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Citation

Wang, M., Oppedijk, B. J., Caspers, M. P. M., Lamers, G. E. M., Boot, M. J., Geerlings, D. N. G., ... Duijn, B. van. (1998). Spatial and temporal regulation of DNA fragmentation in the aleurone of germinating barley. *Journal Of Experimental Botany*, 49(325), 1293-1301.
doi:10.1093/jxb/49.325.1293

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Spatial and temporal regulation of DNA fragmentation in the aleurone of germinating barley

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Received 29 August 1997; Accepted 13 February 1998

Abstract

During germination of barley grains, the appearance of DNA fragmentation started in aleurone cells near the embryo and extended to the distal end in a time-dependent manner. DNA fragmentation was demonstrated to occur only after the expression of α -amylase mRNA in the aleurone layer. In addition, cell wall degradation started in cells near the embryo on the sides facing the endosperm. Subsequently cell wall degradation extended to the lateral cell walls and to cells more to the distal end of the grain. A typical alteration of the nucleus was observed by electron microscopy and an almost complete degradation of DNA was found in the nucleus while the nuclear envelope remained intact. The results indicate that programmed cell death occurred in aleurone cells during germination. A model is proposed for the regulation of programmed cell death in aleurone cells during germination involving ABA levels and cell wall degradation.

Key words: Barley aleurone, programmed cell death, hydrolytic enzymes, cell walls.

Introduction

Mature barley grains mainly consist of the embryo and the starchy endosperm. The endosperm is surrounded almost completely by a three cell layers thick gland-like

tissue, the aleurone. The embryo and the endosperm are separated by the scutellar epithelium which is one cell layer thick and has a dual function (secretion subsequently followed by absorption). Upon imbibition, rehydration of the grain acts as a switch to start germination. During germination nutrients required for the growth of the embryo are initially obtained from the store in the embryo itself, but are subsequently mobilized from the storage materials in the endosperm, which consists mainly of starch and protein granules. The latter process occurs by the action of a wide spectrum of hydrolytic enzymes and is induced through plant hormone production by the embryo during germination. The hormones stimulate the production and secretion of hydrolytic enzymes by aleurone and scutellum cells. The secreted glucanases and xylanases degrade cell walls of aleurone and scutellum as well as endosperm cells. The production of α -amylases, other glycosidases, proteases, peptidases, acid phosphatases, DNases, RNases etc, serve the breakdown of the cell content of the endosperm (Gibbons, 1980; Palmer, 1982; Deikman and Jones, 1985; Briggs, 1987; Jacobsen and Chandler, 1987). The starchy endosperm will be degraded into sugars and other small molecules which can be readily taken up by the embryo. In this way, both scutellum and aleurone provide the machinery to obtain the required nutrients for the embryo. This process is controlled in time in such a way that the young plant can reach the stage of self surviving. The ability of aleurone activation is one of the limiting factors in germination, since it has been recently demonstrated that in dormant

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barley grains, not only is the embryo unable to initiate the germination processes, but that the aleurone is impaired in its activities as well (Schuurink *et al.*, 1992; Wang *et al.*, 1995).

After contributing their hydrolytic enzymes, the aleurone cells will not become part of the young plant but are sacrificed. The death of aleurone cells may be caused by the massive production of hydrolytic enzymes, the simultaneous loss of the cell walls and an increased osmotic pressure caused by degradation of starchy endosperm into sugars and other small molecules. Understanding the sequential events during germination in the aleurone will help not only in the search for molecular switches in the control of germination, but may also be of great help in industrial applications for endosperm modification.

It was recently demonstrated that during the processes of germination, DNA fragmentation (about 200 bp ladder) occurred in aleurone cells. The appearance of DNA fragmentation started in the aleurone cells near the embryo and extended to the distal end of the grain during germination. This DNA fragmentation could be dramatically accelerated by application of cell wall degrading enzymes to aleurone *in vitro* (Wang *et al.*, 1996a). Degradation of nuclear DNA into about 200 bp is considered to be one of the hallmarks of apoptosis, a form of programmed cell death seen in vertebrates with characteristic features such as a shrinking nucleus, condensed chromatin and fragmented DNA (Wyllie, 1980). Therefore, it is likely that a form of apoptosis also occurs in aleurone of germinating barley. Programmed cell death in plants is apparently involved in anther, megagametophyte and vascular tissue development and in at least one mechanism of sex determination (DeLong *et al.*, 1993; Jones and Dangel, 1996; Mittler *et al.*, 1997; Yeung and Meinke, 1993). Recently, more evidence that environmental stress as well as fungal infection induce apoptosis has accumulated (Ryerson and Heath, 1996; Wang *et al.*, 1996b; Mittler *et al.*, 1997). Both the above evidence and the reported apoptosis in diploid parthenogenesis and early somatic embryogenesis of Norway spruce (Havel and Durzan, 1996) suggest that, as in animal systems, apoptosis may also play an important role in plants.

In a previous paper it was shown that both α -amylase production and DNA fragmentation are activated over the aleurone in a spatial-temporal way (Wang *et al.*, 1996a). The activity of both processes first started near the embryo and then spread over the aleurone layer to the distal end of the grain in time. However, the sequence of the different events and cellular morphology was not resolved in this study. To enable improved correlation of DNA fragmentation with different aleurone cell activities, various microscopy techniques were used in combination with different labellings to study these processes at a single cell level.

Materials and methods

Plant materials and germination tests

Hordeum distichum L. cv. Triumph was obtained from Heineken Technical Services (The Netherlands, harvest 1989). *Hordeum vulgare* L. cv. Himalaya (harvest 1988) was from the Department of Agronomy, Washington state University, Pullman, USA. Germination tests were done in Petri dishes (9 cm in diameter) at 20 °C in the dark: 20–50 grains were germinated on two layers of Whatman paper No. 1 with 2 ml H₂O added.

DNA extraction and analysis and TUNEL cytological staining

DNA extraction from aleurone, analysis and TUNEL staining were carried out as described before (Wang *et al.*, 1996a). Briefly, barley grains were imbibed in water for different periods of time and fixed in 2% glutaraldehyde in PBS (pH 7.3) at room temperature. After dehydration in ethanol, the samples were embedded in Histo-resin (Leica Instruments GmbH, Germany). Sectioning was carried out with an ultra-microtome by using glass microtome knives, and sections were collected on a glass slide (coated with BIOBOND (Biocell)). For TUNEL staining, sections were incubated with an *in situ* cell death fluorescein detection kit, (Boehringer Mannheim GmbH, Germany). TUNEL staining detects DNA fragmentation by adding a fluorescent (FITC)-labelled group to the 3'-ends of broken DNA strands. The more fragmentation the more 3'-ends are available for staining. For nuclear staining, the fluorescent dye 4'-6-diamidino-2-phenylindole (DAPI) which stains DNA, was used. Protoplasts were mixed with DAPI solution to give a final concentration of DAPI of about 1 mg l⁻¹ in phosphate buffer (pH 7.0). In most cases, samples were double-stained with both FITC of the TUNEL kit and DAPI. After mounting on a slide and covering with coverslip, the samples were examined with fluorescence microscopy (Leitz Laborlux). For observation of DAPI staining, filters enabling the use of an excitation wavelength between 334–365 nm and an emission wavelength of 420 nm were used. For observation of TUNEL staining, filters enabling excitation and emission around 515 nm were used. Micrographs were made using a Kodak colour gold film (400 ASA).

Electron microscopy

Isolated aleurone layers were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.2) for 4 h at room temperature. Subsequently, the layers were rinsed (2 h) and post-fixed with 1% osmium tetroxide in the same buffer (2 h). After washing in double-distilled water and dehydration in 20, 30, 50, 70, 80, and 96% (v/v) ethanol for 30 min each, the layers were dehydrated further in 100% ethanol for 90 min. After impregnation with propylene oxide, the aleurone layers were passed through a series of five propylene oxide/Epon mixtures with increasing Epon concentration. The tissue was left in pure Epon overnight before embedding in flat silicon rubber molds. The Epon was polymerized at 60 °C for 48 h.

Ultra-thin cross-sections of the aleurone layers were made using a Reichert-Jung ultra-E microtome equipped with a diamond knife. The sections were stained with saturated aqueous uranyl acetate and lead citrate solutions for 5 min each. The sections were examined using a Phillips 300 electron microscope operating at 60 kV.

In situ hybridization

Tissues were fixed at room temperature in 4% paraformaldehyde and 0.25% glutaraldehyde in 10 mM sodium phosphate buffer

(pH 6.8) containing 100 mM NaCl, dehydrated through a graded ethanol and xylene series, and embedded in Paraclean. Sections (8 μm) were attached to poly-L-lysine-coated slides, deparaffinated with xylene and rehydrated through a graded ethanol series. Subsequently they were pretreated for 30 min at 37 °C with 5 $\mu\text{g ml}^{-1}$ proteinase K in 100 mM TRIS/HCl (pH 7.5), 50 mM EDTA, washed three times with water, incubated for 10 min at room temperature in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8), washed twice in $2 \times \text{SSC}$, and then dehydrated through a graded ethanol series. Linearized pBluescript plasmid containing the full length barley high pI α -amylase cDNA (EMBL acc. no. K02637 or J04203) was used as template for synthesis of digoxigenin-labelled sense or antisense riboprobes with T7 or T3 RNA polymerase according to the manufacturer's instructions (Boehringer Mannheim) except that the digoxigenin-11-UTP:UTP ratio was 2:1. Probes were hydrolysed to an average length of 150 nucleotides in 0.2 M $\text{Na}_2\text{CO}_3/0.2$ M NaHCO_3 for 55 min at 60 °C. Sections were hybridized for 16 h at 42 °C with a 1:1 mixture of hydrolysed and non-hydrolysed riboprobe (0.2–1 $\mu\text{g ml}^{-1}$) in hybridization buffer containing 50% formamide, 300 mM NaCl, 10 mM TRIS/HCl (pH 7.5), 1 mM EDTA, 1 \times Denhardt's solution, 10% dextran sulphate, 60 mM DTT, 500 $\mu\text{g ml}^{-1}$ polyA, 150 $\mu\text{g ml}^{-1}$ yeast tRNA, and 200 U ml^{-1} RNasin (Promega). After hybridization the following washing steps were performed: four times in $4 \times \text{SSC}$, 1 mM DTT at room temperature for 10 min each, once in 50 $\mu\text{g ml}^{-1}$ RNaseA in RNase buffer (10 mM TRIS/HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA) at 37 °C for 30 min, four times in RNase buffer supplemented with 1 mM DTT at 37 °C for 10–20 min each, and once in $0.2 \times \text{SSC}$, 1 mM DTT at 60 °C for 45 min. Detection of hybridized probe was performed as described by Coen *et al.* (1990), except that incubation with antibody-alkaline phosphatase-conjugate was for 2 h and at 1:1000 dilution. Following detection, sections were passed through an ethanol series and xylene and mounted in Eukitt (O Kindler GmbH and Co., Freiburg, Germany).

Results

Spatial-temporal events in hydrolytic enzyme production and DNA fragmentation

During germination, many sequential events occur in the aleurone which all start near the embryo and gradually move along the aleurone layer to the distal end of the grain. This process has been proposed to be due to signal molecules (plant hormones, e.g. GA and ABA) produced by the embryo (McFadden *et al.*, 1988). Rehydration and activation of aleurone to produce hydrolytic enzymes starts near the embryo and prolongs to the entire aleurone (Gibbons, 1980; Palmer, 1982; McFadden *et al.*, 1988; Wang *et al.*, 1996a).

To find out if α -amylase production precedes DNA fragmentation, as proposed previously (Wang *et al.*, 1996a), *in situ* hybridization experiments for α -amylase mRNA were performed and compared with *in situ* TUNEL assays in barley aleurone at different times during germination. Figure 1A shows that the expression of α -amylase mRNA started in scutellar epithelium cells and in the aleurone very near to the embryo after 1 d of

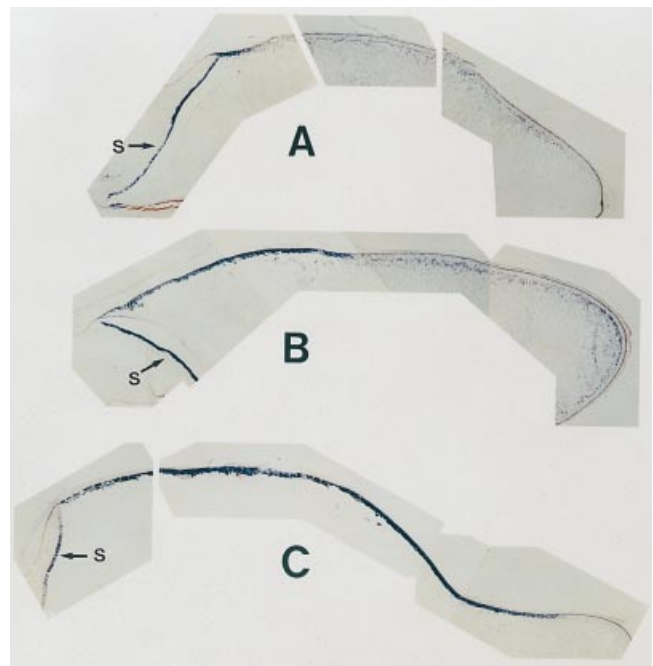


Fig. 1. Localization of α -amylase gene expression during germination of barley aleurone. Longitudinal sections of barley grains (aleurone layers, with the embryo located on the left side) were probed with digoxigenin (DIG)-labelled RNA probes by *in vitro* transcription of the α -amylase cDNA. Antisense probe was synthesized by T7 RNA polymerase. Sense probe was synthesized by T3 RNA polymerase and was used as negative control. Hybridization signals were detected by nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The antisense probe (control) gave no background staining (not shown). S and the arrow indicate the scutellum in each figure. The embryo is located to the left of the scutellum. The aleurone is the cell layer surrounding the endosperm and follows the outline of the grain. Four independent experiments were carried out and one typical experiment is presented. (A) After 1 d of imbibition; (B) after 2 d of imbibition; (C) after 3 d of imbibition.

imbibition. The expression of α -amylase mRNA extended to half of the aleurone layer after 2 d of imbibition and covered almost the whole aleurone layer after 3 d of imbibition (Fig. 1B, C). At 3 d of imbibition (germination), the expression of α -amylase mRNA was decreased in scutellum and aleurone near the embryo (Fig. 1C).

In contrast to the α -amylase mRNA production (Fig. 1), DNA degradation indicated by TUNEL staining in aleurone of germinating grains was not detected after 1 d of imbibition (Fig. 2A). At 1 d of imbibition some positive TUNEL staining could be seen in scutellar epithelium cells and the single layer of germin aleurone cells surrounding the embryo (Fig. 2D, E). TUNEL positive stained aleurone cells could just be detected after 2 d of imbibition in the aleurone very close to the embryo (Fig. 2B) and extended to one-third of the aleurone layer after 3 d of imbibition (Fig. 2C). In addition, this can be seen in Fig. 3 showing the number of aleurone cells with positive α -amylase mRNA expression and DNA fragmentation in aleurone cells in time and position during imbibition (Fig. 3).

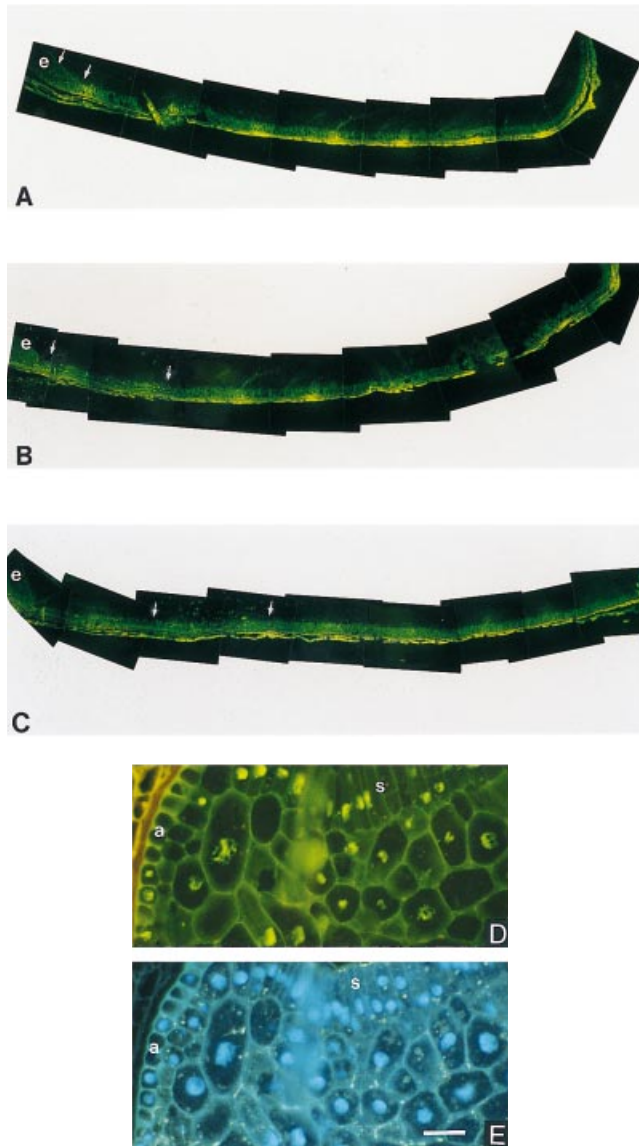


Fig. 2. Localization of DNA fragmentation by TUNEL detection during germination of barley aleurone. TUNEL staining of longitudinal section of barley grains (aleurone layers) during germination. The (e) in (A) (B) and (C) indicates the location of the embryo. The positive TUNEL staining in (A), (B) and (C) can be seen as small yellow dots in a confined region (indicated by the arrows) near the scutellar epithelium cells (in A) and in the aleurone (in B close to the embryo, in C further away from the embryo). Three independent experiments were carried out and one typical experiment is presented. (A) After 1 d of imbibition; (B) after 2 d of imbibition; (C) after 3 d of imbibition; (D) scutellar epithelium cells stained with TUNEL after 1 d of imbibition; (E) scutellar epithelium cells stained with DAPI after 1 d of imbibition. The (a) in (D) and (E) indicates the aleurone layer that surrounds the embryo (germin aleurone), while (s) indicates the scutellar epidermal cells (the outer layer of the scutellum), the other cells mainly are scutellum cells. Bar in Fig. 2E is 80 μ m.

The positive TUNEL staining correlated very well with the appearance of DNA ‘ladders’ with approximately 200 bp intervals on ethidium bromide-stained agarose gels during germination (Fig. 4). After 2 d of imbibition, DNA isolated from aleurone near the embryo showed a

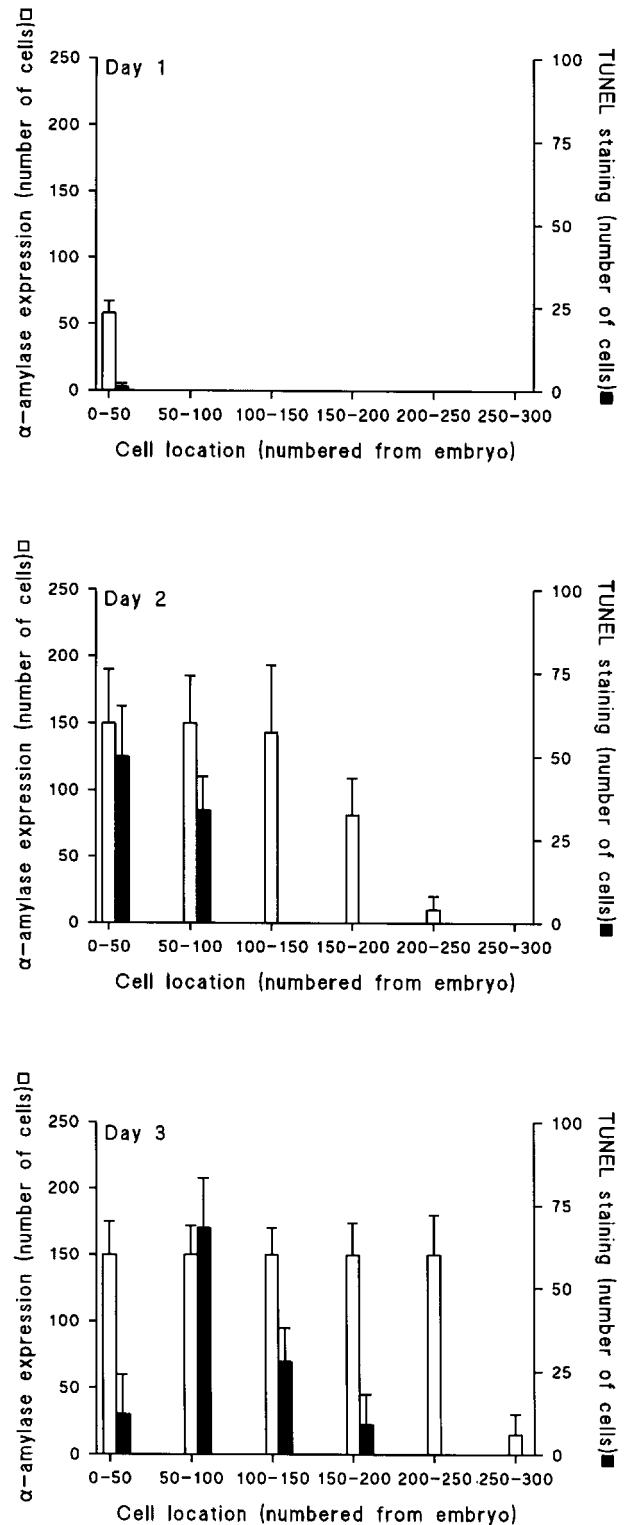


Fig. 3. Comparison of the number of α -amylase expression to the number of TUNEL staining in aleurone during germination. Barley grains were imbibed for 1, 2 and 3 d and samples were collected for *in situ* α -amylase expression and TUNEL staining. The x-axis shows the location of the cells as numbered from the embryo to the distal end of the grain. The y-axes show the number of cells with α -amylase expression and the number of cells showing TUNEL positive staining. Means \pm sem from four barley grains are presented.

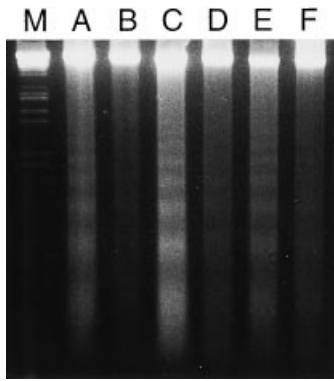


Fig. 4. Agarose gel analysis of barley aleurone DNA fragmentation during germination. Lane M: the DNA marker of lambda DNA digested with *HindII/EcoRI*. DNA isolated from aleurone near the embryo after 2 d (lane A), 3 d (lane C) and 4 d (lane E) of imbibition and DNA isolated from aleurone at the distal end after 2 d (lane B), 3 d (lane D) and 4 d (lane F) d of imbibition. At least three independent experiments were carried out and all gave similar results. One typical experiment is presented.

clear ladder pattern but DNA isolated from aleurone at the distal end showed almost no ladder pattern at all (Fig. 4, lanes A, B). Moreover, after 3 d of imbibition, DNA fragmentation from aleurone cells near the embryo was much more pronounced than in aleurone cells located at the distal region (Fig. 4, lanes C, D). This was also true for aleurone from 4 days of imbibition (Fig. 4, lanes E, F). These data demonstrated that positive TUNEL stained signals in aleurone were due to the fragmentation of DNA into oligomers of about 200 bp fragments. In addition, it was confirmed that aleurone DNA fragmentation occurs only after the production of hydrolytic enzymes has reached a high level during germination in barley aleurone cells.

Morphological changes associated with DNA fragmentation in aleurone nuclei

In order to analyse the cells showing positive TUNEL staining further, higher magnification fluorescence microscopy was used. An overview of the aleurone after 3 d of germination stained with TUNEL is shown in Fig. 5. Aleurone cells located closest to the embryo showed almost all no TUNEL staining any more (Figs 2C, 3, 5), and their cell walls had largely disappeared (Fig. 5A). Eventually these cells became protoplast-like. DAPI DNA staining was still visible in the nuclei of these cells, although with much reduced intensity (Fig. 5B). Aleurone cells located somewhat further away from the embryo showed positive TUNEL staining (Fig. 5C, E). In some aleurone cells there were positive TUNEL stained dots covering the whole cell (Fig. 5C). This suggests that the whole nucleus may have degenerated into small pieces. Cells showing these TUNEL stained dots gave also positive DAPI stained dots and a weakly DAPI stained nucleus (Fig. 5D). These results indicated that clusters of DNA

were out of nuclei and spread into the cytosol. In some aleurone cells, TUNEL staining appeared as brightly stained complete nuclei or as brightly stained spots attached to the nuclear membrane (Fig. 5E). In these cells, a similar DAPI staining pattern was observed as well (Fig. 5E).

Aiming at more detailed information concerning nuclear performance during the process of DNA fragmentation, aleurone cells of germinating grains were studied at the ultrastructural level. Areas of aleurone layers similar to those examined in TUNEL staining were analysed. During very early stages of germination (after 1 d of imbibition), all aleurone nuclei show a characteristic interphase arrangement of the chromosomes. The chromatin is visible as small electron-dense spots, dispersed homogeneously over the nucleoplasm (Fig. 6A). The nuclei are spherical and positioned in the centre of the aleurone cells. However, after 3 d of germination the aleurone cells closer to the embryo showed a gradual condensation of the chromatin (Fig. 6B–D). Although the (double) membrane of such nuclei remained intact (Fig. 6B–E), they frequently lost their spherical appearance (Fig. 6D). Numerous invaginations of the nuclear membrane often resulted in a very irregular nuclear morphology. Coalescence of the condensed chromatin into one, big cluster was also observed in some cells (Fig. 6D). In some cells, the condensed chromatin was located at one side of the nucleus (Fig. 6C). This one side location of chromatin was found with a high frequency in aleurone cells near the embryo and a gradually reduced in frequency in aleurone cells more to the distal end of the grain. In addition, this one side location of chromatin was also observed by fluorescence microscopy (Fig. 5E). Sometimes, an almost empty nucleus with intact nucleoplasm was observed (Fig. 6E). Taken together, these results suggest that in aleurone DNA fragmentation appeared to be associated with condensation of the chromatin, but obviously not with disintegration of the nuclear envelope. Fragmentation of the aleurone cell nuclei into small bodies during later stages of germination was not observed by electron microscopy. Likewise no electron-dense chromatin-like spots were detected in the cytoplasm using electron microscopy. However, the possibility that small degraded DNA fragments became located outside the nucleus, giving rise to the TUNEL staining positive spots dispersed over the cell cytoplasm, can not be excluded, as illustrated in Fig. 5C.

A correlation between cell wall degradation and DNA fragmentation

During germination of barley grains, a range of hydrolytic enzymes involved in the mobilization of the endosperm is produced by the aleurone cells. Starting from the embryo, a gradient of activity can be observed towards

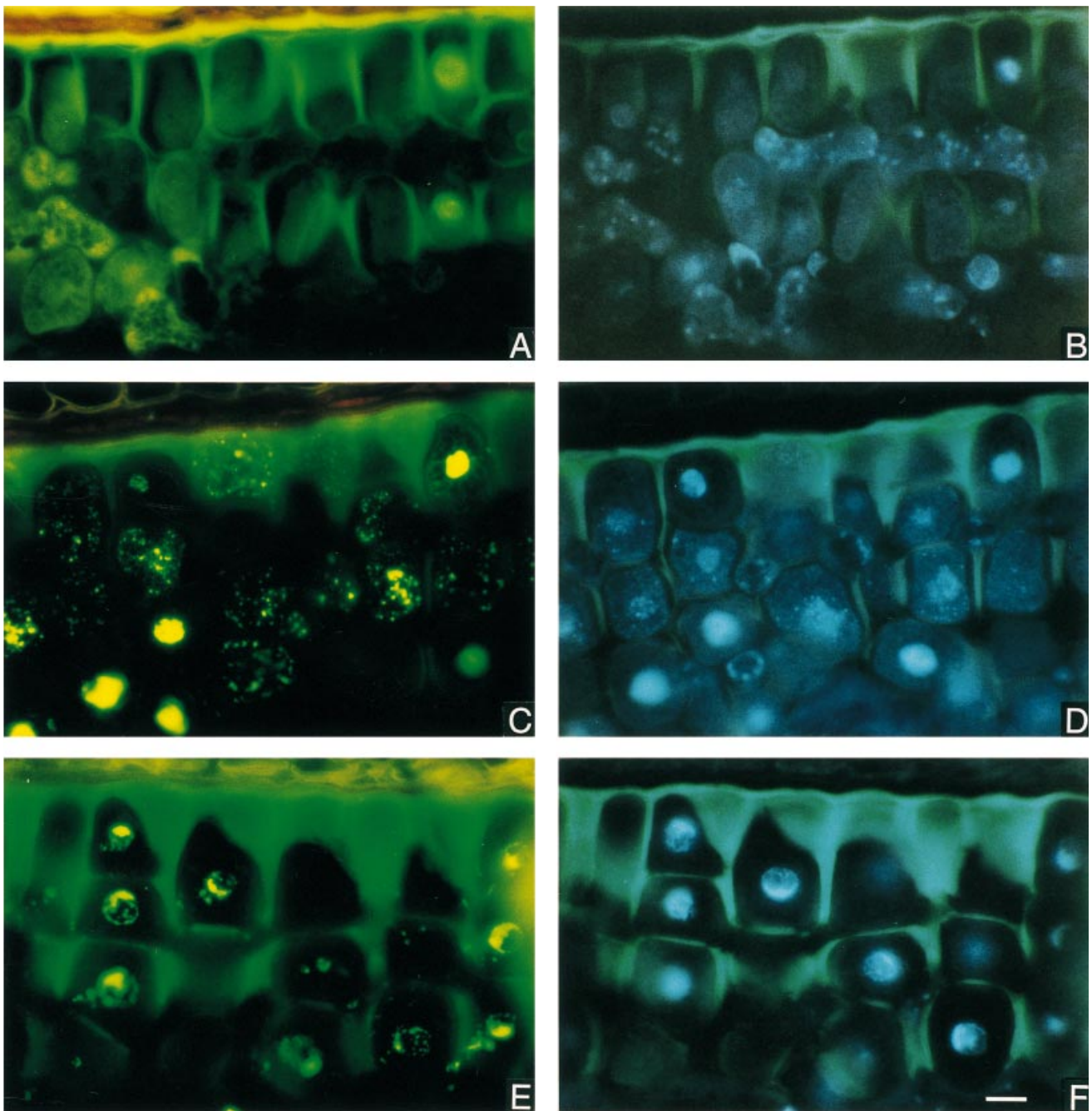


Fig. 5. TUNEL detection of DNA fragmentation in aleurone layers after 3 d of imbibition. TUNEL staining of aleurone section near the embryo (A), aleurone located at about one-third of the grain from the embryo (E) and aleurone between A and E (C). DAPI staining of aleurone section near embryo (B), aleurone located at about one-third of the grain from the embryo (F) and aleurone between A and E (D). At least three independent experiments were carried out and all gave similar results. One typical experiment is presented. Bar in Fig. 5F is 16 μm .

apical aleurone regions. For the cell wall degrading enzyme (1-3,1-4)- β -glucanase it has been demonstrated by McFadden *et al.* (1988) that the mRNAs could be measured firstly in aleurone cells near the embryo. Together with other non-starch polysaccharidases like pentosanases, this enzyme degrades the aleurone cell wall, a prerequisite for the efficient release of starch polysaccharidases. With respect to the activity of (1-3,1-4)- β -

glucanase, this enzyme was found to be first produced in cells near the embryo at day 2 and later (after day 3) in more distal positioned aleurone cells.

Polarized degradation of the aleurone cell wall (Fig. 5) likewise started in the vicinity of the embryo and gradually extended in time to the far distal end of the grain. Examination of the aleurone layer 3 d after the start of germination, therefore, revealed a gradient of stages of

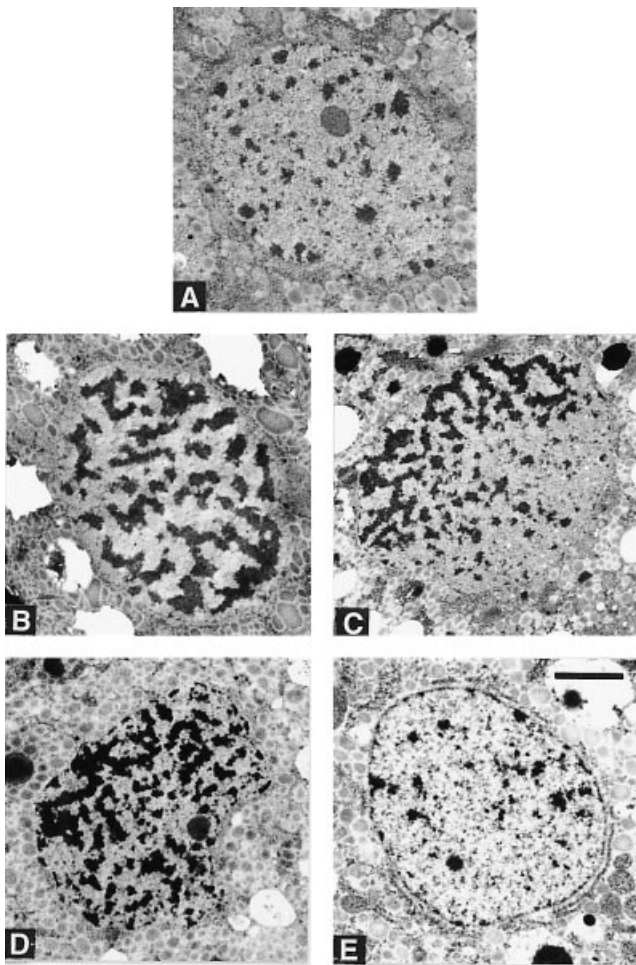


Fig. 6. Electron micrographs of cross-sectioned isolated aleurone layers during germination. (A) Nucleus of aleurone near the embryo from 1 d imbibed barley grains, (B–E) nuclei of aleurone near the embryo from three days imbibed barley grains. Bar in Fig. 6E is 0.42 μm .

aleurone cell activation. The cell wall degradation was intensively present in aleurone cells, first in the walls facing the endosperm (in all three layers of aleurone cells) and then in the lateral cell walls. Evident TUNEL stained nuclei invariably were monitored in the regions in which aleurone cell wall degradative activity was manifest (Fig. 5). This apparent link between *in vivo* aleurone cell wall degradation and the occurrence of apoptosis during germination closely resembles previous observations made during *in vitro*-induced aleurone cell wall degradation by exogenously applied enzymes. Preparation of protoplasts from aleurone dramatically stimulated the process of DNA fragmentation, suggesting that degradation of the cell walls might trigger DNA fragmentation (Wang *et al.*, 1996a). In fact, during germination aleurone cells underwent a kind of auto-protoplastation (Fig. 5A, B). Nuclei with condensed and coalesced chromatin were frequently observed in regions in which extensive aleurone cell wall degradation was prominent.

Discussion

The aleurone function during germination is not only hormonally regulated, but also by diverse environmental factors such as osmotic stress. Activation of aleurone cells and polarized degradation of aleurone cell walls started in the region near the embryo and extended in time to the far distal end of the grain. McFadden and co-workers demonstrated that induction of (1-3, 1-4)- β -glucanase gene expression in the barley aleurone layer progresses from the proximal to the distal end of the grain as a front moving away from, and parallel to, the face of the scutellum (McFadden *et al.*, 1988). The same expression pattern for α -amylase was demonstrated here (Fig. 1). Moreover, the sequence of this pattern corresponds to both endosperm cell wall breakdown and α -amylase secretion in germinating grains (Briggs, 1972; Gibbons, 1980; Palmer, 1982; Nolan and Ho, 1988). Such a pattern indicates that the signal, inducing expression of both α -amylase and (1-3, 1-4)- β -glucanase genes, is probably derived from the embryo and diffuses through the germinating grain. This signal is likely to be the plant hormone gibberellin (GA) produced by the embryo (McFadden *et al.*, 1988), since GA is not only able to induce expression of hydrolytic enzymes such as (1-3, 1-4)- β -glucanase and α -amylase, but is also able to induce expression of lipase and proteases in the aleurone (Jacobsen and Beach, 1985; Mundy and Fincher, 1986; Stuart *et al.*, 1986).

In concert with the spatial-temporal activation of aleurone, the degradation of endosperm occurs first near the embryo and then goes on to the distal end of the embryo. Once the reserves near the embryo are being used, aleurone cells near that part of the endosperm have finished their task. It is supposed that these cells should then enter the cell death programme first, which is supported by the time-course studies of both *in situ* α -amylase expression (Fig. 1) and cellular DNA fragmentation staining (Fig. 2). It is clear that α -amylase mRNA expression in aleurone cells precedes DNA fragmentation.

Programmed cell death or not?

One may ask whether the observed DNA fragmentation in germinating aleurone is due to programmed cell death or that it is perhaps induced by the release of DNases from (dead) aleurone cells. Several observations are in favour of the occurrence of programmed cell death.

First, DNA fragmentation was observed after the detection of α -amylase mRNA expression, but before the decline of α -amylase mRNA level—thus before the cells finished their task (Figs 1, 2, 3).

Second, the occurrence of DNA fragmentation is correlated with cell wall degradation which results in self protoplastation (Fig. 5). In addition, after 3 d of germination, it was observed that DNA fragmentation occurred

in intact cells with no damaged cells in the neighbourhood. It had previously been demonstrated that during *in vitro* protoplast preparation, a strong DNA laddering was observed, which can be inhibited by application of the plant hormone abscisic acid (ABA), while the viability of the protoplasts was identical with or without ABA (Wang *et al.*, 1996a). In addition to this, removal of ABA after the preparation of protoplasts led to DNA fragmentation (Wang *et al.*, 1996a). Moreover, prepared protoplasts (in the absence of ABA) were able to react to the addition of ABA with activation of MAP kinase and expression of Rab mRNA (Knetsch *et al.*, 1996; Van der Veen *et al.*, 1992).

Third, for cell death processes other than apoptosis a smear of broken DNA, instead of a clear fragmentation pattern as seen in our experiments, is usually found (Fig. 4).

Finally, under electron microscopy, an altered morphology of the nucleus and chromatin condensation in the nucleus were observed, which are typical for programmed cell death (Fig. 6). Taking all these arguments together, it was concluded that the observed DNA fragmentation and cellular changes are parts of the processes of programmed cell death in the aleurone layer.

(Ultra)structural observations

The (ultra)structural observations of aleurone cells in the TUNEL positive regions reveal some clear characteristics. Nuclei showed chromatin condensation, but the nuclear envelope apparently remained intact. A similar observation was made by Mittler *et al.* (1997) in TMV-induced programmed cell death in tobacco. Invaginations of the nuclear membrane were observed as well. As in the study by Mittler *et al.* (1997), no apparent apoptotic bodies were seen in the ultrastructural studies (data not shown). The scattering of condensed chromatin over the nucleus and the localization of the condensed chromatin to one side of the nucleus as observed in the ultrastructural observations (Fig. 6C) may be differently appearing forms of the same structure due to cutting of the nuclei under different angles in the preparation. The TUNEL staining, however, argues against this as both forms can also be observed here in a much thicker preparation (Fig. 5). Possibly the localization of the condensed chromatin in one side of the nucleus may be a distinct phase in the apoptotic processes in the nucleus.

The appearing of TUNEL positive-stained spots outside the nucleus could not be confirmed in the ultrastructural study. In addition, the nuclear envelope remained intact, which may indicate that small clusters of fragmented DNA leave the nucleus via the pores in the nuclear envelope. The observation of chromatin free nuclei (Fig. 6E) may be a cutting artefact, but could also indicate that all DNA has been degraded. The fact that, in many cells, no TUNEL

staining was observed but a faint nucleus could still be seen with DAPI staining supports, however, the idea that the DNA is completely degraded in these nuclei. The present ultrastructural observations show that some characteristics found during programmed cell death in animal cells (Wyllie, 1980) and pathogen infected plant cells (Mittler *et al.*, 1997) are also present in aleurone cells during germination.

Triggers for apoptosis

The signal to trigger apoptosis could be the secretion of GA by embryo/scutellum cells (since GA stimulates cell death, Kuo *et al.*, 1996), the osmotic stress which is caused by degradation products of starch in the endosperm (Wang *et al.*, 1996a), the breaking down of the cell walls or a lowering of endogenous ABA levels (Wang *et al.*, 1995). In mammals, over-expression of proteases has been shown to cause apoptosis (see review, Patel *et al.*, 1996). The large production of hydrolases by aleurone cells, specially proteases, may, therefore, be involved in the regulation of apoptosis of these cells as well.

The following hypothesis is proposed for the sequence of events leading to programmed cell death in aleurone during germination. Upon the imbibition, the production of ABA by the embryo prevents premature initiation of hydrolytic enzyme production and apoptosis in the aleurone in order to protect the young seedling under various environmental condition changes. When the environmental condition is suitable to the further growth of the embryo, the synthesis of GA from embryo/scutellum will be a signal to trigger the production of hydrolytic enzymes. Subsequently, when the cells are at the highest level of activity, breaking down their own cell walls, they enter the final processes of programmed cell death.

It is likely that ABA levels play a key role in the regulation of the initiation of programmed cell death. ABA inhibits both α -amylase mRNA expression and DNA fragmentation in aleurone (Mundy and Fincher, 1986; Wang *et al.*, 1996a). In addition, after 4 d of imbibition of dormant barley grains, there were no germination of the grains, no α -amylase production and no positive TUNEL staining in the aleurone (data not shown). This observation was correlated with the findings of a high endogenous ABA level in these dormant grains (Wang *et al.*, 1995). In addition, upon the imbibition, a *de novo* ABA synthesis was observed in these dormant grains as well (Wang *et al.*, 1995). Current investigations focus on the signal transduction cascade by which ABA is able to prevent an aleurone cell entering programmed cell death.

Acknowledgements

We thank RA Schilperoort for stimulative discussion and P Hock for the lay-out of the figures.

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