Cellular Approach to the Study of Androgenesis in Maize Anthers: Immunocytochemical Evidence of the Involvement of the Ubiquitin Degradative Pathway in Androgenesis Induction

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Summary

Maize anthers in the late-uninucleate stage, corresponding to a DH 109×ZK 191/a F1 hybrid line, were induced to produce embryoids by cold pre-treatment and incubation onto a modified YP medium. LM observation of sections of the anthers showed the existence of bicellular microspores two days after starting the culture. Several types of multicellular microspores (MCM) and multinucleate microspores (MNM) appeared from the fifth day after culture onwards, together with many degenerated, non-induced microspores. This abundance of forms reveals the presence of different patterns of division and development. Ultrastructural observation of these cellular forms showed particular features, such as the presence of numerous nuclear pores in early stages of induction and the formation of a thick independent cell wall underneath the microspore intine. A certain degree of polarity in these structures was also frequently observed. Proembryoids appeared after 10–13 days of culture, whereas embryo-like structures were observed several days later (16 days of culture).

Immunocytochemical studies were carried out on semithin sections of the anthers using a commercially available polyclonal antibody to ubiquitin. Slight labeling was consistently found in most cell types, excluding degenerated and non-induced microspores. The intensity of the labeling was found to be substantially greater in putative androgenic MCM. A model for the involvement of the ubiquitin-mediated degradation pathway in pollen cell cycle control and androgenesis induction is proposed.

Key words: androgenesis, immunocytochemistry, ubiquitin, ultrastructure, Zea mays L.

Abbreviations: BCIP = 5-bromo-4chloro-3-indolyl phosphate; BSA = bovine serum albumin; LM = light microscopy; MCM = multicellular microspores; MNM = multinuclear microspores; NBT = 4-nitro blue tetrazolium chloride; RT = room temperature; TBS = Tris buffered saline; TEM = transmission electron microscopy; UBQ = ubiquitin; UBQ-Ps = ubiquitin-conjugated proteins.

Introduction

The microspore of Angiosperms is programmed for the production of gametes after an asymmetric division that takes place during later stages of its differentiation. However, an alternative sporophytic developmental pathway can be induced in this program, usually in response to specific stress signals. The result of this deviation is the formation of haploid plants via embryogenesis (for reviews see Heberle-Bors, 1985, 1989; Raghavan, 1986; Vicente et al., 1991; Reynolds, 1997).

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The production of double haploids through anther cultures is becoming a routinely used technique for the production of homozygous lines in many breeding programs (Raghavan, 1986). Recently, the use of isolated microspores and pollen cultures has begun to emerge as an alternative approach offering in certain cases a number of advantages (Benito-Moreno et al., 1988; Pechan and Keller, 1988; Emons et al., 1991; Hoekstra et al., 1993; Touraev et al., 1996a, b). In both cases, a multitude of treatments and conditions have been found to be effective for the induction of androgenesis, most of them involving several forms of stress (Raghavan, 1986; Touraev et al., 1996c). A number of molecular studies have been carried out in order to characterize the changes in a. o. DNA synthesis, heat shock protein localization and synthesis taking place in pollen during embryogenesis induction in respose to the stress treatment (Binarova et al., 1993; Cordewener et al., 1995; Binarova et al., 1997). To our knowledge, however, no studies have been carried out with the objective of characterizing the presence and localization of ubiquitin and ubiquitin-conjugated proteins throughout this process. Ubiquitin is a small, highly conserved protein present in all eukaryotes either in a free form, or covalently linked to substrate proteins, often targeting them for degradation by the proteasome. Changes in ubiqitin and ubiquitin-protein conjugates, as well as in their mRNAs, have been studied in a variety of plant cellular processes, including cell cycle control (Kampen et al., 1996) and plant senescence and response to stress (Belknap and Garbarino, 1996). A developmentally regulated loss of free ubiquitin and of ubiquitinated proteins during maize pollen maturation has been described by Callis and Bedinger (1994). The formation of embryos from microspores and pollen represents a fundamental switch in normal development which involves both a stress response and the re-entering in the cell cycle of the almost differentiated cells. Consequently, substantial changes in the machinery controlling the cell cycle, including ubiquitination of proteins, are expected to occur during this process.

The aim of this study was to use a highly embryogenic maize genotype in order to study the changes taking place at the levels of UBQ and UBQ-Ps in the various cell types observed after induction. We have used an immunocytochemical approach to this study after observing the diversity of androgenic pathways and the variability present in the anther cultures, which makes biochemical and molecular studies less feasible. A model for the involvement of the ubiquitin-mediated degradation pathway in pollen cell cycle control and androgenesis induction is proposed.

Materials and Methods

In vitro anther culture

Maize tassels, corresponding to a DH 109×ZK 191/a F1 hybrid line, grown in a phytotronic chamber in the «ny2» climatic program designed for maize (Tischner et al., 1997), were collected prior to emergence from the leaf blade and subjected to cold pre-treatment at 7 °C for 10 days. After cold-shock, the tassels were surface-sterilized with 20 % (v/v) sodium hypochlorite for 20 min and then washed three times with sterile distilled water. The anthers with microspores in the late-uninucleate stage were dissected under sterile conditions, and incubated in Petri dishes onto a modified YP medium supplemented with 0.1 mg/l. 2,3,5-triiodobenzoic acid, 5 mg/L charcoal, 500 mg/L casein hydrolysate, 120 g/L sucrose and 2.5 g/L gerlite pH 5.4, at 29 °C in the dark, in order to induce androgenesis.

Preparation of samples for LM/TEM

A minimum of 15 anthers were randomly collected from the Petri dishes 0, 1, 2, 3, 5, 7, 13 and 17 days after the initiation of the culture, and processed for LM/TEM: the anthers were fixed in a mixture of 4 % (w/v) paraformaldehyde and 0.1 % (v/v) glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.2) at 4 °C overnight. After washing in the same buffer, the samples were dehydrated in a graded ethanol series. Throughout dehydration, the temperature was progressively lowered to a final temperature of −25 °C, coinciding the start of the embedding in Unicryl resin (BBInternational, Cardiff, UK). Polymerization was carried out at −25 °C for 3 days under ultraviolet light.

LM/TEM cytological observations

Semithin sections (1 μm thick) were obtained with a Reichert-Jung ultramicrotome, and stained with 0.5 % (w/v) methylene blue and 0.5 % (w/v) toluidine blue in 1 % (w/v) boric acid for 10 min prior to observation with a Zeiss Axioplan photomicroscope.

Ultrathin sections (70 nm thick) were also obtained in a Reichert-Jung ultramicrotome, but transferred to 300-mesh formvar-coated nickel grids and stained with uranyl acetate and lead citrate. Observations were carried out in a Zeiss EM10C transmission electron microscope operating at 60 kV.

Immunolocalization of ubiquitin

Semithin sections obtained as described above were attached to 3-aminopropyl triethoxy-silane-coated slides. The slides were incubated sequentially in TBS: 10 mmol/L Tris, 115 mmol/L NaCl, pH 7.4 (10 min at RT), blocking solution: 2 % BSA in TBS (1 h at RT), rabbit polyclonal anti-ubiquitin antiserum − Sigma, St. Louis, MI, USA − diluted 1 : 10 in blocking solution (overnight at 4 °C), TBS (3 × 20 min at RT), anti-rabbit IgG (Fc)-alkaline phosphatase conjugate − Promega Corporation, Madison, WI, USA − diluted 1/200 (4 h at 4 °C), TBS (3 × 20 min at RT), detection buffer: Tris-HCl 100 mmol/L pH 9.5, NaCl 100 mmol/L, MgCl$_2$ 5 mmol/L, levasimole 1 mmol/l. (20 min at RT), and finally in NBT/BCIP substrate − Amresco Inc., Solon, OH, USA − (5 h at RT in the dark). Control slides were prepared by omitting the anti-ubiquitin antibody.

Results

LM observation of semithin sections of the anthers immediately after starting the culture showed numerous microspores to be present within the anther. The microspores appeared irregularly-shaped and in close contact (Fig. 1 a). Most microspores showed the morphology characteristic of the late uninucleate microspore stage: presence of a large vacuole, and a single nucleus often localized in the periphery of the microspore (Fig. 1 b). The microspore wall was well-structured and heavily colored. The existence of dividing microspores was detected as early as two days after starting the culture, although most microspores remained uninucleate at days 1 and 2 after the onset of the culture (Figs. 1 c and d). From these early days of culture onwards, numerous microspores
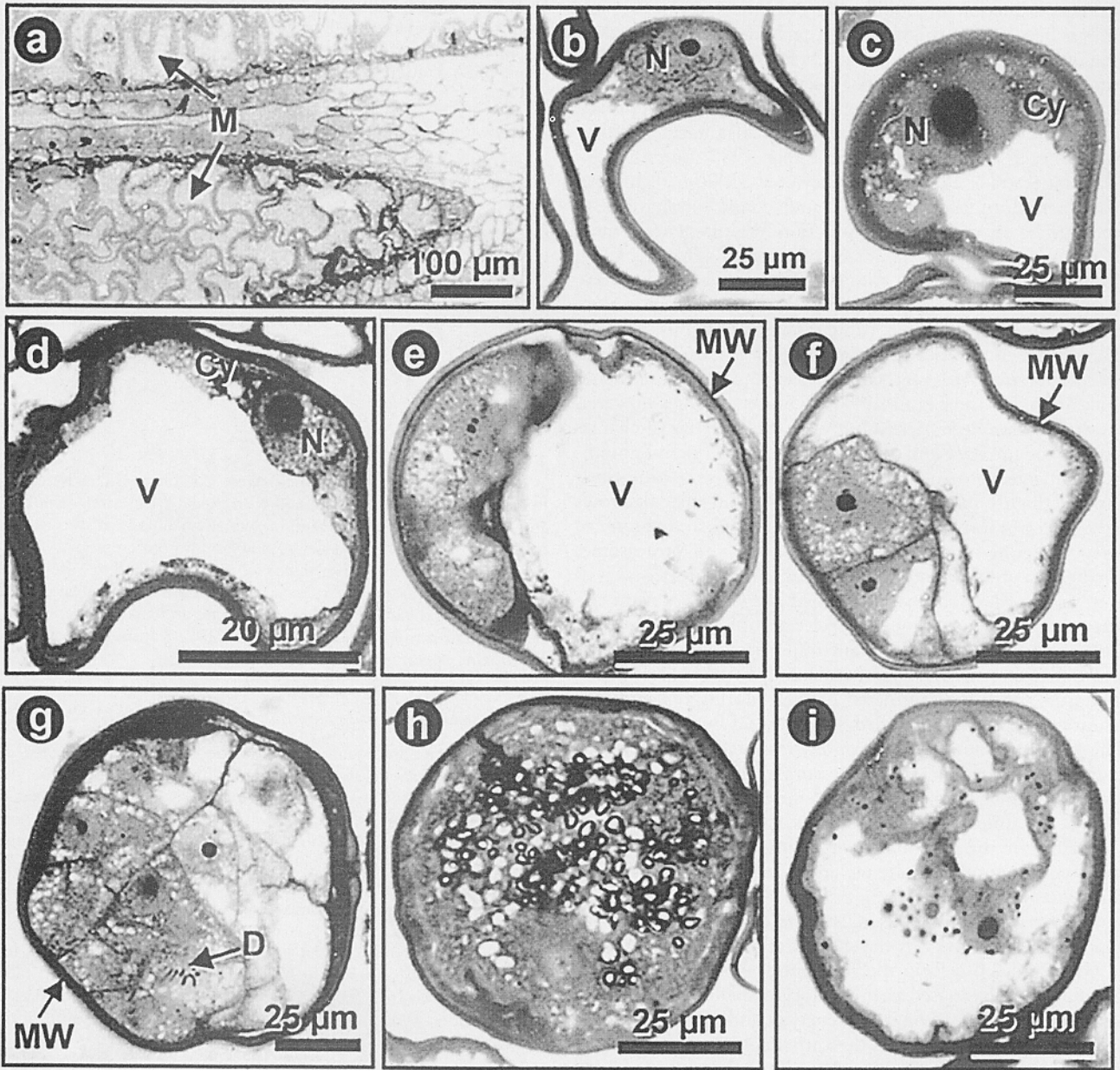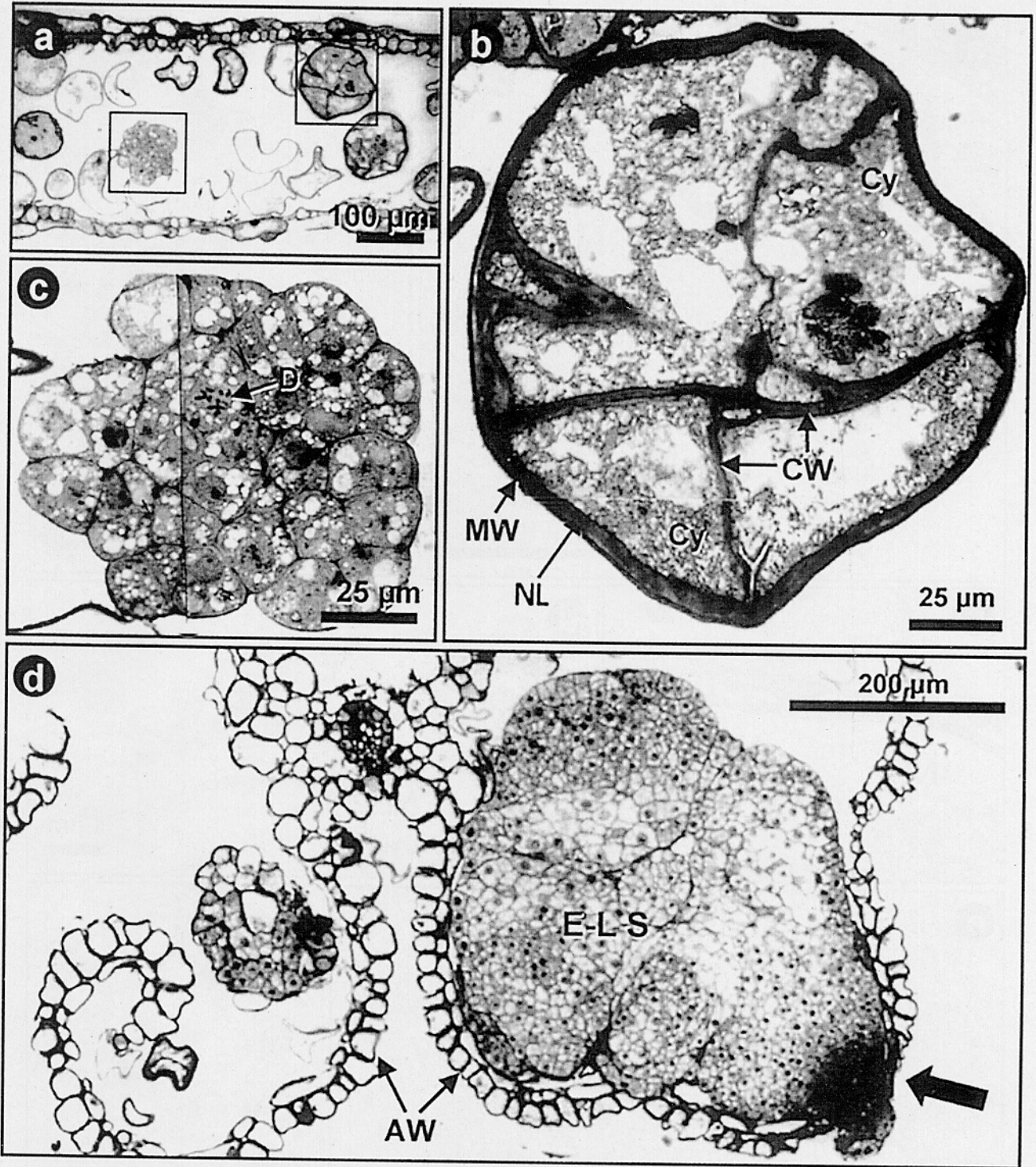
Fig. 1: LM study of androgenesis induction of maize anthers I. **a**: section of an anther processed immediately after cold treatment. **b**: detail of a microspore immediately after cold treatment. **c** and **d**: microspores 1 day after the onset of the culture. **e**: first division within a microspore 5 days after the initiation of the culture. **f**: 4-cell MCM at day 5 of culture. **g**: MCM showing a mitosis (D) at day 7 of culture. **h**: highly vacuolated, starch-rich MCM after 13 days of culture. **i**: MNM at day 13 after the initiation of culture. Cy: cytoplasm, M: microspores, MW: microspore wall, N: nucleus, V: vacuole.

started to swell, becoming rounder. In most cells the cytoplasm also became darker. From the fifth day after culture onwards, the appearance of microspore divisions (Fig. 1e) and multicellular microspores (MCM) (Fig. 1f) was frequent. Multinucleate microspores (MNM) were also observed, although their number was much lower (c.a. 0.5 %). Subsequent cell divisions within MCMs were observed at the seventh day after the onset of the culture (Fig. 1g). Numerous

cell types, MCM and MNM types (Fig. 1g, h, Fig. 2a, b) were observed throughout the progress of the culture. Many degenerated, non-induced microspores were also present. This abundance of forms reveals various androgenic pathways.

Proembryoids were detected after 13 days of culture (Fig. 2c), whereas embryo-like structures showing early symptoms of tissue differentiation (Fig. 2d) were observed thereafter (16 days of culture).

Fig. 2: LM study of androgenesis induction of maize anthers II. **a**: section of an anther after 13 days of culture showing several MCM and a proembryoid. **b**: detail of a MCM still displaying the original microspore wall. Note the new layer close to the intine and the thick cell walls surrounding the different cells. **c**: detail of a proembryoid with cells undergoing division. The original microspore wall is not present in this structure. **d**: embryo-like structure showing early symptomps of tissue differentiatiation after 16 days of culture. The structure is disrupting the anther wall (arrow). AW: anther wall, CW: cell wall, E-L S: embryo-like structure, NL: newly-synthesized layer, MW: microspore wall.
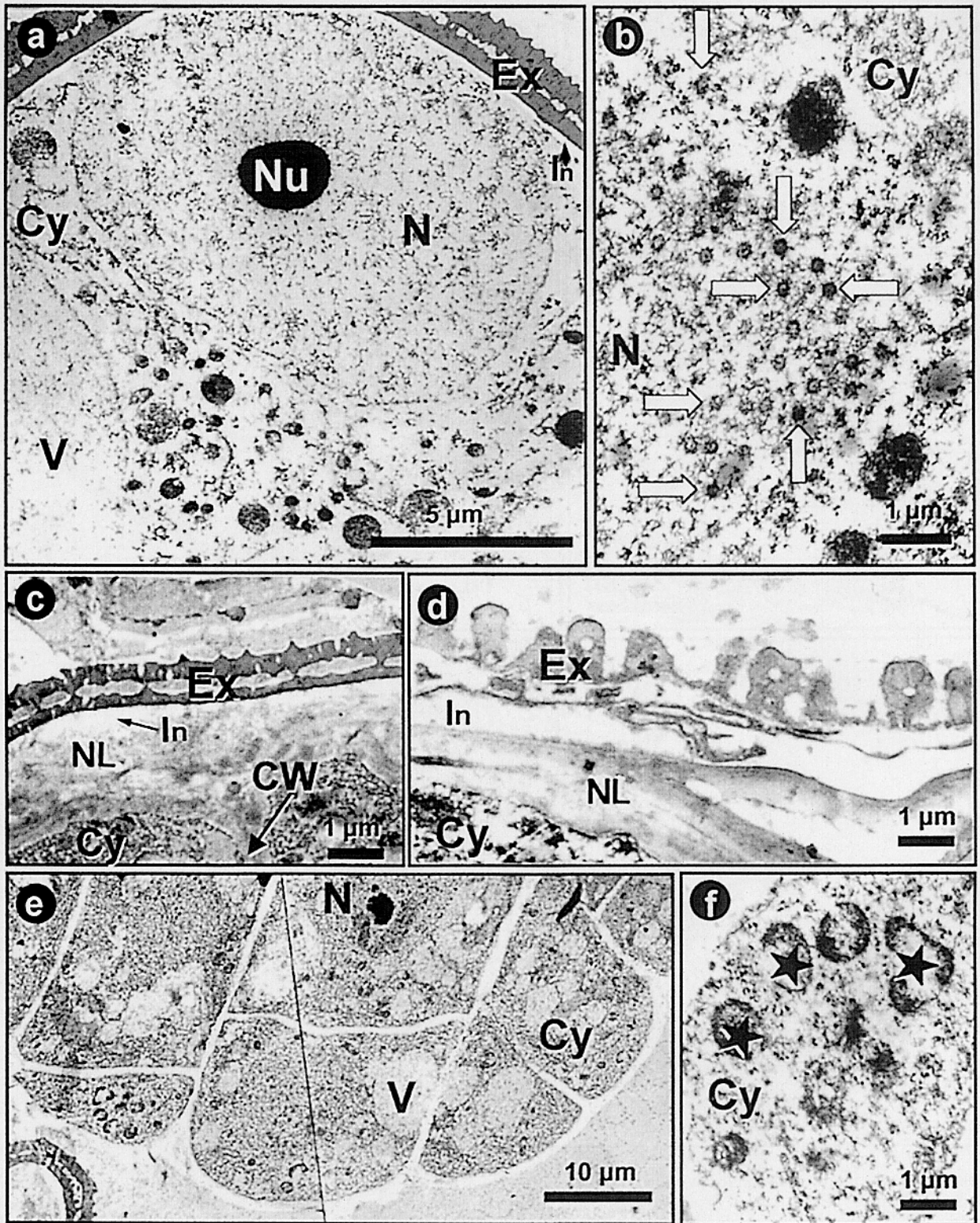
Fig. 3: TEM study of androgenesis induction of maize anthers I. **a:** section of a microspore 1 day after the onset of the culture. **b:** nuclear pores (arrows) decorating the nuclear envelope of a microspore after 1 day of culture. **c** and **d:** original microspore wall at days 7 (c) and 10 (d) after the initiation of culture. Note the thick layer underneath the microspore intine (in both c and d), as well as signs of breakdown of the original wall (in d). **e** and **f:** proembryoid observed after 13 days of culture. Detail showing undifferentiated organelles (asterisks) (f). Cy: cytoplasm, Ex: exine, NL: newly-synthesized layer, In: intine, N: nucleus, Nu: nucleolus, V: vacuole.

Ultrastructural observations

The microspores shortly after the onset of the culture show a cytoplasm with scarce organelle content and a low density of ribosomes (Fig. 3 a). The nucleus shows the chromatin in a decondensed stage, and the nucleolus formed almost exclusively by dense fibrillar component. The pollen wall is composed of a thin intine, and a well-structured exine. Quantitative analysis indicates that the initially low number of nuclear pores decorating the nuclear envelope experiments a rapid increase (2- to 3-fold) throughout the early stages of culture, before the appearance of nuclear divisions (Fig. 3 b).

We have focused our attention on the ultrastructural features of a type of MCM among the various categories observed: this MCM (Fig. 3 c) consisted of four to eight cells, still surrounded by the original microspore wall, and corresponds to the structure and developmental stage shown in Figs. 2 a, b. This structure shows a newly synthesized thick cell wall underneath the original microspore intine (see details in Fig. 3 c). The cytoplasmic content of these cells shows a higher density than that of cells shortly after induction, including a higher population of ribosomes, numerous small-sized mitochondria and plastids with some starch granules and small vacuoles, some of them containing electron dense inclusions. The nuclei display decondensed chromatin and the nucleoli usually present an active configuration, formed by dense fibrillar component, granular component and fibrillar centres intermingled.

Throughout the developmental progress of divisions, and before the establishment of microcalli, the original microspore wall underwent a process of breakdown (compare Figs. 3 c and 3 d) which ends up with its disappearance. Proembryoids and embryo-like structures show ultrastructural features typical for young meristematic cells (Fig. 3 e, f), with frequent divisions, a ribosome-rich cytoplasm, some small-sized vacuoles and undifferentiated organelles (Fig. 3 f).

LM Immunolocalization of ubiquitin

After immunolocalization experiments, a steady low level of UBQ/UBQ-Ps was found to be present in the anthers shortly after induction (0 to 5 days). Labeling appeared lightly in the microspore cytoplasm, and also occurred in locullar material between the microspores, when compared to the negative control sections of Figs. 4 a, b.

The intensity of this initially low labeling in the microspores started to increase in some MCM 5 days after culture (results not shown). The differences among MCMs became evident 13 and 16 days after culture (Figs. 4 c, d): some MCMs then showed an intense precipitate, whereas other MCMs and the undivided, degenerating microspores were devoid of labeling. Early proembryoids were also labeled (Fig. 4 c). Proembryoids formed 16 days after culture, already showing early symptoms of differentiation displayed the colored reaction on the periphery, whereas the inner region of the proembryoids was only weakly labeled (Fig. 4 d). Labeling in the MCMs was localized in the cytoplasmic region of the cells composing the structure, whereas highly vacuolated regions of MCMs were free of labeling (Figs. 4 e, f).

Discussion

Microscopical approaches to the study of plant embryogenesis play an important role in providing information about the cellular changes associated with embryogenic events. However, published reports regarding this matter are relatively scarce, and have focussed on a limited number of systems (Rashid et al., 1982; Barnabas et al., 1987; Zaki and Dickinson, 1990; Pretova et al., 1993; Garrido et al., 1995; Hause and Hahn, 1998). From the study of androgenesis induction in maize reported here, a number of conclusions can be drawn. The cytological characteristics observed in the microspores immediately after the cold pre-treatment suggest that they have undergone an intense degradation (and in some cases collapse) during this process, as is expected in a cell also surviving nutritional stress (Heberle-Bors, 1989). The decreased ribosomal population observed in this stage has also been described in androgenic microspores of *Datura* (Sangwan-Norreel, 1978) and *Nicotiana* (Garrido et al., 1995). However, the cellular swelling, the increasing of the electron-density of the cytoplasm (likely due to the initiation of the ribosome repopulation) and the appearance of numerous nuclear pores, which take place shortly afterwards, during the early stages of the culture, prior to the first division, are relevant cytological symptoms of the re-activation of cell metabolism. Nuclear pores are dynamic structures that change depending on the needs of nucleo-cytoplasmic transport. A significant loss of nuclear pores in the vegetative nucleus of immature pollen grains has been reported in *Nicotiana* during starvation conditions concomitant to the acquisition of embryogenic competence (Garrido et al., 1995).

In all cases observed, the first division of the microspores appeared to be symmetrical, giving way to two identical cells or nuclei. However, this statement has yet to be confirmed by the use of more specific experiments using DAPI staining. This result contrasts with those obtained by Pretova et al. (1993) and Barnabas et al. (in press) after studying different genotypes of maize, who observed a major presence of asymmetric divisions in most cases. Considerations to the putative genotype dependence of this characteristic should be consequently addressed in future works.

Concomitant to the cellular divisions, the formation of a new and thick cell wall between the intine and the plasma membrane, and also surrounding the newly formed cells, accounts for the reactivation of the cell metabolism. A newly synthesized wall, formed during androgenesis in the same localization and denominated fibrillar wall has been described in *Nicotiana* (Rashid, 1982) and *Brassica* (Zaki and Dickinson, 1990). The pressure accumulated as a result of the continuous cellular divisions leads to the breakdown of the original microspore wall, whereas this cell wall remains around the proembryoids.

Two main cytological characteristics of the post-division microspores must be stressed: firstly, the heterogeneity of the diverse cellular forms found even within the same anther, and secondly, (and closely associated with the previous point), the high level of polarity found within a given structure. This heterogeneity has been extensively described by Hause and Hahn (1998) in embryogenic microspore cultures of *Brassica napus*, where 6 groups of uni- or multicellular structures have
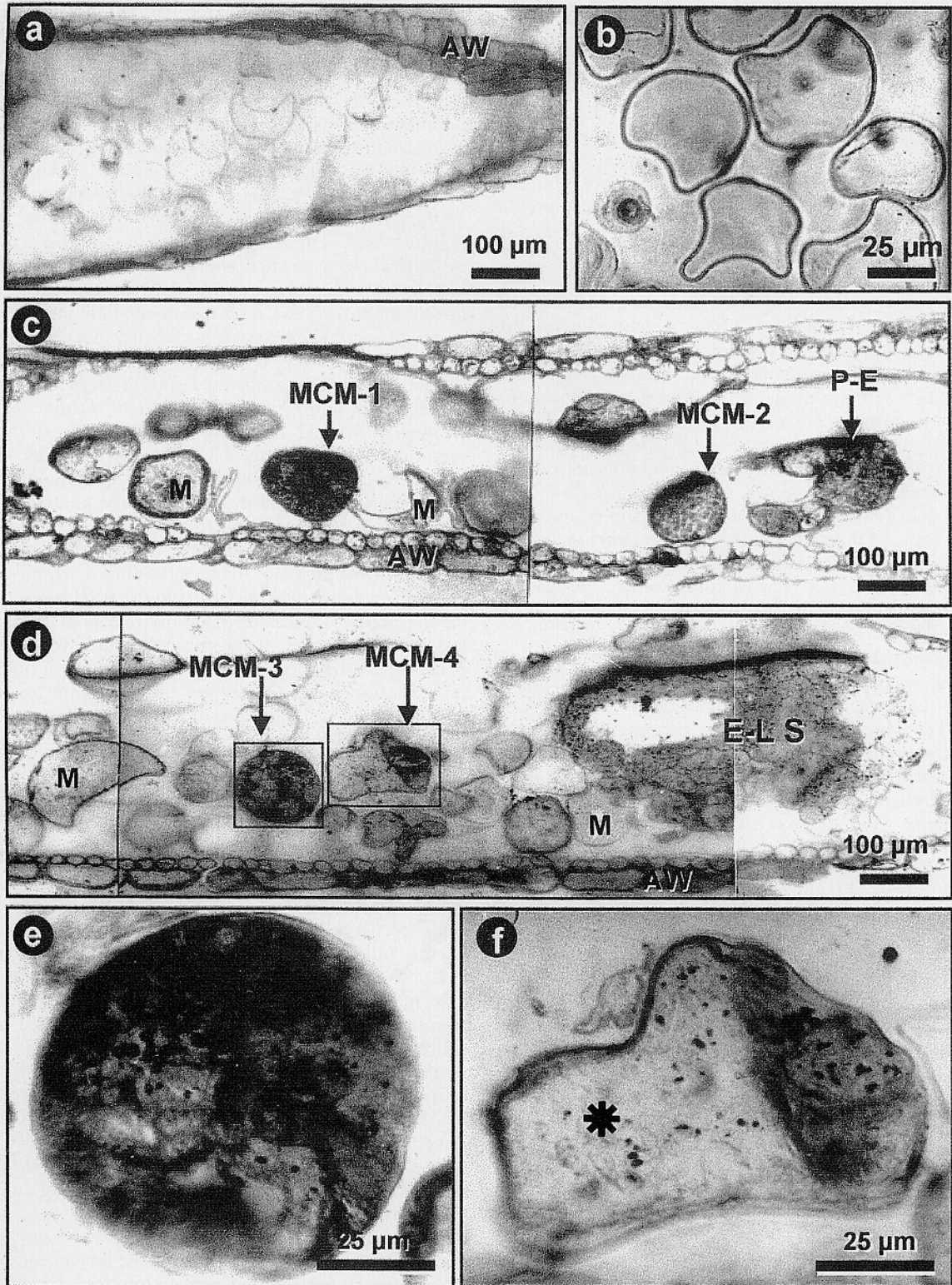
Fig. 4: Immunolocalization of UBQ and UBQ-Ps. **a**: negative control prepared by omitting the primary antibody. **b**: microspores immediately after cold treatment are still negative. **c** and **d**: sections corresponding to anthers after 13 (c) and 16 (d) days of culture. Several MCMs (MCM-1 to MCM-4), proembryoids (P-E) and embryo-like structures (E-L S) showed an intense labeling whereas undivided degenerating microspores (M) were devoid of labeling. **e** and **f**: high magnification of MCMs from Fig. 5 e showing an intense precipitate. Highly vacuolated areas were devoid of labeling (asterisk). AW: anther wall.
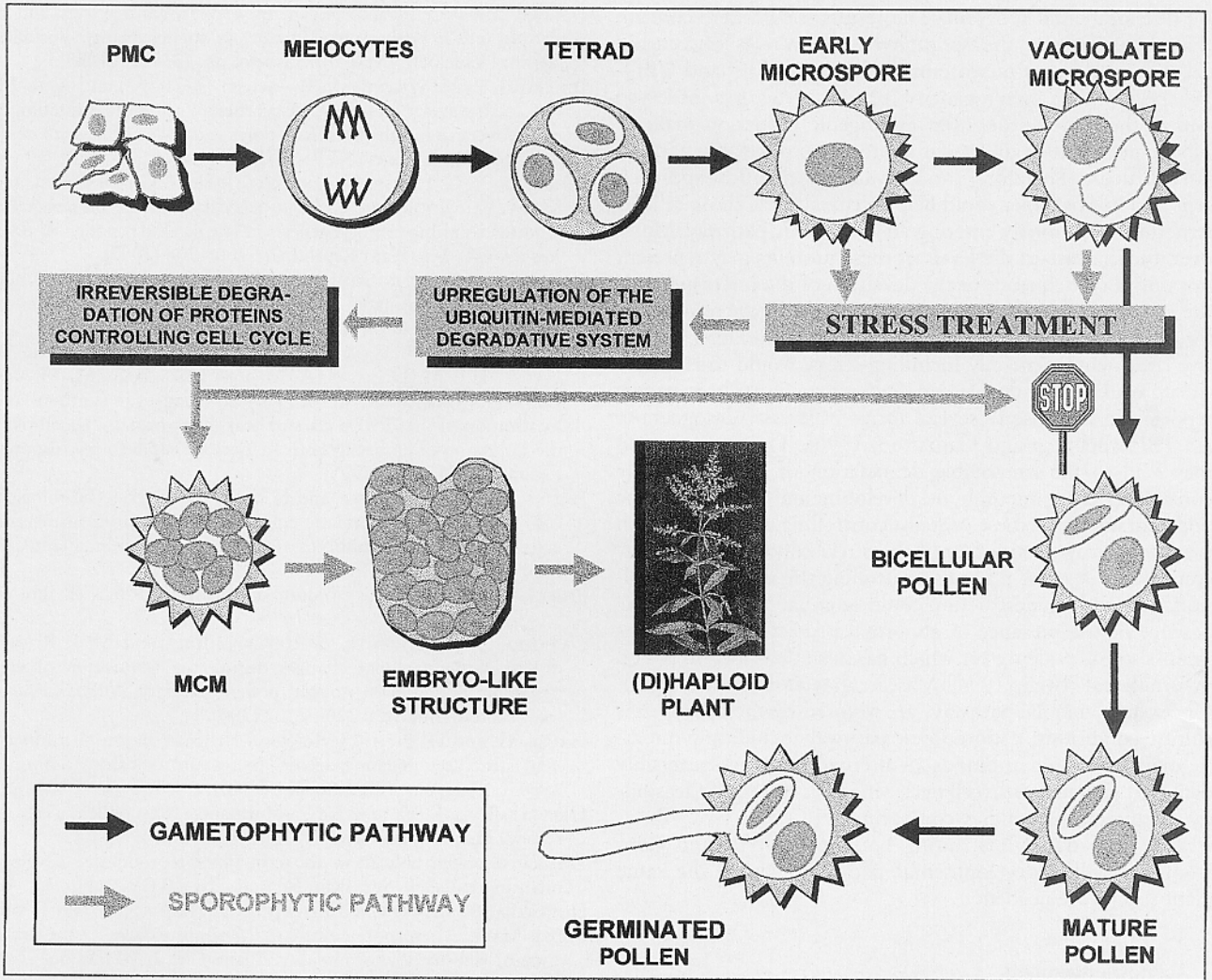
Fig. 5: Proposed model for the involvement of the ubiquitin-mediated degradative pathway in the induction of androgenesis.

been established according to cytological characteristics. In our work, several cellular forms, equivalent to those described in the previously mentioned paper have been found, although only one type of MCM has been studied in detail. This form corresponds to the one most commonly described as capable of progressing through embryogenic development, whereas other forms, mostly including undivided microspores, highly vacuolated MCMs (filled with starch granules), and MNMs do not seem to progress, at least up to the 16th day of the induction culture which has been studied here.

As regards the presence of polarity, putatively androgenic MCMs display a non-homogeneous disposition and size in the various cells conforming their structure, which may further develop in the clear differences and early symptoms of differentiation found in embryoids and embryo-like structures. This very early polarization has also been described by our group in wheat (Dr. A. Olmedilla. Estación Experimental del Zaidín. CSIC. Granada. Spain, pers. comm.).

Localization of ubiquitinated compounds correlated particularly with those MCMs considered potentially androgenic on the basis of their ultrastructural characteristics (dividing cells with rich, non-vacuolated cytoplasm, intact newly-formed layer, intact exine, etc.), to a greater extent than with cells displaying symptoms of elevated proteolytic activity and degradation. Similar results were obtained by Li et al. (1995) in anthers and pistils of *Nicotiana*. These authors found localization of these compounds in the differentiating tissues of the anther, but not in the degenerating tapetal cells. These results confirm that the ubiquitin-mediated pathway is involved in gene expression and regulation of cellular processes (Finley and Chau, 1991), thus characterizing a dynamic state of protein turnover (von Kampen and Wettern, 1992). Pioneer studies on UBQ and UBQ-Ps during maize pollen development (Callis and Bedinger, 1994) showed a developmentally regulated loss of free ubiquitin and ubiquitinated proteins correlated temporally with the commitment to the gametophy-

tic developmental program. The results shown here confirm that the return to the sporophytic pathway is once again accompanied by an increase in the levels of UBQ and UBQ-Ps species. The heterogeneity of the structures observed within the same anther after androgenic induction makes a biochemical and/or molecular approach to this study particularly difficult. Therefore, the immunocytochemical approach reported in this paper could help to characterize those cellular structures presumably entering an androgenic pathway. Moreover, the increase in the levels of these proteins may represent not only a consequence of the deviation of the microspores to the sporophytic pathway, but even a direct factor responsible for androgenesis induction itself, as proposed in Fig. 5. Inductive treatments, generally including stress, would thus trigger the upregulation of this degradative system, as has been widely reported in response to several forms of stress (Almoguera et al., 1995; Belknap and Garbarino, 1996). This upregulation may lead to the irreversible degradation of proteins tightly controlling the gametophytic developmental pathway in the pollen grain, as are these proteins controlling cell cycle (which is momentarily detained until the stress conditions are overcome), and/or those proteins controlling the asymmetric division. This sudden destruction could be enhanced by either the absence or the presence of abnormal protective mechanisms against stress, evidence for which has been described in pollen (Worrall and Twell, 1994; Mascarenhas and Crone, 1996). Once the metabolic pathways are restored by the appropriate culture conditions, a sporophytic pathway including symmetric divisions would appear as an alternative to the irreversibly damaged gametophytic cell cycle. In order to test the feasibility of this model, studies comparing UBQ and UBQ-Ps, as well as their transcripts during both sporophytic and gametophytic pollen developmental pathways within the same plant species are needed.

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