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THE INFLUENCE OF *fie* AND *met1* MUTATIONS AND IN VITRO CULTURE CONDITIONS ON AUTONOMOUS ENDOSPERM DEVELOPMENT IN UNFERTILIZED OVULES OF *ARABIDOPSIS THALIANA*

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In flowering plants, seeds are produced both sexually (double fertilization is required) and asexually via apomixis (meiotic reduction and egg fertilization are omitted). An apomictic-like pattern of endosperm development in planta is followed by *fis* mutants of sexual *Arabidopsis thaliana*. In our experiments in planta, autonomous endosperm (AE) developed in *met1* mutants. Furthermore we obtained autonomous endosperm formation in vitro not only in unfertilized ovules of *fie* mutants but also in wild genotypes (Col-0, *MET1/MET1*, *FIE/FIE*) and *met1* mutants. AE induction and development occurred in all genotypes on the each of the media used and in every trial. The frequency of AE was relatively high (51.2% ovaries) and genotype-dependent. AE induced in vitro represents a more advanced stage of development than AE induced in *fie* mutants in planta. This was manifested by a high number of nuclei surrounded by cytoplasm and organized in nuclear cytoplasmic domains (NCDs), nodule formation, division into characteristic regions, and cellularization. The high frequency of AE observed in homozygous *met1* (*met1/met1*) mutants probably is due to accumulation of hypomethylation as an effect of the *met1* mutation and the in vitro conditions. AE development was most advanced in *FIE/fie* mutants. We suggest that changes in the methylation of one or several genes in the DNA of *Arabidopsis* genotypes caused by in vitro conditions resulted in AE induction and/or further AE development.

Key words: *Arabidopsis*, *fie* and *met1* mutants, apomixis, autonomous endosperm, in vitro culture, methylation.

INTRODUCTION

Induction of embryo and endosperm development as a result of double fertilization is well recognized in flowering plants such as *Arabidopsis thaliana*. In this century several *Arabidopsis* mutants have been isolated in which the endosperm and/or embryo develop in the absence of fertilization (Vinkenoog and Scott, 2001; Vinkenoog et al., 2000). These mutants are known as *mea* (*medea, fis1*, Grossniklaus et al., 1998; Kiyosue et al., 1999), *fis2* (*fertilization-independent seed2*, Luo et al., 1999), *fie* (*fis3*, *fertilization-independent endosperm*, Ohad et al., 1996), *msi1* (*multi-copy supressor of ira1*, Köhler et al., 2003a), *medicis* (*msi1-2*; Guitton et al., 2004), *bga-1* (*borgia*, Guitton et al., 2004), *syl* (*scylla*) and *srn* (*sirène*, Rotman et al., 2008).

In *Arabidopsis* the products of *FIS1/MEA, FIS2, FIS3/FIE* and *MSI1* are components of FIS polycomb repressive complex 2 (FIS PRC2) regulating endosperm development, and *FIS1/MEA* and *FIS2* are imprinted, maternally expressed genes (MEGs; Köhler et al., 2012). Each of these genes controls three functions during endosperm development:

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suppression of proliferation of the central cell before fertilization, control of endosperm organization, and regulation of the number of endosperm nuclei divisions (Lohe and Chaudhury, 2002). Mutation in *FIS* genes causes a distinct gametophytic maternal effect. In the absence of fertilization, the endosperm and fruit develop autonomously. Upon self-fertilization, half of the seeds produced by heterozygous *fis*/+ plants have unusually large *fis*-genotype seeds (Berger, 2006). The proportion of *fis* seeds is the same when *fis*/+ plants are fertilized by wild-type pollen, and maternal transmission of the *fis* allele is very low or nonexistent. In addition to affecting fertilization-independent development, *fie* is also a gametophytic, embryo-lethal mutation. Inheritance of the *fie* allele by a female gametophyte (FG) results in embryo abortion even when the pollen bears the wild-type allele (Ohad et al., 1996). Similarly, the lethality of a *mea* embryo is independent of the paternal contribution and gene dosage (Grossniklaus et al., 1998).

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 proceeded through cellularization and the formation of specific regions (Vinkenoog et al., 2000). Hypomethylation can prevent repression of genes that are expected to be imprinted (silent) in wild-type ovules, supplying the missing paternal genome in the absence of fertilization. In addition, crosses involving *Arabidopsis* mutants combined with hypomethylation of the maternal genome clearly showed that hypomethylation alone does not promote fertilization-independent endosperm proliferation in the wild type with *FIS* gene expression (Vinkenoog et al., 2000); only a combination of maternal genome hypomethylation and loss of the *FIE* function appear to promote autonomous endosperm formation.

Autonomous endosperm development has also been induced in culture of unpollinated flowers, pistils, ovaries or isolated ovules of several wild, ornamental and cultivated plant species (Wijowska et al., 1999a,b; Rojek et al., 2005). Embryogeny failed but autonomous endosperm developed in culture of unpollinated ovaries of two wild-type *Arabidopsis thaliana* L. genotypes: Columbia and Lansberg *erecta* (Rojek et al., 2005; Kapusta et al., 2007). Among *Arabidopsis* mutants in which AE has been observed in planta, Köhler et al. (2003a) noted partly developed AE and embryo-like structure only in unpollinated *MSI1*/*msi1* pistils cultured in vitro. Molecular research on the mechanisms controlling AE development in *fie* and *met1* mutants of *Arabidopsis* strongly suggest that changes in genomic imprinting by DNA and histone methylation are essential for autonomous/apomictic development (Curtis and Grossniklaus, 2008; Köhler and Weinhofer-Molisch, 2010; Schmidt et al., 2013).

In planta, partly developed AE occurs in the heterozygous *fie* and *fis2* genotype. The combination of *fie* (or *mea*) and *met1* mutations (e.g., *fie-1*/*FIE;MET1a*/*s*, Vinkenoog and Scott, 2000; *mea-1*/*MEA;met1-3*/*MET*, Schmidt et al., 2013) results in full AE development. On the basis of those findings we expected that the *met1* mutation combined with in vitro conditions would induce AE development and that the *fie* mutation combined with in vitro conditions would induce full AE development.

To find out whether in vitro conditions induce AE proliferation to full development, we cultured unpollinated ovaries of wild-type, *fie* and *met-1* mutants of *Arabidopsis*, with the goal of better understanding the role of genomic imprinting in endosperm development.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

In this study seeds of Col-0 (obtained from Prof. Jolanta Maluszynska, unavailable from NASC; Maluszynska and Heslop-Harison, 1991), *met1* (*met1-9*; obtained from Prof. Rod Scott; SALK line: N576522; Alonso et. al., 2003; Mathers, 2008) and *fie* (*fie-1*; obtained from Prof. Nir Ohad; unavailable from NASC; Ohad et. al., 1999) were used.

Plants were grown from seeds in a glasshouse under cold fluorescent light (120 μmol/m^2/s) at 21°C and 70% humidity under long days (16 h light) until flowering and seed setting.

The *fie* and *met1-9* genotypes were verified by molecular analysis; additionally, *fie* genotypes were recognized by silique phenotype (e.g., Ohad et al., 1996). Three *met1–9* genotypes were recognized by amplification with primers LSB5.F 5'-AATCTGAACACCTGCCTCACAGGATGC-3', LSB6.R (in intron) 5'-TTATACATCAGCAACAGAAGAAAAAACAGACG-3' and Lba1 5'-TGGTTCACGTAGTGGGCCATCG-3' (to detect genomic WT *MET1*: LSB5.F and LSB6.R primers; ~370 bp fragment size; to detect T-DNA insert: Lba1 and LSB6.R; ~520 bp fragment size; annealing temperature 65°C, extension time: 50 sec).

The PCR protocol for distinguishing the WT *FIE* allele from the mutant allele 547 is based on amplification of genomic DNA with PCR, followed by restriction analysis. The mutant allele (line 547 TTTAAA) is restricted with DraI, while the WT allele (TCTAAA) is not restricted (one band of 866 bp from the WT allele and another two bands of 374 bp and 492 bp from the mutant allele after restriction with

DraI). Primers: 3143 (R) CCTATATGGCAACAGAAAAT and 2277 (F) GCTTGTGGTTCGTTTGTATG were used for PCR.

IN VITRO CULTURE

The explants for in vitro culture were unpollinated pistils removed from flower buds ~1 mm in length. Flower buds were harvested ~24 h before anthesis (24 HBA) at developmental stages 12a/12b of *Arabidopsis* flowering and FG4/FG5 of female gametophyte development as described by Christensen et al. (1997).

The optimal stage at inoculation is an 8-nucleate or 7-celled FG. This corresponds to stages 12a-12c of the flower and stages FG3-FG5/6 of the female gametophyte in *Arabidopsis thaliana* (Smyth et al., 1990; Christensen et al., 1997) and 1.0–1.7 mm flower bud size. To avoid the possibility of accidental self-pollination, the main criterion was the development stage of the pistil (size and appearance of ovary and stigma) and stamens. A poorly formed stigma within a closed flower indicates that the anthers are immature and pollen cannot be shed on the stigma.

Buds were sterilized in 3% hydrogen peroxide and 95% ethanol (1:1 v/v) for 5 min, then rinsed in distilled water 3 times for 5 min each. Pistils were placed on MS (Murashige and Skoog, 1962) medium solidified with Difco Bacto agar (8 g l^{-1}) supplemented with myoinositol (0.01 g l^{-1}), 6% sucrose and phytohormones: P1 – hormone-free, P2 – 0.1 mg l^{-1} NAA + 2 mg l^{-1} BAP, P3 – 1 h incubation in MS6% + 40 mg l^{-1} 2,4-D and then transfer on MS6% (Tab. 1).

NAA, BAP and 2,4-D were added to media prior to autoclaving. 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 $m^{-1}s^{-2}$). Pistils of all genotypes and seeds were cultured 7 days.

IN PLANTA EXPERIMENTS

Flower buds of all genotypes were isolated from open pollination or emasculated and isolated from pollination ~24 h before anthesis (24 HBA). Material was embryologically analyzed after bud fixation at 24 HBA, and at the 3rd or 7th day of the experiment, which means ~48 hours after anthesis (~48 HAA) or ~144 HAA respectively.

EMBRYOLOGICAL STUDY

Material representing all genotypes was used for embryological studies: unpollinated pistils cultured in vitro, flower buds 1.0–1.7 mm in length, depending on genotype (bud size at inoculation, 24 h before anthesis, 24 HBA), flowers after open pollination (24 HBA; analyzed at 3rd or 7th day of experiment (i.e., ~48 HAA or ~144 HAA respectively; *FIE/FIE*, *FIE/fie* and Col-0), non-emasculated but isolated flower buds, and emasculated flower buds isolated from pollination; analyzed 48 HAA or 144 HAA.

Pistils cultured on different media (Tab. 1) were fixed at the 3rd, 5th and 7th day of culture in either acetic alcohol (1:3 glacial acetic acid : 96% ethanol), or FAA (40% formalin : glacial acetic acid : 70% ethanol, 5:5:90, v/v/v). For ovule clearing the material was fixed in FAA overnight at 4°C, rinsed in 70% ethanol, dehydrated in an ethanol series to 100% ethanol and cleared in methyl salicylate : 100% ethanol series to pure methyl salicylate, as described by Mól et al. (1995) or hydrated and cleared in Hoyer's medium (Boisnard-Lorig et al., 2001).

Material fixed in acetic alcohol was prepared by the paraffin method and sectioned 5 μm thick. Sections were stained with Heidenhain's or Erhlisch's haematoxylin combined with alcian blue, ruthenium red or acetic carmine.

Photomicrographs of the embryological slides were taken with a Nikon Eclipse E800 microscope. Pictures were cropped and processed in Adobe Photoshop CS4.

STATISTICS

Statistical analysis of autonomous endosperm frequency induction employed ANOVA [data after angular transformation – $\text{asin}(x^{0.5})$; $\alpha = 0.05$] for all genotypes; each value is the mean of the last three independent replicates.

RESULTS

ENDOSPERM DEVELOPED AFTER CENTRAL CELL FERTILIZATION IN PLANTA – A BASE FOR COMPARISON OF AUTONOMOUS ENDOSPERM STRUCTURE

In planta at ~48 h after anthesis (48 HAA), in ovules, female gametophytes represented different stages of development: 8-nucleate with egg apparatus on micropylar pole, central cell with two polar nuclei and three antipodals on chalazal pole (in *fie* genotypes), mature 4-celled without antipodes which degenerated precociously; with zygotes and few-nucleate endosperm; and with embryos accompanied by endosperm (all genotypes); endosperm was divided in characteristic regions: micropylar endosperm, endosperm around central vacuole and chalazal cyst (Col-0, *FIE/FIE* and *FIE/fie* genotypes). At ~144 HAA the siliques contained immature seeds with embryo and endosperm at different developmental stages: a globular embryo and multinucleate endosperm forming a cyst at the chalazal region

(especially Col-0 and *FIE/fie* genotypes); a heart-stage embryo and endosperm, partly cellularized (Col-0 and *FIE/fie* genotypes); and a torpedo-stage embryo and cellular endosperm. Development of siliques and seeds was slower in *met1/met1* than in the other genotypes. In *FIE/fie*, ~50% of the seeds formed abnormal an embryo and endosperm (delay and disruption in development) as an effect of the *fie* mutation.

INDUCTION OF AUTONOMOUS ENDOSPERM IN PLANTA

To check whether autonomous endosperm develops in unfertilized female gametophyte of investigated genotypes in planta (to the exclusion of *FIE/fie* since the *fie* mutation induced autonomous endosperm development) we analyzed 62 ovaries from flower buds emasculated and isolated from pollination. At ~48 h after anthesis (48 HAA, 3rd day of experiment) siliques and ovules inside Col-0, *MET1/MET1*, *MET1/met1* and *met1/met1* were conspicuously elongated and enlarged. Many ovules still contained an intact mature female gametophytes. All six investigated ovaries of *FIE/FIE* degenerated by the 3rd day. On the 7th day, the elongation and enlargement of siliques were observed only in homozygous *met1/met1*. We noted induction of autonomous endosperm development only once in heterozygous *MET1/met1* at the 3rd day and twice in *met1/met1* at the 7th day (Fig. 1a–c). Autonomous endosperm was few-nucleate (up to 10), with nuclei containing one nucleolus each and clustered in dense cytoplasm in the middle part of the female gametophyte or arranged parietally on cytoplasmic strands.

STAGE OF FEMALE GAMETOPHYTE DEVELOPMENT IN UNFERTILIZED OVULES AT INOCULATION

Flower buds (24 h before anthesis, 24 HBA) 1.0–1.7 mm in length were selected for in vitro culture, under the assumption that the female gametophyte (FG) should present 8-nucleate or 7-celled stages according to Smyth et al. (1990) and Christensen et al. (1997). In fact, at inoculation, 8-nucleate, 7-celled or 4-celled (with the female germ unit only after antipode degeneration) female gametophytes were observed (Fig. 1d–f). In none of the analyzed ovules from wild genotypes, *MET1/met1* and *met1/met1* were embryos and/or endosperm observed.

INDUCTION AND DEVELOPMENT OF AUTONOMOUS ENDOSPERM IN CULTURES OF UNPOLLINATED PISTILS – EFFECT OF GENOTYPE AND MEDIUM

Autonomous endosperm was induced and developed in all studied genotypes (Col-0, *MET1/MET1, MET1/met1, met1/met1, FIE/FIE, FIE/fie*) on all media used, with overall frequency of 51.2% in ovaries and 7.2% in ovules but with variation depending on the genotype and medium. Surprisingly, AE was induced on hormone-free medium (MS6%) in all genotypes, at the highest frequency in *FIE/FIE* (100% of ovaries, 20% of ovules) and *FIE/fie* (92.3% of ovaries, 26.5% of ovules) (Tab. 1).

In the heterozygote *FIE/fie*, autonomous endosperm appeared in almost every ovary on media P1 and P3, but with high frequency (~60%) on medium P2. Interestingly, autonomous endosperm induction in this mutant was lower in ovules (~1/4 of ovules on P1 and P3, less than 10% of ovules on P2). Among the genotypes derived from the *met1* mutant, autonomous endosperm frequency was highest in *MET1/MET1* and *met1/met1* and evidently lower in the heterozygous *MET1/met1*. The relative frequency of autonomous endosperm induction was highest in the heterozygous *FIE/fie* and homozygous *met1/met1*, lower in *FIE/FIE*, and lowest in the ovaries and ovules of *MET1/met1* and Col-0. The best medium for autonomous endosperm induction was MS without hormones and with only 6% sucrose added. In Col-0, autonomous endosperm frequency was highest in ovaries and ovules on P2 with auxin and cytokinin (Tab. 1).

The differences between media in overall autonomous endosperm induction frequency were not significant, indicating that it depended mainly on the genotype but among individual genotypes these differences were significant (e.g., *FIE/FIE, FIE/fie* or Col-0; Fig. 2), showing high interaction between genotype and medium.

STRUCTURE OF AUTONOMOUS ENDOSPERM INDUCED IN VITRO

Initiation of autonomous endosperm (AE) development started from the third day of culture (which corresponds to ~48 HAA) and continued till day 7. AE development was delayed as compared to endosperm development after fertilization in planta.

At day 3 of unpollinated pistil culture, mature female gametophytes (FGs) and also ovules with few- or dozen-nucleate AE were noted, and (very rarely) multinucleate AE. Multinucleate (~100 nuclei) endosperm was usually observed in ovules cultured 7 days.

AE in vitro showed different stages of development depending on the medium and genotype, ranging from the division of the primary nucleus up to multinucleate stages with more than 100 nuclei (in mutant genotypes). Both the young stages of development (from first division to few-nucleate) and older stages (up to ~30 nuclei) were present in all genotypes on all media used (Figs. 3–5). The more advanced stages of AE, from a few dozen to more than 100 nuclei and tissue structures resembling

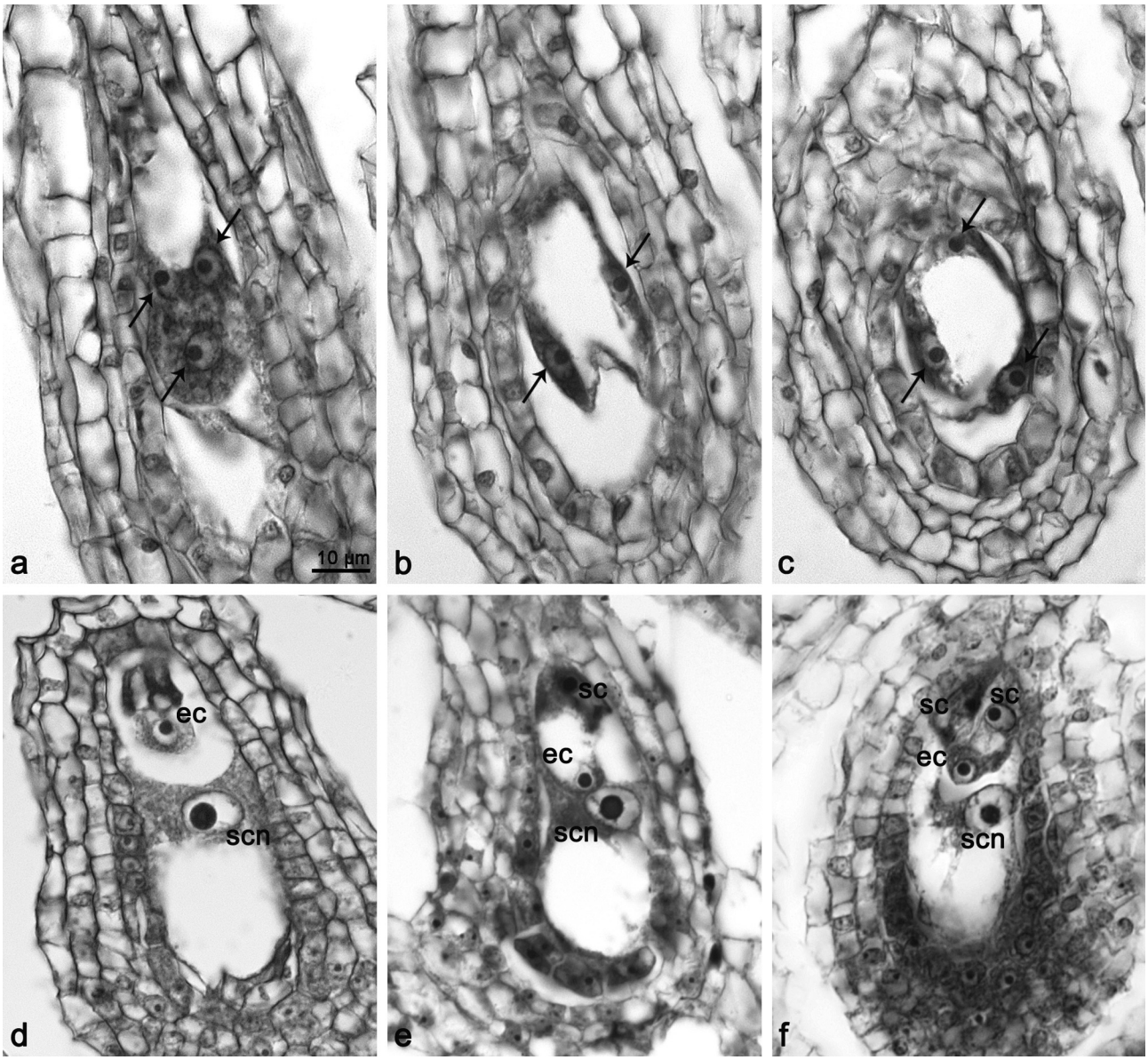

Fig. 1. Longitudinal paraffin sections of *Arabidopsis* ovules inside unpollinated ovaries in planta. Induction of autonomous endosperm (AE) in planta 7 days after bud emasculation and isolation from pollination in *met1/met1* ovule (**a–c**). (**a**) AE nuclei (arrows) in middle or parietal part of female gametophyte (FG) (**b–c**), (**d**) Mature stage of FG in ovules of Col-0 at inoculation for in vitro culture, (**e**) *FIE/fie*, (**f**) *MET1/met1*. FGs with egg cell (eg), synergids (sc) and secondary nucleus (scn). Bar = 10 µm and corresponds to all photographs.

cellular endosperm (tissue-like AE), were observed relatively frequently in Col-0 and *FIE/fie* (Fig. 5).

The first AE nuclei were located at the site of division (near egg cell) or close to each other near the FG wall (where normally after fertilization the FG become horseshoe-shaped, with the chalazal region adjacent to the micropylar region; Fig. 3a,d), or at two opposite poles (one nucleus close to the egg cell, the other at the chalazal pole).

Multinucleate AE induced in vitro showed a variety of developmental patterns: AE nuclei densely arranged close to each other, forming a delicate structure resembling the development stage of endosperm in vivo just before cellularization (Figs. 3b,f, 4d); AE nuclei decreasing in size but increasing in number (Fig. 3f); AE nuclei forming clusters in three distinct regions of the FG (Figs. 3e, 4b,c,f), showing differences in size (Fig. 3c,d) and proliferating over the micropylar region close to a usually enlarged egg cell (Fig. 3e); AE nuclei forming a network (Fig. 4a); AE nuclei parietal and surrounded by cytoplasm forming structures resembling nuclear cytoplasmic

TABLE 1. Induction of autonomous endosperm (AE) in unfertilized ovules (*SE = 1.027) up to day 7 of in vitro culture

Genotype	Medium		No. of non-degenerated			AE frequency (%)			
			ovaries	ovules	ovules per ovary*	ovaries		ovules	
Columbia	P1	MS 6%	53	903	17.0	10	(18.9)	10	(1.1)
	P2	MS6+0.1 mg l^{-1} NAA+2 mg/l BAP	40	630	15.8	23	(57.5)	35	(5.6)
	P3	MS6%+40 mg l^{-1} 2,4-D(1h)→MS6%	35	577	16.5	7	(20.0)	9	(1.6)
MET/MET1	P1	MS 6%	25	500	20.0	12	(48.0)	18	(3.6)
	P2	MS6+0.1 mg l^{-1} NAA+2 mg/l BAP	100	2660	26.6	37	(37.0)	86	(3.2)
	P3	MS6%+40 mg l^{-1} 2,4-D(1h)→MS6%	19	400	21.1	7	(36.8)	12	(3.0)
MET1/met1	P1	MS 6%	20	420	21.0	3	(15.0)	5	(1.2)
	P2	MS6+0.1 mg l^{-1} NAA+2 mg/l BAP	17	357	21.0	3	(17.7)	3	(0.8)
	P3	MS6%+40 mg l^{-1} 2,4-D(1h)→MS6%	15	315	21.0	8	(53.3)	19	(6.0)
met1/met1	P1	MS 6%	18	403	22.4	10	(55.6)	30	(7.4)
	P2	MS6+0.1 mg l^{-1} NAA+2 mg/l BAP	60	1428	23.8	35	(58.3)	128	(8.9)
	P3	MS6%+40 mg l^{-1} 2,4-D(1h)→MS6%	20	420	21.0	10	(50.0)	39	(9.3)
FIE/FIE	P1	MS 6%	15	315	21.0	15	(100.0)	63	(20.0)
	P2	MS6+0.1 mg l^{-1} NAA+2 mg/l BAP	26	582	22.4	7	(26.9)	20	(3.4)
	P3	MS6%+40 mg l^{-1} 2,4-D(1h)→MS6%	21	455	21.7	3	(14.3)	3	(0.7)
FIE/fie	P1	MS 6%	39	842	21.6	36	(92.3)	223	(26.5)
	P2	MS6+0.1 mg l^{-1} NAA+2 mg/l BAP	55	1167	21.2	32	(58.2)	112	(9.6)
	P3	MS6%+40 mg l^{-1} 2,4-D(1h)→MS6%	24	376	15.7	23	(95.8)	97	(25.8)
Total			549	12750	21.2	281	(51.2)	912	(7.2)

domains (NCDs) (Fig. 4e); or AE taking the form of tissue filling the whole FG (tissue-like AE) (Fig. 4g). Embryos were not found in unfertilized ovules cultured in vitro in non-mutant or mutant genotypes. AE developing in vitro often was accompanied by an egg cell and one or two synergids.

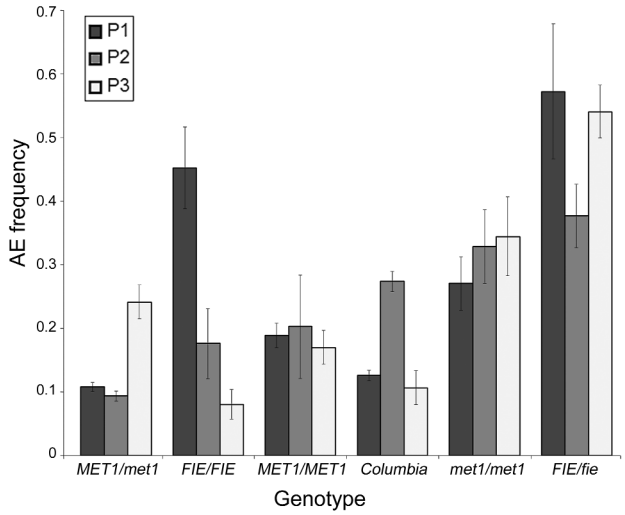

Fig. 2. Correlation between relative frequency of autonomous endosperm (AE) induction and genotype and medium. P1 (MS6%), P2 (MS6% + 0.1 mg l^{-1} NAA + 2 mg l^{-1} BAP), P3 (MS6% + 40 mg l^{-1} 2,4-D for 1h MS6%). SE= error bars. Note that relative frequency of AE induction strongly depends on genotype ($P < 10^{-9}$); the correlation between genotype and medium is also statistically important ($P < 10^{-4}$) but different for each genotype.

DISCUSSION

To induce AE in unfertilized ovules cultured in vitro we selected mutated and non-mutated plants of *Arabidopsis*, including Col-0, which in previous experiments showed a high ability to develop AE in culture (Rojek et al., 2005), and *met1* and *fie* mutants. Plants of *met1* have reduced methylation in all (sporophytic and gametophytic) tissues (Saze et al., 2003; Fitzgerald et al., 2008); *fie* mutants develop partly autonomous endosperm (Ohad et al., 1996; Vinkenoog et al., 2000). Hypomethylation combined with *fie* mutation results in further development of autonomous endosperm-like growth in planta (Vinkenoog et al., 2000; Schmidt et al., 2013).

We expected that the combined effect of mutation and culture conditions would increase the frequency of AE induction in unfertilized ovules of *fie* mutants known to develop AE in planta and/or induce AE development in *met1* unfertilized ovules thought to be incapable of AE production. AE developed in all of the studied genotypes (Col-0, *MET1/MET1*, *MET1/met1*, *met1/met1*, *FIE/FIE*, *FIE/fie*) on all media used.

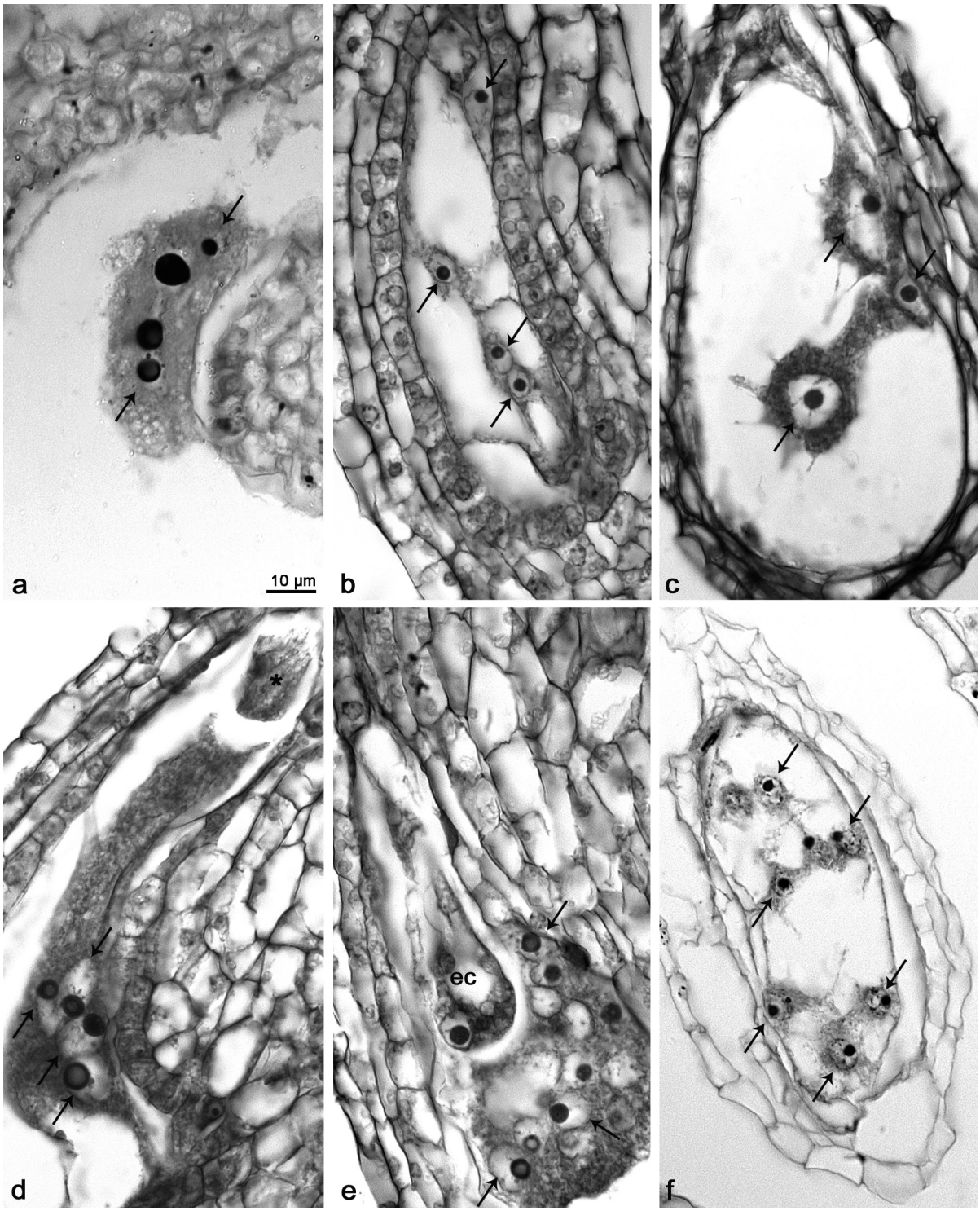

Fig. 3. Longitudinal paraffin sections of *MET1/MET1*, *MET1/met1* and *met1/met1* ovules inside unpollinated ovaries cultured in vitro. Autonomous endosperm (AE) induction and development after 7 days of in vitro culture of unfertilized ovules. (**a**) *MET1/MET1*, (**b–c**) *MET1/met1*, (**d–f**) *met1/met1*. AE induced on P1 (**a, e**), on P2 (**d**), and on P3 medium (**b, c, f**). (**a**) Few-nucleate AE; nuclei (arrows) close to each other in dense cytoplasm near wall in middle part of female gametophyte (FG), (**b**) Multinucleate AE (~50, arrows) accompanied by egg apparatus (not visible), (**c–d**) Few-nucleate AE; large nuclei (arrows) in central part of FG, remnants of egg apparatus mark with star in (d), (**e**) Enlarged egg cell (ec) and mass of dense cytoplasm with many (~50) AE nuclei (arrows), (**f**) Multinucleate AE (arrows) filling the whole FG. Bar = 10 µm and corresponds to all photographs.

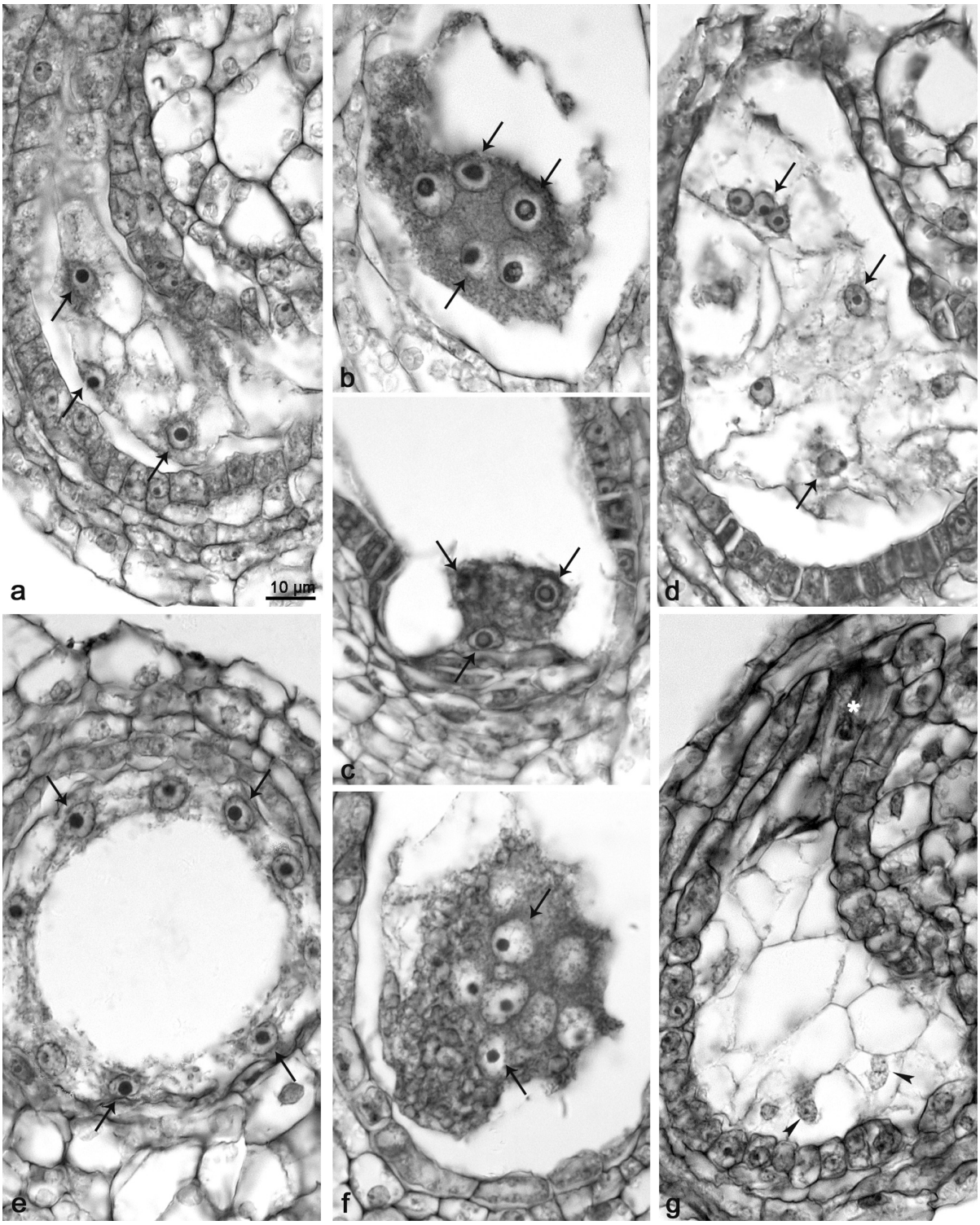

Fig. 4. Longitudinal paraffin sections of *FIE/FIE* and FIE/fie ovules inside unpollinated ovaries, cultured in vitro. Autonomous endosperm (AE) induction and development after 7 days of in vitro culture of unfertilized ovules. (**a–c**) *FIE/FIE*, (**d–g**) *FIE/fie*. AE induced on P1 (**a–d, f**) and on P3 medium (**e, g**). (**a**) Multinucleate AE (arrows) resembling a network, (**b**) Multinucleate AE with nuclei (arrows) clustered in mass of cytoplasm in middle part of female gametophyte (FG), (**c**) Chalazal cyst of AE (arrows), (**d**) AE resembling a network, (**e**) Multinucleate AE with nuclei (arrows) arranged around central vacuole, (**f**) Multinucleate AE; nuclei (arrows) in mass of dense cytoplasm in center of FG, (**g**) Tissue-like AE accompanied by degenerating egg apparatus (star). Bar = 10 µm and corresponds to all photographs.

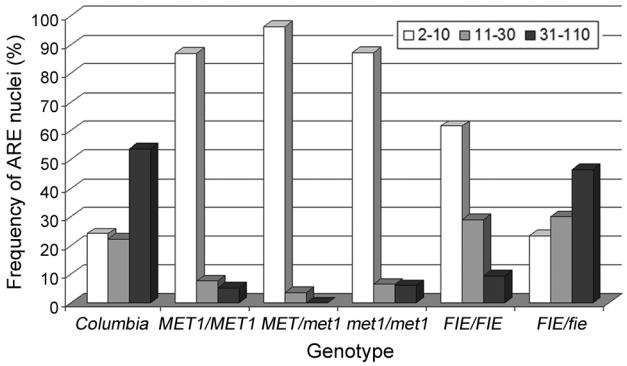

Fig. 5. The frequency of autonomous endosperm (AE) nuclei. Three developmental stages of free-nuclear AE were determined on the basis of AE nuclei number: 2–10, 11–30, 31–110.

INDUCTION AND DEVELOPMENT OF AUTONOMOUS ENDOSPERM IN CULTURE OF UNPOLLINATED PISTILS – EFFECT OF GENOTYPE AND MEDIUM

For all genotypes and media taken together, the rate of autonomous endosperm induction was relatively high (51.2% in ovaries, 7.2% in ovules). This is the highest AE induction frequency seen in culture of unpollinated *Arabidopsis* ovaries (26% in Rojek et al., 2005; 7.8% in Kapusta et al., 2007; 11% in Pawełek-Skoczylas unpubl. data). AE frequency was strongly dependent on genotype. In the heterozygote *FIE/fie* we expected AE induction of at least 50% of the ovules of each ovary, but in fact the frequency was much lower (9.6–26.5% of ovules analyzed, 3.5–6.2 ovules per ovary) in culture conditions. This could be an effect of reduction of ovule number during culture due to degeneration of ovules, also in the *fie* mutation. The almost equal AE frequencies in *FIE/FIE* and *FIE/fie* mutants cultured on medium P1 was unexpected. Perhaps stress in vitro acts through *FIE* and AE induction arose from non-mutated ovules (central cell) in both mutated (*FIE/fie*) and wild (*FIE/FIE*) genotypes. On the other hand, the presence of a mutant allele not only in gametophytic but also in sporophytic tissues of maternal *FIE/fie* plants can cause maternal (or paternal) sporophytic effects in tissues of offspring with the *fie* mutation as well as non-mutated offspring (Ohad et al., 1999). The studied mutations generally increased the relative frequency of AE induction. For *met1/met1* it was the result of reduction of methylation in all tissues of the ovaries and ovules; for *FIE/fie* it was due to the presence of the mutation in ~50% of the ovules at inoculation. The situation is unclear for the *MET1/met1* heterozygote, where decreased DNA methylation would be expected in sporophytic tissues and in ~50% of the female gametophytes (i.e., ~50% of the ovules), in vitro. But AE induction in this genotype was not very high (15–53.3% in ovaries and 0.8–6.% in ovules). The lower AE induction might be explained by changes in the level of DNA methylation occurring in vitro. For this reason, the presence of hemimethylated DNA in the genomes of plant cells, which potentially contains sufficient information to restore full methylation (Takeda and Paszkowski, 2006), must also be taken into account.

Autonomous endosperm developed in each *A. thaliana* genotype on each medium and in each sample. In other species, even from the same family as *Arabidopsis*, AE developed in vitro only in some varieties (e.g., *Brassica napus* cv. Topas, Rojek et al., 2002) and often with low frequency, sporadically or not at all (*Capsella bursa-pastoris*, Trzcińska unpubl. data; *Diplotaxis* sp., Mickiewicz unpubl. data). In *Viola* some species are more susceptible to factors inducing independent cell growth in unfertilized female gametophytes in vitro (e.g., *Viola odorata*, Wijowska et al., 1999a,b) and others less so or not at all (e.g., *Viola tricolor*, Grygiel unpubl. data). Our data indicate that *A. thaliana* has a natural potential ability to initiate development without fertilization.

Autonomous endosperm frequency was relatively high on hormone-free medium (P1) in all genotypes. On this medium, aside from other factors (e.g., development stage, effect of sugar, sterilization), AE formation depended on genotype. Development of autonomous structures (including endosperm) on media without hormones has been reported in both monocotyledonous and dicotyledonous plants (Musial et al., 2005; Rojek et al., 2005; Kapusta et al., 2007).

The ovaries and the ovules inside them were generally enlarged on all the media used, and the ovules resembled seed-like structures without embryos. Developmental delay, enlarged FGs and overproliferation of AE may be considered characters of hypomethylated mutants in planta: *fie-1/FIE;MET1a/s/MET1*a/s and *mea-1/MEA;met1-3/MET1* (with AE but without embryo, Vinkenoog et al., 2000, Schmidt et al., 2013) and *met1-3/met1-3* (no embryos and no endosperm, FitzGerald et al., 2008). The results from in vitro culture of *fie* and *met* mutants (almost fully developed AE in *FIE/fie* and *met1/met*) indicate that the combination of culture conditions, genotype and specific hormones can influence genomic imprinting and that some imprinted paternally expressed genes (PEGs) become active in unfertilized female gametophytes, leading to induction and full development of autonomous endosperm. This finding is in accordance with recent reports on epigenetic regulation (by FIS PRC2 and MET1 interaction) of imprinted plant genes (Köhler, 2012; Schmidt et al., 2013), but how gene expression changes and which gene or genes are involved remain to be settled.

STRUCTURE OF AUTONOMOUS ENDOSPERM FORMED IN CULTURE VERSUS ENDOSPERM DEVELOPED AFTER CENTRAL CELL FERTILIZATION IN PLANTA

In culture, AE development was delayed as compared to findings in planta that at 12 hours after pollination (12 HAP) most seeds of wild-type *Arabidopsis* contain binucleate endosperm and at 24 HAP the vast majority of seeds have 8-nucleate endosperm and an undivided zygote (Faure et al., 2002). Wei and Sun (2002) reported that 30 hours after anthesis the number of endosperm nuclei is nearly 100, at 48 HAA the embryo reaches the globular stage, and cellularization initiates in multinucleate endosperm. In our experiments, AE development in vitro was only slightly delayed versus in planta at day 3 of culture (lacking stages of typical cellular endosperm), and significant dispersion of developmental stages was noted at day 7. All developmental stages from secondary nucleus to multinucleate AE were present at that time (Rojek et al., 2005). The differences might be due to growth conditions and/or the origin of the plants.

It was difficult to establish the timetable of AE development in vitro. Generally, the mature female gametophyte, the first division of the central cell, and bi- or few-nucleate AE were most often observed in ovules cultured 3–5 days. Multinucleate stages of AE were dominant at day 7 of culture. We noted a 2–3-day delay of central cell division, and a 5–6-day delay of the multinucleate stage as compared to endosperm development after fertilization in planta. The multinucleate (~100 nuclei) stage of AE and cellularization symptoms (AE nuclei and cytoplasm arranged in NCDs around central vacuole; AE resembled a network, tissue-like AE) usually appeared only at day 7 of culture. The AE development pattern in vitro differed from that of endosperm formed after fertilization, and was more like that of endosperm of *fis* mutant seeds and also seeds produced by crosses of diploids with tetraploids/hexaploids (Adams et al., 2000; Vinkenoog et al., 2000).

The presence of an egg cell, synergid(s) and AE nuclei in the same female gametophyte is strong evidence for the autonomous origin of this tissue. The structure of AE nuclei indicates its origin from the secondary nucleus, supporting previous primary research on the origin of AE in *Arabidopsis* wild types as well as in other species (Rojek et al., 2005; Kapusta et al., 2007). Binucleate AE with nuclei located at opposite poles is an often-observed pattern of AE development. Such an alignment of endosperm nuclei is characteristic for *Arabidopsis* endosperm development in planta after fertilization (~12 HAP, Faure et al., 2002; 2–4 HAF, Mansfield and Briarty 1990), and has been reported in heterozygous *FIE/fie* mutants after emasculation and blocking of flower bud pollination (Ohad et al., 1996) as well as in ecotype C24 after single fertilization by mutated pollen *cdka;1* (Ungru et al., 2008).

In contrast to endosperm in vivo, in which one pattern of development is common (e.g., Brown et al., 1999), multinucleate AE in vitro showed a variety of developmental types. Some of them were very similar to the type(s) present in planta; others were clearly reminiscent of autonomous endosperm in *fie* mutants in planta, with normal (*fie-1/FIE*) and low (*fie-1/FIE, METI* a/s) levels of DNA methylation (Vinkenoog et al., 2000). It should be added that no embryo was present in unfertilized ovules of *met1*, *fie* and *mea* mutants in planta, nor in unfertilized ovules of *Arabidopsis* genotypes grown in vitro. Thus we can conclude that in vitro conditions induce an effect similar to that exerted by DNA hypomethylation, and culture promotes development of endosperm but not the embryo. However, the presence of an egg apparatus elements in most of the ovules suggests that factors in other systems (especially those selecting the type and concentration of hormones or Ca^{2+}) could lead to development of an unfertilized egg. Unlike in animal egg cells, an increase of the intracellular Ca^{2+} concentration is not enough to trigger parthenogenesis in plants (Curtis and Grossniklaus, 2008). In *fis* mutants in planta, simultaneous development of autonomous endosperm and embryo-like structure in the same FG was observed only in unpollinated ovaries of *MSI1/msi1* (Köhler et al., 2003a).

CHANGES IN *FIS* AND *MET1* GENE EXPRESSION AS PROBABLE INDUCTOR OF AUTONOMOUS ENDOSPERM DEVELOPMENT IN VITRO

The PcG protein complex, which includes FIE, MEA and DNA methylation, is recognized as the major component of regulation of imprinting in the endosperm (Schmidt et al., 2013). The important role of FIE in the complex is confirmed by the absence of these complexes (called PRC2-like complexes) in *fie* mutants (Köhler and Grossniklaus, 2002) and autonomous (without fertilization) development of endosperm, and it is very similar to the autonomous endosperm induced in our experiments in vitro. The endosperm of the *fie* mutant (e.g., Vinkenoog et al., 2000) and AE formed in vitro had very stunted growth. This underdevelopment was repaired in the *fie* mutant of *Arabidopsis* with a low-methylated genome in which endosperm development proceeded normally in the absence of fertilization (Vinkenoog and Scott, 2001). Our preliminary analyses of DNA methylation in *FIE* of Col-0 suggest changes in *FIE* methylation under specific in vitro conditions; *FIE* exhibited relatively high activity in

ovary tissues cultured in vitro (Rojek et al., unpublished data). In vivo, *FIE* activity is high before and especially just after fertilization (Curtis and Grossniklaus, 2008) and perhaps regulation of its expression occurs, as in the case of *MEA*, by antagonistically acting proteins, such as methyltrasferase MET1 and demethylase DME. Thus, the low level of *FIE* methylation both in vitro and in vivo may indicate the initiation and development of endosperm after fertilization and endosperm formed independently. We will continue investigations of methylation in *FIE* and other *FIS* genes, analyses of H3K27 histone methylation and *FIS* and *MET1* genes expression, in ovules cultured in vitro, to determine the inductor of AE development.

CONCLUSIONS

This study of autonomous endosperm in the model plant, *Arabidopsis thaliana* (L.) Heynh (Brassicaceae), showed that under specific conditions in vitro and also in planta the established epigenetic mechanism can fail, leading to independent divisions of the central cell before fertilization.

The results of AE induction in vitro in non-mutated and mutated *A. thaliana* genotypes, and in planta/in vivo, indicate that changes in the expression of several genes like *FIE* are one of the mechanisms responsible for AE induction.

The results presented in this paper may help efforts to exploit apomixis mechanisms in economically important plants that reproduce sexually.

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