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Review

The role of vacuole in plant cell death

I Hara-Nishimura*,[1] and N Hatsugai[2]

Almost all plant cells have large vacuoles that contain both hydrolytic enzymes and a variety of defense proteins. Plants use vacuoles and vacuolar contents for programmed cell death (PCD) in two different ways: for a destructive way and for a non-destructive way. Destruction is caused by vacuolar membrane collapse, followed by the release of vacuolar hydrolytic enzymes into the cytosol, resulting in rapid and direct cell death. The destructive way is effective in the digestion of viruses proliferating in the cytosol, in susceptible cell death induced by fungal toxins, and in developmental cell death to generate integuments (seed coats) and tracheary elements. On the other hand, the non-destructive way involves fusion of the vacuolar and the plasma membrane, which allows vacuolar defense proteins to be discharged into the extracellular space where the bacteria proliferate. Membrane fusion, which is normally suppressed, was triggered in a proteasome-dependent manner. Intriguingly, both ways use enzymes with caspase-like activity; the membrane-fusion system uses proteasome subunit PBA1 with caspase-3-like activity, and the vacuolar-collapse system uses vacuolar processing enzyme (VPE) with caspase-1-like activity. This review summarizes two different ways of vacuole-mediated PCD and discusses how plants use them to attack pathogens that invade unexpectedly.
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Plant Vacuoles as Lytic and Storage Compartments

Most mature plant cells have vacuoles that occupy a large part of the cell volume. This feature is unique to plant cells. There are two types of vacuoles, lytic vacuoles and protein storage vacuoles.[1] Lytic vacuoles contain hydrolytic enzymes to degrade cellular materials that are no longer required, whereas protein storage vacuoles accumulate large amounts of various proteins such as defense proteins, and storage proteins for seed germination and subsequent seedling growth. As lytic vacuoles and protein storage vacuoles have opposite functions, degradation and storage, respectively, these two vacuoles have been distinguished using marker proteins such as tonoplast intrinsic proteins. However, recent studies suggest that both types of vacuoles share machinery for the intracellular trafficking of vacuolar proteins.[2,3] Most vacuolar soluble proteins are synthesized on the endoplasmic reticulum as larger precursors and then transported into vacuoles, where precursor proteins are converted into their respective mature forms by vacuolar processing enzyme (VPE).[4–8] The machinery in plant cells is used to accumulate a variety of proteins in both types of vacuoles, hydrolytic enzymes including aspartate proteinases,[9] cysteine proteinases,[10,11] and nucleases[12] required for non-selective degradation of cellular components during programmed cell death (PCD), and defense proteins including pathogenesis-related proteins (PR proteins),[13] myrosinases,[14] toxic proteins,[15] and lectins[16,17] for defense against invading pathogens.

Non-Destructive *versus* Destructive Vacuole-mediated Cell Death

As plants do not have mobile immune cells, they have evolved unique immune systems with different defense strategies for different pathogens.[18] One of these strategies is the hypersensitive response (HR), which confers broad-spectrum disease resistance in plants. The HR is often accompanied by rapid and localized PCD, known as hypersensitive cell death, at the infection site to prevent the growth and spread of pathogens into healthy tissues.[19,20] This response is initiated by the direct or indirect recognition of a pathogen avirulence (Avr) factor by a plant resistance gene product and is controlled by multiple signal transduction pathways.[21]

Hypersensitive cell death triggered by some pathogens is caused by vacuole-mediated cell death, which is a type of plant-specific PCD. Using vacuoles for defense-related cell death makes sense for plants, because vacuoles exist in each cell of plants. The question is, how are vacuoles used for cell death? There are two different ways of vacuole-mediated cell death, a destructive type triggered by vacuolar membrane collapse[22–26] and a non-destructive type involving no vacuolar membrane collapse[27] (Figure 1). The non-destructive way, which commences with the vacuolar membrane and cytoplasm intact, were recently observed in avirulent bacteria-induced hypersensitive cell death.[27] The cell death by non-destructive way is caused by membrane fusion of the vacuolar membrane and the plasma membrane (Figure 1, upper). Membrane fusion discharges vacuolar hydrolytic enzymes

[1]Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan and [2]Photonic Bioimaging Section, Research Center for Cooperative Projects, Hokkaido University, Kita-ku, Sapporo 060-8638, Japan
*Corresponding author: I Hara-Nishimura, Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kitashirakawa, Kyoto 606-8502, Japan. Tel: +81 75 753 4142; Fax: +81 75 753 4142; E-mail: ihnishi@gr.bot.kyoto-u.ac.jp
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Abbreviations: VPE, vacuolar processing enzyme; PR proteins, pathogenesis-related proteins; HR, hypersensitive response; PCD, programmed cell death
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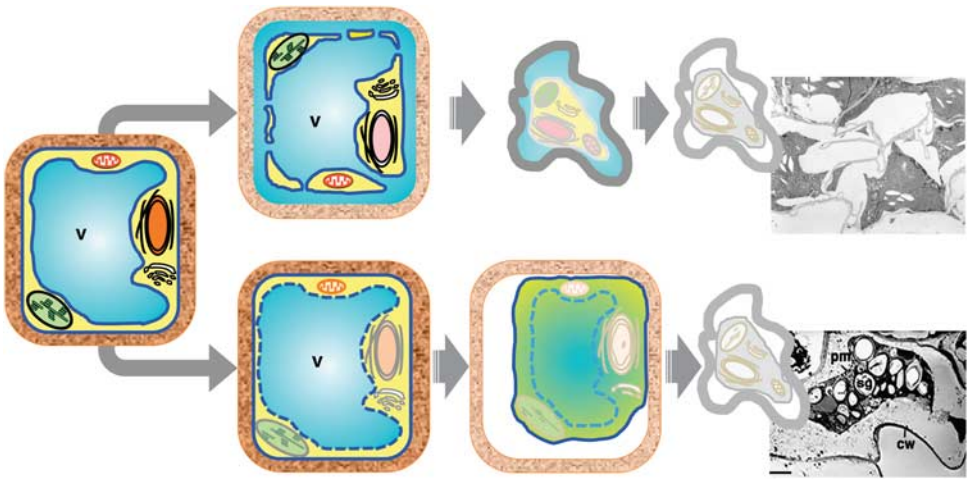

Figure 1 Two different ways of vacuole-mediated cell death: a destructive way triggered by vacuolar membrane collapse and a non-destructive way involving no vacuolar membrane collapse. The non-destructive way involves fusion between the vacuolar membrane and the plasma membrane leading to discharge of vacuolar hydrolytic enzymes outside of the cell, resulting in indirect cell death (upper). The destructive way is caused by vacuolar membrane collapse followed by the release of vacuolar hydrolytic enzymes into the cytosol, resulting in rapid and direct cell death (lower). V, vacuole; cw, cell wall; pm, plasma membrane

into the extracellular matrix, resulting in cell death. On the other hand, the destructive way is initiated by the collapse of the vacuolar membrane, which releases vacuolar hydrolytic enzymes directly into the cytosol to degrade cytoplasmic components, resulting in rapid and direct cell death (Figure 1, lower). They are effective for the digestion of viral pathogens proliferating in the cytosol,[23] for susceptible cell death induced by fungal toxins,[24] and for developmental cell death to generate integuments (seed coats)[25] and tracheary elements.[26]

Membrane Fusion-mediated Cell Death without Destruction of Vacuolar Membrane

Non-destructive way. *Arabidopsis thaliana* plants are resistant to *Pseudomonas syringae* pv. *tomato* that have the Avr gene *avrRpm1* (*Pst* DC3000/*avrRpm1*)[28] and *avrRpt2* (*Pst* DC3000/*avrRpt2*).[29,30] Leaf cells infected with *Pst* DC3000/*avrRpm1* were intact at 3 h, but exhibited cell shrinkage and cytoplasmic aggregation, which are characteristic of hypersensitive cell death, at 12 h. Ultrastructural analysis of infected cells showed that the membrane of the large central vacuole is uniformly and frequently fused with the plasma membrane 3 h after infection (Figures 2b and c).[27] The membrane fusion occurred nearly simultaneously in 83% of the cells examined at 3 h and in most of the cells at 6 h. Another avirulent strain, *Pst* DC3000/*avrRpt2*, caused membrane fusion at 7.5 h after inoculation. On the other hand, membrane fusion was not detected in cells of the *rpm1* mutant even at 12 h after the inoculation of *Pst* DC3000/*avrRpm1*. Similarly, membrane fusion was not caused by the inoculation of *Pst* DC3000, which has neither *avrRpm1* nor *avrRpt2*. Taken together, these results indicated that an interaction between RPM1 (a plant *R* gene product) and AvrRpm1 (a pathogen Avr factor) is required for membrane fusion.

The fusion resulted in the interconnection of vacuoles and the outside spaces of the plasma membrane in leaf cells, which made it possible to discharge vacuolar contents outside of the cells (Figure 2a). A vacuolar-localized fluorescent protein became detectable outside the cells after the inoculation of avirulent strains *Pst* DC3000/*avrRpm1* and *Pst* DC3000/*avrRpt2*. Vacuolar proteolytic enzymes were detected in extracellular fluid from the leaves at 3 h after the inoculation of *Pst* DC3000/*avrRpm1*, and their levels increased at 4.5 h. Interestingly, extracellular fluid from infected leaves exhibited both antibacterial activity and cell death-induction activity.

Vacuolar defense proteins are known to accumulate after the infection of bacterial pathogens.[31] How these proteins attack the bacteria, which proliferate in the extracellular space, has been a mystery. This issue was solved with the finding that fusion of the vacuolar plasma membrane allows vacuolar defense proteins to be discharged into the extracellular space where the bacteria proliferate. Formation of a tunnel from the inside to the outside of the cell wards off the bacteria.

Proteasome-dependent membrane fusion. Membrane fusion between the central vacuole and the plasma membrane does not occur under normal conditions. This leads to the hypothesis that a fusion suppressor exists in the cells. Bacterial infection could trigger degradation of this fusion suppressor, resulting in membrane fusion (Figure 2a). This hypothesis is supported by the finding that proteasome function is required for membrane fusion, followed by hypersensitive cell death in response to avirulent bacterial infection.[27]

The *Arabidopsis* proteasome has three catalytic subunits, PBA1, PBB, and PBE. Treatment with a PBA1 inhibitor (Ac-APnLD-CHO) suppressed not only membrane fusion, but also the discharge of vacuolar proteins outside the infected

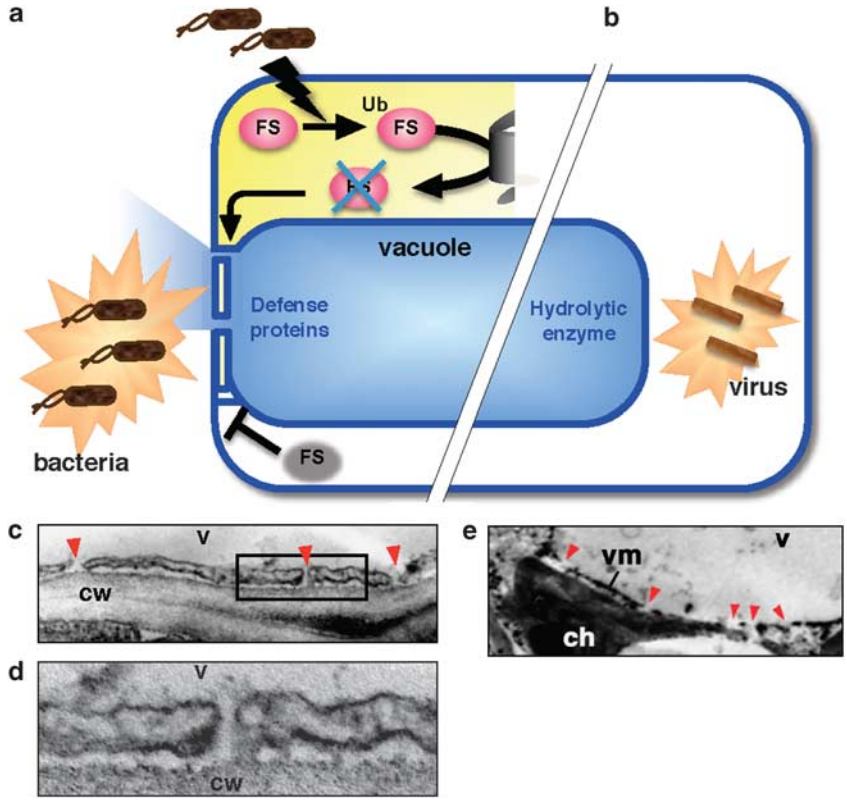

Figure 2 Two types cell autonomous immune systems through vacuole-mediated cell death. Membrane fusion-mediated hypersensitive cell death against bacterial pathogens (**a**) and vacuolar collapse-mediated hypersensitive cell death against viral pathogens (**b**). Electron microscopic pictures show bacterial infection-induced membrane fusion (**c** and **d**) and viral infection-induced vacuolar membrane collapse (**e**). FS, a fusion suppressor; Ub, ubiquitin

cells.[27] Silencing each gene of *PBA1*, *PBB*, and *PBE* blocked membrane fusion after inoculation with avirulent bacteria (*Pst* DC3000/*avrRpm1*).[27] All of the RNAi lines became sensitive to bacterial infection, suggesting that deficiency of one subunit causes a defect in proteasome function, and that proteasome function is required for disease resistance.[27] This is consistent with suggestions on the involvement of ubiquitination in disease resistance.[32,33] Proteasome-dependent degradation of some factor might trigger membrane fusion, followed by the discharge of vacuolar contents for the purpose of attacking bacteria (Figure 2a).

The next question is whether the proteasome has a role in hypersensitive cell death induced by bacteria. Hypersensitive cell death can be monitored by either trypan-blue staining of dead cells, or by measuring ion leakage from dead cells. Results from both experiments reveal that deficiency of each proteasome subunit suppresses hypersensitive cell death in response to the infection of either *Pst* DC3000/*avrRpm1* or *Pst* DC3000/*avrRpt2*, although it did not affect susceptible cell death caused by the virulent strain, *Pst* DC3000.[27] *PBA1* deficiency does not suppress the induction of the NADPH oxidases (*AtrbohD* and *AtrbohF*) responsible for generating reactive oxygen intermediates, or PR-1 and PR-2.[27] Proteasome function confers cell-autonomous immunity to bacterial pathogens through hypersensitive cell death.

Involvement of caspase-3-like activity. PCD is a basic physiological process that occurs under various stresses and during the development in plants and animals, and some regulatory mechanisms underlying PCD are thought to be conserved in both organisms. Apoptotic cell death in animals is regulated by cysteine proteinases called caspases. Many studies have shown that activities similar to those of caspases are required for various types of cell death of plants.[34–36] As plants lack genes homologous to caspases, the question is, what proteinases are responsible for caspase-like activity?

Previously, the vacuolar enzyme VPE was reported to be a proteinase that exhibits caspase-1-like activity (discussed below). However, neither VPE deficiency nor a caspase-1 inhibitor blocks bacterially-induced hypersensitive cell death. Instead, it is effectively blocked by a caspase-3 inhibitor, as described above. This indicates that the membrane fusion system for bacterially-induced cell death involves caspase-3-like activity. The proteinase responsible for caspase-3-like activity is found to be a proteasome β1 subunit, which is one of three catalytic subunits (β1, β2, and β5).[37,38]

In *Arabidopsis*, the genes for these three subunits are *PBA1*, *PBB,* and *PBE*, respectively.[39] *Arabidopsis* RNAi lines, in which the *PBA1* gene was specifically suppressed, had reduced caspase-3-like activity (DEVDase), which is highly correlated with PBA1 activity. A pull-down analysis with biotin-DEVD-fmk, followed by an immunoblot with anti-PBA1 antibody showed that PBA1 is responsible for the DEVDase activity. This analysis unveiled PBA1 as a

previously unidentified plant enzyme that has long been reported to function in hypersensitive cell death.

A model of membrane fusion-mediated cell death. Proteasome-dependent cell death involves three processes (Figure 2a). The first process concerns the fusion of a large central vacuole with the plasma membrane after bacterial infection. Bacterial infection may trigger ubiquitination of an unknown fusion suppressor in proteasome-dependent degradation.

The second process entails the discharge of vacuolar contents, including antibacterial proteins and hydrolytic enzymes, to the outside of the plasma membrane, as well as the production of defense proteins in the cell after membrane fusion. Topologically, membrane fusion between the vacuolar membrane and the plasma membrane does not damage the cytoplasm. Even after membrane fusion, cells remain alive and their ability to produce antibacterial materials is maintained for about 12 h after infection by *Pst* DC3000/*avrRpm1*, until the time of death.

In the third process, cells induce hypersensitive cell death by actions of hydrolytic enzymes released from vacuoles to the outside of the cell. Unlike animal pathogens, phytopathogenic bacteria do not enter host cells; instead, they proliferate in the intracellular spaces of the leaves. This novel defense strategy involving proteasome-regulating membrane fusion between the vacuolar and plasma membranes provides plants with a mechanism for attacking intercellular bacterial pathogens that invade through stomata on the leaves. The immune system complements another vacuolar defense mechanism in which viral propagation inside the cell is checked by vacuolar collapse, as described below.

VPE-Dependent Cell Death Through the Destruction of Vacuoles

Destruction of vacuoles. The second type of vacuole-mediated cell death is associated with the collapse of the vacuolar membrane, resulting in the release of vacuolar hydrolytic enzymes into the cytosol (Figure 1, lower). It is an efficient defense against viruses proliferating in the cytosol (Figure 2d). Tobacco mosaic virus induces typical visible lesions with shrinkage of cells in the leaves of *Nicotiana benthamiana*, in which the vacuolar membrane is disrupted[23] (Figure 2e). The vacuolar enzymes, including nucleases and proteinases, easily degrade the virus composed of RNA and proteins.

The destruction of vacuoles also leads to direct cell death through the degradation of various organelles, including the nucleus. How is the nuclear DNA degraded? Most PCDs are accompanied by the fragmentation of nuclear DNA. Formation of a nucleosomal DNA ladder is known to be a typical feature of apoptosis.[40] However, some plant PCDs do not involve DNA ladder formation.[41,42] Nuclear DNA of the TMV-infected leaves was cleaved into $\sim$50-kb fragments,[23] which is inconsistent with the formation of a nucleosomal DNA ladder. The fact that vacuolar nucleases and proteinases are involved in the non-selective digestion of nucleosomes composed of DNA and histones, respectively, makes the formation of such a ladder unlikely. It is concluded that destructive cell death is not necessarily accompanied by the formation of a nucleosomal DNA ladder.

Virus-induced cell death is a HR involving rapid and localized cell death at infected regions to prevent infestation of pathogens.[23] Plants lacking macrophages use vacuoles to degrade cellular materials and viruses through vacuolar destruction.

VPE-dependent hypersensitive cell death. Vacuole-mediated cell death through destruction is initiated by a vacuolar enzyme, VPE.[22,23,43] *VPE*-gene silencing completely suppresses lesion formation in TMV-infected leaves, vacuolar collapse, and DNA fragmentation. Although VPE deficiency prevents these typical characteristics, it does not interfere with the production of defense proteins (PR proteins).[23] This means that the process of vacuole-mediated cell death is independent of defense-protein production. Considering that VPE appears rapidly at the beginning of the TMV-induced HR and declines before forming visible lesions,[23] VPE is essential in an early step of virus-induced cell death.

VPE-dependent cell death through destruction is effective in eliminating viruses within the cell. However, it is not effective in preventing bacteria from proliferating outside cells. Plants have evolved a cell-autonomous immune system based on membrane fusion to inhibit proliferation of bacterial pathogens, and a vacuolar-collapse system to limit the spread of systemically viral pathogens.

VPE-dependent susceptible cell death. VPE-dependent cell death is also involved in the fungal toxin-induced susceptible cell death of *Arabidopsis thaliana*.[24] Toxin-induced cell death is a strategy of pathogens for infection. Some compatible pathogens secrete toxins to kill host cells and trigger lesion formation at the infection site. Fungal toxins have been shown to induce PCD in animal and plant cells.[44] A host-selective mycotoxin, fumonisin B1 (FB1), which is produced by fungal pathogens, forms lesions on *Arabidopsis* leaves and induces collapse of the vacuolar membrane.[24] An *Arabidopsis* VPE-null mutant, lacking all four *VPE* genes, exhibits neither lesion formation nor vacuolar collapse in the leaves after FB1 infiltration. It should be noted that VPE mediates both hypersensitive cell death as a plant defense strategy, and toxin-induced cell death as a pathogen strategy. During evolution, pathogens might have overcome the plant defense response by utilizing a VPE-dependent cell death system in plants.

VPE-dependent developmental cell death. The *Arabidopsis* genome has four VPE homologues: α*VPE*, β*VPE*, γ*VPE*, and δ*VPE*. The vegetative-types, αVPE and γVPE, are upregulated during tracheary element differentiation, during leaf senescence, and after treatment with salicylic acid.[7,45] Accordingly, VPE is involved in both pathogen-induced cell death and developmental cell death.

Arabidopsis δVPE is specifically and transiently expressed in two cell layers of the seed coat (ii2 and ii3) at an early stage of seed development.[25] At this stage, cell death accompanying vacuolar collapse occurs in the ii2 layer, followed by cell death in the ii3 layer (Figure 3). In a δVPE-deficient mutant,

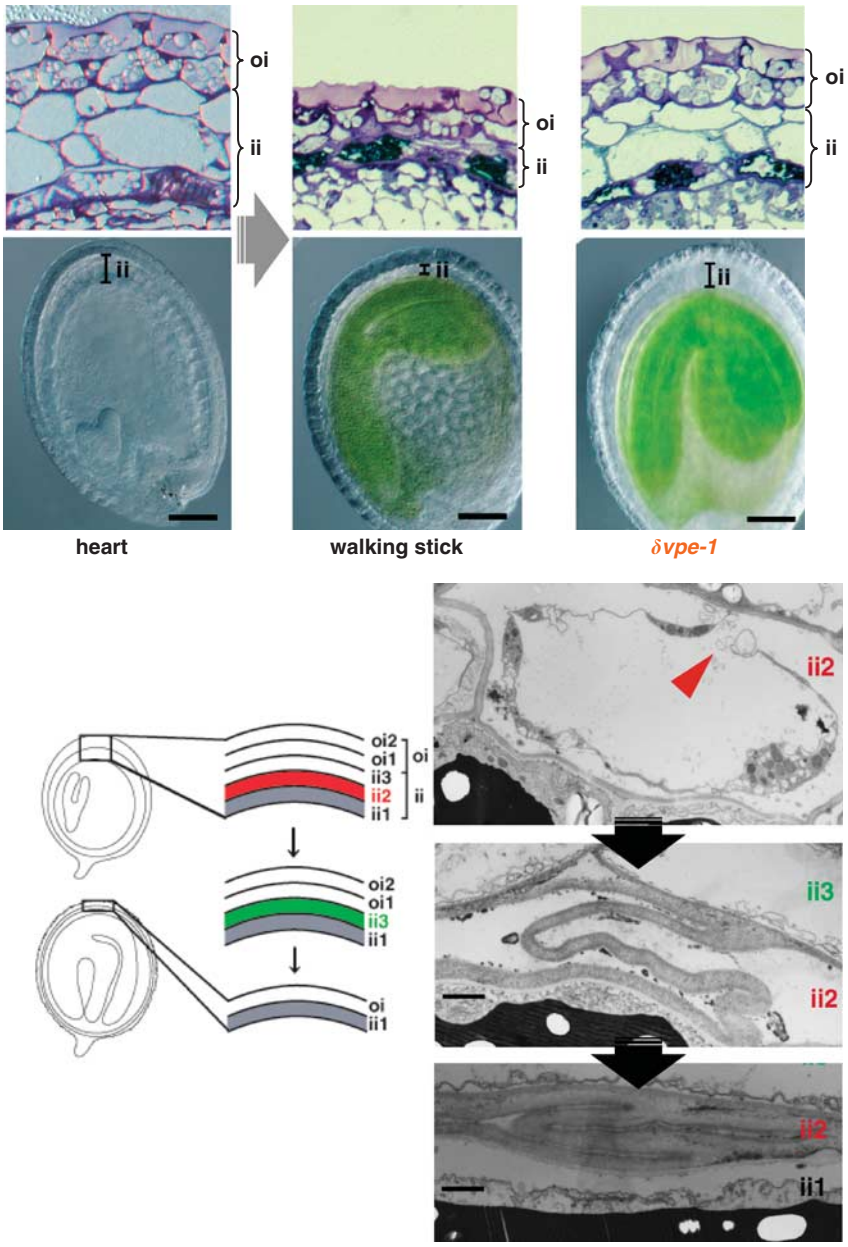

Figure 3 VPE-dependent vacuole-mediated cell death for development of seed coats. At an early stage of seed development, cell death accompanying vacuolar collapse and cell shrinkage occurs in the ii2 layer, followed by cell death in the ii3 layer. δVPE is involved in the cell death of the limited cell layers, the purpose of which is to form a hard seed coat

cell death of the two layers of the seed coat was delayed. Immunocytochemical analysis localized δVPE to electron-dense structures inside and outside the walls of seed coat cells undergoing cell death. At the early stage of seed development, δVPE is involved in the cell death of limited cell layers; the purpose of this process is to form a seed coat.[25]

Vacuolar collapse has also been shown to trigger degradation of the cytoplasmic structures during the differentiation of tracheary elements, leading to cell death.[46] VPE-dependent vacuolar cell death, which degrades dying cells, is useful in plants, because plant cells surrounded with rigid cell walls must degrade materials internally.

Involvement of caspase-1-like activity. As is caspase-3-like activity (described above), caspase-1-like activity is also involved in various types of plant PCDs including the above described VPE-dependent cell death. The *Arabidopsis* VPE-null mutant (*αvpe βvpe γvpe δvpe*) has been shown to lack YVADase activity,[24] and the VPE activity has been shown to be nicely correlated with the YVADase activity in tobacco VPE-silenced plants.[23] Accordingly, VPE was identified as the proteinase responsible for caspase-1-like activity in *Arabidopsis* and tobacco plants. Although VPE is not related to the caspase family or the metacaspase family,[47] VPE and caspase-1 share several enzymatic properties of the catalytic dyad, the active site, and substrate Asp

pocket.[48–50] Both VPE[49,51,52] and caspase-1[48] are subject to self-catalytic conversion/activation from their inactive precursors. A key difference between VPE and caspase-1 is that VPE is localized in vacuoles, unlike caspases, which are localized in the cytosol. Although plants and animals both rely on YVADase activity for cell death, VPE-dependent cell death differs from caspase-1-dependent animal cell death.

Vacuolar processing enzyme/asparagine endopeptidase (VPE/AEP). Vacuolar collapse is initiated by a vacuolar enzyme, VPE,[22–25,43] which was originally identified as a processing enzyme responsible for the maturation of seed storage proteins in protein storage vacuoles.[5,53] Studies indicate that plant VPE is responsible for the maturation or activation of vacuolar proteins.[4–8,54–56] In developing pumpkin seeds, VPE functions in the production of multifunctional proteins, including cytotoxic peptides and trypsin inhibitors, that may act in defense by processing a larger precursor protein, PV100.[15] Although VPE is a proteolytic enzyme, it also functions as a transpeptidase to make a new peptide bond, in association with cleaving a peptide bond at the C-terminal side of asparagine or aspartic acid residue.[57]

VPE is widely distributed in plants and animals and is also referred as to AEP (asparagine endopeptidase) in animals. Analyses with AEP-null mice have shown that AEP is required for the maturation of lysosomal proteinases (cathepsins B, L, and H), and that AEP has a critical role in the endosomal/lysosomal degradation of kidney cells.[58] Recently, it was reported that APE is involved in neuronal cell death, whereby AEP appears to degrade a DNase inhibitor (SET), which is a caspase substrate, and trigger DNA damage in the brain.[59] A similar VPE/AEP-dependent mechanism may function in animal PCD.

Conclusions

Using vacuoles especially for defense cell death makes sense for plants, because plants lack immune cells and so, each cell has to provide its own defense against pathogens. Plants use the vacuoles depending on the type of pathogenic organism involved. Membrane fusion, which is normally suppressed, was triggered in a proteasome-dependent manner by the bacterial infection. However, the membrane fusion system does not work on viruses, because most of defense proteins do not stop viral propagation. The membrane fusion strategy provides plants with a mechanism for attacking extracellular bacterial pathogens, and complements a vacuolar-collapse strategy. Plants have evolved two types of vacuole-mediated cell death as the cell-autonomous immune system, a membrane fusion-mediated system to kill bacterial pathogens, and a vacuolar collapse system to stop viral proliferation. The latter system, which causes rapid degradation of cellular materials, can be used not only for differentiation of cells and tissues in plants, but also for non-apoptotic cell death in animals.

Conflict of Interest

The authors declare no conflict of interest.

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