**Dicer-like 5 deficiency confers temperature-sensitive male sterility in maize**

Chong Teng[^1^,5], Han Zhang[^2^,5], Reza Hammond[^3], Kun Huang[^3], Blake C. Meyers[^1,4][✉] & Virginia Walbot[^2][✉]

Small RNAs play important roles during plant development by regulating transcript levels of target mRNAs, maintaining genome integrity, and reinforcing DNA methylation. *Dicer-like 5* (*Dcl5*) is proposed to be responsible for precise slicing in many monocots to generate diverse 24-nt phased, secondary small interfering RNAs (phasiRNAs), which are exceptionally abundant in meiotic anthers of diverse flowering plants. The importance and functions of these phasiRNAs remain unclear. Here, we characterized several mutants of *dcl5*, including alleles generated by the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system and a transposon-disrupted allele. We report that *dcl5* mutants have few or no 24-nt phasiRNAs, develop short anthers with defective tapetal cells, and exhibit temperature-sensitive male fertility. We propose that DCL5 and 24-nt phasiRNAs are critical for fertility under growth regimes for optimal yield.

[^1]: Donald Danforth Plant Science Center, St. Louis, MO 63132, USA.  
[^2]: Department of Biology, Stanford University, Stanford, CA 94305, USA.  
[^3]: Delaware Biotechnology Institute, University of Delaware, Newark, DE 19716, USA.  
[^4]: Division of Plant Sciences, University of Missouri—Columbia, Columbia, MO 65211, USA.  
[^5]: These authors contributed equally: Chong Teng, Han Zhang.  
[^✉]: Email: bmeyers@danforthcenter.org; walbot@stanford.edu
Three major classes of endogenous small RNAs (sRNAs) exist in plants: microRNAs (miRNAs), heterochromatic small interfering RNAs (hc-siRNAs), and phased, secondary small interfering RNAs (phi-siRNAs). From extensive sRNA sequencing in plants, numerous loci generating phasiRNAs have been reported; in grasses, phasiRNAs are enriched in flowers, particularly male reproductive organs1-5. In this context, phasiRNA production is initiated by miRNA-mediated cleavage of RNA polymerase II transcripts of two classes of PHAS loci. Subsequently, the 3′ portion of cleaved transcripts is converted to double-stranded RNA, a substrate for precise chopping by DICER-LIKE 4 (DCL4) yielding 21-nt products; a distinct, proposed role for DCL5 is generation of 24-nt phasiRNAs6-8. The 21-nt phasiRNAs are highly abundant during initial cell fate setting in maize anthers 1 and are important for male fertility in rice9. The 24-nt phasiRNAs accumulate coincident with meiotic start, peak during meiosis, and persist at lower levels afterwards; this pattern has generated speculation that they regulate meiosis8-9.

In angiosperms, five DICER-LIKE (DCL) proteins have been described and partially characterized10. DCL1 is important for miRNA biogenesis, as illustrated by regulation of meristem determinacy in maize inflorescences11. In Arabidopsis thaliana, DCL2 processes viral-derived and transgene-derived siRNAs12; DCL3 produces 24-nt hc-siRNAs, which then direct DNA methylation of target loci13. In many plants including Oryza sativa (rice), DCL4 generates 21-nt trans-acting siRNAs and phasiRNAs4. Although the functions of these four DCL genes are well conserved in flowering plants, these genes have partially overlapping functions12,14. A fifth, more recently described and named gene, Dcl5, is monocot-specific15. To better understand the function of DCL5 and its proposed role in 24-nt phasiRNA biogenesis1, we characterized maize dcl5 mutants generated using either a high efficiency CRISPR-mediated gene editing system15 or transposon-based mutagenesis. Here, we report that dcl5 mutants have few or no 24-nt phasiRNAs, exhibit defects in tapetal cell differentiation, and show temperature-sensitive male fertility. We propose that DCL5-mediated generation of 24-nt phasiRNAs is required for fertility at temperatures that are optimal for maize yield.

Results

Null mutants of the maize Dcl5 gene confer male-sterility. Using a CRISPR system targeting the middle of the encoded protein15, we generated four alleles for analysis (Fig. 1a), and from T0 plants we developed stable lines that show Mendelian inheritance of dcl5-1 (Fig. 1b, Supplementary Table 1 and Supplementary Fig. 1). dcl5-1 and dcl5-4 are frameshift mutants with transcript levels reduced to about a third of their wild type sibs; by contrast, dcl5-2 (3 bp deletion) and dcl5-3 (12 bp deletion, 1 bp substitution) have similar or higher transcript levels compared to control siblings (Fig. 1b and Supplementary Fig. 2). Wild type and heterozygous dcl5-1/Dcl5 plants greenhouse-grown at 32 °C maximum day/21 °C minimum night were identical in whole plant architecture, anther morphology, and fertility. Under the same conditions, homozygous dcl5-1 plants were male sterile (Fig. 1c). Compared to their fertile siblings, sterile dcl5-1 plants lacked visible differences in tassels and spikelets, however, the sterile anthers were shorter, contained shrunken pollen, and did not exert from the spikelets (Fig. 1c and Supplementary Fig. 3). Sporadically, a few anthers exerted and shed viable pollen. Under field conditions, all four alleles showed male sterility and we did not observe any differences in phenotype between alleles. No genomic editing was detected in the homologous sequence in Dcl5 which is the most likely potentially off-target locus (Supplementary Fig. 4). To further, independently verify the observation of male sterility, we acquired an additional allele from the transposon-based Illumina-Mu population16 (dcl5-mu03, line mu-illumina_139042.7). dcl5-mu03 showed obvious male sterility in both greenhouse and field conditions (Supplementary Fig. 5 and Supplementary Table 1). We concluded that Dcl5 is required for robust male fertility.

dcl5-1 mutants exhibit tapetal defects. To investigate whether dcl5-1 plants display defects in anther cell patterning, we used confocal microscopy and found normal somatic layer architecture without defects in initial cell differentiation (Supplementary Fig. 6). In addition, chromosome pairing, alignment, and meiotic progression were normal in dcl5-1 meiocytes (Supplementary Fig. 7a–l). The dcl5-mu03 meiocytes also routinely completed meiosis and produced haploid gametophytes (Supplementary Fig. 7m, n). Transmission electron microscopy was utilized to visualize nuclei and other cell organelles at higher resolution. During the mid-meiosis stage (2–2.5 mm anthers), normal tapetal cells were densely packed with dark-staining materials and were mostly binucleate (Fig. 2a and Supplementary Fig. 8). By contrast, the tapetal cells in dcl5-1 were pale and mostly mononucleate, and many cell organelles were not clearly resolved (Fig. 2b and Supplementary Fig. 8). Whole mount, fluorescence microscopy was used to quantify binucleate status in dcl5-1 and fertile anthers. In 1.5 mm anthers (prophase I), there were five-fold fewer binucleated tapetal cells in dcl5-1; a significant difference persisted at 2.5 mm (meiosis II) and a large difference remained at 3.0 mm (post-meiosis) (Fig. 2 and Supplementary Fig. 9). In extensive confocal microscopic examination of dcl5-1 anthers, we observed no significant or consistent differences in cell number or volume compared to fertile siblings. About one week after the completion of meiosis (2.5 mm stage), the tapetum starts programmed cell death, vacuolates, collapses, and disappears in the dcl5-1/Dcl5 anthers (Supplementary Fig. 10); by contrast, the tapetum is developmentally delayed and retained in the dcl5-1 anthers (Supplementary Fig. 10). The middle layer in dcl5-1/Dcl5 collapses and disappears, yet it is largely retained in dcl5-1 (Supplementary Fig. 10). Collectively, these observations indicate that tapetal development is delayed or arrested in dcl5 mutants and that the dcl5 tapetal cells are therefore likely defective in conducting post-meiotic functions supporting pollen maturation.

Tapetum-specific and meiocyte-specific Dcl5 expression. To obtain a comprehensive spatiotemporal profile of maize Dcl5 expression, we queried microarray, published and newly-generated RNA-seq (Supplementary Table 2) and proteomics data. Dcl5 transcripts are highly enriched in tassels, cobs, embryos, and seeds17,18, as well as fertile anthers. By contrast, DCL5 protein is low in 1.0 mm, pre-meiotic anthers and extremely high in 2.0 mm mid-meiosis anthers, but is undetectable in ear, embryo, or endosperm19. Microarray analysis of laser-microdissected anther cell types20,21 and in situ hybridization analysis of anther lobes1,22 established that Dcl5 transcripts are highly enriched in the tapetum and are present at lower levels in pre-meiotic pollen mother cells and meiocytes. RNA-seq confirmed Dcl5 expression in isolated maize meiocytes, however, quantitatively there are even higher levels in whole anthers23, as confirmed by newly-generated anther data (Supplementary Table 2). Therefore, Dcl5 is expressed much more highly in one or more somatic cell types than in meiocytes23. Integrating prior observations that 24-nt phasiRNA biogenesis is contingent on a normal tapetum1, with the peak of Dcl5 and DCL5 expression in meiotic anthers, we conclude that the 24-nt phasiRNA pathway is localized in tapetal cells. We hypothesize that this localization and
timing is of functional importance for the redifferentiation of tapetal cells into secretory cells during the meiotic period.

24-nt phasiRNA abundance is greatly reduced in $dcl5$ mutants.

To investigate if 24-nt phasiRNA biogenesis is affected in $dcl5$ mutants, sRNA libraries were constructed from anthers or spikelets of each $dcl5$ allele (Supplementary Table 2). Previously, we found that 24-nt phasiRNAs were readily detected from 176 24-PHAS loci in W23 inbred anthers1. The 24-nt sRNAs produced from all these loci were reduced dramatically in plants homozygous for each $dcl5$ allele compared to fertile siblings even though $dcl5-2$ and $dcl5-3$ encode proteins that lack only a few amino acids (Fig. 3 and Supplementary Fig. 5c). Other sRNAs, including 24-nt hc-siRNAs and 21-nt phasiRNAs were retained (Supplementary Fig. 11). We conclude that $Dcl5$ is required for 24-nt phasiRNA biogenesis and that the $dcl5-2$ and $dcl5-3$ mutations define amino acids essential for $DCL5$ function. Analysis of 24-PHAS precursors in RNA-seq anther libraries from the same plants showed greater or nearly the same levels of precursor abundance in each of the $dcl5$ mutant alleles (Supplementary Fig. 12). Therefore, the absence of functional DCL5 severely disrupts processing of 24-nt phasiRNA precursors with a modest impact on their accumulation.

Analysis of these RNA-seq data to characterize downstream transcriptional pathways impacted in $dcl5$ mutants was uninformative, consistent with prior results that failed to identify mRNA targets for 24-nt phasiRNAs1. The transcriptional changes in $dcl5-1$ mutants were minimal (23 transcripts altered two-fold) and mainly corresponded to genes expressed in later tapetal stages (Supplementary Table 3). Alterations in the transcript population are likely indirect and reflective of defective tapetal development.

dcl5 mutants are temperature-sensitive male sterile. During summer, 2016, $dcl5-1$ field-grown plants exhibited variable fertility under typical summer conditions in Stanford, CA: 25/18 °C interrupted by multi-day heat waves exceeding 32 °C and short periods of cooler weather. To test whether temperature is a restrictive condition, three controlled greenhouse regimes were chosen — 28/22 °C, 26/20 °C, and 23/20 °C (14 h day/10 h night temperature) — and maintained within narrow limits over the life cycle (Fig. 4 and Supplementary Fig. 13). At 28/22 °C, $dcl5-1$ mutants had shorter anthers that never exerted, while heterozygous siblings developed normally. By contrast, under both the 26/20 °C and 23/20 °C regimes, $dcl5-1$ mutants were partially to fully fertile (see “Methods” section for details of tassel development and Supplementary Fig. 13 for quantification of anther emergence); this conditional male sterility was also observed in the transposon-disrupted $dcl5$-mu03 allele (Supplementary Fig. 14). Under permissive conditions, $dcl5-1$ pollen was viable based on Alexander staining despite a prolonged life cycle and delayed flowering date under cooler temperatures...
(Supplementary Fig. 15); importantly, ears pollinated by such pollen yielded viable progeny seed. At least one aspect of normal tapetal development was also restored: the percentage of binucleated tapetal cells at 1.5 and 2.5 mm in *dcl5-1* anthers was similar to their normal siblings (Supplementary Fig. 16). By contrast, in comparing *dcl5-1* transcript levels, 24-nt phasiRNA production, and PHAS precursor accumulation in *dcl5-1* plants under restrictive and permissive temperatures, we observed no significant differences; fertility-restored plants did not accumulate 24-nt phasiRNAs (Fig. 3, Supplementary Figs. 2 and 12). Our interpretation is that the functions of Dcl5 and 24-nt phasiRNAs are dispensable for tapetum development and maize male fertility at low temperatures (23/20 °C) but are required at higher temperatures. Cool temperatures slow the pace of development and may allow alternative pathways independent of the 24-nt phasiRNAs to support tapetal cell redifferentiation.

Maize tassels contain anthers representing seven days of development, and we wondered if sporadic anther exertion in *dcl5-1* plants reflected a short phenocritical period when some anthers experienced permissive conditions. Homozygous *dcl5-1* plants were greenhouse-grown at 28/22 °C until the tassel inflorescence formed (~30 days), then plants were moved into two walk-in chambers: permissive 23/20 °C or restrictive 28/22 °C regimes. In the next three weeks, 14 sets of three plants were swapped between the two regimes for 3, 6, 9, 12, 15, 18, or 21 days, and then all plants finished their life cycle in the 28/22 °C regime.

*Fig. 2 Tapetal cells in *dcl5-1* plants are delayed or arrested in achieving binucleate status.* a Transmission electron microscopy (TEM) of a fertile *dcl5-1/Dcl5* 2.0 mm anther lobe. At this stage, the tapetal cells (TPM) are packed with dark staining materials, likely exine components later secreted onto haploid microspores. The middle layer (ML), endothecial (EN), and epidermal (EPI) cells are highly vacuolated. The orange boxes mark two binucleated tapetal cells in which both nuclei were visible (red dots). b TEM of a sterile *dcl5-1* anther lobe at the same stage, demonstrating distended, pale-staining, and mononucleate tapetal cells. Two anthers as replicates from *dcl5-1/Dcl5* and two anthers from *dcl5-1* were used for TEM study. The other replicate is shown in Supplementary Fig. 8. c *dcl5-1/Dcl5* and d *dcl5-1*: three-dimensional reconstructions of cleared, 2.5 mm anthers stained with the nuclear marker Syto13. Individual nuclei of mononucleate tapetal cells are marked in purple, and binucleated cells are marked in yellow. e Quantification of binucleated cells in *dcl5-1*, *dcl5-1/Dcl5*, and *Dcl5/Dcl5* siblings at the meiotic I (1.5 mm) and meiotic II (2.5 mm) stages, in a family segregating 1:2:1. Statistics: two-tailed Student’s *T*-test. Three replicates were used for 1.5 mm anthers of each genotype; five replicates were used for 2.5 mm anthers of each genotype. Gray dots represent individual replicates (see Source Data File). Error bars indicate standard error of the mean (s.e.m.).
temperature regimes do not restore 24-nt phasiRNA abundances in either fertile (permissive) or sterile (restrictive) abundance in each genotype. Other colored dots are as indicated in the key, lower right.

Higher yields associated with warmer temperatures within this range.25,26. That growth between 20 and 29 °C generates optimal yield, with day temperature for the U.S. corn belt; historical data indicate development at elevated temperatures. Surprisingly, the continued presence of 24-nt phasiRNAs in normal anthers.1,25,26. These stages also encompass the initiation, peak, and meiosis and post-meiotic stages in the most mature part of the main tassel spike, con

Fig. 3 Near absence of 24-nt phasiRNAs in CRISPR-derived dcl5 mutant alleles. a Total 24-nt phasiRNAs from 176 24-PHAS loci are highly abundant in both 1.5 and 2.0 mm fertile heterozygous anthers, but abundance is reduced by >99% in dcl5-1, dcl5-2, and dcl5-4, and by >90% in dcl5-3 anthers. Altered temperature regimes do not restore 24-nt phasiRNA abundances in either fertile (permissive) or sterile (restrictive) dcl5-1 anthers (see main text). W23 inbred fertile anthers have similar 24-nt phasiRNA abundance under both temperature regimes. Error bars indicate standard error of the mean (s.e.m.). b, inset Representative 24-PHAS locus #12 (B73, v4; chr 1, 178454619 bp) in a genome browser showing ~1.5 kbp with sRNAs in the fertile heterozygote (left) and dcl5-1 (right). Orange dots are individual 24-nt phasiRNAs; x-axis is genome position on the top or bottom strand, and the y-axis depicts abundance in each genotype. Other colored dots are as indicated in the key, lower right.

greenhouse (Supplementary Fig. 17). Plants in the 23/20 °C regime from the start of meiosis through the release of mononucleate microspores (~9 day period, swaps 7–11, Supplementary Fig. 17) were more fertile. Sample plants were assessed during the 6th through 9th days to check anther staging in the main tassel spike, confirming that this interval corresponds to meiosis and post-meiotic stages in the most mature part of the tassel. These stages also encompass the initiation, peak, and continued presence of 24-nt phasiRNAs in normal anthers.1. Plants in the permissive temperature for fewer than nine days during this interval had reduced fertility (swaps 1–6, 12–14); we hypothesize that this extended interval is required to allow all anthers to proceed through the phenocritical period to ensure full fertility. The temperature swap experiment demonstrates that there is a short period in which DCL5 is required to “buffer” development at elevated temperatures. Surprisingly, the restrictive temperature is similar to what is considered optimal day temperature for the U.S. corn belt; historical data indicate that growth between 20 and 29 °C generates optimal yield, with higher yields associated with warmer temperatures within this range.25,26.

Discussion

Maize DCL5 is essential for robust male fertility: dcl5 plants are temperature-sensitive during meiosis, show arrested tapetal development at this and later stages, and lack 24-nt phasiRNAs. In rice, perturbed 21-nt, premeiotic phasiRNAs confer photoperiod and temperature-sensitive male fertility.27,28. Two of these loci, PMS1 and PMS3, encode long non-coding RNA;28,29. PMS1 is a PHAS locus that generates 21-nt phasiRNAs associated with male fertility under long-day conditions.28. PMS3 also produces a small RNA of 21-nt.29. The rice rna-dependent rna polymerase 6-1 (rrd6-1) mutant displays various degrees of spikelet defects at high temperatures.31. These findings suggest that long non-coding RNAs and small RNAs are important regulators of male fertility controlled by cross-talk between genetic pathways and environmental factors. Abiotic stress-induced male sterility has been associated with alterations in tapetal development in many plant species.30. For example, TMS10 and its close homolog TMS10L encode two rice receptor-like kinases and are essential for tapetal degeneration and male fertility under high temperatures.31.

In maize, conditional or partial male sterility has been reported, but not extensively studied. The maize ocl4-1 and ocl4-2 mutants both show varying degrees of male sterility, and the severity of the phenotype is strongly influenced by environmental conditions.32. OCL4 is a HD-ZIP IV transcription factor,32. and ocl4 mutants lack 21-phasiRNAs.1. The OCL4 regulation of 21-nt phasiRNA biogenesis and the fact that male sterility in the dcl5 mutants is also sensitive to temperature indicate that the functions of some secondary small RNAs support normal development and cellular functions during environmental fluctuations outside the optimal temperature regime. The finding that dcl5-1 mutants are sterile under the 28/22 °C regime suggests that 24-nt phasiRNA biogenesis is essential for high corn yield under optimal U.S. summer farming conditions.

We can only speculate why the incredibly diverse and abundant 24-nt phasiRNAs are required to support normal tapetal development under mild temperature stress. Plants were well-watered under all growth conditions, and there were no obvious indicators of water stress such as leaf rolling or leaf damage. Under warm temperatures, plant development is accelerated relative to cooler conditions: the transition to flowering is earlier and pollen shed is precocious. In the case of the temperature
regimes we employed, pollen shed was about 10 days earlier under the 28/22 °C growth condition than under cooler daytime temperatures (Fig. 4). It is possible that during more rapid growth, there is a more stringent requirement for coordination of developmental processes, requiring more rapid and complete switches from one developmental state to another. Tapetal cells redifferentiate during meiosis, evidenced by accumulation of future exine components and binucleate status. Because this is the only anther cell type with obvious defects at peak 24-nt phasiRNA production at 2.0 mm, we hypothesize that the abundant phasiRNAs are used to reset tapetal capabilities. Because 24-nt phasiRNAs lack a high degree of complementarity to mRNAs other than their own precursors, one hypothesis is that it is their sheer abundance that permits a resetting of development. For example, the 24-nt phasiRNAs could bind to diverse AGO proteins, in effect displacing miRNAs or other sRNAs involved in gene regulation associated with pre-meiotic tapetal development. The 24-nt phasiRNAs could thus contribute to rapid redifferentiation of tapetal cells during meiosis by eliminating gene expression regulation persisting from an earlier developmental state. It is also possible that 24-nt phasiRNAs perform this or another role in meiocytes or in other anther cell types, although their abundance is much less than in non-tapetal cells. Alternatively, the functions of 24-nt phasiRNAs may involve targeted action utilizing Argonautes or other RNA-binding proteins with as-yet uncharacterized activities required in tapetal differentiation.

Fig. 4 dcl5-1 anther fertility is temperature sensitive. Three sets of dcl5-1 and sibling dcl5-1//Dcl5 plants grown in greenhouses with differing temperature regimes: 28/22 °C, 26/20 °C, or 23/20 °C (day/night). a–c Temperatures were recorded every 2 h for 60 days (gray dots, average in red; day/night indicated as white or black, below). d–f Heterozygous dcl5-1//Dcl5 siblings were fully fertile under all three regimes. g–i dcl5-1 plants in the restrictive regime (g) were completely male sterile, while those in permissive conditions were partially (h) or fully (i) fertile. Images were taken at 60 ± 3 days after planting (DAP) (top row), 70 ± 2 DAP (middle row), and 71 ± 3 DAP (bottom row); the pace of tassel development is temperature-dependent and full anther exertion occurs at different days after planting, for example f was photographed on the first day of anther exertion, a seven-day process (see “Methods” section). The scale bar in d is approximate and pertains to all tassel images.
belt. As climate disruption, including more extensive heat waves, and a general warming trend increase field temperatures, buffering maize tapetal development from adverse effects may be possible by enhancing 24-nt phasiRNA functions. The 24-nt phasiRNAs may permit development during other untested environmental challenges. Given the high degree of variation typically observed across maize inbreds for many traits, the dcl5 mutant phenotype may also vary across inbreds. More generally, it is unknown how many crop plants utilize reproductive phasiRNAs and whether their tapetal health and functions could benefit by introduction or optimization of these or other siRNA pathways.

**Methods**

**Dcl5 mutant generation and characterization.** To increase the CRISPR-based mutagenesis frequency in the targeted region, two target sites were selected by 76 bp were selected for guide RNA (gRNA) construction, designed using the CRISPR Genome Analysis Tool. Rice U6 small nuclear RNA gene promoters (P(U6)1 and P(U6)2) were used to drive the expression of each gRNA. Two guide RNAs (first gRNA, 5’-GGTTATCTTGCACAGTCGTTCTC-3’; second gRNA, 5’-CTGGTTTGCCTCTCTCCTTCACT-3’) were selected: these complementary oligonucleotides were designed for each guide RNA (gzmDmDcl5-F1, 5’-gtgattgtGTTTCTGTTCTTGTTGAAC-3’; gzmDmDcl5-R1, 5’-aacaGTTTCCGAGCTACGATGAAAC-3’; gzmDmDcl5-F3, 5’-ggttgcTGCAGGTTTCTGTTCTTTAA-3’; gzmDmDcl5-R3, 5’-aacaGTTTCCGAGCTACGATGAAAC-3’). These constructs were annealed to double-stranded DNA oligos, then inserted into a pENTR vector using BgelI and BsdI (New England Biolabs) and transformed into Escherichia coli. Three candidates from the Illumina-Mu population were identified: one from UniformMu and two from AcclMu (mu-illumina_139042.7, mu-illumina_139041.7), consistent with Cas9 action in a single cell. Leaves from mature Mu plants were genotyped for a second time using a unique primer amplification and Sanger sequencing to confirm the mutations. Over several generations families were established that segregated 1:1 for dcl5+/+ and dcl5−/− homozygous individuals using standard maize genetic procedures.

**Microscopy and imaging.** For anther wall structure analysis with confocal microscopy, the anthers were dissected and fixed in 70% ethanol for fixation. Fixed anthers were then stained by propidium iodide and visualized on a Leica SP8 confocal microscope. Hoechst was extruded and stained with DAPI (4',6-diamidino-2-phenylindole). The Alexander’s staining solution was used to test the viability of the pollen grains in the permissive conditions. For TEM, anthers were dissected and fixed in fresh 0.1 M PIPES buffer (pH 8.6). A 2% osmium tetroxide stain was applied with brief washes, followed by dehydration in an acetone gradient. Specimens were infiltrated using a gradient of Spurr’s resin (Sigma-Aldrich) and embedded. The sections were cut using a Leica UC7 ultramicrotome, stained in urayl and lead salts, and observed using a Zeiss TEM instrument with a LEO 912 AB energy dispersive X-ray detector.

**RNA-seq and RNA-seq library construction and sequencing.** Total RNA for RNA-seq and RNA-seq libraries was isolated using the PureLink Plant RNA Reagent (ThermoFisher). Total RNA quality was assessed by denaturing agrose gel electrophoresis and quantified by the Qubit RNA BR Assay Kit (ThermoFisher). For RNA-seq, 20 to 30 ng of total RNA was examined for proper library preparation, 20 to 30 ng of total RNA were examined for proper library preparation. Two guide RNAs (first gRNA, 5’-GGTTATCTTGCACAGTCGTTCTC-3’; second gRNA, 5’-CTGGTTTGCCTCTCTCCTTCACT-3’) were selected: these complementary oligonucleotides were designed for each guide RNA (gzmDmDcl5-F1, 5’-gtgattgtGTTTCTGTTCTTGTTGAAC-3’; gzmDmDcl5-R1, 5’-aacaGTTTCCGAGCTACGATGAAAC-3’; gzmDmDcl5-F3, 5’-ggttgcTGCAGGTTTCTGTTCTTTAA-3’; gzmDmDcl5-R3, 5’-aacaGTTTCCGAGCTACGATGAAAC-3’). These constructs were used to drive the expression of each gRNA. Two guide RNAs (first gRNA, 5’-GGTTATCTTGCACAGTCGTTCTC-3’; second gRNA, 5’-CTGGTTTGCCTCTCTCCTTCACT-3’) were selected: these complementary oligonucleotides were designed for each guide RNA (gzmDmDcl5-F1, 5’-gtgattgtGTTTCTGTTCTTGTTGAAC-3’; gzmDmDcl5-R1, 5’-aacaGTTTCCGAGCTACGATGAAAC-3’; gzmDmDcl5-F3, 5’-ggttgcTGCAGGTTTCTGTTCTTTAA-3’; gzmDmDcl5-R3, 5’-aacaGTTTCCGAGCTACGATGAAAC-3’). These constructs were annealed to double-stranded DNA oligos, then inserted into a pENTR vector using BgelI and BsdI (New England Biolabs) and transformed into Escherichia coli. Three candidates from the Illumina-Mu population were identified: one from UniformMu and two from AcclMu (mu-illumina_139042.7, mu-illumina_139041.7), consistent with Cas9 action in a single cell. Leaves from mature Mu plants were genotyped for a second time using a unique primer amplification and Sanger sequencing to confirm the mutations. Over several generations families were established that segregated 1:1 for dcl5+/+ and dcl5−/− homozygous individuals using standard maize genetic procedures.

**Scalep clearing of maize anthers.** Maize anthers were fixed directly into 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), and then prepared following the Scalep protocol. After 8 h KOH treatment, anthers were stained for a week at room temperature with 1 mM Calcofluor white for imaging of cell walls and 3 µM Syto 13 (ThermoFisher) for imaging of nucleic acids in the heavy metal stained sections. The anthers were imaged with a Zeiss 880 multi photon confocal microscope with a 40× LD C-Apochromat water lens (numerical aperture of 1.1; working distance of 0.62 mm). The 3D rendering was performed with Amira (FEI, ThermoFisher) software. Tapetal cells were classified as mononucleate or binucleate manually for the 2-D images, or segmented automatically for the 3-D images, and then artificially colored.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
Requests for materials should be addressed to wball@stanford.edu. Raw data were submitted to GEO under GSE123449 (including sRNA-seq as GSM3466686 to GSM3466700 and GSM4180400 to GSM4180404; and RNA-seq data as GSM3466701 to GSM3466714, and GSM180405 to GSM180408), and the processed data are available via our maize genome browser at https://mpsa.danforthcenter.org. Other supporting data are available from the corresponding authors upon request. The data underlying Figs. 2–4 as well as Supplementary Figs. 2, 5, 9, 11, 12, 16, and 17 are provided as a Source Data file. Source data are provided with this paper.

Received: 30 June 2018; Accepted: 12 May 2020; Published online: 09 June 2020

References
1. Zhai, J. et al. Spatiotemporally dynamic, cell-type-dependent premeiotic and meiotic phased siRNAs in maize anthers. Proc. Natl Acad. Sci. USA 112, 3146–3151 (2015).
2. Xia, R. et al. 24-nt reproductive phased siRNAs are broadly present in angiosperms. Nat. Commun. 10, 627 (2019).
3. Johnson, C. et al. Clusters and superclusters of phased small RNAs in the developing inflorescence of rice. Genome Res. 19, 1249–1240 (2009).
4. Song, X. et al. Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. Plant Cell 19, 462–474 (2012).
5. Song, X. et al. Rice RNA-dependent RNA polymerase 6 acts in small RNA biogenesis and spikelet development. Plant J. 71, 378–389 (2012).
6. Nonomura, K.-I. Small RNA pathways responsible for non-cell-autonomous regulation of plant reproduction. Plant Reprod. 31, 21–29 (2018).
7. Ono, S. et al. EATI transcription factor, a non-cell-autonomous regulator of pollen production, activates meiotic small RNA biogenesis in rice anther tapetum. PLoS Genet. 14, e1007238 (2018).
8. Margis, R. et al. The evolution and diversification of Dicers in plants. FEBS Lett. 580, 2442–2450 (2006).
9. Thompson, B. E. et al. The dicer-like homolog fuzzy tassel is required for the regulation of meristem determinacy in the inflorescence and vegetative growth in maize. Plant Cell 26, 4702–4717 (2014).
10. Parent, J. S., Bouteiller, N., Elmayan, T. & Vaucheret, H. Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in phasiRNA biogenesis and spikelet development. Plant J. 70, 223–232 (2015).
11. Daxinger, L. et al. A stepwise pathway for biogenesis of 24-nt secondary siRNAs and spreading of DNA methylation. EMBO J. 28, 48–57 (2009).
12. Gasciolli, V., Mallory, A. C., Bartel, D. P. & Vaucheret, H. Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. Curr. Biol. 15, 1494–1500 (2005).
13. Char, S. N. et al. An Agrobacterium-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. Plant J. 79, 117–122 (2019).
14. Wang, L. et al. Characterization and fine mapping of a necrotic leaf mutant in maize (Zea mays L.). J. Genet. Genom. 40, 307–314 (2013).
15. Komey, B. et al. RNA interference in Arabidopsis. Adv. Genet. 553, 1–39 (2013).
16. Kersey, P. J. et al. Ensembl Genomes 2016: more genomes, more complexity. Nucleic Acids Res. 44, D574–D580 (2016).
17. Tello-Ruiz, M. K. et al. Gramene 2018: unifying comparative genomes and pathway resources for plant research. Nucleic Acids Res. 46, D1181–D1189 (2018).
18. Mathioni, S. M., Kakrana, A. & Meyers, R. C. Characterization of plant small RNAs by next generation sequencing. Curr. Protoc. Plant Biol. 2, 39–63 (2017).
19. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120 (2014).
20. Kreyser, P. et al. AssemblyGenomes 2016: more genomes, more complexity. Nucleic Acids Res. 44, D5312–D5318 (2016).
21. Trapnell, C., Pachter, L. & Salzberg, S. L. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111 (2009).
22. Trapnell, C. et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28, 511–515 (2010).
23. Liao, Y., Smyth, G. K. & Shi, W. FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930 (2014).
24. Overdevries, E. & Gendrel, A. V. PlantCV v2: image analysis software for high-throughput plant phenotyping. Peerj 5, e4088 (2017).
25. Schlenker, W. & Roberts, M. J. Nonlinear effects of weather on corn yields. Rev. Agric. Econ. 28, 391–398 (2006).
26. Schlenker, W. & Roberts, M. J. Nonlinear temperature effects indicate severe damages to U.S. crop yields under climate change. Proc. Natl Acad. Sci. USA 106, 15594–15598 (2009).
27. Zhou, H. et al. Photoperiod- and thermo-sensitive genic male sterility in rice are caused by a point mutation in a novel noncoding RNA that produces a small RNA. Cell Rep. 22, 649–660 (2012).
28. Fan, Y. et al. PMST1, producing phased small-interfering RNAs, regulates photoperiod-sensitive male sterility in rice. Proc. Natl Acad. Sci. USA 113, 15144–15149 (2016).
29. Ding, J. et al. A long noncoding RNA regulates photoperiod-sensitive male sterility, an essential component of hybrid rice. Proc. Natl Acad. Sci. USA 109, 2654–2659 (2012).
30. Storme, N. D. & Geelen, D. The impact of environmental stress on male reproductive development in plants: biological processes and molecular mechanisms. Plant Cell Environ. 37, 1–18 (2014).
31. Yu, J. et al. Two rice receptor-like kinases maintain male fertility under changing temperatures. Proc. Natl Acad. Sci. USA 114, 12327–12332 (2017).
32. Kelliher, T. & Walbot, V. Maize germinal cell initials accommodate tapetum. Plant J. 69, 1684–1697 (2014).
33. Zhang, H. et al. Stable integration of an engineered megabase repeat array into the maize genome. Plant J. 70, 357–365 (2012).
34. Alexander, M. P. Differential staining of aborted and nonaborted pollen. Plant Physiol. 58, 819–825 (1976).
35. Wang, L. et al. Characterization and fine mapping of a necrotic leaf mutant in maize (Zea mays L.). J. Genet. Genom. 40, 307–314 (2013).
36. Kersey, P. J. et al. Ensembl Genomes 2016: more genomes, more complexity. Nucleic Acids Res. 44, D574–D580 (2016).
37. Tello-Ruiz, M. K. et al. Gramene 2018: unifying comparative genomes and pathway resources for plant research. Nucleic Acids Res. 46, D1181–D1189 (2018).
38. Mathioni, S. M., Kakrana, A. & Meyers, R. C. Characterization of plant small RNAs by next generation sequencing. Curr. Protoc. Plant Biol. 2, 39–63 (2017).
39. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120 (2014).
40. Kreyser, P. et al. AssemblyGenomes 2016: more genomes, more complexity. Nucleic Acids Res. 44, D5312–D5318 (2016).
41. Overdevries, E. & Gendrel, A. V. PlantCV v2: image analysis software for high-throughput plant phenotyping. Peerj 5, e4088 (2017).

Acknowledgements
The authors declare no competing interests.
**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-16634-6.

Correspondence and requests for materials should be addressed to B.C.M. or V.W.

Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer review reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020