UNIVERSITY
OF CRETE

“The role of Metacaspases and Metacaspase-like complexes in plant physiology”

M.Sc. Thesis

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Abstract

Proteins are degraded or cleaved into smaller peptides through a catabolic process knows as proteolysis, executed by a large number of proteases. Metacaspases are caspase-like, cysteine-depended proteases found in plants, fungi and protists but not in animals. Their distant homolog, separase, is also a caspase-like protease which shares same proteolytic targets with metacaspases, especially the Plant Elicitor Peptides (PEPs). The plant Arabidopsis has nine metacaspase genes categorized into two types, I (Atmc1 to 3) and II (Atmc4 to 9) based on their structure, and a single separase gene. Despite that many functional studies focused on metacaspases, their physiological role remains elusive due to their genetic and functional redundancy among them. To overcome the genetic and functional redundancy of metacaspases, our lab created a sextuple mutant with the ablation of all type II metacaspases. This mutant showed low germination in long-term stored seeds and was susceptible to salt stress leading us to hypothesize that there is seed coat or seed filling deficiency. In this study, I tested these hypotheses with phenotypic, microscopical and biochemical approaches, by investigating the metacaspase mutant seed coat structure and seed feeling by estimating nitrogen remobilization efficiency to the seed. The metacaspase mutants, however, showed no differences in either seed coat structure or nitrogen remobilization. In addition, I estimated reactive oxygen species (ROS) accumulation in the metacaspase mutant during salt stress, which revealed increased ROS accumulation. I also established vectors for PEP4 and 7, two PEPs especially expressed in the root tip where several metacaspases and separase are expressed, to track spatiotemporal activation of these proteases.

Introduction

Aspects of proteolysis

Proteolysis is a vital process by which cell maintains protein homeostasis by regulating protein levels, removing misfolded peptides or supplying amino acids for new proteins. Proteolysis refers usually to **protein degradation** or to the cleavage of proteins into smaller fragments (**limited proteolysis,** see below). Protein cleavage by proteases aims to activate, deactivate or change the function of the targeted protein (Pham et al., 2014). The turnover rate of individual proteins can vary, with protein half-lives ranging from minutes to weeks.

The activation of proteolytic mechanisms for protein degradation can differ, depending on the developmental stage of the cell/organism (Genschik et al., 1994; Ribeiro et al., 1995) or as a stress response (Garbarino et al., 1992). More specifically,

proteolysis is a mean by which cells can regulate developmental transitions or adapt to environmental conditions. The adaptation or developmental transition can be achieved by interfering with many pathways, such as targeting specific proteins that are implicated in cell cycle regulation or metabolism.

In eukaryotes, there are three major pathways of degrative proteolysis (Image 1). The first one is known as autophagy-mediated proteolysis or simply autophagy. Autophagy is a catabolic process in which the cytoplasmic components are moved in vacuoles (in plants) or lysosomes for hydrolysis. Autophagy is distinguished in microautophagy, in which the targeted component fuses directly with the lysosome (Li et al., 2011), and macroautophagy where the component is enclosed in a membrane called the phagophore before fusing with the vacuole/lysosome (Feng et al., 2014) and in chaperon-mediated autophagy. The other pathway is the ubiquitin-mediated proteolysis. Degradation of a protein via the ubiquitin-mediated proteolysis involves two steps. First, multiple ubiquitin molecules are attached on a lysine residue of the targeted protein in an ATP-dependent reaction (Image 1). These reactions are catalyzed by 3 enzymes: E1, E2 and E3 and without them, covalent attachment between ubiquitin and protein will not occur. Then the ubiquitinated protein can be detected and degraded by a very specific protease complex called 26S proteasome in an energy-dependent way (Ciechanover and Schwartz, 1994). The third pathway is the proteolytic system of chloroplasts and mitochondria, in which there are present several proteases of bacterial origin (Mayer, 2000; Adam, 2015)

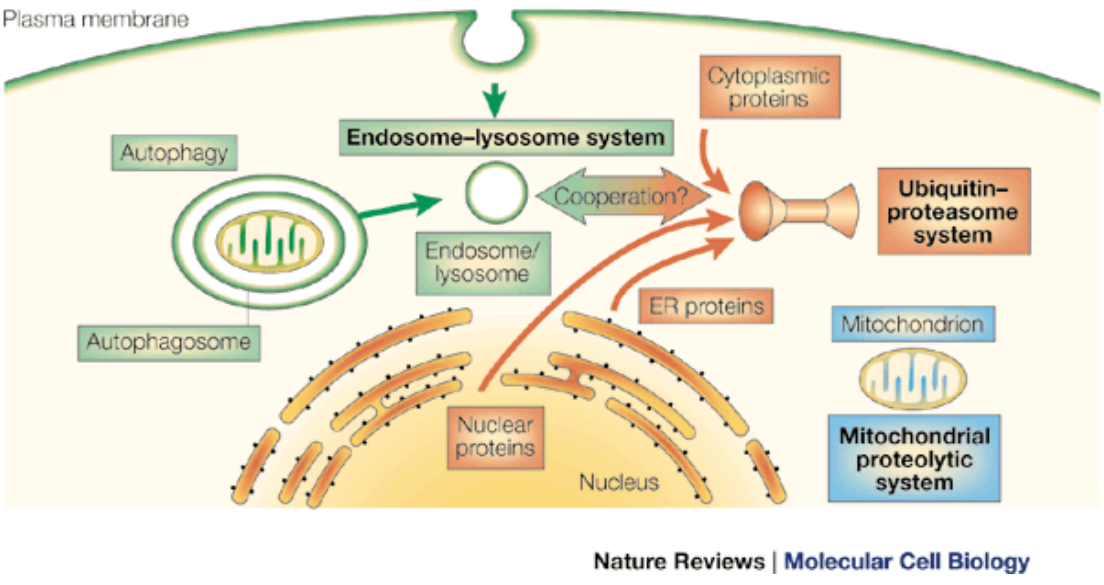


Image 1:: Diagrammatic Representation of the major proteolytic pathways. Green: Autophagy. Red: Ubiquitin proteolytic pathway and Blue: Mitochondrial/Choloroplast pathways (Mayer, 2000)

In addition, there is a proteolytic pathway which is characterized as a fine-tuned post-translational modification known as limited proteolysis or proteolytic

processing (Neurath 1989). This process occurs for several proteins and contains variable changes on peptides through cleavage, such as the removal of N-terminal methionine or the cleavage of inactive precursor peptides in order to activate them (Hirel et al., 1989). The major difference between limited proteolysis and the degrative proteolytic pathways that were mentioned above is that in the first, the substrate is cleaved and produces smaller, usually functional peptides while in the other two, the outcome of the cleavage is the production of amino acids.

In both degrative and limited proteolysis, proteases are involved. Proteases are enzymes that catalyze the hydrolysis of peptide bonds. They can be found in almost every living organism, including viruses. Proteases can be categorized according to many factors, such as their substrate specificity and residues in their catalytic site (Vierstra et al., 1996). Regarding the substrate specificity, proteases can either trim the N or C terminus of a peptide (exopeptidases) or the internal amino acids (endopeptidases). Regarding the catalytic site, proteases are classified in 5 categories according to the residue used to attack the peptide bond of the substrate: Serine, threonine, aspartic, cysteine proteases and there is also a category named metalloproteases due to the involvement of metals in their catalytic mechanism (Clark and Pazdernik, 2016). Furthermore, there are proteases that cleave substrates via unknown mechanisms.

Characteristics of the metacaspase family and their relation with caspases

Caspases are a conserved family of cysteine endoproteases found in animal cells, implicated in many cellular pathways such us Programmed Cell Death (PCD), inflammation and diseases (McIlwain et al., 2013). Caspases are absent in yeast, protozoa and plants. This fact intrigued many researchers to search for caspase orthologs in plants as well as metazoan,protozoa and parazoa based on predicted models for structural homologies with the catalytic domain of caspases. Many years of studies led to the identification of metacaspase sequences in plants, fungi and protozoa (Uren et al., 2000). There is a major difference in catalytic function between caspases and metacaspases: metacaspases, prefer arginine (R) or lysine (K) at the P1 position of the cleavage site (P3'-P2'-P1'--P1-P2-P3 where the dash corresponds to the peptidic bond being cleaved) instead of aspartate (D), the preferable P1 site for caspases in animals (Vercammen et al., 2007).

Metacaspases comprise cysteine-dependent endoproteases and are classified in 2 major categories, type I and II according to their overall structure (Uren et al., 2000; Baskett et al., 2012). Type I metacaspases comprise metacaspase domain in their C-terminus and can have or not an N-terminal prodomain rich in proline followed by a Zn finger motif (Fagundes et al., 2015) (Image 2). On the other hand, type II metacaspases lack both the zinc finger motif and the prodomain but have an insert (linker) between the p20 and p10 subunits (Fagundes et al., 2015) (Image 2). Recently, a new class of metacaspases has been described, dubbed type III, present only in algae which have undergone secondary endosymbiosis (Klemenčic and Funk, 2018). In

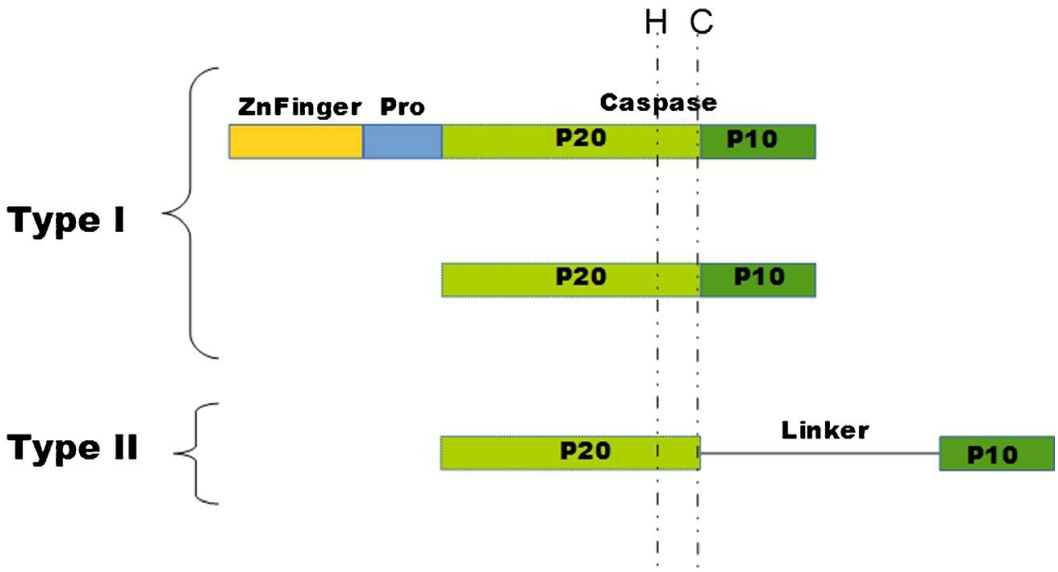


Image 2: The two types of metacaspases according to their structure. The catalytic amino acid regions containing histidine (h) and cysteine (c) residues are shown as dotted lines (Fagundes et al., 2015).

Arabidopsis thaliana there are 9 metacaspases (AtMC 1-9) and the first 3 belong to type I while the other 6 are type II (Tsiatsiani et al., 2011). Metacaspases 1,4,5,6,7 and 8 are all in chromosome 1 and 4-7 are tandemly distributed on it. AtMC 2 is on chromosome 4 while AtMC 3 and 5 are on the 5th chromosome. The tandem distribution of AtMC4-7 on chromosome 1 has so far confounded their genetic and functional studies, as it is almost impossible to identify high-order (>single) loss-of-function insertional mutants for *AtMC4-7* genes. Thus, the role of AtMCs in plants is largely unknown with controversial many controversial studies suggesting a potential role in embryo development and cell death.

Metacaspases activation requires auto-processing similar to caspases, but unlike caspases, many metacaspases are Ca^{2+}-dependent (Vercammen et al., 2004). Concerning their role, type I AtMCs associate with PCD and pro-survival functions, as for example clearance of cellular aggregates to promote protein homeostasis and cell survival (Coll et al., 2014). In addition, particular metacaspases, for example AtMC1, can play a role in resistance against pathogens, mostly during the hypersensitive response (HR) (Coll et al., 2014), a type of PCD that resembles the mammalian

pyroptosis. Although metacaspases are widely known for their role in cell death, it is just one pathway out of many that they are implicated, not only in plants but also in fungi and protozoa. More specifically, proteome analysis in yeast revealed a role of yeast metacaspase called Yca1, which clears protein aggregates in normal and stress conditions (Lee et al., 2010) while in protozoa can play an essential role in cell cycle progression (Ambit et al., 2008).

Metacaspase-like complexes

Recently, the Moschou Lab discovered the Kinesin7/Separase Complex (KISC). KISC relates to metacaspases as separase is an evolutionarily conserved caspase-related protease (Moschou et al., 2013, Minina et al., 2017). More specifically, it is a cysteine-dependent protease with a caspase-like catalytic domain at C-terminus. This protease is associated with microtubule dynamics and cell polarity regulation by interacting with Centromere-associated protein E (CENP-E) related kinesins that belong to Kinesin 7 subfamily (Moschou et al., 2016). However, the exact mechanism by which KISC regulates cell polarity remains unknown.

An interactor of KISC is the lipid transfer protein known as Sec14, firstly described in yeast (Novick et al., 1980; Bankaitis et al., 1989). Sec14 regulates lipid turnover and signalling at the plasma membrane. More specifically, members of the Sec14 family has been also studied in other organisms as well (e.g. *Arabidopsis thaliana*) where genetic approaches using mutant lines indicated the importance of Sec14 members in the formation of secretory vesicles at Golgi network (Fang et al., 1996) and cell polarity (Campos and Shaaf, 2017). Thus, KISC effector of cell polarity might be the Sec14 protein. However, how KISC associates with Sec14 in the cell and the molecular mechanism by which the KISC/Sec14 nexus regulates polarity remain to be established.

ProPEPs are common targets of metacaspases and separase

In Arabidopsis, separase and metacaspases share a group of potential cleavage targets, the precursor molecules of Plant Elicitor Peptides (PEPs) (Liu et al., 2017). PEPs are small peptides that derive from the proteolytic maturation of the C-terminus of 8 precursor proteins known as proPEPs 1 to 8. PEPs are also known as “danger indicator peptides” and are involved in immune and developmental responses (Bartels et al., 2013).

The expression pattern of proPEPs varies in a spatial and temporal manner in Arabidopsis. The promoters of proPEP 1 to 3 showed similar patterns of activity and were active mainly in roots excluding root tip while those of 5 and 8 were only active in root vascular tissue, as well as in flower tissues (Bartels et al., 2013). PROPEP 4 and

7 promoters showed activity only in primary and lateral root tips. Bartels et al. categorized the promoters of proPEPs in 2 major groups: group 1 comprises proPEP1,2,3,5 and 8 and shows expression in the roots and slightly in the leaf vasculature and they were also inducible by wounding. Group 2 comprises proPEP4 and 7 which were not inducible by wounding and their expression was restricted in root tips (Bartels et al., 2013). ProPEPs are thought to be cleaved, mostly by metacaspases, upon stress and have been associated with plant pattern-triggered immunity (Gully et al., 2015; Shen et al., 2019).

In Arabidopsis, PEP cleavage requires a major uptake of Ca^{2+} which activates AtMC4 and executes the cleavage of proPEP1 to release the active PEP1 (Hander et al., 2019). PEP1 is then released into the apoplast following wounding and is perceived as a systemic signal by neighbouring cells. Likewise, earlier proteomic studies from the Moschou Lab showed that proPEP7 is likely cleaved and matured into the active PEP7 in the Arabidopsis root tip, by a proteolytic pathway that involves separase (Liu et al., 2017). The functional significance of PEP7 remains unclear.

Aim of the study

My aim here is to study the role of AtMCs in plant physiology with genetic, molecular and phenotypical approaches at different developmental stages of *Arabidopsis thaliana.* Furthermore, I established vectors for proPEP4 and 7 expression with fluorescent tag that will further expedite the analysis of their maturation by AtMCs or KISC/Sec14. Previously the Moschou Lab, have generated sextuple and nonuple mutants of AtMCs in Arabidopsis, via Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) to overcome the difficulty of generating high-order mutants due to their tandem arrangement (see above). As described below, the CRISPR approach allowed to overcome the genetic redundancy among type II AtMCs, by ablating the AtMCs 4 to 9. Herein, I focused on the phenotypic characterization of the sextuple mutant using two individual plant lines (transformation events), referred hereafter as mc93-3 and mc93-6. In parallel, to check whether MC-like complexes such as KISC behave in a similar manner as the AtMCs, I used a series of mutants of Sec14 gene that its protein was found to interact with KISC (Sec14-2g single mutant, Sec14-2g-3g and Sec14-2g-4g double mutants) where Sec14-2g: At2g21520, Sec14-3g: At3g24840 and Sec14-4g: At4g39170

Materials and Methods

Plant materials

Arabidopsis thaliana seeds of Col-0, 2 lines of sextuple mutants of metacaspases (mc 93-3 and mc 93-6) and a sec14 double mutant (2g-4g, see above) were used. All harvested seeds were dried at 37 ^{o}C overnight and then stored at 4 ^{o}C.

Growth conditions

Seeds were placed in ddH_2O ON for vernalization and scarified with 10 % bleach followed by 3 washes with ddH_2O to remove bleach. Then they were placed in Petri dishes containing 1/2 Murashige-Skoog (MS) medium supplemented with 1% sucrose and 0.8 % plant agar. Petri dishes were then moved in a Conviron growth chamber at 22 ^{o}C with a photoperiod of 18h light - 6 h dark and 100 µE light intensity.

Scanning Electron Microscopy (SEM)

The images were captured with a JEOL JSM-6390 LV scanning electron microscope after seed spattering by incubating with Argon gas at 40mA for 40 seconds with a Bal-tec SCD 050 sputter coater.

Measurements

Measurements of relative fluorescence, size and length were made with the use of Imagej - Fiji software v1.52q.

Statistic Analyses

All statistical data analysis and plot representation were made with the use of R programming package (R studio) version 3.6.1. The statistical significance was evaluated with Student's t-test.

Cloning of ProPEP4 and 7

Constructs were first design with Snapgene software and cloning of proPEP 4 and 7 was performed using the Gibson assembly method, which allows the joining of multiple DNA fragments in a single reaction. In these reaction we added 3 fragments to each construct: a promoter named propep 4 or 7, a VenusYFP tag and the exons of proPEPs named proPEP 4 or 7 (Image 3). Primers used in PCR for each DNA fragment are described in table 1. In-Fusion kit (Clonetech Takara) and the vector pGWB601

were used, followed by transformation into Stellar™ competent cells provided also by Clonetech through heat shock.

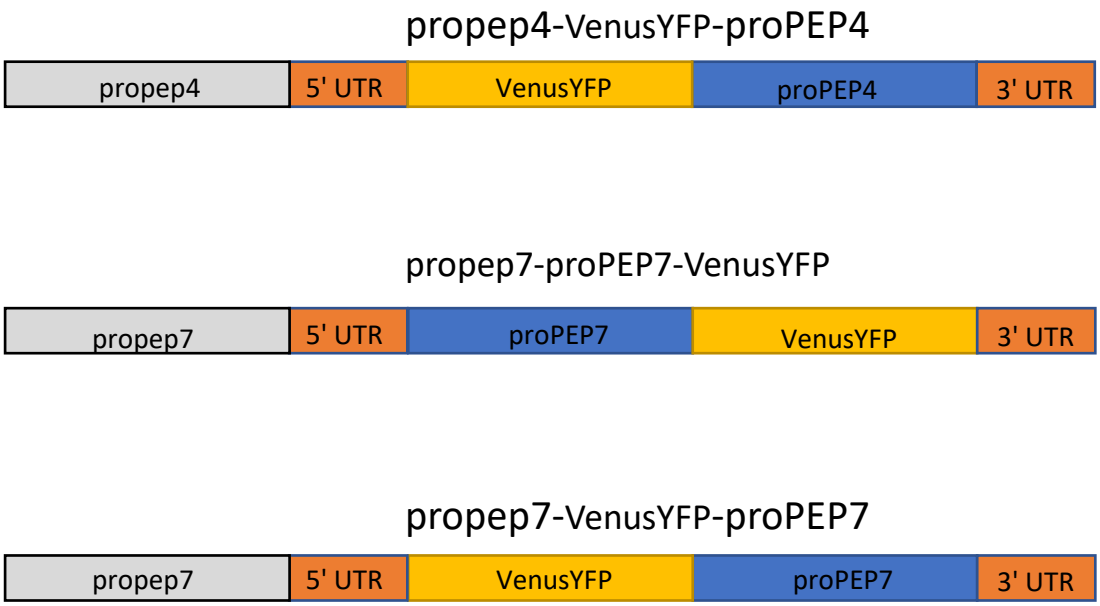


Image 3: The arangment of the 3 inserted fragments for each construct . Gray boxes: promoter. Red boxes: 3' and 5' untranslational regions.Orange boxes: tag VenusYFP. Blue boxes: proPEP 4 or 7 exons.

Construct and Fragment	Primer Name	Sequence 5' ⟶ 3'
pp7-PP7-YFP (promoter and exons)	Propep7-YFP Oligo 1 (Fw)	TCACTATGGCGGCCCCTCGAGTAAG CATTTGTTAGAAAGCCATCG
pp7-PP7-YFP (promoter and exons)	Propep7-YFP Oligo 2 (Rv)	TGCTCACCATGTTGGTACCTCCACCC TTCCC
pp7-pp7-YFP (VenusYFP)	Propep7-YFP Oligo 3 (Fw)	AGGTACCAACATGGTGAGCAAGGGC GAGG
pp7-pp7-YFP (VenusYFP)	Propep7-YFP Oligo 4 (Rv)	AGGAAGGTGCCTTGTACAGCTCGT CCATGCC

pp7-pp7-YFP (3′ UTR)	Propep7-YFP Oligo 5 (Fw)	GCTGTACAAGGCACCTTCCTAATTTTAGCTTCCA
pp7-pp7-YFP (3′ UTR)	Propep7-YFP Oligo 6(Rv)	TGAACGATCGGGGAAATTCGCAGCACTATTATTTACAGTCACAAT
pp7-YFP-PP7 (promoter)	YFP-propep7 Oligo 1 (Fw)	TCACTATGGCGGCCCCTCGAGTAAGCATTTGTTAGAAAGCC
pp7-YFP-PP7 (promoter)	YFP-propep7Oligo 2 (Rv)	TGCTCACCATTTTTTTTCTTCTTTCTTCTAACTAC
pp7-YFP-PP7 (VenusYFP)	YFP-propep7Oligo 3 (Fw)	AAGAAAAAAAATGGTGAGCAAGGGCGAGG
pp7-YFP-PP7 (VenusYFP)	YFP-propep7Oligo 4 (Rv)	CTCCCTCCATCTTGTACAGCTCGTCCATGCC
pp7-YFP-PP7 (Exons and 3′ UTR)	YFP-propep7Oligo 5 (Fw)	GCTGTACAAGATGGAGGGAGAGGGAAGAAGA
pp7-YFP-PP7 (Exons and 3′ UTR)	YFP-propep7Oligo 6 (Rv)	TGAACGATCGGGGAAATTCGCCAGCACTATTATTTACAGTCACA
pp4-YFP-PP4 (promoter)	YFP-propep4 Oligo 1 (Fw)	TCACTATGGCGGCCCCTCGACAATTCTTATAAGTTAATATGCACG
pp4-YFP-PP4 (promoter)	YFP-propep4 Oligo 2 (Rv)	TGCTCACCATGTTTTTCTTCAATTCTGCTTCG
pp4-YFP-PP4 (VenusYFP)	YFP-propep4 Oligo 3 (Fw)	GAAGAAAAACATGGTGAGCAAGGGCGAGG
pp4-YFP-PP4 (VenusYFP)	YFP-propep4 Oligo 4 (Rv)	CTCTCTCCATCTTGTACAGCTCGTCCATGCC
pp4-YFP-PP4 (Exons and 3′ UTR)	YFP-propep4 Oligo 5 (Fw)	GCTGTACAAGATGGAGAGAGGAGTTTCTT
pp4-YFP-PP4 (Exons and 3′ UTR)	YFP-propep4 Oligo 6 (Rv)	TGAACGATCGGGGAAATTCGCATTTTTCGAGGTAAGAGATCGA

Table 1: The primers used for DNA fragments in order to be used for the Gibson assembly. pp7: promoter of proPEP7. pp4: promoter of proPEP4. PP7: proPEP7. PP4: proPEP 4.

Reactive oxygen species (ROS)

ROS induction performed by incubating 7 days old seedlings in 200mM NaCl for 1 hour. Then for visualization of ROS, seedlings were washed with ddH_2O and incubated in 10µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) for H_2O_2 or 10µM dihydroethidium (DHE) for O_2^- detection. Seedlings were observed immediately in a Leica SP8 inverted confocal microscope.

Nitrogen remobilization

50-days old plants were watered with N^{15} (150ml) 3 times per week for 2 weeks. Then samples from young/old seeds and leaves were collected and performed protein extraction with 100µl of extraction buffer (99.875% Methanol and 0.125% Formic Acid). The protein samples were analyzed with LC-MS/MS by a Bruker Maxis Impact ESI-QTOFMS

Transient expression in *Nicotiana benthamiana*

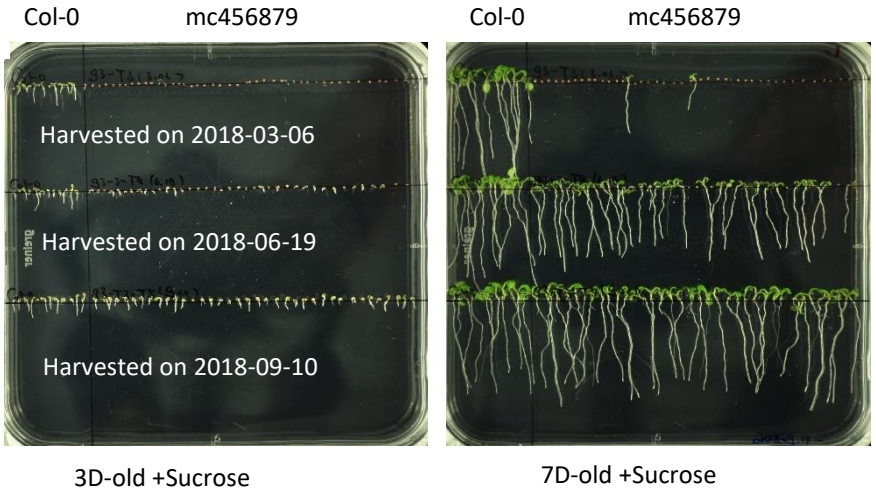
ProPEPs constructs were transformed in *Agrobacterium tumefaciens* GV3101 cells through heat shock method in which competent cells were snap freeze on liquid nitrogen for 1 minute and then placed on 42 ^{o}C for 10 minutes (heat shock). After heat shock cells were incubated on ice for 5 minutes, followed by addition of 400 µl of YEP medium and incubation at 28 ^{o}C for 3 hours. Then bacteria are plated in YEP plates with 100 µg/ml Spectinomycin and 50 µg/ml Rifampycin for selection. For the expression I used 3 weeks-old *Nicotiana benthamiana* wild type plant and the induction of *Agrobacterium tumefaciens* strains took place with an induction buffer containing 10mM NaCl, 10mM MES and 200µM acetosyringone. After infiltration, plants were watered and moved in a Phytotron growth chamber at 22 ^{o}C for 4 days and then were observed in Leica SP8 inverted confocal microscope. For control I used eGFP construct GATEWAY™ pB7FWG2 (Karimi et al., 2002).

Results

Phenotypic analysis of AtMCs mutants reveals defects in growth

In order to check for physiological differences between the wild-type, AtMC sextuple mutants (93-3 and 93-6) and *sec14 2g-4g* double mutant (as an additional control showing normal seed development but compromised overall plant growth), I analyzed their phenotype at different developmental stages. More specifically, the analysis of seed was decided due to the fact that previously in our lab has been

A)



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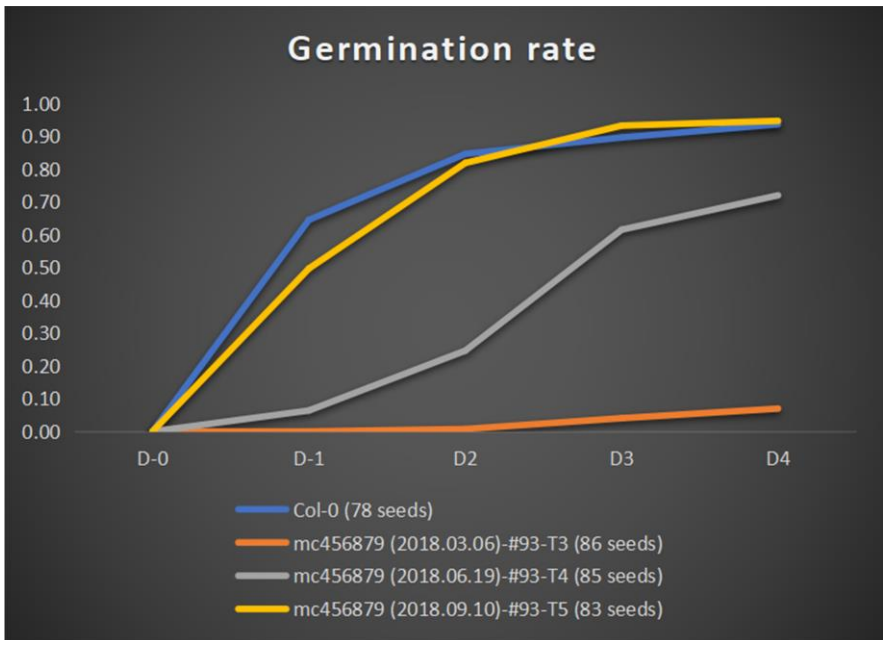

Figure 1: A) The difference in germination between seeds that are stored for different time period. B) The germination rate between 3 generations of sextuple mutant (T3,T4 and T5) compared to Col-0. Data from Chen Liu, unpublished.

observed that long-term stored seeds showed reduced germination (Figure1, data from Chen Liu, unpublished)

The previous observation led me to formulate 3 hypothesis. The first focus on a potential abnormality in the structure of seed testa and dormancy. Seed dormancy is an evolutionary adaptation that prevents seeds from germinating, allowing it to overcome periods that do not favour seed-establishment (Bewley, 1997). The second hypothesis may relate to reduced seed longevity meaning that the seeds have shorter lifespan, implying that seeds are unable to regulate their protein homeostasis and are cannot germinate due to the accumulation of toxic protein aggregates. The third hypothesis suggests a potential defect in seed filling. Particularly, seed filling is the

process of massive storage reserve of oil, proteins and starch (Baud et al., 2009). Significant changes of gene expression levels during seed filling include many proteases (Ruuska et al., 2002). During seed filling, the accumulation of proteins and other micro- and macro-molecules that are vital for germination relies on the nitrogen (N) supply from the mother plant. The previous process suggests that the mother plant functions as a source of N and the seed as a sink.

Imaging of long-term stored seeds with SEM and quantification with ImageJ (Figure 2) showed small non-significant differences in surface structure of the deposition of cellulose on seed testa (seed coat) as well as size, suggesting that there

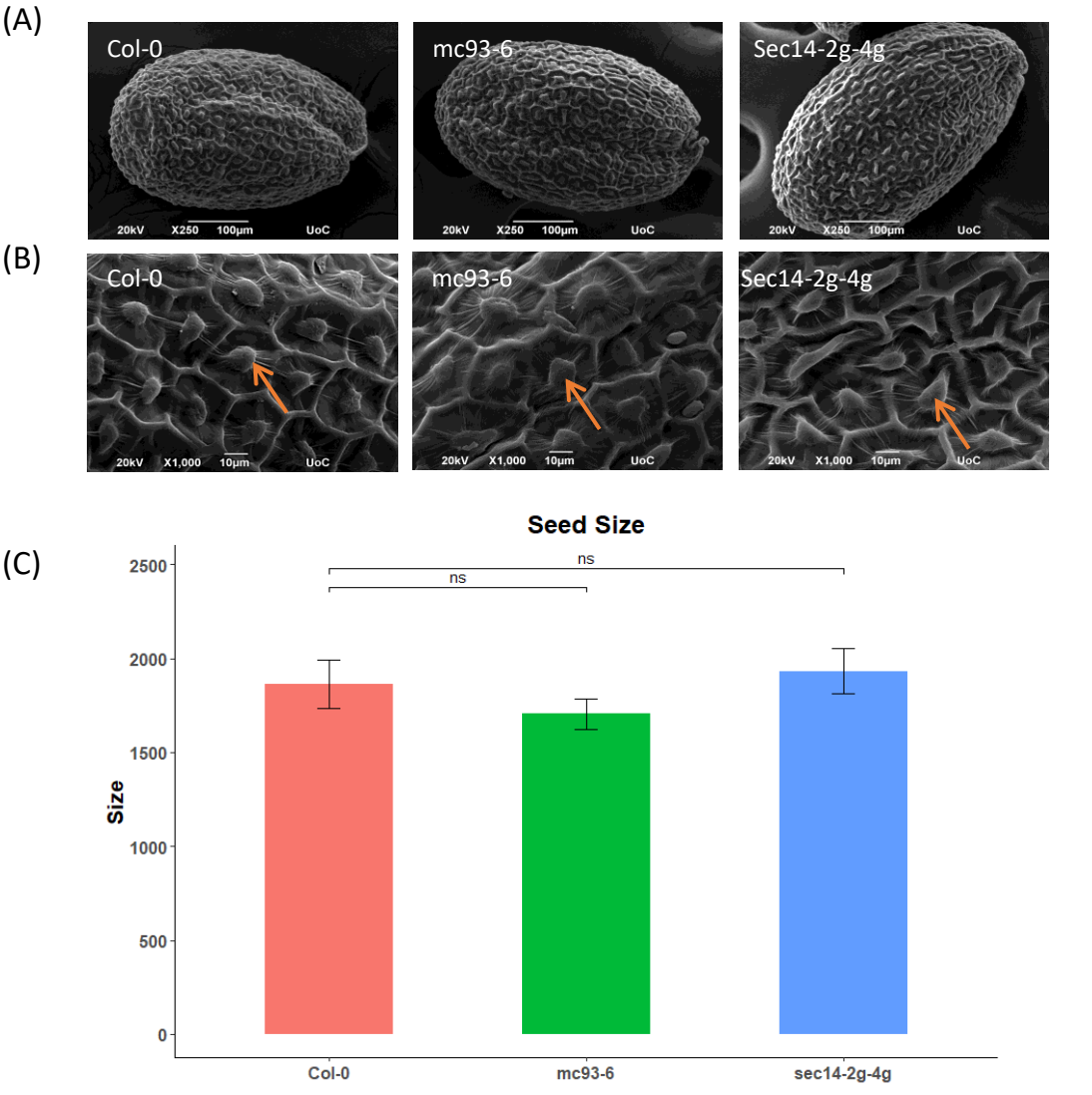

Figure 2: (A): Scanning electron microscopy of whole seeds for size measurement in each genotype (B): Scanning electron microscopy of mature seed coat cells. Arrows indicating the cellulose structure which showed no differences between Col-0 and mutants. (C): Quantification of seed size showed small differences that are non significant. 10 seeds of each genotypes have been measured in 2 biological replicates. Statistical significance was evaluated with Student's t-test

is no defect on testa. As control in these experiments, I used *sec14* mutant with normal germination rate.

After the generation of sextuple mutant, plants on soil appeared to be relatively smaller than the wild-type. For this reason we decided to measure leaf length of full-grown plants (40 days after sowing), in order to see whether the lack of type II AtMCs affects the development and growth. Differences concerning leaf size were observed between wild-type and 2 lines of AtMCs sextuple mutants (Figure 3) but line mc93-6 had smaller differences comparing to wild-type than line mc93-3

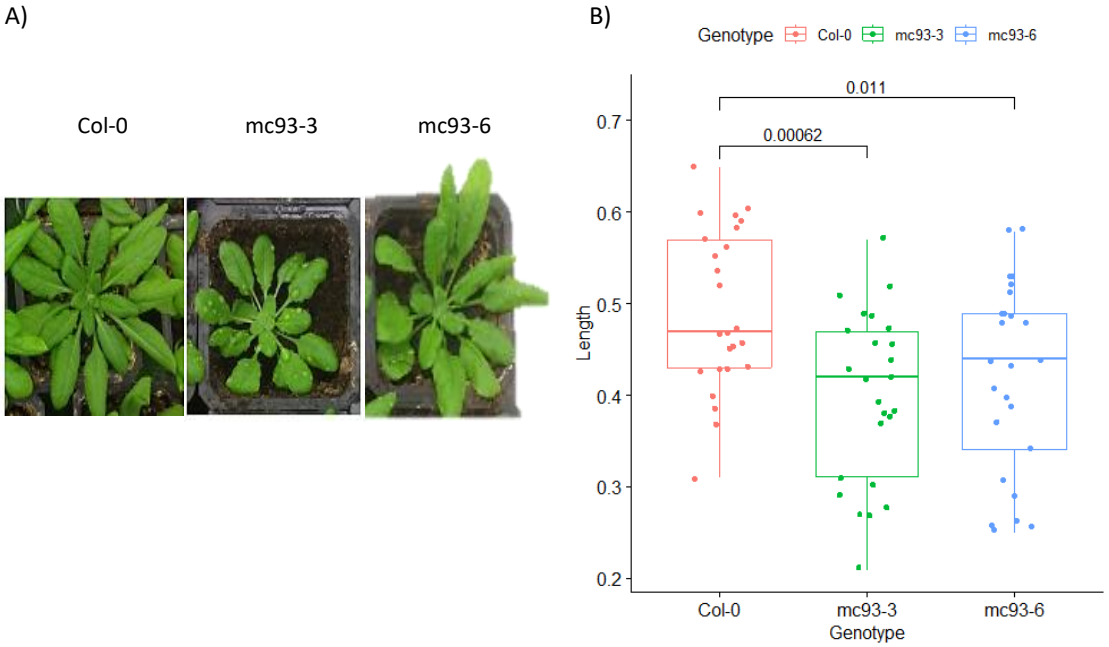

Figure 3: A): phenotypes of 40 days old plants on which leaf length has been measured. B): Quantification of leaf length in every genotype, with v=5 were v is the number of plants 5 leaves of each plant have been measured. Statistical significance was evaluated with Student's t-test and the bars are referring to p-values.

Visualization of Reactive Oxygen Species (ROS) in AtMCs mutants reveals their involvement in ROS homeostasis

AtMCs sextuple mutant are more susceptible in salt stress than wild type (Chen Liu, unpublished; Figure 4A). Salt stress can enhance ROS production in plant cell, which are utilized as mediators of the stress signal leading to PCD (Gechev et al., 2006). Some MCs have been also implicated in PCD in several studies in different organisms and this PCD is triggered by ROS (Madeo et al., 2002; He et al., 2008; Sundström et al., 2009; Coll et al., 2010). For this reason, our next question was whether there is a

difference in ROS accumulation between MCs and wild-type during salt stress. Furthermore, increased ROS production correlates with higher protein aggregation and reduced protein homeostasis. After induction of ROS in seedlings by incubating them in 200mM NaCl solution for 1 hour, ROS were visualized with the use of two fluorescent ROS probes. The first ROS probe is 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) which detects H_2O_2 and becomes fluorescent upon oxidation (Shin et al.,2005). The second ROS probe is dihydroethidium (DHE)which detects O_2^- and when oxidized it becomes red (Wojtala et al., 2014). In both treatments, mc93-3 showed increased relative fluorescence although in DHE the increase was much higher than in H2DCFDA. These results suggest that in mc seedling there was higher ROS accumulation, especially O2.-, than in wild-type during NaCl treatment (Figure 4).Nitrogen remobilization is not altered in AtMCs mutants

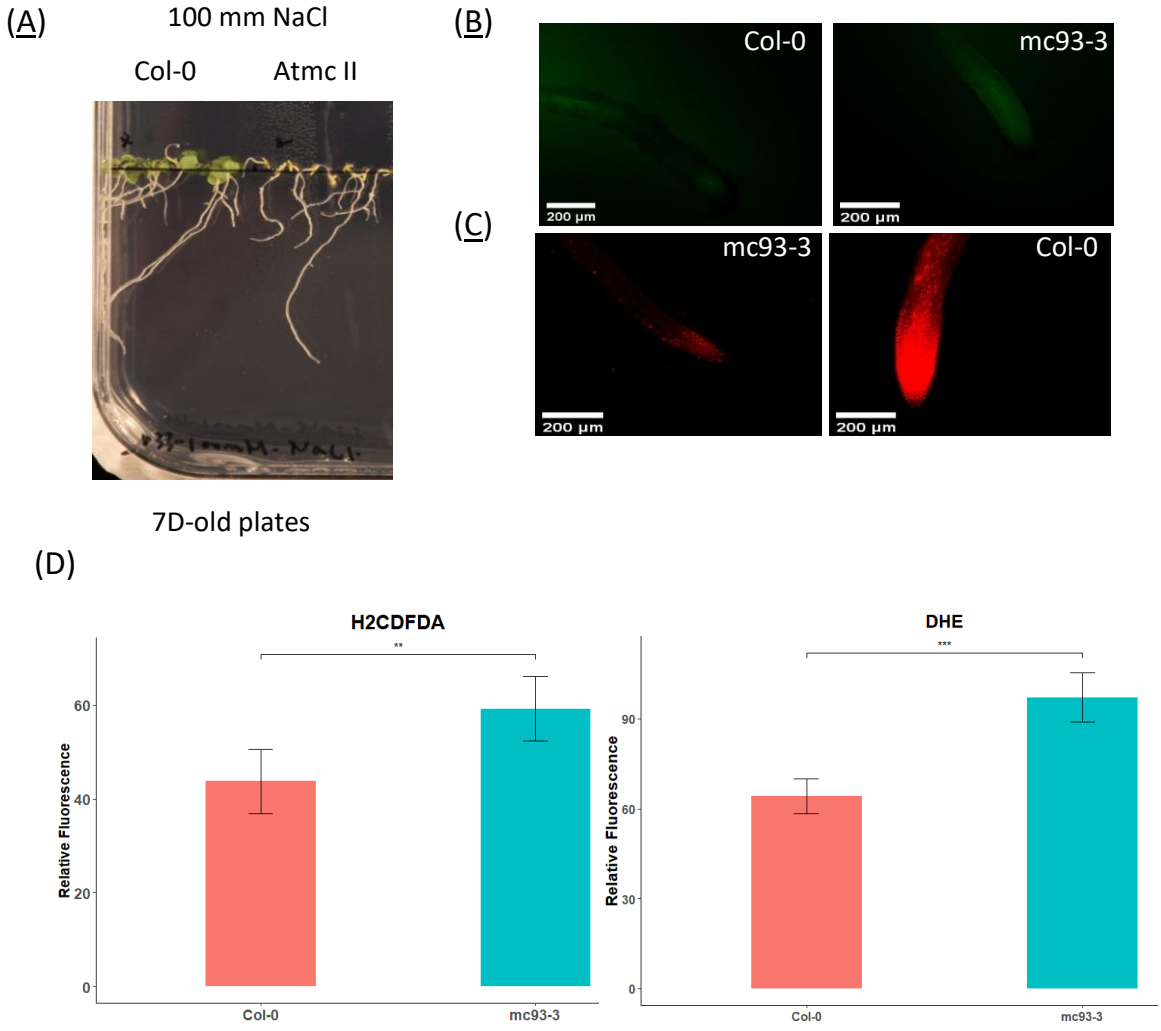

Figure 4: (A): Difference in salt stress tolerance between Col-0 and metacaspase mutant (B) and (C): Relative fluorescence intensity of ROS dyes H2CDFDA (B) and DHE (C) in plants treated with NaCl. (D): Quantification of relative fluoresence for each ROS dye. In both treamtent were performed 2 replicates of each genotype with 10 fluorescence measurements each. Bars indicating standard errors.

The pathway of nitrogen (N) recycling and remobilization in sink and source vegetative organs is a vital process for the whole-plant and seed production quality (Have et al., 2017). The transport of N is affected by the ability of the source cells to export as well as the cells that functions as a sink to import it. Proteases are involved in the source-to-sink remobilization by mobilizing fixed N on proteins through their cleavage and the concomitant degradation (Peoples and Dalling, 1988). Protein degradation is a prerequisite for an efficient N remobilization from senescencing plant parts. Once a protein is cleaved by a protease in source cells, it can be further processed by proteasome and dipeptidases to release amino acids which can be transferred to other sink cells to serve as N sources there. We thus tested the hypotheses that the low germination of long-term stored seeds was due to seed filling defects. This led us to perform the following experiment, in which we checked the nitrogen mobilization, by comparing enrichments between old and young leaves and seeds. This took place through the tracking of N^{15} and its incorporation in glutamate and removing the endogenous N^{15} (Figure 5). Metacaspase mutant 93-3 showed no differences comparing to col-0 in either leaves or seeds. These data suggests that seed filling is not responsible for the low germination of metacaspase seeds.

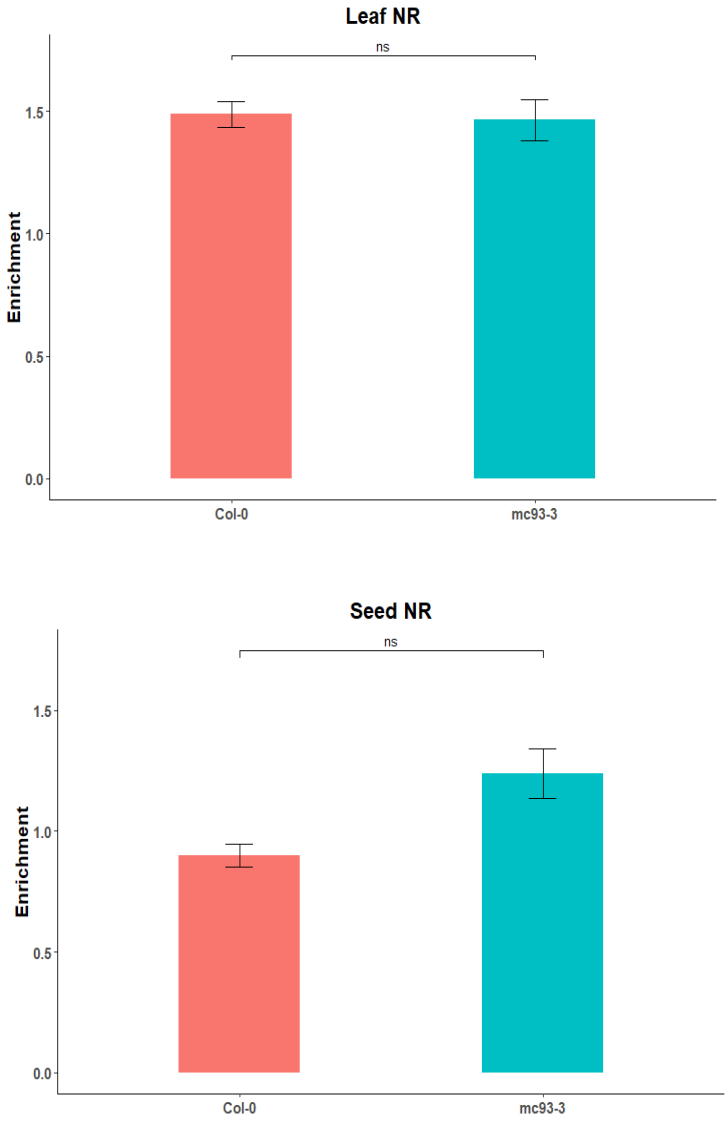

Figure 5: A) Enrichment of young leaves compared to old in mc93-3 and Col-0. Used 2 plants for each genotype with 2 young and 2 old leaves from each replicate. B) Enrichment of young seeds compared to old in mc93-3 and Col-0. For this experiments, I used 2 plants for each genotype with 2 young and 2 old siliques from each. The statistical significance was evaluated with Wilcoxon-test for both experiments. NR stands for Nitrogen Remobilization.

ProPEP4 and 7 show cytoplasmic localization

ProPEPs are potential cleavage targets not only for AtMCs but also for proteases involved in metacaspase-like complexes such as KISC. Previously in Moschou lab proteomic studies showed preliminary evidence that the KISC component separase can cleave these peptides (Liu et al., 2017). In addition, proPEP3 is cleaved during salt stress to release the mature form PEP3. Thus, I decided to monitor *in vivo* cleavage of proPEP4 and 7 on root tips, in the sextuple mutant as well as in *radially swollen 4* (*rsw4*), which corresponds to a weak point mutant allele of separase (Moschou et al., 2016). Three new constructs were generated, two for

proPEP7, in which the tag Venus is either on N- (propep7-Venus-proPEP7) or C-terminus (propep7-proPEP7-Venus) and one for proPEP4 with the Venus tag in the N-terminus (propep4-Venus-proPEP4) where propep is the promoter of each peptide and proPEP the downstream to the promoter gene sequence. After confirming that the constructs were correct through digestions and sequencing, the next step was to check them with transient expression through agroinfiltration in *N. benthamiana* mesophyll cells. All 3 ProPEPs seems to localize in cytoplasm and ER but the exact subcellular location may not correspond to the localization of the native protein in *A. thaliana* (Figure 6). Thus, we cannot reach a safe conclusion unless stable transgenic lines expressing proPEPs are produced (experiments in progress).

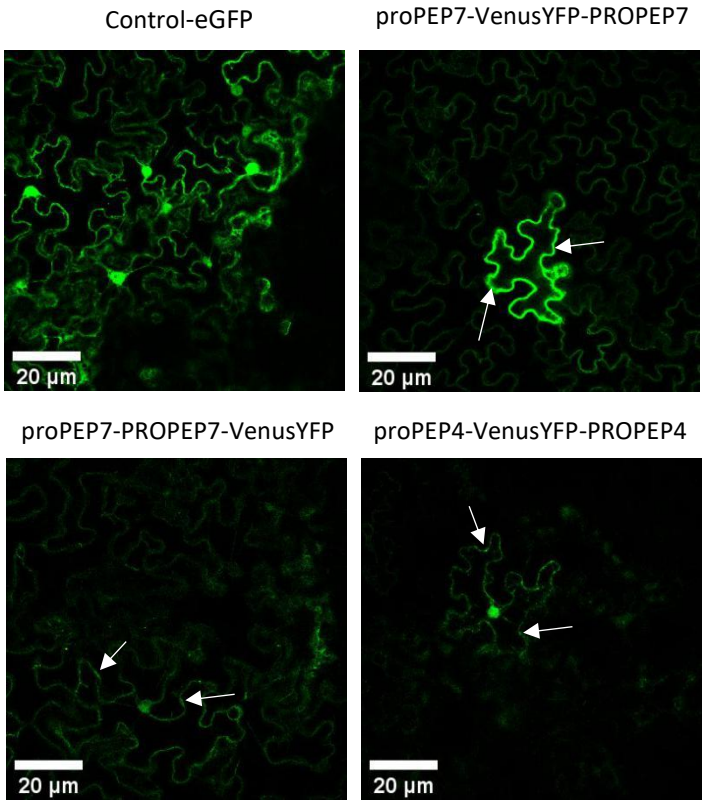

Figure 6: Transient expression of proPEP4 or 7 in *Nicotiana benthamiana* leaves. Control represents the expression of eGFP. Leaves were observed using confocal microscopy 4 Days Post Infiltration (DPI). Data are from single experiment. Arrows indicating the expression of ProPEPs in leaf epidermal cells. The nuclear signal observed may correspond to free GFP released upon proteolytic cleavage.

Discussion

Metacaspases are associated with several cellular pathways, due to their structural homology to caspases. The generation of sextuple AtMCs mutants overcomes genetic limitations of previous studies such as the genetic redundancy among type II AtMCs and can be used to expand our knowledge concerning cell protein homeostasis, potential interactions and impact in plant physiology from the stage of seed to old plants. Although long-term stored AtMCs seeds showed reduced germination, seed analysis in this study did not show major differences between size, or testa structure. However, the fact that the full-grown plants are relatively smaller than wild-type shows developmental deficiencies that their origin remains elusive.

An interesting result was the higher ROS accumulation in mc93-3 during salt stress, both in H2DCFDA (H2O2) and DHE (O2.-). The pathway of abiotic stress-induced PCD needs further study to understand the molecular relation between AtMCs and ROS, due to the fact that many MC genes have been implicated in PCD during salt stress triggered from ROS (Wang et al., 2018). Regarding the nitrogen remobilization tracking of N^{15}, seed filling does not appear to be the factor of low germination in AtMC seeds as data showed no deficiency. Although there were no statistically significant differences between wild-type and AtMC mutant, this was a pilot experiment with a relatively small number of replicates and has to be repeated in order to draw any safe conclusions from this.

Regarding proPEPs, to monitor their cleavage in root tip we need stably transformed proPEP4 and 7-expressing lines in wild-type, AtMCs sextuple and rsw4 mutants. After screening those transformed plant lines, we will be able to monitor if these peptides are matured through the direct involvement of AtMCs and KISC/sec14 nexus. Furthermore, using the same lines, we will be able to decipher the intracellular localization of proPEPs.

Despite the fact that many studies have been made over the last decade, the molecular role of AtMCs remains unclear. This study was the first step for further understanding their molecular functions but there are still many aspects of their nature and function that we do not understand. One of the most important questions is the search of new interactors of AtMCs or KISC/sec14 nexus, revealing new targets and roles for these proteins and contribute to their understanding.

Regarding future work in our lab, apart from the ongoing proPEP experiment, we have established different methods such as immunoprecipitations and yeast two hybrid assays, that will help us to further understand the role of AtMCs and MC-like complexes. More specifically, we search for interactions between kin7.3 with homologues of sec14 such as sec3a. In addition, new experimental setups for seed germination should be prepared, especially with proteomic approaches to find new key proteins or signal peptides which would possibly reveal the cause of the low germination phenotype on metacaspase sextuple mutant.

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