A Vacular Processing Enzyme, δVPE, Is Involved in Seed Coat Formation at the Early Stage of Seed Development

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Vacuolar processing enzyme (VPE) is a Cys proteinase responsible for the maturation of vacuolar proteins. Arabidopsis thaliana δVPE, which was recently found in the database, was specifically and transiently expressed in two cell layers of the seed coat (i2 and i3) at an early stage of seed development. At this stage, cell death accompanying cell shrinkage occurs in the i2 layer followed by cell death in the i3 layer. In a δVPE-deficient mutant, 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 that undergo cell death. Interestingly, δVPE in the precipitate fraction from young siliques exhibits caspase-1–like activity, which has been detected in various types of plant cell death. Our results suggest that, at the early stage of seed development, δVPE is involved in cell death of limited cell layers, the purpose of which is to form a seed coat.

INTRODUCTION

Most vacuolar proteins are synthesized on rough endoplasmic reticulum as prorprotein precursors and then transported to the vacuoles, where they are converted into the respective mature forms. Previously, we identified an enzyme responsible for the maturation of various vacuolar proteins and designated it vacuolar processing enzyme (VPE) (Hara-Nishimura et al., 1991, 1993; Hiraika et al., 1993). VPEs belong to a novel family of Cys proteinases (C13; EC 3.4.22.34) (Hara-Nishimura, 1998). VPE homologs are found in various organisms, including plants (Hara-Nishimura et al., 1998; Hara-Nishimura and Maeshima, 2000), mammals (Chen et al., 1998; Shirahama-Noda et al., 2003), and a protozoan, Schistosoma mansoni (Caffrey et al., 2000). We reported that mouse VPE, which is also referred to as legumain, plays a critical role in the endosomal/lysosomal degradation of kidney cells (Shirahama-Noda et al., 2003).

VPE is synthesized as an inactive proprotein precursor. The C-terminal and N-terminal propeptides are sequentially removed autocatalytically to produce the active mature forms (Hiraika et al., 1997, 1999). The removal of the C-terminal propeptide is necessary for activation of VPE (Hara-Nishimura et al., 1993). The C-terminal propeptide acts as an autoinhibitory domain that masks the catalytic site (Kuroyanagi et al., 2002). No other factor is necessary for activating VPE molecules. Therefore, VPE is a key enzyme in the vacuolar-processing system, which is responsible for maturation and/or activation of various vacuolar proteins.

Plant VPE homologs are separated into two types: vegetative-type VPEs and seed-type VPEs (Hara-Nishimura, 1998; Hara-Nishimura et al., 1998). Three VPE genes, αVPE, βVPE, and γVPE, were found in Arabidopsis thaliana (Kinoshita et al., 1995a, 1995b). An RNA gel blot analysis and a histochemical analysis with β-glucuronidase reporter constructs showed that αVPE and γVPE are expressed in vegetative organs, whereas βVPE is expressed in seeds (Kinoshita et al., 1999). By analyzing VPE-deficient Arabidopsis mutants, we demonstrated that seed-type βVPE is essential for the proper processing of storage proteins (Shimada et al., 2003). On the other hand, vegetative-type αVPE and γVPE are upregulated in association with various types of cell death and under stressed conditions (Kinoshita et al., 1999; Hara-Nishimura and Maeshima, 2000). VPEs are also induced by wound treatment, and the slow wound response of VPE is regulated by endogenous salicylic acid (Yamada et al., 2001). Theses results implied that vegetative-type VPEs are involved in cell death of plants. Recently, we found that tobacco (Nicotiana tabacum) VPEs, which exhibit caspase-1–like activity, are involved in Tobacco mosaic virus–induced hypersensitive cell death (Hatsugai et al., 2004).

A fourth VPE, designated δVPE, was found in the Arabidopsis genome database. Gruis et al. (2002) (2004) observed the expression of δVPE mRNA in young developing seeds and germinating seeds. However, the physiological function of δVPE is unknown. Here, we report that δVPE is specifically expressed in the seed coat and is associated with cell death. In angiosperm seeds, the embryo and endosperm are surrounded by the seed coat, which protects the embryo from mechanical damage, pathogen attack, and UV damage and maintains the dehydrated dormant stage until proper germination conditions.
exist. The seed coat also functions as the maternal conduit to the embryo and some metabolites flow from the seed coat to the embryo (Weber et al., 1995; King et al., 1997; Sheen et al., 1999; Wobus and Weber, 1999). The seed coat of cruciferous plants consists of two integuments (the outer and inner integuments) of the ovule. The generation of the integuments has been described in Arabidopsis (Gasser and Robinson-Beers, 1993; Gaiser et al., 1995; Schnetz et al., 1995).

During seed development, each integument goes through a dramatic differentiation process to form the mature seed coat: some of the tissues that are generated in the early and middle stages of seed development disappear at the later stages in Brassicaceae seeds (Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). As a major part of the differentiation process, the inner integument becomes compressed and impregnated with pigments imparting the characteristic brown color. However, the molecular mechanisms underlying the process are poorly understood. In this report, we show that \( \delta \text{VPE} \) functions in the cell death of the integument of seeds to form the seed coat.

RESULTS

A Novel Type of VPE and an Arabidopsis Mutant Lacking \( \delta \text{VPE} \)

VPEs of higher plants have previously been divided into two types: seed-type VPEs and vegetative-type VPEs. A phylogenetic tree of plant VPEs (Figure 1A) shows the existence of a third type of VPE, which includes Arabidopsis \( \delta \text{VPE} \) (At3g20210), tomato \((\text{Lycopersicon esculentum}) \) VPE (CAB51545), tobacco \( \text{NtPB1} \) (CAB42650), tobacco \( \text{NtPB3} \) (CAE84598), and rice \( \text{(Oryza sativa)} \) VPE (NP_910213). We propose that these VPEs constitute a novel type of VPE (discussed below).

To determine its function, we used a reverse genetic approach to identify an Arabidopsis mutant that is defective in the gene. We isolated two mutants, \( \delta \text{vpe-1} \) and \( \delta \text{vpe-4} \), in which T-DNA insertions disrupt the \( \delta \text{VPE} \) gene in the first exon and in the promoter region, respectively (Figure 1B). In an immunoblot analysis, specific antibodies against \( \delta \text{VPE} \) reacted with a 38-kD protein from wild-type siliques (Figure 1C). Siliques of the \( \delta \text{vpe-1} \) and \( \delta \text{vpe-4} \) mutants lacked the band (Figure 1C), indicating that the \( \delta \text{VPE} \) protein is absent in these mutants.

\( \delta \text{VPE} \) Is Specifically Expressed in the Outer Two Cell Layers of the Inner Integument of Developing Arabidopsis Seeds

An immunoblot analysis with anti-\( \delta \text{VPE} \) antibodies revealed that the 38-kD \( \delta \text{VPE} \) is specifically expressed in siliques (Figure 2A). In siliques at the walking-stick-shaped-embryo stage, \( \delta \text{VPE} \) was expressed in the seed coat, but not in the embryo or the pericarp (Figure 2B). The localization of \( \delta \text{VPE} \) is quite different from that of knockout mutants \( \delta \text{vpe-1} \) and \( \delta \text{vpe-4} \) with antibodies directed against \( \delta \text{VPE} \).
βVPE, which is found in the embryo (Kinoshita et al., 1999; Shimada et al., 2003).

The Arabidopsis seed coat is derived from the maternal parent and is composed of two integuments (the outer and inner integuments) (Leon-Kloosterziel et al., 1994; Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). The outer integument consists of two cell layers and the inner integument consists of mostly three cell layers (i1, i2, and i3) (Figure 2K). βVPE was detected in the inner integument, but not in the outer integument of early developing seeds (Figures 2C to 2H). βVPE was localized in the i2 and i3 layers of the inner integument, but not in the i1 layer (Figure 2H). No immunopositive green fluorescence was detected in wild-type seeds with preimmune serum (Figure 2I) or in βvpe-1 seeds with anti-βVPE antibodies (Figure 2J). This indicates the localization of βVPE is limited to the outer two cell layers (i2 and i3) of the inner integument of the seed coat of developing Arabidopsis seeds.

βVPE Is Transiently Expressed at the Early Stage of Seed Development

An additional 52-kD band was detected on the immunoblot of the developing seeds in the very early stage (Figure 3A, top). This band was not detected on the blot of the developing βvpe-1 seeds (Figure 3A, bottom). This indicates that the 52-kD band corresponds to a precursor of βVPE, which we have named proβVPE, and that the 38-kD band corresponds to the mature form. This result is consistent with our previous result that VPE is synthesized as an inactive proprotein, which is then processed to produce the active form (Hiraiwa et al., 1997; Kuroyanagi et al., 2002).

βVPE levels rapidly and transiently increased in the early stage of embryogenesis, reaching a maximum in the heart-shaped-embryo stage, and then slowly decreased until the full-sized-embryo stage, when storage proteins started to accumulate (Figure 3B). This suggests that βVPE functions in the early stage of seed development. In contrast with βVPE, which was expressed early and transiently, another Arabidopsis VPE, βVPE, increased during the late stage of seed development (Figure 3B). The increase of 37- and 27-kD forms of βVPE was accompanied by the accumulation of seed storage proteins, such as 12S globulins. βVPE expression was consistent with our previous result that βVPE is responsible for the maturation of seed storage protein synthesis.
proteins (Shimada et al., 2003). The finding that dVPE and bVPE function at different stages suggests the functional differentiation of VPEs in developing seeds: dVPE functions in the shrinkage of the seed coat, whereas bVPE functions in storage protein processing in developing cotyledons.

**ΔVPE Deficiency Prevents Shrinkage of the Inner Integument and the Degradation of Nuclei of the ii2 and ii3 Cell Layers**

The above results imply that ΔVPE functions in the formation of seed coats. Therefore, we attempted to clarify the effect of ΔVPE deficiency on the development of the inner integument of the seed coat. In wild-type plants, the thickness of the inner integument, in which ΔVPE is localized, decreased from 60 to 70 μm at the early-embryo stage (Figure 4A) to less than half this value at the walking-stick-shaped-embryo stage (Figure 4B). No reduction in the thickness of the outer integument was observed in this period (Figures 4A and 4B). Interestingly, unlike the inner integument of the wild type, the inner integument of the ΔVPE-deficient mutants Δvpe-1 and Δvpe-4 remained thick throughout embryogenesis (Figures 4C to 4F). At the early-embryo stage, the inner integument of Δvpe seeds, like that of wild-type seeds, had

Figure 3. ΔVPE Is Expressed at an Early Stage in Developing Arabidopsis Seeds, whereas βVPE Is Expressed in the Late Stage in Association with the Accumulation of Seed Storage Proteins.

(A) Developmental changes in the levels of the precursor and mature forms of ΔVPE during seed development. The wild-type and Δvpe-1 siliques were harvested at various stages, and one-tenth of total proteins from developing seeds in each one were subjected to immunoblotting with anti-ΔVPE antibodies. Developmental stages are indicated by shapes of embryo in the seeds: octant, four- to eight-celled embryo (lane 1); heart, heart-shaped embryo (lanes 2 and 3); torpedo, torpedo-shaped embryo (lanes 4 to 6); walking stick, walking-stick-shaped embryo (lanes 7 and 8), full size, almost full-sized embryo (lanes 9 and 10), accumulation stage, embryo accumulating seed storage proteins (lanes 11 to 15); dry seed (lane 16). The molecular masses are given at the right in kilodaltons.

(B) Comparison of the expression pattern of ΔVPE with that of βVPE during seed development. The wild-type siliques were harvested at the stages indicated in (A), and one-tenth of total proteins from developing wild-type seeds in each silique was subjected to immunoblotting with anti-ΔVPE antibodies (anti-ΔVPE) or anti-βVPE antibodies (anti-βVPE) and to Coomassie blue staining (CBB). The 27- and 37-kD bands are two forms of βVPE. 12S globulin is a major storage protein. Asterisk indicates a nonspecific signal.

proteins (Shimada et al., 2003). The finding that ΔVPE and βVPE function at different stages suggests the functional differentiation of VPEs in developing seeds: ΔVPE functions in the shrinkage of the seed coat, whereas βVPE functions in storage protein processing in developing cotyledons.

Figure 4. Thickness of Inner Integuments of the Seed Coats Is Reduced in the Wild-Type Seeds at the Early Stages, whereas It Is Not Reduced in the Δvpe Seeds.

Differential interference contrast images of developing seeds at an early-embryo stage (A), (C), and (E) and walking-stick-shaped-embryo stage (B), (D), and (F) from the wild type (A and B), Δvpe-1 (C and D), and Δvpe-4 (E and F). ii, inner integument. Bars = 100 μm.
In the walking-stick-shaped-embryo stage, the inner integument was compressed in the wild type (Figure 4B), but not in \( d\) (Figures 4D and 4F).

We examined the possibility that the decrease of the wild-type inner integument during development is associated with programmed cell death (Figure 5). In wild-type plants, nuclei in the ii2 and ii3 cell layers, as revealed by DAPI (4'-6-diamidino-2-phenylindole) staining, are clearly visible at the globular embryo stage (Figures 5A to 5C) but disappear at the heart-shaped embryo stage (Figures 5D to 5F). This result is consistent with the developmental aspects of wild-type seeds as shown in Figures 4A and 4B and indicates that the cells of the ii2 and ii3 layers of developing wild-type seeds are programmed to die. On the contrary, the nuclei were detected in developing \( d\) -1 seeds with heart-shaped, torpedo-shaped, and even full-sized embryos (Figures 5G to 5R). This result is consistent with the developmental aspects of \( d\) seeds as shown in Figures 4C to 4F and shows that the cells of ii2 and ii3 layers in \( d\) -1 seeds are still alive. These results suggest that \( d\) is involved in cell death. Therefore, \( d\) deficiency prevents the degradation of nuclei of the ii2 and ii3 cell layers.

**Formation of Seed Coat Is Accompanied by the Sequential Shrinkage of the Outer Two Cell Layers of the Inner Integument**

Figures 6A to 6D show the cytological changes in the inner integuments of developing wild-type seeds at the torpedo-shaped-embryo stages. At first, cells in the ii2 layer started shrinkage and plasmolysis (Figure 6A). The plasma membrane and tonoplast are partially disrupted (indicated by an asterisk in Figure 6A). At this stage, cells in the ii1 and ii3 layers remained in their normal shape. Then, the plasma membrane and tonoplast of the cells in the ii2 layer disappeared (Figure 6B). At this stage, disrupted organelles and cellular materials from the dead cells formed large aggregates. Cell layers that had lost their contents were compressed, resulting in folded cell walls (Figures 6C and 6D). Subsequently, cells in the ii3 layer started to shrink, so that the ii3 layer became compressed like the ii2 layer (data not shown). The final seed coat consisted of a one-cell-layer inner integument (ii1) and a one-cell-layer outer integument (data not shown). The cells of the ii1 layer were filled with large, electron-dense compartments (Figures 6A, 6C, and 6D). The cells of the ii2 and ii3 layers had no such subcellular feature, although they each had a developed central vacuole. The sequential shrinkage of two cell layers in the inner integument is summarized in Figure 6G.

On the other hand, cell shrinkage and plasmolysis did not occur in ii2 and ii3 cell layers of developing \( d\) -1 seeds at the torpedo-shaped-embryo stages, and the plasma membrane and tonoplast were intact (Figures 6E and 6F). This result is consistent with the results in Figures 4 and 5 and indicates that \( d\)-deficiency prevents the sequential shrinkage of the ii2 and ii3 layers.

**\( d\) Is Localized to Novel Electron-Dense Structures Inside and Outside the Cells in the Inner Integument**

After cell shrinkage, small electron-dense structures appeared in the space between the plasma membrane and the cell wall.
(Figure 6A, indicated by arrowheads) and in the extracellular space (Figure 6B, indicated by an asterisk) in the ii2 and ii3 cell layers of the inner integument. Immunofluorescence indicating the localization of δVPE was detected in granules that are distributed in the peripheral region of the cells (Figure 7A). Immunogold particles were detected on electron-dense structures in the space between the plasma membrane and the cell wall (Figure 7B, indicated by arrowheads) and in the extracellular space (Figures 7B and 7C, indicated by asterisks). A control experiment with preimmune serum showed no gold particles in the electron-dense structures (data not shown). Therefore, the electron-dense structures correspond to the granules detected on the immunofluorescence micrograph. These results indicate that δVPE is localized in the electron-dense structures, which appear after cell shrinkage (Figures 6A and 6B), in the ii2 and ii3 cell layers. The electron-dense structures were also observed in δvpe-1 seeds (Figure 7D). This suggests that the structures are aggregates composed of δVPE and other proteins.

δVPE in Insoluble Aggregates Has Proteolytic Activity

Our observations implied that δVPE plays some role in the disappearance of the ii2 and ii3 cell layers of the inner integuments. This raises the question whether δVPE exhibits proteolytic activity in the electron-dense structures. We attempted to isolate the electron-dense structures. Homogenized young siliques were separated into two fractions by centrifugation, the supernatant and pellet fractions. An immunoblot analysis of each fraction with anti-δVPE antibodies showed that δVPE was present in the pellet fraction but not in the supernatant fraction (Figure 7E). This suggests that δVPE aggregates to form the electron-dense structures in the inner integument.

No activity toward Z-AAN-MCA was detected in the siliques of the VPE-null mutant in which all four VPE genes are disrupted (δvpe-1 δvpe-3 γvpe-1 δvpe-f), indicating that VPEs are responsible for the activity detected in the wild-type siliques. δvpe-1 siliques had ~24% of the VPE activity in wild-type siliques, suggesting that δVPE is responsible for ~76% of the activity, and the other VPEs are responsible for ~24% of the activity. When homogenized siliques were separated into the pellet fraction and supernatant fraction, ~70% of the activity was in the pellet, and ~30% of the activity was in the supernatant (Figure 7E). Considering no δVPE in the supernatant (Figure 7E, immunoblot), the VPE activity in the pellet might be derived from δVPE, and the activity in the supernatant might be derived from other VPEs in siliques. These findings suggest that the protease activity that was observed in the electron-dense aggregates was due to δVPE.

δVPE Exhibits Caspase-1–Like Activity

Evidence from extensive studies (discussed below) indicates that caspase activity is also involved in various types of plant programmed cell death. Our recent data show that vegetative-type VPEs of tobacco exhibit caspase-1–like activity and are involved in hypersensitive cell death (Hatsugai et al., 2004). We examined the effects of various proteinase inhibitors on the activity of VPE. Figure 8A shows that the VPE activity was strongly inhibited by biotin-YVAD-fmk (a caspase-1 inhibitor) and
also by Ac-DEVD-fmk, as is mammalian caspase-1 (Ekert et al., 1999). None of pepstatin A (an aspartic proteinase inhibitor), E-64 (a papain-type Cys proteinase inhibitor), or AEBSF (a Ser proteinase inhibitor) had any effect on VPE activity. The result implied that \( dVPE \) has caspase-1–like activity.

To detect the protein responsible for the caspase-1–like activity, we developed a biotinylated inhibitor blot analysis with an irreversible caspase-1 inhibitor, biotin-YVAD-fmk (Figure 8B). In this analysis, an enzyme that conjugates with the inhibitor becomes visible on the blot with streptavidin-conjugated horse-radish peroxidase. Three major bands of 38, 35, and 30 kD were detected on the blot of the extract from developing wild-type seeds with a torpedo-shaped embryo (Figure 8B, lane 1). We examined the competitive effects of various inhibitors on in vitro formation of the enzyme inhibitor complex with extracts from seeds (Figure 8B, lanes 2 to 6). When 0.2 or 1.0 mM Ac-YVAD-CHO was added as a competitor with biotin-YVAD-fmk, the 38-kD band completely disappeared on the blot, but the 35- and 30-kD bands did not disappear (Figure 8B, lanes 5 and 6). This result indicates that the protein that has caspase-1–like activity is a 38-kD protein and that the other two bands are nonspecific. The 38-kD band is consistent with the molecular mass of \( dVPE \) (Figure 8C). This suggests that the caspase-1–like activity is due to \( dVPE \). To examine this possibility, an extract from wild-type or \( dvpe-1 \) developing seeds with a torpedo-shaped embryo was subjected to a biotinylated inhibitor blot analysis (Figure 8D) and an immunoblot analysis (Figure 8E). In the biotinylated inhibitor blot analysis, as in the immunoblot analysis, a 38-kD band was detected in the extract from wild-type seeds, but not in the extract from \( dvpe-1 \) seeds. These results indicate that the 38-kD protein is \( dVPE \) and that \( dVPE \) is an authentic proteinase exhibiting caspase-1–like activity that appears in association with the cell death of the inner integument.

**DISCUSSION**

**\( dVPE \) Is Involved in Cell Death during Seed Coat Formation**

The Arabidopsis seed coat just after fertilization consists of an outer integument made up of two cell layers and an inner integment made up of three layers. After desiccation of the matured seeds, the seed coat consists of one-cell-layered outer integument and one-cell-layered inner integument (ii1). The ii1 layer accumulates pigments (Devic et al., 1999). The elimination of the cell layers (ii2 and ii3) of the inner integument starts at the heart-shaped-embryo stage in Arabidopsis (Figure 4) as reported in *Brassica napus* (Wan et al., 2002). Cell shrinkage and vacuolar disruption occur in these layers at the early stage of seed development (Figure 6). DNA fragmentation, a typical feature of animal apoptosis, was detected in the inner integment of *B. napus* at an early stage of seed development with DAPI stain and a terminal deoxynucleotidyltransferase-mediated \( \text{dUTP-biotin nick end labeling assay} \) (Wan et al., 2002). We found that degradation of the nuclei of the ii2 and ii3 cell layers of developing Arabidopsis wild-type seeds occurred during an early seed development (Figure 5). These results show that the cell death to eliminate the inner integument cell layers is programmed
during seed development. Our findings clearly show that δVPE is specifically and transiently expressed in the cell layers of the inner integument that are eliminated (Figure 2) and that δVPE deficiency delays the targeted cell death and nuclear degradation in the ii2 and ii3 cell layers of the inner integument (Figures 4 to 6). We obtained two δvpe mutants, δvpe-1 and δvpe-4, neither of which expressed the δVPE protein in developing seeds (Figure 1C). Both mutants exhibited the identical phenotype of disintegration of the ii2 and ii3 cell layers. Therefore, δVPE is a key player in the developmental cell death of the outer two cell layers, which occurs during seed coat formation.

We observed that the inner integuments of the δvpe single mutant and VPE-null mutant were finally diminished at the full-sized-embryo stage, resulting in formation of thin seed coats in the dry seeds (data not shown). It is possible that the compres- sion of the seed coats at the final stage is caused by necrotic cell death as a result of mechanical pressure by growing embryo and desiccation of seeds, rather than programmed cell death. There is no difference in the physical appearances of the dry seeds between δvpe mutants and the wild type. δVPE deficiency did not affect seed dormancy or germination (data not shown).

Caspase-1–Like Activity of δVPE

Some regulatory mechanisms that underlie programmed cell death are thought to be conserved in plants and animals, and many studies have provided evidence that programmed cell death in both kingdoms shares components that include activities of caspases. Caspase-1–like activity has been detected in Arabidopsis suspension cultured cells during nitric oxide–induced cell death (Clarke et al., 2000) and in tobacco BY-2 cells during isopentenyladenosine-induced apoptosis (Mlejnek and Prochazka, 2002). Caspase-3–like activity has been detected in tobacco suspension cells during heat shock–induced apoptosis (Tian et al., 2000) and in embryonic suspension cells of barley (Hordeum vulgare) (Korthout et al., 2000). Both caspase-1– and caspase-3–like activities were observed in tomato suspension cells during chemically induced apoptosis (De Jong et al., 2000). Both caspase-1 and caspase-3 inhibitors abolish cell death in various plants (del Pozo and Lam, 1998; Clarke et al., 2000; De Jong et al., 2000; Korthout et al., 2000). A caspase-1 inhibitor abolished the DNA laddering induced by carrot (Daucus carota) cell extracts (Zhao et al., 1999), and a caspase-3 inhibitor abolished the DNA laddering during cell death induced by menadione in tobacco protoplasts (Sun et al., 1999).

Together, these results indicate that caspase activity is also involved in programmed cell death of plants.

Despite much effort, however, the plant proteinases responsible for the observed caspase activity have not been identified. Our findings demonstrate that δVPE has caspase-1–like activity and is involved in the developmental cell death of the seed coat. These results are consistent with our recent observation that tobacco VPEs exhibiting caspase-1–like activity are involved in Tobacco mosaic virus–induced hypersensitive cell death (Hatsugai et al., 2004). VPEs might be responsible for the caspase-1–like activity that has been detected in a variety of plant cell death. There is no sequence similarity between VPEs and caspases. However, VPE recognizes a caspase-1 substrate,
and VPE activity is inhibited by a caspase-1 inhibitor. VPE cleaves a peptide bond at the C-terminal side of Asp (Becker et al., 1995; Hiraiwa et al., 1999) as does caspase-1, although VPE is known to be an asparaginyl endopeptidase (Hara-Nishimura, 1998; Hara-Nishimura and Maeshima, 2000).

Electron-Dense Structure in Which ðVPE Is Localized

Previously, we reported the subcellular localization of seed-type VPE in protein storage vacuoles (Hara-Nishimura et al., 1993; Hiraiwa et al., 1993) and that of vegetative-type VPE in lytic vacuoles (Kinoshita et al., 1999). Unexpectedly, we found that the electron-dense structures in which ðVPE is localized occur outside of the cells of the ii2 and ii3 layers during the targeted cell death (Figure 7). A possible explanation for the extracellular localization of ðVPE is that ðVPE is selectively secreted from the cells because it lacks the C-terminal region of 15 amino acids that are found in other types of VPEs and that probably include a vacuolar targeting signal.

Based on what is known about VPE, ðVPE might function in maturation and activation of some hydrolases, which are upregulated during cell death. It is possible that the electron-dense structures are the site at which the dining cells of the seed coat are degraded. In animals, dying cells are packaged into apoptotic bodies and then engulfed by phagocytes, such as macrophages and neutrophils (Franc, 2002; Geske et al., 2002). However, plants do not have phagocytes, and cells surrounded by a rigid cell wall must degrade their own contents. The plant system for degrading dying cells is different from the animal one. Plants appear to have evolved a VPE-mediated system, whereas animals have evolved a caspase-mediated system.

Different Expression of ðVPE from Those of the Other VPEs

Our results clearly show that ðVPE is specifically localized in two cell layers of the inner integuments of the Arabidopsis seed coat (Figure 2). This is consistent with a histochemical analysis with a β-glucuronidase reporter gene that was driven by a ðVPE promoter (S. Nakane and I. Hara-Nishimura, unpublished data). The highly specific expression of ðVPE in particular cell layers of the seed coat is different from the expression of other Arabidopsis VPE homologs: aVPE and ðVPE are expressed in the vegetative organs and ðVPE is expressed in the developing embryo (Kinoshita et al., 1999). Transcripts of LeVPE1, which is a putative ortholog of ðVPE, were found to increase during the early phases of fruit development, reach a maximum at the end of the expansion phase (15 to 20 d after anthesis), and then slowly decrease (Lemaire-Chamley et al., 1999). The expressions of two tobacco VPEs (NIPB1 and NIPB3), which are also putative orthologs of ðVPE, were found in early seed development (Zakharov and Muntz, 2004). These features are similar to the expression pattern of ðVPE (Figure 3).

In maturing Arabidopsis seeds, two major storage proteins, 12S globulin and 2S albumin, are synthesized as precursors. ðVPE is essential for proper processing of the precursor molecules into the mature storage proteins (Shimada et al., 2003; Gruis et al., 2004). Interestingly, vegetative-type VPEs, aVPE and ðVPE, partly compensate for ðVPE deficiency in the ðvpe seeds (Shimada et al., 2003; Gruis et al., 2004). Triple VPE mutant (ðvpe-1 ðvpe-3 ðvpe-1) seeds accumulate no properly processed mature storage proteins (Shimada et al., 2003; Gruis et al., 2004). This result suggests that ðVPE is not involved in processing storage proteins. This is consistent with the result that ðvpe seeds normally process storage proteins as do wild-type seeds (Gruis et al., 2002, 2004). Even though both ðvpe and ðVPE are accumulated in developing seeds, the tissues in which they are expressed are completely different: ðVPE is expressed in the maternal tissues and ðVPE is expressed in tissues of the next generation (embryo). Unlike ðVPE or ðVPE, ðVPE has no ability to compensate for the deficiency of ðVPE.

METHODS

Plant Materials and Growth Conditions

Seeds of Arabidopsis thaliana (ecotype Columbia [Col-0]) were surface sterilized with 95% ethanol and then sown onto 0.8% agar in MS medium (Wako, Tokyo, Japan). After a 4-d incubation at 4°C to break seed dormancy, the seeds were germinated and grown at 22°C under continuous light (100 μE s−1 m−2) for 21 d. The seedlings were transferred onto vermiculite for subsequent growth.

Isolation of ðvpe Mutants and Generation of a VPE-Null (Quadruple) Mutant of Four VPE Genes

We used two T-DNA insertion mutants of Arabidopsis. ðvpe-1 was isolated from a large population of Arabidopsis plants provided by the Kazusa DNA Institute (Chiba, Japan) by PCR-based screening. We used the following primers: ðVPE-kFW, 5′-TCATGCAAGGTGCTTATG-TGTGA-3′; ðVPE-kRV, 5′-CTGCCGTTACATACGTGGTA-3′; P06RB, 5′-TTCCCTTAAATCTCCTGGTCATGATC-3′ for the T-DNA right border.

ðvpe-4 (SALK_009856) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Insertion mutant information was obtained from the SALK Institute Genomic Analysis Laboratory Web site (http://signal.salk.edu). These mutants have a Columbia background. We crossed the triple mutant (ðvpe-1 ðvpe-3 ðvpe-1; Shimada et al., 2003) and the single mutant ðvpe-1 with each other and isolated a VPE-null mutant (ðvpe-1 ðvpe-3 ðvpe-1 ðvpe-1).

Polyclonal Antibodies against ðVPE

The cDNA of Arabidopsis ðVPE (AVS55212) was donated by the Kazusa DNA Research Institute (Asamizu et al., 2000). A PCR-amplified DNA fragment encoding the putative mature region (amino acids 40 to 407) of ðVPE was inserted into the pET32a vector (Novagen, Madison, WI). A fusion protein with a His-tag was synthesized in E. coli BL21 (DE3) cells and purified on a Ni²⁺ column. The purified protein (1 mg) in 1 mL of 50 mM Na-phosphate buffer, pH 7.2, and 6.4 M urea was emulsified with an equal volume of Freund’s complete adjuvant. The emulsion was injected subcutaneously into a rabbit. After 3 weeks, three booster injections with incomplete adjuvant were given at intervals of 7 d. One week after the final booster injection, blood was drawn and antibody was prepared. The antibodies exhibited strong specificity for ðVPE-related proteins (Figure 4) and were of a titer sufficiently high to detect ðVPE-related proteins (data not shown).

Immunoblot Analysis

The siliques and other organs of Arabidopsis were homogenized in 20 mM Tris-HCl, pH 8.0, and 2% (w/v) SDS before centrifugation to collect
soluble proteins. The proteins were subjected to SDS-PAGE and were transferred electrophoretically to a polyvinylidene difluoride membrane (0.22 μm; Nihon Millipore, Japan). The membrane was incubated with anti-ΔVPE antibodies (diluted 5000-fold) in TBS-T (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% [v/v] Tween 20) after blocking with 5% (w/v) skim milk in TBS-T. Horseradish peroxidase-conjugated donkey antibodies directed against rabbit IgG (diluted 1:5000; Amersham Pharmacia Biotech, Tokyo, Japan) were used as the secondary antibodies. The ΔVPE-related proteins were visualized with an enhanced chemiluminescence kit (ECL system; Amersham Pharmacia Biotech). We also used antibodies directed against βVPE (Shimada et al., 2003).

Immunofluorescence Analysis
The developing seeds of Arabidopsis that had walking-stick-shaped embryos were fixed for 40 min in 7.2% (v/v) formaldehyde, 0.1% (v/v) Nonidet P-40, 10% (v/v) dimethyl sulfoxide, and 50 mM Na-phosphate buffer, pH 7.2, washed twice with TBS-T for 5 min, incubated in TBS-T containing 5% (w/v) Cellulase Onozuka R-10 (Yakult, Tokyo, Japan) and 2% (w/v) Pectolyase Y-23 (Kikkoman, Tokyo, Japan) for 20 min at 30°C, washed twice with TBS-T, incubated in blocking buffer (2% [w/v] BSA and TBS-T) for 30 min, incubated with anti-ΔVPE antibodies (diluted 500-fold) in the blocking buffer for 40 min, washed three times for 5 min each, incubated for 1 h with goat anti-rabbit IgG antibodies conjugated with Alexa Fluor 488 (absorbance, 495 nm; emission, 519 nm; Molecular Probes, Eugene, OR), washed three times for 5 min with TBS-T, and mounted.

Ultrastructural Analysis and Immunogold Labeling
The developing seeds with torpedo-shaped embryos were vacuum infiltrated for 1 h with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.06 M sucrose in 0.05 M cacodylate buffer, pH 7.4. The tissues were cut into slices of <1 mm in thickness with a razor blade and treated for another 2 h with freshly prepared fixative. Procedures for ultrastructural studies were essentially the same as described previously (Hara-Nishimura et al., 1993).

Immunogold labeling procedures were essentially the same as described previously (Hara-Nishimura et al., 1993), except for the use of anti-ΔVPE antibodies. Postfixation was omitted for immunoelectron microscopy. The samples were dehydrated in a graded dimethylformamide series at –20°C and embedded in London Resin White (London Resin Co., Basingstoke, Hampshire, UK). Blocks were polymerized under a UV lamp at a Reichert ultramicrotome (Leica, Heidelberg, Germany). The ultrathin sections were incubated with 1% (w/v) BSA in PBS for 1 h at room temperature and then incubated with anti-ΔVPE antibodies (diluted 1:5000) in blocking solution overnight at 4°C. The sections were washed with PBS and then incubated for 30 min at room temperature with a solution of protein A-gold (10 or 15 nm; Amersham Pharmacia Biotech) that had been diluted 1:30 in the blocking solution. The sections were washed with distilled water and then were stained with uranyl acetate and lead citrate. After staining, all sections were examined with a transmission electron microscope (model 1200EX; JEOL, Tokyo, Japan) at 80 kV.

DAPI Staining
The young wild-type or Δvpe-7 siliques were fixed for 40 min in 7.2% (v/v) formaldehyde, 0.1% (v/v) Nonidet P-40, 10% (v/v) dimethyl sulfoxide, and 50 mM Na-phosphate buffer, pH 7.2. The fixed tissues were dehydrated through a series of ethanol solutions (50, 50, 60, 70, 85, 100, and 100%) for 30 min each, infiltrated with a t-butyl alcohol/Paraplast Plus (Oxford Labware, St. Louis, MO) mixture and then embedded in Paraplast. Thin sections (7-μm thick) were cut on a microtome (RM2155; Leica) and mounted on slides. The samples were dried overnight at 40°C and incubated in xylene to remove Paraplast from the tissue sections. The sections were washed in 100% ethanol twice and stained by 1 μg/mL DAPI in methanol for 30 min.

Enzyme Assays for VPE Activity and Caspase-Like Activity
We used fluorogenic substrates conjugated with a-(4-methyl-coumaryl-7-amide) (MCA; Peptide Institute, Osaka, Japan): Z-AAN-MCA (VPE-specific substrates), Arabidopsis siliques frozen in liquid nitrogen were ground using a mortar and pestle and were homogenized with 50 mM Na-acetate buffer, pH 5.5, containing 50 mM NaCl, 1 mM EDTA, and 100 mM DTT. We used the whole extract as enzyme solution. Alternatively, the soluble and insoluble fractions were obtained by centrifugation at 15,000g for 5 min. The insoluble fraction was resuspended with the same buffer. The reaction was started by adding each fluorogenic substrate (to a final concentration of 500 μM) to the homogenate, and an increase in the fluorescence of the 100-μL reaction mixture was measured at 465 nm with a fluorescence spectrophotometer (GENios; Tecan, Tokyo, Japan). The inhibitors used were as follows: 0.1 mM pepstatin A, 0.1 mM E-64, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 20 to 100 μM biotin-YVAD-fmk, and 20 to 100 μM biotin-DEVD-fmk.

Biotinylated Inhibitor Blot Analysis
We used the extract from developing seeds with torpedo-shaped embryo. For a biotinylated inhibitor blot analysis, the sample solutions were preincubated with or without each inhibitor as described above and incubated in 100 mM Na-acetate, pH 5.5, containing 100 mM DTT with a final concentration of 20 μM biotin-YVAD-fluoromethylketone (biotin-YVAD-fmk; Calbiochem, San Diego, CA) for 60 min. The resulting complex of enzyme and biotin-YVAD-fmk was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was treated with a blocking solution and then with streptavidin-conjugated horseradish peroxidase (diluted, 2000-fold; Amersham Pharmacia Biotech) for 30 min. Detection was performed with an enhanced chemiluminescence kit (ECL system).

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