Identification of a nuclear-localized nuclease from wheat cells undergoing programmed cell death that is able to trigger DNA fragmentation and apoptotic morphology on nuclei from human cells

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PCD (programmed cell death) in plants presents important morphological and biochemical differences compared with apoptosis in animal cells. This raises the question of whether PCD arose independently or from a common ancestor in plants and animals. In the present study we describe a cell-free system, using wheat grain nucellar cells undergoing PCD, to analyse nucleus dismantling, the final stage of PCD. We have identified a Ca^{2+} / Mg^{2+} nuclease and a serine protease localized to the nucleus of dying nucellar cells. Nuclear extracts from nucellar cells undergoing PCD triggered DNA fragmentation and other apoptotic morphology in nuclei from different plant tissues. Inhibition of the serine protease did not affect DNA laddering. Furthermore, we show that the nuclear extracts from plant cells

triggered DNA fragmentation and apoptotic morphology in nuclei from human cells. The inhibition of the nucleolytic activity with Zn^{2+} or EDTA blocked the morphological changes of the nucleus. Moreover, nuclear extracts from apoptotic human cells triggered DNA fragmentation and apoptotic morphology in nuclei from plant cells. These results show that degradation of the nucleus is morphologically and biochemically similar in plant and animal cells. The implication of this finding on the origin of PCD in plants and animals is discussed.

Key words: nucellus, nuclease, nucleus dismantling, programmed cell death, protease, wheat grain.

INTRODUCTION

PCD (programmed cell death) is a process of organized destruction of cells, essential for successful development and to maintain the cellular homeostasis of multicellular organisms [1]. Of the different forms of cell death occurring in animals, apoptosis is a well-defined morphological and biochemical process [2]. Morphologically, apoptosis is characterized by nuclear DNA fragmentation and the formation of apoptotic bodies, which are finally engulfed by neighbouring cells [3–5]. Biochemically, it is a process characterized by the participation of caspases, a family of cysteine proteases able to cleave motifs containing aspartic acid [6]. Among the caspases participating in apoptosis, upstream caspases are known as initiators, whereas downstream caspases, which include caspase 3 and 7, are known as executioners because their activity leads to DNA degradation, chromatin condensation and nuclear membrane blebbing [7]. Caspases induce DNA degradation through the activation of nucleases known as CAD (caspase-activated DNase) or DFF40 (DNA fragmentation factor 40 kDa) [8–10].

As in animals, PCD is also an essential process for plant development [11,12]. In addition, PCD is important for the hypersensitive response produced during plant–pathogen interaction [13,14]. Cell death in plants occurs with a different morphology compared with apoptosis in animal cells. The cell wall of plant cells implies that no apoptotic bodies are formed, neither does engulfment by neighbouring cells occur [15]. Furthermore, vacuoles, organelles specific to plants [16], play an important role in PCD execution in different plant systems such as the formation of tracheary elements [17] and cereal aleurone cells [18].

Given the morphological differences of cell death between plants and animals, an important question is whether the process of PCD arose independently or from a common origin in both kingdoms [19]. To address this question, the biochemical features of cell death have been analysed in animal and plant cells in the search for common mechanisms. A major effort has been focused on the identification of caspases, the proteases characteristic of apoptosis, in plants. Despite the initial identification of a caspase-like activity in plants [20], no genes encoding caspases have been identified in the plant genomes sequenced so far. Further work on the proteases involved in plant PCD identified two groups of proteases, VPEs (vacuolar processing enzymes) and metacaspases. Direct evidence has been reported for the involvement of VPEs in plant cell death [19,21–24].

The cereal grain has become a model system for the study of PCD in plants because different tissues undergo PCD both during development and germination. Very early after fertilization, the nucellus, a maternal tissue surrounding the developing endosperm, undergoes a process of PCD [25]. Later on, during grain maturation, endosperm cells, excluding the aleurone layer, undergo PCD [26,27]. Following germination, the aleurone cells, which play an essential role secreting hydrolytic enzymes for the mobilization of the starchy endosperm, initiate a gibberellin-activated process of death [28]. Morphologically, PCD in nucellar and aleurone cells can be divided into two phases [25,29]. Initially, the cytoplasm is progressively vacuolarized and shows symptoms of intense degradation. The nucleus adopts an irregular morphology but appears essentially intact. This initial phase is followed by nucleus dismantling, including chromatin condensation, internucleosomal fragmentation of DNA and nuclear

Abbreviations used: AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride; CAD, caspase-activated DNase; dpa, days post anthesis; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; ICAD, inhibitor of CAD; PCD, programmed cell death; PEPC, phosphoenolpyruvate carboxylase; VPE, vacuolar processing enzyme.

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envelope disorganization. The enzymes involved in this process in plants are poorly known. In tracheary elements, a nuclease, ZEN1, has been shown to degrade nuclear DNA without forming the ladder characteristic of internucleosomal fragmentation [30]. In contrast, dying aleurone cells display internucleosomal fragmentation of DNA, which is associated with the presence of a nucleolytic activity localized to the nucleus [29].

Thus, the comparison of plant PCD and apoptosis shows important differences regarding the morphological changes occurring in the dying cells and the enzymes involved in the process. However, the final execution of the process, DNA fragmentation and nuclear disorganization, has similarities in plant and animal cells, suggesting that it might have evolved from a common mechanism. To test this hypothesis, we have developed a cell-free system to analyse degeneration of the nucleus using nucellar cells from wheat grains after 8–12 dpa (days post anthesis). We identified a $\text{Ca}^{2+}/\text{Mg}^{2+}$ nuclease localized to the nucleus of nucellar cells undergoing PCD. Nuclear extracts of these cells trigger DNA fragmentation in nuclei from different types of plant cells. DNA fragmentation requires an intact chromatin structure and occurred along with morphological modifications in plant nuclei resembling apoptosis of animal cells. Moreover, we show that the plant nuclear extract triggers DNA fragmentation and apoptotic morphology in nuclei isolated from human cells, thus showing the similarity in the process of nucleus dismantling in plant and animal PCD.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

Wheat (*Triticum aestivum* cv Chinese Spring) plants were grown in a greenhouse under a 16 h day/8 h night cycle at 22–25 °C. Developing grains were harvested at 8–12 dpa and endosperm and maternal tissues were dissected, frozen in liquid nitrogen and kept at –80 °C until required. For dissection of scutellum and aleurone tissues, mature grains were sterilized in 2% (v/v) sodium hypochlorite for 20 min, washed twice with sterile water, once with 0.01 M HCl and then washed extensively with sterile distilled water. Grains were imbibed on sterile filter paper soaked with water for 2 days. The human HCT116 colon carcinoma cell line, cultivated as described previously [31], was kindly provided by Dr F. Romero (Department of Microbiology, Faculty of Biology, University of Sevilla, Sevilla, Spain), and U937 cells previously grown as described [32], were kindly provided by Dr J. C. Reyes (Instituto de Bioquímica Vegetal y Fotosíntesis, Sevilla, Spain).

Isolation of plant and human nuclei and preparation of nuclear and cytoplasmic extracts

Plant nuclei were isolated from maternal tissues dissected from wheat grains at 8–12 dpa or from scutellum and aleurone cells from wheat grains after 2 days of imbibition. Dissected tissues were ground with a pestle and mortar with liquid nitrogen and resuspended in 5 ml of homogenization buffer [0.25 M sucrose, 10 mM NaCl, 10 mM Mes/NaOH (pH 6.0), 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM PMSF, 2 mM 2-mercaptoethanol and 0.25% (v/v) Triton X-100]. The homogenate was clarified by centrifugation at 100 g for 1 min and filtered through a nylon mesh (60 µm pore size; Millipore). Cellular fractionation was performed by carefully placing the clarified homogenate on an equal volume of 30% (v/v) Percoll prepared in the same buffer and centrifuging at 3000 g for 15 min. The upper phase was conserved as the cytoplasmic fraction, the

Percoll phase was discarded and the nuclei-enriched pellet was harvested, washed in homogenization buffer and resuspended in 100 µl of extraction buffer [25 mM KH_2PO_4 (pH 7.8), 40 mM KCl, 20% (v/v) glycerol and 1% (v/v) plant protease inhibitor cocktail (Sigma)]. Nuclear extracts were obtained by adding NH_4SO_4 to the solution containing the nuclei up to a final concentration of 0.4 M. After extraction on ice for 30 min and centrifugation at 13 000 g for 20 min at 4 °C, the supernatant constituted the nuclear extract.

For preparation of intact human cell nuclei, HCT116 or U937 cells were subjected to hypotonic shock by incubation for 10 min at 4 °C in buffer A {10 mM Hepes (pH 7.9), 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM DTT (dithiothreitol), 1 mM AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride], 10 µg/ml aprotinin}. Nuclei were harvested by centrifugation at 2000 g for 10 min, the supernatant being the cytoplasmic fraction. Pelleted nuclei were washed in buffer A, centrifuged at 25 000 g for 20 min at 4 °C and then resuspended in buffer B [20 mM Hepes (pH 7.9), 25% (v/v) glycerol, 1.5 mM MgCl_2 , 0.25 mM Na/EDTA (pH 8.0), 0.5 mM DTT, 1 mM AEBSF, 10 µg/ml aprotinin and 0.14 M NaCl] and kept at –20 °C until required.

In-gel nuclease activity assay

The in-gel nuclease activity assay was performed as reported previously [29]. Cytoplasmic contamination of plant nuclear extracts was routinely analysed by Western blot analysis using PEPC (phosphoenolpyruvate carboxylase) as a cytoplasmic marker. Affinity-purified polyclonal maize PEPC antibodies were purchased from Rockland.

In vitro endonuclease activity assay

An *in vitro* endonuclease activity assay was carried out according to the method described by Ito and Fukuda [30], with modifications. In brief, plant nuclei were isolated from either developing endosperm from wheat grains at 8–12 dpa or from scutellum and aleurone layers from wheat grains after 2 days of imbibition. Apoptosis in U937 human cells was induced by incubation with 50 µg/ml etoposide for 7 h. Human nuclei were isolated as described above. Intact plant or human nuclei (25 µg of DNA) were incubated with the plant or human nuclear or cytoplasmic extracts (4 µg of protein) described above for 2 h at 30 °C in 100 mM Mops/KOH (pH 7.0), 5 mM CaCl_2 (omitted for human cell extracts) and 5 mM MgCl_2 . Reactions were stopped by adding an equal volume of 2× lysis buffer [100 mM Tris/HCl (pH 8.0), 200 mM NaCl, 100 mM EDTA and 2% (w/v) SDS] and were incubated for 1 h at 55 °C. After extraction with phenol/chloroform/isopentanol (25:24:1, by vol.), DNA was precipitated with two volumes of absolute ethanol, resuspended in TE buffer [10 mM Tris/HCl (pH 8.0) and 1 mM EDTA], ethanol-precipitated again and finally resuspended in 25 µl of TE buffer. Contaminating RNA was removed by incubation for 3 h at 37 °C in the presence of RNase A (60 µg/ml, final concentration). DNA was then ethanol-precipitated, resuspended in TE buffer, resolved on 2% (w/v) agarose gels and stained with ethidium bromide.

In-gel protease activity assay

An in-gel protease activity assay was performed according to the method described previously [33], with modifications. In brief, nuclear extracts (100 µg of protein) were subjected to electrophoresis on 12% (w/v) polyacrylamide gels co-polymerized with 0.1% (w/v) gelatine. After electrophoresis, SDS was removed by incubation in 1% (v/v) Triton X-100 (Sigma) for 30 min at room temperature. Gels were then incubated overnight

Figure 1 Characterization of nucleolytic activities in wheat maternal tissues undergoing PCD

(A) DNA (20 μ g) isolated from dissected endosperm (E) and maternal tissues (M) of developing wheat grains harvested at 8–12 dpa was fractionated on 2% (w/v) agarose gels. DNA markers, in kbp, are indicated on the left. (B) Dissected endosperm (E) or maternal tissues (M) were fractionated into cytoplasmic (C) and nuclear (N) extracts, and aliquots from both fractions (100 μ g of protein; upper panel) were analysed by in-gel nuclease assays by overnight incubation at 37 °C in 100 mM Mops/KOH (pH 7.0), 5 mM CaCl₂ and 5 mM MgCl₂, or by Western blot analysis (15 μ g of protein; lower panel) probed with polyclonal anti-PEPC antibodies. Molecular-mass markers, in kDa, are indicated on the left. (C) Effect of divalent cations on nuclease activities. Protein extracts (100 μ g) from nuclear or cytoplasmic fractions were subjected to in-gel nuclease activity by overnight incubation at 37 °C in 100 mM Mops/KOH (pH 7.0) in the absence of added cations (–) or in the presence, as indicated, of 2 mM CaCl₂ (Ca²⁺), 2 mM MgCl₂ (Mg²⁺), 2 mM MnCl₂ (Mn²⁺), 4 mM ZnCl₂ (Zn²⁺). EDTA was added at a final concentration of 0.2 mM.

at 40 °C in 100 mM Mops/KOH (pH 7.0) and 0.5 mM DTT. When indicated, incubation was carried out in the presence of AEBSF (5 mM), leupeptin (10 μ g/ml) or E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] (50 μ M).

Confocal microscopy

For confocal microscopy analysis, nuclei from the *in vitro* endonuclease activity assays described above were treated with 0.5 μ g/ml propidium iodide and analysed with a confocal laser-scanning microscope system Leica TCS SP2 (excitation light of 488 nm, and emitted light between 560 and 700 nm).

RESULTS

We have shown previously [25] that nucellar cells undergo a process of PCD at early stages of grain development. The DNA isolated from this tissue shows the characteristic laddering (Figure 1A) indicative of the internucleosomal fragmentation of DNA associated with PCD. In contrast, the DNA extracted from

the developing endosperm, a tissue actively growing at this stage of grain development, remained intact (Figure 1A). Maternal and endosperm cells were fractionated into nuclei and cytoplasm, and extracts from both cell fractions were subjected to in-gel analysis of nucleolytic activity. A band with nuclease activity at approx. 50 kDa was detected in nuclear extracts from maternal cells (Figure 1B). These cells contained high nucleolytic activity in the cytoplasmic fraction (Figure 1B), whereas no significant activity was detected in nuclear or cytoplasmic extracts from developing endosperm cells (Figure 1B, lanes E). Possible contamination of nuclear fractions with cytoplasmic proteins was ruled out by routinely testing for PEPC, a cytoplasmic enzyme [34], by Western blot analysis (Figure 1B).

The nucleolytic activity from both nuclear and cytoplasmic extracts required Ca²⁺ and Mg²⁺, which could be replaced by Mn²⁺ for optimum activity (Figure 1C). However, the presence of Zn²⁺ in the assay completely inhibited activity (Figure 1C). Consistent with these results, the addition of EDTA completely inhibited the activity of both extracts. The addition of Ca²⁺ or Mg²⁺ fully recovered the nuclear-localized activity, whereas the cytoplasmic activity required both Ca²⁺ and Mg²⁺ for full activity (Figure 1C). Therefore the nucleolytic activity associated with PCD in the nucleus of maternal tissues of wheat grains is a Ca²⁺/Mg²⁺-type nuclease, hence showing the same cation requirements as the nuclease identified in nuclei of dying aleurone cells [29]. However, both nucleases show a different electrophoretic mobility, thus indicating the participation of different nucleases in the different tissues undergoing PCD in wheat grains.

To characterize the process of nucleus dismantling during plant PCD further, we set up a cell-free assay consisting of the incubation of either cytoplasmic or nuclear extracts from nucellar cells undergoing PCD with intact nuclei isolated from different non-dying tissues of wheat grains. The co-incubation of nuclear extracts with intact nuclei from endosperm cells resulted in the internucleosomal fragmentation of DNA (Figure 2A, lane N); however, the nucleolytic activity of the cytoplasmic extracts did not produce any DNA fragmentation in intact nuclei (Figure 2A, lane C), nor increase the activity of the nuclear extracts (Figure 2A, lane N + C). In agreement with the cation requirements described above, the presence of either Zn²⁺ or EDTA completely inhibited the fragmentation of DNA by the nuclear extracts (Figure 2A). Both cytoplasmic and nuclear extracts were assayed on naked DNA, obtained after phenol-extraction of nuclei from endosperm cells. This naked DNA was degraded by both extracts, however degradation appeared as an unspecific DNA smear (Figure 2B), not as the characteristic laddering observed on intact nuclei.

We considered the possibility that the fragmentation of DNA carried out by the nuclear-localized nucleolytic activity required the concerted participation of proteases. To test this possibility, the proteolytic activity of the nuclear extracts was analysed by in-gel protease assays. As expected, the highest proteolytic activity was detected in the cytoplasmic fraction of dying maternal cells (Figure 3A), in agreement with the vacuolarization and lytic activity in the cytoplasm of these cells reported previously [25]. In contrast, developing endosperm cells showed a low content of proteases in the cytoplasmic fraction (Figure 3A). This analysis allowed the identification of an endoprotease of approx. 60 kDa in the nucleus of maternal cells (Figure 3A). This proteolytic activity was completely inhibited by treatment with AEBSF, an inhibitor of serine proteases, and was not affected by either leupeptin or E-64 (Figure 3A). Therefore this analysis identified a serine protease in the nuclei of nucellar cells undergoing PCD. This in-gel protease assay was based on the hydrolysis of gelatine, an artificial substrate for plant proteases. We therefore analysed the

Figure 2 *In vitro* analysis of nuclear DNA fragmentation

(A) Protein extracts (4 μ g of protein) from nuclear (N), cytoplasmic (C) or a mixture of both fractions (N + C) from nucellar cells undergoing PCD were incubated with intact nuclei isolated from developing endosperm. All incubations were performed for 2 h at 30°C in 100 mM Mops/KOH (pH 7.0), 5 mM CaCl₂ and 5 mM MgCl₂. Where indicated, the assay buffer was supplemented with 5 mM ZnCl₂ (Zn²⁺) or 10 mM EDTA. Following incubation, nuclear DNA was isolated and aliquots (20 μ g) were fractionated on 2% (w/v) agarose gels. (B) Naked DNA (20 μ g) obtained after phenol extraction of intact nuclei from endosperm cells was incubated with protein extracts (4 μ g of protein) from nuclear (N), cytoplasmic (C), or a mixture of both fractions (N + C). Incubation was carried out for the times indicated at 30°C in 100 mM Mops/KOH (pH 7.0), 5 mM CaCl₂ and 5 mM MgCl₂. DNA was fractionated on 2% (w/v) agarose gels. DNA markers, in kbp, are indicated on the left.

capacity of the serine protease to hydrolyse proteins present in the nucleus of nucellar cells. The pattern of nuclear proteins after 3 h incubation in the absence of AEBSF showed the disappearance or clear decrease of several proteins (Figure 3B, arrowheads), showing that they were targets of the serine protease identified in the present study. However, the pattern of DNA laddering when nuclear extracts were co-incubated with intact endosperm nuclei was not altered regardless of the presence of AEBSF (Figure 3C), indicating that this proteolytic activity was not required for DNA fragmentation.

We took advantage of the cell-free assay to analyse morphological changes during *in vitro* nucleus dismantling. *In vitro* DNA fragmentation under these assay conditions was a relatively rapid process since DNA laddering was clearly detected after 60 min of incubation, the amount of fragmented DNA increasing progressively up to 180 min (Figure 4A). Parallel analysis of propidium-iodide-stained nuclei by confocal microscopy revealed that the progressive fragmentation of DNA correlated with the formation of nuclear bodies reflecting chromatin condensation (Figure 4B). The same pattern of DNA fragmentation and nuclear morphology was observed when nuclear extracts from nucellar cells

were incubated with nuclei from different tissues of wheat grains, scutellum (Figure 4C) and aleurone cells (Figure 4D).

The detection of DNA laddering and the morphological changes observed during dismantling of nuclei from plant cells described in the present study resembles the morphology of nuclei during apoptosis of animal cells [7]. To test this similarity functionally, we analysed whether the plant nuclear extracts from nucellus cells were able to trigger DNA fragmentation and the corresponding changes of nuclear morphology in intact nuclei from HCT116 human cells. The incubation of nuclear extracts with nuclei from human cells resulted in the characteristic DNA laddering (Figure 5A, lane N), indicative of internucleosomal fragmentation of DNA. In agreement with the results obtained with nuclei from plant cells, the cytoplasmic extracts did not show any activity (Figure 5A, lane C) nor did they increase the fragmentation produced by the nuclear-localized nuclease (Figure 5A, lane N + C), hence showing exclusively that the nuclear-localized nuclease was able to trigger DNA fragmentation in human nuclei. Inhibition of the activity by Zn²⁺ and EDTA confirmed that the mechanism of DNA laddering was common in nuclei from human and plant cells. Moreover, DNA fragmentation was clearly detected after 30 min of incubation (Figure 5B), even more rapid than the corresponding activity on plant nuclei (Figure 4A). The observed fragmentation of DNA correlated with the morphological changes of the nucleus of human cells (Figure 5C), as observed with plant cell nuclei. Furthermore, when the nuclease activity was inhibited in the presence of Zn²⁺ and EDTA, no alteration of the nuclear morphology was observed (Figure 5D), showing the key role of DNA fragmentation in triggering nuclear morphology.

To analyse further whether the basic mechanisms of nuclear dismantling are conserved among plants and animals, we tested the effect of nuclear extracts from human cells undergoing apoptosis in plant intact nuclei. Apoptosis was induced in U937 human cells by treatment with etoposide, which produced the characteristic DNA laddering and nuclear morphology (Figure 6A). Etoposide-treated and non-treated control cells were fractionated into nuclear and cytoplasmic extracts, and extracts from both fractions were incubated with intact nuclei isolated from wheat endosperm cells. When incubations were performed with either nuclear or cytoplasmic fractions from control cells, no DNA laddering or alteration of nuclear morphology was observed (Figure 6B). However, nuclear extracts, but not cytoplasmic extracts, from etoposide-treated cells triggered DNA laddering and produced chromatin condensation in endosperm nuclei (Figure 6B).

DISCUSSION

The comparison of the morphology of PCD in plants and apoptosis in animal cells reveals clear differences, which may be due to the particular characteristics of both cell types, specifically the presence of cell wall and vacuoles in plant cells. Biochemically, knowledge of the enzymes executing plant PCD is scarce when compared with apoptosis in animal cells, but relevant differences have emerged, the most relevant one being the lack of caspase genes in plants. These morphological and biochemical differences lead to the question of whether or not the process of cell death evolved from a common mechanism in both kingdoms [19]. Since the nucleus of animals and plants have a conserved structural organization [35] and DNA laddering may be a universal and convenient way to degrade DNA [36], we have analysed the final stage of cell death, nucleus degradation, by developing a cell-free system which allows the *in vitro* study of the process. Our

Figure 3 In-gel analysis of proteases in nuclear extracts from nucellar cells

(A) Dissected endosperm (E) and maternal tissues (M) from developing wheat grains harvested at 8–12 dpa were fractionated into cytoplasmic (C) and nuclear (N) extracts, and aliquots (100 μ g of protein) from both fractions were analysed by in-gel protease assays using gelatine as the substrate. After electrophoresis, gels were incubated overnight at 40 °C in 100 mM Mops/KOH (pH 7.0) and 0.5 mM DTT in the absence (control) or presence of protease inhibitors (5 mM AEBSF, 10 μ g/ml final concentration of leupeptin or 50 μ M E-64), as indicated. (B) Nuclear extracts (15 μ g of protein) from maternal tissues undergoing PCD were subjected to autolysis for 3 h at 37 °C in 100 mM phosphate buffer (pH 7.2), 10 mM EDTA and 0.5 mM DTT, in the absence or presence of the serine protease inhibitor AEBSF (5 mM final concentration). After incubation, proteins were analysed on SDS/PAGE (12% polyacrylamide gel) and stained with Coomassie Brilliant Blue. Molecular-mass markers, in kDa, are indicated on the left. (C) Protein extracts (4 μ g of protein) from nuclear (N), cytoplasmic (C) or a mixture of both fractions (N + C) from nucellar cells undergoing PCD were incubated with intact nuclei isolated from developing endosperm for 2 h at 30 °C in 100 mM Mops/KOH (pH 7.0), 5 mM CaCl_2 and 5 mM MgCl_2 in the absence or presence of the serine protease inhibitor AEBSF (5 mM final concentration). Ser-EP, serine endoprotease.

results show that the dismantling of the nucleus from different plant tissues is biochemically and morphologically similar to apoptosis in animal cells. In addition, nuclear extracts from plant nucellar cells undergoing PCD trigger DNA laddering and apoptotic morphology in nuclei from human cells, showing that the essential elements required for human nucleus dismantling are present in the nucleus of dying plant cells. Moreover, nuclear extracts from etopoxide-treated human cells undergoing apoptosis trigger DNA laddering and apoptotic morphology on nuclei from wheat endosperm cells. These results suggest that the biochemical mechanism of nucleus dismantling is common in plant and animal cells.

The enzymes involved in nucleus dismantling in plants are still poorly known. Several types of endonucleases have been identified in cells undergoing PCD [37]; however, evidence on their direct involvement in cell death has been reported only for some of them. This is the case with ZEN1, a Zn^{2+} -dependent nuclease directly implicated in the degradation of nuclear DNA in *Zinnia* tracheary elements [30]. ZEN1 is localized to vacuoles which collapse before DNA is degraded [17]. However, ZEN1 activity did not produce the characteristic DNA laddering shown by the nucleases executing DNA fragmentation in apoptotic animal cells [8–10]. Based on the biochemical differences of ZEN1 and the nucleases involved in apoptosis [30], it was proposed that plants and animals have evolved independent systems of nuclear DNA degradation during cell death.

In contrast with tracheary elements, the tissues undergoing PCD in cereal grains show the characteristic DNA laddering indicative

of internucleosomal fragmentation of DNA [25–27,29,38], which is a hallmark of apoptosis in animal cells [3]. Recently, we identified a $\text{Ca}^{2+}/\text{Mg}^{2+}$ endonuclease localized to the nucleus of wheat aleurone cells undergoing PCD, which was detected prior to DNA laddering [29]. In the present study we show that a different wheat tissue, the nucellus, which undergoes PCD at early stages of grain development [25], presents a nuclear-localized nuclease with identical cation requirements, but with a different electrophoretic mobility than the aleurone nuclease. These results suggest that, as occurs in animal apoptosis [7], there is more than one nuclease involved in plant PCD. Indeed, a Mg^{2+} -dependent nucleolytic activity has been identified in the inter-membrane space of mitochondria responsible for the generation of 30 kb DNA fragments in *Arabidopsis* [39].

Nucellar cells undergoing PCD contain high nucleolytic activity in the cytoplasm, as expected of the high hydrolytic activity taking place in this cell compartment [25]. Interestingly, the nucleolytic activity of the cytoplasmic fraction did not have any effect on intact nuclei or produce an unspecific smear, compared with the nuclear-localized nuclease, on naked DNA (Figure 2). The lack of activity of the cytoplasmic nuclease on intact nuclei may indicate that chromatin DNA is not a substrate for this nuclease. Therefore the nucleolytic activity of the cytoplasmic fraction is most probably involved in the active degradation of the contents of the cytoplasm taking place during PCD.

The cell-free system used in the present study revealed that DNA degradation in plant nuclei occurs in parallel with morphological changes similar to those seen in the nuclei of apoptotic cells

Figure 4 *In vitro* analysis of DNA fragmentation and nuclear morphology triggered by nuclear extracts from nucellar cells

Nuclear extracts (4 μ g of protein) from nucellar cells undergoing PCD were incubated with intact nuclei from developing endosperm at 30 °C in 100 mM Mops/KOH (pH 7.0), 5 mM CaCl_2 and 5 mM MgCl_2 . At the indicated times, DNA was isolated and fractionated on 2% (w/v) agarose gels (**A**) or nuclei were stained with 0.5 μ g/ml propidium iodide and visualized by confocal microscopy (**B**). (**C** and **D**), Experimental conditions as in (**A**) and (**B**) but with nuclei isolated from scutellum (**C**) or aleurone (**D**) cells from wheat grains after 2 days of imbibition. Incubation was carried out for 3 h. DNA markers, in kbp, are indicated on the left.

[40]. Our finding that nuclear extracts from plant cells undergoing PCD induce apoptotic morphology in nuclei from human cells and that nuclear extracts from etopoxide-treated human cells have the same effect in plant nuclei reveals that the essential components of nucleus dismantling are common in animal and plant cells. However, no genes encoding CAD, the nuclease that degrades nuclear DNA during apoptosis [8], have been found in the plant genomes sequenced so far. In animal cells, CAD forms a complex with its inhibitor, ICAD, and the caspase-activated cleavage of ICAD allows CAD to enter the nucleus and degrade DNA. We have tested whether ICAD-S (M_r 35 000 isoform) or ICAD-L (M_r 45 000 isoform) from mammalian cells inhibited DNA laddering triggered by the plant nuclease in human or plant nuclei, but no effect was observed (results not shown). Therefore, despite the functional similarity of the process in plant and animal cells, the plant enzymes involved and their corresponding genes seem to have specific features.

Inhibition of the nuclease activity with Zn^{2+} or EDTA treatment had the additional effect of blocking the morphological changes of the nucleus (Figure 5), suggesting that DNA fragmentation is required for the morphological changes occurring in the nucleus.

Figure 5 *In vitro* analysis of DNA fragmentation and nuclear morphology in nuclei from human cells triggered by plant nuclear extracts

Protein extracts (4 μ g of protein) from nuclear (N), cytoplasmic (C) or a mixture of both fractions (N + C) from nucellar cells undergoing PCD were incubated with intact nuclei isolated from HCT116 human cells for 2 h (**A** and **D**), or the indicated time (**B** and **C**) at 30 °C in 100 mM Mops/KOH (pH 7.0), 5 mM CaCl_2 and 5 mM MgCl_2 . When indicated, assay buffer was supplemented with 5 mM ZnCl_2 (Zn^{2+}) or 10 mM EDTA. After the reaction, DNA was extracted and fractionated on 2% (w/v) agarose gels, or nuclei were stained with 0.5 μ g/ml propidium iodide and visualized by confocal microscopy. DNA markers, in kbp, are indicated on the left.

In animal cells, nucleus dismantling is a complex process that requires the contribution of several factors [41], so additional elements present in the nuclear extracts are most probably necessary for plant nucleus dismantling. The search for proteolytic activities identified a serine protease specifically localized to the nucleus of nucellar cells undergoing PCD. However, inhibition of this activity did not affect DNA degradation (Figure 3) or apoptotic morphology (results not shown). These results do not rule out a possible role for this serine protease in previous phases of plant PCD. A nuclear-localized metacaspase activity described recently [42] is required to complete the process of nucleus dismantling, which may act in concert with the nucleolytic activity described in the present study.

Two phases may be distinguished in the progression of cell death in plants: degradation of the cytoplasm and dismantling of the nucleus. For cytoplasm degradation, vacuoles play the important role of lytic compartments [17,18,25,29]. Consistent with the high hydrolytic activity during this phase, plant PCD is associated with the expression of genes encoding most types of proteases [43–47]. However, these proteases are most probably involved in the mechanism of autophagy proposed for cytoplasm degradation [15], rather than the organized and processive role of caspases in animal cell apoptosis.

Despite the absence of genes encoding caspases, it is known that caspase inhibitors block the progression of PCD in plants [20] and a caspase 3-like protease has been described in the

Figure 6 *In vitro* analysis of DNA fragmentation and nuclear morphology in wheat endosperm nuclei triggered by nuclear extracts from etopoxide-treated human cells

(A) DNA (20 µg) isolated from control and etopoxide-treated U937 human cells was fractionated on 2% (w/v) agarose gels. (B) Protein extracts (4 µg of protein) from nuclear (N) or cytoplasmic (C) fractions from control and etopoxide-treated human cells were incubated with intact nuclei isolated from developing wheat endosperm for 2 h at 30 °C in 100 mM Mops/KOH (pH 7.0) and 5 mM MgCl₂. After the reaction, DNA was extracted and fractionated on 2% (w/v) agarose gels, or nuclei were stained with 0.5 µg/ml propidium iodide and visualized by confocal microscopy. Untreated DNA from intact endosperm nuclei (U) was also loaded onto the gel. DNA markers, in kbp, are indicated on the left.

cytoplasm of *Chara* cells [48], suggesting that caspase-like activity is important for plant PCD. Several types of proteases have been proposed to play an important role in plant PCD. These include a protease containing an active-site serine residue, termed saspase [49], VPEs [21,22] and metacaspases [42,50,51]. VPE is localized to the vacuole [21], thus reinforcing the major role of this organelle in plant PCD [24]. The relationship of VPE activity and the dismantling of the nucleus remains to be established. In contrast, the metacaspase mclII-Pa is not a vacuolar enzyme and accumulates in the nucleus of *Picea* embryo suspensor cells undergoing PCD [42]. This result and our finding that plant nuclear extracts trigger DNA fragmentation and apoptotic morphology in nuclei from human cells shows that the basic mechanism for nucleus dismantling is common to plant PCD and animal cell apoptosis.

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