

Structure, DNA content and DNA methylation of synergids during ovule development in *Malus domestica* Borkh.

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Abstract — DNA content and DNA methylation of synergids of *Malus domestica* Borkh have been evaluated in two different stages before fertilization. A monoclonal antibody against 5-methylcytidine has been used to evidence the level and distribution of DNA methylation. In the first stage, both synergids were alike in structure, with a large chalazal vacuole, a large nucleus and nucleolus and a well developed filiform apparatus. One of the two cells was localized closer the micropilar region of embryo sac. At this stage both cells showed a 2C DNA content and a similar level and pattern of DNA methylation. The two synergids in *Malus* had different fates. In the second stage, near the time of fertilization, the micropilar synergid showed signs of degeneration: a reduced size, a compact chromatin and a more darkly stained cytoplasm. DNA methylation of this synergid was twice as much as the other cell and a different distribution of DNA methylation was also evidenced between the two cells, while their DNA content was unaltered. These observations are discussed in relation to the hypothesis of a different gene expression in the two synergids, thus supporting the assumption of a preprogramming for the degeneration of one of the two cells.

Key words: DNA content, DNA methylation, fertilization, *Malus domestica*, PCD, synergid.

INTRODUCTION

Synergids are probably the most dynamic and structurally complex cells of the female gametophyte. Walls of mature synergids may be partially or entirely absent over the chalazal end and are characterized over the micropylar end by a cell wall proliferation, the filiform apparatus. Histochemical properties, organelle composition and anatomical features indicate that synergids function as active secretory cells (TILTON 1981), involved in the last steps of pollen tube growth (KASAHARA *et al.* 2005) and in the process of fertilization (RUSSELL 1982; FOLSOM and CASS 1992; SHIMIZU and OKADA 2000; HIGASHIYAMA *et al.* 2001). A chemiotropic attractant of pollen tube is produced by the synergids, as demonstrated by experiments of laser ablation of gametophyte cells of *Torenia fournieri* (HIGASHIYAMA *et al.* 2001). Ca^{2+} has been suggested to be the putative substance because of its high concentration in the synergids of some plants (CHAUBAL and REGER 1990; 1992; HUANG and RUSSELL 1992) before

and after their degeneration. In *Torenia fournieri*, however, the synergids emit a not still identified diffusible signal that is species specific and acts at a short range (HIGASHIYAMA *et al.* 2003), in contrast to Ca^{2+} , that functions over long distances. Afterwards Marton *et al.* (2005) have reported that a small maize protein, EA1, is required for pollen tube guidance; however, this protein is not likely to be an universal attractant because no homolog of *ea1* is present in *Arabidopsis* or other dicotyledons.

Degeneration of one or occasionally of both synergids near the time of fertilization in angiosperm has been widely documented (JENSEN and FISHER 1968; TILTON 1981, FOLSOM and CASS 1992), even if, in some species, one or both synergids can survive for a long time and may undergo cycles of DNA endoreduplication (D'AMATO 1984). The degenerating synergid becomes predisposed to facilitate entry of pollen tube in the embryo sac. Two aggregates of actin filaments, the "coronas", presumably guiding the pathway of the male gametes, have been identified in the embryo sac of tobacco and one corona forms at the chalazal end of the degenerating synergid (HUANG and RUSSELL 1994; HUANG and SHERIDAN 1998; HUANG *et al.* 1999; YE *et al.* 2002), suggesting a role for actin in the reception

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of the pollen tube and in the double fertilization process.

The problem to be cleared up is whether a specific synergid degenerates or the degeneration may occur by chance between the two cells. In *Helianthus annuus* the degenerating synergid is closer to the micropyle and contains more calcium than the other cell (HE and YANG 1992) and in tobacco TIAN and RUSSELL (1997) have also evidenced a difference between calcium in the two synergids after pollination and less conspicuous differences in unpollinated flowers, suggesting that some aspects of synergid degeneration are preprogrammed and this event is accelerated, but not determined by pollination.

As DNA methylation levels are specific to a particular cell type and may be different not only between times but also within different genes (BROWN 1989), we have tried to put in evidence differences in the pattern of distribution of DNA methylation between the two synergids of *Malus domestica* Borkh., observed in two different stages before fertilization, using a monoclonal antibody against 5-methylcytidine (5-mCyt). It is known that methylation plays a crucial role in the regulation of gene expression and in the control of genome stability in higher eukaryotes (ADAMS 1990; CEDAR and RAZIN 1990; BIRD 1992; ZHANG *et al.* 2006; ZILBERMAN *et al.* 2006) and is essential for the normal development of most multicellular organisms (KLOSE and BIRD 2006). Mutations in any of three known DNA methyltransferase genes of the mouse are lethal either during embryogenesis or soon after (LI *et al.* 1992; OKANO *et al.* 1999); the reduction in methylation in *Arabidopsis thaliana* results in developmental abnormalities and partial female sterility (MITTELSTEN SCHEID and PASZKOWSKI 2000) and *met1* mutation alters the epigenetic inheritance during plant gametogenesis (SAZE *et al.* 2003).

MATERIALS AND METHODS

Flowers of *Malus domestica* Borkh., cv. Golden Delicious, were sampled in different stages from trees grown at the experimental farm of San Piero (University of Pisa) between April and May.

DNA measurements - Ovules, in two different stages before fertilization, were isolated and fixed in Carnoy (ethanol and acetic acid, 3:1), dehydrated through a graded ethanol series, incubated overnight in 100% ethanol, infiltrated and em-

bedded in LR White resin (Sigma). The material was polymerized at 60°C for 24 h. 3 µm serial sections were cut with a LKB Ultratome Nova and stained with Feulgen (O'Brien and McCully 1981) for DNA content determination. Feulgen staining was preceded by a treatment of material with a solution of 0.5% dimedone for 24 h. DNA measurements were performed by means of a Leitz MPV3 microdensitometer. Relative DNA content of synergid nuclei was estimated by adding the absorption of the same nucleus in all the serial sections, in which it appeared. The relative DNA content of embryonic metaphase cells was also determined to evaluate the 4C DNA content. Twenty ovules were considered for each stage.

Cytological analysis - Isolated ovules were fixed in FAA (10% formaldehyde, 5% acetic acid, 45% ethanol), dehydrated, embedded in resin and sectioned as previously described. 3 µm sections were stained with periodic acid-Schiff's reagent (PAS) (O'Brien and McCully 1981) and 1% aniline blue black (Sigma) in 7% acetic acid (FISHER 1968), for detection of insoluble polysaccharides and total protein, respectively. A part of material was stained with 0.05% toluidine blue O (TBO, BDH Chemicals) in 0.1 M acetate buffer, pH 4.4 (FEDER and O'BRIEN 1968) to obtain a differential staining of the different cellular components.

Immunological localization of the 5-mCyt rich regions - Isolated ovules, fixed in FAA (10% formaldehyde, 5% acetic acid, 45% ethanol) were treated as previously described. 3 µm thick sections were denatured in 4 N HCl for 20 min at room temperature and washed twice in distilled water and once in 0.1 M phosphate buffer solution (PBS) at pH 7.4 + 1% bovine serum albumin (BSA) + 0.1% Tween 20 (solution A). The slides were covered with 0.1 M PBS at pH 7.4 + 1% BSA + 1.5% normal goat serum (NGS) to block non-specific reactions for 20 min at room temperature. They were subsequently incubated in a solution with primary monoclonal antibody anti 5-mCyt diluted 1:200 in solution A for 2 hours in a moist chamber at room temperature. Then they were washed with solution A. The slides were exposed to secondary antibody gold conjugate (1:200 in solution A) (Bio Cell Research Laboratories) for 1 hour. Unbound secondary antibody gold conjugate was removed by six washes, the first in solution A for 5 min, the others in H₂O for the same time. The gold signal was silver enhanced to make it visible in the light microscope: three drops of initiator and three drops of en-

hancer (Bio Cell Research Laboratories) were mixed, and the slides were covered with this solution in the dark. The reaction was stopped after 6 min with tap water. Sections were stained with 0.05% toluidine blue O, air-dried and mounted in DPX. A silver grains count was carried out to evaluate the amount of DNA methylation in synergid nuclei in seriate sections: the count was made adding up the number of silver grains in each synergid nucleus for all the sections in which it appeared.

Immunochemicals controls - Conventional controls for the immunohistochemical method were processed in parallel in each experiment. Specificity of the immunolabelling was tested by the following controls: (1) replacement of the first antibody by solution A; (2) dilution of the primary antibody; (3) treatment with a non immune rabbit serum at the same dilution as primary antibody; and (4) omission of the DNA denaturation step.

Monoclonal antibody preparation - The preparation and specificity test of the monoclonal antibody directed against 5-mCyt was described in detail by PODESTÀ *et al.* (1993).

Statistical analysis - The data of DNA methylation and DNA content were analyzed statistically using the Student's t-test. The differences were considered significant at $p < 0.01$ and $p < 0.05$.

RESULTS

Malus domestica Borkh. female gametophyte undergoes *Poligonum* type development and consists of seven cells: three antipodes, a central cell, two synergids and one egg (Fig. 1).

Before fertilization, in the first stage (Stage I), mature synergids did not show structural differences. The micropylar end of *Malus* synergids had a filiform apparatus, that stained intensely with PAS and ABB (Fig. 2A), suggesting the occurrence of polysaccharides and proteins, and contained most of the cytoplasm, while the chalaza was occupied by a large vacuole (Fig. 2C, 3A). As seen in Fig. 2B, 2C and 3A, the nucleus of both synergids was very large, lied in the mid portion of the cell and contained a large spherical nucleolus; the chromatin, organized in chromocenters, had a peripheral localization close to the nuclear envelope. The cytoplasm was granular and very dense. The wall between the two synergids, chalazal to the filiform apparatus, became segmented into re-

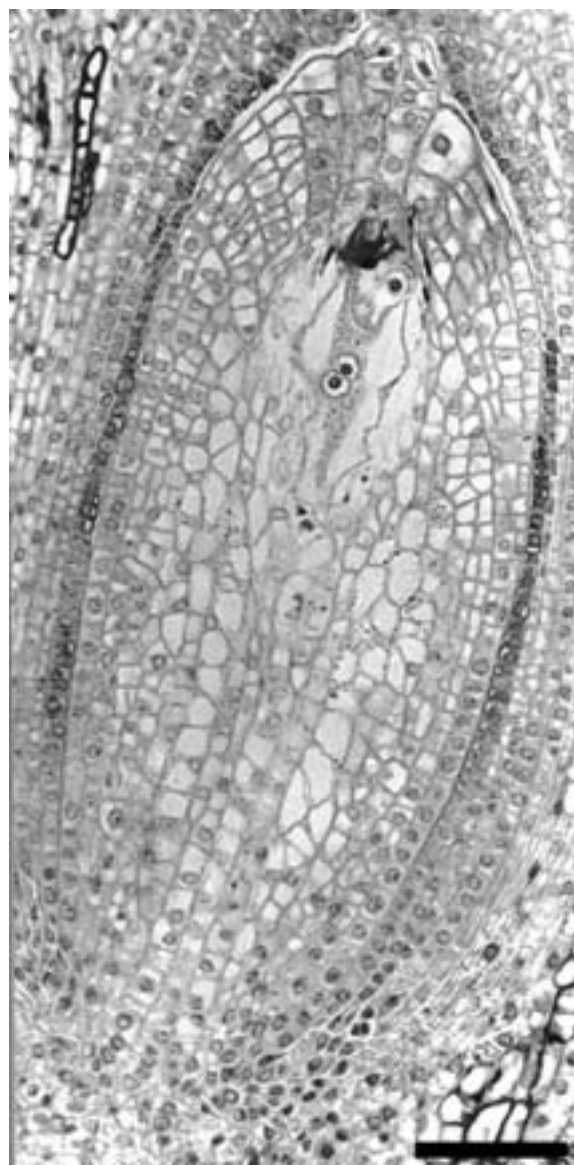


Fig. 1 — Longitudinal section of an ovule of *Malus domestica* Borkh. showing the embryo sac. Staining by PAS + ABB. Scale bar= 60µm.

gions separated by areas of membrane contact; the wall was interrupted also between the chalazal end of synergids and the egg cell (Fig. 2A and 2B).

A difference regarded the reciprocal position of the two synergids, as one was positioned closer to the micropylar end of embryo sac shaping sometimes a small hook (Fig. 2B).

At this stage DNA nuclear measurements of both synergids showed a 2C DNA content, evidencing that both cells were in the post synthetic period (Table 1, Stage I).

No differences were evidenced in level (Table 2, Stage I) and pattern of distribution of DNA

Table 1 — Mean relative DNA content (\pm Standard Error) per synergid nucleus. Stage 1: synergids (Sy 1, Sy 2) are morphologically similar. Stage 2: a synergid (Sy 2) is degenerating. None of the two stages denotes significant difference at $P < 0.05$. t is the value of the Student's t -test.

Embryo sac stage	Sy 1	Sy 2	F.D.	t
Stage I	100.1 \pm 0.53	100.3 \pm 0.36	19	0.31
Stage II	100.0 \pm 0.72	100.05 \pm 0.55	19	0.055

Table 2 — Mean number of silver grains (\pm Standard Error) per synergid nucleus. Stage 1: synergids (Sy 1, Sy 2) are morphologically similar. Stage 2: a synergid (Sy 2) is degenerating. Means \pm s.e. of stage I denotes difference significant at $P < 0.01$ not significant. Means \pm s.e. for stage II significantly greater than controls in a one-tailed test ($P < 0.01$). t is the value of the Student's t -test.

Embryo sac stage	Sy 1	Sy 2	F.D.	t
Stage I	25.8 \pm 0.74	26.6 \pm 1	19	1.45
Stage II	23.8 \pm 1.94	46.1 \pm 3.8	19	10.628

methylation between the two synergids; the labelling was localized in the heterochromatic regions (localized DNA labelling) in both cells and nucleolus was not labelled (Fig. 3A).

Malus synergids showed different fates. As seen in Fig. 2D, near the time of fertilization (Stage II), a synergid, the micropylar one, began to degenerate; the nucleus showed a compact chromatin and the nucleolus was not present, cytoplasm stained more darkly, vacuole disappeared and the cell was reduced in size. No areas of contact were evident between the plasma membrane of the two synergids. At this stage it was enhanced the micropylar position of the degenerating synergid in the embryo sac; the same cell showed a zone around the micropylar end that flared out laterally, forming a big hook (Fig. 2E). At this stage DNA content of both synergids was unaltered; both cells, in fact, showed a 2C DNA content as in the first stage (Table 1, Stage II), while the level and pattern of distribution of DNA methylation were different between the two cells; the degenerated synergid showed a nucleus with a diffuse and more intense labelling than the persistent synergid (Fig. 3B and 3C) and its level of DNA methylation was twice as much than the other cell (Table 2, Stage II), that presented a pattern of methylation similar to that before fertilization (localized labelling of DNA, Fig. 3C).

The results of DNA densitometric analysis showed no significant difference between mean relative DNA content of synergids at $P < 0.05$ in both the stages I and II.

The results of DNA methylation showed no significant differences between synergids in stage I and significant differences ($P < 0.01$) in stage II.

DISCUSSION

Before fertilization mature synergids of *Malus domestica* exhibited similar structural features and an intensely active metabolism. A strict functional relation between the two cells was evidenced, moreover, by the presence of areas of contact between the plasma membranes, interrupted by wall packets, which in soybean were suggested to be involved in some type of Golgi mediated activity (FOLSOM and CASS 1990). The partial lack of cell wall over the chalazal end of synergids in *Malus* formed areas of contact also between synergids and egg cell. This wall peculiarity and the presence of the filiform apparatus are favourable, in this developmental stage, to the hypothesis of a common role of transfer cells for *Malus* synergids, that may be, like in other plants, involved in the transport of nutrients needed for growth and development of the embryo sac (PRITHCARD 1964; FOLSOM and CASS 1990), in the expression of plant defensins genes (CORDTS *et al.* 2001) and in the secretion of chemiotrophic attractant of pollen tube (TILTON 1981; HIGASHIYAMA *et al.* 2001; 2003). In regard to the last point it has been demonstrated that the gene MYB 98 controls a branch of the synergid cell gene regulatory network, involved specifically in pollen tube guidance and the formation of the filiform apparatus (KASAHARA *et al.* 2005). The attraction frequency of the pollen tube has been demonstrated to depend on the presence of both synergids besides the number of pollen tubes and, when a synergid is ablated, the embryo sac retains the ability to attract a pollen tube, but the attraction is less than the complete embryo sac (HIGASHIYAMA *et al.* 2001).

Before fertilization both *Malus* synergids exhibited a similar pattern of distribution and level of DNA methylation; both these aspects, like as the same structural features, indicate that the two cells share the same functional activity.

Near the time of fertilization a synergid, the micropylar one in *Malus* embryo sac, degenerated before the pollen tube arrival and the persistent synergid remained intact until some time after fertilization. At this stage there were no evident areas

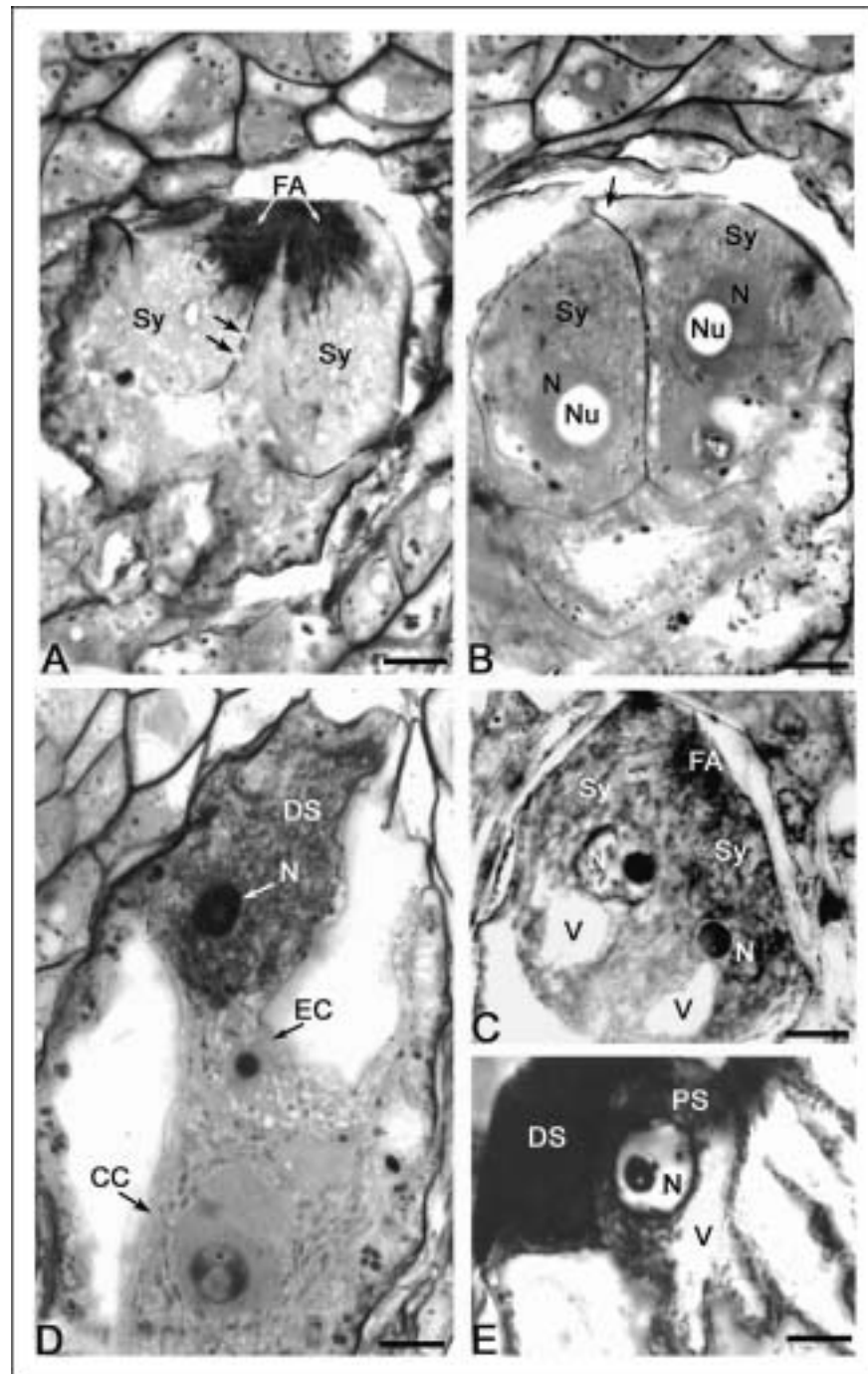


Fig. 2 A-E — Micropylar region of the embryo sac of *Malus domestica* Borkh. before fertilization. Stage I: figs A, B and C; Stage II: figs D and E. (A) Micropylar synergid walls have developed a prominent filiform apparatus. The wall between the two synergids is partially interrupted (arrows). (B) The two synergids do not show morphological differences; both cells show a large nucleus with a large spherical nucleolus and a dense cytoplasm. A synergid is positioned closer to the micropylar end of embryo sac where it shapes a small hook (arrow). (C) Both synergids show nuclei with chromatin organized in chromocenters, a large nucleolus and a large chalazal vacuole. (D) The degenerating synergid is deeply stained with a dense cytoplasm; the chromatin is condensed and intensely stained. It may be observed the egg cell with the nucleus and nucleolus and the central cell with both fusing nuclei. (E) The degenerating synergid is intensely stained, deeply contracted and shows a big hook, no internal structures are visible. The persistent synergid shows no evident morphological alteration: nucleus, nucleolus and vacuole are well structured. A, B and D: staining by PAS + ABB; C and E: staining with TBO. Sy = synergid; FA = filiform apparatus; N = nucleus; Nu = nucleolus; V = vacuole; CC = central cell; EC = egg cell; DS = degenerating synergid; PS = persistent synergid. Scale bar = 9 μ m.

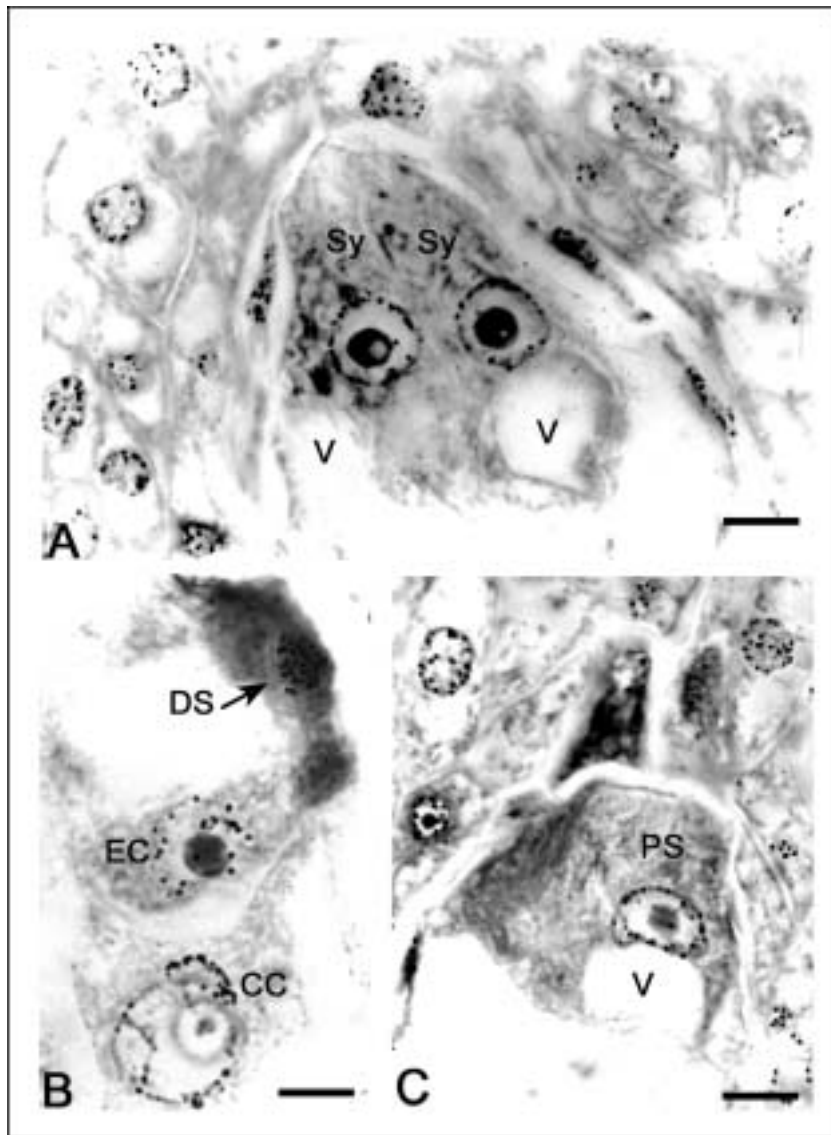


Fig. 3 A-C — Micropylar region of the embryo sac of *Malus domestica* Borkh. from ovules treated with the anti 5-methylcytidine antibody revealed by immunogold technique. (A) Stage I. Nuclei of both synergids show a similar pattern of labelling with the silver grains localized on the chromocenters. (B) Stage II. Degenerated synergid shows a condensed chromatin and an intense scattered labelling. (C) Stage II. Persistent synergid from the same ovule of Fig. 3B. The nucleus of this synergid shows a pattern of labelling similar to the nucleus of synergids of stage I; the silver grains are localized on the chromocenters. Staining by TBO. Sy = synergid; DS = degenerated synergid; PS = persistent synergid; CC = central cell; EC = egg cell; N = nucleus; V = vacuole. Scale bar = 9 μ m.

of contact between plasma membranes of the two cells. The degenerating synergid appeared shrunk and exhibited a more intensely staining for protein, a level of DNA methylation higher than the persistent synergid and a different pattern of distribution of DNA methylation, while DNA content was unaltered. ZHANG *et al.* (2006) have demonstrated the fundamental role of DNA methylation in the regulation of gene expression and genome stability on a genome wide scale, but they have, also, emphasized that subtle and perhaps

short lived changes in the DNA methylation of specific tissues or cell types that play key roles in development may be overlooked. As just one example, the authors reported that DNA methylation analysis of single cell types in maize demonstrated that particular imprinted loci have a different DNA methylation status in the egg and in the central cell, which are located right next to each other in the ovule. Cell-specific changes in DNA methylation coincide with a change in expression of these loci that is essential for development. Sin-

gle differentially methylated regions (DMRs), moreover, are correlated with allele-specific expression of two maternally expressed genes in the seed and one DMR is differentially methylated between gametes (GUTIERREZ-MARCOS 2006). The increase of DNA methylation in the degenerating synergid in comparison with the persistent synergid of *Malus* may indicate that a *de novo* DNA methylation activity occurred in this cell. *De novo* DNA methylation has been well documented during mammalian germ-cell development and early embryogenesis, when many DNA methylation marks are reestablished after phases of genome demethylation (BIRD 1999; REIK *et al.* 2001). A reprogramming of DNA methylation in the degenerating synergid is, probably, needed to enable the process of reproduction and it is conceivable that in this cell different sequences become methylated at different time points, as observed during oocyte growth in mammalian (REIK *et al.* 2001). It may be suggested that the *de novo* DNA methylation in the degenerating synergid of *Malus* may induce the repression of some genes, while DNA demethylation of other specific genes, for example those encoding apoptogenic factors, allow their expression (HOEBERICHTS *et al.* 2001). SCHWARTZ *et al.* (1990) have related selective repression and activation of particular genes in the tobacco hawkmoth *Manduca sexta* to programmed cell death and also VANYUSHIN *et al.* (2004) have related this phenomenon to the derepression of definite terminal genes inducing senescence and death. The increase and the different pattern of DNA methylation of degenerating synergid of *Malus* may be one of the several signals that switch for the activation of a differentiative pathway that may be responsible of the phenomenon of the senescence of this cell. One mutant of *Arabidopsis*, *gfa2*, has a defect in synergid cell death and, additionally, a defect in fusion of the polar nuclei; *gfa2* is targeted to mitochondria in *Arabidopsis*, suggesting a role for mitochondria in cell death in plants (CHRISTENSEN *et al.* 2002). In peculiar plants tissues, that are determinant in specific phases of plant development, DNA methylation seems to increase with the senescence, as evidenced in the suspensor cells of *Phaseolus coccineus* (ANDREUCCI *et al.* 1994; PIEROTTI *et al.* 1998) and in the tapetum of *Malus domestica* (FORINO *et al.* 2003). The high level of DNA methylation, of these cells, like as in the degenerating synergid of *Malus*, might have the role to improve the processes related to plant reproduction and to the first phases of embryo development, leading the cells to synthesize only those substances, that are necessary for par-

ticular metabolic requirements. For tissues, characterized by high levels of DNA methylation, it has been demonstrated (embryo suspensor, WREDDLE *et al.* 2001) or supposed (anther tapetum, PAPINI *et al.* 1999; FORINO *et al.* 2003) a process of genetically programmed death (PCD). Also in *Malus* the death of the synergid might occur by a distinct PCD morphology (LAM 2004), as suggested by the cell shrinkage and chromatin condensation during the degeneration.

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