Characterization of *Petunia x hybrida* 'Mitchell Diploid' Metacaspases during Petal Senescence

DISSERTATION

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Abstract

Senescence is the final stage of plant development. It is a genetically controlled process leading to organ or whole plant death. The death process at the cellular level is called programmed cell death (PCD). Caspases play a key role in PCD in animals. Although caspase-like activities have been reported in plants, caspase homologs have not been identified in plant genomes. Instead, metacaspases, which are structurally similar to caspases, have been found in fungi and plant genomes *in silico*. In this study, a type I (PhMCA1) and a type II (PhMCA2) metacaspase were identified from *Petunia x hybrida* 'Mitchell Diploid' and characterized at the molecular level. Both Petunia metacaspases possessed similar structural properties characteristic of caspases including small and large subunits and a highly conserved histidine/cysteine catalytic dyad. When a catalytic cysteine residue was replaced by alanine, enzymatic activities of both metacaspases were reduced. Caspases cleave at aspartate residue, however petunia metacaspases cleave at an arginine residue. Inhibitor studies using a cysteine specific inhibitor showed that both petunia metacaspases were cysteine proteinases. Transcript levels of *PhMCA1* and *PhMCA2* were quantified by real-time RT-PCR. Expression of *PhMCA1* increased as petal senescence progressed, while *PhMCA2* transcripts were most abundant early during petal development. Western blots showed that protein abundance correlated with RNA

abundance of both metacaspases during petal senescence. The PhMCA1 protein appeared to undergo processing, while processing of PhMCA2 was not observed. Catalytic cysteine mutants of *PhMCA1* and *PhMCA2* expressed in *E. coli* showed no enzymatic activity due to the lack of protein processing. Overexpression and underexpression studies using RNAi and VIGS (Virus Induced Gene Silencing) showed no delayed or accelerated petal longevity compared to wild type flowers. In tomato two type I and one type II metacaspases were identified, a close relative of petunia. Findings in this study will lay the groundwork for future identification of the direct substrate(s) of the petunia metacaspases and enhance our understanding of the function of metacaspases in petal senescence.

Dedication

To my Lord, parents, brother, sisters and wife

Acknowledgments

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Fields of Study

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Chapter 1:

INTRODUCTION

Overview of Petal Senescence

Senescence is the final stage of plant development leading to the ultimate death of

organs or the whole plant. Floral senescence in flowering plants is important to reduce

energy usage and to recycle macromolecules. Floral senescence also protects the plant

from the pathogen attack because the stigma often serves as an entry point for pathogens

(Rogers, 2006). During petal senescence, a plethora of genes associated with nutrient

remobilization and responses to environmental stresses (including transcription factors

and signal transduction regulators) are up- or down-regulated (Jones et al., 2009).

Among those genes that are associated with petal senescence, no single gene responsible

for cell death has been identified and the functional role of only a few genes that are

associated with transcriptional regulation and signal transduction during petal senescence

are known (van Doorn and Woltering, 2008; Jones et al., 2009).

There are two hypotheses on what triggers petal senescence. Tonoplast rupture is

a well studied cause of cell death during plant development. In petal senescence it is also

speculated to be the immediate cause of cell death (van Doorn and Woltering, 2008).

During petal senescence, hydrolytic enzymes in vacuoles are released into the cell and

rapidly degrade most of the contents including the plasma membrane. In *Iris* the

cytoplasm contents and most of the organelles, including the endoplasmic reticulum

(ER), attached ribosomes and golgi apparatus, are lost (van Doorn et al., 2003). The

nucleus, some mitochondria and plastids remain intact until a later stage of petal

senescence in carnation (Yamada et al., 2006a, 2006b; van Doorn et al., 2003). Nuclear

fragmentation was also found in the petals of ethylene sensitive (*Petunia x hybrida*)

(Langston et al., 2005) and insensitive (*Argyranthemum frutescens*) flowers, but not in

the petals of *Antirrhinum majus* (Yamada et al., 2006b). Disappearance of the tonoplast

membrane preceded that of the plasma membrane (van Doorn et al, 2003).

Another possible cause of cell death is autophagy. Autophagy is a highly

conserved process among eukaryotes by which the macro and micronutrients in the cell

are remobilized (van Doorn and Woltering, 2010). Occurrence of autophagy in plants is

strongly suggested by morphology of senescing cells, biochemical, and gene expression

data during senescence (Verheye et al., 2007). Several experiments have revealed the

function of autophagy genes. Knockout autophagy (ATG) mutant lines in Arabidopsis

showed delayed cell death and accelerated leaf yellowing (Hofius et al., 2009; Wada et

al., 2009). Leaf yellowing symptoms were suspected as a result of stress due to the

absence of autophagy (van Doorn and Woltering, 2008).

Regulation of Senescence

Senescence can be regulated at the level of transcription and translation as well as

at the post-translational level including protein activation, phosphorylation by kinases,

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and protein degradation (van Doorn and Woltering, 2008; Jones et al., 2009). The eukaryotic translation initiation factor 5A (eIF-5A) is an example of the post-translational regulation of senescence (Duguay et al., 2006). In order for eIF-5A to be activated, a conserved lysine residue is converted to hypusine by deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DHH) (Wang et al., 2001). eIF-5A is a translation initiation factor, and it is involved in the regulation of senescence by selective recruitment of mRNAs from the nucleus (Xu et al., 2004, van Doorn and Woltering, 2008).

Protein activation is required for certain proteases to become active. For example, cysteine proteases need to be activated through autocatalytic activation in the vacuole which triggers protein degradation in the proteasomes (van Doorn and Woltering, 2008). Another example of protein activation is the processing of metacaspase proenzymes. Inactive metacaspases are activated by cleavage of the unprocessed zymogen into a small and large subunit (Watanabe and Lam, 2005).

Ethylene Signal Transduction and Regulation of Petal Senescence

Ethylene exists in the gaseous state and is produced from essentially most parts of plants, including leaves, stems, roots, flowers, fruits, tubers, and seedlings. Ethylene is biologically active at lower concentrations and regulates many important aspects of plant life including growth, development, germination, responses to various stresses and senescence (Yang and Hoffman, 1984). In ethylene-sensitive petal senescence, a rapid production of ethylene accompanied by increased respiration coincides with the onset of visible wilting. It has been speculated that a signal triggered by pollination causes the

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ethylene production that results in accelerated flower senescence, (van Doorn and Woltering, 2008). Ethylene is synthesized from methionine and ATP to make *S*-adenosylmethionine (SAM) by SAM synthetase, which is converted to aminocyclopropanecarboxylate (ACC), the direct precursor of ethylene, by ACC synthase. ACC oxidase converts ACC to ethylene. ACC synthase and ACC oxidase genes in this pathway are upregulated during ethylene-sensitive petal senescence (Jones, 2004; Jones et al., 2009; Hoeberichts et al, 2007; van Doorn and Woltering, 2008). Prolonged flower longevity was reported by decreasing the level of *ACC oxidase* transcripts in cut carnation flowers (Kosugi et al, 2002) and by decreasing the abundance of *ACC synthase* transcripts in transgenic carnation flowers (Michael et al., 1993; van Doorn and Woltering, 2008).

Ethylene is perceived by ethylene receptors located in the plasma membrane and they play a major role in the ethylene signaling pathway. There are five ethylene receptors in Arabidopsis (ETR1, ETR2, ERS1, ERS2 and EIN4) (Chang et al., 2003; Mason and Schaller, 2005). Overexpression of mutated ethylene receptors in *Petunia hybrida* (Wilkinson et al., 1997), *Dianthus caryophyllus* (Bovy et al., 1999), and *Nemesia strumona* (Cui et al., 2004) petals delayed senescence. Increased abundance of *DC-ETR1* and *DC-ERS2* mRNA was observed in carnation petals when flowers are fully opened, and level of *DC-ETR1* expression did not change while transcripts of *DC-ERS2* decreased in senescing petals. Application of exogenous ethylene did not change mRNA abundance of these two genes although petal senescence was accelerated. These results indicated that *DC-ETR1* and *DC-ERS2* are ethylene independent (Shibuya et al., 2002).

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Ethylene was suggested to be the causal signal for petal senescence and the ovary might be the source of this ethylene. Expression of ACC synthase and ACC oxidase was first observed in ovary, then in the style and lastly in the petals when excised flower parts of unpollinated cut carnation flowers were examined (ten Have and Woltering, 1997; van Doorn and Woltering, 2008). However, the production of ethylene in petunia petals after pollination was first observed in styles, then ovaries and lastly corollas (Jones et al., 2009).

A breakthrough discovery of a role of the ovary during petal senescence came from developing a rather simple technique of carpel removal (Shibuya et al., 2004). Removal of the carpel by hand instead of cutting off by forceps or scissors delayed petal senescence in carnations without carpels and a lower level of ethylene production was observed. On the other hand, when the carpel was cut off, the flowers showed accelerated wilting and a high level of ethylene production, indicating that wounding seemed to cause ethylene production. These experiments strongly suggested that the carpel is the source of ethylene production which triggers the senescence of carnation petals (Shibuya et al., 2000; Nukui et al., 2004; van Doorn and Woltering, 2008).

It has been suggested that in unpollinated flowers ethylene production leading to petal senescence depends on the combination of sensitivity of cells to their basal level of ethylene and cytokinin reduction in ethylene sensitive petal senescence (van Doorn and Woltering, 2008; Jones et al., 2009). Overexpression of isopentenyltransferase (*ipt*) in petunia resulted in prolonged life of petunia flowers. In *ipt* overexpressing flowers, induction of endogenous ethylene production by pollination was delayed and ethylene

sensitivity was reduced(Chang et al., 2003). According to previous experiments, carpels have been known as a central regulator of petal senescence and it is strongly desired to investigate the relationship between ethylene production and levels of cytokinins in carnation carpels (van Doorn and Woltering, 2008).

Programmed Cell Death

Programmed cell death (PCD), a term which was coined by Lockshin and colleagues in 1964, is a genetically controlled developmental and stress response event. Tight genetic control of this program ensures that initiation and execution of PCD occurs only in the required cells at the right time (Sanmartin et al., 2005). Deregulation of PCD in plants is often associated with developmental aberration or lethality (Lorrain et al., 2003). PCD is known to be ubiquitous throughout eukaryotes including single cell organisms (Sanmartin et al., 2005). Functional and morphological similarities of PCD exist in animals and plants. In plants, PCD is associated with embryo morphogenesis, abscission, phloem and xylem formation and defense against pathogens (Woltering et al., 2002; Sanmartin et al., 2005). Discovery of some of the molecular components of PCD in animals and plants strongly suggests a common origin of PCD (Sanmartin et al., 2005). Expression of genes associated with PCD from animals exhibit similar effect on PCD in plants or yeast (Sanmartin et al., 2005; Watanabe and Lam, 2005).   Lacomme and Santa Cruz (1999) reported that Bax, a death-promoting member of the Bcl-2 family, triggered cell death when expressed in plants. Mitsuhara et al. (1999) showed that Bcl-x(L), animal cell-death suppressors, inhibited cell death in tobacco plants, and AtBI-1, a plant

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homologue of Bax inhibitor-1, suppressed Bax-induced cell death in yeast (Sanchez et al., 2000). Homologs associated with apoptosis control [*Defender against Apototic Death-1* (*DAD-1*)] and *Bax-inhibitor-1* in animals are also found in plant genomes and they are also involved in PCD (Sanmartin et al., 2005). When the Bax inhibitor was over-expressed in rice, it suppressed the fungal elicitor-induced cell death (Matsumura et al., 2003). Danon et al. (2004) reported that PCD induced by ultraviolet-C overexposure in Arabidopsis could be suppressed by caspase inhibitors, p35 and Dad. Caspases are known to be the central prosecutor in animal PCD, yet no animal caspase homologs have been found in plants or fungi (Sanmartin et al., 2005).

Caspases & Caspase-like Activity in Plants

Caspases are cysteine containing aspartate specific proteinases in animals, metazoans, insects and nematodes (Shi, 2002). Inactive, unprocessed caspase zymogens are self-processed to active caspases by PCD promoting signals. Caspases activate the cell death program via an irreversible proteolytic cascade. Even though there have been no caspases identified from plant genome data bases, PCD in most plants studied so far is induced by caspase-like activities and can be inhibited by caspase inhibitors (Sanmartin et al., 2005; Lam and del Pozo, 2000).

No caspase homologs have been found in plant genomes but proteases containing caspase-like features have been identified. Examples are the vacuolar processing enzyme (VPE)(Nakaue et al., 2005), metacaspases (Vercammen et al., 2004), saspases (Coffeen and Wolpert, 2004) and phytaspase (Chichkova et al., 2010). Although there is limited

sequence homology between caspases and caspase-like proteases, they share similar structural properties.

VPEs are involved in the processing of seed storage proteins (Shimada et al., 2003) and regulating protein degradation during senescence (Rojo et al., 2003). VPEs have caspase-like activity and have been shown to regulate cell death in *Nicotiana tabacum* and *Arabidopsis thaliana* (Hatsugai et al., 2004; Rojo et al., 2004). Caspase activity of VPEs from Nicotiana may initiate PCD during TMV infection by promoting vacuolar rupture (Sanmartin et al., 2005). Four members of the VPE gene family (VPE$_{\alpha - \delta}$) were identified in the Arabidopsis genome. Predicted modeling of the catalytic residues of Arabidopsis VPEγ aligns with those of caspase-8 (Sanmartin et al., 2005). VPEγ is activated inside of the vacuole through autoprocessing and then it activates vacuolar proteases including cysteine proteases. Increased ion leakage was observed after Arabidopsis overexpressing VPEγ were challenged with *Pseudomonas syringae*. These findings indicated that VPE was involved in plant pathogen interaction through a vacuole mediated PCD (Rojo et al., 2003, 2004; van Doorn and Woltering, 2008). Caspase inhibitors inhibited self-maturation of VPEγ from Arabidopsis and presumably inhibited all of the downstream *in vivo* activities (Rojo et al., 2004). Another gene, VPE$_\delta$, was transiently expressed in a tissue specific manner in Arabidopsis seed coat cell layers. When VPE$_\delta$ was mutated, cell death of the specific layers of the seed coat was delayed (Nakaune et al., 2005). VPE transcripts were up-regulated during carnation petal senescence (Hoeberichts et al., 2007). Thus, VPEs appear to be involved in plant PCD processes including biotic stress response and developmental processes.

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Another type of caspase-like protease, saspase or serine-dependent aspartate-specific protease was discovered as one of the proteases that is involved in cleavage of Rubisco during victorin-induced PCD (Coffeen and Wolpert, 2004). Saspases are expressed constitutively and exhibit caspase specificity, unlike the other serine proteases that utilize aspartate as a substrate at the P1 position. As the name suggests, saspase activity is inhibited by serine protease inhibitors, which are not effective on caspases (Coffeen and Wolpert, 2004). The role of saspase activity in plant senescence remains unknown.

Recently, Chichkova et al., 2010 discovered novel PCD related subtilisin-like proteases, phytaspase or plant aspartate-specific protease, from tobacco and rice. Phytaspase possesses a caspase specificity and requires processing for activation. Phytaspase localizes in the apoplast prior to being re-imported back into the cell during PCD. Although phytaspase is known to be essential for PCD-related responses to tobacco mosaic virus and abiotic stresses, the role of phytaspase in senescence is unknown (Chichkova et al., 2010).

Regulation of Caspase Activity in Plants

Animal caspases exist as inactive zymogens, which are later processed and activated, leading to irreversible PCD in response to death signals. The regulation of caspases in animals is mostly posttranslational. In plants, VPEs and metacaspases are upregulated after reception of external cues such as pathogen attack, senescence, or accumulation of reactive oxygen species, suggesting the regulation of their expression is

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partly at the transcriptional level (Rojo et al., 2004). It is also possible that they are post-translationally regulated by processing of the zymogens. A processing requirement for metacaspase activities has been shown in recombinant AtMCA1b, AtMC4, AtMCA2b and AtMC9 (Vercammen et al., 2004; Watanabe and Lam 2005). Thus, regulation of VPE and metacaspases may be either posttranslational or transcriptional in plants (Sanmartin et al., 2005). It has been speculated that VPEs maintained in an unprocessed form in intermediate non-acidic compartments of the secretory pathway may be transferred to the vacuole where they are activated and promote vacuolar collapse (Sanmartin et al., 2005). Induction of the expression of caspase-like enzymes may be even before the death signal triggers PCD and caspase-like activity may depend on processing of inactive zymogens into the active form or by subcellular relocation (Sanmartin et al., 2005)

Localization of Caspases-like Proteins

Most animal caspases are cytoplasmic proteins with the exceptions of caspase-2 and caspase-12 (Sanmartin et al., 2005). Caspase-12 is localized to the endoplasmic reticulum (ER) and activated by membrane ER stress (Nakagawa et al., 2000), while caspase-2 is localized in the nucleus (Lassus et al, 2002). In the cytosol, a proteolytic cascade is initiated after all animal caspases are activated. This cascade results in the activation of downstream enzymes that degrade cellular components and eventually kill the cell (Sanmartin et al., 2005). All type II metacaspases (AtMCP2a through AtMCP2f) in Arabidopsis are predicted to be found in the cytosol due to a lack of signal peptides or

transmembrane domains, while type I metacaspases are predicted to be localized in the

mitochondria (AtMCP1a and AtMCP1c) and the chloroplast (AtMCP1b) (Sanmartin et

al., 2005). Localization of these enzymes needs to be experimentally confirmed due to

the low confidence level of this prediction. For example, VPEs contain signal peptides

for insertion into the endomembrane system but have been found in protease precursor

vesicles (PPVs) or vacuoles (Rojo et al., 2003).

Metacaspases

Metacaspases are structurally related to the caspases and have been identified in

plant, fungal and protozoan genomes by iterative PSI-BLAST searches (Uren et al.,

2000). Metacaspases are classified as type I and type II. The type I metacaspases differ

from the type II metacaspases in that there is an N-terminal prodomain consisting of a

zinc finger motif and proline-rich domain, while no obvious prodomain is found in the

type II metacaspases. Both metacaspases consist of a large (p20) and small (p10)

subunits (Figure 1.1). Three type I and six type II metacaspases were identified from the

Arabidopsis genome. *In silico* evaluations have reported that *AtMCP1a* and *AtMCP2f*

are induced in senescing flower (Sanmartin et al., 2005). Two *Arabidopsis* metacaspases

(*AtMCP1b* and *AtMCP2b*) activate apoptosis-like cell death in yeast under H_2O_2 stress

and aging (Watanabe and Lam, 2005), and *LeMCA1* (tomato type II metacaspase) is

upregulated during *B. cinerea* infection (Hoeberichts et at., 2003). mc*II-Pa* metacaspases

(type II) from *Picea abies* have caspase-like activity during embryo pattern formation

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(Bozhkov et al., 2004). The experimental data suggested that metacaspases play a role in developmental or stress-induced PCD in plants (Sanmartin et al., 2005).

The type I and II metacaspases from Arabidopsis (AtMCP1a, AtMCP2a) are known to cleave their substrates at arginine/lysine but not caspase-specific substrates (Vercammen et al., 2004; Watanabe and Lam, 2005). These findings suggest that metacaspases could activate downstream caspase-like activities required to mediate cell death activation (Watanabe and Lam, 2005) and they are not directly responsible for caspase-like activities in plants (Vercammen et al., 2004).

We investigated whether petunia metacaspases played a role during flower development. Petunias are an excellent model system for studying flower senescence. Petunias have a well defined petal senescence scheme and large corollas. It is relatively easy to transform Petunias using Agrobacterium mediated transformation. A broad range of molecular tools are currently available, including genomic and EST information, microarray data and cDNA and insertion libraries (Jones et al., 2009; Gerats and Vandenbussche, 2005).

The goal of this study was to investigate the role of petunia metacaspases during petal senescence. The first part of this study was to clone and characterize metacaspases from petunia petals and the second part of the research was to investigate the biochemical properties of the metacaspases using recombinant proteins. The specific objectives for the first part of the study were (chapter 2):

1. To clone and sequence metacaspases from *Petunia x hybrida* 'Mitchell Diploid';

2. To investigate expression of metacaspases in ethylene sensitive and insensitive genotypes;

3. To investigate protein abundance and processing during petunia petal senescence;

4. To localize metacaspases in petunia petals; and

5. To elucidate the functional role of metacaspases utilizing RNAi, VIGS and overexpression techniques.

The specific objectives of the second part of the study were (chapter 3):

1. To investigate the enzymatic activity of petunia metacaspases (PhMCA1 and PhMCA2) utilizing fluorogenic substrates and protease inhibitors;

2. To determine optimum pH of PhMCA1 and PhMCA2; and

3. To investigate the effect of autoprocessing on enzymatic activity utilizing mutants containing mutations at catalytic cysteine residues.

The information obtained from this study will enhance our current knowledge of the role of metacaspase in petal senescence and it will also allow us to engineer petunias with increased blooming time.
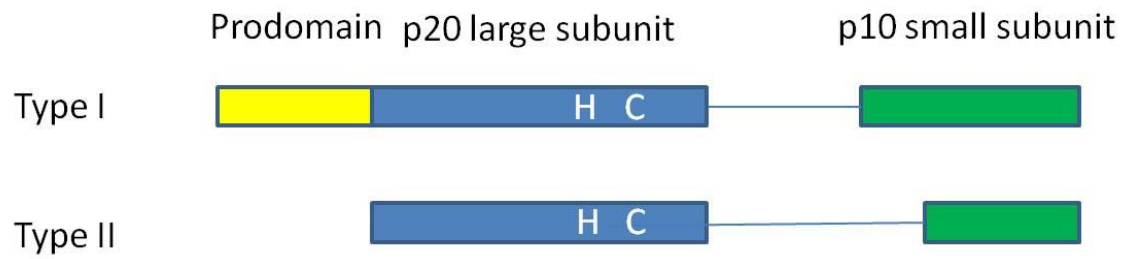
Figure 1.1. Schematic diagram of type I and type II metacaspases. Prodomain, p20 large subunit and p10 small subunit are depicted in yellow, blue and green, respectively. H and C indicate a histidine and a cysteine residue of dyad, respectively.

Chapter 2:

CLONING AND FUNCTIONAL ANALYSIS OF *PETUNIA X HYBRIDA* 'MITCHELL

DIPLOID' METACASPASES DURING PETAL SENESCENCE

Abstract.

Previous research has suggested "caspase-like" activity might be involved in

programmed cell death processes during plant development including organ senescence

and abiotic and biotic stress responses. Metacaspases, saspases (serine proteases) and

legumain family proteases (vacuolar protease enzymes, or VPEs), have been identified as

putative candidates for "caspase-like" enzymes. The metacaspases, which are structural

homologs of caspases in animals, are found in plants, fungi and protozoans. Two

putative metacaspases were cloned from petunia and sequence alignment analysis

revealed that they were a type I and a type II metacaspase. Expression analysis during

petal development showed that *PhMCA1* transcripts increased at a later stage of petal

senescence, while transcript abundance of *PhMCA2* was higher at the early to mid stage

of flower development. Western blot analysis suggested the occurrence of

autoprocessing in PhMCA1, while PhMCA2 did not appear to undergo autoprocessing.

Immunolocalization localized both metacaspases to the vascular bundles. Over-/under-

expression of petunia metacaspases did not result in phenotype changes in flower longevity and the metacaspases seem unlikely to play a key role in regulating petal senescence. The role of PhMCA1 and PhMCA2 in petal senescence of petunia awaits further investigation.

Key words: metacaspases, PCD (programmed cell death), petunia, senescence

Introduction

Plants constantly remove unnecessary cells to recycle their resources, to respond to biotic or abiotic stimuli or to undergo differentiation through programmed cell death (PCD). PCD in plants plays an important role during embryogenesis (Filonova et al., 2000), xylogenesis (Suarez et al., 2004), self-incompatibility and senescence (Thomas and Franklin-Tong, 2004; Carimi et al., 2004). Senescence, a type of programmed cell death (PCD) at the organ level, is a genetically controlled event and the last stage of the plant development. The gaseous hormone ethylene is a key regulator in plant senescence.

Caspases, cysteine dependent aspartic acid specific proteases, play a key role in PCD in animals. Initiator caspases activate downstream caspases (executioners) and these executioner caspases activate various proteins involved in PCD signal pathways leading to cell death. In plants, caspase-like activities have been observed, but their corresponding gene(s) have not been identified. The candidate caspase-like enzymes include vacuolar processing enzymes (VPE) (Hatsugai et al., 2004), saspases (serine protease), phytaspases and metacaspases (Chichkova, 2010).

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Metacaspases were discovered by a structure-based iterative search PSI-BLAST in plants and yeast (Uren et al., 2000). As suggested from structural similarity between caspases and metacaspases, there are common characteristics including the presence of a large subunit (p20) and a small subunit (p10). Cleavage of procaspases (processing) is required for their activity in animals and a similar processing event using recombinant metacaspases of yeast (Madeo et al., 2002) and Arabidopsis (Vercammen et al., 2004 and Watanabe and Lam 2005) has been reported. Another similar characteristic between metacaspases and caspases is the presence of a highly conserved histidine/cysteine dyad at the catalytic site. Mutation of this cysteine residue abolishes enzymatic activity by inhibiting processing (Madeo et al., 2002; Vercammen et al., 2004; Watanabe and Lam, 2005).

According to the structural differences, metacaspases are divided into two groups (type I and type II). Type I metacaspases possess a prodomain at the N terminus, which is either short or not present in type II metacaspases. The prodomain contains a zinc finger motif, which is also present in LSD1, a regulator of the hypersensitive response in plants (Dietrich et al., 1997) and is speculated to interact with other proteins. Due to the structural similarity, metacaspases have been predicted as a functional analog to caspases. Madeo et al., (2002) demonstrated that the single yeast metacaspase YCA1 exhibited caspase-like autoprocessing of the protein and increased caspase-like activity was correlated to induced apoptosis during aging and oxidative stress. Overexpression of YCA1 increased cell death, which was prevented by the caspase-specific inhibitor zVAD-fmk. Disruption of YCA1 abolished apoptosis caused by oxidative stress or

aging. Despite the structural and functional similarities with caspases, however, metacaspases have a different substrate preference. They cleave target sites with alkaline amino acids, arginine or lysine, at the P1 position instead of aspartate as caspases utilize (Degterev, 2003, Vercammen et al., 2004, Watanabe and Lam, 2005, He et al., 2008).

Metacaspases in PCD have been suggested to be involved in abiotic/biotic stress responses or developmental processes. Overexpression of *AtMC1b* (*AtMC2*) and *AtMC2b* (*AtMC5*) in the yeast YCA1 mutant accelerated apoptotic cell death, suggesting Arabidopsis metacaspases could complement the YCA1 mutant defective in PCD during oxidative stress and aging (Watanabe and Lam, 2005). *AtMC1b* (Castillo-Olamendi et al., 2007) and *LeMCA1* (tomato type II metacaspase, Hoeberichts et al., 2003) were up-regulated when plants were challenged with *Pseudomonas syringae* and *Botrytis cinerea*, respectively. The functional role of Arabidopsis metacaspases in oxidative stress was suggested. Overexpression of *AtMC8* accelerated PCD induced by UVC or H_2O_2, while reduced cell death was observed in protoplasts when *AtMC8* was knocked-out (He et al., 2008).

Involvement of metacaspases in plant development was also demonstrated in a series of experiments in Norway spruce. Bozhkov et al. (2004) demonstrated that VEIDase activity (cleavage at the aspartate residue) coincided with embryonic cell death during somatic embryogenesis, while inhibition of the VEIDase activity resulted in abnormal embryo development. Metacaspase (*mcII-Pa*) was subsequently isolated and the expression of *mcII-Pa* was observed by *in situ* hybridization in suspensor cells that are committed to cell death (Suarez et al., 2004).

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Petunia x hybrida 'Mitchell Diploid' was chosen as a model plant to investigate the role of metacaspases in flower senescence. Petunia flowers have advantages over Arabidopsis flowers in that petunias have larger corolla and petunia petals wilt when they senesce while Arabidopsis flowers abscise before wilting. Petunias have a well defined schedule of petal senescence and are relatively easy to transform (Gerats and Vandenbussche, 2005). During petal senescence, proteases, oxidative enzymes and nucleases are activated and such activation results in a loss of membrane permeability, increase in reactive oxygen species, degradation of proteins and nucleic acids, and decrease in protective enzyme activity (van Doorn and Woltering, 2008; Jones et al., 2009; Sachs and Vartapetian, 2007).

During senescence in petunia, cell death is accelerated by ethylene. The increased production of ethylene stimulates petal senescence resulting in wilting and discoloration of the petals. In order to investigate the effect of ethylene on the expression pattern of the metacaspases, an ethylene insensitive petunia line (*35S:etr1-1*, ethylene response gene) was also included. The Petunia *35S:etr1-1* line was generated by overexpressing the mutated ethylene receptor gene isolated in Arabidopsis linked to the CaMV35S promoter, conferring ethylene insensitivity (Chang et al., 1993). We hypothesized that petunia metacaspases play a role in regulating petal senescence. We have cloned and sequenced one type I (*PhMCA1*) and one type II (*PhMCA2*) metacaspase from petunia and their expression pattern in ethylene sensitive and insensitive (*35S:etr1-1*) petunias was examined during petal senescence. Protein abundance and processing events were investigated by Western blot analyses and immunolocalization was conducted to localize

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the metacaspases in senescing and non senescing petals. We also performed functional

analysis using RNAi, VIGS (Virus Induced Gene Silencing) and overexpression of both

metacaspases.

The goal of this study was to clone and characterize petunia metacaspases

during petal senescence. The specific objectives to achieve the research goal were:

1. To clone and sequence metacaspases from *Petunia x hybrida* 'Mitchell Diploid';

2. To investigate metacaspase gene expression in ethylene sensitive and insensitive

   petunias;

3. To investigate protein abundance and processing during petunia petal senescence;

4. To localize metacaspases in petunia petals; and

5. To elucidate the functional role of metacaspases utilizing RNAi, VIGS and

   overexpression techniques.

Materials and Methods

Cloning of Petunia Metacaspases

For *PhMCA1* cloning, a coding region of *PhMCA1* was amplified by PCR using cDNA

prepared from senescing petunia corollas (7 days after anthesis) as a template with

primers indicated in Table 2.1. RNA was isolated using TRIzol® (Invitrogen, Carlsbad,

CA) according to the manufacturer's instruction after 0.5 g of corollas was ground in

liquid nitrogen. The insert was digested and ligated into pET28a(+) vector at BamHI and

HindIII sites. The construct (pET28a(+)::PhMCA1) was made and the entire coding

region and the cloning sites were sequenced and confirmed. For *PhMCA2* cloning,

partial sequences of 5' (562 bp; 117 bp of 5' untranslated and 445 bp of coding regions)

and 3' (338 bp; 197 bp of coding and 141bp of 3' untranslated regions) ends of petunia

type II metacaspase (TC2403 and TC3508/866, respectively) were obtained from TIGR

data base (TGIP Computational Biology and Functional Genomics Laboratory,

http://compbio.dfci.harvard.edu/) by a key word "metacaspase" search. A pair of primers

(F-PhMCA2_3 and R-PhMCA2_4) was designed to obtain the *PhMCA2* sequences. PCR

product was sequenced and a pair of primers were designed to clone 5' and 3' ends of

PhMCA2 by RACE (5′ and 3' RACE System for Rapid Amplification of cDNA Ends,

Version 2.0, Invitrogen Cat. No. 18374-058). A pair of primers (F-PhMCA2pET and R-

PhMCA2pET) were used to clone the entire coding region of *PhMCA2*. AtMC1

(GenBank Accession AY322525) and AtMC9 (GenBank Accession AY322531) were

obtained from the Arabidopsis Biological Resource Center (ABRC, The Ohio State

University) and re-cloned by PCR using primers with BamHI and XhoI site extensions

(Table 2.1 for primers). Primers were designed to clone the coding regions of AtMC1

and AtMC9, respectively. Each PCR fragment was ligated into pET28a(+) at BamHI and

XhoI sites. pET28a(+)::AtMC1 and pET28a(+)::AtMC9 were constructed and verified

by sequencing.

Alignment of Metacaspases

Coding regions of metacaspases from tomato (*Lycopersicon esculentum* AY114141),

Arabidopsis (*Arabidopsis thaliana* AtMC1 AY322525; AtMC2 AY322526; AtMC3

AY322527;AtMC 4 AY322528; AtMC5 AY322532; AtMC6 AY322533; AtMC7

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AY322529; AtMC8 AY322530; AtMC9 AY322531;), Scots pine (*Pinus sylvestris* EU513166), Norway spruce (*Picea abies* AJ534970), rice (*Oryza sativa* AC092075), and corn (*Zea mays* EU973061) were obtained from NCBI. Sequencing of the coding regions of the petunia metacaspases was performed at the Molecular and Cellular Imaging Center at The Ohio State University (Wooster, OH). Alignment of metacaspase sequences and unrooted tree (Phylip's Drawtree) were generated by ClustalW.

Expression Analysis of PhMCA1 and PhMCA2 During Petal Senescence

Plant Materials: *Petunia x hybrida* 'Mitchell Diploid' (MD) and *35S::etr1-1* (line 44568) were grown in the greenhouse. *35S::etr1-1* was generated by overexpressing a mutated form of the ethylene receptor (Wilkinson et al, 1997). Greenhouse temperatures were maintained at 21 to 24ºC during the day and 18 to 16ºC at night. Natural light was supplemented by high-pressure sodium and metal halide lamps (GLX/GLS e-systems GROW lights, PARSource, Petaluma, CA). Plants were watered daily with 150 mg N·L^{-1} 20N-3P-19K (Jack's Professional^{TM} Water-Soluble Petunia FeED; J.R. Peters, Inc., Allentown, PA). Six corollas from anthesis until petals wilted (every day up to 10 days for MD and every 3rd day up to 22 days for *35S:etr1-1* lines) were collected in the morning between 10:00 and 11:00 and petal tissues were immediately frozen in liquid nitrogen. Half of collected corollas were ground in liquid nitrogen into a fine powder and total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA). The remaining MD corollas were used for protein preparation for Western blot analysis. cDNAs were synthesized using Omniscript® RT Kit (Qiagen, Valencia, CA) according to the

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manufacturer's protocol. Quantitative RT-PCR was performed using B-R SYBR® Green

SuperMix for iQ (Quanta BioSciences, Inc, MD, 170-8882BR) on Bio-Rad iQ™5 thermo

cycler. The temperatures and run time used for denaturing, annealing and polymerization

were 95°C for 15 sec, 60°C for 15 sec and 72°C for 20 sec, respectively in 20 μl reaction

volume. *PhACTIN* was used as an internal control. Parameters for designing primers for

qRT-PCR included primer/probe T_m, complementarity, and secondary structure as well as

amplicon size. Identical nucleotide runs was also avoided. Petunia expression data were

analyzed by normalization to the expression of *PhACTIN* using Bio-Rad iQ™5 optical

system software version 2.1.

Antibody Production and Western Blot Analysis of PhMCA1 and PhMCA2

Total proteins were prepared from a half of corollas collected for expression analysis.

Corollas were ground in liquid nitrogen to a fine power and the ground tissues were

dissolved in ice-cold 100mM sodium phosphate buffer (pH 6.2) with 20 mM DTT. After

removing debris by centrifugation at 10,000 g at 4°C for 15 minutes, the supernatant was

stored at -80°C. To raise antibodies specific to the p20 subunits of the petunia

metacaspases, amino acid sequences were selected from the conserved regions of the p20

subunits of PhMCA1 (CPKMLKGSVTDALSM) and PhMCA2

(CITDDDFRELVDKVP). Peptides were synthesized and antibodies were generated by

EZBiolab polyclonal antibody service (Westfield, IN) in rabbits. In similar way anti

PhMCA1 and PhMCA2 antibodies using the amino acid sequences selected from the

conserved regions of p10 subunits were generated. They did not yield any positive

signals and they were not used in this study. A cysteine residue was added to the N-terminal end of each peptide for KLH conjugation and affinity purification. Twenty micrograms of total protein was loaded in each lane on a 15% polyacrylamide gel (Bio-Rad Criterion Tris-HCl, Cat. No. 345-0019, Hercules, CA). After staining with Coomasie blue and destaining with deionized water, the proteins were transferred onto PVDF membrane (Bio-Rad Immuno-Blot PVDF membrane Cat. No. 162-0177, Hercules, CA) using the Genie transfer system (IdeaScientific, Minneapolis, MN) for 1.5 hrs at 12 Volts. Signals were detected using SuperSignal West Pico complete Rabbit IgG Detection Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instruction. Primary antibodies of PhMCA1 and PhMCA2 were diluted 500 and 1000 times, respectively and they were incubated with the membrane for 2 hours. ImmunoPureAntibody Goat Anti-Rabbit IgG Horseradish Peroxidase (Thermo Scientific, Waltham, MA) was used as secondary antibody and a working dilution was made at 1:60,000 or 0.6 µg/ml. Signal was visualized on a ChemiDoc XRS using Quantity One software version 4.6.7 (Bio-Rad, Hercules, CA).

Immunolocalization of PhMCA1 and PhMCA2 in Petunia Corollas

Petunia corollas were collected at anthesis and at 8 d after anthesis (late senescence stage). Petunia corollas were cut into 5x5 mm sections. Samples were fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer pH7.2 (PB) for 2-3 hours at room temperature prior to dehydration. Dehydrated samples were infiltrated with paraffin before being imbedded in paraffin blocks. Paraffin blocks were sliced into

40 µm thick ribbons using a TBS-Cut Rotary microtome (Leica ultracut UCT, Germany) at the Molecular and Cellular Imaging Center (OARDC, Wooster, OH). Twenty to thirty thin sections were placed on glass slides on the warm plate overnight at 30°C. Each glass slide contained two sections and one section was incubated with pre-immune serum as a negative control. At least 6 slides per treatment were prepared. Slides were de-paraffinized using xylene and then subsequently washed with decreasing concentrations of ethanol from 100% to 0%. Areas around the sections were dried while samples were kept wet. The section area was delimited with a Pap Pen (Invitrogen Zymed, CA). The slide was washed with PBS (10 mM of KH_2PO_4, pH7.4 and 150 mM of NaCl) or PBST (0.002% of Tween 20 added to PBS) once for 2-3 min before adding Blocking Solution (0.2% BSA, 5% Normal Goat Serum, 10 mM Sodium Azide in PBS) for 1-2 hrs at room temperature. The slides were washed briefly with Incubation Buffer (same as blocking solution without Normal Goat Serum) once for 2-3 min, then incubated with primary antibody at 1:400-800 in Incubation Buffer overnight at 4°C (or 1-3 hours at RT) in a humid chamber. After the sections were washed at least 6 times (for 2-3min each) with PBST, they were incubated with secondary antibody (AlexaFluor488 goat anti-rabbit IgG, 1:600 dilution, Invitrogen, Carlsbad, CA) in Incubation Buffer for 2-3 hours at room temperature in a humid chamber. The sections were washed at least 6 times (for 2-3min each) with PBST. Sections were incubated with propidium iodide (Invitrogen) for less than 2 min to stain nuclei, then washed twice with PBST. Coverslips were placed on the sections with Gel/Mount (Biomeda, Foster City, CA). Images were visualized at 20X

magnification at gain 2 setting with Leica MD IRB epifluorescent microscope (Wetzlar, Germany). These settings were applied to all samples.

RNAi, VIGS and Overexpression of PhMCA1 and PhMCA2

For RNAi, PCR products from the *PhMCA1* coding region (450 bp; 563 bp to 1013 bp) were ligated in the inverted orientation flanking the GUS stuffer region in pGSA1276 shown in Figure 2.7 (ChromDB vector developed by Carolyn Napoli and Ross Atkinson, www.chromdb.org). PCR fragments of *PhMCA1* were ligated at the AscI and SwaI sites in the sense orientation and at the BamHI and SpeI sites in the antisense orientation. Leaf fragments (1 x 1 cm) of *Petunia x hybrida* 'Mitchell Diploid' were transformed with pGSA1276::PhMCA1 using *Agrobacterium tumefaciens* LBA4404 using a modified method from Jorgenson et al., 1996. Callus was moved to shooting media and plantlets that emerged from callus were moved to rooting media. Those plantlets that rooted were transferred to soil in a plastic crisper under the light bench for acclimation. Prior to further acclimation in greenhouse conditions, a leaf was collected for PCR verification of successful transformation. The primers for PCR verification were designed to contain an intron in the *PhMCA1* so that PCR amplification could distinguish between the endogenous gene and the transgene. PCR was performed to screen positive transgenic plants. PCR primers for semi quantitative RT-PCR were designed from the 3' UTR to be gene specific (227bp; 1212bp to 1438bp) and used for expression analysis. All samples were taken after 25 cycles of PCR, which was within a linear amplification range and 5 µl of each PCR products were loaded on a gel for expression analysis. Expression levels

were determined by band intensities on the ethidium bromide stained gel. Control plants contained the vector only.

Virus induced silencing (VIGS) constructs were made using pTRV2 (gift from Dr. Dinesh-Kumar SP, Yale University) as a vector. PCR fragments of *PhMCA1* and *PhMCA2* using F-PhMCA1 VIGS and R-PhMCA1 VIGS, and F-PhMCA2 VIGS and R-PhMCA2 VIGS, respectively, were generated. PCR fragments of *PhMCA1* (356 bp in length of coding region) and *PhMCA2* (403 bp in length of coding region) contained XbaI and BamHI sites for *PhMCA1*, and BamHI and XhoI sites for *PhMCA2*. These fragments were cloned into the pTRV2::CHS construct at respective cloning sites to generate pTRV2::CHS-PhMCA1 or pTRV2::CHS-PhMCA2. For the double metacaspase knock-out construct, the PCR fragment of PhMCA2 was ligated at BamHI/XhoI sites in the pTRV2::CHS-PhMCA1 construct. All constructs were confirmed by sequencing. Control plants contained a pTRV2::CHS only. Longevity was determined by the number of days from anthesis until petal wilting. When CHS is down-regulated by VIGS, the petals of *Petunia x hybrida* 'Fantasy Blue' lose the blue pigmentation to become white petals depending on the severity of down-regulation of CHS (Napoli et al., 1990). For VIGS experiment, only petunias with white petals were evaluated.

Overexpression constructs were made using pJL36 (gift from Dr. John Lindbo, Campbells Seed, Sacramento, CA). The pJL36 vector contains a 35S promoter driven tobacco mosaic virus (TMV) expression vector that is designed to deliver a gene of interest by agroinfiltration (Lindbo, 2007). Fragments containing the entire coding

regions of *PhMCA1* and *PhMCA2* were generated by PCR using primers F-/R-pJL36PhMCA1 and F-/R-pJL36PhMCA2, respectively. Each fragment was cloned into the pJL36 vector at PacI/NotI sites to make pJL36::PhMCA1 and pJL36::PhMCA2, respectively. These two constructs were sequenced for confirmation. Control plants contained vector only (pJL36).

  Petunia x hybrida 'Fantasy Blue' were grown in the greenhouse from seeds. Greenhouse temperatures were 21 to 24ºC during the day and 15 to 18ºC at night. Natural light was supplemented by high-pressure sodium and metal halide lamps (GLX/GLS e-systems GROW lights, PARSource, Petaluma, CA). Petunias were grown until they had 4-5 true leaves. Agobacteria containing each construct was infiltrated from underside of the leaves using needless syringes.   agroinfiltration technique for both VIGS and overexpression experiments according to the procedure described in Lindbo, 2007. For simultaneous overexpression of two metacaspases, each agrobacteruim containing pJL36::PhMCA1 or pJL36::PhMCA2 construct was co-infiltrated through the underside of the leaves. Six to ten petunias with three to five young leaves per plant were infiltrated with agrobacteruim containing the corresponding construct described earlier. pTRV1 containing RNA dependant RNA polymerase was co-infiltrated with VIGS construct containing target gene(s) and p19 suppressor of RNA silencing was co-infiltrated for overexpression analysis.The longevity was measured by the number days from anthesis until flowers wilted. Longevity data were means of eighteen flowers per treatment in a completely randomized block design. Plants containing each construct were blocked by construct treatments and by replication for VIGS and overexpression.

Longevity data were analyzed by using Proc GLM (generalized linear model) with Tukey's standardized range test. Data were analyzed by SAS (Statistical Analysis System, SAS Institute, Inc., Cary, NC; SAS Institute, Inc., 2002–2003). Semi quantitative RT-PCR (semi qRT-PCR) was performed for 25 cycles to examine the expression levels of petunia metacaspases using cDNAs prepared from corollas which wilted at less than 13 days (short) and at longer than 17 days (long).

Results

Alignment of Metacaspases

The nucleic acid sequences of metacaspases from *Arabidopsis thaliana* (*AtMCP1* to *AtMCP9*), tobacco, rice, corn, wheat, Scots Pine, Norway spruce (*mcII-Pa*), and tomato (*LeMCA1*) were obtained using the NCBI database and two metacaspases were cloned and sequenced from *Petunia x hybrida* 'Mitchell Diploid'. The alignment of the corresponding amino acid sequences of metacaspases and the dendrogram is shown in Figure 2.1 and Figure 2.2, respectively. PhMCA1 was aligned with three of the *Arabidopsis* type I metacaspase proteins (AtMC1, AtMC2, and AtMC3) and amino acid sequences of AtMC3 were most closely aligned with those of PhMCA1. PhMCA2 was aligned with type II metacaspases (Figures 2.1 and 2.2). The highly conserved signature histidine/cysteine dyad of caspases was also found in all metacaspases aligned in this study. The sequence context of the catalytic histidine residue SG**H**G was conserved in all metacaspases aligned, and cysteine residues in DA**C**XSGT and SDS**C**HSGGLI were also conserved in all type II metacaspases aligned. Putative conserved aspartic acid residues in the substrate P1 position were also found as indicated by ▲. PhMCA1 possessed an N-terminal prodomain about 90 amino acids in length and type I metacaspases in Arabidopsis contained predicted lengths of 80 to 120 amino acids. Two putative zinc finger motifs (C*XX*C) were also found in PhMCA1 and are the other type I metacaspases aligned in this study. The prodomains of *Arabidopsis* metacaspases were proline (AtMC1 and AtMC2) or glutamine (AtMC3) rich, while the PhMCA1 prodomain did not contain either a proline or glutamine rich region. All type II metacaspases

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possessed a short or no prodomain. In both type I and type II metacaspases, there were

conserved regions of about 150 amino acids corresponding to the p20 and p10 subunits of

mammalian caspases (Vercammen et al., 2004). There was a linker region between these

putative p20 and p10 domains. The length of this region of type I metacaspases was

shorter than that of type II metacaspases, which varied in size from 90 to 150 amino

acids. The predicted processing site (Vercammen et al., 2004) at either arginine or lysine

residues were also conserved among type II metacaspases. The findings from the

alignment strongly suggested that PhMCA1 and PhMCA2 encode a type I and a type II

metacaspases, respectively.

Expression Analysis of PhMCA1 and PhMCA2 During Petal Senescence

Transcript levels of *PhMCA1* and *PhMCA2* were measured to investigate the pattern of

expression and abundance of petunia metacaspases during petal development using qRT-

PCR (Figure 2.4). The longevity of *35S::etr1-1* flowers was two or three times longer

than that in MD. Corollas were collected everyday and every third day from anthesis

until wilting from MD and *35S::etr1-1* lines, respectively. Expression of *PhMCA1* was

significantly increased at the wilting stage of petal development in MD, while transcript

levels of *PhMCA2* were higher at the early to mid stage of petal development (Figure

2.4). *PhMCA1* expression was maintained at the relatively low level until visible petal

senescence occurred in both genotypes and the level of *PhMCA1* expression was lower in

etr1-1 corollas at the wilting stage. Transcript levels of *PhMCA2* decreased as petal

senescence progressed in both genotypes. Overall expression of *PhMCA2* was lower in

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etr1-1 lines than MD. Transcripts of *PhMCA2* were more abundant than that of *PhMCA1*

in petunia petals, except at the wilting stage in both genotypes.

Western Blot Analysis of PhMCA1 and PhMCA2

Western blot analysis was conducted using total proteins isolated from petunia corollas to

investigate whether protein levels of *PhMCA1* and *PhMCA2* coincided with transcript

levels and to determine if processing occurred *in vivo*. Total proteins were prepared from

Petunia x hybrida 'Mitchell Diploid' corollas collected everyday from anthesis until

visible petal wilting. PhMCA1 was not detected until the wilting stage (lane 9 in Figure

2.5A), while protein levels of PhMCA2 were higher at the early to middle stage of petal

development (lanes 3 through 5 in Figure 2.5B). Protein abundance patterns of PhMCA1

and PhMCA2 correlated to the respective transcript patterns (Figures 2.4 and 2.5).

PhMCA1 was ~28 kDa, this was approximately 10 kDa smaller than the expected size of

the unprocessed protein (38.2 kDa). PhMCA2 was ~45 kDa, which was the expected

size of the unprocessed protein. These results suggested that PhMCA1 may undergo

processing, but that processing of PhMCA2 did not occur in petunia petals.

Immunolocalization of PhMCA1 and PhMCA2 in Petunia Petals

Localization of PhMCA1 and PhMCA2 was studied using anti-PhMCA1 and anti-

PhMCA2 antibodies specific to the p20 subunits. Both PhMCA1 and PhMCA2 were

detected around the vascular bundle in young (similar to the petal at anthesis in Figure

2.6) and senescing petals (similar to the petal at wilting in Figure 2.6). At anthesis both

metacaspases were visible throughout the petal including vascular bundles. PhMCA2

localized to epidermal cell layers and the vascular bundle, while PhMCA1 was detected

primarily around the vascular bundle at the wilting stage.

RNAi, VIGS and Overexpression of PhMCA1 and PhMCA2

To investigate the function of petunia metacaspases during petal senescence, expression

of PhMCA1 and PhMCA2 was either up-regulated by overexpression or down-regulated

by RNAi and/or VIGS. For VIGS, chalcone synthase (CHS) was used as a marker.

Flowers of the control (pTRV2::CHS) petunias showed longevity of 14.9 d $\pm$ 2.0 and the

longevity of flowers from all other treatments was not different from control flowers

(P>0.05). Longevity of petunia corollas was not altered regardless of manipulation and

target gene(s) using VIGS or overexpression (Table 2.2). The longevity of flowers,

either short (up to 13 days) or long (longer than 17 days), did not correlate with the

transcript levels of *PhMCA1*, *PhMCA2* or both *PhMCA1* and *PhMCA2* in VIGS or

overexpression analyses (Fig. 2.8). In addition to the transient downregulation of

PhMCA1, stable transformants were also generated using RNAi technology. The positive

RNAi lines, containing the inserts integrated into the genomic DNA, were screened by

PCR and their expression levels were examined. Three lines (6, 53, and 65) with near

knockout transcript levels of *PhMCA1* at T0 were chosen to be advanced to the T2

generation. The transcript levels of 6, 53 and 65 were 0.6%, 12.9%, and 5.7% of MD

control levels in senescing corollas at 48 h after pollination. The longevity of 6, 53 and

65 were 8.6 $\pm$ 1.39, 8.8 $\pm$ 1.63, and 7.5 $\pm$1.68, respectively and it was similar to that of

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the control plants (9.6 ± 1.34) (Figure 2.9).

Discussion

Metacaspases were structurally similar to caspases and they were speculated to be responsible for "caspase-like" activity in plants. Since the discovery of metacaspases in plants, research has focused on the biochemical characterization of these proteins, while the functional characterization of metacaspases *in vivo* is scarce. We identified one type I and one type II metacaspase from *Petunia x hybrida* 'Mitchell Diploid'. These petunia metacaspases contain the histidine/cysteine dyad, that is highly conserved among all caspases. PhMCA1 aligned with known type I metacaspases in Arabidopsis. PhMCA2 aligned with type II metacaspases found in Solanaceae (tomato and tobacco), monocots (rice, wheat and corn) and trees (pine and Norway spruce). PhMCA1 contains a prodomain which is found in Arabidopsis type I metacaspases. Although the role of these extended amino acids is not clear in plant metacaspases, they are speculated to play a role in protein-protein interactions between metacaspases and other proteins needed to activate downstream target proteins as in animal initiator caspases (Vercammen et al., 2004). The conserved zinc coordinating cysteine residues were identified in the prodomain in PhMCA1 and all type I Arabidopsis metacaspases by sequence comparison. These cysteine residues are also found in *LSD1* (Lesion Simulating Desease1), which is a negative regulator of the host-pathogen interaction in plants (Dietrich et al., 1997), suggesting that type I metacaspases are involved in the hypersensitive response. This assumption was reinforced by the finding that the upregulated expression of *AtMC1b*, one of the type I metacaspases in Arabidopsis, coincided with increased cell death responding to *P. syringae* infection (Castillo-Olamendi et al., 2007).

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The expression level of *PhMCA1* and *PhMCA2* was investigated during petal senescence. Petunia petal senescence is ethylene dependent and we included an ethylene insensitive line of petunia (35S::*etr1-1*), which expressed the mutated ethylene receptor, to determine whether ethylene played a role in regulating petunia metacaspases. Transcript abundance of *PhMCA1* and *PhMCA2* was reduced in ethylene insensitive lines, suggesting that ethylene is a positive regulator of the petunia metacaspase during petal development. However, expression of *PhMCA2* decreased in both genotypes during the progression of petal senescence, when ethylene production was expected to increase. These data suggested that the regulation of metacaspases during petal development was more likely controlled by multiple factor(s) involved in developmental process as well as ethylene perception. Timing of peak expression of *PhMCA1* is nearly at the end of petal wilting, indicating that *PhMCA1* may not play a role as a regulator during petal senescence, while *PhMCA2* could be involved in initiating the senescence process due to its relatively early expression.

Abundance of metacaspases detected by anti PhMCA1 and anti PhMCA2 antibodies correlated to the transcript abundance of both metacaspases (Figures 2.4 and 2.5), suggesting that synthesis of metacaspases during petal senescence is controlled at the transcriptional level.

The plant metacaspases characterized so far demonstrated that autoprocessing was required for their activation. We investigated whether PhMCA1 and PhMCA2 underwent autoprocessing during flower senescence by Western blot assay. The size of a single band corresponding to PhMCA1 (~29 kDa) appeared at the later stage of petal

senescence and was smaller than the expected size of the zymogen (38.2 kDa). This size

corresponded to the expected size of the prodomain plus the p20 subunit (Figure 2.5A),

indicating PhMCA1 may have undergone autoprocessing. It is, however, interesting to

note that the expected zymogen or unprocessed protein band was not detected. This

could be due to the quick processing of the zymogen and processing may have been

completed within 24 hours, which was the interval of sampling. The size of bands

detected by the anti PhMCA2 antibody corresponded to the expected size of the

PhMCA2 zymogen (45.4 kDa) (Figure 2.5B). Thus, PhMCA2 did not seem to undergo

autoprocessing, which could result in an inactive PhMCA2 during petal senescence.

Processing of the type I Arabidopsis metacaspase AtMC1b was required for cell death in

yeast (Watanabe and Lam 2005), while processing was not required for activity of

metacaspase 2 from *Trypanosoma brucei* (Moss et al., 2007). Therefore, investigation of

PhMCA2 activity *in vivo* and identification of the upstream factor(s) for PhMCA2

activation are desired for better understanding of the functional role of PhMCA2.

To investigate where petunia metacaspases are localized in corollas, we

conducted immunolocalization assays using corollas at anthesis and wilting (i.e

senescing) stages. Both metacaspases localized around vascular bundle in petunia petals,

suggesting that they may be involved in vascular differentiation. Involvement of

metacaspases in vascular cell differentiation was investigated by Suarez et al. (2004) and

Castillo-Olamentdi et al. (2007). The expression of the type II metacaspase (*mcII-Pa*) in

mature embryos of Norway spruce (*Picea abies*) coincided with the formation of

procambial strands which are precursors of all vascular cells leading to differentiation of xylem (Suarez et al., 2004). Castillo-Olamentdi et al. (2007) suggested the involvement of type I metacaspases in phloem sieve tube formation. They cloned the promoter region of *AtMCP1b* and found that it contains predicted motifs involved in the vascular gene expression. GUS expression driven by the promoter was detected mainly in the phloem tissue. Our localization data also suggested that petunia metacaspases may be involved in phloem differentiation.

Under- or overexpression of PhMCA1, PhMCA2 or both metacaspases was attempted to investigate whether petunia metacaspases were involved in regulation of flower longevity. Longevity of petunia corollas was not altered in RNAi, VIGS or overexpression experiments. Expression analyses showed that longevity of petunia corollas did not correlate with the expression level of either metacaspase. PhMCA1 RNAi lines with more than 80% reduction in transcript abundance compared to that of control lines at T2 generation also did not show prolonged longevity of flowers. The possible existence of gene or functional redundancy could prevent phenotype change from occurring. Genome search using tomato WGS scaffold 2.1 BLASTN (nucleotide to nucleotide) and coding sequence of *PhMCA1* as a query sequence indicated that there were two copies of type I metacaspase. In Arabidopsis genome there are three members of type I metacaspases. For VIGS or RNAi experiment, even though the inserts to down-regulate metacaspases were designed from the highly conserved regions of the p20 subunits of either metacaspase, they may not contain the sufficient length of exact

matching sequences to down-regulate possible additional metacaspase family members. Gene(s) that are functionally similar to metacaspase without sequence homology cannot be down-regulated with the VIGS technique. Although overexpression of a gene may be able to overcome the problem caused by either redundancy, overexpression does not always result in a phenotypic change. Post-transcriptional and/or post-translational control could play a role in regulating the activity of metacaspases. For example, overexpression of *AtMC8* did not result in spontaneous cell death, although expression of *AtMC8* was highly elevated by UVC and oxidative stresses (He et at., 2008). Another example of post-translational regulation of metacaspase activity is *S*-nitrosylation, the covalent bonding between a nitrogen monoxide group and the thiol side chain of cysteine residue through NO signaling pathway. Zymogens of AtMC9 were *S*-nitrosylated at the catalytic cysteine residue resulting in suppression of autoprocessing (Belenghi et al., 2007).

In Western blot analysis, PhMCA2 does not appear to undergo autoprocessing, which is known to be required for metacaspase activation in plants. PhMCA2 may not be able to be activated due to the absence of up-stream activator(s) or signal(s) for processing in the corollas. For PhMCA1, the expression of PhMCA1 was dramatically increased only at the wilting stage during petal senescence, suggesting that PhMCA1 may not play a role during petal senescence as an "initiator" in caspase cascade to activate down-stream proteins involved in programmed cell death process although the role as an "executioner" cannot be excluded. As suggested by the existence of a putative zinc

finger motif involved in hypersensitive response in the prodomain of type I metacaspases, PhMCA1 may play a role in responding to biotic stresses rather than in developmental process (Castillo-Olamendi et al., 2007). Thus, identification of the target substrates and up-stream signal(s) or activator(s) of PhMCA1 and PhMCA2 will greatly help to understand the functional role(s) of petunia metacaspases.

Conclusion

One type I (*PhMCA1*) and one type II (*PhMCA2*) petunia metacaspases were cloned and verified by sequencing. The amino acid alignment showed that they consisted of a large subunit (p20) and a small subunit (p10). Like caspases a highly conserved histidine/cysteine dyad was found in both petunia metacaspases. PhMCA1 possessed a prodomain, while no prodomain was found in PhMCA2. Petunia metacaspases were regulated differently during petal development. As petal senescence progressed, *PhMCA1* expression was increased, while expression of *PhMCA2* was decreased. The expression levels of both metacaspases were lower in ethylene insensitive lines (*etr1-1*) compared to MD background, while the patterns were similar to that observed in wild type petunias.   Thus, other developmental factor(s) in addition to ethylene were suggested to be involved in regulating both metacaspases. Western blot analysis revealed that protein abundance of petunia metacaspases was correlated to the transcript abundance during petal senescence, suggesting that their regulation was at the transcriptional level. The autoprocessing, which is known to be required for metacaspase activity, was not observed in PhMCA2, while PhMCA1 seemed to undergo

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autoprocessing. Over-/underexpression of either metacaspase did not yield a significant change in flower longevity. Both metacaspases were localized around the vascular bundles in the petals, suggesting that that petunia metacaspases might be involved in vascular formation or nutrient remobilization. Petunia metacaspases were identified and characterized at the molecular level. Although the exact functional role of either metacaspase during petal senescence is still not fully understood, the identification of direct substrate(s) of the metacaspases will allow us to elucidate the role of metacaspases.

Table 2.1. Primers used for cloning and characterization of petunia metacaspases.

Primer Names	Sequences
F-PhMCA2 3	TGTTTAGTCAACCGTTTCGGATTT
R-PhMCA2 4	CATAAACCTCCTGCTTAGCTTCAACAT
R-PhMCA25RACE	GAACTTTGTCCACAAGCTCTCTAAAATCA
F-PhMCA23RACE	GCAGGAGGAGATTCTTATGGTGCTC
F-PhMCA1pET	AATTAGGATCCGACGGCAGGAGATACAAATGCAAGTG
R-PhMCA1pET:	TTATGAAGCTTGCAAACGTACCTGAGTTTCTTTGGCACC
F-PhMCA2pET:	AAATTGGATCCGCAAAAAAAGCAGTGTTAATAGGAATCAACTATCC
R-PhMCA2pET:	CAGCAGAGCTCGCACACGAAAGGAGCATCAACG
F-PhMCA1 VIGS	GTCACTCTAGACCAACCAAGGCCAATATCAGATCAGC
R-PhMCA1 VIGS	ATAAAGGATCCGTACCTTTGTATGAGCTGATACGATGGTCTTC
F-PhMCA2 VIGS	TTGTAGGATCCACTGATGATTCTTACACACAACCGACAG
R-PhMCA2 VIGS	ACCGCCTCGAGCAAATGCATCTTCAACACTTCGACGT
F-pJL36PhMCA1	GTCTTAATTAAATGGACGGCAGGAGATACAAA
R-pJL36PhMCA1	ATTGCGGCCGCTTACAAACGTACCTGAGTTTC
F-pJL36PhMCA2	GTATTAATTAAATGGCAAAAAAAGCAGTGTTAA
R-pJL36PhMCA2	ATTGCGGCCGCTTAGCACACGAAAGGAGCATC

Table 2.2. Number of days from anthesis until flower wilting. *PhMCA1*, *PhMCA2* or both metacaspases were downregulated by VIGS or upregulated by overexpression. Control plants were infiltrated with pTRV2::CHS only for VIGS and empty vector (pJL36) for overexpression. *Petunia xhybrida* 'Fantasy Blue' was used as host plants.

Manipulation	Gene Targeted			
	Control	PhMCA1	PhMCA2	PhMCA1 & PhMCA2
VIGS	14.9 ± 2.0	13.9 ± 1.2	14.0 ± 1.6	14 .0 ± 2.0
Overexpression	14.4 ± 2.0	14.9 ± 1.9	13.4 ± 2.0	13.9 ± 1.9

Longevity was determined by the number of days from anthesis until wilting. At least 6 plants were infiltrated with each construct and more than 12 flowers were tagged per plant. This experiment was repeated at least twice with similar results. Data are the means of three replicates ± SE

Figure 2.1. Multiple alignment of amino acid sequences of metacaspases from Petunia (PhMCA1 and PhMCA2), Arabidopsis (AtMC1 – 9), Tomato (LeMCA1; *Solanum lycopersicum*), *Nicotiana tabacum*, rice (*Oryza sativa*), wheat (*Tricicum aestivum*), corn (*Zea mays*), Norway spruce (*Picea abies*) and scots pine (*Pinus sylvestris*). Putative His and Cys catalytic residues are designated by ♦. Cysteine residues in Zn finger motif of the prodomains of type I metacaspases are indicated by Δ. Putative conserved aspartic acid residues in substrate P1 coordination are indicated by ▲• Sequence alignment and generation of dendrogram was performed using ClustalW. Predicted cleavage site is indicated by ●.

```
PhMCA1      ----------MDGRRYKCKWCGTKMAAPIGAPSICCPRCQSVTEL--QPRNNNGYAKMAA
AtMC1       MYPPPPSSIYAPPMLVNCSGCRTPLQLPSGARSIRCALCQAVTHIA-DPRTA--------
AtMC2       ----------MLLLVDCSSCRTPLHLPPGATRIRCAICHAFTLIAPEPRLQSHASASPF
AtMC3       ---------MASRREVRCR-CGRRMWVQPDARTVQCSTCHTVTQLYSLVDIARGANRIIH
PhMCA2      ------------------------------------------------------------
LeMCA1      ------------------------------------------------------------
AtMC4       ------------------------------------------------------------
AtMC5       ------------------------------------------------------------
AtMC6       ------------------------------------------------------------
AtMC7       ------------------------------------------------------------
AtMC8       ------------------------------------------------------------
AtMC9       ------------------------------------------------------------
Nicotiana   ------------------------------------------------------------
Oryza       ------------------------------------------------------------
Triticum    ------------------------------------------------------------
Zea         ------------------------------------------------------------
Picea       ------------------------------------------------------------
Pinus       ------------------------------------------------------------
                            Δ   Δ               Δ   Δ


PhMCA1      PIANVVNNMINSAVNTGFYPAMQGRIPNGNNYQQQP--------------------Q
AtMC1       -----------------PPPQPSS------APSP----PPQI-------------HA
AtMC2       PFPNS-----SPAPSTFIYPPPTPSPYTHAPHAPSPFNHAPPDSYPFTHAPPASSPFNHA
AtMC3       GFQQL--------------LRQHPQHHEQQQQQMMAQPPPRL----------------
PhMCA2      ------------------------------------------------------------
LeMCA1      ------------------------------------------------------------
AtMC4       ------------------------------------------------------------
AtMC5       ------------------------------------------------------------
AtMC6       ------------------------------------------------------------
AtMC7       ------------------------------------------------------------
AtMC8       ------------------------------------------------------------
AtMC9       ------------------------------------------------------------
Nicotiana   ------------------------------------------------------------
Oryza       ------------------------------------------------------------
Triticum    ------------------------------------------------------------
Zea         ------------------------------------------------------------
Picea       ------------------------------------------------------------
Pinus       ------------------------------------------------------------


PhMCA1      PFMRPPAVHGRKRAVLCGITYRGHPKMLKGSVTDALSMRQLLVGKLGFPNASVVVLTEDE
AtMC1       PPGQLPHPHGRKRAVICGISYRFSRHELKGCINDAKCMRHLLINKFKFSPDSILMLTEEE
AtMC2       PPGPPPPVHGQKRAVIVGVSYKNTKDELKGCINDANCMKFMLMKRFQFPESCILMLTEEE
AtMC3       -LEPLPSPFGKKRAVLCGVNYKGKSYSLKGCISDAKSMRSLLVQQMGFPIDSILMLTEDE
PhMCA2      ---------MAKKAVLIGINYPGTKAELKGCINDVTRMYSCLVNRFGFSEDDITVLIDTD
LeMCA1      ---------MAKKAVLIGINYPGTKAELRGCINDVKRMYNCLLNRYGFAEEDITVLIDTD
AtMC4       ---------MTKKAVLIGINYPGTKAELRGCVNDVRRMYKCLVERYGFSEENITVLIDTD
AtMC5       ---------MAKKAVLIGINYPGTKAELRGCVNDVRRVHKSLVDRFGFSERNITELIDTD
AtMC6       ---------MAKKALLIGINYVGTKAELRGCVNDVRRMRISLVERYGFSEENIKMLIDTD
AtMC7       ---------MAKRALLIGINYPGTTEELQGCVNDVHRMHKCLVDRFGFAEEDITVLIDTD
AtMC8       ---------MAKKALLIGINYPGTAVELRGCVNDVHRMQKCLIELYGFANKDIVIMIDTD
AtMC9       --MDQQGMVKKRLAVLVGCNYPNTRNELHGCINDVLAMKETILSRFGFKQDDIEVLTDEP
Nicotiana   ---------MAKKAVFIGINYPGTKAELKGCINDVKRMYSCLINRFGFSEEDITVLIDTD
Oryza       ----MERGQKKKLATLVGCNYAGTPNELQGCINDVAAMRDALVARFGFAPADVTVLTDDR
Triticum    --------MGRKLALLVGINYPGTKAELKGCHNDVDRMHKCLVDRFGFDEDNITVLLDRG
Zea         --------MGQKRALLVGINYLGTDGELKGCLNDVARMRRCLVGRFGFDEADIRVLADAD
Picea       ---------MTKKAVLVGCNYPGTKAELKGCVNDVNRMYKCLVERYGFEEENIRVLIDTD
Pinus       ---------MTRKAVLVGCNYPGTKAELKGCVNDVNRMYKCLVERFGFKEENIRVLIDTD
                                          ▲
```

45

Figure 2.1 continued

```
PhMCA1      KDPCKIPTKANIRSALRWLVQGCQP--GDSLVFFYSGHGTRVRDRDGDEVD--GHDESLC
AtMC1       TDPYRIPTKQNMRMALYWLVQGCTA--GDSLVFHYSGHGSRQRNYNGDEVD--GYDETLC
AtMC2       ADPMRWPTKNNITMAMHWLVLSCKP--GDSLVFHFSGHGNNQMDDNGDEVD--GFDETLL
AtMC3       ASPQRIPTKRNIRKAMRWLVEGNRA--RDSLVFHFSGHGSQQNDYNGDEID--GQDEALC
PhMCA2      DSYTQ-PTGRNVRKALSDLVGSAEP--GDFLFVHYSGHGTRLPAETGEQDDT-GFDECIV
LeMCA1      DSYTQ-PTGRNIRKALSDLVGSAES--GDCLFVHYSGHGTRLPAETGEEDDT-GFDECIV
AtMC4       ESSTQ-PTGKNIRRALADLVESADS--GDVLVVHYSGHGTRLPAETGEDDDT-GFDECIV
AtMC5       ESSTK-PTGKNIRRALLNLVESAKP--GDVLVVHYSGHGTRLPAETGEDDDT-GYDECIV
AtMC6       SSSIK-PTGKNIRQALLDLVEPAKS--GDVLFVHYSGHGTRLPAETGEDDDT-GYDECIV
AtMC7       ESYTQ-PTGKNIRQALSELIKPAKS--GDVLFVHYSGHGTRVPPETGEEDDT-GFDECIV
AtMC8       KSCIQ-PTGKNICDELDNLIASGQS--GDFLVFHYSGHGTRIPPGIEDSEDPTGFDECIT
AtMC9       ESKVK-PTGANIKAALRRMVDKAQAGSGDILFFHYSGHGTRIPSVKSAHPFK-Q-DEAIV
Nicotiana   DSYTQ-PTGRNIRKALSDLVGSAEE--GDSLFVHYSGHGTRLPAETGEDDDT-GYDECIV
Oryza       GSPVL-PTGANIKRALADMVARAAP--GDVLFFHYSGHGTLVPPVKGRRHGHGECDEAIV
Triticum    SSGPQ-PTGANIRHALAKLVGDARR--GDFLFFHYSGHGTRLPAETGQDDDT-GYDECIV
Zea         PSTPP-PTGANIRLELERLVAGARP--GDALFFHYSGHGLQPLAETGEDDDT-GYDECIV
Picea       DSYPQ-PTGANVRRALKTLVEGSRA--GDIIFFHYSGHGVRLPAETGDQVDT-GYDECIV
Pinus       DSYPQ-PTGANIRRAWKTLAEGARP--GDIIFFHYSGHGVRLPAETGDQDDT-GYDECIV
                    .                              ♦
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PhMCA1      PVDYESEGRILDDEINATIVRPLPRGATLHGIIDTCFSGTFLD-----------------
AtMC1       PLDFETQGMIVDDEINATIVRPLPHGVKLHSIIDACHSGTVLD-----------------
AtMC2       PVDHRTSGVIVDDEINATIVRPLPYGVKLHAIVDACHSGTVMD-----------------
AtMC3       PLDHETEGKIIDDEINRILVRPLVHGAKLHAVIDACNSGTVLD-----------------
PhMCA2      PCDMN----LITDDDFRELVDKVPEGCQITIVSDSCHSGGLIDKAKEQIGESTKPA----
LeMCA1      PCDMN----LITDDDFRELVDKVPEGCQITIVSDSCHSGGLIDKAKEQIGESHKQG----
AtMC4       PCDMN----LITDDDFRDLVDKVPPGCRMTIISDSCHSGGLIDEAKEQIGESTKKE----
AtMC5       PCDMN----LITDDEFRDLVEKVPKEAHITIISDSCHSGGLIDEAKEQIGESTKKK----
AtMC6       PSDMN----LITDDDFRDLVDMVPKDCPITIISDSCHSGGLIDEAKEQIGESTKKK----
AtMC7       PSDLN----PIPDDDFRDLVEQVPEGCQITIVSDSCHSGGLIDEAKEQIGESTTTK----
AtMC8       PCDMN----LIKDQQFREMVSRVKEGCQLTIISDSCHSGGLIQEVKEQIGESHMKP-VDK
AtMC9       PCDFN----LITDVDFRELVNQLPKGTSFTMISDSCHSGGLIDKEKEQIGPSSVSS----
Nicotiana   PCDMN----LITDDDFRELVDKVPEGCRITIVSDSCHSGGLIDKAKEQIGESHKQG----
Oryza       PCDFN----LITDVDFRRLVDRVPRGASFTMVSDSCHSGGLIDLEKEQIGPSVLAA----
Triticum    PSDMN----LITDQDFRDLVQKVPDGCIFTIVSDSCHSGGLLDKAKEQIGNSTRQN---Q
Zea         PCDLN----LIKDQDFTDLVAKVPDGCRFTMVSDSCHSGGLIDKTKEQIGNSTKQNRTQQ
Picea       PCDMN----LITDDDFREFIDKVPRDCFCTIVSDSCHSGGLIERAKEQIGESTKDG----
Pinus       PCDMN----IITDDDFRDFIDKVPRDCLCTIVSDSCHSGGLIERAKEQIGESTKDG----
                                              ▲ ♦
```

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PhMCA1      ------------------------------------------------------------
AtMC1       ------------------------------------------------------------
AtMC2       ------------------------------------------------------------
AtMC3       ------------------------------------------------------------
PhMCA2      -----DDNESHGSGFGFKNFLRRSVEDAFESRGIHIPRRHHRGEEEDENFAESSVIETED
LeMCA1      -----DDDEGHGSGFGFKNFLRRNVEDAFESRGIHLPGRHHRREEEEENFAESSVIETED
AtMC4       -AEDEDESEESSRFGFRKFLRSKVEGAIESRGFHIGGN--KKDEDEAEEIETKEIELED
AtMC5       ----PKKESGGSSGLGIKGFVREAVEEALESKGIAIP-----HHKDEKDENKTKELKLED
AtMC6       ------------------------------------------------KDSGDSSTINKETE
AtMC7       ---PNRESKVSSFEFEFKNCLHSIFVKLLAFCGI-----------GSSHVETREIVEVG
AtMC8       VKEQIEESHMKQPKLGIASYFLNIVMNLLATCGVSKS----QRDRGGGEESFRGEIELEK
AtMC9       -------------------------------------------------------NISPAIE
Nicotiana   ----DDENEGHGSGFGFKKFLRRSVEDAFESRGIHIPRRHDRREEEEESFAESSVIETED
Oryza       -------------------------------------------------GAAPAAAAAST
Triticum    TQSREPKEERSHSGSGFRSFLKETVRDAFESHSRH--------------GGEDQDEQP
Zea         RREREMKPPPPAPGSALRVSLARIVRGVLESLGC------------IHCSRCRVQQQGN
Picea       ---DERPEHKKQGGFGFRDLLSESVHGAFESRGIHLPSRHEHKDRRESDEGETQEIEYH-
Pinus       ---DEQPEQNKHSRFGFRDLLSESAHDAFESRGVHLPSHHEHKDRHEFDEGETREIEYR-
```

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Figure 2.1 continued

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PhMCA1       ----------LPFMCRIN----REGYFMWEDHRISS--YK--------------------
AtMC1        ----------LPFLCRMN----RAGQYVWEDHRPRSGLWK-------------------
AtMC2        ----------LPYLCRMD----RLGNYEWEDHRPKTGMWK-------------------
AtMC3        ----------LPFICRME----RNGSYEWEDHRSVRA-YK-------------------
PhMCA2       GDQVHAKNKSLPLSTLIEILKQTGKDDIDVGKLRPALFDVFGEDASPKVKKFMK-VIFN
LeMCA1       GDQVHVKSKSLPLSTLIEILKQTGKDDIDVGKLRPTLFDVFGEDASPKVKKFMK-IIFN
AtMC4        GETIHAKDKSLPLQTLIDILKQQTGNDNIEVGKIRPSLFDAFGDDSSPKVKKFMK-VILG
AtMC5        GAKVHVVNKSLPLQTLIDILKQNTGNNDIEVGKIRPTLFNVFGEDASPKVKKFMK-VILT
AtMC6        AEIIEVGNRSLPLETLIDMLKQETGNDDIEVGKIRTTLFDMFGDDSSPKVKKFMN-VILS
AtMC7        EGDEVVRSRYLPLERFIELLKQQTGQDNIEIGKIRPTLFDVFGEDSSPKIKKFMK-VILT
AtMC8        DETLDIKTRYLPFESYLSLLKEQTGQTNIEPVRIRQTLLKLFGEDPSPNRQRGLSDLGNC
AtMC9        TTNKTITSRALPFKAVLDHLSSLTG---ITTSDIGTHLLELFGRDAGLKFRLPAM-DLMD
Nicotiana    GDQVHVKNKSLPLSTLIEILKQTGKDDIDVGKLRPTLFDVFGEDASPKVKKFMK-VIFN
Oryza        TATRATAARFLPYAAVVEHLSGVSGVD--AAHHVADHLLALFGADASAKFHR--------
Triticum     TGDGLTKNRSLPLSTLIEMLKEQTGKDDIEEGSIRLTLFNVFGDDASPKIKKFMK-VMLD
Zea          SNSSSISNRSLPLSTFIQMLRDKTGRHDVGVGSIRTTLFHHFGDEASPKVKRFMKAMLLG
Picea        -GKGRAKNRSLPLSTLIEILNEKTGRHDIDVGKIRPTLFDMFGDDASPKVKKFVK-VLVT
Pinus        -GRGHAKNRSLPLSTLIEILNEKTGRHDIDVGKIRPALFDIFGDDASPKVKKFVK-VLVT
                      •


PhMCA1       ----------------------------------------------------------
AtMC1        ----------------------------------------------------------
AtMC2        ----------------------------------------------------------
AtMC3        ----------------------------------------------------------
PhMCA2       KLQHGNGEGE--SGGFLGIVGNLAQEFLKQKLDENDE--GYAKPAMETHVEAKQEVYAG-
LeMCA1       KLQKNNEQGG--GGGFMGMVGNLAQEFLKQKLDENDE--SYAKPAMETHVEGKQEVYAG-
AtMC4        KLQAGNGE----EGGLMGMLGKLASGFLEGKLNDE----DYVKPAMQTHVGSKEEVYAG-
AtMC5        KLQEGKT-----EGGILGMIGKLAQEFLKHKLNDDE---EYVKPAMKTHVGNKQEVYAG-
AtMC6        NLQETTTTIQTVSDEVLGSVENLAQEFLEQKLSDD------VKPAI-------QDVYAG-
AtMC7        KLRKTND-----QSTLLGKIEESARGYIEETLNDE----HYMKPAMQAQVKSDREIYGG-
AtMC8        EVDAGDS---------------------------------------------------
AtMC9        LLE-------------------------------------------------------
Nicotiana    KLQHGNEEGE--SGGFLGMVGNLAQEFLKQKLDENDE--SYAKPAMETHVEGKQEVYAG-
Oryza        ----------------------------------------------------------
Triticum     KFHEGASGE---QGGVMGMVGSLAHQFLKAKLEGTEE--ETFKPAIEQDMGSAEEAYA--
Zea          KLRHDGK-----EAEEQSRVPREAE---------------VEETLAQDAHSVEEVYAGP
Picea        KLQSSGN-----EGGLLGAIGNLAQQFLKQKLDGSDGGSEYVKPALQEEVSSTQEIYAG-
Pinus        KLQSADN-----KGGLLGAIGNLAQQFLKQKLDGSDEGSEYVKPALKEKVSSTQEVYAG-


PhMCA1       --GTNGG-----TAISISACDDHQNSGDTTAFTGFP-TGALTYSFIKTLEQ-EPKLSYGR
AtMC1        --GTAGG-----EAISISGCDDDQTSADTSALSKITSTGAMTFCFIQAIERSAQGTTYGS
AtMC2        --GTSGG-----EVFSFTGCDDDQTSADTPQLSGSAWTGAMTYAFIQAIER-GHGMTYGS
AtMC3        --GTDGG-----AAFCFSACDDDESSGYTPVFTGKN-TGAMTYSFIKAVKTAGPAPTYGH
PhMCA2       --SGNRG--LPDSGILISGCQTDQTSADATP------AG--------------GDSYGA
LeMCA1       --SGNRG--LPDSGILVSGCQTDQTSADATP------AG--------------GESYGA
AtMC4        --GSRGSVPLPDSGILISGCQTDQTSADATP------AG-------------KPTEAYGA
AtMC5        --ASNGS--LADNGILISGCQTDQTSADASP------QG-------------HPEMAYGA
AtMC6        --AINGA--LPDNGILISGCQTDQTSSDASP------PG-------------HPELAYGA
AtMC7        --RSSNGL-FPDRGILLSGCQTDETSADVKK------KG----------------EAFGA
AtMC8        --GASRLNAVTDNGILLSGCQTDQRSEDVYV------TR-------------TGKAYGA
AtMC9        --TMTAREKHVDSGILMSGCQADETSADVG-------VG-------------NGKAYGA
Nicotiana    --SGSRG--LPDSGILVSGCQTDQTSADATP------AG--------------GDSYGA
Oryza        --DAEQPVRTDDDGILLSGCQTDETSADVPGDDEVA-AG-------------GKACGA
Triticum     --GTKSW--APNNGILISGCQTSQTSADATI------PG-------------GTSFGA
Zea          AAAAAAAARVPPRNGVLISGCQTDETSADATT------AD-------------GMSYGA
Picea        --SGKRG--LPDMGILVSGCQSDQTSADANP------TG------------NPAHAYGA
Pinus        --SGNRG--LPDMGILASGCQSDETAADANP------TG------------KPDHAYGA
                      ▲
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Figure 2.1 continued

PhMCA1      LLMSMHNKIHE-----------AQKGVGLTGAKE-----------------------T
AtMC1       LLNSMRTTIRN--TGNDGG-----GSGGVVTTVLSMLLTGGS---------AIGGLRQEP
AtMC2       LLNAMRSTVHEIFDKNKGRELVEVGGADFLSTLLGLLILGASPPDEEEEVNQAPQKTQEP
AtMC3       LLNLMCSAIRE--AQSRLA-----FNGDYTSSDASAE--------------------P
PhMCA2      LSNAIQEILSE--SD--GP----ITNEELVRKARKKL------------QRQGFTQRP
LeMCA1      LSNAIQEILAE--SD--GP----VTNEELVSKARKKM------------QKQGFTQRP
AtMC4       MSNSIQTILEE--TD--GE----ISNREMVTRARKAL------------KKQGFTQQP
AtMC5       FTNAVQIILEE--TK--GM----ITYKELVLKARKLL------------KKQGFSQRP
AtMC6       LTNAIQIIIGE--TK--GK----ISNKDLVLKARKLL------------RKQGFDQRP
AtMC7       FSNAIQMVLSE--TDHKDK----ITNKEMVLRAREIL------------KKQMFIQRP
AtMC8       FSDAIQMILSA--PRKDKK---KITNKELVSEARVFL------------KKRGYSQRP
AtMC9       FSNAIQRVLNE--NE--GA----MKNKQLVMMARDVL------------ERLGFHQHP
Nicotiana   LSNAIQEILAE--SD--GP----ITNEEVVTKARKKL------------QKQGYTQRP
Oryza       FSNAIQTVLAS--HP--AP----VSNRQLVSMARAVL------------SDQGFEQHP
Triticum    MSNAIQAILAS--DD--GK----ITNKDLVMKARAAL------------SKQGYTQQP
Zea         LSNVIQTILAG--DGKKRGVALAVTNRELVVRARELL------------SRQGYTQQP
Picea       LSNAIQTILAE--HD--GP----IPNRQLVMKARQIL------------SKQGFAQRP
Pinus       LSNAIQTILAE--HD--GP----IPNRQLVMKARQML------------SKQGFKQRP


PhMCA1      QVRL-------------------------------
AtMC1       QLTACQTFDVYAKPFTL--------------------
AtMC2       QLSANEAFAVYEKPFSL--------------------
AtMC3       LLTSSEEFDVYATKFVL--------------------
PhMCA2      GLYCGDHHV--DAPFVC--------------------
LeMCA1      GLYCDDHHV--DAPFVC--------------------
AtMC4       GLYCHDGYA--NAPFIC--------------------
AtMC5       GLYCSDSFV--NAPFIC--------------------
AtMC6       GLYCNDAYV--NARFIC--------------------
AtMC7       GLYCNDRFV--NAPFIC--------------------
AtMC8       GLYCHDRFV--DKPFICY-------------------
AtMC9       CLYCSDQNA--DATFLSQP------------------
Nicotiana   GLYCSDHHV--DAPFV---------------------
Oryza       CLYCSDGNA--ETPFLWQEEDKKKTVTAAAEQAMTAL
Triticum    GLYCSDEHV--HVAFIC--------------------
Zea         GLYCSDEHA--TLPFIC--------------------
Picea       GLYCTDRLA--NAPFICD-------------------
Pinus       GLYCTDRLA--NAPFICD-------------------
```
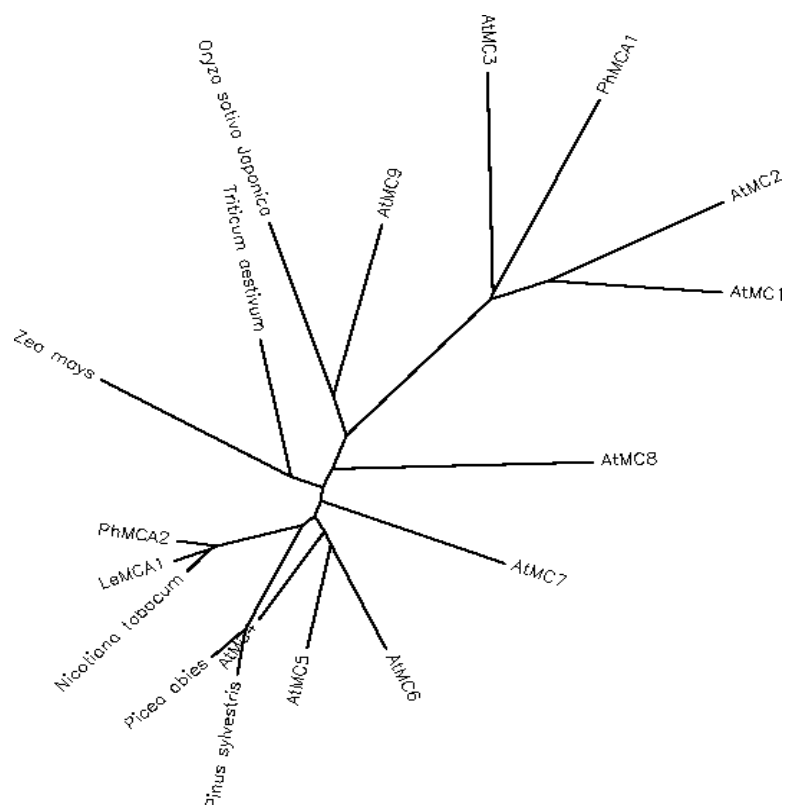
48

Figure 2.2. Dendrogram of metacaspases using the amino acid sequences of the coding regions from Petunia (PhMCA1 and PhMCA2), Arabidopsis (AtMC1−9), Tomato (LeMCA1; *Solanum lycopersicum*), *Nicotiana tabacum*, rice (*Oryza sativa*), wheat (*Tricicum aestivum*), corn (*Zea mays*), Norway spruce (*Picea abies*) and scots pine (*Pinus sylvestris*).
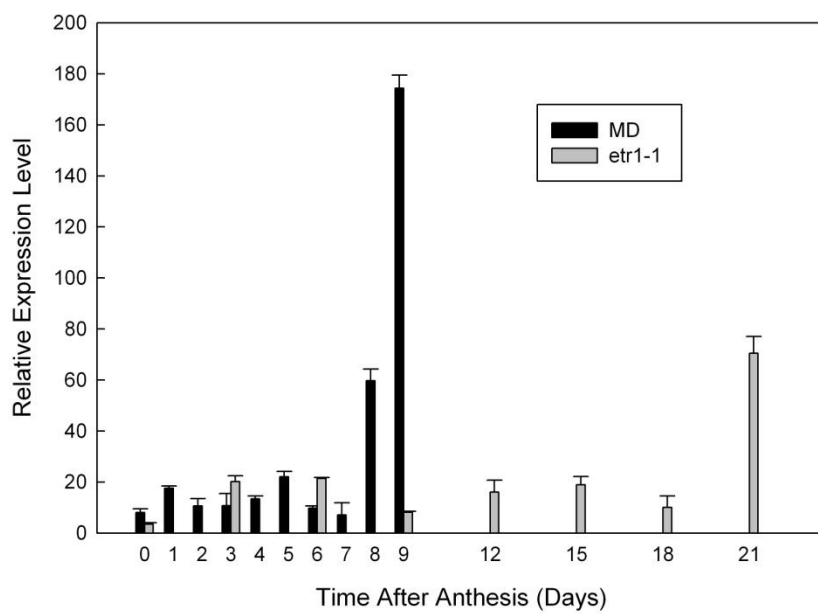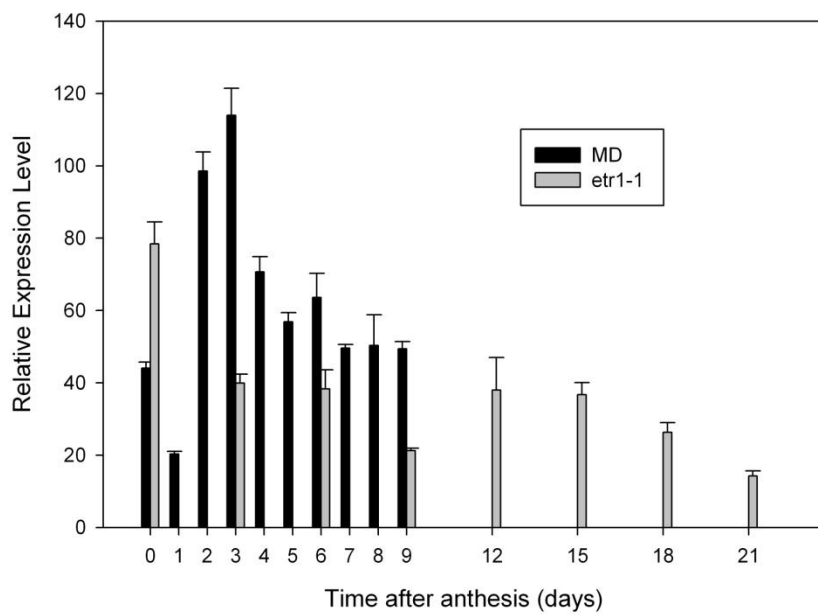
Figure 2.3. Images of corollas of *Petunia xhybrida* 'Mitchell Diploid' (MD) and ethylene insensitive line (35S::etr1-1, line 44568) from anthesis until wilting. Numbers indicate number of days after anthesis.

Figure 2.4. Relative gene expression of *PhMCA1* (A) and *PhMCA2* (B) in *Petunia xhybrida* 'Mitchell Diploid' (MD) and 35S::*35S:etr1-1* transgenic corollas during petal development. Relative abundance of mRNA (*PhMCA1*/*PhACTIN* or *PhMCA2*/*PhACTIN*) was determined by quantitative RT-PCR. Data are the means of three replicates ± SE. The experiment was performed twice with similar results.
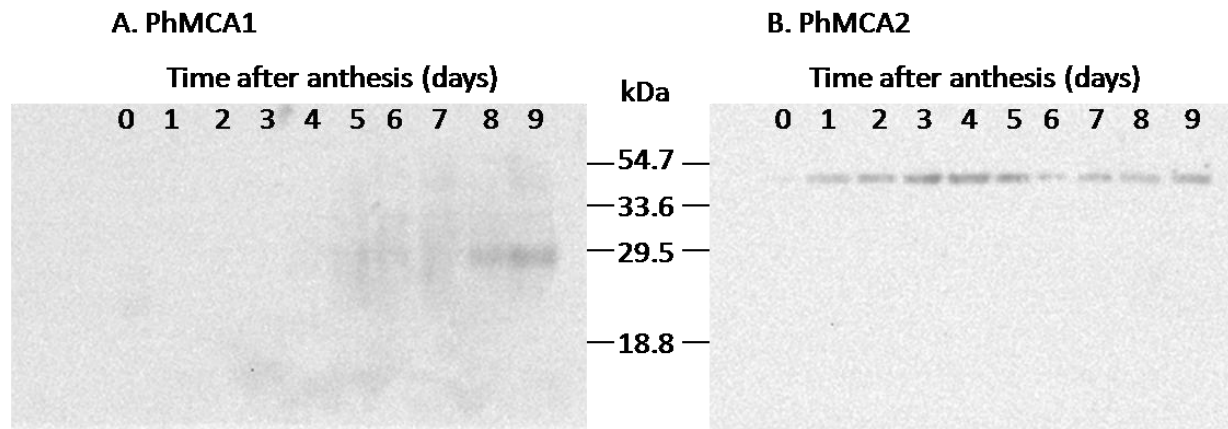
A. PhMCA1

B. PhMCA2

A. PhMCA1

Time after anthesis (days)

0 1 2 3 4 5 6 7 8 9

B. PhMCA2

Time after anthesis (days)

0 1 2 3 4 5 6 7 8 9

kDa

—54.7 —
—33.6 —
—29.5 —
—18.8 —

Figure 2.5. Western blot analysis of total protein isolated from *Petunia xhybrida* 'Mitchell Diploid' during petal development. Signals were detected using anti PhMCA1 p20 antibody (A) and anti PhMCA2 p20 antibody (B) as primary antibodies. Numbers 0-9 indicates the number of days after anthesis; Proteins isolated at anthesis (lane 0) until wilting (9 days; lane 10). Protein standard sizes are shown in kDa.

53

A   B

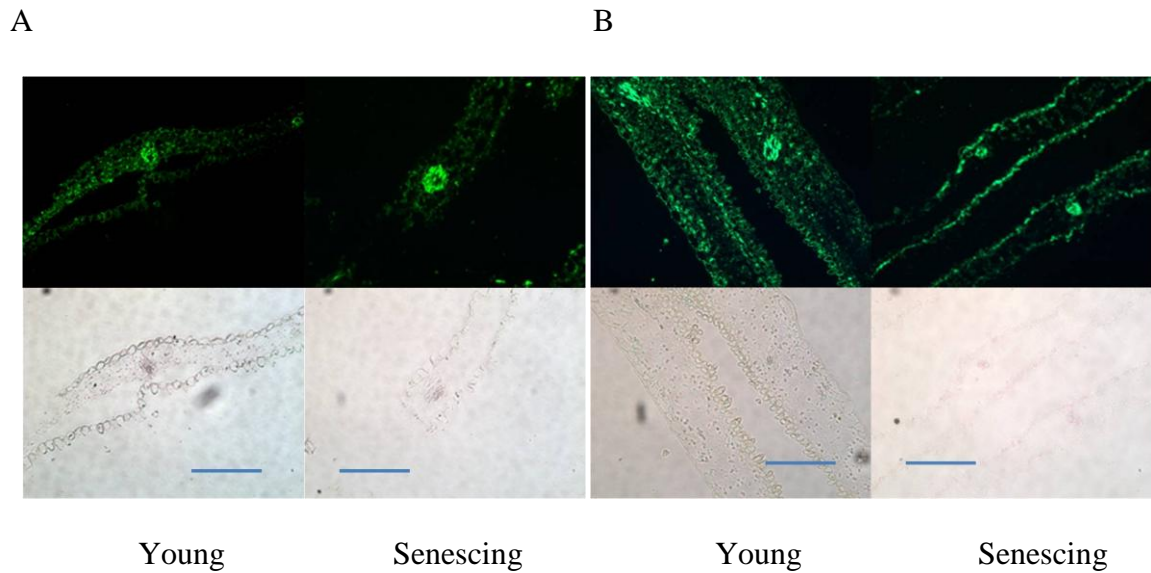Young   Senescing   Young   Senescing

Figure 2.6. Immunolocalization of PhMCA1 (panel A) and PhMCA2 (Panel B) in
Petunia xhybrida 'Mitchell Diploid' petals. Anti-PhMCA1p20 and anti-PhMCA2 p20
antibodies were used as primary antibodies and AlexaFluor488 goat anti-rabbit IgG used
as secondary antibody. Images taken under white light are shown in the lower section of
each panel. Epifluorescent images were viewed using 45 µm sections with a 20X
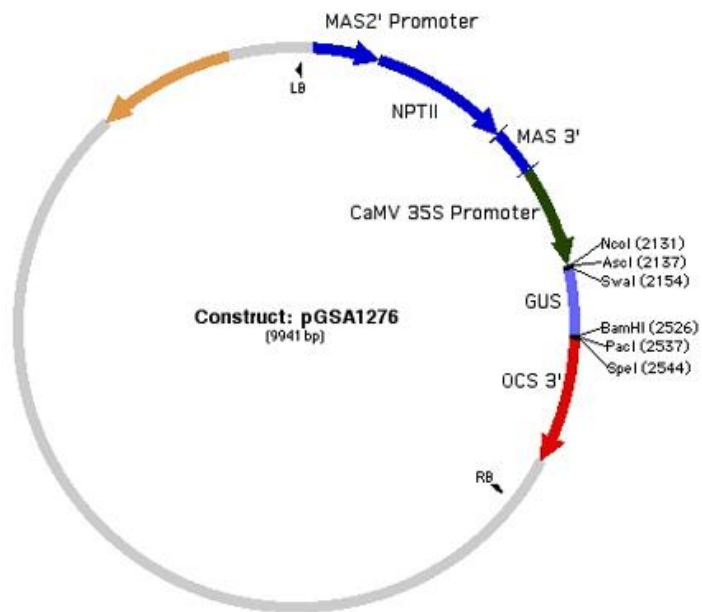magnification. Bars = 0.5 mm

Figure 2.7. Map of pGSA1276. A sense fragment was cloned into AscI/SwaI and an antisense fragment was cloned into BamHI/SpeI sites.
(http://www.chromdb.org/rnai/pGSA1276.html)

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Figure 2.8 . Semi quantitative RT-PCR showing expression levels of *PhMCA1, PhMCA2* or both *PhMCA1* and *PhMCA2* in VIGS (panel A) and overexpression (panel B) analyses. *PhACTIN* was used as a control. Expression levels of *PhMCA1*, *PhMCA2* or both *PhMCA1* and *PhMCA2* from control, longer longevity (17 days or longer) and shorter longevity (up to 13 days) corollas are indicated as 'Control', 'Short' and 'Long' with underlines, respectively. Control plants were infiltrated with Agrobacterium containing pTRV2::CHS for VIGS and empty vector (pJL36) for overexpression.
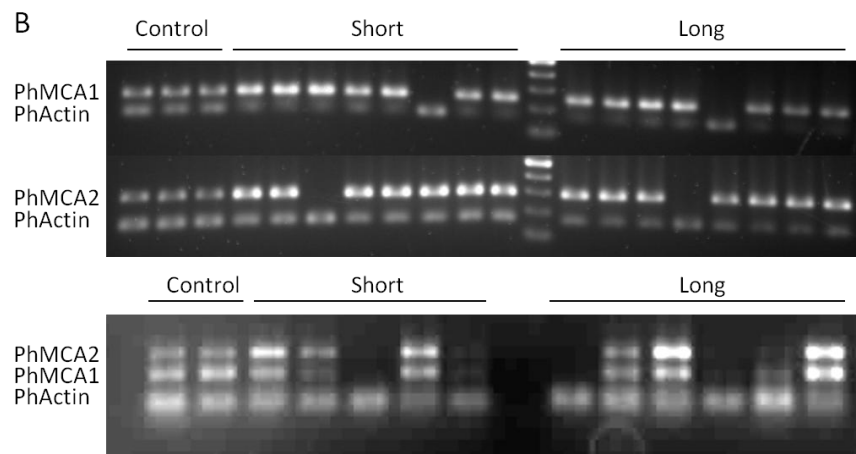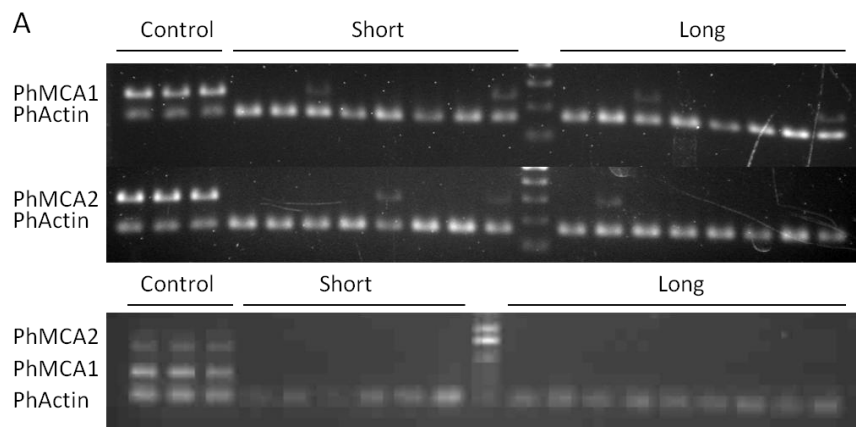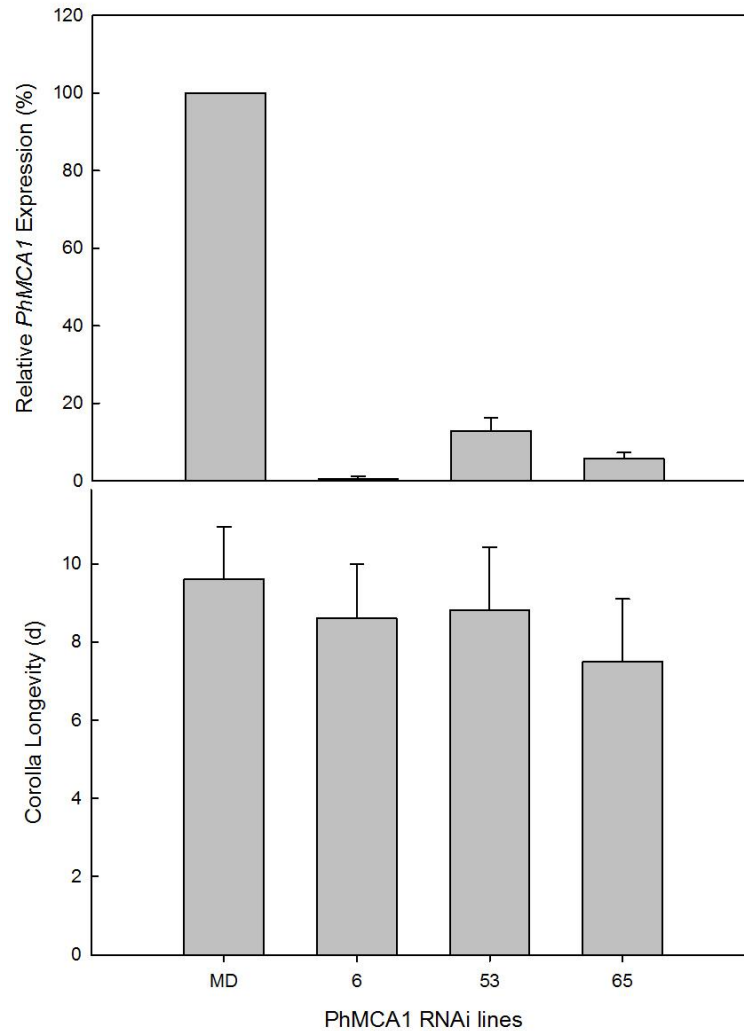
Figure 2.9. Quantitative RT-PCR analysis and longevity of *PhMCA1* RNAi lines at T2 generation. Expression levels of *PhMCA1* RNAi lines (6, 53 and 65) were measured by qRT-PCR at 48 hours after pollination. Corolla longevity was measured from anthesis until wilting. Plant numbers in respective lines were indicated in the parentheses. *PhACTIN* was used as a control. Control plants were untreated petunia (MD). This analysis was performed at least three times with similar results. Data are the means of three replicates ± SE

CHAPTER 3:

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF RECOMBINANT
METACASPASES FROM *PETUNIA X HYBRIDA* 'MITCHELL DIPLOID'
EXPRESSED IN *E. COLI*

Abstract.

Caspases play a critical role in the animal cell death program, but no caspase
homologs have been identified in plants. Structurally similar caspase-like proteases
called metacaspases have been found in plants and fungi genome data bases.
Metacaspases contain a highly conserved histidine/cysteine dyad and processing is
required for their activation, similar to the animal caspases. Two metacaspases from
petunia were cloned and sequenced. Previous sequence analysis revealed that both the
type I (*PhMCA1*) and type II (*PhMCA2*) metacaspase contained an expected catalytic
dyad (described in chapter 2). Recombinant PhMCA1 and PhMCA2 metacaspases
produced in *E. coli* exhibited autoprocessing and mutations of the cysteine residue at the
catalytic dyad blocked autoprocessing and nearly abolished the enzymatic activity. Both
metacaspases cleaved protease substrates with an arginine residue at the P1 site and the
cysteine specific inhibitor iodoacetamide and arginal protease inhibitor leupeptin nearly
abolished the activity of both petunia metacaspases. The pH optimum of PhMCA1 and
PhMCA2 was 8. We concluded that the petunia metacaspases were similar to other
metacaspases known from plant models and they are not likely to be responsible for

"caspase-like" activity due to different substrate specificity than that of caspases, which utilize an aspartate as a cleavage residue.

Introduction

Programmed cell death (PCD) is a genetically controlled suicide process and one of the integral processes for development and homeostasis, biotic and abiotic stress responses. In animal cells, caspases (cysteine dependent aspartate specific proteases) play a pivotal role in PCD. All caspases contain a highly conserved histidine/cysteine catalytic dyad and hydrolyze peptide bonds at the C-terminal side of an aspartate residue (P1 site). Caspases consist of a small (p10) and a large (p20) subunit and are regulated at the post-translational level. Caspases are synthesized as an unprocessed inactive form (zymogen) and are activated by cleavage of the zymogen into a large and a small subunit. Caspases consist of, according to their functional properties, initiator and executioner caspases. Initiator caspases possess a longer prodomain, while executioner caspases posses shorter or no prodomain. The prodomain often contains a caspase recruit domain (CARD), or a death effector domain (DED), and they interact with the ATP/cytochrome-*c,* the apoptotic protease-activating factor 1 (Apaf-1) protein or other proteins to regulate their activation (Vercammen et al., 2004). Activation of initiator caspases is achieved by heterodimerization of initiator caspases' two different subunits after cleavage at the caspase recognition site by other caspases (Meergants et al., 2000).

Although "caspase-like" activities are documented in various plant models, to date no caspase homologs have been identified in plants, fungi, or protozoan data bases. However, proteases that are structurally similar to caspases have been found (Uren et al., 2000) in plants, fungi and protozoa. Metacaspases are classified as a CD clan of cysteine peptidases with six other families, including caspases and paracaspases (Mottram et al.,

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2003). Metacaspases are not only structurally similar to caspases but also share similar enzymatic properties. There are two types of metacaspases, type I and type II. Both types of metacaspases contain a small (p10) and a large (p20) subunit and a catalytic dyad similar to animal caspases. Type I metacaspases possess prodomains which contain zinc-finger motifs similar to that found in the hypersensitive response protein LSD1 (Lesion Simulating Desease1). Their function is unclear, but they are speculated to be involved in biotic stress responses (Vercammen et al., 2004). Recombinant metacaspases produced in *E. coli* and yeast revealed that autoprocessing was required for their enzymatic activity (Watanabe and Lam, 2005; Vercammen et al., 2004) although a metacaspase from the protozoan parasite *Leishmania* did not require processing for its activity (Lee et al., 2007).

Numerous fluorogenic synthetic peptide substrates and inhibitors composed of 3 to 5 amino acid residues specific to caspases have been developed and widely used to determine the properties of caspases. When a fluorogenic moiety is cleaved off by a specific protease, fluorescent signals are emitted and intensity of the emitted signals is measured for enzymatic activity. These synthetic substrates are N-terminally protected from degradation by aminopeptidases (Witt, 1991). Such synthetic fluorogenic substrates and inhibitors were also used to investigate caspase-like activities including metacaspases in plants (Bozhkov et al., 2004;, Hoeberichts and Woltering 2003; Lam and del Pozo 2000; Watanabe and Lam 2005; Vercammen et al., 2004), yeasts (Madeo et al., 2002, 2004; Thrane et al., 2004) and protozoans (Kosec et al., 2006).

Caspases cleave an aspartate residue as their target site, while metacaspases utilize an arginine or lysine residue at the P1 position (Vercammen et al., 2004). Recombinant *Arabidopsis* metacaspases (AtMC2, AtMC4, AtMC5 and AtMC9) expressed in *E. coli* were not able to utilize caspase-specific substrates but recognized an arginine or lysine residue as their target site (Vercammen et al., 2004; Watanabe and Lam, 2005). While caspase-specific inhibitors were not able to block these metacaspases, the plant recombinant metacaspases (AtMC4, AtMC9 from Arabidopsis and mcII-Pa from Norway Spruce) and yeast metacaspase YCA1 were sensitive to arginal or serine protease inhibitors (Vercammen et al., 2004; Bozhkov et al., 2005; Watanabe and Lam 2005; van Door and Woltering 2008).

Little is known about the enzymatic properties of plant metacaspases other than those found in a few model systems including Arabidopsis (Watanabe and Lam, 2005; Vercammen et al., 2004; Lee et al., 2007). We have characterized two metacaspases of *Petunia x hybrida* 'Mitchell Diploid'. The goal of this study was to investigate the biochemical properties of metacaspases from petunia and the specific objectives were:

1. To investigate the enzymatic activity of petunia metacaspases utilizing fluorogenic substrates and protease inhibitors;

2. To determine the optimum pH of PhMCA1 and PhMCA2 metacaspases; and

3. To investigate the effect of autoprocessing on enzymatic activity using mutants containing an alanine at the catalytic cysteine residue.

Materials and Methods

Cloning of Metacaspase from Petunia x hybrida 'Mitchell Diploid' and Construction of

Metacaspase Mutants

The coding regions of *AtMC1, AtMC9, PhMCA1 and PhMCA2* were cloned as described

in Chapter 2. The cysteine residues of the catalytic dyad were converted to alanine

(AtMC1^{C220A}, in AtMC9^{C147A}, PhMCA1^{C237A} and PhMCA2^{C139A}). An additional mutant

(PhMCA1^{S116C}) was also constructed in PhMCA1. All mutations were made using

overlapping PCR technique. Two pairs of primers (Table 3.1) were designed so each

PCR fragment contained a desired mutation. For example, PCR fragments amplified

using F-PhMCA1pET/R-PhMC1C237A primers contained a point mutation converting a

cysteine at 237th residue to an alanine and these fragments were served as the 5' end of

the PhMCA1 coding region with a mutation. In order to amplify the 3' end of the

mutated fragments, another pair of primers (F-PhMC1C237A/R-PhMC1pET) were used.

These PCR fragments contained an alanine residue at 237th cysteine site. 5' and 3' PCR

fragments overlapped each other where the mutation was created. The entire coding

region with a mutation was then amplified using the third set of primers with BamHI and

HindIII restriction enzyme extensions (F-PhMCA1pET/R-PhMCA1pET). In this way,

PCR fragments containing a mutation were generated for all mutant constructs. The PCR

fragments were digested with the corresponding restriction enzymes (BamHI and HindIII

for PhMCA1, PhMCA1^{C237A}, PhMCA1^{S116C}, BamHI and SacI for PhMCA2 and

PhMCA2 ^{C139A}, BamHI and XhoI for AtMC1, AtMC1 ^{C220A}, AtMC9 and AtMC9 ^{C147A})

and they were ligated into pET28a(+) vector for each construct. Using this overlapping

PCR technique pET28a(+)::AtMC1^{C220A}, pET28a(+)::AtMC9^{C147A} ,

pET28a(+)::PhMCA1^{S116C}, pET28a(+)::PhMCA1^{C237A}, pET28a(+)::PhMCA2^{C139A} were

constructed. All constructs were verified by sequencing. Phusion proof reading

polymerase was used with F-518 5X Phusion HF reaction buffer (Finnzymes, Woburn,

Massachusetts).

Metacaspase Enzyme Activity Analysis

Induction and Isolation of recombinant metacaspases: A single colony for each construct,

including a control (*E. coli* with pET28a(+) vector alone), was picked and grown in 5 ml

of LB liquid media containing Kanamycin (50mg/ml) overnight at 37°C. Three ml of

overnight culture was inoculated in 50 ml LB with Kanamycin in a 250 ml Erlenmeyer

flask and incubated at 37°C (3 – 3.5 hrs) until OD$_{600}$ was 0.6-1.0. IPTG was added to a

final concentration of 1 mM and cultures were incubated for 2-3 hrs at 37°C or up to 6 hrs

at 30°C. After the flasks were put on ice for 5 min, the cells were harvested by

centrifugation at 5,000 x g for 5 min at 4°C. The cells were resuspended in 0.25 volume

of cold 20 mM Tris-HCl pH 8.0, and centrifuged at 5,000 x g for 5 min. Pellets were

stored frozen at -80°C or purification was continued. Metacaspases containing poly

histidine tag were purified according to the manufacturer's instruction using TALON

Metal Affinity Resin (BD Biosciences Clontech, Mountain View, CA). Thoroughly

resuspended 100 µl of TALON resin was transferred to a sterile tube and centrifuged at

700 x g for 2 min to pellet the resin. The supernatant was discarded. One milliliter of

provided 1X Equilibration/Wash Buffer was added and mixed briefly to pre-equilibrate

the resin. The resin was washed twice by centrifugation at 700 x g for 2 min to pellet the

resin and discard the supernatant. The protein samples (10 – 100 µl) were added to the

resin. Samples were gently agitated at room temperature for 20 min on a platform shaker

at 50 rotations per minute to allow the polyhistidine-tagged protein to bind the resin.

After the sample protein bound to the resin was centrifuged at 700 x g for 5 min, the

supernatant was carefully removed without disturbing the resin pellet. The resin was

washed by adding 1 ml of 1X Equilibration/Wash Buffer. Then, the resin was gently

agitated at room temperature for 10 min on a platform shaker at 50 rotations per minute

to promote thorough washing. The resin was washed twice by repeating centrifugation at

700 x g for 5 min and removing the supernatant. One bed volume of the 1X

Equilibration/Wash Buffer (100 µl) was added to the resin, and resuspended by

vortexing. The resin was transferred to a 2 ml gravity-flow column with an end-capin

place, and the resin was allowed to settle out of suspension for 5 minutes. The end-cap

was removed, and the buffer was allowed to drain until it reached the top of the resin bed.

The column was washed once with 500 µl of bed volumes of 1X Equilibration/Wash

Buffer. The polyhistidine-tagged protein was eluted by adding 500 µl of the provided

Elution Buffer to the column. Five protein samples were collected in 250 µl fractions.

Protein amount was determined in each fraction using UV absorbance at 280 nm using

NanoDrop (ThermoScientific, Wilmington, DE).

The amount of purified metacaspase was measured by Bio-Rad Protein Assay

(Hercules, CA). Purified recombinant protein (0.1 µg) and 100 µM peptidyl substrates or

inhibitors were mixed in 100 µl of reaction buffer (150 mM NaCl, 10 mM $CaCl_2$, 10%

glycerol (w/v), 0.1% CHAPS, 10mM DTT and pH adjustment agents). The pH of the

reaction buffers was adjusted with 100 mM glycine (pH 4 and pH 5), 100 mM MES (pH

6), 50mM HEPES-KOH (pH 7 and pH 8), 100 mM Tris (pH 9) for pH optimum analysis.

All other experiments were conducted at pH 8 except for AtMC9 and AtMC9^{C147A} which

was at pH 5 for substrate and inhibitor assays. The reaction mix was incubated for 30

min at 30°C and 100 µl of reaction stop solution (10% acetic acid in 150 mM sodium

acetate) was added to stop the reaction. A portion of the reaction mix (30-45 µl) was

diluted with nanopure water to 1.5 ml. Enzyme activity was measured by the amount of

AMC liberated using a fluorescence spectrophotometer (DTX880 Multimode Detector,

Beckman Coulter Inc., Austria) at 370 nm excitation and 450 nm emission wavelengths.

Substrates included Boc-Gly-Arg-Arg-AMC and Ac-Asp-Glu-Val-Asp-AMC (Bachem,

Torrance, CA) and inhibitors included 10 mM Iodoacetamide, 1 mM

phenylmethanesulfonylfluoride (PMSF) and 1 µM Leupeptin (Sigma-Aldrich, St. Louis,

MO). Data were analyzed using Multimode analysis software version 3.2.0.6 (Beckman

Coulter Inc., Austria). Relative metacaspase activities were analyzed by using Proc GLM

(generalized linear model) with Tukey's standardized range test with three replications.

Data were analyzed with SAS (Statistical Analysis System, SAS Institute, Inc., Cary, NC;

SAS Institute, Inc., 2002–2003).

Western Blot Analysis of Recombinant Petunia Metacaspases

Total proteins from *E. coli* cells containing pET28a(+)::PhMCA1^{S116C},

pET28a(+)::PhMCA1^{C237A}, pET28a(+)::PhMCA2 ^{C139A} were isolated as described

previously. Ten micrograms of recombinant proteins were loaded on a gel (Bio-Rad

Criterion Gradient Precast Gel 10-20%) and electrophoresis was performed at 100 Volts

for 2.5 hrs. After the gel was stained with GelCode® Blue Stain Reagent (Thermo

Scientific) for 2 hrs and washed in deionized water for 1 hr, the proteins were transferred

onto PVDF membrane (Bio-Rad Immuno-Blot PVDF membrane). Western blot analysis

was performed and the image was recorded as described in Chapter 2.

Results

Metacaspase Enzyme Activity Analysis

Enzymatic assays were performed to determine whether petunia metacaspases also utilize

arginine as a target residue at the P1 site by incubating purified recombinant

metacaspases with synthetic fluorogenic oligopeptide P1 arginine *t*-butyloxycarbonyl-

Gly-Arg-Arg-amindo-4-methylcoumarin (Boc-GRR-AMC) or caspase substrate Asp-

Glu-Val-Asp-amindo-4-methylcoumarin (Ac-DEVD-AMC). The enzyme activity was

determined by measuring the amount of fluorescence generated during the assay from

release of AMC. Activities of petunia metacaspases (PhMCA1 and PhMCA2) and two

Arabidopsis metacaspases (AtMC1 and AtMC9) as controls were measured and relative

enzymatic activities were calculated as the percentage of the activity compared to the

highest metacaspase activity (PhMCA2 at pH8). All metacaspases in this analysis

cleaved substrates containing an arginine residue (Boc-GRR-AMC in panel A), but no

cleavage of the caspase-specific substrate containing an aspartic acid residue (Ac-DEVD-

AMC in panel B) was observed (Figure 3.1). In order to determine whether *E. coli* DE3

strain had trypsin-like activity, enzymatic assays were also performed from bacterial

lysates prepared from cells containing only pET28a(+) and only background protease

activity was detected in this assays. Activities of AtMC9 and PhMCA2 (type II

metacaspases) were higher than those of AtMC1 and PhMCA1 (type I metacaspases),

respectively (Figure 3.1A). Petunia metacaspases PhMCA1 and PhMCA2 and

Arabidopsis AtMC1 showed optimal activity at pH 8 (detectable range from pH 7 to 9),

while the pH optimum of AtMC9 was 5.

The effect of various protease inhibitors on recombinant metacaspase activity was assessed using Boc-GRR-AMC as the substrate (Figure 3.2). Results showed that incubation with iodoacetamide, a cysteine protease inhibitor (10 mM), and leupeptin, an arginal inhibitor (10 μM), showed near complete inhibition (about 95% and 90%, respectively) of all metacaspase activity tested, while the serine proteinase inhibitor, PMSF (1 mM), was the most inefficient inhibitor of metacaspase activity (30 – 50% inhibition).

Investigation whether the putative cysteine residues of the dyad in PhMCA1 and PhMCA2 were catalytic residues was performed. Catalytic mutants of AtMC1, AtMC9, PhMCA1 and PhMCA2 were constructed by replacing the putative catalytic cysteines with alanines and they are AtMC1^{C220A} (AtMC1*), AtMC9 ^{C147A} (AtMC9*), PhMCA1 ^{C237A} (PhMCA1*), and PhMCA2 ^{C139A} (PhMCA2*). When the putative catalytic cysteine residues of AtMC1, AtMC9, PhMCA1 and PhMCA2 were replaced with alanine, nearly all metacaspase activity was abolished (Figure 3.3). In the petunia metacaspase PhMCA1, a serine residue was found at the putative second catalytic cysteine site found in type II metacaspases. Investigations were also conducted to determine if this serine residue affected PhMCA1 activity. A serine at 116th amino acid to cysteine mutant of PhMCA1 (PhMCA1 RV) was constructed. Metacaspase activity of PhMCA1 RV, serine to cysteine mutation at the putative second catalytic cysteine residue in type II metacaspase, was marginally but not significantly increased compared to that of PhMCA1 ($P = 0.0648$) (Figure 3.3). In conclusion, the putative cysteine residues of dyad in petunia metacaspases were critical for their enzymatic activities.

Western Blot Analysis of Recombinant Petunia Metacaspases

To investigate whether petunia metacaspases expressed in *E. coil* undergo processing and if the catalytic cysteine is responsible for the processing, recombinant metacaspases of PhMCA1 and PhMCA2 along with mutant metacaspases containing an alanine instead of a cysteine at the dyad (PhMCA1 [C237A], PhMCA2 [C139A]) were prepared for Western blot analysis. An additional catalytic cysteine residue was discovered in type II metacaspases as mentioned earlier. A mutant (PhMCA1 [S116C], serine at 116th residue replaced with cysteine) was also constructed to investigate whether this serine residue was responsible for autoprocessing. Western blot analysis was conducted using antibodies generated from the p20 subunits of PhMCA1 (Figure 3.4A) and PhMCA2 (Figure 3.4B), respectively. PhMCA1 and PhMCA2 containing no mutation (Figure 3.4) showed two distinct bands, a putative p20 subunit and an unprocessed zymogen. The expected sizes of zymogens of PhMCA1 and PhMCA2 expressed in pET28a(+) vector were 42.8 kDa and 50.1 kDa, respectively. The expected sizes of the p20 subunits of PhMCA1 and PhMCA2 were 33 kDa and 28 kDa, respectively. When the catalytic cysteine residue of the dyad was converted to alanine (PhMCA1[C237A] and PhMCA2 [C139A]), processing was blocked in both metacaspases (Figure 3.4). The second putative catalytic residue in PhMCA1, however, did not seem to be involved in protein processing (Figure 3.4). Thus, autoprocessing of recombinant PhMCA1 and PhMCA2 was observed but the catalytic mutants of PhMCA1 and PhMCA2 did not undergo autoprocessing (Figure 3.4) as previously found in Arabidopsis metacaspases (Vercammen, et al., 2004; Watanabe and Lam 2005).

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Discussion

Substrate specificity of metacaspases has been shown to be either arginine or lysine at the P1 position of the substrates, which is different from caspase substrates containing aspartic acid at the P1 position (Vercammen et al., 2004; Watanabe and Lam, 2005; Bozhkov et al., 2005). Our data showed that petunia metacaspases PhMCA1 and PhMCA2 also utilized substrate site having a basic amino acid residue, arginine, at the P1 position as a target cleavage site. This finding indicated that petunia metacaspases also did not possess caspase-like protease activity as has been shown in other plant metacaspases. These results were in accordance with previous experiments using oligopeptide substrates containing either arginine or lysine at the P1 position (Vercammen et al, 2004; Watanabe and Lam 2005). Our results also showed that petunia metacaspases were relatively insensitive to a serine protease inhibitor, while they were highly sensitive to cysteine (iodoacetamide) or arginal (leupeptin) inhibitors. These findings suggested that petunia metacaspases were cysteine dependent proteases and an arginine residue could be used as an autoprocessing site as indicated in Vercammen et al. (2004). These results strongly suggested that petunia metacaspases were not responsible for "caspase-like" proteases activity in plants as demonstrated in Arabidopsis (Vercammen et al., 2004; Watanabe and Lam 2005).

Alignment of metacaspases previously reported showed that all type II metacaspases possessed either arginine or lysine at the putative processing site as reported by Vercammen et al. (2004). When the putative autoprocessing site (arginine at

72

183rd amino acid) of AtMC9 or the catalytic cysteine residues of Arabidopsis

metacaspases were replaced by alanines, autoprocessing was blocked and the

metacaspase activity was abolished (Watanabe and Lam, 2005). These experiments

demonstrated that processing is required for their activities (Vercammen et al., 2004;

Watanabe and Lam 2005). Our data were in line with these findings, showing that

metacaspase activities of PhMCA1 and PhMCA2 were nearly abolished in mutants

containing an alanine residue instead of a putative catalytic cysteine residue.

The processing site of type II metacaspase (AtMC9) has been identified in

Arabidopsis (Vercammen et al., 2004) and the putative cleavage site for processing of

PhMCA2 was predicted by the amino acid alignment with AtMC9, allowing us to

calculate the putative size of the p20 subunit of PhMCA2. Even though no known

processing site has been identified for type I metacaspases, amino acid alignment data

also allowed us to estimate the approximate sizes of p10 and p20 subunits of PhMCA1.

According to the estimated sizes of processed and unprocessed petunia metacaspases, we

were able to determine whether PhMCA1 and PhMCA2 underwent autoprocessing in *E.

coli*. The results indicated that PhMCA1 and PhMCA2 underwent autoprocessing, while

mutant strains did not undergo autoprocessing. Our results showed that the catalytic

cysteine residues of the dyad in petunia metacaspases were required for autoprocessing

and metacaspase activity. These results agreed with previous findings in Arabidopsis

metacaspases (Vercammen et al., 2004; Watanabe and Lam, 2005).

In the Arabidopsis AtMC9, there is an additional catalytic cysteine residue (at 29th

amino acid residue), and this cysteine is conserved in all type II metacaspases

(Vercammen, et al., 2007). PhMCA2 contained this conserved cysteine residue as shown in amino acid alignment in Chapter 2. Although this second catalytic cysteine residue was reported only in type II metacaspases, this cysteine residue was also conserved in all three type I Arabidopsis metacaspases. In contrast, the amino acid sequence of PhMCA1 showed a serine residue instead of cysteine at this second catalytic site. Our experiments indicated that the serine residue in type I metacaspase did not affect the metacaspase activity of PhMCA1. PhMCA1 and PhMCA1 RV also showed autoprocessing, suggesting that type I metacaspase (PhMCA1) may not contain the second catalytic residue as type II metacaspases do.

Conclusion

      Characterization of metacaspases was performed using recombinant petunia metacaspases. Both metacaspases cleaved oligo amino acid substrates at an arginine residue as a target site, but unlike caspases, neither metacaspase was able to cleave the substrate at an aspartic acid residue. Therefore, petunia metacaspases did not seem to be responsible for "caspase-like" activity. When a catalytic cysteine residue of the dyad in either metacaspase was replaced with an alanine residue, metacaspase activities were substantially reduced and autoprocessing did not occur, indicating that the catalytic cysteine was required for the enzymatic activity and autoprocessing. PhMCA1 contained a serine residue instead of the conserved cysteine residue at the putative second catalytic cysteine residue found in type II metacaspases. When this serine was converted to cysteine, enzymatic activity was not significantly altered and autoprocessing was still

observed. Thus, this cysteine residue did not appear to be essential for the activity or autoprocessing of the type I metacaspase PhMCA1. Autoprocessing was required for enzymatic activity.

Acknowledgements

Table 3.1. Primers used for cloning of recombinant metacaspases.

Primer Names	Sequences
F-AtMC1pET	ATTTGGATCCTACCCGCCACCTCCCTC
R-AtMC1pET	AAATCTCGAGTGAGAGTGAAAGGCTTTGCATAGACATCG
F-AtMC1C220A	TGCTGCCCATAGTGGTACCGTTCTGGATTT
R-AtMC1C220A	AAATCCAGAACGGTACCACTATGGGCAGCATCGATAATTGAATGGAGCT
F-AtMC9pET	ATTTGGATCCGATCAACAAGGGATGGTCAAGAAAAGACTAG
R-AtMC9pET	ATTGCTCGAGTAGGTTGAGAAAGGAACGTCGC
F-AtMC9C147A	TTCTGCCCATAGTGGTGGTCTCATCGACA
R-AtMC9C147A	TGTCGATGAGACCACCACTATGGGCAGAATCAGAGATCATGGTAAAGCTTGTTCC
F-PhMCA1pET	AATTAGGATCCGACGGCAGGAGATACAAATGCAAGTG
R-PhMCA1pET	TTATGAAGCTTGCAAACGTACCTGAGTTTCTTTGGCACC
F-PhMCA1C237A	ACTGCCTTCAGTGGAACTTTCCTAGATTTGCCATTT
R-PhMCA1C237A	GGCAAATCTAGGAAAGTTCCACTGAAGGCAGTATCGATTATGCCATGAAGTGTTGC
F-PhMCA1S116C	GGATGCGTTACTGATGCTTTATCCATGAGACA
R-PhMCA1S116C	TGTCTCATGGATAAAGCATCAGTAACGCATCCTTTCAGCATCTTTGGA
F-PhMCA2pET	AAATTGGATCCGCAAAAAAGCAGTGTTAATAGGAATCAACTATCC
R-PhMCA2pET	CAGCAGAGCTCGCACACGAAAGGAGCATCAACG
F-PhMCA2C139A	CAGCACACAGTGGTGGCCTCATTGATAAGGCT
R-PhMCA2C139A	AGCCTTATCAATGAGGCCACCACTGTGTGCTGAGTCGGAGACAATTGTGATCTGGC

Figure 3.1. Substrate specificity of recombinant AtMC1, AtMC9, PhMCA1 and
PhMCA2 metacaspases using fluorogenic substrate (Boc-GRR-AMC in panel A) and
caspase substrate (Ac-DEVD-AMC in panel B) at different pH. Relative enzymatic
activities of metacaspases are measured as the percentage of the activity compared to the
highest metacaspase activity (PhMCA2 at pH 8). Data are the means of three replicates $\pm$
SE. The experiment was performed twice with similar results.
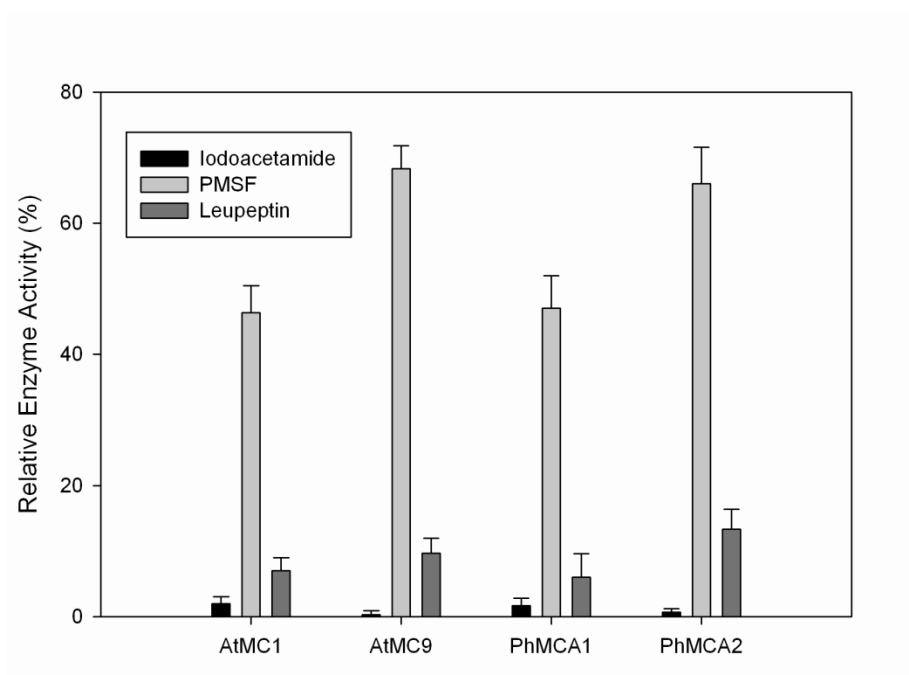
A. Boc-GRR-AMC
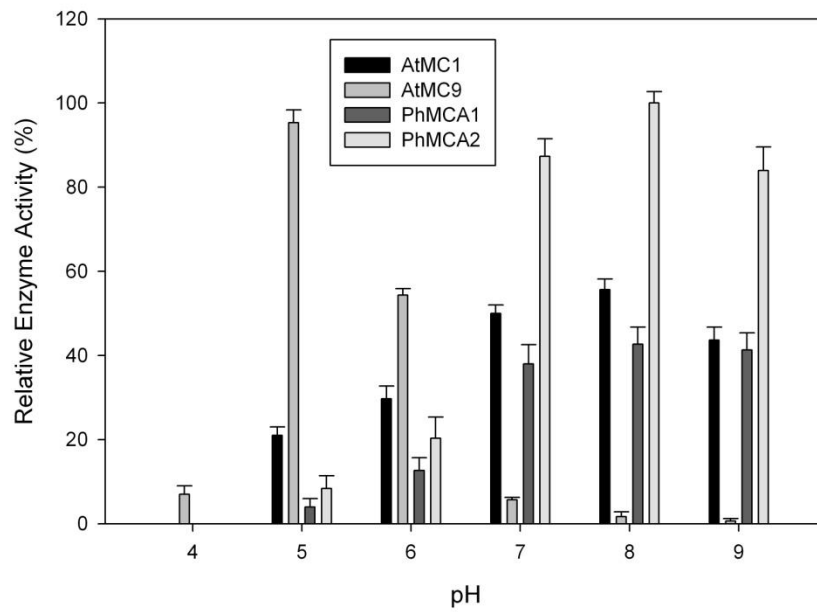
B. Ac-DEVD-AMC
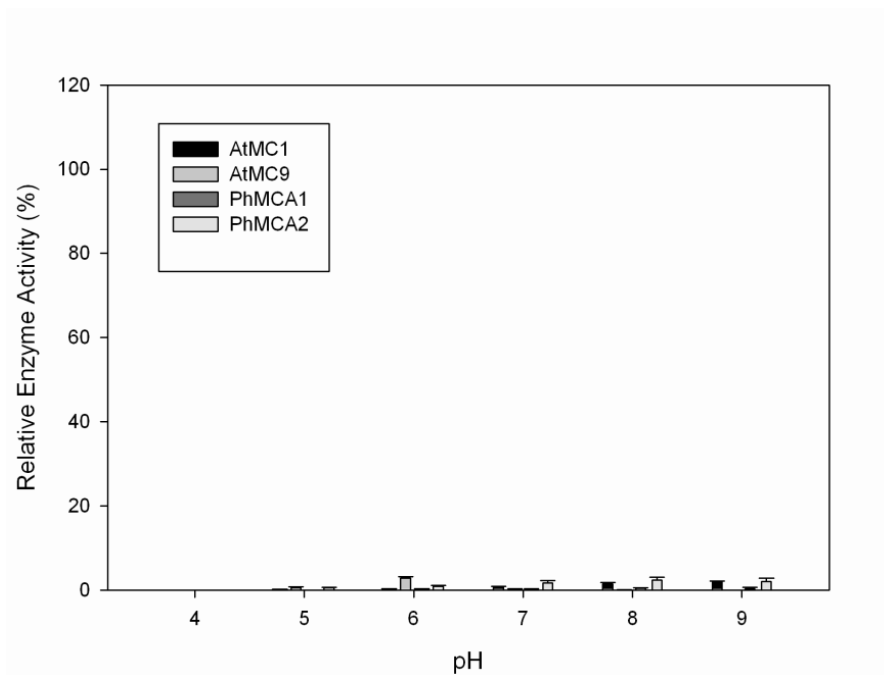
Figure 3.2. Effect of cysteine inhibitor (iodoacetamide), serine/threonine inhibitor (PMSF), and arginal inhibitor (leupeptin) on the activities of recombinant PhMCA1 (pH 8), PhMCA2 (pH 8), AtMC1 (pH 8) and AtMC9 (pH 5) using Boc-GRR-AMC as substrate at their optimum pH as indicated in parenthesis. Enzymatic activities of PhMCA1, PhMCA2, AtMC1 and AtMC9 were expressed as the percentage of the relative activity compared to activities without inhibitors, respectively. Data are the means of three replicates ± SE. The experiment was performed twice with similar results.
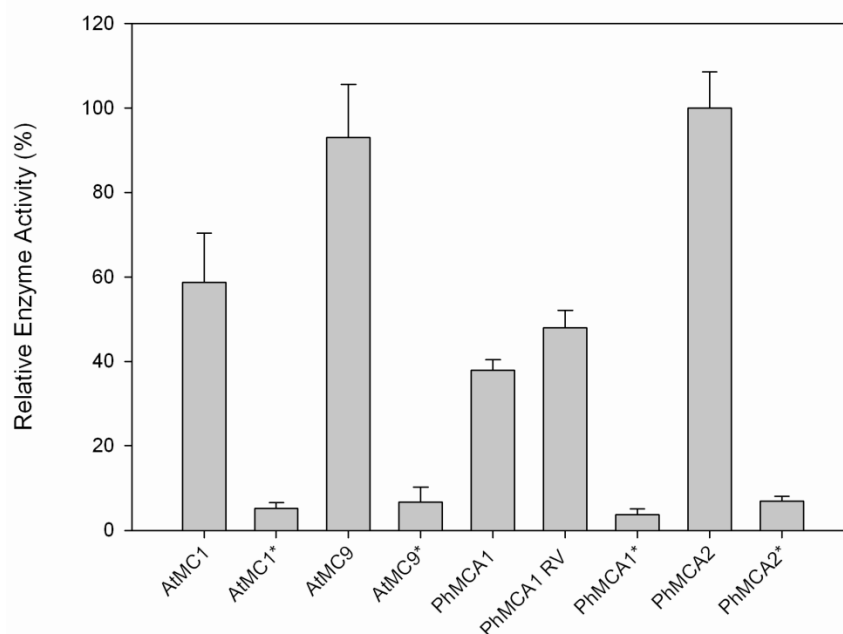
Figure 3.3. Metacaspase activity assay using catalytic mutants. * indicates catalytic mutants and RV indicates serine to cysteine mutant of PhMCA1. Enzyme assay was conducted using fluorogenic substrate (Boc-GRR-AMC) in the reaction buffer at pH 8 except AtMC9 and AtMC9* at pH5. Relative enzymatic activities of metacaspases are measured as the percentage of the activity compared to the highest metacaspase activity (PhMCA2 at pH8). Data are the means of three replicates ± SE (n=3). The experiment was performed twice with similar results.
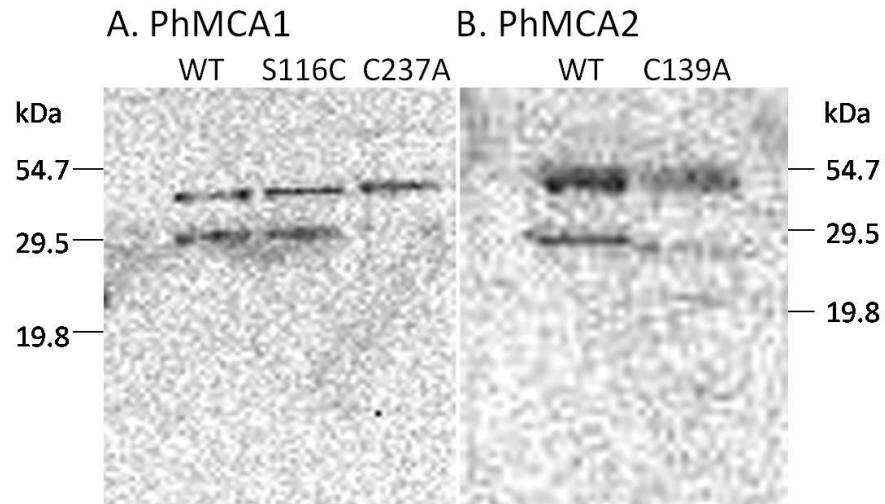
Figure 3.4. Western blot analysis using recombinant proteins from PhMCA1, PhMCA1 ^{S116C}, PhMCA1 ^{C237A} using anti PhMCA1 p20 antibody (panel A), and PhMCA2 and PhMCA2 ^{C139A} using anti PhMCA2 p20 antibody (panel B).

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