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2 **BREAKTHROUGH REPORT**

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4 **Endosperm development is an autonomously programmed process**
 5 **independent of embryogenesis**

6

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21

22 **Abstract**

23 The seeds of land plants contain three genetically distinct structures: the embryo,
 24 endosperm, and seed coat. The embryo and endosperm need to interact and exchange
 25 signals to ensure coordinated growth. Accumulating evidence has confirmed that
 26 embryo growth is supported by the nourishing endosperm and regulated by signals
 27 originating from the endosperm. Available data also support that endosperm
 28 development requires communication with the embryo. Here, using single-fertilization
 29 mutants, *Arabidopsis dmp8/9* and *gex2*, we demonstrate that in the absence of a zygote
 30 and embryo, endosperm initiation, syncytium formation, free nuclear cellularization,
 31 and endosperm degeneration are as normal as in the wild type in terms of the
 32 cytological process and time course. Although rapid embryo expansion accelerates
 33 endosperm breakdown, our findings strongly suggest that endosperm development is an
 34 autonomously organized process, independent of egg cell fertilization and embryo–
 35 endosperm communication. This work confirms both the altruistic and self-directed
 36 nature of the endosperm during coordinated embryo-endosperm development. The
 37 findings provide novel insights into the intricate interaction between the two
 38 fertilization products and will help to distinguish the real roles of the signaling between
 39 endosperm and embryo. These finding also shed new light on agro-biotechnology for
 40 crop improvement.

41

42

43 **Key words**

44 Endosperm, embryo, seed development, cell–cell communication, autonomous
 45 development

46 INTRODUCTION

47 Seed development in flowering plants is initiated by double fertilization, which
 48 leads to the formation of a diploid zygotic embryo and triploid endosperm. These two
 49 genetically distinct “siblings” then develop concomitantly within the surrounding
 50 maternal tissues, the seed coat, to form a seed (Lafon-Placette and Kohler, 2014). The
 51 endosperm plays an important role in supporting embryo growth by supplying nutrients
 52 and other factors during seed development and germination (Ingram, 2020; Li and
 53 Berger, 2012). Several endosperm-expressed genes, such as *EMBRYO SURROUNDING*
 54 *FACTOR 1 (ESF1)*, *ABNORMAL LEAF SHAPE1 (ALE1)*, and *ZHOUP1 (ZOU)* (Costa
 55 et al., 2014; Tanaka et al., 2001; Yang et al., 2008) have been reported to regulate
 56 embryo development. Endosperm cellularization also defines an important
 57 developmental transition for embryo development (Hehenberger et al., 2012). In
 58 mutants of *fertilization independent seed 2 (fis2)* and *endosperm defective 1 (ede1)* that
 59 fail to undergo endosperm cellularization, embryo development is arrested. A recent
 60 work first clearly describes a pathway for the communication between the endosperm
 61 and embryo, in which *TWISTED SEED1 (TWS1)* acts as a ligand of the receptor-like
 62 kinases GSO1 and GSO2 in the embryo and this sulfated peptide needs to be cleaved by
 63 ALE1 in the neighboring endosperm to release the active peptide, which then triggers
 64 GSO1/2-dependent cuticle reinforcement in the embryo (Doll et al., 2020). This
 65 strongly suggests that normal endosperm is essential for embryo development.
 66 Conversely, some embryo-derived factors have also been reported to regulate
 67 endosperm development, reflecting the impact of embryo development on endosperm
 68 (Aw et al., 2010; Nowack et al., 2006; Xu et al., 2015). Mutant analysis using defective
 69 kernel mutations in maize also provided examples showing that the normal embryo
 70 could enhance the mutant endosperm development (Neuffer and Sheridan, 1980). It seems
 71 gradually accepted that embryo and endosperm development depend on each other.
 72 Since embryogenesis and endosperm development are also involved in the seed coat

development, it is critical to further clarify the relationship among them and especially between the two fertilization products to recognize the roles of cell-cell communications in their coordinated development.

Two membrane proteins, GAMETE-EXPRESSED 2 (GEX2) (Mori et al., 2014) and DOMAIN OF UNKNOWN FUNCTION 679 membrane protein 8/9 (DMP8/9) (Cyprys et al., 2019; Takahashi et al., 2018), have been reported to be involved in double fertilization and loss of their functions caused single central cell fertilization, offering a unique opportunity to exclude an embryonic effect on endosperm development and to investigate the need of an embryo for endosperm development at every critical stage, as well as the seed coat development in an embryo-free seed.

RESULTS AND DISCUSSION

To address this, we first created *AtDMP8/9* CRISPR/Cas9 gene-edited double mutants, and found that these mutants showed serious defects in the seed set, similar to those previously reported (Figures 1A to 1F) (Cyprys et al., 2019). Obviously, knockout of *DMP8/9* could produce embryo-free seeds by single fertilization (Figures 1G to 1J). Thus, based on the mutants, we then characterized the main features of early endosperm development by clearing seeds at successive development stages when the embryo was not present (Figure 2). We found that when only the central cell was fertilized, the primary endosperm cell divided normally, indicating that the initiation of endosperm development does not require a message from the zygote and an unfertilized egg cell does not negatively affect endosperm development. After the first divisions of the primary endosperm nucleus, one nucleus migrated along the micropylar-chalazal axis to near the unfertilized egg cell, just like its counterpart in Col-0 ovules moves toward the zygote. After the third nuclear division, one or two nuclei were located at the chalazal pole of the embryo sac, leading to eight endosperm nuclei evenly distributed along a curved tube-like embryo sac. During the following cycles of syncytial division, the

larger nuclei were observed in the posterior endosperm pole, which is an early marker of the chalazal endosperm. Then, the syncytial endosperm continued to divide until the endosperm nuclei fully distributed in the periphery rejoin of the embryo sac. These results confirm that the embryo-free seeds undergo the same endosperm development pattern and follow the same time course as the wild type (WT) in the syncytial phase, in terms of endosperm initiation, nuclear migration, and free nucleus distribution (Figure 2; Supplemental Figure 1A). In addition, the Green fluorescent protein (GFP) reporters of four endosperm marker genes (Li et al., 2010; Portereiko et al., 2006; Steffen et al., 2007) were expressed in embryo-free seeds (Figures 3A to 3D) and the micropylar endosperm, which occupies a domain called the embryo-surrounding region (ESR), was also marked by the basic helix loop helix factor-*ZHOUP1* (*ZOU*) (Yang et al., 2008), and its expression pattern and levels in embryo-free seeds remained normal, as in the WT (Figures 3E and 3F). These observations suggest that the formation or presence of an embryo is not required for early syncytial endosperm development.

As in many angiosperms, *Arabidopsis thaliana* endosperm development consists of two main phases: an initial syncytial phase followed by a cellularized phase. In the embryo-free seeds, auto-fluorescence analysis revealed that the initiation and progression of endosperm cellularization occurred normally (Figures 4A and 4C; Supplemental Figure 1B). Endosperm cell walls were present in the embryo-free seeds, as in WT seeds at 6 days after pollination (DAP), indicating that the initiation and progress of endosperm cellularization are independent of embryo–endosperm communication, more like an autonomous developmental process. *AGL62*, a Type I MADS domain protein (Kang et al., 2007), functions as a major negative regulator of endosperm cellularization in *Arabidopsis* and is exclusively expressed during the syncytial phase and then declines abruptly just before cellularization (Figure 4B). Interestingly, when the embryo was absent, the expression of *AGL62-GFP* was identical to that in the WT (Figures 4B and 4D). GFP signals were detectable at 3 DAP, but not

127 at 5 DAP, indicating the disappearance of *AGL62* expression according to the normal
128 programmed time schedule, confirming normal endosperm cellularization in
129 embryo-free seeds.

130 In *Arabidopsis*, after cellularization, the endosperm eventually experiences cell
131 death and is gradually absorbed by the embryo, which lives on to form the plant of a
132 new sporophyte generation. Previous work using *dek1-3* and *atml1-3 pdf2-2* mutants
133 reported that endosperm breakdown requires embryo growth in which embryo
134 development arrests at the globular stage, and then the endosperm remains intact
135 (Fourquin et al., 2016). Here, in embryo-free seeds, which completely exclude the
136 influence of embryo growth and the risk of gene expression leakage, we found that the
137 endosperm cell wall was still present at 9 DAP, as previously reported, while in
138 phenotypically WT seeds, the endosperm had been eliminated (Supplemental Figure
139 2A). This means that rapid endosperm breakdown indeed involves embryo growth in
140 *Arabidopsis*. To understand whether the initiation of endosperm programmed cell death
141 (PCD) relies on embryo development due to spatial competition or embryo-derived
142 signals, we used terminal deoxynucleotidyl transferase dUTP nick end labeling
143 (TUNEL) to follow DNA degradation *in situ*, which highlights ongoing PCD in the
144 endosperm cells. In the WT, TUNEL signals began to appear in endosperm cells at 5
145 DAP and became widespread at 6 DAP (Figure 4E). Surprisingly, although the embryo
146 was absent, the endosperm showed the same TUNEL signal patterns, a sign of the
147 initiation of endosperm degeneration, suggesting the embryo-independent initiation of
148 DNA fragmentation. Although the mechanism underlying endosperm breakdown
149 remains unclear, *ZOU* was reported to be responsible for endosperm breakdown (Yang
150 et al., 2008) and triggered cell death by regulating the expression of cell-wall-modifying
151 enzymes (Fourquin et al., 2016). Studies have also demonstrated that different cases of
152 developmental PCD share a set of cell death-associated genes (Olvera-Carrillo et al.,
153 2015). Therefore, we characterized the expression of *ZOU* and six of these potential

154 PCD markers in the endosperm (Supplemental Figure 2B) during seed development
155 using qRT-PCR (Figure 4F). This showed that except for *CEP1*, the expression of *ZOU*
156 and the other five developmental PCD markers is not altered in embryo-free seeds
157 compared with the control, confirming the embryo-independent initiation of endosperm
158 breakdown.

159 Previous work has demonstrated that co-ordination between the endosperm and the
160 seed coat is necessary for successful seed development (Roszak and Kohler, 2011;
161 Garcia et al., 2003; Wang et al., 2010; Luo et al., 2005) and thus we also observed the
162 process of seed coat development in different fertilization types of seeds when
163 pollinated with *dmp8/9* pollen (Figure 5). In seeds containing only an embryo, seed coat
164 development was not initiated and the integument began to degenerate at 4DAP, similar
165 to that of unfertilized ovules (Figures 5C to 5E). This finding is consistent with
166 previously reported results that the embryo alone is not sufficient for the initiation of
167 seed coat growth (Roszak and Kohler, 2011). In *Arabidopsis*, upon double fertilization,
168 the surrounding integuments will undergo a process of growth and differentiation that
169 will lead to the formation of five cell-layered seed coat: two layers derived from the
170 outer integument and three layers derived from the inner integument. Obviously, the
171 seed coat in all embryo-free seeds was well initiated and normally developed. The seed
172 coat was composed of five layers, the same as that in WT seeds (Figures 5A, 5B, 5E
173 and 5F), suggesting that seed coat development mainly depends on endosperm
174 development and conversely confirming normal endosperm development when the
175 embryo is absent. In fact, we could not morphologically distinguish the embryo-free
176 seeds from WT seeds until 6 DAP. Later, the embryo-free seeds showed smaller in size
177 (Figures 5G; Supplemental Figures 3B and 3C), indicating that when endosperm PCD is
178 initiated, the embryo plays an increasingly important role in seed expansion and further
179 development, revealing an interesting coordination between seed coat and a late embryo
180 although the molecular signaling between them remains to be elucidated. In

181 *Arabidopsis*, seed coat growth is mostly driven by cell elongation (Garcia et al., 2005)
182 and this was also illustrated by our findings that the cell number of the outermost seed
183 coat layer is not changed but the average cell length is increased during the progress of
184 seed coat development, no matter in normal seeds or embryo-free seeds (Supplemental
185 Figure 3A).

186 In summary, our results strongly support the conclusion that the embryo is not
187 required for endosperm development, although embryo growth acts in rapid endosperm
188 elimination. In *Arabidopsis*, the endosperm is an ephemeral tissue that breaks down
189 almost completely to provide space for embryo expansion physically and to recycle the
190 nutrients stored in the endosperm tissues to fuel embryo growth. When an embryo is not
191 present, endosperm elimination seems meaningless. Surprisingly as we observed,
192 endosperm cells begin PCD as usual and ultimately break down almost completely at a
193 low speed (Figure 4E and Supplemental Figure 2C). Thus, endosperm development is
194 actually an autonomously programmed process, independent of embryo development.
195 This work provides direct evidence for an “altruistic” nature of the endosperm in the
196 relationship with its “sibling”—the zygotic embryo, and also a self-directed role in
197 embryo-endosperm coordinated development.

198 Recently, auxin has been reported to be a signal involved in the dialogue among
199 the endosperm, embryo, and seed coat. In *Arabidopsis*, auxin production after
200 fertilization in the central cell is sufficient to trigger endosperm proliferation
201 (Figueiredo et al., 2015; Batista et al., 2019). Intriguingly, auxin efflux from the
202 endosperm has been reported to drive seed coat development (Figueiredo et al., 2016).
203 Furthermore, auxin derived from the integument appears to be required for correct
204 embryo development (Robert et al., 2018). Although direct links between
205 endosperm-derived auxin and embryo development remain elusive, current knowledge
206 suggests a central controller role of the endosperm in seed development. Thus, it is not
207 surprising that the endosperm self-programs all its critical developmental processes and

208 promotes seed coat development. In addition, considering the origin of double
209 fertilization, as seen in *Ephedra*, sperm cells fuse with identical female nuclei to
210 produce an embryo and a supernumerary embryo (Friedman et al., 1998). Both embryos
211 develop independently and the development of the second fertilization product is
212 characterized by an initial period of free nuclear proliferation followed by a process of
213 cellularization, just similar to that of endosperm in flower plants. Thus, our finding
214 seems favor the homologous theory, which can well explain the independence of
215 endosperm development.
216

218 **METHODS**

219 **Plant materials and growth conditions**

220 *Arabidopsis thaliana* (accession Col-0) were grown in greenhouse under a photoperiod
221 of 16 h light and 8 h dark at 22°C. The T-DNA insertion lines *gex2-2* (FLAG_441D08)
222 (Mori et al., 2014) was obtained from the Nottingham Arabidopsis Stock Centre
223 (NASC). The background of all Arabidopsis marker lines was Col-0.

224

225 **Constructs and plant transformation**

226 For the construct of *ProZOU::H2B-GFP*, a 1.5kb promoter was amplified from
227 genomic DNA using the primer pair (*ZOU-H2B-S/A*) and cloned into destination of the
228 vector pART27 upstream of H2B-GFP after the digest of KpnI and AvrII. For the
229 double marker lines carrying *pDD45::GFP* and *pDD22::CFP*, the length of promoter
230 used in this study is according to the previous report (Steffen et al., 2007). For the
231 *DMP8/9* CRISPR-Cas9 vector, *DMP8* and *DMP9* were targeted by one sgRNA and
232 generated using a robust CRISPR/Cas9 vector system according to the reported methods
233 (Ma et al., 2015).

234 All constructs were verified by sequencing and subsequently transformed into
235 *Arabidopsis* (Col-0) by floral dip methods (Clough and Bent, 1998). Gene editing
236 events for *DMP8/9* were analyzed by amplifying the genomic region that flanks the
237 sgRNA target site by PCR, followed by sequencing. *dmp8/9-1* was used for subsequent
238 experiments.

239

240 **Cytological observation**

241 Ovule clearing was performed as previously reported (Boisnard-Lorig et al., 2001) and
242 ovule autofluorescence observation was used to analysis endosperm cellularization (Li
243 et al., 2017). As for TUNEL assays, a reported method was adapted using the

244 DeadEnd™ Fluorometric TUNEL System (Promega) (Wang et al., 2019). For
245 Propidium iodide-staining seeds, Schiff reagent with propidium iodide (P4170; Sigma)
246 was used like previously described (Shi et al., 2019) and finally the samples were
247 observed under a confocal microscope (Leica SP8 CLSM). The area and length was
248 calculated using the “measure”-tool with ImageJ.

249

250 **RT-qPCR**

251 At 6 DAP, the ovules which smaller than siblings side by side were dissected from
252 siliques when *dmp8/9-1* as a pollinator. Totally RNA of about 250 ovules a sample was
253 extracted using RNeasy Plant Mini Kit. After digestion with DNase I (Qiagen),
254 first-strand cDNA synthesis was performed using an M-MLV First-Strand Kit
255 (Invitrogen, Carlsbad, CA, USA). RT-qPCR analysis was conducted according to the
256 protocol previously described (Czechowski et al., 2005). The data were normalized to
257 three housekeeping genes (*AT4G0532*, *AT1G13320* and *AT4G34270*), and each
258 experiment was repeated three times.

259

260 **Accession Numbers**

261 The Arabidopsis Genome Initiative accession numbers for the genes and gene products
262 mentioned in this article are as follows: At1g09157 (*DMP8*), At5g39650 (*DMP9*),
263 AT5G49150 (*GEX2*), At2g06090 (*DD19*), At5g38330 (*DD22*), At5g48670 (*AGL80*),
264 AT5G60440 (*AGL62*), AT2G15890 (*AtCBP1*), AT1G49770 (*ZOU*), AT4G04460
265 (*PASPA3*), AT1G11190 (*BFN1*), AT4G18425 (*DMP4*), AT5G50260 (*CEP1*),
266 At1g26820 (*RNS3*) and At3g45010 (*SCPL48*).

267

268 **Supplemental Data**

269 **Supplemental Figure 1.** Phenotype analysis of endosperm in Col-0 and embryo-free
270 seeds

271 **Supplemental Figure 2.** Embryo growth accelerates endosperm breakdown

272 **Supplemental Figure 3.** Integument cell elongation is responsible for seed coat growth
273 and embryo-free seeds show smaller sizes at 6 DAP

274 **Supplemental Table 1.** Primers used in this study

275

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278 and *AGL80-GFP*) and Wei-Cai Yang for the *CBP1-3xGFP* maker line. We thank Y.G.
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282 **AUTHOR CONTRIBUTIONS**

283 H.X., W.W., and MX.S. designed the experiments. HX.X. and W.W. performed the
284 experiments. W.W, H.X. and MX.S. analyzed the data and wrote the manuscript. All
285 authors approved the final version of the manuscript.

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287

288

Figure legends

Figure 1. Knock-out of *DMP8* and *DMP9* causes single-fertilization.

(A) Targeted mutagenesis in *Arabidopsis* using the CRISPR/Cas9 system by one target site. The black boxes showed the target site and disrupted *DMP8* and *DMP9* coding sequences just after ATG in three different homozygous mutants.

(B-E) Unfertilized ovules (arrowheads) and aborted seeds (arrows) were frequently observed in three *dmp8/9* double mutants generated by CRISPR/Cas9. The seed set rates (means ± SD) were shown at the top right. n=568, 723, 539 and 467 seeds, respectively. Scale bar represents 0.5mm.

299 **(F)** Statistics of various types of seed abortion in different crossing groups for Col-0 and
 300 *dmp8/9-1*. For each crossing group, from left to right, n=631, 551, 610 and 627 seeds,
 301 respectively. Data are the means $\pm$ SD.

302 **(G-J)** DMP8/9 are required for fertilization. The phenotype of non-fertilization **(H)** or
 303 single-fertilization **(I and J)** were observed in *dmp8/9-1* seeds (n=391). The data (means
 304 $\pm$ SD) were shown at the top right. Scale bars=20 μ m. Abbreviations: em, embryo; ec,
 305 the egg cell; ccn, the central cell nuclei.

306

Figure 2. Early endosperm development does not depend on the presence of an embryo. Cleared seeds before 4 DAP. The ovules were pollinated with Col-0, *dmp8/9*, or *gex2* pollen. Note that when only the central cell was fertilized, endosperm development initiates and proceeds normally, just as in Col-0 seeds. The insets in the lower right indicate egg cell nuclei (arrows), zygotes or embryos (arrowheads). Hollow arrowheads indicate larger nuclei in the chalazal endosperm. Scale bars, 20 μ m.

Figure 3. Embryo-free seeds express different endosperm-cell fate markers.

(A–F) Confocal laser scanning microscopy (CLSM) images of seeds expressing different endosperm reporters: *pDD22::CFP* in (A), *pDD19::GFP* in (B), *pAGL80::AGL80-GFP* in (C), *pCBP1::CBP1-3xGFP* in (D), and *pZOU::H2B-GFP* in (E) and (F). The insets in the lower right indicate the egg cell (arrows), zygotes or embryos (arrowheads). Note that at 3 DAP, the *pZOU::H2B-GFP* reporter expression is largely confined to the embryo-surrounding region (ESR) in both normal and embryo-free seeds. Scale bars, 20 μm.

Figure 4. Endosperm cellularization is a cell-autonomous process and the embryo is not required for the initiation of endosperm cell PCD.

328 **(A)** Autofluorescence of seeds seen by confocal microscopy after pollination with Col-0
329 or *dmp8/9* pollen. The insets in the lower left indicate the egg cell.

330 **(B)** Seeds expressing *pAGL62::AGL62-GFP* after pollination with Col-0 or *dmp8/9*
331 pollen. Note that *AGL62*, which suppresses cellularization, is expressed during syncytial
332 endosperm development and becomes undetectable before cellularization in both
333 normal and embryo-free seeds. The egg cell and embryo are marked by *pDD45::GFP*.

334 **(C)** Autofluorescence-analysis of endosperm cellularization in embryo-free seeds
335 pollinated with *gex2* pollen. The insets in the lower left indicate the egg cell.

336 **(D)** Embryo-free seeds pollinated with *gex2* pollen show a *pAGL62::AGL62-GFP*
337 expression pattern similar to that of Col-0 seeds. The egg cell is marked with
338 *pDD45::GFP*.

339 **(E)** TUNEL signals in seeds pollinated with Col-0, *dmp8/9*, or *gex2* pollen. Propidium
340 iodide staining was used to stain cells and the TUNEL-positive signal is indicated by
341 asterisks, and shown by the yellow fluorescence (green + red). Note that PCD signals
342 begin to appear at 5 DAP in embryo-free seeds, similar to the control. Arrows indicate
343 the embryo; arrowheads indicate the egg cell. Scale bars, 20 μ m in **(A–E)**.

344 **(F)** qRT-PCR analysis of *ZOU* and developmental PCD markers in embryo-free seeds
345 compared with the WT (Col-0) at 6 DAP. Data are the means $\pm$ SD of three biological
346 replicates. Significant differences ($*P < 0.05$, two-sided Student's *t*-test) are indicated.

347

348

349 **Figure 5.** Seed coat development in embryo-free seeds.

350 (A) Propidium iodide-stained seeds in WT at 2, 4 and 6 DAP.

351 (B-D) Propidium iodide-stained seeds after pollination with *dmp8/9* pollen at 2, 4 and 6

352 DAP. Please note that the seed coat development in embryo-free (em-free) seeds (B)

353 appeared to be similar to normal seeds (A). Meanwhile seed coat development in seeds

354 containing no endosperm (en-free) was not initiated and the integuments collapsed as

355 that in unfertilized ovules (un-fer) at 4 DAP (C-D). Arrows indicate the embryo and

356 arrowheads indicate the egg cell. Scale bars, 20 μm.

357 **(E-G)** The distribution of seed coat and embryo sac sizes in seeds after pollination with
358 Col-0 or *dmp8/9* pollen at 2, 4 and 6 DAP, respectively.
359

Supplemental Data

Supplemental Figure 1. Phenotype analysis of endosperm in Col-0 and embryo-free seeds.

(A) Distribution of endosperm nuclei number in Col-0 and embryo-free seeds shown in Figure 3A. At 1 DAP, embryo-free seeds pollinated with *dmp8/9* pollen do not have defects in endosperm proliferation. (B) Statistics of endosperm cellularization in seeds at 4-6 DAP.

Supplemental Figure 2. Embryo growth accelerates endosperm breakdown.

(A) Toluidine Blue-stained paraffin sections showing endosperm structure at 9 DAP.

Please note that in wild-type seeds, the endosperm had been eliminated. However, in embryo-free seeds, the endosperm was intact. Scale bars, 20 μ m.

(B) Expression data for *BFN1*, *CEP1*, *DMP4*, *PASPA3*, *RNS3* and *SCPL48* downloaded from the Seed Gene Network resource (<http://seedgenenetwork.net/>).

(C) Dry seeds of Col-0 and embryo-free seeds in *dmp8/9*. Scale bars, 0.5 mm.

Supplemental Figure 3. Integument cell elongation is responsible for seed coat growth and embryo-free seeds show smaller sizes at 6 DAP.

(A) Quantification of cell number and length in the outermost seed coat layer shown in Figure 5. **(B)** Cleared *dmp8/9* ovules, 6 days after pollination. Developing embryo and endosperm (left); endosperm but no embryo (right). Scale bars = 50 μm. **(C)** Quantification of seed sizes shown in **(B)**, indicating that the embryo-free seeds are smaller. Data are the means ± SD, with n= 38, 48 seeds from left to right. Significant differences (**** P < 0.0001, two-sided Student's t-test) are indicated. Abbreviations: un-fer: un-fertilized; en-free: endosperm-free; em-free: embryo-free; em: embryo; ec: egg cell; enn: endosperm nuclei; cze: the chalazal endosperm.

390 **Supplemental Table 1. Primers used in this study**

Name	Forward (5'-3')	use
<i>ZOU-H2B-S</i>	NNNNGGTACCTGTGGTGGCATAATACGAAAATC	vector cons.
<i>ZOU-H2B-A</i>	NNNNCCTAGGATTGAATTGAATGCTCATTTTAC	vector cons.
<i>CRI-S-1</i>	ATTGAGAAAACAGAGGAAAGCGT	CRISPR-Cas9
<i>CRI-A-1</i>	AAACACGCTTTCCTCTGTTTCT	CRISPR-Cas9
<i>PASPA3-RT-S</i>	GGACGGGTGCTATTTCTGGT	RT-PCR
<i>PASPA3-RT-A</i>	GGATCTTTCGGGTACGGTTAA	RT-PCR
<i>BFN1-RT-S</i>	GGGGATACAAAGGCGTCAAG	RT-PCR
<i>BFN1-RT-A</i>	TGGCAGCAACACCAGCAA	RT-PCR
<i>DMP4-RT-S</i>	CGTTATTCGTGTTTGGTGCG	RT-PCR
<i>DMP4-RT-A</i>	TGCTTCTGCTGACGGTGATG	RT-PCR
<i>CEP1-RT-S</i>	GAAGCGATTTATTGTTCTTGCG	RT-PCR
<i>CEP1-RT-A</i>	CACCGTTCGTATAGCTCCAC	RT-PCR
<i>RNS3-RT-S</i>	CAATGATGGTATGAAGTTTGGACA	RT-PCR
<i>RNS3-RT-A</i>	TTGGTAAGGGCATGAAGGAGA	RT-PCR
<i>SCPL48-RT-S</i>	CGGTGCGGAAGGCATTAG	RT-PCR
<i>SCPL48-RT-A</i>	CCATTTCGTGAACCCACTTTG	RT-PCR
<i>ZOU-RT-S</i>	CATCATCATCCTCTTCTCCAACA	RT-PCR
<i>ZOU-RT-A</i>	TCCCACAGATAGTCAGCACCAC	RT-PCR
<i>AGL62-RT-S</i>	TCATAAACAATAACCCTCTACCTCCT	RT-PCR
<i>AGL62-RT-A</i>	AGATAACGCAAGTTCCTCAACG	RT-PCR
<i>SUC5-RT-S</i>	TTACCACAGTGACCGATGCG	RT-PCR
<i>SUC5-RT-A</i>	GAAAACCGAGACGGCTACGA	RT-PCR
<i>DD36-RT-S</i>	TTTTGGTCATCACATCTAATCTTGG	RT-PCR

<i>DD36-RT-A</i>	AGAATCCTCCCCTGCGACA	RT-PCR
<i>IKU1-RT-S</i>	ATGTTTCAGTCAGATGTATGGTGGAT	RT-PCR
<i>IKU1-RT-A</i>	ATGAGAAAGTTTGGGGTAAGTGGT	RT-PCR
<i>BBM-RT-S</i>	GTAACAAAGACCTCTACTTGGGAACT	RT-PCR
<i>BBM-RT-A</i>	CAGTGTTTTGCCAACCGCTA	RT-PCR
<i>AT4G05320-S</i>	GGCCTTGTATAATCCCTGATGAATAAG	RT-PCR
<i>AT4G05320-A</i>	AAAGAGATAACAGGAACGGAAACATAGT	RT-PCR
<i>AT1G13320-S</i>	TAACGTGGCCAAAATGATGC	RT-PCR
<i>AT1G13320-A</i>	GTTCTCCACAACCGCTTGGT	RT-PCR
<i>AT4G34270-S</i>	GTGAAAACGTGTTGGAGAGAAGCAA	RT-PCR
<i>AT4G34270-A</i>	TCAACTGGATACCCTTTCGCA	RT-PCR

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Parsed Citations

Aw, S.J., Hamamura, Y., Chen, Z., Schnittger, A., and Berger, F. (2010). Sperm entry is sufficient to trigger division of the central cell but the paternal genome is required for endosperm development in Arabidopsis. Development 137: 2683-2690.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Batista, R.A., Figueiredo, D.D., Santos-Gonzalez, J., and Kohler, C. (2019). Auxin regulates endosperm cellularization in Arabidopsis. Genes Dev. 33: 466-476.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, W., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J., and Berger, F. (2001). Dynamic analyses of the expression of the HISTONE :: YFP fusion protein in Arabidopsis show that syncytial endosperm is divided in mitotic domains. Plant cell 13: 495-509.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-743.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Costa, L.M., Marshall, E., Tesfaye, M., Silverstein, K.A., Mori, M., Umetsu, Y., Otterbach, S.L., Papareddy, R., Dickinson, H.G., Boutiller, K., VandenBosch, K.A., Ohki, S., and Gutierrez-Marcos, J.F. (2014). Central cell-derived peptides regulate early embryo patterning in flowering plants. Science 344: 168-172.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Cyprys, P., Lindemeier, M., and Sprunck, S. (2019). Gamete fusion is facilitated by two sperm cell-expressed DUF679 membrane proteins. Nat plants 5: 253-257.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant physiol. 139: 5-17.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Doll, N.M., Royek, S., Fujita, S., Okuda, S., Chamot, S., Stintzi, A., Widiez, T., Hothorn, M., Schaller, A., Geldner, N., and Ingram, G. (2020). A two-way molecular dialogue between embryo and endosperm is required for seed development. Science 367: 431-435.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Figueiredo, D.D., Batista, R.A., Roszak, P.J., and Kohler, C. (2015). Auxin production couples endosperm development to fertilization. Nat plants 1: 15184.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Figueiredo, D.D., Batista, R.A., Roszak, P.J., Hennig, L., and Kohler, C. (2016). Auxin production in the endosperm drives seed coat development in Arabidopsis. eLife 5.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fourquin, C., Beauzamy, L., Chamot, S., Creff, A., Goodrich, J., Boudaoud, A., and Ingram, G. (2016). Mechanical stress mediated by both endosperm softening and embryo growth underlies endosperm elimination in Arabidopsis seeds. Development 143: 3300-3305.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Friedman, W.E. (1992). Evidence of a pre-angiosperm origin of endosperm: implications for the evolution of flowering plants. Science 255: 336-339.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Garcia, D., Fitz Gerald, J.N., and Berger, F. (2005). Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in Arabidopsis. Plant cell 17: 52-60.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Garcia, D., Saingery, V., Chambrier, P., Mayer, U., Jurgens, G., and Berger, F. (2003). Arabidopsis haiku mutants reveal new controls of seed size by endosperm. Plant physiol. 131: 1661-1670.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Hehenberger, E., Kradolfer, D., and Kohler, C. (2012). Endosperm cellularization defines an important developmental transition for embryo development. Development 139: 2031-2039.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Ingram, G.C. (2020). Family plot: the impact of the endosperm and other extra-embryonic seed tissues on angiosperm zygotic embryogenesis. F1000Res. 9.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Kang, I.H., Steffen, J.G., Portereiko, M.F., Lloyd, A., and Drews, G.N. (2008). The AGL62 MADS domain protein regulates cellularization during endosperm development in Arabidopsis. Plant cell 20: 635-647.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Lafon-Placette, C., and Kohler, C. (2014). Embryo and endosperm, partners in seed development. Curr Opin Plant Biol. 17: 64-69.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Li, G., Zou, W.X., Jian, L.F., Qian, J., Deng, Y.T., and Zhao, J. (2017). Non-SMC elements 1 and 3 are required for early embryo and seedling development in Arabidopsis. J Exp Bot. 68: 1039-1054.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Li, H.J., Zhu, S.S., Zhang, M.X., Wang, T., Liang, L., Xue, Y., Shi, D.Q., Liu, J., and Yang, W.C. (2015). Arabidopsis CBP1 Is a Novel Regulator of Transcription Initiation in Central Cell-Mediated Pollen Tube Guidance. Plant cell 27: 2880-2893.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Li, J., and Berger, F. (2012). Endosperm: food for humankind and fodder for scientific discoveries. The New Phytol. 195: 290-305.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Luo, M., Dennis, E.S., Berger, F., Peacock, W.J., and Chaudhury, A. (2005). MINISEED3 (MINI3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis. Proc Natl Acad Sci U.S.A. 102: 17531-17536.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., Lin, Y., Xie, Y., Shen, R., Chen, S., Wang, Z., Chen, Y., Guo, J., Chen, L., Zhao, X., Dong, Z., and Liu, Y.G. (2015). A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. Mol Plant 8: 1274-1284.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Mori, T., Igawa, T., Tamiya, G., Miyagishima, S.Y., and Berger, F. (2014). Gamete attachment requires GEX2 for successful fertilization in Arabidopsis. Curr Biol. 24: 170-175.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Neuffer, M.G., and Sheridan, W.F. (1980). Defective kernel mutants of maize. I. Genetic and lethality studies. Genetics 95: 929-944.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Nowack, M.K., Grini, P.E., Jakoby, M.J., Lafos, M., Koncz, C., and Schnittger, A. (2006). A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. Nat Genet. 38: 63-67.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Olvera-Carrillo, Y., Van Bel, M., Van Hautegeem, T., Fendrych, M., Huysmans, M., Simaskova, M., van Durme, M., Buscaill, P., Rivas, S., Coll, N.S., Coppens, F., Maere, S., and Nowack, M.K. (2015). A Conserved Core of Programmed Cell Death Indicator Genes Discriminates Developmentally and Environmentally Induced Programmed Cell Death in Plants. Plant Physiol. 169: 2684-2699.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Portereiko, M.F., Lloyd, A., Steffen, J.G., Punwani, J.A., Otsuga, D., and Drews, G.N. (2006). AGL80 is required for central cell and endosperm development in Arabidopsis. Plant cell 18: 1862-1872.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Robert, H.S., Park, C., Gutierrez, C.L., Wojcikowska, B., Pencik, A., Novak, O., Chen, J.Y., Grunewald, W., Dresselhaus, T., Friml, J., and Laux, T. (2018). Maternal auxin supply contributes to early embryo patterning in Arabidopsis. Nat Plants 4: 548-553.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Roszak, P., and Kohler, C. (2011). Polycomb group proteins are required to couple seed coat initiation to fertilization. Proc Natl Acad

Sci U.S.A. 108: 20826-20831.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Shi, C., Luo, P., Du, Y.T., Chen, H., Huang, X., Cheng, T.H., Luo, A., Li, H.J., Yang, W.C., Zhao, P., and Sun, M.X. (2019). Maternal control of suspensor programmed cell death via gibberellin signaling. Nat Commun. 10: 3484.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Steffen, J.G., Kang, I.H., Macfarlane, J., and Drews, G.N. (2007). Identification of genes expressed in the Arabidopsis female gametophyte. Plant J 51: 281-292.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Takahashi, T., Mori, T., Ueda, K., Yamada, L., Nagahara, S., Higashiyama, T., Sawada, H., and Igawa, T. (2018). The male gamete membrane protein DMP9/DAU2 is required for double fertilization in flowering plants. Development 145.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Tanaka, H., Onouchi, H., Kondo, M., Hara-Nishimura, I., Nishimura, M., Machida, C., and Machida, Y. (2001). A subtilisin-like serine protease is required for epidermal surface formation in Arabidopsis embryos and juvenile plants. Development 128: 4681-4689.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Wang, A., Garcia, D., Zhang, H., Feng, K., Chaudhury, A., Berger, F., Peacock, W.J., Dennis, E.S., and Luo, M. (2010). The VQ motif protein IKU1 regulates endosperm growth and seed size in Arabidopsis. Plant J 63: 670-679.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Wang, W., Xiong, H., Lin, R., Zhao, N., Zhao, P., and Sun, M.X. (2019). A VPE-like protease NtTPE8 exclusively expresses in the integumentary tapetum and is involved in seed development. J Integr Plant Biol. 61: 598-610.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Xu, T.T., Ren, S.C., Song, X.F., and Liu, C.M. (2015). CLE19 expressed in the embryo regulates both cotyledon establishment and endosperm development in Arabidopsis. J Exp Bot. 66: 5217-5227.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Yang, S., Johnston, N., Talideh, E., Mitchell, S., Jeffree, C., Goodrich, J., and Ingram, G. (2008). The endosperm-specific ZHOUP1 gene of Arabidopsis thaliana regulates endosperm breakdown and embryonic epidermal development. Development 135: 3501-3509.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)