24-nt phasiRNAs move from tapetal to meiotic cells in maize anthers

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Abstract

In maize, 24-nt phased, secondary small interfering RNAs (phasiRNAs) are abundant in meiotic stage anthers, but their distribution and functions are not precisely known. Using laser capture microdissection we analyzed tapetal cells, meiocytes, and other somatic cells at several stages of anther development to establish the timing of 24-*PHAS* precursor transcripts and the 24-nt phasiRNA products. By integrating RNA and small RNA (sRNA) profiling plus single-molecule and sRNA FISH (smFISH or sRNA-FISH) spatial detection, we demonstrate that the tapetum is the primary site of 24-*PHAS* precursor and *Dcl5* transcripts and the resulting 24-nt phasiRNAs. Interestingly, 24-nt phasiRNAs accumulate in all cell types, with the highest levels in meiocytes, followed by tapetum. Our data support

the conclusion that 24-nt phasiRNAs are mobile from tapetum to meiocytes and to other somatic cells. We discuss possible roles for 24-nt phasiRNAs in anther cell types.

In plants, small RNAs (sRNAs) belong to three main categories: microRNAs (miRNAs) involved in target mRNA cleavage, heterochromatic small interfering RNAs (hc-siRNAs) involved in RNA-directed DNA methylation (RdDM), and phased, secondary small interfering RNAs (phasiRNAs). PhasiRNAs were initially reported in male reproductive organs of maize and rice¹⁻⁶, and more recently in diverse monocots and eudicots⁷⁻¹⁰; they are found primarily in anthers. In most species, 21-nt phasiRNAs predominate during early anther development, while 24-nt phasiRNAs are abundant during meiosis.

PhasiRNA biogenesis starts with transcription of long noncoding precursors (*PHAS* transcripts) by RNA polymerase II. Subsequently, the *PHAS* transcripts are capped and polyadenylated, translocated to the cytoplasm, and then bound by ribosomes¹¹. In the polysomal complex, an Argonaute-associated 22-nt miRNA complementary to sequences in the *PHAS* transcript binds to this precursor molecule and triggers cleavage. miR2118 is utilized for most 21-nt *PHAS* precursors and miR2275 for most 24-nt *PHAS* precursors based on the presence of complementary sequences; a fraction of precursors lacks such complementarity and may be processed utilizing other miRNAs. Then, the 3' portions of cleaved transcripts are converted to double-stranded RNA by RNA-DEPENDENT RNA POLYMERASE 6 (RDR6), and subsequently processed by Dicer-like proteins (DCL4 for the 21-nt and DCL5 for the 24-nt type) to yield the phasiRNAs^{1,2,4,12}. Despite the expectation of equal stoichiometry of all the products from one *PHAS* transcript, individual phasiRNA abundances derived from the same transcript

can differ by more than 1000-fold indicating differential stabilization^{6,13}. It is possible that only these few abundant products generated from one precursor have functions.

In maize the 21-nt phasiRNAs are distributed throughout immature anther lobes based on *in situ* hybridization with one or a few examples, although the production of miR2118 is restricted to the epidermis¹. Several observations associate phasiRNAs with male sterility. First, the absence of specific 21-nt phasiRNAs are implicated in causing male sterility in rice^{14,15}. Second, in numerous male-sterile mutants of maize, either the 21-nt or 24-nt phasiRNAs are absent reflecting the tight control of the normal timing of their appearance during development^{1,2,16,17}. More specifically addressing the role of 24-nt phasiRNAs, *dicer-like5* mutants unable to process most 24-nt *PHAS* precursors are male-sterile and exhibit aberrant tapetal development under normal growing conditions². In maize, the expression of *PHAS* precursors and miR2275 as well as the accumulation of 24-nt phasiRNAs occur mainly in the tapetum at the prophase I stage in anthers¹. Male-sterile mutants with tapetal developmental defects evident during early meiotic stages are missing the 24-nt phasiRNAs^{2,17}. Interestingly, the male sterility cases in rice and maize exhibit temperature-dependent male sterility: in appropriate growing conditions, the plants are male-fertile despite the absence of specific or an entire class of phasiRNAs^{2,15,16}. This observation has led to the hypothesis that phasiRNAs may buffer anther development against environmental perturbation, particularly in the tapetum. In addition to the expectation that phasiRNAs could modulate mRNA half-life of still poorly defined targets, the 24-nt phasiRNAs result in increased CHH DNA methylation at 24-*PHAS* loci in maize, as assessed in whole anthers¹³. In analysis of isolated meiocytes, 24-nt phasiRNAs have been detected, and there is an increase in CHH DNA methylation, leading to the hypothesis that these phasiRNAs might regulate meiosis¹⁸ by an unknown mechanism related to *cis*

methylation of their own loci. To date, the precise role of 24-nt phasiRNAs in regulating maize anther development has remained elusive.

sRNAs can function by travelling within and between organs: from the site of synthesis, they can spread both to neighboring cells and systemically over long distance¹⁹⁻²¹. Cell-to-cell movement of sRNAs is probably through plasmodesmata (PD), a plasma membrane-lined pore acting as an intercellular channel that connects the cytoplasm of adjacent cells²². To date, the potential movement of 24-nt phasiRNAs from the site of biogenesis to the neighboring cell layers in maize anthers has not been well studied. A major concern with our previous localization analysis for 24-nt phasiRNAs and biogenesis components primarily to the tapetum is that only a few examples were evaluated, and the probes can adhere to the callose coat surrounding the germinal cells, yielding a non-specific signal¹. Therefore, in meiotic stage maize anthers, two major questions should be addressed: 1) do meiocytes contain all the components for generating 24-nt phasiRNAs? And, 2) using RNA-seq and small RNA-seq, what is the timing of synthesis and the distribution of these components in anther lobes?

To explore the distribution of 24-nt phasiRNA biogenesis components and the resulting small RNAs, we used laser capture microdissection (LCM) to isolate meiocytes (ME), tapetum (TAP), and other somatic cells (OSC) from two anther developmental stages for low input RNA-seq and sRNA-seq and for a more comprehensive developmental analysis using RT-qPCR. By integrating comprehensive RNA/sRNA profiling and single-molecule or sRNA FISH (smFISH/sRNA-FISH) spatial detection, we demonstrate that TAP contain the most 24-*PHAS* primary transcripts, whereas ME ultimately accumulate more 24-nt phasiRNAs at meiotic prophase I. We discuss these results in a model in which 24-nt phasiRNAs move from TAP to ME during meiosis.

Results

LCM collections from meiotic stage maize anthers

Control of fertility in maize anthers, the male reproductive floral organs, is important for hybrid seed production as an economic way to improve grain yield. Maize tassels produce hundreds of spikelets, each of which contains an upper and lower floret, and each floret contains three anthers (Fig. 1a). The lower floret stamens develop approximately 1 day later than the upper floret stamens within the same spikelet. At the beginning of meiosis, each anther contains four lobes; each lobe consists of four somatic cell types that are required to support the meiotic cell development (Fig. 1a). The high regularity of maize anther development, numerous anthers, and a detailed staging system allow rapid dissection of carefully staged anthers for cytological and molecular analysis²³ (Fig. 1b, c). To explore the spatial distribution of 24-nt phasiRNAs and their pathway components in the W23 inbred (Fig. 2a), LCM was used to isolate three samples (ME, TAP, and OSC) from two stages of meiotic anthers (1.5 mm zygotene and 2.0 mm pachytene) (Supplementary Fig. 1 and Supplementary Table 1). Collections from additional stages were used in RT-qPCR analysis to provide finer detail of the timing of biogenesis of a subset of 24-*PHAS* precursor and *Dcl5* transcripts.

24-*PHAS* precursors accumulate in tapetal cells

Seventeen RNA-seq libraries were built from ME, TAP, and OSC recovered from 1.5 mm and 2.0 mm stage anthers plus single, fixed anthers (FA) (Supplementary Table 2). To check the reproducibility of the datasets, a correlation heatmap and a Principal Component Analysis (PCA) plot were constructed (Fig. 1d, e). Biological replicates are highly similar and distinct from other cell collections at both stages, confirming the high quality of our LCM sample collections.

(TPM > 0) of 176 previously discovered 24-*PHAS* precursor types were identified in FA and LCM cell sample collections¹ (Supplementary Table 3); *PHAS* precursors that overlapped protein coding genes were excluded. As shown in Fig. 2b, 118 of these loci had sufficient and reliable expression (TPM reads > 5 in at least one sample) for further analysis. Overall, the 24-*PHAS* precursors were most abundant in TAP at the 1.5 mm stage, showing an expression pattern and high abundance similar to that in 1.5 mm FA (Fig. 2b); therefore, the majority of 24-*PHAS* precursors in whole anthers are found in the tapetal cells. The average total expression levels of 24-*PHAS* loci are approximately 65 times lower in ME and 20 times lower in OSC at this zygotene stage (Fig. 2b and Supplementary Table 3). Single cell RNA-seq (scRNA-seq) analysis of individual ME detected a low abundance of 24-*PHAS* loci during the pre-zygotene/zygotene stages in a A188/B73 maize hybrid stock²⁴. The total average abundance of these 24-*PHAS* transcripts per zygotene sample in A188/B73 is less than that in the pooled meiocyte LCM samples at 1.5 mm from W23 maize anthers. Furthermore, our previous scRNA-seq data also identified extremely low abundances of 24-*PHAS* precursors in premeiotic AR and PMC in W23 maize anthers²⁵. These data suggests that the TAP is the main source for 24-*PHAS* precursors in zygotene stage maize anthers.

To further verify our LCM RNA-seq data and to quantify transcripts in more stages, we randomly selected three highly expressed 24-*PHAS* precursors (*PHAS15*, *PHAS116*, and *PHAS296*) for smFISH and RT-qPCR assays (Fig. 2c). In the LCM RNA-seq data, transcripts for all three loci were significantly enriched in TAP at the 1.5 mm stage compared to ME and OSC (Supplementary Fig. 2). smFISH images confirmed that TAP contain most of these three 24-*PHAS* transcripts (yellow dots resulting from dye AF647 labeling) at the 1.25 mm and 1.5 mm stages during late leptotene/pre-zygotene and zygotene stages in W23 anthers (Fig. 2d, g and

Supplementary Fig. 3a). Quantification counts (transcript count) demonstrated that TAP is significantly higher at both stages (Fig. 2e, h and Supplementary Fig. 3b). Almost no expression was detected at earlier or later stages (Fig. 2d, e, g, h and Supplementary Fig. 3a, b) or in control hybridization assays (Supplementary Fig. 4). For RT-qPCR using W23 anthers and LCM samples, *ZmCyanase* was used as the internal reference; it is consistently expressed across all anther stages and is expressed in all cell layers based on smFISH detection (Supplementary Fig. 5) compared to the no label control hybridization test (Supplementary Fig. 6). In 0.75-2.5 mm W23 maize anthers, the expression patterns of the three 24-*PHAS* loci showed a similar pattern, with high expression levels in both 1.25 mm and 1.5 mm anthers and with significant enrichment in TAP compared to ME and OSC (Fig. 2f, i and Supplementary Fig. 3c).

To further solidify the conclusion that TAP is the primary site of 24-*PHAS* transcript biogenesis, we utilized the *dcl5-mu3* mutant. Several *dcl5* mutants have been documented to contain normal 24-*PHAS* precursor levels but lack virtually all the 24-nt phasiRNAs². We reasoned that in the absence of the processing machinery, the large 24-*PHAS* precursors would accumulate in the cells where they were synthesized. This was indeed true as the *PHAS116* transcripts were found to be highly enriched in TAP at the 1.75 mm stage (this is a slightly later stage than in W23 anthers) in homozygous *dcl5* (*dcl5-mu03//dcl5-mu03*) anthers (mixed inbred background) using smFISH (Fig. 2j).

Overall, these data demonstrate that maize tapetal cells are the main site for 24-*PHAS* precursor accumulation and that accumulation starts at early meiotic prophase I and peaks during zygotene. We also established that accumulation is already substantial at the initial stages of meiosis in 1.25 mm anthers, approximately one day earlier than detected previously by analysis of 1.0 mm and 1.5 mm W23 whole anthers¹.

The spatial distribution of 24-nt phasiRNA biogenesis pathway components

Argonaute (AGO) proteins, binding partners of miRNAs and phasiRNAs, play essential roles in phasiRNAs biogenesis and RNA silencing (RNAi)^{26,27}. AGOs are assumed to be involved at two steps in 24-nt phasiRNA biogenesis and function: a specific AGO as the binding protein for the miR2275 trigger and one or several AGOs as the binding proteins for the diverse 24-nt phasiRNAs (Fig. 2a). We identified transcripts encoding 18 AGO proteins in LCM cell collections (Fig. 3a). Nine AGO protein transcripts were expressed in one or two cell collections, and the other nine AGO proteins were detected in all three cell collections. None of the AGO isotype transcripts were specific to tapetal cells. Fifteen of 18 AGO members were expressed in TAP at the 1.5 mm stage, providing a list of AGOs that could be involved in 24-nt phasiRNA biogenesis (Fig. 3a). Among these, AGO1 family members have already been designated as candidates in previous studies in maize and rice^{5,7,28}, and now three AGO members (AGO10b, AGO2b, and AGO5c) not expressed in 1.5 mm TAP can likely be excluded as candidates for 24-nt phasiRNA biogenesis (Fig. 3a). In meiocytes, transcripts were found for 11 AGO proteins, many of which were more highly expressed in ME than other cell types, but none were exclusive to ME (Fig. 3a). Several of these AGO types were predicted in previous transcriptome profiling and spatial localization studies to be potential 24-nt phasiRNA binding partners, such as AGO18 family members^{1,5,29} and AGO2b⁵ based on the timing of their expression. It is noteworthy that we detected abundant *AGO5c* transcripts, and these were enriched in ME and absent in TAP (Fig. 3a). *AGO5c* is encoded by the maize *male-sterile28* gene and is homologous to rice MEL1³⁰, which is the binding partner for a subset of 21-nt phasiRNAs in rice¹⁴.

We investigated the transcript abundance of *Dcl5* and *Rdr6* in the LCM RNA-seq data. *Dcl5* was significantly higher in TAP at the 1.5 mm stage than any other samples (Fig. 3b). In

contrast, *Rdr6* was expressed in all three cell collections, with slightly higher expression levels in ME, followed by TAP at both stages (Fig. 3b). To precisely pinpoint the timing of expression, RT-qPCR demonstrated that *Dcl5* transcripts were significantly higher in TAP samples (Fig. 3c); furthermore, the expression pattern of *Dcl5* is similar to the three 24-*PHAS* precursors (*PHAS15*, *PHAS116*, and *PHAS296*), with the highest expression levels detected in TAP compared to ME or OSC at both 1.25 mm and 1.5 mm stages (Fig. 3c). As shown in Fig. 3d-f with smFISH detection, *Dcl5* transcripts (yellow dots resulting from dye Texas Red labeled smFISH probes) were prominently localized in TAP at the 1.25 mm, 1.5 mm, and 1.75 mm stages in either W23 anthers or heterozygous *Dcl5//dcl5* fertile anthers. Far fewer yellow dots were detected in ME or OSC, and almost no signal was detected in the negative control samples (Fig. 3f and Supplementary Fig. 6). An additional smFISH analysis of *Dcl5* using AF647 labeled smFISH probes confirmed that *Dcl5* transcripts were localized mainly in TAP at 1.25 mm and 1.5 mm (Supplementary Fig. 7). These data suggest that TAP is the main site for *Dcl5* accumulation at early meiotic prophase I in maize.

Previous studies have reported that four basic helix-loop-helix (bHLH) factors (*Ms23*, *Ms32*, *bHLH52*, and *bHLH122*) are required for normal tapetal cell specification and differentiation in maize^{17,31}. *Ms23* is the master transcription factor (TF) and is required for expression of 24-*PHAS* precursors, miR2275, and 24-nt phasiRNAs¹. Here, *Ms23* and *bHLH51* were highly expressed in TAP at 2.0 mm, whereas *Ms32* and *bHLH122* were more abundant in TAP at 1.5 mm (Supplementary Fig. 8). These TF expression patterns are consistent with our previous work in maize anthers, particularly the absence of *bHLH122* transcripts at 2.0 mm¹⁷. Importantly, the TAP is significantly higher for these four TFs compared with the other two cell collections (Supplementary Fig. 8). Collectively, the distribution of key TFs by RNA-seq and

localization data provide further evidence that TAP is the main site for transcription of *PHAS* precursors and their processing into 24-nt phasiRNAs. Based on the timing of 24-*PHAS* accumulation, heterodimers of *Ms32* and *bHLH122* are candidates for direct regulation of 24-*PHAS* loci. Because *Ms23* is essential for expression of *bHLH122*, it is also a critical factor.

24-nt phasiRNAs are abundant in all three cell collections

To investigate the spatial distribution of 24-nt phasiRNAs in meiotic stage anthers, we collected ME, TAP, and OSC by LCM from 1.5 mm and 2.0 mm W23 maize anthers for low input sRNA-seq (Supplementary Table 1, 2). We first classified sRNAs by length (18 nt to 30 nt) in FA and LCM cell collections. Confirming previous results¹, the 24-nt size sRNA class is the most abundant at both stages (Supplementary Fig. 9a). The 21-nt size sRNA class is also detectable, but with lower expression levels (Supplementary Fig. 9a). Using the set of 118 24-*PHAS* loci for analysis (Fig. 2b), 24-nt phasiRNAs from these loci were more abundant in all three cell collections at 2.0 mm than at the 1.5 mm stage (Fig. 4a and Supplementary Table 3). The expression patterns of 24-nt phasiRNAs and 22-nt miR2275 family members are very similar, with the highest abundance in ME at 2.0 mm during pachytene, followed by TAP at this stage (Fig. 4a). A previous sRNA-FISH co-localization study reported that miR2275 and one 24-nt phasiRNA had higher abundances in AR/ME and TAP in maize anthers³². Here, two of the miR2275 family members (miR2275a and miR2275b) were highly expressed, indicating they might play key roles in 24-*PHAS* precursor cleavage (Fig. 4b). Although the precursors are most abundant in TAP cells (Fig. 2b, c and Supplementary Fig. 2), the 24-nt phasiRNAs accumulate to higher levels in the ME (Fig. 4a, c and Supplementary Fig. 9b). Individual 24-nt sRNA abundances from products of the same locus (*PHAS15*, *PHAS116*, and *PHAS296*) are not

uniform: only a few of the 24-nt sRNAs show high abundance despite the expectation of equal stoichiometry from a single precursor molecule (Fig. 4d, e and Supplementary Fig. 10a). Furthermore, sRNAs localization studies illustrated that 24-nt phasiRNAs from three 24-*PHAS* loci (*PHAS15*, *PHAS116*, and *PHAS296*) were expressed in all three cell types, with higher expression levels in ME and TAP, lower in OSC from 1.25 mm to 2.0 mm (Fig. 4f, g and Supplementary Fig. 10b). Almost zero expression, considered to be background, was detected in control hybridization assays (no labeled control and mouse HKH RNA control) (Fig. 4f, g and Supplementary Fig. 10c). These distribution patterns lead us to hypothesize that the TAP is the site for nearly all 24-nt phasiRNAs biogenesis and that the presence of 24-nt phasiRNAs in ME and OSC depends on transport during early meiotic prophase I in maize.

Discussion

In anthers, TAP is the innermost of the four somatic layers, adjacent to the germinal cells. The TAP plays essential roles in providing nutrients, enzymes, and pollen wall components to support AR, ME, and microspore (MSP) development. Defects in tapetal function or the abnormal differentiation of TAP usually leads to meiotic arrest or to later pollen abortion^{2,17,31,33-35}. 24-nt phasiRNAs have been reported as abundant in meiotic stage maize anthers from hundreds of unique copy loci¹. In TAP-defective, male-sterile mutants lacking key tapetal-expressed TFs, almost all the 24-nt phasiRNAs are missing^{1,17}, which indicates that TAP is likely to be involved in 24-nt phasiRNA biogenesis. Our low-input RNA-seq data for ME, TAP, and OSC demonstrated that the TAP is the main source for the 118 24-*PHAS* precursor transcripts as well as *Dcl5* transcripts in 1.5 mm stage anthers (Fig. 2b, c and Fig. 3b), confirming a previous study in the same W23 background that 1.5 mm anthers were the peak stage for 24-*PHAS* loci

production with 1.0 mm anthers lacking expression¹. We further refined the timing using smFISH and RT-qPCR assays, demonstrating that three selected 24-*PHAS* loci (*PHAS15*, *PHAS116*, and *PHAS296*) and *Dcl5* transcripts were initially expressed at 1.25 mm during the leptotene-zygotene transition and were continuously expressed at 1.5 mm or 1.75 mm during zygotene (Fig. 2d-i, 3d-f and Supplementary Fig. 3, 7). Moreover, the earlier timing for 24-nt phasiRNA generation corresponds to the stage just prior to and including the leptotene-zygotene transition (Fig. 4f, g and Supplementary Fig. 10b), which is a key checkpoint in meiosis. A previous study has reported that CHH DNA methylation is elevated in isolated ME, specifically at the phasiRNA-producing loci¹⁸. Therefore, a logical hypothesis is that some of the 24-nt phasiRNAs generated in the TAP and transported to ME might induce methylation in the corresponding loci. It is unclear how such *cis* methylation could contribute to meiotic regulation. Furthermore, in *dcl5* mutants, meiosis is normal in the near complete absence of 24-nt phasiRNAs², indicating that whatever role the 24-nt phasiRNAs play, it is dispensable.

By using LCM-purified cell collections, our data demonstrate that TAP cells are the primary site of 24-*PHAS* precursor accumulation and 24-nt phasiRNA biogenesis. sRNA-seq data for ME, TAP, and OSC illustrated that 24-nt phasiRNAs and miR2275 family members were found in all three cell collections, with higher expression levels in ME at 2.0 mm during pachytene, followed by TAP at the same stage (Fig. 4a-c). Furthermore, sRNA-FISH detection confirmed that the 24-nt phasiRNAs originating from three 24-*PHAS* loci (*PHAS15*, *PHAS116*, and *PHAS296*) were present in all three cell layers from 1.25 mm to 2.0 mm (Fig. 4f, g and Supplementary Fig. 10b). Our major new conclusion is the proposal that 24-nt phasiRNAs generated in the TAP move to other cell types. This conclusion is summarized in a model of 24-nt phasiRNA biogenesis and mobility in maize anthers during meiotic prophase I (Fig. 5). We

demonstrated that TAP is the main site of 24-nt phasiRNAs biogenesis, as TAP contain the most of 24-*PHAS* precursors, *Dcl5* transcripts, adequate miR2275, and *Rdr6*. To understand why ME later contain the highest levels of 24-nt phasiRNAs, we note that there are approximately 30 tapetal cells touching each ME, whereas only approximately 0.6 tapetal cell touch neighboring OSC²³. Utilizing the LCM cell numbers we recovered and the total 24-nt phasiRNA reads we generated (Supplementary Table 1, 3b), we calculated that each tapetal cell “donates” approximately 5% of its 24-nt phasiRNAs to ME or OSC during meiotic prophase I in maize anthers. If each TAP cells exports 5% (5 units) of its 24-nt phasiRNAs to ME cells, each ME would have 5 units x 30 = 150 units of 24-nt phasiRNAs, therefore reaching a higher level than the source TAP cells. In contrast, export of 5 units of 24-nt phasiRNAs to the OSC, would result in a low level of 24-nt phasiRNAs in the immediate receiving middle layer cell with further dilution as 24-nt phasiRNAs move throughout the OSC group. A challenge for future research will be documenting the path of 24-nt phasiRNA movement. Other cases of plant intercellular movement of sRNAs are probably through PD, regulating tissue or organ development by genes silencing and RdDM²². PD are membrane-lined intercellular channels that connect the cytoplasm of adjacent cells, such as between TAP and PMC/ME or non-TAP somatic cells in anthers^{36,37}. Therefore, we speculate that 24-nt phasiRNAs move from the biogenesis site in TAP to ME or OSC through PD in meiotic prophase I maize anthers.

In *Arabidopsis*, tapetal-derived 24-nt siRNAs induce DNA methylation in the germline and maintain genome integrity by silencing corresponding genes and transposons³⁸. Here, 24-nt phasiRNAs are generated in TAP and then exported to ME (Fig. 5). These exported 24-nt phasiRNAs are likely required for the elevated CHH DNA methylation at 24-*PHAS* loci in isolated ME in maize¹⁸. In *Arabidopsis*, the 24-nt siRNAs appear to have a broader role in

modifying the ME genome and gene expression than the 24-nt phasiRNAs have in maize. This is particularly true for any impact 24-nt phasiRNAs might have on gene expression, because no mRNA targets for this class of small RNAs have been identified. Nonetheless, it is interesting that small RNAs synthesized in the TAP are transferred to maize ME. Analysis of *dcl5* maize mutants establishes that in the absence of 24-nt phasiRNA biogenesis, tapetal cells fail to differentiate properly at normal maize growing temperatures (28 °C), while meiosis proceeds normally². These observations indicate that the 24-nt phasiRNAs play an essential role in the TAP -- not in ME -- during the meiotic stages; later, microspore failure renders *dcl5* mutants male-sterile, a fact that we attribute to the failure of the tapetum to supply nutrients and wall components to the developing uninucleate microspores. Mutants grown at low temperature (21 °C) are fertile and tapetal development is slow but sufficient indicating that 24-nt phasiRNAs are not required under these conditions. Considering these observations *in toto*, it is possible that in maize the transfer of 24-nt phasiRNAs to meiocytes is an evolutionary relic that no longer serves (or fully serves) a required function and that the primary role of the 24-nt phasiRNAs is to support rapid tapetal differentiation.

Methods

Plant materials

Maize (*Zea mays*) W23 *bz2* inbred line plants were grown in Stanford, CA, USA under greenhouse conditions (31 °C/21 °C, 14 h day/10 h night light cycle with supplemental LED and UV-A lamps providing ~50% of summer sun conditions). Anthers were dissected from tassels and then measured using a micrometer (Fisher Scientific).

Homozygous *dcl5-mu03//dcl5-mu03* plants are temperature-sensitive male sterile. They are male sterile under optimized growth condition (28 °C/22 °C day/night temperature, 14 h light, 400 µmol/m²/s) in the greenhouse. *dcl5-mu03//dcl5-mu03* male sterile and *dcl5-mu03//Dcl5* fertile siblings were grown in St. Louis, MO, USA under the optimized growth conditions in a greenhouse.

Laser Capture Microdissection (LCM)

ME, TAP, and OSC were isolated from 1.25 mm, 1.5 mm, and 2.0 mm W23 anthers by LCM as described previously³⁹, with minor modification. Fixed anthers were cryoprotected in 10% (2-3 days) and 15% sucrose/PBS (phosphate buffered saline) (5 to 7 days) to better preserve tapetal cell layer morphology. Total RNA was isolated from each cell type using the RNeasyTM-Micro Kit (Invitrogen); RNA quality was evaluated on an Agilent Bioanalyzer using the RNA 6000 Pico Kit (Agilent Technologies).

Cytological staging and imaging

AR/PMC/ME from W23 anthers (from the 0.75 mm through 2.25 mm anther length stages, recovered at 0.25 mm intervals) were extruded, classified using a micrometer, and then

appropriately sized anthers were stained with 10 µg/mL Hoechst 33342 (Sigma-Aldrich) according to our previous report²⁵. Confocal image stacks for the germinal cells were taken with a Leica SP8 microscope using a 93X Glycerol immersion objective. ImageJ⁴⁰ was used for processing the fluorescent microscopy images. Cytological stages during meiotic prophase I were assigned using established criteria^{25,41}.

RNA-seq and sRNA-seq library construction and sequencing

Total RNA was isolated from three different LCM cell samples and FA samples at 1.5 mm and 2.0 mm using the RNAqueous™-Micro Kit (Invitrogen). RNA-seq libraries were prepared using CEL-seq2⁴² based on a modified protocol²⁵. Primers were synthesized by phosphoramidite chemistry at the Stanford Protein and Nucleic Acid Facility (Stanford, CA) (Supplementary Table 4). All RNA-seq libraries were sequenced on an Illumina HiSeq 4000 instrument at the Stanford Genome Sequencing Service Center (Stanford, CA) with paired-end 150 bp reads.

For small RNA library preparation, RealSeq®-AC kits (SomaGenics) were used for ultra-low amounts of total RNA starting with 100 ng from whole anthers or 10 ng from LCM cell samples. Sequencing in a single-end mode on an Illumina NextSeq (University of Delaware) yielded 75 bp reads for sRNA-seq.

Data handling and bioinformatics

Paired-end RNA-seq Read1 contained a 10 bp unique molecular identifier (UMI)⁴³ and sample barcodes, and Read2 contained the transcript sequence. Each UMI was processed and attached to the Read2 metadata using fastp⁴⁴. Barcodes were used to demultiplexed Read2 into a separate file for each library using fastq-multx⁴⁵. After trimming the adapters by Trim Galore, the reads

were mapped to version 4 of the B73 maize genome using Hisat2⁴⁶. RNA-seq reads were normalized by dividing by the total number of transcripts (UMIs) in each sample and multiplying by one million (transcripts per million normalization).

sRNA-seq data were processed as previously described⁴⁷. In brief, Trimmomatic version 0.32⁴⁸ was used to remove the linker adaptor sequences. Then, the trimmed reads were mapped to version 4 of the B73 maize genome using Bowtie⁴⁹. Read counts were normalized to one million to allow for the direct comparison across libraries. PhasiRNAs were designated based on the criteria of a 24-nt length and mapping coordinates within the previously identified 176 24-*PHAS* loci¹ that were updated to version 4 of the B73 genome using the assembly converter tool⁵⁰.

Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR for multiple stage anthers (0.75 mm – 2.0 mm; 0.25 mm intervals) and LCM cell sample collections (ME, TAP, and OSC) was performed using the Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs). Quantitative PCR was performed using TaqMan primers synthesized by Integrated DNA Technologies (Table S5) on a CFX96 C1000 Touch Real-Time PCR Detection System (BioRad). *ZmCyanase* expression was used to normalize among biological samples, because this gene is highly expressed at all stages of anther development (Supplementary Fig. 5). Each sample type was tested in two biological and three technical replicates.

Single-molecule fluorescence *in situ* hybridization (smFISH) for mRNAs and sRNAs

382 Maize spikelets of various sizes were dissected from the central spike and fixed in 20 mL glass
383 vials using 4% paraformaldehyde in 1x PHEM buffer (5 mM HEPES, 60 mM PIPES, 10 mM
384 EGTA, 2 mM MgCl₂ pH 7). Fixation was done three times in a vacuum chamber at 0.08 MPa, 15
385 min each. After fixation, samples were sent for paraffin embedding at the histology lab in the
386 Nemours/Alfred I. duPont Hospital for Children (Wilmington, DE). Embedded paraffin samples
387 were sectioned using a paraffin microtome and dried on poly-l-lysine-coated 22 x 22 mm #1.5
388 coverslips (Carl Zeiss Microscopy, LLC, Cat# 474030-9020-000). Samples were then de-
389 paraffinized using Histo-Clear (Fisher Scientific, 50-899-90147) and re-hydrated by going
390 through an ethanol series of 100, 95, 80, 70, 50, 30, 10% (vol/vol) (30 sec each) and finally water
391 (1 min) at room temperature. After protease (Sigma, P5147) digestion (20 min, 37°C), samples
392 were neutralized in 0.2% glycine (Sigma-Aldrich, G8898) for 2 min. After two washes in 1x
393 PBS buffer, samples were dehydrated and then hybridized with smFISH probes. About 20-nt
394 smFISH probes were designed to bind specifically across the length of each target RNA in a non-
395 overlapping series⁵¹. For small RNA detection, probes were designed reverse complemented to
396 ~19 nt of the phasiRNA sequences generated from each locus. Briefly, 23-51 probes (total
397 coverage of about 800 nt for mRNAs or sRNAs) were designed for each target, and each probe
398 was synthesized with a 3' amino modification (LGC Biosearch Technologies, CA)
399 (Supplementary Table 6). All the probes of one set were pooled and *en masse* coupled with
400 Alexa Fluor 647 (Thermo Fisher) or Texas Red; the labeled probe fraction was purified using
401 reverse phase HPLC⁵². The probes were diluted to a concentration of 20 ng/ul in a hybridization
402 buffer containing 10% formamide, 1 mg/mL yeast tRNA, 10% dextran sulfate and 2 mM
403 Vanadyl ribonucleoside complex (New England Biolab, Ipswich, MA, Catalogue Number,
404 S142), 0.02% RNase free BSA (Ambion, AM2618). The biological samples were hybridized

with hybridization mix overnight in a humid chamber at 37°C. The samples were then washed two times with 2X SSC buffer (sodium saline citrate) containing 10% formamide, and a final wash was done using 1X TBS buffer (Tris-buffered saline). Samples were mounted using ProLong™ Glass Antifade Mountant with NucBlue™ Stain (ThermoFisher Scientific, P36981). Fluorescence was detected by spectral unmixing of autofluorescence spectra using laser scanning confocal microscopy on a Zeiss LSM 880 multiphoton confocal microscope. We used 30% power of the 633 nm laser and an alpha Plan-Apochromat 100x/1.46 oil lens to acquire RNA *in situ* images. Pure dye was used as positive control, and autofluorescence from maize tissue was used as the negative control for spectral bleed-through. After images were acquired, each image was spectra unmixed. The brightness and contrast of images in the same figure panel were adjusted equally and linearly in Zen 2010 (Carl Zeiss). For quantification, the numbers for each localization event were calculated using ImageJ (SpotQuant). Two replicates for each Z-stack of images were used for calculating the copy number for each gene's transcripts.

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Author contributions

X.Z., B.C.M, and V.W. conceived and designed the project. X.Z. performed most of the experiments and bioinformatics. K.H. and M.B. designed and synthesized smFISH probes. K.H. performed smFISH and imaging analysis. T.C. constructed sRNA libraries and performed raw data processing. X.Z. and V.W wrote the manuscript with editing by B.C.M. and K.H. and with contributions from all co-authors.

Competing interests

The authors declare no competing interests.

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Fig. 1. Staging of maize anthers and quality evaluation of LCM cell sample collections. (a) Male inflorescence structures. From left to right: maize tassel; central spike; single spikelet; three

566 lower and three larger upper florets; transverse confocal microscopy view of a single anther (near
 567 the start of meiosis). **(b)** The timeline of maize anther development from initial cell specification,
 568 proliferation, and differentiation to pollen maturation. Approximately 10 days of development
 569 occur between anther initiation and the start of meiosis at 1.2 mm; meiosis requires 6 days and
 570 finishes at 2.5 mm with the tetrad stage; an additional 15-16 days are required for microspore
 571 and then pollen development (note that this portion of the timeline is not drawn to scale).
 572 Tracings of confocal images of single anther lobes are shown through the dyad stage. The
 573 germinal cell stages are abbreviated as follows: mitotic archesporial cells (AR); post-mitotic
 574 pollen mother cells (PMC); meiocytes (ME); uninucleate microspore (UM); binucleate
 575 microspore (BM); and mature pollen (MP). **(c)** Fluorescence microscopy images of germinal
 576 cells visualized with Hoechst staining. During the meiotic proliferation period (0.75 mm anther),
 577 archesporial cells have a central nucleolus (N) and a smaller volume than meiotic nuclei. At
 578 premeiotic interphase/ early leptotene, the knobs (arrowhead) are round, the nucleoli are located
 579 centrally, and chromatin threads are not visible or visible only as localized patches. During
 580 leptotene leading to pre-zygotene, the spherical knobs become elongated (arrowhead) coincident
 581 with nucleolar movement to the periphery. During zygotene, the knobs are paired (red dashed
 582 oval); chromosomes are observed as condensation proceeds. Fully paired bivalents (4 strands per
 583 thread) and finally a centrally located nucleolus were observed at pachytene. Scale bar represents
 584 2 μ m. **(d)** Sample-sample correlation analysis in fixed anthers and LCM cell sample collections
 585 at the 1.5 mm and 2.0 mm stages. As shown in the heatmap, the biological replicates of the same
 586 sample type are highly correlated at both stages. The scale based on the Pearson correlation is
 587 from lightest (no correlation) to darkest (1.0 correlation). Abbreviations: fixed anthers (FA);
 588 meiocytes (ME); tapetum (TAP); and other somatic cells (OSC). **(e)** Principal component
 589 analysis (PCA) to evaluate the similarity of biological replicates.

Fig. 2. 24-PHAS precursor distribution in maize anthers. (a) 24-nt phasiRNA biogenesis

pathway. miR2275 is complementary to a conserved motif in precursors, resulting in precise cleavage; cleaved products are converted to double stranded molecules by RNA-dependent RNA polymerase 6 (RDR6). Unknown Argonaute (AGO) proteins participate in biogenesis and bind at least a subset of the 24-nt phasiRNA products. **(b)** Heat map depicting the abundances of 118 24-*PHAS* precursors (TPM > 5 in at least one sample) in fixed anthers and LCM cells. Solid bubbles on the left represent the total abundance of 24-*PHAS* precursors in that sample. **(c)** Boxplot depicts the distribution of the 20 most abundant 24-*PHAS* precursors in LCM cell collections. Arrowheads point to the three 24-*PHAS* loci (*PHAS15*, *PHAS116*, and *PHAS296*) chosen to represent different abundances for RT-qPCR and single-molecule FISH (smFISH) verifications. **(d)** and **(g)** smFISH was performed using probes conjugated with dye Alexa Fluor 647 (AF647) against *PHAS116* and *PHAS296* in W23 maize anthers (1.0-2.0 mm). The fluorescent micrographs were taken using laser scanning confocal microscopy. Below the images are enlargements of area in red rectangles. Yellow smFISH spots correspond to precursor transcripts, which primarily accumulate in TAP. Scale bars = 10 μ m. **(e)** and **(h)** Quantification analysis of images in panel **d** and **g**. Transcript counts show the binding events for each cell type at different anther stages using spot count in ImageJ. Two biological and three technical replicates were performed for each stage. Statistical analysis was performed with Prism 8 software. *P* values were determined using one-way ANOVA and Tukey tests. Differences among three cell sample collections within the same anther stage were considered significant for *P* values ≤ 0.05 (* *P* ≤ 0.05 ; ** *P* ≤ 0.01 ; *** *P* ≤ 0.001 ; **** *P* ≤ 0.0001). **(f)** and **(i)** Quantification of localization of *PHAS116* and *PHAS296* in whole anthers (0.75- 2.5 mm) and LCM cells (1.25 and 1.5 mm) by RT-qPCR. The expression patterns of these 24-*PHAS* precursors are very similar, with high expression levels in tapetum at 1.25 mm, with a peak at the 1.5 mm stage. The tapetal cell sample is significantly higher than either meiocytes or other somatic cells. *P* values were determined using one-way ANOVA and Tukey tests in Prism 8 software. Differences among the samples were considered significant for *P* values ≤ 0.05 (* *P* ≤ 0.05 ; ** *P* ≤ 0.01 ; *** *P* ≤ 0.001 ; **** *P* ≤ 0.0001). Note that there is a different scale for each locus. The reference gene (*ZmCyanase*) was used to normalize expression for the accurate assessment of target 24-*PHAS* locus expression. **(j)** smFISH detection of *PHAS116* in homozygous *dcl5-mu03//dcl5-mu03* anthers. The red boxes labeled I and II are enlarged in the adjacent image to the right. No signal was detected in control hybridization assays (*Dcl5* probes) at 1.75 mm stage anthers. Scale bars = 10 μ m.

Fig. 3. Evaluation of Argonaute, *Dcl5*, and *Rdr6* expression and *Dcl5* transcript localization in maize anthers. (a) RNA abundance from the 18 maize loci encoding Argonaute proteins (AGO). At least one AGO participates in phasiRNA biogenesis by binding miR2275 and a few others have been shown to bind 24-nt phasiRNAs (see text). (b) Transcript abundances in LCM cells for two biogenesis genes from RNA-seq datasets. *Dcl5* was highly expressed in 1.5 mm

TAP, and *Rdr6* was enriched in both ME and TAP. Differences among the LCM cell samples were considered significant for P values ≤ 0.05 (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$) using one-way ANOVA followed by Tukey tests. (c) RT-qPCR analysis for *Dcl5* at multiple anther stages and in LCM cell samples. The tapetal cell samples are significantly higher than ME and OSC (** $P \leq 0.01$). Note that each graph has a different scale. (d) and (e) smFISH detection and quantification analysis for *Dcl5* transcripts in W23 maize anthers (1.0-2.0 mm). Probes are conjugated with Texas Red dye. The images below each panel are the enlarged portions of red rectangle areas. Yellow smFISH spots correspond to *Dcl5* transcripts are significantly higher in TAP (*** $P \leq 0.001$). Scale bars = 10 μm . (f) smFISH detection of *Dcl5* transcripts in homozygous *dcl5-mu03//dcl5-mu03* anthers. Probes are conjugated with Texas Red dye. The red boxes designated as I and II indicate the area enlarged in the adjacent image. To validate the lack of *Dcl5* transcripts, no signal was detected in a control hybridization assay utilizing *Dcl5* probes applied to homozygous *dcl5-mu03//dcl5-mu03* anthers. Scale bars = 10 μm .

Fig. 4. 24-nt phasiRNA distribution in maize anthers. (a) Heat map depicting 24-nt phasiRNA abundance from 118 24-*PHAS* loci in fixed anthers and LCM cell sample collections. Solid bubbles on the left represent the total abundance of miR2275 family members and 24-nt

phasiRNAs in that sample. The highest levels of 24-nt phasiRNAs and miR2275 family members are in 2.0 mm meiocytes, followed by tapetum at this stage. **(b)** The abundance of miR2275 family members in three LCM cell sample collections. **(c)** Box plot depicting the distribution of 24-nt phasiRNAs from the 20 most abundant 24-*PHAS* loci (same as panel **2c**). **(d)** and **(e)** 24-nt sRNA distributions from *PHAS116* and *PHAS296* in FA and LCM cells. The dotted lines represent the miR2275 cleavage sites, and each of the grid lines are spaced 24- nucleotide apart. The accumulation of 24-nt sRNA fragments generated from the same locus are not uniform: only a few of the 24-nt sRNAs show high abundance despite the expectation of equal stoichiometry from a single precursor molecule (15). Overall, accumulation patterns are similar in fixed anthers and LCM cells at each locus. **(f)** 24-nt phasiRNA localization from *PHAS116* in W23 maize anthers (1.25-2.0 mm). Probes are conjugated with Texas Red dye. The red box in each image is enlarged in the panel immediately below each image. Below are negative control images obtained with probes against mouse Hexokinase H (HKH) RNA in W23 maize anthers (1.25-2.0 mm). Yellow smFISH spots corresponding to 24-nt phasiRNAs are expressed in all three cell types. Scale bars = 10 μ m. **(g)** 24-nt phasiRNA localization from *PHAS296* in W23 maize anthers (1.25-1.75 mm). Probes are conjugated with Texas Red dye. The red box in each image is enlarged in the panel immediately below each image. Yellow smFISH spots corresponding to 24-nt phasiRNAs are expressed in all three cell types. No signal was detected in an unlabeled control hybridization. Scale bars = 10 μ m.

Fig. 5. Model of 24-nt phasiRNAs biogenesis and mobility in maize anthers. On the left is a colored confocal image as a transverse section of a single anther. On the right is an enlargement of a portion of a single lobe using the same color scheme. During the leptotene-zygotene transition, 24-nt phasiRNAs are generated in TAP and are proposed to move to other cell types. The 30 TAP cells in contact with each meiocyte are proposed to each export a small fraction of their 24-nt phasiRNAs to ME; similarly, a small amount of TAP 24-nt phasiRNA is exported to first to the adjacent Middle Layer (ML) and then distributed to other OSC.

Supplementary information

Supplementary Figures

Supplementary Fig. 1. Sequential application of LCM to isolate ME, TAP, and OSC in 1.5 mm and 2.0 mm W23 anthers.

Supplementary Fig. 2. Transcript abundances of three *24-PHAS* loci (*PHAS15*, *PHAS116*, and *PHAS296*) in LCM cell sample collections in RNA-seq data.

Supplementary Fig. 3. *PHAS15* localization in maize anthers.

Supplementary Fig. 4. Negative control hybridization assay.

Supplementary Fig. 5. *ZmCyanase* localization in maize anthers.

Supplementary Fig. 6. No labeled control hybridization assay.

Supplementary Fig. 7. *Dcl5* localization in maize anthers.

Supplementary Fig. 8. The distribution of four tapetal-enriched TFs (*Ms23*, *Ms32*, *bHLH51*, and *bHLH122*) in LCM cell sample collections at 1.5 mm and 2.0 mm in RNA-seq data.

Supplementary Fig. 9. sRNA distribution in maize anthers.

Supplementary Fig. 10. 24-nt phasiRNAs localization in maize anthers.

Supplementary Tables

Supplementary Table 1. List of LCM cell sample collections from 1.5 mm and 2.0 mm maize anthers.

Supplemental Table 2. Summary of RNA-seq and sRNA-seq libraries prepared from FA and LCM cell sample collections.

Supplementary Table 3. Coordinates and abundance of the 126 *24-PHAS* loci and their corresponding 24-nt phasiRNAs in FA and LCM cells.

Supplementary Table 4. Primer sequences for CEL-seq 2 library preparation.

Supplementary Table 5. Primer sequences for RT-qPCR by the TaqMan assay.

Supplementary Table 6. Probes sequences (5'-> 3') used for smFISH or sRNA-FISH.