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Programmed cell death of the mung bean cotyledon during seed germination

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ABSTRACT. The cotyledon of the mung bean (*Vigna radiata*) undergoes programmed cell death (PCD) during seed germination. Death of the cotyledon storage parenchyma cells was preceded by reserve mobilization and vacuolation. During the dismantling phase of PCD, the storage parenchyma cells exhibited progressive autolysis, resulting in the formation of a large central vacuole. Complete lysis of the cytoplasm and nucleus occurred, and condensation of the protoplast away from the cell wall appeared at the final stage of cell death. Cytochrome c translocation from the mitochondria to the cytosol and internucleosomal DNA fragmentation were also observed in cotyledon senescence during seed germination. Nuclease activity was detected *in vitro* during mung bean cotyledon PCD, and the activity of this nuclease was repressed by the caspase-3 inhibitor Ac-DEVD-fmk. Caspase-like protease (CLP) activity was detected in cotyledons during seed imbibition and after seed germination. CLP activity reached a peak in day 3 cotyledons. These CLP activities were inhibited by a caspase-3-specific inhibitor but were resistant to caspase-1-specific inhibitors. This study demonstrates that the programmed cell death in mung bean cotyledons involves nuclease induction, nuclear DNA fragmentation, cytochrome c releasing and CLP participation. These PCD hallmarks establish the important paradigm for further understanding of PCD in higher plant.

Keywords: Caspase-like protease; Cytochrome c releasing; Internucleosomal DNA fragmentation; Mitochondria; Programmed cell death; Reserve mobilization and vacuolation.

INTRODUCTION

Programmed cell death (PCD) is a genetically determined process present in all multicellular organisms by which cells activate their own demise (Lam, 2004). PCD occurs in various types of plant cells during development and in response to environmental stimuli (Greenberg, 1996; Mittler et al., 1996; Pennell and Lamb, 1997; McCabe and Leaver, 2000).

The most widely studied form of PCD in animal cells is apoptosis, which is characterized by distinct morphological and biochemical features. The common morphological changes associated with apoptosis include cell shrinkage, cytoplasmic membrane blebbing, nuclear condensation and fragmentation (Steller, 1995). Comparative studies of PCD in plants and animals reveal both similarities and distinct features (Lam and del Pozo, 2000; Bonneau et al., 2008; Reape et al., 2008; Reape and McCabe, 2010). Although the mechanisms of PCD in plants are not well understood, the release of apoptogenic proteins from mitochondria and the involvement of caspase-like molecules appear to be key factors. A better understanding of PCD in plants was achieved by two recent discoveries, namely, that plant cell death is dependent on proper proteasome functioning (Hatsugai et al., 2009) and that the *in vivo* substrate of a type II metacaspase is associated with cell viability (Sundstrom et al., 2009).

Based on the morphological hallmarks of the cell-dismantling phase, programmed cell death has been categorized into three major morphotypes in animal PCD: apoptotic cell death (morphotype 1), autophagic or vacuolar cell death (morphotype 2) and degenerative cell death (morphotype 3). During normal plant growth and developmental PCD, it is most likely that morphotype 2 morphological changes are involved in developmental plant PCD. During PCD, a large vacuole forms and then ruptures to release hydrolytic enzymes to degrade the cellular contents (reviewed by Beers, 1997; van Doorn and Woltering, 2005). The protoplast then retracts from the cell wall in the last step of PCD (Reape et al., 2008).

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Much of the concept of PCD in plants derives from the observation of DNA fragmentation, a feature of both plant and animal PCD. Internucleosomal-sized fragments have been found in a variety of plant tissues undergoing PCD (Mittler and Lam, 1997; Balk et al., 2003). The internucleosomal DNA degradation that occurs during apoptosis is accompanied by the activation of endogenous Ca^{2+}/Mg^{2+}-dependent endonucleases (Mittler and Lam, 1995; Xu and Hanson, 2000; He and Kermode, 2003a). In plants, nucleases are regulated both developmentally and in response to endogenous and exogenous stimuli (reviewed by Sugiyama et al., 2000).

Mitochondria can play a role in the initiation of PCD by releasing cytochrome c from the intermembrane space into the cytoplasm in response to a variety of death-promoting stimuli, such as heat shock, D-mannose and menadione treatment (Balk et al., 1999; Stein and Hansen, 1999; Sun et al., 1999; Vacca et al., 2006). Moreover, cytochrome c release was observed in the PCD that occurs during tracheary element (TE) formation in zinnia cells and during anther development in sunflowers (Balk and Leaver, 2001; Yu et al., 2002).

Proteases are known to play important roles in PCD (reviewed by Beers, 1997; Lam and del Pozo, 2000). Numerous studies have indicated the involvement of caspase-like proteases in the regulation of plant PCD (reviewed by Bonneau et al., 2008; Reape and McCabe, 2010). Activation of caspase-like proteases (CLPs) was observed in stressed and normal developmental plant PCD. Caspase-specific inhibitors abolished the induction of plant PCD both in normal development and in response to bacteria, fungal elicitors and various forms of stress. However, no caspase-like protease genes have yet been isolated from plants.

This study assessed programmed cell death in mung bean (*Vigna radiata*) cotyledons by examining the processes of reserve mobilization, vacuolation and the dismantling of cotyledon cells during seed imbibition and after germination. Nuclease induction and nuclear DNA fragmentation were also investigated, and the role of mitochondria in the PCD process was characterized by detecting the release of cytochrome c. Finally, we examined the involvement of proteases and CLPs in PCD as well as the effects of caspase inhibitors on protease and CLP activities.

MATERIALS AND METHODS

Plant materials

Mung bean (Vigna radiata, Tainan No. 5) seeds were immersed in water either at 4° C or 27°C for 12 h. Seeds, which began to germinate after imbibition at 27°C for 12 h, were further incubated at 27°C in vermiculite for 0.5, 1.5, 2.5, 4.5, 6.5, or 8.5 days at which the corresponding seeds were classified, respectively, as at the stage of day-1, -2, -3, -5, -7 and -9 germination. Cotyledons were dissected from the imbibed seeds or seeds at each one of these germination stages, frozen in liquid nitrogen and stored at -80°C until use.

Microscopic and electron microscopic analysis

Whole cotyledons were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, at 4°C overnight. After three 20-min buffer rinses, the cotyledons were postfixed in 1% osmium tetraoxide in the same buffer for 4 hr at room temperature and then rinsed three times with the same buffer. The cotyledons were dehydrated in an acetone series, embedded in Spurr's resin and sectioned with a Leica Ultracut E ultramicrotome. Semi-thin sections (1 μm) for light microscopy were stained with 1% toluidine blue for 1 min at 60°C on a hot plate and observed using Zeiss Axioplan microscopy. Thin sections (90 nm) were stained with uranyl acetate and lead citrate and were examined using a Philips CM 100 transmission electron microscope at 80 KV.

DNA extraction and Southern hybridization

Genomic DNA was purified from the cotyledons of mung bean seeds (Wang et al., 1996) and Southern hybridized with a radioactive probe prepared from the mung bean nuclear DNA (Dai et al., 2005).

Preparation of cytosolic and mitochondrial extracts

Cotyledons (with the embryo completely removed) were frozen in liquid nitrogen and ground to a fine powder. After adding extraction buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 1 mM dithiothreitol, and 1 tablet of EDTA-free protease inhibitor [Roche] per 7 ml of buffer), cytosolic extracts were obtained after centrifugation to remove cellular debris and nuclei. Cotyledon mitochondria purification was performed using the same procedures as previously reported with some minor modifications in tissue blending (Dai et al., 2005). A modified Lowry method was used for protein quantification in the cotyledon cytosolic and mitochondrial fractions.

Gel assay of DNase activity

The detection of DNase activity was based on the method of Thelen and Northcot (1989) with minor modifications. For the in-gel nuclease assay, cytosolic extracts (15 μg protein per lane) were heated in sample buffer (2% SDS, 50 mM Tris-HCL, pH 6.8, 12% glycerol and 0.01% G-250) at 55°C for 10 min and run out in a 16.5% (w/v) polyacrylamide gel. The resolving gel contained 50 μg/ml bovine serum albumin and 50 μg/ml of ss- or dsDNA substrate. Following electrophoresis, the gel was washed twice for a total of 30 min with basic buffer (10 mM Tris-HCl, pH 7.5) consisting of 25% isopropanol, followed by two 30-min buffer washes at room temperature. To detect DNase activity, the gel was incubated overnight at 37ºC in basic buffer plus 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Nucleic acids in the gel were visualized by staining with 10 mM

Tris-HCl and 0.5 μg/ml ethidium bromide for 1 h. Upon UV illumination, DNase activity was visualized as a clear band in a stained field.

For the nicking and linearization of supercoiled plasmid PBR 322 by DNase, the cytosolic fractions were diluted to a final protein concentration of 2 mg/ml in 56 μl of buffer A (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.25 M sucrose) plus 4 μl (2 μg) of supercoiled plasmid PBR 322. The reactions were incubated for 30 min at 30ºC and were then stopped and analyzed by agarose gel electrophoresis. The presence of endonuclease activity was detected by following the conversion of the plasmid from its supercoiled (CCC) to open-circle (OC) and linear forms. Analyses of nuclease activity present during cotyledon PCD were repeated three times and identical results were obtained.

Protease inhibitor effects on endonuclease activity

The irreversible specific peptide caspase-3 inhibitor Acetyl-Asp-Glu-Val-Asp-fluoromethylketone (Ac-DEVD-fmk, Calbiochem) was dissolved in 1% dimethyl sulfoxide (DMSO, Merck). Aliquots (10 μg-protein equivalent) of cytosolic fractions were preincubated with 20 mM Ac-DEVD-fmk for 2 h at 37°C and then exposed to supercoiled plasmid PBR 322 (0.5 μg). The reactions were incubated for 22 min at 30°C in a final volume of 15 μl of buffer A. The reactions were stopped and analyzed by agarose gel electrophoresis.

Western blot analysis

Mitochondrial protein (5 μg) and cytosolic protein (10 μg) were separated in 12.5% (w/v) SDS-PAGE gels. After the proteins were transferred to a PVDF membrane, immunoblotting analysis was carried out using a cytochrome c antibody (Pharmingen).

Protease activity assay

Mung bean cotyledons (approximately 3 g) were frozen in liquid nitrogen, ground to a fine powder and then dissolved in extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 20 mM NaCl, 0.1% Triton X-100, 60 mM sucrose). The samples were centrifuged at 12000 × *g*, and the supernatants were used for the protease activity assay. In-gel activity assays were carried out according to He and Kermode (2003b) with minor modifications. Briefly, 20-μl protein extracts were mixed with an equal volume of gel sample buffer and incubated at room temperature for 20 min, followed by fractionation in a 12.5% SDS-PAGE containing 0.1% gelatin in the separating gel. The gel was soaked for 30 min in renaturing buffer (50 mM sodium acetate, pH 5.5) containing 0.1% Triton X-100. Protease activities were visualized by incubating the gel in the developing buffer (50 mM sodium acetate, pH 5.5, 1 mM DTT, 5 mM $CaCl_2$) at 37°C for 4 h, followed by staining with Coomassie blue R250. For the protease inhibitor analysis, an inhibitor was mixed with protein extracts and incubated at room temperature for 30 min before electrophoresis. The inhibitors applied were 1 mM PMSF (serine protease inhibitor), 6 μM Aprotinin (serine protease inhibitor), 50 μM Pepstetin A (aspartic protease inhibitor), 40 μM Leupeptin (cysteine protease inhibitor), 35 μM E-64 (cysteine protease inhibitor), 100 μM Ac-VAD-CHO (caspase-1 inhibitor), 200 μM Ac-YVAD-CHO (caspase-1 inhibitor) and 50 μM Ac-DEVD-fmk (caspase-3 inhibitor).

Caspase-like protease activity assay

Caspase-like protease activity assays were performed according to He and Kermode (2003b). The reactions were adjusted to 400 μg of cotyledon protein in 50 μl extraction buffer containing 75 μM Ac-DEVD-AMC (caspase-3 substrate). After a 3-h incubation at 30°C in a 96-well black plate, the reaction was stopped by adding 20 μl of 1 M HCl. The fluorescence was measured with Labsystems Fluoroskan II at excitation/emission wavelengths of 355/460 nm. The relative activities of CLPs were determined according to He and Kermode (2003b). Each assay was carried out in triplicate. Inhibition of CLP activity was performed by adding different inhibitors as described above.

RESULTS

Morphological changes and the mobilization pattern of protein reserves in the cotyledons after seed germination

The morphological changes of the mung bean cotyledon during seed germination are shown in Figure 1. To observe the mobilization pattern of protein reserves in the mung bean cotyledons, transverse sections of cotyledon were stained with toluidine blue (TB, Figure 2). In cotyledons incubated at 27°C for 12 h, most cells were stained with toluidine blue, indicating that little mobilization of seed proteins occurred in the cotyledons at this seed-germinating stage (Figure 2a). The epidermal cells became

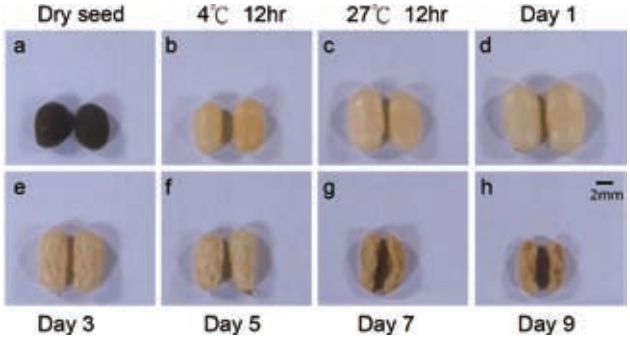

Figure 1. Morphological changes of the mung bean cotyledon during seed imbibition and germination. a, Dry seeds. b, seeds imbibed in water at 4°C for 12 h. c, seeds beginning to germinate after imbibition for 12 h in water at 27°C. d, e, f, g and h, seed germination for 1 day (imbibition at 27°C for 12 h plus 12 hr incubation in vermiculite), 3 days, 5 days, 7 days and 9 days, respectively.

TB-negative in one-day-old cotyledons (Figure 2b). As seedling growth continued, there was a progressive decline of storage organelles across the storage parenchyma tissue, indicating the mobilization of carbohydrate and protein reserves (Figure 2c-e). The degradation of storage proteins and starch organelles had reached the cells of the vascular bundle on day 7 (Figure 2e). The cotyledons had lost nearly all of their storage proteins and starch organelles by day 9 (Figure 2f). Loss of the storage parenchyma cell structure was evident at the later stages of cotyledon senescence.

Ultrastructure of cotyledon cells during PCD

The protein reserves of the storage parenchyma cells in *V. radiata* are located in protein bodies (PB, Figure 3a), spherical organelles measuring 2-10 μm in diameter and consisting of a dense protein matrix. Ultrastructural studies have shown that the dense protein matrix gradually disappears in cotyledons during seed germination and that the protein bodies become protein storage vacuoles (Figure 3b-c, arrow). As post-germinative seedling growth continued, there was a progressive decline of storage proteins across the parenchyma tissue, indicating that mobilization of the protein reserves was continuous. More cells became vacuolated and displayed an autolytic mechanism of protoplast degradation (Figure 3d-f). The cytoplasm and organelles were gradually engulfed and lysed by a high number of growing vacuoles. In storage parenchyma cells, the vacuoles progressively destroyed the cytosol and organelles, leading to the formation of a

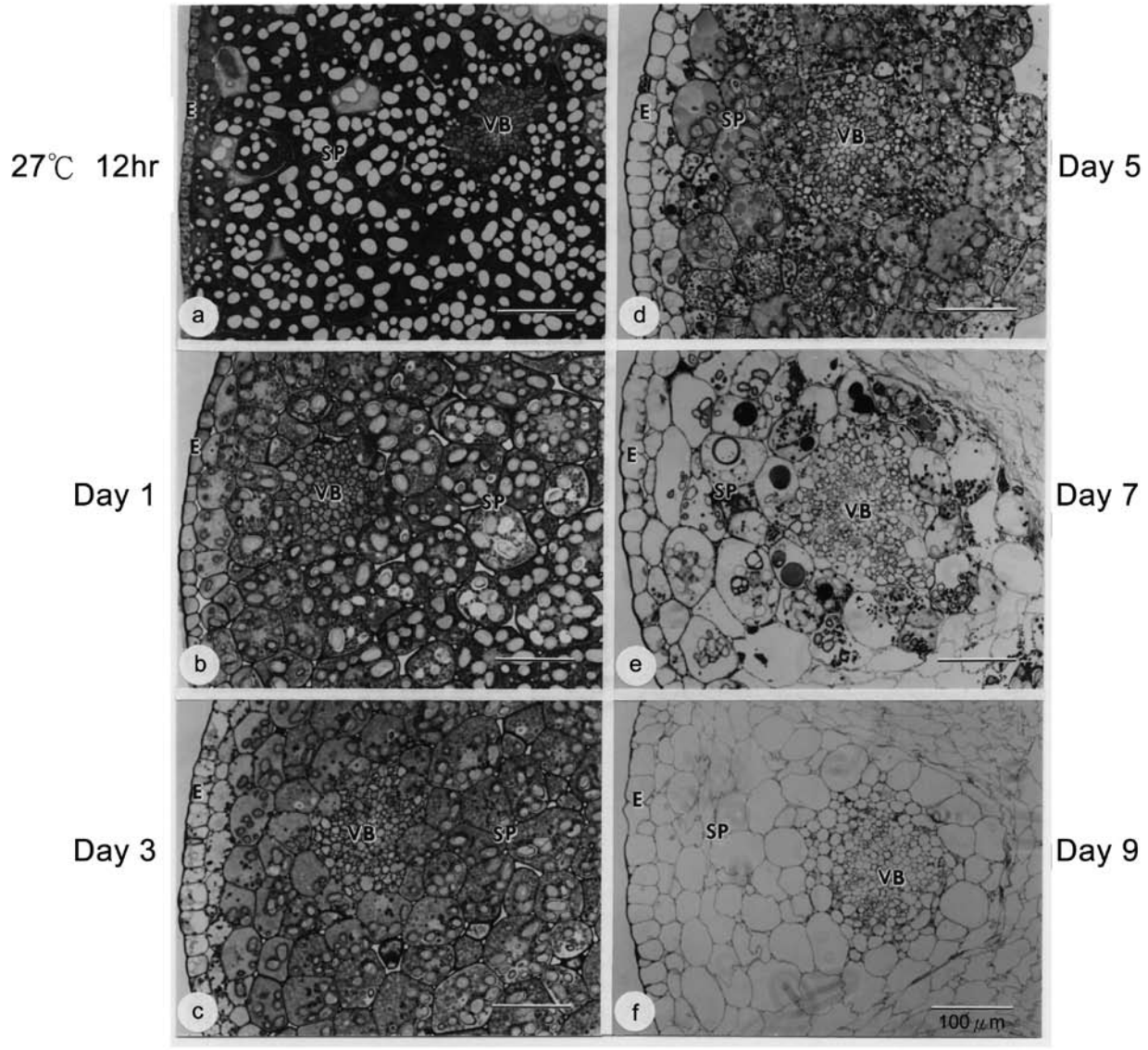

Figure 2. Toluidine blue staining of thin sections of mung bean cotyledons of imbibed and germinated seeds. a, Cotyledon of seeds imbibed at 27°C for 12 h. b, c, d, e and f represent day 1, day 3, day 5, day 7 and day 9 cotyledons, respectively, as described in Figure 1. Thin sections (1 μm) of cotyledons stained with toluidine blue show that the mobilization of storage materials starts with epidermal cells at the regions farthest from the vascular bundles and gradually reaches the center of the vascular bundle region. VB, vascular bundles; E, epidermis; SP, storage parenchyma. Scale bar = 100 μm

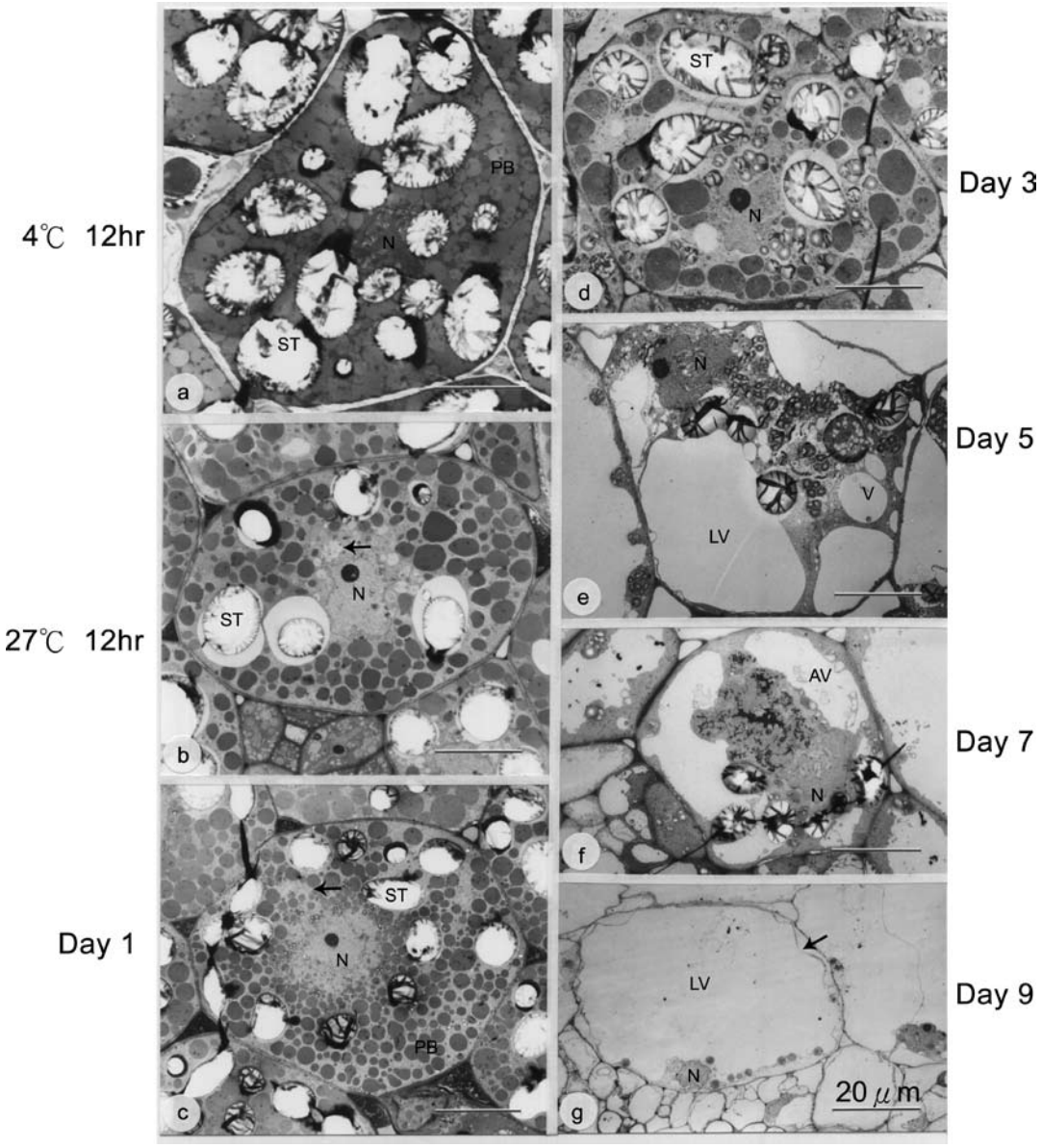

Figure 3. Ultrastructures of storage parenchyma cells in the cotyledon of mung bean seeds during PCD. a to g present cotyledons of plants incubated at 4°C for 12 h, 27°C for 12 h, 1 day, 3 days, 5 days, 7 days and 9 days, respectively. Parenchyma cells are in the process of losing their reserves, as shown in b and c. The protein bodies are transforming to protein storage vacuoles. Arrow indicates protein storage vacuoles converting from protein bodies. The formation of numerous large vacuoles (LV) and lobing of the nucleus is exhibited in the day 3 cotyledon (d). Larger vacuoles held against the cell wall may result from coalescence of protein storage vacuoles (ST) in the day 5 cotyledon (e). The day 7 cotyledon is in the late stage of PCD (f). Near-complete lysis of the cytoplasm and nucleus is shown by collapsing autolytic vacuoles. The plasma membrane is shrunken and detached from the cell wall in the day 9 cotyledon (g).

large central vacuole. This vacuole occupied virtually all of the cytoplasm (Figure 3e-f). Cells did not die before this highly vacuolated configuration formed. The earliest morphological sign of nuclear degradation was the appearance of crenellated and lobed nuclei, as shown in Figure 3c-d. The tonoplast rupture was delayed until the cytoplasm and organelles, including the nucleus, were almost completely lysed (Figure 3f). Once this occurred, the protoplasm disappeared, leaving a cellular corpse comprising the cell wall and the retracted protoplast (Figure 3g, arrow).

Internucleosomal DNA degradation during cotyledon PCD

Genomic Southern hybridization revealed that 180-200 bp DNA ladder fragments appeared in the cotyledon cells as early as the mung bean seeds began to germinate (Figure 4, panel A). The sizes of these DNA ladder fragments were similar to those of mono- and oligo-nucleosomal DNA and thus suggested us that they were the products of the internucleosomal DNA degradation. The intensity of these DNA ladder fragments increased significantly in the cotyledon cells from day-5 to day-9 germinating seeds.

Cytochrome c release from the mitochondria to the cytosol during cotyledon PCD

Purified cotyledon mitochondria and cytosolic proteins were obtained from the cotyledons of seeds immersed at 4°C for 12 h and day 1 to day 9 seedlings. An immunoblotting analysis with a cytochrome c antibody is shown in Figure 4. Panel B represents cytochrome c in the mitochondria and Panel C represents cytochrome c released into the cytosol. Cytochrome c was present in the mitochondria, but not in the cytosol, of seeds immersed at 4°C for 12 h and of day 1 cotyledons (Figure 4, panels B and C, respectively). Cytosolic cytochrome c was detected in day 2 cotyledons and observed continuously in day 3 to day 9 cotyledons. Mitochondrial cytochrome c gradually decreased from day 5 to day 9.

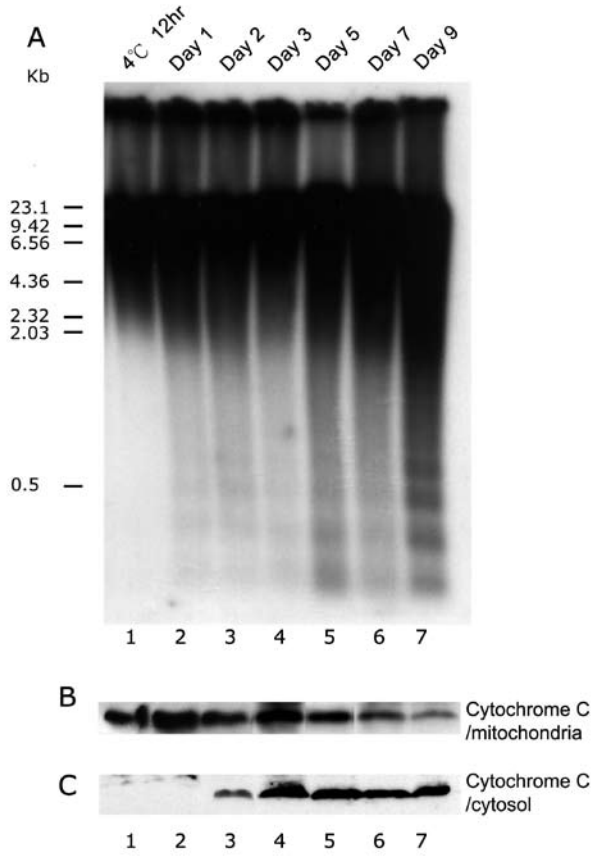

Figure 4. Southern blot analysis of genomic DNA fragmentation and immuno-detection of cytochrome c in mitochondrial and cytosolic fractions of mung bean cotyledons during PCD. Panel A, Equal amounts of genomic DNA harvested from cotyledons were separated in an agarose gel and then transferred to a nylon membrane. The DNA was hybridized to a radioactive probe purified from mung bean total nuclear genomic DNA. Panel B and C, mitochondrial and cytosolic proteins from cotyledons were fractionated by SDS-PAGE electrophoresis and then subjected to western blot analysis with a cytochrome c antibody. Lanes 1 to 7 present samples harvested from 4°C for 12 h and day 1 to day 9 cotyledons, as indicated at the top of the image. Nuclear DNA fragmentation appeared in day 1 cotyledons and was continuously exhibited up to day 9. The release of cytochrome c from mitochondria to the cytosol was detected in day 2 to day 9 cotyledons.

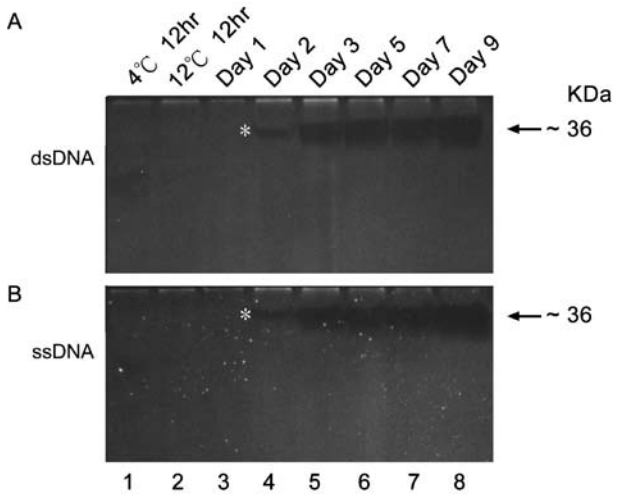

Figure 5. In-gel nuclease activity of cytosolic enzymes extracted from mung bean cotyledons. Equal amounts of cotyledon protein from the indicated time points were resolved by SDS-PAGE electrophoresis. The gel contained either double-stranded (A) or single-stranded (B) salmon sperm DNA. After gel fractionation, nuclease detection was subsequently processed and followed by ethidium bromide staining. The band indicated by a star (about 36 kDa) represents the location of the nuclease activity in the gel matrix. Cytosolic extracts of cotyledon from dry seeds, seeds imbibed for 12 h at 4°C, seeds imbibed for 12-h at 27°C, 1-day-old, 3-day-old, 5-day-old, 7-day-old and 9-day-old seedlings were used for the nuclease activity analysis.

Endonuclease activity during cotyledon PCD

In gel assay of nuclease activity in the cytosolic extracts of cotyledon cells detected a protein band with endonuclease activity and the size around 36 kDa that appeared at day-2 germinating seeds (Figure 5, panels A and B). The activity of this endonuclease increased quantitatively with the number of days after seed germination (Figure 5, panels A and B).

Caspase-3 inhibitor blocks endonuclease activity during cotyledon PCD

The activity of the cotyledon endonucleases was further confirmed by their effect on a DNA plasmid. Figure 6, Panel A indicates that supercoiled plasmid was progressively converted to its open-circle and linear forms by endonuclease digestion and finally became a smear of DNA fragments when exposed to the protein extract of day 7 cotyledons. The irreversible caspase-3 inhibitor, Ac-DEVD-fmk, was tested for its ability to inhibit cotyledon endonuclease activity (Figure 6, Panel B). Degradation of the plasmid DNA by the inhibitor-treated cytosolic fraction of the cotyledon was partially inhibited by Ac-DEVD-fmk. As shown in Figure 6, Panel B, the DNA degradation caused by the cytosolic extracts from day-3 and day-5 germinating seeds was inhibited by the addition of Ac-

DEVD-fmk (compare lane 5 to lane 4 and lane 7 to lane 6, respectively).

Protease activities of cotyledons upon germination of mung bean seeds

Protease activities of cotyledons were examined during seed imbibition and germination (Figure 7, Panel A). The protease activity was shown to be limited in seeds imbibed at 4°C for 12 h (lane 1), but it increased gradually up to day 7 (lane 2 to lane 5). An in-gel protease activity assay was carried out using gelatin in-gel with different protease inhibitors applied to day 3 cotyledon protein extracts. Figure 7B shows that proteases a (~52 kDa), b (30 kDa) and c (20 kDa) were inhibited by Ac-DEVD-fmk, Ac-VAD-CHO and E-64 but not by Ac-YVAD-CHO, pepstatin, aprotinin or PMSF. It is interesting that loupeptin inhibited proteases a and c of day 3 cotyledons but tended to enhance the activity of protease b in day 3 cotyledon protein extracts.

Caspase-like protease activity during cotyledon PCD in seed germination

To determine whether the cotyledons of the mung bean contain caspase-like proteolytic activity during PCD, we tested the CLP activities of the cell extracts of cotyledons at different developmental stages. Ac-DEVD-AMC, a fluorogenic substrate specific for caspase-3 in animal cells, was applied to test for cotyledon CLP activity. We found that CLP activity was present in the cotyledon extracts of seeds imbibed at 27°C for 12 h, reached a peak three days after germination, and then decreased as the cotyledons aged (Figure 8, lanes 1-9). Various caspase inhibitors were tested to assign this caspase activity to a specific class of protease. Day-3 cotyledon CLP activities were inhibited by Ac-DEVD-CHO (a specific inhibitor of caspase-3, lane 10) but not by Ac-YVAD-CHO (a specific inhibitor of caspase-1, lane 11), Ac-VAD-CHO (caspase-1 inhibitor, lane 12) and E-64 (cysteine protease inhibitor, lane 13).

DISCUSSION

The earliest hallmarks of PCD in cotyledons during mung bean seed imbibition and after germination were progressive vacuolation of the cytoplasm (Figure 3b) and nuclear DNA fragmentation (Figure 4A, lane 2), which were observed in seeds immersed at 27°C for 12 h and day 1 seeds, respectively. However, most differentiated plant cells normally contain vacuoles that play active roles in the creation and maintenance of turgor, the storage of metabolic products and cell elongation (Zakeri et al., 1995). Vacuoles are therefore not exclusive attributes of a dying cell in plants. A large number of growing vacuoles was observed during the engulfment and digestion of the protoplast portion and of other organelles, and eventually they formed a large central vacuole by coalescence. The slow phase of cell dismantling was followed by a brief terminate phase triggered by the large vacuole collapse, which

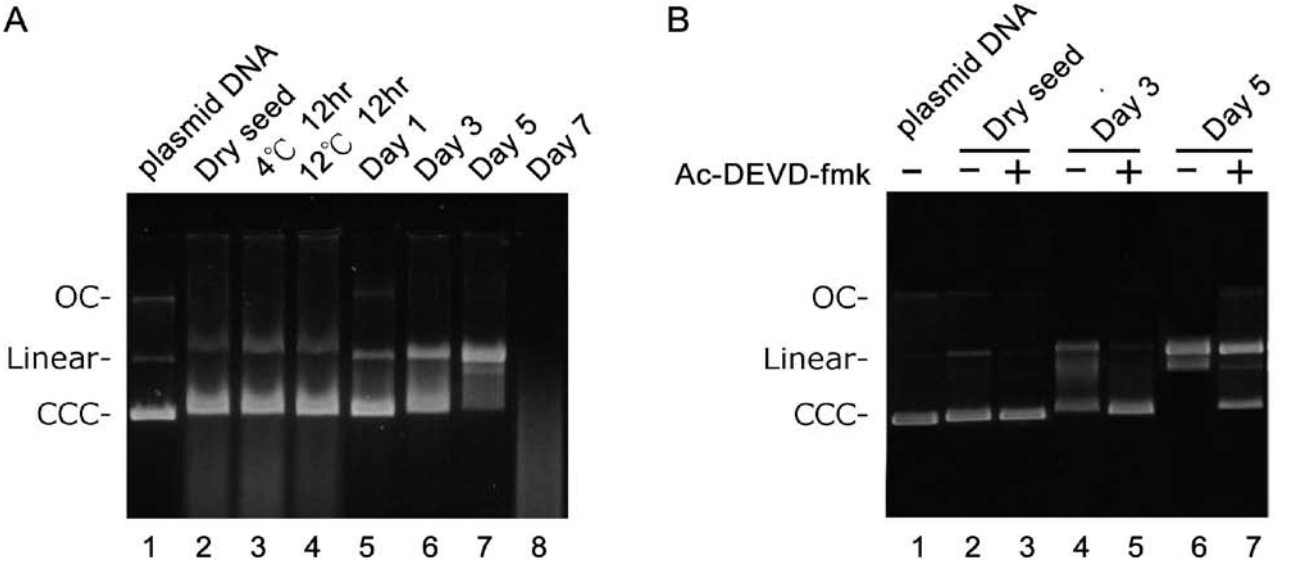

Figure 6. *In vitro* detection of cotyledon endonuclease activity and the effect of a caspase-3 inhibitor (Ac-DEVD-fmk) on endonuclease activity during cotyledon PCD. Panel A: Cotyledon proteins (1.5 µg) were incubated with 2 µg of plasmid DNA (pBR 322) at 37°C for 0.5 h, followed by agarose gel electrophoresis. Endonuclease activity was detected through the conversion of plasmid DNA from its supercoiled (CCC) to open-circle (OC) and linear forms, as indicated to the right of the panel. Lane 1, control plasmid DNA. Lanes 2-9 present endonuclease activity of cotyledon cell extract from the indicated stages of germinating seeds. Panel B: Aliquots of cytosolic fractions were preincubated with Ac-DEVD-fmk for 2 h at 37°C followed by the endonuclease assay as described in Panel A. Lane 1, plasmid DNA. Lanes 2-3, cytosolic proteins obtained from cotyledon of dry seeds. Lanes 4-5, cytosolic proteins from day 3 cotyledon, lanes 6-7, cytosolic proteins from day 5 cotyledon. Lanes 3, 5 and 7, reaction in the presence of 20 mM Ac-DEVD-fmk. Lanes 2, 4 and 6, reaction without inhibitor.

led to complete protoplast removal (Figures 2 and 3). This PCD pattern has been widely reported during developmental PCD of seed tissues, and vacuole collapse plays a critical role in autolysis and nuclear degradation (Sugiyama et al., 2000; Jones, 2001; Ito and Fukuda, 2002).

In plants, PCD is often associated with the cleavage of DNA into oligonucleosomal fragments through the activation of an endogenous nuclease. In the present study, internucleosomal DNA cleavage in cotyledons started in day 1 seedlings (Figure 4A, lane 2). The fragmentation of nuclear DNA began before the storage parenchyma cells reached a highly vacuolated stage. The endonuclease activity in day 2 cotyledons was detected by an in-gel nuclease activity assay (Figure 5A and 5B). However, some endonuclease activity was seen in day 1 cotyledons in an *in vitro* plasmid degradation assay (Figure 6A, lane 5). We believe that our in-gel activity assay of cotyledon endonucleases (Figure 5) may not be sufficiently sensitive to reflect the nuclear DNA fragmentation and laddering exhibited by day 1 cotyledons (Figure 4, Panel A). The in-gel nuclease activity assay allowed the identification of a single 36-kDa DNase. This DNase was primarily characterized by its requirement for Ca^{2+} and Mg^{2+} and was strictly inhibited by Zn^{2+} and ATA (unpublished results). Our data showed a strong correlation between the accumulation of nuclease activity and other markers of PCD such as internucleosomal DNA fragmentation and cytochrome c release (Figure 4, Panels B and C). The 36-kDa endonuclease was the sole endonuclease by in-gel nuclease activity assay during mung bean cotyledon PCD in this study.

Mitochondria are key players in the regulation of mammalian cell death, and the release of cytochrome c from

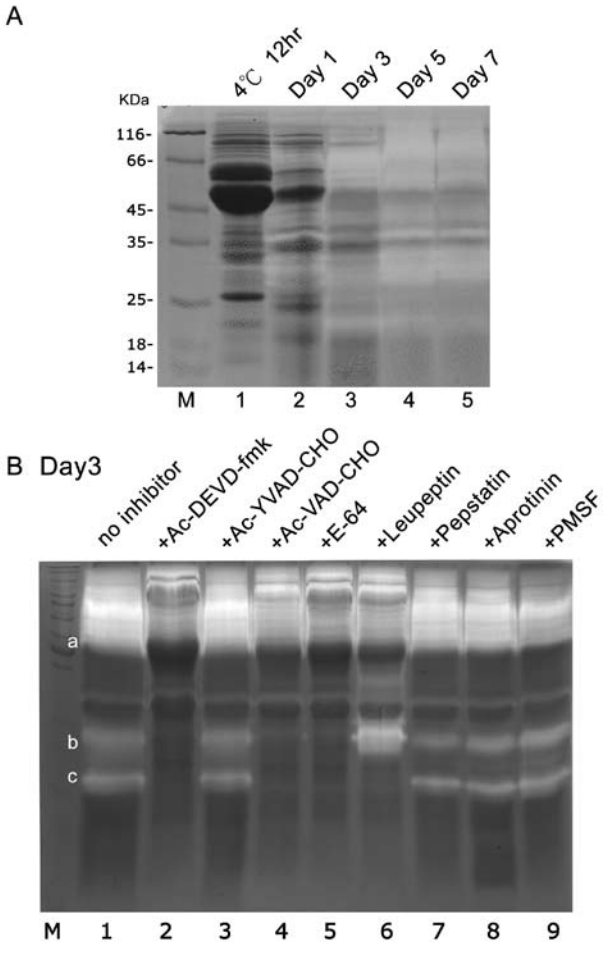

Figure 7. Protease activities and inhibition of protease activities during cotyledon PCD. Panel A: Protein extracted from cotyledons at different stages post-germination were run out by SDS-PAGE containing 1% gelatin. After electrophoresis, the gel was soaked in renaturing buffer. Protease activities were investigated by incubating the gel in the developing buffer, followed by staining with Coomassie blue. The proteins with protease activities are detected as clear bands against a dark background. Panel B: Inhibition of protease activities of day 3 cotyledons by various protease inhibitors. Inhibitors applied to each reaction were as follows: Ac-DEVD-fmk (a specific inhibitor of caspase-3), Ac-YVAD-CHO (caspase-1 inhibitor), Ac-VAD-CHO (caspase-1 inhibitor), E-64 (cysteine protease inhibitor), Leupeptin (cysteine protease inhibitor), Pepstetin A (aspartic protease inhibitor), Aprotinin (serine protease inhibitor) and PMSF (serine protease inhibitor). a, b and c marked on the right of lane 1 indicate three proteases with molecular masses of approximately 52, 30 and 20 kDa, respectively, that were affected by various protease inhibitors.

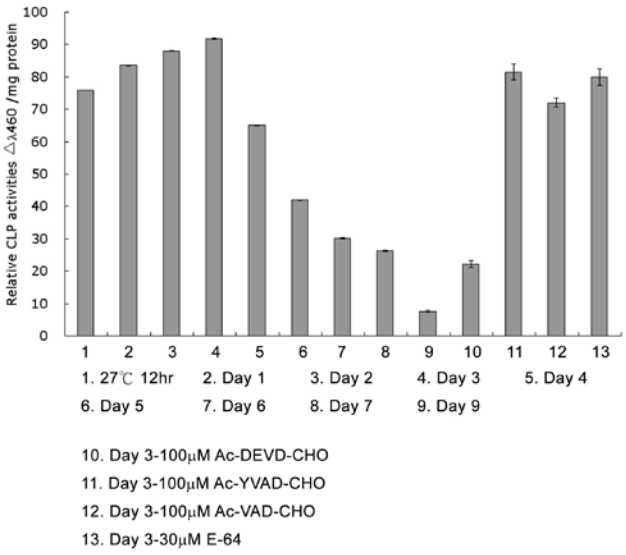

Figure 8. Caspase-like protease activity analysis of mung bean cotyledons undergoing PCD. Lanes 1 to 9 show CLP activity of cotyledon proteins extracted from seeds imbibed at 27°C for 12 h, day 1, day 2, day 3, day 4, day 5, day 6, day 7 and day 9 seedlings, respectively. CLP activity appears in the cotyledons from seeds imbibed at 27°C for 12 h and peaks in day 3 cotyledons. CLP activity continuously declines between day 3 and day 9 cotyledons. The protein extract of day 3 cotyledons, which showed the highest CLP activity, was further analyzed by addition of different protease inhibitors: Ac-DEVD-CHO (lane 10), Ac-YVAD-CHO (lane 11), Ac-VAD-CHO (lane 12) and E-64 (lane 13), as indicated in the figure. The caspase-3-specific inhibitor appears to inhibit CLP activity during cotyledon PCD, as shown in lane 10.

mitochondria to the cytosol is regarded as a key regulatory event of animal PCD. Cytochrome c acts in the cytosol by recruiting a group of proteases termed caspases, which serve to degrade critical cell components (reviewed by Reape and McCabe, 2010). It remains unclear to what extent mitochondria are involved in PCD in plant cells. The release of cytochrome c from the mitochondria to the cytosol was first detected in day 2 cotyledons, was significantly elevated in day 3 cotyledons and was maintained at the same level in day 9 cotyledons. Mitochondrial cytochrome c started to decline in day 5 cotyledons. Nuclear DNA fragmentation, starting in day 1 cotyledons, appeared earlier than the mitochondrial cytochrome c release, which began in day 2 cotyledons in this analysis. It is possible that our immunoblotting analysis for cytochrome c in the cotyledon cytosol may not be as sensitive as the DNA ladder detection. Another possibility is that cytochrome c release may not be directly related to nuclear DNA fragmentation in cotyledon PCD. Despite the cell collapse and DNA fragmentation, mitochondria remained morphologically intact until the late stage of cell lysis in our study (unpublished data). Therefore, we suggest that during cotyledon PCD, cytochrome c may be released from the mitochondria via transient pores rather than by the swelling of the matrix and the rupture of the outer mitochondrial membrane. In various plant systems, the release of cytochrome c from the mitochondria to the cytosol precedes cell death. In this study, we also demonstrated that PCD during mung bean cotyledon senescence involves the common components of apoptosis in animal cells, including decreased mitochondrial membrane integrity and cytochrome c release.

The role of proteases during PCD has been well documented for animal systems, and proteases have often been suggested to play a role in plant PCD. The evident participation of proteases, especially caspase in the regulation of animal PCD, implies that caspase-like proteases might also be involved in the regulation of plant PCD (He and Kermode, 2003b; Reape and McCabe, 2010). In this study, we found that proteases a (~52 kDa), b (30 kDa) and c (20 kDa) were inhibited by Ac-DEVD-fmk, Ac-VAD-CHO and E-64 but not by Ac-YVAD-CHO, pepstatin, aprotinin or PMSF (Figure 7B). It is not yet clear why loupeptin inhibited proteases a and c but tended to enhance the activity of protease b in day 3 cotyledon protein extracts. When we measured the CLP activities of cotyledon protein extracts, we found that CLP activity was present in the cotyledons at a very early stage, the extract of seeds imbibed at 27º C for 12 hr, reached a peak in day 3 cotyledons, and then decreased during cotyledon senescence (Figure 8). Various caspase inhibitors were tested to assign this DEVDase activity to a specific class of protease. Cotyledon CLP activities were inhibited by Ac-DEVD-CHO (a specific inhibitor of caspase-3) and were resistant to Ac-VAD-CHO (a specific inhibitor of caspase-1), Ac-YVAD-CHO (a specific inhibitor of caspase-1) and E-64 (cysteine protease inhibitor). Our results suggest that caspase-like proteolytic activity plays a role in mung bean cotyledon PCD. Indeed, numerous evidence from inhibitor studies and biochemical approaches has implicated CLPs in cell death control in higher plants (reviewed by Lam and del Pozo, 2000; Reape and McCabe, 2010; Woltering, 2010).

Taken together, the hallmark features of programmed cell death, including vacuolar-collapse-mediated cell death, nuclear DNA fragmentation, mitochondrial cytochrome c release and caspase-like protease activity, were found in cotyledon PCD during mung bean seed germination. However, it remains unclear how endonuclease, cytochrome c and caspase-like proteases interact with each other in mung bean cotyledon PCD.

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綠豆發芽過程中之子葉細胞凋亡

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在綠豆發芽過程中，其子葉進行細胞凋亡。細胞貯存物質之流失及大量液泡化造成了子葉貯藏薄壁細胞的死亡，當細胞凋亡進入細胞瓦解之階段，貯藏薄壁細胞逐漸自動溶解，形成巨大的中心液泡，細胞質、細胞核完全溶解，原生質體由細胞壁向內萎縮，進入了細胞凋亡的最後階段。在此凋亡過程中，細胞色素 C 由粒線體釋放至細胞質中，細胞核 DNA 片段化亦明顯發生。細胞凋亡過程中核酸酶之活性可由體外測試証明，此核酸酶之活性可被 caspase-3 抑制劑 Ac-DEVD-fmk 所抑制。Caspase-like protease (CLP) 之活性亦可在子葉凋亡過程中偵測到。CLP 之活性在種子發芽第三天之子葉中活性最高，此 CLP 之活性可被 caspase-3 專一抑制劑所抑制，對 caspase-1 專一抑制劑則有抗性。

關鍵詞：Caspase-like protease；細胞色素 c 釋放；Internucleosomal DNA 片段化；線粒體；程序性細胞凋亡；貯藏物質之溶解和液泡化。