Coordinate involvement of cysteine protease and nuclease in the executive phase of plant apoptosis

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Abstract We have developed an oat cell-free apoptosis system to investigate the execution mechanisms of plant apoptosis. Cell extracts derived from oat tissues undergoing toxin (victorin)-induced apoptosis caused nuclear collapse and internucleosomal DNA fragmentation in isolated nuclei. Pharmacological studies revealed that cysteine protease, which is E-64-sensitive but insensitive to caspase-specific inhibitors, is a crucial component in the morphological change of isolated nuclei, and that nuclease and the cysteine protease act cooperatively to induce the apoptotic DNA laddering. Interestingly, this finding is contrasted with those in well-studied animal cell-free systems in which an apoptotic endonuclease is solely responsible for the DNA fragmentation.

Keywords: Apoptosis; Programmed cell death; Oat; Victorin; Cysteine protease; Nuclease

1. Introduction

Plants evoke innate immune responses against pathogenic attack early in the infection process. The defense reaction often includes the rapid and localized collapse of the challenged cells, known as the hypersensitive response (HR) [1]. The HR displays several biochemical and morphological features of animal apoptosis [2–6], which is a process of programmed cell death (PCD) essential in development, tissue homeostasis and response to environmental stresses [7].

Animal apoptosis can be initiated by a variety of stimuli and is executed via an intrinsic cellular suicide program that leads to internucleosomal DNA fragmentation (DNA laddering) and nuclear condensation [7,8]. Several apoptogenic effectors, such as caspases (cysteine proteases) [9–12], CAD/DFF40 (caspase-activated DNase/40 kDa DNA fragmentation factor) [9–12], endoG (mitochondrial endonuclease G) [13], DNase I [14], DNase II [15], AIF (apoptosis-inducing factor) [16] and Acinus (apoptosis chromatin condensation inducer in the nucleus) [17], have been implicated in mediating these nuclear disintegration processes using a cell-free system. In plants, chromatin condensation and DNA laddering are induced during PCD in response to biotic/abiotic stresses and developmental factors [4,6,18–22]. However, only limited information is available about plant effectors, especially those involved in the executive phase of plant PCD. Several lines of evidence implicate cysteine proteases and nucleases in the commitment to plant PCD during oxidative stress- [5] and mannose-induced cell death [22], tracheary element differentiation [23] and the HR [24]. However, it is not yet clear whether those enzymes are directly involved in the executive phase in plant PCD or in upstream signaling pathways.

In oat plants, victorin, a host-selective toxin (HST) produced by *Cochliobolus victoriae*, induces apoptotic cell death, characterized by DNA laddering, chromatin condensation and a TUNEL-positive response [18–20]. In this study, we established a cell-free system comprising a combination of oat nuclei and lysates from cells treated with victorin. This system offers an excellent tool for identifying apoptogenic effectors in plants. Using the cell-free system with a pharmacological approach, we showed that cysteine protease and nuclease act cooperatively to induce apoptotic events in oat nuclei.

2. Materials and methods

2.1. Plant materials and reagents

The oat (*Avena sativa* L.) cultivar Iowa X469 carrying the *Vb* gene to be sensitive to the HST victorin produced by *Cochliobolus victoriae* was grown in vermiculite under a 16 h photoperiod at 20 °C, as described previously [25]. Primary leaves of 7-day-old seedlings were used for the present study. Victorin C was supplied by Dr. T.J. Wolpert (Oregon State University, Corvallis, OR, USA). Aprotinin A and E-64 were purchased from Nacalai Tesque (Kyoto, Japan). Aurintricarboxylic acid (ATA) was obtained from Calbiochem (San Diego, CA, USA). Caspase and Granzyme specific inhibitors used were Ac-YVAD-CHO (caspase-1) (Calbiochem), Ac-VDVAD-CHO (caspase-2) (Alexis Biochemicals, San Diego, CA, USA), Ac-DEVD-CHO (caspase-3) (Sigma, St. Louis, MI, USA), Ac-LEVD-CHO (caspase-4) (Alexis Biochemicals), Ac-WEHD-CHO (caspase-5) (Sigma), Ac-VEID-CHO (caspase-6) (Sigma), Ac-IETD-CHO (caspase-8) (Sigma), Ac-LEHD-CMK (caspase-9) (Alexis Biochemicals) and Z-AAD-CMK (granzyme B) (Calbiochem).

2.2. Preparation of cell lysates from oat leaves treated with victorin

The lower epidermis was peeled from the primary leaves and 5 cm segments were taken 1–6 cm from the leaf top. The segments were placed in 60 × 15 mm glass petri dishes in contact with 3 ml of 5 ng ml^{-1} victorin solutions. After incubation at 20 °C under illumination for 3 h,

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Abbreviations: ATA, aurintricarboxylic acid; DAPI, 4′,6-diamidino-2-phenylindole; HR, hypersensitive response; PCD, programmed cell death

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the leaf segments were ground to a fine powder under liquid nitrogen and suspended in buffer A (20 mM Mes–KOH, pH 5.5, 50 mM KCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$). After centrifugation at $10000 \times g$ for 20 min at 4 °C, the resulting supernatants were used for the cell-free apoptosis induction assay.

2.3. Preparation of oat leaf nuclei

Oat nuclei were isolated from protoplasts prepared from primary leaves of Iowa X469. The epidermis layer of oat leaves was stripped, and the leaf segments were then floated for 2 h at room temperature on an enzyme solution composed of 20 mM Mes–KOH, pH 5.7, 2% (w/v) cellulase 'Onozuka' R-10 (Yakult Pharmaceutical, Tokyo, Japan), 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), 10 mM $CaCl_2$ and 0.5 M sorbitol, pH 5.6. The protoplasts were released by gentle shaking after addition of buffer B (15 mM Pipes–NaOH, pH 7.4, 250 mM sucrose, 80 mM KCl, 15 mM NaCl, 0.5 mM spermidine and 0.2 mM spermine) and were separated from tissue by filtration through four layers of gauze. All further procedures were carried out at 4 °C. The resulting protoplast suspension was washed twice with buffer B and was collected by centrifugation at $90 \times g$ for 3 min. Then, 10% (w/v) of Nonidet P-40 was added up to 2.4% (w/v) to the protoplast suspension (10^6 cells ml^{-1}) and filtrated through a 20 μm metal mesh (Iida Seisakusyo, Osaka, Japan). Liberated nuclei were then layered onto 8 ml of buffer B containing 30% sucrose and centrifuged at $1000 \times g$ for 10 min, followed by resuspension in buffer B at a concentration of 1.5×10^7 nuclei ml^{-1}. Nuclei were used immediately after preparation.

2.4. In vitro apoptosis assay

Aliquots of 100 μl of 2.5 μg-protein μl^{-1} lysates from victorin-treated oat leaves plus 15 μl of purified oat nuclei were incubated with or without the inhibitors shown above at 25 °C for 0–180 min. After incubation, 300 μl of 2% CTAB solution (100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA and 2%, w/v, cetyltrimethylammonium bromide) was added to the reaction and incubated at 65 °C for 30 min. An equal volume of chloroform–isoamylalcohol (24:1) was added and gently mixed by inversion for 5 min. The aqueous phase was recovered by centrifugation at $10000 \times g$ for 15 min at room temperature, and 1.5 volumes of 1% CTAB precipitation solution (50 mM Tris–HCl, pH 8.0, 10 mM EDTA and 1%, w/v, cetyltrimethylammonium bromide) was added. After centrifugation at $8000 \times g$ for 10 min at room temperature, the precipitate was dissolved in 400 μl of 1 M CsCl and subjected to alcohol precipitation with a twice volume of 100% ethanol. The DNA pellet was collected by centrifugation at $10000 \times g$ for 10 min at 4 °C, washed with ice-cold 70% ethanol, and dissolved in 20 μl of Tris–EDTA buffer containing 0.1 mg ml^{-1} RNase. After incubation at 37 °C for 30 min, DNA was recovered by phenol extraction and ethanol precipitation. The resulting DNA was run on 2% (w/v) agarose gels and visualized with 0.5 μg ml^{-1} ethidium bromide under UV illumination. To control equal loading of DNA in each lane, concentration of DNA samples was measured using a spectrophotometer before electrophoresis.

For the assessment of nuclear morphological changes, 2.3×10^5 nuclei were incubated with the cell lysates alone or plus the inhibitors as described above. Nuclei were then fixed in 2% paraformaldehyde and stained with 2 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI) that selectively labels DNA. The nuclei were observed under an Olympus IX70 (Tokyo, Japan) fluorescence microscope with a MicroMax 12-bit CCD camera system (Princeton Instruments, Inc., Trenton, NJ) and MetaMorph software (Universal Imaging Co., Downingtown, PA, USA).

2.5. Nuclease activity

To detect endonuclease activity and its molecular mass, an in-gel nuclease assay was performed as described previously with some modifications [19]. Samples (100 μg protein per lane) were run on a 12% (w/v) SDS polyacrylamide gel containing 200 μg ml^{-1} heat-denatured calf thymus DNA (Nacalai Tesque). Following electrophoresis, the gel was washed twice with 20 mM Mops–KOH, pH 6.5, in 1% (v/v) Nonidet P-40 at 50 °C for 30 min, and was then incubated in 20 mM Mops–KOH, pH 6.5, containing 2 mM $CaCl_2$ for 20 h at room temperature. To visualize nuclease activities, the gel was stained with 0.5 μg ml^{-1} ethidium bromide for 30 min and observed under UV light. Nuclease activities were identified as dark bands without DNA.

3. Results and discussion

3.1. Induction of apoptotic events in isolated oat nuclei by cell-free extraction from victorin-treated oat leaves

Cell-free systems provide powerful tools to identify apoptotic effectors involved in the execution phase of nuclear apoptosis, which is characterized by features such as DNA laddering and heterochromatin condensation. In mammals, cell-free systems have been used to identify crucial regulators of apoptosis, such as cytochrome *c*, AIF, CAD/DFF40 and endoG [9,12,13,16,26]. We employed the same system to explore regulators responsible for nuclear degradation and chromatin condensation during apoptotic cell death in oat plants. A cell lysate was prepared from primary leaves of victorin-sensitive oat cultivar Iowa X469 treated with 5 ng ml^{-1} victorin, by which apoptotic cell death is induced within 2 h [19]. Isolated nuclei were incubated with 2.2 mg protein ml^{-1} of cell lysate for 0–180 min and then DNA was analyzed by agarose gel electrophoresis. DNA fragmentation was observed from 30 min after incubation and was clearly detected as a ladder of approximately 180-bp units by 60 min (Fig. 1A). The intensity of DNA laddering was increased by the cytosolic extract in a dose-dependent manner (data not shown).

Typical apoptotic changes, such as heterochromatin condensation and a TUNEL-positive response, are observed in sensitive cells treated with victorin in vivo [19,20]. To assess the validity of the cell-free system, we examined whether the same apoptotic morphological changes occurred in the isolated nuclei using fluorescence microscopy after DNA staining with DAPI. Normal nuclei exhibited a weak and homogeneous blue fluorescence distributed evenly over the nuclear structure (Fig. 1B, panel a). By 15 min after incubation with the lysate, the bright regions began to aggregate into large masses (panel b), indicating irregular chromatin condensation. The nuclei decreased in size and the chromatin was further condensed within 60 min (panels c–e). By 90 min, the condensed chromatin had formed small discrete granules that showed a uniform and bright fluorescence and finally blebbed off from the nuclear surface (panels f and g). The morphological changes in the oat nuclei treated with the lysates are in agreement with the apoptotic features reported in animal systems, and the timings of the changes correlated closely with the occurrence of DNA laddering. These results indicated that the oat cell-free system would enable us to identify execution factors that participate in the induction of plant apoptosis. In animal cell-free systems, the induction of nuclear apoptosis requires some co-factors, such as dATP, ATP and Mg^{2+} [26,27]. The addition of co-factors was not required to initiate the apoptotic processes in our system. This may be due to the presence of sufficient co-factors in the oat cell lysate, or to the difference in the co-factor requirement for apoptotic effectors between plant and animal systems.

3.2. Requirement of a heat-labile molecule(s) in apoptotic nuclear events

To characterize apoptosis-inducing factor(s) in the oat cell lysate, we tested the effects of heat treatment on the activity of the cell lysate to induce DNA laddering and nuclear morphological changes in the cell-free system. The oat cell lysate was pre-treated by heating at temperatures of 40, 60 or 80 °C for 10 min and then subjected to the nuclear apoptosis

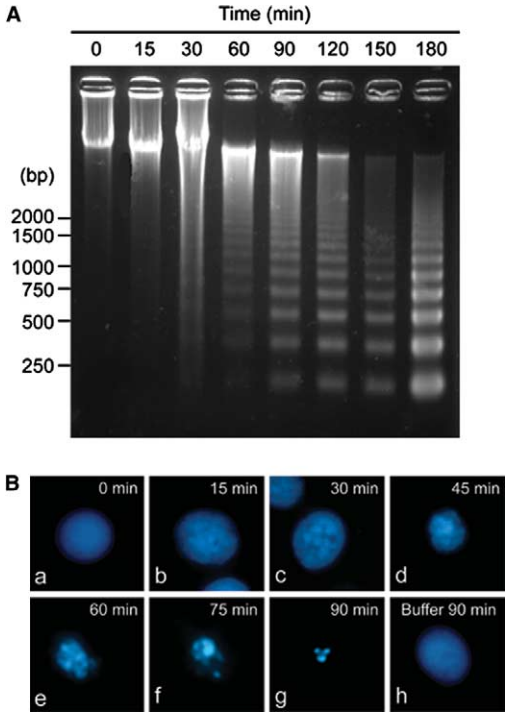

Fig. 1. Induction of apoptotic DNA fragmentation and morphological changes in oat nuclei by victorin-treated cell lysates in vitro. (A) Time course of DNA fragmentation. Nuclei (2.3×10^5) from oat leaves were incubated at 25 °C for a period of 0, 15, 30, 60, 90, 120, 150 and 180 min with the lysate (2.2 mg ml^{-1}) from oat leaves treated with 5 ng ml^{-1} victorin. After incubation, chromosomal DNA was analyzed by agarose gel electrophoresis. (B) Time course of nuclear morphological changes. Nuclei were incubated at 25 °C for 0, 15, 30, 45, 60, 75 and 90 min with the lysate (lane a–g) or for 90 min with buffer alone (lane h) and stained with DAPI to monitor apoptotic nuclear changes.

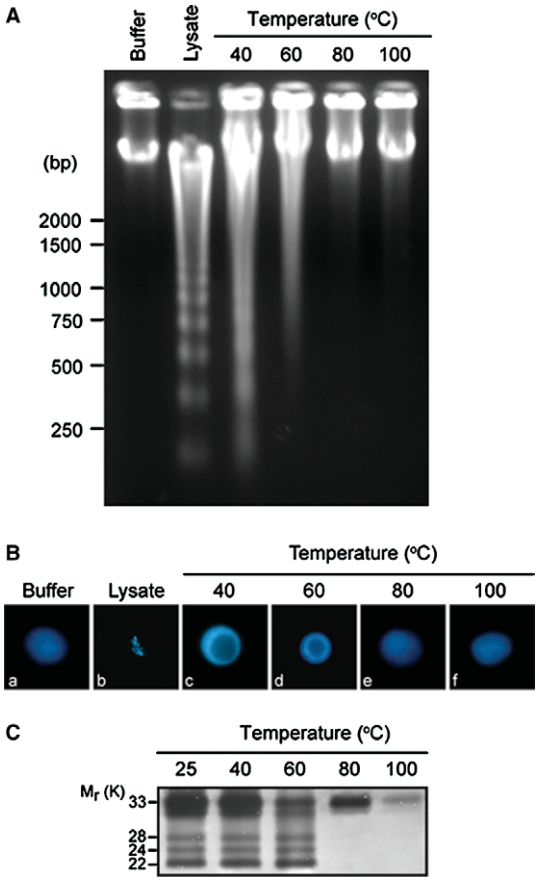

Fig. 2. A heat-labile molecule(s) is responsible for inducing in vitro apoptosis in oat. (A) Effect of heat treatment on DNA fragmentation activity in lysates from victorin-treated oat leaves. Lysates were heated at a range of temperatures (40, 60, 80 and 100 °C) for 10 min and chilled on ice immediately. Nuclei were incubated at 25 °C for 2 h with the heated or unheated lysates and then DNA was visualized by agarose gel electrophoresis. (B) The effect of heat-treated lysates on morphological changes in isolated nuclei. The lysates heated at temperature ranges from 40 to 100 °C for 10 min (lanes c–f), buffer (lane a) or unheated lysate (lane b) were incubated with nuclei at 25 °C for 2 h, and the nuclear changes were viewed with DAPI staining. (C) Effect of heat treatment on nuclease activities in lysates from leaves treated with victorin. The lysates were heated at a series of temperatures (25, 40, 60, 80 and 100 °C) for 10 min. After cooling down on ice, the heated or unheated lysates were subjected to an in-gel nuclease assay as described in Section 2.

assay with isolated oat nuclei. DNA laddering was suppressed by heating the lysate at 40 °C and was abolished completely by heating above 80 °C (Fig. 2A). Similarly, no detectable morphological changes were observed in the isolated nuclei when the nuclei were incubated with lysate that was heat-treated above 80 °C (Fig. 2B). When the lysate was heated at 40 or 60 °C, the treated nuclei exhibited a uniform chromatin ring around the nuclear periphery, which was an entirely distinct morphological change from that observed in the isolated nuclei treated with the unheated lysate (Fig. 2B). Of note, treatment of the lysate with 100 μg ml^{-1} proteinase K also neutralized the activities for apoptotic nuclear events (data not shown). These results suggested that a heat-labile protein(s) is essential in the executive phage of oat apoptosis.

Previously, we reported that victorin treatment of oat leaves induced the activity of a 28 kDa endonuclease (p28), and that this preceded the DNA laddering and heterochromatin condensation. The p28 activity was also markedly increased in parallel with the rate of DNA fragmentation and cell death [19]. In addition to p28, an inducible nuclease, p24 (24 kDa), and four constitutive nucleases, p22 (22 kDa), p31 (31 kDa), p33 (33 kDa) and p35 (35 kDa), have been detected in oat cell lysates using an in-gel assay for nuclease activity [19]. Although the inducible p28, p24 and the other nucleases retained their activity at 60 °C, the progression of the nuclear apoptotic events was strongly suppressed at the temperature (Fig. 2C). Furthermore, the activities for p31 and p33 were still detected in the lysate heated at 80 °C, the temperature at which the in vitro apoptosis induction was completely abrogated (Fig. 2C). The simplest explanation for the results would be that the heat-labile mediator(s) other than nucleases is involved in the cleavage of genomic DNA and the progression of chromatin condensation into discrete masses, even though we cannot rule out the possibility here that a heat-labile nuclease whose activity is not detectable in the in-gel assay is solely responsible for the DNA laddering.

3.3. Synergistic induction of nuclear apoptosis by protease and nuclease

We showed previously that cysteine proteases, serine proteases and nucleases were involved in the victorin-triggered signaling pathway that mediates nuclear DNA laddering and

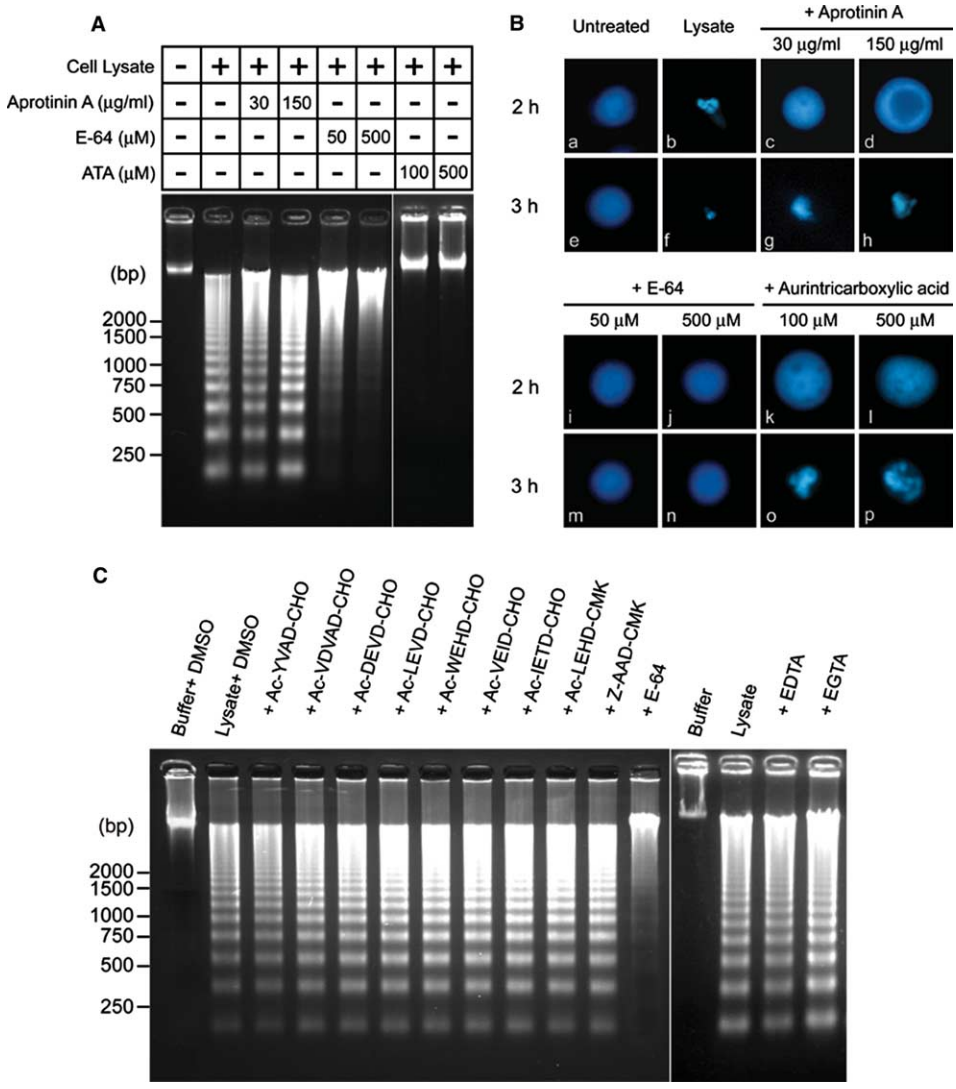

Fig. 3. Cysteine protease and nuclease are key factors to perform nuclear apoptosis in oat. (A) Effect of inhibitors on DNA fragmentation in the oat cell-free apoptosis system. Nuclei were incubated at 25 °C for 2 h with the lysates in the absence or the presence of aprotinin A, E-64 and ATA, and DNA was detected by agarose gel electrophoresis. (B) Effect of inhibitors on in vitro morphological changes in nuclei. Nuclei were incubated at 25 °C for 2 or 3 h with the lysates in the absence or the presence of aprotinin A, E-64 and ATA and stained with DAPI to evaluate nuclear morphology. (C) Effects of caspase and granzyme B specific inhibitors and divalent cation chelators on DNA fragmentation in the oat cell-free apoptosis system. Specific inhibitors used were Ac-YVAD-CHO (for caspase-1), Ac-VDVAD-CHO (caspase-2), Ac-DEVD-CHO (caspase-3), Ac-LEVD-CHO (caspase-4), Ac-WEHD-CHO (caspase-5), Ac-VEID-CHO (caspase-6), Ac-IETD-CHO (caspase-8), Ac-LEHD-CMK (caspase-9) and Z-AAD-CMK (granzyme B). All the specific inhibitors were treated at a concentration of 200 μM. E-64 is used as a positive inhibitor at a concentration of 500 μM. The divalent cation chelators, EDTA and EGTA, were used at a concentration of 2 mM.

chromatin condensation in oat leaves [19,20]. To determine whether these enzymes directly participate in the executive phase of oat apoptosis, isolated nuclei were incubated for 2 h with the lysates prepared from victorin-treated leaves in the presence of a cysteine protease inhibitor, E-64, a serine protease inhibitor, aprotinin A or a nuclease inhibitor, ATA. E-64 and ATA effectively suppressed the formation of DNA laddering within a range of 50–500 and 100–500 μM, respectively, whereas up to 150 μg ml^{-1} aprotinin A had no effect on DNA degradation, suggesting that cysteine proteases and nucleases were essential for nuclear DNA fragmentation in the executive phase of oat apoptosis (Fig. 3A).

We next examined the effects of these inhibitors on nuclear morphology during the apoptotic processes (Fig. 3B). In the presence of 50 μM E-64, no detectable morphological changes were induced in the nuclei by the lysate even after 3 h incubation, indicating that cysteine proteases are crucial for the apoptotic events occurring in oat nuclei. ATA and aprotinin A also showed negative effects on the morphological changes induced in the nuclei by the lysate. The addition of either ATA (100 and 500 μM) or aprotinin A (30 and 150 μg ml^{-1}) delayed chromatin condensation and nuclear collapse induced by the lysate. However, neither aprotinin A nor ATA treatment fully prevented the nuclear collapse. Of note, oat nuclei treated with the lysate in the presence of 150 μg ml^{-1} aprotinin A displayed an abnormal morphological feature in the form of a peripheral chromatin ring, which was similar to that observed with heat-treatment at 40 or 60 °C (Fig. 2C).

To gain further insights into the types of protease and nuclease responsible for the apoptotic events in oat nuclei, specific

inhibitors of caspase and granzyme B, and chelating reagents were employed in the nuclear apoptosis assay at the concentrations indicated in Fig. 3C. Surprisingly, none of the inhibitors or chelators was effective at suppressing DNA laddering (Fig. 3C) and nuclear morphological changes (data not shown) induced by the oat cell lysate. This suggests that the E-64-sensitive cysteine protease and the aprotinin A-sensitive serine protease in oat cells may belong to different classes of protease from caspase and granzyme B. It has been reported that caspase-specific inhibitors effectively suppress plant cell death induced by biotic and abiotic stresses [28,29]. Actually, victorin-induced proteolysis of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) can be prevented by caspase-specific inhibitors and general protease inhibitors such as E-64 [30]. Therefore, it might be possible that a caspase-like protease is involved in upstream apoptosis signaling pathways triggered by victorin but not directly in nuclear apoptotic events. Another intriguing point is that nucleolytic activities in the lysate were not inhibited by the presence of cation chelators such as EGTA and EDTA, indicating that the nuclease responsible for nuclear DNA laddering in oat cells does not require divalent cation co-factors such as Ca^{2+}, Mg^{2+} and Zn^{2+}. This implies that the oat nuclease may belong to the DNase II family, which shows an acidic pH optimum, and is divalent cation-independent. In fact, DNase II has been recognized as an important effector of apoptosis in animal systems [15,31].

Although some plant PCD programs induce chromatin condensation and fragmentation of nuclear DNA into a nucleosomal ladder [18–21], little is known about intracellular components and mechanisms involved in these events in plants. In animal cells, each apoptotic endonuclease such as CAD/DFF40, endoG and DNase II is solely capable of inducing DNA laddering and/or chromatin condensation [10–13,15]. In contrast, our in vitro analyses showed that the induction of DNA laddering requires at least both an E-64-sensitive cysteine protease and an ATA-sensitive nuclease. In addition, cysteine proteases appear to be indispensable for mediating the apoptotic morphological changes in isolated nuclei, while the nuclease and the aprotinin A-sensitive serine protease partially contributed to the execution of rapid and complete nuclear changes. These protease activities may be involved in modifications and breakdown of nuclear apoptotic substrates, such as histones, lamins and actin, which subsequently allow the nuclease to act on genomic DNA in the plant nuclei. In Jurkat T lymphocytes, a nuclear scaffold-associated serine protease is strongly associated with in vitro nuclear apoptosis; the inhibition of this protease can suppress lamin B1 cleavage, as well as chromatin breakdown [32].

In conclusion, our results implicate multiple players in apoptotic DNA fragmentation and chromatin condensation in plants. The machinery responsible for the executive phase of victorin-triggered PCD seems to be consistent with well-studied animal apoptosis systems in some components and different in others. We believe that our oat cell-free apoptosis system provides an excellent tool for identifying mechanistic components in plant apoptosis and to examine conservation and differences between apoptotic mechanisms across plant and animal kingdoms.

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