*ACTA BIOLOGICA CRACOVIENSIA Series Botanica* 49/2: 53–59, 2007

# INDUCTION OF AUTONOMOUS ENDOSPERM DEVELOPMENT IN OVULES OF UNPOLLINATED PISTILS OF ARABIDOPSIS THALIANA VAR. LANDSBERG CULTURED IN VITRO

MAŁGORZATA KAPUSTA, JOANNA ROJEK, AND JERZY BOHDANOWICZ

*Department of Genetics and Cytology, University of Gdańsk, ul. Kładki 24, 80-822 Gdańsk, Poland*

Received May 15, 2007; revision accepted September 20, 2007

The paper reports induction of autonomous endosperm (AE) development in ovules of unpollinated pistils of *Arabidopsis thaliana* var. Landsberg cultured in vitro. The basic nutritive medium was hormone-free Murashige and Skoog (MS) medium with 6% sucrose added. Unpollinated pistils were also cultured on MS medium supplemented with growth regulators: naphthylacetic acid (NAA; 0.1 mg $l^{-1}$) combined with benzylaminopurine (BAP; 2 mg $l^{-1}$) and 2,4-dichlorophenoxyacetic acid (2,4-D; 20 mg $l^{1}$ or 40 mg $l^{-1}$). AE development was induced on all the media used, in 7.8% of the cultured ovaries (9 ovules in 9 ovaries). The frequency of AE induction was highest on MS medium with 20 mg $l^{-1}$2,4-D (12.5%), and lowest on MS medium containing NAA and BAP (3.6%). On the hormone-free MS and MS with 40 mg $l^{-1}$ 2,4-D the AE induction frequency was 6.25% and 8.70%, respectively. The number of AE nuclei ranged from 2 to 9, depending on the length of culture and the medium used. Cellularization and differentiation for the characteristic region of endosperm typical in wild-type *Arabidopsis thaliana* was never observed. AE probably originated from the secondary nucleus, as indicated by nuclear size and structure. Medium containing a high concentration of synthetic auxin (40 mg $l^{-1}$ 2,4-D) induced initiation of antipodal cell divisions, whereas in planta antipodals degenerated when the female gametophyte of *Arabidopsis* matured. Induction of parthenogenetic development of the egg cell was not observed.

**Key words:** *Arabidopsis*, in vitro culture, unpollinated ovaries, autonomous endosperm (AE).

## INTRODUCTION

In sexual plants, the embryo and endosperm are the result of double fertilization. After the nuclei of the sperm cell and the central cell fuse, development of the endosperm proper depends on the correct proportion (2:1) of maternal to paternal genomes. Disturbance of this relation usually causes seed damage. In some species where the proportion of parental genomes differs from 2:1, endosperm develops normally, demonstrating the operation of other mechanisms involved in endosperm and seed development (reviewed by Rojek and Kuta, 2002). It also confirms that fertilization-independent endosperm can emerge by an autonomous path. In nature this state is quite common among apomictic plants, but very rare among sexual ones. Autonomous endosperm can also be induced in special culture conditions. Nowadays research is focused on the genetic aspect of endosperm development and the influence of genomic imprinting (Kuta and Rojek, 2004).

Wild-type *Arabidopsis* endosperm undergoes the nuclear type of development (Mansfield and Briarty, 1990a). Early endosperm development is characterized by a series of synchronized nuclear divisions not followed by cytokinesis, and three domains are established: the anterior micropylar, peripheral and posterior chalazal domains. Cellularization begins in the micropylar domain where endosperm cells surround the embryo, proceeds across the peripheral domain, and ends in the chalazal region where a multinucleate mass of cytoplasm forms a coenocytic cyst (Olsen, 2004). The site and structure of the cyst indicate its haustorial function (Nguyen et al. 2000). In the peripheral domain, spherical, multinucleate structures called nodules result from fusions of nucleocytoplasmic domains (NCDs). The cellularized micropylar endosperm (MCE) and peripheral endosperm (PEN) undergo two series of divisions, and the chalazal endosperm (CZE) develops with proliferation in both the cyst and the nodules (Sørensen et al., 2001).

*e-mail: rojek@biotech.ug.gda.pl

PL ISSN 0001-5296

The results of new research examining genomic imprinting show that several genes are responsible for seed and thereby endosperm development. This epigenetic phenomenon results in suppression of one of the parental alleles, and nonequivalent expression of the maternal and paternal genes. Imprinting in plants affects the endosperm but not embryo development. The mechanisms responsible for genomic imprinting in plants and mammals alike are cytosine methylation and chromatin condensation, but paramutations and transgene silencing could also be involved. A few gametophytic genes are very important for proper endosperm development. The group of *Arabidopsis*' genes *MEDEA/ FIS1/EMB173* (*Fertilization Independent Seed 1*), *FIS2* (*Fertilization Independent Seed 2*), and *FIE* (*Fertilization Independent Endosperm*)/ *FIS3* prevent seed development in the absence of fertilization. Mutations of these genes induce the development of nuclear or cellular endosperm, seed coat, and partial development of the embryo (Vinkenoog et al., 2003). In *fie1* mutants, no cellularization or distinct separation between endosperm compartments were observed (Vinkenoog et al., 2000; Vinkenoog and Scott, 2001). However, when the *fie* mutation was combined with hypomethylation of the maternal genome (demethylation of *fie-1/FIE* heterozygotes using *MET I* a/s construct), autonomous endosperm development was complete, with cellularization and formation of specific regions. In the *cdc2a* mutant of *Arabidopsis*, where only one sperm is produced, unfertilized endosperm development can also be induced by a previously unrecognized positive signal from egg cell fertilization (Nowack et al., 2006).

Previous research demonstrated autonomous development of endosperm in cultured ovules of the Columbia ecotype (Rojek et al., 2005). Our research investigated whether autonomous development is unique to *Columbia* or occurs in other ecotypes and might therefore be a general feature of *Arabidopsis* and perhaps of higher plants.

## MATERIALS AND METHODS

### PLANT MATERIAL

Plants of *Arabidopsis thaliana* (L.) Heynh. var. Landsberg (La–0, wild ecotype) were grown from seed (Lehle Seeds, Round Rock, TX, U.S.A.) in a greenhouse under a 16 h photoperiod at 20°C and 35% humidity. For in vitro culture, flower buds 1–1.5 mm long were harvested at developmental stages FG3 (late 2-nucleate) to FG5 (8-nucleate) of female gametophyte development and 12b, 12b/12c of *Arabidopsis* flowering, as described by Christensen et al. (1997). At these stages the petals are longer than the stamens but shorter than the sepals. The petals are milky white and the anthers are yellow-green. Anthesis has not occurred and thus emasculation was not needed. Pistils were excised and rinsed in 70% ethanol for 30 sec, sterilized for 7 min in commercial bleach diluted with distilled water (1:2) and rinsed three times in sterile water prior to culture.

### IN VITRO CULTURE

The medium, growth regulator combinations and concentrations were as reported for experiments on *A. thaliana* var. Columbia (Rojek et al., 2005). Pistils were placed on MS (Murashige and Skoog, 1962) medium solidified with agar (8 g $l^{-1}$) and supplemented with 6% sucrose, myoinositol (0.1 g $l^{-1}$) and growth regulators (Tab. 1). The pH was adjusted to 5.7–5.8 and phytohormones were added before autoclaving at 121°C and 108 kPa for 20 min. In the case of medium supplemented with 20 and 40 mg $l^{-1}$ 2,4-D, the explants were kept for 1 h on the medium with hormone (auxin shock), and then transferred to hormone-free MS medium. All cultures were maintained under the above-mentioned conditions. Light was supplied by cool white fluorescent tubes (70–100 M photons $m^{-1}s^{-2}$). Pistils were cultured for 3, 5, 7 and 12 days.

### EMBRYOLOGICAL STUDY

Ovaries cultured on four media (Tab. 1) were harvested after 3, 5, 7 and 12 days of culture. Sampled ovaries were fixed either in acetic alcohol (1:3 glacial acetic acid/96% ethanol), or FAA (5:5:90 40% formalin/glacial acetic acid/70% ethanol), After 1 to 2 weeks the material was placed in 70% ethanol. Permanent slides were prepared using the paraffin method. Microtome sections (5, 10, 15 μm) were stained with Heidenhain's hematoxylin combined with Alcian blue or Fast Green FCF, or with safranin combined with Fast Green FCF (Ruzin 1999), and mounted in DPX (Fluka).

For controls, flower buds 1–1.5 mm long (bud size at inoculation) and flowers pollinated in planta (with embryo and endosperm) were used. Plant material was fixed in acetic alcohol, and paraffin sections (10 μm) were stained with Heidenhain's hematoxylin combined with Alcian blue and mounted in DPX. Another part of the material was hydrated and cleared with Hoyer's medium (Liu and Meinke, 1998), placed on slides and observed with a differential interference contrast microscope. Microphotographs of the embryological slides were taken with a Nikon Eclipse E800 microscope equipped with a Nikon DS–5Mc camera and analyzed with the Lucia Image program.

TABLE 1. *Arabidopsis thaliana* var. Landsberg. Autonomous endosperm (AE) induction recorded in unpollinated ovaries / ovules after 12 days of in vitro culture; % in brackets

| Medium | Embryological study of in vitro material | | | |
|---|---|---|---|---|
| | No. of pistils inoculated | No. of ovaries/ ovules analyzed | Frequency of ovaries/ ovules with AE | No. of ovules with divisions of antipodal cells |
| MS | 40 | 32 / 635 | 2 (6.3) / 2 (0.3) | – |
| MS+2 mg $l^{-1}$BAP + 0.1mg $l^{-1}$ NAA | 40 | 28 / 595 | 1 (3.6) / 1 (0.2) | – |
| MS+20 mg $l^{-1}$ 2.4-D (1h) | 40 | 32 / 732 | 4 (12.5) / 4 (0.5) | – |
| MS+40 mg $l^{-1}$ 2.4-D (1h) | 30 | 23 / 474 | 2 (8.7) / 2 (0.4) | 1 |
| Total | 150 | 115 / 2463 | 9 (7.8) / 9 (0.4) | 1 |

## RESULTS

### PISTIL CULTURE

A total 150 pistils were cultured on four media (Tab. 1). In all cultured pistils the ovaries were enlarged after 10–12 days. Most of the ovaries did not degenerate (96.65%) and were conspicuously enlarged. The ovary walls proliferated during culture, especially in ovaries cultured on medium with synthetic auxin (at both concentrations). Explants cultured on medium supplemented with a combination of NAA and BAP became lavender-colored, but their viability was not affected. Callus induction was not observed.

All of the ovules excised from enlarged ovaries in 3-day-old culture were green and viable. During further culture the frequency of enlarging and viable ovules declined to 30% of the ovules examined at 12 days.

### EMBRYOLOGICAL STUDY OF CONTROL MATERIAL

The ovule of *Arabidopsis thaliana* is anatropous, bitegmic and tenuinucellate, and undergoes the Polygonum type of megagametophyte development. The female gametophyte is monosporic, 8-nucleate and 7-celled (Mansfield et al., 1991). In the ovaries at inoculation (1–1.5 mm), 28–32 ovules were observed in three stages of female gametophyte (FG) development: 2-, 4-, and 8-nucleate. The most frequent of them contained four nuclei of similar size and structure.

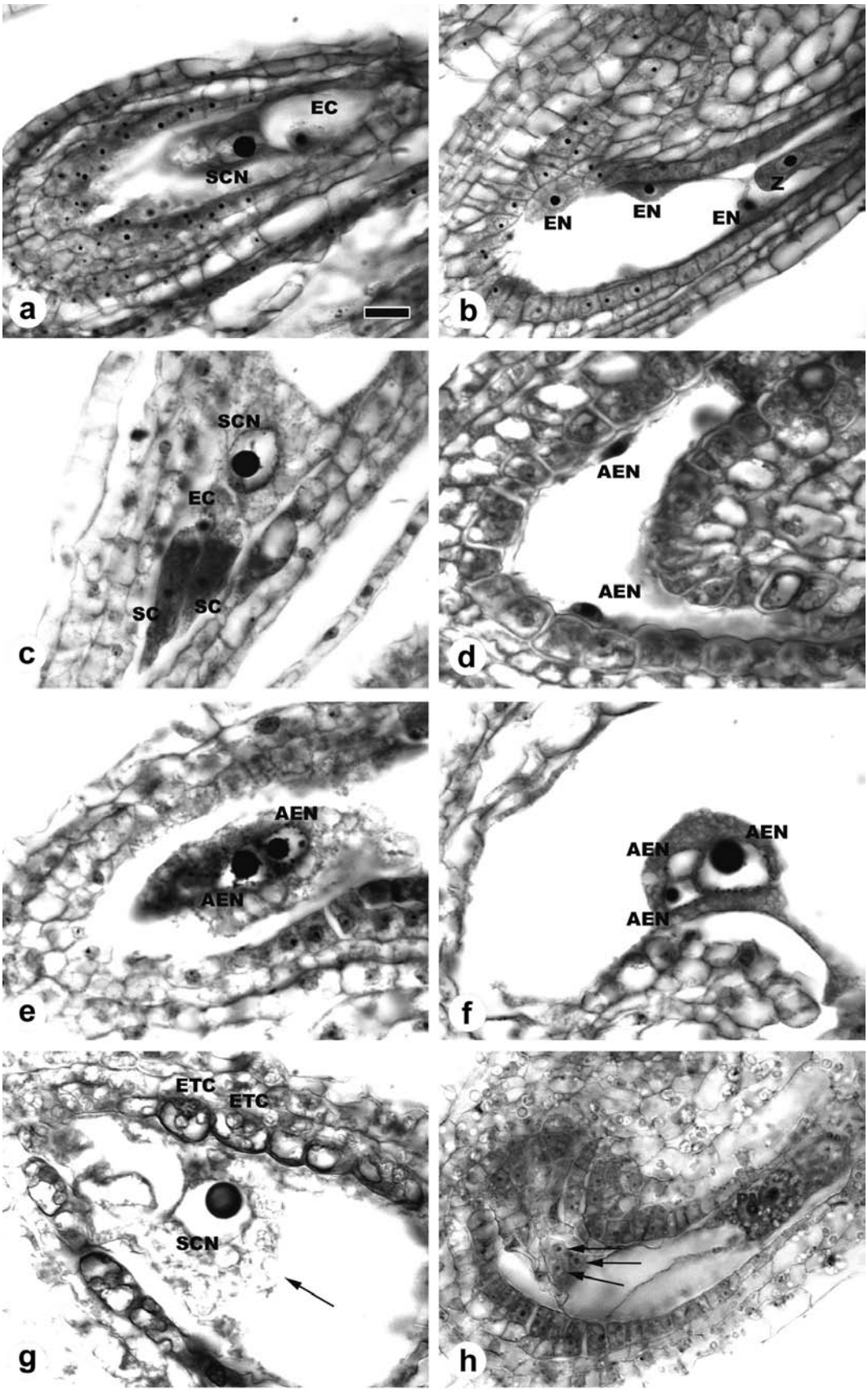
The mature FG showed an egg apparatus at the micropylar pole, and two polar nuclei or a secondary nucleus situated close to the egg cell (Fig. 1a). Antipodal cells as ephemeral structures did not occur.

In ovules from pollinated flowers, a zygote or 2-celled embryo was accompanied by nuclear endosperm (Fig. 1b). Fusion of two polar nuclei was observed sporadically. In a few cases a 2-nucleate structure with dense cytoplasm at the chalazal pole of the embryo sac was identified as a young chalazal endosperm cyst. At this stage all the endosperm nuclei were of the same size, and all contained a single large nucleolus, not accompanied by the many micronucleoli characteristic of the secondary nucleus.

### EMBRYOLOGICAL STUDY OF MATERIAL CULTURED IN VITRO

A total 2463 ovules (115 ovaries) were examined embryologically. The results varied depending on the medium used. After 3 days of culture all the ovules on all media were enlarged. Three FG developmental stages (2-, 4- and 8-nucleate) were observed on hormone-free MS medium; on media containing growth regulators the FG reached an 8-nucleate or 7-celled stage. In enlarged 5-day-old ovules, all female gametophytes reached maturity and contained an egg apparatus and a secondary nucleus, and antipodal cells were still present. At this time the frequency of viable ovules decreased to 75%. In enlarged 7-day-old ovules the mature structures were visible (Fig. 1c), without antipodal cells but with a high concentration of starch grains close to the secondary nucleus. Some ovules contained empty gametophytes squeezed by growing integuments. After 12 days of culture only 30% of the ovules were enlarged and viable. Some single structures of mature FG could be observed, but most of them had degenerated. Until this time the integuments remained very distinct, with the cells filled with starch (Fig. 1g). The proliferating somatic cells distorted the female gametophytes.

Partly autonomous endosperm development was detected on all four media. The frequency of AE was highest on medium containing 20 mg $l^{-1}$ 2,4-D (12.5% of ovaries had AE). The total number of AE nuclei ranged from 2 to 9 depending on the number of days in culture and the medium used. In some cases AE nuclei were located in peripheral cytoplasm (Fig. 1d); others were at the site where the secondary nucleus occurred (Fig. 1e,f).


EC
SCN
a
EN
EN
EN
Z
b
SCN
EC
SC
SC
c
AEN
AEN
d
AEN
AEN
e
AEN
AEN
AEN
f
ETC
ETC
SCN
g
h

Neither cellularization of autonomous endosperm nor separation into different domains, characteristic of wild-type *Arabidopsis* endosperm, occurred after fertilization.

## DISCUSSION

In nature, the endosperm of a flowering plant originates from the central cell of the embryo sac, which is fertilized by one of two male gametes. The second fertilization generates the embryo (Berger, 2003). In some cases endosperm can develop by an autonomous path as described for apomictic plants (for review see: Grossniklaus, 2001). Endosperm is a very important plant tissue responsible for nutrition of the embryo and germinating seed. For sexual plants, the correct proportion of parental genomes is necessary for proper development (Quarin, 1999). Current experiments are focused on understanding how to induce AE in sexual plants. Many studies have shown that autonomous endosperm can be induced in cultures of unpollinated flowers, pistils, ovaries or isolated ovules of several species. AE in vitro originates from a secondary nucleus or polar nuclei, but in some cases endosperm-like structures can result from atypical female gametophyte development. In vitro, AE forms independently or in combination with parthenogenesis (Rojek et al., 2005).

In this study the development of autonomous endosperm was induced in cultures of unpollinated ovaries of *Arabidopsis thaliana* var. Landsberg (La-0). We observed the greatest AE induction response on medium supplemented with two different high concentrations of synthetic auxin, 2,4-D (12.5% and 8.7% of ovaries), but the 2,4-D doses caused abnormal growth of the ovary wall and somatic tissue of the ovules. Such a situation could unbalance transport of nutritive substances, resulting in disturbed organization of the embryo sac (the presence of an additional egg cell; differences in the size of the polar nuclei), observed in ovules cultured on medium with the higher concentration of 2,4-D. Adding synthetic hormones may influence female gametophyte organization (Mól, 1999).

The frequency of explants with AE was also relatively high (6.25%) on hormone-free medium MS plus 6% sucrose, confirming earlier results (Rojek et al., 2002) and demonstrating that exogenous hormones are not needed to stimulate divisions of the central cell. Higher concentrations of sucrose (6–8%) induce gynogenesis in vitro (Bogunia and Przywara, 1999), and perhaps high osmolarity of the medium also affects AE development. As in *A. thaliana* var. Columbia in vitro (Rojek et al., 2005), AE obtained in culture was free-nuclear, with no more than 8–9 nuclei after 7 days of culture and a significant delay in development (Fig. 1a-f). In planta 4–8 h after anthesis, endosperm has several free nuclei (Wei and Sun, 2002).

At the first stages of development (after the first and second divisions), in terms of nuclear size and structures the AE nuclei resembled secondary nuclei (Fig. 1c). Each of them contained one big nucleolus and many micronucleoli, often observed in AE nuclei in other Brassicaceae such as *Brassica napus* (Rojek et al., 2002) or *A. thaliana* var. Columbia (Rojek et al., 2005). Similarly to the nuclear endosperm stage in wild-type *Arabidopsis*, AE nuclei were situated at the periphery (Fig. 1d) and in the center of the embryo sac, but later on in culture the formation of domains characteristic of in planta endosperm was never found. Also not noted were endosperm-like structures observed earlier in culture of unfertilized ovules of cotton, rice (for review see: Kuta and Rojek 2002) and rape (Rojek et. al., 2002).

After 3 days of culture on MS medium with 40 mg $l^{-1}$ 2,4-D five cells with similar-sized nuclei formed in the chalazal region of the female gametophyte (Fig. 1h). It should be emphasized that divisions of antipodal cells were noted for the first time in vitro. Antipodals are ephemeral structures in wild-type *Arabidopsis* in vivo. They degenerate before the fusion of the polar nuclei, and are not present in further FG development (Christensen et al., 1997). Thus it is all the more interesting that, in the absence of antipodals in mature FG of control material, antipodal cells were still visible in ovules after 3 days of in vitro culture or even later, on all the media used. The early stage (2- and 4-nucleate)

**Fig. 1**. *Arabidopsis thaliana* var. Landsberg. Longitudinal sections of ovule. Control (**a–b**) and cultured in vitro (**c-h**). Bar = 10 μm. (**a**) Secondary nucleus (SCN) and egg cell (EC) in mature embryo sac (ES), (**b**) Endosperm nuclei (EN) and zygote (Z) in ovule from flower bud fixed after anthesis, (**c**) Secondary nucleus (SCN) and egg apparatus: egg cell (EC) and synergids (SC) in ovule after 5 days of culture on hormone-free MS medium, (**d**) Autonomous endosperm (AEN) after 7 days of culture after 1 h incubation on MS+40 mg $l^{-1}$ 2,4-D, followed by transfer to hormone-free medium, (**e**) Two-nucleate autonomous endosperm (AEN) after 7 days of culture on hormone-free MS medium, (**f**) Three-nucleate autonomous endosperm (AEN) after 7 days of culture after 1 h incubation on MS + 20 mg $l^{-1}$ 2,4-D, followed by transfer to hormone-free medium, (**g**) Secondary nucleus (SCN) with surrounding starch grains (arrow) and endothelium cells (ETC) in ovule after 7 days of culture after 1 h incubation on MS + 40 mg $l^{-1}$ 2,4-D, followed by transfer to hormone-free medium, (**h**) Divided antipodal cells; three of five cells visible (arrows) and polar nuclei (PN) after 3 days of culture after 1 h incubation on MS + 40 mg $l^{-1}$ 2,4-D, followed by transfer to hormone-free medium.

of FG development at which inoculation took place may explain the phenomenon of nondegenerating antipodals.

Considerable growth of endothelium occurred during culture (Fig. 1g). After 3 days, integumental cells with dark content were most evident when cultured on medium with synthetic auxin. After 5 and 7 days of culture the endothelium cells became enlarged, strongly vacuolated and filled with starch. Towards the end of the experiment, starch and tannins were observed in the degenerating endothelium cells; this is characteristic of in vivo development (Mansfield and Briarty, 1990a). Starch grains were also observed inside all viable embryo sacs cultured on all media, especially the cytoplasm surrounding the secondary nucleus; this is also characteristic of the mature female gametophyte in vivo (Mansfield et al., 1991).

None of the 115 analyzed ovaries cultured in vitro contained ovules showing parthenogenetic development of the egg cell. Neither pollen tubes nor sperm nuclei were detected, confirming other work showing that pollination does not take place at developmental stages 12b, 12b/c of *Arabidopsis* flowering and FG3-FG5 of female gametophyte development (Christensen et al., 1997). During culture, a nondegenerating egg cell apparatus with both synergids or a single one, or an egg cell alone, was observed even to day 12, showing the potential of the egg cell to achieve parthenogenetic development. Parthenogenetic development of the egg cell in culture has been noted in several cases (Mól, 1999). The presence of undegenerated egg cells and AE nuclei in the same female gametophytes suggests that the two structures follow independent developmental pathways, but can feature some similarities in culture. This is supported by the results of in vitro culture of unfertilized *Allium cepa* ovules, indicating that AE development and parthenogenetic development of the egg cell can occur simultaneously (Musiał et al., 2001). On the other hand, Nowack et al. (2006) constructed the mutant *Arabidopsis thaliana Cdc2*, homolog of *CDC2A* (also called *CDKA;1*), which had a parental effect. In *cdc2a* mutant pollen, only one sperm cell is produced instead of two. Mutant pollen fertilized only the egg cell but the central cell started to develop autonomously. Remembering that endosperm proliferation is also regulated by the action of the *FIS* gene complex (Chaudhury et al., 2001; Guitton et al., 2004), these new data suggest that early seed development is regulated by two signal pathways: the release of the FIS-dependent proliferation block of the central cell, and a positive signal from the fertilization of the egg cell (Nowack et al. 2006). Culture conditions (e.g., the presence of phytohormones, high concentration of sucrose, auxin shock) possibly influence or supplant one or both of the two signal pathways and initiate partial development of unfertilized endosperm.

In this study, the success in AE induction on all the media used suggests that *Arabidopsis thaliana* var. Landsberg, like ecotype Columbia, is a good model for further investigation of fertilizationless endosperm development. The similarity of observations relating to the structure and origin of AE nuclei in the two ecotypes suggests that the same genetic and epigenetic mechanisms are responsible for their development. To verify this suggestion, molecular analysis should focus on changes in the level of cytosine methylation, which are a well-known phenomenon in culture of plant tissue in vitro, as they constitute one of the mechanisms involved in somaclonal variation and gene expression. Hypomethylation of the genome of *Arabidopsis* mutant *fie1* (*fertilization independent endosperm1*) leads to complete autonomous endosperm development in the absence of fertilization (Vinkenoog et al., 2000; Vinkenoog and Scott, 2001).

Besides the many similarities, the two ecotypes of *Arabidopsis* (Columbia and Landsberg) showed some differences in the frequency of AE induction. For ecotype Columbia the results were best (59%) on medium with NAA combined with BAP; in contrast, in this study the frequency was highest (12.5%) on MS medium with 20 mg $l^{-1}$ 2,4-D. Further embryological and molecular study is needed to explain the differences in AE induction frequency. It will be interesting to determine whether the differences are related to the genotype of the studied ecotypes or rather to the different experimental conditions.

## ACKNOWLEDGEMENTS

We thank Professor Elżbieta Kuta for stimulating discussions regarding interpretation of the results, and the reviewers for valuable remarks.

## REFERENCES

Berger F, and Gaudin V. 2003. Chromatin dynamics and *Arabidopsis* development. *Chromosome Research* 11: 277–304.

Bogunia H, and Przywara L. 1999. Rola cukrowców w roślinnych kulturach in vitro. *Wiadomości Botaniczne* 43: 25–26.

Chaudhury AM, Koltunow AM, Payne T, Luo M, Tucker MR, Dennis ES, and Peacock WJ. 2001. Control of early seed development. Annual. *Review of Cell and Developmental Biology* 17: 677–691.

Christensen CA, King EJ, Jordan JR, and Drews GN. 1997. Megagametogenesis in *Arabidopsis* wild type and Gf mutant. *Sexual Plant Reproduction* 10: 49–64.

GROSSNIKLAUS U. 2001. From sexuality to apomixis: molecular and genetic approaches. In: Savidan Y, Carman JG, and Dresselhaus T [eds.], Flowering of apomixis: From mechanisms to genetic engineering, 168–211. CYMMIT, IRD & RTD FAIR Programme.

GUITTON A-E, PAGE DR, CHAMBRIER P, LIONNET C, FAURE J-E, GROSSNIKLAUS U, and BERGER F. 2004. Identification of new members of Fertilization Independent Seed Polycomb Group pathway involved in the control of seed development. *Development* 131: 2971–2981

KUTA E, and ROJEK J. 2002. Bielmo-tkanka odżywiająca zarodek II. Autonomiczny rozwój bielma u roślin okrytonasiennych (Angiospermae). *Kosmos* 51: 85–97.

KUTA E, and ROJEK J. 2004. Imprinting genomowy u roślin. *Postępy Bioliogii Komórki* 31: 353–3 71.

LIU CM, and MEINKE DW. 1998. The titan mutants of *Arabidopsis* are disrupted in mitosis and cell cycle control during seed development. *Plant Journal* 16: 21–23.

MANSFIELD SG, and BRIARTY LG. 1990a. Development of free-nuclear endosperm in *Arabidopsis thaliana* L. *Arabidopsis Information Service* 27: 53–64.

MANSFIELD SG, BRIARTY LG, and ERNI S. 1991. Early embryogenesis in *Arabidopsis thaliana*. I. The mature embryo sac. *Canadian Journal of Botany* 69: 447–460.

MÓL R. 1999. Embryological aspects of in vitro gynogenesis in plant organ cultures. *Acta Biologica Cracoviensia Series Botanica* 41: 67–74.

MURASHIGE T, and SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.

MUSIAŁ K, BOHANEC B, and PRZYWARA L. 2001. Embryological study on gynogenesis in onion (*Alium cepa* L.). *Sexual Plant Reproduction* 13: 335–341.

NGUYEN H, BROWN RC, and LEMMON BE. 2000. The specialized chalazal endosperm in *Arabidopsis thaliana* and Lepidium virginicum (Brassicaceae). *Protoplasma* 212: 99–100.

NOWACK MK, GRINI PE, JACOBY MJ, LAFOS M, KONCZ C, and SCHNITTGER A. 2006. The positive signal from fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. *Nature Genetics* 38: 64–67.

OLSEN OA. 2004. Nuclear endosperm development in cereals and *Arabidopsis thaliana*. *Plant Cell* 16: S214–S227.

QUARIN CL. 1999. Effect of pollen source and pollen ploidy on endosperm formation and seed set In pseudogamous apomictic *Paspalum notatum*. *Sexual Plant Reproduction* 11: 331–583.

ROJEK J, and KUTA E. 2002. Bielmo-tkanka odżywiająca zarodek I. Bielmo u roślin okrytonasiennych (Angiospermae) jako wynik podwójnego zapłodnienia. *Kosmos* 51: 69–83.

ROJEK J, KUTA E, and BOHDANOWICZ J. 2005. In vitro culture promotes partial autonomous endosperm development in unfertilized ovules of wild-type *Arabidopsis thaliana* var. Columbia. *Sexual Plant Reproduction* 18: 29–36.

ROJEK J, KUTA E, and PRZYWARA L. 2002. Autonomous endosperm development in unpollinated ovaries in *Brassica napus* L. cv. Topas cultured in vitro. *Acta Biologica Cracoviensia Series Botanica* 44: 195–202.

RUZIN SE. 1999. Plant Microtechnique and Microscopy. New York Oxford University Press. Annals of Botany Company, Book reviews 546.

SØRENSEN MB, CHAUDHURY A, ROBERT H, BANCHREL E, and BERGER F. 2001. Polycomb group genes control pattern formation in plant seed. *Current Biology* 11: 277–281.

VINKENOOG R, SPIELMAN M, ADAMS S, FISCHER RL, DICKINSON HG, and SCOTT RJ. 2000. Hypomethylation promotes autonomous endosperm development and rescues post-fertilization lethality in fie mutants. *Plant Cell* 12: 2271–2282.

VINKENOOG R, and SCOTT RJ. 2001. Autonomous endosperm development in flowering plants:how to overcome the imprinting problem? *Sexual Plant Reproduction* 14: 189–194.

VINKENOOG R, BUSHELL C, SPIELMAN M, ADAMS S, DICKINSON HG, and SCOTT RJ. 2003. Genomic imprinting and endosperm development in flowering in plants. *Molecular Biotechnology* 25: 149–184.

WEI J, and SUN MX. 2002. Embryo sac isolation in *Arabidopsis thaliana*: A simple and efficient technique for structure analysis and mutant selection. *Plant Molecular Biology Reporter* 20: 141–148.