority of eukaryotic organisms including plants that govern cell death in a well-maintained and genetically regulated fashion, causing the death of specific cells, tissues, and whole organs for the developmental homeostasis of the organisms (Dickman et al., 2017; Kabbage et al., 2017). To maintain cellular or tissues homeostasis, PCD regulates the number of cells by removing damaged or unwanted cells. In plants, PCD stimulates the hormone signals, Reactive Oxygen Species (ROS) production, and also the expression of several transcriptional factors, proteases, lipases, nucleases, and transporters in plants, which have roles in PCD signalling pathways, cell differentiation, nutrition mobilization, protein maturation, and destruction (Kabbage et al., 2017; Maizel, 2015; Stael et al., 2019).

PCD occurs both during development and in reaction to its environment. Developmental PCD can be seen at various stages of vegetative and reproductive organ and tissue differentiation as well as during the senescence of leaves and floral organs. Developmental PCD regulates xylem tissues differentiation, phloem maturation, aerenchyma formation, root cap formation, floral organ abortion in unisexual flowers, gametogenesis, embryogenesis, seed maturation, leaf, sepal and petal senescence (Bennett et al. 2010; Filonova et al. 2002; Gadjev et al. 2008; Huysmans et al. 2017; Maizel 2015). Xylem tracheary elements (TEs), transporting water and minerals, is one of the most suitable examples of tissue developed via PCD (Watanabe & Lam, 2005). Plants also use cell death as a defence mechanism when they are exposed to adverse abiotic and biotic conditions (Huysmans et al., 2017; Kurusu & Kuchitsu, 2017; Van Hautegem

et al., 2015). The most established model of PCD in animals is known as apoptosis, which is characterized by chromatin condensation, membrane blebbing, chromosomal DNA breaking, cytoplasm condensation, and nucleus fragmentation. Contrary to these, plant PCD mainly goes through cytoplasmic shrinkage and nucleus fragmentation (Greenberg & Yao, 2004; Woltering, 2010). Where as in plants, PCD is also linked with vacuolar collapse (Bonneau et al., 2008), which is an important feature of PCD during TE formation which suggests that plant PCD is quite different from apoptosis in animals. PCD also plays an important role during plant defence responses against various pathogens and is also regulate in gametic self-incompatibility (Bonneau et al. 2008; Love et al. 2008). In addition, PCD is the key mechanism that occurs asymmetrically between the abscission zone proximal and distal side of the abscission progression (Bar-Dror et al., 2011). PCD also occurs in response to virus-induced hypersensitive responses (Hatsugai et al., 2004). The oxidative damage caused by $H_2O_2$, UV radiation and methyl viologen also induce cell death in plants (He et al., 2008). Cell death in plants is facilitated by proteases that can cause proteolysis. This research focuses on these enzymes and their role in plant development.

### 1.2 PCD and Proteases

Protein degradation, also known as proteolysis, is the process of breaking down proteins by enzymes called proteases to cleave them. Cleavage is a regulatory mechanism that can either stimulate or inhibit protein activity or can destroy it. The active site of each protease is made up of a catalytic dyad or trio including S1 Pocket. The substrate binds to the pocket, causing the peptide to be cleaved by the catalytic dyad/ triad. Water penetrates the active site, causing the remaining substrate inside the pocket to be released. Protease inhibitors, such as Inhibitors of Apoptosis (IAPs) and serpins, are used by cells to limit protease activity and consequently the amount and rate of PCD. (Vercammen et al., 2006). In PCD and plant defence against pathogens and herbivores, protease inhibition is used as a regulatory step. Proteases come in a variety of forms, such as serine, aspartate, and cysteine proteases. Proteases have been demonstrated to have varied substrate specificities, as well as different protease cleavage preferences, after certain amino acids. A histidine amino acid deprotonates a nucleophilic cysteine in cysteine proteases, and subsequently the cysteine nucleophile cleaves the substrate's peptide link. The presence of an asparagine residue helps to orient the histidine side chain in the right direction. Cysteine proteases include enzymes like papain and caspases, which are implicated in mammalian apoptosis.

## 1.3 Caspase-like Proteases

Caspases are cysteine-dependent aspartate-specific proteases found in metazoans. After an aspartate amino acid residue, caspases cleave substrates. *Caenorhabditis elegans* produced the first caspase, and fourteen distinct caspases have since been identified in mammals, with seven of those involved in apoptosis. Caspases are synthesized as zymogen, which is an inactive pro-enzyme. Caspases are then auto-processed (auto-cleaved), resulting in the formation of p20 and a small p10 subunit, which causes structural change that allows the substrate to reach the active site for cleavage. Caspase-like activities have been shown to occur in a variety of ways in plants (Bonneau et al. 2008). These caspase-like activities in plants are named as YVADase, DEVDase, VEIDase, and Saspase based on target substrates (Woltering, 2010). Although the whole spectrum of caspase- activities in plants is difficult to identify, it is thought that up to eight distinct activities have been found in different plant species (Bonneau et al. 2008). In plants, caspase-like activity has been discovered in cell organelles which include vacuole, cytosol, and nucleus. However, whether the caspase-like actions identified in plants are actually related to PCD has been a source of debate (Bonneau et al., 2008). In plants, identity of cysteine proteases with caspase-like activity has only been determined for YVADase, which is dependent on vacuolar processing enzymes (VPEs) to some extent (Hatsugai et al., 2004). The VPEs are held inactive in the lytic vacuoles until they are activated after collapse of vacuoles that causes PCD (Bonneau et al., 2008).

## 1.4 Identification of Metacaspases (MCs)

After identification of caspases in metazoans, similar proteases were also discovered in other organisms including plants. The two of the most interesting caspase-like proteases discovered were paracaspases (PCs) and metacaspases (MCs) ( a G. Uren et al., 2000). PCs are reported to exist in metazoans and slime molds, whereas MCs only found in plants, algae, fungi, chromista and protozoa ( a G. Uren et al., 2000). Initially, only two types of MCs (type I and type II) were thought to exist, however, recently a third type was also discovered, termed type III MCs (Choi & Berges, 2013). In addition to MCs, which all contain p20 and p10 domains, two further classes containing only the catalytic p20 domain have also been identified. The metacaspase-like proteins have also been identified in bacteria (Choi & Berges, 2013; a G. Uren et al., 2000). Bacterial metacaspase like proteases have recently been named orthocaspases (Klemenčič & Funk, 2018). Type I MCs contain a proline-rich prodomain which is structurally similar to the prodomain found in metazoa caspases. Type I MCs are found in

all evolutionary lineages between algae to higher plants and also exist in protozoa, fungi and chromista (Cambra et al. 2010) Type II MCs, on the other hand, lack a prodomain and instead have a longer linker region connecting the p20 and p10 domains than type I MCs. Type II MCs are not present in fungi, chromista, or any other evolprotozoa; they are only present in plants and algae (with the exception of a few algae) (Cambra et al. 2010; Uren et al. 2000). The dearth of type II MCs in the few algae is assumed to be owing to environmental adaptation-induced gene loss (Cambra et al. 2010). There is currently no known relationship between the number of type I and type II MCs in different plant species. The inclusion of an LSD1-like finger N-terminal motif in type I is another significant variation between MCs. LSD1 has been found to prevent cell death when the hypersensitive response is initiated, and all known *Arabidopsis thaliana* type I MCs can strongly interact with LSD1. The *Arabidopsis* type II MCs can only weakly interact with LSD1 (Coll et al., 2010).

## 1.5 Role of Metacaspases (MCs)

### 1.5.1 Role of yeast metacaspases

Yeast genome encodes for MC, YCA1 and its loss of function be directly linked with insoluble protein aggregates during physiological growth. This accumulation of the protein aggregates leads to induction of the autophagic pathway (Lee et al., 2010). Also, the presence of the poly-Q motif in the YCA1 prodomain dictates its role for localization in protein aggregates (Lee et al., 2010). In *Aspergillus fumigates,* deletion of the two MC genes (*CasA and CasB*) causes hypersensitivity to ER stress (Richie et al., 2007), however, no effect on growth was found with the ΔcasA/ΔcasB mutants under induced oxidative stress conditions. These results indicate that some of the MCs may involve in a clearing of the protein aggregates and enhancing the survival of cells and organisms. Besides these, some MCs may be important for the cleavage of specific cellular targets that can have an important role in controlling ER stress.

### 1.5.2 Role of plant metacaspases

Identification of MCs in fungi, protozoa, and plants has offered the opportunity to understand their potential roles in cell death pathways. The first functional characterization of MC has come from a study in yeast, where only MC, named YCA1 displays induced caspase-like proteolytic activity when yeast cells were stimulated by $H_2O_2$ to undergo apoptosis (Madeo et al., 2002). The role of the first plant MC was reported in Norway spruce where silencing of MCII-Pa causes suppression of terminal cell differentiation and cell death in embryo suspensor cells resulting in developmental arrest during embryogenesis (Bozhkov et al., 2005).

Characterization of plant metacaspases was done in *Arabidopsis* and some other plants. In the last decade, several reports have uncovered the roles of MCs in *Arabidopsis*. Its type II MC, AtMC8 shown to be involved in the regulation of PCD induced by oxidative stress (He et al., 2008). Another *Arabidopsis* AtMC4 plays a positive regulatory role in biotic and abiotic stress-induced PCD (Watanabe & Lam, 2011). The Arabidopsis type I MCs, AtMC1 and AtMC2, antagonistically control PCD, which means AtMC1 acts as a positive regulator, whereas AtMC2 negatively regulates cell death. It has been shown that manipulation of the Arabidopsis type I MC can nearly eliminate the hypersensitive cell death response (HR) activated by plant intracellular immune receptors (Coll et al., 2010). In *Capsicum annuum*, CaMC9 plays a role in pathogen-induced cell death (Kim et al., 2013). TaMCA4, a novel wheat MC gene functions in PCD induced by a fungal pathogen (Wang et al., 2012). In tomato MC, LeMCA1 is upregulated during PCD in *Botrytis cinerea* infected leaves (Hoeberichts et al. 2003) however its precise role in *B. cinerea* induced PCD is yet to be characterized. Out of nine MCs reported in *Arabidopsis,* AtMC9 does not show any requirement for calcium for auto-activation *in vitro* (Vercammen et al., 2004). It shows optimal activity at acidic pH of 5.5, whereas AtMC4, AtMC5 and AtMC8 all exhibit optimal activity at around pH 7.5 and require calcium for their activity (He et al., 2008; Watanabe & Lam, 2011). By yeast two-hybrid system AtSerpin1 (serine protease inhibitor) has been identified as a known substrate inhibitor of activated AtMC9. Both AtMC9 and AtSerpin1 were localized in apoplast supporting their interaction (Vercammen et al., 2006). However, the dynamics of the interaction between AtMC9 and AtSerpin and the level of their binding/ interaction remain unexplored. In zymogen form, a specific S-nitrosylation of site 2-1 cysteine residue occurs *in vivo* with overexpression of AtMC9 in *Arabidopsis.* This post-translational modification was reported to suppress auto processing as well as proteolytic activity of AtMC9 (Belenghi et al., 2007). Whether this nitrosylation process regulates other type II MCs as well as Type I MCs needs to be determined. In AtMC4 the peptide mapping after calcium activation has been found that a basic residue (K225) in the core region of a linker is required for AtMC4, and a point mutation inhibits autolytic activation of recombinant AtMC4 (Watanabe & Lam, 2011). The Calcium dependence and subsequent loss of activity in vitro have also been found in type I MCs of *Trypanosoma brucei* (Moss et al., 2007). The detailed study of these processes in MCs could uncover their mechanistic similarities.

### 1.5.3 Non death role of metacaspases

Other than the PCD related roles, a non-death role for MCs has also been reported. In the protozoa, *T. brucei* non-death roles of MCs were found (Shrestha and Megeney 2012). However; how this non-death role is regulated is very poorly understood. It would be interesting to uncover any such role of MCs in plants.

## 1.6 Tomato crops and their genetic resources

Tomato is one of the most important vegetable crops belonging to the Nightshade family Solanaceae. It is extensively used as a genetic model system to study the development, physiology, biochemistry, genetics and ripening of tomato fruits (Giovannoni, 2001). Tomato is a diploid plant with 12 pairs of chromosomes and the size of its genome is 799.09 Mb (Su et al., 2021). The complete genome of tomato cultivar Heinz 1706 was sequences that encoded for 34,384 predicted protein-coding genes (Su et al., 2021). The center of origin of tomato, has been confined to the Andes mountains and the Pacific coast region. Based on work from the Tomato Genome Consortium 2012, the three wild species, viz., red-fruited species *S. pimpinellifolium* and the orange-fruited species, *S. galapagense* and *S. cheesmaniae* are considered most closely related to cultivated tomato (Menda et al. 2013).

In the year 2019, total production of tomatoes were 181 million tones worldwide, with China producing 35%, followed by India and Turkey as main tomato-producing countries (https://www.fao.org/). Tomatoes are one of the three most important crops on the Indian government's "Top" priority list of crops, along with onions and potatoes.

Other than the tomato genome database several other genetic resources and tools are also available for tomato crop improvements and genetic research which includes, tomato wild species and mutant collections; various genetic stocks; multiple marker collections; BAC libraries, TILLING populations; tomato microarrays, and other expression data; (Barone et al., 2008).

Tomato cultivars are exposed to various diseases. The most common diseases of tomato are late blight, early blight, verticillium wilt, fusarium wilt, nematodes, tobacco mosaic virus, damping off, Septoria leaf spot, bacterial wilt, bacterial leaf spot, Tomato leaf curl, tomato spotted wilt and grey mould.

Many important commercial fruits, like strawberries, apples, melons, mango and bananas share more or less similar characteristics to the developmental processes of tomato fruits. Therefore,

it is most likely that the availability of tomato genome sequences and genetic resources may allow the identification of novel genetic regulators which could be used to further improve the qualities of tomatoes and all other fruits.

### 1.7 Tomato metacaspases

In the tomato genome, eight metacaspases have been identified (Dubey et al., 2019; Liu et al., 2016), out of which six are type I and two are type II metacaspases and function of none these have been characterized in plants. Some of these metacaspases are shown to be differentially expressed during development and are induced by various environmental stresses (Hoeberichts et al., 2003; Liu et al., 2016). Despite work done so far, none of the tomato metacaspases have been characterized in plants. Functional characterization of these metacaspases could increase our understanding of this important family of proteases. Some of these may likely have roles in developmental PCD and others could be parts of stress-induced cell death responses in plants. These metacaspases could also be targeted to improve various plant traits such as seedless fruits, delaying leaf and flower senescence, delayed fruit ripening, male sterility, and disease resistance in tomatoes.

### 1.8 Aim and Objectives

In the present work, the role of type II MCs was analysed in tomato by using molecular genetics and biochemical approaches.

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.