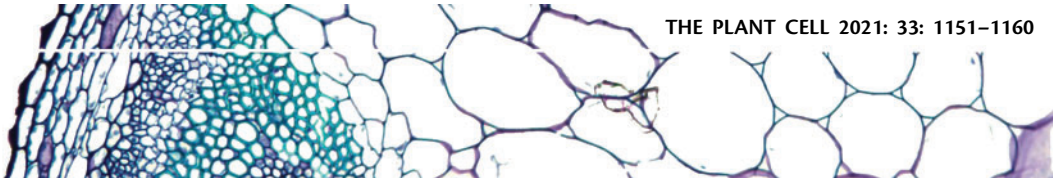
doi:10.1093/plcell/koab007



THE PLANT CELL 2021: 33: 1151–1160

Endosperm development is an autonomously programmed process independent of embryogenesis

Hanxian Xiong [1,†] Wei Wang [1,†] and Meng-Xiang Sun [1,*]

1 State Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Wuhan 430072, China

*Author for correspondence: mxsun@whu.edu.cn
†These authors contributed equally.

H.X., W.W., and M.-X.S. designed the experiments, analyzed the data, and wrote the manuscript. H.-X.S. and W.W. performed the experiments. All authors approved the final version of the manuscript.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plcell) is: Meng-Xiang Sun (mxsun@whu.edu.cn).

Abstract

The seeds of flowering plants contain three genetically distinct structures: the embryo, endosperm, and seed coat. The embryo and endosperm need to interact and exchange signals to ensure coordinated growth. Accumulating evidence has confirmed that embryo growth is supported by the nourishing endosperm and regulated by signals originating from the endosperm. Available data also support that endosperm development requires communication with the embryo. Here, using single-fertilization mutants, *Arabidopsis thaliana dmp8 dmp9* and *gex2*, we demonstrate that in the absence of a zygote and embryo, endosperm initiation, syncytium formation, free nuclear cellularization, and endosperm degeneration occur as in the wild type in terms of the cytological process and time course. Although rapid embryo expansion accelerates endosperm breakdown, our findings strongly suggest that endosperm development is an autonomously organized process, independent of egg cell fertilization and embryo–endosperm communication. This work confirms both the altruistic and self-directed nature of the endosperm during coordinated embryo–endosperm development. Our findings provide insights into the intricate interaction between the two fertilization products and will help to distinguish the physiological roles of the signaling between endosperm and embryo. These findings also open new avenues in agro-biotechnology for crop improvement.

Introduction

Seed development in flowering plants is initiated by double fertilization, which leads to the formation of a diploid zygotic embryo and triploid endosperm. These two genetically distinct "siblings" then develop concomitantly within the surrounding maternal tissues—the seed coat—to form a seed (Lafon-Placette and Kohler, 2014). The endosperm plays an important role in supporting embryo growth by supplying nutrients and other factors during seed development and germination (Li and Berger, 2012; Ingram, 2020). Several endosperm-expressed genes, such as *EMBRYO SURROUNDING FACTOR 1* (*ESF1*), *ABNORMAL LEAF SHAPE1* (*ALE1*), and *ZHOUPI* (*ZOU*; Tanaka et al., 2001; Yang et al., 2008; Costa et al., 2014), regulate embryo development. Endosperm cellularization also defines an important developmental transition for embryo development (Hehenberger et al., 2012). In mutants of *fertilization independent seed 2* (*fis2*) and *endosperm defective 1* (*ede1*) that fail to undergo endosperm cellularization, embryo development is arrested (Chaudhury et al., 1997; Pignocchi et al., 2009; Hehenberger et al., 2012). Recent work clearly describes a pathway for communication between the endosperm and embryo, in

Received August 31, 2020. Accepted January 4, 2021. Advance access publication January 25, 2021

which *TWISTED SEED1* (*TWS1*) acts as a ligand of the receptor-like kinases GSO1 and GSO2 in the embryo and this sulfated peptide needs to be cleaved by ALE1 in the neighboring endosperm to release the active peptide, which then triggers GSO1/GSO2-dependent cuticle reinforcement in the embryo (Doll et al., 2020). This signaling pathway strongly suggests that normal endosperm is essential for embryo development. Conversely, some embryo-derived factors have also been reported to regulate endosperm development, reflecting the influence of embryo development on the endosperm (Xu et al., 2015). Mutant analysis using *defective kernel* mutations in maize have also provided examples showing that the normal embryo could enhance the mutant endosperm development (Neuffer and Sheridan, 1980). Based on this information, it has gradually been accepted that embryo and endosperm development depend on each other. However, some other mutants with embryos arrested at the globular stage display a nearly normal process of endosperm proliferation and cellularization (Johnson et al., 2005; Lid et al., 2005; Garcion et al., 2006; San-Bento et al., 2014). Notably, endosperm final breakdown in these mutants requires embryo growth (Fourquin et al., 2016). Thus, it is not yet clear whether embryogenesis is essential for endosperm development. Given that embryogenesis and endosperm development are also involved in seed coat development, it is critical to further clarify the relationship among them and especially between the two fertilization products to assess the roles of cell–cell communication in their coordinated development.

Three membrane proteins, GAMETE-EXPRESSED 2 (GEX2; Mori et al., 2014), DOMAIN OF UNKNOWN FUNCTION 679 membrane protein 8 (DMP8), and DMP9 (Takahashi et al., 2018; Cyprys et al., 2019), have been reported to be involved in double fertilization, and loss of their functions causes 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. Thus, by creating single-fertilization mutants we finally demonstrated that endosperm development is an autonomously programmed process independent of embryogenesis.

Results

Confirmation of central cell single fertilization in *dmp8 dmp9*

To understand the possible influence of embryogenesis on endosperm development, we created *AtDMP8* and *DMP9* CRISPR/Cas9 gene-edited double mutants (named as *dmp8 dmp9*). These mutants showed serious defects in the seed set, similar to those previously reported (Figure 1, A–F; Cyprys et al., 2019). In selfed *dmp8 dmp9* mutant plants, seeds aborted at a high frequency (about 57%). The reciprocal crosses between wild type (WT) and *dmp8 dmp9-1* revealed that fertility was affected in the male, but not the female. Then, we examined the development of the seeds in self-fertilized *dmp8 dmp9* siliques and observed that 36.8% seeds showed normal embryo and endosperm development, 28.7% seeds showed no embryo and endosperm, 10.4% showed no endosperm and 24.1% showed no embryo (Figure 1, G–J). These data confirm previously reported defects in seed set and the proposed roles of DMP8 and DMP9 in fertilization (Takahashi et al., 2018; Cyprys et al., 2019). To further explore whether single fertilization in the central cell by *dmp8 dmp9* sperm cell could occur, a double-marker line (*pDD45::GFP/pDD22::CFP*) was pollinated with *dmp8 dmp9-1* pollen (Figure 1, K–O). The seeds at 30 h after pollination were observed and the results showed that the single fertilization of central cell occurred at a frequency of 20.5%, which matches the central cell single-fertilization frequency (24.5%) in *dmp8,9*^{C} knock-out lines (Cyprys et al., 2019) and is slightly more than 17.6% in *dmp9* knock-down lines (Takahashi et al., 2018).

Early endosperm development does not depend on zygote development and embryogenesis initiation

Since *dmp8 dmp9* and *gex2* could produce embryo-free seeds by single fertilization (Mori et al., 2014), we used these two mutants to characterize the main features of early endosperm development by clearing seeds at successive development stages when the embryo was not present (Figure 2). We observed 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 seeds 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 at the chalazal 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 region 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 WT in the syncytial phase, in terms of endosperm initiation, nuclear migration, and free nucleus distribution (Figure 2 and Supplemental Figure S1, A). In addition, the green fluorescent protein (GFP) reporters of four endosperm marker genes (Portereiko et al., 2006; Steffen et al., 2007; Li et al., 2015) were expressed in embryo-free seeds (Figure 3, A–D). The micropylar endosperm, which occupies a domain called the embryo-surrounding region (ESR), was also marked by the basic helix loop helix factor *ZHOUPI* (*ZOU*; Yang et al., 2008), and its expression pattern and levels in embryo-free seeds remained similar to those in the WT (Figure 3, E and F). These observations suggest that

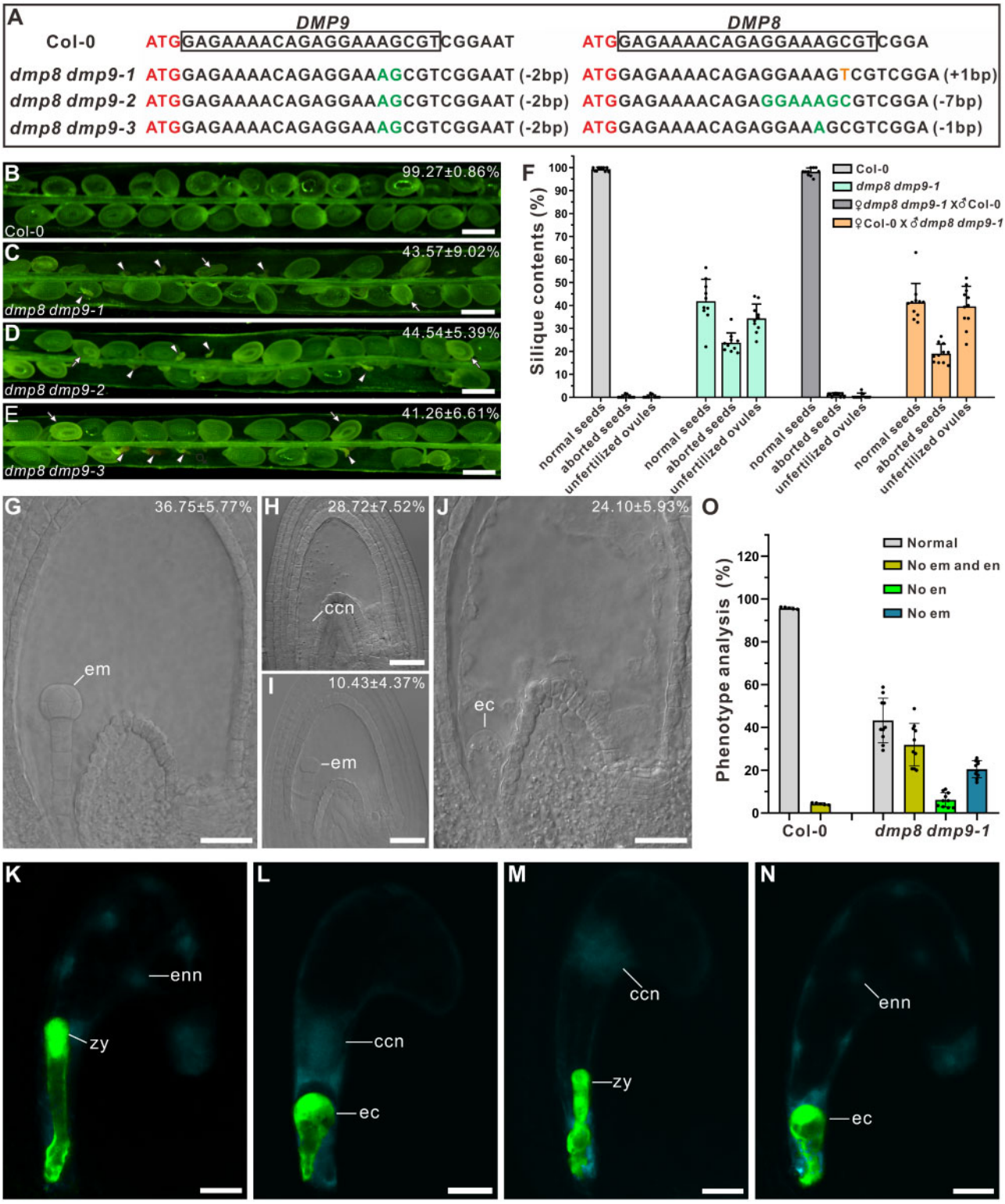


Figure 1 Knock-out of *DMP8* and *DMP9* causes single fertilization. A, Targeted mutagenesis in *Arabidopsis* using the CRISPR/Cas9 system. The gRNA target sites are indicated by the black boxes. *DMP8* and *DMP9* coding sequences are disrupted just after ATG in three different homozygous mutants. B–E, Unfertilized ovules (arrowheads) and aborted seeds (arrows) were frequently observed at 9–11 DAP in three *dmp8 dmp9* 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 0.5 mm. F, Statistics of various types of seed abortion in different crossing groups for Col-0 and *dmp8 dmp9-1*. For each crossing group, from left to right, n = 631, 551, 610, and 627 seeds, respectively. Data are the means ± SD. G–J, DMP8 and DMP9 are required for fertilization. The phenotype of non-fertilization (H) or single fertilization (I and J) were observed in *dmp8 dmp9-1* cleared seeds (n = 391) at 3 DAP. The data (means ± SD) were shown at the top right. K–N, Ovules or seeds co-expressing *pDD45::GFP* and *pDD22::CFP* at 30 h after pollination with *dmp8 dmp9-1* pollen. K, Developing zygote and endosperm. L, Unfertilized egg cell and central cell. M, Zygote but no endosperm. N, Endosperm but no zygote. O, Frequencies of phenotypes (means ± SD) shown in K–N. Col-0, n = 236 seeds; *dmp8 dmp9-1*, n = 357 seeds. Scale bars = 20 μm. ccn, the central cell nucleus; ec, the egg cell; em, embryo; en, endosperm; enn, endosperm nucleus; and zy, zygote.

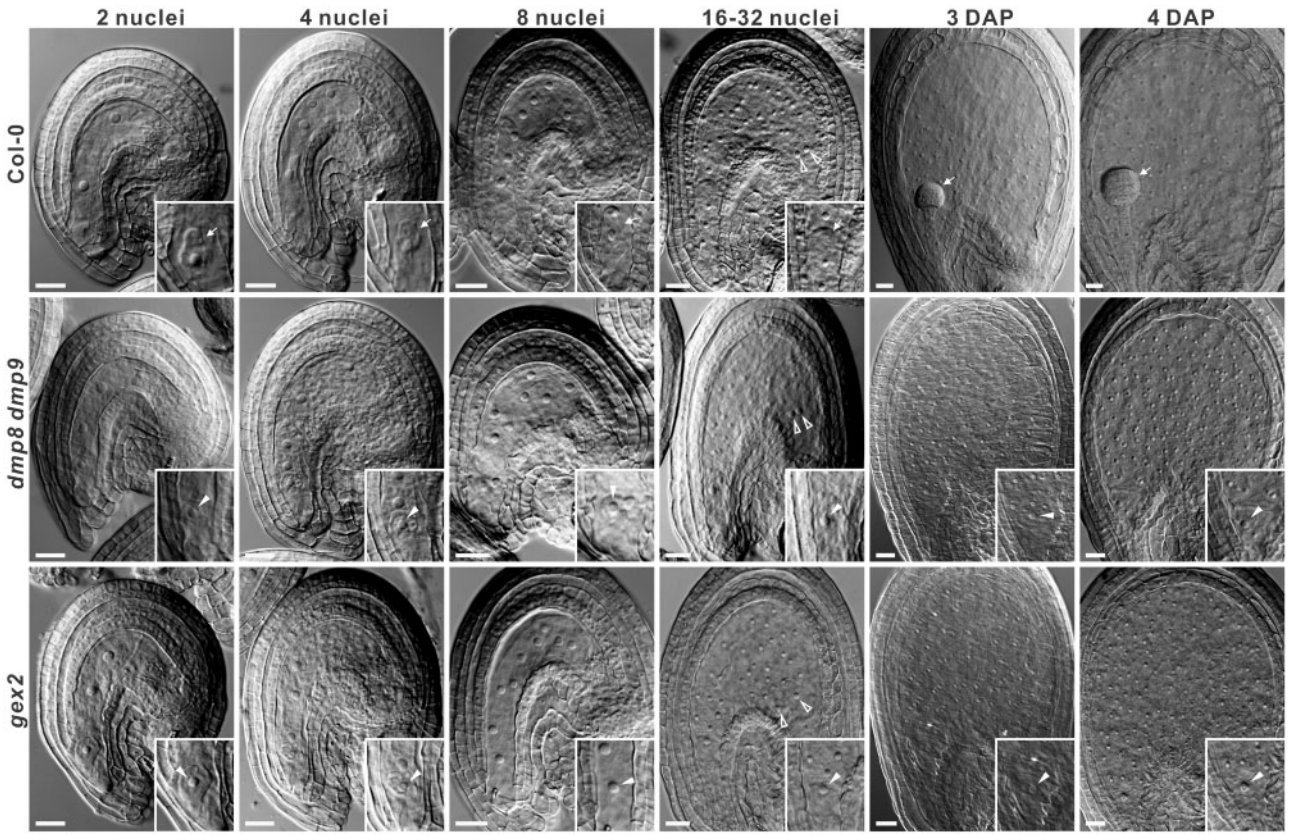


Figure 2 Early endosperm development does not depend on the presence of an embryo. Cleared seeds before 4 DAP. The ovules were pollinated with Col-0, *dmp8 dmp9*, or *gex2* pollen. Note that when only the central cell is fertilized, endosperm development initiates and proceeds normally, just as in Col-0 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 μm.

the formation or presence of an embryo is not required for early syncytial endosperm development.

Endosperm cellularization is self-programmed

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 (Figure 4, A and C and Supplemental Figure S1, B). 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., 2008), 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 4, B). Interestingly, when the embryo was absent, the expression of *AGL62-GFP* was identical to that in the WT (Figure 4, B and D). GFP signals were detectable at 3 DAP, but not at 5 DAP, indicating the disappearance of *AGL62* expression according to the normal programmed time schedule, confirming normal endosperm cellularization in embryo-free seeds.

Initiation of endosperm degeneration does not require messages from the embryo

In *Arabidopsis*, after cellularization, the endosperm eventually experiences cell death and is gradually absorbed by the embryo, which lives on to form the plant of a new sporophyte generation. Previous work using *dek1-3* (Johnson et al., 2005; Lid et al., 2005) and *atml1-3 pdf2-2* mutants (San-Bento et al., 2014) reported that endosperm breakdown requires embryo growth in which embryo development arrests at the globular stage, and then the endosperm remains intact (Fourquin et al., 2016). Here, in embryo-free seeds, which completely exclude the influence of embryo growth and the risk of gene expression leakage, we observed that the endosperm cell wall was still present at 9 DAP as previously reported, while in phenotypically WT seeds, the endosperm had been almost eliminated (Supplemental Figure S2, A). This means that rapid endosperm breakdown indeed involves embryo growth in *Arabidopsis*. To understand whether the initiation of endosperm programmed cell death (PCD) relies on embryo development due to spatial competition or embryo-derived signals, we used terminal deoxynucleotidyl transferase dUTP nick end labeling

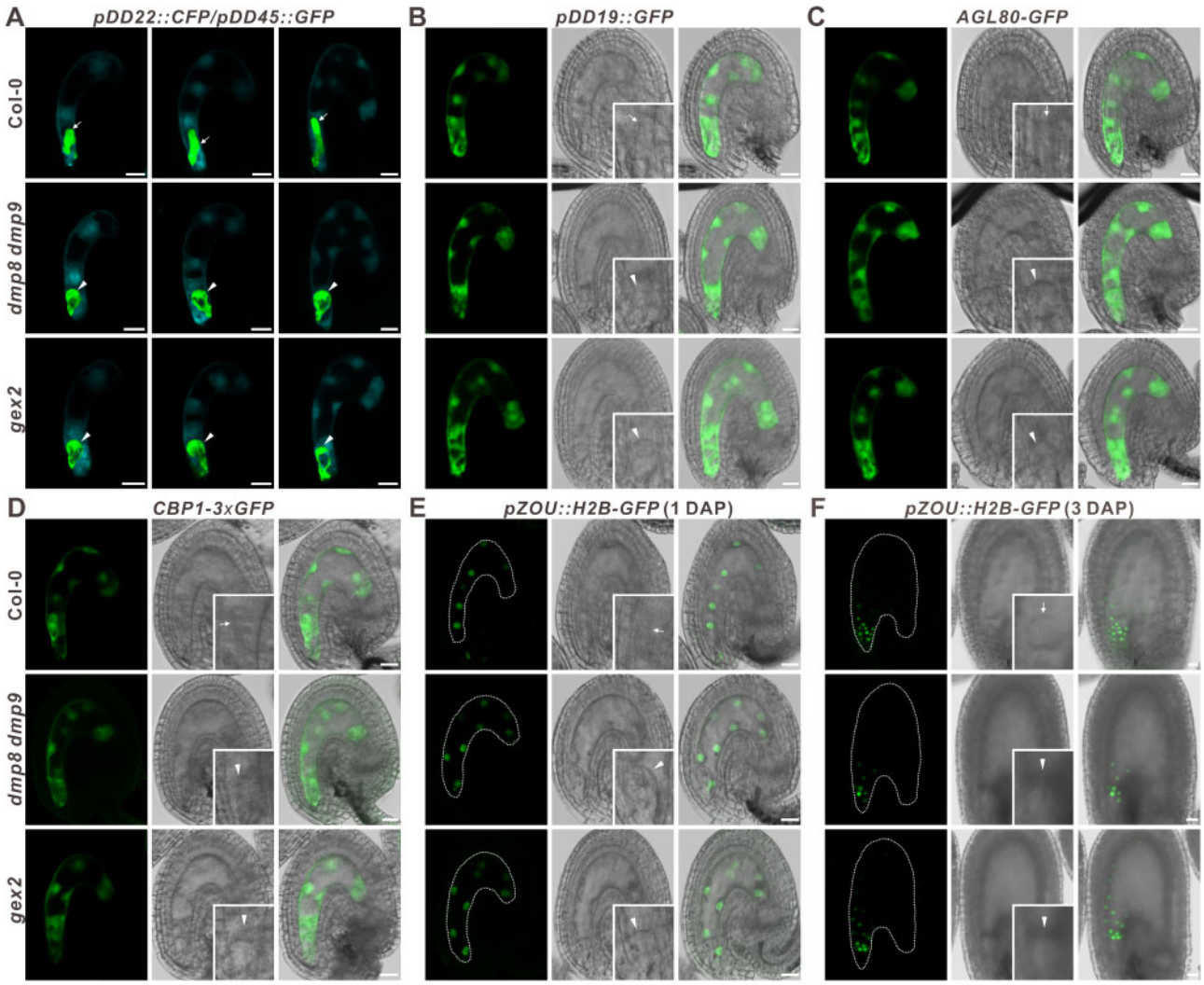


Figure 3 Embryo-free seeds express different endosperm-cell fate markers. A–F, Confocal laser scanning microscopy (CLSM) images of seeds expressing different endosperm reporters: *pDD22::CFP* in (A), *pDD19::GFP* in (B), *pAGL80::AGL80-GFP* in (C), *pCBP1::CBP1-3xGFP* in (D), and *pZOU::H2B-GFP* in (E) and (F). The insets in the lower right indicate the egg cell (arrows), zygotes, or embryos (arrowheads). The egg cells and zygotes are marked by *pDD45::GFP* in (A). Note that at 3 DAP, the *pZOU::H2B-GFP* reporter expression is largely confined to the ESR in both normal and embryo-free seeds. Scale bars, 20 μm.

(TUNEL) to follow DNA degradation in situ, which highlights ongoing PCD in the endosperm cells. In the WT, TUNEL signals began to appear in endosperm cells at 5 DAP and became widespread at 6 DAP (Figure 4, E). Surprisingly, although the embryo was absent, the endosperm showed the same TUNEL signal patterns, suggesting the embryo-independent initiation of DNA fragmentation, a sign of the initiation of endosperm degeneration. Although the mechanism underlying endosperm breakdown remains unclear, *ZOU* was reported to be responsible for endosperm breakdown (Yang et al., 2008) and trigger cell death by regulating the expression of cell-wall-modifying enzymes (Fourquin et al., 2016). Studies have also demonstrated that different cases of developmental PCD share a set of cell death-associated genes (Olvera-Carrillo et al., 2015). Therefore, we characterized the expression of *ZOU* and six of these potential PCD markers in the endosperm (Supplemental Figure S2, B) during seed development using RT-qPCR (Figure 4, F). The expression levels of the six dPCD indicators were enhanced as the process of endosperm breakdown no matter in Col-0 or in embryo-free seeds. Furthermore, except for *PASPA3* (at 5 DAP), *CEP1* (at 6 DAP), the expression of *ZOU* and the other four developmental PCD markers was not altered in embryo-free seeds compared with the control, confirming the embryo-independent initiation of endosperm breakdown.

Seed coat development is normal in seeds with only endosperm

Previous work has demonstrated that co-ordination between the endosperm and the seed coat is necessary for successful seed development (Garcia et al., 2003; Luo et al., 2005; Wang et al., 2010; Roszak and Kohler, 2011), and therefore we also observed the process of seed coat development in

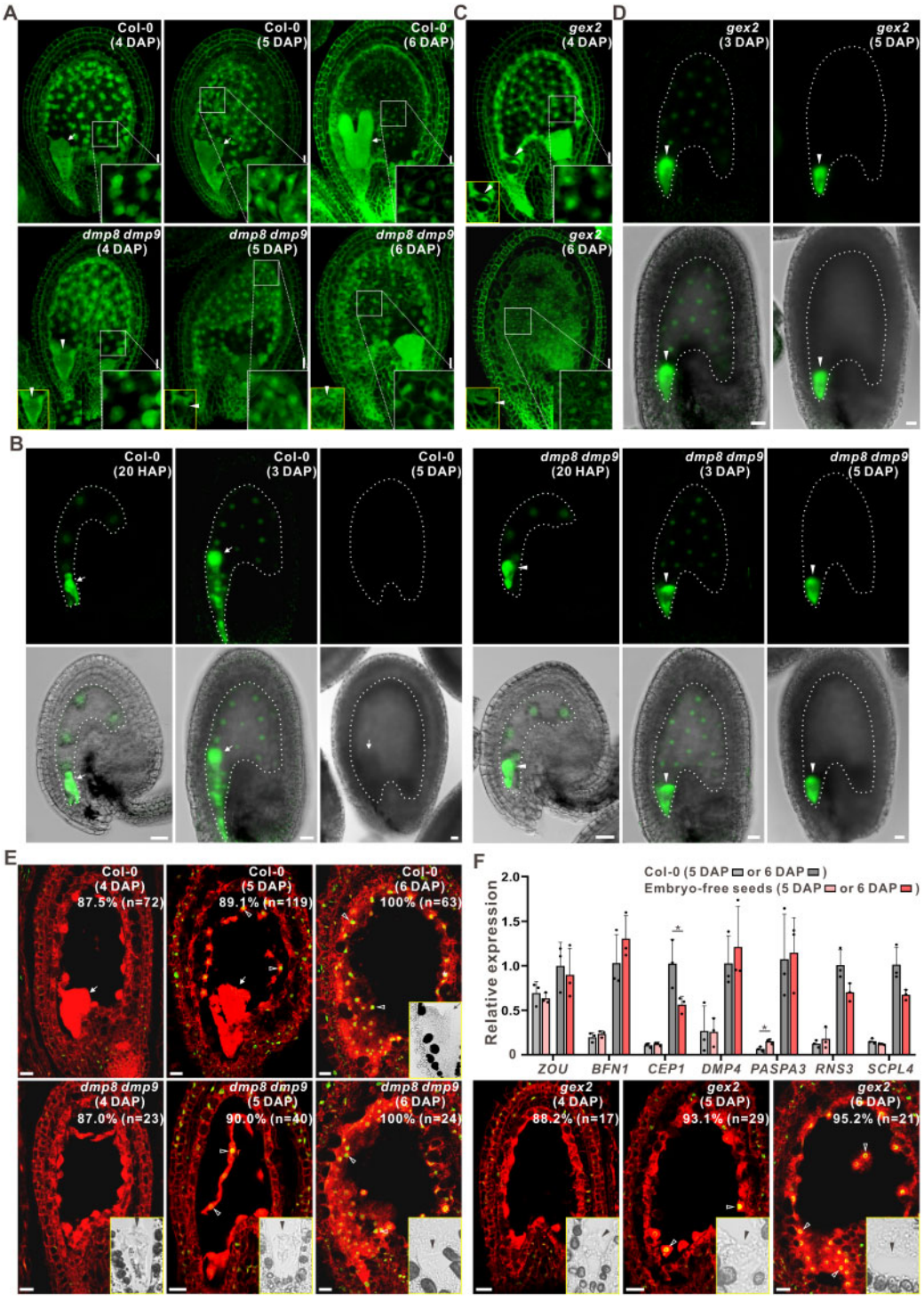


Figure 4 Endosperm cellularization is a cell-autonomous process and the embryo is not required for the initiation of endosperm cell PCD. A, Autofluorescence of seeds seen by confocal microscopy after pollination with Col-0 or *dmp8 dmp9* pollen. The insets in the lower left indicate the egg cell. B, Seeds expressing *pAGL62::AGL62-GFP* after pollination with Col-0 or *dmp8 dmp9* pollen. Note that *AGL62*, which suppresses cellularization, is expressed during syncytial endosperm development and becomes undetectable before cellularization in both normal and embryo-free seeds. The egg cell and embryo are marked by *pDD45::GFP*. C, Autofluorescence analysis of endosperm cellularization in embryo-free seeds pollinated with *gex2* pollen. The insets in the lower left indicate the egg cell. D, Embryo-free seeds pollinated with *gex2* pollen show a *pAGL62::AGL62-GFP* expression pattern similar to that of Col-0 seeds. The egg cell is marked with *pDD45::GFP*. E, TUNEL signals in seeds pollinated with Col-0, *dmp8 dmp9*, or *gex2* pollen. Propidium iodide staining was used to stain cells and the TUNEL-positive signal is indicated by hollow arrowheads and shown by the yellow fluorescence (green + red). Note that PCD signals begin to appear at 5 DAP in embryo-free seeds, similar to the control. Arrows indicate the embryo; arrowheads indicate the egg cell. Scale bars, 20 μm in (A–E). F, RT-qPCR analysis of *ZOU* and developmental PCD markers in embryo-free and Col-0 seeds at 5 and 6 DAP. The transcription level of Col-0 at 6 DAP is set as 1. Error bars are s.d. from three biological replicates. Significant differences (*$P < 0.05$, two-sided Student's *t* test) are indicated.

different fertilization types of seeds when pollinated with *dmp8 dmp9*pollen (Figure 5). In seeds containing only an embryo, seed coat development was not initiated, and the integument began to degenerate at 4 DAP, similar to that of unfertilized ovules (Figure 5, C–E). This finding is consistent with previously reported results that the embryo alone is not sufficient for the initiation of seed coat growth (Roszak and Kohler, 2011). In *Arabidopsis*, upon double fertilization, the surrounding integuments undergo a process of growth and differentiation that will lead to the formation of five cell-layered seed coat: two layers derived from the outer integument and three layers derived from the inner

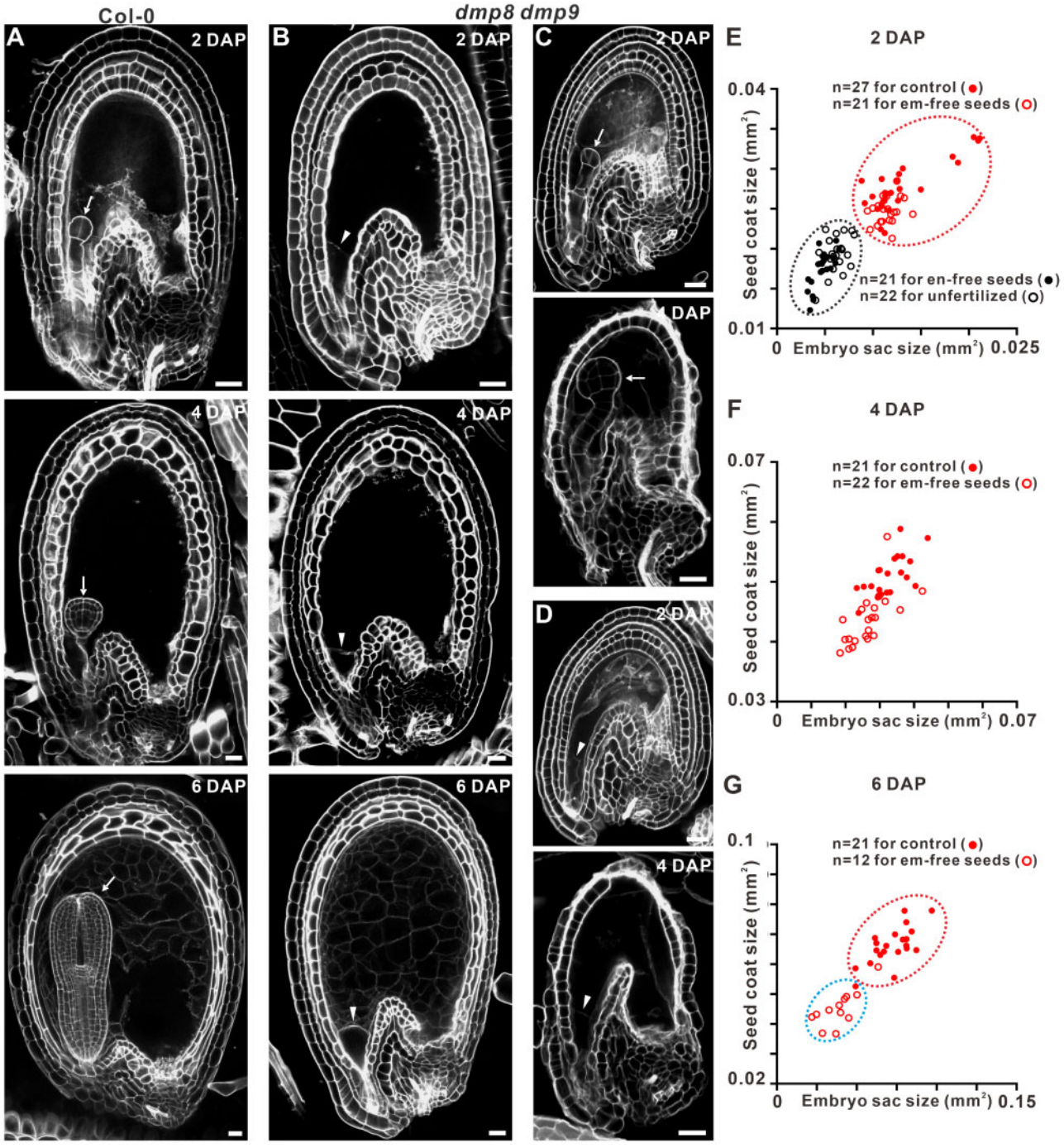


Figure 5 Seed coat development in embryo-free seeds. A, Propidium iodide-stained seeds in WT at 2, 4, and 6 DAP. B–D, Propidium iodide-stained seeds after pollination with *dmp8 dmp9* pollen at 2, 4, and 6 DAP. Note that the seed coat development in embryo-free (em-free) seeds (B) appeared to be similar to that in normal seeds (A). Meanwhile, seed coat development in seeds containing no endosperm (en-free) was not initiated and the integuments collapsed as that in unfertilized ovules (un-fer) at 4 DAP (C–D). Arrows indicate the embryo and arrowheads indicate the egg cell. Scale bars, 20 μm. E–G, Distribution of seed coat and embryo sac sizes in seeds after pollination with Col-0 or *dmp8 dmp9* pollen at 2, 4, and 6 DAP, respectively.

integument. The seed coat in all embryo-free seeds was well initiated and normally developed. The seed coat was composed of five layers, the same as that in WT seeds (Figure 5, A, B, E, and F), suggesting that seed coat development mainly depends on endosperm development and conversely confirming normal endosperm development when the embryo is absent. In fact, it is very difficult to morphologically distinguish the embryo-free seeds from WT seeds until 6 DAP. Later, the embryo-free seeds showed smaller in size (Figure 5, G and Supplemental Figure S3, B and C), indicating that when endosperm PCD is initiated, the embryo plays an increasingly important role in seed expansion and further development, revealing an interesting coordination between the seed coat and the late embryo, although the molecular signaling between them remains to be elucidated. In *Arabidopsis*, seed coat growth is mostly driven by cell elongation (Garcia et al., 2005), and this was also illustrated by our findings that the cell number of the outermost seed coat layer is not changed but the average cell length is increased during the progress of seed coat development, no matter in normal seeds or embryo-free seeds (Supplemental Figure S3, A).

Discussion

Although some works suggested that embryo might be necessary for endosperm development (Neuffer and Sheridan, 1980; Xu et al., 2015), the embryo-defective mutants previously reported actually imply distinct conclusions (Johnson et al., 2005; Lid et al., 2005; Garcion et al., 2006; San-Bento et al., 2014). Here, our results strongly support the conclusion that the embryo is not required for endosperm development, although embryo growth acts in rapid endosperm elimination. In *Arabidopsis*, the endosperm is an ephemeral tissue that breaks down almost completely to provide space for embryo expansion physically and to recycle the nutrients stored in the endosperm tissues to fuel embryo growth. When an embryo is not present, endosperm elimination seems unnecessary. Surprisingly as we observed, endosperm cells begin PCD as usual and ultimately break down almost completely at a low speed (Figure 4, E and Supplemental Figure S2, C). Thus, endosperm development is actually an autonomously programmed process, independent of embryo development. This work provides direct evidence for an "altruistic" nature of the endosperm in the relationship with its "sibling," the zygotic embryo, and also a self-directed role in embryo–endosperm coordinated development.

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

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (accession Col-0) were grown in greenhouse under a photoperiod of 16 h light and 8 h dark at 22°C with a light intensity of ~170 μmol/m^2/s using LED bulbs (Philips GreenPower TLED W 20W and DR/W/FR 15W). The T-DNA insertion lines *gex2-2* (FLAG_441D08; Mori et al., 2014) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The background of all Arabidopsis marker lines was Col-0.

Constructs and plant transformation

For the construct of *ProZOU::H2B-GFP*, a 1.5-kb promoter was amplified from genomic DNA using the primer pair (*ZOU-H2B-S/A*) and cloned into destination of the vector pART27 upstream of H2B-GFP after the digest of KpnI and AvrII. For the double marker lines carrying *pDD45::GFP* and *pDD22::CFP*, the length of promoter used in this study is according to the previous report (Steffen et al., 2007). For the *DMP8/DMP9* CRISPR-Cas9 vector, *DMP8* and *DMP9* were targeted by one sgRNA and generated using a robust CRISPR/Cas9 vector system according to the reported methods (Ma et al., 2015). All constructs were verified by sequencing and subsequently transformed into *Arabidopsis* (Col-0) by floral dip methods (Clough and Bent, 1998).

For *DMP8/DMP9* gene editing experiments, transgenic seedlings were selected on plates with half strength MS medium supplemented with 25 μg/mL hygromycin B. The primer pairs (*DMP8/DMP9-sequence-S/A*) were used to amplify the genomic region that flanks the sgRNA target site by PCR. The fragments were sequenced to identify genome editing events. Finally, three different double-homozygous mutants were acquired and *dmp8 dmp9-1* was used for subsequent experiments.

Cytological observation

Ovule clearing was performed as previously reported (Boisnard-Lorig et al., 2001) and ovule autofluorescence observation was used to analyze endosperm cellularization (Li et al., 2017). For TUNEL assays, a reported method was adapted using the DeadEnd Fluorometric TUNEL System (Promega; Wang et al., 2019). For propidium iodide-staining seeds, Schiff reagent with propidium iodide (P4170; Sigma) was used like previously described (Shi et al., 2019) and finally the samples were observed under a confocal microscope (Leica SP8 CLSM). The area and length were calculated using the "measure" tool with ImageJ.

The settings used for confocal microscopy were as follows (in nm: excitation [ex] and emission [em]): for CFP, ex 448, em 468–546; for GFP, ex 488, em 500–535; for PI, ex 552,

em 590–700; for TUNEL, ex 488, em 515–565; and for autofluorescence, ex 488, em 500–535.

RT-qPCR

At 6 DAP, the seeds which smaller than siblings side by side were dissected from siliques when *dmp8 dmp9-1* as a pollinator. Total RNA of about 250 seeds a sample was extracted using RNeasy Plant Mini Kit. After digestion with DNase I (Qiagen), first-strand cDNA synthesis was performed using an M-MLV First-Strand Kit (Invitrogen, Carlsbad, CA, USA). RT-qPCR analysis was conducted according to the protocol previously described (Czechowski et al., 2005). The data were normalized to three housekeeping genes (*AT4G0532*, *AT1G13320*, and *AT4G34270*), and each experiment was repeated three times.

Accession numbers

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

Supplemental data

Supplemental Figure S1. Phenotype analysis of endosperm in Col-0 and embryo-free seeds.

Supplemental Figure S2. Embryo growth accelerates endosperm breakdown.

Supplemental Figure S3. Integument cell elongation is responsible for seed coat growth and embryo-free seeds show smaller sizes at 6 DAP.

Supplemental Table S1. Primers used in this study

Acknowledgments

The authors thank Prof. Gary Drews for kindly providing maker lines (*pDD19::GFP*, *AGL62-GFP* and *AGL80-GFP*) and Prof. Wei-Cai Yang for the *CBP1-3xGFP* maker line. They also thank Prof. Y.G. Liu for providing the CRISPR/Cas9 system used in this work.

Funding

This work was supported by two grants from the National Natural Science Foundation of China [31991201 and 31800264].

Conflict of interest statement. None declared.

References

Batista RA, Figueiredo DD, Santos-Gonzalez J, Kohler C (2019) Auxin regulates endosperm cellularization in *Arabidopsis*. Genes Dev **33**: 466–476

Boisnard-Lorig C, Colon-Carmona A, Bauch W, Hodge S, Doerner P, Bancharel E, Dumas C, Haseloff J, 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

Chaudhury AM, Ming L, Miller C, Craig S, Dennis ES, Peacock WJ (1997) Fertilization-independent seed development in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **94**: 4223–4228

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16**: 735–743

Costa LM, Marshall E, Tesfaye M, Silverstein KA, Mori M, Umetsu Y, Otterbach SL, Papareddy R, Dickinson HG, Boutiller K, et al. (2014) Central cell-derived peptides regulate early embryo patterning in flowering plants. Science **344**: 168–172

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

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol **139**: 5–17

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

Figueiredo DD, Batista RA, Roszak PJ, Kohler C (2015) Auxin production couples endosperm development to fertilization. Nat Plants **1**: 15184

Figueiredo DD, Batista RA, Roszak PJ, Hennig L, Kohler C (2016) Auxin production in the endosperm drives seed coat development in *Arabidopsis*. eLife **5**

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

Garcia D, Fitz Gerald JN, 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

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

Garcion C, Guilleminot J, Kroj T, Parcy F, Giraudat J, Devic M (2006) AKRP and EMB506 are two ankyrin repeat proteins essential for plastid differentiation and plant development in *Arabidopsis*. Plant J **48**: 895–906

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

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

Johnson KL, Degnan KA, Walker JR, Ingram GC (2005) AtDEK1 is essential for specification of embryonic epidermal cell fate. Plant J **44**: 114–127

Kang IH, Steffen JG, Portereiko MF, Lloyd A, Drews GN (2008) The AGL62 MADS domain protein regulates cellularization during endosperm development in *Arabidopsis*. Plant Cell **20**: 635–647

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

Li G, Zou WX, Jian LF, Qian J, Deng YT, 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

Li HJ, Zhu SS, Zhang MX, Wang T, Liang L, Xue Y, Shi DQ, Liu J, Yang WC (2015) *Arabidopsis* CBP1 is a novel regulator of transcription initiation in central cell-mediated pollen tube guidance. Plant Cell **27**: 2880–2893

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

Lid SE, Olsen L, Nestestog R, Aukerman M, Brown RC, Lemmon B, Mucha M, Opsahl-Sorteberg HG, Olsen OA (2005) Mutation in the *Arabidopisis thaliana DEK1* Calpain gene perturbs endosperm and embryo development while over-expression affects organ development globally. Planta **221**: 339–351

Luo M, Dennis ES, Berger F, Peacock WJ, 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

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y, et al. (2015) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant **8**: 1274–1284

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

Neuffer MG, Sheridan WF (1980) Defective kernel mutants of maize. I. Genetic and lethality studies. Genetics **95**: 929–944

Olvera-Carrillo Y, Van Bel M, Van Hautegem T, Fendrych M, Huysmans M, Simaskova M, van Durme M, Buscaill P, Rivas S, Coll NS, et al. (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

Pignocchi C, Minns GE, Nesi N, Koumproglou R, Kitsios G, Benning C, Lloyd CW, Doonan JH, Hills MJ (2009) ENDOSPERM DEFECTIVE1 is a novel microtubule-associated protein essential for seed development in *Arabidopsis*. Plant Cell **21**: 90–105

Portereiko MF, Lloyd A, Steffen JG, Punwani JA, Otsuga D, Drews GN (2006) AGL80 is required for central cell and endosperm development in *Arabidopsis*. Plant Cell **18**: 1862–1872

Robert HS, Park C, Gutierrez CL, Wojcikowska B, Pencik A, Novak O, Chen JY, Grunewald W, Dresselhaus T, Friml J, et al. (2018) Maternal auxin supply contributes to early embryo patterning in *Arabidopsis*. Nat Plants **4**: 548–553

Roszak P, 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

San-Bento R, Farcot E, Galletti R, Creff A, Ingram G (2014) Epidermal identity is maintained by cell–cell communication via a universally active feedback loop in *Arabidopsis thaliana*. Plant J **77**: 46–58

Shi C, Luo P, Du YT, Chen H, Huang X, Cheng TH, Luo A, Li HJ, Yang WC, Zhao P, et al. (2019) Maternal control of suspensor programmed cell death via gibberellin signaling. Nat Commun **10**: 3484

Steffen JG, Kang IH, Macfarlane J, Drews GN (2007) Identification of genes expressed in the *Arabidopsis* female gametophyte. Plant J **51**: 281–292

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

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

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

Wang W, Xiong H, Lin R, Zhao N, Zhao P, Sun MX (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

Xu TT, Ren SC, Song XF, Liu CM (2015) *CLE19* expressed in the embryo regulates both cotyledon establishment and endosperm development in *Arabidopsis*. J Exp Bot **66**: 5217–5227

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