Modern Phytomorphology **5**: 25–30, 2014

EFFECT OF ESTRONE ON SOMATIC AND FEMALE GAMETOPHYTE CELL DIVISION AND DIFFERENTIATION IN *ARABIDOSPIS THALIANA* CULTURED *IN VITRO*

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Abstract. The aim of the study was to determine the effect of the mammalian female sex hormone estrone on differentiation of somatic tissues and on induction of autonomous endosperm in culture of female gametophyte cells of *Arabidopsis thaliana* ecotype Columbia (Col-0).

In culture, estrone-stimulated development of autonomous endosperm (AE) occurred in 14.7% of unpollinated pistils. The AE represented development stages similar to those of young endosperm after fertilization and AE of *fis* mutants *in vivo*. In the majority of ovules the AE was in a few-nucleate young stage. Some ovules showed more advanced stages of AE development, with nuclei and cytoplasm forming characteristic nuclear cytoplasmic domains (NCDs). Sporadically, AE was divided into regions characteristic for *Arabidopsis* endosperm formed after fertilization.

Direct organogenesis (caulogenesis, rhizogenesis), callus proliferation and formation of trichome-like structures were observed during *in vitro* culture of hypocotyls and cotyledons of 3-day-old seedlings cultured on medium supplemented with estrone for 28 days. Histological analysis showed adventitious root formation and changes in explant anatomy caused by estrone.

Key words: *Arabidopsis,* ovules, autonomous endosperm, hypocotyl, cotyledon, female mammalian hormone, *in vitro* culture

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Introduction

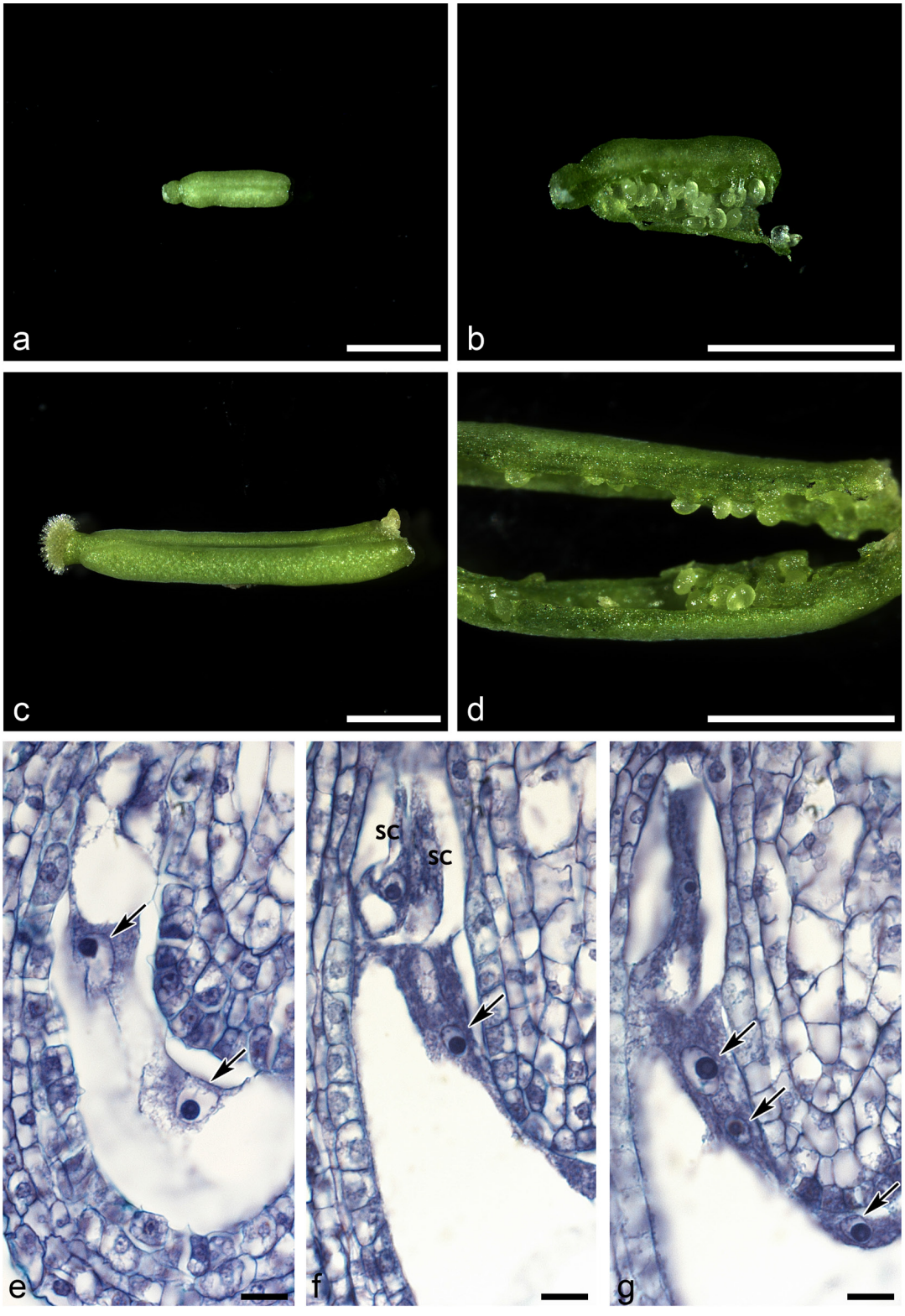
Mammalian sex hormones (androgens, estrogens, progesterone) play a key role in mammalian reproduction. They also are synthesized by plants, in 128 species representing more than 50 families (Janeczko & Skoczowski 2005, and references therein). Selected steroids applied exogenously to plants stimulate cell division, pollen maturation, growth and flowering (Janeczko *et al.* 2003, 2013). In *Arabidopsis thaliana*, estrone stimulated induction of flowering *in vitro* (Janeczko & Filek 2002).

The aim of this study was to examine the effect of estrone on somatic and female gametophyte cell division and differentiation in culture of *Arabidopsis thaliana* (Columbia-0 ecotype, Col-0) hypocotyls, cotyledons and unfertilized ovules.

Material and methods

In vitro culture of female gametophyte cells

Unpollinated pistils (~1.2 mm long) of Col-0 were removed from flower buds and sterilized in a solution of 3% hydrogen peroxide and 95% ethanol (1:1, v/v) for 5 min, then rinsed in sterile distilled water 3 times for 5 min each. Pistils were placed on MS medium solidified with Difco Bacto agar (8 $g \cdot l^{-1}$) supplemented with myoinositol (0.01 $g \cdot l^{-1}$), 6% sucrose and 1 μM estrone. The steroid hormone was dissolved in 50% ethanol and stored in stock (2 mg/1 ml) at –20°C. The concentration of pure (99.8%) ethanol added to the medium was 6 $\mu l \cdot l^{-1}$ and this concentration did not influence the experiment. The hormone was sterilized through a 0.22 μm syringe filter (Millipore) and added to the medium after autoclaving.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All cultures were maintained under a 16 h photoperiod at 21±3°C. Light was supplied by cool white fluorescent tubes (avg. 70-100 M photons $m^{-1} \cdot s^{-2}$). Pistils were cultured 7 days.

Embryological study

Unpollinated pistils cultured *in vitro* were fixed on 3rd, 5th, and 7th days of culture in acetic alcohol (1:3 glacial acetic acid : 96% ethanol) or FAA (40% formalin: glacial acetic acid : 70% ethanol, 5:5:90, v/v/v). Fixed material was prepared by the paraffin method and sectioned 5 μm thick. Sections were stained with Heidenhain's or Ehrlich's hematoxylin combined with alcian blue. Micrographs of the embryological slides were taken with a Nikon Eclipse E800 microscope fitted with a DS.-5Mc camera. The images were cropped and reprocessed in Adobe Photoshop CS4.

In vitro culture of somatic tissues

Seeds of the *Arabidopsis* Columbia-0 ecotype (CS60000, Col-0) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK). To obtain material for *in vitro* culture (hypocotyls, cotyledons) the seeds were sterilized by vortexing in 70% ethanol for 3-5 min and then in a 50% solution of commercial bleach (Ace) with a few drops of Triton X-100 detergent for 3-5 min, followed by 3 rinses in sterile distilled water. Seeds were refrigerated at 4°C for 3 days on 0.15% agar solution and germinated on MS medium (Murashige & Skoog 1962). To prepare medium supplemented with 3.7 μM estrone, the steroid hormone was dissolved in absolute alcohol, sterilized using a 0.22 μm syringe filter (Millipore) and added to MS basic medium after autoclaving. Control medium with ethanol was also prepared.

Hypocotyls and cotyledons of 3-day-old seedlings grown on MS medium were excised under a laminar flow hood and placed in medium-filled Petri dishes (10 explants per dish); 50 hypocotyls and 50 cotyledons were cultured *in vitro*. Cultures were maintained in a growth chamber at 25±3°C under a 16 h photoperiod (cool-white fluorescent lamps, 60-90 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Hypocotyls and cotyledons were cultured 28 days.

For histological analysis the material cultured *in vitro* was fixed and prepared according to the procedure described by Żabicki *et al.* (2013). Hypocotyl and cotyledon sections were photographed with a Zeiss Axio Cam MRc digital camera using Zeiss Axio Vision 3.1. Gimp 2.6.11 and Open Office Draw 3.1 were used for image reprocessing.

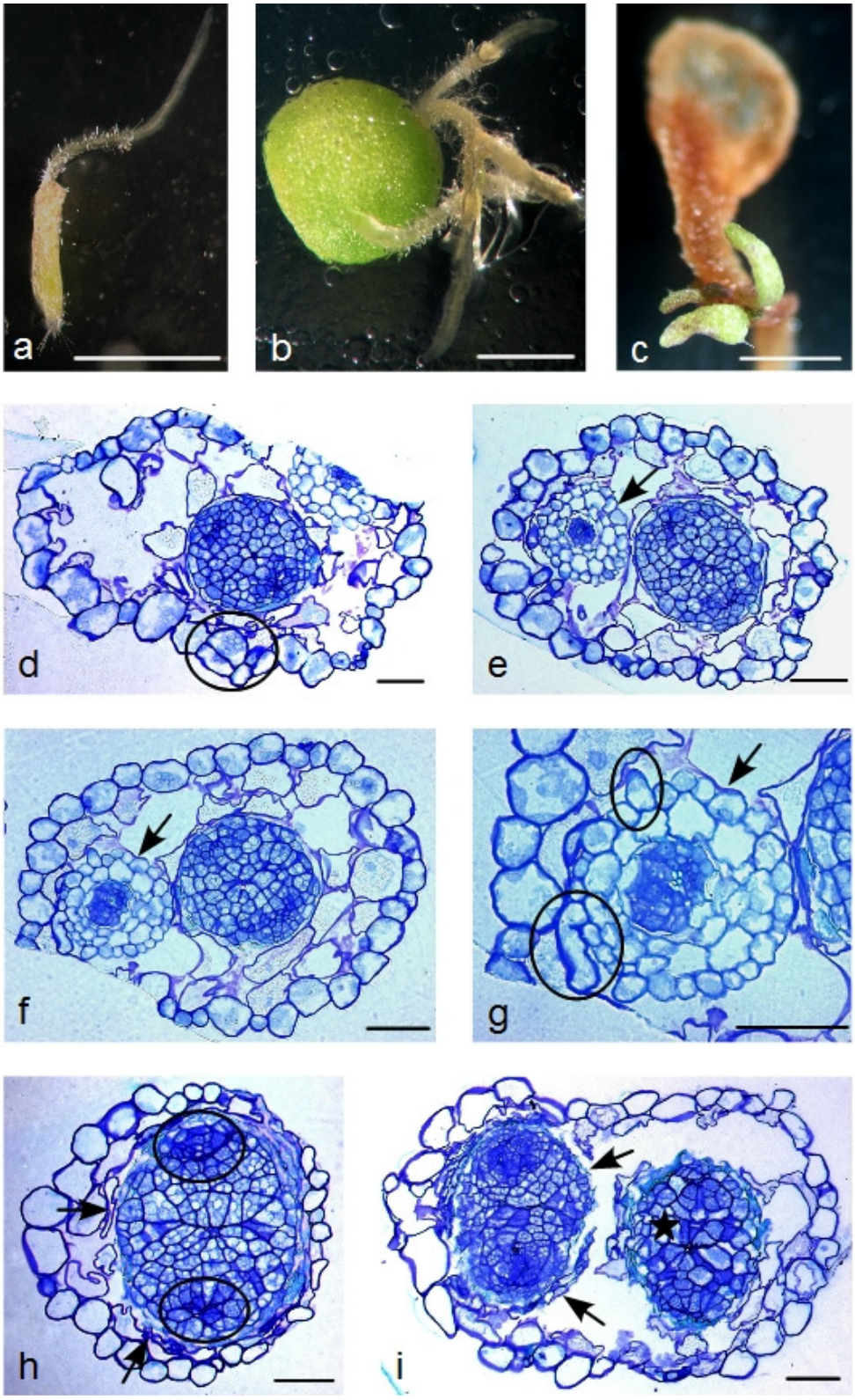
Results

Induction and development of autonomous endosperm in vitro

Unpollinated pistils cultured on medium with estrone added were conspicuously enlarged at day 7 of culture (Fig. 1 a-d). Explant viability was 86%; ovule viability was markedly lower at 70%.

On the third day of culturing the female gametophytes (FGs) were enlarged and contained an egg apparatus and secondary nucleus or autonomous endosperm nuclei. The AE were few-nucleate; the nuclei were of similar size, small and clustered together in dense cytoplasm. At day 7 of unpollinated pistil culture, secondary nuclei and intact egg apparatuses were still observed in FGs, and also few- to multinucleate AE. The first AE nuclei moved into two opposite poles (one nucleus close to the egg cell, the other at the chalazal pole) (Fig. 1 e). The nuclei of few- to multinucleate AE were of different size, parietally arranged close to egg apparatus (Fig. 1 f-g). Rarely the AE nuclei were organized in nuclear cytoplasmic domains (NCDs) and formed regions typical for *Arabidopsis* endosperm developed *in vivo* after fertilization.

◂ **Fig. 1.** Unpollinated *Arabidopsis* Col-0 pistils at inoculation and cultured on MS 6% + estrone 1 μM for 7 days (**a-d**), and longitudinal paraffin sections of ovules inside unpollinated ovaries (**e-g**): **a, b** – pistils at inoculation; enlarged pistils (**c**) and ovules inside ovaries (**d**) after 7 days of culture; **e** – AE with two distant nuclei (**arrows**); **f, g** – few nucleate AE (**arrows**) accompanied by egg apparatus, visible synergids (**sc**). Bar – 1 mm (**a-d**) and 10 μm (**e-g**).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Somatic cell division, differentiation and organ formation

Trichome-like structures were formed on 4% of the hypocotyl explants on the third day of culture (Fig. 2 a). On the 9th day of culture, adventitious shoots developed on 10% of the hypocotyl explants, and roots with root hairs developed on 5% of them. Callus proliferation started on the 14th day on 3% of the explants. Histological analysis of plant material cultured *in vitro* showed division and differentiation of epidermis (cells differed in size and shape, and underwent periclinal divisions) (Fig. 2 d), disintegration of the parenchyma cortex due to proliferation of other tissues, stele enlargement through parenchyma cell division, and the formation of meristematic centers (Fig. 2 d-i). Adventitious roots with root hairs were formed near the stele as additional structures inside the explants (Fig. 2 e-g, i).

Cotyledons cultured *in vitro* produced callus on the cut end (12%) and trichome-like structures (4%) on the 14th day of culture. Adventitious roots with root hairs (Fig. 2 b) and nodular structures (Fig. 2 c) were formed on 28th day of culture. There was revealed no significant effect of estrone on cotyledon explants. The anatomy of cultured cotyledons was similar to their normal anatomy.

Discussion

Based on molecular data on the signaling pathway of steroid plant hormones (Janeczko 2012) and their role in auxin redistribution, vernalization, flowering, and regulatory mechanisms in response to environmental stress (Janeczko *et al.* 2013), we expected estrone to stimulate somatic and female gametophyte cell division, proliferation and differentiation. In fact, we observed direct organogenesis induced by this mammalian hormone in both hypocotyl and cotyledon explants. Organogenesis differed between the two types of explant: callus proliferated and adventitious roots formed on hypocotyls and cotyledons, but adventitious shoots formed only on hypocotyls.

Estrone induced division of the female gametophyte secondary nucleus without fertilization. It is well known that in sexual angiosperms the endosperm is formed after fertilization as a nutrient source for the developing embryo. *Fertilization-independent seed* (*fis*) mutants of sexual *Arabidopsis* develop endosperm without fertilization. Rojek *et al.* (2013) obtained AE induction in *Arabidopsis* Col-0 in culture of unpollinated pistils (ovules) regardless of the media used, suggesting that the *Arabidopsis* Col-0 genotype has the ability to develop endosperm independently as autonomous apomictic plants do.

Acknowledgements

This work received funding from the Polish Ministry of Science and Higher Education (project no. N30300731/0281).

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◂ **Fig. 2.** Hypocotyl (**a**) and cotyledons (**b, c**) of *Arabidopsis* Col-0 cultured *in vitro* on MS + 3.7 μM estrone for 14 (**a**) and 28 (**b, c**) days, and transverse sections of hypocotyls cultured for 10 (**d-g**), 14 (**h**) and 28 (**i**) days: trichome-like structures (**a**), adventitious roots with root hairs (**a, b**) and nodular structures (**c**) visible on explants; **d** – periclinal division of epidermis cell (**circle**), epidermis cells of different sizes and shapes, cortex cells disintegrated, stele enlarged by divisions of parenchyma; **e-g** – adventitious root-like structure (**arrow**) formed inside hypocotyl explant, visible root hairs (**circles**) (**g**); **h** – enlarged, modified stele with meristematic centers (**circles**) surrounded by epidermis with cells of different sizes and shapes, cortex parenchyma crushed (**arrows**); **i** – structure difficult to identify (**star**) accompanied by modified stele (**arrows**) inside hypocotyl. Bar – 1 mm (**a-c**) and 100 μm (**d-i**).

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