Somatic small RNA pathways promote the mitotic events of megagametogenesis during female reproductive development in *Arabidopsis*

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Summary

Female gamete development in *Arabidopsis* ovules comprises two phases. During megasporogenesis, a somatic ovule cell differentiates into a megaspore mother cell and undergoes meiosis to produce four haploid megaspores, three of which degrade. The surviving functional megaspore participates in megagametogenesis, undergoing syncytial mitosis and cellular differentiation to produce a multicellular female gametophyte containing the egg and central cell, progenitors of the embryo and endosperm of the seed. The transition between megasporogenesis and megagametogenesis is poorly characterised, partly due to the inaccessibility of reproductive cells within the ovule. Here, laser capture microdissection was used to identify genes expressed in and/or around developing megaspores during the transition to megagametogenesis. *ARGONAUTE5* (*AGO5*), a putative effector of small RNA (sRNA) silencing pathways, was found to be expressed around reproductive cells during megasporogenesis, and a novel semi-dominant *ago5-4* insertion allele showed defects in the initiation of megagametogenesis. Expression of a viral RNAi suppressor, *P1/Hc-Pro,* driven by the *WUSCHEL* and *AGO5* promoters in somatic cells flanking the megaspores resulted in a similar phenotype. This indicates that sRNA-dependent pathways acting in somatic ovule tissues promote the initiation of megagametogenesis in the functional megaspore. Notably, these pathways are independent of *AGO9*, which functions in somatic epidermal ovule cells to inhibit the formation of multiple megaspore-like cells. Therefore, one somatic sRNA pathway involving AGO9 restricts reproductive development to the functional megaspore and a second pathway, inhibited by *ago5-4* and *P1/Hc-Pro*, promotes megagametogenesis.

Introduction

Unlike animals, where male and female gametes are derived from germline stem cells, plants produce their gametes from somatic cells that are directed onto specialised pathways based on their position within the reproductive

organ, rather than their lineage (Huala and Sussex, 1993; Nonomura et al., 2007). Formation of the female gametophyte (FG) in *Arabidopsis* ovules begins with megasporogenesis when a single sub-epidermal somatic cell differentiates from nucellar tissue located at the distal tip of the immature ovule, and is specified as a megaspore mother cell (MMC; Fig. 1A,B). The diploid MMC subsequently initiates meiosis to produce four haploid megaspores (Fig. 1C). The most proximal of these, termed the functional megaspore, initiates megagametogenesis and undergoes three rounds of syncytial mitosis eventually giving rise to the mature female gametophyte (Fig. 1A).

Transcriptome profiling in *Arabidopsis* has identified pathways acting in the megaspore mother cell (Schmidt et al., 2011) and dividing or mature female gametophytes (Yu et al., 2005; Johnston et al., 2007; Jones-Rhoades et al., 2007). Little is known, however, about pathways involved in the transition from megasporogenesis to megagametogenesis. Mutations in the *INNER NO OUTER* and *WUSCHEL* (*WUS*) transcription factors induce somatic defects in ovule tissues that compromise early stages of gametophyte development, suggesting that inter-regional signalling is important (Elliott et al., 1996; Lieber et al., 2011). Mutations in components of small RNA (sRNA) silencing pathways also influence gametophyte initiation. Ovules lacking function of *ARGONAUTE9* (*AGO9*), *RNA-DEPENDENT RNA POLYMERASE6* (*RDR6*) or *SUPPRESSOR OF GENE SILENCING3* produce multiple functional megaspore-like cells in pre-meiotic ovules that express the pFM1:GUS marker (Olmedo-Monfil et al., 2010). AGO9 protein binds 24nt siRNAs and accumulates in nucellar epidermal cells, suggesting that it promotes a non-cell-autonomous signal that restricts gametophyte identity in sub-epidermal cells (Olmedo-Monfil et al., 2010).

Arabidopsis ovules were dissected by Laser Capture Microdissection (LCM) and profiled to identify pathways involved in the transition from megasporogenesis to megagametogenesis. Analyses revealed genes up-regulated in distal parts of the ovule, including the nucellus and megaspores, relative to other ovule tissues. Characterisation of a unique insertion allele for one of the genes, *ARGONAUTE5* (*AGO5*), suggested that sRNA pathways acting in somatic nucellar cells promote the initiation of megagametogenesis

in the functional megaspore. This was supported by tissue specific expression of viral RNAi suppressor proteins, and shown to be independent of *AGO9*. These results indicate that at least two somatic sRNA pathways contribute to gametophyte development. One pathway restricts reproductive potential to the functional megaspore and another promotes the initiation of megagametogenesis in this cell.

Results and Discussion

LCM and transcriptional profiling of *Arabidopsis* ovule cells

To identify genes involved in the transition from megasporogenesis to megagametogenesis, cells from the distal tip of developing ovules undergoing megaspore selection were captured using LCM (Stage 2-V, Fig. 1C-E; Schneitz et al., 1995). The laser system used could not be sufficiently focused to capture developing functional megaspores. However, by identifying genes highly expressed in the “nucellus” region, comprising the nucellar epidermis, developing megaspores and flanking sub-epidermal nucellar cells (NUC2), relative to other ovule tissues (OV2), components of both cell-autonomous and non-cell-autonomous pathways promoting megagametogenesis might be identified. Three biological replicates of both captured regions were profiled and 1524 genes were differentially expressed between the two samples (FC≥2.0, p≤0.01, Sup Figure 1A, Sup Table 1). The NUC2 subset (563 genes) was enriched for transcriptional regulators (agriGO, Sup Table 2; Su et al., 2010) including positive controls *WUS* (Fig. 1F-G) and *SPOROCYTELESS* (*SPL*; Yang et al., 1999), and comprised diverse gene expression clusters indicative of multiple developmental functions (Sup Figure 1B). Qualitative comparisons of the NUC2 subset were made to lists of genes down-regulated in *sporocyteless* ovules lacking functional nucelli (Johnston et al., 2007), genes expressed in the integuments (Skinner and Gasser, 2009) and genes up-regulated in a dividing female gametophyte (FG3-4) sample generated in this study (Sup Table 3). Approximately 26% of the NUC2 genes were present in the *spl* and/or FG3-4 datasets, while only 2% were detected in the integument dataset (Fig. 1F, Sup Table 4), indicative of minimal integument

contamination. The high proportion (~70%) of unique NUC2 genes possibly reflects the different annotation versions and array systems used to generate the datasets, but also the ability of the LCM method to enrich for RNAs from specific cell types in complex tissues.

To assess whether these transcriptome data were indicative of expression dynamics *in planta*, promoter:fluorophore fusions were generated for multiple NUC2 candidates and examined in transgenic plants (Sup Table 5). Diverse expression patterns were conferred by the promoter fragments in ovules, including combinations of expression in the nucellar epidermis, functional megaspore, inner integument and female gametophyte (Fig. 1G-P). In most cases, the expression dynamics fit the criteria of being up-regulated in the distal tip of the ovule relative to other ovule tissues during the transition from megasporogenesis to megagametogenesis. The LCM approach utilised was therefore considered successful in defining a unique set of genes and developmental markers expressed at this stage.

An insertion in At2g27880 inhibits the initiation of megagametogenesis

Expression of At2g27880 (*AGO5*; Fig 1K) in somatic cells surrounding the MMC and megaspores (Sup Fig. 2 and 3) was reminiscent of *AGO9*, which accumulates in nucellar epidermal cells and inhibits gametophyte development in sub-epidermal cells (Olmedo-Monfil et al., 2010). Conserved in plants and animals, AGO proteins bind sRNAs and induce repression of complementary RNA targets (Mallory and Vaucheret, 2010). T-DNA insertion lines were examined to investigate whether *AGO5* might be involved in gametophyte formation. Two *ago5* insertion alleles (Sup Fig. 4A), *ago5-1* (Katiyar-Agarwal et al., 2007) and *ago5-2* (Takeda et al., 2008), showed no defects in ovule or gametophyte development. A third line, SALK_050483, containing an insertion closer to the 5' end of *AGO5* (Sup Fig. 4A) showed defects in seed development and is hereafter referred to as *ago5-4*. Heterozygous *ago5-4*/+ plants produced siliques containing aborted ovules (51±6%), white seeds (1±1%) and green seeds (48±6%; Fig. 2A, n=563), compared to 95±5% green seeds in wild-type (WT) siblings (Fig. 2A, n=543).

Homozygous *ago5-4/-* plants were not identified, possibly due to defects in embryogenesis (Sup Fig. 4B).

Emasculated flowers from *ago5-4/+* plants showed different ovule phenotypes at anthesis. Approximately 51% of the ovules contained a mature WT-like female gametophyte containing an egg and central cell nucleus (Fig. 2B,C). In the remainder, a one-nucleate female gametophyte (FG1) was observed (Fig. 2D), indicating that FG development had terminated prior to the first mitotic division. Although a 1:1 WT:mutant ratio is characteristic of gametophytic-effect mutations, reciprocal crosses between *ago5-4*/+ and WT suggested that *ago5-4* is not a typical gametophytic mutation. Approximately 36% (n=150) of the F1 plants produced from *ago5-4/+* females crossed with WT males inherited the mutation, indicating that the presence of *ago5-4* in the female gametophyte only slightly diminishes fecundity.

Expression of marker genes in the megaspore mother cell, megaspores (pKNU:nlsYFP, pKNU:nlsGUS; Payne et al., 2004) and functional megaspore (pFM1:GUS; Acosta-Garcia and Vielle-Calzada, 2004) indicated ovule development was normal in *ago5-4*/+ until FG1 abortion (Fig. 2E-J). By contrast, a marker for the mature female gametophyte (pMYB64:GFP; Wang et al., 2010) was not detected in aborted *ago5-4*/+ ovules (Fig. 2K,L). This suggests that *ago5-4/+* FG1 abortion is not caused by an obvious change in MMC or megaspore identity. Moreover, *ago5-4/+* does not form extra megaspore-like cells, and thus is phenotypically distinct from *ago9* mutants (Olmedo-Monfil et al., 2010).

AGO5-4 induces female sterility from somatic cells

Sequencing of the *ago5-4* allele showed that the T-DNA is inserted immediately after the coding sequence for the sRNA-binding PAZ domain (Sup Fig. 4). Quantitative-PCR indicated that *AGO5* mRNA levels are only slightly reduced (~89%) during early stages of *ago5-4*/+ fruit development, with 20% of expression originating from the *ago5-4* allele compared to <2% in *ago5-1* and *ago5-2* (Fig. 3A-C). To test whether the *ago5-4/+* phenotype might be due to expression of transcript from the *ago5-4* locus, the predicted *AGO5-4* cDNA fragment was cloned, fused to the *AGO5* promoter and

transformed into plants. Multiple (5/10) transgenic lines containing *pAGO5:AGO5-4* showed similar defects in ovule development to *ago5-4*/+ and aborted at the FG1 stage (Fig. 4A,B) with frequencies ranging from 27-74% (Fig. 3D).

Comparable results were obtained for a *pAGO5:YFP-AGO5-4* gene, which induced FG1 abortion and accumulated YFP-AGO5-4 protein in the cytoplasm of nucellar epidermal and inner integument cells (Fig. 4O,P). Notably, YFP-AGO5-4 was absent from the megaspores, similar to YFP-AGO5 (Fig. 4M,N) and pAGO5:YFPer (Sup Fig. 2 and 3). This is consistent with the somatic effect of the *ago5-4*/+ mutation and suggests the *ago5-4* phenotype is unlikely to result from movement of AGO5-4 into the MMC or megaspores. Supporting this, restricted expression of *pWUS*:*AGO5-4* in somatic nucellar epidermal cells (Fig. 4E) induced FG1 abortion in 25-51% of ovules (Fig. 3D, 4F), and increased the frequency of FG1 abortion in *ago5-4/*+ to 63±5% (n=460), indicative of a dose-dependent effect on a somatic target. By contrast, expression of *AGO5-4* in the MMC and megaspores via the *KNUCKLES* (*KNU*) promoter (Fig. 4I) did not alter gametophyte abortion in WT or *ago5-4/*+ plants (Fig. 3D, 4J).

To address whether *ago5-4*/+ competes with residual AGO5 activity, extra copies of *AGO5* were transformed into *ago5-4*/+ plants. However, this failed to alleviate *ago5-4*/+ defects (Fig. 3D). Moreover, removal of WT AGO5 protein in trans-heterozygous *ago5-4/ago5-1* and *ago5-4/ago5-2* plants failed to alter the frequency of FG1 abortion (Fig. 3D). Similar results were obtained for double mutants with *ago1-27* and *ago10-1* (Fig. 3D), which encode ovule-expressed *AGO5* homologues with affinity for microRNAs (Mallory et al., 2009).

Collectively, these results suggest that the *ago5-4* insertion generates a semi-dominant *AGO5* gene that acts in somatic cells to inhibit pathways required for the initiation of megagametogenesis. Although a complex level of redundancy cannot be discounted, the finding that *ago5* knockouts have no obvious effect on FG development in WT or *ago5-4* background suggests that the unique *ago5-4* insertion compromises pathway(s) not normally targeted by *AGO5*. One possibility is that *ago5-4* generates a truncated, tissue-specific AGO5 protein with altered sRNA binding efficiency, which induces de-

repression of complementary sRNA-targets and disrupts a novel pathway required for the transition to megagametogenesis.

Tissue-specific *P1/Hc-Pro* expression induces phenotypes similar to *ago5-4*/+

The predicted structure of *AGO5-4*, retaining the AGO5 PAZ domain but lacking the MID-domain and catalytic PIWI-domain, is reminiscent of viral suppressor proteins such as P1/Hc-Pro and P19 that bind sRNAs and inhibit AGO-dependent pathways (Kasschau et al., 2003; Dunoyer et al., 2004). Although both proteins have a negative effect on miRNA function, P19 also inhibits function of 21 and 22nt siRNAs (Voinnet et al., 2003) while P1/Hc-Pro inhibits function of 21 and 24nt siRNAs (reviewed in Li and Ding, 2006).

To examine if viral suppressor proteins can impair ovule development similarly to *ago5-4*/+, the *P1/Hc-Pro* polyprotein sequence from Turnip Mosaic Virus and *P19* sequence from Tomato Bushy Stunt Virus were expressed from the tissue-specific *AGO5*, *WUS, KNU* (Fig. 4) and constitutive *35S* promoters. Transgenic plants expressing *35S:P1/Hc-Pro* or *35S:P19* showed vegetative defects similar to previous reports (Kasschau et al., 2003; Dunoyer et al., 2004). The other lines appeared indistinguishable from WT, but an examination of ovule development showed that the *pAGO5:P1/Hc-Pro* and *pWUS:P1/Hc-Pro* genes induced FG1 abortion similar to *ago5-4*/+ (Fig. 3D and 4C,G). By contrast, the *pAGO5:P19, pWUS:P19* and reproductive cell-specific *pKNU:P1/Hc-Pro* and *pKNU:P19* suppressor constructs had no effect on ovule or gametophyte development (Fig. 3D and 4D,H,K,L). These results suggest that specific, somatic sRNA pathways inhibited by P1/Hc-Pro but not P19, are required for initiation of megagametogenesis.

Based on their structural and phenotypic similarities, it is likely that *P1/Hc-Pro* and *AGO5-4* inhibit similar pathways. Consistent with the inhibitory activity of *P1/Hc-Pro*, AGO5 predominantly binds 24nt siRNAs and some 21 and 22nt sRNAs (Mi et al., 2008; Takeda et al., 2008). Interestingly, AGO9 also acts through 24nt siRNAs, but preferentially binds those with a 5' terminal adenosine (Havecker et al., 2010) compared to the 5' terminal cytosine preferred by AGO5 (Mi et al., 2008). Neither *ago9* nor *rdr6* mutations altered

the frequency of FG1 abortion in *ago5-4*/+ plants, which might be expected if they competed for targets or acted in the same pathway (Fig. 3D). Furthermore, the frequency of *ago9* ovules containing extra megaspore-like cells was not altered by *ago5-4*/+, although their subsequent development was inhibited irrespective of their origin (Sup Fig. 5).

Collectively, these data suggest that at least two sRNA pathways act in somatic cells flanking the MMC and megaspores to regulate female reproductive development in *Arabidopsis* (Fig 4Q). One pathway depends on *AGO9* and 24nt siRNAs to prevent sub-epidermal cells from adopting megaspore-like identity (Olmedo-Monfil et al., 2010). A second, independent sRNA pathway is required to promote the transition to megagametogenesis in the functional megaspore. This pathway is inhibited by the semi-dominant *ago5-4* mutation and P1/Hc-Pro polyprotein and may also act through 24nt siRNAs. Notably, silencing pathways inhibited by P19, P1/Hc-Pro and *ago5-4* either do not act or have limited cell-autonomous functionality in the early reproductive cells examined here. This highlights the key role played by somatic ovule cells in regulating the events of early female gametophyte development.

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Materials and Methods

Plant Material

Plants were grown as per previous (Goetz et al., 2006).The *ago1-27, ago5-1*, *ago5-2, ago5-4* (SALK_050483; Alonso et al., 2003), *ago9-2 and ago10-1* alleles are in the Columbia background, while *rdr6-3* is in Landsberg *erecta*.

LCM

Flowers were fixed in 3:1 ethanol:acetic acid and embedded in butyl-methyl-methacrylate (Rasheed et al., 2006). Sections (5µM) were placed on membrane slides (Leica), treated with acetone and dissected using an AS-LMD (Leica). NUC2 and OV2 samples were captured from the same slides in three independent replicate collections. RNA was extracted using a Picopure kit (Acturus) and amplified twice using MessageAmp III (Applied Biosystems), similar to previous methods (Schmidt et al., 2011). RNA was labelled using an ULS aRNA labelling kit (Kreatech) and hybridised to Agilent 4x44K arrays (IMB, Brisbane).

Microarray Analysis

Data was analysed in R (http://www.r-project.org/) using bioconductor (http://www.bioconductor.org) packages Agi4x44PreProcess and limma (Smyth, 2004). For each probe, background median intensities were subtracted from mean probe signal and subtracted values less than 0.5 were set to 0.5. Values were normalized across samples using quantile normalisation and log2 transformed. Agilent quality flags were used to identify probes robustly detected in all replicates of at least one sample group. This requires the difference between probe signal and local background to be more than 1.5 times the local background noise with acceptable spot image morphology. Replicates showed good correlation as evidenced by comparison of the median coefficient of variation from all probes which was 2.8% across all NUC2/OV2 samples decreasing to 1.9% within sample groups. In total, 20,732 probes passed quality filtering and were tested for differential expression using the limma package, allowing for the paired nature of the experimental design. Finally 1725 probes, corresponding to 1524 gene identifiers showed evidence of differential expression ($P \leq 0.01$, $FC \geq 2$). Probes were annotated through alignment to TAIR10. Heatmaps were generated in R using the heatmap.2 function in the gplots package.

qPCR

RNA was extracted from frozen tissue using the RNeasy Kit (Qiagen). Quantitative PCR was performed on Superscript III-generated cDNA

(Invitrogen) and normalised using *eIF4a*. Primer sequences are listed in Sup Table 6.

Constructs

Details of all constructs are available upon request. At least 10 transgenic lines were examined for each construct.

Microscopy

Ovules were dissected from flowers and cleared in 0.25% chloral hydrate, 30% glycerol for phenotypic analysis or 10% glycerol for fluorescence analysis on a Zeiss M1 Imager as per previous (Tucker et al., 2008).

Figure 1: Identification of candidates via LCM. (A) Megasporogenesis and megagametogenesis in *Arabidopsis* ovules. Yellow shading indicates the nucellus. Dotted lines outline epidermal cells. (B) Pre-meiotic ovule. (C) Post-meiotic ovule. (D,E) Sectioned flower containing ovules before and after NUC2 (red line) capture. Remaining tissue was captured as OV2 (blue line). (F) Venn diagram (http://bioinfogp.cnb.csic.es/tools/venny/index.html) comparing ovule gene expression datasets listed in Sup Table 4. (G-P) Candidate promoter expression patterns in ovules. (G,H) pWUS:GFP. (I) pAt2g43150:3xnls-vYFP. (J) pAt5g54570:YFP. (K) pAt2g27880:3xnls-vYFP. (L) pAt1g21670:3xnls-vYFP. (M) pAt3g05460:erYFP. (N) pAt5g53250:YFP (O) pAt3g2100:3xnls-vYFP. (P) pAt5g40730:3xnls-vYFP. ne, nucellar epidermis; mmc, megaspore mother cell; f, funiculus; oi, outer integument; ii, inner integument; fm, functional megaspore; ds, degenerating megaspores; ms, megaspores; FG, female gametophyte; MFG, mature FG; ccn, central cell nucleus; ecn, egg cell nucleus*; spl*, *sporocyteless*. Scale bars: 10µm.

Figure 2: Ovule development in *ago5-4/+* (A) Comparison of seeds in mature wild-type (left) and *ago5-4*/+ (right) siliques. (B) Wild-type anthesis ovule. (C) Wild-type-like *ago5-4*/+ anthesis ovule. (D) Phenotypic *ago5-4*/+ anthesis ovule showing FG1 abortion. (E,F) pKNU:nlsYFP in wild-type and *ago5-4*/+ pre-meiotic ovules. (G,H) pKNU:nlsGUS in wild-type and *ago5-4*/+ anthesis ovules. Inset shows expression in a post-meiotic ovule. (I,J)

pFM1:GUS in wild-type and *ago5-4*/+ anthesis ovules. Inset shows expression in a post-meiotic ovule. (K,L) MYB64:GFP in wild-type and *ago5-4*/+ anthesis ovules. Aborted FGs are outlined with dashed lines. Labels as per Fig. 1. FG1, one-nucleate FG Scale bars: 10µm.

Figure 3: *AGO5* expression and analysis of double mutants and transgenic lines. (A) *AGO5* mRNA levels during gynoecia development (B) *AGO5* and *ago5-4* allele-specific expression as a proportion of total *AGO5* expression in *ago5-4*/+ gynoecia. (C) *AGO5* expression derived from *ago5-1*, *ago5-2* and *ago5-4* alleles. (D) FG abortion in double mutants and complementation lines +/- standard deviation. PM, pre-meiosis; ME, meiosis; AN, anthesis; PF, post-fertilisation.

Figure 4: Cell-specific expression of *AGO5-4* and RNAi suppressor proteins. (A-L) Expression of *YFPer*, *AGO5-4*, *P1/Hc-Pro* and *P19* directed by (A-D) *pAGO5*, (E-H) *pWUS* and (I-L) *pKNU*. Aborted FGs are outlined with dashed lines. (M-N) YFP-AGO5 fusion protein is located in the cytoplasm of nucellar epidermal and inner integument cells. (O-P) YFP-AGO5-4 accumulates similarly to YFP-AGO5 in M and N. Dotted lines outline the position of the megaspores. (Q) Model for sRNA function during megasporogenesis and megagametogenesis. AGO9 acts through 24nt siRNAs in nucellar epidermal cells to inhibit reproductive development of sub-epidermal cells. A second somatic sRNA pathway involving one or more AGOs (AGOx), promotes megagametogenesis (arrows) in the functional megaspore, likely through a different class of 24nt siRNAs. This pathway could influence megagametogenesis by transmitting a sRNA into the MMC or megaspores, by repressing movement of a mRNA or downstream metabolite from the nucellus or by indirectly influencing nucellus development. Labels as per Figs. 1 and 2. sy, synergid; ec, egg cell. Scale bars: 10µm except 5µm in B,C,F and G.

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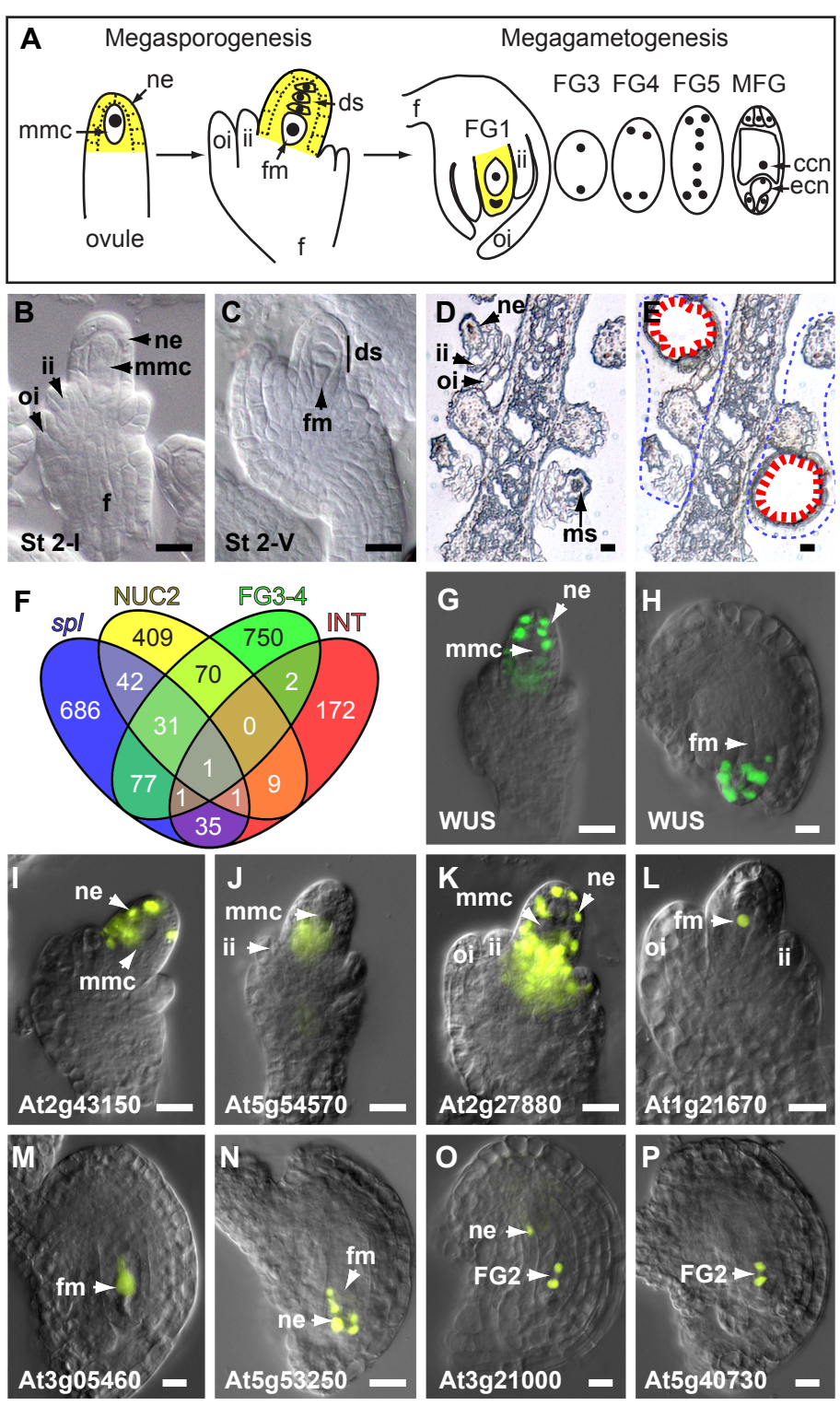
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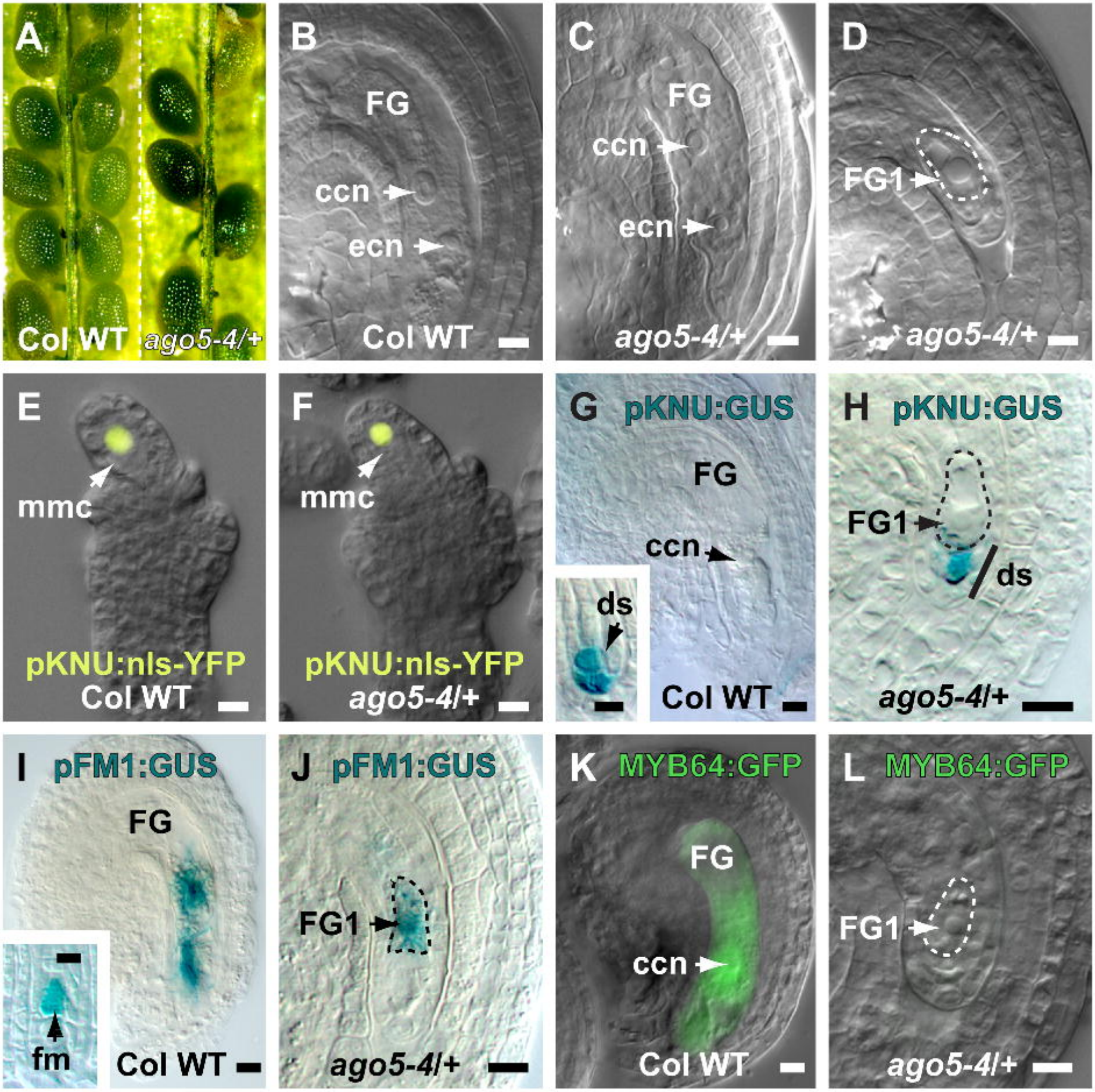
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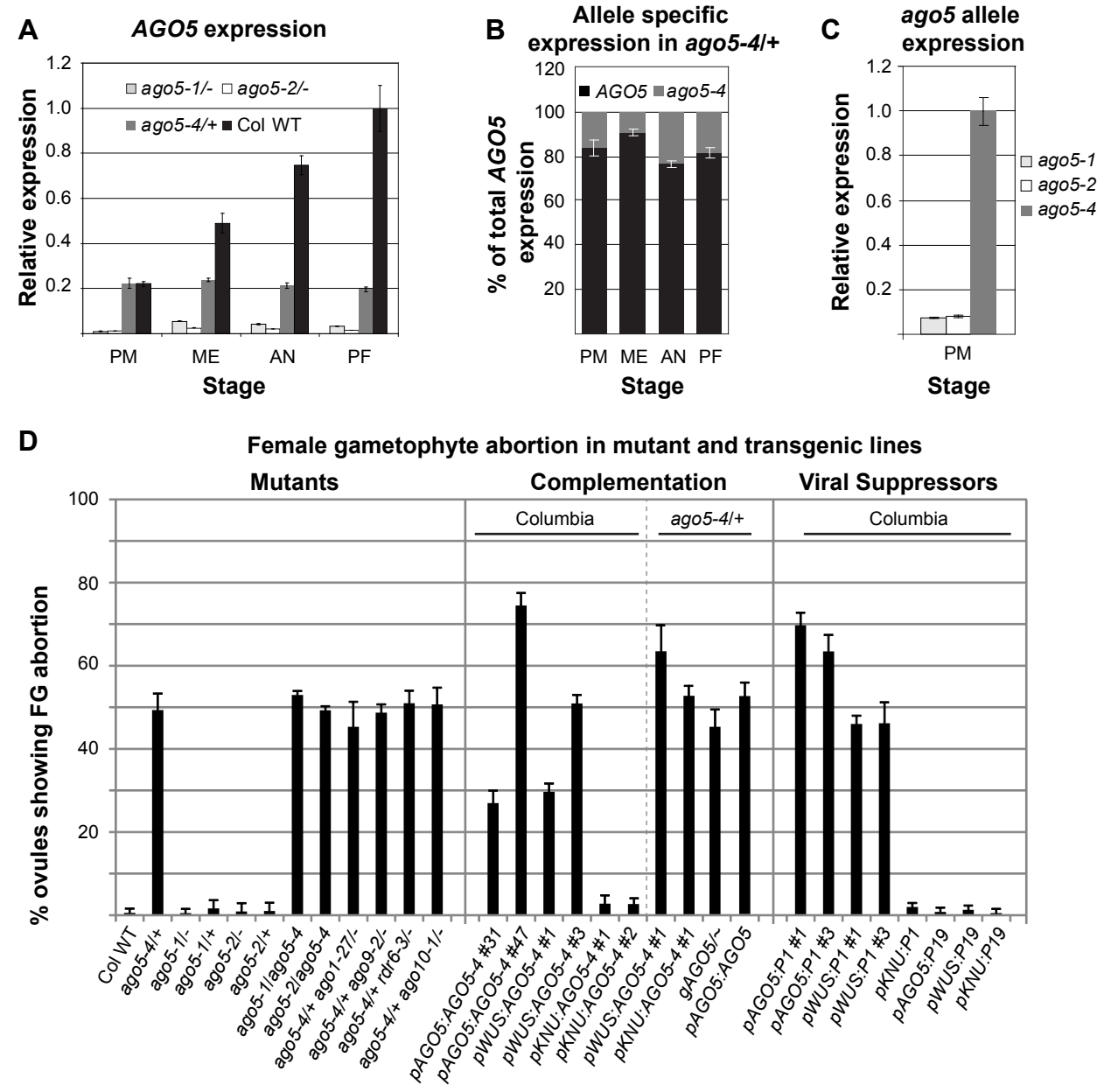
A
AGO5 expression
Relative expression
ago5-1/- ago5-2/-
ago5-4/+ Col WT
PM ME AN PF
Stage
B
Allele specific expression in ago5-4/+
% of total AGO5 expression
AGO5 ago5-4
PM ME AN PF
Stage
C
ago5 allele expression
Relative expression
ago5-1
ago5-2
ago5-4
PM
Stage
D
Female gametophyte abortion in mutant and transgenic lines
Mutants
Complementation
Viral Suppressors
Columbia
ago5-4/+
Columbia
% ovules showing FG abortion
Col WT
ago5-4/+
ago5-1/-
ago5-1/+
ago5-2/-
ago5-2/+
ago5-1/ago5-4
ago5-2/ago5-4
ago5-4/+ ago1-27/-
ago5-4/+ ago9-2/-
ago5-4/+ rdr6-3/-
ago5-4/+ ago10-1/-
pAGO5:AGO5-4 #31
pAGO5:AGO5-4 #47
pWUS:AGO5-4 #1
pWUS:AGO5-4 #3
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pKNU:AGO5-4 #1
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pAGO5:AGO5
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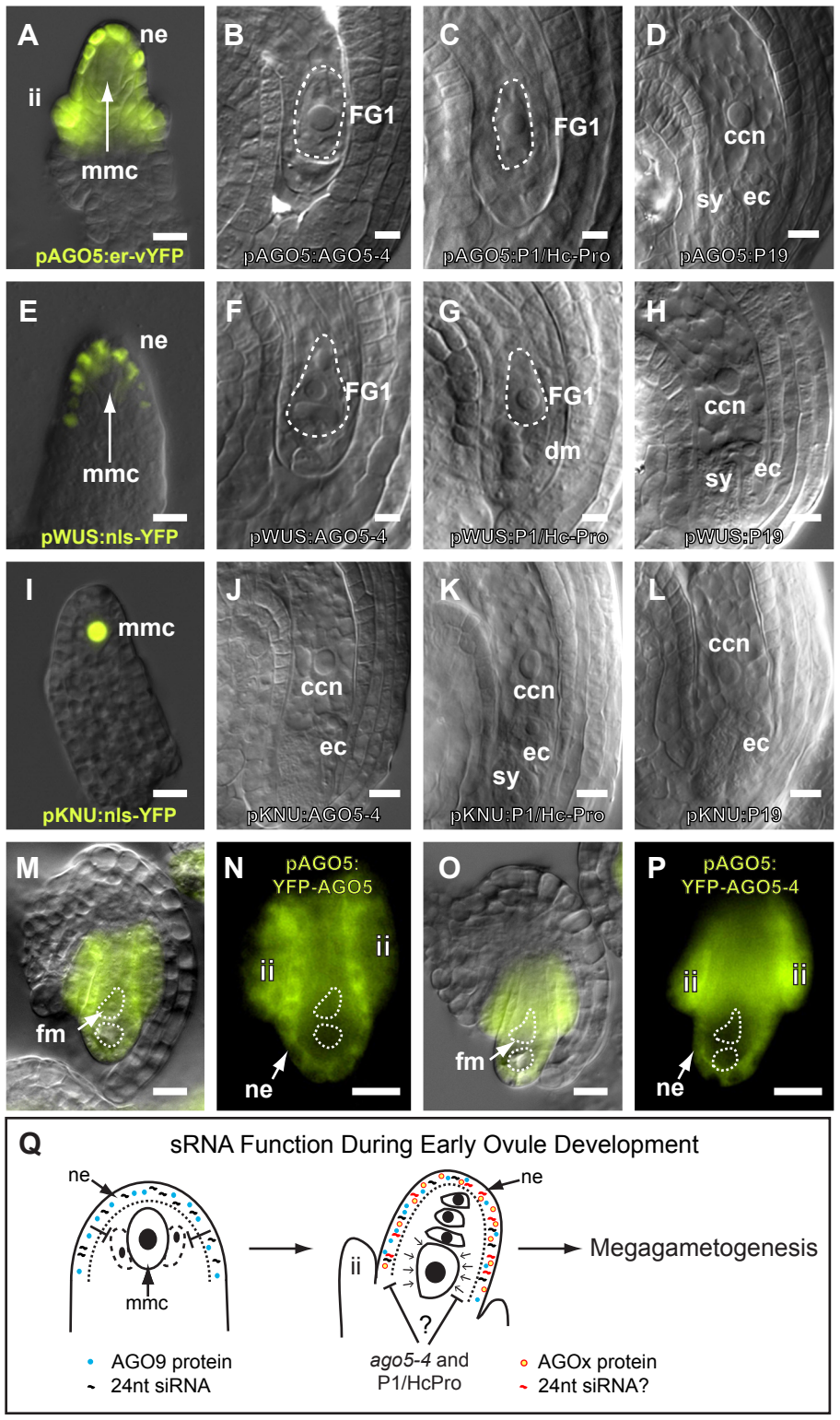
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H
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Q
sRNA Function During Early Ovule Development
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?
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AGO9 protein
24nt siRNA
ago5-4 and
P1/HcPro
AGOx protein
24nt siRNA?