Downregulating a Type I Metacaspase in *Petunia* Accelerates Flower Senescence

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ABSTRACT. Metacaspases are cysteine proteases from plants, fungi, and protozoans that have structural similarity to metazoan caspases. They play a critical role in programmed cell death (PCD) induced by developmental cues and environmental signals. In this study, a type I metacaspase (*PhMC1*) was identified and characterized from *Petunia ×hybrida* ‘Mitchell Diploid’ (MD) (petunia). The recombinant PhMC1 had activity against the metacaspase substrate Boc-GRR-AMC (GRR). Activity was highest at pH 7–9 and was dependent on the active site C²³⁷. Quantitative polymerase chain reaction (qPCR) showed that *PhMC1* transcripts increased at a later stage of petal development, when corollas were visibly senescent in both pollinated and unpollinated flowers. Gene expression patterns were similar to that of the senescence-related gene *PhCP10*, a homolog of *Arabidopsis thaliana* (arabidopsis) *AtSAG12*. *PhMC1* transcripts were upregulated in the petals by ethylene treatment. This ethylene regulation did not require protein synthesis, indicating that *PhMC1* is a primary ethylene response gene. Metacaspase-like activity against Boc-GRR-AMC increased in protein extracts from senescing petals. RNAi was used to knock down the expression of *PhMC1*. Transgenic PhMC1 petunias had no abnormal, vegetative growth phenotypes under normal greenhouse conditions, but flower senescence was accelerated by an average of 2 days.

Programmed cell death is a highly ordered process whereby plants or animals can remove unneeded or damaged cells or tissues during development or in response to abiotic or biotic stresses (Beers and McDowell, 2001). PCD in animals is characterized by the activation of a family of cysteine-dependent aspartate specific proteases or caspases. Caspases proteolytically activate downstream caspases or cleave substrates that ultimately lead to cell death (Fischer et al., 2003). The search for plant caspase-like enzymes led to the identification of a structurally related family of cysteine proteases called the metacaspases, which are also present in protozoans and fungi (Uren et al., 2000). Metacaspases have been shown to play a critical role in developmental and stress-induced PCD in plants (Fagundes et al., 2015; Lam and Zhang, 2012). Plant metacaspases may be positive or negative regulators of PCD, and they are classified as type I or type II (Coll et al., 2010). Type I metacaspases are distinguished by a proline- or glutamine-rich N-terminal prodomain containing a zinc finger motif. Type II metacaspases have no prodomain, but contain a linker between the p20 and p10 caspase-like domains. Whereas the caspases are aspartic acid-specific cysteine proteases, the metacaspases are cysteine proteases that cleave target substrates after arginine and lysine residues (Bozhkov et al., 2005; Tsiatsiani et al., 2013; Vercammen et al., 2004, 2006; Watanabe and Lam, 2005).

Characterization of gene expression patterns for multiple metacaspase family members has been conducted in arabidopsis

(Kwon and Huang, 2013; Tsiatsiani et al., 2011), *Vitis vinifera* [grape (Zhang et al., 2013)], *Oryza sativa* [rice (Huang et al., 2015; Wang and Zhang, 2014)], *Hevea brasiliensis* [rubber tree (Liu et al., 2016a)], and *Solanum lycopersicum* [tomato (Liu et al., 2016b)]. The arabidopsis genome contains three type I and six type II metacaspases (Tsiatsiani et al., 2011), whereas the tomato genome has six type I and two type II metacaspases (Liu et al., 2016b). Expression analysis shows spatiotemporal regulation of individual metacaspase genes within the plant, as well as differential regulation by plant hormones, pathogens, and environmental stresses (Ahmad et al., 2012; Hoeberichts et al., 2003; Huang et al., 2015; Kim et al., 2013; Kwon and Huang, 2013; Liu et al., 2016a, 2016b; Tsiatsiani et al., 2011; Wang et al., 2012; Wang and Zhang, 2014; Zhang et al., 2013). Two type I metacaspases in arabidopsis, AtMC1 and AtMC2, work antagonistically to regulate cell death during the hypersensitive response (HR). AtMC1 is a positive regulator of HR-induced PCD, whereas AtMC2 functions as a negative regulator (Coll et al., 2010). Ethylene treatment induces multiple metacaspase genes in both rubber tree and tomato plants (Liu et al., 2016a, 2016b). Metacaspase genes have also been shown to be differentially regulated by abiotic stresses including cold, heat, drought, salt, ozone, and ultraviolet light (Ahmad et al., 2012; He et al., 2008; Huang et al., 2015; Kwon and Huang, 2013; Liu et al., 2016a, 2016b; Wang et al., 2012; Wang and Zhang, 2014).

Both plant development and adaptation to biotic and abiotic stresses require the induction of PCD to remove unwanted or unneeded cells and tissues. A recent study by Coll et al. (2014) reports a dual prodeath/prosurvival function for the type I metacaspase AtMC1. Although AtMC1 positively regulates pathogen-induced cell death in young tissues, it negatively regulates senescence in older tissues. The *atmc1* mutants exhibit premature leaf senescence, which is accompanied by earlier expression of the senescence marker *AtSAG12* (Coll et al., 2014). These *atmc1*-deficient plants are also

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hypersensitive to reactive oxygen species (ROS), and they cannot restrict cell death after infection by pathogens.

Although cysteine proteases have been one of the most common proteins identified in screens for petal senescence-associated genes (SAGs) (Jones et al., 2009), no studies to date have investigated the role of metacaspase proteases in petal senescence. This article presents the cloning and characterization of a type I metacaspase from petunia (*PhMC1*). Transgenic RNAi *PhMC1* petunias had reduced flower longevity, suggesting that *PhMC1* may function as a negative regulator of senescence in petals.

Materials and Methods

CLONING A METACASPASE FROM PETUNIA. Petunia sequences with similarity to the arabidopsis metacaspases were identified from BLAST searches of a petunia transcriptome database within the Sol Genomics Network [SGN (Fernandez-Pozo et al., 2015)]. Searches identified petunia clone PP-8-D05 as a putative metacaspase. The PP-8-D05 (*PhMC1*) insert cloned into pBluescript (Fermentas Intl., Burnie, MD) was obtained from the freezer stocks. Insert size analysis by gel electrophoresis and sequencing confirmed that the *PhMC1* clone was full length and contained the entire coding region.

The full length clone was sequenced [capillary sequencing, Molecular and Cellular Imaging Center, The Ohio State University/Ohio Agricultural Research and Development Center (OARDC), Wooster] to at least 6× coverage to assure accurate sequence data. Sequences were analyzed with ChromasPro (Technelysium, South Brisbane, Australia) and with the BLAST algorithm from the National Center for Biotechnology Information (NCBI, Bethesda, MD) nonredundant database (NCBI Resource Coordinators, 2017). Clustal Omega was used for multiple amino acid sequence alignments (Sievers et al., 2011). Coding regions of metacaspases from tomato (SIMC1 Solyc01g088710, SIMC2 Solyc03g094160, SIMC3 Solyc05g052130, SIMC4 Solyc01g105320, SIMC5 Solyc01g105300, SIMC6 Solyc01g105310, SIMC7 Solyc09g098150, and SIMC8 Solyc10g081300) and arabidopsis (AtMC1 AY322525, AtMC2 AY322526, AtMC3 AY322527, AtMC 4 AY322528, AtMC5

AY322532, AtMC6 AY322533, AtMC7 AY322529, AtMC8 AY322530, and AtMC9 AY322531) were obtained from SGN (Fernandez-Pozo et al., 2015) and NCBI (NCBI Resource Coordinators, 2017).

CONSTRUCTION OF RECOMBINANT PROTEIN MUTANTS. The coding region of *PhMC1* was amplified by PCR using primers with *Bam*HI and *Xho*I site extensions [mutagenesis primers *PhMC1pET* (Table 1)]. PCR fragments were ligated into the expression vector, pET28a(+) (Novagen, Darmstadt, Germany) at the *Bam*HI and *Xho*I sites and transformed into *Escherichia coli* strain BL21(DE3) pLysS (New England Biolabs, Ipswich, MA). All constructs were verified by sequencing. Recombinant proteins that contained a mutation in the cysteine residue of the catalytic dyad were created using the QuikChange II site-directed mutagenesis kit (Agilent, Santa Clara, CA). Cysteine was converted to alanine (rPhMC1^{C237A}). The entire coding region including the introduced mutation was then amplified using a set of primers with restriction enzyme extensions (Table 1). The PCR fragments were digested with the corresponding restriction enzymes, ligated into pET28a(+) vector, and transformed into *E. coli* strain BL21(DE3) pLysS (pET28a(+):PhMC1^{C237A}). All constructs were verified by sequencing.

RECOMBINANT METACASPASE ENZYME ACTIVITY ANALYSIS. A single colony for each construct, including a control [*E. coli* with pET28a(+) vector alone] was picked and grown in 5 mL of Luria-Bertani (LB) liquid media containing kanamycin (50 mg·mL⁻¹) overnight at 37 °C. Three milliliters of overnight culture was inoculated in 50 mL LB with kanamycin and incubated at 37 °C (3–3.5 h) until OD₆₀₀ was 0.6–1.0. Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM and the cultures were incubated for 2–3 h at 37 °C or up to 6 h at 30 °C. After the flasks were put on ice for 5 min, the cells were harvested by centrifugation at 5000 g_n for 5 min at 4 °C. The cells were resuspended in a 0.25 × volume of cold 20 mM Tris-HCl pH 8.0, and centrifuged at 5000 g_n for 5 min. Pellets were stored frozen at –80 °C, or purification was continued.

Metacaspases were purified according to the manufacturer's instruction using TALON Metal Affinity Resin (BD Biosciences Clontech, Mountain View, CA). The amount of purified

Table 1. Primers used for cloning and characterization of *Petunia ×hybrida* metacaspase *PhMC1*.

Primer name	Sequences	Notes
F-PhMC1pET	AATTAGGATCCGACGGCAGGAGATACAAATGCAAGTG	Cloning <i>PhMC1</i> into pET28a(+)
R-PhMC1pET	TTATGAAGCTTGCAAACGTACCTGAGTTTCTTTGGCACC	
F-PhMC1C237A	ACTGCCTTCAGTGGAACCTTTCCTAGATTGCCATT	Introducing a point mutation at aa 237 of <i>PhMC1</i>
R-PhMC1C237A	GGCAAATCTAGGAAAGTTCCACTGAAGGCAGTATCGAT-TATGCCATGAAGTGTTGC	
F-PhMC1 RT	AGGAGCCTCAACTATCCTCGTA	qPCR of <i>PhMC1</i>
R-PhMC1 RT	CATGATCAAGGTTACACAGAA	
F-PhCP10 RT2	ACTTTGTGGACTTGCAACGGAAGC	qPCR <i>PhCP10</i>
R-PhCP10 RT2	CCAAGCCTATCTCAATCCCATACA	
F-PhACTIN RT	AGCCAACAGAGAGAAGATGACCCA	qPCR of Petunia actin, reference gene
R-PhACTIN RT	ACACCATCACCAGAGTCCAACACA	
F-PhRPS13 RT	CAGGCAGGTTAAGGCAAAGC	qPCR of Petunia RPS13, reference gene
R-PhRPS13 RT	CTAGCAAGGTACAGAAACGGC	
F-PhMC1RNAi	TGGAACACTAGTGGCGCGCCGATGGAGATGAGGTTGAT	Creating RNAi construct
R-PhMC1RNAi	GCTACCGGATCCATTTAAATGGCCTACTCCTTTCTGTGCTT	
F-PhMC1RNAi2	GGGAAGACCATCGTATCAGC	Confirming transgenic plants contain RNAi construct
R-PhMC1RNAi2	GGCCTACTCCTTTCTGTGCTT	

metacaspase was quantified using the Bradford Protein Assay (Bio-Rad, Hercules, CA). The purified recombinant protein (0.1 μ g) and 100 μ M peptidyl substrates or inhibitors were mixed in 100 μ L of reaction buffer [150 mM NaCl, 10 mM CaCl₂, 10% glycerol (w/v), 0.1% CHAPS, 10 mM DTT, and pH adjustment agents]. The pH of the reaction buffers was adjusted with 100 mM glycine (pH 4 and pH 5), 100 mM MES (pH 6), 50 mM HEPES-KOH (pH 7 and pH 8), or 100 mM Tris (pH 9) for optimum pH analysis. All other substrate and inhibitor assays were conducted at pH 8. The reaction mix containing 50 μ M of the metacaspase substrate *t*-butyloxycarbonyl-Gly-Arg-Arg-7-amido-4-methylcoumarin (GRR, lot no. 0519975; Bachem, Torrance, CA) or the caspase substrate Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC, lot no. 2000367; Bachem) was incubated for 30 min at 30 °C. One hundred microliters of 10% acetic acid in 150 mM sodium acetate was added to stop the reaction. A 30- μ L aliquot of the reaction mix was diluted with nanopure water to 1.5 mL.

Enzyme activity was measured as the amount of AMC liberated using a fluorescence spectrophotometer (DTX880 Multimode Detector; Beckman Coulter, Indianapolis, IN) at 370 nm excitation and 450 nm emission wavelengths. Substrates included GRR and Ac-DEVD-AMC, and inhibitors included 10 mM iodoacetamide and 1 μ M leupeptin (Sigma-Aldrich, St. Louis, MO). All reactions were performed in triplicate and data were analyzed using Multimode Analysis Software (version 3.2.0.6, Beckman Coulter).

METACASPASE-LIKE ACTIVITY IN PETUNIA COROLLAS. Protein was extracted from corolla samples by methods previously reported (Watanabe and Lam, 2011) and quantified with Pierce Coomassie Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA). Metacaspase activity against GRR substrate was assayed as described previously using 50 mM HEPES-KOH pH 8.0. Fluorescence units measured at 370-nm excitation and 450-nm emission wavelengths after a 10-min incubation at 30 °C are reported.

PLANT MATERIALS. *Petunia $\times$ hybrida* MD were started from seed and grown in the greenhouse at the OARDC unless otherwise noted. The growing environment was 24/20 °C (day/night) with a 13-h photoperiod supplemented by high-pressure sodium and metal halide lights (GLX/GLS e-systems GROW lights; PARSource, Petaluma, CA). Plants were fertilized at each irrigation with 20N–1.3P–15.8K (Jack's Professional Petunia FeED; J.R. Peters, Allentown, PA) at 150 mg·L⁻¹ N.

FLOWER DEVELOPMENT AND ETHYLENE TREATMENTS. *Petunia* flowers were tagged and emasculated 1 d before flower opening (i.e., anthesis) to prevent self-pollination. Corollas from unpollinated flowers were collected every day from anthesis (0 d) to senescence (7 d) to investigate gene expression during petal development. Six corollas were collected from at least three different plants at 1000 HR each day. To study pollination-induced responses, flowers were pollinated on the day of anthesis, and corollas were collected at 0, 1, 3, 6, 12, 18, 24, and 48 h after pollination ($n = 6$). For ethylene and cycloheximide treatments, flowers that were at 1 d after anthesis were detached from the plants, and peduncles were cut to equal lengths. Peduncles were placed in vials of 0 μ M (nanopure H₂O) or 50 μ M cycloheximide for 3 h. After cycloheximide treatment, flowers (all in vials of H₂O) were placed into sealed 24-L chambers. The chambers were injected with ethylene to a final concentration of 0, 0.1, 1.0, or 10.0 μ L·L⁻¹. Six flowers were included in each treatment. The chamber with 0 μ L·L⁻¹ was the

control chamber and included a sachet containing potassium permanganate to absorb any ethylene produced by the flowers. After 4 h, the chambers were opened, and the corollas were collected for subsequent RNA extraction.

RNA EXTRACTION AND GENE EXPRESSION ANALYSIS BY QUANTITATIVE PCR. All collected tissue was immediately frozen in liquid nitrogen and stored at –80 °C before RNA extraction. Plant tissue was ground to a fine powder in liquid nitrogen, and total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI), and cDNA was synthesized from 2 μ g RNA using the Omniscript Reverse Transcriptase kit (Qiagen, Valencia, CA). A qPCR was performed in a 20- μ L reaction volume using SYBR Green Supermix (Bio-Rad). One microliter cDNA was used as template, and all reactions were performed in triplicate. PCR was conducted for 40 cycles of 94 °C for 10 s, 62 °C for 30 s, and 72 °C for 30 s using a thermocycler (iQ5, Bio-Rad). Primers specific to *PhMC1* and *PhCP10* [*PhMC1* RT, *PhCP10* RT (Table 1)] were designed using IDT SciTools (PrimerQuest; Integrated DNA Technologies, Coralville, IA). Amplification specificity was confirmed with melt curve analysis. Normalized relative quantitation was calculated using arithmetic mean of relative quantity for each target gene with *PhACTIN* (CV299322) and *PhRPS13* (SGN-U209515) as reference genes (Hellemans et al., 2007). These genes have been previously determined to serve as reference genes in petunia flower tissue (Chapin and Jones, 2009; Mallona et al., 2010).

PhMC1 RNAi TRANSGENIC PETUNIAS. *PhMC1* was down-regulated using RNAi in transgenic petunias. For RNAi constructs, PCR products from the *PhMC1* coding region [a 450-bp fragment from 563 to 1013 bp; primers F-*PhMC1*RNAi and R-*PhMC1*RNAi (Table 1)] were ligated in the inverted orientation flanking the GUS stuffer region in the pGSA1276 binary vector containing a kanamycin-resistance selectable marker. PCR fragments of *PhMC1* were ligated at the *AscI* and *SwaI* sites in the sense orientation and at the *BamHI* and *SpeI* sites in the antisense orientation. Leaf pieces (1 $\times$ 1 cm) of *P. hybrida* MD were transformed with pGSA1276::*PhMC1* using *Agrobacterium tumefaciens* LBA4404 following the method of Jorgensen et al. (1996). A leaf was collected from each resulting plantlet for PCR verification of successful transformation. The primers for PCR verification [F-*PhMC1*RNAi2 and R-*PhMC1*RNAi2 (Table 1)] contained an intron to distinguish between the endogenous gene and the transgene. Plants positive for the transgene and with decreased expression of *PhMC1* as compared with wild type MD were selected to carry on to the next generation. Plants were self-pollinated and seeds were collected to generate *T*₁ and then *T*₂ plants. *T*₂ plants from three independent transformation events (line F05-53, F05-6, and F05-65) were used in all experiments. Three flowers per plant, from five plants per line, were emasculated and tagged as previously described ($n = 15$). Flower longevity was measured as the number of days from anthesis until unpollinated flowers were visibly wilted. Flowers for longevity evaluations and RNA extraction were collected in April, and the greenhouse temperatures during this collection averaged 22.6 $\pm$ 2.0/18.6 $\pm$ 2.7 °C (day/night). The expression of *PhMC1* was compared between transgenic and wild type corollas at 48 h after pollination. Flowers were pollinated on the day of flower opening with pollen from another flower on the same plant. RNA extraction and gene expression analyses were

conducted as described in previous sections. Greenhouse experiments used a completely randomized design and plants were grown under the conditions described previously. All experiments with transgenic plants were repeated at least twice.

STATISTICAL ANALYSIS. Enzyme activity, flower longevity, and gene expression were analyzed in SAS (version 9.3; SAS Institute, Cary, NC) using the Proc GLIMMIX (generalized linear mixed model) algorithm with least squared means separation evaluated at $P \leq 0.05$.

Results

PhMC1 IS A TYPE I METACASPASE. *PhMC1* (GenBank accession no. JQ314004) included an open reading frame of 1044 bp and encoded a protein with 347 aa and a predicted MW of 38.2 kDa. Alignment of the predicted aa sequence of PhMC1 with other metacaspases (Supplemental Fig. 1) indicated that the highly conserved signature histidine/cysteine dyad of caspases was present in the petunia clone. PhMC1 contained a 90 amino acid N-terminal prodomain, which is characteristic of the type I metacaspases (Supplemental Fig. 1). This prodomain contained one plant-specific *LSD1*-like zinc finger motif defined by the sequence CxxCRxxLMYxxGASxVxCxxC. The prodomains of the arabidopsis metacaspases were proline (AtMC1 and AtMC2) or glutamine (AtMC3) rich, whereas the PhMC1 prodomain did not contain either a proline or a glutamine rich region. Among the tomato and arabidopsis metacaspases, PhMC1 shares the highest amino acid identity with SIMC6 (77.98%) and AtMC1 (52.6%), respectively (Table 2). These are both type I metacaspases.

RECOMBINANT PhMC1 HAS METACASPASE-LIKE ACTIVITY. Purified recombinant metacaspases were incubated with a metacaspase substrate, the synthetic, fluorogenic oligopeptide P1 arginine GRR, or a caspase substrate Ac-DEVD-AMC (DEVD). The enzyme activity was determined by measuring the amount of fluorescence generated by the release of AMC, and relative

Table 2. Comparison of the percent amino acid identity between arabidopsis and tomato metacaspases and petunia metacaspase1 (PhMC1).

Metacaspase	Identity (%) ²
SIMC6	77.98
SIMC5	73.73
AtMC1	52.60
SIMC1	50.64
SIMC2	48.87
AtMC3	47.29
SIMC3	45.66
AtMC2	43.07
SIMC4	36.81
AtMC5	33.90
AtMC4	33.47
AtMC6	32.77
SIMC7	32.77
AtMC7	30.77
AtMC9	29.87
SIMC8	29.60
AtMC8	29.36

²Percent identity was obtained from a pairwise comparison of amino acid sequences using Clustal Omega (Sievers et al., 2011).

Fig. 1. Characterization of substrate specificity and pH optima for *Petunia x hybrida* metacaspase1 (PhMC1). Relative enzymatic activity in each sample is presented as the percentage of the activity compared with the sample with the highest activity. All data are the means of three replicates $\pm$ SE ($n = 3$). The experiment was performed twice with similar results. Different letters represent statistically significant difference between the means ($P \leq 0.05$). (A) The activity of the recombinant PhMC1 against the fluorogenic metacaspase substrate Boc-GRR-AMC (GRR) and the fluorogenic caspase substrate Ac-DEVD-AMC (DEVD) was determined at various pH. (B) The effect of cysteine protease inhibitor (iodoacetamide) and arginal protease inhibitor (leupeptin) on the activity of recombinant PhMC1 using GRR as substrate was evaluated at pH 8.0. All GRRase activity of the recombinant metacaspases in the presence of inhibitors is expressed as a percentage of the relative activity without inhibitors. (C) A recombinant metacaspase mutant, PhMC1^{C237A}, was created with the catalytic cysteine converted to alanine. The activity of this mutant was determined using the substrate GRR at pH 8.0.

Fig. 2. Gene expression was analyzed in petals of unpollinated *Petunia ×hybrida* ‘Mitchell Diploid’ flowers. (A) Photos show flowers from anthesis (0 d) through senescence and corolla wilting (7 d). Numbers indicate the days after anthesis. Transcript levels of (B) *PhMC1* and (C) *PhCP10* (mean $\pm$ SD, $n = 3$) in petals during flower development. Relative mRNA abundance compared with two reference genes (*PhACTIN* and *PhRPS13*) was determined by reverse transcription quantitative polymerase chain reaction. Different letters represent statistically significant difference between the means ($P \leq 0.05$).

enzymatic activities were calculated as the percentage of activity compared with the sample with the highest metacaspase activity. *E. coli* BL21 (DE3) containing only the control vector pET28a(+) was used to determine background activity with both substrates. PhMC1 cleaved the GRR substrate

containing an arginine residue at the P1 position (Fig. 1A). There was barely detectable activity against the caspase-specific substrate (DEVD) containing an aspartic acid residue at the P1 position. The petunia metacaspase had optimal GRRase activity at pH 7 to 9. Iodoacetamide (10 mM),

a cysteine protease inhibitor, and leupeptin (10 μM), an arginal protease inhibitor, resulted in near complete inhibition (about 95% and 90%, respectively) of all GRRase activity (Fig. 1B). Recombinant mutants that had the putative catalytic cysteine replaced with an alanine (rPhMC1^{C237A}) were used to determine if the conserved cysteine was required for GRRase activity. The GRRase activity of PhMC1 was nearly abolished when the catalytic site cysteine was mutated (Fig. 1C).

PhMC1 GENE EXPRESSION IS UPREGULATED DURING PETAL SENESCENCE AND BY ETHYLENE. Quantitative PCR data indicated that *PhMC1* gene expression was upregulated in flower petals (Figs. 2 and 3). Expression of *PhMC1* was low in unpollinated petals from 0 to 5 d after anthesis and increased during senescence (6 and 7 d after anthesis) when the petals were visibly wilted (Fig. 2A and B). *PhCP10*, a homolog of arabidopsis *SAG12*, which is often used as a senescence marker (Jones et al., 2005; Noh and Amasino, 1999), was also upregulated at 6 and 7 d (Fig. 2C). In petunia flowers, pollination accelerates petal senescence. *PhMC1* was also upregulated in petals from pollinated flowers at 48 h when the corollas were visibly wilted (Fig. 3A and B). Increases in *PhCP10* transcript abundance were detected at 24 h after pollination when corollas were not yet visibly wilted and continued to increase at 48 h (Fig. 3C). *PhMC1* transcript abundance also increased in the petals when detached flowers were treated with ethylene (Fig. 4A). There was no effect of treatment with 0.1 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 4 h, but transcript abundance increased 4.5-fold when flowers were treated with 1.0 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene. Gene expression continued to increase slightly (though not significantly) with the 10 $\mu\text{L}\cdot\text{L}^{-1}$ treatment. Cycloheximide treatment did not prevent or reduce the increases in *PhMC1* transcript abundance induced by ethylene treatment. Transcript abundance of *PhCP10* in petals also increased 27.7-fold with 1.0 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene treatment compared with untreated control flowers, but this induction was less with 10 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene treatment (Fig. 4B). In contrast to *PhMC1*, cycloheximide treatment significantly inhibited the ethylene-induced increase in expression of *PhCP10*.

Fig. 3. Gene expression was analyzed in petals of pollinated *Petunia x hybrida* 'Mitchell Diploid' flowers. (A) Photos show flowers at 0, 12, 24, and 48 h after pollination. Transcript levels of (B) *PhMC1* and (C) *PhCP10* (mean $\pm$ SD, $n = 3$) in petals from pollinated flowers. Relative mRNA abundance compared with two reference genes (*PhACTIN* and *PhRPS13*) was determined by reverse transcription quantitative polymerase chain reaction. Different letters represent statistically significant difference between the means ($P \leq 0.05$).

METACAPASE-LIKE ACTIVITY INCREASES IN SENESCING PETALS. The GRR substrate was used to measure metacaspase-like activity in petal protein extracts from newly open flowers

Fig. 4. Gene expression was analyzed in *Petunia x hybrida* 'Mitchell Diploid' petals from flowers treated with ethylene following pretreatment with cycloheximide or water. Detached flowers were treated with 0, 0.1, 1.0, or 10.0 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 4 h after pretreating with either 0 (water) or 50 μM cycloheximide for 3 h ($n = 6$). Transcript levels of (A) *PhMC1* and (B) *PhCP10* (mean $\pm$ SD, $n = 3$) in petals. Relative mRNA abundance compared with two reference genes (*PhACTIN* and *PhRPS13*) was determined by reverse transcription quantitative polymerase chain reaction. Different letters represent statistically significant difference between the means ($P \leq 0.05$).

(anthesis), fully open flowers (nonsenescing), and senescing flowers (Fig. 5). GRRase activity increased 2.5-fold in the nonsenescing petals and 7-fold in the senescing petals when compared with the low level of activity detected in petals on the day of flower opening.

PhMC1 RNAi TRANSGENIC PETUNIAS HAVE ACCELERATED PETAL SENESCENCE. To investigate the functional role of *PhMC1* in petal senescence, gene expression was downregulated in transgenic petunias using RNAi. Under optimal greenhouse growing conditions, *PhMC1* RNAi transgenic plants had no obvious vegetative phenotypes (data not shown). Plants from three independent lines of the T_2 generation were analyzed for flower longevity phenotypes (Fig. 6). Flower longevity was evaluated in naturally senescing (i.e., unpollinated) corollas, and the time from anthesis to corolla wilting was reduced by ≈ 2 d in *PhMC1* RNAi transgenics (Fig. 6A). Average corolla longevity for MD flowers in these experiments was 9.7 d, whereas the longevity of F05-53, F05-6, and F05-65 corollas

Fig. 5. Metacaspase-like activity was quantified in *Petunia x hybrida* 'Mitchell Diploid' petals. Activity against the fluorogenic metacaspase substrate Boc-GRR-AMC was analyzed in total petal protein extracts from three flower developmental stages (anthesis, nonsenescing, and senescing). Fluorescence units are reported and bars represent the mean $\pm$ SD ($n = 3$). Different letters represent statistically significant difference between the means ($P \leq 0.05$).

was 7.9, 7.8, and 6.9 d, respectively. In senescing petals (at 48 h after pollination), *PhMC1* transcript levels were reduced by 30% to 96% in the transgenic lines compared with wild type MD petals (Fig. 6B).

Discussion

Metacaspases are key regulators of cell death in plants, and as such, they play an integral role during development and in response to biotic and abiotic stresses (Coll et al., 2010; He et al., 2008; Hoeberichts et al., 2003; Tsiatsiani et al., 2011; Vercammen et al., 2007; Watanabe and Lam, 2011). Sequence and structural similarities support the conclusion that *PhMC1* encodes a type I metacaspase (Supplemental Fig. 1; Table 2). RT-qPCR showed that *PhMC1* transcript abundance increased during petal senescence in both pollinated and unpollinated flowers (Figs. 2 and 3). The expression pattern was similar to that of the senescence-related marker *PhCP10* (Jones et al., 2005). We confirmed that the *PhMC1* recombinant protein had activity against the metacaspase substrate GRR, which was inhibited by the arginal protease inhibitor, leupeptin (Fig. 1). Leupeptin has been shown to be a potent inhibitor of the arabidopsis metacaspases (Vercammen et al., 2004; Watanabe and Lam, 2005). Like *PhMC1*, most metacaspases that have been characterized have a neutral to slightly basic pH optimum (Bozhkov et al., 2005; He et al., 2008; Vercammen et al., 2004). Flower senescence in petunia was also accompanied by an increase in metacaspase-like activity (GRRase activity) in the corollas (Fig. 5).

The activity of some plant metacaspases is regulated post-translationally, but the transcriptional regulation of gene expression also modulates metacaspase activity during plant development (Belenghi et al., 2007; Lam and Zhang, 2012; Wen et al., 2013; Zhang and Lam, 2011). In *Picea abies* (norway spruce), mclI-Pa is an essential component of the cell death machinery in the developing embryo (Bozhkov et al., 2005; Suarez et al., 2004). MclI-Pa is expressed in the embryo suspensor cells during early embryogenesis and in the

Fig. 6. Corolla senescence was evaluated in *Petunia x hybrida* *PhMC1* RNAi transgenic lines. The time to flower senescence and *PhMC1* gene expression were evaluated in T₂ plants from three independent transgenic lines (F05-53, F05-6, and F05-65) compared with ‘Mitchell Diploid’ (MD, wild type). (A) Flower longevity was determined as the time from flower opening (anthesis) until corolla wilting in unpollinated, naturally senescing flowers ($n = 15$). (B) Relative gene expression of *PhMC1* in corollas at 48 h after pollination ($n = 3$) was determined by reverse transcription quantitative polymerase chain reaction. Transcript levels are expressed relative to *PhACTIN* and *PhRPS13*. Bars represent mean $\pm$ SD, and different letters represent statistically significant difference between the means ($P \leq 0.05$).

procambial strands during xylem differentiation (Böhlhoner et al., 2013; Suarez et al., 2004). It is involved in the execution of nuclear disassembly in cells destined for elimination during embryo development (Bozhkov et al., 2005). In grape, four of the six metacaspases show tissue-specific expression (Zhang et al., 2013). *VvMC6* is expressed in floral tissue with very low levels detected in other organs. Zhang and colleagues (2013) conclude that this type II metacaspase (*VvMC6*) might have a role in flower formation and ovule development in grape. The expression of *VvMC1*, *VvMC3*, and *VvMC4* increases during endosperm abortion in seedless grapes, and these metacaspases may be involved in PCD and disassembly of aborted ovules during stenospermocarp (Zhang et al., 2013). The programmed senescence of petals allows the plant to remobilize nutrients from dying to developing tissues such as the seeds (Bai et al., 2010; Chapin and Jones, 2007, 2009; Jones et al., 2009). Gene expression patterns suggest that *PhMC1* may have

a role in the programmed degradation of the corolla during flower senescence.

Like the senescence-associated cysteine protease *PhCP10*, *PhMC1* expression was induced in petals by exogenous ethylene treatment (Fig. 4). Ethylene and other plant hormone signaling leads to the rapid protein synthesis-independent induction of primary response genes, also called immediate-early genes, and the later induction of protein synthesis-dependent secondary response genes. Many primary response genes are induced within minutes of a stimulus, but some have delayed kinetics, and they are referred to more specifically as delayed primary response genes. A great majority of the early induced primary response genes encode transcription factors, whereas delayed primary response genes usually encode other proteins in the genome (Tullai et al., 2007). The loss of fresh weight and petal inrolling that accompanies flower senescence in *Dianthus caryophyllus* (carnation) is prevented by pretreatment with the protein synthesis inhibitor cycloheximide (Wulster et al., 1982). Similarly, cycloheximide prevents petal abscission in pollinated *Pelargonium xhortorum* (geranium) flowers (Hilioti et al., 2000). These studies show that the normal progression of ethylene-induced petal senescence (both wilting and abscission) requires protein synthesis.

In arabidopsis, the induction of *SAG12* by ethylene is age-dependent (Grbic and Bleecker, 1995). *AtSAG12* transcription is regulated directly by developmentally controlled senescence pathways, and the response to ethylene is secondary (Noh and Amasino, 1999). The upregulation of *PhCP10* was prevented by pretreating the flowers with cycloheximide (Fig. 4B). Similar to the regulation of *AtSAG12*, the induction of *PhCP10* is a secondary response to the ethylene stimulus. By contrast, we found that *PhMC1* expression was a primary response to ethylene, which did not require de novo protein synthesis, and was therefore not inhibited by cycloheximide (Fig. 4A). Because *PhMC1* does not encode a transcriptional regulator, it may be functioning as a late primary response gene rather than an early primary response gene. This would be more consistent with its upregulation during the later stages of petal senescence and a role in mediating senescence timing.

Metacaspase genes from multiple plants have been shown to be differentially regulated by plant hormones including abscisic acid, salicylic acid, jasmonic acid, and ethylene (Huang et al., 2015; Liu et al., 2016a, 2016b). The amino acid sequence of *PhMC1* is closest to that of tomato metacaspases *SIMC6* (78% identical) and *SIMC5* (74% identical). *SIMC5* and *SIMC6* transcripts increase during leaf senescence, and these genes are also upregulated by ethylene treatment (Liu et al., 2016b). Two of eight metacaspases characterized from rice show increased expression after treatment with 1-aminocyclopropane-1-carboxylic acid (ACC, the precursor to ethylene) (Huang et al., 2015). Although many articles report expression of metacaspase gene family members in flowers, none have specifically investigated expression in senescing petals or ethylene treated flowers (Kwon and Huang, 2013; Liu et al., 2016b; Zhang et al., 2013).

A recent study by Coll et al. (2014) suggests a model by which *AtMC1* and autophagy pathways are functioning in parallel to positively regulate HR-induced cell death in young tissues and negatively regulate senescence in older tissues. Similar to the autophagy deficient mutants, they report that *atmc1* mutants have premature senescence and are hypersensitive to ROS (Coll et al., 2014). The prosurvival function of

AtMC1 may be due to its role in protein aggregate clearance during senescence. The *atmc1* plants overaccumulate insoluble protein aggregates as they age, which may lead, in part, to their premature senescence. *PhMC1*-deficient transgenic petunias also had an early flower senescence phenotype, evidenced by premature corolla wilting. *PhMC1* may similarly function as a negative regulator of senescence in petunias. After pollination or age-related cues, the expression of *PhMC1* may be needed to coordinate the timely senescence of petals and allow for complete cellular dismantling and remobilization of components from the petals before the final collapse or abscission of the corolla (Chapin and Jones, 2007). A recent search of the *Petunia inflata* and *Petunia axillaris* genome databases at SGN has identified four putative metacaspases (Fernandez-Pozo et al., 2015). This research lays the foundation for further work characterizing additional metacaspase family members in petunia.

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AtMC1 MYPPPPSSIIYAPPMLVNC^ΔSGCRTPLQLPSGARSIR^ΔCALCQAVTHIADPRTA-----
AtMC2 -----MLLLVD^ΔCSSCRTPLHLPPGATRIRCAICHAF^ΔTLIAPEPR^ΔLQSHASASPF
PhMC1 -----MDGRRYK^ΔCKWCGTKMAAPIGAPSI^ΔCCPRCQSVTELQPRNNNGYAKMAAPI
AtMC3 -----MASRREVRCRCGRRMWVQPDARTVQC^ΔSTCH^ΔTVTQLYSLVDIA-----
AtMC9 -----
AtMC8 -----
AtMC4 -----
AtMC7 -----
AtMC5 -----
AtMC6 -----
Δ Δ Δ Δ

AtMC1 -----PPQPS-----SAPSPPQIHAPP
AtMC2 PFPNSSPAP--STFIYPPPTSPSYTHAPHAPSFPFNHAPPDSYPFTHAPPASSFPFNHAPP
PhMC1 ANVVNNMINS^ΔAVNTGFYPAM----QGRIP-----NGN----NYQQ-----QP---QPF
AtMC3 -RGAN-----RIIHGFQQLL----RQHQP-----QHH----EQQQQQMMAQPPP---RLL
AtMC9 -----
AtMC8 -----
AtMC4 -----
AtMC7 -----
AtMC5 -----
AtMC6 -----

AtMC1 GQLPHPHGRKRAVICGISYRFSRHELKGCINDAKCMRHLLINKFKFSPDSILMLTEEETD
AtMC2 GPPPPVHGQKRAVIVGVSYKNTKDELKGCINDANCMKFMMLKRFQFPESCILMLTEEAD
PhMC1 MRPPAVHGRKRAVLCGITR^ΔGH^ΔPKMLKGSVTDALSMRQLLVGKLGFPNASVVVLTEDEKD
AtMC3 EPLPSFPGKKRAVLCGVNYK^ΔGKSYSLKGCISDAKSMRSLVQQM^ΔGFID^ΔSILMLTEDEAS
AtMC9 MDQQGMVKRLAVLVGCNYPNTRNELHGCINDVLAMKETILSRFGFKQDDIEVL^ΔTDEPE-
AtMC8 -----MAKKALLIGINYPGTAVELRGCVNDVHRMQKCLIELYGFANKDIVIMIDTDK-
AtMC4 -----MAKRALLIGINYPGTTELQGC^ΔVNDVHRMHKCLVDRFGFAEEDITVLIDTDE-
AtMC7 -----MTKKAVLIGINYPGTKAEELRGCVNDVRRMYKCLVERYGFSEENITVLIDTDE-
AtMC5 -----MAKKALLIGINYVGTKAELRGCVNDVRRMRISLVERYGFSEENIKMLIDTDS-
AtMC6 -----MAKKAVLIGINYPGTKAEELRGCVNDVRRVHKS^ΔLVDRFGFSE^ΔRNITELIDTDE-
: * : * . * * : ■ : * : : : * : : :

AtMC1 PYRIPTKQNMALYWL^ΔVQGC--TAGDSL^ΔVFHYSGHSGSRQ^ΔRNYNG--DEV^ΔDGYDETLCPL
AtMC2 PMRWPTKNNITMAMH^ΔWLVLSC--KPGDSL^ΔVFHFSGHGNNQMD^ΔDNG--DEV^ΔDGFDETL^ΔLPV
PhMC1 PCKIPTKANIR^ΔSALRWLVQGC--QPGDSL^ΔVFFYSGHGTRV^ΔDRDRG--DEV^ΔDGHDES^ΔLCPV
AtMC3 PQRIPTKRNIRKAMRWLVEGN--RARD^ΔSLVFHFSGHGSQ^ΔQNDYNG--DEIDGQDEALCPL
AtMC9 SKVKPTGANIKAALRRMVDKAQAGSGDILFFHYSGHGTRIPSVKS--AHPFKQDEAIVPC
AtMC8 SCIQPTGKNICDEL^ΔDNLIASG--QSGD^ΔFLVFHYSGHGTRIPPGIEDSE^ΔPTGFDECITPC
AtMC4 SYTQPTGKNIRQALSEL^ΔKIPA--KSGD^ΔVL^ΔFVHYSGHGTRVPPETG--EEDDTGFDECIVPS
AtMC7 SSTQPTGKNIRRALDLVESA--DSGD^ΔVLV^ΔVHYSGHGTRLPAETG--EDDDTGFDECIVPC
AtMC5 SSIKPTGKNIRQALLDLVEPA--KSGD^ΔVL^ΔFVHYSGHGTRLPAETG--EDDDTGYDECIVPS
AtMC6 SSTKPTGKNIRRALNLVESA--KPGD^ΔVLV^ΔVHYSGHGTRLPAETG--EDDDTGYDECIVPC
* * * : : : * * . . . * ♦ * . . * * : *

AtMC1 DFETQGMIVDDEINATIVRPLPHGVKLH^ΔSIIDACHSGTVLDLPFLCR-----MNRAGQ
AtMC2 DHRTSGVIVDDEINATIVRPLPYGVKLHAI^ΔVDACHSGTVMDLPYL^ΔCR-----MDRLGN
PhMC1 DYSEGRILDDEINATIVRPLPRGATLHGII^ΔDTCFSGTFLDLPFMC^ΔR-----INREGY
AtMC3 DHETEGKIIDDEINRILVRPLVHGAKLHAVIDACNSGT^ΔVL^ΔDL^ΔPFICR-----MERNGS
AtMC9 DFN----LITD^ΔVDFREL^ΔVNL^ΔPKGTSFTMISDSCHSGGLIDKEKEQIGPSSVSSNIS^ΔP--
AtMC8 DMN----LIKDQ^ΔQFREMVS^ΔR^ΔVKEGCQLTII^ΔSDSCHSGGLIQEVKEQIGESHMKPVDK^ΔVE
AtMC4 DLN----PIPD^ΔDDFRDLVEQVPEGCQITIVSDSCHSGGLIDEAKEQIGESTTTKPNRESK
AtMC7 DMN----LITD^ΔDDFRDLVDKVP^ΔPGCRMTII^ΔSDSCHSGGLIDEAKEQIGESTKKEAEDEDE
AtMC5 DMN----LITD^ΔDDFRDLVDMVPKDCPITII^ΔSDSCHSGGLIDEAKEQIGESTKKKKK^Δ----
AtMC6 DMN----LITD^ΔD^ΔFRDLVEKVPKEAHITII^ΔSDSCHSGGLIDEAKEQIGESTKKKPKKE--
* . : * : * : : : ♦ * * . . :

Supplemental Fig. 1. Multiple alignment of predicated metacaspase amino acid sequences from *Petunia ×hybrida* PhMC1 (GenBank JQ314004) and *Arabidopsis thaliana* (Arabidopsis AtMC1–9; AtMC1, AY322525; AtMC2, AY322526; AtMC3, AY322527; AtMC 4, AY322528; AtMC5, AY322532; AtMC6, AY322533; AtMC7, AY322529; AtMC8, AY322530; AtMC9, AY322531. Cysteine residues in the zinc finger motif of the prodomain of type I metacaspases are indicated in yellow and by an open triangle (Δ). The putative histidine and cysteine catalytic residues are designated in dark blue and by a closed diamond (♦). The second cysteine involved in activity and autolysis is green and indicated by a closed square (■). The lysine or arginine involved in the predicted autocatalytic cleavage site is pink and indicated by a closed circle (●). Sequence alignment was performed using Clustal Omega (1.2.4) multiple sequence alignment software (Larkin et al., 2007). Identical amino acids are indicated with as asterisk. Double dots indicate conserved substitutions and single dots indicate semi-conserved substitutions.

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AtMC1 -----YVW-----
AtMC2 -----YEW-----
PhMC1 -----FMW-----
AtMC3 -----YEW-----
AtMC9 -----AIETTNKT
AtMC8 QIEESHMKQPKLGIASYFLNIVMNLATCGVSKSQR--DRGGGEESFRGEIELEKDETLD
AtMC4 V-----SSFEFEFKNCLHSIFVKLLAFCGIGSSH-----VETREIVEVGGGDEV
AtMC7 S-----EESSSRFGFRKFLRSKVEGAIESRGFHIGGNKKDEDVAEEIETKEIELEDGETIH
AtMC5 -----SGDSSTIN-----KETEAEIIE
AtMC6 -----SGGSSGLGIKGFVREAVEEALESKGIAIPHHKDEK---DENKTKELEKLEDGAKVH

AtMC1 -----EDHR-----
AtMC2 -----EDHR-----
PhMC1 -----EDHR-----
AtMC3 -----EDHR-----
AtMC9 ITSRLPFFKAVLDHLSSLTGITT---SDIGTHLLELFGRDAGLKFRLPAMDLMDLLET--
AtMC8 IKTRYLPFESYLSLLKEQTGQTNIEPVIRQTLKLFGEDESPNRQRLSDLG-----N
AtMC4 VRSRYLPLERFIELLKQQTQDNIEIGKIRPTLFDVFGEDSSPKIKKFMKVILTCLRKTN
AtMC7 AKDKSLPLQTLIDILKQQTGNDNIEVGKIRPSLFDAGDDSSPKVKKFMKVILGKLQAGN
AtMC5 VGNRSLPLETLIDMLKQETGNDNIEVGKIRTTTDFDMFGDDSSPKVKKFMNVILSNLQETT
AtMC6 VVNKSLPLQTLIDILKQNTGNDNIEVGKIRPTLFNVFGEDASPKVKKFMKVILTCLQEGK
      •                               *

AtMC1 -----PRSGLWKG---TAG---
AtMC2 -----PKTGMWKG---TSG---
PhMC1 -----I-SSYKG---TNG---
AtMC3 -----SV-RAYKG---TDG---
AtMC9 -----MTAREKH
AtMC8 -----CEVDAGDSGASRLNAV
AtMC4 D-----QSTLLGKIEESARGYIETLND-EHYMKPAMQAHVKS DreIYGG--RSSNG-LF
AtMC7 G-----EEGGLMGLMGLKGLASGFLEGLKND-EDYVKPAMQTHVGSKEEVYAG--GSRGSVPL
AtMC5 TTIQTVSDEVLSGVENLAQEFLEQLSDDVK---P-----AIQDVYAG--AINGA--L
AtMC6 T-----EGGILGMIGKLAQEFLLKHLNDD EYVKPAMKTHVGNKQEVYAG--ASNGS--L

AtMC1 -GEAISISGDDDDQTSADTSALSKITSTGAMTFCFIQAIERSAQGTTYGSLLSNMRTTIR
AtMC2 -GEVFSFTGDDDDQTSADTPQLSGSAWTGAMTYAFIQAIERG-HGPTYGSLLSNAMRSTVH
PhMC1 -GTAISISACDDHQNSGDTTFTGTG-PTGALTYSFIKLEQ-EPKLSYGRLLMSMHNKIH
AtMC3 -GAAFCSACDDDESSGYTPVFTGK-NTGAMTYSFIKAVKTAGPAPTYGHLNLNMCASIR
AtMC9 VDSGILMSGCQADETSADVGVG-----NGKAYGAFSNAIQRVLN
AtMC8 TDNGILLSGCQTDQRSEDVYVT-----RTGKAYGAFSDAIQMILS
AtMC4 PDRGILLSGCQTDQTSADVKKK-----G-----EAFGAFSNAIQMVLS
AtMC7 PDSGILISGCQTDQTSADATPA-----GKPT EAYGAMSNSIQTILE
AtMC5 PDNGILISGCQTDQTSADAPP-----GHP ELAGALTNAIQIIG
AtMC6 ADNGILISGCQTDQTSADASPQ-----GHP EAYGAFTNAVQIILE
      . : : : * : : * . : : * : : :

AtMC1 NTGNDGGG-----SGGVVTTVLSMLLTGGS AI-----GGLRQEPQLTACQT
AtMC2 EIFDKNG--RELVEVGADFLSTLLGLLILGASPPDEEEVNQAPQKTQEPQLSANE
PhMC1 EAQKGVL-----TGAK-----TQVRL-----
AtMC3 EAQSR LAF-----NGDYT-----SSDASAEPLLTSS EE
AtMC9 EN---EGAMKNQLVM-----MARDVLERLGFHQHPCLYCSDQ
AtMC8 APRKDKKKITNKELVS-----EARVFLKKRGYSQRPGLYCHDR
AtMC4 ET-DHKDKITNKEMVL-----RAREILKKQMF IQRPGLYCNDR
AtMC7 ET-D--GEISNREMT-----RARKALKKGFTQQPGLYCHDG
AtMC5 ET-K--GKISNKDLVL-----KARKLLRKQGFDRPGLYCND
AtMC6 ET-K--GMITYKELVL-----KARKLLKKQGFSGRPGLYCSDS

AtMC1 FDVYAKPFTL--
AtMC2 FAVYEKPFSL--
PhMC1 -----
AtMC3 FDVYATKFVL--
AtMC9 NA--DATFLSQP
AtMC8 FV--DKPFICY-
AtMC4 FV--NAPFIC--
AtMC7 YA--NAPFIC--
AtMC5 YV--NARFIC--
AtMC6 FV--NAPFIC--

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Supplemental Fig. 1. Continued.