# Paternal microRNA159 promotes endosperm nuclear division during *Arabidopsis* seed development

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**One sentence summary:** Paternal miR159 is necessary for seed development by facilitating early endosperm nuclear division.

**Author Contribution:** S.W. performed most of experiments; Y. Z. conducted material planting and statistical analysis of seeds; W.W. preformed the northern blot assay; S.W. and B.Z. designed the experiments and analyzed the data; B.Z. conceived the project and wrote the article with contributions of all the authors and S.W. complemented the writing.

**Abstract**

MicroRNA (miRNAs) are a class of 20~24-nucleotide endogenous small RNAs that repress gene expression of targets. In plants, miRNAs play an important role in developmental and physiological processes. Highly conserved miRNAs, such as miR159, exist in *Arabidopsis* pollen which contains a vegetative cell and two sperm cells, and miR159 is significantly enriched in sperm cells. However, based on the fact that *DUO1*, a miR159 target, is co-expressed in sperm cells with miR159, the biological significance of sperm cell-enriched miR159 remains poorly understood. Here, we showed that paternal miR159 promotes early endosperm nuclear division during *Arabidopsis* seed development. miR159 had dispensable effects on pollen development, pollen germination, and pollen tube function. However, loss of paternal miR159 caused severely arrested or progressively delayed endosperm nuclei division. Furthermore, *MYB33* and *MYB65*, two major targets of miR159, highly accumulated in central cell. After fertilization, the expression of *MYB33* and *MYB65* was hardly detectable once the endosperm divisions begin. But in the absence of paternal miR159, both MYB33 and MYB65 maintained in supposed endorsperm nuclei in which nuclear division was arrested or delayed. Since *MYB33* and *MYB65* were reported to disrupt cell proliferation in vegetative tissue, it's highly possible that paternal miR159 promotes endosperm nuclei division through repressing the expression of maternal *MYB33* and *MYB65* in early endosperm. Collectively, our results show that paternal miR159 plays an important role in early seed development by repressing maternal miR159 targets to facilitate early endosperm nuclei division, and thus uncover the biological significance of miRNA in sperm cells.

**Key words:** Paternal; endosperm nuclear division; seed development; microRNA

## Introduction

Fertilization triggers the onset of development of the new generation, and plant life cycle initiates from seed development, mainly including embryogenesis and endosperm development (Grossniklaus et al., 2001). In *Arabidopsis,* the endosperm development begins from the endosperm nuclear division (Grossniklaus et al., 2001). A previous study showed that sperm entry is sufficient to trigger the division of the central cell and paternal factors are necessary for the endosperm development (Aw et al., 2010). Central questions are what factors are inherited from sperm cells, and by which such factors trigger early division events.

It is known that paternal RNAs play a role in early development (Ostermeier et al., 2004; Rassoulzadegan et al., 2006). In mouse, interruption of sperm RNA delivery induced embryonic lethality (Liu et al., 2012; Stoeckius et al., 2014; Yuan et al., 2016). In plants, sperm-delivered *SSP* (Short Suspensor) RNA triggered the first zygotic division (Bayer et al., 2009). MicroRNAs are 20-24 nucleiotide small RNAs that play an essential role in different biological processes (Rogers and Chen, 2013). Intriguingly, high throughput sequencing analysis show that many miRNAs, such as one of canonical miRNAs, miR159, enriched in sperm (Grant-Downton et al., 2009a; Grant-Downton et al., 2009b; Borges et al., 2011). To our surprise, DUO1, one of miR159 target, specifically accumulates in sperms, to ensure proper sperm formation by initiating the second mitosis during pollen development (Rotman et al., 2005; Brownfield et al., 2009; Zheng et al., 2011), raising the question: why miR159 and its target co-exist in sperms? Considering that the endosperm nuclear division occurs earlier than zygotic division (Aw et al., 2010), implies that the early endosperm nuclear division might be preferentially dependent on those factors inherited from parents. However, with the only exception (Bayer et al., 2009), whether and how the paternal inherited transcripts regulates early seed development is less well understood.

Given that the enrichment of miR159 in sperm cells (Grant-Downton et al., 2009a; Grant-Downton et al., 2009b; Borges et al., 2011) and that the lack of miR159 activity caused reduced fertility (Allen et al., 2007; Rubio-Somoza and Weigel, 2013), we used miR159 as an example to explore the function of paternally inherited miRNA

90 in seed development in this study. We showed that paternally inherited miR159 is
91 required for seed development by promoting early endosperm nuclear division.
92 Furthermore, paternally-inherited miR159 was destined to inhibit its
93 maternally-inherited targets in the endosperm, uncovering a previously unknown role
94 of a specific sperm-enriched miRNA during early seed development.
95
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## Results

### Both maternal and paternal miR159 is required for seed development

Previous studies showed that miR159 accumulation was almost abolished in sporophytic tissues in the *mir159abc* mutant, in which three independent T-DNAs were inserted in the pri-miRNA regions of *MIR159a*, *MIR159b*, and *MIR159c*, respectively, causing either no pri-miRNA transcription or no pri-miRNA processing (Allen et al., 2007; Allen et al., 2010). To investigate the biological significance of miR159 enriched in sperm cells, we first confirmed that miR159 accumulated in mature pollen of wild type plants by both northern blot (Figure S1A) and RT-qPCR (Figure S1B), but miR159 accumulation was significantly reduced in *mir159abc* pollen (Figure S1A, S1B**)**, indicating it is feasible to compare the differences of sperm cell function-related developmental processes between wild type plants and the *mir159abc* mutant. Together with the fact that the fertility was significantly impaired by the loss of miR159 (Allen et al., 2007; Allen et al., 2010), we hypothesized that miR159 enriched in sperm cells might play a role in fertility.

To investigate whether maternal and/or paternal miR159 regulates seed development, we performed reciprocal crosses between Col-0 and *mir159abc*. Consistent with previous studies (Allen et al., 2007; Allen et al., 2010), hand-pollinated self-fertilized *mir159abc* mutant exhibited severely reduced seed set (Figure 1A; Table 1). Although the reduced seed set seen in the absence of miR159 was variable (Figure 1A), in general, ~78% of F1 siliques from hand-pollinated *mir159abc* mutant, ~70% of F1 siliques from *mir159abc* as the female, and ~52% of F1 siliques from *mir159abc* as the male, respectively, showed reduced seed set with varying extents (Figure 1A). Notably, much more (30%) of F1 siliques from Col-0 as the female and *mir159abc* as the male showed less than 30% seed set (Figure 1A, 1B). In contrast, only 10% of F1 siliques from *mir159abc* as the female and Col-0 as the male showed less than 30% seed set (Figure 1A). In other words, the loss of maternal miR159 caused less severe effects on seed set (Figure 1A), although it led to more broad effects by affecting more seeds (Figure 1A). Taken together, our results indicate that not only maternal miR159 but also paternal miR159 is required for seed

**Figure 1**

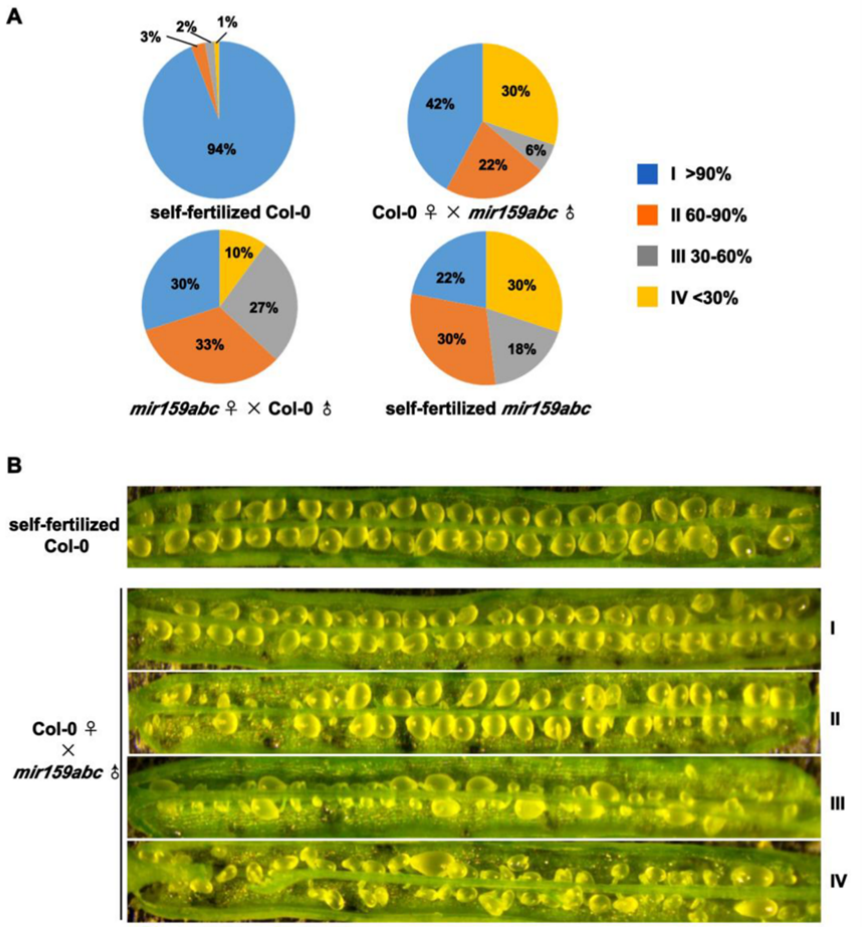


**Fig 1.** Abnormal seed development caused by loss of paternal miR159.

(A) Percentage of siliques with indicated ratio of normal seed sets in reciprocal crosses between Col-0 and *mir159abc*. F1 siliques were divided into 4 categories according to the percentage of seed set per silique, I, II, III, IV indicate seed set >90% (blue), 60-90% (dark orange), 30-60% (grey), <30% (light orange), respectively. Percentage of siliques in each category is shown in pie charts. 150 siliques were examined for each genetic background.

(B) Representative F1 siliques from hand-pollinated self-fertilized wild-type (Col-0) and crossed plants with Col-0 ♀ × *mir159abc* ♂. I, II, III, IV indicate representative siliques shown in (A).

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development. Notably, previous studies showed that seed development are predominantly maternally controlled (Garcia et al., 2003; Olsen, 2004; Autran et al., 2011), it is unremarkable that maternally-inherited miR159 regulates seed

development, our later analysis will thus focus on how paternally-inherited miR159 promotes seed development.

**miR159 is dispensable for pollen development and pollen tube function**

To investigate which step was affected in male penetrance of *mir159abc*, we first examined pollen viability by Alexander red staining. We found that pollen grains of the *mir159abc* triple mutant were indistinguishable from Col-0 pollen (Figures S2A). Then, we want to know whether miR159 enriched in sperm cells aims to repress its targets. It is known that there are 8 miR159 targets that belong to *MYB* transcription factors in *Arabidopsis* (Palatnik et al., 2007). Although microarray comparisons of the transcriptomes of pollen and sperm cells showed that three of them including *MYB65*, *MYB97*, and *MYB101* were expressed in sperm cells (Borges et al., 2008; Table S1), protein fusions of MYB97, MYB101, and MYB120 (Leydon et al., 2013; Liang et al., 2013) and MYB33 and MYB65 (Figure S2B) demonstrated that these MYBs were absent in sperm cells. However, several studies showed that *DUO1/MYB125*, another miR159 target, was specifically expressed in sperm cells to ensure proper sperm cell formation (Rotman et al., 2005; Brownfield et al., 2009; Zheng et al., 2011; Zheng et al., 2014; Figure S2B). Moreover, we did not detected increased expression of miR159 targets in *mir159abc* pollen (Figure S2C), further indicating that the undetectable expression of miR159 target genes was not due to the repression role of miR159. Subsequent DAPI staining showed that the pollen grains from *mir159abc* had an intact vegetative nucleus and two sperm cell nuclei, similar to that of Col-0 pollen grains (Figure 2A, 2B). Taken together, these results indicated that the enrichment of miR159 in sperm cells is not destined to silence its targets to regulate sperm cell formation.

We then examined whether pollen germination and pollen tube growth were affected in the *mir159abc* triple mutant. *In vitro* pollen germination assay showed that pollen germination rate and pollen tube length of *mir159abc* were comparable to that of Col-0 (Figure 2C, 2D). Although the *mir159abc* triple mutant was normal in pollen viability, pollen development, pollen germination and pollen tube growth, the

**Figure 2**

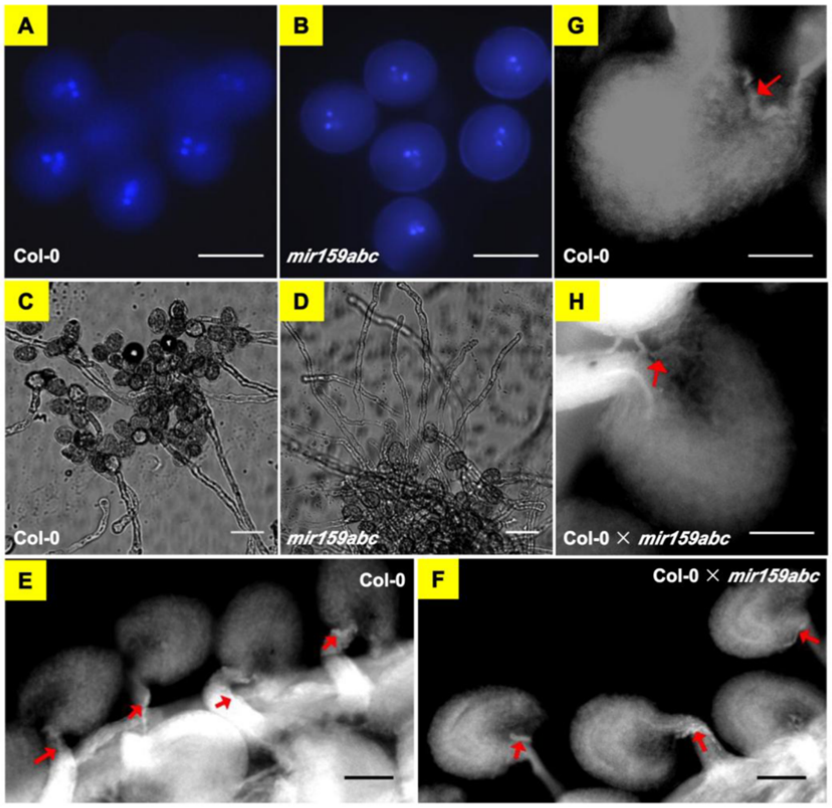


**Fig 2.** The *mir159abc* triple mutant shows normal pollen development, pollen germination, and pollen tube guidance and reception.

(A and B) Mature pollen in Col-0 (A) and *mir159abc* (B) by DAPI staining. >500 pollen from 10 randomly picked flowers for each genotype were observed, and representative images were shown.

(C and D) *In vitro* pollen germination assay was performed for Col-0 (C) and *mir159abc* (D). Photos were taken after incubation for 7 hours.

(E, F, G, and H) *In vivo* pollen tube growth, guidance, and reception for Col-0 (E, G) and *mir159abc* (F, H). Pollen tubes were examined by ovule clearing and Decolorized Aaniline blue staining. >300 ovules from 10 randomly picked pollinated pistils for each genotype were observed, and representative images were shown. Arrowheads indicate the growing pollen tubes. Scale bars are 20 μm for (A, B, E, F, G, and H) and 200 μm for (C and D).

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defective male penetrance of the *mir159abc* triple mutant could be caused by abnormal pollen tube guidance and/or the failure of pollen tube perception, the latter is important for sperm discharge. Recent studies showed that defective sperm delivery

usually exhibits uncontrolled pollen tube growth in synegid cells (Leydon et al., 2013; Liang et al., 2013). *In vivo* Decolorized Aniline Blue (DAB) staining showed that the entry and growth in the synergid cells of pollen tubes from *mir159abc* was quite similar to that of Col-0 (Figure 2E-2H). Taken together, these results indicate that miR159 was not involved in pollen development and sperm delivery.

**Paternal miR159 is necessary for early endosperm nuclear division**

Since miR159 had no detectable role in sperm cell formation and sperm discharge, we hypothesized that sperm-enriched miR159 might act in early embryo or endosperm development. To test this, we hand-pollinated Col-0 pistils with pollen of Col-0 or *mir159abc*, and then observed the process of seed development using DIC imaging. At 8 hours after pollination (HAP), seeds from both hand-pollinated self-fertilized Col-0 and Col-0 ♀ × *mir159abc* ♂ contained an undivided endosperm nucleus and an undivided zygote (Figure S3A, S3B). At 16 HAP, seeds from hand-pollinated self-fertilized Col-0 generally harbored 4 endosperm nuclei (Figure 3A), indicating that endosperm had finished two rounds of nuclear divisions. In contrast, seeds from Col-0 ♀ × *mir159abc* ♂ showed great variation in endosperm nuclear division, with only 48% of the seeds with endosperm nuclei that divided normally (Figure 3B). In more than 50% of Col-0 ♀ × *mir159abc* ♂ seeds, the early division of endosperm nuclei was either delayed (Figure 3C) or arrested (Figure 3D). Progressively more severe defects were found at 24 HAP in Col-0 ♀ × *mir159abc* ♂ seeds (Figure 3E-H). Strikingly, endosperm nuclei in more than 30% of Col-0 ♀ × *mir159abc* ♂ seeds remained undivided at 48 HAP (Figure S3C) and at 72 HAP (Figure S3D). To further confirm that paternally-inherited miR159 promotes early endosperm nuclear divisions, we compared nuclei number of seeds from hand-pollinated Col-0, Col-0 ♀ × *mir159abc* ♂, *mir159abc* ♀ × Col-0 ♂, and hand-pollinated *mir159abc* at 24 HAP. In contrast to that of *mir159abc* as the male, the percentage of seeds containing 8-endosperm nuclei in the case of *mir159abc* as the female was much closer to that of Col-0 (Figure 3I), indicating that maternal miR159 plays a minor role in promoting endosperm nuclear division. Moreover, the complementation of paternal miR159

**Figure 3**

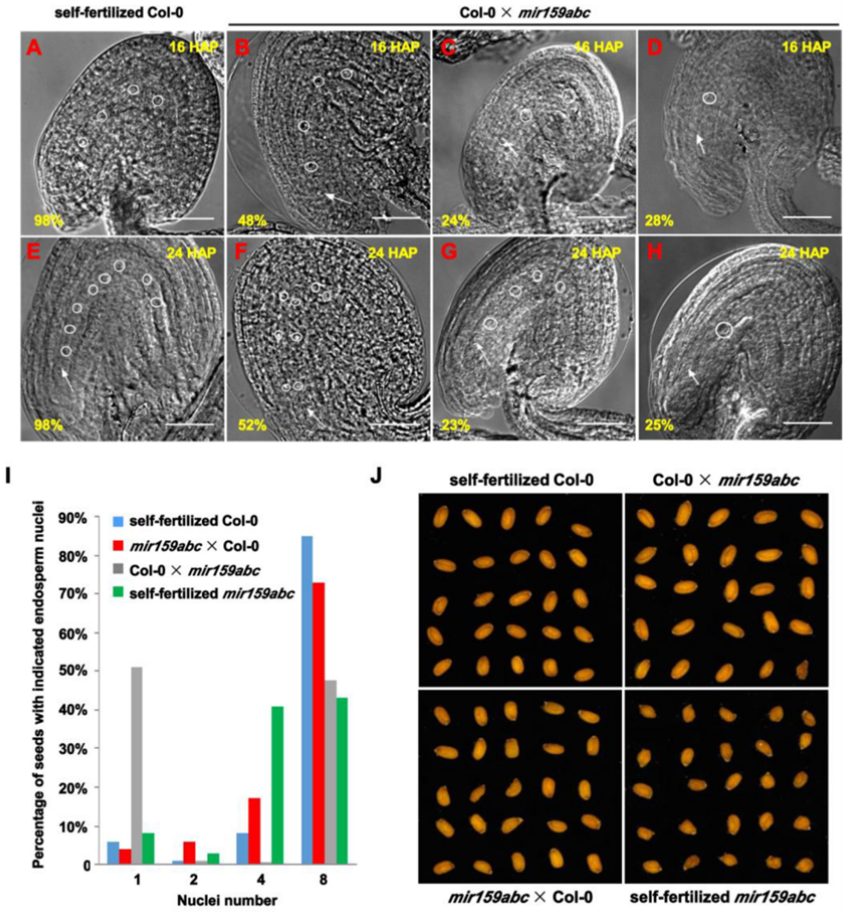


**Fig 3.** Lack of paternal miR159 caused defective endosperm nuclear division.

(A-H) Seeds of Col-0 and progeny from Col-0 ♀ × *mir159abc* ♂ at 4 hr after fertilization (A-D) and 12 hr after fertilization (E-H). Scale bar, 20 μm.

(I) Statistical analysis of numbers of endosperm nuclei from hand-pollinated self-fertilized Col-0 (blue), *mir159abc* ♀ × Col-0 ♂ (red), Col-0 ♀ ×*mir159abc* ♂ (grey), and hand-pollinated self-fertilized *mir159abc* (green) at 12 hr fertilization. >200 seeds from 10 siliques were examined for each genetic background.

(J) Mature F1 seeds of hand-pollinated self-fertilized Col-0, Col-0 ♀ × *mir159abc* ♂, *mir159abc* ♀ × Col-0 ♂, and hand-pollinated self-fertilized *mir159abc*. 500 F1 seeds were examined for each genetic background at each time point. White circles indicate the location of endosperm nuclei. The arrows indicate undivided zygotes.

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significantly rescued the reduced endosperm nuclear divisions in the *mir159abc* mutant, further supporting that paternally-inherited miR159 plays an important role in early endosperm nuclear division (Figure 3I).

To examine whether subsequent endosperm cellularization was affected by paternal miR159, we compared the endosperm size among progeny from different crosses. When the embryo development from each genotype reached the dermatogen stage, the endosperm size from Col-0 ♀ × *mir159abc* ♂ (Figure S3F) was obviously smaller than that of hand-pollinated self-fertilized Col-0 (Figure S3E). The complementation of paternal miR159 using Col-0 as the pollen donor (Figure S3G) partially rescued the smaller size endosperm phenotype in *mir159abc* (Figure S3H). Consistent with the contribution of endosperm cellularization in seed size control (Garcia et al., 2003; Olsen, 2004), we showed that compare to that of the *mir159abc* mutant, seeds from crosses between *mir159abc* ♀ ×Col-0 ♂ were slightly bigger, indicating that the complementation of paternal miR159 partially rescued the small seed phenotype of *mir159abc* (Figure 3J). Notably, although a previous study showed that maternal miR156 is required for early embryogenesis (Nodine and Bartel, 2010), the embryo development of seeds in the lack of either maternal or paternal miR159 was indistinguishable from that of Col-0 (Figure S3E-H), indicating that miR159 had minor role in embryogenesis. Taken together, these results indicate that paternally inherited miR159 was required for seed development by facilitating early endosperm nuclear divisions.

**Paternal miR159 represses maternally inherited MYB33 and MYB65**

Since paternally inherited miR159 promotes endosperm nuclear division, then how miR159 regulates the endosperm nuclear divisions? Because miRNA usually functions by repressing expression of its target genes (Rogers and Chen, 2013), we thus examined the expression of all eight miR159 targets before and after double fertilization in female tissues. RT-PCR analysis showed that only *MYB33* and *MYB65* were expressed in unpollinated pistils (Figure S4A). Similar to that in pollen, no increase of these targets was detected in *mir159abc* unpollinated pistils (Figure S4A), indicating that either no miR159 exists or the miR159 activity might be limited in unpollinated pistils. By analyzing the promoter activities of three *MIR159* genes in unpollinated ovules, we showed that all *MIR159a*, *MIR159b*, or *MIR159c* was not

expressed before fertilization (Figure S4B), indicating that miR159 biogenesis is not active in unfertilized female gametophytes.

To further monitor the subcellular localization of MYB33 and MYB65, we constructed transgenic plants expressing MYB33-GFP and MYB65-RFP fusion proteins driven by their native promoters. By fluorescence microscopy, both MYB33-GFP and MYB65-RFP signals were obviously detectable in the central cell but not in the egg cell (Figure 4A, 4D). In contrast, MYB33-GFP and MYB65-RFP signals were almost abolished when the endosperm nuclear divisions begin (Figure 4B, 4E). To investigate if maternally inherited MYB33 and MYB65 were repressed by paternal miR159, we hand-pollinated MYB33-GFP and MYB65-RFP transgenic plants with pollen of Col-0 or *mir159abc*. When Col-0 as the pollen donor, both MYB33 (Figure 4B) and MYB65 (Figure 4E) accumulation were eliminated at 16 HAP. In contrast, both MYB33-GFP (Figure 4C) and MYB65-RFP (Figure 4F) still accumulated in the undivided endosperm nucleus when miR159 was paternally disrupted, indicating that the removal of MYB33 and MYB65 might be necessary for endosperm nuclear division. qRT-PCR analysis further confirmed that paternally-inherited miR159 was sufficient to repress *MYB65* after fertilization (Figure S4C). Therefore, although it has a limited role in repressing its targets in sperms, paternal miR159 inhibits its maternally inherited targets in the endosperm, further supporting that sperm-enriched miRNAs are destined to regulate early seed development.

**Figure 4**

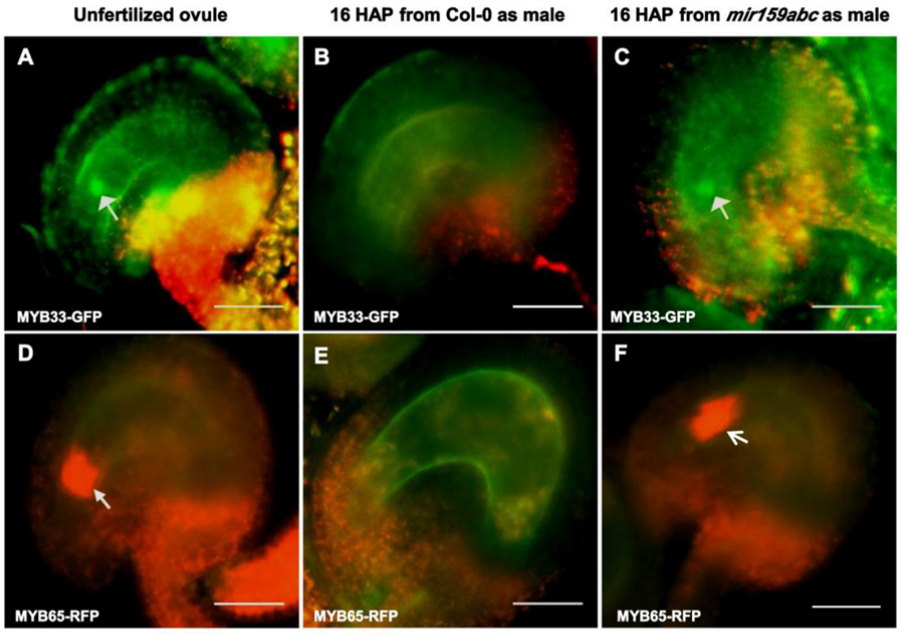


**Fig 4.** Paternal miR159 represses maternally inherited *MYB33* and *MYB65*. MYB33-GFP (A-C) and MYB65-RFP (D-F) in unfertilized ovules (A, D) and fertilized seeds (B, C and E, F). White arrows indicate GFP and RFP in the central cell and presumed endosperm, respectively. Scale bar in, 20 µm.

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## Discussion

Both in animals and plants, parental gene products delivered into the newly fused cell regulate embryonic development before activation of the zygotic genome (Meyer and

Scholten, 2007; Autran et al., 2011; Nodine and Bartel, 2012). Plant development is initiated from seed development, in which the endosperm nuclear division but not zygotic division occurs earlier, implicating that parental factor might play much more important roles in early endosperm development. However, how the newly fused cell by the central cell and sperm perceives these factors to initiate the first nuclear division is less well understood. We have identified paternal miR159 that promotes the initiation and/or the progression of early endosperm nuclear division. Together with that mouse sperm-borne miRNAs are required for early embryogenesis (Wagner et al., 2008; Yuan et al., 2016), these findings implied that the involvement of sperm-delivered miRNA in early development might be evolutionarily conserved.

DUO1, as a miR159 target, specifically accumulates in sperm cells (Rotman et al., 2005), and expression of *DUO1* and *MYB101* in the *mir159abc* mutant pollen was comparable to that of Col-0 pollen (Figure S2C), implying that the biological significance of miRNA enriched in sperms is not supposed to silence its targets locally. Previous studies showed that miRNA activity is globally suppressed in mouse oocytes (Ma et al., 2010; Suh et al., 2010), findings of co-existence of miR159 and its targets in plant sperms (Rotman et al., 2005; Borges et al., 2011) and no increase of *DUO1* and *MYB101* in the absence of miR159 (Figure S2C) indicated that the repression activity of miRNA might be evolutionarily limited in germlines. However, although it has a limited role in repressing its targets in sperm cells, paternal miR159 inhibits its maternally inherited targets efficiently in the endosperm, further supporting the idea that sperm-enriched miRNAs are destined to regulate early zygotic division and/or endosperm nuclear division triggered by fertilization.

In the absence of paternal miR159 the endosperm showed a premature arrest in size that caused precocious cellularization of the syncytial endosperm (Figure S3E-H), which might explain small seed observed in the *mir159abc* triple mutant (Allen et al., 2007). Together with the finding that *MYB33* and *MYB65* were reported to disrupt cell proliferation in vegetative tissue (Alonso-Peral et al., 2010), it is most likely that paternal miR159 promotes endosperm nuclear divisions through inhibiting maternally inherited *MYB33* and *MYB65*. The remarkable role of paternal miR159 in early

endosperm development but not in embryogenesis could be explained because global transcription of the zygotic genome has not been activated yet during the initiation stage of endosperm nuclear division (Autran et al., 2011; Nodine and Bartel, 2012).

Collectively, we showed that sperm-enriched miR159 was not involved in sperm cell formation and sperm discharge (Figure 2), to our surprise, loss of paternal miR159 resulted in the retention of maternally inherited MYB33 and MYB65 in the early endosperm (Figure 4), and caused delayed or arrested endosperm nuclear divisions (Figure 3), which finally led to seed abortion (Figure 1). These findings provide strong evidence that paternal miR159 play a role in early seed development by promoting endosperm nuclear division. Although high-throughput technologies have provided a glance at miRNAs in sperms (Grant-Downton et al., 2009a; Grant-Downton et al., 2009b), the biological significance of these sperm-enriched miRNAs remain largely unknown. Future studies focused on the functional aspects of these miRNAs in the developing seeds will further strengthen the correlation between paternal factors and seed development.

## Material and methods

### Plant material and growth conditions

Col-0 was used as wild type, and transgenic plants of MYB33-GFP and MYB65-RFP are the Col-0 background. The *mir159abc* triple mutant was kindly provided by Dr. Anthony A. Millar, which was constructed by three independent T-DNA insertional mutants (SAIL_430_F11 for *mir159a*; SAIL_770_GO5 for *mir159b*; and SAIL_248_G11 for *mir159c*). Seeds of transgenic plants for proDUO1:DUO1-RFP were kindly provided by Dr. David Twell.

### Plasmid construction

To generate the *MYB33p::MYB33*-GFP construct, the *MYB33* genomic region from 1832 bp upstream of the ATG to the end of ORF (without stop codon) was amplified from Col-0 genomic DNA with primers pro*MYB33* F4/*MYB33* R2 using Q5 DNA polymerase (NEB), and the resulting fragment was cloned into TSK108 modified

from pENTR-D/TOPO (Invitrogen). LR reaction was performed with the destination vector pMDC107. Similarly, the *MYB65* genomic region containing the coding sequence (without the stop codon) and 2449 bp upstream was amplified with primers pro*MYB65* F5/*MYB65* R3 and cloned into TSK108 modified from pENTR-D/TOPO. LR reaction was performed with the destination vector pMDC163-RFP. Primer information is listed in Table S1.

**Reciprocal cross**

Due to the delay in vegetative growth of the *mir159abc* mutant, 8-week-old mutant plants were used for crosses, while Col-0 plants were 5-week-old. Flowers at stage 12 were emasculated and pistils were left to grow for 12-24 hours for maturation. Then pistils were hand-pollinated with pollen grains of Col-0 or the *mir159abc* mutant. For calculating the ratio of aborted seeds, pistils at 4 days after pollination (DAP) were dissected and the numbers of normal and undeveloped seeds were counted under a Leica dissecting microscope.

**miRNA northern blot**

miRNA northern blot was examined as previously described (Zheng et al., 2011). Total RNA of mature pollen was extracted using ZYMO Plant RNA MiniPrep (Cat.R2024) from Col-0 pollen. 20 μg total RNA was separated by denaturing 15% (w/v) PAGE and transferred to a nylon membrane. 5'biotin_labeled-oligo nucleotides of miR159 sequences complementary to miR159 were synthesized as probes. Oligo information is listed in Table S2.

**Microscopy**

For examination of embryo and endosperm with differential interference contrast (DIC) microscopy, ovules from seeds at 2 days after pollination (DAP) were mounted in clearing solution containing chloral hydrate, water, and glycerol (ratio w/v/v: 8:3:1). For examination of seeds before 48 hours after pollination (HAP), ovules were fixed in FAA fixative solution (3.7% formaldehyde, 5% acetic acid and 50% ethanol) for

6-8 hours and then mounted in clearing solution for DIC imaging. The microscope used was an Olympus BX53 equipped with a Sony ICX285 CCD camera. Ovules from pistils within 48 hours after pollination were examined with UPlanFLN 40X objectives, while ovules from pistils older than 2 days after pollination were examined with UPlanFLN 20X objectives. For fluorescence analysis, ovules were dissected and were immediately mounted in water. Fluorescence microscopy analysis was carried out with an Olympus BX53 microscope (image acquisition software: QCapture Pro7; objectives: UPlanFLN 40X). *In vitro* pollen germination were examined under bright field using UPlanFLN 20X objectives. DAPI staining of pollen was examined under UV channel using UPlanFLN 40X objectives. Images were further processed using Adobe Photoshop and Image J.

**Phenotypic characterization of mature pollen grains**

DAPI staining of pollen grains (Zheng et al., 2011), and *in vitro* pollen germination (Boavida and McCormick, 2007) were performed as previously described.

**Decolorized aniline blue staining of pollen tubes *in vivo***

Flowers at stage 12 were emasculated and pistils were left to grow for 12–24 hr to achieve maturation. Then pistils were hand pollinated with pollens of Col-0 or the *mir159abc* mutant. After 12 hr, pistils were dissected and fixed in Carnoy's fixative solution (75% ethanol and 25% acetic acid) for 6 hours. The pistils were then washed in water for 2 min twice, and then mounted in clearing solution containing chloral hydrate, water and glycerol (ratio w/v/v: 8:3:1) for half an hour. The pistils were then washed twice in water and stained with 0.1% decolorized aniline blue (pH 8.0 in 0.1 M $K_3PO4$) for 6 hr. The stained pistils were observed using an Olympus BX53 microscope equipped with an ultraviolet filter set.

**Looped RT-PCR to detect mature miRNAs in pollen**

Primers were designed and looped RT-PCR was performed according to published protocols (Varkonyi-Gasic et al., 2007; Turner et al., 2013). Total pollen RNA was

treated with DNase RQ1 before reverse transcription. Purified RNA (200 ng) was reversely transcribed with a miR159-specific RT primer (miR159 slRT) and an U6-specific RT primer (U6 slRT) using PrimeScript II Reverse Transcriptase and Oligo d (T). cDNA was diluted (1:10) before PCR amplification. Primers miR159 epF/miR uniRT were used to amplify miR159; U6 amplified with U6 epF/miR uniRT was used as an internal control. PCR reactions were run for 25 cycles, and PCR products were analyzed on a 4% agarose gel. Primer information is listed in Table S2.

**RT-PCR and qRT-PCR analysis**

For quantification of *MYBs* transcripts in pistils, Col-0 flowers at stage 12 were emasculated and pistils were left to grow for 12–24 hr for maturation. Then pistils were pollinated with pollen of Col-0 or the *mir159abc* mutant. Pistils were collected quickly in liquid nitrogen at 0, 16, 36, 48, 72, 96 HAP. About 300 pistils were collected at each time point up to 48 HAP. About 150 pistils were collected at each time point after 48 HAP. RNA was extracted from pistils using Trizol reagent. Total RNA was treated with DNase RQ1 for 1 hr at 37℃ before reverse transcription. 2 ug purified RNA was reversely transcribed using PrimeScript II Reverse Transcriptase and Oligo d (T). RT products were diluted 1:10 before the PCR reaction. qRT-PCR assays were performed using iQ$^{TM}$ SYBR Green Supermix and Bio-Rad CFX96 Real-Time PCR detection system. Primer information is shown in Table S2.

**Supplemental information**

**Fig S1.** Validation of miR159 in mature pollen by northern blot and RT-qPCR.

**Fig S2.** Pollen viability analysis and expression of miR159 targets.

**Fig S3.** Seed development in F1 progeny from crosses of Col-0 and *mir159abc*.

**Fig S4.** Expression of miR159 targets and *MIR159* genes in pistils and seeds.

**Table S1.** RNA abundance of eight miR159 target genes in pollen and sperm cells.

**Table S2.** Primer sequences used in this study.

**Acknowledgments**

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**Table 1.** Reduced seed set in the *mir159abc* triple mutant.

| Female | Col-0 | Col-0 ♀ | *mir159abc* ♀ | *mir159abc* |
|---|---|---|---|---|
| | × | × | × | × |
| Male | Col-0 | *mir159abc* ♂ | Col-0 ♂ | *mir159abc* |
| Normal seeds | 7380 | 4721 | 3894 | 3184 |
| Undeveloped seeds | 150 | 2542 | 1917 | 2502 |
| Seed set | 98% | 65% | 67% | 56% |
| number (seeds) | 7531 | 7264 | 5812 | 5687 |

Average number of seeds per Col-0 silique was 50, while average number of the *mir159abc* mutant was 40. ~150 siliques from >50 plants were examined for each genetic background.

## Figure legends

**Fig 1.** Abnormal seed development caused by loss of paternal miR159.

(A) Percentage of siliques with indicated ratio of normal seed sets in reciprocal crosses between Col-0 and *mir159abc*. F1 siliques were divided into 4 categories according to the percentage of seed set per silique, I, II, III, IV indicate seed set >90% (blue), 60-90% (dark orange), 30-60% (grey), <30% (light orange), respectively. Percentage of siliques in each category is shown in pie charts. 150 siliques were examined for each genetic background.

(B) Representative F1 siliques from hand-pollinated self-fertilized wild-type (Col-0) and crossed plants with Col-0 ♀ × *mir159abc* ♂. I, II, III, IV indicate representative

siliques shown in (A).

**Fig 2.** The *mir159abc* triple mutant shows normal pollen development, pollen germination, and pollen tube guidance and reception.

(A and B) Mature pollen in Col-0 (A) and *mir159abc* (B) by DAPI staining. >500 pollen from 10 randomly picked flowers for each genotype were observed, and representative images were shown.

(C and D) *In vitro* pollen germination assay was performed for Col-0 (C) and *mir159abc* (D). Photos were taken after incubation for 7 hours.

(E, F, G, and H) *In vivo* pollen tube growth, guidance, and reception for Col-0 (E, G) and *mir159abc* (F, H). Pollen tubes were examined by ovule clearing and Decolorized Aaniline blue staining. >300 ovules from 10 randomly picked pollinated pistils for each genotype were observed, and representative images were shown. Arrowheads indicate the growing pollen tubes. Scale bars are 20 μm for (A, B, E, F, G, and H) and 200 μm for (C and D).

**Fig 3.** Lack of paternal miR159 caused defective endosperm nuclear division.

(A-H) Seeds of Col-0 and progeny from Col-0 ♀ × *mir159abc* ♂ at 4 hr after fertilization (A-D) and 12 hr after fertilization (E-H). Scale bar, 20 μm.

(I) Statistical analysis of numbers of endosperm nuclei from hand-pollinated self-fertilized Col-0 (blue), *mir159abc* ♀ × Col-0 ♂ (red), Col-0 ♀ ×*mir159abc* ♂ (grey), and hand-pollinated self-fertilized *mir159abc* (green) at 12 hr fertilization. >200 seeds from 10 siliques were examined for each genetic background.

(J) Mature F1 seeds of hand-pollinated self-fertilized Col-0, Col-0 ♀ × *mir159abc* ♂, *mir159abc* ♀ × Col-0 ♂, and hand-pollinated self-fertilized *mir159abc*. 500 F1 seeds were examined for each genetic background at each time point. White circles indicate the location of endosperm nuclei. The arrows indicate undivided zygotes.

**Fig 4.** Paternal miR159 represses maternally inherited *MYB33* and *MYB65*.

MYB33-GFP (A-C) and MYB65-RFP (D-F) in unfertilized ovules (A, D) and

454 fertilized seeds (B, C and E, F). White arrows indicate GFP and RFP in the central cell
455 and presumed endosperm, respectively. Scale bar in, 20 μm.
456
457

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