Sex Plant Reprod
DOI 10.1007/s00497-008-0087-7

ORIGINAL ARTICLE

Histological comparison between wheat embryos developing in vitro from isolated zygotes and those developing in vivo

Ferenc Bakos · László Szabó · Adela Olmedilla · Beáta Barnabás

Received: 18 July 2008 / Accepted: 17 September 2008
© Springer-Verlag 2008

Abstract There is currently great interest shown in understanding the process of embryogenesis and, due to the relative inaccessibility of these structures in planta, extended studies are carried out in various in vitro systems. The culture of isolated zygotes in particular provides an excellent platform to study the process of in planta embryogenesis. However, very few comparisons have been made between zygotic embryos grown entirely in cultures and those grown in vivo. The present study analyses the differences and similarities between the in vitro and in vivo development of wheat zygotic embryos at the level of morphology and histology. The study was possible thanks to an efficient culture system and an appropriate method of preparing isolated wheat zygotes for microscopy. The in vitro embryos were fixed, embedded and sectioned in the two-celled, globular, club-shaped and fully differentiated stages. Embryos developing in vitro closely followed the morphology of their in planta counterparts and their cell types and tissues were also similar, demonstrating the applicability of the present culture system for studying the process of zygotic embryogenesis. However, some important differences were also detected in the case of in vitro development: the disturbance of or lack of initial polarity led to changes in the division symmetry of the zygotes and subsequently to the formation of uniform cells in the globular structures. Presumably, differences between the in vitro and in planta environments resulted in a lower level of differentiation and maturation in in vitro embryos and in abundant starch and protein accumulation in the scutellum.

Keywords Embryogenesis · Isolated zygote culture · Meristem · Reserve material deposition · Wheat

Abbreviations

CBB	Coomassie Brilliant Blue R250
CFW	Calcofluor white
DAP	Days after pollination
DAPI	4′,6-Diamidino-2-phenylindole
ELS	Embryo-like structure
HAP	Hours after pollination
PAS	Periodic acid-Schiff
RAM	Root apical meristem
SAM	Shoot apical meristem

Communicated by Scott Russell.

F. Bakos · L. Szabó · B. Barnabás (✉)
Plant Cell Biology Department, Agricultural Research Institute, Hungarian Academy of Sciences, P.O. Box 19, 2462 Martonvásár, Hungary
e-mail: barnabasb@mail.mgki.hu

F. Bakos
e-mail: bakosf@freemail.hu

A. Olmedilla
Department of Plant Biochemistry, Cell and Molecular Biology, EEZ (CSIC), Professor Albareda 1, 18008 Granada, Spain

Present Address:
L. Szabó
Chemical Research Center, Hungarian Academy of Sciences, P.O. Box 17, 1525 Budapest, Hungary

Introduction

Culture of isolated zygotes provides an excellent system for studying the process of embryogenesis. Such cultures resulting in plant development have already been established in maize (Kranz and Lörz 1993; Leduc et al. 1996), barley (Holm et al. 1994), wheat (Holm et al. 1994; Kumlehn et al. 1998, 1999; Bakos et al. 2003a, b), rice (Zhang et al. 1999; Uchiumi et al. 2006, 2007b) and

tobacco (He et al. 2004), and even in the parthenogenetic Salmon wheat line (Kumlehn et al. 2001), wheat × maize (Bakos et al. 2005) and wheat × rice (Bakos 2007). Although plant regeneration has been achieved in a large number of species from microspore and somatic cell cultures (see Herman 2008), compared to zygote cultures, these methods only offer more indirect ways to study embryo development.

Numerous studies concerning the histology and cytology of in planta zygotic embryos have been published to date (for example, barley: Norstog 1972; maize: Randolph 1936; van Lammeren 1981; wheat: Batygina 1969, 1997; Bennett et al. 1973; Smart and O'Brien 1983; You and Jensen 1985; rice: Jones and Rost 1989a; pearl millet: Taylor and Vasil 1995). However, the developmental pattern of in vitro embryos was only compared with that of their in planta counterparts in the case of somatic embryogenesis (e.g., Jones and Rost 1989b; Taylor and Vasil 1996; Thijssen et al. 1996; Alemanno et al. 1997; Kärkonen 2000; Tereso et al. 2007; Kurczynska et al. 2007) and androgenesis (e.g., Hause et al. 1994; Indrianto et al. 2001; Testillano et al. 2002; Maraschin et al. 2005; Pulido et al. 2005; Pretová et al. 2006; Seguí-Simarro and Nuez 2008; Supena et al. 2008). The development of somatic or microspore-derived embryos closely follows that of in planta zygotic embryos, and efficient regeneration has been reported in numerous cases, but histological aberrations and genetic instability frequently occur in embryogenic cultures. Such abnormalities have a negative influence on the number of regenerants and on their vigour (for reviews, see: Rani and Raina 2000; Gaj 2004; Tahir and Stasolla 2006).

As no detailed comparisons of zygotic embryos developing in vitro have been made, only limited, mainly morphological, information is available about the differences between in vitro and in planta zygotic embryogenesis (Kranz and Lörz 1993; Kovács et al. 1995; Leduc et al. 1996; Kumlehn et al. 1999; Bakos et al. 2003a, b; He et al. 2007). In wheat, the main differences known are the first zygotic division in vitro appears morphologically symmetrical, and the embryos do not have a dormancy phase. In addition, the development of zygotic embryos is somewhat slower in vitro than in planta. In cultures, the first division of wheat zygotes takes place 1 day after pollination (DAP). Approximately 4 days are needed for the development of globular embryos and 8–10 days for club-shaped ones. These embryos become ready for transfer onto regeneration medium and for germination in approximately 20 days (Kumlehn et al. 1998; Bakos et al. 2003b). In planta, the first zygotic division occurs at approximately the same age as in vitro (approximately 21–24 h after pollination; HAP), but globular embryos develop within 2–3 days and club-shaped ones with an embryonal axis are established in 6 days. Morphologically complete embryos ready to enter the dormancy phase develop within 14 days (Batygina 1969, 1997).

Single cells are difficult to manipulate and few efficient zygotic culture systems exist, but some tools are already available for structural and molecular studies on individual isolated cells and on the structures originating from them. Methods have only been published for the fixation, embedding and sectioning of isolated zygotes in the case of maize (Faure et al. 1992) and tobacco (Ning et al. 2006). At the molecular level, gene expression studies are already possible on individual cells (Le et al. 2005; Steffen et al. 2007). Transcriptome and proteome analyses on isolated egg cells, zygotes and proembryos developing in vitro reveal several genes and proteins that play important roles during the initial phases of embryogenesis in wheat (Sprunck et al. 2005; Szűcs et al. 2006), maize (Okamoto et al. 2004, 2005; Okamoto and Kranz 2005; Yang et al. 2006), tobacco (Ning et al. 2006) and rice (Uchiumi et al. 2007a). The aim of this research is to model the development of in planta zygotic embryos, which can only be accessed with difficulty (for review, see Kranz and Scholten 2008; Nawy et al. 2008; Wang et al. 2006). In order to set up a complete and reliable model, in addition to optimal culture systems and molecular research, histological and cytological studies to compare in vitro and in planta embryogenesis will be required.

In the present work, therefore, the development of wheat zygotes in an efficient culture system was analysed using semithin sections and compared with that of their in planta counterparts.

Materials and methods

Plant material

Spring wheat (*Triticum aestivum* L.) cultivars Siete Cerros and Chinese Spring, and the winter wheat cultivar Mv9kr1 were grown in phytotron chambers as described previously (Bakos et al. 2003a).

In vitro culture of isolated wheat zygotes

In order to establish a nurse culture for zygotes, wheat pistils were dissected 3–4 days before anthesis and pre-cultured in 6-well plates (15 pistils, 1.5 ml medium/well, ∅ = 3 cm) for 3–4 days at 26°C in dim light (50 µmol m^{-2} s^{-1}) using N6Z medium (Kumlehn et al. 1998). The zygotes were isolated in 0.18 M glucose + 0.36 M mannitol + 2 mM $CaCl_2$ solution 1–2 h after pollination as described by Kovács et al. (1994). Zygotes were placed into Millicell transwell inserts

(∅ = 9 mm, 10–40 zygotes/insert) previously inserted into 6-well plates. The cultures were incubated at 26°C in the dark. After 2 weeks the pistils and 600 µl N6Z medium were replaced by freshly precultured ones. The preculture was done as described above. At 20–28 days, embryos measuring at least 1 mm were transferred to solid N6D regeneration medium and processed as described by Kumlehn et al. (1998). The viability of the zygotes and the structures derived from them was determined based on membrane integrity and cytoplasmic density viewed on an M35 inverted microscope (Zeiss, Germany).

Processing samples for microscopy

Since embryo maturation occurs at different rates in vitro and in planta, embryos were compared using size and developmental phase as criteria for maturational stage. Accordingly, examinations were made on isolated egg cells, zygotes after the first division (26 HAP for both in vitro and in planta), globular embryos (4 and 2.5 DAP, for in vitro and in planta, respectively), club-shaped embryos (10 DAP, in vitro), and fully differentiated embryos (20 and 14 DAP, for in vitro and in planta, respectively). All the structures from three whole cultures (i.e. a minimum of 40 structures/in vitro sample) and a minimum of 50 structures/in vivo sample were fixed in each case. The fixation and subsequent washing steps were carried out using MSB solution (Hoshino et al. 2004) modified to contain 450 mM glucose instead of 600 mM mannitol. In addition, the fixative contained 4% paraformaldehyde and 0.1% glutaraldehyde.

Smaller samples (structures up to 10 days) were first transferred into N6Z droplets, after which the liquid in the droplets was gradually replaced entirely with fixative using a micropipette over the course of 30 min. The cells were then left in the fixative droplets for 2 h in a humid environment. Subsequently the fixative was replaced by modified MSB as described above, but over a 10 min period. Then, 7 µl of 4% low-melting point agarose solution (in modified MSB) at 37°C was added to the approximately 20-µl droplets, and the solutions were carefully mixed immediately. After solidification, the agarose droplets were cut into 1-mm cubes, which were then transferred into small glass vials containing 1 ml modified MSB. The entire procedure was monitored under an inverted microscope. From this point, the vials were shaken at 20 rpm and two subsequent washes were done with modified MSB (for 30 min each). The dehydration (ethanol series) and embedding (Unicryl, BioCell) steps were done according to the manufacturer's instructions.

During the processing of larger samples (14-day-old half kernels containing the embryo and 20-day-old in vitro embryos, for example) differed in that 0.05 M cacodylate buffer was used instead of modified MSB; all the fixation and washing steps were done in glass vials, and the incubation times were 3 days for fixation (fresh fixative added each day) and 2 days for the other solutions.

Finally, all the samples were polymerised under UV light at −20°C. An inverted microscope was used for the localisation of small structures embedded in the resin (Fig. 1). Semithin (1 µm) sectioning was done using an Ultracut E (Reichert-Jung) ultramicrotome.

Histochemical staining

For labelling with CFW (Calcofluor white, for cell walls) and DAPI (4′,6-diamidino-2-phenylindole, for nucleic acids) the sections were incubated in 20 µg/ml DAPI and 1 mg/ml CFW (in 0.1 M phosphate–citrate buffer, pH 4.4) for 3 min, then rinsed with distilled water. The sections were mounted in Vectashield (Vector) with coverslips and immediately observed using an Olympus BX 51 epifluorescence microscope. Besides the clear, intensive labelling of the cell walls, CFW also labelled other organelles at various intensities at this concentration, so it also provided information about general cell structure.

For the visualisation of general anatomy, sections were stained with 0.5% Toluidine blue O solution. The slides were incubated on a hot plate for 20 s, then the staining solution was removed with distilled water. After drying, the slides were covered with DEPEX.

To visualise accumulated reserve materials, carbohydrates were first stained with PAS (periodic acid-Schiff

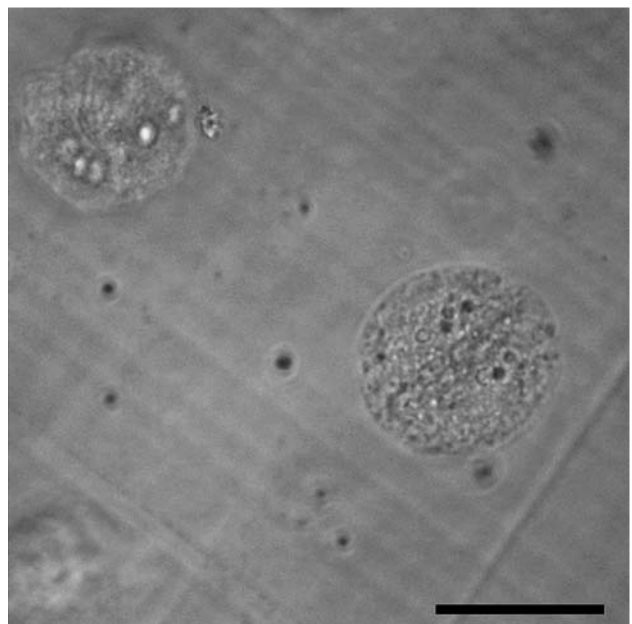

Fig. 1 Identification of small structures (bicellular proembryos in vitro embedded in resin) using an inverted microscope. *Bar* 50 µm

staining; McManus 1948), protein bodies were stained with CBB (Coomassie Brilliant Blue R250) according to Pulido et al. (2006), based on the method of Cawood et al. (1978), and finally Light Green was applied as contrast staining (McManus 1948). The slides were rinsed with distilled water between stains. Finally, the sections were dried and covered with DEPEX.

Results

Wheat zygote cultures

A nurse culture system involving N6Z medium (Kumlehn et al. 1998) and wheat pistils (Bakos et al. 2003b) led to plant regeneration from approximately 60% of the isolated wheat zygotes. The culture system had similar efficiency for all three genotypes examined (Siete Cerros: 59%, Chinese Spring: 54%, Mv9kr1: 61%, with a total of 148, 35 and 33 zygotes, respectively, each of which was monitored throughout the culture period). Of the 148 cultured zygotes of Siete Cerros (Fig. 2a) 126 divided (85%, Fig. 2b), 93 developed into globular embryo-like structures (63%, Fig. 2c), 92 reached the club-shaped phase (62%, Fig. 2d), and 87 became mature embryos or embryo-like structures (ELSs, Fig. 2e). Of these, 52 were single ELSs and 35 were twin (multiple) ELSs. Altogether 87 plants (representing 59% of the cultured zygotes, Fig. 2f) have been regenerated from the single and twin ELSs. Although 12 ELSs regenerated only after a delay via organogenesis, all the plantlets grew into fertile vigorous plants. The pattern and speed of in vitro development (Fig. 2) closely followed that described in earlier studies (Kumlehn et al. 1998; Bakos et al. 2003a, b).

In the next phase of the work, in vitro development of the zygotes was studied histochemically and compared with in planta embryogenesis.

First zygotic division in planta and in vitro

After the first zygotic division, proembryos showed great similarity in cellular architecture, except that the shape of the daughter cells was consistently different in vitro and in planta (Fig. 3). In all examined cases, the first division resulted in two cells with identical shape in vitro (Fig. 3a, b; 48 sectioned proembryos), in contrast to the basal and apical cells separated by a perpendicular cell wall found in vivo (Fig. 3c, d; 14 sectioned proembryos). In both cases, the cytoplasm around the nuclei was rich in organelles, and small vacuoles were visible at the periphery of the daughter cells (Fig. 3a, c). These features are very similar to the cytoplasmic architecture of isolated egg cells (Fig. 3e). The egg cells had no cell wall an hour after isolation, while this was clearly visible in the proembryos. The two-celled proembryos were hardly bigger than the egg cells (Fig. 3a, c, e). Starch grains occurred mostly perinuclearly in the proembryos both in vitro and in vivo (Fig. 3b, d), and they were larger and more numerous than in the egg cells (Fig. 3f).

Globular stage of embryogenesis

Globular embryos were compared when they reached a size of 60–80 μm and contained approximately 10–20 cells (Fig. 4). Embryos in vitro were basically composed of meristematic, cytoplasm-rich cells (Fig. 4a). Starch accumulation showed a slight gradient, which could be interpreted as a sign of embryo polarity (Fig. 4b). In vivo,

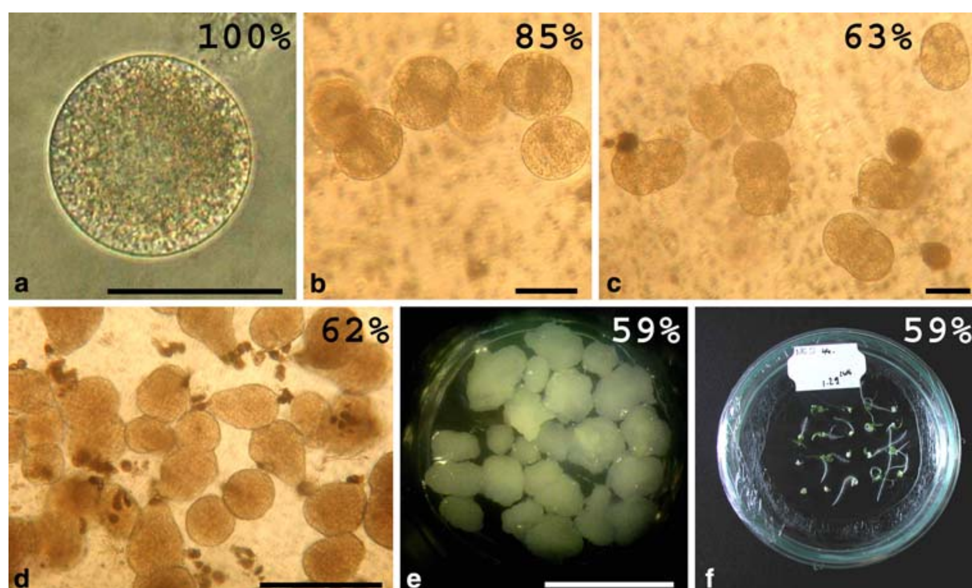

Fig. 2 **a** Freshly isolated zygote, 1 HAP. **b** The first cell division of the zygotes is symmetrical in vitro, 26 HAP. **c** Globular embryo-like structures, 4 DAP. **d** Club-shaped embryos closely resembling those formed in planta, 8 DAP. **e** Embryos and embryo-like structures ready for transfer onto regeneration medium, 20 DAP. **f** Regenerating plantlets, 30 DAP. *Percentages* (%) demonstrate the efficiency of differentiation at the given stages. *Bar* **a–c**: 50 μm; **d**: 500 μm; **e**: 5 mm

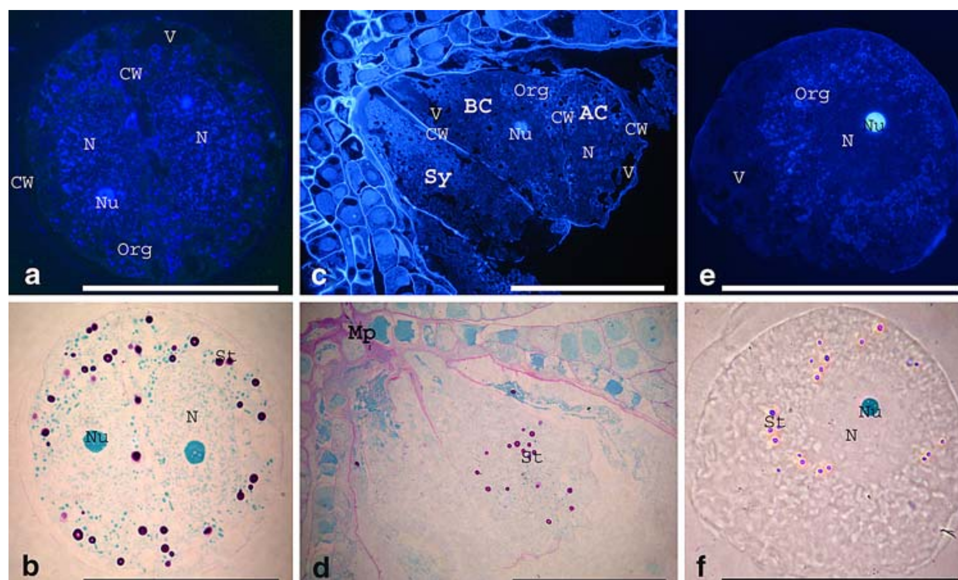

Fig. 3 Two-celled proembryos at 26 HAP developed in vitro (**a**–**b**) and in vivo (**c**–**d**). **e**–**f** Freshly isolated mature egg cell. **a**, **c**, **e** DAPI + CFW staining. **b**, **d**, **f** PAS + CBB staining with Light Green contrast staining. *AC* Apical cell, *BC* basal cell, *Mp* micropyle, *Sy* persistent synergid, *CW* cell wall; *N* nucleus, *Nu* nucleolus, *Org* organelle-rich cytoplasmic area, *St* starch grain, *V* vacuole. *Bar* 50 μm

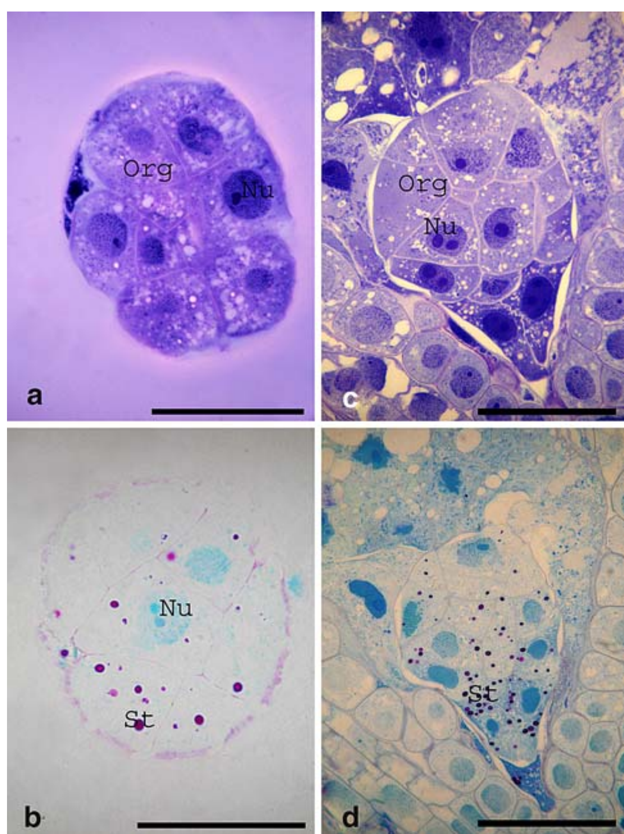

Fig. 4 **a**–**b** Globular embryos developed in vitro at 4 DAP. **c**–**d** Globular embryos developed in planta at 60 HAP. **a**, **c** Toluidine blue staining. **b**, **d** PAS + CBB staining with Light Green contrast staining. *Nu* Nucleolus, *Org* organelle-rich cytoplasmic area, *St* starch grain *Bar* 50 μm

however, the polarity was expressed not only by the higher starch accumulation at the basal pole (Fig. 4d), but also by the intense staining of the basal cells and lighter staining of the apical cells (Fig. 4c). The plurinucleolar nuclei were an indication that the cells were intensely dividing in both the in vitro and in planta embryos.

Embryo axis differentiation

The differentiation of the embryonal axis starts in approximately 10-day-old embryos in vitro (Fig. 5a), which are almost identical to 6-day-old embryos in planta (Batygina 1969, 1997). The shoot primordia can be seen as polar protrusions consisting of cells rich in cytoplasm, and the root primordia as another group of meristematic cells in a central position. The slightly vacuolated cells around the embryonal axis show the differentiation of the scutellum, while the relatively more vacuolated ones at the basal part resemble the suspensor region in planta.

Morphology of mature embryos

The 20-day-old embryos usually grew to 1.5–2 mm in vitro and their size corresponded to that of 14-day-old embryos in planta (Fig. 5b, c). These in vitro embryos were mature enough for transfer onto regeneration medium, where they developed into small plantlets in 6–10 days (Fig. 2f); however, they exhibited a lower degree of differentiation compared to the in planta embryos. All of the organs of the embryonal axis developed, but they were smaller and the arrangement of the cells was less organised than in vivo. The leaf primordia were conical in shape in contrast to the well-developed, sheath-like leaf primordia in vivo. The coleoptile was less distinct and did not envelop the shoot apex in vitro, in contrast to the well-formed, closed coleoptile of the in vivo embryos. The provascular strand was well-differentiated in both environments, but the scutellar

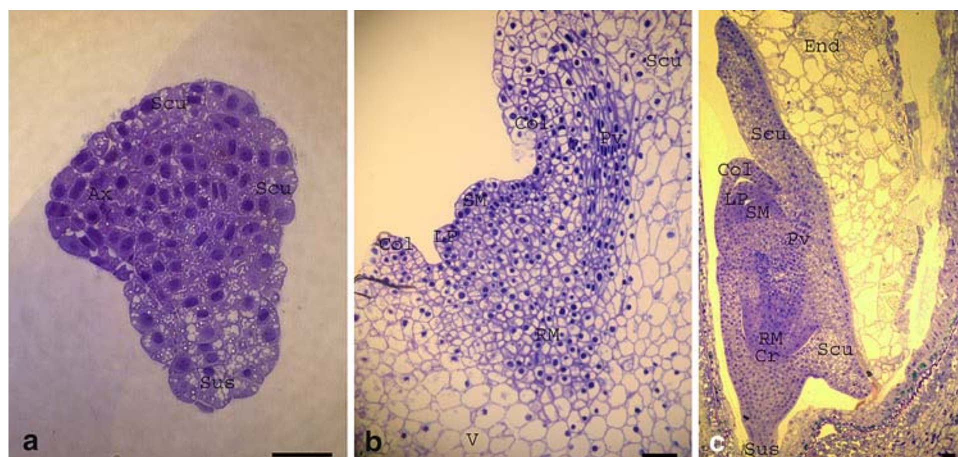

Fig. 5 **a** Club-shaped embryo developed in vitro at 10 DAP. **b** The axis of an embryo developed in vitro at 20 DAP. **c** Morphologically complete embryo in vivo at 14 DAP. **a**–**c** Toluidine blue staining. *Ax* Embryonal axis, *Col* coleoptile, *Cr* coleorhiza, *End* endosperm, *LP* leaf primordium, *PV* provascular strand, *RM* root apical meristem, *SM* shoot apical meristem, *Scu* scutellum, *Sus* suspensor, *V* vacuole. *Bar* 50 μm

morphology and cell types differed greatly (Figs. 5b, c, 7a, b).

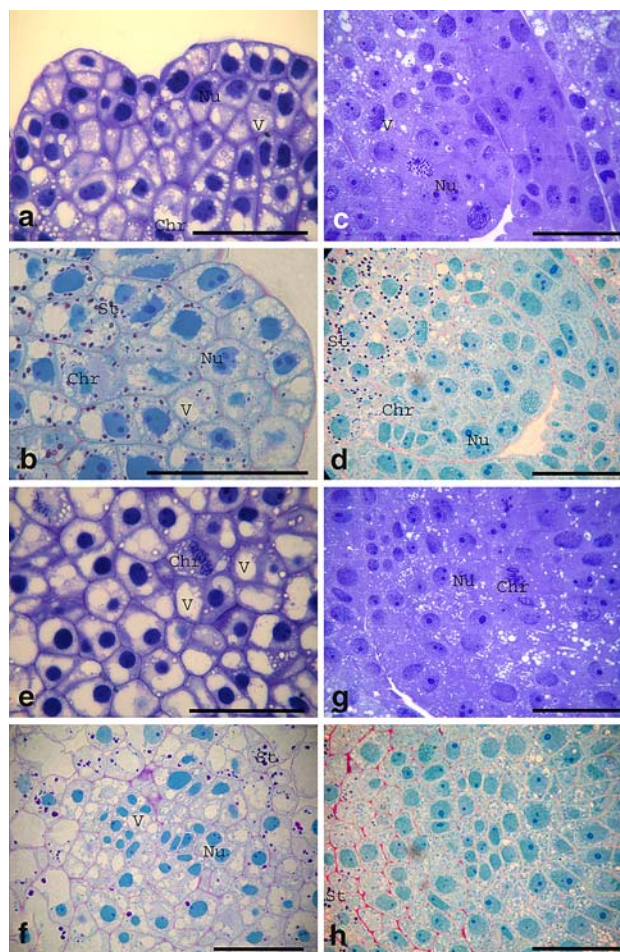

Fig. 6 Details of the meristematic regions of the embryos. **a**–**b**, **e**–**f** In vitro embryos at 20 DAP, **c**–**d**, **g**–**h** in vivo embryos at 14 DAP. **a**–**d** Shoot apex, **e**–**h** root apex. **a**, **c**, **e**, **g** Toluidine blue staining, **b**, **d**, **f**, **h** PAS + CBB staining with Light Green contrast staining. *Nu* Nucleolus, *Org* organelle-rich cytoplasmic area, *St* starch grain, *V* vacuole, *Chr* chromosomes in dividing cells. *Bar* 50 μm

Meristems

A closer observation of the meristems (Fig. 6) revealed that the structure of the shoot apical meristem (SAM) was very similar in both environments (Fig. 6a−d): the small, isodiametric cells were rich in cytoplasm and had relatively large, frequently plurinucleolar nuclei. Cells in M-phase were present in abundance. All the cells were very close together, without intercellular spaces (Fig. 6a, b). The only differences were that the cells were slightly more vacuolated and contained a few small starch grains in vitro (Fig. 6a, b), whereas very few small vacuoles and practically no starch grains occurred in vivo (Fig. 6c, d).

The root apical meristems were less well organised in vitro than in planta (Fig. 6e–h). The cells are more vacuolated and their size and shape more heterogeneous than in vivo. However, these cells were still relatively isodiametric and rich in cytoplasm, and did not accumulate starch. Furthermore, they were frequently plurinucleolar or dividing. Intercellular spaces were not formed between the cells in the meristematic regions.

Scutellar cell types and the accumulation of reserve substances

The neighbourhood of the meristematic region in embryos developed in vitro characteristically containing cells relatively rich in cytoplasm, starch grains and vacuoles. These cells resembled those of the scutellum in vivo (Figs. 5b, c, 6a–d). The other cells of the scutellum were either highly vacuolated (Fig. 5b) or accumulated large amounts of starch and protein in vitro (Fig. 7a, b). The former type were in a central position and resembled the vacuolated region of the in planta endosperm (Fig. 7c), whereas the latter were more characteristic of the periphery and had properties similar to those of cells in the starchy endosperm (Fig. 7d). The accumulation pattern of reserve materials

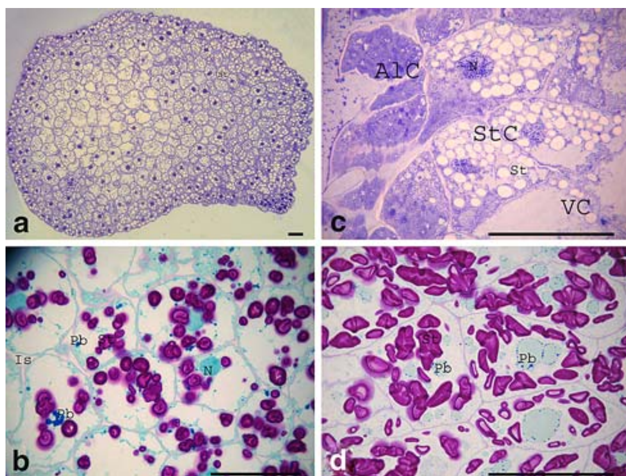

Fig. 7 Reserve material accumulation in the embryos in vitro (20 DAP) and in the endosperm in vivo (14 DAP). **a** An entire section from the scutellum of an embryo developed in vitro. **b** Details of cells accumulating high amounts of starch and protein. **c** Details of the in vivo endosperm near the basal part of the embryo. Note the presence of aleurone (*AlC*), starchy (*StC*) and vacuolated (*VC*) cell types. **d** Reserve material storage in the starchy endosperm. **a**, **c** Toluidine blue staining, **b**, **d** PAS + CBB staining with Light Green contrast staining. *Is* Intercellular space, *PB* protein body, *N* nucleus, *St* starch grain. *Bar* 50 µm

was very similar in the periphery of the scutellum in vitro and in the endosperm in vivo, but slight differences could be observed: in vitro the starch grains were generally more spherical and slightly smaller, and the protein bodies were less composite and were stained more intensely and homogeneously. Small intercellular spaces were present in the scutellum in vitro (Fig. 7b).

Discussion

Technical conditions for the study of wheat zygote development in vitro

Before histochemical studies on the in vitro development of wheat zygotes could be initiated, an efficient culture system had to be constructed. In the newly developed culture system, although some zygotes perished during the first day of culture, once they developed into globular structures, their growth potential and regeneration ability were very high, vying with that described earlier by Kumlehn et al. (1998). Furthermore, direct microscopical observations indicated that the pattern and rate of development were practically the same in all studies focusing on wheat zygote cultures (Kumlehn et al. 1998, 1999; Bakos et al. 2003a, b), although the rate of in vitro development was always somewhat slower than in planta (Batygina 1969; Bennett et al. 1973, 1975). Based on the morphological information from all these studies, it can be assumed that the observed characteristics of the in vitro zygotic embryogenesis in the present study are pertinent not only to the present culture system but also to all wheat zygote cultures hitherto applied.

Another bottleneck faced in this study was the fixation, embedding and sectioning of very small isolated cells and proembryos: the smallest structures only measured approximately 60 µm. To process these cells, they were immobilized in small agarose droplets in a similar manner to that previously described for maize egg cells (Faure et al. 1992). The macroscopic markers required in the case of the even smaller (20–30 µm) tobacco zygotes (He et al. 2006) were not used.

Comparison of zygotic embryogenesis in vitro and in planta: polarity during the initial phase of embryogenesis

It has already been observed that the division of wheat zygotes is morphologically symmetric in vitro and asymmetric in vivo (Kranz and Kumlehn 1999; Kumlehn et al. 1998, 1999; Bakos et al. 2003a, b), but the reason for this distinction is still not clear. In planta, the first division of the zygote is usually asymmetrical: the apical cell of the 2-celled proembryo is rich in cytoplasm, whereas the basal cell is more vacuolated in most higher plants (maize: Randolph 1936; Schel and Kieft 1986; barley: Norstog 1972; rice: Jones and Rost 1989a, b; pearl millet: Taylor and Vasil 1995; *Arundo formosana*: Jane 1999). In wheat, however, these two cells differ only in shape, whereas their morphological architecture and reserve material deposition are very similar (You and Jensen 1985; Naumova and Matzk 1998; Fig. 3c, d). Although Bennett et al. (1973) observed some variation in the morphology of the zygotic daughter cells in wheat cv. Chinese Spring grown in greenhouse conditions, such variation was not found in the present study with the cv. Siete Cerros grown in phytotron.

One explanation for the symmetric cell division in vitro might be that the wheat zygotes lose their cell walls and consequently become spherical protoplasts during the dissection process (Kovács et al. 1994; Kumlehn et al. 1999), leading to a disturbance in polarity. The role of the cell wall in the maintenance of zygotic polarity is also demonstrated by the fact that the retention of the original cell wall is essential for the appropriate differentiation of the daughter cells in tobacco (He et al. 2007). Only cultured zygotes having the original cell wall with its proper chemical gradients reproduced the in planta division pattern. Zygote protoplasts that synthesised cell wall in vitro divided symmetrically and only became similar to in planta embryos much later, if at all. Similarly, the late establishment of polarity was observed in the present wheat zygote culture.

Polarity at the initiation of embryogenesis is decisive not only in zygotic embryos but in all embryogenic systems (Fehér et al. 2003; Hause et al. 1994). Polarity resembling that of the two-celled in planta proembryo was observed in “proembryos” formed from rice scutellar epithelial cells (Jones and Rost 1989b). These somatic embryos also followed in planta embryogenesis very well in later stages. Vasilenko et al. (2000) demonstrated that somatic embryos of orchardgrass mimicking their in planta counterparts developed from mesophyll cells if the first division was periclinal, whereas a random or anticlinal first division resulted in an embryogenic cell mass without any polarity. Recently, a culture system was developed for rape seed, where even microspore-derived structures exactly reproduced the pattern of zygotic embryogenesis following mild heat shock pretreatment instead of the usual prolonged heat shock treatment (Supena et al. 2008). In these cases, the appropriate polarity was established de novo in the embryogenic cell.

In the majority of embryogenic cultures, however, the polarity of the embryos is only established during the globular stage (somatic embryogenesis: Fernandez et al. 1999; Fransz and Schel 1991, 1994; Emons and Kieft 1991; Kärkonen 2000; Kurczynska et al. 2007; Taylor and Vasil 1996; androgenesis: Bonet and Olmedilla 2000; Indrianto et al. 2001; Hause et al. 1994). This developmental pattern bears a close resemblance to that of the isolated wheat zygote protoplasts. In the present study, up to the proembryo stage, the only features common to wheat zygotic structures developing in vitro and their in planta counterparts were the organelle-rich meristematic cells (Fig. 4), and polarity was first detected as a gradient in the starch accumulation (Fig. 4b).

Embryo axis formation in vitro with appropriate meristem integrity

Following the establishment of polarity, the axis of the in vitro embryo was first clearly distinguishable at 10 DAP (Fig. 5a). The fact that these embryos had basically the same morphology as their in planta counterparts at 6 DAP proved that, although they developed at a slower rate, the biochemical gradients leading to differentiation were able to function properly in the present culture system.

By 20 DAP, the embryos were ready for transfer to regeneration medium. All the embryonic organs had differentiated by this time, but they were less well developed than in in vivo embryos of the same size at 14 DAP (Fig. 5b, c). Moreover, twin embryos and distorted ones with multiple organs also developed in the cultures, as reported by Kumlehn et al. (1998). The tendency towards delayed regeneration via organogenesis or precocious germination, abnormal embryo maturation and/or the distortion of the in vitro embryos was also reported for many other in vitro systems developed to support somatic (Felföldi and Purnhauser 1992; Emons and de Does 1993; Taylor and Vasil 1996; Shayakhmetov and Shakirova 1996; Tahir and Stasolla 2006) or zygotic embryogenesis (Leduc et al. 1996; Kumlehn et al. 1998, 1999; Kranz and Kumlehn 1999; Uchiumi et al. 2007b), some of them very efficient.

The most important factor in plant regeneration ability is the maintenance of the integrity of the meristems, especially the SAM (Taylor and Vasil 1996; Nickle and Yeung 1993; Yeung and Stasolla 2000; Tahir and Stasolla 2006); however, meristem cells tend to differentiate into parenchymous vacuolated cells in many culture systems and this deterioration of SAM severely reduces the conversion of the embryos into plants (Tahir and Stasolla 2006). Recently the integrity of the SAM and thus the plant regeneration frequency were successfully improved by the appropriate optimisation of cultures using ABA (Emons et al. 1993) or via the ectopic influence of the redox potential (Belmonte et al. 2006; Belmonte and Stasolla 2007). Because of its relevance, the organisation of the meristems was also compared in wheat embryos in vitro and in vivo (Fig. 6). In the in vitro embryos, the presence of small isodiametric, cytoplasm-rich cells with large nuclei and mitotic forms, and the lack of intercellular spaces were evidence of the well-preserved integrity of both the shoot and root apical meristem. The intense metabolism of the meristems (Raghavan and Olmedilla 1989) was confirmed in the present study by the lack of significant reserve material deposition either in vitro or in vivo. However, the relative disorganisation of the cells and the moderate extent of vacuolisation were signs of slight meristematic disfunction in vitro.

Since all the embryos were converted into vigorous plants in the present culture system, none of the morphological and cytological aberrations observed proved to have any grave consequences. Presumably, these abnormalities during the embryo maturation phase could be explained by differences between the in vitro and in planta environment.

Abundant starch and protein accumulation by the scutellum in vitro

Intense starch and protein accumulation was found in the scutellum (Fig. 7b), which was probably the consequence of the highly metabolisable sugar (glucose) concentration in the medium. As the starch accumulation could be lowered by reducing the amount of metabolisable sugars in the culture medium in different *Brassica* species, leading to an improvement in embryo quality (Ferrie and Keller 2007; Supena et al. 2008), similar culture parameters should be tested in wheat before drawing conclusions on whether the

appearance of tissues resembling the starchy wheat endosperm can be attributed to the scutellum taking over the role of the endosperm, or simply the consequence of the high glucose concentration. Nevertheless, it should be noted that significant starch deposition was detected in a polar pattern both in wheat somatic embryos (Fernandez et al. 1999) and in the scutellum of bamboo somatic embryos (Godbole et al. 2004). Moreover, the high amylase accumulation and activity detected in the bamboo scutellum is characteristic of the aleurone layer of the endosperm in planta (Godbole et al. 2004). The authors speculated that because the endosperm is lacking in somatic embryos, its role was taken over by the scutellum. Besides the tissue resembling starchy endosperm, vacuolated cells were also observed both in the present and in other embryogenic cultures (Emons and Kieft 1991; Taylor and Vasil 1996).

Summary

The present study provides the first histological characterisation of zygotic embryos developing entirely in vitro. The distinctions observed between the in vitro and in planta development of wheat zygotic embryos were probably due to differences in their initial polarity as well as in the in vitro and in planta environments. In order to further elucidate the differences and similarities between in vitro and in planta zygotic embryogenesis and to exactly mimic zygotic embryogenesis in culture systems, the synthesis of results from detailed analytical, physiological and molecular biological studies will be required.

Acknowledgments The authors wish to thank Emese Békné and Mónika Fehér for their excellent technical assistance. This work was financed by a grant from the National Scientific Research Fund (OTKA K67987) and by a Spanish–Hungarian Cooperation (Project, REF2006HU0004).

References

Alemanno L, Berthouly M, Michaux-Ferriere N (1997) A comparison between *Theobroma cacao* L. zygotic embryogenesis and somatic embryogenesis from floral explants. In Vitro Cell Dev Biol Plant 33:163–172

Bakos F (2007) In vitro embryo development in gametophytic cell lines of cereal species. Ph.D thesis, Faculty of Science, Eötvös Loránd University, Budapest

Bakos F, Darkó É, Pónya Z, Barnabás B (2003a) Regeneration of fertile wheat (*Triticum aestivum* L.) plants from isolated zygotes using wheat microspore culture as nurse cells. Plant Cell Tissue Organ Cult 74:243–247

Bakos F, Darkó É, Pónya Z, Barnabás B (2003b) Application of wheat (*Triticum aestivum* L.) microspore culture and ovaries to raise wheat zygotes in vitro. Acta Biol Crac Bot 45:107–110

Bakos F, Jäger K, Barnabás B (2005) Regeneration of haploid plants after distant pollination of wheat via zygote rescue. Acta Biol Crac Bot 47:167–171

Batygina TB (1969) On the possibility of separation of a new type of embryogenesis in angiosperms. Rev Cytol Biol Veg 32:335–341

Batygina TB (1997) Embryogenesis in *Gramineae*. In: Batygina TB (ed) Embryology of flowering plants, terminology and concepts, vol 2. World and Family, St. Petersburg, pp 528–538

Belmonte MF, Stasolla C (2007) Applications of DL-buthionine-[S, R]-sulfoximine deplete cellular glutathione and improve white spruce (*Picea glauca*) somatic embryo development. Plant Cell Rep 26:517–523

Bennett MD, Rao MK, Smith JB, Bayliss MW (1973) Cell development in the anther, the ovule, and the young seed of *Triticum aestivum* L. var. Chinese Spring. Philos Trans R Soc London Ser B 226:39–81

Bennett MD, Smith JB, Barclay I (1975) Early seed development in the Triticeae. Philos Trans R Soc London Ser B 272:199–227

Belmonte MF, Ambrose SJ, Ross AR S, Abrams SR, Stasolla C (2006) Improved development of microspore derived embryo cultures of *Brassica napus* cv Topaz following changes in glutathione metabolism. Physiol Plant 127:690–700

Bonet FJ, Olmedilla A (2000) Structural changes during early embryogenesis in wheat pollen. Protoplasma 211:94–102

Cawood AH, Potter U, Dickinson HG (1978) An evaluation of Coomassie blue as a stain for quantitative microdensitometry of protein in section. J Histochem Biochem 26:645–650

Emons AMC, Kieft H (1991) Histological comparison of single somatic embryos of maize from suspension cultures with somatic embryos attached to callus cells. Plant Cell Rep 10:485–488

Emons AMC, de Does H (1993) Origin and development of embryo and bud primordia during maturation of embryogenic calli of *Zea mays*. Can J Bot 71:1349–1356

Emons AMC, Samallodroppers A, Vandertoorn C (1993) The influence of sucrose, mannitol, L-proline, absicisic acid and gibberellic acid on the maturation of somatic embryos of *Zea mays* L from suspension cultures. J Plant Physiol 142:597–604

Faure JE, Mogensen HL, Kranz E, Digonnet C, Dumas C (1992) Ultrastructural characterization and three-dimensional reconstruction of isolated maize *(Zea mays* L.) egg cell protoplasts. Protoplasma 171:97–103

Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74:201–228

Felföldi K, Purnhauser L (1992) Induction of regenerating callus cultures from immature embryos of 44 wheat and 3 triticale cultivars. Cereal Res Comm 20:273–277

Fernandez S, Michaux-Ferriere N, Coumans M (1999) The embryogenic response of immature embryo cultures of durum wheat (*Triticum durum* Desf.): histology and improvement by $AgNO_3$. Plant Growth Regul 28:147–155

Ferrie AMR, Keller WA (2007) Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the *Brassicaceae*. In Vitro Cell Dev Biol 43:348–355

Fransz PF, Schel JHN (1991) An ultrastructural study on the early development of *Zea mays* somatic embryos. Can J Bot 69:858–865

Fransz PF, Schel JHN (1994) Ultrastructural studies on callus development and somatic embryogenesis in *Zea mays* L. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. (Maize vol 25). Springer, New York, pp 50–65

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regul 43:27–47

Godbole S, Sood A, Sharma M, Nagar PK, Ahuja PS (2004) Starch deposition and amylase accumulation during somatic embryogenesis in bamboo (*Dendrocalamus hamiltonii*). J Plant physiol 161:245–248

Hause B, Vanveenendaal WLH, Hause G, van Lammeren AAM (1994) Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L. cv topas. Bot Acta 107:407–415

He YC, Sun MX, Yang HY (2004) Regeneration of fertile plants from isolated tobacco zygotes by in vitro culture. Chin Sci Bull 49:810–814

He YC, He YQ, Tang M, Sun MX (2006) An improved method for the preservation and location of single cells during the embedding procedure for transmission electron microscopy. S Afr J Bot 72:298–301

He YC, He YQ, Sun MX, Yang HY (2007) Tobacco zygotic embryogenesis in vitro: the original cell wall of the zygote is essential for maintenance of cell polarity, the apical-basal axis and typical suspensor formation. Plant J 49:515–527

Herman EB (ed) (2008) Media and Techniques for Growth, Regeneration and Storage: 2005–2008. Volume 12 of "Recent Advances in Plant Tissue Culture". Agricell Report, Shrub Oak

Holm PB, Knudsen S, Mouritzen P, Negri D, Olsen FL, Roué C (1994) Regeneration of fertile barley plants from mechanically isolated protoplasts of the fertilized egg cell. Plant Cell 6:531–543

Hoshino Y, Scholten S, von Wiegen P, Lörz H, Kranz E (2004) Fertilization-induced changes in the microtubular architecture of the maize egg cell and zygote—an immunocytochemical approach adapted to single cells. Sex Plant Reprod 17:89–95

Indrianto A, Barinova I, Touraev A, Heberle-Bors E (2001) Tracking individual wheat microspores in vitro: identification of embryogenic microspores and body axis formation in the embryo. Planta 212:163–174

Jane WN (1999) Ultrastructure of embryo development in *Arundo formosana* Hack. (Poaceae). Int J Plant Sci 160:46–63

Jones TJ, Rost TL (1989a) Histochemistry and ultrastructure of rice (*Oryza sativa*) zygotic embryogenesis. Am J Bot 76:504–520

Jones TJ, Rost TL (1989b) The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. Bot Gaz 150:41–49

Kärkonen A (2000) Anatomical study of zygotic and somatic embryos of *Tilia cordata*. Plant Cell Tissue Organ Cult 61:205–214

Kovács M, Barnabás B, Kranz E (1995) Electro-fused isolated wheat (*Triticum aestivum* L.) gametes develop into multicellular structures. Plant Cell Rep 15:178–180

Kovács M, Barnabás B, Kranz E (1994) The isolation of viable egg cells of wheat (*Triticum aestivum* L.). Sex Plant Reprod 7:311–312

Kranz E, Lörz H (1993) In vitro fertilisation with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. Plant Cell 5:739–746

Kranz E, Kumlehn J (1999) Angiosperm fertilisation, embryo and endosperm development in vitro. Plant Sci 142:183–197

Kranz E, Scholten S (2008) In vitro fertilization: analysis of early post-fertilization development using cytological and molecular techniques. Sex Plant Reprod 21:67–77

Kumlehn J, Lörz H, Kranz E (1998) Differentiation of isolated wheat zygotes into embryos and normal plants. Planta 205:327–333

Kumlehn J, Lörz H, Kranz E (1999) Monitoring individual development of isolated wheat zygotes: a novel approach to study early embryogenesis. Protoplasma 208:156–162

Kumlehn J, Kirik V, Czihal A, Altschmied L, Matzk F, Lörz H, Bäumlein H (2001) Parthenogenetic egg cells of wheat: cellular and molecular studies. Sex Plant Reprod 14:239–243

Kurczynska EU, Gaj MD, Ujczak A, Mazur E (2007) Histological analysis of direct somatic embryogenesis in *Arabidopsis thaliana* (L.) Heynh. Planta 226:619–628

Le Q, Gutierrez-Marcos JF, Costa LM, Meyer S, Dickinson HG, Lörz H, Kranz E, Scholten S (2005) Construction and screening of subtracted cDNA libraries from limited populations of plant cells: a comparative analysis of gene expression between maize egg cells and central cells. Plant J 44:167–178

Leduc N, Matthys-Rochon E, Rougier M, Mogensen L, Holm PB, Magnard JL, Dumas C (1996) Isolated maize zygotes mimic in vivo embryonic development and express microinjected genes when cultured in vitro. Dev Biol 177:190–203

Maraschin SF, de Priester W, Spaink HP, Wang M (2005) Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. J Exp Bot 56:1711–1726

McManus JCF (1948) Histological and histochemical uses of periodic acid. Stain Technol 23:99–108

Naumova TN, Matzk F (1998) Differences in the initiation of the zygotic and parthenogenetic pathway in the Salmon lines of wheat: ultrastructural studies. Sex Plant Reprod 11:121–130

Nawy T, Lukowitz W, Bayer M (2008) Talk global, act local—patterning the *Arabidopsis* embryo. Curr Opin Plant Biol 11:28–33

Nickle TC, Yeung EC (1993) Failure to establish a functional shoot meristem may be a cause of conversion failure in somatic embryos of *Daucus carota* (Apiaceae). Am J Bot 80:1284–1291

Ning J, Peng XB, Qu LH, Xin HP, Yan TT, Sun M (2006) Differential gene expression in egg cells and zygotes suggests that the transcriptome is restructed before the first zygotic division in tobacco. FEBS Lett 580:1747–1752

Norstog K (1972) Early development of barley embryo fine structure. Am J Bot 59:123–132

Okamoto T, Higuchi K, Shinkawa T, Isobe T, Lörz H, Koshiba T, Kranz E (2004) Identification of major proteins in maize egg cells. Plant Cell Physiol 45:1406–1412

Okamoto T, Kranz E (2005) In vitro fertilization—a tool to dissect cell specification from a higher plant zygote. Curr Sci 89:1861–1869

Okamoto T, Scholten S, Lörz H, Kranz E (2005) Identification of genes that are up- or down-regulated in the apical or basal cell of maize two-celled embryos and monitoring their expression during zygote development by a cell manipulation- and PCR-based approach. Plant Cell Physiol 46:332–338

Pretová A, Obert B, Bartosová Z (2006) Haploid formation in maize, barley, flax, and potato. Protoplasma 228:107–114

Pulido A, Bakos F, Castillo A, Vallés MP, Barnabás B, Olmedilla A (2005) Comparison of cytological and ultrastrucutral changes induced in anther and isolated-microspore cultures in barley: presence of Fe deposits in isolated-microspore cultures. J Struct Biol 149:170–181

Pulido A, Hernando A, Bakos F, Méndez E, Devic M, Barnabás B, Olmedilla A (2006) Hordeins are expressed in microspore-derived embryos and also during male gametophytic and very early stages of seed development. J Exp Bot 57:2837–2846

Raghavan V, Olmedilla A (1989) Spatial patterns of histone mRNA expression during grain development and germination in rice. Cell Differ Dev 27:183–196

Randolph LF (1936) Developmental morphology of the caryopsis in maize. J Agric Res 53:881–916

Rani V, Raina SN (2000) Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. In Vitro Cell Dev Biol Plant 36:319–330

Schel JHN, Kieft H (1986) An ultrastructural study of embryo and endosperm development during in vitro culture of maize ovaries (*Zea mays*). Can J Bot 64:2227–2238

Seguí-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore derived embryogenesis. Physiol Plant. doi: 10.1111/j.1399-3054.2008.01113.x

Shayakhmetov IF, Shakirova FM (1996) Somatic embryogenesis in wheat cell suspension cultures in the presence of abscisic acid. Russian J Plant physiol 43:88–90

Smart MG, O'Brien TP (1983) The development of the wheat embryo in relation to the neighboring tissues. Protoplasma 114:1–13

Sprunck S, Baumann U, Edwards K, Langridge P, Dresselhaus T (2005) The transcript composition of egg cells changes significantly following fertilization in wheat (*Triticum aestivum* L.). Plant J 41:660–672

Steffen JG, Kang IH, Macfarlane J, Drews GN (2007) Identification of genes expressed in the *Arabidopsis* female gametophyte. Plant J 51:281–292

Supena EDJ, Winarto B, Riksen T, Dubas E, Van Lammeren A, Offringa R, Boutilier K, Custers J (2008) Regeneration of zygotic-like microspore-derived embryos suggests an important role for the suspensor in early embryo patterning. J Exp Bot 59:803–814

Szűcs A, Jurca M, Jäger K, Barnabás B, Fehér A (2006) Genomic approaches to reveal gene expression changes during fertilization and early seed development in wheat. In: Jäger K, Barnabás B (eds) From gamates to genes. Book of abstracts. XIXth International Congress on Sexual Plant Reproduction. 11–15 July 2006, Budapest, p 65

Tahir M, Stasolla C (2006) Shoot apical development during in vitro embryogenesis. Can J Bot 84:1650–1659

Taylor MG, Vasil IK (1995) The ultrastructure of zygotic embryo development in pearl-millet (*Pennisetum glaucum*, Poaceae). Am J Bot 82:205–219

Taylor MG, Vasil IK (1996) The ultrastructure of somatic embryo development in pearl millet (*Pennisetum glaucum*; Poaceae). Am J Bot 83:28–44

Tereso S, Zoglauer K, Milhinhos A, Miguel C, Oliveira MM (2007) Zygotic and somatic embryo morphogenesis in *Pinus pinaster*: comparative histological and histochemical study. Tree Physiol 27:661–669

Testillano PS, Ramírez C, Domenech J, Coronado MJ, Vergne P, Matthys-Rochon E, Risueño MC (2002) Young microspore-derived maize embryos show two domains with defined features also present in zygotic embryogenesis. Int J Dev Biol 46:1035–1047

Thijssen MH, Spoelstra P, Emons AMC (1996) Immunodetection and immunolocalization of globulin storage proteins during zygotic and somatic embryo development in *Zea mays*. Physiol Plant 98:539–549

Uchiumi T, Komatsu S, Koshiba T, Okamoto T (2006) Isolation of gametes and central cells from *Oryza sativa* L. Sex Plant Reprod 19:37–45

Uchiumi T, Shinkawa T, Isobe T, Okamoto T (2007a) Identification of the major protein components of rice egg cells. J Plant Res 120:575–579

Uchiumi T, Uemura I, Okamoto T (2007b) Establishment of an in vitro fertilization system in rice (*Oryza sativa* L.). Planta 226:581–589

van Lammeren AAM (1981) Early events during embryogenesis in *Zea mays*. Acta Soc Bot Pol 50:289–290

Vasilenko A, Mcdaniel JK, Conger BV (2000) Ultrastructural analyses of somatic embryo initiation, development and polarity establishment from mesophyll cells of *Dactylis glomerata*. In Vitro Cell Dev Biol Plant 36:51–56

Wang YY, Kuang A, Russell SD, Tian HQ (2006) In vitro fertilization as a tool for investigating sexual reproduction of angiosperms. Sex Plant Reprod 19:103–115

Yang HP, Kaur N, Kiriakopolos S, McCormick S (2006) EST generation and analyses towards identifying female gametophyte-specific genes in *Zea mays* L. Planta 224:1004–1014

Yeung EC, Stasolla C (2000) Somatic embryogenesis—apical meristems and embryo conversion. Korean J Plant Tissue Cult 27:299–307

You R, Jensen WA (1985) Ultrastructural observations of the mature megagametophyte and the fertilization in wheat (*Triticum aestivum*). Can J Bot 63:163–170

Zhang J, Dong WH, Galli A, Potrykus I (1999) Regeneration of fertile plants from isolated zygotes of rice (*Oryza sativa*). Plant Cell Rep 19:128–132