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LETTERS

Bypassing genomic imprinting allows seed development

Moritz K. Nowack[1], Reza Shirzadi[2], Nico Dissmeyer[1], Andreas Dolf[3], Elmar Endl[3], Paul E. Grini[2] & Arp Schnittger[1]

In developing progeny of mammals the two parental genomes are differentially expressed according to imprinting marks, and embryos with only a uniparental genetic contribution die[1–3]. Gene expression that is dependent on the parent of origin has also been observed in the offspring of flowering plants, and mutations in the imprinting machinery lead to embryonic lethality, primarily affecting the development of the endosperm—a structure in the seed that nourishes the embryo, analogous to the function of the mammalian placenta[4]. Here we have generated *Arabidopsis thaliana* seeds in which the endosperm is of uniparental, that is, maternal, origin. We demonstrate that imprinting in developing seeds can be bypassed and viable albeit smaller seedlings can develop from seeds lacking a paternal contribution to the endosperm. Bypassing is only possible if the mother is mutant for any of the *FIS*-class genes, which encode Polycomb group chromatin-modifying factors. Thus, these data provide functional evidence that the action of the FIS complex balances the contribution of the paternal genome. As flowering plants have evolved a special reproduction system with a parallel fusion of two female with two male gametes, our findings support the hypothesis that only with the evolution of double fertilization did the action of the *FIS* genes become a requirement for seed development. Furthermore, our data argue for a gametophytic origin of endosperm in flowering plants, thereby supporting a hypothesis raised in 1900 by Eduard Strasburger.

Flowering plants (angiosperms) have evolved to be one of the predominant life forms on earth with more than 250,000 extant species. An important feature, and probably one of the main reasons for this evolutionary success, is the development of an embryo along with a second fertilization product, the endosperm. The endosperm is usually triploid because a homo-diploid female central cell fuses with one of the male gametes. The exact genome dosage of typically two maternal and one paternal genomes seems to be crucial for endosperm development. Raising the maternal contribution in the endosperm has been found to result in smaller seeds comprising fewer endosperm cells and smaller embryos. In contrast, increasing the paternal input results in larger seeds[5–7]. These results have been interpreted in light of the parental conflict theory (kinship theory) according to which mothers and fathers have a different interest in allocation of resources to their offspring[8,9]. Hence, it has been proposed that paternal genes promote seed growth, whereas maternal genes rather reduce growth; or, conversely, that in the maternal genome growth promoting factors are inactivated.

Indeed, flowering plants have been found to imprint certain genes during endosperm development, which is similar to the situation observed for the placenta of mammals[10–14]. Interestingly, the imprinting machinery seems to regulate itself[15–17]. Imprinting involves the recognition of methylated DNA and histone modifications, leading to chromatin rearrangements. In flowering plants, the FIS complex has been implicated in mediating imprinting, and mutants for individual components—for example, *medea* (*mea*), *fertilization-independent seed 2* (*fis2*), *fertilization-independent endosperm* (*fie*)—show an over-proliferation phenotype of the endosperm, resulting in embryo lethality later in seed development[18–21]. The molecular nature of this mutant phenotype, however, has not been resolved, and a growth factor that is specifically expressed during seed development has not been found. Also, although isolated gametes could be fused *in vitro*, attempts to dissect double fertilization and imprinting at the organ level have not yet been successful[22]. Thus, the actual role of imprinting in seed development remains obscure.

Previously we have characterized a mutant for the *Arabidopsis thaliana* $Cdc2^{+}$/*Cdc28* homologue, *CDKA;1*, in which pollen with only one gamete is produced[23]. This mutant pollen causes a paternal effect, leading to seed abortion. Because this pollen was found to exclusively fertilize the egg cell and not the central cell, the *cdka;1* mutant offers a unique possibility to study the action of the paternal genome and the importance of imprinting during seed development.

To trace the fate of plants fertilized with *cdka;1* mutant pollen, we generated a marker line by fusing the *CDKA;1* complementary DNA to the yellow fluorescent protein gene (*YFP*) under the control of the *CDKA;1* promotor. This construct could completely rescue the *cdka;1* mutant phenotype (Supplementary Fig. 1a, b). Next, we selected plants that were homozygous *cdka;1* mutant at the endogenous locus and contained only one allele of the *CDKA;1–YFP* rescue construct; hereafter referred to as *cdka;1–yfp*$^{+/-}$. These plants mimicked heterozygous *cdka;1* mutant plants with half of the pollen showing a mutant phenotype (Supplementary Fig. 1c, d). In contrast to the wild-type-like pollen and its progeny after fertilization, this mutant pollen and its fertilization products are indicated by the absence of YFP fluorescence (Supplementary Fig. 1d, and data not shown). Consistent with previous studies, ovules fertilized with YFP-negative *cdka;1* mutant pollen aborted around 3 days after pollination (d.a.p.) at an early globular embryo stage, surrounded by an underdeveloped endosperm (Fig. 1a–f)[23].

We have previously shown that seed development is coordinated by two independent signals: a positive signal coming from the fertilization of the egg cell and the release of a negative signal regulated by the action of the *FIS*-class genes[23]. Fertilization of only the egg cell with the *cdka;1* mutant pollen generates a positive signal that triggers a few rounds of central cell divisions. To promote further endosperm proliferation, we combined the *cdka;1* mutant with the *mea* mutant, in which the block of central cell proliferation is released due to the lack of FIS–Polycomb group complex function.

In self-fertilized *mea* mutants, the endosperm typically over-proliferates and embryo development arrests at the heart stage (Fig. 1g–i)[18,19,21]. We analysed embryo and endosperm development in

[1]University group at the Max Planck Institute for Plant Breeding Research, Max-Delbrück-Laboratorium, Department of Botany III, University of Cologne, Carl-von-Linné-Weg 10, D-50829 Cologne, Germany. [2]Department of Molecular Biosciences, University of Oslo, PO Box 1041 Blindern, N-0316 Oslo, Norway. [3]Institute of Molecular Medicine and Experimental Immunology, Sigmund-Freud-Straβe 25, D-53105 Bonn, Germany.

mea$^{-/-}$ plants crossed with *cdka;1–yfp*$^{+/-}$ plants. Strikingly, approximately 20% of these seeds (63 out of 329) not only overcame the paternally conferred *cdka;1*-abortion point around 3 d.a.p., but also bypassed the maternally derived *mea* arrest around 6–7 d.a.p. (Fig. 1j–l). This restoration of seed development gave rise to the unforeseen hypothesis that these seeds complete embryogenesis with only a diploid endosperm. Consistently, we found that the majority of seeds arrested with a heart-stage embryo were YFP-positive, whereas developing seeds were YFP-negative (Supplementary Fig. 1g, h).

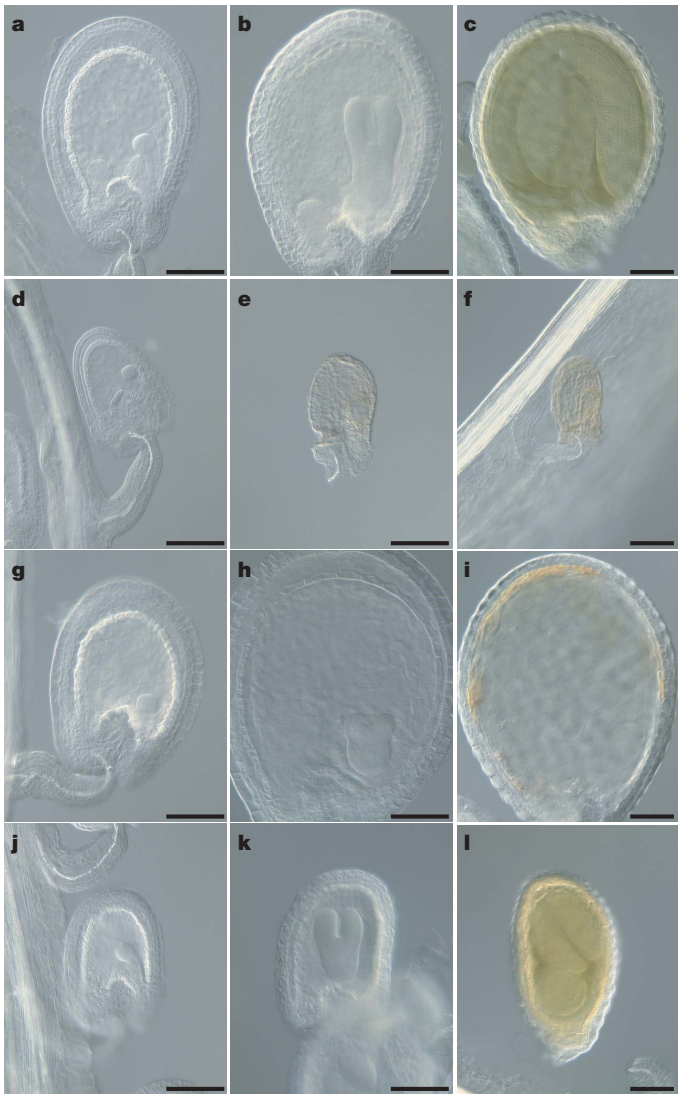

Figure 1 | Rescue of seed abortion in *mea*$^{-/-}$ × *cdka;1–yfp*$^{+/-}$ crosses. Micrographs of cleared seeds. **a–c**, Seed development in L*er* × *CDKA;1–YFP*$^{+/+}$. **a**, Globular-stage embryo. **b**, Torpedo-stage embryo. **c**, Seed nearing maturity. **d–f**, Seed development in L*er* × *cdka;1–yfp*$^{+/-}$ (YFP-negative seeds). **d**, Aborting seed with embryo arrested at early globular stage. The aborted seeds decay (**e**) and (**f**). **g–i**, Seed development in *mea*$^{-/-}$ × *CDKA;1–YFP*$^{+/+}$. **g**, Globular-stage embryo with syncytial endosperm nuclei, similar to wild type. **h**, Seed with an abnormal heart-stage embryo typical for *mea*-conferred seed abortion. **i**, Seed decaying after *mea*-conferred abortion. **j–l**, Seed development in *mea*$^{-/-}$ × *cdka;1–yfp*$^{+/-}$ (YFP-negative seeds). **j**, Globular stage embryo with syncytial endosperm nuclei. **k**, About 25% of the seeds ($n = 112$) are smaller than wild type but embryo development continues. **l**, About 10% of the seeds ($n = 134$) contain small, yet viable-looking embryos. d.a.p., days after pollination. *CDKA;1–YFP*$^{+/+}$ is homozygous for *Pro*$_{CDKA;1}$-*CDKA;1–YFP* in *cdka;1*$^{-/-}$. *cdka;1–yfp*$^{+/-}$ is heterozygous for *Pro*$_{CDKA;1}$*:CDKA;1–YFP* in *cdka;1*$^{-/-}$. YFP-negative, seeds without the *CDKA;1–YFP* transgene. Scale bars, 100 μm.

YFP-negative seeds with a *mea*$^{-/-}$ maternal mutant background showed a characteristic reduction in size in comparison to wild type that became already obvious at 4–5 d.a.p. (Fig. 1j–l; Supplementary Fig. 3a). To examine whether this small seed size phenotype is restricted to the *mea* allele, two other *fis*-class mutants, *fis2* and *fie*, were crossed with *cdka;1*$^{+/-}$ mutants. Similar to *mea* mutants, *fis2* and *fie* have a strong maternal effect and embryos abort around the heart stage[18,20]. However, in the progeny of all crosses we observed a new class of seeds that completed embryogenesis but had an obvious reduction in size similar to that seen for the rescued *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds (data not shown).

We verified that the central cell in the small seeds of the *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ crosses did not receive a paternal genome by performing laser dissection microscopy and subsequent PCR analysis for an accession-specific simple sequence length polymorphism (SSLP) (Fig. 2a–d). In control crosses of *mea*$^{-/-}$ (in the L*er* genetic background) with Col, both maternal and paternal genomes could be detected in both the embryo and the endosperm in approximately 90% of cases (Fig. 2d). In embryos from small *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds, both bands could also be obtained in about 90% of cases. Conversely, in the endosperm of these seeds only the maternal L*er* band could be amplified in 90% of cases (Fig. 2d).

In addition, we measured ploidy levels of developing seeds by flow cytometry to determine whether the endosperm of these small seeds is indeed homoparental diploid rather than biparental triploid. In contrast to the combination of diploid and triploid peaks in wild-type seeds, we observed only diploid nuclei in small *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds (Fig. 2e–f, respectively).

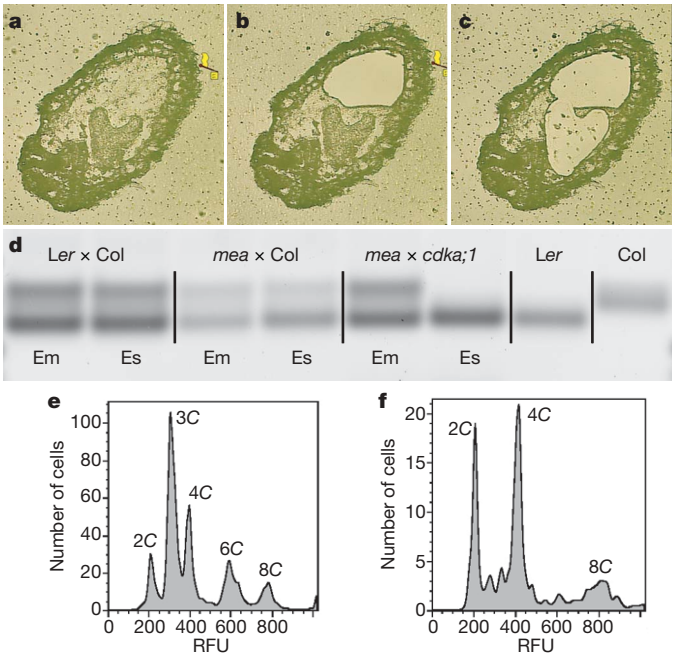

Figure 2 | The small *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds develop with a uniparental diploid endosperm. **a–c**, Micrographs of a *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ rescued-seed section mounted for laser microdissection. **a**, Before dissection. **b**, After collecting endosperm tissue. **c**, After collecting embryo tissue. **d**, Agarose gel showing the products of an SSLP-PCR. The L*er* genome produces a smaller fragment; the Col genome a larger fragment. Ninety per cent of L*er* × Col seeds ($n = 16$) and of *mea*$^{-/-}$ × Col seeds ($n = 19$) have both accession-specific bands in the endosperm. In contrast, 90% of *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds ($n = 16$) contained endosperms only producing a L*er*-band. **e**, **f**, Flow cytometry ploidy analysis of *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds at 6 d.a.p. **e**, DNA profile of wild-type seeds, showing a mixture of diploid (2*C*, 4*C*, 8*C*) and triploid (3*C*, 6*C*) peaks. **f**, DNA profile of small *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ rescued-seeds lacking triploid peaks. L*er*, Landsberg *erecta*; Col, Columbia; *mea*, homozygous *medea* mutant; *cdka;1*, heterozygous *cdka;1* mutant; Em, embryo; Es, endosperm; RFU, relative fluorescence units.

The endosperm in fertilized *fis*-class mutants shows a heterochronic defect; that is, it never reaches maturity[24]. To address the fate of the endosperm in the small seeds originating from the different *fis*-class mutants crossed with *cdka;1*$^{+/-}$, various marker lines were used that are expressed in a development-specific manner. The analysis of these markers revealed that endosperm development in the small *fis*-class-mutant × *cdka;1*$^{+/-}$ seeds is distinct from fertilized *fis*-class mutant seeds and qualitatively resembles wild-type endosperm (Supplementary Fig. 2a–f). A hallmark of endosperm differentiation in *Arabidopsis* is cellularization, which is initiated when the embryo reaches the heart stage (Supplementary Fig. 2g). Whereas cell walls are absent in the endosperm of *mea* mutants (Supplementary Fig. 2h), we found that in small *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds the endosperm was completely cellularized at 6 d.a.p. (Supplementary Fig. 2i). Therefore, the paternal genome does not seem to be required for the adoption of wild-type endosperm qualities, and the *FIS*-class genes are dispensable for the development of a functional endosperm. The diploid endosperm of *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds is genotypically identical to the autonomous endosperm in unfertilized *fis*-class mutant ovules in which the central cell also starts to proliferate. The major difference between these ovules is the presence of a developing embryo. Thus, our data stress a function of the embryo in directing seed growth and suggest repeated cross-talk between embryo and endosperm throughout seed development. First, during very early stages of seed development the zygote stimulates initial central cell divisions as described previously[23]. Here, a second round of embryonic signalling is unravelled because in *fis*-class mutants further differentiation of the diploid endosperm seems to be possible only if an embryo is present.

Except for their reduced size in comparison to wild type, YFP-negative embryos in small *mea*$^{-/-}$ × *cdka;1–yfp*$^{+/-}$ seeds resembled wild-type embryos (see also Fig. 1j–l). *YABBY* messenger RNA, which is a marker for abaxial cell fate, was typically expressed in the two cotyledons at early heart stage (Supplementary Fig. 4a, b). The shoot apical meristem seemed to be properly established, as demonstrated by detection of *WUSCHEL* and *SHOOT MERISTEMLESS* mRNA at a typical region in the axis between the two cotyledons (Supplementary Fig. 4c–f). After completion of embryogenesis, the small seeds that developed with a solely maternal endosperm were found to germinate at frequencies similar to wild-type on agar plates (Supplementary Fig. 3b). However, the size difference of these embryos seemed to be maintained during postembryonic development, and *mea*$^{-/-}$ × *cdka;1–yfp*$^{+/-}$ seedlings were also smaller than controls, as judged by their reduced root lengths after germination (Supplementary Fig. 3c–e). Also, further growth and development of the *mea*$^{-/-}$ × *cdka;1–yfp*$^{+/-}$ plants are delayed, as indicated by smaller rosettes (Supplementary Fig. 3f–l) and a delayed flowering (Supplementary Table 1). Nonetheless, *mea*$^{-/-}$ × *cdka;1–yfp*$^{+/-}$ plants were fertile and could produce viable offspring. At the end of their lifespan, no significant difference in biomass could be detected between *mea*$^{-/-}$ × *cdka;1–yfp*$^{+/-}$ YFP-positive and YFP-negative F_1 plants hatching from small seeds, as judged by shoot dry weight (Supplementary Fig. 3m).

Table 1 | Transmission frequencies of *cdka;1* and the *fis*-class mutant alleles

Genotype of cross	Genotype of viable progeny (%)			Association factor	*n*
	cdka;1$^{+/-}$	*CDKA;1*$^{+/+}$	*fis*-class$^{+/-}$		
Col × *cdka;1–yfp*$^{+/-}$	6	94	NA	NA	412
mea$^{-/-}$ × *cdka;1–yfp*$^{+/-}$	92	8	100	92	186
Col × *cdka;1*$^{+/-}$	10	90	NA	NA	120
mea$^{-/-}$ × *cdka;1*$^{+/-}$	92	8	100	92	142
fis2-4$^{+/-}$ × *cdka;1*$^{+/-}$	39	61	ND	ND	144
fie-11$^{+/-}$ × *cdka;1*$^{+/-}$	36	64	18	100	138

cdka;1–yfp$^{+/-}$ is a homozygous *cdka;1* mutant heterozygously complemented with a $Pro_{CDKA;1}$-*CDKA;1–YFP*$^{+/-}$ transgene; *n*, number of F_1 plants scored; *fis*-class, mutant alleles for *mea*, *fis2* or *fie*; NA, not applicable; ND, not determined; association factor, number of F_1 plants carrying both a *fis*-class mutant and a *cdka;1* mutant allele divided by the total number of plants carrying a *fis*-class mutant allele in per cent.

Embryo development supported by a diploid endosperm is not a rare event in *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ crosses, as conferred by the transmission rate of the *cdka;1* mutant allele. Among the progeny of wild-type plants pollinated with *cdka;1*$^{+/-}$ mutants, only 10% of the siblings carried the mutant allele owing to the paternal effect conferred by the *cdka;1* pollen (Table 1). Strikingly, we found that in *mea*$^{-/-}$ mutants pollinated with *cdka;1*$^{+/-}$ plants the majority of the offspring (92%, $n = 142$) carried the *cdka;1* mutant allele (similar results were obtained using the *cdka;1–yfp*$^{+/-}$ pollen) (Table 1). This fertilization bias was not gene or allele specific because the viable progeny of crosses with other *mea* alleles and other *fis*-class mutants, that is *fis2*$^{+/-}$ and *fie*$^{+/-}$, showed a similar increase in the *cdka;1* allele frequency (Table 1). Notably, a *fie* mutant allele only could be transmitted through the mother in combination with a fertilization by *cdka;1* mutant pollen.

Restoration of the gametophytic maternal effect in *fis*-class mutants by single fertilization of the egg cell suggests that FIS-mediated imprinting counterbalances the activity of the paternal genome. To investigate this hypothesis we analysed the expression levels of the MADS-box transcription factor *PHERES1* (*PHE1*) by quantitative PCR with reverse transcription (qPCR). *PHE1* is one of the few known genes in *Arabidopsis* to be predominantly expressed from the paternal genome in the endosperm and is a target of the FIS complex[25]. Expression data of wild-type control crosses were compared with *mea*$^{-/-}$ × wild-type and *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ crosses (Fig. 3). Consistent with previous findings, *PHE1* expression is strongly upregulated in *mea* mutants[26]. This effect is more pronounced at 4 d.a.p. than at 2 d.a.p. In contrast, *mea*$^{-/-}$ pollinated with *cdka;1*$^{+/-}$ pollen showed a significant reduction in *PHE1* expression when compared to *mea*$^{-/-}$ pollinated with wild-type pollen. Even though previous experiments have shown *phe1* mutants to be indistinguishable from wild-type plants, expression of *PHE1* in antisense orientation could partially rescue the mutant effect of *mea* and elevate the viability of mutant seeds[25,26]. Thus it is conceivable that the rescue of the *mea* mutant phenotype by *cdka;1*$^{+/-}$ pollen is at least partially due to a reconstitution of a more balanced gene dosage of *PHE1* and related genes.

Here we have demonstrated that sexually reproducing plants can be supported by a homoparental diploid endosperm. The finding that viable seeds can develop without a paternal genome contributing to the endosperm demonstrates that imprinting can be bypassed during seed development. On the basis of the monitoring of *PHE1*

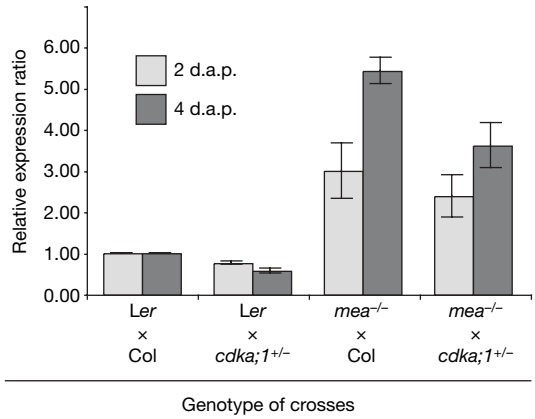

Figure 3 | Quantitative real-time PCR monitoring *PHE1* expression in *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds. Expression levels of *PHE1* were measured by real-time PCR at 2 d.a.p. (light grey bars) and 4 d.a.p. (dark grey bars). A pool of six siliques per biological replica was analysed. The bars represent data from replicate no. 1 (±s.d. of 4 repeats), all biological replicates showed similar results. In the *mea*$^{-/-}$ background, the *PHE1* expression is strongly upregulated, which is most pronounced at 4 d.a.p. On the contrary, in *mea*$^{-/-}$ × *cdka;1*$^{+/-}$ seeds the *PHE1* upregulation at 4 d.a.p. is significantly reduced. For abbreviations, see legends of Fig. 1 and Fig. 2.

expression levels we conclude that the final gene dosage and expression patterns are crucial for seed development: a result consistent with the kinship theory[8,9]. The contribution of the paternal genome and the action of the *FIS*-class genes seem to be opposing mechanisms and might represent sequential layers of regulation that can be stripped off to reveal an ancient core. With this, our findings might also shed light on the evolutionary origin of endosperm.

In the other clade of seed plants, the gymnosperms, offspring are produced only by a single fertilization event. Nonetheless, gymnosperms also support embryo growth with a nourishing tissue of maternal origin, called the prothallium, which is usually well-developed before sperm cell entry[27]. Two hypotheses have been raised to explain the appearance of the endosperm in the angiosperm clade. The first suggests the transformation of a second fertilized embryo into an altruistic nourishing tissue; that is, the endosperm. Thus, endosperm would be of sporophytic origin[28]. Because we have shown here that solely maternally derived endosperm (that is, of gametophytic origin) shows characteristics of wild-type endosperm and is sufficient to sustain complete embryo development, our data provide functional support for the second hypothesis raised by Eduard Strasburger. He proposed, in 1900, that during evolution the female gametophyte was reduced to the central cell of modern angiosperms and that the fusion of the second sperm cell is used as a trigger to start endosperm development[29].

METHODS SUMMARY

Arabidopsis plants used in this study were grown in standard greenhouse conditions. The following genotypes were used: wild-type plants, either the Columbia-0 (Col) or the Landsberg *erecta* (L*er*) accession. Throughout this work the previously characterized *cdka;1-1* allele (SALK_106809.34.90.X) was used[23]. Seeds homozygous for the *mea* allele were obtained from A. Chaudhury and are in the L*er* background[18]. Line KS22 in $fie^{+/-}$ and the enhancer trap GUS line G222 in *mea* were contributed by F. Berger[24]. All genotypes were determined by PCR, by resistance to BASTA or by presence of fluorescent proteins.

Seed morphology was analysed by light microscopy using differential interference contrast optics. Fluorescence in seeds and pollen was analysed with a confocal laser scanning microscope. qPCR was performed on cDNA synthesized from total RNA from siliques. All samples and reference controls were performed in three independent biological replicates and repeated four times.

For a paternity test of the endosperm in *mea* × *cdka;1* seeds, samples of endosperm and embryo tissue were separately collected by laser microdissection and used as templates for SSLP-PCRs discriminating between polymorphisms of the parent accessions L*er* and Col.

For ploidy analysis of the endosperm in *mea* × *cdka;1* seeds, three biological replicates of the nuclei of whole seeds were extracted and analysed with a three laser LSRII analytical flow cytometer.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Plant material and growth conditions. *Arabidopsis* plants used in this study were derived from the Columbia-0 (Col) and the Landsberg *erecta* (L*er*) accessions. Throughout this work the previously characterized *cdka;1-1* allele (SALK_106809.34.90.X) was used[23]. Seeds homozygous for the *mea* allele were obtained from A. Chaudhury and are in the L*er* background[30]. Lines KS117 and KS22 in $fie^{+/-}$, and the enhancer trap GUS line G222 in *mea*, *fis2-4* and *fie-11* were contributed by F. Berger[24]. For genotyping, seeds were germinated on soil or half-strength MS medium and grown under standard green house conditions or in a growth chamber. All genotypes were determined by PCR, by resistance to BASTA or by presence of YFP.

DNA and RNA work. All primers and probes are listed in Supplementary Table 2. For the rescue construct a region of 2,000 base pairs 5′ upstream of the *CDKA;1* start codon together with the *CDKA;1* cDNA fused to a yellow fluorescent protein (YFP) was used.

Total RNA was isolated from siliques using the RNeasy Plant Minikit (QIAGEN) and treated with DNase (TaKaRa) according to the manufacturer's protocol. The RNA concentration was measured twice using a Nanodrop ND-1000 instrument and 3.5 μg of total RNA was used to synthesize cDNA by reverse transcription using Superscript III (Invitrogen). After RNase H treatment at 37 °C for 20 min, a 1:1 dilution of the synthesized cDNA was used in quantitative real-time PCR (qPCR).

qPCR was performed on a Light-cycler LC480 instrument (Roche) according to the manufacturer's protocol. To ensure that the primer combinations did not produce any undesired PCR fragments or primer dimers, a SYBER-GREEN qPCR with melting point analysis was performed using the LightCycler 480 SYBR Green I Master Kit (Roche). Probe-based qPCR with these primers was performed using Universal Probe Library (UPL) hydrolysis probes (Roche) UPL probe no. 147, catalogue no. 04694333001 (*PHE1*) and UPL probe no. 102, catalogue no. 04692209001 (*ACTIN2*) and the LightCycler 480 Probes Master Kit (Roche). All samples and reference controls were performed in three independent biological replicates and repeated four times. The qPCR efficiency was determined independently in all replicates and duplicates by series of tenfold dilutions for each experiment. Calculation of relative expression ratios was performed according to a model previously described[31].

Histology. Pistils and siliques of different developmental stages were prepared as described previously[32]. Dissected siliques were fixed and mounted on microscope slides in a chloral hydrate clearing solution. Light microscopy was performed with a Zeiss Axiophot microscope using DIC optics.

Whole-mount GUS assays were performed as previously described[33] followed by chloral hydrate clearing as described above.

YFP fluorescence in seeds and pollen was analysed with a Leica TCS SP2 AOBS confocal laser scanning microscope. Mature pollen at the stage of anther dehiscence was stained with a 4,6-diamidino-2-phenylindole (DAPI) solution (2.5 μg ml^{-1} DAPI in 50 mM PBS, pH 7.2, with 0.01% Tween20 and 5% DMSO) for one hour.

For the analysis of endosperm cellularization, seeds were fixed and embedded in LR-White plastic resin after a modified protocol[34]; semi-thin sections (0.7 μm) of seeds were prepared with a Reichert Ultracut R microtome and mounted in Canada balsam.

Paternity test of embryo and endosperm. *Arabidopsis* L*er* plants and *mea* mutants (in a L*er* background) were pollinated with pollen from Col plants and *cdka;1* mutants (in a Col background). Six days after pollination, the seeds were fixed in ethanol:acetic acid (3:1) and embedded in Paraplast+ (Kendall) following a standard procedure for plant tissue preparation.

The embedded seeds were dissected using a Rotationsmikrotom 1512 microtome (Leitz). The sections (11 μm thick) were fixed on plastic-coated MembraneSlides (Palm) and stored at 4 °C. Before further handling, the embedding medium was removed using xylole. After rehydration in an ethanol series (100%; 96%; 70%; 50% ethanol in water; finally pure water for 2 min each), the slides were dried and used for laser dissection microscopy. For this, the seed sections were analysed and dissected with a MircoBeam laser dissection microscope (Palm) and fragments of embryos or endosperms of individual seeds were separately collected and stored at −20 °C. Subsequently, the samples were sonicated using an ultrasonic water bath (Bransonic 42 by Branson) for 6× 30 s and applied as template for an accession-specific PCR using a marker (NGA6) to detect simple sequence length polymorphisms between L*er* and Col. The PCR consisted of 55 cycles of product amplification and was performed with LA Taq polymerase (TaKaRa).

Flow cytometry for seed tissue ploidy analysis. For flow cytometry analysis, seeds were crushed with a pistil in 2 ml test tubes with nuclear extraction buffer (CyStain UV-precise kit by Partec). Seeds were identified by their smaller size. All preparations were subsequently filtered through a 30 μm nylon mesh and stained with nuclear staining solution (CyStain UV-precise kit by Partec) containing DAPI. Flow cytometry was performed on a three laser LSRII analytical flow cytometer (BD Biosciences) using the 405 nm solid state laser for excitation and a 440/40 band pass filter for recording of DAPI fluorescence. The ploidy level, represented by the mean peak position in a DAPI fluorescence intensity histogram, was calibrated against the 2*C* nuclear DNA content peak derived from a preparation of young rosette leaves. Doublets were excluded from the analysis by gating on single nuclei in a DAPI-width versus DAPI-area display according to ref. 35. Data were presented using Flowjo analysis software (Tree Star).

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