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CELL STRUCTURAL REORGANIZATION DURING INDUCTION OF ANDROGENESIS IN ISOLATED MICROSPORE CULTURES OF TRITICALE (×*TRITICOSECALE* WITTM.)

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Upon stress treatment, isolated microspores of triticale (×*Triticosecale* Wittm.) were directed towards sporophytic development (androgenesis). We used fluorescence microscopy to study the cell structural reorganization associated with the process. Changes in the developmental pathway coincided with the character of the microtubular cytoskeleton configuration, the number and direction of nuclear divisions, changes in vacuolization, the distribution of mitochondria, ER and starch grains, and the architecture of new cell wall formation. A band of diffused fluorescence surrounding the nucleus was observed before the first symmetric division of microspores. This structure most likely represents a preprophase band (PPB). Successive mitotic divisions within the microspore wall led to the formation of multinucleate or multicellular structures consisting of one or two domains of cells differing in size. They were later released from the sporoderm and continued further development with features typical for a monocotyledonous embryo. The pattern of internal architecture of androgenic structures depended on their developmental phase. Before and after release from the microspore wall, cortical microtubules (MTs) exhibited various configurations without preferential orientation. They formed a denser network in the region opposite to the sporoderm rupture site. Released multicellular structures showed both intensely fluorescing cortical MTs and more dispersed endoplasmic MTs radiating along the cytoplasmic strands from the nuclear region to the cell cortex. Up to globular stage, isotropically expanding cells of androgenic embryos showed a random pattern of MTs. This is the first report that successive events of androgenic development of triticale microspores are associated with MT reorganization. The results support the view that changes in cytoskeleton architecture are critical during induction of androgenesis.

Key words: Androgenesis, microspores, triticale, cytoskeleton, microtubules.

INTRODUCTION

Isolated and in vitro cultured microspores or immature pollen grains of many angiosperms enter the sporophytic developmental pathway (androgenesis) under specific stress conditions (Cordewener et al., 1994; Custers et al., 1994; Reynolds, 1997; Touraev et al., 1997). Embryogenesis from isolated immature pollen or microspores is regarded as the most efficient system for production of doubled haploid plants, and is routinely used in many broad-acre crops such as wheat, barley, maize and rye (Kasha et al., 2003; Pulli and Guo, 2003; Zheng et al., 2003). Doubled haploid technology offers a tool for rapid production of homozygous breeding lines which are either multiplied and released as cultivars or else used as recombinant inbred lines for molecular mapping or as parents in breeding programs. Isolated microspore cultures can be used as alternative systems in biochemical analysis and molecular genetics (Mordhorst et al., 1997; Touraev et al., 1997; Pauk et al., 2000, 2003; Boutilier et al., 2002) and as a model for cytological and physiological studies of embryo development (Magnard et al., 2000; Indrianto et al.,

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2001; Ramírez et al., 2001; de Maraschin, 2005; Massoneau et al., 2005; Pulido et al., 2005; Supena et al., 2008). Due to homologies between early phases of zygotic and microspore embryogenesis (Testillano et al., 2002; Supena et al., 2008), the results of cellular and ultrastructural studies in isolated microspore cultures can be related to embryo development in planta (Ramírez et al., 2001).

In *Brassica napus* it has been shown that changes in the position of the mitotic spindle coincide with the change of developmental pathway of microspores, leading to their androgenic development (Hause et al., 1993; Simmonds and Keller, 1999). However, it is not known how the cytoskeleton configuration (and changes in it) are involved in the switch to androgenesis in the monocot microspore. Testillano et al. (2002) showed immunofluorescence of [beta]-tubulin in cytoplasm of proembryo-like multicellular structures derived from microspores, but did not obtain images showing the cytoskeleton configuration. In this work we used the monocot species triticale to study the structural organization of the microtubular cytoskeleton during androgenic development in isolated microspore and young bicellular pollen culture.

Microtubules (MTs) and actin filaments (AFs) are dynamic structures playing an integral role in determination of cell architecture and in the process of cell division (Volkmann and Baluška, 1999; Staiger et al., 2000; Wasteneys, 2002). The network of MTs underlying the plasmalemma, known as cortical microtubules (CMTs), and MTs penetrating the cell cavity, known as endoplasmic microtubules (EMTs), help to regulate the establishment of the direction of cell division and cell shape. They also take part in intracellular transport and determine the polarity of cell expansion (Straight and Field, 2000; Verma, 2001; Stoeckel and Takeda, 2002). CMTs are involved in cellulose-microfibril deposition in the cell wall (Giddings and Staehelin, 1991; Goddard et al., 1994). The MT cytoskeleton is present throughout the cell cycle of plant cells in four distinct arrays. The basic array of CMTs and EMTs is clearly distinguishable in interphase. Beside the interphase array one can distinguish (1) the preprophase band (PPB), usually seen as a dense ring of MTs surrounding the nucleus prior to division and marking the future division plane, (2) the mitotic apparatus, segregating the chromosomes during division, and (3) the phragmoplast, involved in production of the new cell wall following nuclear division.

During gametophytic pollen development the peripheral position of the nucleus is secured by MT and AF organization (Hause et al., 1992). This microspore asymmetry seems to be very important for gametophytic development. Treatment with substances like colchicine or cytochalasin D, which depolymerize microtubules and result in the nucleus taking a central position, can trigger microspore reprogramming (Zaki and Dickison, 1991; Barnabás et al., 1999; Simmonds, 1994; Zhao et al., 1996; Gervais et al., 2000; Obert and Barnabás, 2004). Studies on isolated microspore cultures of tobacco and rape seed (Telmer et al., 1995; Touraev et al., 1996a, 1997) led to the idea that the direction of microspore development is determined by the pattern of the first mitotic division. According to this scenario, asymmetrical division of the nucleus results in gametophytic microspore development, while disruption of polarity followed by symmetric division induces sporophytic development. Simmonds and Keller (1999) suggested that symmetric division is important for formation of preprophase bands and then the cell wall. However, microspores can develop into pollen grains even after the first symmetrical division (Eady et al., 1995), and colchicine treatment does not always lead to symmetric microspore divisions (Barnabás et al., 1999). Nevertheless, the involvement of cytoskeleton rearrangement in induction of microspore embryogenesis is supported by much data (Zaki and Dickison, 1991; Barnabás et al., 1991; Simmonds, 1994; Zhao et al., 1996; Gervais et al., 2000; Obert and Barnabás, 2004). According to Zoriniants et al. (2005), the MT cytoskeleton may also influence microspore reprogramming indirectly by interacting with cell cycle-dependent kinases.

To our knowledge, our study presents the first documentary data showing changes in MT configuration during microspore embryogenesis of monocotyledonous plants. To obtain clear images of MT we optimized the immunolocalization protocols for sectioned material to improve preservation and visualization of cytoskeletal MTs in the triticale microspore during androgenesis at successive stages leading to the formation of embryo-like structures (ELSs). Our aim was to show whether the changes in the MT configuration during androgenesis are specific to this process.

MATERIAL AND METHODS

DONOR PLANTS AND GROWTH CONDITIONS

The spring cultivar 'Wanad' of hexaploid triticale (×*Triticisecale* Wittmack) used in this experiment was selected for its relatively good response to microspore culture protocols. Donor plants were grown in a greenhouse at 25°C with a 16 h under natural light supplemented with sodium lamps, providing total light intensity of 800 μmol (hv) $m^{-2} \cdot s^{-1}$ PAR, in pots containing peat/soil/sand (3:2:1, v:v:v), watered daily, and fertilized twice a week with Hoagland's liquid medium (pH 6.7) as described by Wedzony (2003).

SPIKE PRETREATMENT, MICROSPORE ISOLATION AND CULTURE

Tillers were cut when the majority of microspores were at late uninucleate to early bicellular stage. Cut tillers were placed immediately in jars of water and kept at 5°C in darkness for three weeks. Then the spikes were released from the flag leaf and surface-sterilized (1 min with 70% ethanol, 1 min with 0.05% mercury chloride, 15 min with Domestos commercial bleach diluted 1:5 with distilled water) and rinsed five times with sterile deionized water. We used the microspore culture protocol described by Pauk et al. (2000), with minor modifications. Microspores were isolated from spikes with a Waring LB 20EG laboratory blender (Torrington, Conn.) using 0.3 M mannitol. The microspore suspension was filtered through a 70 μm metal sieve, pelleted (100 × g, 7 min) and washed by density gradient centrifugation (0.3 M mannitol/ 0.58 M maltose; 80 g, 10 min). The fraction of viable microspores located in a band at the mannitol/maltose interphase was collected, resuspended in 0.3 M mannitol and again pelleted (100 × g, 7 min). Finally the microspores were suspended in 190-2 medium (Zhuang and Xu, 1983, as modified by Pauk et al., 2000). Microspore suspensions (1.5 ml aliquots) at density of $7 \cdot 10^4$ microspores per cm^3 were plated in 35 × 15 mm Petri dishes in co-cultures with immature ovaries derived from the same spike at the time of microspore isolation (10 ovaries per plate) and kept in darkness at 26°C.

VISUALIZATION OF CELL COMPONENTS

DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich, D-9564) was used to visualize chromatin in whole mount preparations of suspension culture and in sectioned material, but with two different DAPI protocols applied. Chromatin in suspension culture was stained according to Custers et al. (1994). Chromatin in Steedman's wax sections was stained with 200 μl/per slide 0.001% DAPI solution in PBS (sodium phosphate buffer: 0.14 M NaCl, 2.7 M KCl, 1.5 mM KH_2PO_4, 6.5 mM $Na_2HPO_4 \cdot 2H_2O$) for 10 min in darkness. DAPI staining followed slide incubation in the secondary antibody and preceded followed washing in PBS, toluidine blue staining and enclosing under cover slips. DAPI fluorescence was studied at absorption 365 nm and emission 420 nm (blue fluorescence). Propidium iodide (PI) (Sigma-Aldrich, P-4170) (0.001% solution in water) (absorption 536 nm, emission 623 nm, red fluorescence) was alternatively used for chromatin visualization in suspension according to the protocol of Kallioniemi (1988).

Cellulose was visualized with Calcofluor White ST (4,4'-bis [4-anilino-6-bis (2-hydroxyethyl) amino-s-trizin-2-ylamino]-2-2'-stilbenedisulfonic acid) (BioChemika, Fluka 18909) (0.01% solution in water) (absorption 365 nm, emission 420 nm, blue fluorescence) according to Benziman et al. (1980). Mitochondria and ER were shown with $DiOC_6$ (3,3-dihexyloxacarbocyanine iodide) (Molecular Probes; D-273) (absorption 483 nm, emission 501 nm, green fluorescence) (0.0001% solution in water) (Terasaki, 1994).

All the above-mentioned dyes and reagents (DAPI, PI, Calcofluor White ST or $DiOC_6$) were added directly onto the slide to the same volume of fresh samples of suspension (1:1 :: v:v). The slides were kept in a humid chamber in darkness for a minimum 15 min before microscope observations.

Starch grains were stained with J/KJ (Lugol solution) (0.0001%, solution in water); 50 μl J/KJ solution was added directly to the same volume of fresh sample of microspore suspension culture. Staining was done on microscope slides kept in a humid chamber in darkness for ~15 minutes before microscope observations.

FIXATION AND IMMUNOLABELLING PROTOCOL

Samples of triticale microspore culture suspension (3 cm^3) were taken on the day of isolation and the 3^{rd}, 5^{th}, 7^{th}, 10^{th} and 20^{th} days of in vitro culture. Cells were collected in Eppendorf tubes by spinning at 80 × g at room temperature (RT) for 5 min. Immediately after centrifugation a fixative solution was added to the pellet.

Vitha et al.' (2000) protocol with major modifications was used for immunodetection of α-tubulin in Steedman's wax sections. Fixation was performed with a solution of 3% paraformaldehyde (PFA, Sigma-Aldrich,76240) and 0.025% glutaraldehyde (GA, Sigma-Aldrich, 49626) in microtubule stabilizing buffer [MSB: 50 mM 1.4-piperazinediethane sulfonic acid (PIPES, Sigma-Aldrich P-1851), 5 mM EGTA (Sigma-Aldrich, O-3778), 5 mM $MgSO_4$, adjusted with 5M KOH to pH 7.0]. The samples were fixed overnight in darkness at 26°C on an orbital shaker. Then they were rinsed with MSB/0.025% Triton (Sigma-Aldrich, 93443) (30 min). After washing, the samples were treated with freshly prepared 0.05 M NH_4Cl and 0.05 M $NaBH_4$ in MSB/0.025% Triton (5 min) to reduce autofluorescence caused by free aldehydes generated during fixation. Only the first component was advised by Vitha et al. (2000). After washing in MSB/0.025% Triton (30 min) and in PBS (sodium phosphate buffer: 0.14 M NaCl, 2.7 M KCl, 1.5 mM KH_2PO_4, 6.5 mM $Na_2HPO_4 \cdot 2H_2O$) for 30 min, the samples were embedded in 6–8% low-gelling-point agarose (Sigma-Aldrich, A-6560) at 37°C and cooled to RT. Agarose blocks were cut into 2 mm pieces and transferred to 30% ethanol in 1 M PBS. Then the material was dehydrated at 4°C in series of ethanol in 1 M PBS solutions with increasing ethanol concentra-

tions (30% for 30 min, 50% for 30 min, 70% overnight, 90% for 30 min) and finally in 0.1% toluidine blue in 95% ethanol for 10 min at RT to make the material visible during further processing.

Then the material was warmed up to 37°C in 95% EtOH/1 M PBS for 30 min. Next it was placed in mixtures of Steedman's wax with 95% EtOH in proportions of 1:2, 1:1 and 2:1, for two hours in each mixture. Finally it was kept at 37°C in Steedman's wax overnight. On the next day the material was embedded into molds at 37°C and left to cool at RT. Sections (6 μm thick) were attached to slides coated with poly-L-lysine (Sigma-Aldrich, P-8920) in order to minimize tissue loss during the subsequent procedure.

IMMUNOLABELLING

Slides were rehydrated in an ethanol series (3 × 10 min 95% ethanol in 1 M PBS, 10 min 90% ethanol in 1 M PBS, 10 min 50% ethanol in 1 M PBS, PBS 10 min) and put into MSB for 30 min. Rehydrated and washed material was incubated with 2% acetylated BSA in MSB for 30 min at 30°C to prevent unspecific binding of antibodies. Slides were incubated with primary antibody anti α-tubulin (Clone DM1A, Sigma-Aldrich T-9026, dilution 1:1000 in 3% BSA in MSB) overnight at RT. After washing with MSB/0.025% Triton (4 × 10 min), the cells were incubated with secondary antibody raised in goat against mouse (GaM/IgG) conjugated with Alexa 488 (Molecular Probes A-11001, dilution 1:100) in 3% BSA/MSB for 3 h at 37°C. Finally the samples were washed with MSB/0.025% Triton (15 min) and in 1 M PBS (3 × 15 min). Between the next two washings in PBS (10 min), samples were stained for 10 min with 0.001% DAPI in PBS (details in "Visualization of cell components") and 0.01% Toluidine blue in PBS to quench autofluorescence of cellulose cell walls. Slides were enclosed in anti-fading solution (Citifluor Ltd. in glycerol, AF2, Enfield Cloisters).

MICROSCOPE OBSERVATIONS

Microscopic analyses were performed on the day of isolation and on the 3rd, 5th, 7th, 10th, and 20th days of in vitro culture. Cell morphology and progress in culture development were examined in Petri dishes containing culture suspension with an inverted light microscope (Olympus ATM3) with phase contrast. The percentages of cells and structures with different features were calculated for the total 500 objects per analysis. The experiment was based on eight biological replicates (each Petri dish was considered one biological replicate).

Microscope slides were examined under a Nikon Eclipse-E600 equipped with a high pressure 100 W mercury lamp with a DIC system. Fluorescence was examined under the same microscope equipped with a high pressure mercury lamp and filters EX 365/DM 400/BA 420 EF: (DAPI and Calcofluor White); EX 470-490/DM 510 BA/515 EF (Alexa 488 and DiOC6) and EX 510-560/DM 580 BA/590 EF for PI. Images were collected with a Nikon DXM1200F digital camera. Whenever a fluorescence image was taken from the same object as the DIC image, the fluorescence image was always taken first. Images were processed with Imaging Analysis Systems Lucia G ver. 4.81 (Laboratory Imaging Ltd., Czech Republic) and Corel PhotoPaint 9.0.

RESULTS

FIRST STAGES OF SPOROPHYTIC DEVELOPMENT IN ISOLATED MICROSPORE AND YOUNG POLLEN GRAIN CULTURE

The suspension received as the result of the isolation procedure was initially a mixture of cells at various stages of development. Degenerated cells presented ~37% of the isolated objects, ~48% had cytological features typical for late stages of uninucleate microspores, and 11% had characteristics of young bicellular pollen grains. Besides them, tricellular pollen grains and maturing pollen grains accumulating starch occurred at low frequency. Since only microspores and bicellular pollen grains switched to androgenic development, they were the subjects of further cytological analysis. Figures 1–4 show the development of triticale microspores and bicellular young pollen grains during culture in vitro.

On the day of isolation the majority of microspores were at the late developmental stage, each having a big central vacuole and the nucleus located close to the sporoderm at the pole opposite to the microspore operculum (Fig. 1 a1). Mitochondria and ER gave strong fluorescence after $DiOC_6$ staining in the vicinity of the nucleus and in the thin layers of peripheral cytoplasm (Fig. 1 a2). A dense network of long thick bundles of CMTs adjacent to the microspore wall was observed (Fig. 1 b). In some microspores only a band of weak diffused fluorescence surrounding the nucleus and thus closer to the nuclear pole of the cell was visible. Fluorescence coming from those microtubules showed parallel orientation and therefore resembled a preprophase band (PPB, Fig. 1 c1). The start of prophase was confirmed by condensation of chromosomes visible on the same section stained with propidium iodide (Fig. 1 c2).

On the day of isolation, bicellular pollen grains had a small generative cell located close to the sporoderm wall, and the vegetative cell occupied

most of the pollen volume (Fig. 1 d1). Mitochondria and ER in proximity to both nuclei gave strong fluorescence (Fig. 1 d2).

When samples collected on the day of isolation and on the 3rd and 5th days are compared it is seen that some of the isolated microspores and young pollen grains became enlarged from ~20 μm to 40 μm in diameter; others remained about the size of freshly isolated ones (~20 μm diam.). Enlargement is considered a sign of a change of developmental pathway. In samples collected on the 5th day of culture, vacuole fragmentation and the shift of the nucleus towards the center of enlarged microspores were often visible (Fig. 1 e1). This resulted in the emergence of cell morphology typical for star-like structures (SLSs). Mitochondria and ER in SLSs gave strong fluorescence around the nucleus and in cytoplasmic strands (Fig. 1 e2).

In the induced bicellular pollen, the big central vacuole also became fragmented. The generative cell was usually located at the periphery of the grain, and the vegetative nucleus was positioned close to it in a cytoplasmic pocket (Fig. 1 f1). Mitochondria and ER gave strong fluorescence in the cortex cytoplasm and in the radiating cytoplasmic strands of the vegetative cell (Fig. 1 f2). We identified this type of structure as bicellular star-like structure (bicellular SLS).

FIRST SYMMETRIC DIVISION

On the 7th day of culture, ~30% of the microspores had already divided regardless of the peripheral or central position of the nucleus. The first division led to the formation of two nuclei of equal size and chromatin condensation, which made them distinct from those that follow regular generative development (i.e., leading to mature pollen grain). This first division led to the formation of binucleate or bicellular structure inside the sporoderm (Fig. 2).

When the excentrally located nucleus divided, two symmetric daughter nuclei were lying in the vicinity of the sporoderm (Fig. 2 a2). Division of nuclei adjacent to the wall usually was not accompanied by the process of new cell wall formation (Fig. 2 b1, b2). The nuclei were surrounded by a dense network of CMTs radiating from the surface adjacent to the wall of the microspore towards the center of the cell. At the same time some CMTs were observed at the opposite pole of the cell (Fig. 2 a1). After $DiOC_6$ staining, mitochondria and ER gave strong fluorescence around the nuclei and in the thin layer of cytoplasm underlying the sporoderm (Fig. 2 b1).

The first mitosis in SLSs with centrally located nuclei was also symmetrical (Fig. 2 c). In this case, however, a new cellulose wall was formed, giving rise to two cells of similar size (Fig. 2 d). The nuclei were surrounded with dense cytoplasm and many cytoplasmic strands crossed the vacuole (Fig. 2 e1,e2). After $DiOC_6$ staining, the mitochondria and ER gave strong fluorescence in the cytoplasm surrounding nuclei and in the cytoplasmic strands (Fig. 2 e1).

MITOTIC DIVISIONS WITHIN THE SPORODERM WALL

In samples collected on day 10 of culture, many structures contained usually numerous nuclei (Fig. 3) of similar size and similar chromatin condensation (Fig. 3a), but it was not clear whether all those structures would develop further by cell wall formation to create androgenic multicellular structures. Synchronized divisions were visible (Fig. 3b) and the formation of numerous CMTs began (Fig. 3 c1, c2). Although some structures reached 60 μm in diameter, they did not rupture. A network of especially thick bundles of randomly arranged CMTs underlay the sporoderm (Fig. 3d). Numerous CMTs were seen lining the cell walls of multicellular structures, implying their role in cellulose deposition at this stage of development (Fig. 3 e1, e2). Mitochondria and ER gave strong fluorescence in the cytoplasm of most cells after $DiOC_6$ staining (Fig. 3 f).

It should be noted that cellulose deposition in new cell walls was not uniform in some structures (Fig. 3 g,h). Cell division synchrony sometimes was lost, leading to the occurrence of cells of different sizes and structures with uneven distribution of nuclei (Fig. 3 k2). Also, mitochondria, ER and starch grains were observed in two patterns. In some structures they were evenly distributed (Fig. 3 f) and in others the distribution of organelles was polarized (Fig. 3 k3). In asymmetric structures, all cells were separated by cellulose walls (Fig. 3 g,h,l).

SPORODERM WALL RUPTURE AND RELEASE OF MULTICELLULAR STRUCTURES

At about two weeks of culture, regardless of the structure of internal organization, the multicellular structures ruptured the sporoderm wall (Fig. 4), which burst out close to the pollen germ pore (Fig. 4 a1, a2). At this stage of androgenic structure development, the distribution of starch grains was not symmetrical (Fig. 4 b,c). Starch grains were seen in the majority of cells but were concentrated at one pole of the cell or were unevenly distributed within cells (Fig. 4 c). In the latter case they were detected mainly at the pole opposite to the sporoderm rapture. Many two-domain structures were observed, composed of small and densely filled cells and large vacuolated cells. The sporoderm wall was always opened at a site adjacent to the domain of larger and more vacuolated cells. The fluorescence coming from the labelled MTs was diminished at that site

(Fig. 4 d1, d2). At the opposite pole of the structure, CMTs formed a dense network in cells (Fig. 4 d1).

The emerged structures followed a series of intensive mitotic divisions and formed cell masses. Cells of the released multicellular structures showed both intensively fluorescing CMTs close to the cell walls and more dispersed EMTs running through the cytoplasmic strands from the nuclear region to the cell cortex (Fig. 4 e). Further development and differentiation led to the formation of more or less compact structures resembling proembryos (Fig. 4 d2). The orientation of CMTs remained random up to the moment when globular androgenic structures were formed (Fig. 4 d1). Often the cells of the structures differed in size and continuously formed two zones: one containing small cells with dense cytoplasm and the other containing large, highly vacuolated cells. The big cells had thick cellulose walls, and the smaller ones thinner cell walls (Fig. 4 f). At day 20 of culture, the last day of microscopy observations in this study, neither structure showed progress of development further then globular embryo-like structure.

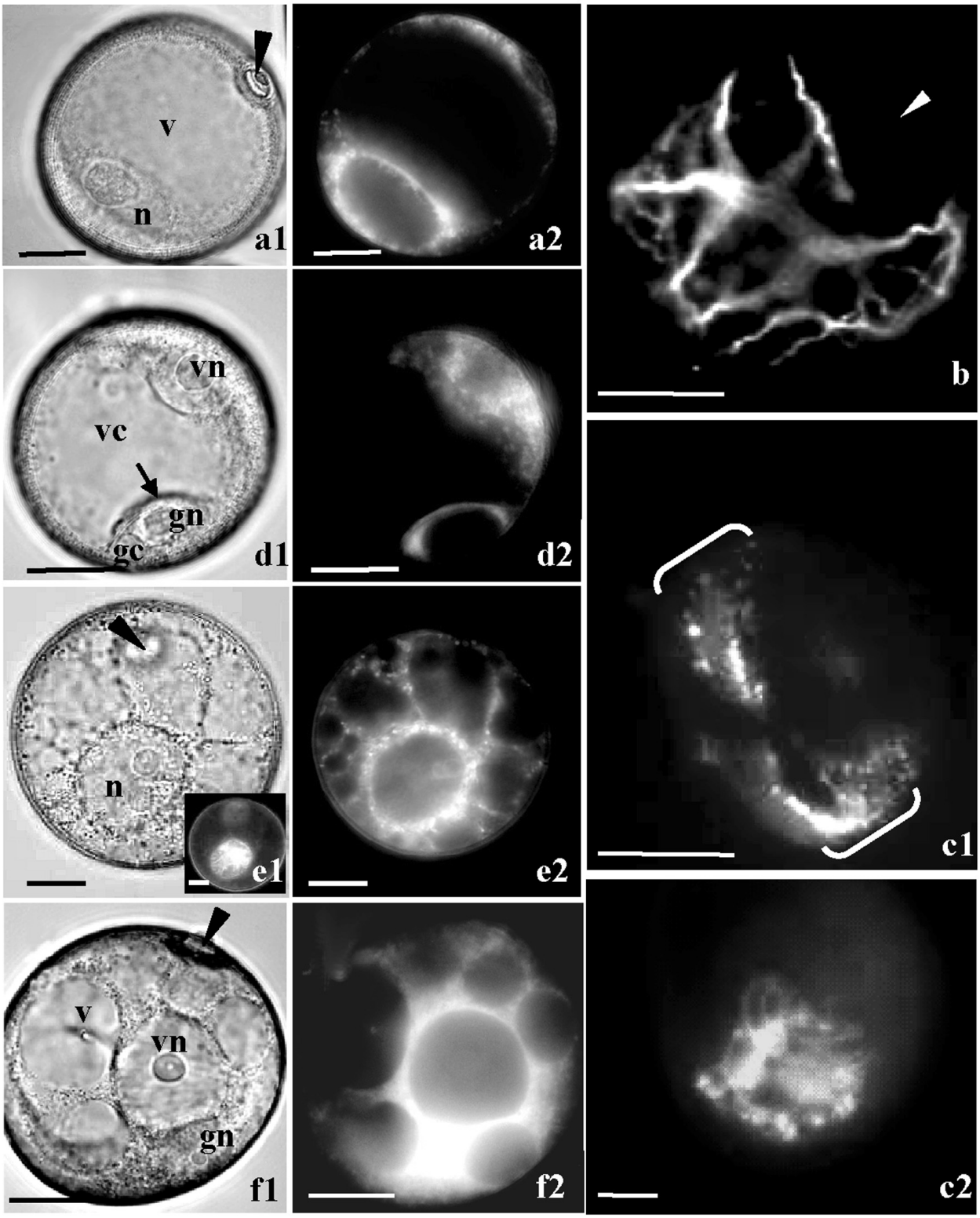
DISCUSSION

Double haploid technology is increasingly incorporated into triticale breeding and biotechnology. Among other steps forward, great progress in isolated microspore culture technique has been made (Pauk et al., 2000; Oleszczuk et al., 2004; Eudes and Amundsen, 2005). Microspore suspension culture of triticale cv. Wanad, used in this study, was earlier successfully used for optimization of procedures for double haploid technology and for identification of physiological parameters important for effective induction of androgenesis (Zur et al., 2008, 2009). Microspores and structures induced toward androgenic development were observed at 63% frequency, with 2.2–11.4% initial mitotic divisions (Zur et al. in preparation, personal communication). Our data coincide with results given by Oleszczuk et al. (2004) for hexaploid triticale cv. Bogo. That is why the Wanad culture system was deemed suitable for the cytological and morphological studies presented here.

MORPHOLOGICAL CHANGE ACCOMPANYING DEVELOPMENT OF ISOLATED TRITICALE MICROSPORES

As described in the results, the suspensions of microspores isolated from triticale anthers consisted of cells differing in developmental phase and morphology, with late uninucleate microspores predominating. The morphological changes observed in induced triticale microspores and immature pollen grains were generally in agreement with published data. The variability of cells isolated from anthers of spikes at the same developmental stage is in accord with previous observations by Pechan and Keller (1988) and many others that pollen development does not proceed uniformly even within a single anther. As in other studies of monocotyledonous microspore suspensions (Oleszczuk et al., 2004; de Maraschin 2005; Massoneau et al., 2005; Pulido et al., 2005), we observed here that isolated microspores swell and their cytoplasm undergoes structural reorganization upon stress treatment. The morphology resulting from these changes, called star-like structure, has been observed in other species, both dicots such as tobacco (Garrido et al., 1995; Touraev et al., 1996a, 1997) or rape seed (Zaki and Dickinson, 1990) and monocots such as wheat (Touraev et al., 1996b), barley (de Maraschin et al., 2005 a,b), rice (Bajaj, 1990; Raina and Irfan, 1998), maize (Testillano et al., 2002; Obert et al., 2005) and triticale (Oleszczuk et al., 2004). This star-like morphology has been recognized as the initial step of microspore embryogenic development (Indrianto et al., 1999; Touraev et al., 2000). However, in a study tracking individual cells in culture of wheat microspores, Indrianto et al.

Fig. 1. Development of triticale (×*Triticosecale* Wittm.) microspores and immature pollen grains in conditions inducing androgenic development in vitro. **(a1, a2)** Uninucleate microspore at late vacuolated stage. Arrowhead points to operculum **(a1)**. Mitochondria and ER **(a2)** visible in thin layer of cytoplasm and around excentrally located nucleus, **(b)** Randomly oriented bundles of cortical microtubules. Arrowhead points to operculum, **(c1)** Weak diffused fluorescence of microtubules resembles preprophase band (brackets) positioned closer to the nuclear pole of the cell, **(c2)** Section of the same cell as in c1, with excentral nucleus showing features of early prophase, **(d1)** Structure resembling bicellular pollen grain. Generative cell wall marked with arrowhead, **(d2)** Same cell as in d1, with mitochondria and ER visualized in cytoplasm, **(e1)** Uninucleate microspore with fragmented vacuole – star-like structure (SLS). Operculum marked with arrowhead. Insert: excentral nucleus of the same cell is visible, **(e2)** Same cell as in e1, with mitochondria and ER visualized in cytoplasmic strands, **(f1, f2)** Bicellular structures. Formation of cytoplasmic strands around one of the two nuclei, **(f1)** Operculum indicated with arrowhead, **(f2)** Same structure as in f1, with mitochondria and ER visualized in cytoplasm. Bar = 20 μm. n – nucleus; vc – vegetative cell; vn – vegetative nucleus; gc – generative cell; gn – generative nucleus; v – vacuole. **a1, d1, e1, f1** – DIC image; **a2, d2, e2, f2** – mitochondria and ER visualized with $DiOC_6$; **b,c1** – microtubules in microspores visualized immunocytochemically with conjugated antibody; **e1** – insert: chromosomes after PI staining; **b, c1, c2** – Steedman's wax sectioned material; **a1, a2, d1, d2, e1, e2, f1, f2** – whole mount staining.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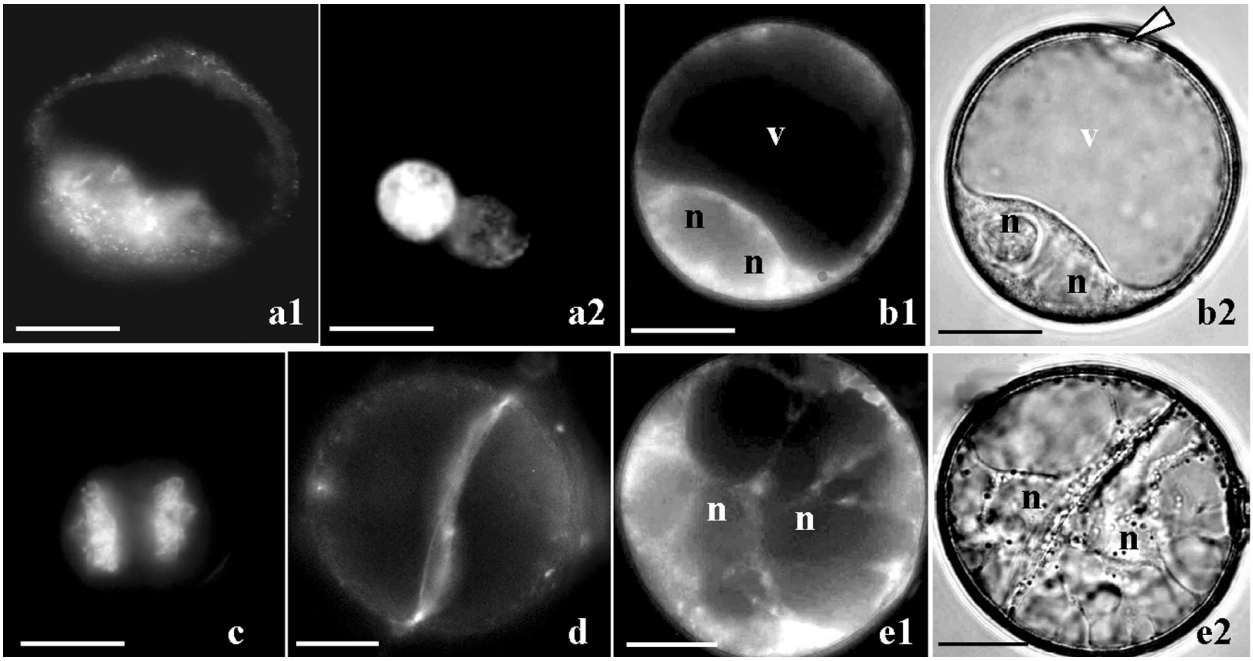


Fig. 2. First nuclear division in microspores of triticale (×*Triticosecale* Wittm.) switched to androgenic development during suspension culture. (**a1, a2, b1, b2**) Two-nucleate structures with peripherally located symmetric nuclei and large vacuole. **(a1)** CMTs, **(a2)** Nuclei of the same structure shown in a1, **(b1)** Mitochondria and ER in cytoplasm surrounding the nuclei and underlying the sporoderm, **(b2)** Same structure as in b1, operculum indicated by arrowhead, **(c, d, e1, e2)** Structures consisting of two cells of the same size, **(c)** Two nuclei located symmetrically in the center of the structure, **(d)** Cellulose wall separating two cells of similar size, **(e1)** Mitochondria and ER in cytoplasmic strands of two-cell structure visible in e2, **(e2)** Nuclei enclosed in cytoplasmic pocket. Bar = 20 μm. n – nucleus; v – vacuole. **a1** – Microtubules visualized immunocytochemically with FITC (Fluoreswcein isothiocyanate) conjugated antibody; **a2, c** – Nuclei visualized with DAPI; **b1**, **e1** – mitochondria and ER visualized with $DiOC_6$; **b2**, **e2**. – DIC images; **d** – cellulose walls after Calcofluor white staining; **a1**, **a2** – Steedman's wax sectioned material; **b1**, **b2**, **c**, **d**, **e1**, **e2** – whole mount staining.

(2001) found that microspores with peripherally positioned nuclei also had embryogenic potential, and they suggested that both types of microspores represent successive stages in a continuous process of sporophytic development. Features they regarded as phenomena connected to switching of the developmental pathway were also observed during our study: cell enlargement, vacuole fragmentation, enrichment of the cell lumen with cytoplasm, mitotic divisions of a frequency and symmetry different from that typical for pollen development, and the emergence and later polarization of granular structures (ER, starch grains). In contrast to other reports (Sunderland, 1973; Gonzáles and Jouve, 2005), we relatively often observed the first symmetric mitotic division in the vicinity of the sporoderm, but it was not clear whether those microspores would develop further into multinuclear or multicellular structures. During microspore culture of triticale, the first mitotic division of microspores is reported to be usually symmetric and followed by the formation of two vegetative-like nuclei or cells of similar size (Gonzáles and Jouve, 2005).

The pattern of cell divisions in barley, maize and wheat leads to the formation of two different cell domains within the multicellular structure enclosed in the sporoderm: a smaller domain composed of small, dense cells, and a larger domain composed of multinucleate cells (Huang, 1986; Bonet and Omedilla, 2000; Magnard et al., 2000; Ramírez et al., 2001; Testillano et al., 2002; de Maraschin et al., 2005b). Although two domains were also observed in our studies, both were cellular.

The site of sporoderm rupture can be marked by granule deposition in androgenic structures. In wheat, androgenic structures accumulate starch grains at the site opposite the place where the sporoderm is ruptured (Indrianto et al., 1999). Hause et al. (1994) found a similar distribution of starch grains in *Brassica napus* microspores. These data correspond with our observations. The results obtained so far indicate that the uneven concentration of ER, mitochondria and starch deposition mark the establishment of polarity necessary for localization of the site of sporoderm rupture and orientation of the androgenic embryo body axis.

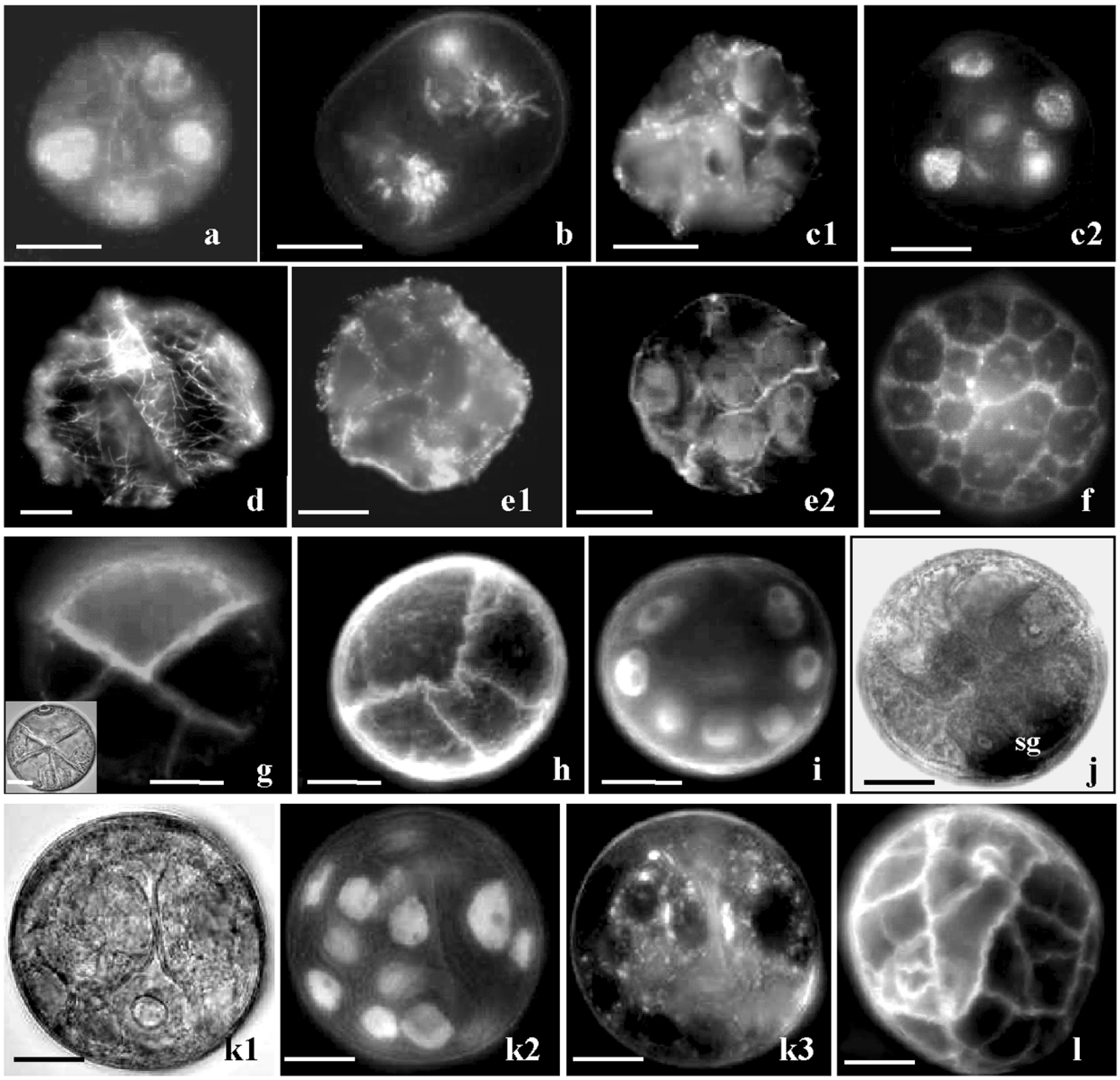
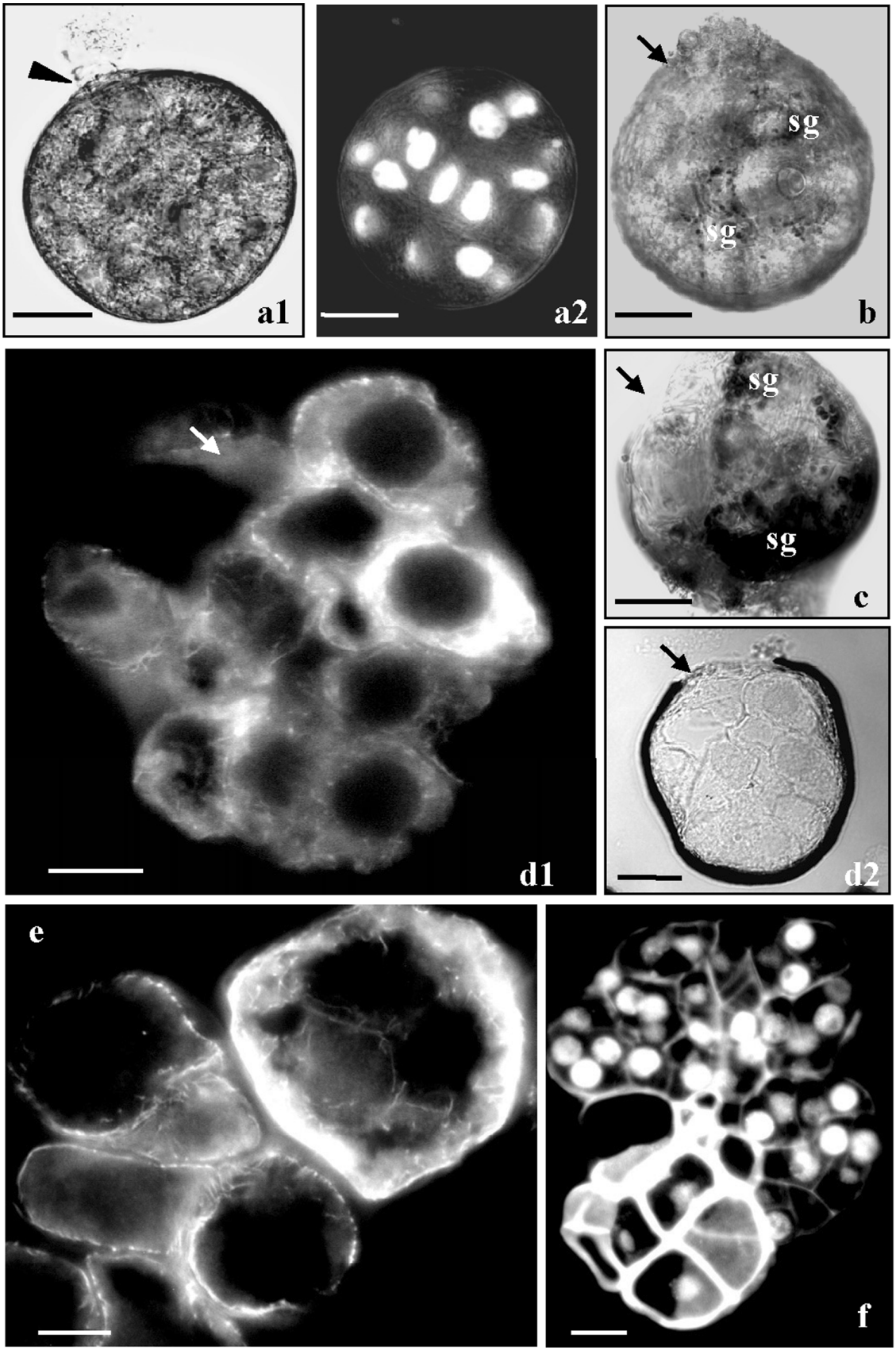


Fig. 3. Multiple divisions in microspores of triticale (×*Triticosecale* Wittm.) switched to androgenic development during suspension culture. **(a)** Symmetric nuclei, **(b)** Synchronized mitosis in multinuclear structure, **(c1)** Numerous CMTs underlying cell walls of multicellular structure, **(c2)** Nuclei of multicellular structure shown in c1. **(d)** Network of especially thick bundles of randomly arranged CMT underlying sporoderm, **(e1)** Cortical microtubules underlying cell walls of multicellular structure, **(e2)** Nuclei of multicellular structure immunolabelled in e1, **(f)** Mitochondria and ER visualized in multicellular structure, **(g, h)** Cellulose walls of multicellular structures, **(i)** Multiple nuclei of similar size underlying sporoderm, with some thin walls separating nuclei visible, **(j)** Starch grains condensed at one pole of multicellular structure, **(k1, k3)** Multicellular structure surrounded by sporoderm wall, **(k1)** Clear image of some walls and cytoplasmic strands, **(k2)** Multiple nuclei of similar size and chromatin condensation, visualized in the same structure as in k1, **(k3)** Mitochondria and ER visualized in the same structure as in k1 and k2. **(l)** Cellulose walls of multicellular structure. Two domains, one with thinner and the other with thicker cell walls. Bar = 20 μm. sg – starch grains. **c1, d, e1** – microtubules visualized immunocytochemically with FITC conjugated antibody; **a, c2, e2** – nuclei visualized with DAPI; **b, i, k2** – nuclei visualized with PI; **f, k3** – mitochondria and ER visualized with $DiOC_6$; **g, h, l** – cellulose walls after Calcofluor white staining. Insert in g: DIC image; **j** – starch grains stained with J/KJ, DIC image. **a, c1, c2, d, e1, e2** – Steedman's wax sectioned material; **b, f–l** – whole mount staining.


a1
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MICROTUBULE REARRANGEMENT DURING EARLY STAGES OF ANDROGENIC STRUCTURE FORMATION

This is the first report describing MT cytoskeleton rearrangements at successive phases of triticale microspore embryogenesis in culture. Testillano et al. (2002) reported concentrations of tubulin in some cells of multicellular structure as a marker of embryogenic development in androgenic maize derived embryo-like structures, but did not give details of cytoskeleton architecture as they were able to detect only diffuse fluorescence in the cytoplasm of multicellular structures, with higher intensity in the larger cell domain. Our experience suggests to us that they probably encountered difficulties in fixing and labelling MTs due to cell wall impermeability.

Our modifications introduced to Vitha et al.'s (2000) protocol for α-tubulin labelling of Steedman's wax sections resulted in successful immunodetection of microtubules in triticale microspores and androgenic structures. The protocol was originally developed for F-actin visualization. A few hours of fixation with 4% PFA, as given in the original protocol, was not sufficient for proper preservation of MTs and the cytoarchitecture of microspores and androgenic structures. Overnight fixation on an orbital shaker reduced cell plasmolysis, preserved intact cytosol and transvacuolar cytoplasmic strands, and maintained the stability of the MT cytoskeleton in the studied objects, while retaining their antigenicity. Since the thick sporoderm wall of triticale microspores is not permeable to antibodies and hampers penetration of fluorescent dyes, we used tissue sections.

Our work revealed the presence of cortical and endoplasmic MTs (at each stage of androgenic structure formation). Androgenic induction and differentiation of structures derived from microspores and immature pollen grains was accompanied by MT rearrangements. We saw similarities between the MT configurations and some observations reported in *Brassica napus* (Hause et al., 1993; Simmonds and Keller, 1999). Similarly, the first symmetric division in the microspore was preceded by PPB formation around the nucleus. In triticale, however, the nucleus is not always displaced to the center, and a band of weak, diffused fluorescence surrounded the nucleus in microspores. We suggest that the band of fluorescence is a preprophase band. PPBs were never observed during gametophytic microspore development leading to pollen formation (Van Lammeren et al., 1985) or in microspores in culture not induced toward embryogenic development (Simmonds and Keller, 1999), so its formation could be used as a marker of induction of microspore embryogenesis.

Here we described two daughter nuclei surrounded by a dense network of CMTs in the vicinity of the sporoderm, formed as a result of symmetric division of an excentrally located nucleus. This division can be regarded as the start of androgenic structure formation. There was intense fluorescence of a CMT network at the surface of both daughter nuclei adjacent to the sporoderm wall, as found in *Brassica* (Telmer et al., 1993; Simmonds and Keller, 1999).

Numerous CMTs without preferential orientation were present in triticale multicellular structures enclosed in the sporoderm wall and later up to the stage of embryo-like globular structures, which can be interpreted as a marker of isodiametric enlargement of cells. Random patterns of cytoskeleton structure organisation have been observed in cells that expanded isotropically (Webb and Gunning, 1991; Seagull, 1992).

In both types of cells found in two-domain structures, the CMT network was dense and randomly oriented. Possibly the denser network of cortical MTs formed at the site opposite to the sporoderm rupture participates in the formation of cellulosic walls and helps the structure to exit the sporoderm wall, as described by Zaki and Dickinson (1990). One explanation of the weak fluorescence coming from MTs in cells adjacent to the sporoderm rapture site might be tubulin depolymerization in

Fig. 4. Release from the sporoderm wall and further development of multicellular structures obtained in suspension culture of triticale (×*Triticosecale* Wittm.) microspores and immature pollen grains switched to androgenic development during culture. **(a–d)** Multicellular structures surrounded by bursting sporoderm wall, **(a1)** Sporoderm wall bursting close to pollen germ pore (arrowhead), **(a2)** Multiple nuclei visualized in the structure presented in a1, **(b, c)** Starch grains located away from the rupture site in sporoderm (arrowhead). Starch-rich domain contrasting with starch-free domain is visible in c. Arrowhead points to site of sporoderm rupture, **(d1)** Cortical microtubules underlying cell walls of multicellular structure partially released from sporoderm. Weak fluorescence from MTs in the region of sporoderm wall rupture (arrow), **(d2)** The same section as in d1, showing more clearly the region of sporoderm rupture (arrow), **(e)** Cortical and endoplasmic MTs in multicellular structure released from sporoderm wall, **(f)** Two-domain multicellular structure released from sporoderm, one part consisting of small cells with thin walls and relatively large nuclei, the other consisting of several large cells with thick cell walls. Bar (a–d, f) = 20 μm; (e) = 10 μm. sg – starch grains. **a1, b, c, d2** – DIC images; **a2** – nuclei visualized with PI; **f** – nuclei visualized with DAPI; **d1, e** – microtubules visualized immunocytochemically with FITC conjugated antibody; **b,c** – starch grains stained with J/KJ; **d1, d2, e, f** – Steedman's wax sectioned material; **a1, a2, b, c** – whole mount staining.

those cells. During exine wall rupture in barley androgenesis, however, the small cells adjacent to the rupture site died prior to the rupture and thus marked the rupture site. Such cell death showed typical features of plant programmed cell death (de Maraschin et al., 2005c). Thus the weak labelling of MTs in cells protruding from the rupture site could also be a sign of cell degeneration.

Here we confirmed several previously formulated hypotheses concerning androgenesis induction in monocotyledonous plants, but a few questions remain open. Such problems as the role of two-domain or one-domain structures in the formation of androgenic embryos can be resolved only by cell-tracking experiments. When such methods are combined with newly improved techniques for fixation, sectioning and labelling, we will gain more insight into androgenic processes.

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