RESEARCH PAPER

The scutellum of germinated wheat grains undergoes programmed cell death: identification of an acidic nuclease involved in nucleus dismantling

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Abstract

Programmed cell death (PCD) is a crucial phenomenon in the life cycle of cereal grains. In germinating grains, the scutellum allows the transport of nutrients from the starchy endosperm to the growing embryo, and therefore it may be the last grain tissue to undergo PCD. Thus, the aim of this work was to analyse whether the scutellum of wheat grains undergoes PCD and to perform a morphological and biochemical analysis of this process. Scutellum cells of grains following germination showed a progressive increase of DNA fragmentation, and the TUNEL assay showed that PCD extended in an apical-to-basal gradient along the scutellum affecting epidermal and parenchymal cells. Electron-transmission microscopy revealed high cytoplasm vacuolation, altered mitochondria, and the presence of double-membrane structures, which might constitute symptoms of vacuolar cell death, whereas the nucleus appeared lobed and had an increased heterochromatin content as the most distinctive features. An acid- and Zn2+-dependent nucleolytic activity was identified in nuclear extracts of scutellum cells undergoing PCD. This nuclease was not detected in grains imbibed in the presence of abscisic acid, which inhibited germination. This nucleolytic activity promoted DNA fragmentation in vitro on nuclei isolated from healthy cells, thus suggesting a main role in nucleus dismantling during PCD.

Key words: cell death, germination, nuclease, scutellum, seed, Triticum aestivum (wheat).

Introduction

The programmed elimination of unwanted cells is an essential process of development of animals and plants. The two most common forms of cell death in animals are apoptosis and autophagy, which can be distinguished by morphological and molecular features (Conradt, 2009). Apoptosis is characterized by a series of well-defined morphological changes including cell shrinkage, cytoplasm contraction, and chromatin condensation prior to the final engulfment by phagocytic cells (Taatsjes et al., 2008). At the molecular level, apoptosis is characterized by activation of caspases (cysteinyl, aspartate-specific proteases) and nuclear DNA fragmentation (Kitazumi and Tsukahara, 2011). In contrast, autophagy is characterized at the morphological level by the presence of autophagic vesicles (autophagosomes) within the dying cells and the absence of engulfment by phagocytes during early stages of the cell-death process (He and Klionsky, 2009). Despite these differences between the two mechanisms of cell death, genetic studies carried out in different animal models have identified genes involved both in autophagy and apoptosis (Conradt, 2009). Besides caspases, there are a variety of apoptogenic effectors supporting the cellular suicide programme that leads to internucleosomal DNA fragmentation and nuclear condensation, such as caspase-activated DNase (CAD), mitochondrial endonuclease G (EndoG), DNaseI, apoptosis-inducing factor (AIF) (for review, see Samejima and Earnshaw, 2005), and apoptosis chromatin condensation inducer in the nucleus (Acinus) (Sahara et al., 1999).
In plants, programmed cell death (PCD) is both an important process of development (Kuriyama and Fukuda, 2002) and a mechanism of defence against pathogens (Lam, 2004). Whilst plant PCD shares some similarities with apoptosis of animals, such as internucleosomal fragmentation of DNA, chromatin condensation, and activation of caspase-like proteases (Bai et al., 2010), PCD in plant cells also exhibit distinctive features. The presence of chloroplasts, a prominent vacuole, and the cell wall are unique characteristics of plant cells, which affect PCD (Williams and Dickman, 2008). In the case of the chloroplasts, which constitute an important source of reactive oxygen species production in plant cells, it was proposed that these organelles may have a signalling function of some plant PCD responses (Zapata et al., 2005). Moreover, a combination of the function of the vacuole during cell death and autophagy may represent a plant alternative to the phagocytosis system of apoptosis (Hatsugai et al., 2006; Bassham, 2007), which has a specific morphology termed ‘vacuolar cell death’ (van Doorn et al., 2011). At the molecular level, although there is increasing evidence which connects the participation of proteases and nucleases in plant PCD, the enzymes directly involved in the execution of nucleus dismantling in plants (chromatin condensation, internucleosomal fragmentation of DNA, and nuclear envelope disorganization) are yet poorly known.

PCD plays an essential role in the processes of development and germination of cereal grains and, thus, the cereal grain has become one of the model systems for the study of PCD in plants. At initial stages of grain development, maternal tissues such as the nucellus and the nuellar projection cells degenerate by a process of PCD associated with characteristic proteolytic and nucleolytic activities (Domínguez and Cejudo, 1998, 2006; Domínguez et al., 2001). Then the starchy endosperm, the tissue specialized in the accumulation of storage compounds, undergoes PCD during maturation (Young et al., 1997; Young and Gallie, 1999, 2000). Germination and postgermination of cereal grains occurs by an ordered sequence of events, which are subjected to hormonal regulation and may be summarized as follows: gibberellins are synthesized at the scutellum and diffuse to the starchy endosperm (Appleford and Lenton, 1997). The hormone is perceived by the aleurone cells, which induce the synthesis and secretion of hydrolytic enzymes, including α-amylases, proteases, and glucanases, and also the acidification of the starchy endosperm, a process that occurs with a well-established spatiotemporal pattern, as described for the wheat grain (Domínguez and Cejudo, 1999). Once the aleurone cells have carried out their essential role, these cells initiate a process of PCD, which is also under the control of gibberellins (Fath et al., 2000; Domínguez et al., 2004). Besides its initial role to produce gibberellins, the major function of the scutellum in the germinated grain is the transfer of sugars and amino acids to the growing seedling (West et al., 1998; Aoki et al., 2006). In addition, the scutellum is itself a storage tissue, the contents of which might be used to feed the seedling once the transfer function is finished. So far, the analysis of PCD in the scutellum has been limited to studies of embryogenesis during maize kernel development (Giuliani et al., 2002; Consonni et al., 2003) or differentiating vascular tissue of germinated grains (Domínguez et al., 2002). However, it is not yet known whether the scutellum undergoes a massive process of PCD during grain germination. The present study addressed whether scutellar cells suffer PCD in germinated wheat grains and the identification of nucleolytic activities involved in nucleus dismantling. The aim was to compare the morphological and biochemical features of this death process with those of other tissues undergoing PCD in cereal grains, such as starchy endosperm, aleurone, or nuellar cells. The relevance of this process of PCD of the scutellum in the context of grain germination is discussed.

Materials and methods

Plant material

Wheat (Triticum aestivum cv. Chinese Spring) grains were sterilized in 2% (v/v) NaOCl for 20 min and washed twice with sterile water, once with 0.01 M HCl and then thoroughly with sterile distilled water. Sterile grains were allowed to germinate at room temperature on sterile filter paper soaked with water. Treatments with hormones and inhibitors of hormone synthesis were carried out on filter paper soaked with 20 mM MOPS-KOH pH 7.0 supplemented with 10 mM CaCl₂. Hormones and inhibitors were added at the following concentrations: gibberellic acid, GA₃, 5 µM; abscisic acid (ABA), 25 µM; paclobutrazol (PCB), 500 µM; 24-epibrassinolide (EBL), 1 nM, and α-(2-aminoethoxyvinyl)glycine (AVG), 10 µM. GA₃, ABA, EBL, and AVG were purchased from Sigma Chemical and PCB from Duchefa Biochimie.

Isolation of DNA and electrophoresis

Scutellum discs, dissected from wheat grains imbibed for up to 7 days, were ground in liquid nitrogen with a mortar and pestle to a fine powder and homogenized in 5 ml of extraction buffer [50 mM TRIS-HCl pH 8.0, 100 mM NaCl, 50 mM EDTA, 1% (v/v) 2-mercaptoethanol, and 2% (w/v) SDS]. For DNA isolation, extracts were incubated at 45 °C for 15 min, at room temperature for 30 min, and then mixed with 5 ml of phenol/chloroform (1:1, v/v). Samples were centrifuged at 10,000 g for 10 min and the upper phase was precipitated at –20 °C for 30 min with 2 volumes of ice-cold ethanol. After centrifugation, the DNA pellet was air dried, dissolved in 250 µl TE buffer (10 mM TRIS-HCl pH 8.0, 1 mM EDTA), and quantified spectrophotometrically. RNase A (1.5 µl of a stock of 10 mg ml⁻¹) was added and incubated at 37 °C for 3 h. After this treatment, DNA was again precipitated and dissolved in TE buffer. Finally, DNA samples (20 µg) were analysed on a 2% agarose gel and stained with ethidium bromide. DNA ladders (500 or 100bp, Gibco) were used to estimate DNA size.

Preparation of nuclear and cytoplasmic extracts

Scutellum discs dissected from grains imbibed for up to 7 days were ground in a mortar with liquid nitrogen and resuspended in 5 ml 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, 20 mM 2-mercaptoethanol, and 2% (w/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). Fractionation was performed by adding the filtered supernatant to homogenization buffer containing 30% Percoll and centrifugation at 3000 g for 15 min. The upper phase was collected as the cytoplasmic extract, the Percoll phase was discarded, and the nuclei-enriched pellet was washed in homogenization buffer and resuspended in 100 µl extraction buffer [25 mM sodium phosphate pH 7.8, 40 mM KCl, 20% glycerol, 1% plant protease inhibitor cocktail (Sigma), 0.4 M (NH₄)₂SO₄]. After addition on ice for 30 min, the supernatant of the subsequent centrifugation (13,000 g, 20 min, 4 °C) was collected as the nuclear extract.
TUNEL assay

Wheat grains harvested at different days after imbibition (DAI) were longitudinally sectioned after removing shoots and roots, immediately fixed in 10% formaldehyde/acetic acid/ethanol (3:7:5-50, v/v), and embedded in Paraplast Plus (Sigma). In situ detection of DNA fragmentation was carried out as previously described (Dominguez et al., 2001). Paraplast Plus was removed from the grain sections by treatment with xylol, and the sections were then dehydrated with a decreasing ethanol series, treated with proteinase K (20 μg ml⁻¹) in PBS (10 mM sodium phosphate buffer, 130 mM NaCl), and rinsed twice with PBS. Endogenous peroxidase activity was then quenched by incubation in 1% (v/v) H₂O₂ in methanol for 30 min and rinsed twice with PBS. For labelling, sections were incubated for 60 min at 37 °C in the presence of terminal deoxynucleotidyl transferase (TdT) with the In situ Cell Death Detection Kit (Roche Applied Systems), according to the manufacturer’s instructions. Controls were performed in which TdT was omitted.

Electron microscopy

For morphological analysis, small fragments of wheat grains harvested at 1 or 5 DAI were fixed in 4% (v/v) glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7.2) for 3 h at 4 °C. The samples were dehydrated in an acetone series and embedded in Epon (an epoxy embedding medium). Toluidine blue-stained semi-thin sections used as control were viewed in a Leica (Aristoplan) light microscope. Thin sections (60–80 nm) were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron microscope.

In-gel nuclease activity assay

The in-gel nuclease activity assay was performed as reported previously (Dominguez et al., 2004) with modifications. Cytoplasmic and nuclear extracts (50 μg protein) obtained as described above were fractionated on SDS-PAGE gels containing 0.3 mg ml⁻¹ salmon sperm DNA at 4 °C and 20 mA/plate. After electrophoresis, the gels were washed at 4 °C and 20 mA/plate. After electrophoresis, the gels were then incubated overnight in 25 mM sodium acetate-acetic acid buffer (pH 5.5, containing 1 mM ZnSO₄ and 0.2 mM DTT) or 100 mM MOPS-KOH (pH 7.0, containing 5 mM CaCl₂ and 5 mM MgCl₂) at 37 °C. False nuclease activities associated with DNA-binding proteins were discarded by incubating the gels in 1% (w/v) SDS for 2 h at room temperature and then washed in water for 10 min. Finally, gels were stained with 1 μg ml⁻¹ ethidium bromide on a UV light box. Cytoplasmic contamination of plant nuclear extracts was routinely analysed by Western blot analysis using phosphoenolpyruvate carboxylase (PEPC) as a cytoplasmic marker (González et al., 1998). Affinity-purified polyclonal maize PEPC antibodies were purchased from Rockland.

In vitro endonuclease activity assay

In vitro endonuclease activity assay was carried out according to the method described by Ito and Fukuda (2002) with modifications. In brief, isolated nuclei from scutellar tissue were incubated with nuclear or cytoplasmic extracts from scutellar isolated from grains at 7 DAI. Incubation was performed for 2 h at 30 °C in 25 mM sodium acetate-acetic acid buffer (pH 5.5) or 100 mM MOPS-KOH (pH 7.0). Reactions were stopped by adding an equal volume of lysis buffer (100 mM TRIS-HCl pH 8.0, 200 mM NaCl, 100 mM EDTA, 2% SDS) and incubation for 1 h at 55 °C. After extraction with phenol/chloroform/isoamylalcohol (25:24:1, v/v/v), DNA was precipitated with two volumes of absolute ethanol, resuspended in TE buffer, precipitated again, and finally resuspended in 25 μl TE buffer. Contaminating RNA was removed by incubation for 3 h at 37 °C in the presence of RNase A (final concentration 60 μg ml⁻¹). DNA was then ethanol-precipitated and resuspended in TE buffer, resolved on 2% (w/v) agarose gels, and visualized using ethidium bromide.

Results

The scutellum of wheat grains following germination undergoes PCD

With the aim of testing whether the scutellum of germinated wheat grains undergoes PCD, this study analysed the internucleosomal fragmentation of genomic DNA, a hallmark of PCD. DNA laddering was first observed in grains after 4 DAI and increased progressively up to 7 days (Fig. 1A). A more precise identification of scutellar cells undergoing PCD was performed with the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay. No labelling was observed in sections of grains at 1 DAI (Fig. 1B) thus revealing the absence of PCD in scutellar cells at these early stages; however, the TUNEL assay showed labelling of nuclei of the parenchymal and epidermal cells of grains at 7 DAI (Fig. 1C). In the central region of the scutellum, TUNEL staining of the epithelial and parenchymal cells was first observed in grains after 4 DAI and increased progressively up to 7 DAI (Fig. 1D–H), in agreement with the detection of DNA laddering (Fig. 1A). No labelling over background was observed in control sections in the absence of TdT (Fig. 1I).

Previous analysis of postgerminative processes in wheat grains revealed important spatiotemporal gradients affecting starchy endosperm acidification, aleurone gene expression, and PCD (Dominguez and Cejudo, 1999; Dominguez et al., 2004). Thus, with the aim of testing whether scutellum PCD takes place with any spatiotemporal pattern, ultrathin sections of wheat grains at 1 and 5 DAI were analysed. A morphological symptom of cell death, the increase of vacuolization, progressed from the upper part of the scutellar epithelium in contact with the aleurone layer to the lower part, which is indicative of a gradient of PCD in this scutellar tissue (Fig. 2A, 2B). It was noticed that PCD was initiated in scutellar cells once the aleurone cells close to the scutellum had completed the process of PCD and were almost empty (Fig. 2B). The TUNEL assay confirmed this pattern of PCD since in wheat grains at 7 DAI most cells of the upper part of the scutellum showed an intense labelling, whereas staining of cells of the lower part was less intense (Fig. 2C). These results suggest that the spatial progression of PCD in the scutellum occurs with an apical-to-basal gradient.

Morphology of scutellum cells undergoing PCD

To study the morphological features of scutellar PCD, this study focused on the analysis of epithelium and parenchyma cells of grains at 5 DAI, considering separately cytoplasmic and nuclear events. Characteristic features of the cytoplasm of cells undergoing death, as observed in the epithelium, are the formation of vacuoles originated from Golgi cisternae or endoplasmic reticulum-derived bodies (Fig. 3A, 3B), which appear in great number and probably assume the role of hydrolytic enzymes storage, until these vacuoles fuse with the central vacuole.
This death process was also characterized by double-membrane vesicles sequestering portions of cytoplasm (Fig. 3D, 3E), which resembled autophagosomes of animal cells undergoing autophagy. Moreover, several alterations of mitochondria could be observed including irregular shape, enlargement, and broken cristae (Fig. 3D–F). In the cytoplasm, another autolytic compartment characterized by an electron-translucent cytoplasm could be distinguished: storage vacuoles evolving to lytic vacuoles (Fig. 3E). In addition, characteristic membranous structures could be observed in dying scutellum cells such as multilamellar structures (Fig. 3G) or the whorls formed from cytoplasmic membranes (Fig. 3H).

Concerning the nucleus, the characteristics observed in parenchymal scutellum dying cells include high heterochromatin...
content and deep invaginations (Fig. 4A), so that narrow layers of cytoplasm are confined between nuclear segments (Fig. 4B; white arrows). A clear symptom of nuclear degradation is the presence of remnants of heterochromatin inside provacuoles (Fig. 4C, white arrowheads), leaking to the central autolytic vacuole (Fig. 4C, black arrowheads). Overall, the morphological

**Fig. 3.** Morphological analysis of cytoplasm of epithelial scutellum cells in germinated wheat grains at 5 days after imbibition. (A, B) Scutellar epithelium cell showing a vacuolated cytoplasm in the proximity of the heterochromatinized nucleus: nu, nucleus; mt, mitochondria; pv, provacuole. (C) A central autolytic vacuole (cv) is formed. (D–G) Appearance of disturbed mitochondria (dmt) and double-membrane vesicles (dmv) at the onset of programmed cell death: in G, note the multilamellar body (mlb) sequestering part of the cytoplasm; psv, protein storage vacuole. (H) Characteristic membrane structures localized in dying cells: wh, whorl formed by the cytoplasmic membrane. Bars, 1 μm (A, B, C, D, H), 2 μm (E), and 0.5 μm (F, G).

**Fig. 4.** Morphological analysis of nuclei of parenchymal scutellum cells in germinated wheat grains at 5 days after imbibition. (A, B) Detail of a nucleus with invaginations (white arrows) and protein storage vacuoles in its proximity. (C) Remnants of condensed chromatin inside provacuoles (white arrowheads) or central vacuole (black arrowheads); cv, central vacuole; nu, nucleus; psv, protein storage vacuoles; pv, provacuole. Bars, 0.5 μm (A), 5 μm (B), and 1 μm (C).
features identified suggest that scutellum epithelial and parenchymal cells of wheat grains following germination undergo vacuolar cell death, as described in other plant tissues (van Doorn et al., 2011).

**A nuclear-localized acid endonucleolytic activity in scutellum cells undergoing PCD**

As shown above, DNA fragmentation was identified as a hallmark of scutellum PCD. To characterize this process at the biochemical level, the nucleases localized in the nucleus of cells undergoing PCD were analysed by in-gel activity assays. For that purpose, scutellum cells were fractionated into nuclear and cytoplasmic fractions according to the scheme depicted in Supplementary Fig. S1A (available in *JXB* online). Nuclei isolated from scutellar cells at early stages (1–4 DAI) appeared intact, whereas at 7 DAI showed a lobed and fragmented appearance (Supplementary Fig. S1B). Protein extracts from both cytosolic and nuclear fractions were subjected to analysis of nucleolytic activity. A band showing endonuclease activity, with a molecular mass of approximately 70 kDa, was detected in nuclear extracts from scutellum cells of wheat grains at 7 DAI when assayed at acid pH, but not at neutral pH (Fig. 5A, 5B). In contrast, cytoplasmic fractions showed no detectable nucleolytic activity when assayed at acidic pH, but showed different neutral nuclease bands (Fig. 5A, 5B). Possible contamination of nuclear fractions with cytoplasmic proteins was ruled out by routinely testing for PEPC, a cytoplasmic enzyme, in the Western blot analysis (Fig. 5C).

To further characterize the process of DNA fragmentation, cell-free assays were carried out by incubating either the nuclear or cytoplasmic extracts from scutellum cells undergoing PCD (at 7 DAI) with intact nuclei isolated from healthy scutellum cells (at 1 DAI). The nucleolytic activity of the nuclear extracts triggered the internucleosomal fragmentation of DNA in intact nuclei at acid pH in contrast to the cytoplasmic extracts, which did not produce any DNA fragmentation or increase the activity of the nuclear extract (Fig. 5D). In agreement with the in-gel activity results, the nuclear-localized nucleolytic activity is acidic, as shown by the low activity detected at neutral pH (Fig. 5E). In addition, the requirement of cations of this nuclear-localized nucleolytic activity was analysed using both in-gel and *in vitro* assays. Fig. 6A shows the activating effect of Zn$^{2+}$ on the nuclease, whereas Ca$^{2+}$ and Mg$^{2+}$ had no effect. *In vitro* assays confirmed the activating effect of Zn$^{2+}$ (Fig. 6B). The cation requirement of the nuclear-localized nucleolytic activity was in contrast with the cytoplasmic activities, which required Ca$^{2+}$ and/or Mg$^{2+}$ and were strongly inhibited by Zn$^{2+}$ (Supplementary Fig. S2A). Although the nucleolytic activities of the cytoplasmic extracts did not produce DNA fragmentation in intact nuclei, as shown in the cell-free assay (Fig. 5D, 5E), these activities effectively degraded naked DNA, producing an unspecific DNA smear (Supplementary Fig. S2B). Therefore, the nucleolytic activity of nuclear extracts is associated with PCD and is able to produce DNA fragmentation on an intact chromatin structure, being Zn$^{2+}$- and acid pH-dependent.

**Fig. 5.** Identification of an acid nuclease in the nucleus of scutellum cells undergoing programmed cell death. (A–C) Scutellum discs dissected from wheat grains at 1, 4, or 7 days after imbibition (DAI), as indicated, were fractionated into cytoplasmic and nuclear extracts, and aliquots from both fractions (25 µg protein) were analysed by in-gel nuclease assay at the indicated pH. (D, E) *In vitro* analysis of nuclear DNA fragmentation. Protein extracts (4 µg protein) from nuclear (N), cytoplasmic (C), or a mixture of both (N+C) fractions obtained from scutellar cells of grains at 7 DAI were incubated with intact nuclei isolated from wheat grains at 1 DAI at the indicated pH. Following incubation, nuclear DNA was isolated and aliquots (20 µg) were fractionated on 2% (w/v) agarose gels. DNA marker, in kbp, is indicated on the left. Assays were repeated at least three times with similar results and representative results are shown.
ABA inhibits germination and DNA fragmentation of scutellum cells

The finding of a spatiotemporal pattern of PCD in the scutellum (Fig. 2C), and the fact that PCD starts once the proximal aleurone cells have undergone PCD, suggested that scutellum PCD is tightly regulated. As hormones play an important role in the control of grain germination and early seedling growth, the effect of hormones and inhibitors of hormone synthesis on scutellum PCD was analysed. For that purpose, wheat grains were imbibed in the presence of different hormones (GA3, ABA, or the brassinosteroid EBL) and PCB, an inhibitor of GA synthesis, or AVG, an inhibitor of ethylene synthesis. As expected, ABA,
and to lower extent PCB, exerted an inhibitor effect on wheat grain germination and seedling growth, whereas GA3 and AVG did not significantly affect the postgerminative process and EBL treatment reduced root elongation (Supplementary Fig. S3). The analysis of DNA fragmentation of scutellum cells showed that only ABA treatment exerted a clear inhibitory effect (Fig. 7A). The other treatments, including PCB or EBL, which affected the postgerminative process, did not show any significant effect (Fig. 7A). In agreement with these results, the in-gel nuclease assay identified the acid nucleolytic activity in nuclear extracts from scutellum cells, with the exception of the ABA-treated grains (Fig. 7B). Similarly, ABA caused a significant inhibition of the nuclear-localized nucleolytic activity, as detected by in vitro assays (Fig. 7C). Thus, only ABA treatment, which had a strong effect on germination, was effective to inhibit the biochemical symptoms of PCD of the scutellar cells.

**Discussion**

The success of cereal grain germination and initial stages of seedling growth depends on the precise organization of events taking place during this process. Because the aleurone cells are able to perceive gibberellins and induce the synthesis and secretion of hydrolytic enzymes, these cells play a central role to mobilize the storage material of the starchy endosperm and have received more attention than any other grain tissue. Interestingly, once the aleurone cells have performed their important function, enter in a process of PCD, which is also activated by gibberellins (Bethke *et al.*, 1999), thus allowing the use of the aleurone cellular contents for seedling growth. Although the scutellum has received less attention, it is clearly a tissue essential for germination. Indeed, gibberellins, the hormones activating germination, are synthesized in the scutellum (Appleford and Lenton, 1997). Moreover, scutellum epithelium cells participate at very initial steps of starchy endosperm mobilization by the secretion of hydrolytic enzymes together with the aleurone layer (Okamoto *et al.*, 1980; Cejudo *et al.*, 1995; Domínguez and Cejudo, 1995).

Nevertheless, the major function of the scutellum of germinated grains is to serve as transfer route for peptides (West *et al.*, 1998) and sugars (Aoki *et al.*, 2006) for the growing seedling. The present study addressed whether the scutellar cells undergoing PCD show vacuolization in the cytoplasm and a proactive intramembrane system (Figs. 2B and 3) linking the intracellular secretory pathway to a process of vacuolar cell death (van Doorn *et al.*, 2011). The presence of precursor protease vesicles and autolytic compartments derived from the endoplasmic reticulum (Toyooka *et al.*, 2000; Greenwood *et al.*, 2005) and Golgi cisternae (Filonova *et al.*, 2000) are considered as features of plant cell death, resembling morphological features of autophagy in animal cells. Although the role of autophagy in cell death is still subject of discussion (Kroemer and Levine, 2008), both morphological and biochemical evidence suggests that autophagy has a pro-death function either in developmental (Bozhkov *et al.*, 2005a) or pathogen-induced PCD in plants (Liu *et al.*, 2005; Hofius *et al.*, 2009). A feature of scutellum PCD is the appearance of different degrees of structural alterations of mitochondria (Fig. 3F). In other eukaryotic cells, mitochondria membranes have been described as origin of autophagosomes (Hernández *et al.*, 2003; Ning *et al.*, 2006; Luo *et al.*, 2009; Hailey *et al.*, 2010). This role of mitochondria in autophagy-type PCD is different from the role that these organelles have in apoptosis-type PCD, in which the disruption of the mitochondria promotes the translocation of cytochrome c and other apoptogenic factors to the cytoplasm (Lam, 2004).

Concerning the nucleus, it adopts a characteristic lobed morphology and a higher heterochromatin content (Fig. 4) as the most relevant features. However, remnants of heterochromatin could be detected in the central autolytic vacuoles (Fig. 4C), as observed in dying cells of somatic embryos of Norway spruce (Filonova *et al.*, 2000), which suggests that the nucleus is dismantled as cell death progresses. The identification of biochemical components participating in nucleus dismantling was another objective addressed in this study.
At the molecular level, the knowledge of enzymes involved in the execution of PCD in plants is much lower than in animals. Despite the absence of genes encoding caspases in plants, it appears that caspase-like activities are important (del Pozo and Lam, 1998). Of the different proteases proposed to participate in plant PCD, only some of them seem to be essential components for nucleus dismantling. This is the case of metacaspase mcII-Pa, which is translocated to the nucleus in cells undergoing PCD during embryogenesis (Bozhkov et al., 2005b) and participates in cleavage and activation of TSN, a phylogenetically conserved multifunctional regulator of gene expression involved in PCD (Sundström et al., 2009). The present study used the wheat grain as a model system to identify nuclear-localized factors involved in the final steps of PCD execution, critically on DNA fragmentation and nucleus dismantling. Biochemical analysis allowed the identification of two Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases, which were localized, respectively, to the nuclei of aleurone cells (Domínguez et al., 2004) and nucellus cells (Domínguez and Cejudo, 2006) undergoing PCD. Although both endonucleases showed the same catalytic requirements, the different electrophoretic mobility suggested that each tissue of wheat grains undergoes PCD with the participation of different nucleases. The identification of a Zn$^{2+}$-dependent endonuclease in the nucleus of wheat scutellum cells undergoing PCD, which produced internucleosomal fragmentation of DNA (Figs. 5–7), is in agreement with the proposal of the participation of different nucleases in different grain tissues. Among the endonucleases identified in cells suffering PCD, only some have been directly involved in nucleus dismantling. This is the case of ZEN1, a Zn$^{2+}$-dependent nuclease implicated in the degradation of nuclear DNA in Zinnia tracheary elements (Ito and Fukuda, 2002). ZEN1 is localized to vacuoles which collapse before DNA is degraded (Obara et al., 2001). However, ZEN1 activity did not produce the characteristic DNA laddering shown in animal apoptosis. In plants, it was proposed that nucleus-localized nucleases are neutral whereas vacuolar nucleases are acidic (Sugiyama et al., 2000). Thus, the identification of an acidic Zn$^{2+}$-dependent endonuclease in the nucleus of wheat scutellum cells undergoing PCD may be considered an exception to this rule, to be added to the previously reported acidic Zn$^{2+}$-dependent nuclease responsible for DNA laddering identified in rice root tip cells undergoing PCD in response to salt stress (Jiang et al., 2008).

The appearance of the nuclear-localized nucleolytic activity was completely inhibited in ABA-treated grains (Fig. 7), which might suggest an inhibitory effect of ABA on nuclease expression. However, it is well known that the success of germination depends of a spatiotemporal sequence of events. Most probably, the strong inhibitory effect of ABA on germination (Supplementary Fig. S3) occurs because it counteracts the activating effect of gibberellins, thus arresting germination at early stages. As a consequence, the rest of events taking place thereafter, including scutellum PCD, will not take place in ABA-treated grains.

**Supplementary material**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Cytoplasm and nuclei fractionation and visualization of isolated nuclei

**Supplementary Fig. S2.** Characterization of nucleolytic activities in the cytoplasm of scutellum cells undergoing PCD

**Supplementary Fig. S3.** Effect of hormones on root and shoot emergence and elongation

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**References**

Aoki N, Scofield GN, Wang X-D, Offer CE, Patrick JW, Furbank RT. 2006. Pathway of sugar transport in germinating wheat seeds. *Plant Physiology* **141**, 1255–1263.

Appleford NEJ, Lenton JR. 1997. Hormonal regulation of α-amylose gene expression in germinating wheat (*Triticum aestivum*) grains. *Physiologia Plantarum* **100**, 534–542.

Bai S, Willard B, Kinter M, Chapin LJ, Francis D, Stead A, Jones ML. 2010. Proteomic analysis of post-pollination senescence. *Journal of Experimental Botany* **61**, 1089–1109.

Bailly C. 2004. Active oxygen species and antioxidants in seed biology. *Seed Science Research* **14**, 93–107.

Bassham DC. 2007. Plant autophagy – more than a starvation response. *Current Opinion in Plant Biology* **10**, 587–593.

Beligni MV, Fath A, Bethke PC, Lamattina L, Jones RL. 2002. Nitric oxide acts as an anti-oxidant and delays programmed cell death in barley aleurone layers. *Plant Physiology* **129**, 1642–1650.

Bethke PC, Lonsdale JE, Fath A, Jones RL. 1999. Hormonally regulated programmed cell death in barley aleurone cells. *The Plant Cell* **11**, 1033–1045.

Bozhkov PV, Filonova LH, Suárez MF. 2005a. Programmed cell death in plant embryogenesis. *Current Topics in Developmental Biology* **67**, 135–179.

Bozhkov PV, Suárez MF, Filonova LH, Daniel G, Zamyatin AA, Rodríguez-Nieto S, Zhivotovsky B, Smertenko A. 2005b. Cysteine protease mcII-Pa executes programmed cell death during plant embryogenesis. *Proceedings of the National Academy of Sciences, USA* **102**, 14463–14468.

Cejudo FJ, Cubo MT, Baulcombe DC. 1995. Amy I expression during wheat seed germination. *Plant Science* **106**, 207–213.

Conradt B. 2009. Programmed cell death during animal development. *Annual Review of Genetics* **43**, 493–523.

Consonni G, Aspesi C, Barbante A, Dolfini S, Giuliani C, Giuliani A, Hansen S, Brettcsneider R, Pili R, Gavazzi G. 2003. Analysis of four maize mutants arrested in early embryogenesis reveals an irregular pattern of cell division. *Sexual Plant Reproduction* **15**, 281–290.

del Pozo O, Lam E. 1998. Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Current Biology* **8**, 1129–1132.
Domínguez F, Cejudo FJ. 1995. Pattern of endoproteolysis following wheat grain germination. Physiologia Plantarum 95, 253–259.

Domínguez F, Cejudo FJ. 1998. Germination-related genes encoding proteolytic enzymes are expressed in the nucellus of developing wheat grains. The Plant Journal 15, 569–574.

Domínguez F, Cejudo FJ. 1999. Patterns of starchy endosperm acidification and protease gene expression in wheat grains following germination. Plant Physiology 119, 81–87.

Domínguez F, Cejudo FJ. 2000. Programmed cell death in cereal aleurone. Physiologia Plantarum 116, 677–688.

Domínguez F, Cejudo FJ. 2001. The nucellus degenerates by a process of programmed cell death during the early stages of wheat grain development. Planta 213, 352–360.

Domínguez F, Moreno J, Cejudo FJ. 2002. A gibberellin-induced nuclease is localized in the nucleus of wheat aleurone cells undergoing programmed cell death. Journal of Biological Chemistry 279, 11530–11536.

Fath A, Bethke PC, Lonsdale J, Meza-Romero R, Jones RL. 2000. Programmed cell death in cereal aleurone. Plant Molecular Biology 44, 255–266.

Filonova LH, Bozhkov PV, Brukhin VB, Daniel G, Zhivotovsky B, von Arnold S. 2000. Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. Journal of Cell Science 113, 4399–4411.

Giuliani C, Consonni G, Gavazzi G, Colombo M, Dolfini S. 2002. Programmed cell death during embryogenesis in maize. Annals of Botany 90, 287–292.

González MC, Osuna L, Echevarría C, Vidal J, Cejudo FJ. 1998. Expression and localization of phosphoenolpyruvate carboxylase in developing and germinating wheat grains. Plant Physiology 116, 1249–1258.

Greenwood JS, Helm M, Giet C. 2005. Ricinosomes and endosperm transfer cell structure in programmed cell death of the nucellus from Ricinus seed development. Proceedings of the National Academy of Sciences, USA 102, 2238–2243.

Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J. 2010. Mitochondria supply membranes for autophagosomal biogenesis during starvation. Cell 141, 656–667.

Hernández LD, Pypaert M, Flavell RA, Galán JE. 2003. A Salmonella protein causes macrophage cell death by inducing autophagy. Journal of Cell Biology 163, 1123–1131.

Hatsugai N, Kuroyanagi M, Nishimura M, Hara-Nishimura I. 2006. A cellular suicide strategy of plants: vacuole-mediated cell death. Apoptosis 11, 905–911.

He C, Klionsky DJ. 2009. Regulation mechanisms and signalling pathways of autophagy. Annual Review of Genetics 43, 67–93.

Hofius D, Schultz-Larsen T, Joensen J, Tsitsigianis DI, Petersen NHT, Mattsson O, Jorgensen LB, Jones JDG, Mundy J, Petersen M. 2009. Autophagic components contribute to hypersensitive cell death in Arabidopsis. Cell 137, 773–783.

Ito J, Fukuda H. 2002. ZEN1 is a key enzyme in the early degradation of nuclear DNA during programmed cell death of tracheary elements. The Plant Cell 14, 3201–3211.

Jiang A-L, Cheng Y, Li J, Zhang W. 2008. A zinc-dependent nuclear endonuclease is responsible for DNA laddering during salt-induced programmed cell death in root tip cells of rice. Journal of Plant Physiology 165, 1134–1141.

Kitazumi I, Tsukahara M. 2011. Regulation of DNA fragmentation: the role of caspases and phosphorylation. FEBS Journal 278, 427–441.

Kroemer G, Levine B. 2008. Autophagic cell death: the story of a misnomer. Nature Reviews Molecular Cell Biology 9, 1004–1010.

Kuriyama H, Fukuda H. 2002. Developmental programmed cell death in plants. Current Opinion in Plant Biology 5, 568–573.

Lam E. 2004. Controlled cell death, plant survival and development. Nature Reviews Molecular Cell Biology 5, 305–315.

Liu Y, Schiﬀ M, Czymmek K, Tallocco Z, Levine B, Dinesh-Lumar SP. 2005. Autophagy regulates programmed cell death during the plant innate immune response. Cell 121, 567–577.

Luo S, Chen Q, Cebollero E, Xing D. 2009. Mitochondria: one of the origins for autophagosomal membranes? Mitochondrion 9, 221–231.

Mylona PV, Polidoros AN, Scandalios JG. 2007. Antioxidant gene responses to ROS-generating xenobiotics in developing and germinated scutella of maize. Journal of Experimental Botany 58, 1301–1312.

Ning G, Haldeman RA, Haimovich B. 2006. Shigella ﬂexneri induces cell death by damage to and autophagy of host cell mitochondria: an electron-microscopic study of infected human monocyte derived macrophages. Microscopy and Microanalysis 12 (Supp. 2), 322–323.

Obara K, Kuriyama H, Fukuda H. 2001. Direct evidence of active and rapid nuclear degradation triggered by vacuole rupture during programmed cell death in Zinnia. Plant Physiology 125, 615–626.

Okamoto K, Kitano H, Akazawa T. 1980. Biosynthesis and excretion of hydrolases in germinating cereal seeds. Plant and Cell Physiology 21, 201–204.

Pulido P, Cazalis R, Cejudo FJ. 2009. An antioxidant redox system in the nucleus of wheat seed cells suffering oxidative stress. The Plant Journal 57, 132–145.

Sahara S, Aoto M, Egushi Y, Imamoto N, Yoneda Y, Tsujimoto Y. 1999. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. Nature 401, 168–173.

Samejima K, Earnshaw WC. 2005. Trashing the genome: the role of nucleases during apoptosis. Nature Reviews Molecular Cell Biology 6, 677–688.
Serrato AJ, Cejudo FJ. 2003. Type-h thioredoxins accumulate in the nucleus of developing wheat seed tissues suffering oxidative stress. *Planta* **217**, 392–399.

Stacy RAP, Nordeng TW, Culiáñez-Maciá FA, Aalen R. 1999. The dormancy-related peroxiredoxin anti-oxidant, PER 1, is localized to the nucleus of barley embryo and aleurone cells. *The Plant Journal* **19**, 1–8.

Sugiyama M, Ito J, Aoyagi S, Fukuda H. 2000. Endonucleases. *Plant Molecular Biology* **44**, 387–397.

Sundström JF, Vaculova A, Smertenko AP, et al. 2009. Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome. *Nature Cell Biology* **11**, 1347–1354.

Taatjes DJ, Sobel BE, Budd RC. 2008. Morphological and cytochemical determination of cell death by apoptosis. *Histochemical and Cell Biology Reviews* **129**, 33–43.

Toyooka K, Okamoto T, Minamikawa T. 2000. Mass transport of proform of a KDEL-tailed cysteine protease (SH-EP) to protein storage vacuoles by endoplasmic reticulum-derived vesicle is involved in protein mobilization in germinating seeds. *Journal of Cell Biology* **148**, 453–463.

van Doorn WG, Beers EP, Dangl JL, et al. 2011. Morphological classification of plant cell deaths. *Cell Death and Differentiation* **18**, 1241–1246.

**West CE, Waterworth WM, Stephens SM, Smith CP, Bray CM.** 1998. Cloning and functional characterization of a peptide transporter expressed in the scutellum of barley grain during the early stages of germination. *The Plant Journal* **15**, 221–229.

Williams B, Dickman M. 2009. Plant programmed cell death: can’t live with it; can’t live without it. *Molecular Plant Pathology* **9**, 531–544.

Wu M, Huang J, Xu S, Ling T, Xie Y, Shen W. 2011. Haem oxygenase delays programmed cell death in wheat aleurone layers by modulation of hydrogen peroxide metabolism. *Journal of Experimental Botany* **62**, 235–248.

Young TE, Gallie DR. 1999. Analysis of programmed cell death in wheat endosperm reveals differences in endosperm development between cereals. *Plant Molecular Biology* **39**, 915–926.

Young TE, Gallie DR, DeMason DA. 1997. Ethylene mediated programmed cell death during maize endosperm development of Su and sh2 genotypes. *Plant Physiology* **115**, 737–751.

Young TE, Gallie DR. 2000. Regulation of programmed cell death in maize endosperm by abscisic acid. *Plant Molecular Biology* **42**, 397–414.

Zapata JM, Guerra A, Esteban-Carrasco A, Martin M, Sabater B. 2005. Chloroplasts regulate leaf senescence: delayed senescence in transgenic ndhi-defective tobacco. *Cell Death and Differentiation* **12**, 1277–1284.