

Programmed cell death or desiccation tolerance: two possible routes for wheat endosperm cells

Elena A. Golovina[1,2*], Folkert A. Hoekstra[2] and Adriaan C. van Aelst[3]

[1]Timiryazev Institute of Plant Physiology, Botanicheskaya 35, Moscow, 127276, Russia; [2]Laboratory of Plant Physiology, Department of Plant Sciences, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, the Netherlands; [3]Laboratory of Experimental Plant Morphology and Cell Biology, Department of Plant Sciences, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, the Netherlands

Abstract

The fate of cells in the endosperm of developing wheat kernels was investigated under normal conditions and upon premature slow drying on the cut ear. To follow the changes in membrane integrity and cellular ultrastructure, an electron spin resonance (ESR) spin probe technique and low temperature scanning electron microscopy (LTSEM) were used. ESR data indicated that during development, the relative amount of cells with intact membranes decreased and became almost zero at the stage of mass maturity, i.e. several days before the onset of maturation drying. This suggests that the death of starchy endosperm cells is a developmental phenomenon rather than one induced by water loss. Even at 8 days after anthesis (daa), early in the differentiation phase, a considerable proportion of endosperm cells had already lost plasma membrane integrity. Comparison of ESR data with LTSEM micrographs revealed that the loss of plasma membrane integrity occurred early in the starch accumulation process, from which it was concluded that the programmes of starch accumulation and developmental death are simultaneously switched on. When the differentiation into starchy endosperm was arrested by premature slow drying of kernels on the cut ear, meristematic cells (aleurone initials) acquired desiccation tolerance as assessed by the combination of the spin probe technique and LTSEM. In contrast, fast drying caused immediate death of these cells. Thus, meristematic endosperm cells have the competence to acquire desiccation tolerance. This occurs upon premature slow drying or during normal differentiation into aleurone cells, but is lost when the cells differentiate into starchy endosperm.

Keywords: Desiccation tolerance, development, endosperm, programmed cell death, starch accumulation, *Triticum aestivum*

*Correspondence
Fax: +31–317–484740
Email: elena.golovina@guest.pf.wau.nl

Introduction

The development of wheat endosperm was described in detail at the beginning of the last century (Brenchley, 1909; Percival, 1921), and electron microscopy studies have provided new information (Buttrose, 1963; Evers, 1970; Bennett *et al*., 1973; Mares *et al*., 1975, 1977; Morrison *et al*., 1975; Morrison and O'Brien, 1976). The basic mode of cereal endosperm development seems highly conserved and can be divided into four main stages – syncytial, cellularization, differentiation, and maturation (Olsen *et al*., 1992). After fertilization of the two polar nuclei of the central cell by the second male gamete, nuclear divisions without cell formation result in a multinucleate endosperm syncytium, undergoing subsequent cellularization. In wheat kernels, these processes are completed at about 5 days after anthesis (daa) (Bennett *et al*., 1973; Mares *et al*., 1975; Batygina, 1987). After cellularization there is a brief period of mitotic activity in the central region of the endosperm and a more prolonged period of meristematic activity in the peripheral layers (Evers, 1970). At the end of the differentiation stage, aleurone layer cells and starchy endosperm cells are formed. Accumulation of

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storage substances commences at the differentiation stage and continues during the maturation stage. It was supposed that at the end of the maturation stage the starchy endosperm cells die, whereas the aleurone layer cells remain alive and survive dehydration (Olsen *et al.*, 1992).

For a long time it was believed that starchy endosperm cells die because of the filling with starch granules followed by water loss (Brenchley, 1909; Percival, 1921; Simmonds and O'Brien, 1981). With the advent of molecular genetic techniques and the availability of different development-defective mutant seeds, a renewed interest in endosperm development arose (Lopes and Larkins, 1993). Recently, it has been shown that starchy endosperm cells of maize undergo programmed cell death during development (Young *et al.*, 1997). Since then, the idea of developmental death of cereal endosperm cells has gained support (Buckner *et al.*, 1998; Olsen *et al.*, 1999). Berger (1999) even defined death as the fourth stage in endosperm development instead of maturation. Programmed cell death of endosperm cells can therefore be considered an integral part of seed development. Other seed tissues may also undergo programmed cell death, such as the suspensor (Marsden and Meinke, 1985; Schwartz *et al.*, 1994) and seed coat cells (Colombo *et al.*, 1997). Aleurone layer cells undergo programmed death much later, a few days after germination (Kuo *et al.*, 1996; Wang *et al.*, 1996; Bethke *et al.*, 1999).

The molecular genetics approach has proven to be very successful in understanding the regulation of seed development. However, direct information about cell functioning still remains valuable. Information about membrane integrity as the main marker of cellular viability can shed additional light on programmed death and the acquisition of cellular desiccation tolerance during endosperm development.

Although cereal endosperm is a simple tissue, consisting of only two main cell types (aleurone layer and starchy endosperm cells), it is rather difficult to study their fate during development. Because of problems with tissue fixation, only the early stages of endosperm development (cellularization and onset of differentiation) have been studied by electron microscopy (Simmonds and O'Brien, 1981). Thus, there is no clear ultrastructural characterization of how starchy endosperm cells die during development. The only attempt previously made to follow the progress of starchy endosperm death was for maize, using the vital stain, Evans blue, a dye which is excluded from living cells by an intact plasmalemma (Young *et al.*, 1997).

Here we present the results of a study into the differential fates of the different endosperm cells during wheat kernel development, obtained by applying electron spin resonance (ESR) spectroscopy and low temperature scanning electron microscopy (LTSEM). To study cell membrane integrity we have developed a sensitive ESR spin probe technique (Golovina *et al.*, 1997, 1998, unpublished results), by which direct information about cell membrane intactness can be obtained even when the ratio of living cells to nonviable cells is very low. This has a particular advantage when investigating developmental cell death and acquisition of desiccation tolerance in developing cereal endosperm. The advantage of LTSEM is that only cryofixation is required, which gives unique possibilities to examine developmental stages of seeds without changing the water content (Yaklich *et al.*, 1994, 1996). One objective of this study was to follow changes in membrane integrity and the related changes in cellular structure during normal endosperm development. Another objective was to elucidate the potential ability of non-differentiated endosperm cells to acquire desiccation tolerance when normal endosperm differentiation is arrested by premature drying.

Materials and methods

Plant material

Plants of spring wheat (*Triticum aestivum* cv Priokskaya) were grown in pots under greenhouse conditions (16 h light/8 h dark and an average day temperature of 22°C). Ears were tagged at the beginning of anthesis. Kernels were collected every day from 4 to 36 days after anthesis (daa) and studied immediately. Alternatively, plants were cut at different times after anthesis and left to dry at approx. 23°C and 30% relative humidity for 1 month reaching a moisture content (MC) of approximately 9–10% on a fresh weight (FW) basis. Dry kernels were removed from the ear and allowed to imbibe on filter paper in Petri dishes at 23°C until fully hydrated. Water content was analyzed by weighing samples before and after heating at 96°C for 36–48 h.

ESR spin probe study

Membrane integrity was measured by an ESR spin probe technique, particularly suitable for small samples (Golovina *et al.*, 1997, 1998, unpublished results). Fresh or rehydrated kernels were cut longitudinally. The embryo and outer pericarp were discarded. A small piece of starchy endosperm from one kernel (about 15 mm^3) was carefully excised from the kernel and immediately loaded into a capillary (2 mm diameter) for ESR measurements. The same procedure was followed for the remainder of the tissues consisting of inner pericarp, seed coat, and peripheral layers of the endosperm attached to the

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seed coat. Then 2–3 µl of a 1 mM solution of the stable free radical 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPONE; Sigma, St Louis, MO, USA), containing 120 mM of the broadening agent $K_3Fe(CN)_6$ was added to the sample in the capillary. After 5 min of incubation, ESR spectra were recorded at room temperature with an X-band ESR spectrometer (Bruker, Rheinstetten, Germany, model 300E). Microwave power was 2 mW, and the modulation amplitude was 1 G (conditions that exclude saturation and overmodulation of ESR spectra). One to four scans were accumulated depending on signal intensity. Every scan took slightly more than 1 min.

Principle of the ESR spin probe technique used to determine cellular membrane integrity

The spectrum of 1 mM TEMPONE in water contains three equidistant narrow resonance lines. The addition of K^+-ferricyanide to a final concentration of 120 mM causes broadening of the spectrum to apparent invisibility because of paramagnetic interactions (Eaton and Eaton, 1978). Placing a sample containing cells with intact membranes in this broadened solution of TEMPONE causes the reappearance of the aqueous ESR signal due to the differential permeability of the plasma membranes to TEMPONE molecules and ferricyanide ions. TEMPONE easily penetrates cells because of its amphiphilic nature and relatively small size. In contrast, the charged $Fe(CN)_6^{-3}$ ions cannot pass through intact plasma membranes (Keith and Snipes, 1974; Morse, 1977; Berg and Nesbitt, 1979). Thus, such a spectrum has an intracellular origin (aqueous cytoplasm), whereas the signal from TEMPONE located outside cells is still broadened by ferricyanide ions. In the case of plasma membrane disruption, ferricyanide ions can penetrate cells and broaden the aqueous cytoplasmic signal. Nevertheless, if cellular membranes are disrupted, there still can be some areas in the cells such as oil bodies and polymers, which are not accessible to ferricyanide ions. The ESR spectra of TEMPONE molecules located in such surroundings are not broadened by ferricyanide and can be observed even in dead tissue (Golovina *et al.*, 1997). However, these spectra have a different shape and can be easily distinguished from spectra originating from the cytoplasm of living cells. Thus, the presence and relative amount of cells with intact plasma membranes in a sample also containing cells with disrupted plasma membranes can be determined by ESR in TEMPONE–ferricyanide solution. The sensitivity of the method allows detection of a few living cells in a sample in which most cells are nonviable.

Multi-component spectra originating from TEMPONE molecules at different locations in a sample can be decomposed to properly estimate each individual component. Decomposing was carried out by spectral titration, i.e. subtraction after adjusting for position and amplitude of the peaks (Berliner, 1976). To be able to compare the intensities of the spectral components obtained from different samples, a normalizing procedure on the basis of MC or dry matter was applied. This procedure enabled the comparison of spectra obtained from different samples (see Results section).

LTSEM

LTSEM (JEOL, model 6300 F, Tokyo, Japan) was used to examine the material. Fresh or rehydrated kernels were mounted on stubs with conductive carbon cement (Leit-C, Neubauer Chemikalien, Münster, Germany) in a position suitable for cross-sectioning. The stubs were placed on a specimen holder and subsequently frozen in liquid nitrogen (–196°C). The frozen samples were transferred into a cryo-transfer unit (CT 1500 HF, Oxford Instruments, Oxon, UK). This unit consisted of a cryo-preparation chamber at high vacuum (10^{-6} Pa) attached to the LTSEM and a cryo-stage inside the microscope. The specimen was placed inside the cryo-chamber at –85°C, kept there for 2 min to sublimate the contaminating water vapour, and was then cross-sectioned with a cold sharp knife. Finally the sample was sputter-coated with 3 nm platinum. The coated specimen was placed inside the LTSEM and observed at 1–5 kV. The temperature of the specimen inside the LTSEM was kept at –180°C.

Results

Time-table of wheat kernel development

To characterize the different stages of wheat kernel development with respect to the growth conditions of the particular variety under study, a time-table was constructed on the basis of the kernel DWs and FWs with the staging according to Rogers and Quatrano (1983) (Fig. 1). Because endosperm development precedes embryo development to a certain extent (Bennett *et al.*, 1973; Batygina, 1987) and because most of the dry matter in mature wheat kernels is accumulated by endosperm tissue, the time-table mainly refers to the changes in weight and MC of the endosperm. The amount of water per kernel increased up to 14 daa, which is caused by kernel growth (endosperm cell division and enlargement) (Fig. 1A). The end of growth is caused by the differentiation of peripheral meristematic cells into aleurone layer cells (Evers, 1970). The increase in dry matter continued up to 25 daa (Fig. 1B), when the stage of mass maturity was achieved. Between 14 and 25 daa there was a slight water loss, which might be

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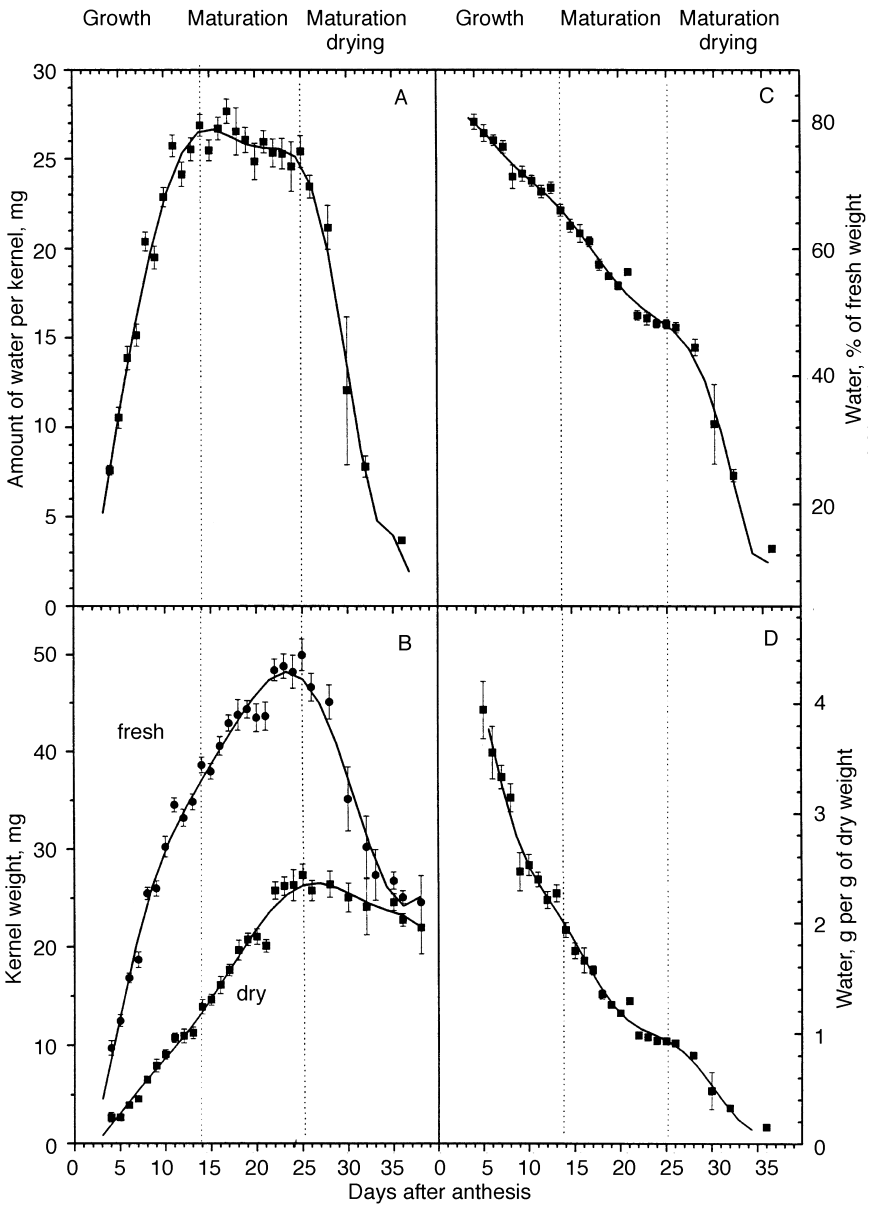


Figure 1. Time-table of wheat kernel development. The following changes of the main characteristics during wheat kernel development are given: (A) weight of water (mg) per kernel; (B) fresh weight (FW) and air-dry weight (DW); (C) moisture content (% of FW); (D) moisture content (g water/g DW). SE values are indicated when they exceed the symbol size (n=6–20). Staging is according to Rogers and Quatrano (1983).

caused by the substitution of aqueous vacuoles by plastids. Maturation drying, as characterized by rapid water loss, began after 25 daa (Figs 1A–D). Because the cellular water potential remains constant up to maturation drying (Barlow *et al.*, 1980), the gradual decrease of MC before 25 daa (Figs 1C and D) is driven by dry weight (DW) accumulation.

Membrane integrity in mature endosperm

The endosperm in the mature wheat kernel consists of two major tissues, the nonviable starchy endosperm and the viable aleurone layer. To study the fate of endosperm cells during normal development and during premature drying, we first applied the spin probe technique to the mature kernel tissues, as a reference.

Figure 2A shows an ESR spectrum of TEMPONE from the starchy endosperm of a mature kernel in the presence of ferricyanide. Three broadened lines of different height were observed. Similar spectral shapes were observed for the major endosperm

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components, gluten (Fig. 2B) and starch (Fig. 2C). This suggests that these storage polymers are responsible for the shape of the typical endosperm spectrum. Such spectra can be observed in the presence of ferricyanide since TEMPONE molecules in the vicinity of polymers are not accessible to collisions with the ferricyanide ions. The splitting factor of the spectra (distance between lines) was approximately 14–15 G, indicative of a relatively hydrophobic environment for the spin probe. This may be caused by TEMPONE molecules located in the vicinity of so-called starch lipids (less than 2% of DW), consisting of lysophospholipids (89%), and nonpolar lipids and glycolipids (10–12%) (Morrison, 1978). These TEMPONE molecules escaped from the interaction with ferricyanide because of the inaccessibility of lipid to this polar reagent. The different heights of the lines and some line broadening suggest that the motion of TEMPONE molecules is restricted and anisotropic (Berliner, 1976).

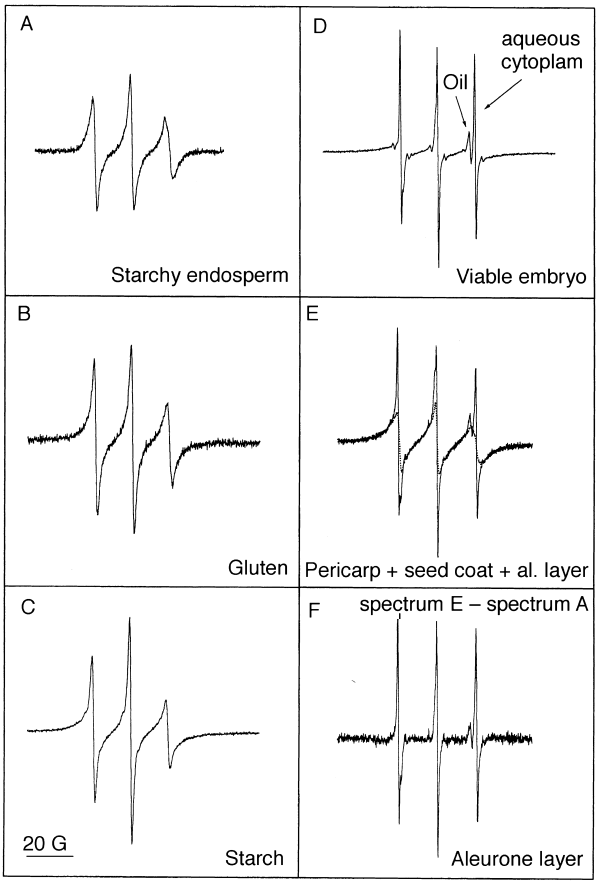


Figure 2. ESR spectra of TEMPONE in the presence of ferricyanide from wheat kernel tissues and related polymers: (A) starchy endosperm from mature kernel; (B) wheat gluten; (C) wheat starch; (D) viable mature embryo; (E) outer tissues of mature kernel (pericarp + seed coat + aleurone layer + remainders of starchy endosperm); (F) aleurone cells. Spectrum F was obtained by subtraction of spectrum A from spectrum E after spectral titration.

For comparison, a spectrum from a viable mature embryo is shown in Fig. 2D. In contrast to the spectrum from mature endosperm (Fig. 2A), that from the viable embryo was composed of two clearly distinguishable components that were well resolved in the high-field (right side) region of the spectrum. The components originate from TEMPONE located in oil bodies (splitting factor 14 G) and aqueous cytoplasm (splitting factor 17 G) of viable cells with intact membranes (Golovina *et al.*, 1997, 1998). Dead embryos with disrupted membranes generally lack the aqueous component (Golovina *et al.*, 1997).

The absence of the narrow cytoplasmic component in the spectrum from mature starchy endosperm (Fig. 2A) can be considered the result of plasma membrane disruption. No oil component was observed in the spectrum, which is in agreement with the reported absence of noticeable amounts of oil bodies in wheat starchy endosperm (Morrison, 1978).

Figure 2E shows the ESR spectrum of TEMPONE from a 24-h imbibed, mature wheat kernel after removal of starchy endosperm and embryo. The sample contained inner pericarp, seed coat, aleurone layer and some remainders of the starchy endosperm. All these tissues except the aleurone layer are known to be nonviable in mature wheat kernels and are characterized by the same broad ESR spectra (spectra not shown) as the starchy endosperm depicted in Fig. 2A. Thus, the broad component of this ESR spectrum has to originate from TEMPONE located in these dead tissues. The spectrum obtained after subtraction of the broad component (Fig. 2F) has two narrow components – one originating from TEMPONE located in lipid bodies, and the other from TEMPONE in the aqueous cytoplasm of cells with intact membranes, through which ferricyanide could not pass. Because the aleurone layer is the only viable tissue in such a sample, we attribute this spectrum to TEMPONE located in the cytoplasm and oil bodies of aleurone cells.

Membrane integrity in developing endosperm cells

Cellular membrane integrity was studied in developing endosperm during differentiation and maturation. Figures 3A–D show ESR spectra of TEMPONE from starchy endosperm excised from fresh kernels at 8, 14, 18 and 25 daa. These spectra differ from the spectrum of TEMPONE from mature endosperm in that they contain a narrow component in addition to a broad component. The origin of this broad component is likely the same as that in the mature endosperm (Fig. 2A), i.e. storage polymers. To obtain the narrow component, the broad component has to be subtracted from the original spectrum. For that purpose the spectrum from the mature

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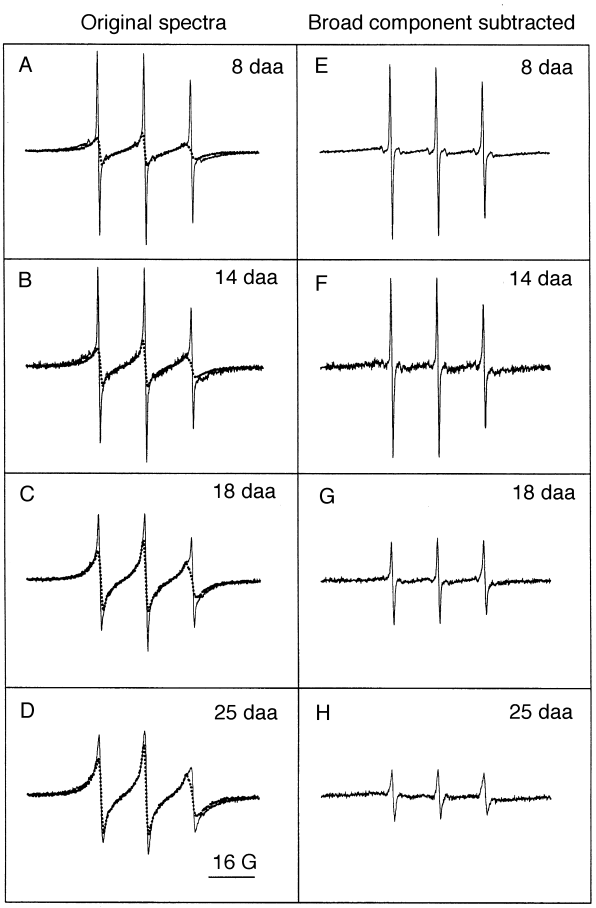


Figure 3. Representative ESR spectra of TEMPONE (in the presence of ferricyanide) from developing wheat starchy endosperm: (A) 8 daa; (B) 14 daa; (C) 18 daa; (D) 25 daa. To obtain the aqueous cytoplasmic component for living cells from these spectra, the broad component (from Fig. 1A) (dashed line) was subtracted from the original spectra after adjustment for amplitude and peak position. The resulting spectra of the aqueous cytoplasm are shown in (E–H) for 8, 14, 18, and 25 daa, respectively. Spectra (A–D) are normalized to the dry matter of the kernel at each stage of development.

endosperm (Fig. 2A) was used after fitting for amplitude (see dashed lines in Figs 3A–D). Figures 3E–H show the narrow components obtained after the subtraction. The spectra in Figs 3A–D were plotted in such a way that the amplitude of the broad component correlated with the DW of the kernel. Therefore, the isotropic spectra (Figs 3E–H) obtained after the subtraction of the broad component reflect the total cytoplasmic volume enclosed by intact plasma membranes in the whole starchy endosperm.

The data in Figs 3E–H can be interpreted in two different ways. On the one hand, the gradual decrease of the aqueous cytoplasmic volume might have resulted from the filling of the cells with proteins and starch. On the other hand, the reduced aqueous volume might be the result of a progressive membrane disruption that permitted penetration of ferricyanide ions into the cytoplasm, followed by quenching of the spectrum. To investigate both possibilities, ESR spectra of TEMPONE were recorded in mature (dead) starchy endosperm being rehydrated to MCs in the range 70–20% (FW basis), in the absence of ferricyanide and in the presence of ferricyanide. The appropriate amounts of solution (1 mM TEMPONE, or 1 mM TEMPONE/120 mM ferricyanide) were added to pieces of air-dry mature endosperm loaded in capillaries which, after sealing, were allowed to

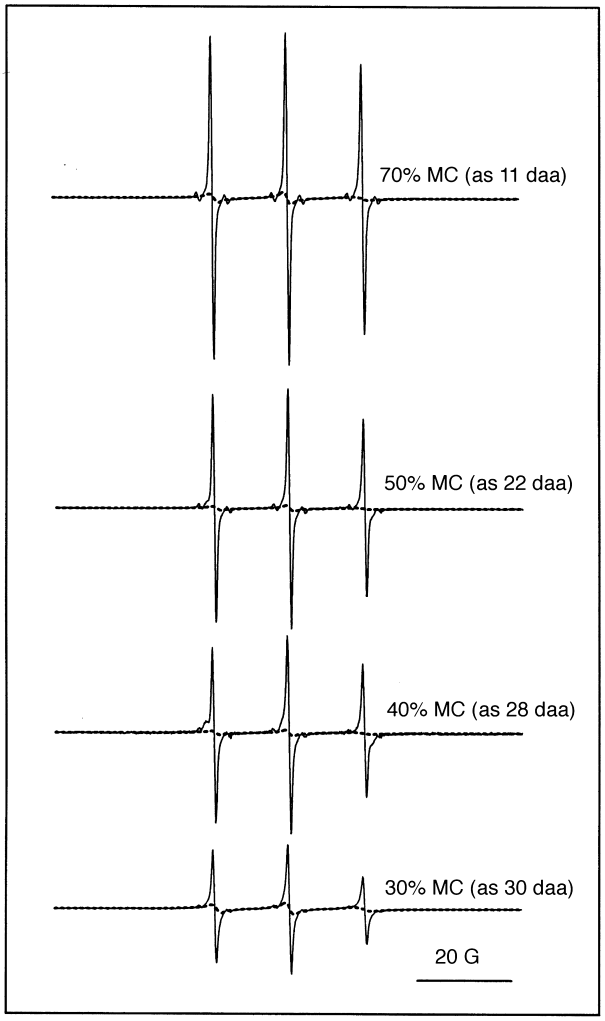


Figure 4. ESR spectra of TEMPONE in the absence of ferricyanide from mature starchy endosperm rehydrated to different MCs (equilibrated for 24 h). Spectra were normalized to MC. The dashed spectra are those of the same samples in the presence of ferricyanide adjusted for spectral position and amplitude of the shoulders. The indicated developmental periods (daa) were derived from the MC–developmental period relationship presented in Fig. 1C as if the spectra were derived from developing endosperm.

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equilibrate for 24 h. The use of TEMPONE without ferricyanide mimics the situation in which all starchy endosperm cells are alive. Figure 4 shows how the ESR spectra of TEMPONE in the endosperm would change during the accumulation of storage substances and maturation drying if all endosperm cells remained alive. The spectra were normalized to the relative MC of the samples. To be able to compare these spectra with spectra from developing fresh endosperm such as those in Figs 3A–D, the corresponding developmental periods were derived from the MC–developmental period relationship presented in Fig. 1C. Thus, assuming retention of membrane integrity, the deposition of storage substances in the endosperm could not have changed the shape of the spectra as dramatically as was observed for the developing starch endosperm cells of comparable MC (cf. Figs 3A–D and Fig. 4).

The ESR spectra of TEMPONE in the presence of ferricyanide in mature endosperm having MCs in the range 70–20% had the same shape as in Fig. 2A (spectra not shown). Since the spectral shape did not change with the decrease in MC to 20%, the mobility of TEMPONE molecules in the vicinity of polymers is not affected by a decrease in MC. In the spectra from endosperm without ferricyanide (Fig. 4), the broad component associated with the presence of biopolymers also had to be present, but was largely masked by the considerable narrow component (see dashed spectra in Fig. 4).

The spectra of TEMPONE in starchy endosperm at different stages of development (Figs 3A–D) con-

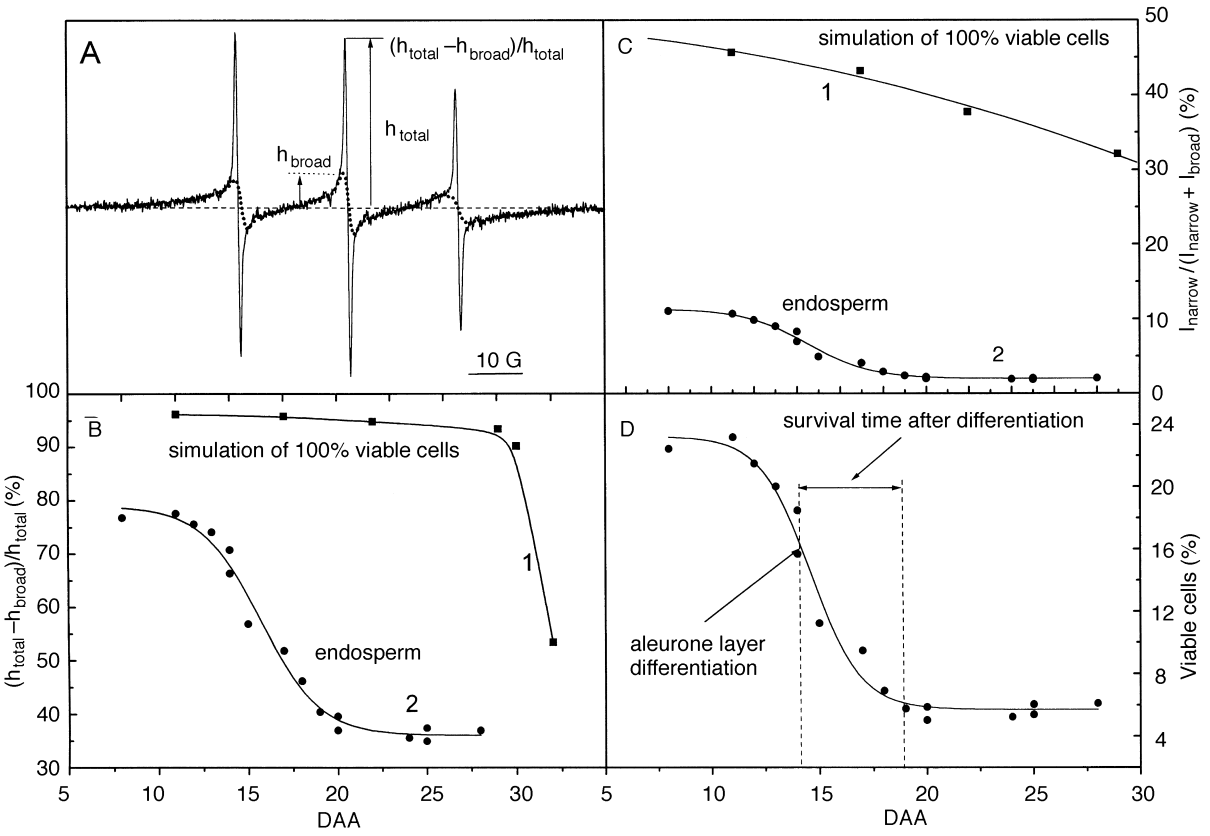


Figure 5. Calculation of the proportion of cells with intact membranes in developing wheat endosperm. (A) Example of an ESR spectrum of TEMPONE from fresh starchy endosperm (13 daa; solid line) and from the adjusted broad component (from mature endosperm in TEMPONE/ferricyanide solution; dashed line). The total amplitude (h_{total}) and the amplitude of the broad component (h_{broad}) were determined as indicated. The proportion of the amplitude of the narrow component was calculated as $(h_{total}–h_{broad})/h_{total}$. (B) This proportion was plotted against daa for samples simulating 100% cells with intact membranes (mature endosperm rehydrated in TEMPONE solution without ferricyanide to different MCs) (1), and samples of developing starchy endosperm (2). (C) After conversion of amplitude (h) into integral intensity (I) with conversion factors determined for both types of spectra ($I_{narrow} = 2.94 \times h_{narrow}$ and $I_{broad} = 87.6 \times h_{broad}$), the proportion of the amount of TEMPONE molecules was plotted for (1) and (2) as in (B). (D) Viable cells in developing starchy endosperm calculated by expressing the data of curve 2 (in C) as a percentage of the data in curve 1 (in C).

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tained both narrow and broad components. The reason why the broad component in these spectra was visible stems from the much greater reduction of the narrow aqueous component than could be expected if all endosperm cells had been alive (Fig. 4).

Proportion of cells with intact plasma membranes in developing endosperm

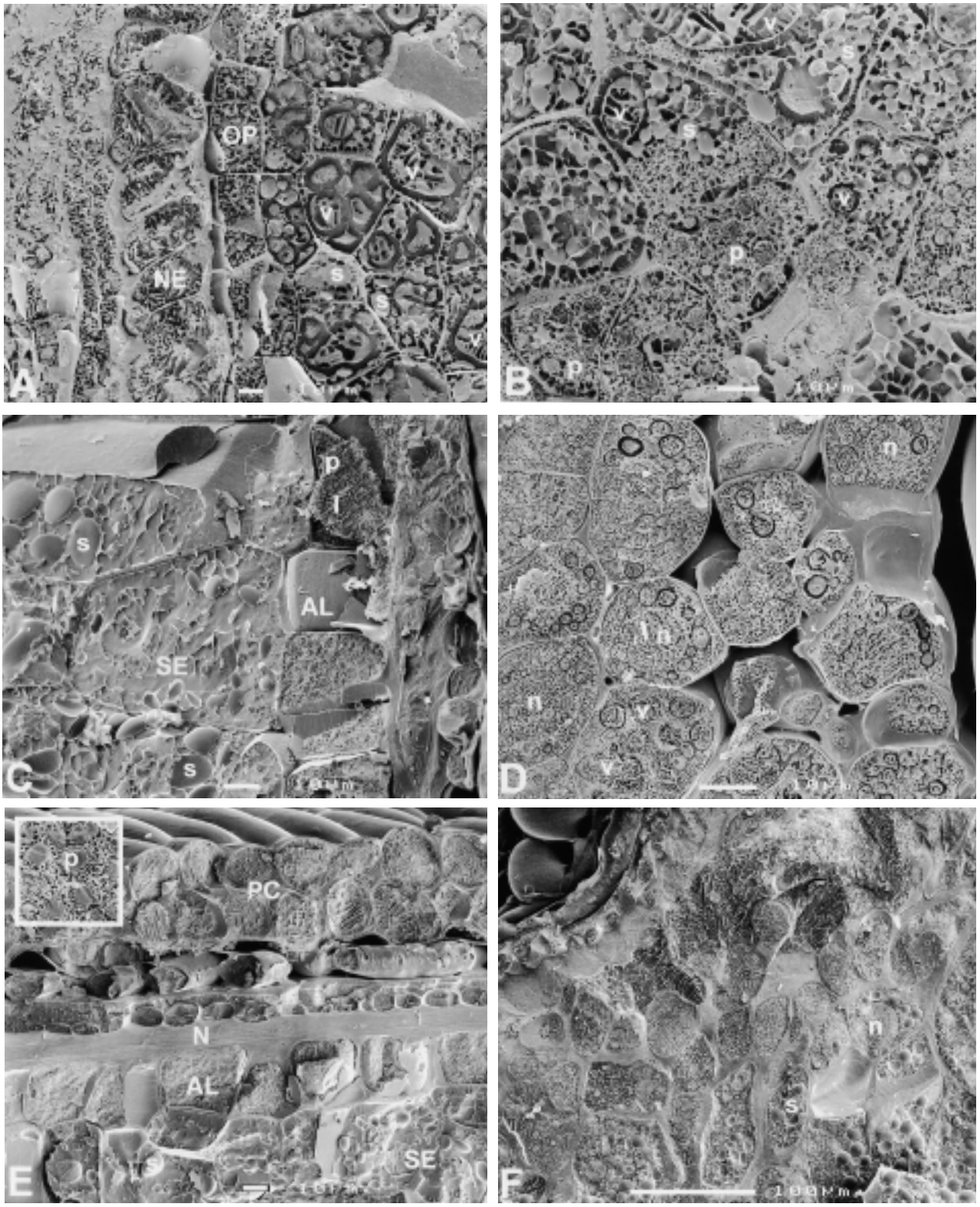
To estimate the first proportion of living cells in developing endosperm, the relative amplitude of the narrow component of the central line was calculated, as indicated in Fig. 5A. The calculation was performed for spectra obtained from developing endosperm (similar to those presented in Fig. 3) and for spectra simulating 100% viable endosperm cells at different MCs (similar to those in Fig. 4). The central line was selected for calculations because it is less influenced by changes in viscosity than the other lines of the ESR spectrum (Morse, 1977). The results of the calculations are presented in Fig. 5B. The data pertaining to the mature endosperm samples at different MCs with simulated 100% living cells were plotted against the developmental age as if they were derived from developing endosperm (daa were derived from the time-table [Fig. 1C]). Figure 5B shows that if all starchy endosperm cells were alive, the decrease in MC (expressed as developmental age) would have only a slight effect on the proportion of the amplitude of the narrow component to the total amplitude of the central line of the ESR spectra (from 96% to 93%) up to the onset of maturation drying (40% MC). Figure 4 shows also that the shape of the spectra did not change dramatically in the range 70–40% MC. However, this amplitude proportion in spectra from developing endosperm (Fig. 5B, curve 2) was considerably lower at 8 to 11 daa (77%) and rapidly decreased between 12 and 20 daa. Although the proportion of the amplitude of the narrow component to the total amplitude is a reflection of the proportion of viable cells in a sample, this parameter cannot be used to directly express the percentage of viable cells. This is because the peak heights of the broad and narrow components do not reflect the number of TEMPONE molecules participating in the signals. However, from the relationship between amplitude and number of TEMPONE molecules (integral intensity), better estimates can be obtained for the proportion of living cells in a sample. We found coefficients for the ratio between amplitude and integral intensity of 2.94 for the narrow component (1 mM TEMPONE in water) and 87.6 for the broad component. Using these coefficients the relative proportion of TEMPONE molecules in cells with intact plasma membranes (narrow component of the spectrum) was calculated for developing endosperm and for the sample with the simulated 100% living endosperm cells (Fig. 5C). Comparison of these proportions allowed calculation of the percentage of viable cells at all stages of endosperm development studied (Fig. 5D). The data presented in Fig. 5D show that at an early stage of development (8 daa) approximately a quarter of the endosperm cells were alive, and that the proportion of viable cells progressively declined with development, to almost zero just before 20 daa. Starchy endosperm cells thus lose membrane integrity long before maturation drying. It is evident that desiccation is not the reason for the death of starch endosperm cells, from which we conclude that programmed cell death occurs during starch accumulation.

Ultrastructure of developing endosperm cells

To get an insight into the ultrastructural changes associated with programmed cell death, an LTSEM study of the developing starchy endosperm was conducted. ESR data indicate that noticeable membrane disruption occurs in starchy endosperm as early as 8 daa (Figs 3A and 5D). At this stage we were able to find few starch granules in the peripheral layers of the endosperm (Fig. 6A) and considerably more in the centre of the endosperm along with protein bodies (Fig. 6B). The cells of the 8-daa starchy endosperm differed from those found at 14 daa (Fig. 6C) in that they still contained aqueous cytoplasm that formed ice crystals after cryo-fixation, whereas the 14-daa starchy endosperm cells had starch granules embedded in an amorphous matrix. The cells in Figs 6B and 6C probably represent the onset and completion of starch accumulation, respectively. The latter type probably characterizes dead cells because of the similarity in appearance to mature starchy endosperm cells (micrograph not shown). The micrograph, Fig. 6D, shows cells from a viable 25-daa embryo that was dried and rehydrated. The ultrastructure of these living cells is completely different from that in Fig. 6C and can be used as a reference for viable cells in wheat kernels. Because cell division at the peripheral layer coincides with starch accumulation in the more centrally located layers, cells at various stages of starch accumulation

Figure 6. Representative LTSEM micrographs of cryo-fractured wheat endosperm at 8 daa at the periphery (A; bar = 10 μm) and in the centre (B; bar = 10 μm), at 14 daa at the periphery (C; bar = 10 μm); embryo, 25 daa dried and fully rehydrated (D; bar = 10 μm); fresh endosperm 18 daa at the periphery (E, bar = 10 μm) and in the vicinity of the vascular bundle (F; bar = 100 μm). Insert in (E) shows a detail of the ultrastructure of an aleurone cell. AL = aleurone layer, N = nucellus, NE = nucellar epidermis, OP = outermost peripheral endosperm cells, PC = pericarp, SE = starchy endosperm cells, l = lipid body, n = nucleus, p = protein body, s = starch granule, v = vacuole.

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can be encountered within the same endosperm (Evers, 1970). However, the proportions of the various types of cells change with kernel developmental age. Even at 18 daa, when ESR data indicated considerable membrane disruption (Figs 3C and 5D), we found cells that had completed starch accumulation (Fig. 6E) as well as cells at earlier stages of starch accumulation (in the area of thick-walled cells close to the vascular bundle; Fig. 6F).

Desiccation tolerance of endosperm cells

From the fact that starchy endosperm cells are dead in mature kernels, it does not automatically follow that these cells are not desiccation tolerant at early stages of development. As was shown above, these cells die during starch accumulation before maturation drying. To test whether starchy endosperm cells are nevertheless desiccation tolerant in the immature kernel before they become nonviable because of developmental death, the spin probe technique was applied to prematurely dried starchy endosperm.

Figure 7 shows the ESR spectra from 14-daa starchy endosperm, before (Fig. 7A) and after (Fig. 7B) fast drying and rehydration. The narrow component which was present in the spectrum from the fresh tissue had completely disappeared after rapid drying and rehydration. Thus, young starchy endosperm cells are not tolerant to fast drying, from which we conclude that during development desiccation tolerance is not acquired in starchy endosperm cells. This was not unexpected because these cells are vacuolated and do not have the typical appearance of desiccation-tolerant cells (micrograph not shown).

From the fact that aleurone layer cells are alive in mature cells, it follows that these cells are desiccation tolerant. Differentiation of the outermost layer of meristematic cells, also called aleurone initials (Olsen *et al.*, 1999), into the aleurone layer was found as early as 14 daa (Fig. 6C). The aleurone cells had similar appearance to desiccation-tolerant embryonic cells (Fig. 6D), which did not change with further development of the kernel (Fig. 6E at 18 daa). It is, therefore, concluded that differentiation of the outermost peripheral layer of endosperm into aleurone layer cells goes together with the acquisition of desiccation tolerance.

Thus, the differentiation of meristematic cells of the periphery of the endosperm is definitive – if the cells differentiate into starch accumulating cells, they

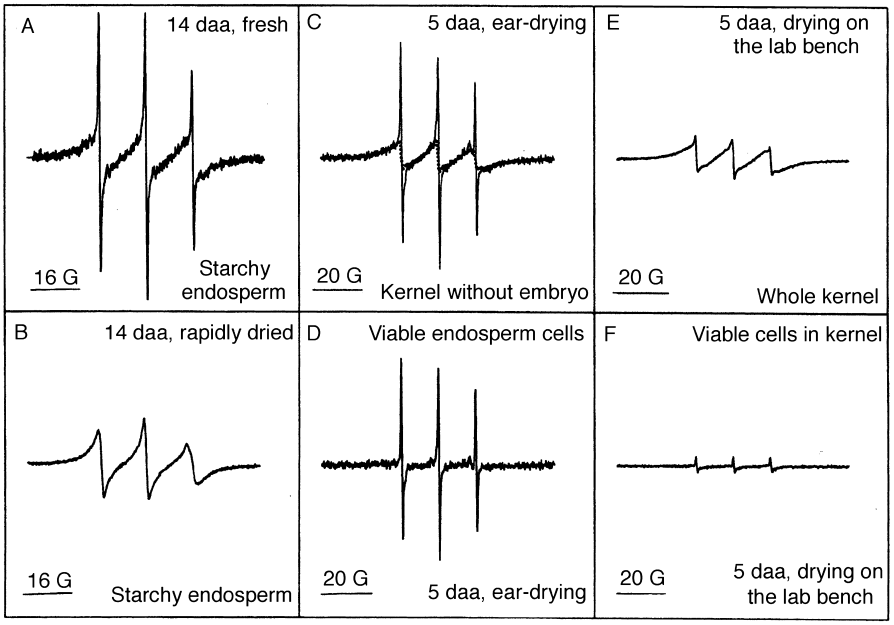


Figure 7. Representative ESR spectra of TEMPONE (in the presence of ferricyanide) from wheat endosperm after drying and rehydration: (A) fresh starchy endosperm at 14 daa; (B) after rapid drying of excised 14-daa starchy endosperm; (C) kernel slowly dried on an ear cut at 5 daa and rehydrated (dwarf embryo removed); (D) the resulting spectrum after subtraction of the broad component (dashed line in C) from spectrum C (solid line); (E) a whole 5-daa wheat kernel rapidly dried on the laboratory bench (1.5 days); (F) the resulting spectrum after subtraction of the broad component (dashed line in E) from spectrum E, (solid line) which represents the cytoplasmic signal of TEMPONE from a few viable cells in such a kernel.

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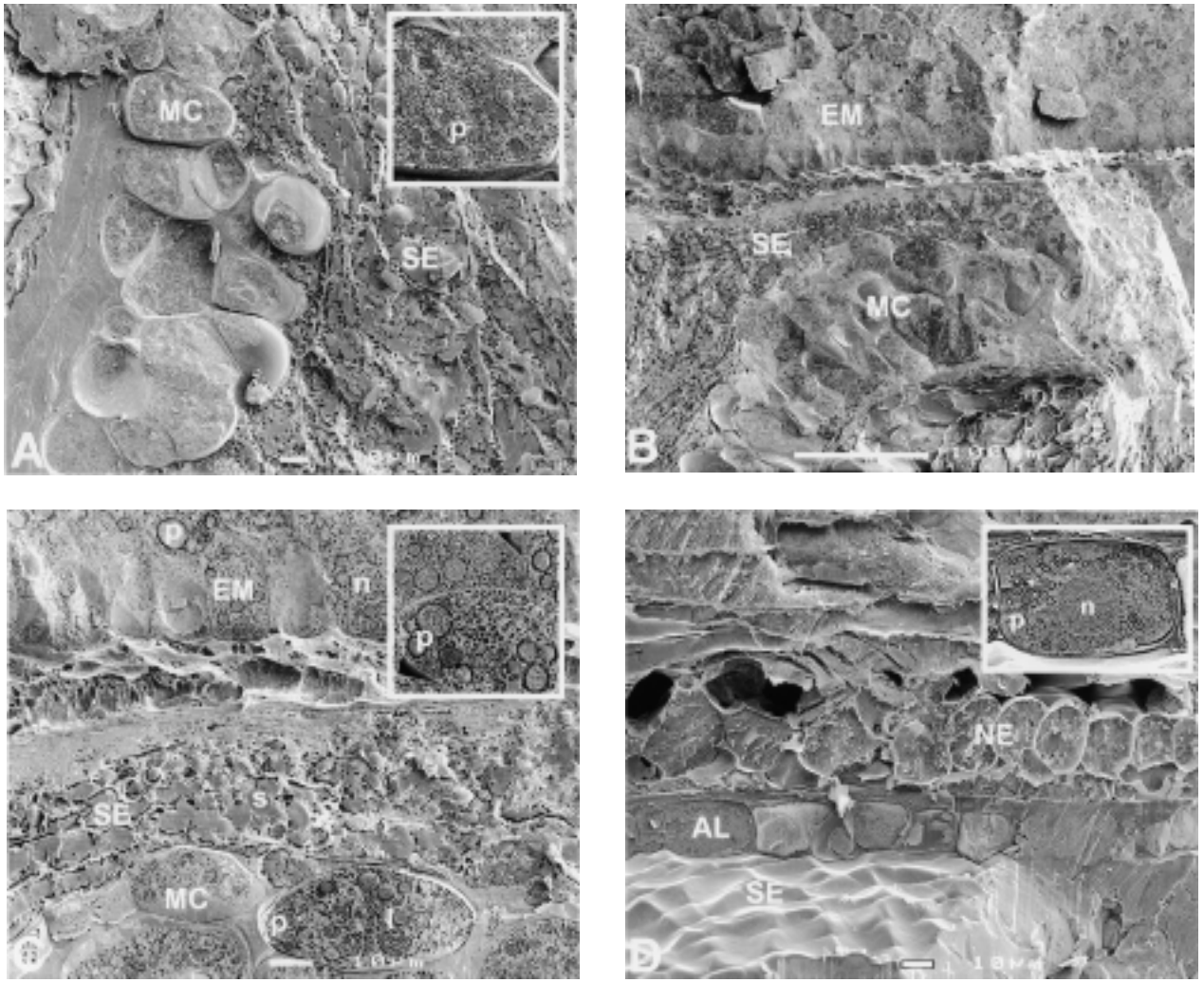


Figure 8. LTSEM micrographs of a cryo-fractured wheat kernel slowly dried on an ear cut at 5 daa and rehydrated: (A) peripheral layers of the endosperm (bar = 10 µm); (B) endosperm in the vicinity of a developed dwarf embryo (bar = 100 µm); (C) area from (B) in more detail (bar = 10 µm); (D) differentiated aleurone layer in a kernel slowly dried on an ear cut at 7 daa and rehydrated (bar = 10 µm). Inserts to (A) details of a meristematic cell, to (C) details of an embryonic cell, and to (D), details of an aleurone cell. AL = aleurone layer, EM = embryo, MC = peripheral meristematic cell, NE = nucellar epidermis, SE = starchy endosperm cells, l = lipid body, n = nucleus, p = protein body, s = starch granule.

are desiccation sensitive, and if they differentiate into aleurone layer, they acquire desiccation tolerance. Meristematic cells, therefore, have the competence to acquire desiccation tolerance, which is lost with the differentiation into starch accumulating cells. The process of differentiation can be arrested by premature slow drying (Golovina *et al.*, unpublished results). To investigate whether meristematic cells can acquire desiccation tolerance in this case, we studied wheat kernels that were prematurely dried, either slowly or rapidly.

Figure 7C shows an ESR spectrum of TEMPONE in a wheat kernel that was slowly dried on the ear cut at 5 daa. The outer pericarp and dwarf embryo which developed (Golovina *et al.*, 2000), were removed. The narrow component in this spectrum was prominent, which is indicative of the presence of live cells. After subtraction of the broad component, the contribution of live cells is shown in spectrum D (Fig. 7D). In contrast, the 5-daa kernel that was dried on the laboratory bench (the process of drying took 1–1.5 days) had not developed a dwarf embryo, and the contribution of the narrow component to the ESR spectrum from such a kernel was almost negligible (Figs 7E and F). This means that during premature slow drying on cut ear there are a considerable

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number of cells in the endosperm which acquired desiccation tolerance. The micrograph (Fig. 8A) of such a wheat kernel shows two to three layers of large cells at the endosperm periphery instead of one layer of aleurone cells as observed in normally developed kernels (Figs 6C and E). These large cells are turgid and have an appearance typical of desiccation-tolerant cells (insert to Fig. 8A). In the vicinity of the developed dwarf embryo (Fig. 8B), even bigger clusters of such cells were found. Higher magnification (Fig. 8C) shows that these cells have an ultrastructure similar to those of the dwarf embryo (insert to Fig. 8C) which is alive and can germinate (Golovina *et al.*, unpublished results). Thus, in the case of premature slow drying, when differentiation into starchy endosperm cells is arrested, the meristematic cells acquire desiccation tolerance. However, kernels dried on the ear cut at 7 daa had a normally differentiated aleurone layer (Fig. 8D; insert to Fig. 8D for ultrastructural details). Drying on the cut ear after 7 daa permitted the completion of differentiation of the peripheral meristematic cells, whereas drying on the ear cut at 5 daa caused the arrest of differentiation.

Discussion

The loss of membrane integrity in starchy endosperm cells during kernel development

Although it is well known that starchy endosperm cells of cereals are dead at maturity, the timing and characteristics of the degeneration process during kernel development have not been studied (DeMason, 1997). Here we present data concerning the loss of plasma membrane integrity of the cells in developing starchy endosperm based on ESR spin probe technique. The study concerned the entire period of wheat endosperm development from early differentiation to the attainment of mass maturity, even though starchy endosperm cells are supposed to die late in maturation (Buckner *et al.*, 1998; Berger, 1999; Olsen *et al.*, 1999).

The attainment of mass maturity of the kernels (Fig. 1B) coincided with the complete loss of plasma membrane integrity in starchy endosperm cells (Figs 3D and 5B–D). One could conclude from these data that as soon as an individual starchy endosperm cell completes starch accumulation, it loses its membrane integrity. The presence of a narrow (cytoplasmic) component in ESR spectra before the attainment of mass maturity is indicative of the presence of cells still with intact membranes. The gradual decrease of this narrow component during endosperm development could be interpreted to mean that the proportion of cells that had completed their starch accumulation gradually increased. In developing endosperm, cells at various stages of starch accumulation can be encountered (Evers, 1970). The further they are situated from the periphery, the more advanced they are in starch accumulation. Indeed, Figs 6A, B and F show cells at various stages of starch accumulation in the same endosperm.

The proportion of viable cells in starchy endosperm as calculated from ESR spectra decreased rapidly between 12 and 19 daa (Fig. 5D), from a quarter to almost zero. Even at 8 daa, only a few days after completion of cellularization, the proportion of cells with intact plasma membranes was estimated to be surprisingly low, also around 25% (Fig. 5D). If the previous suggestion that membrane disruption coincides with the cessation of starch accumulation is correct, then 75% of the endosperm cells should have the same appearance as those shown in Figs 6C and E. Using LTSEM we found endosperm cells only at the early stages of starch accumulation (Fig. 6B), even in the central area where cells are supposed to be more advanced in starch accumulation (Evers, 1970). Therefore, we conclude that the plasma membranes of endosperm cells begin to lose their barrier properties at earlier stages of starch accumulation.

From 8 to 14 daa the further reduction of the cytoplasmic component of the ESR spectra was not dramatic (Figs 3A and B and Fig. 5D), probably because the ongoing loss of membrane integrity associated with starch accumulation in the inner part of the kernel was partly compensated by the increase in the number of live cells produced by cell division at the periphery. Beyond 14 daa, the decrease of the cytoplasmic component of ESR spectra as an indication of cell disruption progressed faster (Fig. 5D). This may be linked to the cessation of cell division associated with the final differentiation of the outermost meristematic cells into aleurone cells (Figs 6A and C), which also coincided with the cessation of water accumulation (Fig. 1A). Moreover, the percentage of cells with intact plasma membranes decreased to a very low level over the 5 days following the differentiation of the aleurone layer (Fig. 5D). From 14 daa onward, no meristematic activity leading to differentiation into starchy endosperm cells could be observed. From this it can be concluded that it takes 5 days for all the starchy endosperm cells to lose plasma membrane integrity. The low level of intact cells at 8 to 12 daa (25%) could be caused by the fact that the majority of starchy endosperm cells at this stage originate from the cellularized syncytium and not from gradually differentiating meristematic cells. Therefore, these cells could have lost plasma membrane integrity simultaneously a few days after cellularization.

Recently, it was proposed that endosperm cells in developing maize kernels undergo programmed cell

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death late in development (Young *et al.*, 1997). Based on data obtained with the vital stain, Evans blue, these authors concluded that endosperm cells die coincident with mass starch deposition. In this paper, the simultaneous use of ESR and LTSEM enabled us to deduce that the loss in membrane barrier properties occurs early in starch accumulation, i.e. soon after differentiation rather than late in maturation. Cells may be accessible to Evans blue only when they have totally collapsed. However, the ESR spin probe technique allows the detection of changes in membrane barrier properties, even before cell death (Golovina *et al.*, 1997). The data presented in this paper support the idea of programmed cell death in developing cereal endosperm. Moreover, the fact that the loss of membrane barrier properties occurs at an early stage of endosperm differentiation indicates that programmed cell death may be an intrinsic part of starchy endosperm differentiation.

The competence of meristematic endosperm cells to acquire desiccation tolerance

The differentiation of the outermost meristematic endosperm cells into aleurone cells is characterized by the formation of one layer of rectangular cells with a dense and granular cytoplasm at the periphery of the endosperm (Percival, 1921). In the present investigation this differentiation into aleurone cells with dense cytoplasm, abundant in protein and oil bodies, was detected at 14 daa at the earliest (Fig. 6C). Such an ultrastructure characterizes desiccation-tolerant cells (see Fig. 6D). The desiccation-tolerant aleurone cells of the mature endosperm also have similar ultrastructure (micrograph not shown). These facts allow us to assume that the aleurone cells observed in 14-daa fresh kernels are also desiccation tolerant. In contrast, those cells of the starchy endosperm which are still alive at 14 daa (see narrow component of the ESR spectra in Figs 3B and F) are desiccation sensitive. This can be concluded from the disappearance of the narrow component in the ESR spectrum from the fresh 14-daa starchy endosperm upon rapid drying and rehydration (Figs 7A and B). Thus, the differentiation of meristematic cells into starch accumulating cells seems to be incompatible with the acquisition of desiccation tolerance. This could be expected if such a differentiation initiates the programme of cell death as discussed above. The programmes of cell death and acquisition of desiccation tolerance are not expected to run simultaneously.

Meristematic cells themselves are apparently unable to survive rapid drying. This was inferred from the disappearance of the narrow component in the ESR spectra (Figs 7E and F) from the 5-daa wheat kernels that were dried on the laboratory bench (during 1.5 days). However, slow drying on the ear cut at 5 daa (1 month) induced the acquisition of desiccation tolerance in wheat endosperm (Figs 7C and D). Slow drying on the cut ear permitted the continuation of seed development as long as the MC was maintained above a critical level–presumably 75% (FW basis) (Brunori, 1967). As soon as the MC decreased below this level, further development would be arrested, whereas other programmes, such as the acquisition of desiccation tolerance, would continue. Thus, at an early stage of development, it is possible to uncouple the programme of histo-differentiation from the programme of desiccation-tolerance acquisition. During slow drying of wheat kernels on the ear cut at 5 daa, the typical aleurone layer did not develop. Instead, two to three layers of large cells at the periphery of the endosperm (Fig. 8A) or even bigger clusters (Fig. 8B) developed. These cells have similar ultrastructural features to embryo cells (Fig. 8C and insert) and are responsible for the narrow component in the ESR spectrum (Fig. 7D). All cells containing starch had the appearance of dead cells (Figs 8A–C). During premature slow drying of 5-daa kernels on the ear, the programme of differentiation of the peripheral layer of meristematic cells into starch accumulating and aleurone layer cells was not completed. Those meristematic cells that did not differentiate into starch accumulating cells acquired desiccation tolerance during slow drying. Those cells that differentiated into starch accumulating cells died, either before drying by undergoing programmed death, or during drying because of desiccation sensitivity. In contrast, slow drying of wheat kernels on an ear cut slightly later (at 7 daa), allowed the outermost peripheral layer of the endosperm to differentiate into the typical, one-cell layer thick, desiccation-tolerant aleurone layer (Fig. 8D and insert).

Endosperm cells in cereals appear to have the same competence to acquire desiccation tolerance as embryonic cells, in spite of their different destinies. This competence in both endosperm (this paper) and embryonic cells (Golovina *et al.*, unpublished results) can find its expression either during premature slow drying, or during very early normal development, long before maturation drying. However, upon differentiation of meristematic endosperm cells into starch accumulating cells, this competence is lost and the programme of cell death is initiated. This similarity between endosperm meristematic cells and embryonic cells supports the idea proposed by Friedman (1992, 1995) that the endosperm evolved from a second embryo present in the predecessors of angiosperms.

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