Characterization of programmed cell death in the endosperm cells of tomato seed: two distinct death programs

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Abstract: Programmed cell death (PCD) is a requisite, genetically controlled process in plants resulting in the death of particular cells and tissues and the recycling of the cellular constituents back to the organism. PCD in the lateral and micropylar endosperm cells during and following germination of tomato (Solanum lycopersicum L.) seeds was characterized by transmission electron microscopy, by terminal d-UTP nick-end labelling of nuclei, and agarose gel electrophoretic analysis of genomic DNA. Postgerminative cells of lateral and micropylar endosperm displayed morphologies and terminal d-UTP nick-end labelling positive nuclei consistent with PCD. PCD was not detected in the lateral endosperm in the absence of the embryo. The embryo’s effect on promoting lateral endosperm PCD could be substituted with gibberellic acid at 50 μmol/L. Micropylar endosperm cells undergo PCD irrespective of incubation with or without the embryo; gibberellic acid only hastens the onset of PCD morphology. Precursor protease vesicles, novel endoplasmic reticulum derived organelles considered markers of PCD, were observed in postgerminative lateral and micropylar endosperm cells. Internucleosomal laddering was not detected in endospermic DNA. These results suggest that a late postimbibition gibberellic acid linked mechanism promotes PCD in the lateral endosperm, whereas the promotion of PCD in the micropylar endosperm occurs early in, or prior to, imbibition.

Key words: programmed cell death, tomato seed, precursor protease vesicles, transmission electron microscopy, gibberellic acid.


Mots clés : apoptose, graine de tomate, précurseurs de vésicules protéasiques, microscopie électronique par transmission, acide gibbérellique.

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Introduction

Programmed cell death (PCD) is a biologically ubiquitous phenomenon among multicellular eukaryotes (Lockshin and Zakeri 2004). It is a genetically controlled, active process responsible, for example, for the removal of portions of the organism that have become developmentally unnecessary and recycling of the cellular matter into basic building blocks to be used by surrounding cells and tissues. In plants, pertinent examples of PCD include autumnal leaf senescence, fruit ripening, tracheryelement formation, cereal and castor seed (Ricinus communis L.) endosperm and nucellar degradation, the hypersensitive response to pathogen invasion, and the development of lace plant (Aponogeton madagascariensis van Bruggen) and Monstera obliqua Miq. leaf perforations (Young et al. 1997; Bethke et al. 1999; Schmid et al. 1999; Xu and Chye 1999; Obara et al. 2001;
The degradation of nuclear DNA is a common feature of PCD in most organisms, but the manner by which it occurs is variable, especially in plants. During apoptotic PCD in metazoans, genomic DNA is digested between nucleosomes resulting in a “laddering” of the DNA in 180 base pair multiples that can be readily separated by gel electrophoresis (Wyllie 1980; Nagata et al. 2003). Laddering of DNA is also observed in plant PCD, e.g., during maize (Zea mays L.) endosperm and anther development and during the hypersensitive response to rust fungus infection of cowpea (Vigna unguiculata L.), but is not a consistent feature (Ryerson and Heath 1996; Young et al. 1997; Hiratsuka et al. 2002; Ku et al. 2003; Dominguez et al. 2004). Apoptotic-like DNA laddering is not seen during PCD during treachery element formation nor during lace plant or Monstera leaf perforation formation. The degradation of nuclear DNA is a common feature of plant PCD, harbouring numerous hydrolytic enzymes (Bethke et al. 1998, 1999; Dominguez et al. 2004, 2005), and in nucellar destruction during castor seed PCD (Dominguez et al. 2001, 2004, 2005), and in nucellar destruction during development (Greenwood et al. 2005) in castor seed, during aerenchyma formation in maize (Gunawardena et al. 2001), and leaf perforation formation in lace plant and Monstera (Gunawardena et al. 2004, 2005) even though genomic DNA degradation occurs.

Terminal d-UTP nick-end labelling (TUNEL) incorporates fluorescently labelled nucleotides to 3'-OH cleaved genomic DNA in situ (Gavrieli et al. 1992; Gorczyca et al. 1993). TUNEL-positive nuclei have been used as indicators of plant PCD-associated DNA degradation in a number of studies including endosperm degradation following germination (Schmid et al. 1999) and nucellar degradation during development (Greenwood et al. 2005) in castor seed, during aerenchyma formation in maize (Gunawardena et al. 2001), and leaf perforation formation in lace plant and Monstera (Gunawardena et al. 2004, 2005) among numerous others.

Although TUNEL-positive nuclei may be considered as one indicator of PCD, other cytological factors must be taken into consideration. Ultrastructural characterization of PCD in plants indicates a progressive loss of cellular organization and compartmentalization (Filonova et al. 2000; Dominguez et al. 2001; Gunawardena et al. 2001, 2004, 2005; Greenwood et al. 2005). Cellular and nuclear shrinkage with chromatin condensation is common, as seen during maize aerenchyma formation (Gunawardena et al. 2001), in barley (Hordeum vulgare L.) aleurone layer cell death following germination (Wang et al. 1996), in lace plant and Monstera leaf perforation development (Gunawardena et al. 2004, 2005), and in nucellar destruction during castor seed development (Greenwood et al. 2005). Nuclei often become highly invaginated prior to cell death (Wang et al. 1996; Gunawardena et al. 2001; Greenwood et al. 2005). The cytoplasm, plasma membrane, and organelles also show progressively aberrant morphologies. The cytoplasm becomes highly vesiculated and vacuolate and develops a flocculent appearance, mitochondria and plastids become misshapen and electron translucent, and the plasma membrane withdraws from the cell wall (Filonova et al. 2000; Gunawardena et al. 2001, 2004; Greenwood et al. 2005).

The vacuole plays an important role as an acidic compartment in plant PCD, harbouring numerous hydrolytic enzymes (Bethke et al. 1998, 1999; Swanson et al. 1998; Ito and Fukuda 2002; Otegui et al. 2005). Rupture of the tonoplast occurs as one of the last stages of cell death, acidifying the cytosol and releasing hydrolytic enzymes into an environment favourable for maximal activity (Schmid et al. 1999; Gietl and Schmid 2001; Ito and Fukuda 2002; Greenwood et al. 2005). Novel endoplasmic reticulum derived organelles, precursor protease vesicles (PPVs), are found in cells undergoing PCD in some species and their presence is considered a marker for PCD (Schmid et al. 1998; Chrispeels and Herman 2000; Toyooka et al. 2000; Gietl and Schmid 2001; Greenwood et al. 2005). PPVs are roughly spherical, approximately 0.5 μm in diameter, with the major protein component being a KDEL-tagged cysteine endopeptidase (Schmid et al. 1998, 1999; Toyooka et al. 2000; Hayashi et al. 2001; Greenwood et al. 2005). It is presumed that acidification of the cytosol upon vacuolar collapse causes the PPVs to lyse, releasing the cysteine endopeptidase, which then completes the destruction of the remaining cytosolic proteins (Gietl and Schmid 2001).

Plant hormones, including brassinosteroids (Clouse and Sasse 1998), cytokinins (Carimi et al. 2003), ethylene (Young et al. 1997), and gibberellic acid (GA) (Bethke et al. 1999; Dominguez et al. 2004), have been implicated as inducers of plant PCD. GA appears to be involved also in germination and reserve mobilization in monocot and dicot species (Hooley 1994), activating and promoting the de novo biosynthesis of hydrolases including cysteine proteases (Bewley and Black 1978; Koehler and Ho 1990; Bethke et al. 1998, 1999; Dominguez et al. 2004). In addition, GA causes lumenal acidification of protein storage vacuoles (Swanson and Jones 1996) and has been shown to induce PCD in the aleurone layer cells of cereals (Bethke et al. 1999; Dominguez et al. 2004).

In the present study, we use germinated tomato (Solanum lycopersicum L.) seed as a model system to investigate plant PCD. These seeds are relatively large, facilitating isolation of the embryo from the endosperm (Bewley and Black 1978; Boesewinkel and Bouman 1995; Cooley et al. 1999), and are readily permeable to common chemical fixatives. Toward that end, we employed both transmission electron microscopy and epifluorescence microscopy to determine if lateral and micropylar endosperm cells had cytological and ultrastructural features consistent with PCD, including the occurrence of PPVs and TUNEL-positive nuclei, during and following germination. We show that PCD in the lateral endosperm is influenced by the embryo. The effect of the embryo on lateral endosperm PCD can be mimicked by exogenous GA. Conversely, PCD of micropylar endosperm cells is controlled in a different manner. Micropylar endosperm cells seem predestined to die, following a death program in the absence of the embryo or exogenous GA. Together, these results provide the first characterization of PCD in endosperm cells of tomato seeds, revealing different mechanisms for micropylar and lateral endosperm cell death programs. Models describing control over PCD in the lateral and micropylar endosperm are discussed based on these data.

Materials and methods

Tissue preparation

Solanum lycopersicum ‘Glamour’ seeds (Stokes Seeds, St. Catharines, Ontario) were imbibed for 24 h on Whatman No. 4 filter paper in Parafilm-sealed 100 mm diameter Petri dishes with sufficient sterile ddH₂O to moisten the seeds.
(about 6 mL). At 24 h after imbibition (HAI), seeds were divided into two groups: whole (intact) seeds and embryoless half-seeds. Embryoless half-seeds were produced through sagittal bisection of the intact seeds and excision of the embryos. After dissection, embryoless half-seeds were further incubated in either streptomycin at 0.1 mg/mL (Fisher Scientific, Whitby, Ontario) or GA₃ at 50 μmol/L (Sigma-Aldrich, Oakville, Ontario) supplemented with streptomycin for the duration of the experiments. Whole seeds were imbibed on sterile ddH₂O alone (solutions were replaced every 24 h). Samples were collected from each group of seeds or embryoless half-seeds at 12 h time points between 60 and 84 HAI and at 120 HAI.

**Transmission electron microscopy**

Intact and embryoless half-seeds were harvested and immersed in cold primary fixative (2% (v/v) glutaraldehyde (Fisher Scientific), 1% (v/v) acrolein (Greenwood et al. 1989; Sigma-Aldrich), MgCl₂ at 10 mmol/L (Schmid et al. 1998), 1% (m/v) caffeine (Mueller and Greenwood 1978), in Sorensen’s phosphate buffer 0.025 mol/L, pH 7.2). Intact seeds were bisected to promote the penetration of the fixative. Samples were fixed for 2 h with gentle agitation at 4 °C. After 2 h, samples were washed in Sorensen’s phosphate buffer at 0.025 mol/L, pH 7.2, three times, 50 min each, and then were postfixed for 2 h in 2% (m/v) OsO₄ (Fisher Scientific) in the same buffer. Following an overnight rinse in ddH₂O, samples were dehydrated through a graded ethanol series (50%, 70% (both at 4 °C), 80%, 90%, 95%, and 2× 100% ethanol (v/v) in ddH₂O) at room temperature, 50 min each step. Samples were then dehydrated further in 50:50 (v/v) ethanol – propylene oxide (Acros Chemicals, Oakville, Ontario) and again in 100% (v/v) propylene oxide, each for 30 min. Samples were then infiltrated overnight in 50:50 (v/v) propylene oxide – Spurr’s resin (hard mixture; Spurr 1969) and then through three changes in fresh Spurr’s resin for a total of 4 h. Spurr’s embedded tissues were cured at 65 °C overnight. Sections of 80–90 nm thickness were obtained using an LKB 2188 (Bromma, Sweden) or a Sorval-MT2 (Norwalk, Connecticut) ultramicrotome and collected on 200-mesh copper grids (Canemco, Lakefield, Quebec). The sections were stained for 15 min with saturated uranyl acetate in ddH₂O (Van Bruggen et al. 1960), rinsed in ddH₂O, and stained again for 3 min with Reynolds lead citrate (Reynolds 1963). Sections were viewed with a Philips CM-10 transmission electron microscope (Eindhoven, Holland) at an accelerating voltage of 80 kV.

**Bright-field microscopy**

Bright-field images of micropylar and lateral endosperm were obtained from 1 μm thick sections of samples prepared to correlate with transmission electron microscopy observations (O’Brien and McCully 1981). Thick sections were produced with a Sorval-MT2 microtome and photographed using a Leica epifluorescence microscope (Richmond Hill, Ontario) equipped with a CCD camera (Hamamatsu, Tokyo, Japan). Images were acquired and processed using OpenLab imaging suite version 3.1.7 (Improvision, Lexington, Massachusetts).

**TUNEL assays**

Samples from the various treatments and throughout the time course were fixed in formaldehyde – acetic acid – ethanol (FAA) for 24 h at 4 °C with gentle agitation and then dehydrated through 75%, 95%, and 2× 100% ethanol (v/v) in ddH₂O) at room temperature for 1 h each step. Samples were then infiltrated with Safeclear (Fisher Scientific) through a second graded series: 50%, 75%, and 95% Safeclear – ethanol (v/v), each for 2 h and then overnight in 100% Safeclear. Samples were then infiltrated for 8 h in a 50:50 (v/v) solution of Safeclear and Paraplast (Fisher Scientific) at 56 °C and then with nine changes of pure Paraplast over 72 h at 56 °C (Spence 2001). Sections, 12–14 μm thick, were obtained using a rotary microtome, mounted on Colorfrost coated slides (Fisher Scientific), and dried for 24 h at 45 °C. Sections were dewaxed through three changes of Safeclear, each for 5 min, and then rehydrated through a graded ddH₂O–ethanol series (Satiat-Jeune-maître and Hawes 2001). TUNEL assays were performed using the in situ Cell Death Detection Fluorescein kit (Roche Applied Science, Laval, Quebec) according to the manufacturer’s instructions with the modification that sections were permeabilized with proteinase K at 10 μg/mL (Fermentas, Burlington, Ontario) for 1 h at 37 °C and TUNEL reactions were carried out for 3 h in darkness at 37 °C. Positive controls were performed by incubating sections in 10 units of DNaseI (Ambion, Austin, Texas) for 20 min. Negative controls were performed by omitting the DNA-labelling terminal transferase. The TUNEL reacted sections were observed at 520 nm excitation wavelength with an epifluorescence microscope (Leica) equipped with a CCD camera as described above.

**DNA extractions and laddering assays**

Eighty isolated tomato seed endosperms from each of deembryonated whole seeds incubated in ddH₂O alone and embryoless half-seeds incubated in ddH₂O or GA were immersed in liquid nitrogen and homogenized using a Thermoelectron FP120 homogenizer (Carlsbad, California). DNA was extracted using the FastDNA kit (Qbiogene, Carlsbad, California) according to the manufacturer’s instructions. Samples of apoptotic and nonapoptotic human T-lymphocyte cells (a gift from Dr. R.D. Mosser, University of Guelph) were used as appropriate controls. Briefly, the DNA of approximately 5 × 10⁶ T-lymphocytes was extracted as previously described (Mosser and Martin 1992) with the exception that trichloroacetic acid precipitations were not performed and DNA was precipitated at −20 °C for only 1 h. Equal amounts of DNA from each endosperm treatment and the apoptotic and nonapoptotic T-lymphocyte samples were loaded on a 2% (m/v) agarose gel and electrophoresed at 100 V for 2 h; gel images were captured using a Biorad Gel Doc system (Hercules, California).

**Results**

The mature tomato seed consists of a quiescent embryo surrounded by the polyploid nutritive endosperm that is completely mobilized following germination; both are enclosed by the seed coat or testa (Figs. 1a–1e). The thin-walled micropylar endosperm (Figs. 1a, 1c, and 1d) consists
of a cap surrounding the radicle tip (Fig. 1a). At 60 HAI, reserves within the micropylar endosperm cells adjacent to the radicle tip have been mobilized and the cells are vacuolated. Cytoplasm is appressed to the cell wall (Figs. 1c and 1d) and mobilization of reserves is less advanced in the cells from the periphery of the endosperm cap (Figs. 1c and 1d). The lateral endosperm (Figs. 1a and 1b) comprises the remainder of the endosperm tissue and is primarily nutritive. The cells are large and thick walled and contain abundant nutrient reserves (Fig. 1b).

Morphological characteristics consistent with PCD were observed in postgerminative lateral endosperm cells of tomato seed

Seeds were analysed over a time course beginning prior to (60 HAI) and following (72, 84, and 120 HAI) embryo emergence to determine if the progression of death in the lateral endosperm cells is marked by features consistent with PCD. Electron micrographs of ultrathin sections of lateral endosperm revealed that at 60 HAI, cells were filled with lipid and protein bodies, and cell walls appeared thick and electron dense (Fig. 2a). Tomato seed germination was somewhat asynchronous, and at 72 HAI, tomato seeds had either completed germination or not. Lateral endosperm cells from ungerminated seeds at 72 HAI did not display morphological features consistent with PCD (not shown) and appeared similar to cells found at 60 HAI. At 72 HAI, lateral endosperm cells from germinated seeds displayed increased electron density of the nuclear envelope (Fig. 2b) and chromatin condensation (Fig. 2c). Protein and lipid bodies were either degraded (Fig. 2b) or less abundant (cf. Figs. 2a and 2b), the cytoplasm appeared flocculent, and vacuolation was widespread. PPVs were first observed in the endosperm cells at this time (Figs. 2b and 2c). They were spherical in shape with a diameter of approximately 1 μm and a rough membrane surface and were closely associated with the endoplasmic reticulum (Figs. 2d and 2e). As the time course progressed, the morphological features of PCD became more pronounced; the cytoplasm of lateral endosperm cells at 120 HAI appeared increasingly flocculent (Figs. 2d and 2e) and highly vacuolate (Figs. 2e–2g) and tonoplasts were electron dense (Figs. 2f and 2g). Collapsed cells did not possess any discernible internal structures and showed only coalesced cellular debris (Fig. 2h). Taken together, these results suggest that lateral endosperm cells from germinated tomato seeds developed morphological features consistent with PCD.

DNA degradation occurred as lateral endosperm cells progress through PCD

A series of TUNEL assays were performed to determine if nuclear DNA cleavage was a feature of lateral endosperm cell death. In the presence of the embryo, the lateral endosperm tissue contained many TUNEL-positive nuclei at 60 HAI, peaking by 72 HAI (Figs. 4a and 4b). However, fewer TUNEL-positive nuclei were detected at 84 HAI (Fig. 4c). Nuclei in lateral endosperm tissue from embryoless half-seeds incubated with GA were also TUNEL positive throughout the time series and yielded stronger fluorescence (Figs. 4d–4f). When incubated in water alone, nuclei in lateral endosperm tissue from embryoless half-seeds were only sparsely labelled throughout the time course (Figs. 4g–4i). A DNase-positive control produced TUNEL-positive nuclei coincident with 4′,6-diamidino-2-phenylindole (DAPI) stained nuclei (Figs. 4j and 4k). A negative control failed to produce any detectable signal (not shown). These results suggest that DNA degradation preceded observable cell death morphology (cf. Figs. 4a and 4a), but only in the presence of the embryo or GA. Furthermore, the morphological characteristics consistent with PCD in lateral endosperm cells were coincident with widespread DNA degradation.

PCD in micropylar endosperm cells occurred independently from the embryo or exogenous GA

We examined if exogenous GA was capable of inducing subcellular morphological changes consistent with PCD in micropylar endosperm cells. To that end, whole seeds in water alone and embryoless half-seeds incubated in the absence or presence of exogenous GA were examined over a time course from 60, 72, and 84 HAI by transmission electron microscopy. Prior to radicle emergence, at 60 HAI, the micropylar endosperm cells appeared healthy, the cells were full of lipid and protein bodies, and cell walls appeared electron dense (Fig. 5a). In intact seeds, micropylar endosperm cells were degraded within the 24 h period between 60 and 84 HAI (Figs. 5a–5c). At 72 HAI, cells that were not crushed by the emergence of the radicle were highly vacuolate (Fig. 5b), the cytoplasm was flocculent, and cell walls appeared less electron dense (cf. Figs. 5a and 5b). By 84 HAI, micropylar endosperm cells contained no discernible internal organization and cell walls appeared degraded (Fig. 5c).

In embryoless half-seeds, in the absence of GA, micropylar endosperm cells (Fig. 5g) were comparable with those of the whole seed (Fig. 5a), and within 12 h, these cells had collapsed (cf. Figs. 5g and 5h). By 72 HAI, little discernible

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At 84 HAI, cellular collapse was observed (Fig. 5i). Application of GA at 50 μmol/L hastened the onset of observable morphologies consistent with cell death in micropylar endosperm cells (Figs. 5d–5f, 5k, and 5l). For instance, at 60 HAI, nuclei and mitochondria with aberrant morphologies were observed in cells from embryoless half-seeds incubated in GA. The former displayed chromatin condensation and the latter contained misshapen cristae that appeared electron translucent (Figs. 5d and 5k). Furthermore, protein bodies were degraded resulting in increased vacuolation (Fig. 5d) and PPVs were observed (Fig. 5k). These endosperm cells were comparable with those cells found in intact seeds at 72 HAI (cf. Figs. 5b and 5d). At 72 and 84 HAI, cells appeared increasingly vacuolate, tonoplasts appeared electron dense (Fig. 5e), the cytoplasm was vesiculated (Fig. 5f), and cell walls were electron translucent (Fig. 5e). A remaining nucleus at 72 HAI showed invagination of the nuclear envelope and chromatin condensation (Fig. 5l). By 84 HAI, cells were collapsed (Fig. 5f). These results suggest that GA only hastens micropylar endosperm PCD, but the initiation of PCD occurs in the absence of the embryo and may be due to some other signal or factor.

DNA degradation in micropylar endosperm cells was coincident with PCD morphology

To determine if DNA degradation is also a characteristic of micropylar endosperm cells during and after germination, tissues from intact seeds incubated in water alone and embryoless half-seeds that were incubated in either GA at 50 μmol/L or water were subjected to TUNEL assays at 60, 72, and 84 HAI. Micropylar endosperm cells from intact seeds had TUNEL-positive nuclei at 60 and 72 HAI, peaking in abundance at the latter time point (Figs. 6a and 6b). The number of labelled nuclei had declined sharply by 84 HAI (Fig. 6c). Cells in micropylar endosperm tissue incubated in water contained nuclei that were strongly TUNEL positive throughout the time series (Figs. 6g and 6i). Treatment of embryoless half-seeds with exogenous GA resulted in a pattern similar to that observed in micropylar endosperm cells from intact seeds; TUNEL-positive nuclei were found at 60 HAI, peaked by 72 HAI (Figs. 6d and 6e), and declined at 84 HAI. The decline was less evident than that observed in micropylar endosperm cells from intact seeds at the same time point (cf. Figs. 6c and 6f). A DNaseI-positive control showed that TUNEL-positive nuclei were coincident with DAPI-stained nuclei (Figs. 6j and 6k). The negative control produced no labelling (not shown). These data suggest that micropylar endosperm cells have received some signal(s) initiating PCD prior to 24 HAI. Embryos were removed from embryoless half-seeds at 24 HAI, and DNA degradation proceeded in the micropylar endosperm cells irrespective of the presence of the embryo or GA.

Endospermic DNA did not show internucleosomal laddering

To determine whether the DNA degradation observed by TUNEL assays in endosperms of germinative and postgerminative tomato seeds was apoptotic-like, leading to laddering, DNA was harvested from intact seeds incubated in water alone and from embryoless half-seeds in the absence and presence of GA at 50 μmol/L at 60, 72, and 84 HAI and then resolved by gel electrophoresis (Fig. 7, lanes 1–9).
DNA extracted from normal and heatshocked apoptotic human T-lymphocyte cells (Fig. 7, lanes 10 and 11, respectively) was used as a control. Low molecular mass fragments consistent with internucleosomal laddering were not detected in DNA isolated from endosperm tissue, regardless of treatment. DNA harvested from apoptotic T-lymphocyte cells showed laddering (Fig. 7, lane 11). These results suggest that apoptotic-like internucleosomal laddering is not a feature of endospermic cell death.

**Discussion**

**PCD occurred as an asynchronous progression**

Temporal analysis of ultrastructural characteristics of to-
mato lateral endosperm cells undergoing PCD revealed spatially proximal cells at varying stages of the death program, and, at the tissue level, cell death proceeded in an asynchronous manner. Lateral endosperm cells closest to embryonic tissue, presumably the source of the death signal, underwent PCD first when, at the same time, cells 10 layers distant appeared to be healthy, probably owing to the dilution of that signal. The embryo had its strongest effect on the most proximal endosperm cells.

Gietl and Schmid (2001) described features of cell death in the endosperm cells of germinated castor seed. In this progression, PPVs bud from the endoplasmic reticulum, the cytoplasm becomes vacuolate and marginalized, genomic DNA is degraded, and, with the collapse of the central vacuole, PPVs lyse, releasing enzymes that destroy the remaining cytoplasm. PCD in castor seed endosperm progresses in a wave-like fashion; cells most proximal to the cotyledons die first followed by those increasingly distal to cotyledons (Schmid et al. 1999). Similarly, during castor seed development, there is a wave-like progression of nucellar cell PCD with the advancing growth of the cellular endosperm (Greenwood et al. 2005). The cells lying closest to the expanding endosperm show various hallmarks of PCD, including the development and final lysis of PPVs, whereas those further removed from the endosperm do not. Cell death of endosperm and nucellar cells of castor seed (Schmid et al. 1999).
1999; Greenwood et al. 2005) parallels that observed in the lateral endosperm cells of tomato seed, sharing morphological markers of PCD, including the occurrence of PPVs (Figs. 2b–2h). However, in tomato seed, lateral endosperm cell death did not proceed in a precise wave-like fashion. Although cell death in the lateral endosperm begins in the cells most proximal to the embryo, cells in a discrete area of the tissue undergo death, while those in a neighbouring group, irrespective of directionality from the embryo, were more or less advanced in the program (not shown).

Endospermic DNA degradation occurred without laddering
The morphological progression of PCD in tomato seed endosperm was preceded by or was coincident with the degradation of nuclear DNA as detected by TUNEL assays. In intact seeds, nuclei in lateral endosperm cells nearest the embryo were TUNEL positive prior to and after radicle protrusion with a sharp decline in the number of labelled nuclei, while those in a neighbouring group, irrespective of directionality from the embryo, were more or less advanced in the program (not shown).

Morphology implicates lytic compartments in PCD
The lateral endosperm cells of germinated tomato seed exhibited a PCD morphology that is entirely consistent with many of the characteristics previously documented for maize (Gunawardena et al. 2001), castor seed (Schmid et al. 1998; Greenwood et al. 2005), wheat grains (Dominguez et al. 2001, 2004; Gunawardena et al. 2001, 2004; Greenwood et al. 2005). However, apoptotic-like internucleosomal DNA laddering was not a feature of tomato seed endosperm cell death (Fig. 7, lanes 1–9), consistent with the reports of studies on PCD leading to the formation of lace plant and Monstera leaf perforations (Gunawardena et al. 2004, 2005), tracheary element formation (Ito and Fukuda 2002), and barley aleurone layer cell degradation (Fath et al. 1999). Conflicting reports from studies of PCD related to the hypersensitive response to pathogen infection (Mittler and Lam 1995; Ryerson and Heath 1996) suggest that DNA laddering is not an appropriate gauge of plant PCD. Although the quantity of endospermic genomic DNA loaded (Fig. 7, lanes 1–9) might be insufficient to show laddering, if it existed, extraction and electrophoresis were performed multiple times and laddering consistent with internucleosomal fragmentation was never seen. However, if apoptotic-like fragmentation of DNA does occur during the PCD of the lateral endosperm cells in tomato seed, the fragmented DNA may be sufficiently diluted by intact genomic DNA from cells that have yet to undergo PCD that laddering would be undetected. The lack of synchrony of PCD in the cell population might effectively hide the signal (Wang et al. 1996).
Fig. 5. Series of electron micrographs detailing PCD morphology observed in micropylar endosperm cells. Micropylar endosperm cells from (a–c) whole seeds, and embryoless half-seeds incubated in (d–f, k, and l) GA at 50 μmol/L and (g–j) water are shown. (a) Cells at 60 HAI appeared healthy and full of nutrient reserves with electron-dense cell walls (cw) (arrowhead). (b) By 72 HAI, cells were highly vacuolate with electron-translucent cell walls (arrowhead). (c) At 84 HAI, cells had collapsed, displaying some vesiculation (vs), and cell walls were visibly degraded (arrowheads). (d) By 60 HAI, 36 h after the application of GA, protein bodies (pb) appeared mobilized, cell walls were thinner, and the nucleus (n) was misshapen. (e) By 72 HAI, 48 h after the application of GA, cells were highly vacuolate with darkening tonoplasts (open arrowhead) and cell walls appeared electron-translucent (solid arrowhead). (f) At 84 HAI, cells were collapsed and no discernible internal structure remained. (g) Micropylar endosperm cells of embryoless half-seeds incubated in water were healthy at 60 HAI but had collapsed by (h) 72 HAI and (i) 84 HAI. (j) In cells found with discernible internal structure from embryoless half-seeds incubated in water at 72 HAI, the plasma membrane was retracted from the cell wall (arrows). (k) Cells from embryoless half-seeds incubated with GA at 60 HAI contained PPVs (solid arrowheads) and electron-translucent mitochondria (m). Chromatin within the nuclei showed some condensation (open arrowhead). (l) At 72 HAI, cells from embryoless half-seeds incubated in GA displayed highly invaginated nuclei with chromatin condensation (arrowhead). pb, protein body; v, vacuole. Scale bars = 5 μm for Figs. 5a–5j; scale bars = 1 μm for Figs. 5k and 5l.
2001), and in leaf perforation formation (Gunawardena et al. 2004, 2005). In the lateral and micropylar endosperm cells of tomato seed, both vacuolation and vesiculation were widespread (Figs. 2 and 5). Although the mobilization of protein reserves from protein bodies in tissues of germinated seeds naturally leads to vacuolation, the cells of the ephemeral endosperm tissue exhibited additional features consistent with PCD. The cytoplasm became flocculent, nuclei assumed aberrant morphology, the nuclear envelope and tonoplast became electron dense, chromatin condensed to the nuclear envelope, the cytoplasm became increasingly vacuolate, and PPVs were found throughout the cytoplasm (Figs. 2 and 5). Vacuolation and vesiculation are considered an early marker of PCD in barley aleurone protoplasts (Bethke et al. 1999; Fath et al. 1999), suspensor cell death in somatic embryos of Norway spruce (Picea abies (L.) Karst.) (Filonova et al. 2000), tapetum destruction (Wu and Cheung 2000; Ku et al. 2003), aerenchyma formation (Gunawardena et al. 2001), castor seed nucellar destruction (Greenwood et al. 2005), and tracheary element formation (Fukuda 2000). The occurrence of vesiculation and vacuolation in cells from multiple genera suggests a conservation and importance of these events in PCD. Increasing vacuolation of dying endosperm cells (Figs. 2b–2g, 3b, 3d, 3e, 5b–5f, and 5h–5k) may serve to appress the cytoplasm into a restricted volume of the cell for efficient degradation by hydrolytic enzymes and subsequent elimination. Vacuolar collapse precedes organellar degradation during PCD (Fukuda 1996). This, associated with the prevalence of PPVs found in dying endosperm cells of tomato seed (Figs. 2b–2e, 3b, and 5k), among other species (Toyooka et al. 2000; Gietl and Schmid 2001; Rojo et al. 2003; Greenwood et al. 2005), may provide a means by which endopeptidases are released to facilitate the digestion of remaining cytoplasm (see Schmid et al. 2001).

**GA as a signal for PCD**

Observations of lateral endosperm cells following germination of intact seed strongly suggested that reserve mobilization and PCD in these cells were promoted by embryonic cue(s). Lateral endosperm cells of embryoless half-seeds incubated in water failed to show any features consistent with PCD prior to or after the time when the radicle would normally emerge (Figs. 3a, 3c, 3e, and 4g–4i). Exogenous GA substituted for the embryo resulting in cells having characteristics consistent with PCD including vacuolation, cytoplasmic flocculence, retraction of the plasma membrane from the cell wall, the formation of PPVs, and TUNEL-positive nuclei (Figs. 3b, 3d, 3g, and 4d–4f). In postgerminative monocot seeds, GA induces the aleurone cells to synthesize...
Fig. 7. Endospermic DNA degradation did not yield apoptotic-like nucleosomal laddering. M, 1 kb molecular mass marker. DNA extracted from isolated endosperms at 60 HAI (lanes 1–3), 72 HAI (lanes 4–6), and 84 HAI (lanes 7–9) in the order embryoless half-seeds without GA and with GA and whole seeds revealed high molecular mass fragmentation of endospermic DNA irrespective of treatment. DNA from lymphoblastic T-cell DNA incubated at 37 °C (lane 10) and heat-shocked cells (lane 11) revealed typical apoptotic nucleosomal laddering in the latter (arrowheads).

and release hydrolytic enzymes, promotes lipid catabolism, and promotes reserve (Bewley and Black 1994). Prior to GA-induced death, barley aleurone cell protoplasts show morphology consistent with PCD, becoming highly vacuolate and exhibiting nuclear lobing (Bethke et al. 1999; Fath et al. 2001). In wheat aleurone layer cells, GA induces the production of a nuclease during PCD (Domínguez et al. 2004). GA is also known to acidify protein storage vacuoles in aleurone layer cells (Swanson and Jones 1996) and, particularly, promote the expression of the membrane spanning subunit of a vacuolar H⁺-ATPase in micropylar endosperm cells during tomato seed germination (Cooley et al. 1999). The role of GA in inducing enzymes complicit in PCD and in the acidification of the vacuolar compartment following germination is intriguing. GA may serve to induce both hydrolytic enzyme biosynthesis and vacuolar acidification leading to tonoplast rupture and PPV lysis, resulting in the subsequent release of active cytoseine endopeptidases that destroy the remaining cytoplasm.

Micropylar endosperm cells exhibit a different control over PCD

Dying micropylar endosperm cells exhibit ultrastructural features consistent with PCD (Figs. 5b–5f and 5h–5l) as observed in lateral endosperm cells (Figs. 2b–2h, 3b, 3d, and 3f). However, the control over PCD appeared to be different. Cells of the lateral endosperm developed ultrastructural features consistent with PCD only when in the presence of the embryo or exogenously applied GA. Micropylar endosperm cells appeared healthy at 60 HAI but developed features consistent with PCD and died whether in situ in intact seeds (Figs. 5a–5c) or ex situ in embryoless half-seeds incubated in water (Figs. 5g and 5h) or GA (Figs. 5d–5f). GA only hastened the onset of PCD in micropylar endosperm cells (Figs. 5d–5f). Similarly, the degradation of genomic DNA, as detected by TUNEL, proceeded in the micropylar endosperm cells independent of the embryo or exogenous GA (Figs. 6d–6f). These data suggested that the PCD of micropylar endosperm cells was not controlled by the germinating embryo. The micropyral endosperm cells entered the PCD program prior to radicle protrusion, and any embryonic signal that may have been involved in promoting micropyral endosperm PCD must have been delivered prior to 24 HAI, the time by which the embryo was removed from the embryoless half-seeds. It is quite possible that the micropyral endosperm cells are predestined to die prior to imbibition. A dual mode of PCD control has also been described in the placento-chalazal regions of the developing caryopses of maize (Kladnik et al. 2004) where death of the integument cells was senescence related but that occurring in the nucellus was under control of the filial endosperm.

Two models for endosperm PCD

The established effect of GA on vacuolar acidity (Swanson and Jones 1996) and the propensity of vacuolar collapse in numerous cases of plant PCD (Fukuda 2000; Obara et al. 2001; Hatsugai et al. 2004; Greenwood et al. 2005) would create the acid cytosolic environment in which PPVs lyse, releasing resident cytoseine endopeptidases (Schmid et al. 2001; Greenwood et al. 2005). This would allow for digestion and subsequent mobilization of the cytoplasmic constituents following vacuolar collapse, as suggested by Toyooka et al. (2000), as well as deliver hydrolytic PCD-related enzymes (Greenwood et al. 2005).

We propose two models for PCD in lateral (Fig. 8a) and micropyral endosperm cells (Fig. 8b) of tomato seeds that reflect the different cell death programs. It should be stressed that these are models and are put forward to suggest directions for future research. In lateral endosperm cells, the embryo may initiate a signalling cascade, possibly through GA. Abscisic acid, a known antagonist of GA (Bethke et al. 1999; Fath et al. 2001), may serve to keep lateral endosperm cells in a quiescent state until the appropriate PCD initiating signal(s) is received from the embryo (Fig. 8a1). Upon PCD initiation, vacuolation of the cytoplasm and acidification of vacuoles begin. Reactive oxygen species could be produced through the β-oxidation of stored lipids (Fig. 8a2; also see Figs. 2a–2c, 3a, 3c, 3e, 5a, 5g, and 5h; this study; Bewley and Black 1978; Bethke and Jones 2000). GA sensitization of nutritive cells is a known component of reactive oxygen species mediated cell death (Fig. 8a2; also see Fath et al. 2001). As cells continue their progression toward death, multiple, large vacuoles would form in the cytoplasm and could become acidic upon the activation of vacuolar H⁺-ATPases and (or) H⁺-PPases (Fig. 8a3). PPVs are formed. The rupture of the tonoplast would result in a decrease of cytosolic pH (Fig. 8a4), which in turn would be conducive to the lysis of PPVs, releasing cytoseine endopeptidase(s) that destroy the remaining contents of the cytoplasm, leaving cellular corpses (Fig. 8a5).

In contrast, micropyral endosperm PCD is controlled differently (Fig. 8b). Micropyral endosperm cells might receive a signal from the embryo to initiate PCD, but this occurs prior to the start of germination and possibly during seed drying (Fig. 8b1). The cells require no additional embryonic signal to sustain the death program. Since PCD occurs in
micropylar endosperm cells in the absence of the embryo, embryonic abscisic acid would not likely regulate the process. GA, if it has any affect on the micropylar endosperm at all, acts either prior to or very shortly after the beginning of imbibition. Exogenous GA serves only to hasten the micropylar endosperm death program (Fig. 8b2; compare Figs. 5a–5c with 5d–5f and 6a–6c with 6g–6i). Vacular H^+-ATPases and H^+-PPases are most likely activated prior to imbibition, acidifying the vacuo. Upon rupture of the tonoplast, the cytosol would acidify, causing the lysis of PPVs that release active cysteine endopeptidases (Fig. 8b3), mobilizing any remaining cellular remnants (Fig. 8b4).

**Concluding remarks**

The destruction of ephemeral nutritive tissue provides nutrients and structural material for the preautotrophic seedling. This study is the first characterization of PCD in endosperm cells of tomato seed. Our findings are in agreement with those of other systems that suggest that PCD is involved in the degradation of these nutritive tissues (Fath et al. 1999; Schmid et al. 1999; He and Kermode 2003; Greenwood et al. 2005). Our results showing the temporal and spatial events in PCD of both lateral and micropylar endosperm tissues of tomato seeds will serve to put biomolecular studies into context and also provide a basis from which to investigate the fine control of PCD as it relates to post-germinative endosperm cell degradation.

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