Apoptosis 2006; 11: 905–911

DOI: 10.1007/s10495-006-6601-1

A cellular suicide strategy of plants: vacuole-mediated cell death

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Published online: 13 March 2006

Programmed cell death (PCD) occurs in animals and plants under various stresses and during development. Recently, vacuolar processing enzyme (VPE) was identified as an executioner of plant PCD. VPE is a cysteine protease that cleaves a peptide bond at the C-terminal side of asparagine and aspartic acid. VPE exhibited enzymatic properties similar to that of a caspase, which is a cysteine protease that mediates the PCD pathway in animals, although there is limited sequence identity between the two enzymes. VPE and caspase-1 share several structural properties: the catalytic dyads and three amino acids forming the substrate pockets (Asp pocket) are conserved between VPE and caspase-1. In contrast to such similarities, subcellular localizations of these proteases are completely different from each other. VPE is localized in the vacuoles, while caspases are localized in the cytosol. VPE functions as a key molecule of plant PCD through disrupting the vacuole in pathogenesis and development. Cell death triggered by vacuolar collapse is unique to plants and has not been seen in animals. Plants might have evolved a VPE-mediated vacuolar system as a cellular suicide strategy.

Keywords: caspase; cysteine protease; programmed cell death; vacuolar processing enzyme; vacuole; VPE.

Introduction

Programmed cell death (PCD) is a highly regulated cellular suicide process for growth and survival in eukaryotes. In plants, PCD occurs in development, such as during xylogenesis, embryogenesis, aerenchyma formation, several plant reproductive processes, seed development and leaf senescence.[1] In addition to its role in plant development, cell suicide significantly contributes to defense from environmental stresses including ozone and UV radiation, and to the host defense against pathogen attack.[2,3] Several morphological similarities were found between animal cells undergoing apoptosis and dying plant cells, including cell shrinkage, chromatin condensation, and DNA and nuclear fragmentation.[4,5] In animal cells, mitochondria integrate cellular stress and regulate PCD.[6] Similarly, a role for mitochondria in plant PCD has been suggested.[7–9] Therefore, some regulatory mechanisms underlying PCD are thought to be conserved in animals and plants, but the molecular mechanism that regulates plant PCD remains unclear.

In animals, the process of controlled cell death is well organized by caspases which are cysteine proteases (aspartate-specific proteases).[10] Caspases modify and activate several proteins that have a role in maintaining cell integrity. Extensive studies have provided evidence that PCDs in plants and animals share components that include caspase-like activity.[7,10,11] For example, caspase-like activities were detected in tobacco exposed to virus,[12] in *Arabidopsis* exposed to bacteria,[13] in tomato after chemical-induced apoptosis,[14] in *Arabidopsis* after nitric oxide-induced cell death[15] and in embryonic suspension cells from barley,[16] and these caspase-like activities could be inhibited with caspase inhibitors but not caspase-unrelated protease inhibitors. Furthermore, the caspase inhibitors have been shown to abolish these PCDs.[12,14–16]

However, with the publication of the *Arabidopsis* full genome, it became evident that the *Arabidopsis* genome has no bona fide caspase-encoding genes. This implied that plant PCD relies on other proteases having caspase-like activities. In oats (*Avena sativa*), serine proteases exhibit caspase-like activities and the activities increased when PCD was induced by the fungal toxin victorin.[17] Homology searches revealed the existence of several metacaspases that contain the caspase-conserved domains, in plants and fungi.[18] Silencing of a metacaspase gene reduced caspase-6-like activity and abolished developmental cell death in Norway spruce.[19] However, it was recently reported that metacaspases are arginine/lysine-specific cysteine proteases and do not cleave caspase-specific substrates.[20,21] Activation of caspase-like proteases triggered through either direct or indirect proteolysis by metacaspases might mediate plant PCD.

Recently, we reported that a vacuole-localized protease called VPE exhibits caspase-1-like activity and regulates cell death in both resistance and susceptible responses to pathogen infection.[22,23] The vacuole has an essential role in

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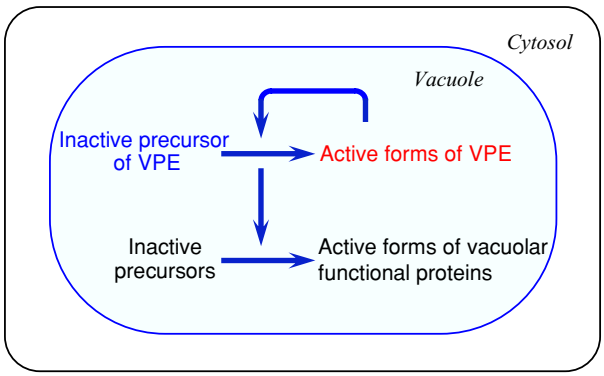
these types of plant PCD, although all plant PCD does not necessarily involve the vacuole. Recent progress of plant PCD has been admirably reviewed.[3,24–26] This review will focus on vacuole-mediated cell death, which is not seen in animals.

Vacuolar-processing system

A variety of vacuolar proteins are synthesized on endoplasmic reticulum as proprotein precursors in plant cells and then are transported to vacuoles. These proproteins are proteolytically processed to produce the respective mature forms. Vacuolar processing is mediated by VPE (Figure 1).[27–29] VPE is a vacuolar cysteine protease responsible for maturation and/or activation of various vacuolar proteins.[30,31] VPE itself is synthesized as an inactive proprotein precursor and then targeted to the vacuole. Within the vacuoles the proprotein precursor of VPE is self-catalytically converted into the active mature form and no other factor is necessary for activating VPE molecules.[32,33] Therefore, VPE is a key enzyme in the vacuolar-processing system. The maturation and activation of VPEs are well characterized.[32,33] *Arabidopsis* has four VPE genes (αVPE, βVPE, γVPE, and δVPE), which are classified into three subfamilies by their homology and expression pattern.[34–39] The pleiotropic functions of the VPE family are also covered in a recent review.[34,40]

An animal ortholog of VPE, asparaginyl endopeptidase (AEP)/legumain, is responsible for processing and maturation of three lysosomal hydrolytic enzymes, cathepsins B, L and H.[41] A similar VPE-dependent processing system functions in mammal lysosomes and/or late endosomes, which are comparable organelles to plant vacuoles.

Figure 1. A hypothetical model for vacuolar-processing system in plants. A variety of vacuolar proteins are synthesized on endoplasmic reticulum as a proprotein precursor in plant cells and then are transported to vacuoles. These proproteins are proteolytically processed to produce the respective mature forms with VPE. VPE itself is synthesized as an inactive proprotein precursor and then targets to vacuoles, where the VPE precursor is self-catalytically converted into the active mature form. No other factors are necessary for activating VPE molecules. VPE functions as a key enzyme of the vacuolar-processing system.



VPE is a cysteine protease that exhibits caspase-1 like activity in plants

Plants, which do not have an immune system, have developed their own strategies for defending against invading pathogens. A typical plant defense strategy is the hypersensitive response (HR), which involves a rapid and localized cell death at the infected sites of host tissues.[42,43] Hypersensitive cell death is thought to prevent pathogens from growing and spreading into healthy tissues. The molecular mechanism underlying execution of the HR is unknown. Many studies have shown the involvement of proteases as central executioners in hypersensitive cell death. During the HR, cell death was suppressed by inhibition of cysteine proteases either through the use of specific chemical inhibitors[5,44] or through overexpression of a protease inhibitor.[45] Recently, we and other groups showed that caspase-specific peptide inhibitors blocked hypersensitive cell death that was triggered by infection of incompatible pathogens.[22,46,47] Caspase-like activity is involved in the hypersensitive cell death.

To detect the proteases that have caspase-like activity, we developed a biotinylated-inhibitor blot analysis with an irreversible caspase inhibitor (biotin-xVAD-fmk). Two positive signals were detected on the blot of virus-infected tobacco leaves and immunodepleted extracts with anti-VPE antibody gave no signal on the blot. This result indicates that the protease exhibiting caspase-1-like activity in plants is VPE.[22] *VPE*-deficient *Nicotiana benthamiana* plants, which were generated by a virus-induced gene silencing system, had reduced caspase-1-like activity in parallel with the reduction of VPE activity.[22] The *Arabidopsis* VPE-null mutant, which lacks all four *VPE* genes ($\alpha vpe\text{-}3$ $\beta vpe\text{-}5$ $\gamma vpe\text{-}1$ $\delta vpe\text{-}1$) from the genome, shows neither VPE activity nor caspase-1 like activity.[23] *Arabidopsis* γVPE recognizes both a VPE substrate with $K_m = 30.3\ \mu M$ and a caspase-1 substrate with $K_m = 44.2\ \mu M$. These values are comparable to those of mammalian caspase-1.[23] Overall the results indicate that VPE is a proteinase that exhibits caspase-1 like activity.

Plant VPEs and caspase-1 share several structural properties

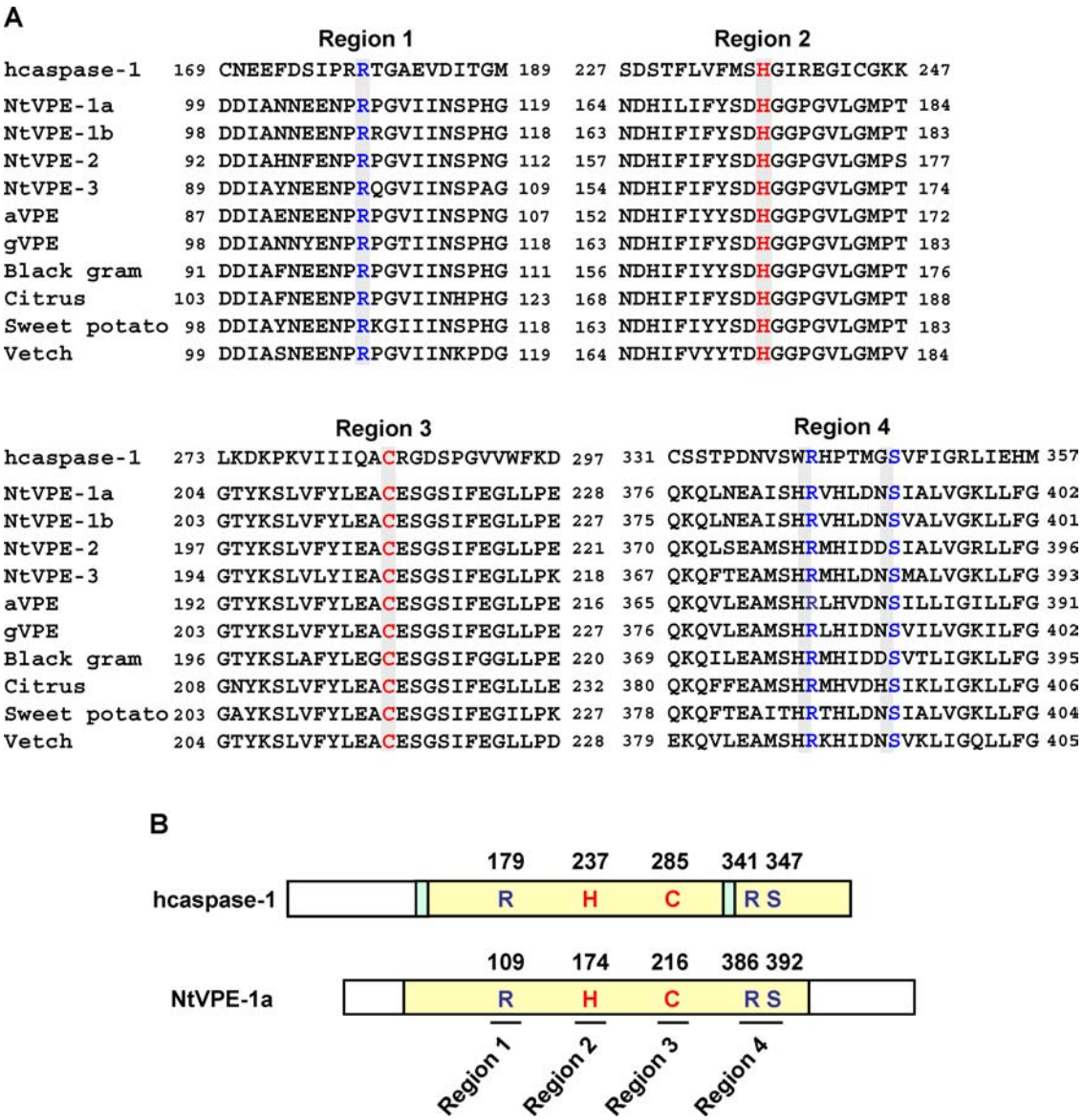
Despite the limited sequence identity, both VPE and caspase-1 share several structural properties. Two residues of the catalytic dyad in VPE (His and Cys, indicated by red-colored H and C, respectively, in Figure 2A) are comparable to His-237 and Cys-285 of the catalytic dyad in human caspase-1.[10,32] A similar correspondence was found between human caspase-8 and *Arabidopsis* γVPE.[48] In addition, each of three crucial amino acids, Arg-179, Arg-341 and Ser-347, which form the Asp pocket of caspase-1,[49] are conserved in VPEs (Figure 2A, indicated by blue-colored R, R and S, respectively). This is the case for all of the more than 20 VPEs that are currently in the

databases. This result suggests that the substrate pocket of VPE is similar to the Asp pocket of caspase-1.[49]

The VPEs of *Arabidopsis*[33] and castor bean[32,50] have been shown to be synthesized as preproprotein precursors that are co-translationally converted into a proprotein precursor with an N-terminal propeptide and a C-terminal propeptide (Figure 2B). The inactive proprotein precursor is self-catalytically activated by sequential removal of the C-terminal and N-terminal propeptides.[32,33] Similarly, caspase-1 is synthesized as an inactive precursor and then is self-catalytically converted into the active mature enzyme.[10] The caspase precursor has no C-terminal propeptide, but it does have a linker peptide (Figure 2B).

VPEs and caspase-1 share several enzymatic properties, although VPEs are not related to the caspase family. While caspases are endopeptidases with a substrate specificity toward aspartic acids,[51,52] VPEs do exhibit activity toward aspartic acids of some peptide substrates,[53] although they are originally regarded as an asparaginyl endopeptidase.[32,54] VPE recognizes the aspartic acid of YVAD, a caspase-1

Figure 2. The essential amino acids for caspase-1-like activity are conserved in VPE homologs from various plants. (A) His-237 and Cys-285 (red colored) form a catalytic dyad in human caspase-1 (hcaspase-1), while His-174 and Cys-216 (red colored) form a catalytic dyad of NtVPE-1a. Three crucial amino acids, Arg-179, Arg-341, and Ser-347 (blue colored) form the substrate pocket of hcaspase-1. The corresponding amino acids are conserved among plant VPEs. References to the published sequences are as follows: hcaspase-1,[10] tobacco VPEs (NtVPE-1a, NtVPE-1b, NtVPE-2 and NtVPE-3),[22] *Arabidopsis* αVPE,[37] *Arabidopsis* γVPE,[36] black gram VmPE-1,[65] citrus VPE,[66] sweet potato VPE (accession number, AF260827–1) and vetch VPE.[54] (B) Both caspase-1 and plant VPEs are synthesized as proprotein precursors and then converted into the respective mature form (yellow boxes) after removal of propeptides (open boxes) and linker peptides (green boxes). The essential amino acids of hcaspase-1 and the corresponding amino acids of tobacco VPE (NtVPE-1a) are shown in yellow boxes.



substrate, but not the aspartic acid of DEVD, a caspase-3 substrate, or ESED, the derivative of a VPE substrate.[23] This suggests that VPE has an affinity for aspartic acid when it is in the sequence YVAD but not necessarily for other aspartic acid residues. The similarity of the substrate specificity between VPE and caspase-1 is consistent with similarities in other characteristics: similar substrate pockets and similar active sites (Figure 2).

Both VPE[10] and caspase-1[32,33] are subjected to self-catalytic conversion/activation from their inactive precursors. Caspase-1 in its active form is comprised of two subunits of 20-kDa and 10-kDa, and the subunits are derived from the single 45-kDa inactive precursor following removal of an N-terminal propeptide and a linker peptide.[10] Removal of the C-terminal propeptide of VPE, which produces a 40-kDa intermediate, is essential for VPE to activate the enzyme.[32,33] Further removal of the N-terminal propeptide to produce the 38-kDa mature enzyme is not required to activate the enzyme. The C-terminal propeptide functions as an auto-inhibitory domain that masks the catalytic site.[33]

VPE-mediated cell death is responsible for both resistance and susceptible responses to pathogen infection

Pathogen-induced host cell death has been observed not only in resistance responses but also in susceptible responses. When host plants are resistant to pathogens, the plants induce hypersensitive cell death to avoid systemic infection (resistance response of plants). In tobacco plants, tobacco mosaic virus (TMV) causes resistance response (HR) in cultivars that carry the *N* resistance gene.[55] The interaction between the tobacco and TMV has often been used as a model system for studying the HR. *VPE* deficient plants prevent the typical characteristics of TMV-induced cell death.[22] Although *VPE* deficiency does not interfere with the induction of defense genes, virus proliferation is markedly increased in the plants.[22] These observations support the idea that the PCD during the HR is really critical for removal of biotrophic pathogens, the growth of which depends on the living host tissues. The model system used in the above study induces cell death synchronously in infected leaves by controlling the *N* resistance gene. During natural infection, the death style in infected cells might be attributed to vacuolar disruption that is mediated by VPE as discussed below. Electron microscopic analysis observed that the cells adjacent to the first-infected cells have the features of chromatin condensation and intact vacuolar membrane,[2] suggesting that the adjacent cells subsequently die by a different mechanism.

On the other hand, when pathogens overcome host plant defense, the plants are infected with pathogens and often suffer from symptoms such as chlorosis and necrosis (susceptible response of plants). The necrotrophic pathogens are able to grow by utilizing the dead tissues of plants. Some necrotrophic pathogens secrete toxins to deliberately kill the plant cells.[56]

Several fungal toxins, such as fumonisin B1 (FB1)[57] and victorin,[58] induce PCD by inhibiting the host metabolism. FB1 is a competitive inhibitor of ceramide synthase, which is involved in sphingolipid biosynthesis[59] and induces plant PCD through a process of disruption of sphingolipid biosynthesis, although the molecular mechanism is unclear. A very recent report shows that FB1-induced cell death was completely abolished in the *Arabidopsis* VPE-null mutant, which lacks all four *VPE* genes.[23] The *γvpe-1* single mutant suppressed lesion formation, although the suppression was not as strong as it was in the VPE-null mutant plant.[23] In contrast, three single mutants (*αvpe-3*, *βvpe-5,* and *δvpe-1*) formed lesions like those on the wild-type plant. Other VPEs might partially compensate for the deficiency of γVPE in *γvpe-1* leaves. Previously, in the case of processing of seed storage proteins, αVPE and γVPE were shown to compensate for the lack of βVPE in the *βvpe* mutant.[60]

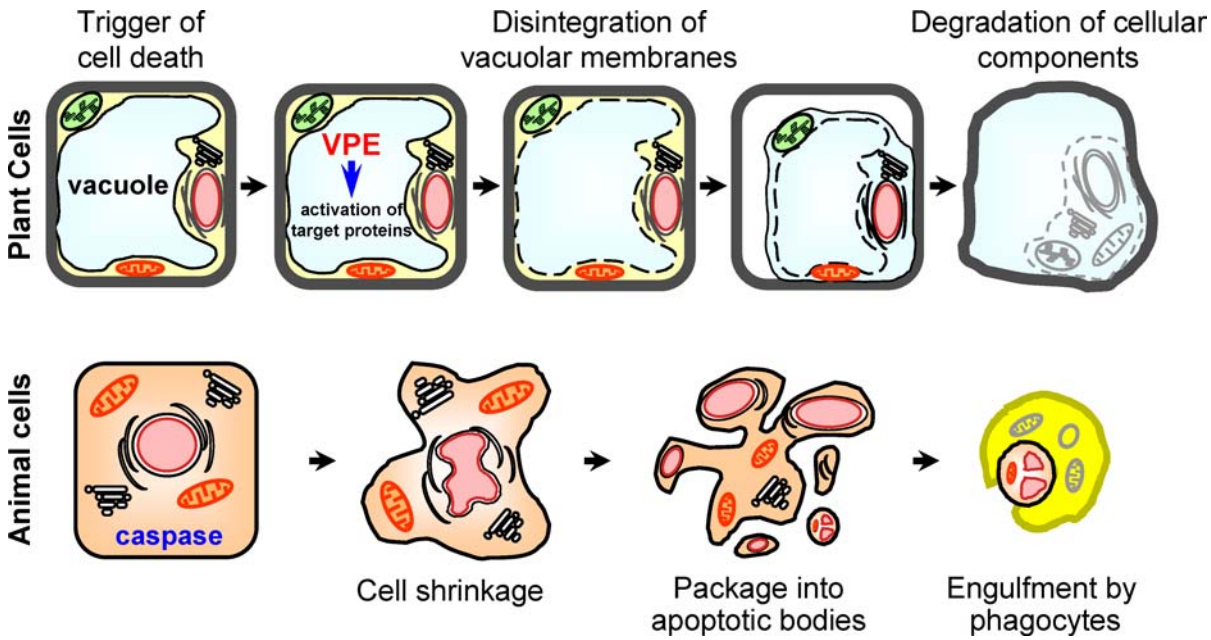
Toxin-induced cell death is a necrotrophic pathogen strategy for infection, whereas hypersensitive cell death is a plant defense strategy against pathogen attack. It appears that these processes are different from each other. However, these findings suggest that both resistance and susceptible responses share the VPE-mediated vacuolar mechanism.

VPE functions as a key molecule in cellular suicide strategy triggered by vacuolar collapse

In spite of the structural and enzymatic similarities between VPE and caspase-1, their subcellular localizations are different: caspase-1 is a cytosolic enzyme[10] and VPE is a vacuolar enzyme.[61,62] In animals, dying cells are packaged into apoptotic bodies and then engulfed by phagocytes such as macrophages. In contrast, in plants, which do not have phagocytes, cells surrounded by rigid cell walls must degrade their materials by themselves. The system for degrading dying cells in plants is thought to be different from that in animals. Because plant vacuoles contain hydrolytic enzymes,[63] vacuoles, like phagocytes, remove unwanted materials during PCD. Disintegration of the vacuolar membrane has been proposed to be the crucial event in plant PCD[64] however the molecular mechanism responsible for vacuolar collapse was not known.

An ultrastructural analysis and a viability assay with protoplasts showed that disintegration of vacuolar membranes occurred in virus-infected leaves before the cells were dead.[22] The disintegration of the vacuolar membranes continued, resulting in complete vacuolar collapse in association with plasmolysis and formation of cytoplasmic aggregations within the cells, as shown in Figure 3. On the contrary, VPE-deficient plants prevented the vacuolar collapse followed by cell death after virus infection.[22] This observation suggests that VPE functions as a key molecule in cell death triggered by vacuolar collapse. To our knowledge, VPE is the first vacuolar component to be identified

Figure 3. VPE processing system mediates a cellular suicide strategy in plants. In animals, dying cells are packaged into apoptotic bodies and then engulfed by phagocytes. In contrast, because plants do not have phagocytes and the cells are surrounded by rigid cell walls, plant cells must degrade their materials by themselves. VPE, which has caspase-1-like activity, is accumulated after perception of death signals such as pathogen infection. VPE is involved in activation of the target proteins to provoke disintegration of the vacuolar membranes. Consequently, the vacuolar hydrolytic enzymes leave the vacuole for the cytosol and degrade cellular components. Plants have evolved a death strategy that is mediated by the VPE processing system, which is not seen in animals.



as a regulator of cell death. Plants have evolved a death strategy that is mediated by a vacuolar system which is not seen in animals (Figure 3).

Future perspective

VPE is involved not only in pathogen-induced PCD but also in developmental cell death in plants. Recently, we found that δVPE is expressed specifically and transiently in two cell layers of seed coats at the early stage of seed development of *Arabidopsis* and is involved in cell death of the limited cell layers, the purpose of which is to form a seed coat.[35] Promoter-GUS analyses showed the up-regulation of *αVPE* and *γVPE* in dying cortex cells next to the emerging lateral root[61] and in dying circular-cells, clusters of anthers that release mature pollen grains (unpublished data), respectively. Both *αVPE* and *γVPE* are also up-regulated in senescing tissues.[61] These results suggest the involvement of VPE in various types of cell death in association with development and senescence of plant organs. To unravel the mechanism underlying VPE-mediated cell death in plants, the molecular machinery triggering vacuolar collapse needs to be clarified. Because VPE acts as a processing enzyme to activate various vacuolar proteins, it might also convert the inactive hydrolytic enzymes to the active forms, which then degrade the vacuoles and initiate the proteolytic cascade in plant PCD. Identification of the VPE-target proteins and/or enzymes will give us valuable insights into plant PCD.

Acknowledgments

This work was supported by CREST and PREST of the Japan Science and Technology Corporation, and Grants-in-Aid for Scientific Research (nos. 16085203 and 17107002) and 21st Century COE Research Kyoto University (A14) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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