In situ detection of programmed cell death in the maize caryopsis

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Abbreviations:
P-C - placento-chalaza
PCD - programmed cell death

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Abstract

Background and Purpose: Programmed cell death (PCD) is one of the major processes in development of multicellular organisms. In this paper we present the use of microscopy methods for the study of PCD in plant tissues.

Materials and Methods: Degradation of nuclei was monitored by a DNA staining fluorochrome DAPI, condensation of chromatin with iodine – crystal violet staining and fragmentation of nuclear DNA with TUNEL reaction, where labeled nucleotides are incorporated into DNA strand breaks. Disruption of cellular integrity was visualized by transmission electron microscopy and endopolyploidy level of cells in the P-C layer was measured with image densitometry using the interphase-peak method.

Results and Conclusions: PCD process in P-C layer starts immediately below endosperm and continues through several cell layers towards vascular tissue in the pedicel. With DAPI staining we detected cells that completed their PCD program and therefore contained no nuclei. The cells immediately below them contained nuclei with completely condensed chromatin. In several layers of cells below the already dead cells, the nuclear DNA was fragmented, as detected with TUNEL reaction. In some TUNEL positive cells we observed apoptotic-like bodies at the periphery of cells. Ultrastructural analyses revealed complete degradation of protoplast, whereas cell walls remained in place. Additionally, cells in the P-C layer enter a special variant of cell cycle, endoreduplication, and reach endopolyploidy level 8 C (1 C equals to DNA content of an unreplicated haploid genome). The microscopy methods described here proved to be highly useful diagnostic tools in the study of PCD in plant tissues.

INTRODUCTION

Programmed cell death (PCD) is a physiological process that leads to the selective elimination of unwanted cells in multicellular organisms. Many developmental processes in animals and plants require removal of cells by PCD (1, 2, 3, 4). In animals, an often used synonym for PCD is apoptosis, which is characterized by a distinct set of morphological features and biochemical reactions (5). On the other hand plant cells and tissues undergo several types of programmed cell death and some of them do not show characteristics of apoptosis (3).

In our previous study we demonstrated that PCD is involved in development of placento-chalazal (P-C) layer in maize caryopsis (6). P-C layer is the maternal tissue just below the basal endosperm cells and above vascular termini in the pedicel (Figure 1). It is believed to play a critical role in transport of water, sugar and other nutrients into devel-
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Figure 1. Maize (Zea mays L.) caryopsis twelve days after pollination, median longitudinal section stained with toluidine blue; em, embryo; en, endosperm; pc, placento-chalazal layer; ped, pedicel; pc; pericarp.

An additional proposed function of the P-C layer is the antimicrobial protection of developing seed with the accumulation of endosperm secreted peptides with antifungal properties (9, 10) and flavonoids (6). In the development of the P-C layer in maize caryopsis, cells degenerate in an orderly manner, layer by layer, starting near endosperm and continue towards vascular termini in pedicel. Our study of this process has shown that the dying of cells has the characteristics of programmed cell death, which occurs in two phases. In the first phase, starting right after the fertilization, cells die rapidly. No fragmentation of DNA, typical for apoptotic types of PCD (11), was detected in nuclei of these cells. However, in the second phase, beginning around eight days after pollination, cell death is slower and intermediary stages of cellular degradation are obvious. Moreover, DNA fragmentation was detected only in this second phase. The result of both phases are cell corpses, where the cell walls remain in place, but the protoplasts are degraded and visible as highly shrunken remains in the cells. The proposed function of the first phase of PCD is the activation of the transport function of the P-C layer, whereas the second phase is thought to be more senescence-related (6). The development of the P-C layer during the first phase of PCD shares several similarities with a formation of tracheary elements (TEs), transport elements of water conducting tissue in plants (12).

Different molecular biology approaches are being used to study PCD in plant tissues. However, we used several microscopy techniques to analyze PCD in the P-C layer of developing maize caryopsis and they proved to be highly useful for the PCD research. We showed distinctive features of PCD by light microscopy using DAPI, iodine – crystal violet and TUNEL staining. Additionally we used image analysis for endopolyploidy measurement and transmission electron microscopy to document disruption of cellular components on ultrastructural level.

MATERIALS AND METHODS

Preparation of plant material for light microscopy

Maize (Zea mays L.) plants of the W22 inbred line were grown in the greenhouse. Carpellate flowers were hand pollinated. Developing caryopses were harvested and immediately fixed in cold FAA fixative (3.7% formaldehyde, 5% acetic acid, 50% ethanol) for 24 hours, followed by dehydration in series of ethanol and tertiary butyl alcohol and embedding in Paraplast Plus (Fisher Scientific). Paraffin embedded caryopses were sectioned to 8–12 micrometers thick sections with a rotary microtome (Microm 325, Carl Zeiss).

DAPI staining

Paraplast embedded sections of developing caryopses were dewaxed in xylene and rehydrated in ethanol series, equilibrated in freshly prepared McIlvaine’s buffer (0.02 M citric acid and 0.16 M Na2HPO4, pH 7.0, mixed before use), followed by staining in 600 nM DAPI (4',6' diamin-2-phenylindole 2 HCl; Molecular Probes) in McIlvaine’s buffer for 15 minutes at room temperature in dark, washed with distilled water, covered with coverslips and observed with UV excitation (365 nm band-pass). Blue fluorescent nuclei were photographed with AxioCam MRc color digital camera (Carl Zeiss Vision).

TUNEL staining

TUNEL (terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling) was performed using In situ Cell Death Detection Kit (Roche Diagnostics), essentially following the manufacturers protocol. Briefly, sections of developing caryopses were dewaxed in xylene and rehydrated in ethanol series, treated with 20 μg/ml Proteinase K (Gibco) in 10 mM Tris pH 7.5, and 5 mM EDTA for 15 minutes at room temperature, followed by incubation in mixture of fluorescein labeled deoxynucleotides and terminal deoxynucleotidyl transferase for 60 minutes at 37°C. After washing the slides with PBS, the coverslips were mounted in aqueous mounting media GelMount (Sigma-Aldrich) containing 600 nM DAPI. The green fluorescein fluorescence of nuclei with fragmented DNA was observed with a microscope with blue light excitation (450–490 nm band-pass), and DAPI fluorescence of all nuclei was observed with UV excitation. Images were taken with AxioCam MRc color digital camera (Carl Zeiss Vision). In addition we found that the omission of Proteinase K step does not affect the TUNEL staining effectiveness, so it was not used in later
experiments. Note that the optimal staining procedure has to be adapted for each tissue and fixation type separately (13).

**Iodine — crystal violet staining**

Staining was performed as described by Jensen (14). Briefly, hydrated sections of developing caryopses were stained with 1% aqueous crystal violet for 15 minutes, rinsed in distilled water, placed in 1% iodine in 2% KI, rinsed in water, dehydrated quickly in 70%, 96% and absolute ethanol, cleared in xylene and mounted with Permount (Electron Microscopy Sciences).

**Transmission electron microscopy**

All materials for electron microscopy processing were purchased from Electron Microscopy Sciences. Tissues were prepared for transmission electron microscopy by fixation in 4% glutaraldehyde (v/v) and 1% paraformaldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2). Fresh caryopses were harvested and immediately processed for fixation on site by placing a small aliquot of chilled fixative on a glass plate and hand sectioning a sagittal section of the caryopsis approximately 2 mm wide with a double edged razor blade. The tissue was then put into a vial of ice cold fixative and placed under vacuum for 5 to 10 minutes to aid fixative infiltration. After vacuuming the samples were placed on a rotating plate overnight, approximately 12 hours at 4°C. After fixation the tissue was rinsed 3 times for 30 minutes each in chilled phosphate buffered saline (PBS), then placed in 2% aqueous osmium tetroxide overnight at 4°C. Once the osmication was completed the samples were rinsed 3 times for one hour each in distilled water, then dehydrated in a chilled acetone series at 20% increments for 1 hour each. After 100% acetone the samples were passed through propylene oxide as a transition solvent, infiltrated and embedded with Spurr’s resin, then polymerized at 56°C for 24 hours. Samples were sectioned on a Sorvall JIB Ultracut ultramicrotome. The sections were cut to approximately 60 nm thickness and picked up on formvar coated copper grids (50 or 100 mesh). The sections were post stained 20 minutes in filtered 2% aqueous uranyl acetate, rinsed 3 times for 1 minute each in distilled water, then were allowed to dry. The sections were then stained 6 minutes in Reynold’s lead citrate, rinsed once in 0.02 M NaOH for 1 minute, then three times for 1 minute each in distilled water. The sections were examined and photographed in a Zeiss 109 or a Zeiss 110 transmission electron microscope.

**Measurement of nuclear DNA amount**

Nuclear DNA amount was measured with image densitometry using the interphase-peak method (15, 16) adapted for use with tissue sections (17, 18). Caryopsis longitudinal sections were dewaxed in xylene, rehydrated through an ethanol series to water, hydrolyzed in 5 N HCl for 75 min at 20°C, stained with the Feulgen reagent for 120 min at 20°C, washed for 45 min in several changes of 0.5% potassium metabisulphite in 50 mM HCl, dehydrated and mounted in DPX (Fisons Scientific Equipment). Integrated optical density (IOD) and coordinates of the nuclei were measured on a series of grayscale images of caryopsis recorded with a 40 X objective. IOD is linearly related to DNA amount and was used to estimate the relative amount of DNA in individual nuclei. The nuclear DNA amount was expressed in C-value units, where 1 C represented the nuclear DNA content of a non-replicated haploid genome.

**RESULTS AND DISCUSSION**

**DAPI staining**

Fluorochrome DAPI (4’, 6’ diamino-2-phenylindole · 2 HCl) binds in the minor groove of double-stranded DNA, preferentially to AT regions and emits strong fluorescence when bound (19), thus DAPI is generally used for specific staining of nuclei. In our study of development of maize caryopsis DAPI was used for rapid monitoring of nuclei degradation, as one of the events during the PCD (6). In sections stained with DAPI we distinguished between cells with nuclei and cells that completed the PCD program and have therefore no nucleus. Several layers of cells without nuclei were seen in the P-C layer of maize caryopsis twelve days after pollination (Figure 2). The number of such layers increased gradually from fourth day after pollination, reaching a maximum of 20–25 cell layers around 24th day after pollination (6). With DAPI staining we observed only the final result of the PCD program, so we used additional microscopy techniques to better describe the PCD process.

**Iodine — crystal violet staining**

Iodine — crystal violet stains condensed chromatin purple, while cytoplasm remains crystal clear. This method is usually used to stain plant chromosomes (14), but we found that it is also useful to identify the extent of chromatin condensation in interphase nuclei. The con-

Figure 2. Median longitudinal sections of maize caryopsis twelve days after pollination showing the loss of nuclei in cells of placento-chalazal layer. Nuclei are stained with DAPI and cell walls are visible due to their autofluorescence when excited with UV illumination (negative image shown); en, endosperm; pc, placento-chalazal layer; ped, pedicel.
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Figure 3. Median longitudinal section of maize caryopsis ten days after pollination, stained with iodine - crystal violet. Complete condensation of chromatin is visible in nuclei of cells just beneath the cells that are already without nuclei (arrow). The nuclei below them show only partially condensed chromatin (arrowheads); en, endosperm; pc, placenta-chalazal layer; ped, pedicel.

Figure 4. Median longitudinal section of maize caryopsis twelve days after pollination, stained with TUNEL reaction. Nuclei with fragmented DNA show stronger green fluorescence and other cellular contents are visible due to their autofluorescence. In some cells the TUNEL positive fragments appear as apoptotic-like bodies at the cell’s perimeter (arrow); en, endosperm; pc, placenta-chalazal layer; ped, pedicel.

densation of chromatin is a characteristic of apoptotic-like cell death (20). Figure 3 depicts a clear progression of changes in nuclear morphology: small nuclei with completely condensed chromatin were readily detectable underneath the cells lacking nuclei (completed PCD); whereas, nuclei in cells proximal to pedicel showed larger nuclei with only partially condensed chromatin.

TUNEL staining
To determine whether the death of P-C cells was apoptotic in its nature, we used TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) reaction on the tissue sections. In the TUNEL reaction the enzyme terminal deoxynucleotidyl transferase is used to incorporate labeled nucleotides into DNA strand breaks with free 3'-OH ends (21). TUNEL reaction combines molecular and anatomical approach in PCD study. With analysis of extracted DNA the positional information about PCD processes in the tissue is lost, whereas TUNEL reaction identifies exactly the cells undergoing PCD in the tissue. Moreover, if the PCD process occurs in only few cells at a time, the DNA fragments would be present in a relatively low concentration when compared to DNA from whole tissue. It is likely that such fragments are undetectable by gel electrophoresis, which is a common method for showing DNA laddering due to DNA fragmentation (20). Since the tissue of interest represented only a few layers of cells in the maize caryopsis, genomic DNA was not checked for laddering in the research of P-C layer, but we relied more to the results of TUNEL reaction (6). Nuclei that contained fragmented DNA, showed stronger fluorescence due to incorporation of fluorescein labeled nucleotides (Figure 4). In the P-C layer twelve days after pollination there were several layers of cells containing nuclei with fragmented DNA, just underneath the cells that were already enucleated. In some instances, the TUNEL-staining pattern appeared as foci concentrated around the periphery of cell (arrow in Figure 4). It is possible that such pattern of staining is due to the membrane-bound apoptotic-like bodies containing fragmented DNA (6) as observed in animal cells undergoing apoptosis (22).

Transmission electron microscopy
The disruption of cellular integrity was most accurately observed by transmission electron microscopy. Ultrastructural analysis of successive P-C layers of caryopsis nine days after pollination showed a progression of cellular change during development (Figure 5). The final stage of PCD was seen in P-C cells directly underneath the basal endosperm. The protoplasts completely degraded and only their shrunken remains were seen within the cell walls that stayed in place in more or less the same shape as before PCD (Figure 5 A). The next layer (distal to basal endosperm cells) was comprised of cells with dense cytoplasm but no discernible nucleus and vacuole, whereas, organelles and large vacuoles were clearly seen in cells further away from the endosperm (Figure 5 B and C, respectively). The vacuole in the upper part of Figure 5 C showed signs of disruption. Moreover, condensation of nuclei, degradation of all organelles and formation of apoptotic-like bodies were also observed during PCD in the P-C layer (6).

Image densitometry by the interphase-peak method
Several recent papers (18, 23, 24) demonstrated the application of image densitometry for measurement of nuclear DNA amount and the estimation of endopolyploidy level. An endopolyploidy level of up to 64 C, resulting from endoreduplication (doubling of DNA without mitosis), has been reported during development of tracheary elements in water conducting tissue of xylem.
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Figure 5. Transmission electron micrographs of placento-chalazal layer in maize caryopsis nine days after pollination, showing spatial progression of programmed cell death in the successive layers. Progressive degradation of protoplasts starts in cells directly underneath the basal endosperm (A) and continues towards cells close to the vascular termini in pedicel (C); en, cytoplasmic remains; cw, cell wall; v, vacuole.

Possible pitfalls of microscopy techniques for PCD detection

Although all applied microscopy techniques in our studies of PCD proved to be reliable and accurate for PCD determination, their drawback may be the use of fixed plant material, since fixation procedure might alter the examined tissue. The advance of imaging techniques could overcome this disadvantage and provide opportunities to monitor processes in living plant cells (28, 29). Although the majority of these assays were developed for human cells, many procedures can also be used on plant tissues. The only method we applied to test viability of cells in living tissue was Evans blue staining, which has allowed detection of PCD progression in maize endosperm (30). Evans Blue dye is excluded from living cells with intact plasma membrane, thus only the cytoplasm of nonviable cells is stained with this dye. However, in our study it was not possible to distinguish among viable and unviable P-C layer cells in fresh hand-cut sections of maize caryopsis stained with Evans blue (data not shown).

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REFERENCES


