Giacomo Bartoli, Cristiana Felici, Monica Ruffini Castiglione*

Female gametophyte and embryo development in *Helleborus bocconei* Ten. (Ranunculaceae)

Department of Biology, University of Pisa, via Ghini 13, Pisa, I-56126, Italy.

*For correspondence. E-mail monica.ruffini.castiglione@unipi.it

Abstract:

In this study we investigated cytohistochemistry, cycle progression and relative DNA content of the female gametophyte cells of *Helleborus bocconei* Ten. before and after fertilization process. The early stages of embryo development were also investigated. *H. bocconei* possesses a monosporic seven-celled/eight-nucleate *Polygonum* type female gametophyte, characterized by a morpho-functional polarity. The cells of the embryo-sac showed abundant reserves of polysaccharides, strongly increasing in the egg cell just before fertilization. With different timing in DNA replication during cell cycle progression, synergids, egg cell and polar nuclei showed a haploid DNA content at the end of their differentiation, while antipodes underwent three DNA endoreduplication cycles. Programmed cell death symptoms were detectable in synergids and antipodal cells.

After double fertilization, the central cell quickly underwent many mitotic cycles forming the endosperm, which exhibited a progressive increase in protein bodies and starch grains. Close to the developing embryo, the endosperm differentiated a well-defined region rich in a fibrillar carbohydrate matrix. The zygote, that does not start immediately to divide after double fertilization, developed in to an embryo that reached the heart stage at fruit maturation time.

A weakly differentiated embryo at this time indicates a morpho-physiological dormancy of seeds, as a survival strategy imposed by the life cycle of this plant with seed dispersal in spring and their germination in the following winter.

Keywords: DNA endoreduplication, embryo, embryo-sac development, hellebore, programmed cell death, relative DNA content

INTRODUCTION

In angiosperms female gametophyte may derive from monosporic, bisporic, and tetrasporic developmental pattern, giving rise, at maturity, to more than 15 alternative cellular arrangements (Maheshwari 1950; Bůžek et al. 1998; Madrid and Friedman 2009).

During the megagametogenesis of monosporic gametophytes (the most frequent in angiosperms), three of the four produced megaspores are genetically programmed to be removed (Cecchi Fiordi et al. 2002; Papini et al. 2011) and the remaining undergoes to three rounds of mitosis without cytokinesis generating eight nuclei in a coenocytic embryo-sac. Auxin gradients influence the chalazal-micropylar polarity of the mature female gametophyte and a battery of transcription factors mediate the specification and the final differentiation of its cells (Sundaresan and Alandete-Saez, 2010). In particular, the eight nuclei are subjected not only to spatial reorganization into the coenocytic embryo-sac but also to wall surrounding and cell differentiation. At the end of the gametogenesis, seven cells are distinguishable: the egg cell, the central cell, two synergids and three antipodes. Other than a specific arrangement along the micropylar-chalazal axis of the embryo-sac, these cells show high specialization degree, distinctive morphologies, different nuclear DNA content and specific biologic significances. The egg cell and the bi-nucleated central cell, after the fertilization, generate a diploid zygote and a triploid central cell respectively. Then, the zygote develops into an embryo and the central cell generates the endosperm, an embryo-nourishing tissue (Reiser and Fischer 1993; Liu et al. 2010). In addition, the ovule gives rise to a seed in which the integuments are converted in seed coat. The two synergids, situated at the micropylar pole of the ovule, at the entrance point of the pollen tube, are characterized by a well-developed wall-ingrowths, named filiform apparatus. The extensive cell wall proliferations increase significantly the surface area of the synergids and make these ones as transfer cells implicated in the translocation of metabolites into and out of the female gametophyte (Punwani and Drews 2008). Synergid cells are also required for both the attraction of the pollen tube and the reception of the sperm cells at level of the degenerating synergid (Higashiyama et al. 2001; Rotman et al. 2003; Amien et al. 2010). The three antipodal cells are confined close to the chalazal pole of the ovule and play a putative role in the transferring of nutrients from the surrounding sporophyte to the female gametophyte (Raghavan 1997). With the exception of several grasses, that replicate their antipodal cells after double fertilization (Heydlauff and Gross-Hardt 2014 and therein references), the other gametophytic cells do not divide again but sometimes can undergo significant increases in nuclear DNA content (Morozova 2002 and therein references) as a way to increase their metabolic performances in the achievement of their function. Consequently, the functional specialization of some embryo-sac cells could be related to the polyploidization of their nuclei (Morozova 2002).

A monosporic female gametophyte that consists in one egg cell, one central cell, two synergids and three antipodal cells is named "*Polygonum* type" (Maheshwari 1948) and it is exhibited by many biologically and economically important

plant groups, including the perennial herb *Helleborus bocconei* Ten. (Ranunculaceae). This plant, native to Europe and Asia and abundantly blooming from late winter to early spring, shows actinomorphic protogynous flowers where female receptivity is long lasting and ceases only if pollination has occurred. Pollination, carried out by insects of the *Bombus* genus, usually occurs in the first days of anthesis, when the flowers are still at the female stage (Vesprini and Pacini 2000). Anthers do not open simultaneously and each anther exposes its pollen for about three days (Vesprini et al. 2002). Interestingly, all the organs produced by the flower whorls are active in photosynthesis and can provide a significant part of the carbon needed for reproduction (Aschan et al. 2005). Especially the green showy elements of the perianth, commonly classified as sepals and persisting beyond floral senescence and seed dispersion, are thought to play a substantial role in the production of photoassimilates (Guitián and Larrinaga 2014). The nectaries are derived from modified petals and secrete nectar continuously for about 20 days after flower anthesis (Vesprini et al. 1999; Guitián and Larrinaga 2014). Since ancient times, *Helleborus bocconei* Ten. is employed in traditional medicine with different therapeutic uses for humans and animals (Puglisi et al. 2009; Maior and Dobrotă 2013) and nowadays it shows potential economic perspectives in terms of ornamental uses (Fascetti et al. 2014).

To the best of our knowledge, in the literature, there are no data regarding the development and the differentiation of female gametophyte in hellebore, especially concerning its cytohistological and cytohistochemical features before fertilization, at fertilization and post-fertilization time. Moreover, little is known about the nuclear DNA content of hellebore gametophytic cells during the different phases of their cell-cycle phases. Therefore, the focus of this investigation was the characterization from cytohistochemical point of view and in terms of nuclear phase and DNA content of *H. bocconei* embryo-sac cells at different developmental stages. The early phases of embryo development were also investigated. The results of this work can provide baseline information functional to taxonomic and phylogenetic investigations as well as for studies on reproduction mechanisms in plants.

MATERIALS AND METHODS

Plant material

Ovules and seeds of *Helleborus bocconei* Ten. were isolated from ovaries and fruits coming from flowers belonging to plants growing at the Botanical Garden of the University of Pisa (Italy) from January to March. The flowers were sampled seven days before anthesis, at anthesis and afterwards every seven days until fruit ripening, for a total of seven collection times. Seven developmental stages (Stage I-VII) were then defined for the study of both gametophyte and embryo development. After isolation, ovules and seeds were properly processed for the different cytohistochemical and cytophotometric investigations.

Histochemistry

Ovules and seeds were fixed in FAA, dehydrated by passing through a graded ethanol series and embedded in LR-White medium Grade (London Resin Company, Reading, Berkshire, UK), according to the manufacturer's instructions. Semi-thin sections (3 μm) were cut with an ultramicrotome (Ultratome Nova LKB Producter, Bomma, Sweden) and were stained using the following histological dyes: toluidine blue O (TBO, 0.05% in 0.1M benzoate buffer at pH 4.4) for general cytological investigations, periodic acid-Schiff (PAS) for non-cellulosic polysaccharides detection (Jensen 1962; Feder and O'Brien 1968), Aniline Blue Black (ABB, 1% in 7% acetic acid) for protein detection (Fisher 1968). Stained sections were cleared in xylene, air dried, mounted in DPX and observed with a LEITZ DIAPLAN light microscope (Wetzlar, Germany). Images of each slide were captured using a Leica DFC 420 (Leica Microsystems, Heerbrugg, Germany). Histological observations were performed on sections from the samples belonging to the different developmental stages and the cytological details were investigated in serial sections on the same slide. Microphotographs were representative of at least 20 samples yielding similar results.

***In situ* detection of DNA fragmentation by TUNEL assay**

Isolated ovules were fixed overnight at 4°C in 4% (w/v) buffered paraformaldehyde (PBS, pH 7.4); then they were dehydrated in ethanol, embedded in Paraplast Plus (Sigma-Aldrich, Milan, Italy) at 60°C for 48 hr, sliced in 10 μm sections and finally collected on poly-lysine coated slides. Deparaffinized sections were rehydrated and subjected to TUNEL assay (Terminaldeoxynucleotidyl transferase (TdT)-mediated dUTPnick-end labelling), by using TACS•XL Blue Label Kit (Trevigen, Gaithersburg, MD, USA) according to Bartoli et al. (2015). The sections were air-dried, mounted with DPX, observed with a LEITZ DIAPLAN light microscope (Wetzlar, Germany) and then captured using a Leica DFC 420 digital camera (Leica Microsystems, Heerbrugg, Germany). The assay was repeated at least three times.

Cytophotometric investigation and DNA quantitation

Ovules and seeds, from ovaries and fruits of flowers collected seven days before anthesis, at anthesis, seven days and fourteen days after anthesis, were fixed in ethanol and acetic acid, 3:1, dehydrated in an ethanol series and embedded in L.R.White resin (Sigma) according to the manufacturer's instructions. 3 μm serial sections were cut with a LKB Ultratome Nova and stained with Feulgen for DNA content determination (Giorgetti et al. 2011).

Measurements were performed by a Leitz MPV3 microdensitometer and the relative DNA content of each nucleus was estimated by adding the absorption of the same in all the serial sections in which it occurred, according to Tagliasacchi et al. (2007).

For each developmental stage, twenty gametophytes were considered and, for each of these, the absorption of each nucleus was recorded. Experiments were repeated three times. The reference standards of mean DNA content for this species were determined starting from root apical meristems. Ten germinating seeds, coming from the same plants used

for this study, were fixed in ethanol and acetic acid, 3:1, and processed with the Feulgen method as previously described (Giorgetti and Ruffini Castiglione 2016).

All the data (expressed as means ± SE) were analyzed by one-way analysis of variance (ANOVA) and Tukey *post hoc* test with values of $P < 0.05$ sufficient to reject the null hypothesis. Statistical analyses were performed by using statistical package Primers of Biostatistics (S.A. Glantz, Statistical Software Program Version 6.0, McGraw Hill 2005).

RESULTS

Cytohistochemical characterization of female gametophyte and embryo

H. bocconei showed anatropous, unitegmic crassinucellar ovules, with gametophytes essentially consisting of seven cells arranged along the major axis of the ovule: one egg and two synergids at the micropylar pole, one central cell at the middle and three antipodal cells at the chalazal pole.

Seven days before flower anthesis (Stage I, Fig. 1 a-f) the cellularisation and the definitive cell positioning into the embryo-sac were accomplished unlike the cell-differentiation that has yet to conclude. Many of the cells of the embryo-sac showed a polar inner organization (mainly due to the position of both nucleus and vacuole in the cytoplasm), a high concentration of starch grains and a nucleus characterized by both a peripherally localized chromatin and a large and well defined nucleolus (Fig. 1 c-f). At this stage, the egg cell showed a large vacuole, as well as the synergid cells that evidenced also a not fully developed filiform apparatus (Fig. 1 c-e), the central cell was still bi-nucleate (Fig. 1 f) and antipodal cells did not show any significant vacuole.

At flower anthesis (Stage II, Fig. 2 a-e), cell differentiation was fully accomplished. Synergids resulted basically indistinguishable (Fig. 2 c) with similar polar organization of the cytoplasm, similar cellular displacement of organelles, a well developed filiform apparatus, comparable nuclear and nucleolar structure. The egg cell increased its size thanks to a large vacuole and accumulated starch grains mainly in perinuclear position (Fig. 2 d). The nucleus of the central cell, showed a large and high vacuolated nucleolus (Fig. 2 e). The antipodal cells were characterized by a clear-cut vacuole and a well-defined nucleus, with the chromatin condensed close to the nuclear envelope (Fig. 2 e).

Seven days after anthesis (Stage III, Fig. 3 a-f), one of the two synergids showed some degeneration signs: the nucleus became irregular in shape with a compact and TUNEL-positive chromatin, the cytoplasm stained more darkly, the vacuole disappeared and the whole cell was reduced in size (Fig. 3 d and e). The persisting synergid, as well as the other gametophytic cells, basically did not show any significant change with respect to the preceding developmental stage (Fig. 3 e-f).

Fourteen days after anthesis (Stage IV, Fig. 4 a-e), the double fertilization had already taken place: the zygote showed a well-developed and thick cell wall, a large micropylar vacuole and some starch grains localized around the nucleus (Fig.

4 c). Mitosis of the central cell formed a nuclear endosperm, organized in a peripheral syncytium surrounding the central vacuole (Fig. 4 b and c). The persisting synergid displayed some cytological degeneration signs (i.e. irregular nuclear shape, signals of chromatin pyknosis, and vacuolar collapse) (Fig. 4 d). Antipodal cells began to degenerate showing nuclear chromatin condensation, DNA fragmentation events and cytoplasm disorganization (Fig. 4 e and f). Afterwards embryo began its development and, at the same time, the cavity of the embryo-sac increased in size. The endosperm was nuclear and localized mainly at the embryo-sac periphery (Stage V, Fig. 5 a-d), then it became cellular and centripetally filled the entire embryo-sac cavity. Starch grains occurred in the cytoplasm of both embryo and endosperm cells (Fig. 5 c and d).

Twenty-one days after anthesis (Stage VI, Fig. 6 a-e), when the development of carpels in young follicles was underway, the embryo reached the globular stage showing a well differentiated embryo-suspensor (Fig. 6 b and c). Both embryo and embryo-suspensor cells stored starch grains in their cytoplasm (Fig. 6 c). The endosperm portion that caps the embryo (proximal region) was cytohistologically well distinguishable from the rest of the endosperm: it consisted of cells with an increased stainability of both cell wall and cytoplasm and in which the plasmalemma withdrawn from the cell wall, occasionally producing significant invaginations (Fig. 6 d). Outside of this peculiar region, the endosperm cells (globally referred as distal region) stored a great deal of both protein bodies and starch grains and sometimes underwent to cell division (Fig. 6 e).

Twenty-eight days after anthesis, at fruit maturation (Stage VII, Fig. 7 a-e), the embryo was at the stage of initial heart stage with a well-developed embryo-suspensor, rich in starch grains (Fig. 7 c). More markedly than the previous stage, the cells of the endosperm capping the embryo evidenced an increased stainability of both cell wall and cytoplasm. Plasmalemma withdrawing from the cell wall (Fig. 7 d) was also detectable in these cells. In this zone, fibrillar carbohydrate matrix accumulates outside the plasmalemma and within intercellular spaces (Fig. 7 d). The cells of the distal endosperm region stored a certain amount of protein bodies and starch grains in their cytoplasm (Fig. 7 e).

DNA content values and cell cycle progression of female gametophyte cells

The mean DNA values, in arbitrary units, corresponding to the DNA content of a haploid genome in the pre-synthetic phase of the cell and of a triploid genome were mathematically obtained from the mean absorption values recorded for mitotic nuclei of cells from apical meristems of *H. bocconei* roots (2C = 2398.5 ± 23.6; 4C = 4662.67 ± 48.26) (Fig. 8 a). Figure 8 a shows the mean nuclear DNA contents of the female gametophyte cells before (Stage I, Stage II and Stage III) and after fertilization (Stage IV), in comparison to the reference standards of mean DNA content corresponding to genomes 1C, 2C, 3C and 4C. Figure 8 b depicts one feasible model of the nuclear events in the cells of hellebore embryo-sac from the Stage I to the Stage IV.

Measurements of the DNA content evidenced not significant differences between the two synergids (Stage I-Stage III), among the three antipodal cells (Stage I-Stage IV) and among the endosperm cells (Stage IV), then in the graph is represented the mean value of the measurements recorded for the cells of the same type.

At the Stage I, synergids, egg cell and central cell nuclei showed a DNA value of 1C corresponding to pre-synthesis phase (G_1) of a haploid nucleus. On the contrary, antipodal cell nuclei displayed a 3C DNA content, corresponding to the S phase between the first and the second endoreduplication cycle.

At the Stage II, the synergids and the egg cell were in synthesis phase (S). The polar nuclei of the central cell were fused and the resulting diploid nucleus was in G_1. The antipodal cells had completed three DNA endoreduplication cycles (16C) and then switched to the G_1/G_0 phase.

At the Stage III, the synergids and the egg cell were in G_2, while the DNA content of central cell and antipodal cells was unaltered with respect to the previous stage.

At the Stage IV, the zygote showed a DNA content of 3C and the triploid endospermatic cells were in G_2 (6C). At this stage, persistent synergid and antipodal cells showed a DNA content of 2C and 16C respectively.

DISCUSSION

The mature female gametophyte of *Helleborus bocconei* Ten. is a polar structure constituted by cells exhibiting an inner polarity, in certain cases even very marked, mainly due to an asymmetric positioning of the nucleus and vacuole into the cytoplasm. In particular, cell polarity establishment occurred early in hellebore embryo-sac development: both gametic and accessory cells evidenced a polar organization before they reached their final cyto-architecture and biological functionality at the end of gametogenesis. Nuclear location within single cells closely correlates with the fate of a given cell (Sprunck and Gross-Hardt 2011) and consequently with its biological function. In *H. bocconei* embryo-sac, as in other plant systems, the synergid and central cell nuclei are localized at the micropylar poles of the cells, and the vacuoles at the chalazal poles. On the contrary, the egg cell exhibited the opposite polarity. The different polarity of egg and central cells might be of strategic importance for the process of double fertilization as they ensure a close proximity of the female gamete nuclei in the region where the sperm cell pair sojourns after delivery into the receptive synergid, and where male and female gamete interactions takes place (Sprunck and Gross-Hardt 2011).

Although the cellular and molecular bases of specification of the embryo-sac cells are largely unknown, emerging evidences support the involvement of both nuclei position-based and lateral inhibition mechanisms in determining the fate of the seven cells of the female gametophyte (Yang et al. 2010; Sprunck and Gross-Hardt 2011).

In *H. bocconei* embryo-sac, the two synergids reached their definitive structural development during Stage II with the formation of a well-developed, fan-shaped filiform apparatus at the micropylar pole, mainly constituted by

polysaccharides. This wall specialization can make the synergids able in metabolite translocation to and from the gametophyte. The accomplishment of the filiform apparatus, and then the ability of solute transport, is one of the latest characteristics acquired by synergids, before double fertilization occurrence (Folsom and Cass 1990). During their development, the two synergids followed the classic cell cycle of a haploid cell, reaching the post-synthetic phase G_2 of the cell cycle in the course of the Stage II and Stage III and no further increases in DNA content by endoreduplication or extrasynthesis events at successive times have been observed. In contrast, some species of the genus *Allium* showed synergids that can experience some endoreduplication cycles, also different in number between the two cells (D'Amato 1984). Although the synergids of hellebore appeared cytohistologically indistinguishable, their functional behaviour was completely different when the female gametophyte gets ready to receive the pollen tube. Only the receptive synergid degenerated seven days after flower anthesis (Stage III), when the pollination was surely occurred, but before pollen tube penetration into the embryo-sac. This suggests the involvement of long-range diffusible signals trigging the synergid commitment towards cell death, as also observed in *Triticum aestivum*, *Gossypium*, *Nicotiana*, *Hordeum vulgare* and in other plant species (Willemse and van Went 1984; An and You 2004; Yadegari and Drews 2004). Afterward, the receptive synergid was penetrated by pollen tube tip for sperm nuclei discharge accomplishment and the persistent synergid remained unaltered until some times after fertilization (Stage IV). In *H. bocconei*, the degeneration of the receptive synergid occurred at specific times of embryo-sac development and it was characterized by significant modifications, including cell shrinking, chromatin condensation associated to DNA fragmentation. These traits, also evidenced in many kinds of synergid degeneration in other plant systems (Heydlauff and Gross-Hardt 2014 and therein references) are typical apoptotic hallmarks, suggesting that synergid removal is a PCD (programmed cell death)-dependent process. PCD has been demonstrated to be a gene-directed highly structured process, occurring during many stages of plant reproduction both in male and in female structures (Wu and Cheun 2000; Papini et al. 1999, 2011). In *H. bocconei*, the early selection of synergid fate (PCD or persistence) seems to be not a positional consequence, as proposed for female gametophytes with differently arranged synergids (Tagliasacchi et al. 2007), but a consequence of a differential inheritance of factors, required to realize cell death at the micropylar pole (Matias-Hernandez et al. 2010) and probably related to cell cycle exit in G_2/G_0.

In *H. bocconei* embryo-sac, the mature egg cell showed an inverted polarity with respect to synergids, with a well-defined nucleus and a vacuolated nucleolus, placed toward the chalazal pole of the embryo-sac and only one vacuole toward the micropylar pole, as in embryo-sac of *Arabidopsis*, *Beta*, *Brassica, Capsella*, *Nicotiana* and many other species (Kapil and Bhatnagar 1981). The same polar organization is also conserved by the zygote after fertilization. Notably, the egg cell is the only cell of the embryo-sac that retain abundant starch grains in its cytoplasm (from the Stage I to the Stage III), especially disposed in perinuclear position. Starch grains, interpreted as dynamic reserve

(Russell 1993), have been also detected in the egg cell of several plants (Mogensen and Suthar 1979; Wilms 1980; Bruun 1989; Russell 1993). Then starch grains persistence in the unfertilized egg cell can suggest a constant metabolic activity of this one unlike the other cells of the female gametophyte, in which the starch grains tend to decrease gradually, until their almost complete disappearance at fertilization time (Stage III). A higher metabolic activity of synergids, central cell and antipodals than the egg cell was observed in many plant systems (Reiser and Fischer 1993).

Cytophotometric results showed that the egg cell completes its cell cycle before fertilization, showing a DNA amount (2C) typical of a haploid cell in the post-synthetic phase (G_2). The zygote showed a DNA content corresponding to 3C value. This unexpected DNA value could be explained if we hypothesize that one of the two male sperm cells was still in pre-synthetic phase (DNA content 1C) at the fertilization time, while the egg cell already completed the DNA replication. This assumption indicates a phase displacement in the cell cycle progression of the male and female gametes at the fertilization time, affecting the possibility of the zygote to promptly undergo mitotic divisions.

In *H. bocconei* the fusion of the two polar nuclei of the central cell is one of the earliest events in the development of the embryo-sac (at Stage II). The diploid central cell remained in pre-synthetic phase (G_1) until the fusion with the sperm nucleus to produce a triploid nucleus. This cell progressed quickly in the post-synthetic phase (G_2) reaching a DNA content of 6C (Stage IV).

This result indirectly can suggest a different behaviour of the two sperm cells in our system: the male gamete involved in the fertilization of the egg cell has yet to complete the DNA replication (1C), while the male gamete, aimed to fuse to central cell, has concluded DNA synthesis as well the central cell (2C and 4C respectively). The different cell fate of the two male gametes, associated in some species with a clear sperm cell dimorphism (Mogensen 1992), has been in the past hypothesized to be driven by a sort of “mutual repulsion” of the two genomes, diverging towards the two distinct fertilization targets (Gerasimova-Navashina 1990), but more recently supplemented by the evidences on actin-based mechanisms of fertilization as pivotal apparatus in such a divergence (Huang and Russell 1994; Kawashima et al. 2014). In *H. bocconei* the different timing in DNA replication could be one of the distinctive feature able to lead the two sperm cells towards two different destinations.

Many mitotic cycles occur to produce a nuclear endosperm when the embryo is still at the stage of zygote or at least composed by few cells (Stage V). Interestingly, as evidenced also in *A. hypochondriacus* (Coimbra and Salema 1999), the divisions of the central cell nucleus occurred before the first division of the fertilized egg cell, suggesting that the central cell is precocious in its post-fertilization development than the zygote. Endosperm cellularization concluded when the embryo reached the globular stage (Stage VI). Then, the endospermatic cells of the region surrounding the embryo undergo significant cytohistological modifications (Stage VI and VII) very similar to those observed in *S. nigrum* (Briggs 1993), in *S. muricatum* (Kopcińska et al. 2004) and in *N. tabacum* (Erdelská 1985). These areas can

provide a path, rich in substances of different nature, released by living (i.e. in *S. nigrum*) or degenerating (i.e. in *N. tabacum*) endospermatic cells where the embryo can grow and develop without being damaged, into the seeds released on the soil after follicular dehiscence.

In *H. bocconei*, the three antipodes showed all the same cytological organization and the same increased DNA content at the end of their differentiation, as also evidenced in some ranunculacean species (D'Amato 1984). In fact, already at Stage I, the content of the nuclear DNA is higher than that estimated for the nucleus of a haploid cell, indicating that these cells began the first cycle of endoreduplication and that they completed three DNA endoreduplication cycles (16C) at Stage II. Many other members of Ranunculaceae evidenced polyploidization events, essentially deriving from disturbed mitosis or from endomitosis (Ünal and Vardar 2006). The high ploidy degree observed in antipodal cells can suggest a high metabolic activity of these ones, putatively related to the trophic role that they can play throughout the period of their persistence in the embryo-sac, as proposed for the ranunculacean *Consolida regalis* (Ünal and Vardar 2006). Interestingly, increases in DNA content are frequently observes in cells with nutritive role (Kozieradzka-Kiszkurno and Bohdanowicz 2003; Greilhuber et al. 2005): by increasing the number of individual loci into the nuclei of these cells, the endoreduplicaton is able to assist in maximizing mRNA and protein synthesis (Lee et al. 2009) necessary for embryogenesis. Endoreduplication, mainly occurring at G_2 phase of cell cycle (D'Amato 1984), can be also considered as part of terminal differentiation (Lee et al. 2009), concluding in this case with the cell death. Indeed the antipodal cells of *H. bocconei* are ephemeral cells as in *Arabidopsis* and in many other flowering plants (Song et al. 2014). Their demise occurred shortly beyond the fertilization (Stage IV), when the differentiating endosperm is preparing to take the full trophic competence, necessary to assist the embryo in its development. Antipodal cells degeneration happened at specific times and involved typical apoptotic hallmarks, suggesting the occurrence of PCD processes as a way to remove these cells become unnecessary, once held their biological function. There is reasonable evidence to assume that the PCD competence of antipodal cells requires both signalling cues provided by the female gametes (Heydlauff and Gross-Hardt 2014) in parallel with antipodal inherent factors, probably expressed exclusively at G_1/G_0 phase cell cycle and differently from that reported for synergids. This hypothesis is supported by previous data that demonstrate a tight correlation between specific cell cycle checkpoints and PCD (Tanaka et al. 2000).

At the time of fruit maturity, the embryo is very weakly differentiated (Stage VII) and it has to complete its development away from maternal sporophyte. In this situation, characterized by rudimentary embryos that must develop further before germination can occur, both the endosperm and the embryo-suspensor can play a crucial role for the accomplishment of embryogenesis on the ground thanks to their protein and starch deposits.

The release of seeds, whose dormancy is due to the dispersal of underdeveloped embryos, is common in some primitive taxa (Tamura 1995; Nikolaeva 1999), including ranunculacean species, having fruits that ripen in spring and seeds that

germinate in the following winter (Atwater 1980; Baskin and Baskin 1994; Engell 1995; Nomizu et al. 2004; Niimi et al. 2006).

Conclusions

In conclusion, we can summarize that *H. bocconei* possesses a monosporic seven-celled/eight-nucleate *Polygonum* type female gametophyte displaying a general morpho-functional polarity characterizing the cells of embryo-sac. Among them, antipodal cells exhibit early endoreduplication events, thought to be crucial for enhancing their metabolic capability and supporting the first phases of embryogenesis. The early different commitment of the two synergids seems to be not a positional effect, but a consequence of specific factors differently inherited. The observed precocious receptive synergid programmed cell death seems to be pollination-dependent and linked to diffusible signals, rather than contact-mediated, and achievable in a precise time of the cell cycle. Interestingly, after double fertilization, central cell underwent many mitotic cycles, when the zygote is delaying its first division. The absence of DNA endoreduplication events in synergids and the delay in the DNA replication of the central cell, together with the poorly differentiated embryo at the fruit maturation, represent archaic features distinctive of the genus *Helleborus*. However, the occurrence of heart shaped embryo at follicle maturation time, indicates a morpho-physiological dormancy of *H. bocconei* seeds and may represent a survival strategy imposed by the life cycle of hellebore, characterized by egg fertilization in late winter, fruit ripening and seed dispersal in spring and seed germination in the following winter.

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Figure captions

Fig. 1 *H. bocconei* at the Stage I. **a** the flower bud seven days before anthesis. **b** longitudinal section of the whole ovule. *Scale bar*= 100 µm. **c-f** embryo-sac seriated sections, showing synergids (S) at the micropylar pole, antipodal cells (A) at the chalazal pole, the egg cell (EC) close to synergids and the still bi-nucleate central cell (CC). Small dark starch grains occur within the cytoplasm of gametophytic cells. *Scale bar*= 20 µm. PAS and TBO double staining

Fig. 2 *H. bocconei* at the Stage II. **a** the flower at anthesis time. **b** longitudinal section of the whole ovule containing the fully developed embryo-sac. *Scale bar*= 100 µm. **c-e** embryo-sac seriated sections, showing the synergids (S) with a well-developed filiform apparatus (FA), the egg cell (EC) with perinuclear starch grains, the nucleus of the central cell (CC) with a large and vacuolated nucleolus, and one antipodal cell (A), with a nucleus showing the chromatin arranged close to nuclear envelope and a well developed nucleolus. *Scale bar*= 20 µm. PAS and TBO double staining

Fig. 3 *H. bocconei* at the Stage III. **a** the flower seven days after anthesis. **b** longitudinal section of the whole ovule showing the embryo-sac before fertilization, at the moment of synergid degeneration. *Scale bar*= 100 µm. **c** and **d** embryo-sac seriated sections, showing the persistent synergid (S), the receptive synergid (RS), the egg cell (EC) with abundant dark starch deposits, the central cell (CC) and one of the three antipodal cells (A). *Scale bar*= 20 µm. PAS and TBO double staining. **e** and **f** histological sections showing TUNEL-positive signal (blue staining) exclusively restricted to the nucleus of receptive synergid and not yet in the nuclei of the persistent synergid or of the antipodal cells. *Scale bar*= 20 µm

Fig. 4 *H. bocconei* at the Stage IV. **a** the flower fourteen days after anthesis. **b** section of the developing seed soon after fertilization. *Scale bar*= 100 µm. **c** and **d** sections of the embryo-sac at the micropylar pole, showing the zygote (Z) with a well-defined wall and small starch grains in its cytoplasm (arrow), the persistent synergid (PS) with an irregularly-shaped nucleus, the residue of the receptive synergid (RS) and the nuclear endosperm (NE). *Scale bar*= 20 µm. **e** section of the embryo-sac at the chalazal pole, showing the antipodal cells (A) with collapsed cytoplasm and highly condensed nuclear chromatin. *Scale bar*= 20 µm. PAS and TBO double staining. **f** histological section showing TUNEL-positive signals (blue staining) in the nuclei of the antipodal cells. *Scale bar*= 20 µm

Fig. 5 *H. bocconei* at the Stage V. **a** the flower twenty-one days after anthesis. **b** section of the developing seed, showing the young embryo and a monolayered peripheral nuclear endosperm. *Scale bar*= 100 µm. **c** and **d** histological

sections, showing the young embryo (EM) and the nuclear endosperm (NE), both with dark starch grains in the cytoplasm of their cells. *Scale bar*= 20 µm. PAS and TBO double staining

Fig. 6 *H. bocconei* at the stage VI. **a** the flower twenty eight days after anthesis. **b** longitudinal section of the developing seed showing the micropylar pole, double stained with PAS-TBO, with the embryo (EM) at globular stage and cellular endosperm differentiated in a proximal region (PR) that caps the embryo and in a distal region (DR). *Scale bar*= 100 µm. **c** section double stained with PAS-ABB showing the globular embryo (EM) with a well differentiated embryo-suspensor (ES). Dark starch deposits in cells of both embryo-suspensor (upper insert, two times magnified) and embryo (lower insert, two times magnified). *Scale bar*= 50 µm. **d** section double stained with PAS-ABB showing the proximal region of the endosperm with disintegrating cells at the embryo-endosperm interface, characterized by plasmalemma withdrawing and invagination events (arrow). *Scale bar*= 25 µm. **e** section double stained with PAS-ABB showing the distal region of the endosperm with cells containing abundant blue protein bodies and small dark starch grains in their cytoplasm; mitoses can also be detectable (asterisk). *Scale bar*= 25 µm

Fig. 7 *H. bocconei* at the stage VII. **a** the flower thirty five days after anthesis, at fruit dehiscence time. **b** longitudinal section of the developing seed, double stained with PAS-TBO, showing the micropylar pole with the embryo at initial heart stage and the cellular endosperm distinctly differentiated in proximal region (PR) and distal region (DR). *Scale bar*= 100 µm. **c** section double stained with PAS-ABB showing the embryo (EM) with a well differentiated embryo-suspensor (ES) containing abundant dark starch deposits (insert, two times magnified) and the proximal region (PR) of the cellular endosperm. Some endospermatic cells collapsed at the embryo-endosperm interface. *Scale bar*= 50 µm. **d** section double stained with PAS-ABB showing the proximal region of the endosperm with cells characterized by significant fibrillar carbohydrate deposits outside the plasmalemma and within intercellular spaces. *Scale bar*= 25 µm. **e** section double stained with PAS-ABB showing the distal region of the endosperm with cells storing a great deal of large light-blue protein bodies and dark starch grains. *Scale bar*= 25 µm

Fig. 8 DNA content and cell cycle progression of female gametophyte cells during its development, from the Stage I to Stage IV representing the early times after the double fertilization. **a** histogram showing the mean DNA contents (arbitrary units) of embryo-sac cells and of both zygote and endosperm cells. **b** schematic drawing showing the nuclear events in the cells of female gametophyte and zygote at the different developmental stages; an hypothetic intermediate stage, showing putative relationships between female gametophyte cells and male gametes, was also represented. s:

synergid, a: antipodal cell, ec: egg cell, cn: central nucleus, cc: central cell, z: zygote, en: endospermatic cell, *sc*: sperm cell, *pt*: pollen tube

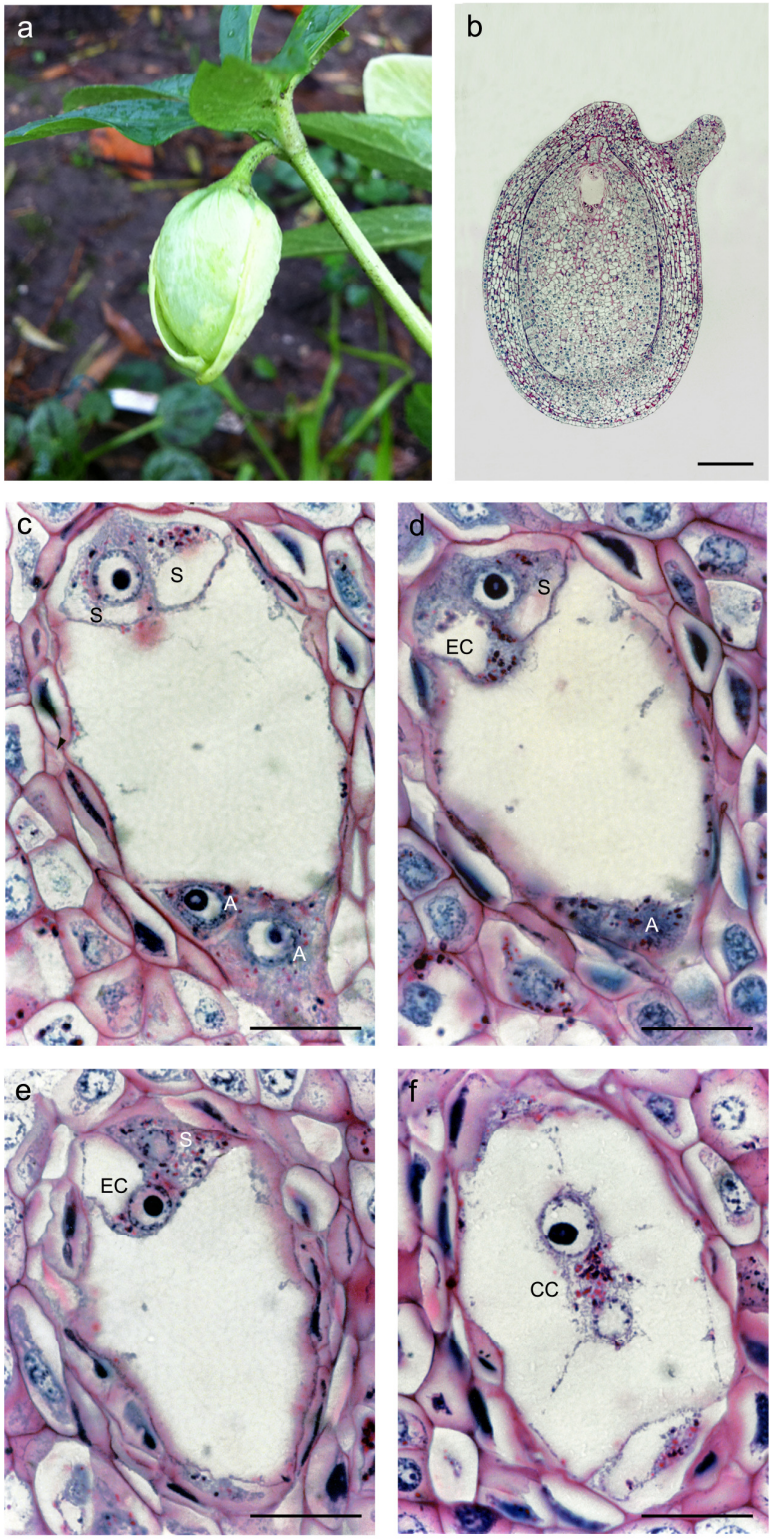

Figure 1

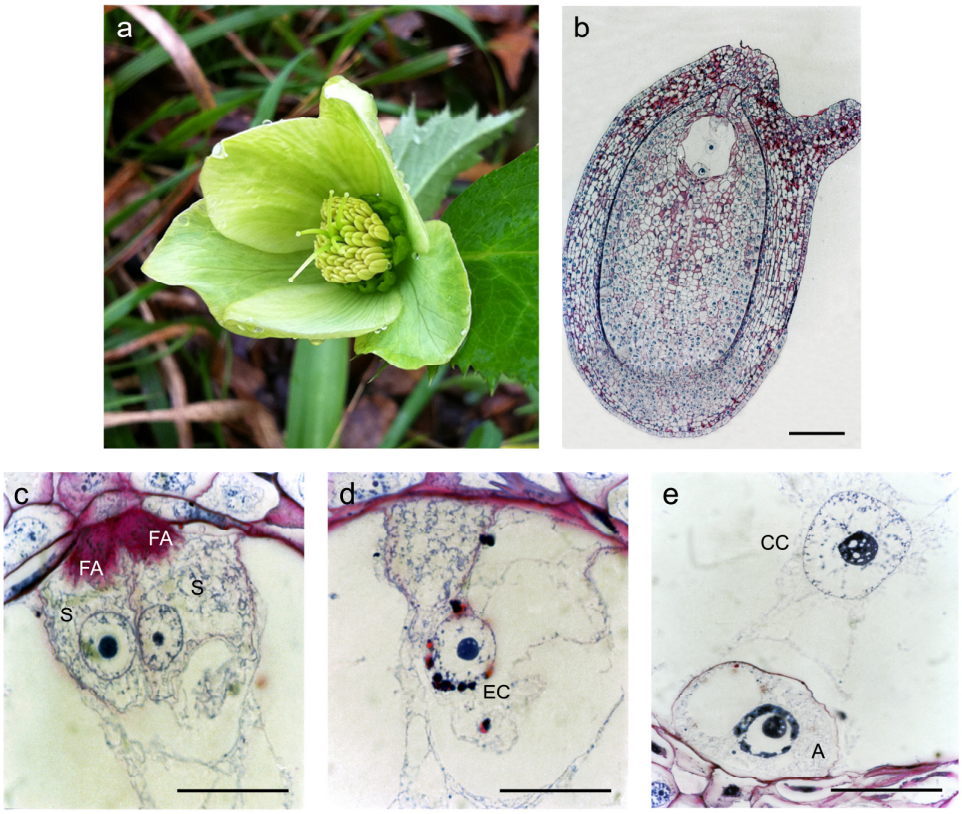

Figure 2

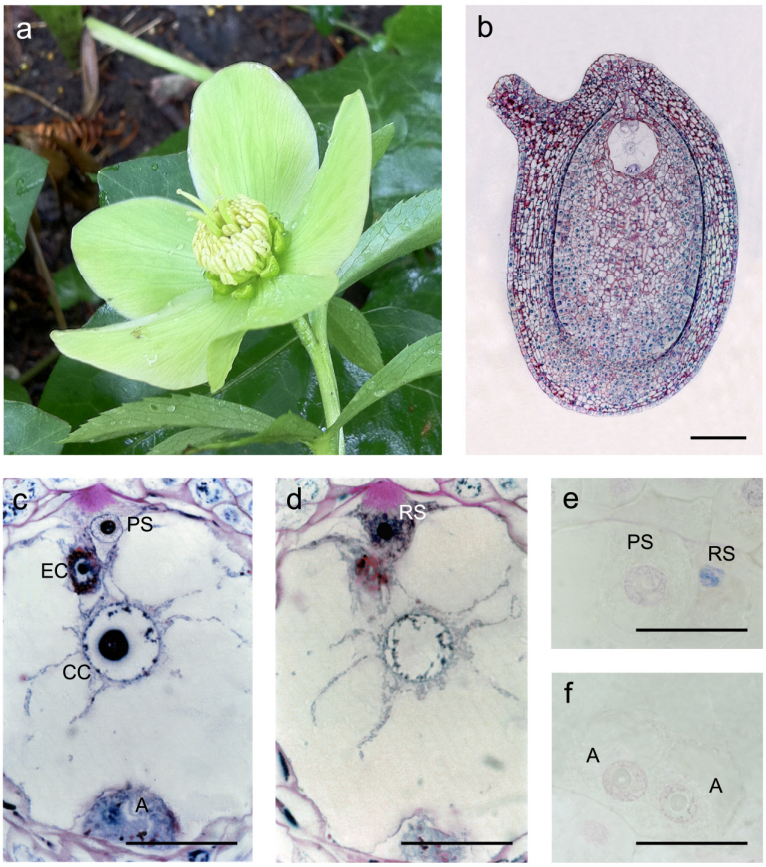

Figure 3

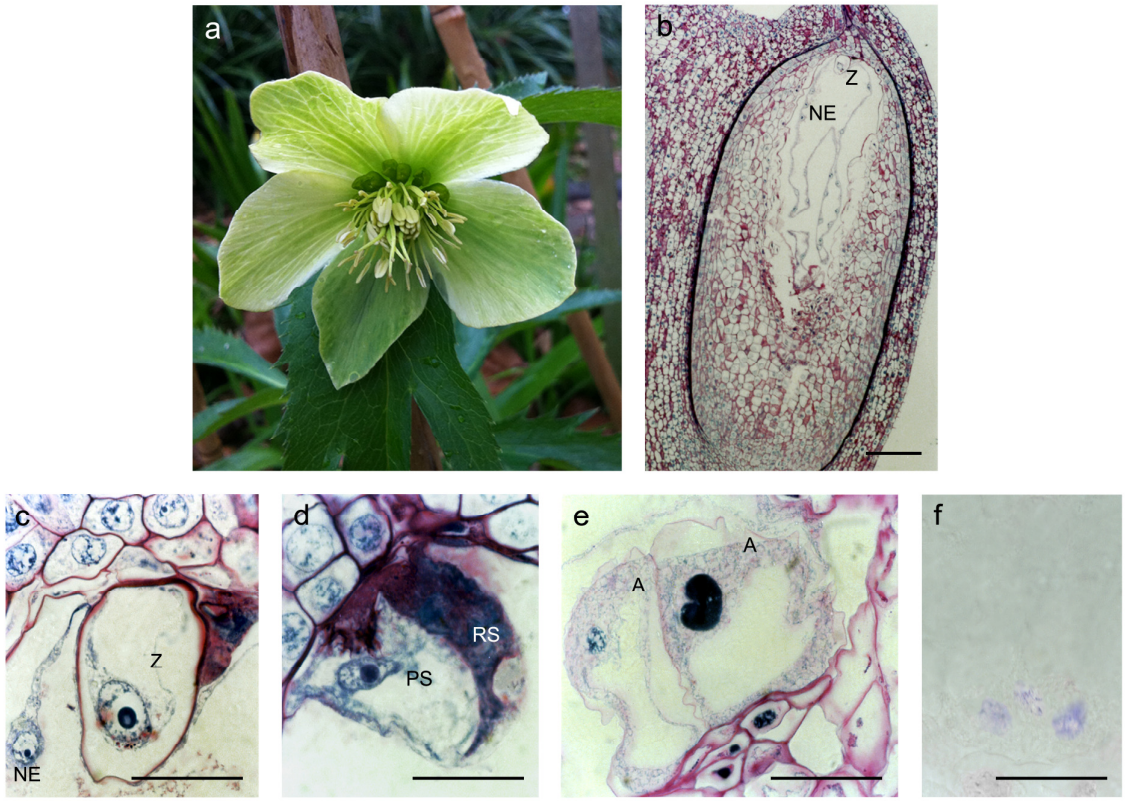

Figure 4

Figure 5

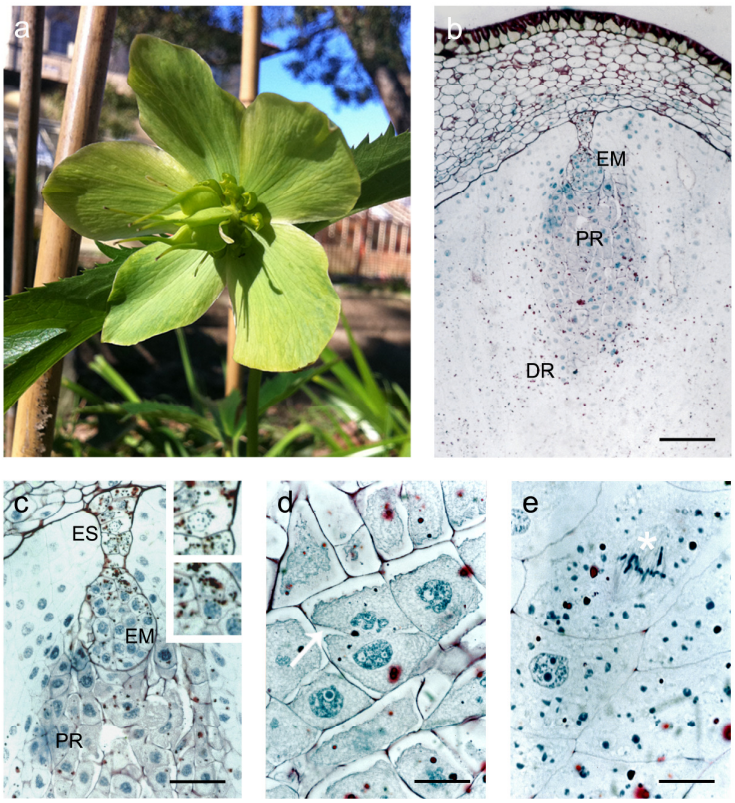

Figure 6

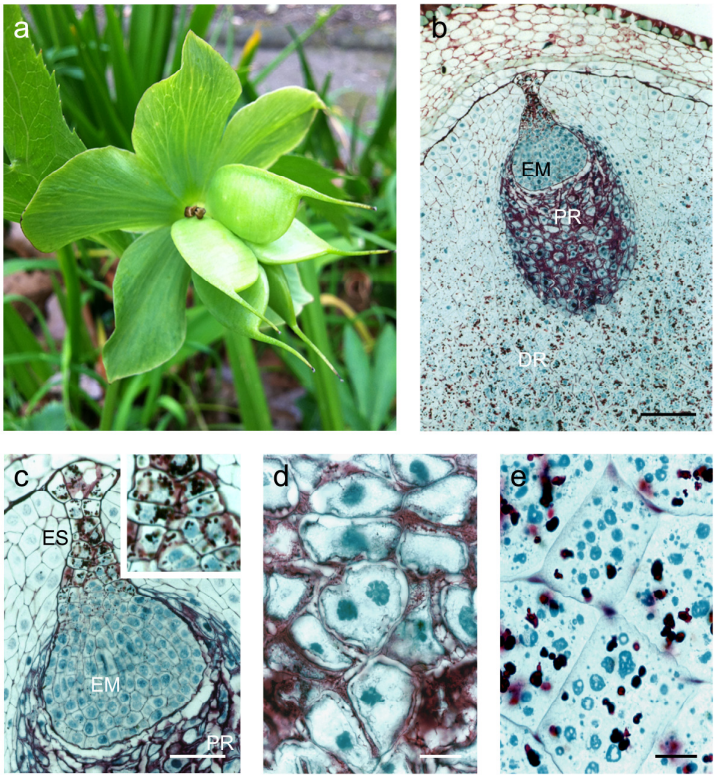

Figure 7

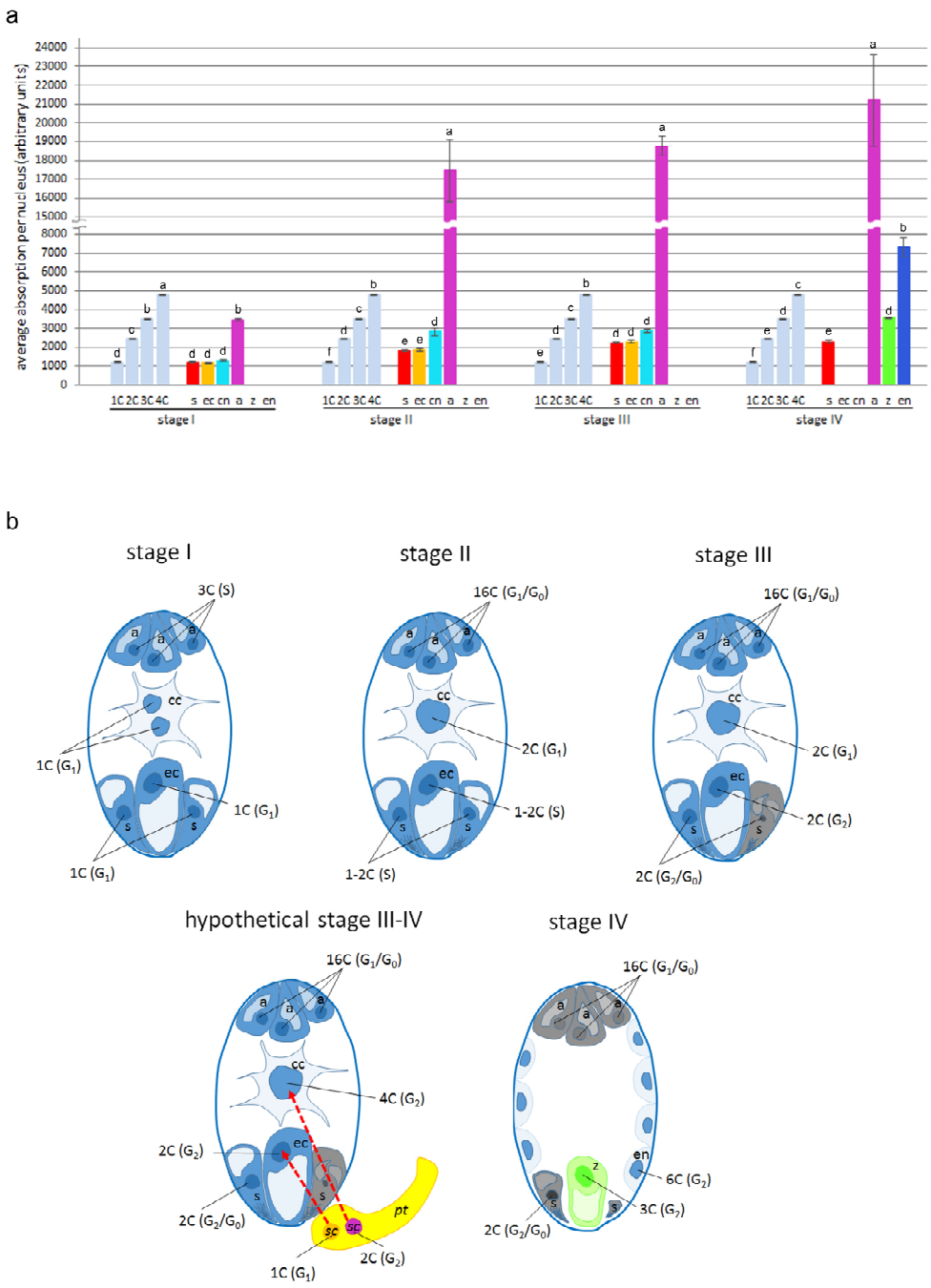
a
average absorption per nucleus (arbitrary units)
1C 2C 3C 4C s ec cn a z en
stage I
stage II
stage III
stage IV
b
stage I
3C (S)
1C (G1)
1C (G1)
1C (G1)
cc
ec
s
stage II
16C (G1/G0)
2C (G1)
1-2C (S)
1-2C (S)
stage III
16C (G1/G0)
2C (G1)
2C (G2)
2C (G2/G0)
hypothetical stage III-IV
16C (G1/G0)
4C (G2)
2C (G2)
2C (G2/G0)
pt
sc
2C (G2)
1C (G1)
stage IV
16C (G1/G0)
en
z
6C (G2)
3C (G2)
2C (G2/G0)

Figure 8