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Characterisation of the metacaspase gene family in Arabidopsis thaliana

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CHARACTERISATION OF THE METACASPASE GENE FAMILY IN *ARABIDOPSIS THALIANA*

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Table of Contents

List of Figures

List of Tables

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Abstract

Caspases are known to be involved in animal programmed cell death (PCD). The objective of this thesis was to use gene expression analysis and reverse genetics to determine if *Arabidopsis* metacaspase (*AtMC*) genes play a role in plant PCD. The majority of *AtMC* genes were found to be expressed nearly constitutively in various tissues, developmental stages, and under various inductive treatments. Transgenic *Arabidopsis* plants generated with AtMCpromoter::AtMCgene::GUS fusions showed expression of the reporter gene in leaves, vasculature, trichomes, siliques, anthers, and during embryo development. Preliminary phenotypic characterization of single and double *Arabidopsis AtMC* loss-of-function mutants suggested that the expression of the AtMC genes are highly functionally redundant. Nevertheless, our results suggest that *AtMC1, 2, 4, 6* and *9* may be directly involved in rosette and/or stem development. Although this study does not provide a definitive role of MCs in plant PCD, it lays the foundation for their further in-depth analysis.

1 Introduction

1.1 Introduction to the components of programmed cell death

1.1.1 Introduction to Programmed Cell Death

Programmed cell death (PCD), also known as apoptosis, is a process which maintains constant cell numbers during development through elimination of unwanted cells (Watanabe & Lam 2005). PCD also creates structures entirely composed of dead cells, for example plant xylem treachery elements (TEs) which function in the transport of water and nutrients from the soil (Greenberg 1996, Cooper 2000). In animals, the active process of PCD is characterized by condensation of the chromatin, membrane blebbing, chromosomal DNA breakage, condensation of the cytoplasm, and fragmentation of the nucleus (Cooper 2000.) Several of these cellular events can be observed in plants as well. For example, plants also undergo cytosolic condensation, and fragmentation of the nucleus (Greenberg 1996, Woltering et al. 2004). However, in plants PCD is also associated with vacuolar collapse (Nieminen et al. 2004, Bonneau et al. 2008) which is especially important during PCD of TEs. Cell death is also important during plant reproduction, senescence, flowering, and defence against pathogens (Greenberg 1996). It has been suggested that PCD may be involved in self incompatibility responses (Bonneau et al. 2008, Love et al. 2008). PCD in plants is enabled by the proteases that can elicit, en masse, cellular proteolytic degradation. These enzymes and their function in plant development are the focus of this study.

1.1.2 Enzymes Involved in PCD

Protein degradation, or proteolysis, is the breakdown of proteins via cleavage by enzymes known as proteases. Cleavage can be a regulatory process which activates or inhibits protein activity, or can destroy it altogether. It is important in regulation of gene expression, in the cell cycle and in PCD (Sheth & Yadlapati 2009). Proteases cleave the proteins' peptide bonds using hydrolysis. Each protease contains a catalytic diad or triad made up of two to three amino acids, plus a pocket (S_1Pocket), which together creates the active site. The substrate binds to the pocket and the catalytic dyad/triad induces

cleavage of the peptide. Water enters the active site and the part of the substrate still located inside the pocket is released (Cooper 2000).

Cells control protease activity and thus the amount and rate of PCD via protease inhibitors (Solomon et al. 1999). Two well known protease inhibitors are Inhibitors of Apoptosis (IAPs) and Serpins (Thornberry & Lazebnik 1998, Vercammen et al. 2006). Protease inhibition is utilised as a regulatory step in processes like PCD and plant defence against pathogens and insects (Ryan 1990). There are several kinds of proteases. The largest protease families are serine proteases, aspartate proteases, and cysteine proteases (Beers et al. 2004). Though not much is currently known, it has been shown that proteases can have different substrate specificities (García-Lorenzo 2007). Each protease has a cleavage preference for a specific amino acid and cleaves the peptide bond after this amino acid. For cysteine proteases, a nucleophilic cysteine is first activated by a histidine amino acid via deprotonation and the cysteine nucleophile cleaves the peptide bond of the substrate (Berg et al. 2005, van der Hoorn 2008). An asparagine residue is present in order to orient the histidine side chain in the appropriate direction (García-Lorenzo 2007). Examples of cysteine proteases are papain, an enzyme in papaya fruit, and caspases, enzymes involved in apoptosis in animals

1.1.3 Caspases and Proteins with Caspase-like activity

Caspases are cysteine dependent aspartate-specific proteases (Lee 2010). As their name implies, caspases cleave substrates after an aspartate (Asp) amino acid residue. The first caspase was isolated from *Caenorhabditis elegans* (*C. elegans*) and since then, fourteen have been discovered in mammals with seven of those playing a role in apoptosis. Caspases are first synthesized as an inactive pro-enzyme, called a zymogen. The caspases undergo autoprocessing (autocleaving) which generates a large (p20) and a small (p10) subunit, which brings about conformational change allowing access of the substrate to the active site for cleavage.

Plants have several different types of caspase-like activities (reviewed in Bonneau et al. 2008). Plant caspase-like activities are named according to the target substrate (eg. YVADase, DEVDase, VEIDase, and Saspase) (Woltering et al. 2004). It is difficult to determine the full spectrum of caspase-like activities in plants, but it is believed that there are up to eight different activities observed in different plant species (see Bonneau et al. 2008 for a complete list). Caspase-like activities have also been demonstrated in several different locations of the cell, such as the vacuole, cytosol and nucleus. It is also currently unclear whether the caspase-like activities observed in plants are directly involved in PCD (Bonneau et al. 2008) and what role they may play. To date, the identity of the plant cysteine proteases with caspase-like activities has only been solved for the caspase1-like YVADase, which is believed to a large extent to be dependent on the activity of plant vacuolar processing enzymes (VPEs) (Hatsugai et al. 2004). The VPEs are stored in an inactive form in the central lytic vacuole, and are activated after the vacuolar collapse which leads to PCD (Bonneau et al. 2008). No other enzymes responsible for the plant caspase-like activities have been described. Their identity and function(s) in plants remain to be discovered and studied.

1.1.4 Introduction to Metacaspases

Following the discovery of caspases in animals, homologous proteases were identified in other organisms. Exhaustive bioinformatic analyses identified two groups structurally similar to caspases, the paracaspases (PCs) and the metacaspases (MCs) (Uren 2000). PCs are present in metazoans, while MCs can be found in plants, bacteria, fungi, chromista, and protozoa (Uren 2000). There are two types of MCs, MC type I and MC type II (Bonneau et al. 2008). MCs type I contain a proline rich prodomain which is structurally similar to the prodomain found in animal caspases involved in PCD initiation and inflammation. MC type I can be found in all lineages between algae to flowering plants, and are also present in protozoa, fungi and chromista as well (Cambra et al. 2010). MC type II, on the other hand, lacks a prodomain but instead contains a longer linker region than that found in type I MCs, which connects the p10 and p20 subunits. MCs type

II are found only in plants and algae (minus two algae) and not in protozoa, fungi or chromista (Cambra et al. 2010, Uren et al. 2000). The lack of MCs in the two algae is believed to be due to gene loss from environmental adaptation (Cambra et al. 2010). Another difference between the two MC types is the presence of a *LSD1*-like-finger N-terminal motif in MC type I. *LSD1* has been shown to halt PCD when the hypersensitive response is initiated, and all three MC type I can strongly interact with *LSD1*. MC type II can only weakly interact with *LSD1* (Coll et al. 2010). There is no known connection between the gene numbers of MC type I vs. MC type II within different species.

1.1.5 Similarities and differences between caspases, and MCs

One common characteristic among caspases and both types of MCs is the presence of a histidine/cystein (His/Cis) dyad (Cambra et al. 2010). Predicted analysis suggests that MCs contain a caspase/hemoglobinase fold consisting of a histidine-cysteine pair, and has thus been placed into the CD cystein protease clan (Aravind & Koonin 2002, Barrett & Rawlings 2001, Vercammen et al. 2004). This clan contains all enzymes that use a catalytic cysteine to hydrolyse peptide bonds of their substrates (Cambra et al. 2010). MCs also share the S_1 pocket forming residues and maturation sites seen within the caspase/hemoglobin fold (Sundström et al. 2009). Similar to caspases, MCs also show the presence of a heterodimer made up of the p20 and p10 subunits (Woltering et al. 2002, Vercammen et al. 2004). Studies have shown that MCs along with caspases need to be activated via autoprocessing and that this process is cysteine dependant (Vercammen et al. 2004, Belenghi et al. 2007, Watanabe & Lam 2011).

Even though caspases and MCs both contain a His/Cis dyad, show a similar secondary structure, and share a basic mechanism of catalysis, there are still defining differences between the two. One of the largest differences between the two protease families is the amino acid after which they cleave (Bonneau et al. 2008). In caspases, cleavage occurs after acidic aspartate, while MCs cleave after either a basic lysine (Lys) or arginine (Arg) residue (Vercammen et al. 2004, Watanabe & Lam 2005, Watanabe & Lam 2011). A second major difference is the low sequence homology between the two

protease families (Cambra et al. 2010). The overall similarity of the two sequences is not high (Belenghi et al. 2007, Cambra et al. 2010), and while MCs have two cysteine residues in their sequence, caspases only have one (Belenghi et al. 2007). Due to these differences, it is considered unlikely that MCs are indeed homologs to caspases (Vercammen et al. 2004, Bonneau et al. 2008). Despite the fact that MCs have similar morphology and secondary structure as caspases, since MCs do not show cleavage specificity for Asp, they can not be defined as a caspase (Woltering et al. 2002).

1.2 Analysis of MC Functions

1.2.1 Enzyme activity and substrate/inhibitor analyses

There are a total of nine *Arabidopsis* MC genes making up a gene family (**Figure 1.1**) which consists of three MC type I (*AtMC1-3*) and six MC type II genes (*AtMC4-9*). *AtMC* mutants do not show obvious phenotypic differences, most likely due to significant functional redundancy (Bonneau et al. 2008). Overexpression of MC genes in transgenic plants have been equally uninformative of their function (Bonneau et al. 2008), likely due to post translational modifications by the serpin enzymes (Vercammen et al. 2006), or because overexpression is merely not creating any visible phenotype.

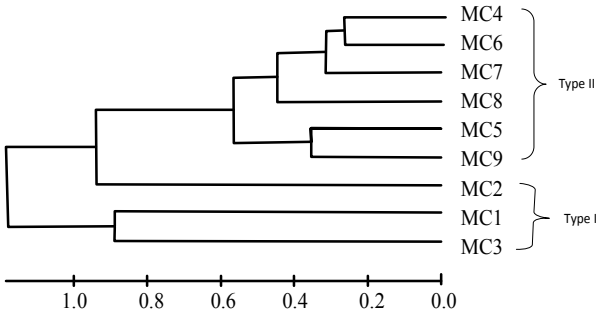


Figure 1.1: AtMC phylogenetic tree. Relationship between protein sequences of the nine MC *Arabidopsis* genes (created by Mega5).

Up until recently, there were no known substrates for MCs. It has been shown that the human Tudor staphylococcal nuclease (TSN) is a substrate for animal caspases (Sundström et al. 2009). Discovery of conserved plant TSN suggested this may be a substrate for MC cleavage. A recent study in *Picea abies (P. Abies)* and *Arabidopsis* has shown that in both plants, TSN was cleaved by MCs at Lys or Arg *in vivo* (Sundström et al. 2009).

In support of the initial discovery that MCs cleave after Arg and Lys, two purified *Arabidopsis* MCs (AtMC4 and AtMC9) were tested using five oligopeptide substrates containing an Arg or a Lys. All five substrates were cleaved by both MCs at the predicted amino acids, while neither of the two MCs was capable of cleaving three chosen caspase-specific oligopeptide substrates containing Asp (Vercammen et al. 2004). The same MC cleavage specificity was observed in *P. abies*, as well. A fluorometric peptide cleavage assay of *Escherichia coli (E. coli)* expressed recombinant mcII-Pa (spruce MC) zymogen showed cleavage after both Arg and Lys, separating the p20 and p10 subunits from the linker region (Bozhkov et al. 2005). There was no cleavage when caspase-specific substrates were added to recombinant mcII-Pa.

To further test the functional homology between caspases and MCs, the effects of protease inhibitors which normally affect caspases were tested on AtMC4 and AtMC9 (Vercammen et al. 2004). These inhibitors were unable to block either MC from binding to their synthetic substrate at very high concentrations (up to 100μM). However, addition of arginal protease inhibitors instead of caspase inhibitors at concentrations as low as 1μM fully prevented AtMC9 from cleaving its substrate, while AtMC4 was moderately blocked (Vercammen et al. 2004). After determining that AtMC4 and AtMC9 were Arg/Lys specific, Vercammen et al. 2006 went one step further and used an *Arabidopsis* Serpin1 inhibitor (AtSerpin1) identified in a yeast two-hybrid system to test AtMC9 substrate specify and discovered that this plant protease inhibitor did indeed inhibit activity of AtMC9.

It was previously believed that MC proteins may be localized in the cytoplasm of plant cells due to their lack of a signal peptide (Woltering et al. 2004). Several studies showed that different AtMCs have different pH optimums. AtMC9 requires a low pH (between 4.5-6.0) while AtMC4 requires high pH (between 6.5-9) for activation (Vercammen et al. 2004). Activation of AtMC8 has been shown to occur at the slightly basic pH of 7.5-8.5 (He et al. 2007). This leads to the speculation that if different AtMCs do localize to the cytoplasm, yet require different pH environments, the function of these MCs must be correlated with events triggering significant ion exchanges which can significantly change the pH of the cytosol. Studies in tobacco show that the elicitor cryptogein induces acidification of the cytosol, while NaCl can significantly lower the pH in less than an hour (Vercammen et al. 2004). It seems likely that AtMCs will have different activation conditions depending on their localization, their signalling pathways and their overall role in plant development.

Another study has suggested that AtMC9 localization takes place in the apoplast. Due to the low pH required for activation of this AtMC, apoplastic localization is logical (Vercammen et al. 2006). It is also possible that AtMC proteins localize in either the nucleus or the vacuole. When *AtMC9* under a 35S promoter was fused to GFP, a strong GFP signal was observed in the nucleus and a weaker signal in the cytosol. No signal was observed in the vacuole, but this could be attributed to masking of the vacuolar signal sequence by the reporter gene fusion (Vercammen et al. 2004). A study using 35S::*AtMC1*::GFP fusion showed that AtMC1 localizes in the chloroplasts (Castillo-Olamendi et al. 2007). To date, no systematic study of all AtMCs' localization has been performed.

The identification of AtSerpin1 as an inhibitor of AtMC9 may suggest a possible regulatory role for AtSerpin1 in *Arabidopsis* MCs' regulation. In addition to AtSerpin1, autoprocessing and S-nitrosylation have also been suggested to play an active role in the control of MCs at both the transcriptional and post-transcriptional levels. Studies on AtMC9 have shown that enzyme activity is conditional on cleavage at Arg183

(Vercammen et al. 2004). It is currently unknown what triggers autoproccessing in MCs but possibilities include initiation by a change in pH, by other proteases, by metacaspases themselves, or by the addition of Calcium (Ca^{2+}). According to Vercammen et al. in 2004 in *Arabidopsis* type II MCs overproduction of all AtMCs in bacteria lead to autoproccessing similar to the way some caspases initiate cleavage. Caspases are capable of cleaving themselves and are broken down into two groups, initiator caspases, and effector caspases. Initiator caspases are auto-activated and are required for activation of effector caspases, (Riedl & Shi 2004). Also shown in this publication was the fact that AtMC9 requires an acidic pH for activation. A recent study has determined that in some AtMCs Ca^{2+} is necessary for conversion of the proenzyme into the catalytically active form by promoting cleavage of the highly conserved Lys-225 residue found in all type II MCs. This Ca^{2+} dependency was observed in AtMC4, but not in AtMC9, suggesting that not all MC type II proteases require Ca^{2+} for activation of the zymogen and thus may have a different function and activation mechanism (Watanabe & Lam 2011).

Another process thought to regulate MC activity is S-nitrosylation. This occurs when the active Cys residue in MCs is nitrosylated in the presence of nitric oxide (NO). Nitric oxide is a signalling molecule that exists in both the intracellular and intercellular regions of plants and is transported via the plant xylem (Ohashi-Ito et al. 2010). When a NO is covalently attached to a cysteine, the protein undergoes post translational modification (Belenghi et al. 2007). S-nitrosylation and its link to enzyme inhibition was first described in animal procaspase 3. MCs can also be regulated by S-nitrosylation. It has been shown that Cys-147 S-nitrosylation impedes AtMC9 autoprocessing (Belenghi et al. 2007). Cys-29 in the same protein, however, is immune to S-nitrosylation and is able to restore the autoprocessing function of the metacaspase (Belenghi et al. 2007).

1.2.2 Developmental Functional characterisation

Saccharomyces cerevisiae (*S. cerevisiae*) (baker's yeast) was the first organism used to study MCs and their relationship with PCD. The single *S. cerevisiae* metacaspase, *yeast caspase 1* (*Yca1*) is required for hydrogen peroxide induced aging and apoptosis,

while overproduction of *Yca1* results in early aging (Madeo et al. 2002). A recent study has shown that *Yca1* also plays a role in the control of aggregate formation of insoluble proteins by controlling their removal (Lee et al. 2010). *P. abies* somatic embryos have been a valuable system in understanding the role of a MC in embryogenesis in general (Helmerssoon 2007). RNAi down-regulation of a *P. abies* type II metacaspase (*mcII-Pa*) led to a decrease in PCD of the embryo suspensor cells (Suarez et al. 2004). Further studies have shown that mcII-Pa moves from the cytoplasm to the nuclei of undifferentiated embryonic cells, leading to DNA fragmentation, and disassembly of the nuclear envelope resulting in PCD of those cells. After silencing *mcII-Pa*, the cells no longer disintegrated suggesting that MCs do play in role in plant PCD (Bozhkov et al. 2005). It has also been shown that *Arabidopsis MC8* is upregulated by H_2O_2 and other types of stresses. In the *AtMC8* loss-of-function mutant, cell death was reduced after H_2O_2 treatment suggesting that *AtMC8* is induced and controls a response to oxidative stress through promoting PCD (He et al. 2007).

It has also been shown that PCD can be activated in response to pathogens. Plants with insertional disruptions in two AtMC type I, and five AtMC type II genes were inoculated with *Botrytis cinerea*, a necrotrophic fungi known to affect a range of different plants. After infection, all seven MC mutants showed a significant reduction in cell death over the Wild-type (van Baarlen et al. 2007). Other studies have suggested differential roles for the two types of MCs in response to bacterial pathogens. Two of the type I *Arabidopsis* MCs (*AtMC1* and *AtMC3*) were upregulated by various bacterial pathogens, while none of the type II MCs showed any induction (He et al. 2007). A different study concluded that all three type I MCs and two type II MCs (*MC5* and *MC8*) were activated upon infection with various bacterial pathogens (Watanabe & Lam 2005).

1.3 My Thesis: The value in determining the function of MC genes and their connection to programmed cell death in *Arabidopsis thaliana*

1.3.1 Value of the project

Manipulation of tree biomass is of substantial interest to timber, bioenergy and pulp/paper industries. Trees provide long-term carbon storage with mitigates the negative effects of CO_2 emissions and associated climate changes. The main biomass of trees resides in the woody tissues of the stem, which is the secondary xylem of the trees. Xylem plays a role in water and nutrient transport as well as providing mechanical strength. It contains three different cell types, parenchyma cells, fibers and treachery elements (TE) (Ohashi-Ito 2010). Parenchyma cells are alive and are only a relatively minor part of xylem total biomass. Fibers are highly lignified and provide mechanical support to the tree. Treachery elements can be either tracheids or vessel elements (VEs). One venue to increase biomass is to manipulate genes responsible for PCD in the tree xylem. Postponing PCD would lead to the development of thicker cell walls in xylem, thus leading to increased biomass.

In order to fully understand, and to be able to manipulate PCD in plants, the players involved in the process of PCD should first be discovered. PCD is a highly organized process and thus the mechanism and interaction between the different players should be subsequently well-understood. This thesis focused only on the first step, identification of the key players. The objective of the thesis was to determine if the distantly related MC genes play a role in plant PCD similar to the way caspases play a role in animal PCD. The long term goal of this project is to prolong the lifetime of xylem fibers through modification of genes involved in PCD to create thicker cell walls leading to higher wood density, and thus higher biomass.

1.3.2 Why study MCs?

The primary interest in MCs is in relation to their putative role(s) in PCD in plants. Although the role of MCs in PCD is yet unclear (He et al. 2008, Cambra et al. 2010) they are the closest known homologs to caspases in animals and thus good putative

targets that can provide an entry point to our understanding of PCD in plants. In addition to playing a direct role in PCD, MCs may also be indirectly involved in PCD via a signalling pathway(s) which ultimately leads to cell death (Bonneau et al. 2008). For example, the deactivation or activation of other proteins could be necessary for PCD, and proteases such as metacaspases could be speeding up the reaction (Bonneau et al. 2008). It is also probable that since MCs comprise a large gene family, some of them may play a more direct role in PCD than others. PCD is not the only known function for caspases, they have been implicated in activation of the immune system and differentiation of different cell types (Lee et al. 2010). This suggests that MCs may have other functions in plant growth and development which are not even speculated. Thus the function of MCs in relation to PCD, and in general, is still a mystery (Cambra et al. 2010). Thus, study of MCs may provide new fundamental insights into plant development and provide useful means for manipulation of woody biomass.

1.3.3 Objectives and goals of the thesis

Due to the many experimental advantages, we choose *Arabidopsis* for the analysis of MCs' roles in plant PCD. Though it is not a tree species, it can produce secondary woody-like xylem in the hypocotyl and thus can be used as proxy for studies of wood development. Of the nine MC genes in *Arabidopsis*, the most heavily analyzed has been *AtMC9*, and yet, information regarding even *AtMC9* is minimal. Some work has been done on *AtMC4* and *AtMC8*, but there is relatively nothing known about the other AtMC genes, other than their structure and the fact that they do indeed fall into the MC category. Therefore, this work focused on a broader and more general characterization of the whole *AtMC* gene family, however, particular focus was placed on *AtMC9* because of its putative importance for wood development. Previous expression data from poplar suggests that a putative aspen ortholog of *AtMC9* is expressed during the stage of PCD of xylem development.

There were three main objectives of this thesis.

- To analyze the expression pattern of the *Arabidopsis* metacaspase genes using the rich expression databases for this species in order to understand the expression pattern of the different genes across different developmental stages and in response to stress and other stimuli.

- Generate and preliminarily analyze transgenic lines expressing translational beta-glucuronidase GUS fusions of each *AtMC* gene. The goal was to produce AtMCpromoter::AtMCgene::GUS lines for each of the AtMCs in *Arabidopsis*. This study would point to organ/tissue specific level localization of each MC genes' activity.

- To study the developmental function of the different members of the *Arabidopsis* metacaspase gene family. This was performed using reverse genetics on both single mutants from each of the gene family members, and on double mutants created by crossing each of the single mutants with the *AtMC9* mutant.

The hypothesis was that AtMC genes do play a direct role in PCD.

2 *Materials and methods*

2.1 Plant Material

Arabidopsis thaliana (*A. thaliana*) ecotype 'Columbia' (Col-O) were used as Wild-type controls. *Arabidopsis* MC homozygous T-DNA insertion mutants were obtained from the Syngenta *Arabidopsis* Insertion Library (SAIL), the Salk Institute for Biological Studies (SALK), and from the GK project (GABI-KAT). See **Table 2.2** for a complete list of single and double mutants analyzed.

2.2 Growth Conditions

Plants were grown in a controlled growth chamber at 22^0C during the day and 19^0C at night. Light conditions were typical long days with 16 hours of light and 8 hours of dark with a PAR of 150µmol/m^{-2}/s^{-1}. More information can be found at http://www.upsc.se/ under "Resources-Controlled environment."

2.3 DNA Extraction

Genomic DNA for PCR was extracted from *A. thaliana* leaves using extraction buffer (See section 2.12), heated and centrifuged to produce a supernatant, precipitated with equal volume of isopropanol, washed with 70% ethanol dried at room temperature and resuspended in 50µl Tris. DNA was analyzed using a Nanodrop spectrophotometer.

2.4 Gene expression studies

We studied the expression of the 9 *AtMCs* using Genevestigator (https://www.genevestigator.com/gv/index.jsp). This meta-profile analysis tool was used to collect expression data on the tissues, developmental stages, and stimulus treatments for the 9 *Arabidopsis* MCs. The data in Genevestigator was compiled from a large collection of Affymetrix microarrays.

2.5 GUS histochemical staining

Leaves were incubated at 37^{0}C for 24h in 1mM $K_4Fe(CN)_6$, 1mM $K_3Fe(CN)_6$, 50mM sodium phosphate buffer (pH7) and 0,1% Triton X-100. After incubation, samples were destained in 70% EtOH, mounted in 50% glycerol and viewed with a Zeiss Axioplan light microscope.

2.6 Genotyping

Prior to genotyping, seeds from *mc9-1 mc4* and *mc9-2 mc4* double mutants were grown on regular MS media and were selected for reduced growth. *mc9-1 mc5* double mutant seeds were plated on antibiotic medium and selected for survival. *mc6* mutant seeds were sown directly into soil. DNA from these mutants was extracted from young leaf tissues as described above. A list of primer sequences can be found in **Table 2.1**.

Table 2.1
Primers used for genotyping. Genomic Col-O was used as negative control

GK forward	CCTTTTTCCTTTTAGAGTACACCAC
GK reverse	TTCGGATTCAAACAAGACGAC
GK LB	GGGCTACACTGAATTGGTAGCTC
Sail 856D05 forward	AACTTCTTCACTTTCGGGCTC
Sail 856D05 reverse	AATGTCTCGTTGAACGGTACG
Sail 856D05 LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
Salk LB1.3	ATTTTGCCGATTTCGGAAC
Salk 006679 forward	AAACCGAGCATTGACATAAGC
Salk 006679 reverse	CCATTACAGTGGACATGGGAC
Salk 145461 forward	ACACATGTTGGGAACAAGCA

2.7 Phenotyping

2.7.1 Mutants and experimental design

Single mutations for all genes, with the exception of *AtMC5* and *AtMC8*, for which there were no mutants available, were crossed into the *mc9-1* or *mc9-2* mutants' background. Plants from **Table 2.2** were sown directly into soil. Trays were rotated around the growth chamber once a week.

Table 2.2
AtMC single and double mutants analyzed in the phenotype screening. Asterisk (*) indicate genes for which mutants were unavailable for pheontyping

Single Mutant T-DNA insertion line	AtMC gene	TAIR Accession Number	AtMC gene number abbreviation	Double Mutant T-DNA insertion line	AtMC gene number abbreviation
Col Wild-type (Control)					
SALK075814 (Salk 14)	*MC9*	At5g04200	*mc9-2*		
GABI 540H06 (GK)	*MC9*	At5g04200	*mc9-1*		
GABI 096A10	*MC1*	At1g02170	*mc1-1*	GABI 540H06 x GABI 096A10	*mc9-1 mc1-1*
SALK 002986	*MC1*	At1g02170	*mc1-2*	*GABI 540H06 x SALK 002986*	*mc9-1 mc1-2*
SALK 009045	*MC2*	At4g25110	*mc2*	*GABI 540H06 x SALK 009045*	*mc-9-1 mc2*
GABI 545D06	*MC3*	At5g64240	*mc3*	*GABI 540H06 x GK 545D06*	*mc9-1 mc3*
SAIL 856D05	*MC4*	At1g79340	*mc4*	*SALK14xSAIL856D05*	*mc9-2 mc4*
*SALK 145461C**	*MC5**	At1g79330	*mc5*	*GABI 540H06 x Salk 145461**	*mc9-1 mc5**
SALK 063453	*MC6*	At1g79320	*mc6-1*	*GABI 540H06 x SALK 063453*	*mc9-1 mc6-1*
SALK 006679	*MC6*	At1g79320	*mc6-2*	*GABI 540H06 x Salk 006679*	*mc9-1 mc6-2*
SALK 127688	*MC7*	At1g79310	*mc7*	*GABI 540H06 x SALK 127688*	*mc9-1 mc7*
	*MC8**	At1g16420	*mc8*		

2.7.2 Phenotyping Data Collection

Data collection on rosette development and stem height was separated into three different experiments. Experiment one contained *mc9-2 mc4* mutants, the second consisted of *mc6-2* mutants and the third analyzed the remaining mutants with the exception of *mc5* and *mc8*. The number of leaves, minus the cotyledons, per plant was counted after three weeks of growth under long days. During week six, data on the number of leaves per rosette, the width of the broadest leaf per rosette, and the size of each rosette was collected. Leaf width was determined by measuring the distance across the widest leaf per mutant per line. Rosette size was determined by measuring the length from the tip of the longest leaf to the tip of the leaf directly horizontal to it. The largest distance per mutant plant per line was recorded. Senescence occurred until week seven and individual plant senescence was analyzed by recording the number of leaves showing yellowing and dividing that number by the total number of leaves on the plant. During week seven the stem of each plant was measured from the base of the rosette to the tip of the main stem. Photographs of each phenotypic characteristic during week six were taken using a Canon EOS 450D. For bolting time, data was not collected from *mc9-2 mc4* or *mc6* and thus one control was used. The percentage of plants in each line showing the presence of bolting during weeks 3-5 were recorded. After week seven, nine out of the ten plants were dissected and the hypocotyls were removed and fixed in FAA (see section 2.12). The remaining plant was grown to full maturity and seeds were harvested.

2.7.3 Data analysis

Excel was used to record, analyze and graph data. In order to combine data from three different experiments each trait in the mutants was calculated as a proportion from the Wild-type in its own particular experiment. Statistical significant differences were determined using a two independent sample two-tailed t-test with 18 degrees of freedom. Lines were considered to be significantly different from the Wild-type control if the p-value was less than or equal to 0.05.

2.8 Production of Promoter::gene::GUS constructs

2.8.1 Primer design and PCR of At*MC1-9*:

The *Arabidopsis* Information Resource (TAIR) database was used to locate the sequences of *AtMC1-9* genes. Primers were designed for the promoter::gene construct using Vector NTI 11 (Invitrogen). The promoter was cloned in order to incorporate the endogenous promoter into the construct while the genes themselves were included in the construct in order to insure that any transcription machinery coded for within the gene would be included along with post-transcriptional regulatory sequences. In the reverse primers, a cytosine was added immediately after the last nucleotide of the attB sequence in order to maintain the proper reading frame. Inset primers (p800) were designed as needed to cover gap regions during sequencing (**Table 2.3**). Phusion® polymerase (Finnzymes) was to amplify the different gene fragments during PCR.

Table 2.3
Forward, reverse and inset primer sequences for cloning in the 5'-3' direction. The additional cytosine is underlined.

Gene	Primer Sequence	Inset Primer Sequence (p800)	Fragment Size (kb)
At1g02170-MC1F	GGGGACAAGTTTGTACAAAAAAGCAGG CTTACCAATGATGTCTCAGAAC	TCAACGACGCCAAGTGCATGC	2.0
At1g02170-MC1R	GGGGACCACTTTGTACAAGAAAGCTGGG TAC<u>C</u>GAGAGTGAAAGGCTTTGC	CGGCGAGCCCTTTTCCTT	
At4g25110-MC2F	GGGGACAAGTTTGTACAAAAAAGCAGG CTTACTTCCCCCTTGATCTTCGTCG	GCGCTCCCTGACAATTGC	2.9
At4g25110-MC2R	GGGGACCACTTTGTACAAGAAAGCTGGG TAC<u>T</u>AAAGAGAAGGGCTTCTCATATA	TTCATGGGTTTCAACAGC	
At5g64240-MC3F	GGGGACAAGTTTGTACAAAAAAGCAGG CTTAAGATACGCAACAGAGTTC	TTCTTGTCGTTCATTACA	2.0
At5g64240-MC3R	GGGGACCACTTTGTACAAGAAAGCTGGG TAC<u>C</u>GAGTACAAACTTTGTCGCG	ATCATCACCAAACGCATCAA	
At5g64240-MC3R	GGGGACCACTTTGTACAAGAAAGCTGGG TAC<u>C</u>GAGTACAAACTTTGTCGCG		

Table 2.3 continued

Gene	Primer Sequence	Inset Primer Sequence (p800)	Fragment Size (kb)
At1g79340-MC4F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TAAGGAAATTTAAATTTAGATCCGGTT	GGGATGCGTCAACGATGT	2.1
At1g79340-MC4R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACACAGATGAAAGGAGCGTT		
At1g79330-MC5F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TATTCATATCCCAAGTACTG	GCTCTCTCCGATCTGCTCTT	2.1
At1g79330-MC5R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACACAAATAAACGGAGCATT		
At1g79320-MC6F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TATTTGACTATTTCTTATAAGC	ATACTGGTTACGATGAGT	1.7
At1g79320-MC6R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACACATATAAACCGAGCATT		
At1g79310-MC7F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TAATCTTACCTTACGGTACA	AATGTTTAGTATTTTAAT	2.3
At1g79310-MC7R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACGCATATAAACGGAGCATT		
At1g16420-MC8F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TATATGGAGGCTTTAGTGGTACAG	AAAGCACTTTTGATAGGAATCA	2.3
At1g16420-MC8R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACGTAGCATATAAATGGTTT		
At5g04200-MC9F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TACATAAATGGTTCGTCTCA	TGAAGACGTTTAATTTCTG	1.6
At5g04200-MC9R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACAGGTTGAGAAAGGAACGT		

2.8.2 Production of constructs using Gateway® cloning

The promoter::gene::GUS constructs were created using the Gateway® manufacturer's instructions. A BP reaction inserted the amplified fragments into the entry clone, pDONOR207, and the LR reaction inserted it into the expression binary vector, pKGWFS7.0. A colony PCR was used to confirm the presence of the entry clone. DNA was isolated using a miniprep and the concentration was measured. Plasmid DNA was

sequence validated and transformed into *Agrobacteria* after the LR reaction as described below.

2.8.3 Transformation of *E. coli* and *Agrobacterium*

Electrocompetent *E. coli* strain DH5α cells and *Agrobacterium* strain GV3101::pMP90RK were transformed using electroporation via a BioRad Gene Pulser. *E.coli* cells were incubated at 37^{0}C on a shaker for 1 hour while *Agrobacterium* was incubated at 28^{0}C for 48 hours. After PCR validation, the starter culture was incubated on a shaker for 24 hours at 28^{0}C. *A. thaliana* Col-O with newly opened flowers were dipped into the respective *Agrobacterium* solution. Three plants per construct were used. Plants matured for 4 weeks until harvesting.

2.9 Sequencing

Plasmid DNA from Entry clones (50-100ng) was sent to Eurofins MWG Operon for sequencing DNA. L1 and L2 primers were provided by Eurofins and p800 inset primers were added to the samples prior to sending in order to cover the middle region of the sequence.

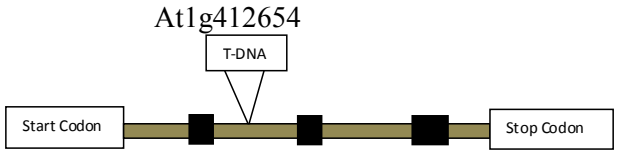
2.10 Sequence Analysis

Each MC nucleotide sequence from the sequencing result was aligned to the original MC nucleotide sequence from Vector NTI using MultAlign (http://multalin.toulouse.inra.fr/multalin/multalin.html). Nucleotide sequences of *AtMCs* from TAIR were converted to protein sequences using the European Molecular Biology EBI: Transequence Nucleotide to Protein Sequence Conversion (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) and a phylogenetic tree was produced using Molecular Evolutionary Genetics Analysis (Mega5) software.

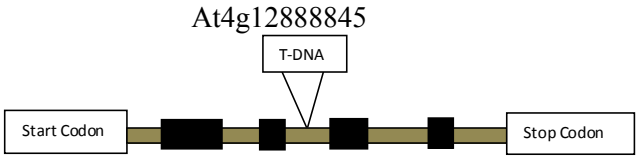
2.11 T-DNA insertion sites

T-DNA insertion sites for all *AtMC* genes were located using http://signal.salk.edu/cgi-bin/tdnaexpress. The relative location inside the gene between the start and stop codons were determined using http://arabidopsis.org/ (**Figure 2.1**).

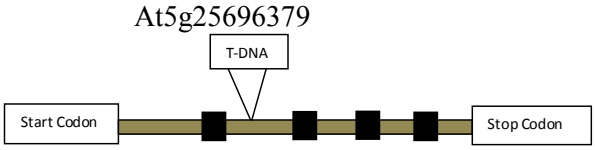
MC1 (At1g392654-432654)



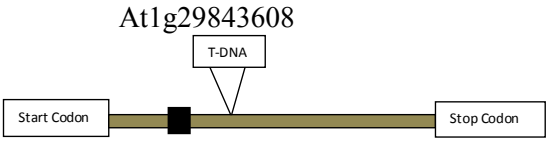
MC2 (At4g12868845-12908845)



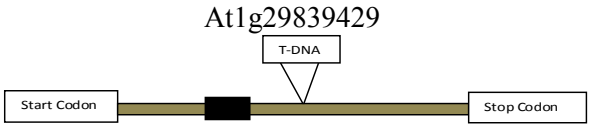
MC3 (At5g25676379-25716379)



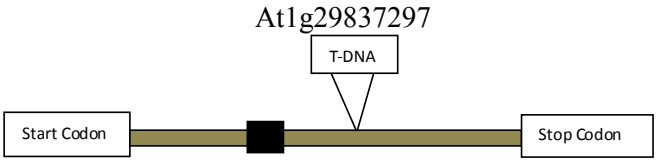
MC4 (At1g29823608-29863608)



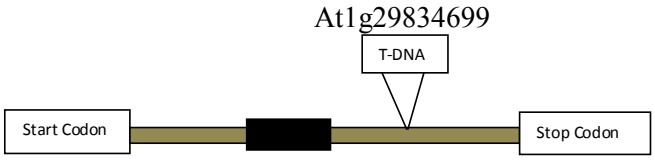
MC5 (At1g29819429-29859429)



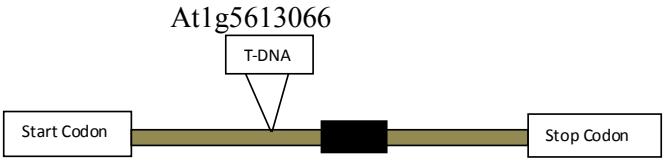
MC6 (At1g29817297-29857297)



MC7 (At1g29814699-29854699)



MC8 (At1g5593066-5633066)



MC9 (At5g1134381-1174381)

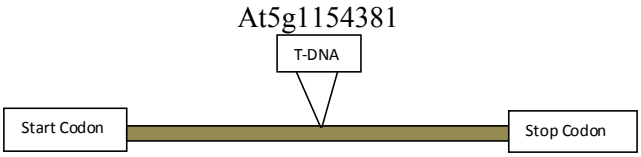


Figure 2.1: Schematic representation of the DNA insertion sites for all *AtMCs*. The size of the gene is noted along with the location on the chromosome where the T-DNA was inserted. Start and stop codons are depicted at the beginning and endings of each gene. Information was obtained from http://signal.salk.edu/cgi-bin/tdnaexpress.

2.12 Supplemental Material and Methods

Extraction Buffer 50ml
200mM Tris-HCL pH 7.5 10ml 1M
250 mM NaCl
2.5ml 5M
2.5ml 0.5M
25 mM EDTA
0.5% SDS 2.5ml 10%

Formalin-Acetic-Alcohol (FAA)
50% EtOH
10% formaldyhyde
5% acetic acid

Plate medium-Plants (pH5.8)

4.4g/L MS media
2.56ml/L MES
10g/L sucrose
10g/L plant agar
1/1000 antibiotic

Plate medium-Bacteria (pH7.2)

1x Luria-Bertani Buljong
15g/l Bacto-Agar
1/1000 antibiotic

PCR reactions-Genotyping

95^0C 1 min.
95^0C 30 sec.
50^0C 20 sec.
72^0C 3 min.
72^0C 10 mins.
8^0C forever
35 cycles

PCR reactions-Cloning

95^0C 1min
95^0C 20 sec.
44-61^0C 20 sec
72^0C 3 min
72^0C 10 min
4^0C forever
35 cycles

3 Results

3.1 Isolation and validation of *AtMC* mutants

Previously, the effect of each T-DNA insertion on gene expression was analyzed for reduction or complete absence of expression (**Table 3.1**). All mutant lines were previously genotyped to confirm homozygous mutations, except for *mc9-1 mc5* which, after genotyping, showed no conclusive data for the presence of a double homozygous mutation.

3.2 In silico expression analysis of the *AtMC* gene family

With the exception of *AtMC4* which showed consistent and relatively similar expression in all developmental stages, each *AtMC* showed a noticeable peak in different developmental stages, in different tissues, and in response to inductive stimuli (**Table 3.2**). High expression values were determined for *AtMC1-4* and *9*, with *AtMC4* having the highest expression levels in all developmental stages. Expression for all *AtMC*s was detected during all stages analyzed. *AtMC9* was highly expressed in root xylem, while *AtMC1-4* showed higher expression values than *AtMC5-8* across all of the tissues studied. *AtMC7* was not expressed in young leaf tissue (**Figure 3.1**). During response to inductive treatment, *AtMC5-8* showed higher expression while *AtMC1-4* and *AtMC9* consistently displayed minimal expression. Neither *AtMC7* nor *AtMC9* were expressed in response to heat treatment (**Table 3.2**, **Figure 3.1**).

Table 3.1

RT-PCR expression analysis of each *Arabidopsis* insertional mutations. (*) represents strong or Wild-type like band, (**) represents full silencing, and (***) represents down regulation. Lines without an asterisk indicate no data to date.

AGI code	Name	Line	Used in Publication
At1g02170	**AtMC1**	GABI_096A10**	Coll et al. 2010
		Salk_002986*	
At4g25110	**AtMC2**	SALK_009045**	van Baarlen 2007, Coll et al. 2010
At5g64240	**AtMC3**	GABI_545D06*	
At1g79340	**AtMC4**	Sail_856_D05**	Watanabe and Lam 2011
At1g79330	**AtMC5**	SALK_145461C	
At1g79320	**AtMC6**	SALK_063453**	
		SALK_006679	
At1g79310	**AtMC7**	SALK_127688 **	
At5g04200	**AtMC9**	SALK14**	van Baarlen 2007
		GK540**	

Table 3.2

AtMCs highest expression in various developmental stages, tissues and in response to inductive stimulus. Data collected from Genevestigator

AtMC gene	Developmental stage where most highly expressed	Anatomical tissue where most highly expressed	Stimulus treatment where most highly expressed
1	Young Flower	Pollen	Cycloheximide, NAA+FLG
2	Young Flower	Adult Leaves	IAA, FAA+FLG, Salicylic acid
3	Young Flower	Root Phloem	Drought, Hypoxia
4	Seedling	Lateral Roots	NAA+FLG, Hypoxia
5	Mature Siliques	Lateral Roots	Salicylic acid, IAA
6	Flowers and Siliques	Lateral Roots, Root Phloem	Cold, Drought
7	Young Rosette	Endoderm, Root Phloem	MeJa
8	Young Flower	Adult Leaves	Clycloheximide, NAA+FLG
9	Mature Siliques	Root Xylem	Heat, MeJa

A.)

B.)

C.)

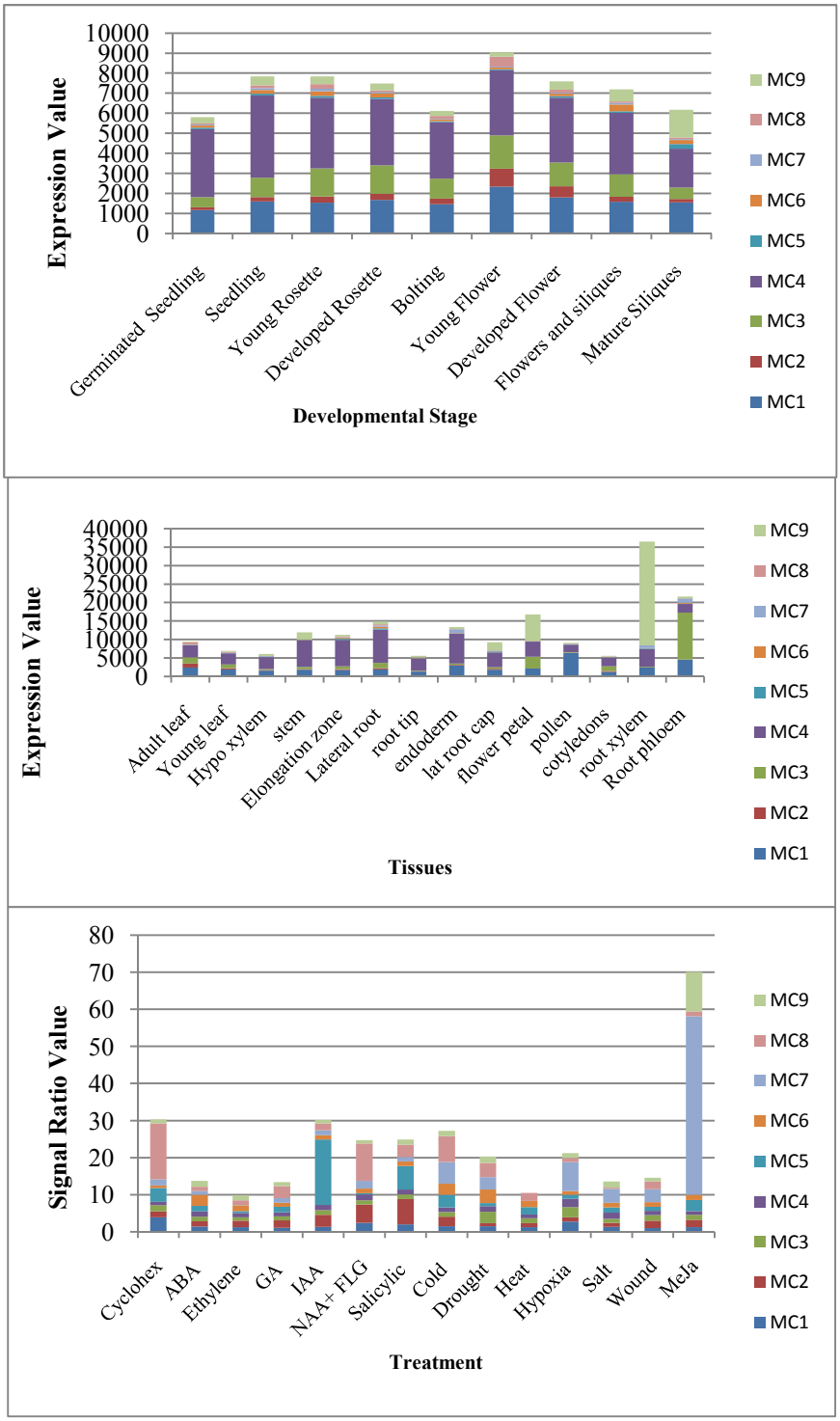
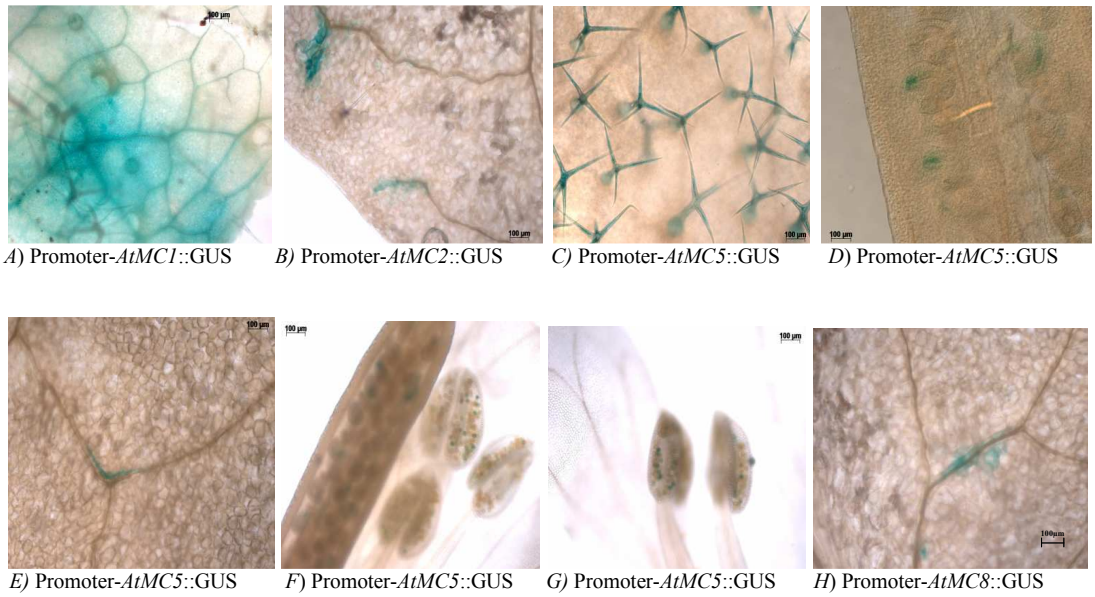


Figure 3.1 Expression Analysis of *AtMC1-9*. *A*, Developmental expression. *B*, Anatomical expression. *C*, Expression in response to stimulus treatment.

3.3 Reporter gene analysis of the *AtMC* gene family

Sequence alignment showed perfect matches for *AtMC*s *1, 2, 4, 5, 8* and *9* constructs. *AtMC3* and *7* were not successfully amplified and *AtMC6* had a single mutation of an adenine to guanine. The mutation was located in the promoter region approximately 300bp upstream from the ATG start codon of the gene. Due to its location in the promoter, it was determined that *MC6* could progress to the transformation stage back into *Arabidopsis*. The constructs containing *AtMC1, AtMC2, AtMC5, AtMC6* and *AtMC8* were transformed into *Arabidopsis* T1 plants and were inspected for localization of the GUS signal. *AtMC1*was expressed in both the leaf tissue and vasculature (**Figure 3.2a**), *AtMC2* was observed at the tips of vascular tissue in leaves (**Figure 3.2b**), and *AtMC8,* to a small degree, appeared in leaf tissue **(Figure 3.2h**). *AtMC5* was expressed in trichomes and in leaf tissue where vascular patterns diverged, along with being expressed in pollen grains within an anther, seeds within a mature silique, and in developing seeds in an embryo **(Figure 3.2c-g**).



A) Promoter-*AtMC1*::GUS *B)* Promoter-*AtMC2*::GUS *C)* Promoter-*AtMC5*::GUS *D*) Promoter-*AtMC5*::GUS

E) Promoter-*AtMC5*::GUS *F*) Promoter-*AtMC5*::GUS *G)* Promoter-*AtMC5*::GUS *H*) Promoter-*AtMC8*::GUS

Figure 3.2: Expression of promoter::gene::GUS constructs in *Arabidopsis*. Pictures depict expression of *AtMC1, AtMC2, AtMC5,* and *AtMC8* in various plant tissues.

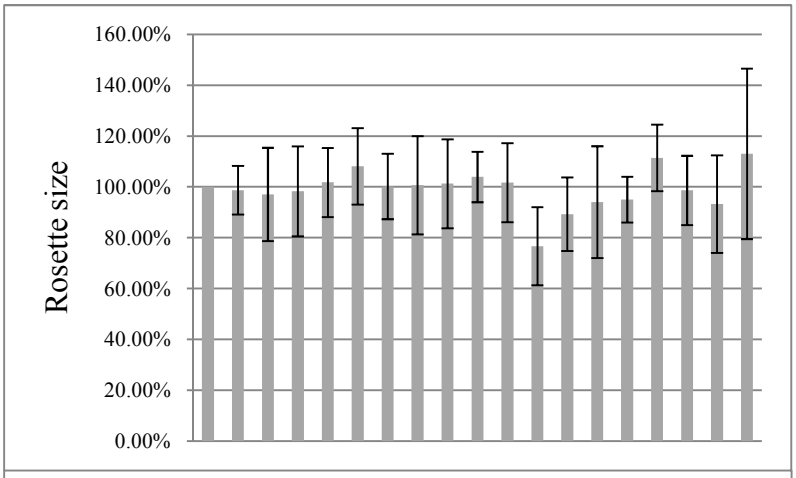
3.4 Functional characterization of the *AtMC* gene family by reverse genetic approaches

There were no statistical differences between the mutants and Wild-type for the size of the rosette, the leaf number per rosette, or in the proportion of senescing leaves. Five of the mutant lines did show significantly different development than the wild type. Significant differences were observed in leaf width, in stem height and bolting time (data not shown) (**Figure 3.3**). These differences are summarized in **Table 3.3**.

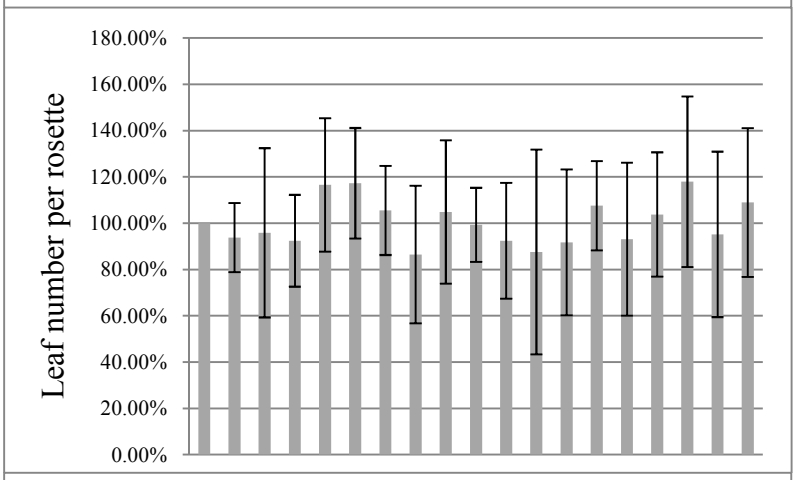
Table 3.3
Mutant lines with statistically significant differences in rosette and stem development and bolting time

Mutant	Characteristic with significantly different phenotype
mc6-2	Smaller leaf number
mc6-2	Smaller stem height
mc9-2 mc4	Smaller stem height
mc9-1 mc1-1	Smaller rosette leaf width
mc2	Faster bolting time
mc9-2	Slower bolting time

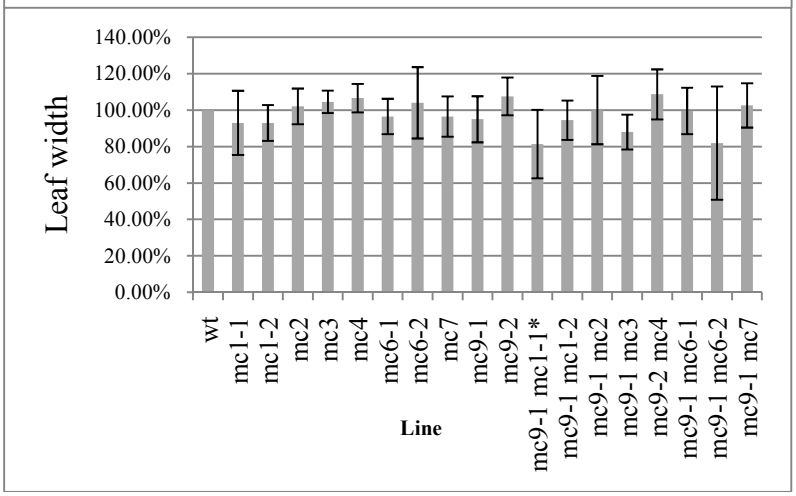
A.)


Rosette size
160.00%
140.00%
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%

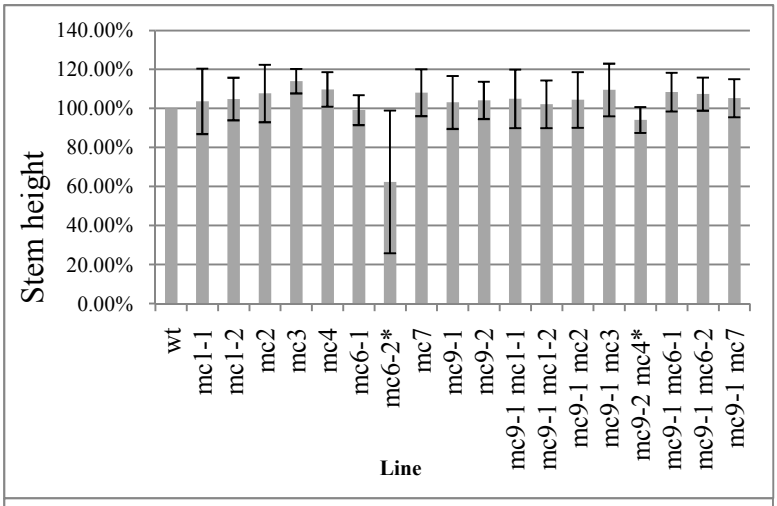
B.)


Leaf number per rosette
180.00%
160.00%
140.00%
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%

C.)


Leaf width
140.00%
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
wt
mc1-1
mc1-2
mc2
mc3
mc4
mc6-1
mc6-2
mc7
mc9-1
mc9-2
mc9-1 mc1-1*
mc9-1 mc1-2
mc9-1 mc2
mc9-1 mc3
mc9-2 mc4
mc9-1 mc6-1
mc9-1 mc6-2
mc9-1 mc7
Line

D.)



E.)

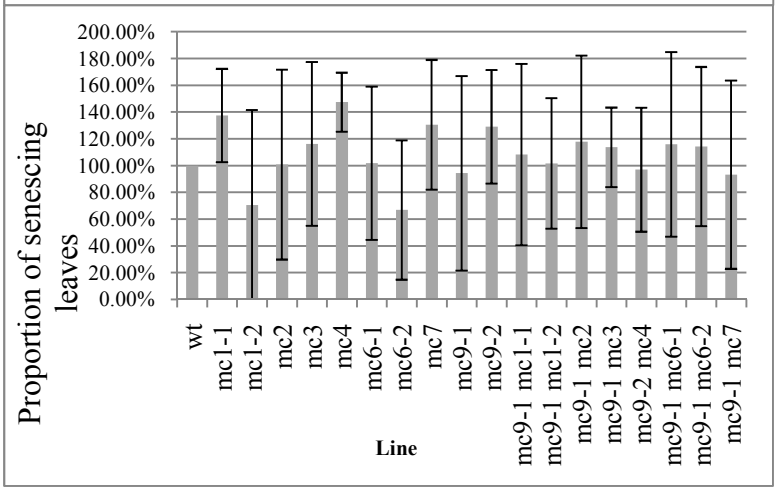


Figure 3.3: Morphological changes in *AtMC* single and double mutants. Data is presented as percentages of the mean value for each mutant line in comparison to the Wild-type (wt) mean value. Significant differences were analyzed using a t-test with a p-value of 0.05 and are marked with an asterisk (*). *A*, The size of the rosette was analyzed by measuring the rosette length from the tip of the longest leaf to the tip of the leaf directly horizontal to it. *B*, The number of leaves per rosette was counted. All leaves that were not fully degraded were scored as one individual leaf. *C,* The width of the leaves was analyzed by measuring the width of each leaf perpendicularly to the leaf petiole, at the middle of the leaf. The leaf per rosette with the largest width was recorded. *D,* The height of the main stem was measured from the base of the rosette to the top of the stem. *E,* The number of leaves showing any amount of senescence was noted per rosette per plant and the number of leaves per rosette was recorded. Next, for each plant, the number of leaves showing senescence was divided by the total number of leaves per rosette. This yielded a percentage of leaves which showed senescence per plant per mutant. This number was then used to determine the percentage value from the Wild-type mean as stated above.

4 Discussion

Following the discovery that caspases play a role in animal PCD, homologous proteases like metacaspases (MCs) were identified in plants and other organisms. Even though caspases and MCs may be structurally homologous, they do not share functional homology. To date, the function of MCs in plants is still unknown. The nine MC genes of *Arabidopsis* were studied in this project in order to try and determine the function of *AtMCs* in various growth stages including plant PCD. The information learned from this project could be applied to other plants as well, such as *Populus spp*., spruce, and wheat and rice in agricultural systems. All of these plants, except spruce, have fully sequenced genomes allowing for more efficient interspecific MC sequence comparison.

In order to help elucidate the function MC genes play in plants, this project utilized expression analysis and reverse genetics. Based on *in silico* expression analysis, all AtMC genes are expressed in developmental stages ranging from seedling, rosette, stem, flower and silique maturation (**Figure 3.1a**). This data was further supported by promoter::gene::GUS experiments. Transformed *Arabidopsis* plants containing these constructs showed that AtMC expression is indeed present in leaves, leaf vasculature, pollen, and siliques, along with trichomes and during what is believed to be embryo suspensor development in the siliques (**Figure 3.2a-h).** If indeed this expression represents embryo development, these findings support previous data from two publications where a type II metacaspase in *P. abies* was down-regulated resulting in a decrease in PCD in cells of the embryo suspensor (Suarez et al. 2004), and supports data which suggests the possibility that MCs may be involved in *P. abies* PCD affecting pattern formation during embryogenesis (Bozhkov et al. in 2005). The reporter gene in this project was detected in mature siliques, though this expression was confined to small localized areas throughout the tissue within some seeds (**Figure 3.2g).** Reporter gene expression was also detected in some pollen grains within the developing anthers (**Figure 3.2g-h**). Our result suggests that PCD could be involved during seed and pollen development.

In silico expression analysis suggested that AtMC genes are not only expressed during various developmental stages, but in individual tissues as well, including leaves, stems, roots, flowers and in the vasculature (**Figure 3.1b**). Transformed *Arabidopsis* plants in this experiment showed that MC expression is indeed present in leaf tissue, in pollen and in leaf vasculature (**Figure 3.2**a-h) Using *Arabidopsis mc* single and double mutants, the function of MCs in rosette, leaf, and stem development was studied. Based on the data collected during these functional analyses, significant differences between the mutants and Wild-type were observed in both leaves and stems **(Table 3.3**, **Error! Reference source not found.**) in five of the nine mutants. According to this data, it is possible that *AtMC1, 2, 4, 6* and *9* may be directly involved in leaf or stem development in *Arabidopsis*.

Even though significant phenotypic differences were not observed for the remaining mutants, roles for these genes during development or in PCD are possible. One reason for a lack of an obvious phenotype in the remaining *mc* mutants could be contributed to the need of correct identification of environmental or external conditions to expose the phenotypic differences. It is possible that at least some of the *AtMC* genes are pseudogenes. This possibility may be supported by the fact that MC type II are completely absent in two algae species (Cambra et al. 2010). This means that only homologues of *AtMC1-3* are completely conserved in the algae and that *AtMC4-9* may play dispensable roles in plants. However, because all MC genes were expressed during the expression analysis of this study, and because algae are much simpler than higher plants, this alternative seems unlikely. The results from this study, together with the published work on the type II MCs have clearly shown that MCs do play some role in higher plant development.

Although it is unlikely that the AtMC genes are pseudogenes, gene redundancy due the close evolutionary relationship between some of the AtMCs (**Figure 1.1**, **Table 2.2**) may account for the lack of significantly different mutant phenotypes for the majority of the mutations. To my knowledge, this is the first report where significant

differences between *mc* mutants and Wild-type have been reported in *Arabidopsis*. Other publications have failed to reveal any major alterations in the growth and development of *mc* mutants (Coll et al. 2010, He et al, 2008, Wantanabe and Lam 2011). According to the expression analysis from this project, *AtMC1* and *AtMC4* show high expression values during development and in various anatomical features, suggesting they may take over the role of other genes. Of course, with the consistently high levels of expression seen in *AtMC4* it may not be completely reasonable to focus attention on the analysis of this gene since it could have other roles deviating from development or PCD. It is also possible that with its ubiquitous expression level throughout the plant, *AtMC4* is playing a role in redundancy leading to the lack of a phenotype. It would be interesting to see if any significant developmental differences exist between an *atmc1 atmc4* double mutant. Also, to better study the roles of AtMC genes, crosses producing plants with more than two MC silenced genes per plant should be analyzed. Due to the interest of this lab in *AtMC9* and xylem formation, it would be interesting to analyze the phenotype of the triple mutant *mc1 mc4 mc9.* Other possible triple mutants of interest according to the expression analysis data would be *mc1 mc3 mc4, mc2 mc4 mc9,* and *mc3 mc4 mc9.* Even with the support of this expression data, the other AtMC genes should not be ignored since even low expression levels located in either specific tissue or within certain cell types could be important, and the lack of a high level of expression in pooled microarray database does not indicate that other MCs are not important.

Even though *AtMCs1-4*, and *9* may be the key players in development and have the highest expression in various tissues, according to this project the other *AtMC*s appear to be most affected by stimulus treatments. As stated above, mutant plants which have been crossed to provide more than two silenced genes per plant could provide valuable information on the role of the genes more highly expressed under stimulus treatments. Unfortunately, there is no chance to create mutant crosses between these genes due to their close proximity to each other on the chromosome (**Table 2.2**). Previous studies have shown that *AtMC8* is highly induced by stress treatments such as UV, and H_2O_2 (He et al

2007). Data collected from database expression analysis in this project supported this hypothesis by showing high expression levels of *AtMC8* in plants treated with cycloheximide, auxin and cold. Surprisingly, in one transformed *Arabidopsis* plant during the project, which contained the *AtMC8* promoter::gene::GUS construct, expression was observed around small areas of vasculature in leaf tissue (**Figure 3.2h**). This could be due to unintentional wounding of the plant tissue prior to analysis, GUS leakage, or it could suggest that *AtMC8* plays more of a role during development than was previously believed. In the future, it would be interesting to study the phenotype of *AtMCs* while under external stimuli after application of exogenous plant hormones, application of stress factors known to promote expression in the various genes (**Figure 3.1c**), or under pathogen infection. Also, transformed plants containing the AtMCpromoter::AtMCgene::GUS constructs could be placed under various stress treatments, or grown with the addition of exogenous hormones, and the effect on GUS expression could be observed.

When the database expression data is combined, it suggests that each MC may have a role to play in plants, and that MC genes not strongly involved in natural plant growth may be induced by external conditions. Due to their structural similarity to caspases, it is believed that MCs may play a role in PCD. It appears from the results of this project that AtMC genes alone may not contribute to PCD in plants as much as was previously believed and that there are other players involved in the process. This would not be surprising since biology is complex and in many systems multiple interconnecting components are necessary to allow for full functionality of the organism. It is possible that AtMC genes may have a complex system among themselves; a system which acts as a sort of checks and balances to provide PCD stability throughout the lifespan of the plant. This idea is supported by a recent study which showed that *AtMC1* and *AtMC2* acted antagonistically with *AtMC1* positively regulating PCD and *AtMC2* negatively regulating it during pathogen attack (Coll et al 2010). It is possible that not one but a combination of all these suggested processes contribute to *Arabidopsis* PCD in various

degrees depending on internal and external conditions. Yet another possibility is that AtMCs do not play any role in PCD which is ongoing during normal growth and development.

4.1 Future work

On a broad scale, obtaining the full genome sequence of spruce will give more insight into the roles of MCs by providing another organism in which to study. The study of Poplar and spruce together could lead to significant discoveries regarding the role of MCs in xylem development since they are woody plants containing a large amount of xylem. Also, different plants contain different numbers of MC genes, for example, there are nine in *Arabidopsis* and four in *Populus*. Within the ancestors of modern plants, cyanobacteria, there are at least 58 MC genes within 33 different species which have been identified (Jiang et al. 2010). The difference in number may or may not influence the way in which MCs interact and the mechanisms behind their roles in each species. The more plants species to be fully sequenced, the more information science can obtain regarding how MC genes have evolved in different species over time, their various roles, and how they function both similarly and differently among species today. This knowledge could allow increased production and conservation techniques in both the forestry and agricultural sectors providing solutions to some of the world's most challenging problems such as declining resources, the need for increased productivity, and yield loss due to pathogen attack.

On a more detailed scale, the expression work performed during this thesis could be continued in a variety of ways. To date, only *AtMC1, 2, 5, 6* and *8* were transformed and observed for GUS expression. The remaining lines should be analyzed and future GUS analysis should include other tissues such as roots. After all *AtMC* constructs are transformed and all tissues are observed for GUS expression, the various expression patterns should be compared to provide insight to possible roles each *AtMC* may play. Another alternative for expression study could include *In situ* hybridization which may

provide supporting information regarding the expression patterns of the AtMC genes. Since the GUS constructs also contained GFP, AtMC protein localization could be analyzed to give further insight and/or support into the function of each gene.

Future phenotyping projects could include the use of RNA interference (RNAi). Theoretically, RNAi would be able to silence anywhere from one to all nine genes at once without the logistical restrictions of genetic crosses. This technique was presently attempted in this laboratory for *AtMC9*, and produced interesting results suggesting a function for *AtMC9* together with still unknown additional members of the MC gene family in the overall growth of the seedlings and secondary cell wall properties (Unpublished). RNAi can target genes that it was not designed to target and it is not always able to fully silence the genes of interest, thus a more gene specific technology such as artificial microRNA (amiRNA) analysis could be a better option.

Combining DNA analysis with proteomics could give a more detailed indication of the functions of AtMCs. Other proteases such as serine proteases and other cysteine proteases are believed to be involved in PCD. A vacuolar cysteine protease, along with an aspartic protease, has been shown to influence proto- and meta-xylem formation in barley through immunohistochemical staining (Runeberg-Roos & Saarma1998). A yeast-two-hybrid may be able to determine which enzymes may also be interacting with MCs.

Initiator caspases contain two domains, a death effector domain (DED) and a caspase recruitment domain (CARD), both of which are located in the prodomain. Caspases which contain these domains can autoprocess while caspases without the prodomain must use another protease for activation (Lee et al. 2010). The yeast MC type 1 gene (*Yca1*), like all other MC type I genes, contains a prodomain, but lacks a DED or CARD motif (Lee et al. 2010), while MC type II genes do not contain any prodomain (Cambra et al. 2010). Currently, it is unknown how MCs in yeast, or other fungi, plants or protists, are activated (Carmona-Gutierrez et al. 2010). Future work could shed some insight on how autoprocesseses in both types of MCs is initiated.

According to database expression analysis in this project and from previous microarray data in Poplar (Courtois-Moreau 2009), *AtMC9* is most dominantly expressed in root xylem. If *AtMC9* does play a direct role in xylem PCD, it could be expected that the mutants would show a reduced rate of PCD causing xylem to develop later than in the wild-type. It is unlikely that full mutations in these genes would completely halt PCD development, though. If this were the case, xylem would not develop and thus the mutants would obtain a lethal-like phenotype. Electron microscopy analysis of cross sectioned roots and hypocotyls could provide data on xylem development and PCD in *Arabidopsis mc9* mutants. An alternative for *mc9* study would be the development of assays which would identify xylem specific mutants. Even though treachery elements (TEs) are the current focus for increasing xylem biomass, it may not be advantageous to the health of the plant to manipulate the rate at which these cells undergo PCD. For example, altered TE development could lower the rate of water transportation, decrease the overall growth rate, or produce plants that are more sensitive to drought. Ultimately we are interested in manipulating the structural components of xylem, the fiber cells, rather than TEs. First, though, the promoters of fiber genes need to be isolated. After identification of these promoters, work can turn to increasing biomass via fiber manipulation rather than TE manipulation.

The work done in this thesis was only a small part of the bigger picture. After determining the role of AtMCs, the next step will be to elucidate the other key players in the signalling pathways and determine the mechanisms behind how they all interact together. To do this, other components need to be identified such as upstream transcription factors which can act as either activators or repressors and the cis-elements of AtMCs, along with discovering which substrates besides Tudor staphylococcal nuclease (Sundström et al. 2009) MCs act on. Other genes involved in PCD could to be studied to determine possible connections between them and AtMCs. Potential candidates could be *ACAULIS5* (*ACL5*), whose mutation has been shown to cause premature death

in xylem, (Muñiz 2008) and *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*), a regulator of xylem PCD (Ohashi-Ito et al. 2010)

4.2 Conclusion

In conclusion, the role of AtMCs in *Arabidopsis* PCD is still unclear. Even after determining where each AtMC protein is expressed within the plant, we still need to discover if AtMCs play a direct role in PCD, if they play a role but interact with other players, or if they do not have a role in PCD at all and have another role entirely. It appears that AtMC genes do play a role in PCD due to their similar structure to caspases, and the data collected to date. Judging by the lack of a strong mutant phenotype, it is likely that they do not act alone, but interact with both each other, and other components, creating a complex interaction whose mechanism is modified depending on different internal and external conditions. The key is to identify those players and to develop a hypothesis on how the players interact in order to regulate PCD so effectively.

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