

Vacuolar Processing Enzyme Is Essential for Mycotoxin-induced Cell Death in *Arabidopsis thaliana**[§]

Received for publication, April 25, 2005, and in revised form, July 25, 2005 Published, JBC Papers in Press, July 25, 2005, DOI 10.1074/jbc.M504476200

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Some compatible pathogens secrete toxins to induce host cell death and promote their growth. The toxin-induced cell death is a pathogen strategy for infection. To clarify the executioner of the toxin-induced cell death, we examined a fungal toxin (fumonisins B1 (FB1))-induced cell death of *Arabidopsis* plants. FB1-induced cell death was accompanied with disruption of vacuolar membrane followed by lesion formation. The features of FB1-induced cell death were completely abolished in the *Arabidopsis* vacuolar processing enzyme (VPE)-null mutant, which lacks all four VPE genes of the genome. Interestingly, an inhibitor of caspase-1 abolished FB1-induced lesion formation, as did a VPE inhibitor. The VPE-null mutant had no detectable activities of caspase-1 or VPE in the FB1-treated leaves, although wild-type leaves had the caspase-1 and VPE activities, both of which were inhibited by a caspase-1 inhibitor. γ VPE is the most essential among the four VPE homologues for FB1-induced cell death in *Arabidopsis* leaves. Recombinant γ VPE recognized a VPE substrate with $K_m = 30.3 \mu\text{M}$ and a caspase-1 substrate with $K_m = 44.2 \mu\text{M}$, which is comparable with the values for mammalian caspase-1. The γ VPE precursor was self-catalytically converted into the mature form exhibiting caspase-1 activity. These *in vivo* and *in vitro* analyses demonstrate that γ VPE is the proteinase that exhibits a caspase-1 activity. We show that VPE exhibiting a caspase-1 activity is a key molecule in toxin-induced cell death. Our findings suggest that a susceptible response of toxin-induced cell death is caused by the VPE-mediated vacuolar mechanism similar to a resistance response of hypersensitive cell death (Hatsugai, N., Kuroyanagi, M., Yamada, K., Meshi, T., Tsuda, S., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2004) *Science* 305, 855–858).

Programmed cell death (PCD)² is a basic physiological process that occurs under various stresses and during the development of animals

and plants. Apoptosis, a type of PCD, is an essential mechanism for eliminating unwanted cells and involves DNA fragmentation, nuclear condensation, membrane blebbing, and cell shrinkage in animals (1). The main executioners in apoptosis are caspases (aspartate-specific cysteine proteinases). Plant PCD often resembles animal apoptotic cell death with respect to some features such as DNA fragmentation and nuclear condensation (2, 3). Extensive studies have shown that plant PCD is regulated by caspase-like activities (4, 5). However, no caspase homologue has been found in the *Arabidopsis* genome (6). Plants might use other proteinases that have caspase-like activity underlying the PCD. Identification of such proteinases is essential to elucidate the molecular mechanism that operates plant PCD and to provide some insights into differences between plant and animal PCDs.

In oats (*Avena sativa*), serine proteinases exhibit caspase-like activities, and the activities increase during fungal toxin victorin-induced PCD (7). Homology searches reveal the existence of several metacaspases in plants and fungi (6). The silencing of a metacaspase gene reduced one caspase-like activities and abolished developmental cell death in Norway spruce (8). These results imply that proteinases exhibiting caspase-like activities are involved in plant PCD. We recently showed that vacuolar processing enzyme (VPE) deficiency abolishes caspase-1 activity in virus-infected tobacco leaves (9).

VPE was originally found as a cysteine proteinase responsible for maturation of seed storage proteins (10). VPEs are involved in maturation and/or activation of not only various vacuolar proteins in plants (11–15) but also lysosomal proteins in the mouse (16). VPE is synthesized as an inactive larger precursor and is self-catalytically converted into the mature form at acidic condition (pH 5.5) (17, 18). The C-terminal propeptide of *Arabidopsis* VPE functions as an autoinhibitory domain of the enzyme, and the self-catalytic removal of the C-terminal propeptide is required to generate an active enzyme (18).

The *Arabidopsis* genome has four VPE genes: α VPE, β VPE, γ VPE, and δ VPE (19, 20). These VPEs can be separated into two subfamilies: vegetative-type VPEs and seed-type VPEs. The vegetative α VPE and γ VPE are induced during senescence, wounding, and pathogen infection and may play a role in the various types of cell death (19, 21). We have found that VPE mediates virus-induced hypersensitive cell death (9). Hypersensitive cell death is accompanied by hypersensitive response and is caused by an interaction between plants and incompatible pathogens.

On the other hand, some compatible pathogens secrete toxins to kill host cells and promote pathogen growth. A fungal pathogen, *Fusarium moniliforme*, causes serious disease symptoms in maize and some other grains. The host-selective mycotoxin fumonisins B1 (FB1) is produced by the pathogen. It has been shown that the toxin induces PCD in animal and plant cells (22), but the mechanisms of the toxin-induced cell death are unclear in plants. FB1, a sphinganine analogue, inhibits ceramide synthase (sphinganine *N*-acyltransferase), which is a key enzyme in

* This work was supported by Core Research for Evolutional Science and Technology of the Japan Science and Technology Corporation, Grants-in-aid for Scientific Research 16085203 and 16657013 and 17107002, 21st Century Center of Excellence Research Kyoto University (A14) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Postdoctoral Fellowship 15008768 from the Japan Society for the Promotion of Science (to M. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1 and 2.

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² The abbreviations used are: PCD, programmed cell death; VPE, vacuolar processing enzyme; E64-d, (L-3-trans-ethoxycarbonyloxirane-2-carbonyl)-L-leucine (3-methylbutyl) amide; FB1, fumonisins B1; MCA, α -4-methyl-coumaryl-7-amide; fmk, fluoromethylketone; mVPE, mature vacuolar processing enzyme; iVPE, intermediate vacuolar processing enzyme; proVPE, precursor of γ VPE; AAL toxin, *Alternaria alternata* f. sp. *lycopersici* toxin.

sphingolipid biosynthesis (23). The sphingolipid synthesis pathway in yeast and animals is known to be a generator of a variety of signals involved in maintaining cellular homeostasis, including cell death (22). It has not yet been clarified whether the signals produced by the sphingolipid synthesis are involved in plant cell death. FB1-induced cell death in *Arabidopsis* protoplasts is light-dependent and requires phytohormones, salicylate, and jasmonate- and ethylene-mediated signaling pathways (24). It seems that toxin-induced cell death is triggered by some complicated signaling pathways.

Toxin-induced cell death is a pathogen strategy for infection, whereas hypersensitive cell death is a plant defense strategy against pathogen attack. The goal of this study was to determine what is an executioner of toxin-induced cell death. Here we report that VPE, which exhibits caspase-1 activity, is also involved in a fungal toxin-induced cell death in the same way that it is involved in cell death during hypersensitive response.

EXPERIMENTAL PROCEDURES

***Arabidopsis vpe* Mutants**—Four single *vpe* mutants of *Arabidopsis thaliana* ecotype Columbia (Col-0) were used (supplemental Fig. 1A). All of *avpe-3* (Syngenta Biotechnology), *γvpe-1* (14), and *δvpe-1* (25) are t-DNA insertion mutants. *βvpe-5* has a GA insertion in the first exon (14).

Generation of a VPE-null (Quadruple) Mutant of Four VPE Genes—We generated a VPE-null mutant from four homozygous single *vpe* mutants of *A. thaliana* ecotype Columbia (Col-0); *avpe-3*, *βvpe-5*, *γvpe-1*, and *δvpe-1* (supplemental Fig. 1A). *avpe-3* was crossed with *γvpe-1*, and *βvpe-5* was crossed with *δvpe-1*. We isolated two double mutants (*avpe-3 γvpe-1* and *βvpe-5 δvpe-1*) from F2 progeny by PCR-based genotyping. We crossed the double mutants with each other and isolated a VPE-null mutant (*avpe-3 βvpe-5 γvpe-1 δvpe-1*). We used the primers for genotyping of *γvpe-1* and *δvpe-1* as described previously (Refs. 14 and 25, respectively). The primers for the genotyping of *avpe-3* were used as follows: *αVPE*-HtRV, 5'-CGAAGCTTATGCCA-GAAATGGACAA-3', and LB3-1, 5'-TAGCATCTGAATTCATA-ACCAATCTCG-3'. To identify the *βvpe-5* genotype, we used the cleaved amplified polymorphic sequence (CAPS) method. A DNA fragment was amplified by PCR with the genome DNA and the following primers: *βVPE*-scrF, 5'-TTCTCGCTGCCATGGCTAAG-3', and *β5*-CAPS, 5'-CACCATCTCTTACACGATAGAT-3'. The PCR product was digested with BsmAI.

Reverse Transcription-PCR—Total RNA was extracted from leaves with an RNeasy plant mini kit (Qiagen) and was treated with DNase. The RNA (0.5 μg) was subjected to reverse transcription with SuperScript II (Invitrogen) in a 20-μl reaction volume containing oligo(dT)₁₂₋₁₈ primer (Invitrogen). The reaction (0.4 μl) was used for PCR in a 40-μl solution with TaKaRa Ex Taq polymerase (Takara). We used each primer set for *γVPE* (14) and *ACT2* (actin 2) (26). We also used each primer set as follows: *αVPE* (sense, 5'-TCTAGAATGACC-ACCGTCGTTTCCTTTCTCG-3', and antisense, 5'-AGATCTTCA-AGCACTGAATCCAC-3'), *βVPE* (sense, 5'-TCTAGAATGGCTAAGTCTTGCTATTTTCAAGAC-3', and antisense, 5'-AGATCTTCAAGCCTATAGCCTAAGAT-3'), and *δVPE* (sense, 5'-ATGTCTAGTC-CTCTTGGTCA-3', and antisense, 5'-GTTTTGCAAATCATTACA-TCGAACAAGCT-3'). PCR was run for 25 cycles with an annealing temperature of 55 °C (*VPEs*) or 60 °C (*ACT2*).

FB1 Treatment—Seeds were sown onto 0.4% agar that contained Murashige-Skoog medium and 1% sucrose and were grown at 22 °C under continuous light. Detached leaves from 5-week-old plants were infiltrated with FB1 (10 μM) in 0.1% methanol as an elicitor to trigger cell death. Methanol used as a solvent of FB1 caused little effect on the lesion

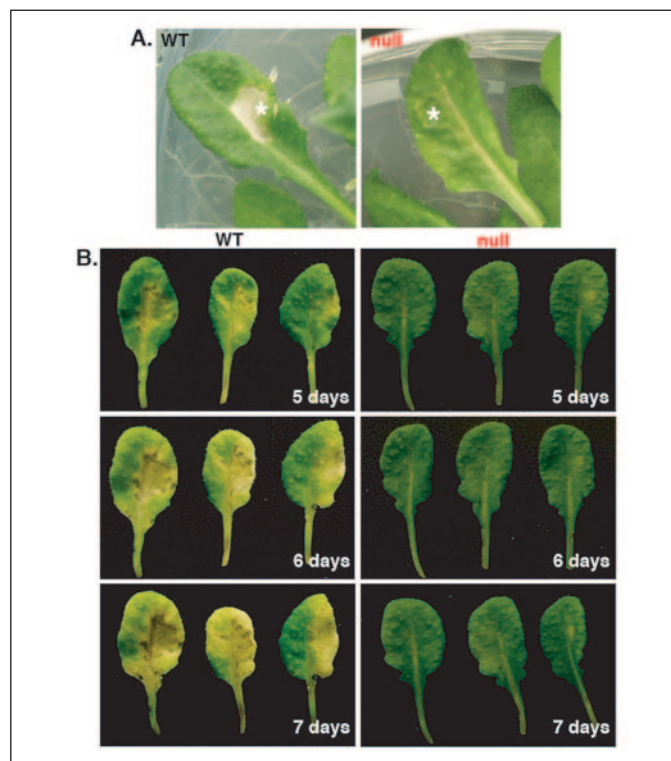


FIGURE 1. VPE deficiency suppresses FB1-induced lesion formation in *Arabidopsis* leaves. A, FB1 was infiltrated into the leaves of wild-type (WT) and VPE-null mutant (*null*) plants that were grown on agar plates. The plants were photographed at 5 days after infiltration. Asterisks, FB1 infiltration sites. B, FB1 was infiltrated into the right halves of the detached leaves of wild-type and VPE-null mutant plants. The developmental changes in the lesion on the leaves were monitored from 5 to 7 days after the infiltration.

formation (supplemental Fig. 1, B and C). The leaves were incubated at 22 °C under 12-h light/12-h dark condition to induce lesion formation as described elsewhere (27). Alternatively, for preparation of RNA, electron microscopy, pulsed-field gel electrophoresis (supplemental Fig. 2), and cellular electrolyte leakage, we used leaves detached from 4-week-old plants. To examine the effects of proteinase inhibitors, we infiltrated a VPE inhibitor (Ac-ESEN-CHO, Peptide Institute) and a caspase-1 inhibitor (biotin-YVAD-fluoromethyl ketone (fmk), Calbiochem) into leaves together with FB1.

Recombinant *Arabidopsis* γ VPE—Sf21 insect cells expressing γ VPE were prepared as described previously (18). The cells were incubated on ice for 1 h in either a neutral solution (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) to obtain the proprotein precursor of γ VPE or an acidic solution (50 mM sodium acetate, pH 5.5, 50 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) to obtain both intermediate and mature forms of γ VPE, as described previously (18). We purified these forms of γ VPE partially.

Enzyme Assay—The recombinant γ VPE (1.6–4.0 μg) was preincubated in 100 mM sodium acetate, pH 5.5, and 100 mM dithiothreitol before adding each fluorogenic substrate of Ac-ESEN- α -(4-methyl-coumarinyl-7-amide) (MCA), Ac-ESED-MCA, Ac-YVAD-MCA, and Ac-DEVD-MCA (Peptide Institute). An increase of the fluorescence was measured at 460 nm with a fluorescence spectrophotometer (RF-5000, Shimadzu). K_m values were determined by using a 80–400 μM concentration of each substrate.

To determine the effects of various inhibitors, we used the recombinant γ VPE and each inhibitor (20 μM): a VPE inhibitor (Ac-ESEN-CHO (Peptide Institute)), three caspase-1 inhibitors (biotin-XVAD-fmk and biotin-YVAD-chloromethylketone (Calbiochem) and Ac-YVAD-CHO (Peptide Institute)), a caspase-3 inhibitor (Ac-DEVD-CHO (Peptide

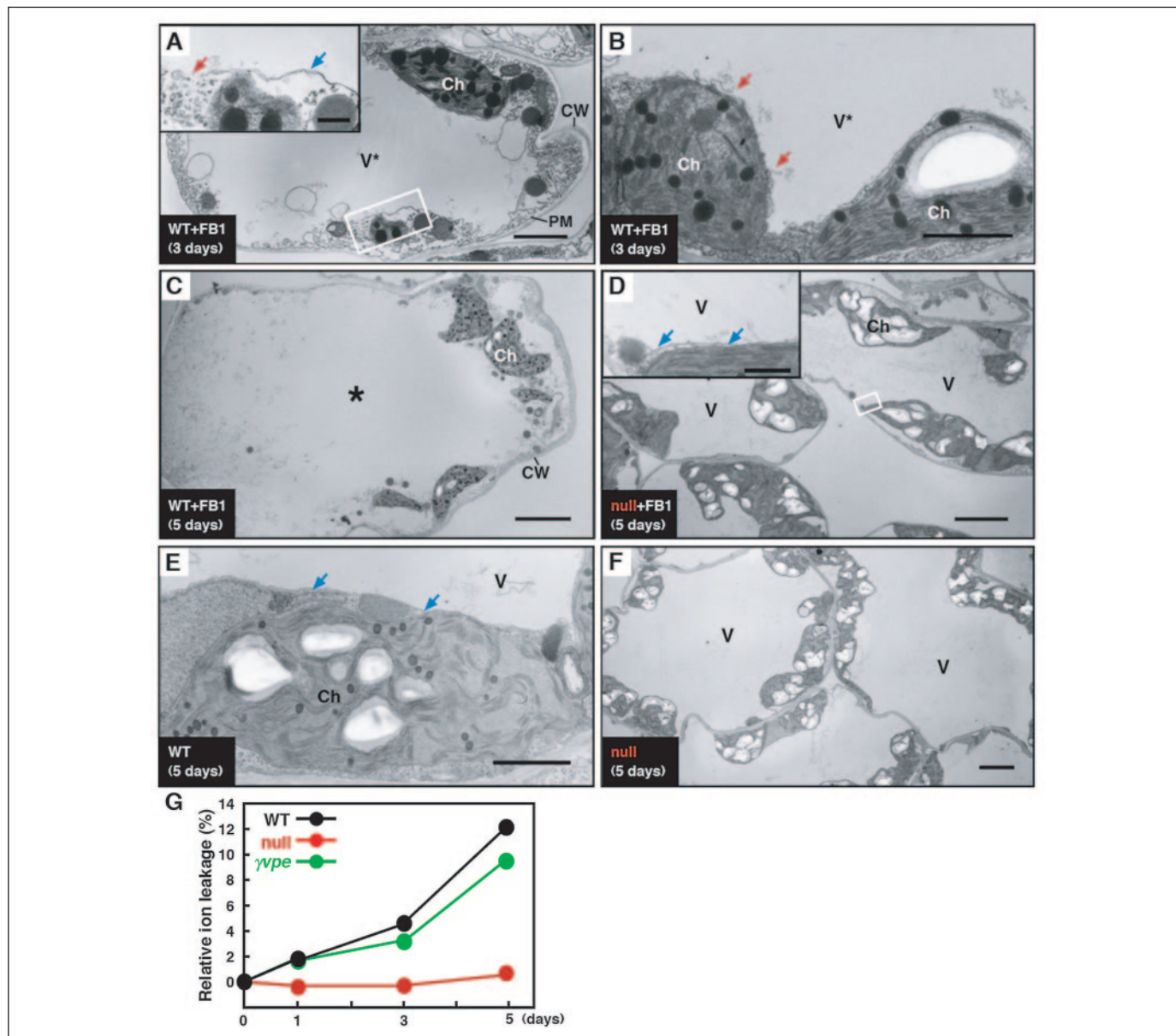


FIGURE 2. VPE deficiency suppresses vacuolar collapse leading to FB1-induced cell death. A–F, electron micrographs of the leaves of wild-type (WT) and VPE-null mutant (*null*) at 3 and 5 days after the FB1 infiltration (+FB1) or no infiltration. Insets in A and D show a more highly magnified view of each boxed area. Blue arrows, intact part of vacuolar membrane; red arrows, disrupting part of vacuolar membrane; V, vacuole; V*, disrupting vacuole; *, a cell with no vacuolar membrane; Ch, chloroplast; CW, cell wall; PM, plasma membrane. Scale bars, 2 μ m (A, B, E); 5 μ m (C, D, F); and 0.5 μ m (insets in A and D). G, ion leakage from the wild-type, VPE-null mutant, and γ vpe single mutant (γ vpe) leaves was measured after FB1 infiltration ($n = 12$).

Institute)), and a cysteine proteinase inhibitor (E64-d ((L-3-trans-ethoxycarbonyloxirane-2-carbonyl)-L-leucine (3-methylbutyl) amide) (Peptide Institute)). Each experiment was repeated three times, and the standard errors were obtained.

We prepared the leaf extract in 100 mM sodium acetate, pH 5.5, containing 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 100 μ M E64-d and measured the VPE activity against benzylloxycarbonyl-AAN-MCA (1 mM, Peptide Institute) with a fluorescence microplate reader (GENios, TECAN). Each experiment was repeated three times, and the standard errors were obtained.

Inhibitor Blot and Immunoblot—Sample solutions containing the recombinant γ VPE were incubated with 20 μ M biotin-XVAD-fmk. The resulting enzyme-inhibitor complex was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (0.22 μ m, Nihon Millipore). The membrane was treated with a blocking solution and then with 20 μ M streptavidin-conjugated horseradish peroxidase

(Amersham Biosciences) for 1 h. Detection was performed with an enhanced chemiluminescence kit (ECL system, Amersham Biosciences). Immunoblot analysis was essentially as described previously (18).

Electron Microscopy—FB1-infiltrated leaves of wild-type and VPE-null mutant plants were vacuum-infiltrated for 1 h with a fixative, dehydrated, and embedded in Epon. Thin sections were stained with 4% uranyl acetate and lead citrate and were examined with a transmission electron microscope (model 1200EX, JEOL).

Detection of Ion Leakage—Cellular electrolyte leakage was measured with an electrical conductivity meter (B-173, Horiba, Japan) as described elsewhere (28).

RESULTS

Arabidopsis VPE Is Involved in FB1-induced Cell Death—FB1 is a fungal toxin produced by the plant pathogen *F. moniliforme*, which has

been shown to induce PCD of the cells of *Arabidopsis* leaves (24, 27). We treated *Arabidopsis* leaves of wild-type plants with FB1. A typical lesion showing cell death was formed on the leaves 5 days after FB1 infiltration (Fig. 1A, WT). To clarify whether VPEs are essential for the FB1-induced cell death, we generated an *Arabidopsis* mutant (VPE-null mutant) that lacks all four VPE genes ($\alpha vpe-3$, $\beta vpe-5$, $\gamma vpe-1$, $\delta vpe-1$) (supplemental Fig. 1A). There is no difference in development and growth between the VPE-null mutant plants and wild-type plants. No typical lesion was formed on the VPE-null mutant leaves 5 days after FB1 infiltration (Fig. 1A, null). The lesions on the wild-type leaves further developed from 5 to 7 days leading to cell death (Fig. 1B, WT). Interestingly, no lesions formed on the leaves of the VPE-null mutant plants even at 7 days after FB1 infiltration (Fig. 1B, null). Our results indicate that VPE is involved in FB1-induced cell death.

VPE Deficiency Suppresses the Disruption of Vacuolar Membranes during FB1-induced Cell Death—Cell death after FB1 infiltration was monitored by measuring cellular electrolyte leakage. A significant ion leakage from the cells was found in the wild-type leaves at 3 and 5 days (Fig. 2G, WT), indicating that FB1-induced cell death started in the leaves. This was followed by typical lesion formation at 5 days. We examined the morphological changes in the cells of the leaves at 3 and 5 days after the FB1 infiltration. At 3 days, the vacuolar membranes were partially disrupted in the leaves and formed some membrane blebs (Fig. 2, A and B, red arrows), whereas the plasma membranes, cell walls, and other organelles remained ultrastructurally intact (Fig. 2, A and B). Normally, turgor pressure forces organelles close to the plasma membrane, but in the FB1-infiltrated leaves, the organelles were separated from the plasma membrane, indicating a loss of turgor pressure in the cells. At 5 days, the vacuolar membranes and plasma membranes were completely disrupted, and the other organelles were loosely distributed in the cell (Fig. 2C).

In contrast to the wild-type leaves, the VPE-null-mutant leaves showed no ion leakage at 3 days or even 5 days after FB1 infiltration (Fig. 2G, null). In the null mutant leaves, vacuoles and vacuolar membranes remained intact at 3 days (data not shown), and even at 5 days after FB1 infiltration (Fig. 2D, blue arrows), and intact organelles were located close to the plasma membrane by turgor pressure (Fig. 2D). This was also the case in the untreated leaves of the wild-type and the null mutant plants (Fig. 2, E and F). These results indicate that VPE deficiency suppresses cell death by preventing the vacuoles from collapsing. Vacuolar membrane fragmentation that is triggered by VPE in toxin-induced cell death is supported by our previous results on fluorescent dye staining of protoplasts from virus-infected tobacco leaves (9). FB1-induced cell death was accompanied by the DNA fragmentation (supplemental Fig. 2). This result suggested that VPE is involved in the DNA fragmentation into ~50 kb by way of vacuolar disruption, leading to cell death. Toxin-induced cell death and hypersensitive cell death may share the VPE-mediated vacuolar mechanism.

There is evidence that caspase-like activities are involved in various types of plant cell death, including the hypersensitive response (4, 29). To determine whether VPE and caspase-like activities are involved in the FB1-induced cell death in *Arabidopsis* plants *in vivo*, we infiltrated FB1 together with each proteinase inhibitor into the right halves of the leaves and examined the lesion formation on the leaves at 4 days after the infiltration. Significant lesions formed on the leaves treated with no proteinase inhibitor (Fig. 3A, left). A VPE inhibitor abolished completely such FB1-induced lesion formation on the leaves (Fig. 3A, middle). This is consistent with the result using the VPE-null mutant (Fig. 1). Similarly, a caspase-1 inhibitor abolished lesion formation (Fig. 3A, right). These results indicate that both VPE and caspase-1 activities are involved in the FB1-induced cell death.

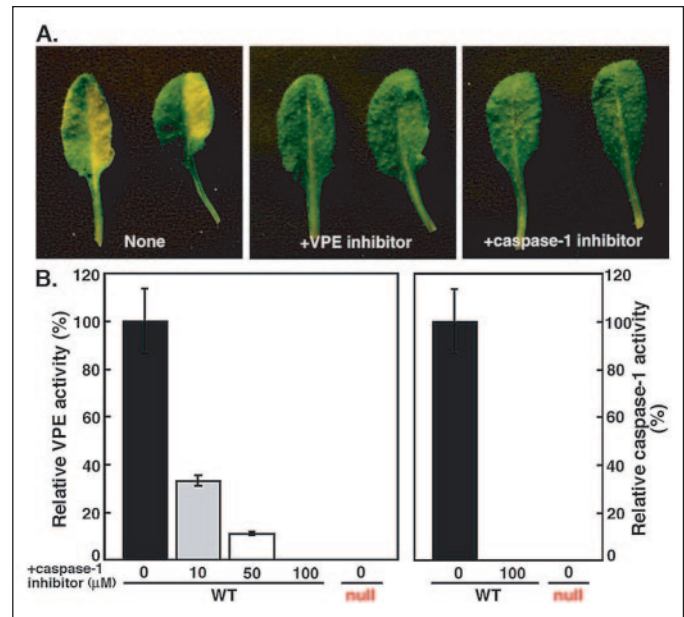


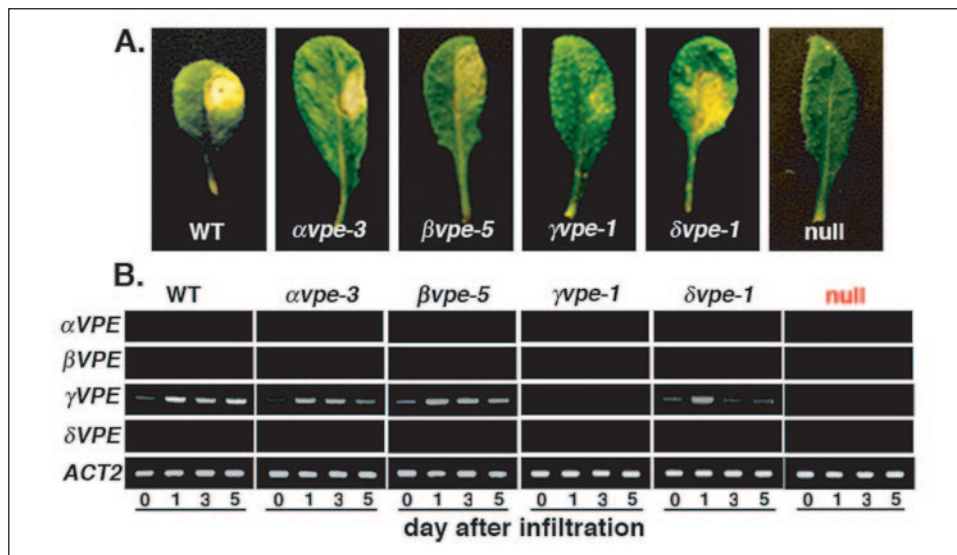
FIGURE 3. Both a VPE inhibitor and a caspase-1 inhibitor suppress FB1-induced lesion formation. A, FB1 was infiltrated into the right halves of the leaves of a wild-type leaf together with or without 1 mM cysteine proteinase inhibitors, a VPE inhibitor (Ac-ESEN-CHO), or a caspase-1 inhibitor (biotin-YVAD-fmk). The leaves were photographed at 4 days after infiltration. B, VPE activity (left) and caspase-1 activity (right) in the wild-type (WT) and VPE-null mutant (null) leaves at 1 day after FB1 infiltration. We also analyzed the effect of the caspase-1 inhibitor on both activities of the wild-type leaves.

We recently showed that VPE has caspase-1 activity in tobacco mosaic virus-induced cell death in tobacco plants (9). However, weak VPE and caspase-1 activities were detected in the VPE-silenced tobacco plants. To confirm that *Arabidopsis* VPE has caspase-1 activity, both VPE and caspase-1 activities were measured in the extracts from the FB1-treated leaves of the wild-type and VPE-null mutant. We found both activities in wild-type extracts (Fig. 3B), and the caspase-1 inhibitor reduced the VPE activity by 67% at a concentration of 10 μM and inhibited it completely at a concentration of 100 μM (Fig. 3B, left). The caspase-1 inhibitor also completely inhibited the caspase-1 activity at a concentration of 100 μM (Fig. 3B, right). Interestingly, neither VPE nor caspase-1 activity was detected at all in the VPE-null mutant (Fig. 3B). This result indicates that VPE is the proteinase that exhibits caspase-1 activity.

γ VPE Functions Primarily in FB1-induced Cell Death in *Arabidopsis* Leaves—To determine which VPE among the four VPE homologues is the most essential for toxin-induced cell death, we examined FB1-induced lesion formation on the leaves of each *vpe* single mutant. Three single mutants, $\alpha vpe-3$, $\beta vpe-5$, and $\delta vpe-1$, formed lesions like those on the wild-type plant (Fig. 4A). On the other hand, the $\gamma vpe-1$ mutant suppressed lesion formation (Fig. 4A). However, the suppression was not as strong as the VPE-null mutant plant. At 8 days after FB1 treatment, there was little difference in the lesion formation between the $\gamma vpe-1$ mutant and wild-type plant (data not shown). γ VPE deficiency may delay the lesion formation. We found the levels of ion leakage from the γvpe leaves at 3 and 5 days after FB1 infiltration were lower than those from the wild-type leaves and were higher than those from the VPE-null mutant leaves (Fig. 2G).

The suppression level of lesion formation in γvpe leaves was higher than that of ion leakage. This result suggests that lesion formation needs a larger amount of VPE than ion leakage. It has been shown that VPE triggers vacuolar collapse (9), and the vacuolar collapse directly causes ion leakage. On the other hand, lesion formation is caused indirectly by a vacuolar proteinase (VPE)-triggered vacuolar collapse. The lesion for-

FIGURE 4. γ VPE is the most essential for FB1-induced lesion formation among four VPEs. A, FB1 was infiltrated into the right halves of the detached leaves of wild-type (WT) and various *vpe* mutants including *α vpe-3*, *β vpe-5*, *γ vpe-1*, *δ vpe-1*, and the VPE-null mutant (*null*). The leaves were photographed at 6 days after the infiltration. B, reverse transcription-PCR showing changes in the mRNA levels of α VPE, β VPE, γ VPE, δ VPE, and ACT2 (actin) in the FB1-infiltrated leaves of wild-type and various *vpe* mutants described in A. The same results were obtained in three independent experiments.



mation is visualized by chlorophyll degradation. The vacuolar collapse may release vacuolar hydrolytic enzymes, which cause degradation of chloroplasts and chlorophylls. These results indicate that cell death is delayed in the *γ vpe* leaves and does not occur in the VPE-null mutant leaves. It is possible that the other VPEs partly compensate for the deficiency in γ VPE in *γ vpe* leaves (discussed below).

γ VPE mRNA was significantly induced in FB1-treated leaves of wild-type and three single *vpe* mutants (*α vpe-3*, *β vpe-5*, and *δ vpe-1*) (Fig. 4B), which formed FB1-induced lesions (Fig. 4A). In contrast, γ VPE mRNA was not induced in the treated leaves of *γ vpe-1* and VPE-null mutants (Fig. 4B), which did not form the lesions (Fig. 4A). This result suggests that lesion formation is coupled with γ VPE gene expression in the FB1-treated leaves. γ VPE mRNA increased rapidly at 1 day after FB1 infiltration in the leaves (Fig. 4B), and then the vacuoles in the leaves started to collapse around 3 days after FB1 infiltration (Fig. 2, A and B). The expression of γ VPE preceded the vacuolar membrane disruption in the cells. Thus, γ VPE primarily functions in FB1-induced cell death.

Recombinant γ VPE Exhibits a Caspase-1 Activity—To determine whether γ VPE has caspase-1 activity, we expressed the precursor of γ VPE in insect cells. The recombinant γ VPE exhibited activities toward a caspase-1 substrate (Ac-YVAD-MCA, $K_m = 44.2 \mu\text{M}$) and a VPE substrate (Ac-ESEN-MCA, $K_m = 30.3 \mu\text{M}$) but not toward a caspase-3 substrate (Ac-DEVD-MCA) or a derivative of a VPE substrate (Ac-ESED-MCA), as summarized in Fig. 5A. The K_m value for the caspase-1 substrate ($44.2 \mu\text{M}$) is comparable with the values reported for mammalian caspase-1 (11 and $23 \mu\text{M}$) (30, 31). Previously, we reported that VPE is a cysteine proteinase with a substrate specificity toward aspartic acids (32) similar to the substrate specificity of caspases (33), although VPE is an asparaginyl endopeptidase (34). We also found that VPE activity and caspase-1 activity were similarly reduced by both a VPE inhibitor (Ac-ESEN-CHO) and caspase-1 inhibitors (Ac-YVAD-CHO, biotin-XVAD-fmk, and biotin-YVAD-chloromethylketone) (Fig. 5B). This result suggests that the substrate pocket of VPE is similar to the Asp pocket of caspase-1. The K_i value of biotin-XVAD-fmk determined for the VPE substrate was 0.1 nM , and that for the caspase-1 substrate was 3.0 nM (Fig. 5A). These values are consistent with the K_i values reported for human and mouse caspase-1 (0.76 and 3.0 nM , respectively) (35). E64-d, a cysteine proteinase inhibitor, did not inhibit either VPE or caspase-1 activity (Fig. 5B). This result is consistent with the finding that caspases are insensitive to E64-d.

As shown in Fig. 5C (upper panel), a precursor of γ VPE (lane 1, proVPE) was self-catalytically converted into an intermediate form

(lane 3, iVPE) and then into the mature form (lane 5, mVPE). To clarify whether each form of γ VPE binds to biotin-XVAD-fmk, we subjected each of proVPE, iVPE, and mVPE to an inhibitor blot analysis. Both iVPE (Fig. 5C, lower panel, lane 10) and mVPE (Fig. 5C, lower panel, lane 12) bound to the inhibitor, whereas the proVPE (Fig. 5C, lower panel, lane 8) did not bind. This indicates that iVPE and mVPE have caspase-1 activity but proVPE does not. It has been shown that a procaspase-1 is self-catalytically converted into the mature enzyme (36). Therefore, VPE has characteristics similar to those of caspase-1.

DISCUSSION

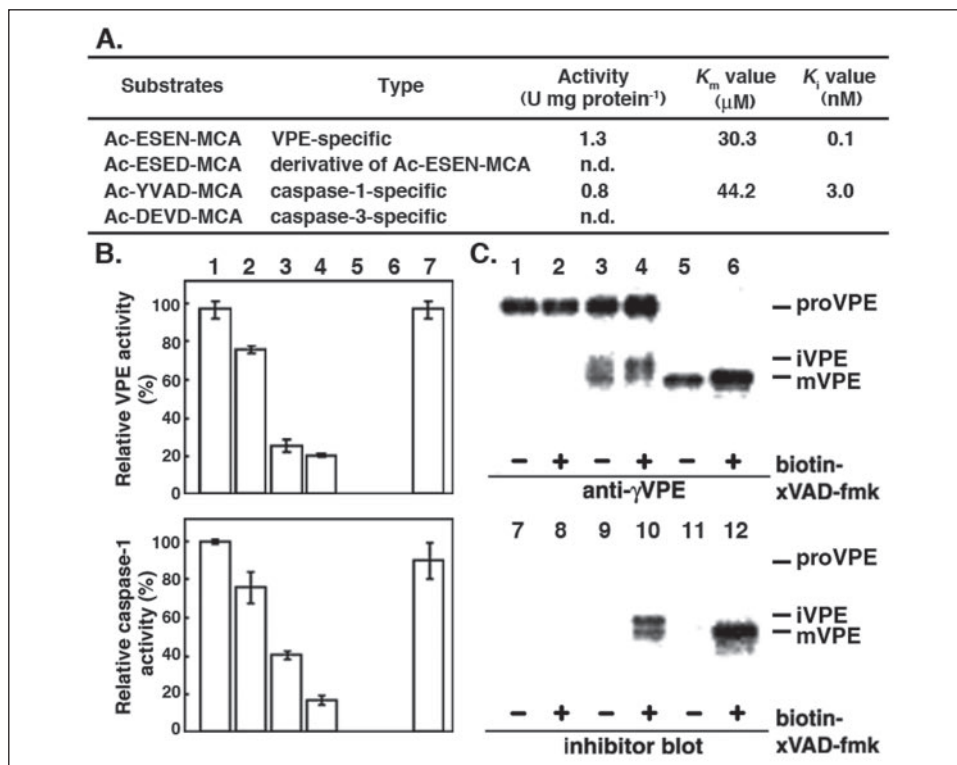
Previously, we detected both VPE and caspase-1 activities in virus-infected tobacco leaves and found that the level of VPE activity completely paralleled that of the caspase-1 activity in each VPE gene-silenced plant (9). This result suggested that VPE is responsible for caspase-1 activity. However, we were unable to prove that VPE is the only proteinase with caspase-1 activity in plants because VPE gene expression was not completely suppressed in the VPE gene-silenced tobacco leaves. To overcome this difficulty in the current study we generated an *Arabidopsis* VPE-null mutant, which lacks all four VPE genes in the genome and used it to demonstrate *in planta* that VPE is responsible for caspase-1 activity. In addition, a recombinant VPE was found to have an enzymatic activity toward the caspase-1 substrate ($K_m = 44.2 \mu\text{M}$), which is comparable with mammalian caspase-1. These are the first *in vivo* and *in vitro* demonstrations that VPE exhibits a caspase-1 activity similar to that of mammalian caspase-1.

VPE resembles mammalian caspase-1 in other ways. The active site (EACE) of VPE is similar to that of human caspase-1 (QACR). Both enzymes are cysteine proteinases, and both are synthesized in a precursor form and then self-catalytically converted into the active enzymes (18, 36). Neither VPE nor mammalian caspase-1 is inhibited by the papain-type cysteine proteinase inhibitor E-64.

The two enzymes also have some differences. First, the entire amino acid sequence of *Arabidopsis* γ VPE exhibits a very low identity (21%) with that of human caspase-1 (36, 37). Second, the optimum pH of VPE (5.5) is lower than that of human caspase-1 (7.5) (38). This is consistent with the different subcellular localizations of these enzymes: VPE is localized in vacuoles, which are acidic compartments (19), and caspase-1 is localized in the cytosol (38).

There are extensive studies showing that caspase-like activities are involved in plant PCD (4, 5). However, most caspase-1 activities in plants were very low compared with the caspase activities in mammals

FIGURE 5. The recombinant γ VPE has caspase-1 activity. A, the proteinase activity toward the indicated substrates was measured with recombinant *Arabidopsis* γ VPE that was expressed in insect cells (see "Experimental Procedures"), to determine K_m and K_i values. Biotin-XVAD-fmk was used for the determination of K_i values. n.d., not detected. B, effects of various inhibitors on VPE activity (upper) and caspase-1 activity (lower). Numbers at the top of each lane represent each inhibitor used (20 μ M except for lane 3): 1, none; 2, Ac-ESEN-CHO; 3, Ac-ESEN-CHO (100 μ M); 4, Ac-YVAD-CHO; 5, biotin-XVAD-fmk; 6, biotin-YVAD-chloromethylketone; 7, E64-d. C, an inhibitor blot of each form of γ VPE with a caspase-1 inhibitor. The recombinant proVPE (lanes 1, 2, 7, 8) was converted into iVPE (lanes 3, 4, 9, 10) and then into mVPE (lanes 5, 6, 11, 12). Each form of γ VPE was incubated in the absence or presence of the caspase inhibitor (biotin-XVAD-fmk) and was subjected to SDS-PAGE followed by either an immunoblot with anti- γ VPE antibodies (lanes 1–6) or an inhibitor blot with streptavidin-conjugated horseradish peroxidase (lanes 7–12).



(39, 40). The low activities might have been the result of their being measured at neutral pH, which is used for mammal caspases. In this study, we measured the activities of both VPE and caspase-1 at pH 5.5 and obtained high levels of these activities. We reported previously that VPE with an acidic optimum pH exhibits little activity at neutral pH (18). Considering that VPE is a proteinase exhibiting caspase-1 activity, the caspase-1 activity should be measured at acidic pH. The activities that were measured at neutral pH do not reflect the full activities in the cells.

The acidic optimum pH of VPE is in agreement with the localization of VPE in vacuoles. This study and our previous study (9) showed that VPE-mediated cell death induces vacuolar disruption. Vacuolar disruption has been shown to trigger degradation of the cytoplasmic structures during the differentiation of tracheary elements, leading to cell death (41). The vacuolar disruption-triggered cell death is unique to plants and has not been seen in animal cell death (42). Our findings show that VPE is a key molecule in the molecular mechanism underlying plant-specific cell death.

FB1 is a competitive inhibitor of ceramide synthase, which is responsible for sphingolipid biosynthesis (22) and induces plant PCD through a process of disruption of sphingolipid biosynthesis, although the molecular mechanism is unclear. Sphingolipid is abundant in vacuolar membranes (43). It is possible that abolishing ceramide synthesis causes the loss of integrity of the vacuolar membrane. Therefore, modulation of the lipid composition of vacuolar membranes might lead to VPE-triggered vacuole collapse.

The fungus *Alternaria alternata* f. sp. *lycopersici* produces AAL-toxin, a chemical congener of FB1 (44). AAL-toxin was shown to induce PCD in tomato plants (45). AAL-toxin-induced cell death was suppressed in tomato plants by overexpressing anti-apoptotic baculovirus p35 (46), which is a caspase inhibitor. We found that the recombinant p35 inhibited VPE activity *in vitro*.³ AAL-toxin-induced cell death might be mediated by VPE, as is the case with FB1-induced cell death.

This suggests that VPE is involved commonly in fungal toxin-induced cell death in plants.

Toxin-induced cell death is a necrotrophic pathogen strategy for infection, whereas hypersensitive cell death is a plant defense strategy against pathogen attack. Apparently these processes are different from each other. However, our results show that toxin-induced cell death as a pathogen strategy (this study) and the hypersensitive cell death as a plant defense strategy (9) are both mediated by VPE.

The expression patterns of the VPE genes in different plant tissues (19, 21) suggest that VPE is involved in various types of plant cell death. We recently found that *Arabidopsis* δ VPE, which exhibits caspase-1 activity, is involved in the cell death of two cell layers during embryogenesis, the purpose of which is to form a seed coat (25). The developmental cell death, as well as pathogen-induced cell death, is regulated by VPE. The results suggest that the VPE-mediated cell death system is a common machinery of plant cell death.

A reverse genetic analysis is a powerful way to elucidate how VPE functions in cell death. However, it should be noted that a single γ vpe mutant exhibits a weak and leaky phenotype on cell death. Neither lesion formation (Fig. 4) nor ion leakage (Fig. 2G) was suppressed in the γ vpe leaves as strongly as in the VPE-null mutant. Also, a γ vpe single mutant exhibited no difference from wild-type plants in lesion formation on *Pseudomonas syringae*-infected leaves, despite some differences in bacterial growth (40). Other VPEs may compensate for the deficiency in γ VPE in γ vpe mutant. Previously, we reported that α VPE and γ VPE compensate for the lack of β VPE in β vpe mutant (14). Further studies with a VPE-null mutant are needed to demonstrate that VPE is involved in the cell death.

Acknowledgments—We thank Syngenta Biotechnology for donating *avpe-3*. We are grateful to Prof. Masashi Tazawa (Fukui University of Technology) for valuable advice and Dr. Ryoichi Tanaka (Hokkaido University) for advice on the measurement of ion leakage.

³ M. Kuroyanagi and I. Hara-Nishimura, unpublished data.

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