

# Paternal miRNA biogenesis contributes to seed development in *Arabidopsis*

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## Abstract

miRNAs are key regulators of gene expression and play important roles in various developmental processes. The development of the plant male gametophyte begins with a microspore, which undergoes two rounds of pollen mitosis to produce mature pollen, consisting of a vegetative nucleus and two sperm cells. Although many miRNAs are known to accumulate in mature pollen, it remains unclear how miRNA biogenesis is regulated during pollen mitosis and whether miRNA biogenesis in mature pollen is necessary for seed development. Here, we focus on DCL1, the major enzyme for miRNA biogenesis in *Arabidopsis*. Hand-pollination using *dcl1-7* mutant pollen results in severe seed development defects, characterized by shortened siliques and approximately 80% aborted seeds. While miRNA genes are primarily transcribed at early stages of pollen development, the core factors of the miRNA biogenesis machinery are expressed throughout pollen development, with preferential expression in the vegetative nucleus. Using an artificial miRNA strategy to conditionally knock down *DCL1* in the vegetative cell and sperm cells, respectively, we demonstrate that miRNA biogenesis in both cell types contributes to fertility control, but results in distinct defects in seed development. Collectively, these results show that miRNA biogenesis in mature pollen plays a significant role in regulating fertility and seed development.

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## Introduction

MicroRNAs (miRNAs) are endogenous non-coding RNAs, 20–24 nucleotides in length, which play a crucial role in regulating gene expression. They are pivotal modulators of developmental processes and responses to environmental stimuli in eukaryotes. The biogenesis of plant miRNAs consists of three major steps<sup>[1,2]</sup>. Endogenous *MIRNA* (*MIR*) genes are transcribed as independent units by Pol II, generating stem-loop structured pri-miRNAs. These pri-miRNAs are then processed into a miRNA/miRNA\* duplex with 2-nucleotide overhangs at the 3' ends by the dicing complex, which consists of the nuclear endonuclease DICER-LIKE 1 (DCL1), the double-stranded RNA-binding protein HYPOASTIC LEAVES 1 (HYL1), and the zinc-finger protein Serrate (SE)<sup>[3–5]</sup>. Next, the nascent miRNA/miRNA\* duplex undergoes 2'-O-methylation at the 3' ends by HUA ENHANCER1 (HEN1)<sup>[6]</sup>. In *Arabidopsis*, mutants with complete miRNA biogenesis defects are embryo-lethal. Embryos lacking DCL1 arrest early in development and exhibit aberrant patterning in most regions of the embryo<sup>[7,8]</sup>. Similarly, embryos of *serrate* and *hyl1* also show aberrant phenotypes at the beginning of the zygote development<sup>[9]</sup>. These abnormal phenotypes in an embryo could be partially attributed to the ovule development defects and reduced female fertility caused by mutations of miRNA biogenesis factors<sup>[10,11]</sup>. However, whether and to what extent the miRNA biogenesis factors in male gametophytes contribute to male fertility and early seed development are still not well understood.

In flowering plants, the male gametophyte microspores undergo two rounds of mitosis. The first asymmetric mitotic division (PMI) produces two daughter cells with different cell

fates: a larger vegetative cell (VC) and a smaller generative cell (GC). The VC exits its cell cycle while the GC undergoes another mitotic division known as pollen mitosis II (PMII) to produce two sperm cells (SCs). This process results in a mature pollen with the two sperm cells embedded in the cytoplasm of the vegetative cell<sup>[12]</sup>. Increasing evidence has revealed the extreme heterogeneity between two cell types in mature pollen, characterized by distinct epigenetic controls<sup>[13–17]</sup>. Interestingly, substantial communication persists between the VC and the GC/SCs, particularly involving the movement of small RNA reported during pollen development<sup>[18–22]</sup>. miRNAs also play a crucial role in these processes by regulating gene and transposable element (TE) transcripts. It has been reported that pollen miRNAs serve dual functions: one is to target genes related to pollen development and germination, while the other is to mediate the biogenesis of TE-derived secondary epigenetically activated siRNAs (easiRNAs), thereby initiating sperm silencing of TEs<sup>[23–25]</sup>. One typical example of the latter is the accumulation of miR845-directed 21, 22-nt easiRNAs in a dose-dependent manner, which mediates hybridization barriers between diploid seed parents and tetraploid pollen parents (triploid block)<sup>[26]</sup>.

The complexity of pollen development and the communication between two types of cells highlight the distinct biological functions of miRNA biogenesis in the VC and the GC/SC. In this study, we explored the spatiotemporal pattern of miRNA biogenesis across various stages of pollen development, focusing on the dynamics of *MIR* gene transcription and the distribution of miRNA biogenesis factors. Using an artificial miRNA strategy driven by cell type-specific promoters, DCL1 was selectively knocked down in the VC and GC/SC. Our findings

underscored the critical roles of miRNA biogenesis in these two cell types of pollen, emphasizing their contributions to fertility control and seed development.

## Materials and methods

### Plant materials and growth conditions

Columbia-0 (Col-0) ecotype was used in this study as wild-type plants. The Arabidopsis mutant lines used in this study, including *dcl1-7* (CS3089) and the *myb33 myb65* double mutant created by introducing a Cas9 deletion of *MYB65* into the *myb33* background (CS851168), are all in the Col-0 background. The reporter lines of *proMIR845a::GFP*, *proMIR845b::GFP*, *proMIR164a::GFP*, and *proMIR2939::GFP* were constructed by our lab, the *proMIR159a::GFP* and *proMIR159b::GFP* lines were described in a previous study<sup>[27]</sup>. Transgenic plants of *proVCK1::amiR\_DCL1* and *proHTR10::amiR\_DCL1* were generated in this study. All plants were grown in the soil with a humidity of 65% under a 16 h light/8 h dark photoperiod at 22 °C.

### Plasmid construction and plant transformation

To generate the constructs of *MIRNA* reporter lines of *proMIR845a::GFP*, *proMIR845b::GFP*, *proMIR164a::GFP*, and *proMIR2939::GFP*, the promoter region of *MIRNA* genes was amplified from Col-0 genomic DNA, cloned into pENTR-D/TOPO, and then transferred into *proGW::NLS-2 × GFP* by LR reaction. For *proVCK1::amiR\_DCL1* and *proHTR10::amiR\_DCL1*, the artificial miRNA were designed by the WMD3 website (<http://wmd3.weigelworld.org>), and the amiR\_DCL1 fragments were amplified from the pRS300-miR319a backbone and cloned to pB7WG2-HTR10 or pB7WG2-VCK1. For *myb65-cas9* mutant, the web tool CHOPCHOP (<http://chopchop.cbu.uib.no>) was used to design the sgRNAs. The primers of MYB65-DT1-BsF, MYB65-DT1-F0, MYB65-DT2-R0, MYB65-DT2-BsR were used to amplify the fragment using plasmid *pCBC-DT1T2* as a template, then the amplified fragment was digested with BsaI and inserted into *pHEE401E*. All primers are listed in [Supplementary Table S1](#).

### Phenotypic analysis

Flowers at stage 12 were emasculated and pistils were left to grow for ~12 h for maturation. Then pistils were hand-pollinated with pollen grains of Col-0 or the *dcl1-7* mutant. To measure the length and seed number of F1 siliques, and pistils at 7 days after pollination (DAP) were dissected and the seed numbers were counted under a Leica dissecting microscope. To calculate the proportion of viable seeds in different T1 lines of the transgenic plants, and self-pollinated pistils at 8 DAP were dissected under a microscope. To observe the pollen tubes growth *in vitro*, pollen grains were spread on the solid pollen germination medium (1 mM CaCl<sub>2</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 1.62 mM H<sub>3</sub>BO<sub>3</sub>, 18% (w/v) sucrose, 1% (w/v) low melting agarose, adjusted to pH 7.0 with 0.1 M KOH) and incubated in a humid chamber at 28 °C for 6 h<sup>[28]</sup>. To examine embryo development with differential interference contrast (DIC) microscopy, the seeds at 7 DAP were mounted in clearing solution (chloral hydrate : water : glycerol, w/v/v, 8:3:1) for DIC imaging with UPlanFLN × 20 objective. Images were further processed using Adobe Photoshop and Image J.

### Microscopy

To visualize the promoter-reporter of *MIRNA* genes, fluorescence microscopy analysis was carried out with an Olympus

BX53 microscope (image acquisition software: QCapture Pro7; objectives: UPlanFLN 40×). DAPI staining of pollen was examined under a UV channel using UPlanFLN 40 × objectives. To visualize the localization of miRNA biogenesis factors, pollen grains were observed by the Leica Stellaris 5 WLL confocal microscope with the lightning mode. Images were processed using LAS X and Image J.

### Statistical analysis

All raw data were imported into GraphPad Prism version 9.5 (GraphPad Software) and then analyzed by Student's *t*-test, one-way ANOVA, or Chi-square test. A value of *p* < 0.05 was considered as statistically significant. \*\*\*\*, \*\*\*, \*\*, and \* indicate *p* < 0.0001, 0.001, 0.01, and 0.05, respectively. ns, no significant difference.

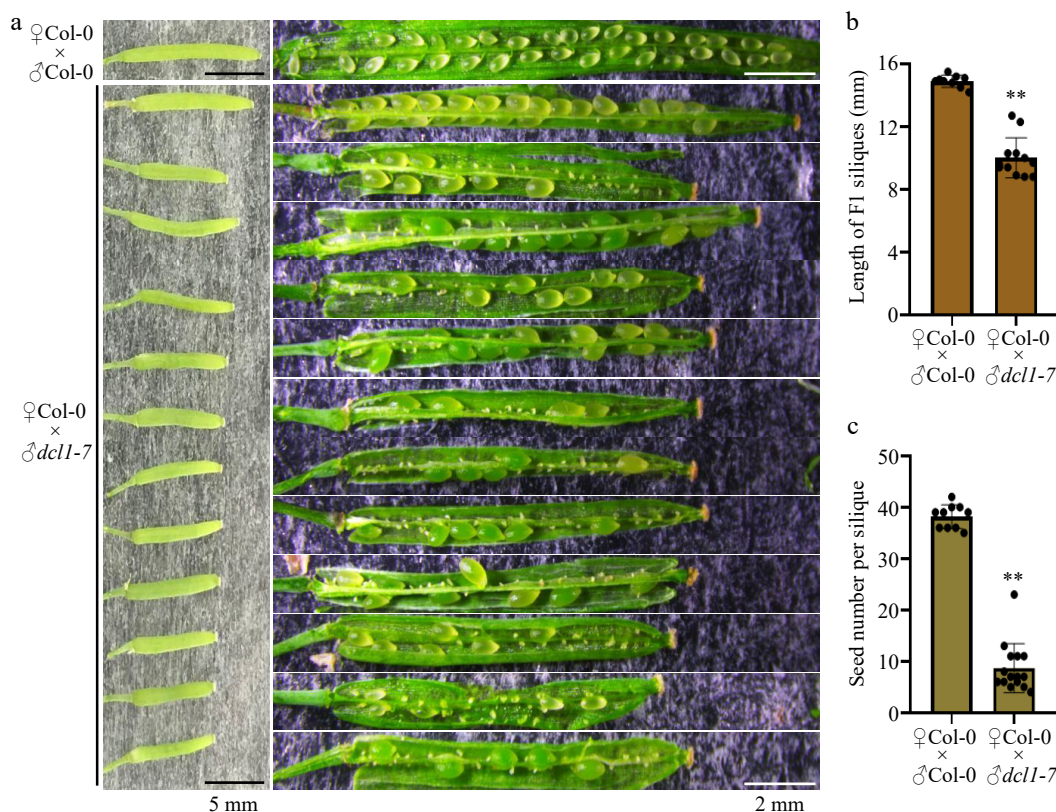
## Results

### Mature pollen-expressed DCL1 is required for subsequent seed development

It has been reported that several *dcl1* mutants, such as *dcl1-3*, *dcl1-4*, *dcl1-5*, *dcl1-10*, *dcl1-7*, and *dcl1-15*, display abnormalities in embryo development<sup>[9]</sup>. Moreover, maternal defects of several *dcl1* alleles significantly contribute to the defects of embryo development<sup>[10,11]</sup>. However, it has been shown that paternal DCL1 from wild-type pollen can partially rescue the embryo-lethal phenotype when *dcl1-5/+* mutants are used as females<sup>[29]</sup>. This finding indicates that paternal DCL1-mediated miRNA biogenesis potentially contributes to embryo development. In this study, a weak allele, *dcl1-7* (CS3089) was used, which allowed for the production of viable homozygous mutant plants, to perform the hand-pollination assay. The *dcl1-7* homozygous plants displayed defects in anther development ([Supplementary Fig. S1a](#)) and a large proportion of pollen grain with low viability ([Supplementary Fig. S1b](#)). Notably, at least 50 pollen grains per anther retained normal viability. Furthermore, DAPI staining revealed that these viable pollen grains in the *dcl1-7* mutant contained a vegetative nucleus and two sperm nuclei ([Supplementary Fig. S1c & d](#), also shown below), similar to wild-type pollen grains. This suggests that the non-viable pollen grains are likely a result of impaired anther (sporophyte) development, rather than defects in male gametophyte development itself. Given that some pollen grains remained viable in the *dcl1-7* mutant, we aimed to investigate whether paternal miRNA biogenesis plays a role in subsequent processes such as double fertilization and early seed development.

To investigate this question, we assessed seed development by pollinating wild-type pistils with excess pollen from both Col-0 wild-type pollen and the *dcl1-7* mutant pollen. Compared with F1 siliques from ♀Col-0 × ♂Col-0 crosses, F1 siliques from ♀Col-0 × ♂*dcl1-7* crosses showed significantly shortened siliques ([Fig. 1b](#)) and severe defects in seed development, resulting in only 20%–30% viable seeds ([Fig. 1c](#)). These observations indicate that paternal miRNA biogenesis machinery in mature pollen contributes to subsequent seed development.

To conclusively demonstrate these results, we followed our previous study, where sperm-delivered miR159 was shown to promote seed development by removing maternal roadblock targets<sup>[30]</sup>. Given the developmental defects observed from early vegetative growth stages in the *mir159abc* triple mutant, we investigated the contribution of paternal-generated miR159 to the phenotypes of shortened siliques and aborted seeds.



**Fig. 1** Hand-pollination by the *dcl1-7* mutant pollen caused defects in seed production. (a) Representative F<sub>1</sub> siliques from ♀Col-0 × ♂Col-0 and ♀Col-0 × ♂*dcl1-7* respectively. The *dcl1-7* mutant pollen as the male causes aborted seeds. Black bars indicate 5 mm length, and the white bars indicate 2 mm length. (b), (c) Statistical analysis showing the (b) length, and (c) the total number of the F<sub>1</sub> siliques. Each point indicates one silique, *p*-value were calculated using an unpaired, two-sided Student *t*-test (\*\* *p* < 0.01). Scale bars are shown.

Consistent with the male germ cell-specific expression of *HTR10*<sup>[31]</sup>, the over-expression of *MIR159a* driven by the *HTR10* promoter did not complement the vegetative growth phenotypes (Supplementary Fig. S2a), but partially rescued the defects on silique length and seed setting rate of the *mir159abc* triple mutant (Supplementary Fig. S2b–d). The defects in endosperm nuclear divisions fertilized by *mir159abc* pollen were attributed to the retention of miR159 targets MYB33 and MYB65 in the central cell after fertilization<sup>[30]</sup>. Moreover, the decreased proportion of viable seeds in F<sub>1</sub> siliques obtained by *mir159abc* pollen as male could be restored when using the *myb33 myb65* double mutant as female instead of Col-0 (Supplementary Fig. S2e–g), although the *myb33 myb65* double mutant exhibited partial shortened silique phenotype in self-pollination (Supplementary Fig. S2f). These results further confirmed our previous observation that sperm-delivered miR159 promotes seed development by repressing maternal MYB33 and MYB65. Collectively, it was conclusively demonstrated that paternal miRNA biogenesis is essential for fertilization and viable seed production.

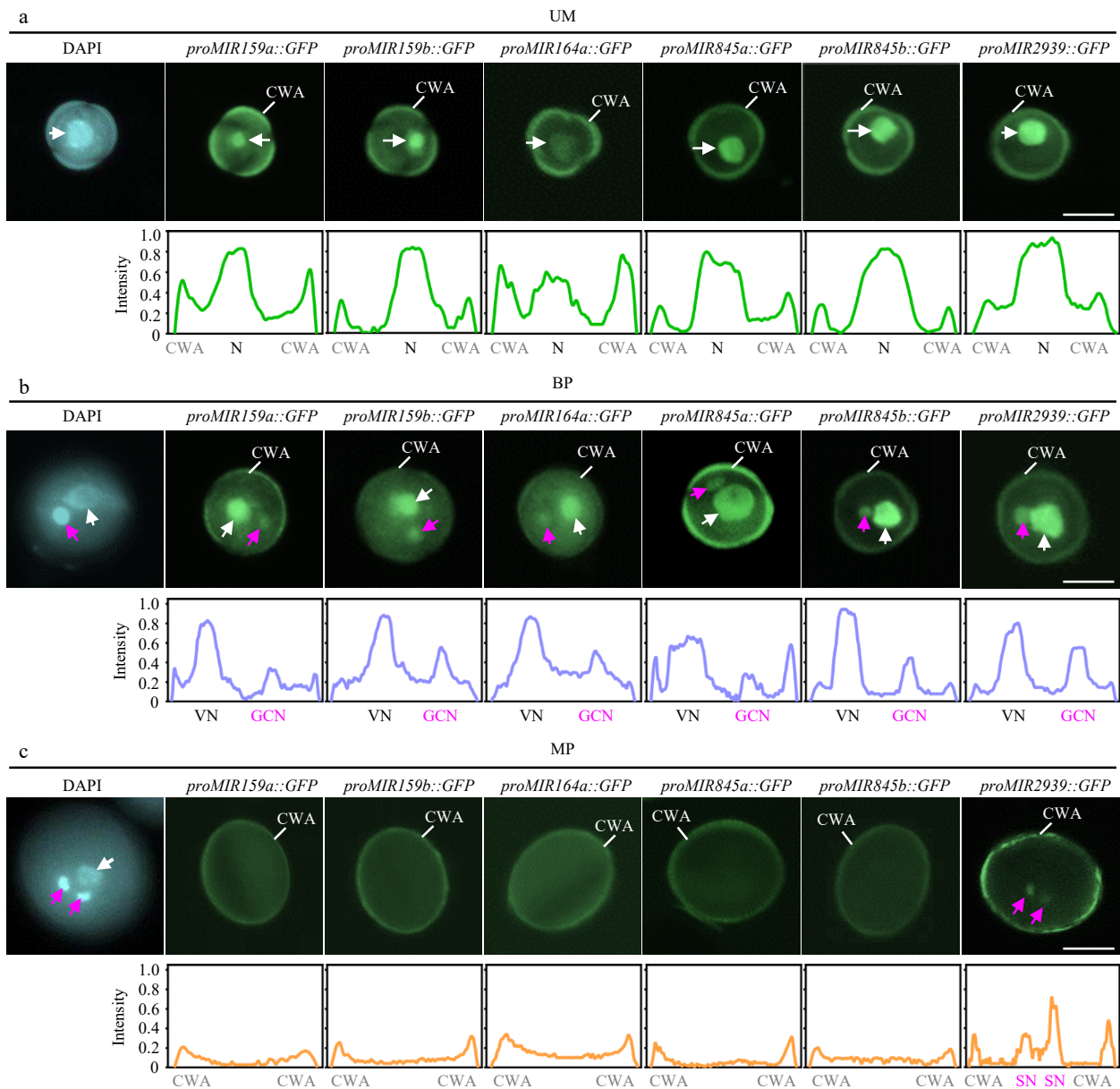
### MIR genes are preferentially transcribed at early stages of pollen development

A recent study has analyzed the miRNA profiles at the microspore, bicellular pollen, and mature pollen stages, thereby providing an atlas of the miRNome during pollen development<sup>[25]</sup>. The overall levels of total miRNAs are similar among microspore, bicellular pollen, and tricellular pollen stages, but slightly increased in mature pollen (Supplementary

Fig. S3a). This suggests that *MIR* transcription and/or miRNA biogenesis may not exhibit significant dynamics during pollen development. To investigate whether this is the case, the timing and location of *MIR* gene transcription during pollen mitoses was first examined, focusing on several well-known abundant miRNAs in mature pollen. Several transgenic lines with GFP reporters driven by endogenous promoters of pollen-enriched miRNAs were constructed, including *MIR159a*, *MIR159b*, *MIR164a*, *MIR845a*, *MIR845b*, and *MIR2939*. The present findings show that all these *MIR* genes initiated their transcription primarily early in the microspore stage before PMI (Fig. 2a). Following PMI, transcription of these tested *MIR* genes continued in both VC GC, with a slightly stronger signal observed in the VC-based on GFP intensity (Fig. 2b). However, the transcriptional signal of five out of the six tested *MIR* genes was completely absent in both VC and GC at the mature pollen stage (Fig. 2c). The exception was *proMIR2939::GFP*, which exhibited weak but robust expression signals in sperm cells (Fig. 2c). Considering that all four miRNAs are relatively steadily produced across pollen development (Supplementary Fig. S3b)<sup>[25]</sup>, the phenomena of *MIR* transcription at early stages of pollen development indicate that miRNAs detected in mature pollen are obtained by either local processing of early transcribed pri-miRNA or inherited from bicellular pollen via PMII.

### miRNA biogenesis factors are ubiquitously expressed during pollen mitosis

Since the transcription of *MIR* genes mainly occurs before the second pollen mitosis, does miRNA biogenesis also exhibit

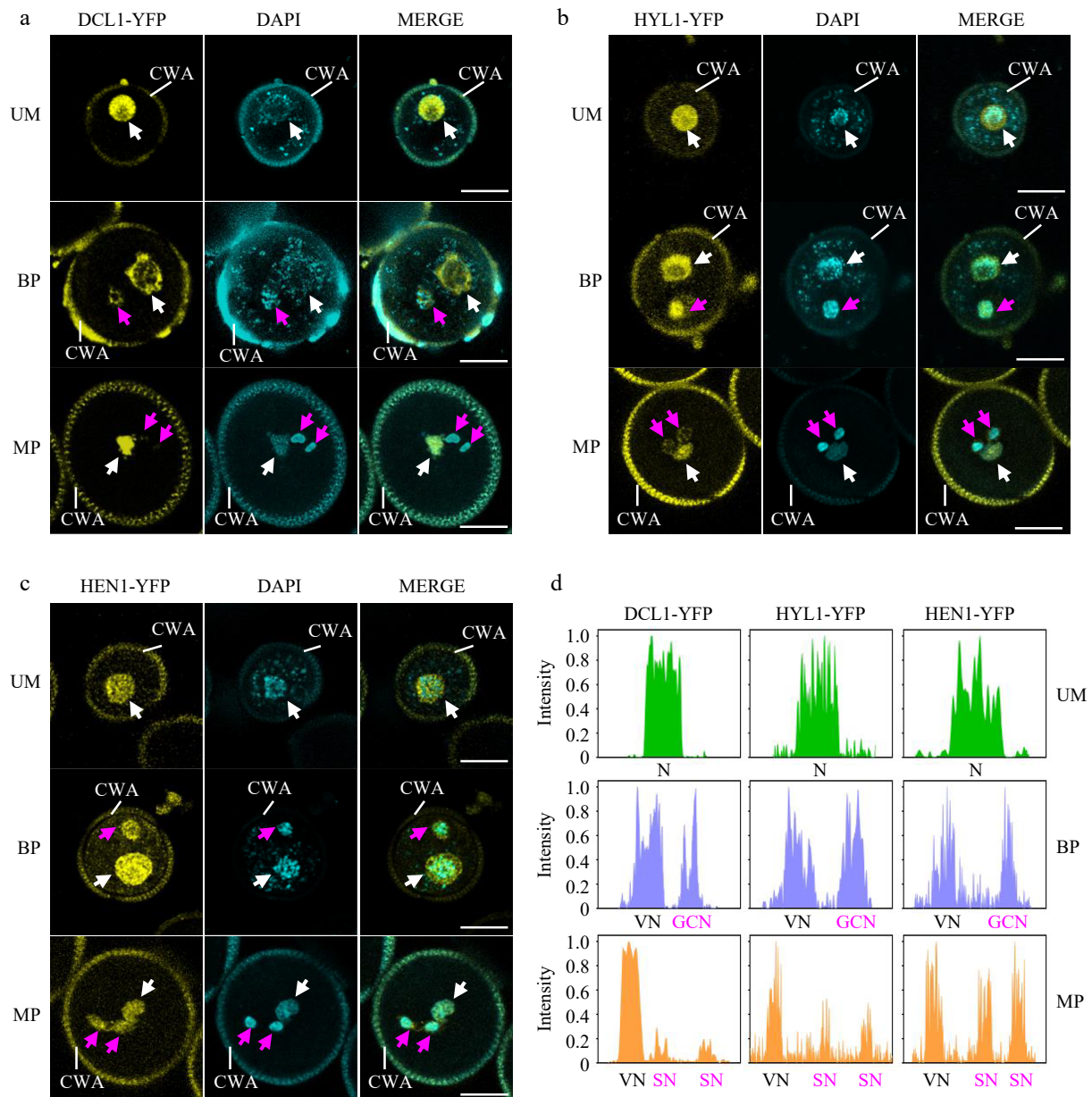


**Fig. 2** Spatiotemporal patterns of *MIR* transcription during pollen development. The expression of *proMIR159a::GFP*, *proMIR159b::GFP*, *proMIR164a::GFP*, *proMIR845a::GFP*, *proMIR845b::GFP*, and *proMIR2939::GFP* was observed during pollen development by fluorescence microscopy. Representative pollen grains show DAPI fluorescence (left, blue) and GFP fluorescence (right, green) at uncellular microspore [(a), UM], bicellular pollen [(b), BP], and mature pollen [(c), MP] stages. White arrowhead indicates N or VN; magenta arrowhead indicates GCN or SN. Relative intensity curve of the GFP signal were shown below the images. VN, vegetative nucleus; GCN, generative cell nucleus, SN, sperm cell nuclei; CWA, cell wall autofluorescence. Scale bar = 10  $\mu$ m.

similar properties? RNA-seq data from the vegetative cells and sperm cells of mature pollen were first analyzed<sup>[32]</sup>, focusing on the expression of several main factors involved in miRNA biogenesis. The results show that while DCL1, SE, and HEN1 are detectable in both sperm cells and the vegetative cell, the overall mRNA levels of these genes are low in mature pollen (Supplementary Fig. S3c). Notably, mRNA of HYL1 is barely detected in mature pollen (Supplementary Fig. S3c). These analyses indicate that the local activity of the intact miRNA biogenesis machinery in mature pollen is low.

To further elucidate the spatiotemporal pattern of miRNA biogenesis during pollen mitosis, we investigated the subcellular

localization of DCL1, HYL1, and HEN1 throughout pollen development using corresponding transgenic plants expressing DCL1-YFP, HYL1-YFP, and HEN1-YFP, each driven by their native promoters<sup>[33]</sup>. The results show that all three proteins are highly detected in the microspore (Fig. 3a–d, top panels), indicating the potential for local processing of pri-miRNA. After the first pollen mitosis, there were no significant differences in DCL1-YFP, HYL1-YFP, and HEN1-YFP signal intensity between the vegetative cell and the generative cell at the bicellular pollen stage (Fig. 3a–d, the middle panels). However, the intensities of DCL1-YFP and HYL1-YFP were decreased in sperm cells at the mature pollen stage, while the intensity of HEN1-YFP in sperm



**Fig. 3** Spatiotemporal patterns of miRNA biogenesis factors during pollen development. (a)–(c) The localization of (a) DCL1-YFP, (b) HYL1-YFP, and (c) HEN1-YFP was observed during pollen development by confocal microscopy. Representative pollen grains show YFP fluorescence (right, yellow) and DAPI fluorescence (right, cyan) at unicellular microspore (UM), bicellular pollen (BP), and mature pollen (MP) stages. White arrowhead indicates N or VN; magenta arrowhead indicates GCN or SN. VN, vegetative nucleus; GCN, generative cell nucleus, SN, sperm cell nuclei; CWA, cell wall autofluorescence. Scale bar = 10 μm. (d) Relative intensity curve of YFP signal in (a) DCL1-YFP, (b) HYL1-YFP, and (c) HEN1-YFP.

cells remained at the same level as in the vegetative cell (Fig. 3a–d, the middle panels). These results indicate that miRNA biogenesis factors persist in the vegetative cell throughout pollen development. Instead, miRNA biogenesis activity may be compromised in sperm cells due to a much lower accumulation of DCL1, suggesting that the miRNAs enriched in sperm cells may be inherited from bicellular pollen or transferred from the vegetative cell at the mature pollen stage. The high signal of HEN1-YFP suggests that sperm-enriched small RNAs may require HEN1-mediated 3' end methylation. Notably, while the miRNA biogenesis machinery has been found to localize in dicing bodies, no nuclear punctate structures were observed

for all three proteins in pollen. This indicates that the subcellular territory of miRNA biogenesis in pollen may differ from that in somatic cells.

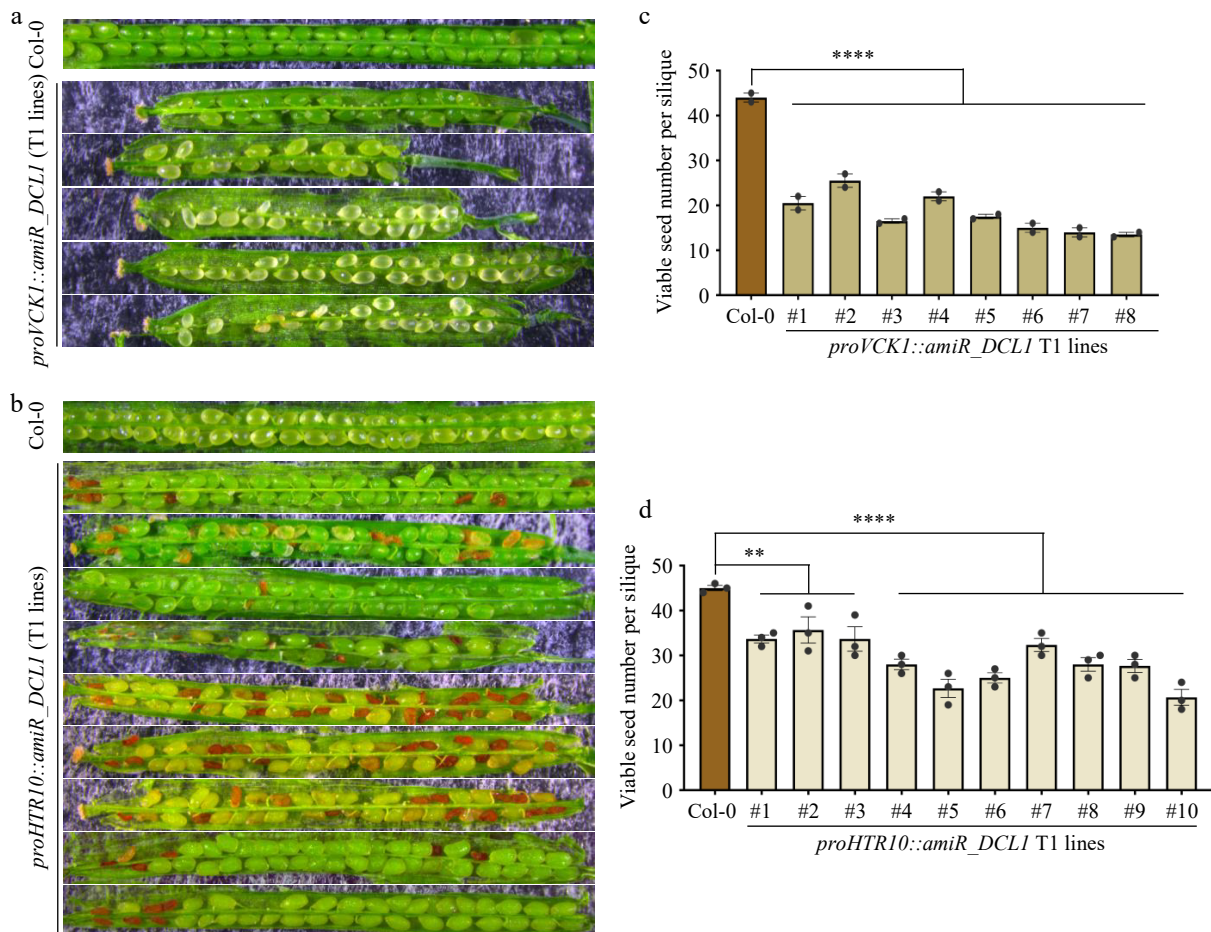
### Conditional knockdown of *DCL1* in VC and GC/SC caused compromised but distinctive defects in seed development

As the transcription of *MIR* genes is mostly completed before the second pollen mitosis (Fig. 2), what is the biological significance of the persistence of the miRNA biogenesis machinery in mature pollen? To investigate the role of miRNA biogenesis factors detected in the vegetative cell and sperm cells of

mature pollen, two types of transgenic lines were generated with cell-type-specific knockdown of *DCL1* in the VC and GC/SC, respectively. Expression of the artificial miRNA targeting *DCL1* was achieved by using the late VC-specific promoter *VCK1*<sup>[34]</sup> and the GC/SC-specific promoter *HTR10*<sup>[31]</sup>, respectively. These constructs were transformed into the *proDCL1::DCL1-YFP* reporter line to primarily evaluate the efficiency of knockdown by the artificial miRNA (Supplementary Fig. S4a). As shown in Supplementary Fig. S4a, the introduction of the VC-expressed artificial miRNA targeting *DCL1* significantly attenuated the signal intensity of *DCL1*-YFP in the vegetative cell. Similarly, the introduction of the GC/SC-expressed artificial miRNA targeting *DCL1* specifically attenuated the signal intensity of *DCL1*-YFP in the sperm cells, while having no effect on the signal in the vegetative cell. When *proVCK1::amiR\_DCL1* was introduced, the faint signal of *DCL1* in the vegetative nucleus could be still observed, indicating a possible leaky expression. Whether the reduced expression of *DCL1* in the vegetative cell and sperm cells affects double fertilization and subsequent seed development were then examined. In eight independent transgenic T1 lines expressing *proVCK1::amiR\_DCL1* in the vegetative cell, we found that these plants exhibited noticeably shortened siliques, reduced seed numbers, and approximately half of the seeds

were unfertilized (Fig. 4a, c), which resembled the phenotypes observed in the F1 silique of ♀Col-0 × ♂*dcl1-7* (Fig. 1a). Interestingly, in ten individual transgenic T1 lines expressing *proHTR10::amiR\_DCL1* in the generative cell and sperm cells, it was observed that approximately 30% of seeds exhibited a typical embryo lethal phenotype (Fig. 4b). Additionally, these plants produced an average of about 30 seeds per silique (Fig. 4d), compared to the Col-0 plants, which produce approximately 50 seeds per silique on average. These results indicating that *DCL1* expressed in sperm is involved in early embryogenesis after double fertilization.

Given the nature of phenotypes in seed development of plants with conditional knockdown of *DCL1* in mature pollen, we hypothesized that the seed development defects in *proVCK1::amiR\_DCL1* plants should be attributed to the fertilization failure rather than post-fertilization impairment. To test this, the *in vitro* pollen germination rate of Col-0 and *proVCK1::amiR\_DCL1* heterozygous plants were preliminarily assessed. Compared to Col-0, five transgenic lines of *proVCK1::amiR\_DCL1* showed a significantly reduced pollen germination rate (Supplementary Fig. S4b & c), indicating that VC-expressed *DCL1* plays an important role in pollen germination. In contrast, the transgenic lines expressing



**Fig. 4** Both VC and GC/SC-knockdown of *DCL1* caused compromised but distinctive defects in seed development. (a) Seed development defects of *proVCK1::amiR\_DCL1* transgenic T1 lines, representative siliques are shown. (b) Seed development defects of *proHTR10::amiR\_DCL1* transgenic T1 lines, representative siliques are shown. (c), (d) Statistical analyses of viable seed number per silique in (c) *proVCK1::amiR\_DCL1*, and (d) *proHTR10::amiR\_DCL1* transgenic T1 lines. Each column indicates one individual T1 line. *p*-values were calculated by one-way ANOVA Dunnett's multiple comparison, \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ .

*proHTR10::amiR\_DCL1* exhibit approximately 30% aborted seeds (Fig. 4b & d). To further investigate the arrested stage of the abnormal seeds in the *proHTR10::amiR\_DCL1* transgenic lines, we observed the embryo development of normal and abnormal seeds in a same *proHTR10::amiR\_DCL1* silique at 7 DAP, when the abnormal seeds had not been completely dried out. DIC microscopy analyses showed that the embryos of the abnormal seeds were arrested at the globular stage, while mature embryos had been developed in the normal seeds of the same silique (Supplementary Fig. S4d). The arrest of embryos at the globular stage is consistent with the embryo abnormalities observed in several *dcl1* mutant alleles<sup>[7–9,29]</sup>, demonstrating that paternal DCL1 contributes to early embryo patterning during seed development. Together, it is concluded that mature pollen-expressed DCL1 is necessary for pollen germination and early embryo development.

## Discussion

The embryo lethal phenotype of *dcl1* mutant alleles highlights the remarkable role of miRNA biogenesis during seed development<sup>[8,9,29]</sup>. Due to the size discrepancy between sperm cells and the egg cell, it is generally believed that substances in egg cells play a major role in early development. However, it is increasingly clear that the male gamete carries additional coding or non-coding RNAs necessary for early development in different species<sup>[35,36]</sup>. Our previous work shows that paternal miR159 is required to promote endosperm nuclear divisions during early seed development<sup>[30]</sup>. In this study, we further demonstrate that mature pollen-expressed miRNA biogenesis factors contribute to both the fertilization process and early embryo development.

The spatiotemporal dynamics of *MIR* transcription and miRNA biogenesis factors through pollen mitosis indicate that the biogenesis of most pollen-enriched miRNAs is completed at early developmental stages before PMII. Furthermore, the miRNA biogenesis patterns between the vegetative cell and male germ cells exhibit both similarities and differences, supported by these observations: (i) pollen-expressed *MIR* genes preferentially complete their transcription before the second pollen mitosis in both the vegetative cell and the generative cell; (ii) miRNA biogenesis factors are localized in both the vegetative cell and generative cell at early developmental stages; (iii) the abundance of DCL1 shows a striking decrease in sperm cells compared to the vegetative cell. The differences in the dicing complex between the vegetative cell and sperm cells might be attributed to the de-condensed chromatin in the vegetative cell and the gradually increasing chromatin condensation in sperm cells<sup>[37]</sup>. Notably, previous study on miRNA profiling in vegetative and sperm cells have shown that specific miRNAs are distinctly enriched between these two cell types<sup>[38]</sup>, indicating that miRNA distribution within mature pollen is tightly regulated. Based on the vegetative cell-preferred localization of DCL1 and the transcription of *MIR* genes before sperm cell formation, the specificity of miRNA distribution in the two cell types within mature pollen may not be determined by the localization of miRNA biogenesis machinery. Instead, likely specific miRNAs are actively transferred from the vegetative cell to the sperm cells<sup>[18–21]</sup>. One typical example is miR159, where the transcription of three *MIR159* genes occur before the second pollen mitosis, but mature miR159 is significantly enriched in sperm cells<sup>[38]</sup>.

Unlike specific miRNAs that function in pollen by inhibiting target genes expressed either in pollen itself or on the maternal side, the fact that reduced expression of *DCL1* in both the vegetative cell and sperm cells causes distinct defects in seed development indicates that mature pollen-expressed *DCL1* is important for specific miRNA biogenesis in pollen and the pollen tube, or is delivered into female gametes *via* fertilization to promote early miRNA biogenesis. The effects of vegetative cell-expressed *DCL1* on pollen germination and sperm cell-expressed *DCL1* on embryo development align with the default functions of the vegetative cells and sperm cells, respectively. The present data showed that a significantly reduced pollen germination rate in vegetative-cell *dcl1* knockdown plants, indicating that the seed development defects in *proVCK1::amiR\_DCL1* plants are likely due to fertilization failure rather than post-fertilization impairment. We suggest that DCL1-mediated miRNA biogenesis in the vegetative cell may also play a role in pollen tube targeting or other processes required for successful double fertilization. Meanwhile, the embryo development defects in *proHTR10::amiR\_DCL1* transgenic lines resemble the embryo abnormalities in *dcl1* mutants<sup>[9,29]</sup>, indicating that not only maternal but also paternal DCL1 contributes to early embryo patterning.

## Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Yang H, Zhao Y, Zheng B; experiments conducted: Yang H, Zhao Y; proMIR2939 reporter construction: Lin Z; phenotypic analysis: Jiang T; DCL1-YFP transgenic plants construction: Ren G; draft manuscript preparation: Yang H, Zheng B. All authors reviewed the results and approved the final version of the manuscript.

## Data availability

All data generated or analyzed during this study are included in this published article and its Supplementary information files.

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## Conflict of interest

The authors declare that they have no conflict of interest. Binglian Zheng is the Editorial Board member of *Seed Biology* who was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of this Editorial Board member and the research groups.

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