Two Arabidopsis Metacaspases AtMCP1b and AtMCP2b Are Arginine/Lysine-specific Cysteine Proteases and Activate Apoptosis-like Cell Death in Yeast

Received for publication, December 1, 2004, and in revised form, February 1, 2005
Published, JBC Papers in Press, February 3, 2005, DOI 10.1074/jbc.M415327200

Naohide Watanabe‡ and Eric Lam§

From the Biotechnology Center for Agriculture and the Environment, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901-8520

Metacaspases in plants, fungi, and protozoa constitute new members of a conserved superfamily of caspase-related proteases. A yeast caspase-1 protein (Yca1p), which is the single metacaspase in Saccharomyces cerevisiae, was shown to mediate apoptosis triggered by oxidative stress or aging in yeast. To examine whether plant metacaspase genes are functionally related to YCA1, we carried out analyses of AtMCP1b and AtMCP2b, representing the two subtypes of the Arabidopsis metacaspase family, utilizing yeast strains with wild-type and the disrupted YCA1 gene (yca1Δ). Inducible expression of AtMCP1b and AtMCP2b significantly promoted yeast apoptosis-like cell death of both the wild-type and yca1Δ strains, relative to the vector controls, during oxidative stress and early aging process. Mutational analysis of the two AtMCPs revealed that their cell-death-inducing activities depend on their catalytic center cysteine residues as well as caspase-like processing. In addition, the phenotype induced by the expression of two AtMCPs was effectively prevented when the cells were pretreated with a broad-spectrum caspase inhibitor, N-benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone. These results suggest that the two subtypes of Arabidopsis metacaspases are functionally related to Yca1p with caspase-like characteristics. However, we found that bacterial and yeast extracts containing AtMCP1b, AtMCP2b, or Yca1p exhibit arginine/lysine-specific endopeptidase activities but cannot cleave caspase-specific substrates. Together, the results strongly imply that expression of metacaspases could result in the activation of downstream protease(s) with caspase-like activities that are required to mediate cell death activation via oxidative stress in yeast. Metacaspases from higher plants may serve similar functions.

Apoptosis or programmed cell death (PCD)† is a conserved and genetically controlled process in multicellular organisms during development, homeostasis, and in response to pathogens and stress signals (1, 2). Activation of caspases (cysteine-containing aspartate-specific proteases) represents a central core execution switch for apoptosis in animal cells (reviewed in Refs. 3–5). Similarly, accumulating evidence in recent years suggest the involvement of caspase-like proteases during the activation of various types of PCD in plants (reviewed in Refs. 6–9). At present, no caspase gene has been identified in the available plant genome sequences, including Arabidopsis and rice, but recent studies have revealed the presence of a family of genes encoding proteins with distant homology to mammalian caspases, designated metacaspases, in plants, fungi, and protozoa (10).

In the Arabidopsis thaliana genome, there are nine predicted metacaspase genes: three of these A. thaliana genes belong to the type-I class (AtMCP1a-1c), containing predicted N-terminal prodomain consisted of a zinc finger motif followed by a proline-rich region (AtMCP1b and AtMCP1c) or by a glutamine- and proline-rich region (AtMCP1a) and a C-terminal caspase-like domain, whereas the other six type II metacaspases (AtMCP2a-2f) possess no obvious prodomain (10, 11). Our Northern blot analysis revealed that transcripts of all type-I metacaspases (AtMCP1a-1c) and two type II metacaspases (AtMCP2b and AtMCP2d) are rapidly up-regulated at a similar fashion upon infection of leaves with bacterial pathogens, suggesting their possible involvement in the activation of cell death.‡ Similarly, the up-regulation of LeMCA1, a type II metacaspase, was found in necrotrophic pathogen-infected tomato leaves, suggesting the link of this class of metacaspase to the induction of plant cell death (12). A type II metacaspase gene, mcII-Pa, in Norway spruce, was very recently shown to be involved in somatic embryogenesis, which is one model system of developmental PCD in plants (13). This metacaspase gene may encode a functional homologue of animal caspase-6 (VEIDase), because silencing of this gene resulted in a significant reduction of VEIDase activity strongly associated with the

7-amino-4-methylcoumarin; AtMCP, Arabidopsis metacaspase; Boc-GRR-MCA, tert-butyloxycarbonyl-Gly-Arg-Arg-4-methylcoumaryl-7-amide; Boc-VKL-MCA, tert-butyloxycarbonyl-Val-Leu-Arg-4-methylcoumaryl-7-amide; DAPI, 4′,6-diamidino-2-phenylindole; DHE, dihydroethidium; DHR, dihydrorhodamine 123; MCA, 4-methylcoumaryl-7-amide; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; Z-RR-2NA, benzoyloxycarbonyl-Arg-β-naphthylamide; Z-ARR-2NA, benzoyloxycarbonyl-Ala-Arg-β-naphthylamide; Z-GRR-2NA, benzoyloxycarbonyl-Gly-Gly-Arg-β-naphthylamide; Z-VKKR-2NA, benzoyloxycarbonyl-Val-Lys-Leu-Arg-β-naphthylamide; Z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; YCA1, yeast caspase-1; 2-NA, β-naphthylamide; mAb, monoclonal antibody; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; VEIDase, VEID-hydrolyzing protease.

† To whom correspondence should be addressed: Biotechnology Center for Agriculture and the Environment, Rutgers, The State University of New Jersey, 59 Dudley Rd., New Brunswick, NJ 08901-8520. Tel.: 732-932-8165 (ext. 220); Fax: 732-932-6535; E-mail: lam@sesop.rutgers.edu.

‡ Supported in part by a postdoctoral research fellowship from the JSPS.

§ To whom correspondence should be addressed: Biotechnology Center for Agriculture and the Environment, Rutgers, The State University of New Jersey, 59 Dudley Rd., New Brunswick, NJ 08901-8520. Tel.: 732-932-8165 (ext. 220); Fax: 732-932-6535; E-mail: lam@sesop.rutgers.edu.

The abbreviations used are: PCD, programmed cell death; AMC,
Initial execution phase for this developmental PCD (13). However, direct biochemical evidence for the activity of the mcl-1-Pa encoded protein is lacking.

The budding yeast Saccharomyces cerevisiae has emerged as a useful system for PCD or apoptosis studies, because it is readily amenable to genetic and molecular analysis (14, 15). Indeed, apoptosis-like cell death of yeast occurs in certain cdc48 mutant (16) or upon treatment with low doses of hydrogen peroxide (17). In addition, ectopic overexpression of pro-apoptotic factors such as Bax induces yeast apoptosis; the effect can be counteracted by the simultaneous overexpression of anti-apoptotic proteins such as Bcl-Xl (18), suggesting a striking parallelism with apoptosis in metazoans. The single metacaspase gene in *S. cerevisiae*, termed yeast caspase-1 (YCA1), was shown to mediate oxidative stress-induced and age-related apoptosis in yeast (19). Overexpression of YCA1 stimulates apoptotic cell death, whereas disruption of this gene prevents it, indicating that this metacaspase is required for apoptosis-like cell death in yeast. Interestingly, the wild-type Yca1p but not one with a point mutation at the catalytic center Cys297 residue was shown to be proteolytically activated upon oxidative stress, which correlated with the appearance of a new caspase-like activities that have similar substrate specificity with initiator caspases (caspase-6 and caspase-8). However, conclusive evidence demonstrating that Yca1p indeed exhibits caspase-like activity using purified or recombinant enzymes is still lacking. On the other hand, in Trypanosoma brucei metacaspase gene family is consisted of five different genes (TbMCA1–5) (20). Of these, heterologous expression of *T. brucei* metacaspase gene family is consisted of five different genes (TbMCA1–5) (20). Of these, heterologous expression of *T. brucei* metacaspase gene in *Saccharomyces cerevisiae* has emerged as a well characterized metacaspase gene (19, 21). Using the wild-type and yca1Δ yeast strains, we performed a detailed analysis of the activities of AtMCP1b, AtMCP2b, and Yca1p during oxidative stress-stimulated and age-related apoptosis in yeast. Furthermore, we performed an initial biochemical characterization of these metacaspases produced in yeast and bacteria, to compare their enzymatic properties, including substrate preference.

**Experimental Procedures**

Yeast Strains, Plasmids, and Transformation—Two yeast strains BY4741 (KF715) (MATa; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ0; lac:LEU2; from EUROSCARF) and yca1Δ mutant (yca1Δ:kanMX4) in the BY4741 background (KF729), and two YCA1 clones pFM21 (YCA1 with C-terminal FLAG tag in pESC-His) and pFM22 (yca1Δ:LEU2 with C-terminal FLAG tag in pESC-His) were kindly provided by Frank Madeo (University of Tubingen, Germany). The two yeast strains were used as hosts for transformation.

For generation of C-terminally V5-epitope and hexahistidine-tagged AtMCP1b, AtMCP2b, and YCA1, 199N11 (expressed sequence tag clone of AtMCP1b; accession no. IT88457), 147M2 (expressed sequence tag clone of AtMCP2b, and YCA1 construct during oxidative stress-stimulated and age-related apoptosis in yeast. Furthermore, we performed an initial biochemical characterization of these metacaspases produced in yeast and bacteria, to compare their enzymatic properties, including substrate preference.

**Experimental Procedures**

Yeast Strains, Plasmids, and Transformation—Two yeast strains BY4741 (KF715) (MATa; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ0; lac:LEU2; from EUROSCARF) and yca1Δ mutant (yca1Δ:kanMX4) in the BY4741 background (KF729), and two YCA1 clones pFM21 (YCA1 with C-terminal FLAG tag in pESC-His) and pFM22 (yca1Δ:LEU2 with C-terminal FLAG tag in pESC-His) were kindly provided by Frank Madeo (University of Tubingen, Germany). The two yeast strains were used as hosts for transformation.

For generation of C-terminally V5-epitope and hexahistidine-tagged AtMCP1b, AtMCP2b, and YCA1, 199N11 (expressed sequence tag clone of AtMCP1b; accession no. IT88457), 147M2 (expressed sequence tag clone of AtMCP2b, and YCA1 construct during oxidative stress-stimulated and age-related apoptosis in yeast. Furthermore, we performed an initial biochemical characterization of these metacaspases produced in yeast and bacteria, to compare their enzymatic properties, including substrate preference.

**Experimental Procedures**

Yeast Strains, Plasmids, and Transformation—Two yeast strains BY4741 (KF715) (MATa; his3 Δ1; leu2 Δ0; met15 Δ0; ura3 Δ0; lac:LEU2; from EUROSCARF) and yca1Δ mutant (yca1Δ:kanMX4) in the BY4741 background (KF729), and two YCA1 clones pFM21 (YCA1 with C-terminal FLAG tag in pESC-His) and pFM22 (yca1Δ:LEU2 with C-terminal FLAG tag in pESC-His) were kindly provided by Frank Madeo (University of Tubingen, Germany). The two yeast strains were used as hosts for transformation.

For generation of C-terminally V5-epitope and hexahistidine-tagged AtMCP1b, AtMCP2b, and YCA1, 199N11 (expressed sequence tag clone of AtMCP1b; accession no. IT88457), 147M2 (expressed sequence tag clone of AtMCP2b, and YCA1 construct during oxidative stress-stimulated and age-related apoptosis in yeast. Furthermore, we performed an initial biochemical characterization of these metacaspases produced in yeast and bacteria, to compare their enzymatic properties, including substrate preference.
Functional Characterization of Arabidopsis Metacaspases

Caspase-like Processing of Two AtMCPs—Most animal caspases are initially produced in cells as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. The inactive zymogens consist of an N-terminal prodomain and a large catalytic subunit (main, a large catalytic subunit of poly(ADP-ribose) polymerase). The inactive zymogens and must undergo proteolytic activation during apoptosis. The inactive zymogens consist of an N-terminal prodomain and a large catalytic subunit (main, a large catalytic subunit of poly(ADP-ribose) polymerase). The inactive zymogens and must undergo proteolytic activation during apoptosis. The inactive zymogens consist of an N-terminal prodomain and a large catalytic subunit (main, a large catalytic subunit of poly(ADP-ribose) polymerase). The inactive zymogens and must undergo proteolytic activation during apoptosis. The inactive zymogens consist of an N-terminal prodomain and a large catalytic subunit (main, a large catalytic subunit of poly(ADP-ribose) polymerase).

RESULTS

AtMCP1b and AtMCP2b Can Be Efficiently Expressed in Yeast—To facilitate a comparative analysis of two subtypes of Arabidopsis metacaspases (AtMCP1b and AtMCP2b) and Yca1p, a plasmid encoding each gene C-terminally tagged with a V5 epitope and hexahistidine sequence under the GAL1 promoter control was created. The inducible expression of each metacaspase was verified by immunoblotting using anti-V5 epitope antibody. When all metacaspases were individually expressed in the yca1Δ mutant, a dominant band of about 47 kDa (AtMCP1b), 53 kDa (AtMCP2b), and 54 kDa (Yca1p), which likely corresponded to the predicted molecular mass of each metacaspase tagged with a V5 epitope and hexahistidine (44.9, 46.7, and 52.4 kDa, respectively) were detected at 6 h after the galactose shift (Fig. 1A). In contrast, no band was detected in the control strain. In the strains expressing AtMCP2b or Yca1p, additional protein bands were also detected at this time point, suggesting the possibility of their partial proteolysis by endogenous yeast protease activities or by itself (autocatalytic processing). The levels of AtMCP1b and AtMCP2b were comparable to the level of Yca1p when expressed in yca1Δ mutant. Similar results were obtained with the wild-type yeast strain (data not shown). From these, we concluded that AtMCP1b and AtMCP2b can be efficiently expressed in yeast.

Immunoblotting—Yeast cells were lysed mechanically as described (19). Bacterial cells were lysed by sonication as described under “Enzyme Assay.” Protein concentration was determined with a Protein assay kit (Bio-Rad) using bovine serum albumin as the standard. Proteins (10–20 μg) were separated by SDS-PAGE (15%, v/v). Separated proteins were electrophotorethically transferred to a polyvinylidene difluoride membrane (0.45 μm, Immobilon-P, Millipore) with a semidry blotter (Bio-Rad). Non-specific binding was blocked with 3% (w/v) bovine serum albumin (Sigma-Aldrich) in TBST buffer (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1% (w/v) tween 20) for 1 h at room temperature. Immunoreactive polypeptides were detected using anti-V5 epitope mAb (1:5000 dilution, Invitrogen) or anti-T7 epitope mAb (1:4000 dilution, Novagen) conjugated with horseradish peroxidase and developed using the enhanced chemiluminescence kit (ECL, Amersham Biosciences). The chemiluminescence was finally detected with a photographic film (Kodak).

Enzyme Assay—To assay for metacaspase activity in crude cell extracts of galactose-induced yeast cultures (15–50 ml), the cells were broken by brief sonication, and insoluble materials were removed by centrifugation (20,000 × g, 20 min, 4 °C). The resulting supernatant was subjected to measurement of in vitro metacaspase activity (see below). To assay enzyme activity of recombinant AtMCP1b, AtMCP2b, and Yca1p produced in Escherichia coli BL21(DE3) strain (Invitrogen), the coding region of each gene, amplified by PCR, was cloned in-frame in the bacterial expression vector pET23a (Novagen), resulting in double fusion proteins (N-terminal fusion with a T7 tag and C-terminal fusion with His6). The recombinant metacaspases were induced by adding 0.4 mM isopropyl-D-galactopyranoside to cell culture with an A600 of 0.4–0.6. Then, the cell culture was shaken (260 rpm) at 28 °C for 3 h (for AtMCP2b) or 3 h (for AtMCP1b and Yca1p). Cells were harvested, washed with distilled water, and resuspended in extraction buffer (50 mM potassium phosphate, pH 7.4, 500 mM NaCl, 0.1 mM dithiothreitol, 1% (w/v) CHAPS, and 5 μg/ml aprotinin). Following cell lysis by vortexing with glass beads, the cell lysates were centrifuged (20,000 × g, 10 min, 4 °C) and the resulting supernatant was subjected to measurement of in vitro metacaspase activity (see below).

Assay of metacaspase activity in crude cell extracts of galactose-induced yeast cultures (15–50 ml), the cells were broken by brief sonication, and insoluble materials were removed by centrifugation (20,000 × g, 20 min, 4 °C). The resulting supernatants were used as active lysates for direct measurement of metacaspase activity in vitro. Alternatively, recombinant AtMCP1b proteins were purified. The soluble extracts from the recombinant AtMCP2b-expressing strain, supplemented with 20 mM imidazole, was mixed with Ni2+-chelating Sepharose FF (Amersham Biosciences) equilibrated with 20 mM imidazole in extraction buffer and incubated for 3 h at 4 °C. After washing with extraction buffer supplemented with 50 mM imidazole, bound metacaspases were eluted with 250 mM imidazole in extraction buffer. The purified samples were further passed through an NAP-10 column (Amersham Biosciences) equilibrated with sample buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 0.1 mM dithiothreitol, 1% (w/v) CHAPS, and 5 (w/v) glycerol), followed by a filtration with a 0.45 μm filter (Kodak). The immunoblotting analysis of AtMCP1b, AtMCP2b, and Yca1p tagged with V5-His6 at their C-terminus. A, extracts from yeasts strains, with plasmid encoding each metacaspase as indicated and were grown in 2% Ura media containing 2% galactose for 0 and 6 h, were immunoblotted and probed with anti-V5 antibody. B, caspase-like processing of AtMCP1b, AtMCP2b, and Yca1p during prolonged culture in yeast. Metacaspase expression in exponentially grown yca1Δ cells, transformed with the indicated constructs, was induced in SC-Ura media containing 2% galactose (t = 0). At the time indicated, aliquots were subjected to immunoblot analysis using anti-V5 antibody.
AtMCP2b might mimic the function of endogenous Yca1p as discussed previously (19). The phenotype progression of cell death were somewhat delayed compared to the wild-type background, no significant loss of cell viability was observed in all strains tested at 24 h after the galactose shift (Fig. 2A). However, after 48 h the viability of AtMCP1b or AtMCP2b-expressing cells gradually started decreasing and finally reached to <20% at 72 h in a manner resembling the effect of overexpressing YCA1 (Fig. 2A). In contrast, the viability of control cells decreased more slowly (61% at 72 h). Similar results were also obtained with yca1Δ cells (Fig. 2B). In yca1Δ background, expression of AtMCP1b or AtMCP2b could accelerate cell death progression in a similar manner to that of YCA1. However, it should be noted that the timing of cell death activation and progression of cell death were somewhat delayed compared with the wild-type background, probably due to the absence of endogenous Yca1p as discussed previously (19). The phenotype induced by AtMCP1b, AtMCP2b, and YCA1 were partially prevented by pan-caspase specific inhibitor zVAD-fmk (Fig. 2C) or by site-specific mutations with the AtMCP1bC220A, AtMCP2bC129A, or yca1ΔC297A variants (Fig. 2A, and additional data not shown), suggesting that cysteine-dependent protease activity of AtMCP1b and AtMCP2b could contribute to activation of cell death as in the case of Yca1p. Therefore, it is possible that inducible expression of AtMCP1b or AtMCP2b can partially restore a yca1Δ mutation by functionally phenocopying YCA1.

We next addressed the question of whether AtMCP1b or AtMCP2b might mimic the function of YCA1 in H2O2-mediated apoptosis. As previously reported, low external doses of reactive oxygen species stimulate induction of yeast apoptosis (17). However, yca1Δ mutant shows better survival after treatment with 1.2 mM H2O2 in comparison to the wild-type, indicating that disruption of YCA1 protected the cells from H2O2-activated apoptosis (19) (Fig. 3A). Conversely, sensitivity of yca1Δ mutant to H2O2 can be restored by overexpression of YCA1 in the mutant (19) (Fig. 3A). Treatment with 1.2 mM H2O2 for 26 h significantly affected the viability of yca1Δ cells expressing AtMCP1b (58%) or AtMCP2b (75%), but their degree of cell viability reduction was apparently lower than those of YCA1-overexpressing strain (25%). In contrast, treatment with 0.4 mM H2O2 did not cause any activation of cell death in yca1Δ mutant harboring AtMCP1b, AtMCP2b, YCA1 and the corresponding vector control after 26 h (data not shown). Furthermore, mutational analysis and inhibitor study using zVAD-fmk suggested that the phenotypes induced by AtMCP1b, AtMCP2b, and YCA1 depend on protease activities (Fig. 3). Similar experiments were also run with the wild-type strain. In the wild-type background, overexpression of YCA1 in synergy with treatment with mild oxygen stress (0.4 mM H2O2) can effectively induce cell death, whereas the vector control shows perfect viability against this treatment (~90%) (Fig. 3C). Interestingly, the degree of cell death in wild-type background induced by expression of AtMCP1b or AtMCP2b in synergy with H2O2 was very similar to the levels observed in the yca1Δ mutant background (data not shown). Therefore, these results strongly suggest that AtMCP1b or AtMCP2b can partially restore the sensitivity of yca1Δ mutant to H2O2 by functionally replacing YCA1 rather than by providing an additional stimulus from a cryptic pathway.

Expression of AtMCP1b and AtMCP2b Induce an Apoptosis-like Cell Death in Yeast—Because overexpression of YCA1 resulted in the activation of apoptosis in yeast (19), we next wanted to investigate whether the loss of viability observed in stationary yeast cells expressing AtMCP1b or AtMCP2b was also related to apoptotic events. However, to address the question of whether AtMCP1b and AtMCP2b can induce apoptosis by itself, we expressed the two AtMCPs and YCA1 in yca1Δ mutant.

One of the key events in triggering yeast apoptosis is the accumulation of ROS (17, 23–25). To detect ROS production, we
intracellular DHR fluorescence was observed by 72 and 96 h of 26 h of galactose-shift in combination with or without 1.2 mM H₂O₂ treatment. B, immunoblot analysis of AtMCP1b and AtMCP2b with or without a mutation at the predicted active site (Cys to Ala). At 26 h after galactose-shift with H₂O₂ treatment, cell extracts from each cell culture were subjected to immunoblot analysis using anti-V5 mAb. Each asterisk shows specific C-terminal fragments produced by apparent self-processing. C, effect of caspase inhibitor zVAD-fmk (20 μM) on cell death induction by metacaspases in synergy with 1.2 mM H₂O₂ treatment for 26 h. Aliquots of the cells were subjected to trypan blue-dye exclusion assay to estimate the cell viability. Data are represented as the mean cell viability (%) ± S.D. (n = 5).

Fig. 3. Expression of AtMCP1b, AtMCP2b, and YCA1 accelerates cell death in yca1Δ cells when challenged with H₂O₂. A, cell viability of yca1Δ cells expressing AtMCP1b, AtMCP2b, YCA1, or the vector control with DHR. In the presence of ROS, DHR is oxidized to fluorescent chromophore rhodamine 123, which depends on the redox status of mitochondrial membrane potential and can then be visualized under a fluorescence microscope. We monitored the formation of ROS in a time course during the early aging process until 96 h. Exponentially growing yca1Δ cells expressing AtMCP1b, AtMCP2b, YCA1, and the corresponding vector control at 24 h showed no DHR-positive fluorescence upon staining (data not shown). At 48 h, DHR-positive cells but with low fluorescence became visible in AtMCP1b-, AtMCP2b-, and YCA1-expressing cells, and strong intracellular DHR fluorescence was observed by 72 and 96 h (Fig. 4A). In contrast, control yca1Δ cells showed weaker fluorescence signal from 72 to 96 h, but the level of ROS accumulation was remarkably lower than that in AtMCP1b-, AtMCP2b-, or YCA1-expressing cells (Fig. 4A). In addition, expression of site-specific mutated variants (AtMCP1bC220A, AtMCP2bC139A, or yca1C297A) did not result in significantly increased ROS level (not shown). As shown in Fig. 4B, similar results were also obtained with another ROS indicator, DHE, which is oxidized specifically by superoxide ion (O₂⁻), and the oxidized form (ethidium) shows red fluorescence in cell cytoplasm (19). From 48 h, the ratio of ROS-accumulating cells increased in AtMCP1b-, AtMCP2b-, and YCA1-expressing strains; after 72 h, >50% cells showed strong ROS level while the control strains did not. It appears that the ratio of DHE-positive cells correlated with increased cell death in all strains (Fig. 2B). In addition, we found that the production of ROS induced by AtMCP1b, AtMCP2b, and YCA1 was partially prevented by pretreatment with zVAD-fmk (Fig. 4B). These results thus suggest that ectopic expression of each AtMCP and overexpression of YCA1 resulted in increased ROS accumulation in yca1Δ cells, which is dependent on downstream caspase-like activities, and promotes yeast apoptosis-like cell death. A positive feed-back loop thus may exist whereby activation of metacaspases by ROS could in turn trigger additional ROS production via zVAD-sensitive proteases.

Other hallmarks of apoptosis in yeast are cleavage of DNA in the nucleus and nuclear fragmentation (16, 23–25). DNA nicking can be visualized by TUNEL staining, which reveals free 3’-OH ends originated by DNA strand breaks, whereas nuclear fragmentation can be detected by DAPI staining (see “Experimental Procedures”). Fig. 4C shows the percentages of TUNEL-stained and trypan-blue staining cells over 96 h in strains expressing each metacaspase or vector control after galactose-shift without H₂O₂ treatment. Initially, ~10% of the cells expressing AtMCP1b, AtMCP2b, YCA1, or the vector control stained positive for TUNEL at 24 h. However, after 48 h populations of TUNEL-positive cells were remarkably increased and in good agreement with the populations of dead cells in each metacaspase-expressing strains but not in the vector control strain (Fig. 4C, upper panel). DAPI staining revealed that the majority of cells expressing the vector control showed a normal and single round-shaped nucleus, whereas an abundance of abnormally shaped and fragmented nuclei (50–70%) was observed in the cells expressing AtMCP1b, AtMCP2b, or YCA1 at 96 h (Fig. 4C, lower panel). In addition, expression of site-specific mutated variants (AtMCP1bC220A, AtMCP2bC139A, or yca1C297A) did not result in significantly increased TUNEL-positive cells or abnormally shaped and fragmented nuclei in yca1Δ cells (not shown). Taken together, the results strongly suggest that ectopic expression of AtMCP1b or AtMCP2b in yeast can induce an apoptotic-like cell death correlated with an increased generation of ROS in a similar manner to that of YCA1.
strates (Ac-YVAD-MCA, Ac-DEVD-MCA, Ac-VEID-MCA, and Ac-IETD-MCA). Also, these metacaspases do not show any sensitivity to caspase-specific inhibitors, including zVAD-fmk (26). Independently of this report, we also found that purified recombinant type II metacaspases (AtMCP2b and AtMCP2d) can cleave arginine/lysine-specific substrates such as Boc-GRR-MCA in a Ca\(^{2+}\)/H\(_{11001}\)-dependent manner (at sub-millimolar range), whereas they were unable to cleave caspase-specific synthetic substrates. Also, we found that the endopeptidase activity of purified recombinant AtMCP2b is strongly inhibited by two arginine protease inhibitors (leupeptin and antipain), oligopeptide inhibitor (benzloxy carbonyl-Ph-e-Lys-trimethylbenzoyloxymethylketone), and tosyl-lysyl-chloromethylketone but not aprotinin and phenylmethylsulfonyl fluoride (serine protease inhibitors), pepstatin (aspartic protease inhibitor), oligopeptide inhibitor (Z-FA-fmk), and zVAD-fmk.2

To examine whether AtMCP1b and Yca1p also have arginine/lysine-specific endopeptidase activities, we first attempted to produce active recombinant proteins as double fusions with T7 epitope-(at the N terminus) and His6-tagged (at the C terminus) in E. coli. Immunoblot analysis using anti-T7 epitope antibodies revealed that molecular masses of the recombinant AtMCP1b, AtMCP2b, and YCA1 in yeast (Fig. 5A), Bacterial extracts containing recombinant AtMCP1b or Yca1p exhibited significantly increased endopeptidase activities hydrolyzing arginine- and/or lysine-containing peptides, which are largely different in degree (1.5- to 4-fold), as compared with control extracts (Fig. 5B). However, their activities were apparently much lower than AtMCP2b (Fig. 5C). Interestingly, strict Ca\(^{2+}\) dependence of AtMCP1b and Yca1p in Boc-GRR-

---

**FIG. 4.** Expression of AtMCP1b, AtMCP2b, and YCA1 in yca1Δ cells induces an apoptosis-like cell death. **A**, detection of ROS accumulation in yca1Δ cells expressing AtMCP1b, AtMCP2b, YCA1, or the corresponding vector control during prolonged culture until 96 h after the galactose shift. Dihydrorhodamine 123 (DHR) staining was performed after 48, 72, and 96 h of galactose-shift. Accumulation of ROS visualized by DHR staining was viewed through EYFP filter set fitted to an epifluorescence microscope. Scale bar = 20 \(\mu\)m. **B**, effect of zVAD-FMK on ROS accumulation in yca1Δ cells expressing AtMCP1b, AtMCP2b, YCA1, or the corresponding vector control during prolonged culture until 72 h after the galactose shift. Dihydroethidium (DHE) staining was performed after 48 and 72 h of galactose-shift. zVAD-FMK was added in cell culture (final concentration, 20 \(\mu\)M) at 24 h after galactose shift and cultured until 72 h. Accumulation of ROS visualized by DHE staining was viewed through DsRed filter set fitted to an epifluorescence microscope. Scale bar = 5 \(\mu\)m. **C**, DAPI and TUNEL staining of yca1Δ cells expressing AtMCP1b, AtMCP2b, YCA1, or the corresponding vector control during prolonged culture until 96 h after the galactose shift. Data are represented as the mean of positive stained cells (%) \(\pm\) S.D. (n = 5). Scale bar = 5 \(\mu\)m.
MCA-hydrolyzing activity was not observed, but these activities were slightly stimulated (2- to 3-fold) under the presence of 10–100 mM Ca2+ (not shown). In addition, bacterial extracts containing recombinant AtMCP1b and Yca1p did not display any increased caspase-like activity against some of caspase-specific substrates tested, including Ac-YVAD-MCA, Ac-DEVAD-MCA, Ac-VEID-MCA, and Ac-IETD-MCA (not shown).

Our results using the bacterial expression system suggested the possibility that enzymatic activities of AtMCP1b and Yca1p are potentially lower than that of type II metacaspases, and/or recombinant AtMCP1b and Yca1p were largely inactive. Therefore, we next assessed the question of whether arginine/lysine-specific endopeptidase activities of all metacaspases examined in this study might be increased in ycaΔ cells with or without low doses of H2O2. We found that AtMCP1b or YCA1 overexpression for 24 h without additional apoptotic stimuli did not result in significant increase of arginine/lysine-specific endopeptidase activities in yeast extracts (data not shown). However, significantly increased endopeptidase activities (3- to 7-fold) toward paired basic amino acids-containing substrates (RR, ARR, GRR, and VKKR) occurred after incubation with low doses of H2O2 (Fig. 6A). In contrast, AtMCP2b expression for 24 h without additional apoptotic stimuli resulted in dramatically increased endopeptidase activities toward all substrates examined in this assay (9- to 280-fold) (Fig. 6B). In addition, we found that expression of site-specific mutated variants (AtMCP1bC1290A, AtMCP2bC1396A, or ycaIC1297A) did not result in any increased arginine/lysine-specific endopeptidase activities (not shown). Taken together, the results shown by using bacterial and yeast expression systems strongly suggest that AtMCP1b, AtMCP2b, and Yca1p have intrinsic arginine/lysine-specific endopeptidase activities.

**DISCUSSION**

Molecular phylogenetic analysis suggests that metacaspases in plants, fungi, and protozoa may be at the evolutionary root of caspases (10). Accumulating evidence suggest that a yeast metacaspase Yca1p serves to regulate yeast apoptosis triggered by some apoptotic stimuli, including oxidative stress (19), low pH (19), and high salinity stress (27). It has also become increasingly clear that there is a connection between YCA1 and age-related apoptosis in yeast cells (19, 21). Although all members of the Arabidopsis metacaspase family contain a caspase-like domain, it remains to be determined whether they are functionally related to YCA1 as a caspase-like enzyme, or have other biological functions. Here we provided evidence that both AtMCP1b and AtMCP2b, representing the two subtypes of Arabidopsis metacaspases, can partially substitute for Yca1p function in mediating oxidative stress-induced and age-related apoptosis in yeast. In yeast apoptosis, cell-death-inducing activity of AtMCP1b and AtMCP2b depends on both cysteine protease activity and caspase-like processing in a similar manner to Yca1p. To our knowledge, this is the first study experimentally implicating that the two subtypes of AtMCP and YCA1 belong to a conserved protease family that has a similar molecular function between plants and yeast. This suggests an evolutionarily-conserved relationship between PCD and the metacaspase family. However, using recombinant enzymes produced in bacteria and yeast, we also found that the two AtMCPs and Yca1p have arginine/lysine-specific endopeptidase activities but failed to cleave caspase-specific synthetic substrates under our in vitro assay conditions. Indeed, various synthetic substrates containing an arginine/lysine residue at the P1 position could be cleaved by all metacaspases examined in this study. These data, in turn, suggest that AtMCPs and Yca1p are not bona fide homologues of animal caspases.

AtMCP1b and YCA1 belong to the same type-I subtype of metacaspase gene family, whereas AtMCP2b is a type II subtype that is not found in yeast. Nevertheless, we found that AtMCP2b can also partially replace the function of YCA1 in mediating oxidative stress-induced and age-related apoptosis in yeast. However, the potency of cell-death-inducing activity of AtMCP2b seems to be lower than that of AtMCP1b and Yca1p (Fig. 3A). This difference between type I and type II metacaspases could be explained by two possibilities: one is that the prodomain of AtMCP1b and Yca1p might contain an important regulatory function to initiate apoptosis effectively in yeast, and the other is that AtMCP2b may have lower activity or different substrate specificity relative to Yca1p and AtMCP1b. Our initial characterization of enzymatic activity of the three metacaspases suggest that AtMCP1b and Yca1p have a similar preference for paired basic amino acid residues at P1 and P2 positions in RR, ARR, GRR, and VKKR substrates and apparently display similar levels of endopeptidase activity (Figs. 5 and 6). In contrast, AtMCP2b has a strong preference for a GRR substrate relative to the others, but apparent endopeptidase activity of this metacaspase against various synthetic substrates was much stronger than that of both AtMCP1b and Yca1p under our enzyme assay condition. Therefore, it seems likely that the strength of endopep-
Proteases such as caspases are synthesized as zymogens that await activation at a suitable time to initiate a cascade of subsequent proteolytic events that act to orchestrate a critical cellular event like PCD (3, 6, 7, 9, 26). The data presented in this study indicate that activation of AtMCP1b and AtMCP2b are initiated by the proteolytic removal of the small C-terminal fragment, which probably corresponds to the p10-like subunit of Yca1p (Figs. 1B and 3B). In the case of AtMCP2b, this processing did not require an additional apoptotic stimuli in yeast and bacteria, thus an autocatalytic mechanism exists (Figs. 1B and 3B). It seems likely that autocatalytic maturation of all type II Arabidopsis metacaspases (Atmc4–9) could occur in bacterial cells (26). Among these metacaspases, it was shown that site-specific processing at Arg-183 of Atmc9 is essential for the conversion of the proenzyme into mature form, resulting in generation of the p10-like subunits of Atmc9 consisting of amino acids 184–325 (∼15.4 kDa). It is noteworthy that the deduced amino acid sequence of the p10-like region shows significant similarity to other type II AtmcPs that possess a conserved putative processing site Arg or Lys. On the other hand, caspase-like processing of AtMCP1b for 24 h requires apoptotic stimuli induced by exposure to sub-lethal dose of H2O2, which is very similar with the case of Yca1p (Fig. 3B). However, this processing could occur spontaneously during prolonged culture (Fig. 1B), suggesting the existence of a certain late-limiting step for their activation in yeast. AtMCP1b contains one zinc-finger domain and one proline-rich domain at its N terminus, whereas Yca1p contains only one proline-rich domain. So far, zinc-finger proteins and proline-rich proteins have been implicated in cytoplasmic protein-protein interaction and/or binding to nuclear DNA (29–31). For example, the human zinc-finger protein (ZNF198) contains a proline-rich region that constitutes a self-association domain and confers the oligomerization of ZNF198 with fibroblast growth factor receptor 1, thereby leading to tyrosine kinase activation of this receptor (31). We speculate that the prodomain of AtMCP1b and Yca1p might mediate homomeric interactions between AtMCP1b or Yca1p, leading to their activations via local increases in their concentrations as a result of oligomerization or aggregation (19). This hypothesis is consistent with the fact that bacterial overexpression could result in AtMCP1b and Yca1p autocatalytic processing with concomitant activation (Fig. 5A). However, the process of how these type-I metacaspases are converted into the mature forms remains to be elucidated.

Enzymatic assays of AtMCP1b, AtMCP2b, and Yca1p in this study have focused only on the repertoires of some synthetic substrates and assay conditions. We found that the amount of Ca2+ required for the activation of AtMCP2b and stimulation of both AtMCP1b and Yca1p is remarkably higher than the estimated physiological concentration of Ca2+ in the cytoplasm of plant cells (32). Similarly, all plant calcium-dependent proteases that have been characterized so far require sub-millimolar levels of Ca2+ for optimal enzyme activation in vitro (33, 34). However, all type II metacaspases may not require Ca2+ as a cofactor or activator, because it appears that at least activation of Atmc9 (AtMCP2f) does not depend on the addition of Ca2+ in the assay mixture (26). Interestingly, Atmc9 has different enzymatic characteristics, including substrate specificity, sensitivity to protease inhibitors, and pH dependence in comparison with Atmc4 (AtMCP2d). Among these differences, it is noteworthy that Atmc9 has an acidic pH optimum and is inactive at pH 7.0–8.0, which is closer to the physiological cytoplasmic pH. Conversely, Atmc4, which is closely related to Atmc9, has a neutral pH optimum and is inactive under acidic pH (26). Therefore, metacaspases may have distinct enzymatic properties, including substrate specificities and requirement of specific reaction conditions. Further comparative studies will be required to scrutinize in more detail the enzyme properties of metacaspases including AtMCP1b and Yca1p by using purified enzymes.

In yeast apoptosis mediated by YCA1, activation of cell death was correlated with appearance of new caspase-like activities that have similar substrate specificity to initiator caspases (with VEID- and IETD-hydrolyzing activities) and could be abolished by a pan-caspase inhibitor zVAD-fmk (19). Several lines of evidence in the present study showed that AtMCP1b, AtMCP2b, and Yca1p do not exhibit caspase-like activity but arginine/lysine-specific endopeptidase activity in vitro (Figs. 5 and 6). Also, their activities could not be inhibited by zVAD-fmk in vitro (not shown). Nevertheless, activation of apoptosis-like cell death in AtMCP1b-, AtMCP2b-, or YCA1-expressing ycaΔ cells was effectively inhibited by pretreatment with this inhibitor (Figs. 2C and 3C), indicative of the contribution of caspase-like protease(s) in yeast apoptosis downstream of the metacaspases. In addition, it was recently reported that activation of caspase-8-like activity (VEIDase) correlated with massive cell death during embryonic pattern formation in plants. Silencing of a type II metacaspase, mcII-Pa, resulted in a significant reduction of both VEID-hydrolyzing activities and cell death (13). Together with our present findings, the above results suggest that these metacaspases may not be directly
responsible for earlier reported caspase-like activities in plants and yeast. Namely, activation of unidentified caspase-like protease(s) triggered through either direct or indirect proteolysis by a metacaspase could mediate execution of yeast apoptosis or plant PCD.

Very little is known about the identities of caspase-like protease(s) in yeast and plants. Recently two investigations identified new types of subtilisin-like proteases (named aspase-A and -B) from oats (35) and a vacuolar processing enzyme from tobacco (36) that may play important roles as caspase-like proteases in the execution of PCD in plants. Interestingly, the two oat aspases possess strong initiator caspase-like activities (caspase-6, -8, and -9) with VKMD (Val-Lys-Met-Asp)-, IETD (Ile-Glu-Thr-Asp)-, LEHD (Leu-Glu-His-Asp)-hydrolizing activity but not DEVD (Asp-Glu-Val-Asp)– and VEID-hydrolizing activity, whereas these enzymes are very sensitive to numerous caspase-specific inhibitors (35). Because aspases do not appear to be cysteine proteases, they are not caspase-like proteases in a strict sense. In contrast, the cysteine protease vacuolar processing enzyme possesses unique characteristics with a caspase-1-like activity (YVAD (Tyr-Val-Ala-Asp)-hydrolyzing activity) and the ability to cleave the peptide bond at the carboxyl side of the asparagine residue responsible for maturation of various seed proteins in protein-storage vacuoles (36). In addition, these enzymes are sensitive to a pan-caspase inhibitor, zVAD-chloromethyl ketone or zVAD-fmk. Therefore, it would be interesting to see if yeast orthologues of plant caspases in plants.

In conclusion, our present work revealed that AtMCP1b and AtMCP2b representing two subtypes of Arabidopsis metacaspases genes have a similar molecular function to that of YCA1, thus implicating a conserved link between PCD and the metacaspase family. Furthermore, results from using bacteria and yeast expression systems in this work show that metacaspase representatives examined so far are arginine/lysine-specific cysteine proteases with no obvious caspase-like activity. Currently, the integrated function of plant metacaspases and the function of each individual member remain largely unknown. Also, there is no evidence of whether the two subfamilies of plant metacaspases act in series or in parallel pathways in plant cells. Using overexpression and/or silenced (knock-out) lines of various plant metacaspases should help in determining their possible roles in the regulation of PCD in plants.

Acknowledgments—We thank Dr. Frank Madeo (University of Tubingen, Germany) for kindly providing the yeast and E. coli strains and protocols and for technical advise and useful suggestions, Drs. Koichi Watanabe and Naohiro Kato (Rutgers University) for technical advise and stimulating discussions. N. W. is grateful to Drs. Akira Isogai and Fang-Sik Che (Nara Institute of Science and Technology, Japan) for stimulating discussions at the initial stage of this work.

REFERENCES

1. Lawen, A. (2003) BioEssays 25, 888–896
2. Lam, E. (2004) Nat. Rev. Mol. Cell. Biol. 5, 305–315
3. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) Annu. Rev. Biochem. 68, 383–424
4. Los, M., Stroh, C., Janicke, R. U., Engels, I. H., and Schulze-Osthoff, K. (2001) Trends Immunol. 22, 31–34
5. Shi, Y. (2002) Mol. Cell 9, 657–670
6. Lam, E., and del Pozo, O. (2000) Plant Mol. Biol. 44, 417–428
7. Woltering, E. J., van der Bent, A., and Hoeberichts, F. A. (2002) Plant Physiol. 130, 1764–1768
8. Lam, E. (2005) Trends Cell Biol, in press
9. Woltering, E. J. (2004) Trends Plant Sci. 9, 469–472
10. Uren, A. G., O’Rourke, K., Arvindiv, L. P., Seshagiri, S., Koonin, E. V., and Dixit, V. M. (2000) Mol. Cell 6, 961–967
11. Watanabe, N., and Lam, E. (2004) Mol. Plant Pathol. 5, 65–70
12. Hoeberichts, F. A., ten Have, A., and Woltering, E. J. (2003) Planta 217, 517–522
13. Suarez, M. F., Filonova, L. H., Smertenko, A., Savchenko, E. V., Clapham, D. H., von Arnold, S., Zhivotovsky, B., and Bozhkov, P. V. (2004) Curr. Biol. 14, R339–R340
14. Jin, C., and Reed, J. C. (2002) Nat. Rev. Mol. Cell. Biol. 3, 453–459
15. Madeo, F., Engelhardt, S., Herker, E., Lehmann, N., Maldener, C., Prokosh, A., Wissing, S., and Frohlich, K.-U. (2002) Curr. Genet. 41, 208–216
16. Madeo, F., Frohlich, E., and Frohlich, K.-U. (1997) J. Cell Biol. 139, 729–734
17. Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S. J. J., Wolf, D. H., and Frohlich, K.-U. (1999) J. Cell Biol. 145, 757–767
18. Gross, A., Pilcher, K., Blachly-Dyson, E., Basso, E., Jockel, J., Bassik, M. C., Kirsonmeyer, S., and Forte, M. (2000) Mol. Cell. Biol. 20, 3125–3136
19. Madeo, F., Herker, E., Maldener, C., Wissing, S., Lachelt, S., Herlan, M., Fehr, M., Lauher, K., Sigrist, S. J. J., Wessellborg, S., and Frohlich, K.-U. (2002) Mol. Cell 9, 911–917
20. Szallies, A., Kubata, B. K., and Duszenko, M. (2002) FRS Lett. 517, 144–150
21. Herker, E., Jungwirth, H., Lehmann, K. A., Maldener, C., Frohlich, K.-U., Wissing, S., Buttnner, S., Fehr, M., Sigrist, S. J. J., and Madeo, F. (2004) J. Cell Biol. 164, 501–507
22. Xu, Q., Jurgensmeier, J. M., and Reed, J. C. (1999) Methods 17, 292–304
23. Lam, E., Pichova, A., Madeo, F., Fuchs, J., Ellinger, A., Kohwehn, S., Dawes, I., Frohlich, K.-U., and Breitenbach, M. (2001) Mol. Microbiol. 39, 1166–1173
24. Mazoni, C., Mancini, P., Verdone, L., Madeo, F., Serafini, A., Herker, E., and Falcone, C. (2003) Mol. Cell Biol. 14, 721–729
25. Fahrenkrog, B., Sauder, U., and Aeblí, U. (2004) J. Cell Sci. 117, 115–126
26. Vercaemmen, D., van de Cotte, B., De Jaeger, G., Eeckhout, D., Casteels, P., Vandepoele, K., Vandenberghe, J., Van Eenum, J., Ince, D., and Van Breusegem, F. (2004) J. Biol. Chem. 279, 45329–45336
27. Wadsog, I., Maldener, C., Prokosh, A., Madeo, F., and Adler, L. (2004) Mol. Biol. Cell. 15, 1436–1444
28. Neurath, H. (1989) Trends Biochem. Sci. 14, 268–271
29. Mackay, J. P., and Crossley, M. (1998) Trends Biochem. Sci. 23, 1–4
30. Ray, B. K., Williamson, M. P., and Sudan, M. (2000) FASEB J. 14, 231–241
31. Xiao, S., McCarthy, J. G., Aster, J. C., and Fletcher, J. A. (2000) Blood 96, 699–704
32. Bush, D. S. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 95–122
33. Reddy, A. S., Safadi, F., Feyette, J. R., and Mykles, D. L. (1994) Biochem. Biophys. Res. Commun. 199, 1089–1095
34. Safadi, F., Mykles, D. L., and Reddy, A. S. (1997) Arch. Biochem. Biophys. 348, 143–151
35. Coffer, W. C., and Wolpert, T. J. (2004) Plant Cell 16, 857–873
36. Hatsugai, N., Kuroyanagi, M., Yamada, K., Meshi, T., Teuda, S., Kondo, M., Nishimura, M., and Haru-Nishimura, I. (2004) Science 305, 855–858