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Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L.

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Abstract

Intra-nucleosomal cleavage of DNA into fragments of about 200 bp was demonstrated to occur in developing anthers, in which microspores had developed into the mid-late to late uni-nucleate stage *in situ*, i.e. at the verge of mitosis. The same was observed, but to a much larger extent, if these anthers were pre-treated by a hyper-osmotic shock. Pretreatment of anthers before the actual culture of microspores was required for optimal androgenesis of microspores. The use of the TUNEL reaction, which specifically labels 3′ ends of DNA breaks, after intra-nucleosomal cleavage of DNA, revealed that DNA fragmentation mainly occurred in the loculus wall cells, tapetum cells and filament cells. TUNEL staining was absent or infrequently observed in the microspores of developing anthers *in situ.* Electron microscopy studies showed condensed chromatin in nuclei of loculus wall cells in the developing anthers. These observations at the chromatin and DNA level are known characteristics of programmed cell death, also known as apoptosis. Features of apoptosis were infrequently found in microspores from freshly isolated mature anthers. However, most tapetum cells had disappeared in these anthers and the remaining cell structures showed loss of cellular content. The viability of microspores in pre-treated anthers was comparable to those in freshly isolated anthers and almost four times higher than in anthers from control experiments. This observation was correlated with three to four times less microspores showing TUNEL staining and a two times higher level of ABA in the anther plus medium samples than in controls. Addition of ABA to the controls enhanced the viability and lowered the occurrence of apoptosis linked characteristics in the microspores. These data suggest that pre-treatment is effective in stimulating androgenesis because it leads to an increase in ABA levels which protects microspores from dying by apoptosis.

Introduction

The development of haploid or double haploid plants from immature pollen or microspores in culture conditions is called (*in vitro*) androgenesis. For optimal androgenesis to occur three requirements have to be met. Firstly, the developmental stage of the microspores is important [7, 8, 11]. For barley the microspores must be at the verge of mitosis, i.e. in their mid-late to late uninucleate stage. This stage can be recognized when the nucleus is located opposite the germ pore. Secondly, the appropriate hormonal treatment is necessary for induction of plant production [9]. Thirdly, a pre-treatment of the anthers is needed in order to switch the normal gametophytic pathway into a sporophytic development of the microspores. The pre-treatment is stringent, including starvation as well as salt and

osmotic stresses [10, 11]. In the present study, an investigation was made at the molecular level to determine the biological processes by which pre-treatment stimulates androgenesis.

It has been found that a pre-treatment for 4 days in a medium containing 10 mM $CaCl_2$ adjusted with mannitol to 440 mOs/kg leads to optimal androgenesis in barley [10]. In the case of tobacco anthers, it was shown that a pre-treatment with mannitol caused an increase in the endogenous ABA level [12]. Furthermore, it has been suggested that in response to certain stress treatments like osmotic shock and cold, ABA levels increase [1, 2]. High endogenous ABA levels are reported to be correlated with high embryogenic potential of *Pennisetum* and carrot cells [15, 20, 21, 22]. These data clearly correlate with the conditions for optimal androgenesis in barley and thus prompted a study of the role of ABA in this process.

Since cell death in anther tissue is observed during pre-treatment, we were also interested to see whether this is due to programmed cell death, which is also called apoptosis, during the hyper-osmotic stress. Apoptosis is a cell type and tissue specific process, which is often triggered by surpassing certain physiological thresholds related, for example, to osmotic stress, temperature, metabolic block, toxins and low oxygen pressure [6, 29]. According to Sunderland *et al.* [25] for induction of androgenesis, degradation of the tapetum as well as properties of the anther wall are critical and are more akin to those of maturing anthers near to anthesis.

Apoptosis is characterized by a shrinking nucleus, condensed chromatin and fragmentation of DNA into oligomers of 200 bp due to intra-nucleosomal cleavage. It plays an important role in embryogenesis and developmental processes in animals [3, 26, 31]. In plants, it has been demonstrated that environmental stress [23] as well as certain fungal infections can induce cell death with apoptotic characteristics [17, 29]. Characteristic features of apoptosis, have been reported also in development of the suspensory apparatus [32], in tomato root cap cells [29], barley aleurone cells [30] and in diploid parthenogenesis and early somatic embryogenesis of norway spruce [6, see review 13].

Plant hormones have a regulating influence on the process of apotosis [see review 18]. It has recently been demonstrated that ABA inhibits intra-nuleosomal cleavage of DNA in barley aleurone cells [30]. Thus, the evidence indicates that, like in animal systems, apoptosis also plays an important role in plants [e.g. 13, 18].

Materials and methods

Materials

Donor plants of *Hordeum vulgare* L. cv. Igri were grown in a phytotron under conditions described previously [8]. Monoclonal antibody to free (+)ABA was purchased from Idetek (San Bruno, CA). Rabbit anti-mouse alkaline-phosphatase conjugate, (+)ABA and bovine serum albumin (grade suitable for enzyme-linked immunosorbent assay, ELISA) were obtained from Sigma (St. Louis, MO). The cell death fluorescein detection kit for the TUNEL assay was obtained from Boehringer (Mannheim, Germany). Fluorescein di-acetate (FDA) and DAPI came from Sigma and Historesin from Leica Instruments (Germany).

Androgenesis

Selection of materials and culture conditions of anthers were as described [10]. Briefly, anthers containing microspores at their mid-late to late uninucleate stage, i.e. at the verge of mitosis, were isolated from spikes of donor plants. They were pre-treated in a medium, containing 0.37 M mannitol, 10 mM $CaCl_2$, 1 mM $MgSO_4.7H_2O$, 1 mM KNO_3, 200 μM KH_2PO_4, 1 μM KI and 100 nM $CuSO_4.5H_2O$ and adjusted with mannitol to 440 mOs/kg, for a period of 4 days in the dark at 25 °C. Samples were taken at day 0, 1, 2, 3 and 4 for various assays. As a control, anthers in the same developmental stage were incubated in above medium but without mannitol. A pre-treatment gives rise to 50 to 100 plants from cultured microspores isolated from 30 anthers. Under control conditions, no plant production is observed upon further culture in culture medium [10].

Cytological staining

Barley anthers were fixed in 2% glutaraldehyde in PBS (pH 7.3) at room temperature. After dehydration in ethanol, the samples were embedded in Historesin. Longitudinal sections were made with an ultra microtome using glass microtome knives. The sections were collected on glass slides, coated with Biobond (Biocell). For TUNEL staining, sections were incubated in reagents from an *in situ* cell death fluorescein

detection kit. Samples were examined with a fluorescence microscope (Leitz Diaplan). Micrographs were made using a Kodak Gold film (400 ASA). For DAPI staining, 0.02 mg/ml was used for above sections. For the viability test, microspores were mixed with a FDA stock solution (5 mg/ml in acetone) to give a final concentration of FDA of about 0.1 μg/ml. After mounting on a slide and covering with a coverslip, the samples were examined with fluorescence microscopy.

Electron microscopy

Anthers 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 anthers were rinsed (2 h) and postfixed with 1% osmium tetroxide in the same buffer (2 h). After washing in double-distilled water and dehydrated in 70, 80, and 96% (v/v) ethanol for 30 min each, the anthers were dehydrated further in 100% ethanol for 60 min. After impregnation with propylene oxide, the anthers were passed through a propylene oxide/Epon mixture for 2 h. The tissue was left in pure Epon overnight before embedding in flat silicon rubber moulds. The Epon was polymerized at 60 °C for 48 h.

Ultra-thin cross sections of the anthers were made and stained with uranyl acetate and lead citrate solutions for 10 min each. The sections were examined under an electron microscope operating at 60 kV.

ABA extraction and ELISA assay

Anthers were put into Eppendorf vials and frozen with liquid N_2. The contents of the Eppendorf vials were freeze-dried. The media in which anthers were incubated were also freeze-dried. Both types of samples were extracted twice in 0.5 ml methanol (containing 100 mg/l butylated hydroxytoluene and 0.5 g/l citric acid monohydrate) at 4 °C with constant shaking at 150 rpm for 24 h. The vials were centrifuged at 8 000 $\times$ g for 15 min at 4 °C and the supernatants of the samples were pooled. The samples were concentrated in a Speed-vac system (Savant) and the volume adjusted to 25 μl with methanol. The samples were stored at -80 °C until assay. ABA content of anthers plus pre-treatment solution was determined by ELISA as described [28].

DNA and RNA isolation

Samples of anthers containing microspores were used for total RNA isolation and genomic DNA isolation. Anthers were frozen in liquid nitrogen and homogenized as described [27]. DNA was isolated from the samples as described [14]. Thirty anthers were frozen in liquid nitrogen and ground to powder. 750 ml extraction buffer (0.1 M Tris/50 mM EDTA/500 mM NaCl, pH 7.5) and 100 μl 10% SDS was added to the powder. The mixture was incubated for 20 min at 65 °C. After incubation, 250 μl 5 M potassium acetate was added and mixed well with the lysed cells. The mixture was kept on ice for 30 min and centrifuged at 14 000 rpm. The supernatant was transferred to a new tube and mixed with one equal volume of iso-propanol and immediately centrifuged for 5 min at 14 000 rpm. The pellet was dissolved in 250 μl TE and 250 μl CTAB buffer (0.2 M Tris pH 7.5, 50 mM EDTA, 2 M NaCl and 2% =Cetyl-N,N,N-triethylammonium bromide, CTAB) was added. Samples were incubated for 15 min at 65 °C and subsequently extracted with one volume of chloroform. After centrifugation the water phase was transferred to a new tube and precipitated with one volume of iso-propanol, followed by immediate centrifugation at 14 000 rpm. The DNA pellet was dissolved in 50 μl TE containing 1 mg/ml RNAse A. RNA was isolated from liquid N_2 disrupted microspores or anthers using a Stratagene micro RNA isolation kit. Quantification of DNA and RNA was carried out by measuring OD_{260} as described by Sambrook *et al.* [24].

Experimental data

The data presented are mean values $\pm$SD unless otherwise stated. Significance of differences in mean values were tested with Student's *t*-test.

Results

DNA fragmentation in anther cells during development in situ

Anthers that have developed into a stage in which the uninucleate microspores are at the verge of mitosis are often used in androgenesis. Such microspores have the largest potency of regeneration into plants [8]. Gel electrophoresis of DNA isolated from freshly isolated anthers in this stage showed a clear banding pattern or 'ladder', demonstrating that intra-nucleosomal cleavage of DNA into about 200 bp oligomers had taken place. Smearing of DNA could also be seen (Figure 1, lane D). The TUNEL reaction [4], which labels the 3′ ends of DNA strand breaks, was used in order

Figure 1. DNA fragmentation in developing and mature anthers. Genomic DNA (1 μg per lane), isolated from developing (D) and mature (F) anthers, were loaded on a 1% (w/v) agarose in TBE buffer, stained with ethidium bromide and separated electrophoretically. M: the molecular weight range 564 bp–21.2 kb marker of lambda DNA digested with *Hin*dII/*Eco*RI. At least three independent experiments with all similar results were carried out and one typical experiment is presented.

to confirm the occurrence of intra-nucleosomal DNA cleavage and to identify the cells in which this process took place (Figure 2A and C). The TUNEL reaction mainly stained the nuclei of cells in the loculus wall cells and tapetum cells but not in microspores (Figure 2A and C). Nuclei of filament cells were also TUNEL stained (Figure 2A and B). In order to give a clear picture of the location of different tissues within an anther, a phase contrast microscopy photo of Figure 2C and a representative drawing of a cross section of an anther are presented in Figure 2D and E. Figure 2D shows the location of tapetum cells which contain almost no cell wall.

Since cell walls of all observed microspores and loculus wall cells showed a positive fluorescent staining, it was necessary to check if this positive signal is due to either non-specific staining or autofluorescence. To this end a control experiment was carried out using anther samples incubated for 4 days without pre-treatment. Some microspores and loculus wall cells under these conditions show intra-nucleosomal DNA cleavage (Figure 3). For each sample two sections were made; one for a TUNEL staining and one without any staining. Fluorescence microscopy clearly established that the section without any staining showed autofluorescence of the cell walls but no fluorescence in the nuclei of cells (Figure 3B). The section on which the TUNEL staining had been applied showed some cells with fluorescence in both cell walls and nuclei (Figure 3A). This demonstrated the specificity of the TUNEL reaction.

Developing anthers *in situ* become mature about 10 to 11 days after mitosis of the microspore. DNA isolated from mature anthers showed no ladder pattern (Figure 1 lane F) after gel electrophoresis. In contrast to the developing anthers, the mature anthers showed disappearance of tapetum cells and only some nuclei of loculus wall cells staining with the TUNEL reaction (Figure 2B). Our observations suggest that during anther development loculus tapetum and wall cells were dying due to apoptosis resulting in the absence of these cells in mature anthers. Partial disappearance of filament cells was also observed (Figure 2B).

Since the presence of condensed chromatin is another characteristic feature in apoptotic cells we analysed the late-developing anther by electron microscopy. At this stage, tapetum cells had disappeared (see arrow in Figure 4A). This demonstrated the presence of condensed chromatin in nuclei of loculus wall cells (Figure 4A–D) of developing anthers. Cells in two different stages of disintegration of the nucleus could be observed in the loculus wall cells (Figure 4A) corresponding to the TUNEL-positive structures (Figure 2C). The condensed chromatin in the nuclei of the developing anther shown by electron microscopy (Figure 4), together with the data obtained with gel electrophoresis and the TUNEL reaction demonstrating intra-nucleosomal DNA cleavage (Figures 1 and 2), indicated that developing anther loculus cells may be in the process of apoptosis indeed.

The fate of anther cells during pre-treatment

Gel electrophoresis of DNA from hyperosmotic stress pre-treated anthers in which the uni-nucleate microspores are at the verge of mitosis, showed a clear 'ladder' pattern in samples taken after 1 and 2 days of incubation (Figure 5). This indicated extensive intra-nucleosomal cleavage of DNA. A smear of DNA degradation products was observed as well (Figure 5). It was evident that the degree of DNA fragmentation during pre-treatment was strongest during the first two days. After 4 days of pre-treatment only a high molecular band was detected. This genomic DNA band must represent DNA from surviving cells and microspores. When anthers were cultured without pre-treatment, much weaker DNA fragmentation 'ladders' were observed at day 1 of incubation (Figure 5). Apparently, cells in anther tissue were dying independently of the pre-treatment conditions. But pre-treatment conditions induced a much stronger DNA fragmentation as compared to control conditions (without pre-treatment).

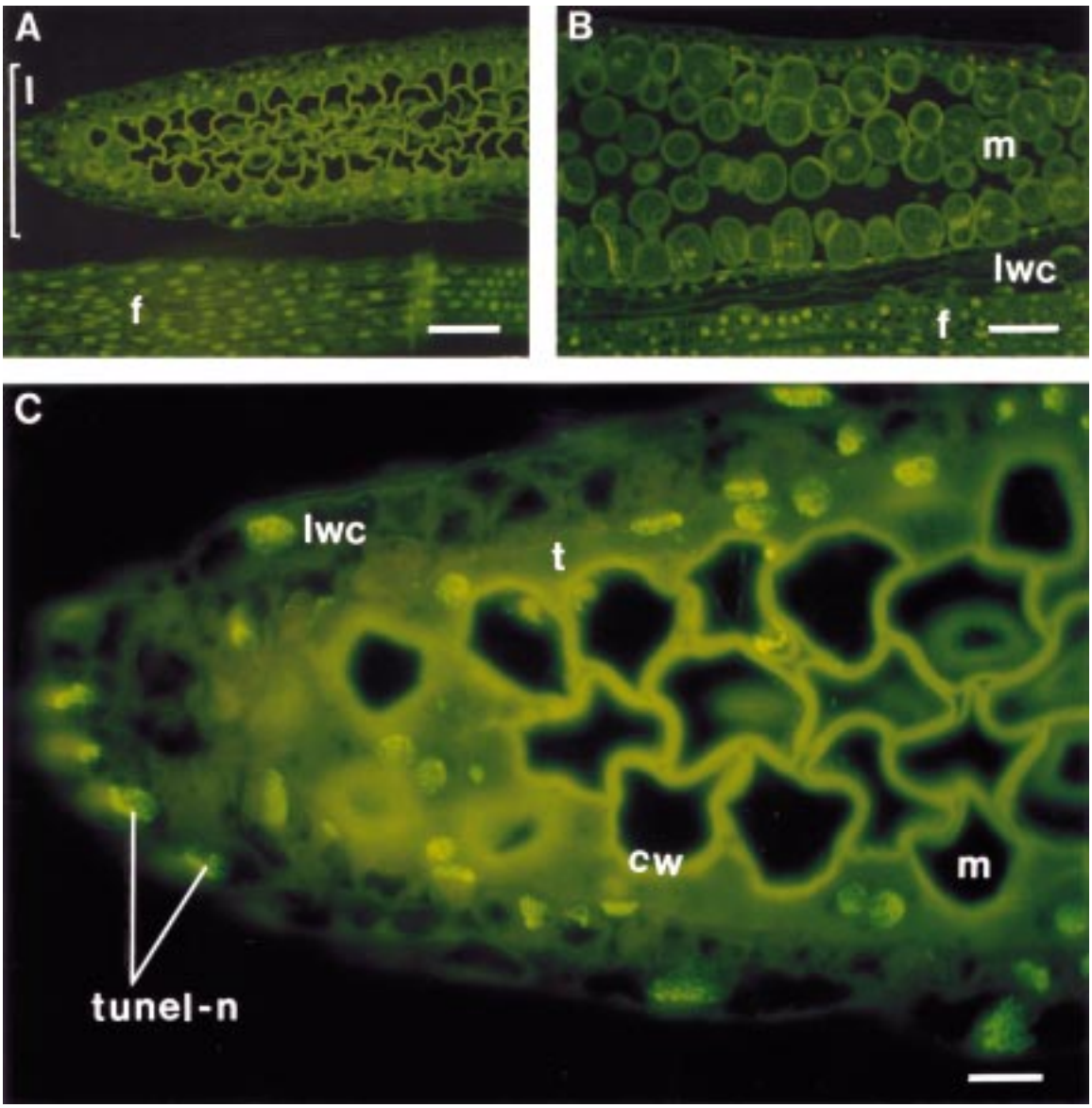

Figure 2. TUNEL staining of developing and mature anthers. TUNEL staining of anther sections from freshly isolated developing anthers (A). Freshly isolated mature anthers (B) and a higher magnification of A (C). A phase contrast microscopy photo of C (D). For each sample, 5 to 10 anthers were analysed and representative examples are presented. cw, cell wall; f, filament, l, loculus; lwc, loculus wall cells; m, microspore; t, tapetum cell; TUNEL-n, TUNEL-stained nucleus. Bars in A and B are 150 μm and in C and D are 30 μm. A representative drawing of a cross section of an anther; c, connective tissue such as filament; e, epidermis; l, anther locule containing epidermis, tapetum, loculus wall cells and microspore etc.; t, tapetum; m, microspore, v, vascular bundle.

Under pre-treatment conditions, also a significant decrease of both DNA and RNA levels was observed (Table 1). After 4 days of pre-treatment, the amount of total RNA obtained from microspores of 30 anthers was 1.5 ± 0.6 μg. This is not significantly different from the amount of RNA isolated (1.7 ± 0.7 μg/30 anthers) from 30 complete anthers after 4 days of the pre-treatment (Table 1). These data suggest that in pre-treated anthers all RNA is derived from the microspores and not from the anther tissue. This means that the genomic band detected after 4 days of pre-treatment represents the DNA from viable microspores, while most or all of the anther cells are degraded. The data from gel electrophoresis and nu-

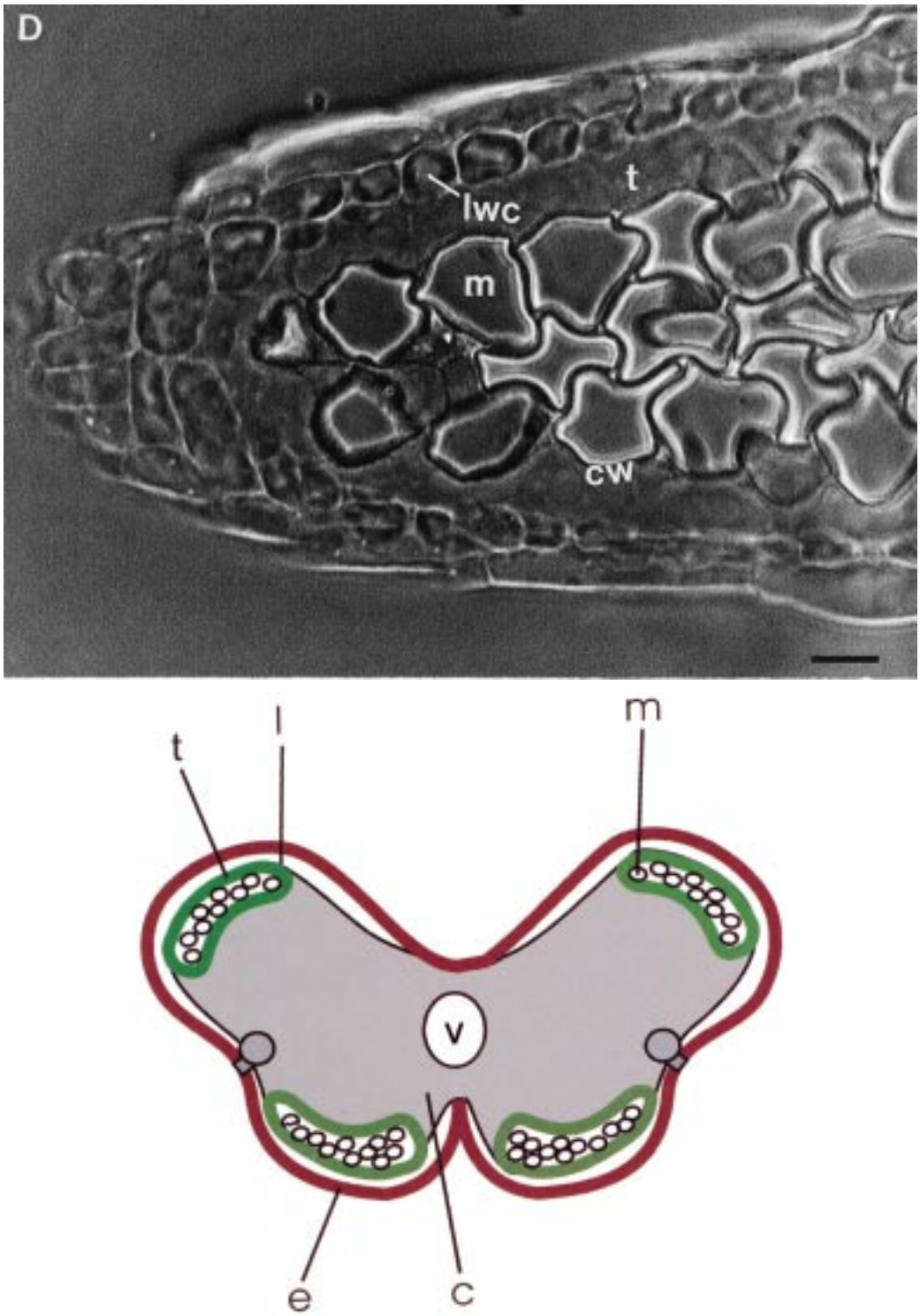

Figure 2. Continued.

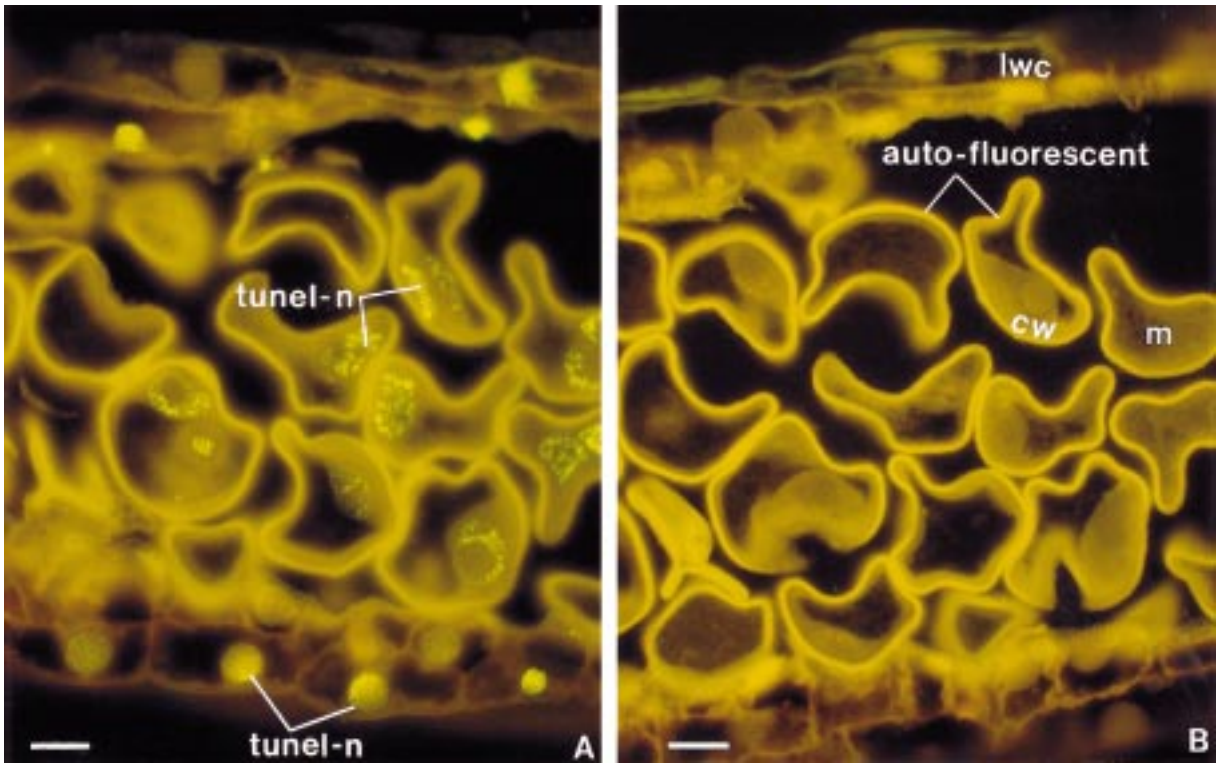

Figure 3. Fluorescence microscopy on sections with or without TUNEL staining. Anther sections from 4 days directly cultured anthers without pre-treatment were used for the observation. A. TUNEL staining. B. The same anther sample but the second section was observed under fluorescence microscopy without any staining. In total, 5 anthers were used. Autofluorescence on cell wall and TUNEL-stained nucleus are indicated in the figure. cw, cell wall; m, microspore; lwc, loculus wall cells; Representative examples are presented and bars in A and B are 30 μm.

Table 1. Quantification of genomic DNA and total RNA (in μg per 30 anthers) isolated from anthers during pre-treatment. Means $\pm$SD of three independent experiments are presented.

Days of pre-treatment	Anther DNA (μg per 30 anthers)	Anther RNA (μg per 30 anthers)
0	28.3 $\pm$ 1.2	24.3 $\pm$ 5.1
1	13.5 $\pm$ 1.3	15.4 $\pm$ 9.2
2	10.4 $\pm$ 1.8	7.6 $\pm$ 4.0
3	2.4 $\pm$ 0.5	2.6 $\pm$ 0.8
4	2.1 $\pm$ 0.3	1.7 $\pm$ 0.7

cleic acid measurements suggest that anther cells have suffered from apoptosis during pre-treatment.

The viability of the microspores in anther tissue with or without pre-treatment was investigated. It was found that the viability of the microspores was constant during pre-treatment, while a pronounced decrease in viability was observed in samples after two days of incubation without pre-treatment conditions (Figure 6A). In order to see whether intra-nucleosomal DNA cleavage had occurred, and to identify the cells suffering from this degradation, longitudinal sections of the anther tissue were stained using the TUNEL reaction. The degradation of anther loculus wall cells could be clearly seen both after 4 days of pre-treatment and without pre-treatment (Figure 7 A and C). The similar morphological appearance was also observed after 2 days of incubation but pre-treatment caused an advanced degradation of anther loculus wall cells (data not shown). We found that in anthers incubated for 4 days without pre-treatment, about one third of the microspore population showed a positive TUNEL-stained nucleus (Figure 7C and D). Anthers pre-treated for 4 days, however, had less than 10% of microspores showing TUNEL-positive nuclei (Figure 7A and B). These data are well in agreement with the viability assays. In addition, some TUNEL positive binuclear cells were observed (Figure 7D) in control (no pre-treatment) conditions. Also different stages of nuclear DNA fragmentation were observed

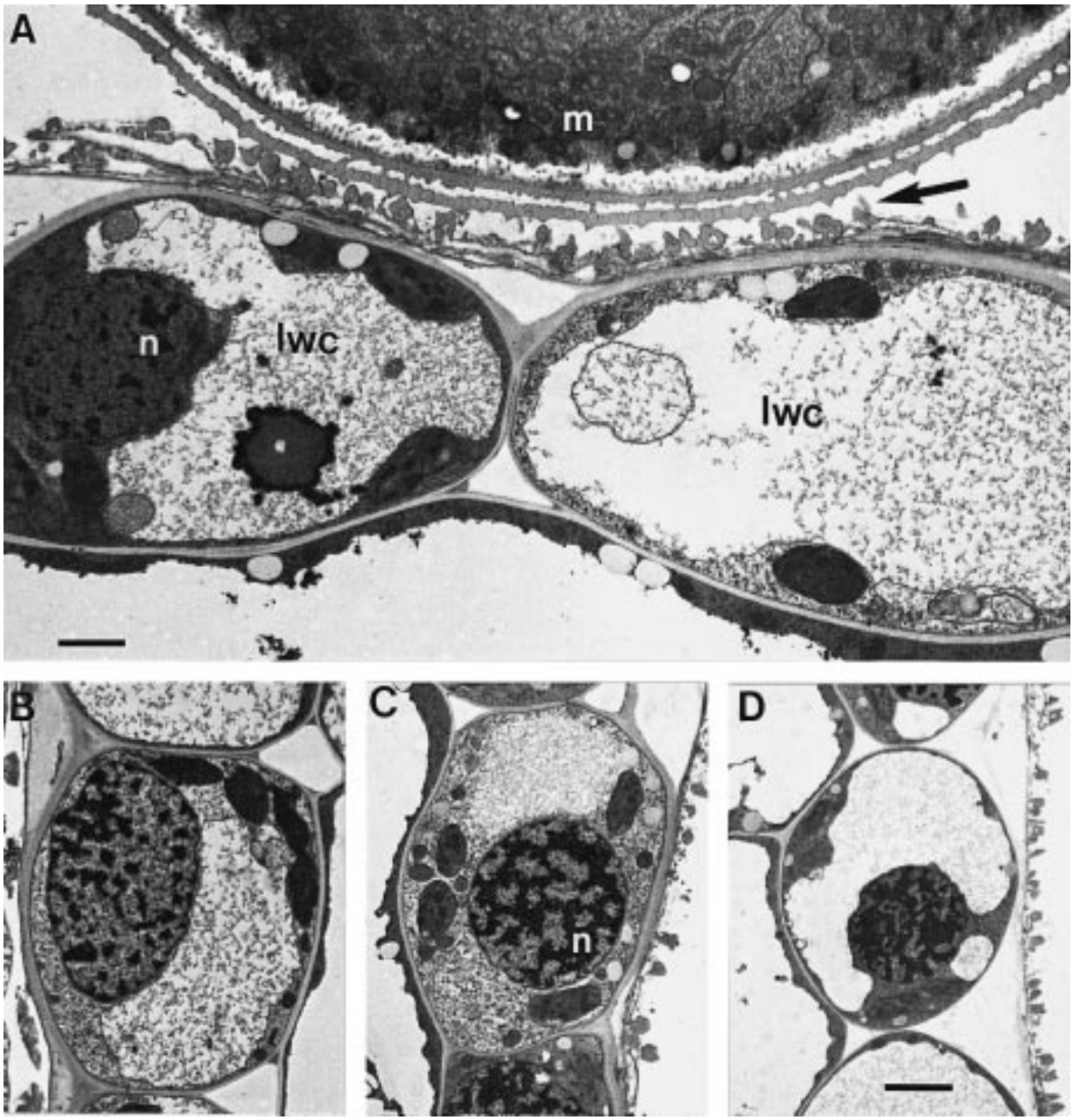

Figure 4. Ultra-structure of anther wall cells. A: Anther wall cells in various stages of disintegration of nucleus with incipient condensation. arrow, remnants of tapetal orbicular; lwc, loculus wall cells; m, microspore; n, nucleus. Bar is 1 μm. B to D. Loculus wall cell with condensed chromatin structure. Early stage with still recognizable organelles and fuzzy nuclear inclusions. Bar is 2 μm.

in microspores incubated under control conditions (without pre-treatment) for 4 days (Figure 7 E–H). The similar morphological appearance of microspores was also observed after two days in incubation (data not shown).

In order to get more quantitative data on the percentages of TUNEL-stained microspores and percentages of viability, the same TUNEL staining experiments were performed on isolated microspores from both anthers with or without pre-treatment and developing anthers. After fixation and embedding in Historesin, sections of the microspores were used for TUNEL and DAPI staining (Table 2). In addition, the viability of microspore in different samples was de-

termined. In each microspore section, the number of TUNEL stained microspores was quantified, and the microspores showing positive nuclear DAPI staining were used as reference. About 18% of the microspores from freshly isolated developing anthers in their mid-late to late uninucleate stage (Table 2), were stained with TUNEL and showed a rather dense cytoplasm. Viability of these microspores was about 56%. Only 7% of the microspores which were isolated from 4 days pre-treated anthers were stained with TUNEL (Table 2, Mannitol) and these microspores were much more vacuolated. Viability of the microspores from 4 days pre-treated anthers was about the same as found for microspores from the freshly isolated anthers. About 33% of microspores isolated from anthers which were cultured without pre-treatment, were positively stained with TUNEL (Table 2, Medium). These microspores showed a similar morphology to microspores from freshly isolated anthers. Viability of microspores cultured without pre-treatment decreased to about 16% (Table 2, Medium).

Furthermore, it can clearly be seen that after 4 days of culture with and without pre-treatment, a loss of anther cells had occurred and that the arrangement of microspores in the anther loculus had changed (Figure 7A and C). Thus, under both conditions, disappearance of tapetum cells and a reduction of the number of loculus wall cells occurred (Figure 7A and B). However, under pre-treatment conditions, a more pronounced reduction of loculus cells took place than in the control without pre-treatment. These data are in good agreement with the DNA fragmentation data (Figure 5).

The role of ABA during pre-treatment

It has been demonstrated that the plant hormone ABA is able to inhibit both intra-nucleosomal DNA cleavage in barley aleurone cells [30] and death of wheat aleurone cells [16]. Since our anther pre-treatment involved both starvation and osmotic stress, which was correlated with less death of microspores, we studied the effect of pre-treatment on ABA levels. Total ABA levels were measured in anthers (extracts from freeze-dried anthers plus incubation medium) with and without pre-treatment. Figure 6B shows that when anthers were cultured with or without pre-treatment, ABA levels increased after one day of incubation under both conditions. However, the ABA level continued to increase during the pre-treatment of the anthers but not in the case of untreated anthers (Figure 6B). After 4

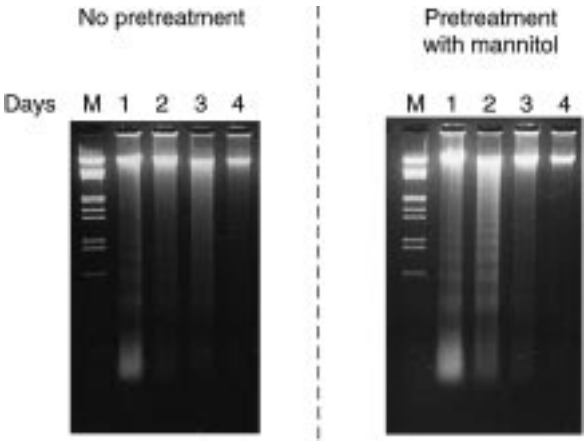

Figure 5. DNA fragmentation in anthers with or without pre-treatment. Genomic DNA (1 μg per lane), isolated from anthers incubated for various times, was loaded on a 1% (w/v) agarose in TBE buffer, stained with ethidium bromide and separated electrophoretically. The number above each lane shows the number of days of pre-treatment. M: the molecular weight range 564 bp–21.1 kb marker of lambda DNA digested with *Hin*dII/*Eco*RI. At least four independent experiments with similar results were carried out and one typical experiment is presented.

days of pre-treatment, the total ABA level in the samples was about twice the ABA level found in samples not pre-treated (Figure 6B).

Since a higher ABA level was found under pre-treatment conditions than under control conditions, we assume that the increase of the ABA level during pre-treatment was due to starvation in combination with salt and osmotic stress. Our observation, showing a correlation between a high ABA level and a constant level of microspore viability in anthers during mannitol pre-treatment, is in line with the reported data on the role of ABA in cell death [16, 30]. In the light of these data we propose that ABA at the higher level acts as an inhibitor of cell death in microspores, preventing a decrease in the level of viability (Figure 6A). Furthermore, we observed by use of the TUNEL reaction that the DNA degradation in the microspores occurred via a characteristic intra-nucleosomal cleavage suggesting that the death of microspores (as shown for the anther cells) was due to programmed cell death. If our hypothesis is indeed correct, an artificial increase of ABA levels in anthers without pre-treatment should directly affect both DNA fragmentation and viability of microspores. Table 2 shows that the addition of ABA to anthers cultured without pre-treatment (Medium + ABA) causes a significant increase in the percentage of viable FDA-stained microspores if we compare the value with that obtained for microspores from anthers

Table 2. Percentages of both positive TUNEL stained and FDA stained microspores isolated from fresh anthers before culture, 4 days of anther culture with and without pre-treatment both in the presence or absence of ABA. Results presented here are from three independent experiments with more than 500 microspores counted in each independent experiment. n.s., not significantly different ($P > 0.95$) from value obtained from samples A. *($P < 0.05$) from value obtained from sample E.

Samples	TUNEL staining as% of DAPI positive microspores (means ± SD)	% FDA staining of microspores (means ± SD)
A. Freshly isolated anthers	18.0 ± 7.1	56.0 ± 3.3
B. Mannitol	7.0 ± 4.0 n.s.	58.0 ± 9.4 n.s.
C. Mannitol+ABA	11.5 ± 4.4 n.s.	55.0 ± 16.0 n.s.
D. Medium	32.6 ± 4.8*	15.5 ± 10.0*
E. Medium+ABA	6.3 ± 4.9 n.s.	34.8 ± 6.0 n.s.

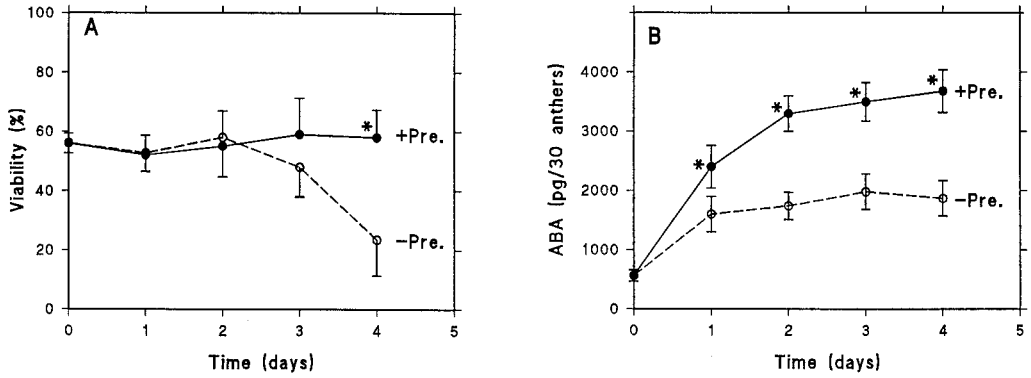

Figure 6. Viabilities of microspores and total ABA content in anthers during pre-treatment. A: FDA staining of microspores during pre-treatment and without pre-treatment. At each time point, 30 anthers were used for both viability test and ABA level assay. For ABA assay, the total extracts from both anther and incubation medium. The means ± SD of three independent experiments are presented in both A and B. *Mean value significantly different ($P < 0.05$) from value obtained at the same time point from control (-Pre.).

not pre-treated (Table 2, Medium). Moreover, in these samples, a significant decrease in the percentage of positively TUNEL-stained microspores was observed as well. Addition of 1 μM ABA to anthers during mannitol pre-treatment, however, had no significant additional effect on the percentages of both viability and TUNEL-stained microspores (Table 2, Mannitol + ABA).

Discussion

According to Sunderland *et al.* [25], for induction of androgenesis, degradation of the tapetum as well as properties of the anther wall are critical and are more akin to those of maturing anthers near to anthesis. Comparison with literature data from microspore development *in vivo*, reveals that in most species the tapetum begins to degenerate shortly after the first pollen mitosis and is usually not present any more at anthesis. Therefore, anther dehiscence *in vivo* involves programmed destruction of specific cell types [5]. The development of barley anthers, starting from the stage around the first microspore mitosis, into mature anthers normally takes about 10 to 11 days (Hoekstra *et al.*, unpublished data).

In developing anthers at the verge of mitosis and even much stronger in anthers after 4 days of pre-treatment, intra-nucleosomal DNA cleavage was observed. The TUNEL assay showed that this kind of degradation mainly involved loculus tapetum and loculus wall cells (Figures 1 and 2). Anthers without pre-treatment also show clearly the loss of loculus tapetum and wall cells and DNA fragmentation by using the TUNEL assay (Figure 7, C and D). This could not very clearly be demonstrated by DNA gel

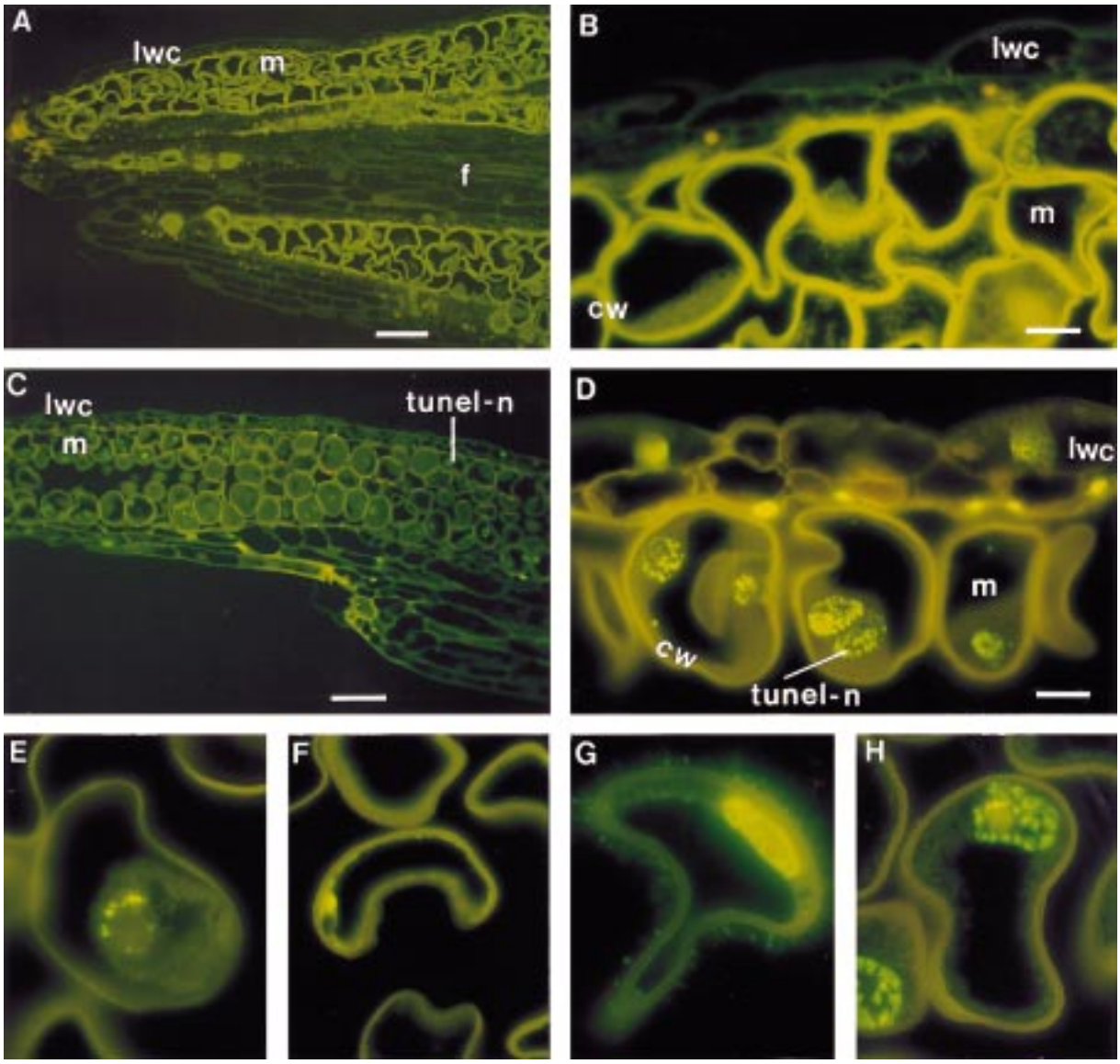

Figure 7. TUNEL detection of DNA fragmentation in anthers with and without pre-treatment for 4 days. TUNEL staining of anthers with mannitol pre-treatment (A, B) and anthers incubated in medium for 4 days without pre-treatment (C, D). TUNEL staining in microspores under control conditions (without pre-treatment) for 4 days (E to H). For each sample, 5 to 10 anthers were analyzed and representative examples are presented. cw, cell wall; m, microspore; lwc, loculus wall cells; TUNEL-n, TUNEL-stained nucleus. Bars in A and C are 150 μm. Bars in B and D are 30 μm.

electrophoresis (Figure 5). Lack of synchronization in DNA fragmentation could clearly affect the DNA banding pattern [30]. Therefore, the results from the DNA gel electrophoresis might in part be explained by less synchronization in anthers without pre-treatment than in anthers with pre-treatment (Figure 5). Our data point out that degradation of loculus tapetum and wall cells occurred in developing barley anthers already before the onset of microspore mitosis.

Detection of broken 3′ ends of DNA by the TUNEL reaction and a 200 bp 'ladder' seen upon gel electrophoresis of DNA, showing fragmentation of DNA into oligomers of oligonucleosome-sized fragments, as observed in the present study, is considered to be a hallmark for programmed cell death, which also is called apoptosis [4, 19]. Raff describes shrinkage or fragmentation of the nucleus and cytoplasm as another characteristic of apoptosis [4]. In our study,

the presence of condensed chromatin in several nuclei of cells of the anther wall cells was demonstrated by EM studies indeed (Figure 4). Thus, all the mentioned criteria for apoptosis are demonstrated in our study. Therefore, the degradation of the tapetum described by Sunderland *et al.* [25] and 'the programmed destruction' of a specific cell type in anthers reported by Goldberg *et al.* [5], therefore, are likely to be due to apoptosis.

Upon harvest of anthers, the connection with the vascular tissue is interrupted and the anthers are placed in a pre-treatment medium containing 10 mM $CaCl_2$ and adjusted with mannitol to 440 mOs/kg. The exposure of anthers to both of these stress conditions apparently stimulated ABA production, since under pre-treatment conditions much higher ABA levels were reached than in the control. As the anther tissue is degrading, the microspores become gradually exposed to the pre-treatment solution containing an increasing level of ABA. The pre-treatment-induced level of ABA probably might act as an inhibitor of apoptosis in ABA susceptible microspores which have enlarged under the osmotic circumstances. The speculated role of ABA during pre-treatment of microspores is supported by the following observations.

- A continuous increase of the ABA level during pre-treatment of anthers (Figure 6B) was correlated with both a constant viability of microspores in these anthers, and a low percentage of microspores showing DNA fragmentation as measured with the TUNEL reaction (Figure 6A, Table 2, Mannitol).
- The relatively low level of ABA in cultured anthers without pre-treatment (Figure 6B) was correlated with a low percentage of viability of microspores and a high percentage of microspores carrying fragmented DNA as indicated by the TUNEL reaction (Figure 6A, Table 2, Medium).
- Addition of ABA to a medium in which anthers were incubated without pre-treatment, significantly increased the percentage of viable microspores and strongly lowered the percentage of microspores showing fragmentation of DNA as indicated by the TUNEL reaction (Table 2, Medium + ABA).
- It has been demonstrated that ABA is able to inhibit both intra-nucleosomal cleavage of DNA and the death of barley aleurone cells [30] and the death of wheat aleurone cells [16].

Our data suggest that pre-treatment is effective in stimulating androgenesis because it leads to an increase in ABA levels which protects ABA susceptible microspores from death by apoptosis. It is not yet known, however, where ABA is synthesized in the anthers. It is also not known whether the increased levels are due to a higher synthesis or less break-down.

The knowledge about the occurrence of apoptosis in microspore regeneration may enable us to develop new tools for improving androgenesis methods and to obtain a better understanding of the signals and molecular switches involved in apoptosis and development.

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