Natural Variation in Epigenetic Pathways Affects the Specification of Female Gamete Precursors in Arabidopsis

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In angiosperms, the transition to the female gametophytic phase relies on the specification of premeiotic gamete precursors from sporophytic cells in the ovule. In Arabidopsis thaliana, a single diploid cell is specified as the premeiotic female gamete precursor. Here, we show that ecotypes of Arabidopsis exhibit differences in megasporogenesis leading to phenotypes reminiscent of defects in dominant mutations that epigenetically affect the specification of female gamete precursors. Intraspecific hybridization and polyploidy exacerbate these defects, which segregate quantitatively in F2 populations derived from ecotypic hybrids, suggesting that multiple loci control cell specification at the onset of female meiosis. This variation in cell differentiation is influenced by the activity of ARGONAUTE9 (AGO9) and RNA-DEPENDENT RNA POLYMERASE6 (RDR6), two genes involved in epigenetic silencing that control the specification of female gamete precursors. The pattern of transcriptional regulation and localization of AGO9 varies among ecotypes, and abnormal gamete precursors in ovules defective for RDR6 share identity with ectopic gamete precursors found in selected ecotypes. Our results indicate that differences in the epigenetic control of cell specification lead to natural phenotypic variation during megasporogenesis. We propose that this mechanism could be implicated in the emergence and evolution of the reproductive alternatives that prevail in flowering plants.

INTRODUCTION

The life cycle of flowering plants alternates between a dominant, diploid, sporophytic generation and a short-lived, haploid, gametophytic generation in specialized reproductive organs. Integration between environmental signals and developmental programs that control flowering initiates development of the female reproductive lineage within the gynoecium. During early formation of the gynoecium in Arabidopsis thaliana, ovule primordia develop from the placenta as finger-like protrusions by active cell divisions in the subepidermal layer (Schneitz et al., 1995; Grossniklaus and Schneitz, 1998; Ferrándiz et al., 1999). A single cell is specified as the archesporial, which directly differentiates into the premeiotic gamete precursor known as the megaspore mother cell (MMC). The MMC subsequently divides by meiosis to produce four haploid cells, one of which is specified as the functional megaspore (FM), the first cell of the female gametophytic phase. The FM develops by three rounds of mitosis into a female gametophyte, containing three antipodal cells, two synergids, the egg, and a binucleated central cell. After double fertilization, the egg and the central cell will develop into the embryo and the endosperm, respectively (Reiser and Fischer, 1993; Grossniklaus and Schneitz, 1998; Drews and Koltunow, 2011).

This pattern of development characteristic of Arabidopsis and the majority of flowering plants is known as the monosporic, Polygonum-type of female gametogenesis (Maheshwari, 1950; Eames, 1961). Although the Polygonum-type prevails in most angiosperms examined to date (Huang and Russell, 1992), there are many examples of naturally occurring variations that affect cell specification in the ovule during female meiosis or gametogenesis. These variations often involve the emergence of several female gamete precursors (Vandendries, 1909; Grossniklaus and Schnitz, 1998; Bacheler and Friedman, 2011), the incorporation of more than one meiotically derived product to the female gametophyte (Maheshwari, 1950; Madrid and Friedman, 2009), the formation of unreduced female gametophytes (Karpechenko, 1927; Bretagnolle and Thompson, 1995; Ramsey and Schenske, 1998), or the formation of seeds through asexual reproduction by a process known as apomixis that bypasses meiosis and fertilization (Grimanelli et al., 2001; Koltunow and Grossniklaus, 2003). Although extensive comparative and morphological reports describing these alternatives are available for many angiosperm taxa, the genetic basis and molecular mechanisms that control this type of reproductive natural variation remain unclear.

Several genes are known to be involved in the control of gamete cell specification and meiosis, and they act either by restricting the number of meiotic precursors or by enabling the progression through the meiotic division (Sheridan et al., 1996; Ferrándiz et al., 1999; Schiefthaler et al., 1999; Nonomura et al., 2003; Lieber et al., 2011). In addition, epigenetic components mediated by the action of small RNAs (sRNAs) have been described as important regulators of cell specification and female gametogenesis in Arabidopsis (Armenta-Medina et al., 2011; Rodríguez-Leal and Vielle-Calzada, 2012). sRNAs are 18- to 30-nucleotide RNA molecules that regulate gene expression at the transcriptional and posttranscriptional level by their association with members of the ARGONAUTE (AGO)
protein family. Several classes of sRNAs have been defined, depending on their mechanisms of biogenesis and action (Ghildiyal and Zamore, 2009). The function of AGO9 and other specific members of the so-called RNA-directed DNA methylation or trans-acting small interfering RNA pathways are important for the correct specification of female gamete precursors. Mutations in genes such as RNA-DEPENDENT RNA POLYMERASE6 (RDR6), DICER-LIKE3, and AGO9 exhibit increased frequency of abnormal gamete precursors that often give rise to more than one female gametophyte developing in the Arabidopsis ovule (Olmedo-Monfil et al., 2010). Additional roles for sRNAs and their interactors in female meiosis and gametogenesis have been described, suggesting that some epigenetic pathways are crucial for the establishment of the gametophytic generation (Nonomura et al., 2007; Garcia-Aguilar et al., 2010; Olmedo-Monfil et al., 2010; Schmidt et al., 2011; Tucker et al., 2012).

Here, we report a detailed analysis of the phenotypic effects of natural variation in the control of gamete precursor specification in the developing ovule of Arabidopsis. We show that in phylogenetically distant ecotypes, the mechanisms that specify gamete precursors are naturally variable, and they often lead to the differentiation of supernumerary cells as meiotic precursors. Moreover, the frequency at which these ectopic cell configurations occur is increased in F1 hybrids of specific ecotypes, and the prevalence of ectopic cells at late stages of meiosis is increased in tetraploid individuals. We also show that the genetic introgression of mutations affecting the function of AGO9 or RDR6 is buffered by allelic interactions in the ovule of ecotypic F1 hybrids and that the complete loss of AGO9 activity disrupts this mitigating effect. Finally, we demonstrate that the patterns of transcriptional regulation and protein localization of AGO9 are variable between ecotypes and that the abnormal gamete precursors found in mutants defective in RDR6 share a cellular identity with the ectopic cells naturally found in specific ecotypes. Our results link previously characterized epigenetic pathways to mechanisms of natural variation that affect the specification of female gamete precursors in Arabidopsis.

RESULTS

Selected Ecotypes of Arabidopsis Show Natural Variation in Cell Differentiation during Megasporogenesis, Which Is Influenced by Intraspecific Hybridization and Ploidy

Previous reports showed that in most cases of meiosis in Arabidopsis, a single subepidermal cell in the ovule primordium is specified as the MMC (Vandendries, 1909; Schnetz et al., 1995). The MMC enters meiosis and gives rise to four haploid cells, of which only one survives to develop into female gametophyte. To further characterize the timeframe of cell differentiation in the apical region of the developing ovule, we correlated premeiotic to postmeiotic cellular differentiation with integumentary growth in five genetically distant ecotypes of Arabidopsis and their respective hybrids (Figure 1). In addition to the reference ecotype Columbia-0 (Col-0), we selected Shakdara-0 (Sha-0), Borky-4 (Bor-4), Cape Verde Islands-0 (Cvi-0), and Monterrosso-0 (Mr-0) as four genetically distant ecotypes with a contrasting pattern of geographic and environmental distribution (Nordborg et al., 2005). As presented in Figure 1A and Table 1, four temporal stages that encompass meiosis were defined to score cell differentiation and division. We defined Stage 1 as corresponding to ovule primordium having a well-defined proximal-distal axis and the absence of integument initiation. Stage 2 comprises ovules that have initiated integument growth and have an inner integument composed of a maximum of two cell layers. In Stage 3 ovules, both integuments have initiated growth and development, and the inner integument has three to four cell layers. Finally, Stage 4 ovules contain an inner integument of at least five cell layers and an adaxial outer integument that reaches the tip of the nucellus.

All four stages encompass the main cellular events occurring during meiosis, up to the differentiation of the FM at the onset of megagametogenesis. For Stages 1 to 3, the majority of ovules in all five ecotypes showed a single conspicuous MMC with a dense cytoplasm, large nucleus, and prominent nucleolus, occupying a preponderant position in direct contact with the apical L1 layer. At these same stages, all five ecotypes also showed variable frequencies of ovules harboring more than one cell reminiscent of the MMC (Figures 1B to 1E); these alternative morphological events were named ectopic configurations. Mr-0 was the most variable ecotype, with nearly 30% of ovules showing ectopic configurations at Stage 1 (Table 1; Supplemental Table 1). Following the developmental progression from Stages 1 to 4, the frequency of ectopic configurations declined in all ecotypes, being almost absent at Stage 4, suggesting developmental competition between neighboring ectopic cells (Table 1). In all cases where ectopic configurations were observed, a single linear arrangement of degenerated cells adjacent to a surviving megaspore was observed at Stage 4, suggesting that a single cell divided meiotically.

We also quantified cell differentiation during meiosis in F1 individuals resulting from intraspecific crosses between Col-0 and each of the additional four selected ecotypes. The results were analyzed using a χ² test. As shown in Table 1 and Supplemental Table 2, the phenotypic frequencies revealed cases of both additive and nonadditive effects, depending on the parental combinations and the developmental stage analyzed. When compared with their mid-parent value (i.e., the average phenotype frequency observed in the parental lines), F1 individuals resulting from crosses between Col-0 and Sha-0 showed additive effects in Stages 1, 3, and 4, while F1 plants from crosses between Col-0 and Bor-4 or Cvi-0 ecotypes exhibited nonadditive phenotypic frequencies at different stages. Whereas for Col-0 × Cvi-0 F1 individuals nonadditivity was confirmed for Stages 1 and 3, Col-0 × Bor-4 F1 hybrids showed the same pattern from Stages 1 to 3. A statistically significant suppressive effect on the frequency of ectopic configurations was observed in the Col-0 × Sha-0 F1 individuals, suggesting that in this cross nonadditive effects are associated with reduction of ectopic configurations. In the case of Col-0 × Mr-0 F1 hybrids, a nonadditive effect was only present across Stages 1 and 2. In all genetic combinations, the trend in phenotypic frequencies was consistent in reciprocal crosses, showing that the selection of an ecotype as a maternal or paternal parent did not influence the F1 results (Supplemental Table 1).

Finally, we quantified cell differentiation during meiosis in tetraploid individuals of Col-0 and Landsberg erecta (Ler). Both ecotypes exhibited high frequencies of ovules showing...
ectopic configurations compared with their diploid counterparts (29.9% for Col-0; 24.2% for Ler at Stage 1). Although in both ecotypes the frequency of ectopic configurations decreased during the progression of megasporogenesis, 10.7% of Col-0 ovules showed ectopic configurations at Stage 4—a frequency significantly higher than diploid ovules of this same ecotype (0.8%) at that stage—suggesting that dosage factors are able to mitigate the developmental mechanisms that favor competition between neighboring gamete precursors. Overall, these results indicate that premeiotic gamete cell specification is naturally variable in the ovule of Arabidopsis, with a strong tendency toward restricting the persistence of ectopic configurations at late stages of megasporogenesis. They also show that specific allelic combinations have a tendency to increase the differentiation of ectopic cells at the onset of meiosis, whereas higher ploidy levels in specific ecotypes tend to increase the prevalence of ectopic cells at late stages of megasporogenesis.

**The Frequency of Ectopic Cells in the Ovule Segregates Continuously among Individuals of Wild-Type F2 Populations**

To determine whether multiple segregating factors could contribute to the variability found in gamete precursor cell specification, the frequency of ovules showing ectopic configurations was quantified in a population of F2 individuals from two different crosses that previously exhibited nonadditive effects (Col-0 × Cvi-0 and Col-0 × Bor-4). For ~50 F2 individuals per cross, close to 100 ovules were cytologically analyzed at Stage 1 in both segregating populations (Figure 2). In both cases, the phenotypic frequencies of ectopic
Ecotypic Variation in Cell Differentiation Is Influenced by the Activity of AGO9

The presence of ectopic configurations at variable frequencies in ecotypes of Arabidopsis is reminiscent of phenotypes found in dominant ago9 and rdr6 loss-of-function mutants. Plants defective in AGO9 or RDR6 showed ectopic differentiation of female gamete precursors and subsequent formation of extranumerary female gametophytes in the developing ovule (Olmedo-Monillo et al., 2010). To investigate a possible involvement of these sRNA-related genes in the natural ecotypic variation during megasporogenesis, we quantified the frequency of ectopic configurations in F1 progeny resulting from the cross of ago9-3 (Col-0) individuals with Bor-4, Cvi-0, and Mr-0 plants (Table 2). Whereas heterozygous F1 individuals resulting from a cross between homozygous ago9-3 and Sha-0 showed an additive increase in ovules exhibiting ectopic configurations (Table 2), F1 plants from homozygous ago9-3 crossed to Bor-4, Cvi-0, and Mr-0 showed no increase in the frequency of ovules showing ectopic configurations compared with heterozygous ago9-3/+ or their corresponding wild-type ecotypic hybrids. A similar tendency was detected in reciprocal crosses between rdr6-15 mutants (Col-0) and Cvi-0, indicating that the effect of dominant ago9 or rdr6 mutations in the ectopic configuration phenotype is buffered in ecotypic hybrids, as the introduction of ago9-3/+ or their corresponding wild-type ecotypic hybrids.

Table 1. Quantitative Analysis of Cell Differentiation during Megasporogenesis in Ovules of Arabidopsis Ecotypes, Their Wild-Type Hybrid, and Tetraploid Lines

| Genotype | Single MMC (%) | Ectopic Configurations (%) | Total | Single MMC (%) | Ectopic Configurations (%) | Total | Functional | Meiotic Segregation of Ovules Showing Ectopic Con (B) Frequency of ovules showing ectopic configurations in F2 Populations Originating from Ecotypic F1 Hybrids. | Total |
|----------|----------------|----------------------------|-------|----------------|----------------------------|-------|-------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| Col-0    | 478 (80.4)     | 51 (9.6)                   | 529   | 578 (89.6)     | 67 (10.4)                  | 529   | 593 (96.1)  | 24 (3.9)                                                                                                                                  | 529   |
| Ler      | 282 (83.7)     | 55 (16.3)                  | 337   | 325 (89.9)     | 38 (10.5)                  | 337   | 245 (96.1)  | 10 (3.92)                                                                                                                                  | 337   |
| Sha-0    | 370 (95.2)     | 19 (4.8)                   | 388   | 705 (96.6)     | 25 (3.4)                   | 398   | 500 (99.2)  | 4 (0.8)                                                                                                                                  | 398   |
| Bor-4    | 620 (89.5)     | 73 (10.5)                  | 693   | 702 (93.6)     | 53 (7)                     | 693   | 335 (95.4)  | 16 (4.6)                                                                                                                                  | 693   |
| Cvi-0    | 314 (88.5)     | 41 (11.5)                  | 355   | 335 (92.5)     | 27 (7.5)                   | 355   | 241 (93.8)  | 16 (6.2)                                                                                                                                  | 355   |
| Mr-0     | 303 (72.7)     | 114 (27.3)                 | 417   | 339 (82.9)     | 70 (17.1)                  | 409   | 339 (96.3)  | 13 (3.7)                                                                                                                                  | 409   |
| Col-0 × Sha-0 F1 | 491 (88.2) | 66 (11.8)                  | 557   | 612 (92.2)     | 13 (8)                     | 625   | 453 (97.4)  | 12 (2.6)                                                                                                                                  | 625   |
| Col-0 × Bor-4 F1 | 323 (78.6) | 88 (21.4)                  | 411   | 311 (79.7)     | 79 (20.3)                  | 390   | 269 (91.2)  | 26 (8.8)                                                                                                                                  | 390   |
| Col-0 × Cvi-0 F1 | 174 (71.3) | 70 (28.7)                  | 244   | 305 (86.9)     | 46 (13.1)                  | 351   | 249 (89.9)  | 28 (10.1)                                                                                                                                  | 351   |
| Col-0 × Mr-0 F1 | 401 (77.4) | 117 (22.6)                 | 518   | 251 (83.9)     | 48 (16.1)                  | 299   | 313 (83.7)  | 21 (6.3)                                                                                                                                  | 299   |
| Col 4N   | 282 (70.1)     | 120 (29.9)                 | 402   | 534 (85.9)     | 88 (14.1)                  | 622   | 355 (97.3)  | 10 (2.7)                                                                                                                                  | 622   |
| Ler 4N   | 292 (75.8)     | 93 (24.2)                  | 385   | 335 (82.7)     | 70 (17.3)                  | 405   | 253 (97.7)  | 6 (2.3)                                                                                                                                  | 405   |

Figure 2. Segregation of Ovules Showing Ectopic Configurations in F2 Populations Originating from Ecotypic F1 Hybrids.

(A) Frequency of ovules showing ectopic configurations in Col-0 × Cvi-0 F2 individuals.

(B) Frequency of ovules showing ectopic configurations in Col-0 × Bor-4 F2 individuals.
heterozygous ago9-3/+ plants introgressed into a Bor-4 background showed frequencies of ectopic configurations in Stage 1 ovules equivalent to those found in homozygous ago9-3 (Col-0) individuals ($\chi^2 = 2.35 < \chi^2_{0.05} = 3.84$; Table 2). Since homozygous ago9-3 mutants were previously shown to lack any AGO9 activity (Olmedo-Monfli et al., 2010), this result indicates that the buffering effect described above is overcome by the complete loss of function of AGO9. Our results suggest that although hybridization between some phylogenetically distant ecotypes perturbs the process of gamete precursor cell specification at quantitative levels similar to those observed in mutants defective in AGO9 function, the introduction of mutations affecting AGO9 or RDR6 activity is buffered by complex allelic interactions in specific ecotypic F1 hybrids. They also show that the complete absence of AGO9 activity disrupts this buffering effect, revealing a genetic interaction between the natural mechanisms that control ecotypic variation during megasporogenesis and the function of AGO9.

Natural Variation in Genomic Regulatory Regions of AGO9

Results in Changes in Transcriptional Regulation and Protein Localization

To determine whether differences in AGO9 expression could help explain differences in the frequency of ectopic configurations found between Col-0 and Mr-0 ecotypes, we conducted mRNA whole-mount in situ hybridization in developing ovules of Col-0 and Mr-0 individuals using a 149-bp antisense RNA probe corresponding to a Col-0 sequence located in the 3’ untranslated region (Supplemental Figure 1). As expected from previous results, in both genetic backgrounds, AGO9 mRNA was localized in all cells of the ovule primordium throughout megasporogenesis. However, under identical experimental conditions, the level of mRNA expression was consistently higher in developing ovules of Col-0 compared with Mr-0 ovules. Because the level of genomic polymorphisms between Col-0 and Mr-0 within the sequence corresponding to the selected probe is not sufficient to cause deficiencies in the formation of antisense RNA:mRNA duplexes to explain this difference (Supplemental Figure 1), these in situ hybridization results suggest that AGO9 mRNA is weakly expressed in Mr-0 ovules. To determine whether this difference in AGO9 expression is related to ecotypic differences in AGO9 transcriptional regulation, the complete 2619-bp intergenic region upstream of the AGO9 coding sequence isolated from either Col-0 or Mr-0 plants was cloned in front of the uidA (β-glucuronidase [GUS]) reporter gene to subsequently transform Col-0 wild-type individuals. Stage 1 ovules of Col-0 plants transformed with a transcriptional fusion that includes the Col-0 AGO9 regulatory sequence (proCol-0AG09:GUS) showed strong GUS expression after 6 h of histochemical incubation, with initial expression in the nucellar cells located at the proximal pole of the MMC (99%, n = 110). At Stage 2, GUS expression was localized in additional nucellar cells and in the L1 layer. At Stage 4, GUS expression prevailed in the chalazal region of the ovule (Figures 3A to 3C). In contrast, ovules of Col-0 plants transformed with a transcriptional fusion that includes the Mr-0 AGO9 regulatory sequence (proMr-0AG09:GUS) showed GUS expression only after 24 to 36 h of histochemical incubation in a pattern antagonistic to developing ovules of proCol-0AG09:GUS transformants. The majority of proMr-0AG09:GUS ovules at Stage 1 (88%, n = 568) showed GUS expression restricted to L1 cells located at the apical (distal) pole of the MMC. Although Stage 2 ovules showed GUS expression restricted to a larger number of L1 cells in the same region, Stage 4 ovules showed GUS expression in a pattern similar but not identical to Stage 4 ovules in proCol-0AG09:GUS transformants (Figures 3D to 3F), since GUS expression in the subependimal apical region remains strong in proMr-0AG09:GUS transformants. We also determined the pattern of AG09 transcriptional regulation in developing ovules of F1 plants generated by crossing individuals of Sha-0, Bor-4, and Cvi-0 to the proCol-0AG09:GUS Col-0 line (Figures 3G to 3L). Whereas the general pattern of GUS expression was reminiscent of the pattern found in ovules of Col-0, ovules of F1 individuals exhibited specific differences at both premeiotic and postmeiotic stages (Stages 1 and 4). At Stage 1, whereas Sha-0 × proCol-0AG09:GUS F1 ovules showed a pattern equivalent to Col-0 (Figure 3G), GUS expression was substantially reduced in Bor-4 × proCol-0AG09:GUS and Cvi-0 × proCol-0AG09:GUS F1 ovules (Figures 3H and 3I). Also, whereas initial GUS expression in Col-0 ovules was in most nucellar cells located at the proximal pole of the MMC, Bor-4 × proCol-0AG09:GUS F1 individuals showed initial expression in a small cluster comprising L2 and L3 cells at the midlateral region of the premeiotic primordium (Figure 3H). At Stage 4, Bor-4 × proCol-0AG09:GUS and Cvi-0 × proCol-0AG09:GUS F1s also showed reduced GUS expression compared with Sha-0 ×

Table 2. Quantitative Comparison among Wild-Type and Mutant Ecotypic Hybrids at Stage 1

| Genotype | Single MMC (%) | Ectopic Configurations (%) | Total |
|----------|----------------|---------------------------|-------|
| Col-0 × Sha-0 F1 | 491 (88.2) | 66 (11.8) | 557 |
| ago9-3 × Sha-0 F1 | 646 (78.4) | 178 (21.6) | 824 |
| Col-0 × Cvi-0 F1 | 174 (71.3) | 70 (28.7) | 244 |
| ago9-3 × Cvi-0 F1 | 228 (76.0) | 72 (24.0) | 300 |
| rdr6-15 × Cvi-0 F1 | 372 (77) | 111 (23) | 483 |
| Col-0 × Mr-0 F1 | 401 (77.4) | 117 (22.6) | 518 |
| ago9-3 × Mr-0 F1 | 303 (73.2) | 111 (26.8) | 414 |
| Col × Bor-4 F1 | 323 (78.6) | 88 (21.4) | 411 |
| ago9-3 × Bor-4 F1 | 560 (78.4) | 154 (21.6) | 714 |
| ago9-3 × BC ago9-3 (Bor-4) −/− F1 | 396 (73.9) | 140 (26.1) | 536 |
| ago9-3+/− | 371 (75.3) | 122 (24.7) | 493 |
| rdr6-15/+ | 369 (68.6) | 169 (31.4) | 538 |
| ago9-3 | 551 (70) | 236 (30) | 787 |
proCol-0AGO9:GUS F1 and Col-0 ovules (Figures 3J to 3L), indicating that in these F1 individuals AGO9 expression is reduced throughout megasporogenesis. Taken together, these results suggest that the factors controlling transcriptional regulation of AGO9 are variable among ecotypes and their hybrids. They also suggest that although divergent regulation of the Mr-0 AGO9 promoter region in the Col-0 background can cause antagonistic changes in the spatial pattern of reporter gene expression, additional genetic factors are likely to compensate these changes to produce similar but not identical spatial patterns of transcriptional regulation at subsequent stages of ovule development.

To determine the pattern of AGO9 protein expression in ovules of selected ecotypes, we conducted whole-mount immunolocalizations using a polyclonal antibody previously reported to specifically recognize an epitope of the AGO9 protein. Previous experiments showed that in wild-type Col-0 ovules undergoing meiosis, AGO9 is localized in discrete cytoplasmic foci of sporophytic cells, preferentially in the apical region of the L1 layer, but not in meiotically dividing cells or in the functional megaspore. The same antibody was used to localize AGO9 in Stage 1 ovules of all five selected ecotypes (Figure 4). In premeiotic Stage 1 ovules of Col-0, Bor-4, and Sha-0, AGO9 was localized in cytoplasmic foci present in most sporophytic cells of the primordium, but also transiently in the nucleus of the MMC at variable expression levels. In these three ecotypes, most Stage 1 ovules also showed a clear pattern of preferential AGO9 localization in a cluster of apical L1 cells located at the distal pole of the MMC, in agreement with previous immunolocalizations conducted in Col-0 at subsequent developmental stages (Figures 4A to 4C). By contrast, most Stage 1 ovules of Mr-0 and Cvi-0 did not show this preferential localization of AGO9 in cells of the L1 layer (Figures 4D and 4E). Whereas the large majority of ovules of Col-0, Bor-4, and Sha-0 showed AGO9 localization in apical cells of the L1 layer (86.4% for Col-0, 97.2% for Bor-4, and 95.8% for Sha-0; total \( n = 119 \)), only close to 50% showed the equivalent pattern in Mr-0 and Cvi-0 (50% for Cvi-0 and 53.4% for Mr-0; total \( n = 94 \)). These results indicate that the ecotypic differences found in the pattern of AGO9 transcriptional regulation are also reflected in the cellular pattern of protein localization.

Abnormal Ectopic Cells in Ovules Defective for RDR6 Share Identity with Ectopic Cells Found in the Analyzed Ecotypes

The nuclear pattern of AGO9 protein localization specifically shown by the MMC was also present in extranumerary cells found in ovules of ecotypes Mr-0, Bor-4, and Cvi-0. Previous studies indicated that heterozygous rdr6-15/+ individuals showed aberrant
cell specification at premeiotic stages, with several female gamete precursors differentiating, growing, and dividing at the apical region of the developing ovule. To determine if the ectopic cells naturally found in selected ecotypes acquire a developmental identity similar to aberrant accessory cells found in ovules defective for RDR6, we conducted AGO9 whole-mount immunolocalizations in ovules of heterozygous rdr6-15/+ plants. Under whole-mount histological observations, the frequency at which Stage 1 rdr6-15/+ ovules exhibited accessory cells was of 31% (n = 538). For rdr6-15/+ ovules, whereas the preferential localization of AGO9 in the apical cells of the L1 layer was absent, AGO9 was expressed in the nucleus of accessory cells present in the nucellus (Figure 4F). Under confocal illumination, the frequency of Stage 1 rdr6-15/+ ovules showing AGO9 localization was of 26% (n = 33). An identical nuclear pattern of AGO9 localization was observed in Stage 1 ectopic cell configurations present in ovules of the Mr-0, Bor-4, and Cvi-0 ecotypes (Figures 4G to 4I), suggesting that naturally occurring ectopic cells in Arabidopsis ecotypes acquire the same identity as abnormal female gamete precursors found in rdr6-15/+ individuals, confirming that natural variation in the specification of female gamete precursors acts through some of the sRNA-dependent epigenetic pathways that prevail in the ovule of Arabidopsis.

DISCUSSION

Intraspecific natural variation in the angiosperms offers opportunities for natural selection to exert evolutionary pressure over the diversity of developmental pathways that are essential for survival, reproduction, or dispersal (Alonso-Blanco et al., 2009; Prasad et al., 2012; Anderson et al., 2014). In some cases, this variation is directly related to quantitative traits that can be mapped and associated with developmental or physiological processes (Atwell et al., 2010; Strange et al., 2011), whereas in others it can be traced to functional multiallelic diversity of a single locus (Todesco et al., 2010). Epigenetic natural variation, manifested through either epimutations or epialleles, is currently under intense investigation to assess its impact in both adaptability and evolution. Although the extent to which epigenetic variation contributes to phenotypic variation remains to be determined, several examples of naturally occurring epialleles that affect plant development have been reported (Brink,

Figure 4. AGO9 Protein Localization in Stage1 Ovules of Arabidopsis.
(A) AGO9 localization in a Col-0 ovule.
(B) AGO9 localization in a Sha-0 ovule.
(C) AGO9 localization in a Bor-4 ovule.
(D) Cvi-0 ovule showing absence of AGO9 localization in the L1 layer.
(E) Mr-0 ovule showing absence of AGO9 localization in the L1 layer.
(F) AGO9 localization in a heterozygous rdr6-15/+ ovule; arrows indicate the presence of gamete precursors.
(G) AGO9 localization in a Mr-0 ovule; arrows indicate the presence of gamete precursors.
(H) AGO9 localization in a Bor-4 ovule; arrows indicate the presence of gamete precursors.
(I) AGO9 localization in a Cvi-0 ovule; arrows indicate the presence of gamete precursors.
(J) AGO9 expression is absent in homozygous ago9-3 ovules.
L1, L1 cell layer; N, nucleus of the MMC. Bars = 10 μm in (A) to (J) and 5 μm in insets in (F) to (I).
Arabidopsis has provided abundant evidence of epigenetic variation through either spontaneous or induced epimutants, including the induction of flowering, seed development, and genomic imprinting (Shindo et al., 2006; Fujimoto et al., 2011; Pignatta et al., 2014); however, the consequences of natural epigenetic variation in gametogenesis have not been investigated.

Our study shows that natural variation among distinct Arabidopsis ecotypes reflects the epigenetic mechanisms that lead to the differentiation of female gamete precursors at the onset of meiosis. Despite significant differences in their intrinsic frequencies of ectopic configurations, crosses between Col-0 and Sha-0 or Mr-0 showed additive effects, suggesting that widespread natural variation is based on conserved and independent genetic factors that control the specification of gamete precursors. By contrast, F1 hybrids between Col-0 and Bor-4 and Cvi-0 gave rise to ovules in which the frequency of gamete precursors was significantly increased compared with the corresponding mid-parent value. This disruption in the control of cell specification, beyond the frequency observed in the parental ecotypes, is reminiscent of nonadditive effects associated with the phenomenon of heterosis or hybrid vigor (Birchler et al., 2010; Chen, 2010). Differences in the phenotypic frequency of ectopic configurations are reminiscent of heterotic responses previously reported for F1 progeny of some Arabidopsis ecotypes (Moore and Lukens, 2011; Groszmann et al., 2014). A similar effect was also found in Medicago sativa, for which only certain parental combinations produced heterotic responses, as revealed by nonadditive gene expression (Li et al., 2009). Although an increase in the number of female gamete precursors does not necessarily affect fertility or seed production, the possibility of increasing the number of cells entering the gametophytic developmental pathway, through divergent allelic combinations present in specific ecotypic hybrids, could represent an adaptation to detrimental conditions that affect female meiosis and cause fertility defects.

Several studies have raised the possibility that alternative reproductive pathways, such as tetraspary or asexual reproduction through seeds (apomixis), evolved as a response to hybridization, genomic collisions, or unstable climatic environment (Carman, 1956; Cubas et al., 1999; Manning et al. 2006). Arabidopsis has effects associated with the phenomenon of heterosis or hybrid vigor (Birchler et al., 2010; Chen, 2010). Differences in the phenotypic frequency of ectopic configurations are reminiscent of heterotic responses previously reported for F1 progeny of some Arabidopsis ecotypes (Moore and Lukens, 2011; Groszmann et al., 2014). A similar effect was also found in Medicago sativa, for which only certain parental combinations produced heterotic responses, as revealed by nonadditive gene expression (Li et al., 2009). Although an increase in the number of female gamete precursors does not necessarily affect fertility or seed production, the possibility of increasing the number of cells entering the gametophytic developmental pathway, through divergent allelic combinations present in specific ecotypic hybrids, could represent an adaptation to detrimental conditions that affect female meiosis and cause fertility defects.

Previous results showed that during megasporogenesis, AG09 protein localization is equivalent in both ecotypes, their initial pattern of transcriptional regulation is antagonistic, revealing that divergent allelic combinations can have an effect at the transcriptional level in the control of AG09 expression. When comparing transcriptional fusions corresponding to Col-0 and Mr-0 alleles, the pattern of GUS expression is similar but not identical at the onset of female gametogenesis, suggesting that a dynamic pattern of transcriptional regulation and possibly mRNA transport prevals during megasporogenesis, since the pattern of mRNA localization coincides with the final pattern of AG09 protein localization. Because AG09 contains a large intron at the 5' region that was not included in the proAG09:GUS fusions tested, it is possible that additional genomic elements present in the transgenic lines could influence the ectopic pattern of AG09 transcription. The transcriptional regulation of AG09 is also altered in some hybrids between ecotypes, particularly in those exhibiting a nonadditive exacerbation of the frequency of ectopic configurations (Col-0 × Bor-4 and Col-0 × Cvi-0 F1 individuals), suggesting that hybridization can also perturb AG09 expression by reducing its transcriptional activity, leading to the differentiation of accessory cells into premeiotic gamete precursors, leading to phenotypic consequences that result in natural variation during female reproductive development.

Previous results showed that during megasporogenesis, AG09 protein localization was confined to discrete foci present in the cytoplasm of sporophytic cells within the nucellus, with abundant expression at the apex of the ovule primordium, in cells of the L1 layer (Olmedo-Monfil et al., 2010). Our results show that the same pattern prevals in Stage 1 ovules at the onset of meiosis; however, the nucleus of the MMC in several ecotypes sporadically
shows AGO9 expression, suggesting that an ephemeral and transient nuclear AGO9 localization can be found in the MMC, a discovery that suggest a dynamic pattern of protein traffic between cytoplasm and nucleus reminiscent of AGO4 dynamics in root cells (Li et al., 2006; Pontes et al., 2006; Ye et al., 2012). In the case of AGO9, nuclear localization is exclusive of the MMC and ectopic (Li et al., 2006; Pontes et al., 2006; Ye et al., 2012). In the case of cytoplasm and nucleus reminiscent of AGO4 dynamics in root cells to integrate an understanding of AGO9 function, sRNA dynamics, (University of Arizona).

**METHODS**

**Plant Material and Growth Conditions**

*Arabidopsis thaliana* ecotypes Bor-4, Sha-0, Cvi-0, and Mr-0 were obtained from the ABRC as part of the collection of 96 ecotypes provided by Joy Bergelson, Martin Kreitman, and Magnus Nordborg (Stock CS22660). Tetraploid Col-0 (CS3176) and Ler were a gift from David Galbraith (University of Arizona), ago9-3 (SAIL_34_G10) and rdr6-15 (SAIL_617_H07) were also obtained from the ABRC. Five rounds of backcrossing were performed to introgress the ago9-3 mutant allele into Bor-4 ecotype. All seeds were surface sterilized with 100% ethanol or with chlorine gas and germinated in Murashige and Skoog medium at 22°C or 25°C under stable long-day (16 h light/8 h dark) or full-day conditions. Plants were grown at 24°C under controlled growth chamber or greenhouse conditions.

**Generation and Analysis of proAGO9:GUS Transforms**

The regulatory region of the AGO9 gene (At5g21150) from Col-0 and Mr-0 ecotypes was transcriptionally fused to the uidA (GUS) reporter gene by amplifying a 2619-bp DNA fragment corresponding to the complete intergenic region located upstream of its coding sequence but excluding the 5’ untranslated region (primers: pAGO9S1_HindIII 5’-AATATGAGCTTGGGAAGACGAAAGTCCGGTGAAGAGAGAC3’ and pAGO9AS1_KpnI 5’-GGCGTCGTTACGTTAATAATAGGTTGTCGTTTATA3’). Amplifications were cloned into pCR8 TOPO TA (Invitrogen) and used as donors in LR recombination (LR Clonase II; Invitrogen) with pMDC162 (Curtis and Grossniklaus, 2003), producing a binary vector that contains the uidA reporter gene. Transgenic Col-0 plants were obtained by floral dipping as previously described (Clough and Bent, 1998). At least 15 T1 individuals were obtained and analyzed for each of the two constructs (Col-0 or Mr-0 version). At least five individuals of 10 independent T2 transformants were cytologically examined to quantify the frequency of GUS expression at different stages of megagametogenesis.

**Cytological and Histological Analysis**

Influences were fixed in FAA (50% ethanol, 10% formaldehyde, and 5% acetic acid) for 24 to 48 h and subsequently dehydrated in 70% ethanol. Immature flower buds were dissected with hypodermic needles (1-mL insulin syringes; TERUMO SS10M2913M) to isolate the developing gynoecium. Several hundred ovules for each of the four stages were obtained by mounting individual gynoecia in regular microscope slides and immersing in Her’s clearing solution (pheno:chloral hydrate:85% lactic acid:xylene:clovce oil in a 1:1:0.5:1 proportion). Histochromosomal localization of GUS activity was performed by incubating gynoecia of 0.5 to 1 mm in length in GUS staining solution (10 mM EDTA, 0.1% Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in 50 mM sodium phosphate buffer, pH 7.4) for 2 to 36 h at 37°C. For propidium iodide staining, gynoecia of 0.5 to 0.7 mm in length were fixed in FPA (10% formaline, 5% propionic acid, and 70% ethanol) overnight at 4°C. After fixation, samples were washed with 100 mM L-arginine, pH 8.0 (Sigma-Aldrich) and stained with 2 mg mL⁻¹ propidium iodide in 100 mM of 4',6-Diamidino-2-phenylindole (ph3.5). Ovule primordia were exposed by gently pressing a cover slip over a conventional slide containing 15 mL of Vectashield (VectorLabs). Serial optical sections were obtained on a Zeiss LSM510 META confocal laser scanning microscope, with single-track configuration for detecting propidium iodide (excitation with a diode-pumped solid-state laser at 568 nm, with emission collected using a band-pass of 575 to 615 nm). Sections were edited using ImageJ software (Schneider et al., 2012). For light microscopy observations, samples were analyzed under Nomarski illumination using a DMR Leica microscope.

**Whole-Mount in Situ Hybridization**

Digoxigenin-labeled RNA probes specific for AGO9 were synthesized by in vitro transcription as previously described (Viele-Calzada et al., 1999; Olmedo-Monfort et al., 2010). Hybridizations were performed as previously described (Garcia-Aguilar et al., 2005). Developing gynoecia of 0.5 to 0.8 mm in length were fixed in paraformaldehyde (4% paraformaldehyde, 2% Triton, and 1× PBS in diethylpyrocarbonate [DEPC]-treated water) for 2 h at room temperature with gentle agitation, washed three times in 1× PBS-DEPC water, and embedded in 15% acrylamide:bisacrylamide (29:1) using
precharged slides (Fisher Probe-On) treated with poly-L-Lys as described (Bass et al., 1997). Gynoecia were gently opened to expose the ovules by pressing a cover slip on top of the acrylamide. Samples were then treated with 0.2 M HCl for 20 min at room temperature, followed by a washing step in 1× PBS-DEPC water. The slides were then incubated with protease K (1 μg mL⁻¹) for 30 min at 37°C. The protease K reaction was stopped by glycine (2 mg mL⁻¹). A postfixation step was performed in 4% formaldehyde for 20 min at room temperature, followed by incubation in hybridization buffer (6× SSC buffer, 3% SDS, 50% formamide, and 0.1 mg mL⁻¹ of yeast tRNA [Roche] for 2 h at 37°C. An overnight incubation was performed using 600 ng of sense or antisense RNA probe against AGO9 in hybridization buffer. After overnight incubation, three washing steps with 0.2× SSC/0.1% SDS at 55°C were performed. Slides were treated with RNase (10 μg mL⁻¹), washed four times in 2× SSC/0.1% SDS at 55°C, and treated with 1× TBS/0.5% blocking agent [Roche] for 2 h at room temperature. Samples were incubated with anti-DIG (Roche) at a concentration of 1:1000 in 1× TBS/0.1% BSA for 2 h at room temperature. After antibody incubation, four washing steps in 1× TBS/0.5% BSA/1% Triton X-100 were performed at room temperature. Slides were incubated in detection buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween, and 1 mM levamisole [Sigma-Aldrich]) for 15 min before adding 10 μL of each nitroblue tetrazolium and 5-bromo-4-chloro-3′-indolylphosphate (AP Conjugate Substrate Kit; Bio-Rad) per milliliter of detection buffer and incubated overnight at room temperature. Slides were mounted in 50% glycerol and visualized using Nomarski illumination under a Leica DMR microscope.

**Whole-Mount Protein Immunolocalization**

Developing gynoecia of 0.5 to 0.6 mm in length were fixed in paraformaldehyde (1× PBS, 4% paraformaldehyde, and 2% Triton), under continuous agitation for 2 h on ice, washed three times in 1× PBS, and embedded in 2% acrylamide: bisacrylamide (29:1) over precharged slides (Fisher Probe-On) treated with poly-L-Lys as described (Bass et al., 1997). Gynoecia were gently opened to expose ovules by pressing a cover slip on top of the acrylamide. Samples were digested in an enzymatic solution composed of 1% droselase, 0.5% cellulase, and 1% pectolase (all from Sigma-Aldrich) in 1× PBS for 60 min at 37°C, subsequently rinsed three times in 1× PBS, and permeabilized for 2 h in 1× PBS:2% Triton. Blocking was performed with 1% BSA [Roche] for 1 h at 37°C. Slides were then incubated overnight at 4°C with AGO9 primary antibody used at a dilution of 1:100 (Olmedo-Monfil et al., 2010). Slides were washed for 8 h in 1× PBS:0.2% Triton, with refreshing of the solution every 2 h. The samples were then coated overnight at 4°C with secondary antibody Alexa Fluor 488 (Molecular Probes) at a concentration of 1:100. After washing in 1× PBS:0.2% Triton for at least 8 h, the slides were incubated with propidium iodide (500 μg mL⁻¹) in 1× PBS for 20 min, washed for 40 min in 1× PBS, and mounted in Prolong medium (Molecular Probes) overnight at 4°C. Serial sections on Stage 1 ovules were captured on a confocal laser scanning microscope (Zeiss LSM 510 META), with multitrack configuration for detecting iodide (excitation with a diode-pumped solid-state laser at 588 nm, emission collected using a band-pass of 575 to 615 nm) and Alexa 488 (excitation with an argon laser at 488 nm, emission collected using a band-pass of 500 to 550 nm). Laser intensity and gain were set at similar levels for all experiments. Projections of selected optical sections were generated using ImageJ (Schneider et al., 2012).

**Accession Numbers**

Sequence data for genes in this article can be found in the GenBank/EMBL database or the Arabidopsis Genome Initiative database under the following accession numbers: AGO9, NM_122122/AED92940 or At5g21150; and RD16, NM_114910/AED78550 or At3g69500. The ago9-3 (SAIL_34_G10) and rd16-15 (SAIL_617_H07) lines were obtained from the ABRC.

**Supplemental Data**

**Supplemental Figure 1.** AGO9 mRNA localization in the developing ovule of Col-0 and Mrt-0 ecotypes.

**Supplemental Table 1.** Quantitative analysis of ectopic configurations during meiosis in selected ecotypes of Arabidopsis.

**Supplemental Table 2.** Quantitative analysis of ectopic configurations in the ovule of ecotype F1 hybrids.

**Supplemental Table 3.** Segregation analysis in F2 populations from Arabidopsis ecotype hybrids.

**ACKNOWLEDGMENTS**

We thank Stewart Gillmor and Shai Lawit for useful comments on the article, Marcella García-Aguilar for technical advice with immunolocalizations, and members of the Group of Reproductive Development and Apomixis for stimulating discussions. All Arabidopsis seed stocks were obtained through the ABRC at Ohio State University. D.R.-L. and G.L.-M. were recipients of a graduate scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACyT). Research was supported by CONACyT, the DuPont Pioneer regional initiatives to benefit local subsistence farmers, and the Howard Hughes Medical Institute.

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Natural Variation in Epigenetic Pathways Affects the Specification of Female Gamete Precursors in *Arabidopsis*
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*Plant Cell*; originally published online March 31, 2015;
DOI 10.1105/tpc.114.133009

This information is current as of August 15, 2017

| Supplemental Data | /content/suppl/2015/03/10/tpc.114.133009.DC1.html |
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