VPE\textsubscript{\gamma} Exhibits a Caspase-like Activity that Contributes to Defense against Pathogens

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Summary

Background: Caspases are a family of aspartate-specific cysteine proteases that play an essential role in initiating and executing programmed cell death (PCD) in metazoans. Caspase-like activities have been shown to be required for the initiation of PCD in plants, but the genes encoding those activities have not been identified. VPE\textsubscript{\gamma}, a cysteine protease, is induced during senescence, a form of PCD in plants, and is localized in precursor protease vesicles and vacuoles, compartments associated with PCD processes in plants.

Results: We show that VPE\textsubscript{\gamma} binds in vivo to a general caspase inhibitor and to caspase-1-specific inhibitors, which block the activity of VPE\textsubscript{\gamma}. A cysteine protease inhibitor, cystatin, accumulates to 20-fold higher levels in vpe\textsubscript{\gamma} mutants. Homologs of cystatin are known to suppress hypersensitive cell death in plant and animal systems. We also report that infection with an avirulent strain of \textit{Pseudomonas syringae} results in an increase of caspase-1 activity, and this increase is partially suppressed in vpe\textsubscript{\gamma} mutants. Plants overexpressing VPE\textsubscript{\gamma} exhibit a greater amount of ion leakage during infection with \textit{P. syringae}, suggesting that VPE\textsubscript{\gamma} may regulate cell death progression during plant-pathogen interaction. VPE\textsubscript{\gamma} expression is induced after infection with \textit{P. syringae}, \textit{Botrytis cinerea}, and turnip mosaic virus, and knockout of VPE\textsubscript{\gamma} results in increased susceptibility to these pathogens.

Conclusions: We conclude that VPE\textsubscript{\gamma} is a caspase-like enzyme that has been recruited in plants to regulate vacuole-mediated cell dismantling during cell death, a process that has significant influence in the outcome of a diverse set of plant-pathogen interactions.

Introduction

Programmed cell death (PCD) occurs through normal development in plants [1], but it can also be induced in response to pathogen attack [2, 3]. Examples of PCD during development include the dismantling of the suspensor during embryogenesis and of the aleurone layer during seed germination, the removal of cells during morphogenesis of the embryo, the differentiation of xylem vessels, the elimination of incompatible pollen, and organ senescence [3, 4]. A well-characterized example of PCD in plant-pathogen interactions occurs during the hypersensitive response (HR), a rapid defense reaction that is activated when particular pathogens infect plants.

Although PCD is involved in many fundamental aspects of plant biology, the components of the PCD machinery have not been identified. At the morphological level, PCD in plants shares many common events with animal apoptosis. Moreover, expression of regulators of animal apoptosis can interfere with PCD in plants [3], and biochemical evidence supports the involvement of conserved components in both animal apoptosis and plant PCD [6]. Caspases are components of the cell death machinery universally involved in animal apoptosis. They are a family of aspartate-specific cysteine proteases that play an essential role in initiating and executing PCD in metazoans by processing and activating enzymes involved in killing and dismantling the cells committed to die [7]. They are key regulatory switches that, once activated, irreversibly trigger the cell death program. To date, no clear homologs for caspases have been found in yeast or plant genomes. Although, a yeast cysteine protease that shares structural homology to caspases has recently been shown to be involved in PCD in this unicellular organism [8]. This indicates that the role of caspase-like proteases in PCD is evolutionarily conserved in organisms outside the animal kingdom. In plants, evidence for the existence of caspase-like activities has been reported, and such activity has been shown to be required for PCD [3, 6, 9–11], but the genes encoding these activities have not been identified.
The genome of Arabidopsis encodes more than 550 putative proteases (http://merops.sanger.ac.uk). A serine protease has recently been shown to encode a caspase-like activity that may be required for PCD in Avena sativa [9]. However, the prime candidates to encode caspase-like activities in plants are the legumains and metacaspases [4, 12], which are members of the cysteine protease superfamily. The legumain family of proteases belongs to a large cysteine protease group that also includes caspases. Although there is very low overall sequence similarity between legumains and caspases, they share a highly conserved motif in the catalytic core and are predicted to possess a similar protein fold [12, 13]. Moreover, besides animal caspases, legumains are the only family from the cysteine protease plants that accumulate both the intermediate and mature forms of PCD in plants [3]. The vacuolar processing enzymes (VPE) α, β, γ, and δ are Arabidopsis legumains essential for the processing of vacuolar proteins [17–19]. VPEγ is the isoform most highly expressed in vegetative tissues [19], where it plays a role in protein degradation during senescence, a form of PCD in plants [18]. VPEγ is localized in precursor protease vesicles (PPVs), hydrolase-containing organelles that are associated with PCD processes [18, 20]. VPEγ is also found in the vacuole [18], an organelle essential for cell dismantling during PCD of plants [3]. Vacuolization of the cytoplasm through autophagy and, in late stages, vacuole disruption is observed in most cases of plant PCD, including somatic embryogenesis, xylem differentiation, elimination of the aleurone layer during germination and the HR, although the extent and the timing of vacuole intervention during different modes of PCD may vary [3]. Many of the hydrolytic activities, including proteases, RNases and DNases [3, 21], that carry out PCD are localized in vacuoles physically separated from their targets. The cell death program induces the collapse of the tonoplast (the vacuolar membrane) by a mechanism that is still not understood and initiates the massive degradation of cellular contents. Because VPEγ regulates the hydrolytic activity of the vacuole [17, 18], it may be essential for this stage of the cell death program.

Here we show that VPEγ has caspase-like activity and that it regulates cell death progression after infection with an avirulent strain of Pseudomonas syringae pv tomato DC3000 (Pst) that expresses the avrRpm1 avirulence gene. VPEγ expression is induced in plants challenged with Pst (avrRpm1), turnip mosaic virus (TuMV), or Botrytis cinerea. Moreover, vpeγ mutants are more susceptible to each of these pathogens. These results suggest that, in plants, caspase-like enzymes regulate cell death whether it is programmed or not, and this has a marked influence on the outcome of plant-pathogen interactions.

Results

VPEγ Has Caspase-like Activity
To determine whether VPEγ has caspase-like activity, we tested its in vivo sensitivity to inhibitors that mimic the recognition site of natural caspase substrates. We analyzed the effects of a general caspase inhibitor and also specific inhibitors for mammalian caspase-1 and caspase-3 (which had been shown to be active in plants [6]) on three VPEγ-dependent activities: maturation of VPEγ, the processing of a serine carboxypeptidase CPY, and the turnover of a vacuolar invertase.

Experiments with recombinant VPEγ expressed in insect cells suggested that processing of VPEγ occurs through autocatalytic self-maturation. VPEγ is processed from its precursor isoform (56 kDa) by sequential removal of an inhibitory C-terminal propeptide (13 kDa) to generate the intermediate isoform (43 kDa) and of an N-terminal propeptide (3 kDa) of unknown function to produce the mature isoform (40 kDa) [22]. Arabidopsis plants accumulate both the intermediate and mature isoforms of VPEγ, the intermediate isoform being the most abundant in young seedlings ([18] and Figure 1). In Figure 1A we show the effect of different protease inhibitors on VPEγ processing. It is important to note that the caspase-1 and general caspase inhibitors used in these experiments bind covalently to their target and thus remain attached after SDS-PAGE (VPEγ + peptide). Treatment with the caspase-1 inhibitors (YVAD-CMK and YVKD-CMK) resulted in the disappearance of the mature isoform, concomitant with an increase in the abundance of the intermediate isoform of VPEγ (Figure 1A; iVPEγ + peptide), suggesting that processing of the N-terminal propeptide was being blocked. However, YVAD-CMK and YVKD-CMK did not appear to affect the processing of the C-terminal propeptide because no accumulation of the VPEγ precursor was observed. Compared to the treatment with the DMSO control, treatment with the caspase-3 inhibitor (DEVD-CHO) or a general cysteine protease inhibitor (LEUP) only slightly increased the level of the intermediate isoform (iVPEγ), whereas the mature isoform was still present (Figure 1A). In addition, general serine (PMSF) and aspartic protease inhibitors (PEPS) had little effect on the accumulation of the intermediate isoform (iVPEγ) of VPEγ in comparison to the DMSO control, demonstrating the unique effect of caspase-1 inhibitors. We also tested the effect of a general caspase inhibitor (VAD-FMK) that blocks most animal caspases, including caspase-1. Treatment with this inhibitor also resulted in the accumulation of the intermediate isoforms (iVPEγ + peptide), whereas no mature isoform was seen, supporting cleavage of the N-terminal propeptide being mediated by a caspase-like activity. Moreover, VAD-FMK treatment resulted in a slight accumulation of the precursor isoform (pVPEγ), indicating that processing of the C-terminal propeptide is also sensitive to this general caspase inhibitor (Figure 1A). Interestingly, in plants treated with general caspase and caspase-1 inhibitors, we observed a shift in mobility of the intermediate isoform of VPEγ, and this observation was consistent with the expected increase in molecular weight upon binding of the inhibitor (Figure 1A, iVPEγ + peptide). In addition, in plants treated with the VAD-FMK inhibitor, we observed an a lower-molecular-weight isoform that may represent an abnormally processed VPEγ. These results indicate that VPEγ is recognizing and binding general caspase and caspase-1 inhibitors, which block the self-processing of the intermediate to the mature isoform of the enzyme. The removal
of the C-terminal propeptide appears to be insensitive to caspase-1 inhibitors but partially sensitive to a general caspase inhibitor, suggesting that a caspase-like protease other than VPEγ is specifically responsible for the removal of the C-terminal propeptide.

In animals, caspases are part of a proteolytic cascade that activates downstream enzymes that carry out the cell death program. VPEγ is also thought to process and activate downstream enzymes. We have previously shown that VPEγ is required for processing of the vacuolar serine carboxypeptidase, CPY [18]. If general caspase and caspase-1 inhibitors are indeed blocking VPEγ activity, an effect in the processing of CPY is expected. As shown in Figure 1B, a reduction in accumulation of the mature isoform of CPY was observed in plants treated with either a general caspase inhibitor (VAD-FMK) or with a caspase-1 inhibitor (YVAD-CMK). As with VPEγ processing, the caspase-3 inhibitor (DEVD-CHO) had only a weak effect on CPY maturation, indicating a higher affinity of VPEγ for caspase-1 rather than for caspase-3 target recognition sites. Treatment with general cysteine, serine, or aspartic protease inhibitors had no effect on the processing of CPY (data not shown).

In these experiments some mature isoform of CPY was still observed in plants treated with the general caspase or caspase-1 inhibitors. This most likely reflects protein that was processed before initiation of the treatments. Indeed, by analyzing de novo processing of radiolabeled CPY, we have determined that caspase-1 and general caspase inhibitors completely block CPY processing in vivo (data not shown).

VPEγ is also involved in degradation of a vacuolar invertase (AtFruct4) in aging plants, possibly by processing and activating degradative vacuolar proteases [18]. Because in young seedlings exogenous overexpression of VPEγ also reduces the levels of AtFruct4 [18] and Figure 1C), we checked the effect of caspase inhibitors on AtFruct4 accumulation in these plants. As shown in Figure 1C, treatment with a general caspase inhibitor (VAD-FMK) completely restored the levels observed in wild-type and vpeγ mutant plants, whereas treatment with caspase-1-specific inhibitors (YVAD-CMK and YVKD-CMK) resulted in a moderate increase of the AtFruct4 accumulation, similar to that observed with a caspase-3 inhibitor (DEVD-CHO) or a general cysteine protease inhibitor (LEUP). This observation suggests that VPEγ may not directly degrade AtFruct4 but rather that it activates other proteases that in turn degrade AtFruct4. The corresponding proteases are probably cysteine proteases; an increase in the AtFruct4 accumulation may be due to an inhibitory effect of both caspase-3 (DEVD-CHO) and general cysteine protease inhibitor (LEUP) on those proteases. Additionally, a VPEγ-dependent degradation of AtFruct4 may not be selectively sensitive to caspase-1 inhibitors but, as is more likely, may be selectively sensitive to inhibitors of other members of the caspase family. This is reflected in a high level of AtFruct4 accumulation in seedlings treated with a general caspase inhibitor rather than exclusively with a caspase-1 inhibitor.

To summarize this section, we have detected binding of general caspase and caspase-1 inhibitors to VPEγ
and have shown that three distinct VPEγ-dependent activities are blocked by caspase inhibitors. Importantly, the autocatalytic and AtCPY-processing activities of VPEγ are examples of direct effects of those inhibitors on VPEγ activity, whereas the AtFruct4 degradation data may reflect the activity of other proteases that are normally activated by VPEγ-dependent processing (e.g., AtFruct4 is likely not a direct target of VPEγ). These data strongly suggest that VPEγ has caspase-like activity in vivo.

**VPEγ Regulates Caspase Activity, Protein Degradation, and Ion Leakage during the Hypersensitive Response to an Incompatible Strain of* Pseudomonas syringae**

As our data indicate, VPEγ is a caspase-like enzyme that shows higher affinity for caspase-1 than for caspase-3 target sites (see above). Induction of caspase-1-like activity has been observed in tobacco plants undergoing the hypersensitive response (HR) to infection with tobacco mosaic virus [6]. Another prototypical example of HR is activated in response to infection by incompatible strains of *P. syringae* in *Arabidopsis* plants. However, it has not been reported whether this type of HR also results in increased caspase-1-like activity. To test this, we analyzed the cleavage activity for a fluorogenic substrate specific for animal caspase-1 (Ac-YVAD-AMC) in extracts from *Arabidopsis* plants infected with the avirulent strain *Pst* (avrRpm1) that elicits an HR in the Col-0 ecotype. As shown in Figure 2A, infected Col-0 plants showed an increase in YVAD-cleaving activity after inoculation with *Pst* (avrRpm1), which was evident 8 hr after inoculation and increased thereafter. To determine whether VPEγ is responsible for the rise of caspase-1-like activity in plants infected with Pst, we analyzed the YVAD-AMC-cleaving activity of extracts from vpeγ mutants and vpeγ mutants complemented with the VPEγ cDNA under the control of the 35S promoter (vpeγ:35SV-PEγ plants). The overexpressing line that we used accumulates a higher amount of VPEγ protein than wild-type plants and shows complementation of the defect in CPY processing and AtFruct4 degradation observed in vpeγ plants (data not shown). As predicted, vpeγ mutants did not show an increase in caspase-1 activity 8 hr after inoculation, whereas a significant increase in caspase-1 activity was observed between vpeγ mutants and wild-type or vpeγ:35SV-PEγ plants (indicated with an asterisk, ANOVA, post-hoc LSD test p < 0.05) 8 hr after inoculation. RFU-relative fluorescence unit.

(B) *Arabidopsis* plants grown in long-day conditions were inoculated with *Pst* avrRPM1, and infected leaves were harvested 8 hr and 24 hr later and assayed for Ac-YVAD-AMC-cleaving activity. The activity of water-inoculated plants collected 8 hr after inoculation (mock) is shown for comparison. Data shown are the means and standard deviation of eleven independent samples. A significant difference was determined by immunoblots with anti-Fruct4 antibodies.

(C) The expression of VPEγ was analyzed by Northern blot in samples from leaves infiltrated with a diluted culture of *Pst* avrRpm1, collected at the indicated times after inoculation.

(D) Soluble proteins were extracted from wild-type and vpeγ plants inoculated with water or with a diluted culture of *Pst* avrRpm1 at the indicated times after inoculation, and the levels of AtFruct4 and VPEγ were determined by immunoblots with anti-Fruct4 antibodies.
inoculation, at which time the activity levels were significantly lower than in the wild-type or in vpeγ:35S VPEγ
plants. However, one day after inoculation the levels of caspase-1 activity were not significantly different be-
tween the three genotypes, suggesting that the activity of caspase-1-like enzymes distinct from VPEγ increased
during the course of the HR. The YVAD-cleaving activity did not increase in plants mock-inoculated with water
even three days after inoculation (data not shown).

These results show that infection with an avirulent strain of Pst induces a caspase-1-like activity in Arabidopsis
and suggest that VPEγ is required for the early increase of activity. Taken together with the inhibitor experiments
shown above, these results suggest that VPEγ encodes a caspase-1-like enzyme that is activated during the HR
elicited by Pst in Arabidopsis. Alternatively, VPEγ may be required for HR-induced activation of another cas-
pase-1-like enzyme, possibly by processing the zymogen.

In tobacco, treatment with caspase-1 inhibitors blocks PCD induced by P. syringae [6]. Similarly, we expected
that Arabidopsis mutants lacking VPEγ activity would be affected in Pst (avrRpm1)-activated cell death, but we
did not detect any macroscopic differences in the development of HR symptoms between vpeγ and Wt or vpeγ:35S-VPEγ
plants (not shown). In the final stages of PCD, the plant plasma membrane ruptures, releasing ions into the extracellular space. Thus, an increase of conductivity of the apoplastic fluid is a measure of the PCD in a given tissue. We therefore determined whether measuring ion leakage in infected plants would detect subtle PCD changes that are not evident by visual examination of the infected plants. All three genotypes underwent a similar rapid increase in ion leakage in infected leaves relative to mock-inoculated leaves (Figure 2B). However, the overexpressing line showed a higher level of ion leakage than the vpeγ null mutant at all post-infection time points (8, 24, and 48 hr), suggesting that VPEγ overexpression may accelerate membrane disruption or increase the extent of PCD. Importantly, at 2 days post-inoculation (dpi), wild-type plants achieved similar ion leakage to that of the overexpressing line. This is consistent with the timing of VPEγ induction after Pst infection (Figure 2C). A specific aspect of plant PCD compared to animal apoptosis is that dying cells must dismantle themselves, and vacuolar hydrolases are thought to be essential for this process. Thus, VPEγ, which is localized in the vacuole and regulates its hy-
drolytic activity [18], may be involved in executing the degradation of cellular components in cells committed
to die. To test this, we analyzed the fate of AtFruct4 in Pst (avrRpm1)-infected plants. As shown in Figure 2D, the levels of AtFruct4 decreased significantly in wild-type plants inoculated with bacteria in comparison to mock-inoculated plants or to bacterially inoculated vpeγ plants. This decrease was not the result of a repression of AtFruct4 mRNA expression (data not shown), suggesting that the reduced levels of AtFruct4 were due to a higher turnover rate in the tissues undergoing PCD that was dependent on VPEγ. In contrast, the levels of VPEγ protein (Figure 2D) increase during the infection. These results suggest that Pst (avrRpm1) infection leads to VPEγ-dependent degradation of proteins in vacuoles and supports the involvement of vacuolar hydrolases in the execution of the HR. Thus, although macroscopically the HR was similar in infected wild-type or vpeγ plants, we were able to detect subtle differences by monitoring caspase-1-like activity and the accumulation of At-
Fruct4.

VPEγ Is Involved in Defense Responses to Turnip Mosaic Virus and Botrytis cinerea

In most cases, pathogen attack has a negative effect on the viability of the infected cells, which in turn influences pathogen growth. Cell death promotion may be the result of activation of PCD, but it can also be the indirect consequence of pathogen-induced cellular stress inflicted by the pathogen. Thus, genes that execute cell death in plants should affect the growth of a broad range of pathogens. To test whether VPEγ is required for pathogen response pathways, we analyzed the response of wild-type, vpeγ, and complemented vpeγ mutants to infection with TuMV, an obligate biotroph that does not induce PCD, and with the necrotrophic fungus Botrytis cinerea, which induces a PCD that may be beneficial for fungal growth [23].
Figure 4. \textit{vpe}γ Mutants Are More Susceptible to TuMV Infection

(A) Twenty-five-day-old plants were inoculated with TuMV-GFP, and the relative GFP mRNA level was measured by real-time RT-PCR. Inoculated leaves from four plants (two leaves per plant) were harvested and pooled for mRNA extraction at the indicated times after inoculation with TuMV-GFP, with ubiquitin serving as a standard reference. Data shown are the means and the standard deviations from triplicates of the real time RT-PCR. The whole experiment was repeated three times with similar results.

(B) The expression of \textit{VPE}/H9253 was analyzed by Northern blot in samples from Col-0 leaves collected at the indicated times after inoculation.

We could detect a slightly higher fluorescence in \textit{vpe}/H9253 mutant plants compared to wild-type or \textit{vpe}/H9253 complemented plants (data not shown). In order to quantify the levels of TuMV-GFP, we conducted real-time PCR with GFP specific primers. We could detect a low but significant increase of GFP mRNA in \textit{vpe}/H9253 mutant plants, but this increase fully reverted to wild-type levels upon ex-

Figure 3. \textit{VPE}γ Is Required for Limiting Bacterial Growth in \textit{Pst} Infected Plants

(A) Bacterial growth was measured in 4-week-old wild-type and \textit{vpe}γ plants inoculated with a diluted culture of \textit{Pst avrRPM1}.

(B) Bacterial growth was measured in 6-week-old wild-type, \textit{vpe}γ and \textit{vpe}γ:35S:VPE\textit{γ} plants that had already flowered (bolts of approx. 10 cm). Bacterial growth in the inoculated leaves was determined 0, 2, and 4 days after inoculation. The reported data are the means and standard deviation of the values obtained in three independent experiments. Values that bear different letters are significantly different at $p < 0.001$ (ANOVA, post-hoc LSD test).

(C) The expression of \textit{PR1} was analyzed by Northern blot in samples from leaves infiltrated with a diluted culture of avirulent (DC3000 \textit{avrRpm1}) \textit{Pst}, collected at the indicated times after inoculation.

To monitor TuMV growth in infected plants, we used a strain that was engineered to express a green fluorescent protein [24] and measured the fluorescence of infected leaves at different time points after inoculation. We could detect a slightly higher fluorescence in \textit{vpe}γ mutant plants compared to wild-type or \textit{vpe}γ complemented plants (data not shown). In order to quantify the levels of TuMV-GFP, we conducted real-time PCR with GFP specific primers. We could detect a low but significant increase of GFP mRNA in \textit{vpe}γ mutant plants, but this increase fully reverted to wild-type levels upon expression of the \textit{VPE}γ cDNA (Figure 4A). Moreover, \textit{VPE}γ expression is induced by TuMV infection (Figure 4B), suggesting that \textit{VPE}γ is part of a regulated disease response to this virus.

To assess the resistance to \textit{Botrytis cinerea}, we scored the size of the lesions formed in the leaves at various times after inoculating with drops of a spore suspension onto the upper epidermis of rosette leaves. As shown in Figure 5A, lesions were significantly larger in the mutant plants compared to wild-type plants. Expression of the \textit{VPE}γ cDNA with the 35S promoter only partially suppressed the enhanced susceptibility to \textit{B. cinerea}. Lesions on \textit{vpe}γ mutant leaves were initially surrounded by chlorotic rings, which then turned into water-soaked areas and eventually extended into the whole leaf, which was completely macerated and de-
cayed by 2 weeks after infection. At these same time points, the leaves of wild-type and vpeγ:35SVPES plants remained green, and the lesions formed did not spread to the whole blade but instead turned into dry necrotic tissue. The expression of the defensin PDF1.2 was similar in the three genotypes (Figure 5B), indicating that the differences in pathogen growth were not due to a general block in defense gene activation but may have been due to a direct function of VPEγ in regulating pathogen proliferation. VPEγ expression is also induced by Botrytis infection (Figure 5C), supporting a direct involvement in resistance.

A Cystatin Accumulates to High Levels in vpeγ Mutants

Using a methodology to quantify specific protein concentration in two parallel samples (see Supplemental Data), we identified from the cystatin superfamily of cysteine protease inhibitors (At2g40880) a protein that accumulates to high levels in vpeγ vacuoles (Figures S1C and S1D). Our LC/MS/MS data indicated that the concentration ratio of cystatin in vpeγ plants to control plants exceeded 20-fold, as determined by the arbitrary TIC (total ion current) counts of a cystatin peptide precursor ion (Figure S1B). Further repetitions with LC/MS only (without MS/MS) confirmed this ratio. The TIC counts of the same cystatin peptide ion were 920 and 20,700 for wild-type and vpeγ, respectively (data not shown). This protein is homologous to a previously characterized Arabidopsis cystatin, AtCYS1 (At5g12140). AtCYS1 has cysteine protease-inhibiting activity, and its overexpression blocks cell death in plants infected with avirulent pathogens [25]. Our results suggest that the cystatin may be a target for VPEγ-dependent degradation and may function as a downstream effector of VPEγ in regulating cell death.

Discussion

Processing and Activation of VPEγ

In animal systems, caspases are synthesized as inactive zymogens. Specific stimuli activate initiator caspases by inducing their self-processing, and they in turn activate downstream effector caspases by processing in trans and thereby triggering dismantling of the cells committed for apoptosis. VPEs are also synthesized as inactive zymogens, which in vitro are self-processed by sequential cleavage of an inhibitory C-terminal propeptide and of an N-terminal propeptide [15, 22]. Our results suggest that in vivo only the processing of the N-terminal propeptide of VPEγ is autocatalytic. The in vivo processing of the N-terminal propeptide from Canavalia ensiformis and Oryza sativa VPEs occurs after an aspartate residue, whereas that of Phaseolus vulgaris and Vicia sativa VPEs occurs after an asparagine [26–28]. Both aspartate and asparagine residues are present in conserved positions in VPEγ, and cleavage at either site would be consistent with the caspase (this study) and asparagyl-endopeptidase [29] activities reported for VPEγ. The N-terminal propeptide has no conserved motifs and no described function. In vitro it has no detectable effect on the enzyme activity, although it could regulate it in vivo. The N-terminal propeptide of human caspase-7 has no effect on the in vitro activity, but its removal is required for efficient activation in vivo [30]. Alternatively, the N-terminal propeptide could have a role in protein stability. This is supported by the increase in the accumulation of the intermediate form of VPEγ in plants treated with caspase inhibitors; this increase is much more pronounced than the correlative decrease in the accumulation of the mature form, pointing to a higher stability of the intermediate form.

Because the C-terminal propeptide of VPEγ has inhibitory activity on the enzyme [15, 22], to understand how VPEγ is activated, it is crucial to identify the protease responsible for the processing and to determine how this maturation is regulated and where it occurs. We have shown that cleavage of the C-terminal propeptide of VPEγ is partially blocked by a general caspase inhibi-
tor that blocks VPEγ activity, but this cleavage is not sensitive to caspase-1 inhibitors. This observation suggests that cleavage of the C terminus is carried out in trans by a caspase-like protease other than VPEγ. Processing by a caspase would be consistent with the cleavage occurring after a conserved aspartate, as proposed by Hiraiwa and collaborators (1997). The C-terminal propeptide also inhibits VPEγ in trans [22], so the turnover of the peptide and not only its processing may determine VPEγ activity in vivo. Turnover of the peptide may not occur in PPVs, and it may only become degraded once it reaches the vacuole, as may be the case for the invertase AtFruct4 [18].

Most caspases in metazoa are localized in the cytoplasm, whereas VPEγ is localized in PPVs, from which it is delivered into vacuoles. Many proteins localized in PPVs have ER retention signals at the C-terminal end of the protein, and these signals are thought to be required for targeting the proteins to PPVs [20]. Interestingly, however, VPEγ lacks this motif. By analogy to vacuolar sorting signals, it is likely that the propeptides of VPEs contain the sorting determinants to target VPEγ into PPVs. Identification of the targeting signal of VPEγ will allow us to test if localization in PPVs plays a role in regulating its function.

The Role of VPEγ in Plant Cellular Responses to Pathogens

During bacterially induced HR, we observed an increase of caspase-1 activity correlated with a decrease of vacuolar AtFruct4 levels. The absence of VPEγ protein in vpeγ mutants reduces caspase-1 activity and blocks AtFruct4 degradation during HR but does not affect the expression of the PR1 gene, a well-defined marker of salicylic acid-dependent defense. AtFruct4 is a downstream target for VPEγ-dependent proteolysis, and its degradation during avirulent bacterial infection supports the participation of VPEγ in activating vacuolar hydrolases during the execution of HR-associated cell death. Higher bacterial proliferation found in vpeγ plants could be due to a higher availability of nutrients in dying cells rather than to a lower proportion of dying cells.

This type of PCD involving caspase activities and cell dismantling by vacuoles is also observed in some specialized forms of apoptosis in metazoa and may constitute an ancestral mode of PCD [31–33]. The function of VPEγ in cell dismantling would resemble the role of animal effector caspases. Moreover, the analogy of VPEγ to effector caspases is extended to the presence of a short N-terminal propeptide and to the activation through processing in trans by other caspases.

Alternatively, or in addition to its role in late stages of cell dismantling, VPEγ may have a role in PCD initiation. In Alzheimer’s disease, caspase-3 localized in granulovesicular vesicles, a type of autophagic vacuole, has been proposed to process the amyloid B-protein and produce the C-terminal fragment that triggers neuronal cell death [34]. By analogy VPEγ may regulate the accumulation of proteins that control PCD initiation. In this regard, it will be interesting to analyze if the cystatin that accumulates in vpeγ mutants regulates cell death initiation as its homologous protein AtCYS1 does [25].

The role of VPEγ in inhibition of virulent and avirulent pathogens through PCD is supported by the increased proliferation of biotrophic pathogens such as Pst and TuMV in vpeγ mutants. However, the explanation of the higher growth of Botrytis cinerea in vpeγ mutants is not so straightforward because PCD has been suggested to be beneficial for Botrytis proliferation [23]. The increased susceptibility observed in vpeγ plants is not attributable to a deficiency in JA-mediated defense responses because the expression level of PDF1.2 after fungal infection is not affected. As in the case of Pst infection or senescence, VPEγ may not be involved in cell death initiation upon Botrytis infection but rather may be involved in degradation of cellular components. Therefore, abrogation of VPEγ activity in the mutant would increase the nutrient availability and result in increased fungal growth.

Redundancy in the Control of Cell Death

Plants and animals may differ in the number of enzymes that exhibit caspase-like activity and affect cell death and apoptosis. Due to a higher number of functionally redundant enzymes in plants, knocking out VPEγ has relatively subtle effects on cell death as compared to similar alterations in caspases in animals. In this regard, in vpeγ plants infected with Pst, caspase-1-like activity was lower at early stages but was observed at wild-type levels at late stages in infection, indicating that enzymes with similar activity to VPEγ are also induced during the HR. In Arabidopsis there are three other vacuolar processing enzymes that are very closely related to VPEγ, and they could compensate for its absence. Indeed, these enzymes have already been shown to be partially functionally redundant for one another [17, 19]. Functional redundancy has been proposed to explain why components of the cell death machinery have not been isolated in the various genetic screens designed to find mutants affected in the HR [5]. Several mutants with alterations in PCD (lsp, acd, and dnd mutants) have been isolated, but none of the mutated genes were found to encode caspase-like enzymes or genes directly involved in executing PCD [5]. In addition to functional redundancy in cell death genes, it has been proposed that PCD in plants may be controlled by several pathways [3]. Each pathway would contribute partially to cell death execution, generating the range of cell death morphotypes observed in plants. The combinatorial nature of cell death in plants may make disruptions within a single pathway difficult to detect.

Conclusions

VPEγ is a caspase-like enzyme that regulates vacuole-mediated cell dismantling during cell death, a process that has significant influence in the outcome of a diverse set of plant-pathogen interactions. When we finalized this manuscript for submission, Hatsugai and colleagues [35] published a paper showing that VPEs from tobacco have caspase-1 activity, mediate TMV-induced hypersensitive cell death, and are involved in suppression of virus proliferation. These results support our findings that implicate VPEγ in cell death progression and defense against pathogens in Arabidopsis.
Experimental Procedures

Plants and Materials
The plant genotypes and antibodies used in this article have been described previously [18]. We used a vpe-2 allele, which is a result of a T-DNA integration in the last intron of VPE. Caspase inhibitors and substrates were purchased from Calbiochem. All other chemicals were from Sigma.

Bacterial Inoculations and In Vivo Growth Curves
The growth of Pseudomonas syringae pv. tomato DC3000 expressing the avirulence gene avrRPM1 was examined by infiltration of leaves with a bacterial suspension of 10^7 colony-forming units (cfu) per ml. Discs of 0.3 cm^2 were excised from each infected leaf with a core borer, pooled in triplicate, and homogenized in sterile water with a plastic pestle. Six replicates were used for each time interval examined. For determination of bacterial populations, dilutions from each sample were plated in King’s medium supplemented with 100 μg/L rifampicin and 10 mg/L tetracycline for selection.

Inoculation of Arabidopsis with B. cinerea
Maturating rosette leaves were detached and placed on 1.5% water agar surface with their petioles embedded into agar. An isolate of B. cinerea from cabbage was cultivated on 39 g/L PDA (Sigma) at room temperature. B. cinerea was inoculated as 5 μl drops of a 5 × 10^6 spores/ml suspension in half-strength potato dextrose broth (Difco). Discs of fungal suspension were applied on the adaxial epidermis of the detached rosette leaves (two drops per leaf) on the right side of the central vein in the middle of the leaves. After inoculation, the leaves were kept at 20°C and 100% relative humidity for 5 days and subsequently at 70% relative humidity.

Ion Leakage Measurements
Leaves of 4-week-old plants were infiltrated with a suspension of 10^7 cfu/ml of Pst DC3000 avrRPM1. For each measurement, eight leaf discs (four disks per plant, two plants per sample) were collected from the inoculated area and floated on 5 ml of distilled water for 6 hr at room temperature. After incubation, the conductivity of the water was measured with a Crinson conductivity meter. Four replicates were used for each time point examined. The units of this measurement are μS/cm, where cm refers to the distance between electrodes.

TuMV-GFP Infection and Real-Time RT-PCR
Leaves of 25-day-old plants were infiltrated with a suspension of 10 μl of TuMV-GFP sap (propagated in Nicotiana benthamiana [24]). Eight inoculated leaves of similar size (two leaves from four plants) were harvested for each genotype at 0, 1, 3, and 5 dpi and pooled so that the differences due to leaf-to-leaf, plant-to-plant variation averaged out. Total RNA was extracted with Qiagen RNeasy kit. A primer (5'-GGC ATGGCAGCTCTGGAAAAAG-3') annealed to the negative strand of VPE and a oligo dT17 were used for reverse transcription. Real-time PCR was performed with ABI PRISM 7000 and SYBR Green I (ABI). Primers were designed with Primer expression 2.0 (ABI), and GFP primers (5'-CTCAGGGCAAGATTGGTGAAG-3' and 5'-GCGATCGGCAGCTCTGGAAAAAG-3') were used for quantifying TuMV-GFP RNA. The reference polyubiquitin (UbQ) primers were 5'-CCG AAGAGACATTACCTCTGGAA-3' and 5'-CAAGTGTGGCAACATCCTCAA-3'. The comparative Ct method was applied (ABI User Bulletin #2).

Protease Activity Assay
For in vitro caspase-1 activity, protein extracts were obtained by homogenization of three leaves from 4-week-old plants inoculated with Pst avrRPM1 (10^7 cfu/ml) in 400 μl of ice-cold extraction buffer (50 mM HEPES-KOH [pH 7.5], 10% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100). Protein (2 μg) was diluted to 10 μl in extraction buffer, added to 90 μl of extraction buffer minus Triton X-100 plus 70 μM Ac-YVAD-AMC (Calbiochem), and incubated at 30°C for 1 hr. The fluorescence was measured in a SpectroMax Gemini fluorometer ( Molecular devices; Sunnyvale, CA). Each sample was analyzed in duplicate.

Supplemental Data
Supplemental Experimental Procedures and supplemental figures are available with this article online at http://www.current-biology. com/cgi/content/full/14/21/1897/DC1/

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