

VPE γ 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 γ , 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 γ binds *in vivo* to a general caspase inhibitor and to caspase-1-specific inhibitors, which block the activity of VPE γ . A cysteine protease inhibitor, cystatin, accumulates to 20-fold higher levels in *vpe γ* 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 *Pseudomonas syringae* results in an increase of caspase-1 activity, and this increase is partially suppressed in *vpe γ* mutants. Plants overexpressing VPE γ exhibit a greater amount of ion leakage during infection with *P. syringae*, suggesting that VPE γ may regulate cell death progression during plant-pathogen interaction. VPE γ expression is induced after infection with *P. syringae*, *Botrytis cinerea*, and turnip mosaic virus, and knockout of VPE γ results in increased susceptibility to these pathogens.

Conclusions: We conclude that VPE γ 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. PCD during the HR is thought to be a defense response that isolates pathogens in dead tissues and restricts their access to nutrients or cellular factors needed to thrive. The analysis of plant mutants affected in the onset and extent of PCD has provided evidence that supports a key role for PCD in the resistance response to HR-inducing pathogens [5]. However, because the mutants analyzed thus far are also affected in other aspects of the defense response in addition to PCD, the relative contribution of PCD to resistance or susceptibility to different pathogens remains unknown [2].

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.

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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 group that has been shown to cleave substrates adjacent to an aspartate residue [14–16].

The vacuolar processing enzymes (VPE) α , β , γ , and δ are *Arabidopsis* legumains essential for the processing of vacuolar proteins [17–19]. VPE γ 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]. VPE γ is localized in precursor protease vesicles (PPVs), hydrolase-containing organelles that are associated with PCD processes [18, 20]. VPE γ 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 VPE γ 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 VPE γ 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. VPE γ expression is induced in plants challenged with *Pst* (*avrRpm1*), turnip mosaic virus (TuMV), or *Botrytis cinerea*. Moreover, *vpe* γ 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

VPE γ Has Caspase-like Activity

To determine whether VPE γ 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 VPE γ -dependent activities: maturation of VPE γ , the processing of a serine carboxypeptidase CPY, and the turnover of a vacuolar invertase.

Experiments with recombinant VPE γ expressed in insect cells suggested that processing of VPE γ occurs through autocatalytic self-maturation. VPE γ 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 VPE γ , 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 VPE γ 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 (iVPE γ + 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 VPE γ (Figure 1A; iVPE γ + 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 VPE γ 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 (iVPE γ), 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 (iVPE γ) of VPE γ 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 (iVPE γ + 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 (pVPE γ), 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 VPE γ , and this observation was consistent with the expected increase in molecular weight upon binding of the inhibitor (Figure 1A, iVPE γ + peptide). In addition, in plants treated with the VAD-FMK inhibitor, we observed a lower-molecular-weight isoform that may represent an abnormally processed VPE γ . These results indicate that VPE γ 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

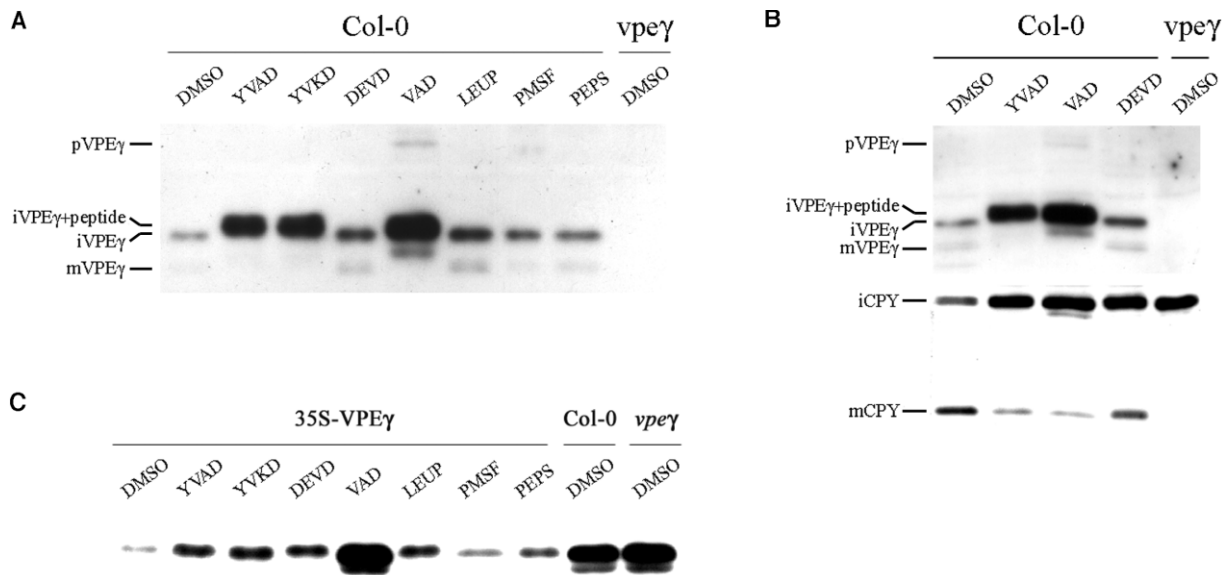


Figure 1. Caspase Inhibitors Bind and Inactivate VPE γ In Vivo

(A) Wt (Col-0) and *vpe γ* mutant seedlings were grown in liquid culture for 6 days. At that time the inhibitors (final concentration 200 μ M) or an equal amount of DMSO was added to the media, and plants were incubated for an additional 4 days. Soluble protein extracts were analyzed by Western blot with anti-VPE γ -specific antibodies. Abbreviations are as follows: YVAD, Ac-YVAD-CMK; YVKD, Ac-YVKD-CMK (caspase-1 inhibitors); DEVD, Ac-DEVD-CHO (caspase-3 inhibitor); VAD, Z-VAD-FMK (general caspase inhibitor); LEUP, leupeptin (general cysteine protease inhibitor); PMSF, phenylmethylsulfonyl fluoride (general serine protease inhibitor); PEPS, Pepstatin A (general aspartic protease inhibitor). DMSO was used as a control. Arrowheads indicate the positions of the precursor (pVPE γ), intermediate (iVPE γ), and mature isoforms of VPE γ (mVPE γ), and the expected position of the complex of iVPE γ and the inhibitor (iVPE γ +peptide).

(B) Seedlings were treated as described in (A), and soluble proteins were analyzed by Western blot with anti-VPE γ and anti-CPY-specific antibodies. Arrowheads indicate the positions of the intermediate (iCPY) and mature isoforms of CPY (mCPY).

(C) Seedlings were treated as described in (A), and soluble proteins were analyzed by Western blot with anti-AtFruct4 antibodies.

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 VPE γ 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. VPE γ is also thought to process and activate downstream enzymes. We have previously shown that VPE γ is required for processing of the vacuolar serine carboxypeptidase, CPY [18]. If general caspase and caspase-1 inhibitors are indeed blocking VPE γ 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 VPE γ processing, the caspase-3 inhibitor (DEVD-CHO) had only a weak effect on CPY maturation, indicating a higher affinity of VPE γ 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).

VPE γ 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 VPE γ 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 *vpe γ* 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 VPE γ 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 VPE γ -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 VPE γ

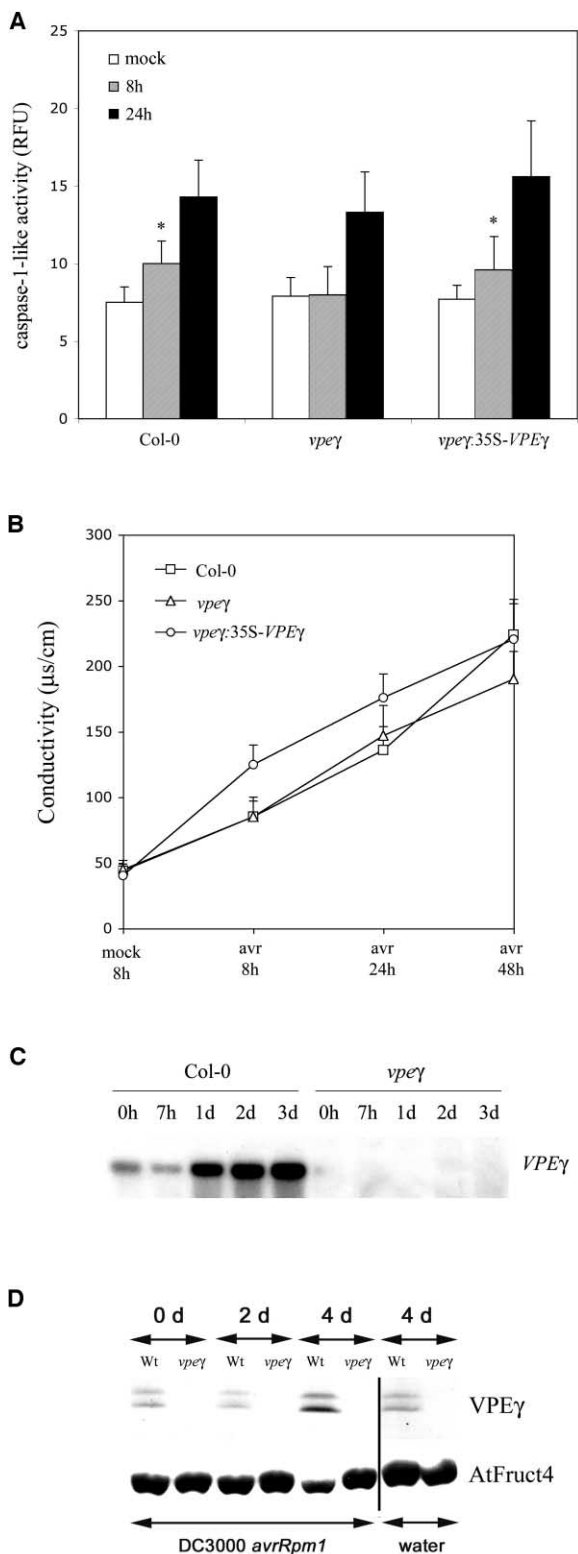


Figure 2. VPE γ Is Involved in Cell Death Progression during the HR
(A) *Arabidopsis* plants grown in long-day conditions were inoculated with *Pst avrRPM1*, and infected leaves were harvested 8 hr and 24 hr later and assayed for Ac-YVAD-AMC-cleaving activity. The activity of water-inoculated plants 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

and have shown that three distinct VPE γ -dependent activities are blocked by caspase inhibitors. Importantly, the autocatalytic and AtCPY-processing activities of VPE γ are examples of direct effects of those inhibitors on VPE γ activity, whereas the AtFruct4 degradation data may reflect the activity of other proteases that are normally activated by VPE γ -dependent processing (e.g., AtFruct4 is likely not a direct target of VPE γ). These data strongly suggest that VPE γ has caspase-like activity *in vivo*.

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

As our data indicate, VPE γ 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 VPE γ 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 VPE γ cDNA under the control of the 35S promoter (*vpeγ:35S-VPEγ* plants). The overexpressing line that we used accumulates a higher amount of VPE γ 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

in caspase-1 activity was observed between *vpeγ* mutants and wild-type or *vpeγ:35S-VPEγ* 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* or mock inoculated with water. Leaf disks were collected from mock-inoculated plants 8 hr after inoculation and from bacterial inoculated plants 8, 24, and 48 hr after inoculation and floated in water for 6 hr, and conductivity was measured. The data shown are the averages and standard deviations in a representative experiment.

(C) The expression of VPE γ 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 VPE γ 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 *vpe γ :35SVPE γ* plants. However, one day after inoculation the levels of caspase-1 activity were not significantly different between the three genotypes, suggesting that the activity of caspase-1-like enzymes distinct from VPE γ 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 VPE γ is required for the early increase of activity. Taken together with the inhibitor experiments shown above, these results suggest that VPE γ encodes a caspase-1-like enzyme that is activated during the HR elicited by *Pst* in *Arabidopsis*. Alternatively, VPE γ may be required for HR-induced activation of another caspase-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 VPE γ 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 *vpe γ* and Wt or *vpe γ :35S-VPE γ* 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 apoplasmic 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 *vpe γ* null mutant at all post-infection time points (8, 24, and 48 hr), suggesting that VPE γ 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 VPE γ 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, VPE γ , which is localized in the vacuole and regulates its hydrolytic 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 *vpe γ* 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 VPE γ . In contrast, the levels of VPE γ protein (Figure 2D) increase during the infection. These results suggest that *Pst* (*avrRpm1*) infection leads to VPE γ -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 *vpe γ* plants, we were able to detect subtle differences by monitoring caspase-1-like activity and the accumulation of AtFruct4.

VPE γ Is Involved in Resistance to an Incompatible Strain of *Pseudomonas syringae*

To test whether the induction of VPE γ expression is important in the defense response to *Pst* (*avrRpm1*), we characterized bacterial growth in wild-type and *vpe γ* plants. As shown in Figure 3A, when we inoculated 4-week-old plants with an avirulent strain of *Pst*, we observed an approximately 2-fold increase in bacterial growth in *vpe γ* plants as compared with the wild-type. However, because of the intrinsic variability of the assay, the significance of these results was low. These small differences are consistent with the subtle effects that *vpe γ* knockout or overexpression have on cell death progression during the HR. We have previously reported that the activity of VPE γ is regulated by the developmental stage of the plant, and its role in protein degradation is only apparent in aging tissues, coincident with its localization in vacuoles [18]. We speculated that the contribution of VPE γ to resistance to *Pst* (*avrRpm1*) could also be developmentally regulated. We therefore tested bacterial growth in 6-week-old plants that had undergone the transition to flowering. In these plants, we observed that, compared to the wild-type (Figure 3B), *vpe γ* mutants supported a significant increase in bacterial growth (7-fold; $p < 0.001$) at 4 dpi. Importantly, wild-type levels of resistance were recovered in *vpe γ :35S VPE γ* plants, demonstrating that the increased susceptibility was due to the lack of VPE γ expression. We tested whether the activation of *PR1*, a well-characterized marker of salicylic acid dependent defenses was altered in *vpe γ* mutants. As shown in Figure 3C, *PR1* expression was similarly induced in wild-type and *vpe γ* mutant plants, suggesting that the differential pathogen growth was not due to inhibition of *PR1* gene activation but most likely was due to a direct effect of VPE γ on cell death progression. However, we noted the *PR1* induction was sustained longer in the wild-type when compared to the *vpe γ* mutant.

VPE γ 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 VPE γ is required for pathogen response pathways, we analyzed the response of wild-type, *vpe γ* , and complemented *vpe γ* 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].

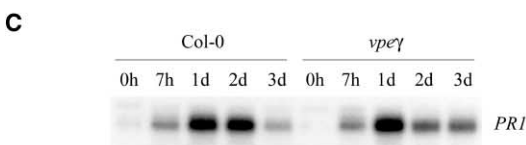
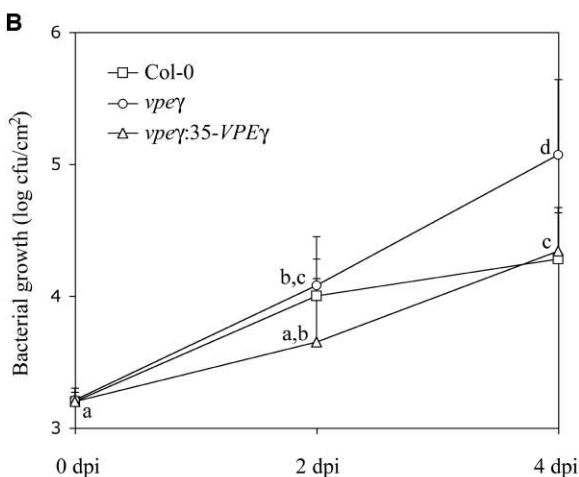
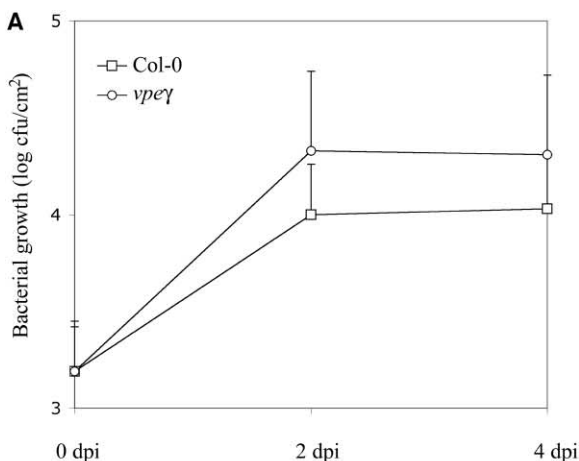


Figure 3. *VPE* γ Is Required for Limiting Bacterial Growth in *Pst*-Infected Plants

(A) Bacterial growth was measured in 4-week-old wild-type and *vpe* γ plants inoculated with a diluted culture of *Pst avrRPM1*. (B) Bacterial growth was measured in 6-week-old wild-type, *vpe* γ and *vpe* γ :35S*VPE* 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 *PR1* was analyzed by Northern blot in samples from leaves infiltrated with a diluted culture of avirulent (DC3000 *avrRpm1*) *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 in-

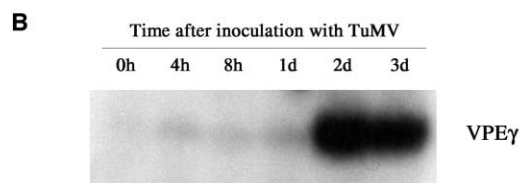
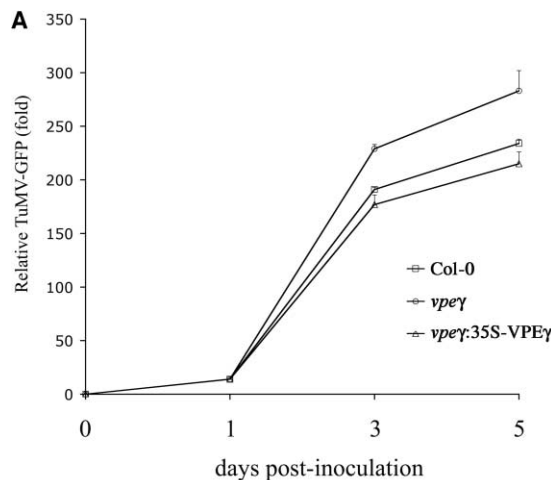


Figure 4. *vpe* γ Mutants Are More Susceptible to TuMV Infection
(A) Twenty-five-day-old plants were inoculated with TuMV-GFP, and the relative GFP mRNA level was measured by real-time RT-PCR. Inoculated leaves from four plants (two leaves per plant) were harvested and pooled for mRNA extraction at the 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 *VPE* γ was analyzed by Northern blot in samples from Col-0 leaves collected at the indicated times after inoculation with TuMV-GFP.

fecting leaves at different time points after inoculation. We could detect a slightly higher fluorescence in *vpe* γ mutant plants compared to wild-type or *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 *vpe* γ mutant plants, but this increase fully reverted to wild-type levels upon expression of the *VPE* γ cDNA (Figure 4A). Moreover, *VPE* γ expression is induced by TuMV infection (Figure 4B), suggesting that *VPE* γ is part of a regulated disease response to this virus.

To assess the resistance to *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 *VPE* γ cDNA with the 35S promoter only partially suppressed the enhanced susceptibility to *B. cinerea*. Lesions on *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-

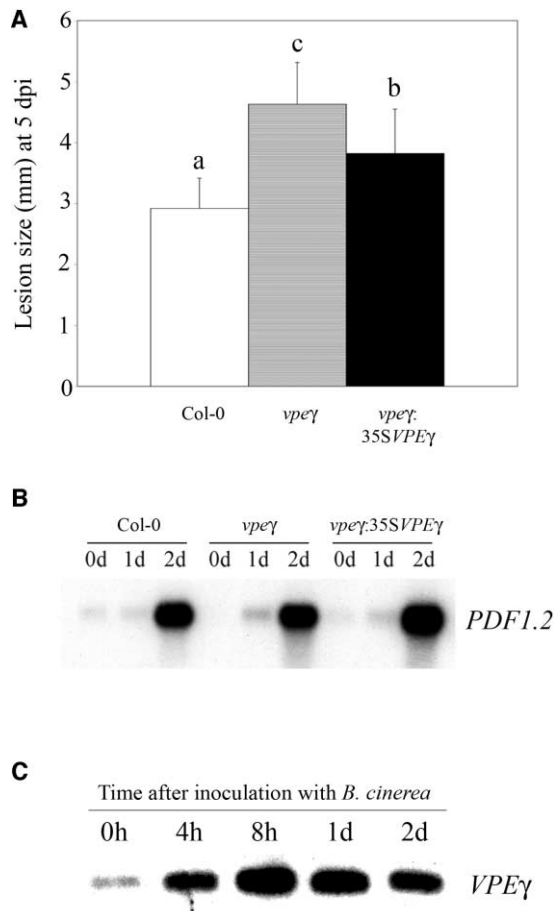


Figure 5. Susceptibility to *Botrytis cinerea* Is Regulated by VPE γ
(A) A suspension of spores of *B. cinerea* was applied on the adaxial epidermis of detached leaves, and the lesion diameter was measured 5 days after inoculation. Values that bear different letters are significantly different at $p < 0.05$ (ANOVA, post-hoc LSD test). This experiment was repeated three more times with similar results.
(B) The expression of *PDF1.2* in inoculated attached leaves was analyzed by Northern blot in samples from the indicated genotypes at different times after infection.
(C) The expression of VPE γ was analyzed by Northern blot in samples from Col-0 leaves collected at the indicated times after inoculation with *B. cinerea*.

cayed by 2 weeks after infection. At these same time points, the leaves of wild-type and *vpe* γ ;35SVPE γ 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 VPE γ in regulating pathogen proliferation. VPE γ expression is also induced by *Botrytis* infection (Figure 5C), supporting a direct involvement in resistance.

A Cystatin Accumulates to High Levels in *vpe* γ 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 *vpe* γ vacuoles (Figures S1C and S1D). Our LC/MS/MS data indicated that the concentration ratio of cystatin in *vpe* γ 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 *vpe* γ , 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 VPE γ -dependent degradation and may function as a downstream effector of VPE γ in regulating cell death.

Discussion

Processing and Activation of VPE γ

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 VPE γ 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 VPE γ , and cleavage at either site would be consistent with the caspase (this study) and asparaginyl-endopeptidase [29] activities reported for VPE γ . 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 VPE γ 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 VPE γ has inhibitory activity on the enzyme [15, 22], to understand how VPE γ 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 VPE γ is partially blocked by a general caspase inhibi-

tor that blocks VPE γ 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 VPE γ . 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 VPE γ in *trans* [22], so the turnover of the peptide and not only its processing may determine VPE γ 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 metazoans are localized in the cytoplasm, whereas VPE γ 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, VPE γ lacks this motif. By analogy to vacuolar sorting signals, it is likely that the propeptides of VPEs contain the sorting determinants to target VPE γ into PPVs. Identification of the targeting signal of VPE γ will allow us to test if localization in PPVs plays a role in regulating its function.

The Role of VPE γ in Plant Cellular Responses to Pathogens

During bacterially induced HR, we observed an increase of caspase-1 activity that correlated with a decrease of vacuolar AtFruct4 levels. The absence of VPE γ protein in *vpe γ* 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 VPE γ -dependent proteolysis, and its degradation during avirulent bacterial infection supports the participation of VPE γ in activating vacuolar hydrolases during the execution of HR-associated cell death. Higher bacterial proliferation found in *vpe γ* 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 metazoans and may constitute an ancestral mode of PCD [31–33]. The function of VPE γ in cell dismantling would resemble the role of animal effector caspases. Moreover, the analogy of VPE γ 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, VPE γ may have a role in PCD initiation. In Alzheimer's disease, caspase-3 localized in granulo-vesicular 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 VPE γ 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 *vpe γ* mutants regulates cell death initiation as its homologous protein AtCYS1 does [25].

The role of VPE γ in inhibition of virulent and avirulent pathogens through PCD is supported by the increased proliferation of biotrophic pathogens such as *Pst* and *TuMV* in *vpe γ* mutants. However, the explanation of the higher growth of *Botrytis cinerea* in *vpe γ* mutants is not so straightforward because PCD has been suggested to be beneficial for *Botrytis* proliferation [23]. The increased susceptibility observed in *vpe γ* 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, VPE γ may not be involved in cell death initiation upon *Botrytis* infection but rather may be involved in degradation of cellular components. Therefore, abrogation of VPE γ 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 VPE γ has relatively subtle effects on cell death as compared to similar alterations in caspases in animals. In this regard, in *vpe γ* 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 VPE γ are also induced during the HR. In *Arabidopsis* there are three other vacuolar processing enzymes that are very closely related to VPE γ , 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 (*Isd*, *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

VPE γ 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 VPE γ 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* γ -2 allele, which is a result of a T-DNA integration in the last intron of VPE γ . 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 10⁵ colony-forming units (cfu) per ml. Discs of 0.3 cm² 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 μ g/L rifampicin and 10 mg/L tetracycline for selection.

Inoculation of *Arabidopsis* with *B. cinerea*

Maturing 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 μ l drops of a 5 \times 10⁵ spores/ml suspension in half-strength potato dextrose broth (Difco). Drops 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 10⁷ 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 μ S/cm, where cm refers to the distance between electrodes.

TuMV-GFP Infection and Real-Time RT-PCR

Leaves of 25-day-old plants were inoculated with 10 μ l 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 ATGGCACTCTTGAAAAG-3') annealed to the negative strand of GFP RNA and an 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'-TCCATGGCCAACTTGTCA-3' and 5'-GGCATGGCACTCTTGAAAAG-3') were used for quantifying TuMV-GFP RNA. The reference polyubiquitin (UBQ3) primers were 5'-CGG AAAGACCTACTCTGGA-3' and 5'-CAAGTGTGCGACCATCCTCAA-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* (10⁷ cfu/ml) in 400 μ l 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 μ g) was diluted to 10 μ l in extraction buffer, added to 90 μ l of extraction buffer minus Triton X-100 plus 70 μ M 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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VPE γ Exhibits a Caspase-like Activity that Contributes to Defense against Pathogens

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Supplemental Experimental Procedures

Vacuole Isolation

Vacuoles were purified from wild-type and *vpe γ* healthy green rosette leaves from 35 day old wild-type and *vpe γ* plants. In brief, 2 g of leaf tissue was cut into thin slices with a razor blade and incubated for 4 hr at 21°C in 30 ml protoplasting solution containing 0.3 g macerozyme R-10 (0.55 U/mg) (Serva, Heidelberg), 0.3 g cellulase "Onozuka R-10" (1.0 U/mg) (Serva, Heidelberg), and 0.12 g CaCl₂ dissolved in 30 ml of wash buffer (0.4 M mannitol/10 mM MES [pH 5.7]). For recovering protoplasts, the protoplasts were filtered away from undissolved tissue, and centrifugation followed for 20 min at 500 rpm (57 × g) in a Beckman GS-6R centrifuge (GH-3.8

rotor). Protoplasts were washed two times in wash buffer and then lysed in a solution containing 0.2 M mannitol/10% ficoll/10 mM EDTA/5 mM NaPO₄ (pH 8.0). Density ultracentrifugation was then performed as previously described [S1] except that bovine serum albumin (BSA) was omitted from all solutions. This method results in the layering of enriched vacuoles and allows for the efficient separation of central vacuoles from other endomembrane compartments [S1]. Yields were approximately 50 μ g of protein per preparation.

Proteomics of Purified Samples

Vacuolar preparations were concentrated with a Millipore 10,000 molecular-weight cutoff centrifugal concentrator, and total protein

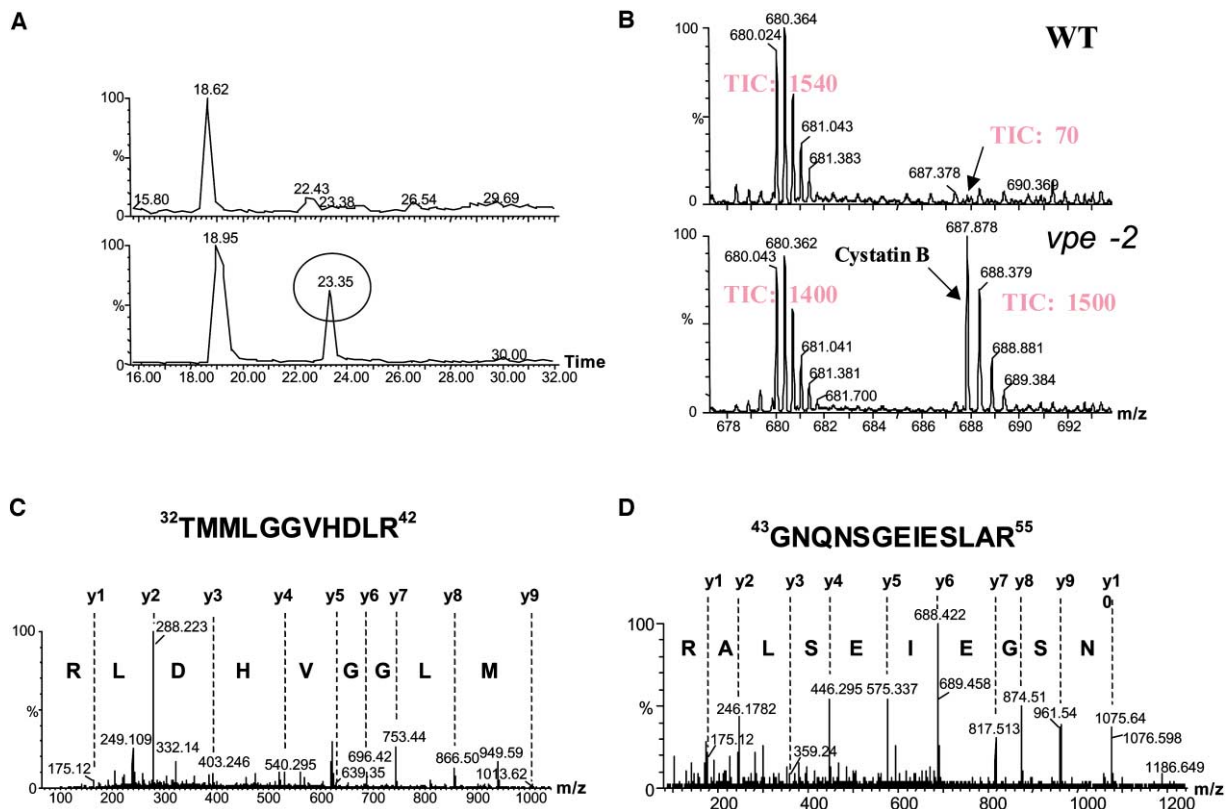


Figure S1. LC/MS/MS Analysis to Identify Cystatin B-like Protein Highly Accumulated in Vacuoles of *vpe γ* Mutants

(A) A base peak ion (BPI) chromatogram of *m/z* 687.88 shows an ion fraction at retention time of 23.35 min that was observed only in the *vpe γ* mutant sample but not in the wild-type.

(B) An MS spectrum of BPI at 23.35 min fraction shows total ion current (TIC) of the precursor ion (*m/z* 687.88, 2+) of the cystatin B-like peptide. TIC counts for *m/z* 687.88 were approximately 20-fold higher in *vpe γ* mutant than in the wild-type, and this was a reproducible measurement. As an internal control, another coeluted peptide precursor (*m/z* 680.04, 3+), whose parent protein was not identified, showed similar TIC counts in the wild-type and the *vpe γ* mutant (approximately 1500).

(C) MS/MS spectrum of a precursor ion (*m/z* 631.32, 2+) that was matched to a cystatin B-like peptide spanning residues 32–42. Only y-series ions are labeled.

(D) MS/MS spectrum of a precursor ion (*m/z* 687.88, 2+) that was matched to a cystatin B-like peptide spanning residues 43–55. Only y-series ions are labeled.

concentration was determined. Two hundred and fifty micrograms of total wild-type and *vpe* γ protein was subjected to standard 18 cm 14% SDS PAGE. The resulting gel was briefly stained with Coomassie blue R-250 and cut into thin horizontal slices with wild-type and *vpe* γ lanes processed pair-wise. Gel slices were then digested with trypsin and processed essentially as described previously [S2].

The in-gel digest of individual gel bands was analyzed by a μ LC-nano ESI-MS/MS with a Micromass Q-TOF API US instrument (Waters, Milford, MA), and wild-type and *vpe* γ samples were analyzed pair-wise so that system variation would be minimized. The Atlantis dC₁₈ column (3 μ m, 100 Å, 100 μ m internal diameter, 15 cm long) was used for LC peptide separation (Waters). A 300- μ m-internal-diameter \times 5-mm-long C₁₈ guard column (Dionex, Sunnyvale, CA) was placed at the front of the analytical column to preconcentrate the tryptic peptides through a 10-port switch valve connection. The μ l pickup sample loading method was configured with a 20 μ l sample loop in the auto-sampler for maximal loading (6.4 μ l) with zero sample loss. Samples were loaded and desalted for 3 min with 0.2% formic acid with a flow rate of 25 μ l/min. The solvent components for peptide elution were as follows: mobile phase A was 0.1% formic acid and 5% acetonitrile, mobile phase B was 0.1% formic acid and 95% acetonitrile. The duration of the complete LC method was for 140 min: 0–3 min, 5% B; at 5 min, 12% B; at 95 min, 45% B; at 107 min, 70% B; at 110–115 min, 90% B; at 120–140 min, 5% B. A 1 hr blank washing method was introduced between each sample. A manually configured flow-splitter was used for applying a 0.4 μ l/min flow rate to the guard and analytical columns, which were connected with a 10 μ m internal diameter PicoTip nanospray emitter (New Objective, Woburn, MA). The MS instrument parameters were tuned so that the highest possible sensitivity and resolution were achieved.

An LC/MS/MS survey scan method was used for analyzing all peptide precursor ions for both wild-type and mutant samples. All MS/MS spectra were processed to generate pkl text files with software provided by the instrument manufacturer. Pkl files were then searched against NCBI nr database of *Arabidopsis* with the MASCOT algorithm for protein identification. To compare the abundance level of this protein between wild-type and mutant, we used a relative-quantification strategy similar to that described in a previous study [S3]). From the MASCOT search results, the precursor ions *m/z* values and charge states for identified peptides were known. The LC retention time for a precursor ion was determined from the auto.txt file in each raw MS data folder. With the retention time known, the precursor ion of the matched peptide was correctly located in the base peak ion (BPI) chromatogram in a manual manner. Finally, the total ion current (TIC) of this peptide ion, representing the relative amount of protein in the samples, was summed from the corresponding BPI fraction. Arbitrary TIC counts were used for comparing the relative abundance of proteins in wild-type and mutant samples as previously described [S3].

Supplemental References

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