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Metacaspase-8 Modulates Programmed Cell Death Induced by Ultraviolet Light and H_2O_2 in *Arabidopsis*[*]

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Programmed cell death (PCD) is a genetically controlled cell death that is regulated during development and activated in response to environmental stresses or pathogen infection. The degree of conservation of PCD across kingdoms and phylum is not yet clear; however, whereas caspases are proteases that act as key components of animal apoptosis, plants have no orthologous caspase sequences in their genomes. The discovery of plant and fungi metacaspases as proteases most closely related to animal caspases led to the hypothesis that metacaspases are the functional homologues of animal caspases in these organisms. *Arabidopsis thaliana* has nine metacaspase genes, and so far it is unknown which members of the family if any are involved in the regulation of PCD. We show here that metacaspase-8 (*AtMC8*) is a member of the gene family strongly up-regulated by oxidative stresses caused by UVC, H_2O_2, or methyl viologen. This up-regulation was dependent of *RCD1*, a mediator of the oxidative stress response. Recombinant metacaspase-8 cleaved after arginine, had a pH optimum of 8, and complemented the H_2O_2 no-death phenotype of a yeast metacaspase knock-out. Overexpressing *AtMC8* up-regulated PCD induced by UVC or H_2O_2, and knocking out *AtMC8* reduced cell death triggered by UVC and H_2O_2 in protoplasts. Knock-out seeds and seedlings had an increased tolerance to the herbicide methyl viologen. We suggest that metacaspase-8 is part of an evolutionary conserved PCD pathway activated by oxidative stress.

In some instances, programmed cell death (PCD)[4] in plants is comparable with animal apoptosis at the cellular level. However, sequencing the *Arabidopsis* genome revealed that very few of the animal PCD regulators are conserved in plants. This suggests a greater divergence of the PCD pathways across kingdoms than thought. Initial reports seemed to provide indirect evidence supporting the existence of caspase orthologues in plants, with several caspase-like activities detected in plant extracts and inhibitor studies that show them to be required for PCD (for review, see Ref. 1). Although several research groups reported the absence of orthologous caspase sequences in plant genomes, a more in depth analysis revealed a greater diversity of caspase-related proteases than previously suspected (2). In particular, two families of predicted proteases were identified that are more closely related to animal caspases than to other proteases: the paracaspases and metacaspases. Paracaspases and caspases appear animal specific, whereas metacaspases are present in other eukaryotes, including plants. Plant metacaspases are subdivided in type I and type II on the basis of their structure; type I have an N-terminal prodomain that is not present in type II. A role for metacaspases in plant PCD was proposed (3) for four reasons; 1) a common origin with caspases, 2) the absence of closer caspase homologues in plants, 3) the proliferation of the genes coding for metacaspases in plant genomes mirrors the pattern of the proliferation and specialization of caspases in animal genomes, and 4) the fusion of type I plant metacaspases with a zinc-finger domain also present in LSD1, a regulator of the hypersensitive response in plants (4). Early observations suggested that metacaspases could be responsible for caspase-like activities detected in plants (5, 6); however, three reports have now demonstrated that metacaspases are unable to cleave caspase substrates. So far, all four recombinant *Arabidopsis* metacaspases tested have arginine as the preferred P1 amino acid in the cleavage site instead of caspase aspartic acid (7). A recombinant metacaspase from pine showed the same preferred cleavage site with Arg in P1 (8). These activities are not inhibited by caspase inhibitors.

Although metacaspases do not have caspase-like activities, there is strong support for a role in PCD. The yeast metacaspase (YCA1) knock-out survives in the presence of H_2O_2 (5), and the down-regulation of a metacaspase suppresses PCD in the suspensor cells of *Picea abies* embryogenic culture (9). Another report using *Arabidopsis* detected small but statistically significant changes in metacaspase KOs; KOs of *Arabidopsis* type II metacaspases had a reduced cell death, whereas KOs of type I metacaspases had an increased cell death when challenged by botrytis (10). A study of botrytis infection in tomato further supported the idea that type II metacaspases may mediate cell

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[4] The abbreviations used are: PCD, programmed cell death; KO, knock out; WS, *Wassilewskija*; Ler, *Landsberg erecta*; MS, Murashige and Skoog; MV, methyl viologen; ORF, open reading frame; RT, reverse transcription; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Q-PCR, quantitative-PCR; AMC, aminomethylcoumarin; MES, 4-morpholineethanesulfonic acid.

death in response to necrotic pathogens, as pathogen exposure led to type II metacaspase up-regulation (11). In contrast, there is no support for a metacaspase role in PCD using a range of stresses in *Trypanosoma brucei* (12) where metacaspases may function in cell proliferation and mitochondria biogenesis (13). A study using metacaspase KO strains in *Aspergillus fumigatus* found metacaspases required for phosphatidylserine exposure, a cellular marker of PCD but not for other markers such as activation of caspase-like activities and loss of viability (14). Finally, it has been reported that *Arabidopsis* plants overproducing selected members of the metacaspase gene family had no obvious cell death-related phenotype (15). Taken together, evidence is conflicting but certainly suggestive that some metacaspases may play a role in PCD, and some may not.

Previously alluded to, the proliferation of metacaspase genes in plants may reflect some form of specialization. *Arabidopsis thaliana* has nine metacaspase genes, and it can be expected that some members of the family are involved in the regulation of PCD, and some may be involved in other processes. As an illustration of that, two type-I metacaspases (At5g64240 and At1g02170) are up-regulated in *Arabidopsis* plants 24 h post-infiltration with bacterial pathogens, whereas type II metacaspases are not significantly affected (7). In addition, metacaspases can have different pH optima, suggesting various subcellular localizations (16), and display different substrate affinities (7). It is, therefore, important to analyze the function of each member of this gene family in *Arabidopsis*. In addition, different PCD pathways activated in plants may be either metacaspase-dependent or metacaspase-independent. This hypothesis is exemplified in yeast, where the single yeast metacaspase (YCA1) mediates PCD induced by H_2O_2 or acetic acid (5), by viruses (17), and by hyperosmotic stress (18), but in contrast, it is not required for PCD during mating (19), triggered by ammonia (20), or induced by Bax expression (21). We show here that *Arabidopsis* metacaspase-8, a type II metacaspase, is up-regulated by the oxidative stress caused by UVC and leading to PCD, and this up-regulation is RCD1-dependent. We have genetic evidence that metacaspase 8 is part of a PCD pathway induced by UVC, H_2O_2, or methyl viologen. This report contributes to our understanding of the organization of the metacaspase family in *Arabidopsis* and the origin of eukaryotic PCD.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions—Three *A. thaliana* ecotypes, Columbia (Col-0), *Wassilewskija* (WS), and *Landsberg erecta* (Ler) were used. Transgenic overexpressing metacaspase-8 were constructed in a Col-0 background. The *AtMC8-1* (FLAG322_G10) line in a WS background was supplied by FLAGDB (FLAGDB, Institut National de la Recherche Agronomique, France), and *AtMC8-2* (JII Gene Trap line GT_3_12679) in a Ler background was purchased from NASC. Salk_047489 Salk_047493 and Salk_063109 were purchased from NASC. The *rcd1-1* mutant was provided by Dr. R. Ahlfors (University of Helsinki). Plants were grown at 22–23 °C in long day (16/8 h) or short day (8/16 h) photoperiod in Perceval growth cabinets. *In vitro* seedlings were grown in a Sanyo cabinet under continuous light at 22 °C.

Plasmids—An *AtMC8* ORF was obtained using RT-PCR and RNA from UV-treated seedlings using primers with Lic adaptors GAC GAC GAC AAG ATG GCG AAG AAA GCA CTT TTG and GAG GAG AAG CCC GGT CCG TAG CAT ATA AAT GGT TTA TCA AC. The full ORF was cloned into the pET-30 Ek/LIC vector (Novagen), which added a His tag at both termini and an S ·tag at the N terminus to give pLIC-AtMC8. A point-mutated form, pLIC -AtMC8^{C140A}, was generated using the QuikChange mutagenesis protocol (Stratagene). The *AtMC8* ORF was excised from pLIC-AtMC8 with the His and S tags using XbaI and SalI and ligated into pDH51 (22) using XboI and SalI to add a cauliflower mosaic virus 35 S promoter and terminator. The resulting plasmid was then restricted with *PVU II* to excise the *AtMC8* coding sequence flanked with the cauliflower mosaic virus 35 S promoter and terminator. This fragment was ligated into the T-DNA vector pZP111b (23) linearized using SmaI and dephosphorylated to give pZP111-AtMC8. pZP111-AtMC8^{C140A} was generated the same way using pLIC-AtMC8^{C140A} as a starting point. The wild type and the point-mutated AtMC8 ORF were cloned into the yeast vector pYES2 (Invitrogen) using XbaI and SacI to give pYES-AtMC8 and pYES-AtMC8^{C140A}.

Expression in Escherichia coli—pLIC-AtMC8 and pLIC-AtMC8^{C140A} were transformed into BL21(DE3) competent cells (Invitrogen). Kanamycin-resistant colonies were grown to an absorbance of 0.5 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside, 2%ethanol for 48 h at 22 °C. Proteins were extracted in ice-cold 500 mM NaCl, 50 mM K_2PO_4 pH7.4, 3 mM dithiothreitol, 10%(w/v) glycerol, 1%(w/v) CHAPS, 5 $\mu l \cdot ml^{-1}$ aprotinin with a ratio of 200 μl/5 absorbance units. Cells were broken using a sonicator (Vibra Cell™, JECONS Sonics & Materials Inc.) with five short bursts of 10 s followed by intervals of 30 s on ice. A supernatant was obtained by centrifugation at 4 °C for 30 min at 16,000 rpm using a refrigerated microcentrifuge (Eppendorf). For purification, cell lysis was performed using a French press at 900 p.s.i. Cell debris were pelleted by ultracentrifugation at 4 °C, 25,000 × *g* for 1 h. His-tag purification was obtained using Talon magnetic beads (Dynabeads, Qiagen).

Yeast Complementation—The yeast deletion strain *yca1*Δ (Y02453; yca1::kanMX4) in YOR197W and the wild type strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) were supplied by Euroscaf. pYES2 (Invitrogen) and pYES-AtMC8 were introduced in Y02453 and BY4741. The survival assay was carried out essentially as published (5). In short, yeast cultures were grown overnight with glucose or galactose, diluted to the same density, and then treated or not with 1.2 mM H_2O_2 (Sigma) for 24 h. Cells were counted using a Thoma double cell "Clearsight" chamber and 1000 cells plated on solid yeast extract/peptone/dextrose medium for each replicate. The survival rate was calculated using the number of colonies formed after 2 days at 30 °C.

Plant Transformation—pZP111-AtMC8 and pZP111-AtMC8^{C140A} were transformed into competent *Agrobacterium tumefaciens* GV3101 using electroporation. *Agrobacterium* strains were grown with appropriate antibiotics (20 $mg \cdot liter^{-1}$ gentamycin, 50 $mg \cdot liter^{-1}$ kanamycin, and 200 $mg \cdot liter^{-1}$ rifampicin) and used to transform *Arabidopsis Col0* by the flo-

ral dip method (24) in 10 mM $MgCl_2$, 300 μl·liter^{-1} Silwet L-77 with 30 s of vacuum infiltration. Transformed seeds were collected and selected on 50 mg.liter^{-1} kanamycin and 1% Bactoagar.

RT-PCR—Total RNA was extracted from 3-week-old seedlings grown *in vitro* using Invisorb Spin Plant RNA mini kit (Invitek, Gmbh) according to the manufacturer's instructions and then treated with RQ1 RNase-free DNase (Promega) for 60 min. The treated RNA was used to perform cDNA synthesis using Reverse-iT^{TM} First Strand Synthesis Kit (***AB***gene) following the protocol supplied with the kit. The resulting cDNA samples were used as templates for PCR reactions with Bioline Taq. To amplify the full-length ORF the following primers were used: MC8-ATG, 5′-TGG CGA AGA AAG CAC TTT TGA-3′; MC8-GGT, GGT CGT TGA CTA TAC CCC CGT. For expression studies the following primers were used: *AtMC1*-At1g02170, CTC CTC CGC AAC CTT CCT and CAT CAA CTT CAT CAC CGT TGT; *AtMC2*-At5g64240, TCT GGT CAT GGA TCT CAG CA and CTG TGT TCT TCC CCG TGA AC; *AtMC3*-At4g25110, TGG ACC ACA GGA CTT CAG GT and AAG CGC CTA AGA TGA GCA AA; *AtMC4*-At1g79310, TGA TTG ACA CCG ACG AAT CT and CTT CCG ATT CCA CAA AAA GC; *AtMC5*-At1g79340, GCT TGC TGA TCT CGT CGA A and GCT GCG CAA GAA CTT CCT AA; *AtMC6*-At1g79330, CGA AGG GCA TTG TTG AAT CT and GCT TCT TCC ACA GCT TCA CG; *AtMC7*-At1g79320, AGA TCT CGT TGA ACC GGC TA and TCC AAT GGC AGA GAT CTG TTT; *AtMC8*-At1g16420; GGA TAA TCT CAT CGC ATC AGG and GCT GCT TCA TAT GGC TTT CC; *AtMC9*-At5g04200, GTG TTC ACG GGG AAG AAC and GCG GAT GCA TCA GAG CTT; *AtDAD1*, GGA TGC TCA GGA TCT ATT TCG and GTG AAA CCT CTA TCC GAG GAA GTT G; *Actin2*, GTT AGC AAC TGG GAT GAT ATG G and CAG CAC CAA TCG TGA TGA CTT GCC C.

Q-PCR—cDNA was synthesized as described in the RT-PCR method and used as templates in Q-PCR reactions using Taqman Master mix (Eurogentec) according to the manufacturer's instruction and an ABI 7000. For *18S* detection, the primers 18 S-29F (5′-GGT CTG TGA TGC CCT TAG ATG TT-3′), 18 S-102R (5′-GGC AAG GTG TGA ACT CGT TGA-3′) and probe 18 S-58T (5′-CGC ACG CGC GCT ACA CTG ATG T-3′) were used. For *Actin2* detection, the primers ACT2-123F (5′-GAG AGA TTC AGA TGC CCA GAA GTC-3′), ACT2-190R (5′-TGG ATT CCA GCA GCT TCC A-3′), and probe ACT2-148T (5′-TCC CAC AAA CGA GGG CTG CAA CA-3′) were used. For *AtMC8* detection, the primers AtMC8-50F (5′-TGG AGT TAC GTG GCT GTG TCA-3′), AtMC8-124R (5′-TGT TGG CGA ACC CGT AAA G-3′), and probe AtMC8-79T (5′-CAC CGG ATG CAG AAA TGC CTA ATC GA-3′) were used. A standard curve using dilutions of *Arabidopsis* genomic DNA was used to calculate the absolute number of cDNA copies in each sample.

H_2O_2 Measurement—The Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen) was used to measure H_2O_2 production in 10^5 protoplasts. After a treatment with 50 kJ·m^{-2}, the samples were kept in the light for 30 min before harvesting, and the Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazine) was used according to the manufacturer's instruction.

Protease Assay—Standard 2× assay buffer was 20 mM HEPES, pH 7.4, 4 mM EDTA, 0.04% NaN_3, 50 mM $CaCl_2$, 6 mM dithiothreitol; the pH range was obtained using 100 mM sodium acetate for pH 4–5, 50 mM MES for pH 5.5–6.5, and 100 mM Tris-HCl for pH 7–9. Protein extracts were carried out in distilled water and 3 mM dithiothreitol. For plant extract the FW/buffer ratio was 1 g/2 ml. For yeast total protein was extracted from yeast cell pellets by adding glass beads and shaking with a Ribolyser. Protease activities were determined by incubation of 48 μl of plant extract (or 20 μl of yeast extract, 10 μl of *E. coli* extract, or 2 μl of purified *AtMC8* made up to 48 μl with sterile distilled water), 50 μl of 2× assay buffer at different pH, and 50 μM *t*-butoxycarbonyl-GRR-aminomethylcoumarin (AMC) and benzyloxycarbonyl-FR-AMC (substrate for papain; Bachem Ltd). Ac-DEVD-AMC (substrate for caspase-3, -6, -7, -8, and -10; MP Biomedicals), Ac-VEID-AMC (substrate for caspase-6), Ac-YVAD-AMC (substrate for caspase-1), Ac-IETD-AMC (substrate for caspase-6 and -8, Granzyme B), and benzyloxycarbonyl-AAN-AMC (substrate for legumain) (Bachem) were used at 50 μM final. The release of AMC was measured every 2 min at 30 °C with a Microtiter Plate Fluorometer (Labsystems, DYNEX Technologies) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Data time points were analyzed by the Fluoroskan Ascent software, and activities were expressed in fluorescence units/min/mg or μg of total protein. Protein concentration was measured using the Bradford reagent (Bio-Rad).

Immunodetection—Antibodies used comprised a rabbit polyclonal raised against purified recombinant AtMC8 (by Eurogentec). Acrylamide gels were cast and run using a Bio-Rad mini gel apparatus. Western analysis was carried out according to Sambrook *et al.* (25) using Hybond P (Amersham Biosciences). For signal detection ECL (Pierce) and Eastman Kodak Co. Biomax film were used.

Ion Leakage—Seedlings were grown in soil under short days for 4 weeks and watered 24 h before the experiment to avoid ion leakage variation linked to variation in water status. 1100 μl of sterile distilled water was added in each well of a 24-well culture plate (Techno Plastic Products). 3-mm diameter discs were punched out from leaves, and 3 discs were floated in each well. At 0.5 h, the conductivity of the water in each well was measured using 100 μl and an HORIBA Twin Cond conductivity meter B-173 (HORIBA Ltd, Kyoto, Japan). Stress treatment were carried by applying UVC radiation using a CL-1000 UV cross-linker (UVP Ltd) or the addition of H_2O_2 (Sigma) to the discs to reach the doses required, and the discs were left in continuous light at 22 °C for 16 h. The conductivity of the liquid in each well was measured again, and the difference before and after treatment was calculated to represent the amount of cell death of each sample. Three wells were used for each treatment.

Protoplasts and Evans Blue—Seedlings grown *in vitro* for 3–4 weeks were incubated with leaf digestion medium (4.41 g·liter^{-1} Murashige and Skoog (MS) medium including Gamborg B5 vitamins (Duchefa), 0.4 M sucrose, 0.96% cellulase R-10,

and 0.64% macerozyme (Yakult Pharmaceutical), pH 5.8) in the dark for 16 h at room temperature. Buoyant protoplasts were harvested by centrifugation for 5 min at 600 rpm and washed 2–3 times in 12 ml of W5 medium (154 mM NaCl, 5 mM KCl, 5 mM glucose, and 125 mM $CaCl_2$, pH 5.6–5.8). The pellet was then resuspended in protoplast culture medium (4.41 g·liter^{-1} MS medium including Gamborg B5 vitamins, 0.4 M sucrose, 0.4 M mannitol, and 0.5g·liter^{-1} MES, pH 5.8) at a density of 1×10^6 protoplasts per ml. For each replicate, 1 ml of protoplasts was used per well of a 6-well culture plate (Techno Plastic Products). Stress treatments were carried by applying UV radiation using a CL-1000 UV cross-linker (UVP Ltd) or exogenous application of H_2O_2 (Sigma) to wells in triplicate, and the protoplasts were left in continuous light for 4 h at 22 °C. The dye Evans blue (Sigma) was added to protoplast suspension to the final concentration of 0.05%. After 5 min of room temperature incubation, an aliquot of the stained protoplasts was mounted on a slide. Pictures of at least four randomly selected fields of view (150–250 cells/field) were taken within 10 min. The ratio of number of Evans blue-stained cells over total cell number was calculated. All the microscopic work was carried using a Leica microscope connected to a digital SPOT camera.

Germination on Methyl Viologen—Seeds were sterilized for 10 min in 70% ethanol and sown on MS media with Gamborg B5 vitamins (Duchefa), 0.5g/liter MES, pH 5.8, 0.7% phytagel (Sigma). Plates were supplemented with 0, 0.25, or 25 μM methyl viologen (Sigma). Seeds were incubated for 3 days at 8 °C and then germinated at 22 °C in continuous light. Germination was scored using a stereomicroscope. Digital pictures were taken with a Nikon D100. Each treatment was carried out in triplicate with at least 100 seeds.

RESULTS

AtMC8 Expression Is Induced by Oxidative Stress and Correlates with Cell Death—We have previously shown that a UVC treatment can induce PCD in *Arabidopsis* (26). Using RT-PCR and gene-specific primers, we analyzed the transcription during UVC-induced PCD of the nine metacaspases (*AtMC1–9*) that are present in the *Arabidopsis* genome. Three-week-old seedlings were treated with 50 kJ/m^2 and kept in the light. Total RNA was extracted 30 or 120 min after treatment. We found that the *AtMC-8* transcript (At1g16420) was the only metacaspase strongly up-regulated, with a peak 30 min after UVC treatment (Fig. 1*A*). We found no transcript for *AtMC6* and *AtMC7*. *AtMC1*, *-2*, *-3*, and *-5* were present and down-regulated by the UV treatment. *AtMC-4* was expressed at a low level and slightly up-regulated with higher expression at 2 h. *AtMC9* was slightly up-regulated. The expression of *AtMC8* was then analyzed using Taqman Q-PCR. Q-PCR results (Fig. 1*B*) showed that a lethal UV dose of 50 kJ/m^2 is required to induce the expression of *AtMC8* 80-fold using *18 S* as a reference gene. Using *ACTIN2* as a reference gene instead of *18S* resulted in a calculated induction of 120-fold (data not shown) because UVC down-regulates *ACTIN2*. A sublethal dose of 1kJ/m^2 was ineffective, and a 10 kJ/m^2 resulted in a 10-fold induction, indicating that expression of the *AtMC8* transcript is proportional to the level of stress. To investigate whether *AtMC8* induction could be linked either to the induction of PCD or linked to the general stress caused by the UV treatment, we analyzed its expression level under heat and light stresses thought to be associated with a UV treatment (Fig. 1*B*). Light stress (1000 μmol/m^2/s), and heat shock (42 °C, 2 h) did not induce the expression of *AtMC8*. Finally, we have shown that UV-induced PCD is light-dependent (27), and our experiments showed that light was required after UVC treatment to induce *AtMC8* expression, suggesting a correlation between the expression of *AtMC8* and the induction of PCD. A light requirement suggested a link with oxidative stress. H_2O_2 production was measured using 10^5 protoplasts treated with 50 kJ·m^{-2} UVC and was found to be 8.5 μM, 30 min after treatment. Untreated protoplasts only generated 0.4 μM H_2O_2. H_2O_2 was found to induce *AtMC-8* expression 23-fold, 5 h after seedlings were transferred on MS media supplemented with 30 mM (Fig. 1*C*). To further investigate a link with oxidative stress and cell death, we analyzed the effect of methyl viologen, an herbicide that induces an apoptotic-like PCD (28) and causes the production of superoxide in the chloroplast, which is converted into H_2O_2. Methyl viologen (MV) was found to induce *AtMC8* expression 64-fold, 5 h after seedlings were transferred on MS media supplemented with 25 μM (Fig. 1*C*). This concentration caused bleaching and

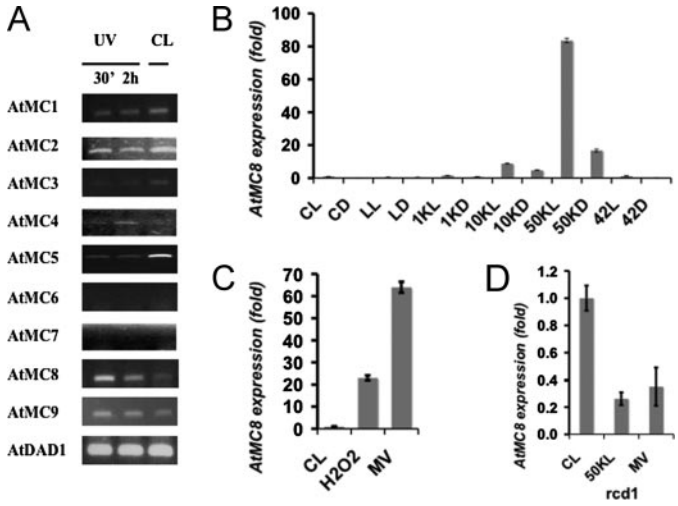

FIGURE 1. **AtMC8 expression is up-regulated by oxidative stress.** *A*, 3-week-old *A. thaliana* Col-0 seedlings were grown *in vitro*, treated with UV-C (50 kJ/m^2), and harvested after 30 min or 2 h in the light; control plants (*CL*) were not treated with UV-C. cDNAs were synthesized from DNase-treated RNA samples, and RT-PCR was performed with gene-specific primers for each member of the metacaspase genes family in *A. thaliana* (*AtMC-1–9*) and *AtDAD1* as a reference gene. *B*, total RNA was extracted from Col-0 seedlings grown *in vitro* for 3 weeks. cDNAs were synthesized from DNase-treated RNA samples and analyzed using Taqman Q-PCR. Transcript levels are given as *AtMC8/18S* copy number ratio using the untreated sample + light (*CL*) as a value of 1. Various treatments were: untreated and kept in light (*CL*) or in the dark (*CD*); 1-h light stress and kept in the light (*LL*) or in the dark for 1 h (*LD*); seedlings irradiated with 1 kJ·m^{-2}, 10 kJ·m^{-2}, or 50 kJ·m^{-2} UV-C and kept in the light (*1KL*, *10KL*, and *50KL*) or kept in the dark for 1 h (*1KD*, *10KD*, and *50KD*); seedlings heat-shocked at 42 °C 2 h and kept in the light (*42L*) or in the dark for 1 h (*42D*). *C*, 3-week-old Col-0 seedlings grown *in vitro* on solid MS medium were transferred on MS medium supplemented with 30 mM H_2O_2 or 25 μM MV for 5 h. cDNAs were synthesized from DNase-treated RNA samples and analyzed using Taqman Q-PCR. *Error bars* indicate ±S.D. of three replicates. *D*, 3-week-old seedlings of the mutant *rcd1-1* grown *in vitro* on solid MS medium were untreated (*CL*), treated with 50 kJ/m^2 UVC (*50KL*), and left in the light for 1 h or transferred on MS medium supplemented with 25 μM MV for 5 h. cDNAs were synthesized from DNase-treated RNA samples and analyzed using Taqman Q-PCR. *Error bars* indicate ±S.D. of three replicates.

cell death of seedlings (not shown). To establish whether the oxidative stress inducing *AtMC8* expression originated from the chloroplast, we analyzed the transcript level of *AtMC8* in the mutant *rcd1-1*. *RCD1* is proposed to regulate specific stress-responsive genes, and *rcd1* is insensitive to ROS produced by the chloroplast (29). A 50-kJ UVC treatment was found to cause an actual 5-fold decrease of the expression of *AtMC8* in the *rcd1-1* background rather than an increase (Fig. 1*D*), similar to the effect of UVC on *ACTIN2* and members of metacaspase gene family. There was a similar reduction of expression when transferring *rcd1-1* seedlings to solid media supplemented with 25 μM MV. This suggested that in the absence of an *RCD1*-mediated induction by oxidative stress, *AtMC8* expression is subject to a general down-regulation of expression by stress. This general down-regulation is seen with some members of the metacaspase gene family (Fig. 1*A*), and our microarray data indicate that most protease transcripts are down-regulated after UV treatment (Affymetrix microarray data[5]). Induction of expression by H_2O_2 and MV added to a requirement for a functional *RCD1* suggested that an oxidative stress generated at the chloroplast level induces *AtMC8* expression. In all, these results show a correlation between *AtMC8* expression, oxidative stress, and cell death. Metacaspase-8 was, therefore, selected for further studies bearing in mind that a correlation at the expression level is not proof of a definitive link between metacaspase-8 and PCD.

Recombinant AtMC8 Cleaves Substrates with R in p1 and Is More Active at pH 7.5–8.5—An *AtMC8* cDNA was obtained using RT-PCR and RNA from UV-treated seedlings. AtMC8, a type II metacaspase, is a cysteine protease with a predicted size of 42.5 kDa. Previous studies have shown there is a clear conservation of the cysteine-histidine dyad in the catalytic center for cysteine proteases (2). To establish whether activity was required for the function of *AtMC8* in cell death, the cysteine residue in position 140 was mutated to create a nonfunctional allele. AtMC8 and AtMC8^{C140A} were cloned in the expression vector PET-30, which enables detection and purification of the recombinant protein using a His tag or an S tag. Western analysis confirmed that the protein was expressed upon induction using isopropyl 1-thio-β-D-galactopyranoside (Fig. 2*A*). Immunodetection using an antibody against AtMC8 confirmed the production of a 50-kDa protein corresponding to the full-length-tagged metacaspase. The soluble protein fraction and the His-purified enzyme presented evidence of activation by cleavage, generating a p20-like subunit at 29 kDa as described for other metacaspases (7, 8, 16). Enzymatic assays on *E. coli* extracts and purified recombinant protein confirmed that AtMC8 has no activity against the caspase substrates DEVD, YVAD, IETD, or VEID (Fig. 2*B*). Of the substrates tested, AtMC8 was able to cleave the papain substrates FR and GRR (Fig. 2*B*). This activity was abolished with the mutation of the predicted catalytic cysteine in the recombinant AtMC8^{C140A}. The optimum pH was determined to be between 7.5 and 8.5 with no activity below pH 6 (Fig. 2*C*). *AtMC8* expressed in yeast using the yeast expression vector pYES2 showed the same substrate specificity as the recombinant AtMC8 produced in *E. coli* (data not shown).

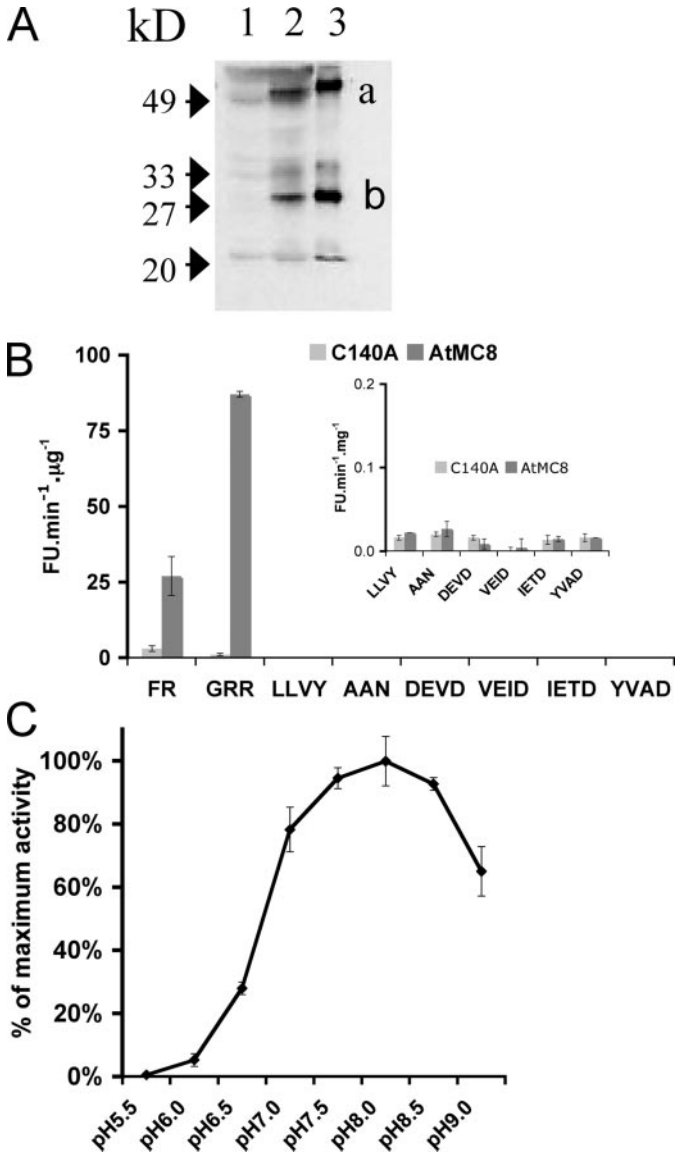

FIGURE 2. **Recombinant AtMC8 shows GRR and FR cleavage activity.** *A*, expression of AtMC8 in *E. coli*. A lysate from non-induced *E. coli* was prepared as a control (*lane 1*). A crude cell lysate was prepared from the induced culture (*lane 2*) and the C- and N-His tag were used to purify AtMC8 using Talon™ beads (*lane 3*). Samples were immunoblotted and analyzed using a polyclonal antibody raised against *AtMC8*. Full-length tagged AtMC8 has an approximate molecular mass of 50 kDa (*arrow a*). The 29-kDa band (*arrow b*) is thought to be the p20-like subunit resulting from activation by autoprocessing. *B*, recombinant AtMC8 and the null allele AtMC8^{C140A} were incubated at pH 8 at 30 °C with 10 μM benzyloxycarbonyl-FR-AMC (*FR*) or *t*-butoxycarbonyl-GRR-AMC (*GRR*), 50 μM caspase substrates Ac-DEVD-AMC (*DEVD*), Ac-YVAD-AMC (*YVAD*), Ac-VEID-AMC (*VEID*), and Ac-IETD-AMC (*IETD*), legumain substrate benzyloxycarbonyl-AAN-AMC (*AAN*), calpain substrate Suc-LLVY-AMC (*LLVY*). Note the change of scale in the *inset*. Protease activity is given as fluorescence unit/min/μg or mg of total protein. *Error bars* represent the S.D. of three replicates. *FU*, fluorescence units. *C*, proteolytic activity of AtMC8 against 50 μM *t*-butoxycarbonyl-GRR-AMC at different pH values. Relative activity is expressed as the percentage of the optimal activity. The *error bar* is the S.D. of triplicates.

AtMC8 Complements a Yeast Metacaspase (YCA1) KO—A complementation experiment was conducted to examine the effect of *AtMC8* in yeast PCD. The *AtMC8* cDNA was cloned into the yeast expression vector pYES2. The construct and the

[5] V. Delorme and P. Gallois, unpublished data.

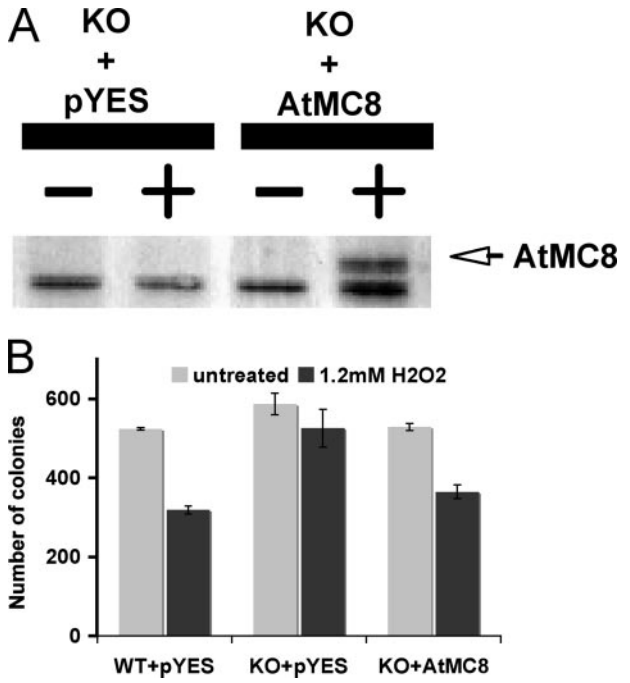

FIGURE 3. **Expression of AtMC8 complements the reduced cell death phenotype of yeast metacaspase knock-out cells.** *A*, detection of AtMC8 in yeast protein extracts. AtMC8 expression was induced in yeast *yca1* knock-out Y02453 (*KO*) by shifting from glucose (−) to galactose (+). pYES is the empty expression vector. Protease extracts were immunoblotted using an antibody against AtMC8. *KO+pYES* is Y02453 + empty pYES2; *KO+AtMC8* is Y02453+ pYES2-*AtMC8*. *B*, complementation. After 24 h of growth in SCD medium, yeast cultures were transferred to a galactose-containing medium for an overnight induction period and then treated with 1.2 mM H_2O_2 for 24 h. The survival was expressed as the number of colonies grown of 1000 cells plated on YPD plates. *WT+pYES* is wild type + empty pYES2; *KO+pYES* is Y02453 + empty pYES2; *KO+AtMC8* is Y02453 + pYES2-*AtMC8*. *Error bars* indicate ±S.D. of triplicates.

empty vector were transformed into two different genetic backgrounds, either wild type or metacaspase (YCA1) KO. Galactose was used to induce the expression of the recombinant proteins, and 1.2 mM H_2O_2 was applied to the yeast cultures for 24 h. Expression of AtMC8 in the knock-out background was confirmed by Western analysis (Fig. 3*A*). The *yca1* KO expressing the empty vector survived the H_2O_2 treatment, and by contrast, both the wild type and the *yca1* knock-out expressing *AtMC8* were shown to have a reduction of survival by 39 and 31%, respectively (Fig. 3*B*). Therefore, AtMC8 is able to complement the cell death function of the yeast metacaspase YCA1, and this prompted us to investigate if AtMC8 was involved in the regulation of PCD induced by H_2O_2 in *Arabidopsis*.

AtMC8 Over-expressers Are Hypersensitive to PCD Induction by H_2O_2 or UVC—The effect of *AtMC8* overexpression on PCD in *Arabidopsis* was studied by generating lines of Col-0 overexpressing AtMC8 or nonfunctional $AtMC8^{C140A}$ under the control of a 35 S promoter. Three lines overexpressing AtMC8 and two lines overexpressing $AtMC8^{C140A}$ were selected for expression levels at least 100-fold above wild type using *AtMC8* primers and Q-PCR (Fig. 4*A*). Homozygous lines were segregated. Increased mRNA level resulted in an increase FR activity measured after UV treatment in the plants overexpressing *AtMC8* (Fig. 4*B*). FR was selected over GRR because it gave a clearer increase of activity in over-expressers possibly because of a reduction in background activity. As expected, the increased activity was absent from the two lines expressing high levels of $AtMC8^{C140A}$ transcript. The increased FR activity measured correlated with the relative expression level in each line. The already high FR activity in wild type is presumably the combined activities of metacaspases and of papains. There was no increase of caspase-like activities (data not shown). There was no obvious morphological phenotype during the development of the over-expresser lines. These homozygous lines were subjected to two death inducers, namely UV and H_2O_2. UV was shown to induce PCD by us (26, 27). H_2O_2 has been shown to induce PCD in *Arabidopsis* protoplasts (30), and the metacaspase YCA1 in yeast is required for H_2O_2-induced PCD (5). The effect of *AtMC8* overexpression on PCD in *Arabidopsis* was quantified using ion leakage from leaf discs. Ion leakage is indicative of a loss of membrane permeability and has been used in hypersensitive response studies in *Arabidopsis*, where it correlates with cell death, *e.g.* see Ref. 31. In addition, the experimental setup used here has shown that *Arabidopsis* transgenic lines overexpressing the PCD suppressor Bax-inhibitor-1 have a reduced ion leakage during PCD induced by Bax expression (32), demonstrating that ion leakage correlates with PCD. Leaf discs from same age leaves were taken from plants grown in soil in short days and floated adaxial side up on sterile distilled water. The doses to be used were first determined to be sublethal or close to sublethal on wild type. Fig. 4*C* shows that when leaf discs were treated with 20 mM H_2O_2 for 16 h, there was up to a 2-fold increase of ion leakage in plants overexpressing *AtMC8* compared with wild type. This was observed in three over-expresser lines. The C140A mutation abolished the sensitivity of the leaf discs to H_2O_2 stress, and $AtMC8^{C140A}$ over-expressers showed an ion leakage similar to the wild type plant. A UV dose of 5 kJ/m^2 increased ion leakage 3- 4-fold in over-expressers. Again, the C140A mutation abolished the ion leakage increase (Fig. 4*D*). The small increased ion leakage in line C140A8 seen in Fig. 4*C* was not consistent across experiments and was, therefore, considered as non-significant.

To confirm those results with a different experimental system, protoplasts were prepared from homozygous lines overexpressing *AtMC8* or $AtMC8^{C140A}$. PCD was then induced by applying H_2O_2. Measurements using Evans blue were conducted after 4 h incubation under continuous light. A dose of 100 μM H_2O_2 did not induce cell death in wild type Col-0; by contrast, in lines overexpressing *AtMC8*, there was a 2-fold increase in cell death, going from 8 to 15%. Overexpressing the inactive metacaspase, $AtMC8^{C140A}$, did not increase protoplast cell death when treated with 100 μM H_2O_2 (Fig. 4*E*). The effect of overexpression was also studied using UV, and protoplasts were prepared from transgenic plants. A dose of 5 kJ/m^2 was used in this experiment, causing no cell death increase in protoplasts prepared from wild type Col-0 seedlings. Similar to PCD induction by H_2O_2, cell death increased 2-fold with protoplasts from over-expressers, and protoplasts overexpressing $AtMC8^{C140A}$ showed a cell death close to that of wild type protoplasts (Fig. 4*F*). Therefore, overexpressing *AtMC8* increased the sensitivity of *Arabidopsis* to the two PCD inducers used in these experiments. Overall, the increased ion leakage or cell death correlated with the increase at the enzymatic level, albeit

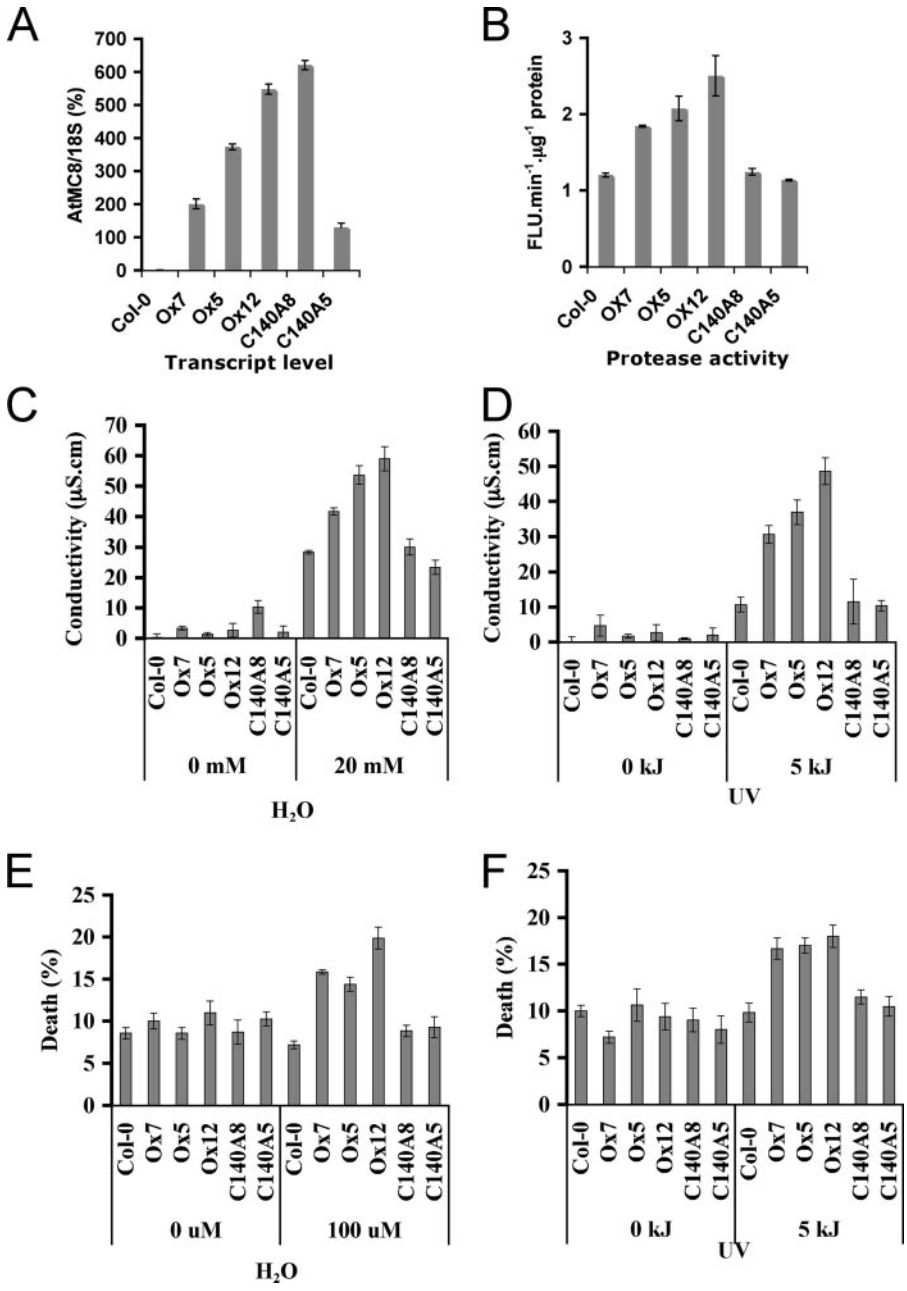

FIGURE 4. **Increased ion leakage and protoplasts cell death in lines overexpressing AtMC8.** Three lines overexpressing *AtMC8* (*Ox5*, *Ox7*, *Ox12*) and two lines overexpressing $AtMC8^{C140A}$ (*C140A5*, *C140A8*) were selected. Col-0 is the genetic background of the over-expressers. *A*, level of *AtMC8* transcript in over-expressers. Total RNA was extracted from leaves. cDNA synthesized from DNase-treated RNA was used to carry out Taqman Q-PCR analysis using 18 S transcript as the internal reference. Expression of *AtMC8* in Col-0 is around 1% of 18 S. *Error bars* indicate ±S.D. of the mean of three replicates. *B*, Phe-Arg-ase activity in over-expresser plants. Seedlings grown *in vitro* for 3 weeks were treated with 50 kJ·m^{-2} UVC and incubated in the light for 1 h, and total soluble proteins were extracted. Protein extracts were incubated in the presence of 10 µM Ac-FR-AMC substrate at 30 °C for 30 min. The activity is expressed as fluorescence units/min/µg of protein; *error bars* indicate ±S.D. of the mean of three replicates. *C* and *D*, 3-mm-diameter leaf discs were punched from 4-week-old plants floated on sterile distilled water. Water conductivity was measured before treatment and 16 h after treatment with 20 mM H_2O_2 (*C*) or 5 kJ·m^{-2} UV (*D*). *E* and *F*, protoplasts isolated from 3–4-week-old seedlings grown *in vitro* were treated with the sublethal doses of 100 µM H_2O_2 (*E*) or 5 kJ·m^{-2} UV (*F*). Protoplasts death was scored using Evans blue uptake. For each replicate at least 200 protoplasts were examined. *Error bars* represent ±S.D. of three replicates.

an attenuation of this correlation in protoplasts. In experiments with increasing H_2O_2 or UV doses, the increased death in the Ox lines became less obvious as the difference with wild type is reduced (data not shown). Therefore, overexpressing *AtMC8* caused an increased sensitivity at the low dose end of the spectrum.

AtMC8 Knockouts Have a Reduced Cell Death Induced by H_2O_2 or UVC—We analyzed five putative KO lines in the *AtMC8* gene and identified two true and independent knockouts. Of the five insertion lines, the lines Salk_047489 and Salk_047493 are downstream of the stop codon, and Salk_063109 is upstream of the ATG. These lines when homozygous did not show a reduced transcript level, indicating that the insertion sites were too remote to affect transcription strongly. The flag line FLAG322_G10 has an insertion in the second exon and was denominated *AtMC8-1*. The insertion can be predicted to cause the expression of a truncated protease that lacks its C terminus (Fig. 5*A*). The transcript sequence downstream of the insertion was not expressed in homozygous lines (RT-PCR data not shown). The JII Gene trap line GT_3_12679 has an insertion in the single intron of the gene. Q-PCR experiments showed that homozygous lines had no detectable transcript, demonstrating that the T-DNA was not excised with the intron, even at a low level (data not shown). This line is, therefore, a true KO line and is denominated *AtMC8-2.* In this line, expression, if any, would also result in a truncated protease that lacks the C-terminal half of the catalytic site (Fig. 5*A*).

There was no obvious morphological phenotype during development of the KO lines compared with their wild type. The wild type and KO lines were then exposed to UV or H_2O_2 in leaf discs or protoplasts. The two different wild type ecotypes, WS and Ler, were shown to have different thresholds of cell death induction, Ler being less sensitive than WS, and doses applied were adjusted accordingly. Experiments using leaf discs and H_2O_2 indicated that knocking out *AtMC8* had little effect on cell death measured using ion leakage as a marker. Only the mutation in the Ler background showed a significant reduction of ion leakage when challenged with UV (Fig. 5, *B* and *C*). By con-

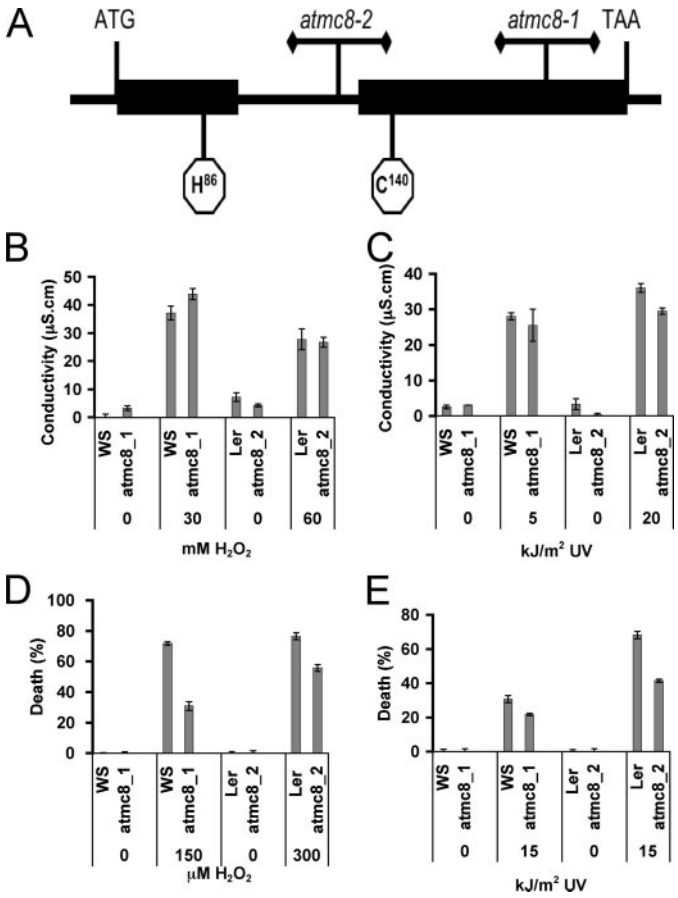

FIGURE 5. **Ion leakage and protoplasts cell death in two *AtMC8* KO lines.** *A*, localization of the selected T-DNA insertions in the *AtMC8* gene. *Closed boxes* represent exons. The positions of the ATG, stop codon, and the two *AtMC8-1* and *AtMC8-2* T-DNA insertions are indicated. The conserved histidine 86 and cysteine 140 of the catalytic site are *highlighted*. *B* and *C*, 3-mm-diameter leaf discs were punched off our week-old plants and floated on sterile distilled water. Water conductivity was measured before treatment and 16 h after H_2O_2 (*B*) or UV (*C*) treatment. *D* and *E*, protoplasts isolated from 3-weeks-old *in vitro* grown seedlings were treated with H_2O_2 (*D*) or UV (*E*). *AtMC8-1* and *AtMC8-2* are KO lines in *AtMC8*; *WS*, genetic background of *AtMC8-1*; Ler, genetic background of *AtMC8-2*. Protoplasts cell death was scored using Evans blue uptake. To make ecotypes comparable, the untreated values have been subtracted from the treated values. For each replicate at least 200 protoplasts were examined. *Error bars* represent ±S.D. of three replicates.

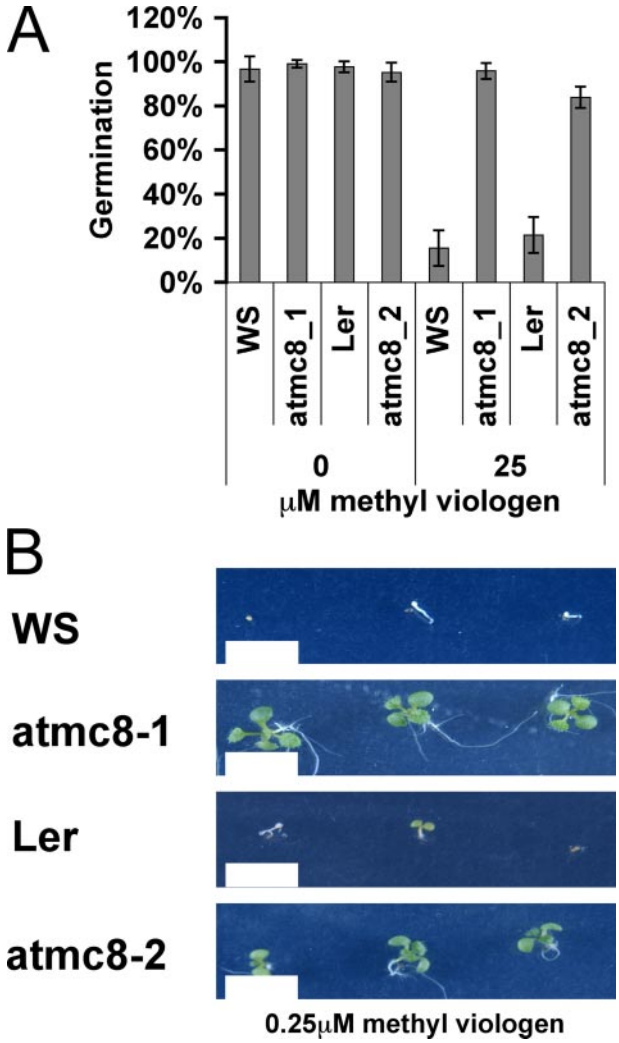

FIGURE 6. **AtMC8 KO lines have reduced sensitivity to methyl viologen.** Sterile seeds were plated *in vitro* on solid MS media supplemented with 0, 0.25, or 25 μM methyl viologen and placed to germinate in continuous light, 22 °C. *A*, germination was scored at 6 days; *error bars* represent S.D. of triplicates. *B*, seedlings of wild types and knock-out lines, photographed at day 9, representative of the 100 seeds plated for each genotype. *White scales* represent 5 mm.

trast, protoplast experiments were very clear. In both KO lines and using either H_2O_2 or UV, knocking the gene in a Ler or WS background resulted in a reduction of cell death by 30–40% (Fig. 5, *D* and *E*).

Seeds and Seedlings of AtMC8 Knockouts Have a Reduced Sensitivity to Methyl Viologen—The reduction of cell death in KO lines as described above places metacaspase-8 in a PCD pathway activated by an oxidative stress caused by UV or H_2O_2. The herbicide MV is a chemical that causes oxidative stress by generating $O_2^{\cdot-}$ and H_2O_2 in chloroplasts and induces *AtMC8* expression. MV has been shown to induce PCD with the formation of a DNA ladder (28). Ion leakage experiments with a range of MV concentrations from 0.1 to 100 μM gave results similar to those obtained with H_2O_2 and did not reveal any phenotypes in the KO lines (data not shown). Cell death assays using protoplasts and MV were unsuccessful because in our hand MV precipitates when added to protoplasts culture medium. For a further test, seeds of wild type and KO lines were plated on MS medium supplemented with various concentrations of MV. Germination on 25 μM was scored after 6 days (Fig. 6*A*). As expected, the two wild type ecotypes seeds germinated below 20% in the presence of the herbicide. By contrast, the two KO lines were able to germinate at 96% for *AtMC8-1* and 89% for *AtMC8-2.* The germinated KO seeds grew a radicle of a few millimeters but did not develop further. When seeds were plated on the lower dose 0.25 μM, the wild type seeds of WS and Ler germinated at 36 and 39%, respectively, whereas the KO seeds of *AtMC8-1* and *AtMC8-2* germinated at 96%. The more striking difference between wild type and KO lines was that 90% of germinated knock-out seeds gave a seedling that developed true leaves at 12 days, whereas 94% of *WS* seedlings and 60% of Ler seedlings did not develop true leaves and bleached partially or totally (Fig. 6*B*).

DISCUSSION

Our study provides strong genetic evidence that *AtMC8* is a member of the *Arabidopsis* metacaspase family that is involved

in the regulation of PCD induced by oxidative stress. So far no other study has demonstrated a major role for an *Arabidopsis* metacaspase in PCD. Our data support the finding during development that metacaspases have a functional role in plant PCD (9). TPMCII-Pa, a metacaspase from pine, was reported involved in the cell death of suspensor cells, and TPMCII-Pa is most likely to be orthologous to AtMC5 (At1g79340), which at present has neither been proved nor disproved to play a role in PCD. AtMC8 (or AtMCP2e; At1g16420) has not been previously studied, possibly because there was no previous evidence of expression (7, 16). Although there was so far no obvious cell death-related phenotype in *Arabidopsis* plants overproducing some of the members of the metacaspase family (15), our findings suggest at least one does. This exemplifies the notion that each member of the *Arabidopsis* metacaspase family should have their biological function determined. It is likely that metacaspases will be shown to carry out various functions in *Arabidopsis*, some linked to cell death and some linked to other processes. This divergence of function would be similar for example to the divergence noticed in the caspase family where distinct members regulate apoptosis or aspects of inflammation (33). In addition, we can expect to find out that some forms of PCD are metacaspase-dependent and some others metacaspase-independent. Interestingly analysis of public microarray data using for example the BAR website (University of Toronto) indicates that *AtMC8* is induced by infiltration of leaves with pathogen elicitors. This possibly illustrates that UVC and pathogen response share common response genes (34).

There is a striking expression pattern of *AtMC8* during PCD induced by UV, which our results show correlated with a PCD phenotype. This correlation is reinforced by the fact that H_2O_2 and MV induce cell death and *AtMC8* expression This gene is the only one in the metacaspase family that is induced strongly by our UV treatment; the transcript is present at very low levels in seedlings, and it is induced as soon as 30 min after a UV treatment leading to PCD. This induction of expression precedes an increase in caspase-3 like activity peaking at 1 h (our data). *AtMC8* induction is dependent of a functional RCD1, and this dependence places *AtMC8* in an oxidative stress pathway activated by ROS produced in chloroplast, possibly after damage by UV radiation. This is consistent with methyl viologen, generating $O_2^{\cdot-}$ and H_2O_2 in chloroplasts that in turn induce *AtMC8* expression. Genetically, *AtMC8* could be downstream of RCD1 as RCD1 has been shown to interact with transcription factors (35) that may in turn interact with the *AtMC8* promoter. Another possibility is that the *rcd1-2* mutant has been reported to up-regulate reactive oxygen species scavenging enzymes (36), and this may explain indirectly why *AtMC8* is not induced in the *rcd1-1* background by UVC or MV. Interestingly, *rcd1-1*, although ozone-sensitive, is resistant to MV (29), reinforcing the idea that RCD1 and AtMC8 are in the same pathway. Crucially, *AtMC8* induction is dependent of the seedlings being kept in the light after the UV treatment, as is cell death. This expression pattern suggests that *AtMC8* is involved in the process of a light-dependent PCD induced here by UV. Light dependence has been shown in several examples of cell death such as developmental cell death in *Monstera* (37), fumonisin (38), singlet oxygen cell death (39), and hypersensitive response (40). Little is yet known as to how light dependence is integrated in plant PCD molecular cascades, but we suggest here that *AtMC8* is part of it.

The over-expressers showed increased cell death both in ion leakage and protoplasts experiments. This increase requires a functional catalytic site. The attenuated correlation between the increased enzymatic activity in Ox plants and protoplasts cell death suggests that additional limiting factors control cell death in protoplasts in addition to metacaspase-8 activity. Compared with the very clear and consistent phenotype obtained using over-expressers, the two independent KO data are more complex and appear to vary with the ecotype used. Germination test on MV using the KO lines showed a clear effect of the mutations with a lower sensitivity to death-inducing doses in both genetic backgrounds. Similarly, the reduction of cell death is obvious using protoplasts again in both genetic backgrounds, albeit some variation; however, the induced cell death is not totally abolished. The simplest explanation is that AtMC8 is probably not the only *Arabidopsis* metacaspase to be involved in the PCD process induced by UV or H_2O_2. Our RT-PCR experiments indicated that after UV treatments, up to seven metacaspases are expressed at various levels with *AtMC2* and *-9* being the most abundant at the transcript level. We are aware that these other members of the metacaspase family that remain constitutively expressed may also contribute to the oxidative stress PCD pathway via activation at the protein level. Unexpectedly, when using leaf discs and ion leakage, only UV and the Ler background show a small significant effect. The absence or small phenotype in leaf discs suggests that removing the cell wall in protoplasts may remove components that compensate for the loss of AtMC8 in the PCD process in leaf discs. Alternatively, metacaspase-8 may be required for loss of viability measured by Evans blue but not required for ion leakage. As a cell death marker, ion leakage may be upstream of Evans blue. This situation is reminiscent of experiments in *A. fumigatus* where metacaspases are required for phosphatidylserine exposure and not for loss of viability (14).

Although our data suggest a link between PCD and *AtMC8*, overexpressing *AtMC8* in *Arabidopsis* is not lethal but makes plants more sensitive to UV or H_2O_2. The viability of over-expresser plants points at a control mechanism that maintains the metacaspase-8 pathway inactive in the absence of a cell death signal. Possibly, there are essential components of the PCD machinery that are downstream of AtMC8 and absent in non-induced plants. Alternatively, *AtMC8* is subject to two levels of regulation, the first at the level of transcription and the second at the level of the proteolytic activity. Members of the serpin family are good candidates for regulating enzymatic activity since a serpin has been shown to inhibit AtMC9 (15). In addition, *S*-nitrosylation has been put forward as a possible mechanism for metacaspase activity inhibition *in planta* (41). This suggests that a critical level of activated metacaspase needs to be reached to trigger PCD; with overexpression, this level is reached at lower doses of the inducer. This control of enzymatic activity would possibly be lost during protein extraction. Finally, it cannot be excluded that metacaspase-8 may be involved in the signaling toward PCD rather than in the execution of it.

We show here that *AtMC8* mediates cell death induced by addition of H_2O_2, and this is similar to the situation in yeast (5). This suggests that H_2O_2 activates an ancestral pathway present in both yeast and *Arabidopsis*. This is reinforced by the fact that when we overexpressed *AtMC8* in a yeast *yca1* knock-out strain, this expression was able to rescue the no-PCD phenotype in the presence of H_2O_2. It should be noted that *AtMC1* (or 1b) and *AtMC6* (or 2b) are also able to complement the same yeast *yca1* knock-out (7).

Using expression in *E. coli*, we showed here that *AtMC8* has an affinity for the substrates FR or GRR and is unable to cleave the caspase synthetic substrates tested. This is supported by our overexpression data in yeast and *Arabidopsis*. Four of the nine *Arabidopsis* metacaspases, *AtMC1*, *-4*, *-6*, and *-9* have also been shown to be arginine/lysine-specific cysteine proteases and unable to cleave caspase-specific synthetic substrate, reinforcing the observation that metacaspases are not plant caspases. We found AtMC8 to be more active at pH 7 to 8.5, suggesting that AtMC8 most likely functions in the cytoplasm or organelles with neutral pH. These observations make AtMC8 clearly different from AtMC9, which has an optimum pH of 5.5 and is localized in the cell wall (15). By virtue of this difference, it seems unlikely that AtMC8 is localized in the cell wall. In addition, overexpressing or ablating AtMC8 expression affects cell death in protoplasts, cells that have been stripped of their cell wall. This phenotype in protoplasts also suggests that AtMC9 is not involved, as AtMC9 should be removed or depleted during protoplast preparation.

In summary, we have shown that in *Arabidopsis* metacaspase-8 mediates cell death induced by reactive oxygen species. This is an important step, but much remains to be discovered about the pathway involved and the position of AtMC8 in the cascade in relation to light and RCD1. The protein targets that are cleaved by metacaspase-8 and the molecular steps that lead to its activation at the gene or protein level remain to be identified and will help defining the pathway.

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