

Type II Metacaspases Atmc4 and Atmc9 of *Arabidopsis thaliana* Cleave Substrates after Arginine and Lysine*

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Nine potential caspase counterparts, designated metacaspases, were identified in the *Arabidopsis thaliana* genome. Sequence analysis revealed two types of metacaspases, one with (type I) and one without (type II) a proline- or glutamine-rich N-terminal extension, possibly representing a prodomain. Production of recombinant *Arabidopsis* type II metacaspases in *Escherichia coli* resulted in cysteine-dependent autocatalytic processing of the proform into large and small subunits, in analogy to animal caspases. A detailed biochemical characterization with a broad range of synthetic oligopeptides and several protease inhibitors of purified recombinant proteins of both metacaspase 4 and 9 showed that both metacaspases are arginine/lysine-specific cysteine proteases and did not cleave caspase-specific synthetic substrates. These findings suggest that type II metacaspases are not directly responsible for earlier reported caspase-like activities in plants.

Primarily based on morphological features, animal cell death is usually referred to as apoptosis or necrosis. Apoptosis is characterized by membrane blebbing, cytosolic condensation, cell shrinkage, nuclear condensation, breakdown of nuclear DNA, and finally, the formation of apoptotic bodies that can easily be taken up by other cells (1). Necrosis, as defined on a microscopic level, denotes cell death in which cells swell, round up, and then suddenly collapse, spilling their contents into the medium. However, in animals, other forms of cell death exist, such as autophagic and autolytic death and intermediate varieties (2).

In plants “programmed cell death” usually denotes apoptosis-like cell death characterized by chromatin aggregation, cell

shrinkage, cytoplasmic, and nuclear condensation, and DNA fragmentation (3–5). Apoptotic characteristics have been observed during hypersensitive response and after abiotic stress, such as exposure to ozone, UV irradiation, chilling, and salt stress (6–10).

On a biochemical level, apoptosis in animals is characterized and commonly defined by the activation of a family of cysteine-dependent aspartate-specific proteases, or caspases (11). Caspases can proteolytically activate downstream caspases or cut various cellular substrates, resulting in a plethora of structural and metabolic alterations, ultimately leading to cell death (11–13).

Caspases hydrolyze peptide bonds at the C-terminal side of an aspartate, the so-called P1 residue. By using a variety of synthetic oligopeptide caspase substrates and inhibitors with an aspartate at this P1 position, caspase-like activity has recently been demonstrated in various plant cell death models (14–17). However, the corresponding genes for these activities have never been identified. Previously, two families of distant caspase homologues in plants, fungi, protozoa, and animals have been reported; that is, paracaspases, restricted to the metazoa, and metacaspases, identified in plants, fungi, and protozoa (18–20). Here, we report the identification, cloning, and biochemical characterization of two metacaspases of *Arabidopsis thaliana* as arginine/lysine-specific cysteine-dependent proteases.

EXPERIMENTAL PROCEDURES

Cloning of Metacaspase Open Reading Frames in *Arabidopsis*—First-strand cDNA was synthesized from pooled RNA obtained from leaves, inflorescences, and roots of young and mature plants with the Superscript II RNase H[−] reverse transcriptase (Invitrogen) according to the manufacturer's instructions and used as template for PCR reactions with PLATINUM Pfx DNA polymerase (Invitrogen) and the forward and reverse primers: 5'-ATGTACCCGCCACCTCC-3' and 5'-CT-AGAGAGTGAAAGGCTTTGCATA-3' for *Atmc1*; 5'-ATGTTGTTGCTGTGGACTG-3' and 5'-TTATAAAGAGAAGGGCTTCTCATATAC-3' for *Atmc2*; 5'-ATGGCTAGTCGGAGAGAAG-3' and 5'-TCAGAGTACAAA-CTTTGTGTCGT-3' for *Atmc3*; 5'-ATGACGAAAAGGCGGTGCTT-3' and 5'-TCAACAGATGAAAGGAGCGTTGG-3' for *Atmc4*; 5'-ATGGCGAAGAAAGCTGTGTTG-3' and 5'-TTACAAATAAACGGAGCATTCAC-3' for *Atmc5*; 5'-ATGGCCAAGAAAGCTTTACTG-3' and 5'-TCAACATATAAACCGAGCATTTGAC-3' for *Atmc6*; 5'-ATGGCAAAGAGAGCGTGTGTTG-3' and 5'-TTAGCATATAAACGGAGCATTCAC-3' for *Atmc7*; 5'-ATGGCGAAGAAAGCACTTTTG-3' and 5'-TTAGTAGCATATAAATGGTTTATCAAC-3' for *Atmc8*; 5'-ATGGATCAACAAGGGATGGTC-3' and 5'-TCAAGGTTGAGAAAGGAACGTC-3' for *Atmc9*. For forward primers, 5'-AAAAAGCAGGCTCCACC-3' was attached to the 5' end to enable subsequent amplification with the *attB1* primer 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'; for reverse primers, the 5' extension was 5'-AGAAAGCTGGGTC-3' to allow annealing with the *attB2* primer 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'. PCR

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AY219826–AY219834.

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products were cloned into pDONR201 (Invitrogen) to generate entry vectors for each metacaspase.

For cloning of human caspase 7 (Ref. 21; accession number NP_001218), the forward and reverse primers 5'-ATGGCAGATGAT-CAGGGCTGT-3' and 5'-CTATTGACTGAAGTAGAGTTCC-3' were used. Extensions were added to allow annealing of *attB1* and *attB2* primers as mentioned above for metacaspase cloning. GenBank™ accession numbers for the reported metacaspase sequences are AY219826-AY219834.

Alignment of Metacaspase Sequences—Sequences were aligned with ClustalX (22) and manually edited with BioEdit (23).

Bacterial Production and N-terminal Peptide Sequencing of Atmc9 Fragments—The open reading frames were cloned into the bacterial expression vector pDEST17 (Invitrogen), resulting in the N-terminal fusion with a His₆ epitope tag. Transformed cultures of *Escherichia coli* strain BL21(DE3) were induced with 1 mM isopropyl β-D-thiogalactopyranoside for 1–3 h. Cells were centrifuged and lysed under denaturing conditions (24). The bacterial cell pellet from a 500-ml culture was lysed with 5 ml of 100 mM Tris-HCl (pH 8.0), 20 ml of 8.0 M urea, and 2.7 ml of 10% (w/v) sodium N-lauroylsarcosinate completed with 1 mM phenylmethylsulfonyl fluoride (PMSF)¹ and 1 mM oxidized glutathione. After sonication and centrifugation, the volume was brought to 50 ml with buffer 1 (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% (v/v) glycerol, 0.1% (w/v) sodium N-lauroylsarcosinate, 1 mM PMSF, and 1 mM oxidized glutathione). The lysate was applied to a 2-ml nickel nitrilotriacetic acid column (Qiagen, Hilden, Germany) equilibrated with buffer 1. The column was first washed with buffer 2 (buffer 1 with 0.1% (v/v) Triton-X100 instead of 0.1% (w/v) sodium N-lauroylsarcosinate), then with buffer 2 supplemented with 10 mM imidazole. Recombinant metacaspases were eluted with 300 mM imidazole in buffer 2 and checked by 12% (w/v) PAGE.

Automated N-terminal Edman degradation of the immobilized Atmc9 fragments and mass determination were performed on a 476A pulsed-liquid sequencer equipped with an on-line phenylthiohydantoin-derivative analyzer and a 4700 proteomics analyzer, respectively (Applied Biosystems, Foster City, CA). Reversed-phase high performance liquid chromatography was done in two steps; the nickel nitrilotriacetic acid-purified bacterially produced protein was applied on a PLRP-S column (Polymer Laboratories, Amherst, MA; 4.6 × 200 mm; eluent A = 0.1% (v/v) trifluoroacetic acid, B = 90% isopropanol in 0.07% (v/v) trifluoroacetic acid) to separate the mixture in two fractions, fractions 1 and 2, between ~80–85 and ~85–95% of eluent B, respectively. The second fraction was separated using a μRPC column (Amersham Biosciences; 4.6 × 100 mm; eluent A = 0.1% (v/v) trifluoroacetic acid; eluent B = 90% (v/v) MeCN in 0.1% trifluoroacetic acid). The p10-like fragment was eluted as a single peak at ~60% of eluent B.

Native Preparation of Purified Recombinant Metacaspase 4 and 9 and Human Caspase 7—The bacterial expression vectors pDEST-17Atmc4, pDEST17Atmc4C/A, pDEST17Atmc9, pDEST17Atmc9C/A, pDEST17Atmc9R/A, and pDEST17HsCasp7 were transformed into *E. coli* strain BL21(DE3)pLysE, and production was induced for 24 h with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside. Cultures were harvested, resuspended in extraction buffer (50 mM phosphate buffer, pH 7.0, 300 mM NaCl, 1 mM oxidized glutathione), and passed through a French press. Cellular debris were removed by centrifugation, and the supernatant was mixed with TALON cobalt affinity resin (BD Biosciences) and incubated overnight at 4 °C. Beads were washed twice in the same buffer, loaded on a column, and washed twice again. Bound protein was eluted with extraction buffer supplemented with 150 mM imidazole.

Metacaspase and Caspase Assays, Substrates, and Inhibitors—All tested fluorogenic substrates were obtained from Bachem (Bubendorf, Switzerland), except for D-VKKR-AMC (Enzyme Systems Products, Livermore, CA) and inhibitors from Sigma-Aldrich (St. Louis, MO), except for the caspase inhibitors, Z-FA-fmk and Z-FK-tbmK (Enzyme Systems Products). Assays were performed in 150 μl with 50 μM substrate in optimized metacaspase assay buffers. For Atmc9, the buffer consisted of 50 mM MES (pH 5.5), 150 mM NaCl, 10% (w/v) sucrose, 0.1%

(w/v) CHAPS, 10 mM dithiothreitol; for Atmc4 the buffer consisted of 50 mM Hepes (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 100 mM CaCl₂, 10 mM dithiothreitol; for caspase activity assays, the buffer consisted of 50 mM Hepes (pH 7.5), 150 mM NaCl, and 10 mM 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane. For the determination of the pH profile of metacaspases, the buffer consisted of 50 mM acetic acid, 50 mM MES, and 100 mM Tris (25). Time-dependent release of free amido-4-methylcoumarin (AMC) was measured on a FLUOstar OPTIMA reader (BMG Labtechnologies, Offenburg, Germany), and activity was expressed as the increase per minute of fluorescence units in each well.

RESULTS

Identification and Cloning of Arabidopsis Metacaspases—A sequence homology search (BLASTP; 26) of the eight published *Arabidopsis* metacaspases (20) against a database of predicted *Arabidopsis* protein-encoding genes (EuGene; Ref. 27) identified one extra putative metacaspase gene, leading to a total of nine metacaspase genes in the genome of *A. thaliana* (designated *Atmc1* to *Atmc9*). The alignment of the corresponding protein sequences is shown in Fig. 1 and the corresponding phylogenetic tree in Fig. 2. Previous genomic analysis has revealed that the *Arabidopsis* genome consists of a large number of duplicated blocks, which might be the results of one or many complete genome duplications (28–30). Comparison of the genomic organization of all nine *Arabidopsis* metacaspases and these duplicated segments shows that the *Atmc8* gene is linked with genes *Atmc4*, *Atmc5*, *Atmc6*, and *Atmc7* by an internal duplication event on chromosome I (data not shown). In addition, these genes (*Atmc4* to *Atmc7*) are present as a tandem within a region of 10.6 kilobases on chromosome I. By combining the phylogenetic tree topology (Fig. 2) and this genomic organization, this metacaspase cluster (genes *Atmc4* to *Atmc7*) originated through a block duplication of the *Atmc8* gene that was followed by a tandem duplication. As shown in Fig. 1, a high degree of similarity exists between the members of this cluster, with amino acid sequence identities ranging from 56% to 71%. We isolated the corresponding open reading frames of all but one genes by reverse transcription-PCR on pooled RNA isolated from different organs of *Arabidopsis*. Despite several attempts we could not isolate a full-length *Atmc8* cDNA fragment. Because no expressed sequence tags corresponding to *Atmc8* are found in public databases, we assume that it is probably a pseudogene.

Three of the *Arabidopsis* metacaspase proteins (*Atmc1*, *Atmc2*, and *Atmc3*) have previously been designated as “type I” metacaspases. They possess N-terminal extensions ranging from ~80 to 120 amino acids in length (Fig. 2; Ref. 20) and could represent a prodomain that is also present in mammalian upstream “initiator” caspases. As such, this domain may be responsible for protein-protein interactions between metacaspases and/or other oligomerizing components of different signaling complexes, leading to subsequent metacaspase activation (11, 31). The *Arabidopsis* metacaspase prodomains are rich in proline (*Atmc1* and *Atmc2*) or glutamine (*Atmc3*), and all contain two putative CXXC-type zinc finger structures, similar to the lesion-simulating disease-1 protein, a negative regulator of the hypersensitive response with homology to GATA-type transcription factors (20, 32). The other metacaspases (*Atmc4* to *Atmc9*) lack a large prodomain, although the existence of a short prodomain cannot be excluded, and were designated type II metacaspases (20).

In both types, a conserved region of ~150 amino acids could correspond with the p20 subunit of mammalian caspases (11). At the C terminus, another conserved domain is reminiscent of the p10 of caspases. In between these putative p20 and p10 domains, a variable region is present that differs considerably between type I and type II metacaspases; this putative loop or linker region is ~20 amino acids long in type I metacaspases,

¹ The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amido-4-methylcoumarin (AMC); Atmc, *A. thaliana* metacaspase; C/A, cysteine-to-alanine mutation; Z-VAD-fmk, benzylloxycarbonyl (Z)-Val-Ala-Asp(O-methyl)-fluoromethyl ketone (fmk); Boc-GRR-AMC, *t*-butyloxycarbonyl-Gly-Arg-amido-4-methylcoumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; tbmK, trimethylbenzoyloxymethyl ketone; TLCK, N^ε-tosyl-L-lysine-chloromethyl ketone; cmk, chloromethyl ketone; E-64, 1-*trans*-epoxysuccinyl-leucylamide-(4-guanido)-butane; MES, 4-morpholineethanesulfonic acid; R/A, arginine-to-alanine mutation.

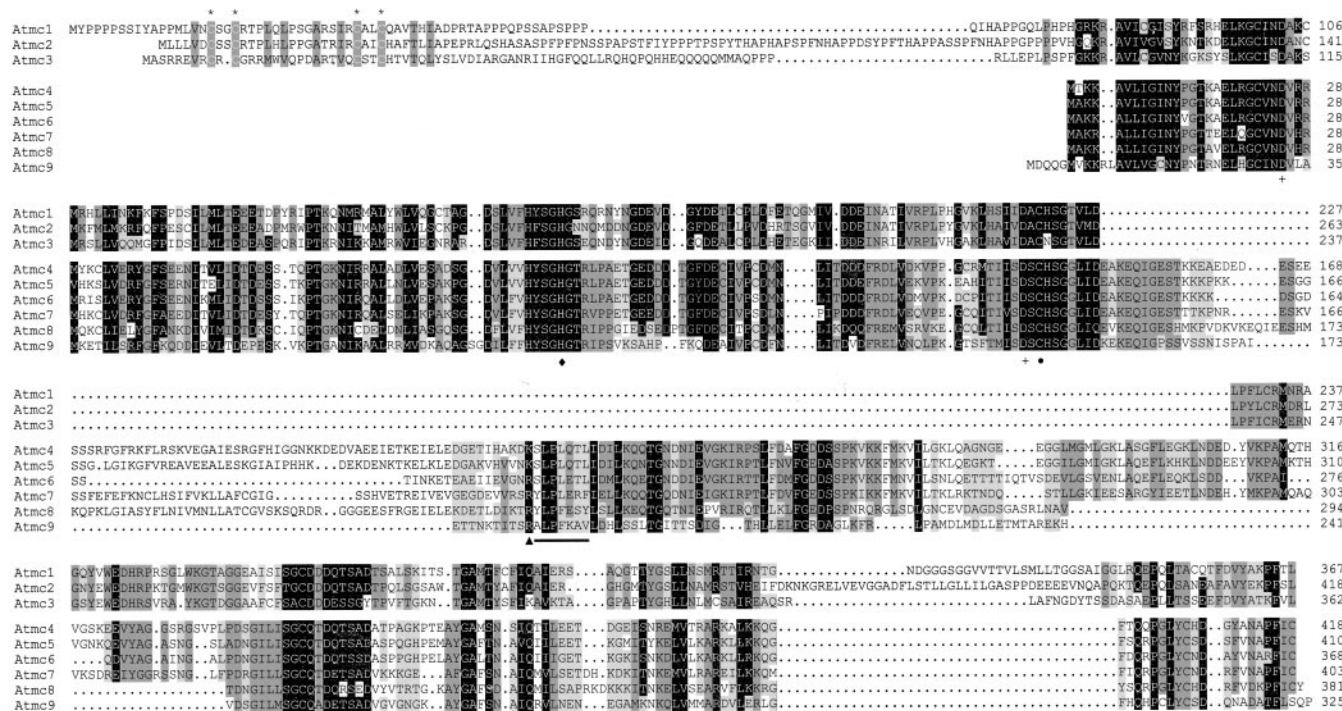


FIG. 1. Multiple alignment of the nine metacaspases in *A. thaliana*. The putative catalytic His and Cys residues are marked by a diamond and a dot, respectively. Zinc finger cysteines in the prodomains of type I metacaspases are indicated by asterisks, and the P1 position for autocatalytic cleavage of Atmc9 is indicated by a triangle. Conserved aspartate residues possibly involved in substrate P1 coordination are marked by a plus sign. The obtained N-terminal peptide sequence for the p15 fragment of Atmc9 is underlined. Shading is based on the amino acid groups described by Wu and Brutlag (51). In addition to identical amino acids, those belonging to the groups PAGST, QNED, KRH, VLIM, and FYW were also scored as highly homologous and are shaded black (all metacaspases) or dark gray (one type only). To determine weakly conserved residues, PAGSTQNEDHKR and CVLIMFYW were used (light gray).

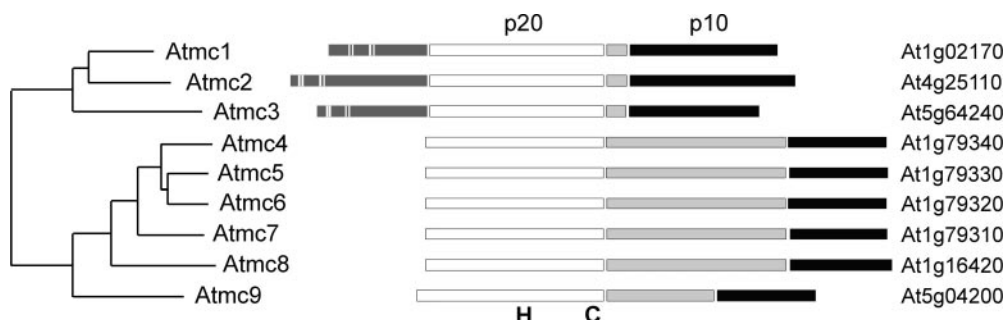


FIG. 2. Unrooted phylogenetic tree of the *Arabidopsis* metacaspase family. For construction of the tree, the alignment of Fig. 1 was subjected to the TREECON software package (52). On the right side a tentative schematic representation of the structure of the nine *Arabidopsis* metacaspases is presented. The putative prodomain is in dark gray, the large subunit (p20) is in white, the small subunit (p10) is in black, and the linker/loop regions between p20 and p10 are in light gray. H and C designate His and Cys residues, respectively. Cysteine residues within the putative prodomain zinc fingers are shown as white stripes. Numbers given at the right correspond to those provided by the Arabidopsis Genome Initiative (28).

whereas in type II metacaspases it varies in size from 90 (for Atmc9) to 150 residues (Atmc4 to Atmc8).

In addition to this structural domain homology, the designation of metacaspases as caspase homologs is based on the presence of a conserved histidine/cysteine dyad in the p20 domain. The sequence context of the catalytic histidine residue in *Arabidopsis* metacaspases is H(Y/F)SGHG, suggesting an intermediate stabilizing role for the adjacent glycine, as in mammalian caspases (11). The catalytic cysteine in caspases is contained in a QAC(R/Q)G context, with the glutamine residue shown to coordinate the P1 aspartate. The metacaspases of *Arabidopsis* have a D(A/S)C(H/N)SG signature, suggesting that this role is now played by an aspartate.

Bacterial Overproduction of Arabidopsis Type II Metacaspases Leads to Cysteine-dependent Autocatalytic Processing—Upon overproduction in bacteria, caspases autoprocess in

a cysteine-dependent manner to generate fully active proteases (11). We initiated a biochemical analysis by overproducing N-terminal His₆-tagged versions of the *Arabidopsis* type II metacaspases in *E. coli* (Fig. 3a). Detection with anti-His₆ antibody revealed that all type II metacaspases could be produced in bacteria and, more interestingly, that this overproduction led to their processing, in analogy to caspases. Only for Atmc6 was cleavage less clear. During this processing, a C-terminal region ranging from 15 to 30 kDa was removed, resulting in a concomitant decrease in the size of the detected His₆-tagged protein.

For a detailed analysis of the type II metacaspases, we chose Atmc4 as a representative of the closely related group encoded by the gene cluster Atmc4 to Atmc7, together with Atmc9. For these two proteins, mutant forms in which the presumed catalytic cysteine (Cys¹³⁹ for Atmc4 and Cys¹⁴⁷ for Atmc9) was

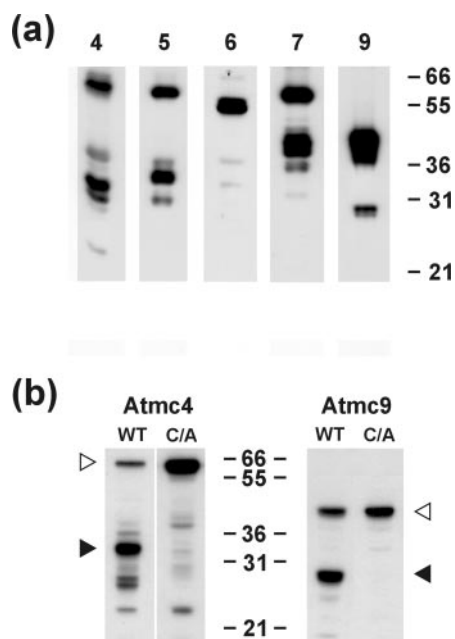


FIG. 3. Production of type II metacaspases in *E. coli*. *a*, bacterial production of type II metacaspases. Bacterial cultures carrying an expression vector for N-terminally His₆-tagged metacaspases were induced for 4 h and total bacterial protein subjected to immunoblotting with anti-HIS. *b*, bacterial production of Atmc4 and Atmc9 and their respective C/A mutants. Bacterial cultures carrying an expression vector for N-terminally His₆-tagged Atmc4 and Atmc9 wild-type (WT) or C/A mutants were induced for 2 h, and lysates were subjected to immunoblotting with anti-His. Open and black triangles indicate full-length proteins and His₆-tagged p20 subunits, respectively.

replaced by an alanine (designated C/A mutation) were also produced. Fig. 3*b* shows immunoblots with anti-His₆ antibodies on bacterial lysates overproducing wild-type and mutated Atmc4 and Atmc9. Overproduction of Atmc4 resulted in the presence of the His₆-tagged full-length protein (apparent molecular mass 60 kDa) and of an N-terminal fragment of 33 kDa. For Atmc9, overproduction led to the detection of the His₆-tagged full-length protein (apparent molecular mass 46 kDa) and an N-terminal fragment of 28 kDa. These patterns suggest that both metacaspases, like caspases, are able to autoprocess, thereby separating the putative large (p20) and small (p10) subunits. For the cysteine mutants, no such processing occurred, demonstrating that it is the result of cysteine-dependent autocatalytic action of the type II metacaspases.

Atmc9 Autoprocesses after an Arginine Residue—Because bacterial overproduction of type II metacaspases is sufficient for autoprocessing, we were able to characterize the putative p20 and p10 subunits of Atmc9 by N-terminal peptide sequencing and by molecular mass determination via mass spectrometry. When His₆ tag-purified Atmc9 was analyzed by PAGE and silver staining, major fragments with apparent molecular masses of 22 and 15 kDa were visible, whereas bands at 38 and 28 kDa were less clear (Fig. 4). Western blot analysis revealed that besides the full-length C/A mutant, only the 28-kDa fragment of wild-type Atmc9 could be detected with the anti-His₆ antibody and, thus, represented the His₆-tagged p20-like subunit. Therefore, the 22-kDa band could represent the mature p20 subunit after removal of the His₆ tag and possibly a very short prodomain, whereas the other fragment (15 kDa) could be the p10-like subunit. It should be noted that, although the p20- and p10-like fragments did not carry a His₆ tag, they probably co-purified in complexes that did so. Furthermore, we observed that processing and concomitant disappearance of the full-length zymogen, which occurred after purification, also re-

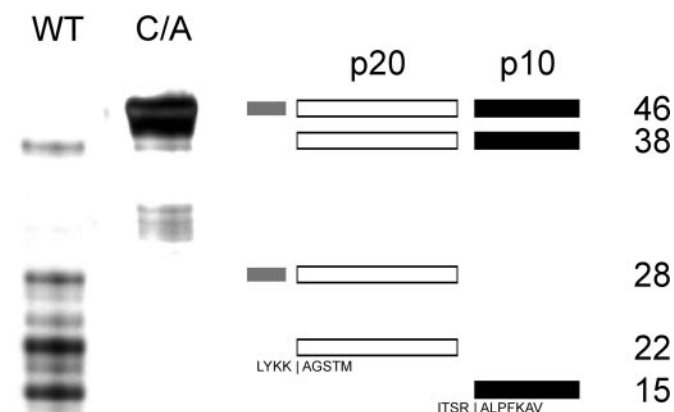


FIG. 4. Characterization of autoprocessing fragments of Atmc9. Bacterially produced Atmc9 and its C/A mutant form were separated by SDS-PAGE and silver-stained. The detected fragments are represented schematically on the right. Gray, N-terminal His₆ tag and putative small prodomain; white, p20-like subunit; black, p10-like subunit. The N-terminal amino acid sequences obtained are shown below the p20-like and p10-like fragments. WT, wild type.

sulted in the presence of these non-tagged fragments. The 15-kDa protein band was sufficiently purified and Edman degradation sequencing resulted in the peptide sequence ALPFKAV, indicating that the fragment was generated by cleavage after Arg¹⁸³. Molecular mass determination by matrix-assisted laser absorption deionization coupled to tandem time of flight revealed the p10-like subunit of Atmc9 had a mass of 15,442 Da, confirming that it consists of amino acids 184–325. As seen on Fig. 1, all type II metacaspases possess either an arginine or a lysine at this position. The nature of the autocatalytic cleavage site of Atmc9 already suggests that, although mammalian caspases and metacaspases are structural homologs, they differ in substrate specificity, with a Lys/Arg specificity at the P1 position for the latter.

N-terminal sequencing of the 22-kDa fragment revealed that it was generated through removal of the His₆ tag at an artificial site, LYKK ↓ AGST, introduced by the Gateway cloning procedure (data not shown). A few residues downstream a similar sequence were present, conserved in both type I and type II metacaspases. Therefore, it is possible that the natural processing site is masked by the artificial one, and hence, no conclusions can be drawn for now on any additional processing on the N-terminal side of the large subunit.

Atmc4 and Atmc9 Cleave P1 Arginine/Lysine Substrates—To study the substrate specificity of Atmc4 and Atmc9 in more detail, the purified recombinant proteins and their respective cysteine mutants were tested for their ability to cleave the synthetic fluorogenic oligopeptide P1 arginine substrate *t*-butyloxycarbonyl-Gly-Arg-Arg-amido-4-methylcoumarin (Boc-GRR-AMC) in a wide pH range. As shown in Fig. 5*a*, both Atmc4 and Atmc9 have prominent activity toward GRR. The corresponding catalytic cysteine mutants were not active at all (see also below). For Atmc4, optimal buffer pH was 7.5–8.0, with detectable activity in the pH range of 6.5 to 9.0. Interestingly, the pH optimum for Atmc9 activity was 5.0–5.5 (pH range 4.5 to 6.0), whereas activity at the physiological pH of the cytoplasm (7.0–7.5) was completely abolished.

To explore substrate P1 preference, proteolytic activities of purified Atmc4 and Atmc9 were tested against additional oligopeptide substrates with an arginine or lysine at the P1 position; benzyloxycarbonyl-Phe-Arg-amido-4-methylcoumarin (Z-FR-AMC), Boc-GRR-AMC, *t*-butyloxycarbonyl-Gly-Lys-Arg-

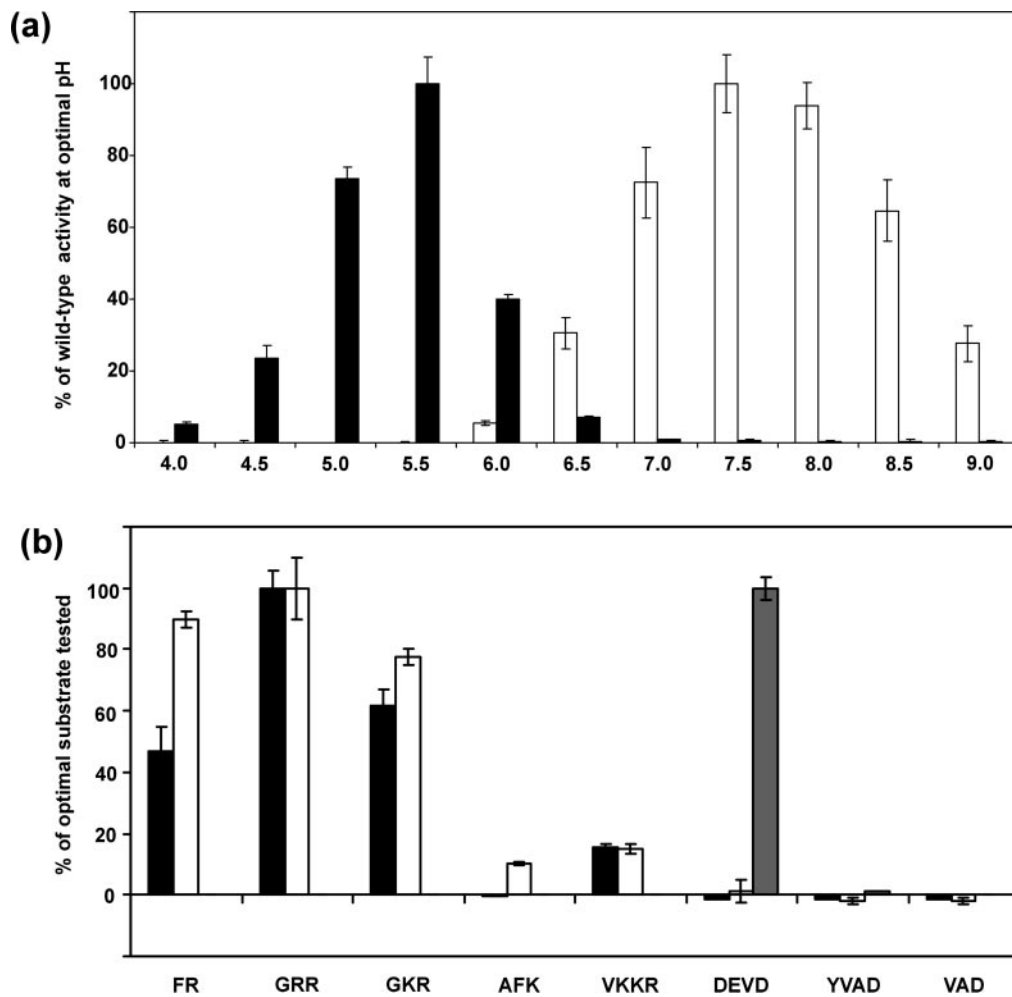


FIG. 5. **pH optimum and substrate specificity of Atmc4 and Atmc9.** *a*, proteolytic activity against Boc-GRR-AMC at different pH of Atmc4 (white bars) and Atmc9 (black bars). Relative activity is expressed as the percentage of the activity of wild-type metacaspases at optimal pH. *b*, proteolytic activity of Atmc4 (white bars) and Atmc9 (black bars) and human caspase-7 (gray bars) against Z-FR-AMC (FR), Boc-GRR-AMC (GRR), Boc-GKR-AMC (GKR), H-AFK-AMC (AFK), D-VKKR-AMC (VKKR), Ac-DEVD-AMC (DEVD), Ac-YVAD-AMC (YVAD), and Z-VAD-AMC (VAD). Relative activity is expressed as the percentage of the activity of wild-type metacaspases with the best substrate tested.

AMC (Boc-GKR-AMC), D-VKKR-AMC, and H-Ala-Phe-Lys-AMC (AFK-AMC). All tested P1 arginine substrates are indeed cleaved by both Atmc4 and Atmc9, albeit at different efficiencies (Fig. 5*b*). Noteworthy, Atmc9 also showed some activity toward the P1 lysine substrate H-AFK-AMC. To confirm that type II metacaspases prefer basic rather than acidic P1 residues, we tested whether Atmc4 and Atmc9 could cleave the caspase substrates Ac-DEVD-AMC, Ac-YVAD-AMC, and Z-VAD-AMC. None of the caspase substrates were cleaved by the metacaspases (Fig. 5*b*). To exclude the possibility that specific experimental conditions would impair the measurement of caspase activity in our assays, we cloned and purified human caspase 7 as a His₆-tag fusion in the same vector as the *Arabidopsis* metacaspases and assessed its potential to cleave caspase substrates. As shown in Fig. 5*b*, recombinant human caspase 7 efficiently hydrolyzed Ac-DEVD-AMC and also Ac-YVAD-AMC, although at hardly measurable efficiency. On the other hand, human caspase 7 did not cleave any of the arginine/lysine substrates. These results demonstrate that caspase substrates cannot be cleaved by *Arabidopsis* Atmc4 and Atmc9 and are, hence, very unlikely to be responsible for the caspase-like activities in plants reported in literature.

Effect of Protease Inhibitors on the Activity of Type II Metacaspases—We assessed the effect on Atmc4 and Atmc9 of several protease inhibitors with Boc-GRR-AMC as substrate (Ta-

TABLE I
Effect of different protease inhibitors on the activity of Atmc4 and Atmc9 with Boc-GRR-AMC as substrate

Percentage of the activity of recombinant Atmc4 and Atmc9 in standard assay buffers (see “Experimental Procedures”). Values are the means of triplicate experiments. TPCK, *N*^α-tosyl-L-phenylalanyl chloromethyl ketone.

Reagent	Concentration	Activity	
		Atmc4	Atmc9
		%	
Z-YVAD-cmk	100 μM	97 ± 5	99 ± 9
Z-DEVD-cmk	100 μM	94 ± 14	94 ± 8
Z-VAD-fmk	100 μM	90 ± 7	103 ± 1
Z-FA-fmk	100 μM	97 ± 4	92 ± 3
Chymostatin	100 μM	14 ± 2	10 ± 1
Soybean trypsin inhibitor	100 μg/ml	34 ± 0	50 ± 4
Pepstatin	100 μM	66 ± 1	69 ± 0
Benzamide	5 mM	2 ± 1	5 ± 1
Iodoacetamide	10 mM	0 ± 0	3 ± 0
PMSF	1 mM	23 ± 0	98 ± 14
E-64	100 μM	44 ± 2	93 ± 6
Aprotinin	5 μg/ml	114 ± 11	113 ± 7
Leupeptin	1 μM	12 ± 1	5 ± 1
Antipain	1 μM	6 ± 0	1 ± 1
Z-FK-tbmK	1 μM	5 ± 0	12 ± 0
TLCK	250 μM	41 ± 5	0 ± 0
TLCK	1 μM	79 ± 5	0 ± 1
TPCK	250 μM	72 ± 0	79 ± 6

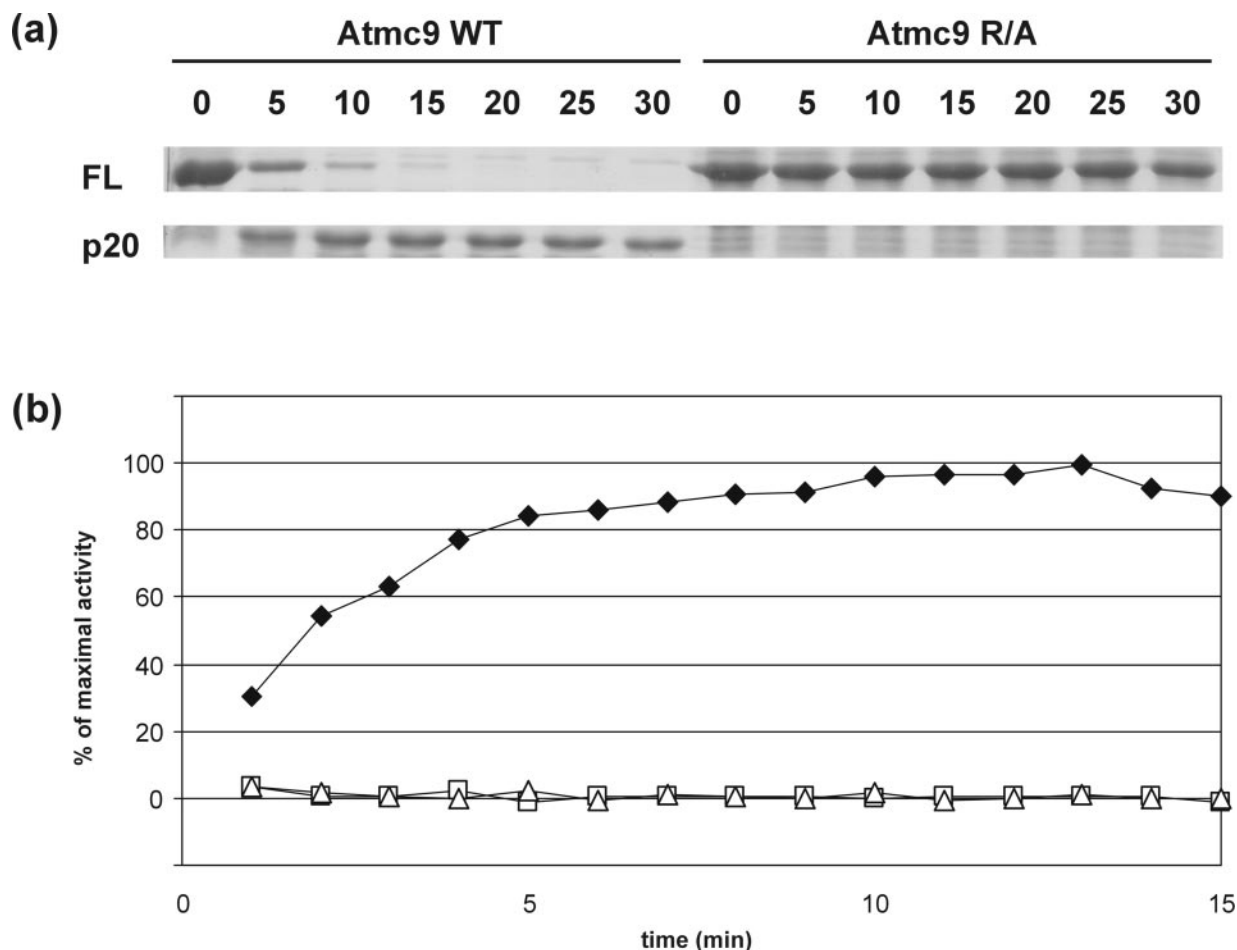


FIG. 6. Requirement of autoprocessing for enzymatic activity of Atmc9. *a*, wild-type (WT) Atmc9 and Atmc9R/A were incubated for different times at pH 5.5 in the presence of 10 mM dithiothreitol and subsequently separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Full-length zymogens (FL) and large (p20) subunits are shown. *b*, proteolytic activity of Atmc9 (black diamonds), Atmc9C/A (white squares), and Atmc9R/A (white triangles) at different time points after incubation with assay buffer and substrate. Relative activity is expressed as the percentage of the maximal activity of Atmc9.

ble I). First, we tested the caspase inhibitors Z-YVAD-chloromethyl ketone (cmk), Z-DEVD-cmk, and Z-VAD-cmk. However, none of these compounds could block metacaspase activity at concentrations up to 100 μ M. Also, the cathepsin B inhibitor Z-FA-fmk had no effect as well at this concentration.

Of the broad-spectrum inhibitors tested, only chymostatin, a reversible serine and cysteine protease inhibitor, and to a lesser extent soybean trypsin inhibitor and pepstatin could block metacaspase activity at 100 μ M. Benzamidin and iodoacetamide inhibited both activities at the millimolar range. PMSF (1 mM) and *l*-trans-epoxysuccinylleucylamide-(4-guano)-butane (E-64, 100 μ M) blocked Atmc4 activity only moderately but not that of Atmc9, whereas the serine protease inhibitor aprotinin had no effect on metacaspase activity at concentrations up to 5 μ g/ml.

We finally tested whether inhibitors with a basic amino acid at the P1 position could block type II metacaspase activity more efficiently. Both Atmc4 and Atmc9 are strongly inhibited by leupeptin and antipain, two arginal protease inhibitors, at concentrations as low as 1 μ M. The oligopeptide inhibitor Z-FK-tbm equally inhibited Atmc4 and Atmc9 activities at the micromolar range. Interestingly, although *N* $^{\alpha}$ -tosyl-L-lysine-chloromethyl ketone (TLCK) had only moderate blocking activity on Atmc4, Atmc9 was fully inhibited at 1 μ M, indicating that both metacaspases differ in their substrate binding properties. In contrast, *N* $^{\alpha}$ -tosyl-L-phenylalanyl chloromethyl ketone, an irreversible inhibitor of chymotrypsin proteases, very weakly

blocked the activities of both Atmc4 and Atmc9. Taken together, these results confirm the specificity of Atmc4 and Atmc9 for basic P1 residues in their substrates.

Processing of Atmc9 Is a Prerequisite for Activity—Mammalian executioner caspases are activated by processing of the zymogen through upstream caspases or by autocatalytic proteolysis upon overproduction in bacteria or cells (33). To find out whether *Arabidopsis* type II metacaspase activity also requires cleavage and concomitant separation of the large and small subunits, we created a mutant form of Atmc9 where the P1 arginine at the autoprocessing site was replaced by an alanine (R/A mutant). To assess whether this mutant could autoprocess, a kinetics experiment was set up in which both wild-type and R/A mutant Atmc9 were incubated for different times at pH 5.5. Fig. 6*a* shows that only the wild-type protein autoprocesses into a large and a small subunit, whereas the R/A mutant remained uncleaved, confirming that autoprocessing of Atmc9 occurs at Arg¹⁸³ and further demonstrates that no secondary autoprocessing sites exist that could substitute for this residue. In parallel, we assessed whether the uncleavable zymogen of Atmc9 showed activity toward Boc-GRR-AMC together with wild-type Atmc9 and Atmc9C/A. Protein preparations were added to assay buffer containing dithiothreitol and substrate, and release of free AMC was immediately measured. Activity of Atmc9 gradually increased to a maximum at ~10–15 min, corresponding to the complete autoprocessing of the proform (Fig. 6*b*). In contrast,

no activity toward Boc-GRR-AMC could be measured with the catalytic mutant (C/A) nor with the zymogen (R/A). Thus, type II metacaspases, like the executioner caspases in animals, appear strongly dependent on (auto)processing in order to be active.

DISCUSSION

Cell death in plants has previously been associated with caspase-like activities. Chemically induced cell death in tomato cells can be blocked by the addition of different caspase inhibitors (15). Tobacco plants infected with the tobacco mosaic virus show protease activity as measured by Ac-YVAD-AMC cleavage, a synthetic substrate for caspase-1. Also, caspase inhibitors co-infiltrated with an incompatible *Pseudomonas syringae* pathovar prevented the hypersensitive response in tobacco (16). Embryonic barley cells contain caspase 3-like activity, as measured with the specific substrate Ac-DEVD-AMC (17). More recently, activity toward VEID-AMC has been reported in cell death during spruce embryo development (14). However, the genes encoding the putative caspase-like proteases have not been identified yet. As reported by Uren *et al.* (20) on their *in silico* discovery of para- and metacaspases, it became evident to check whether plant metacaspases would be responsible for these caspase-like activities.

We cloned and characterized all but one of the reported *Arabidopsis* metacaspases. By mere overproduction of type II metacaspases in bacteria, autocatalytic processing depending on a defined catalytic cysteine results in the formation of p20-like and p10-like fragments. However, this processing pattern was not observed with mere bacterial overproduction of type I metacaspases (data not shown). In animals, the prodomain-containing initiator caspases are located in the upstream regions of signaling pathways, and their activation requires induced oligomerization (31, 34). Possibly, type I metacaspases, like the initiator caspases, similarly need oligomerization to be activated, a hypothesis that we are currently investigating. Furthermore, type II metacaspases may relate to the animal executioner caspases, which are proteolytically activated by active initiator caspases.

N-terminal peptide sequencing of the p10-like fragment generated after bacterial Atmc9 overproduction already suggests that substrate specificity of plant metacaspases is not directed to aspartate at the P1 site but, rather, to arginine and lysine. Furthermore, whereas various synthetic oligopeptide substrates containing a P1 arginine/lysine are efficiently cleaved by Atmc4 and Atmc9, the classical caspase substrates Ac-DEVD-AMC, Ac-YVAD-AMC, and Z-VAD-AMC are not. Also, metacaspase activity is completely blocked by leupeptin and antipain, two arginal inhibitors, and the P1 lysine inhibitors Z-FK-tbmK and TLCK in the case of Atmc9, confirming the arginine/lysine specificity of Atmc4 and Atmc9 and, as a result of the high similarity between Atmc4 to Atmc7, probably of all type II metacaspases. Further indications that all metacaspases are arginine/lysine-specific come from sequence analysis. In caspases, P1 specificity is dictated by arginine and glutamine residues located in both the p20 and p10 subunits, respectively, which help to position the substrate aspartate in the catalytic site (11). Based on the alignment of the *Arabidopsis* metacaspases and mammalian caspases and in analogy with gingipain R (35), Asp³² of Atmc9, which is conserved through all metacaspases in other organisms, could coordinate the P1 Arg/Lys residue of the substrate together with Asp¹⁴⁵, two positions upstream of the catalytic cysteine, and Asp²⁵⁴, located in the p10-like subunit. It is noteworthy that these aspartate residues are conserved in paracaspases as well, suggesting that the latter might also be Arg/Lys-specific proteases (data not shown). For Atmc9, we have shown that processing at

Arg¹⁸³ is necessary for the activation of the zymogen, as in executioner caspases of animals. There, cleavage of the linking loop between the p20 and p10 subunits results in deblocking of the active site cleft, thus allowing binding and hydrolysis of the substrate (36, 37).

Our findings that Atmc9 activity has an acidic pH optimum and is inactive under conditions corresponding to the physiological cytoplasmic pH have some important implications. First, mere overproduction and autoprocessing of the zymogen into the mature protease do not necessarily implicate protease activity but would need additional acidification of its environment. Using Bright Yellow-2 tobacco cells that produce green fluorescent protein fusions of Atmc9 under control of the CaMV35S promoter, we could only detect a strong green fluorescent protein signal in the nucleus and a weaker one in the cytosol (data not shown). However, signal sequences for sorting to the vacuole may have been masked by green fluorescent protein. Therefore, we cannot rule out that Atmc9 is a vacuolar protease. Activation by acidic pH has also been observed for human caspase 3 (38). In this case, a so-called "safety catch" hinders both the autocatalytic maturation and the vulnerability to proteolytic activation by upstream proteases. However, although this activation is stable in the case of caspase 3, *i.e.* once mature the protease shows optimal activity at pH 7.0–8.0 (39), preincubation of Atmc9 at low pH is not sufficient for its irreversible activation (data not shown). Several reports show that acidification of the cytosol is involved in plant cell signaling. For instance, the elicitor cryptogin induces calcium-dependent cytosolic acidification in tobacco cells, which probably results from activation of the plasma membrane NADPH oxidase, although inhibition of a plasma membrane H⁺-ATPase could also provoke sustained pH changes (40). Treatment of tobacco cells with 300 mM NaCl is immediately followed by acidification, the cytosolic pH decreasing from 7.3 to around 6.4 in 40 min (41). In 500 mM NaCl, the cytosolic pH decrease starts at 15 min and, probably due to tonoplast rupture, reaches 5.85 in 40 min, a value well in the active pH range of Atmc9. Tracheary element formation can be regarded as a paradigm for vacuole-mediated cell death (42). In this case, secondary wall thickening of differentiating tracheary elements is followed by fast vacuolar collapse. Interestingly, Minami and Fukuda (43) reported previously on the induction of an acidic cysteine protease with activity toward FR-AMC in *Zinnia* cells during differentiation into tracheary elements. This activity could be strongly inhibited by TLCK, leupeptin, E-64, and less efficiently by PMSF and pepstatin, a profile somewhat reminiscent of the metacaspases. Possibly, Atmc9 plays a role in these acidification-associated cell death systems.

The ability of type II metacaspases to cleave P1 Arg/Lys and not P1 Asp substrates demonstrates that they are probably not directly responsible for the reported caspase activities measured in plants. Recently, Coffeen and Wolpert (44) purified and characterized two subtilisin-like serine proteases from oat, designated saspases, which show caspase activity and which are involved in proteolysis of Rubisco during victorin-induced plant cell death. Therefore, it would be interesting to see whether members of the plant subtilisin family are also responsible for the caspase-like activities observed in other plant cell death models.

With the current knowledge, it is still unclear whether metacaspases play a role in plant cell death. Protease activity during oxidative stress-induced cell death of soybean cells has been detected by several synthetic fluorogenic substrates with an arginine residue at the P1 site (45). Furthermore, one of the metacaspase genes from tomato is up-regulated during *Botrytis cinerea*-induced plant cell death (46). Overproducing a meta-

caspase of *Trypanosoma brucei* in yeast inhibited growth and caused loss of respiratory competence and clonogenicity (19). Although overproduction and concomitant processing of the yeast metacaspase in bakers' yeast as such had little effect, it resulted in apoptotic cell death when combined with mild stresses, such as sublethal concentrations of H₂O₂ or cell culture aging (18). This cell death correlated with enhanced cleavage of the caspase-8-specific substrate Ac-IETD-AMC and could be blocked by the general caspase inhibitor Z-VAD-fmk. An alternative explanation for the detection of caspase-like activity after metacaspase overproduction in yeast could be that caspase-like proteases are activated by the latter either directly by proteolysis or indirectly by disturbance of cellular homeostasis. Therefore, it would be interesting to assess the effect of P1 arginine/lysine inhibitors on caspase activity measurements in metacaspase-overproducing yeast. The question of in which biological processes metacaspases are involved in plants could be addressed by overproducing or suppressing endogenous metacaspase levels. Furthermore, identification of natural substrates and inhibitors could point toward new insights into the functions of metacaspases.

We describe the first biochemical characterization of plant metacaspases. Based on their primary and predicted secondary structures, metacaspases can be classified as clan CD peptidases, a family of proteases characterized by their His/Cys catalytic dyad (47, 48), which include the Arg/Lys-specific clostripain (C11) and gingipains (C25), Asn-specific legumains (C13), Asp-specific caspases (C14), and Arg-specific separase (C50). Regarding their preference for basic P1 amino acids, metacaspases are similar to the other CD clan proteases separase, clostripain, and gingipains. We show that type II metacaspases are also biochemically similar to other clan CD proteases regarding their cysteine-dependent autocatalytic processing. In the case of gingipain R, three sequential autoprocessing steps are required to obtain full activity (49). Likewise, fully active legumain is only obtained after two acidic pH-dependent autocatalytic processing stages (50). Interestingly, like metacaspase 9, activity of legumain strongly depends on low pH. Given their structural and biochemical characteristics, we suggest that metacaspases would be classified as a new family of the CD clan of proteases.

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**Protein Synthesis, Post-Translation
Modification, and Degradation:
Type II Metacaspases Atmc4 and Atmc9 of
Arabidopsis thaliana Cleave Substrates
after Arginine and Lysine**

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