

# The Triploid Endosperm Genome of *Arabidopsis* Adopts a Peculiar, Parental-Dosage-Dependent Chromatin Organization<sup>W</sup>

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The endosperm is a seed tissue unique to flowering plants. Due to its central role in nourishing and protecting the embryo, endosperm development is subject to parental conflicts and adaptive processes, which led to the evolution of parent-of-origin–dependent gene regulation. The role of higher-order chromatin organization in regulating the endosperm genome was long ignored due to technical hindrance. We developed a combination of approaches to analyze nuclear structure and chromatin organization in *Arabidopsis thaliana* endosperm. Endosperm nuclei showed a less condensed chromatin than other types of nuclei and a peculiar heterochromatin organization, with smaller chromocenters and additional heterochromatic foci interspersed in euchromatin. This is accompanied by a redistribution of the heterochromatin mark H3K9me1 from chromocenters toward euchromatin and interspersed heterochromatin. Thus, endosperm nuclei have a specific nuclear architecture and organization, which we interpret as a relaxed chromocenter-loop model. The analysis of endosperm with altered parental genome dosage indicated that the additional heterochromatin may be predominantly of maternal origin, suggesting differential regulation of maternal and paternal genomes, possibly linked to genome dosage regulation.

## INTRODUCTION

During double fertilization, the pollen tube delivers two sperm cells to the female gametophyte: one fertilizes the egg cell giving rise to the embryo, while the other fuses with the central cell forming the endosperm. In most flowering plants, the central cell contains two haploid nuclei, which fuse prior to or at fertilization. The fusion of this homodiploid nucleus with the sperm nucleus results in a triploid primary endosperm nucleus with two maternal and one paternal genome complements (2m:1p). This unusual triploid genetic constitution is typical, although not universal, of flowering plants and has attracted the attention of botanists for over a century (for review, see Birchler, 1993; Friedman, 2001; Baroux et al., 2002a).

“The endosperm is an intriguing botanical tissue” (Birchler, 1993). The endosperm is an ephemeral tissue, restricted to the period of seed production and germination. It is largely consumed either by the developing embryo or after germination by the seedling (Lopes and Larkins, 1993). In addition to storage and nourishing functions (Lopes and Larkins, 1993; Weschke et al., 2003), the endosperm plays a protective role and secretes antifungal peptides (Guo et al., 1999; Serna et al., 2001; Balandin et al., 2005). This central role as an embryo nurse tissue may provide an evolutionary explanation for the unusual genetic constitution of the endosperm. On one hand, the paternal con-

tribution can be viewed as a source of heterozygosity and triploidy as providing polyploid vigor, a genetic state beneficial to the high rate of proliferation and metabolism of the endosperm (for review, see Lopes and Larkins, 1993; Friedman, 2001). On the other hand, Haig and Westoby (1989) proposed that triploidy and biparental origin of the endosperm may be the result of genetic parental conflicts over resource allocation from the maternal sporophyte to its offspring. Double fertilization may have first evolved to convey the interests of the father in promoting its offspring's growth rate, while doubling of the maternal genome may be seen as an evolutionary response to reinforce the interests of the mother to equally partition resources among siblings (Haig and Westoby, 1989; Friedman and Williams, 2003). Furthermore, triploidy seems to have a selective advantage in offering more levels to regulate dose responses in ongoing parental conflicts (Stewart-Cox et al., 2004).

Either as a consequence of such a conflict or as an adaptive trait, several mechanisms arose to control embryo and endosperm growth in a parent-of-origin–specific manner. Endosperm with an imbalanced parental genome contribution, for instance, as a result of crosses between di- and tetraploid plants, often abort (Birchler, 1993). In maize (*Zea mays*) and *Arabidopsis thaliana*, increased maternal genome contribution (e.g., in 4m:1p endosperm) lead to a small endosperm with reduced mitotic activity and precocious cellularization, whereas an increased paternal contribution (e.g., in 2m:2p endosperm) promotes a large endosperm with accelerated proliferation and delayed cellularization (Lin, 1982; Scott et al., 1998). These experiments illustrate distinct functions of the maternal and paternal genomes in the regulation of endosperm growth. However, these parent-of-origin effects may not only arise from the dosage of parental genomes (maternal:paternal genome ratio) but also from the dosage of cytoplasmic components contributed by the maternal gametes in relation to the ploidy of the zygote (von Wangenheim and

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Peterson, 2004; Grossniklaus, 2005). Synthetic maize endosperms with a ploidy higher than  $3n$  and deviating from the  $2m:1p$  constitution abort even within a normal diploid maternal tissue (Lin, 1984), while endosperm of high ploidy but with a  $2m:1p$  ratio develop normally, indicating that the relative dosage of the parental alleles is more important than the overall genome dosage. In addition, deletions or lesions at certain chromosomal loci can cause endosperm failure when inherited from one parent that cannot be complemented when a wild-type copy is introduced through the other parent (Lin, 1982; Grossniklaus, 2005).

Thus, several mechanisms apparently contribute to the unequal influence of parental genomes over endosperm growth regulation and include (1) disproportionate cytoplasmic contributions by the gametes (e.g., of transcripts, proteins, and organelles), (2) imbalanced genomic contributions ( $2m:1p$ ), and (3) differential expression of parental alleles due to genomic imprinting (Baroux et al., 2002b; Dilkes and Comai, 2004). The regulation of the triploid endosperm genome may integrate a complex interplay of stoichiometric parental expression ( $2:1$  maternal:paternal ratio) at certain loci and maternal- or paternal-specific expression at others. Large-scale chromatin modifications during epigenetic regulation of parental genomes in the endosperm may play a role. In maize, the maternal genome is extensively hypomethylated (Lauria et al., 2004), and in *Arabidopsis*, hypomethylation of either the maternal or paternal genome has antagonistic effects on endosperm growth (Adams et al., 2000). Knowledge of the nuclear organization of the triploid endosperm genome during its development, when parent-of-origin effects on seed development are manifest, may contribute to understanding the function of chromatin remodeling in regulating parental genome expression.

We applied a combination of cytogenetic approaches to analyze the nuclear phenotype of the *Arabidopsis* endosperm. The triploid endosperm nuclei display unique features in comparison with diploid differentiated or meristematic nuclei. The genome is less condensed in endosperm than in other types of nuclei and is characterized by the presence of endosperm-specific heterochromatic foci interspersed within euchromatin and by a redistribution of the heterochromatic mark H3K9me1 into euchromatin. We found that the fraction of interspersed heterochromatin increases with higher maternal, but not paternal, genome dosage, suggesting that this new heterochromatin is likely linked to parental dosage and is preferentially, but not exclusively, formed by maternal components.

## RESULTS

### Endosperm Cells Have Larger Nuclei and Less Condensed Chromatin Than Other Cell Types

The endosperm is present several cell layers beneath the maternal tissues of the seed enclosed in siliques. Classical DNA staining procedures that allow analysis of *Arabidopsis* seeds by confocal laser scanning microscopy (CLSM) usually employ Feulgen's stain or glutaraldehyde (Braselton et al., 1996; Scott et al., 1998; Barrell and Grossniklaus, 2005). However, these procedures either involve chromatin denaturing steps or are not

specific to the nuclear chromatin. Thus, we established a new, nondenaturing DNA staining procedure (see Methods) for CLSM analysis of the endosperm nuclei in whole-mount seeds until the early globular embryo stage (Figures 1A and 1B) and for later stages after removal of the seed coat (Figures 1C and 1D). This enabled measurements of nuclear and nucleolar size in relation to the stages of endosperm growth. In *Arabidopsis* seeds, the endosperm develops in two major phases: (1) active growth during proliferation of the nuclei in a syncytium and (2) cellularization and differentiation (Berger, 1999). We followed the nomenclature presented by Boissnard-Lorig et al. (2001) to describe the developmental stage of the endosperm, with the difference that under our conditions the embryo developed a little bit faster (data not shown). Because parent-of-origin effects are manifested during endosperm growth, we analyzed endosperm nuclei before cellularization, corresponding to stages I to IX according to Boissnard-Lorig et al. (2001). The endosperm is subdivided into peripheral, chalazal, and micropylar endosperm (Boissnard-Lorig et al., 2001; Figure 1). Here, we focused on nuclei from the peripheral endosperm. As controls, we studied differentiated nuclei from the maternal seed coat and meristematic nuclei from root primordia of young seedlings, which show a high mitotic activity similar to endosperm nuclei. Nuclear volume and the relative nucleolus size were measured on reconstructed three-dimensional confocal images at different stages of endosperm development (Figure 1D, Table 1; see Supplemental Figure 1 online for the full data set).

Soon after fertilization and before the zygotic nucleus divides, the endosperm undergoes mitosis (Boissnard-Lorig et al., 2001). Up to stage IV, endosperm nuclei have a volume of  $160 \mu\text{m}^3$  (i.e., they are  $\sim 4$  and  $3$  times larger than diploid seed coat and meristematic nuclei, respectively). Our measurements fall into the same range as the measurements reported by Mansfield and Briarty (1990) for early stages, but we find a decrease of nuclear volume at later stages (see Supplemental Figure 1 online). Endosperm nuclei have a large nucleolus that occupies  $\sim 18$  to  $28\%$  of the nuclear volume compared with  $9\%$  in seed coat and  $33\%$  in root meristem nuclei. The large nucleolus is compartmented by visible chromatin strands, a feature of endosperm but also of embryo nuclei (Figures 3C and 3D). Beyond stage IV, endosperm nuclei are still  $\sim 2$  to  $3$  times larger ( $\sim 80$  to  $125 \mu\text{m}^3$ ; Table 1; see Supplemental Figure 1 online) than seed coat or meristematic nuclei.

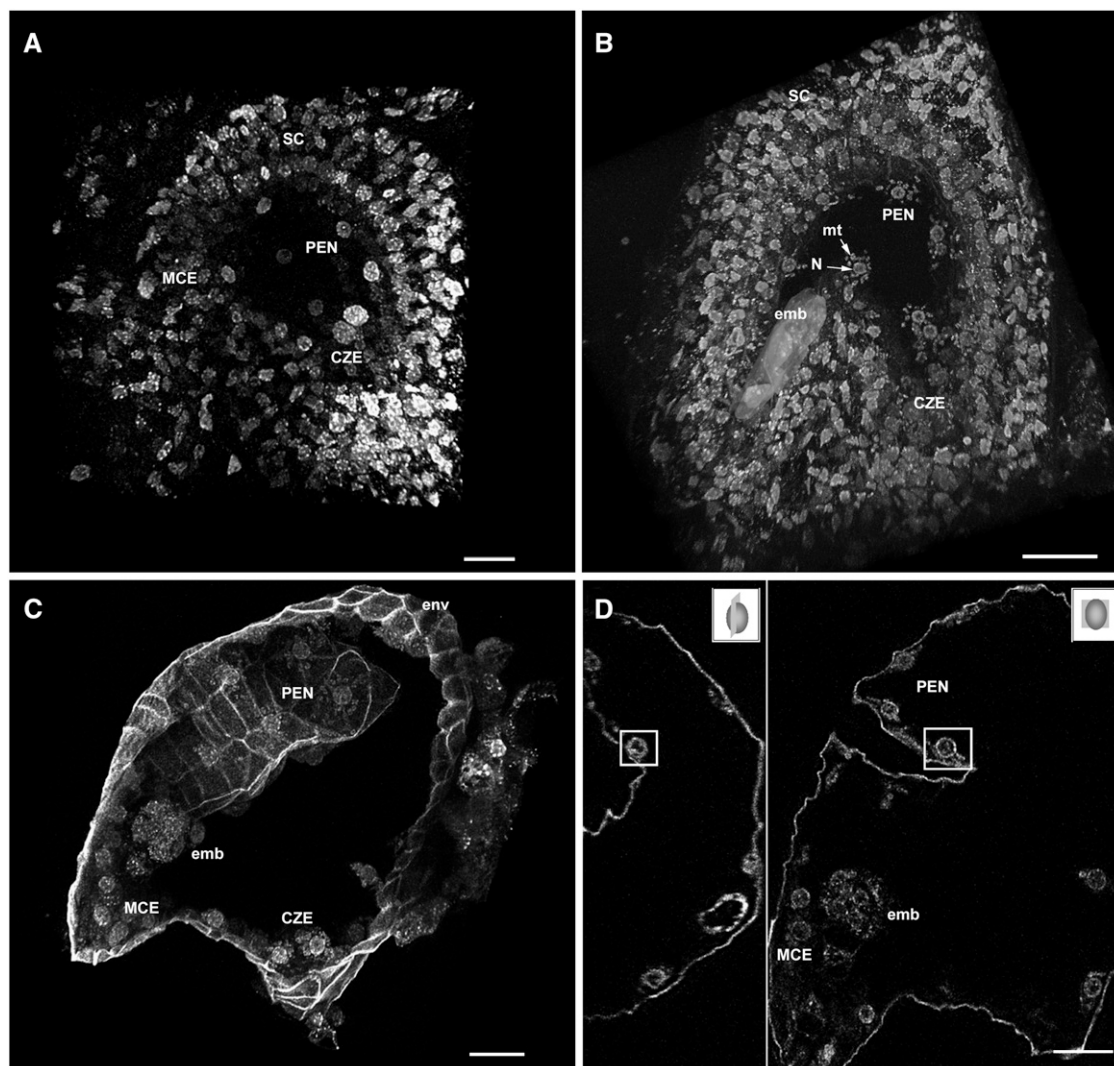
To gain insights into the level of chromatin compaction in interphase endosperm nuclei, we estimated the chromatin volume per  $1C$  content (Table 1). The estimated volume was  $16$  to  $17 \mu\text{m}^3/1C$  DNA content for both diploid tissues and differed significantly from the values of endosperm nuclei ( $P < 0.001$ ), showing  $\sim 41 \mu\text{m}^3/1C$  DNA content during the early growth phase and  $\sim 22$  to  $26 \mu\text{m}^3/1C$  DNA content at later stages (Table 1). Thus, chromatin compaction is lower by a factor of  $1.4$  to  $3.2$  in endosperm compared with other nuclei. This finding was corroborated by the observation that painted chromosome territories on isolated endosperm nuclei often appeared more extended and less compact in  $3C$  endosperm than in  $2C$  leaf nuclei, especially at the early growth phase (Figure 2).

Taken together, these data show that the genome of endosperm nuclei appears to be less condensed compared with that of diploid seed coat or meristematic root nuclei.

### Endosperm Nuclei Present a Novel Interspersed Heterochromatin Type of Predominantly, but Not Exclusively, Maternal Origin

DNA staining of endosperm nuclei in whole seeds revealed differences in euchromatin and heterochromatin distribution compared with seed coat or root meristem nuclei (Figure 3). As described previously for somatic nuclei (Fransz et al., 2002), heterochromatin forms densely stained chromocenters, while euchromatin reveals weaker and uniform staining in seed coat and meristematic nuclei (Figures 3A and 3B). In addition to chromocenters, endosperm nuclei contain heterochromatic foci

of variable size and intensity, interspersed in euchromatin and along chromatin strands that traverse the nucleolus (Figure 3C). These heterochromatic foci are not present in embryo nuclei (Figure 3D) and thus are specifically formed by the endosperm chromatin in *Arabidopsis*. Such endosperm-specific interspersed (ESI) heterochromatin is prominent in nuclei of the young endosperm, while the number of foci tends to decrease, but not disappear, at later stages (see Supplemental Figure 2 online). Overall, it represents 3.7% of the genome (Table 2). ESI heterochromatin was not found in somatic nuclei of higher ploidy (endoreduplicated nuclei) identified either during quantification of nuclear size in whole-mount seeds or in flow-sorted 4C seed



**Figure 1.** Nondenaturing Nuclear Staining in Whole-Mount Seeds.

Three-dimensional reconstructions ([A] to [C]) and single plane projections (D) of propidium iodide (PI)-stained *Arabidopsis* seeds at stage IV ([A]; eight-nuclei endosperm stage), stage V ([B]; two-cell stage embryo delimited by the artificial contour in gray and 16-nuclei endosperm), and stage VIII ([C] and [D]; 16-cell stage embryo and ~60-nuclei endosperm). In (C) and (D), the seed coat has been removed after cellulose and driselase treatment. (D) shows xz (left) and xy (right) plane projections of the specimen shown in (C). The frame shows a nucleus from the peripheral endosperm as used for size measurements. SC, seed coat; emb, embryo; PEN, peripheral endosperm; MCE, micropylar endosperm; CZE, chalazal endosperm; N, nucleus; mt, mitochondria. Bars = 100  $\mu$ m.

**Table 1.** Nuclear and Nucleolar Volumes and Genome Compaction of Endosperm Nuclei Compared with Seed Coat and Meristematic Nuclei

| Nucleus Type       | Nuclear Volume<br>$\mu\text{m}^3$ ( $\pm$ SD) |                  | Nucleolar Volume<br>$\mu\text{m}^3$ (% Nucleus) |                  | Volume per 1C Content<br>$\mu\text{m}^3$ ( $\pm$ SD) <sup>a</sup> |                  |                |
|--------------------|-----------------------------------------------|------------------|-------------------------------------------------|------------------|-------------------------------------------------------------------|------------------|----------------|
| Seed coat (2n)     | 37.8 ( $\pm$ 14.8)                            |                  | 3.3 (8.7%)                                      |                  | 17.2 ( $\pm$ 7.1)                                                 |                  | <i>n</i> = 35  |
| Root meristem (2n) | 48.9 ( $\pm$ 13.7)                            | <i>P</i> = 0.003 | 16.2 (33.1%)                                    | <i>P</i> < 0.001 | 16.3 ( $\pm$ 5.2)                                                 | <i>P</i> = 0.6   | <i>n</i> = 29  |
| Endosperm (3n)     |                                               |                  |                                                 |                  |                                                                   |                  |                |
| Stages II to IV    | 160 ( $\pm$ 42.1)                             | <i>P</i> < 0.001 | 37.7 (23.5%)                                    | <i>P</i> < 0.001 | 40.8 ( $\pm$ 9.7)                                                 | <i>P</i> < 0.001 | <i>n</i> = 69  |
| Stages V to VII    | 80.9 ( $\pm$ 27.9)                            | <i>P</i> < 0.001 | 14.5 (17.9%)                                    | <i>P</i> < 0.001 | 22.1 ( $\pm$ 7.9)                                                 | <i>P</i> < 0.001 | <i>n</i> = 126 |
| Stages VIII to IX  | 105 ( $\pm$ 21.1)                             | <i>P</i> < 0.001 | 28 (26.6%)                                      | <i>P</i> < 0.001 | 25.7 ( $\pm$ 6.7)                                                 | <i>P</i> < 0.001 | <i>n</i> = 9   |

<sup>a</sup> Data are given as (nuclear volume – nucleolar volume)/C value. *n*, number of nuclei analyzed. *P* values were calculated according to a bidirectional *t* test against measurements in seed coat nuclei.

nuclei (data not shown). ESI heterochromatin was also not found in somatic nuclei from tetraploid *Arabidopsis* lines (Figure 3E), indicating that ESI heterochromatin does not result from a higher DNA content in endosperm nuclei.

To elucidate the origin of ESI heterochromatin, we analyzed endosperm nuclei with altered proportions of maternal or paternal genomes (Figures 3F to 3H) and quantified the changes (Table 2). In balanced 2m:1p endosperm nuclei, ESI heterochromatin represents approximately one-fourth (0.25) of the total heterochromatin (Table 2). Crossing of diploid (2n) maternal and tetraploid (4n) paternal plants yielded 2m:2p endosperm nuclei with a fraction of ESI heterochromatin similar to that in balanced nuclei (Figure 3F, Table 2), indicating that the additional paternal genome contributed to the ESI heterochromatin fraction. Interestingly, 4m:1p endosperm nuclei of 4n × 2n crosses are significantly enriched in ESI heterochromatin (Figure 3G), constituting almost half of the total heterochromatin (0.42; Table 2). In *fertilization independent seed (fis)* mutants, endosperm development is initiated in the absence of fertilization and endosperm nuclei have the same 2m:0p constitution (Chaudhury et al., 1997) as sporophytic nuclei. In *fis* endosperm nuclei of the *mea-2* mutant (Grossniklaus et al., 1998), although the paternal genome is not present, ESI heterochromatin is still formed (Figure 3G) but represents a slightly lower fraction of the total heterochromatin (0.18; Table 2).

In summary, we found that ESI heterochromatin is specifically formed in the triploid endosperm genome and is neither a con-

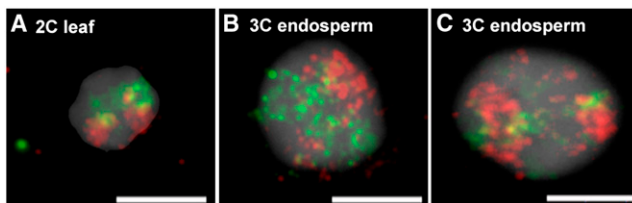
sequence of the higher ploidy level nor a by-product of fertilization. ESI heterochromatin is contributed to by both parental genomes. Importantly, however, additional ESI heterochromatin is formed upon doubling the maternal, but not the paternal, genome, indicating that ESI heterochromatin in *Arabidopsis* is linked to parental genome dosage and may be preferentially formed by maternal components upon increasing the maternal contribution.

#### The Interspersed Heterochromatin of the *Arabidopsis* Endosperm Is a Fraction Quantitatively Distinct from Chromocenters and Does Not Originate from the Dispersion of (Peri)centromeric Repeats

Chromocenters in the *Arabidopsis* endosperm nuclei looked smaller than in differentiated (seed coat) and meristematic nuclei (Figures 3A to 3C). Therefore, we quantified their heterochromatin content. Chromocentric heterochromatin represents ~20% of the nuclear chromatin in seed coat nuclei and 8.8% in meristematic nuclei, which correspond to the highest and lowest range reported for somatic nuclei, respectively (Tessadori et al., 2004). Normally balanced endosperm nuclei and endosperm with increased paternal or maternal genomes showed a similar chromocenter fraction (~11%; Table 2), while endosperm lacking a paternal genome showed variably sized chromocenters but with a heterochromatin content not significantly different from that of 2m:1p endosperm (8.7%, *P* = 0.2).

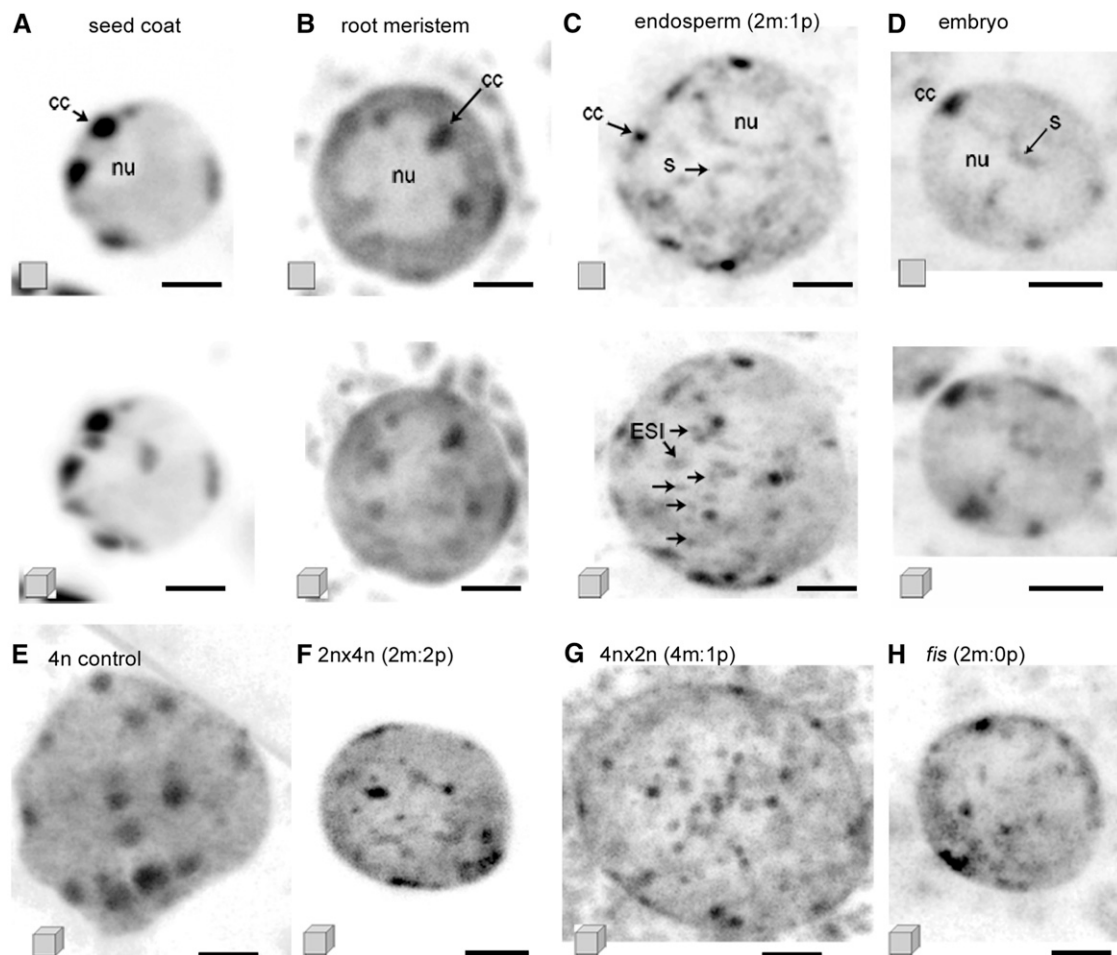
We investigated the possibility that ESI heterochromatin in balanced endosperm nuclei could arise by dispersion of sequences normally located within chromocenters. Chromocenters of *Arabidopsis* nuclei are constituted mainly of centromeric 180-bp repeats, pericentromeric sequences including high-copy transposable elements, and rDNA repeats (e.g., from the nucleolus organizing regions of chromosomes 2 and 4) (Fransz et al., 2003). In endosperm nuclei, centromeric and 45S rDNA repeats form chromocenters as in leaf nuclei (Figures 4A and 4B) and do not disperse as, for instance, in *ddm1-5* mutant nuclei (Probst et al., 2003). Pericentromeric *Athila* transposons (Pelissier et al., 1995) mainly cluster at or around the centromeric repeats in endosperm and leaf nuclei, although some *Athila* signals can be observed in endosperm euchromatin (Figures 4C and 4D).

Taken together, centromeric, pericentromeric, and rDNA repeats do not contribute to ESI heterochromatin, which thus must be formed by other sequences that remain to be identified.



**Figure 2.** Chromosome Territories Appear More Expanded in Endosperm Than in Leaf Nuclei.

Two-color fluorescence in situ hybridization (FISH) painting of chromosome 1 (top arm, red; bottom arm, green) in 2C leaf (A) and 3C nuclei from seeds at the preglobular embryo (<stage V; [B]) or later stages (stages VIII to IX; [C]), isolated by flow cytometry according to their ploidy. Chromosome territories are compact in leaf nuclei and more dispersed in endosperm nuclei. Bars = 2  $\mu\text{m}$ .



**Figure 3.** Endosperm Nuclei Show Specific Heterochromatic Foci Interspersed in Euchromatin That Seem Mostly Contributed by the Maternal Genome.

(A) to (D) Heterochromatin-euchromatin distribution in nuclei of different origin and genetic constitution: nuclei from seed coat (A), root meristem (B), endosperm at stage IV (C), and embryo (D) from a diploid plant.

(E) Sporophytic nucleus from a tetraploid plant.

(F) to (H) Endosperm nuclei with different parental genome ratios as indicated (m, maternal genome; p, paternal genome) at stages IV to VI. Nuclei in (F) and (G) are derived from endosperm of reciprocal crosses between a diploid and a tetraploid line. Endosperm in (H) is derived from unfertilized seeds of the *mea-2* mutant (Grossniklaus et al., 1998).

PI-stained nuclei from whole-mount seeds [(A) and (C) to (H)] or seedlings [(B) and (E)] were fine-scanned along their volume under confocal microscopy. The top panels in (A) to (D) show single planes, and the bottom panels show maximum projections of three-dimensional stacks. (E) to (H) show maximum projections. cc, chromocenter; nu, nucleolus; s, chromatin strands across the nucleolus. Bars = 2  $\mu$ m.

### ESI Heterochromatin Is Associated with H3K9me1 but Not with Other Heterochromatin-Specific Marks

Due to the numerous and small ESI heterochromatic foci, euchromatin-heterochromatin boundaries in endosperm nuclei are not as clearly defined as in somatic nuclei. Therefore, we investigated the distribution of histone and DNA modifications specifically associated with heterochromatin (Figure 5) or euchromatin (Figure 6; see Supplemental Figure 3 online). Despite the compatibility of our DNA staining procedure with histone immunolabeling in whole-mount vegetative tissues (data not shown), the latter was not reliably applicable to *Arabidopsis* en-

dosperm. Therefore, we isolated endosperm nuclei from seeds at the globular to transition embryo stages, during which the endosperm is not cellularized, and analyzed their chromatin in comparison with leaf and seed coat nuclei.

Methylated DNA ( $^5\text{mC}$ ) was found to be clustered around chromocenters in endosperm nuclei as in leaf nuclei (Figure 5A), although in repeated immunostaining experiments, the total labeling intensity seemed to be lower in endosperm than in leaf nuclei. H3K9me1 and H3K9me2 as well as H3K27me1 and H3K27me2 are marks of heterochromatic chromocenters in most somatic nuclei (Fuchs et al., 2006). However, 74% ( $n = 130$ ) of endosperm nuclei showed more H3K9me1 in euchromatin

**Table 2.** Heterochromatin Content in Endosperm, Seed Coat, and Root Meristem Nuclei

| Heterochromatin Type      | Seed Coat            | Root Meristem        | Endosperm            |                      |           |  | Endosperm with Imbalanced Parental Genome Dosage |         |  |                                |
|---------------------------|----------------------|----------------------|----------------------|----------------------|-----------|--|--------------------------------------------------|---------|--|--------------------------------|
|                           |                      |                      | 2m:1p                | 2m:0p                |           |  | 2m:2p                                            |         |  | 4m:1p                          |
| CC                        | 21.3 % ( $\pm 5.3$ ) | 8.8 % ( $\pm 3.5$ )  | 11.3 % ( $\pm 4.1$ ) | 10.0 % ( $\pm 4.0$ ) | P = 0.4   |  | 11.1 % ( $\pm 2.3$ )                             | P = 0.9 |  | 11.7 % ( $\pm 4.0$ ) P = 0.8   |
| ESI                       | 0.1* % ( $\pm 0.0$ ) | 0.7* % ( $\pm 1.2$ ) | 3.7 % ( $\pm 1.2$ )  | 2.2 % ( $\pm 1.7$ )  | P = 0.004 |  | 3.4 % ( $\pm 1.7$ )                              | P = 0.5 |  | 8.4 % ( $\pm 3.1$ ) P < 0.0001 |
| Total <sup>a</sup>        | 21.4 % ( $\pm 3.3$ ) | 9.0 % ( $\pm 3.7$ )  | 15.0 % ( $\pm 4.3$ ) | 12.2 % ( $\pm 5.4$ ) |           |  | 14.5 % ( $\pm 3.4$ )                             |         |  | 20.0 % ( $\pm 5.7$ )           |
| ESI fraction <sup>b</sup> | 0                    | 0.07                 | 0.25                 | 0.18                 |           |  | 0.23                                             |         |  | 0.42                           |
|                           | n = 40               | n = 20               | n = 23               | n = 11               |           |  | n = 12                                           |         |  | n = 11                         |

Heterochromatin content in chromocenter (CC) and ESI foci is expressed as percentage of the total nuclear chromatin ( $\pm$ SD). Endosperm nuclei in wild-type seeds are triploid, with two maternal (m) and one paternal (p) genome complement. Endosperm nuclei with an altered parental genome ratio were obtained from autonomously developing seeds of the *mea-2* mutant (Grossniklaus et al., 1998) in the absence of fertilization (2m:0p) or from F1 seeds of a cross between a diploid maternal and a tetraploid paternal line (2m:2p) or the reciprocal cross (4m:1p). Asterisks indicate small and weakly staining heterochromatic foci counted as ESI-like heterochromatin that were only rarely found.

<sup>a</sup> Total heterochromatin.

<sup>b</sup> Fraction of ESI heterochromatin over total heterochromatin. n, number of nuclei analyzed. P, probability returned by a bidirectional *t* test against the corresponding heterochromatin in 2m:1p endosperm nuclei.

than at chromocenters (Figure 5B), whereas H3K9me2 seemed to be distributed as in leaf nuclei (Figure 5C). H3K27me1 showed enrichment in chromocenters, similar as in leaf nuclei (Figure 5D), in 96.5% of endosperm nuclei ( $n = 163$ ). A few endosperm nuclei (3.5%) showed a loss of chromocentric H3K27me1 enrichment that was not observed in leaf nuclei. H3K27me2, as H3K9me1, showed enrichment mainly in euchromatin in 93% ( $n = 172$ ) of endosperm nuclei (Figure 5E).

To determine whether the different distribution patterns of H3K9me1 and H3K27me2 between 2C leaf and 3C seed nuclei were ploidy specific, seed specific, or endosperm specific, we analyzed the immunolabeling patterns of H3K9me1 and H3K27me2 in leaf and seed nuclei of different ploidy (Figures 5F and 5G). A total of 70 to 90% of seed nuclei ( $n = 172$ ) clearly showed an increased proportion of H3K27me2 within euchromatin compared with leaf nuclei (Figure 5F), among which 20% of nuclei showed simultaneous labeling at the chromocenters. Preferential distribution of H3K9me1 within euchromatin seems to occur in seed but not in leaf nuclei (Figure 5G). In contrast with the situation described for H3K27me2, 73% of 3C endosperm nuclei (and 58% of 6C endosperm nuclei) showed specific formation of H3K9me1 foci in euchromatin at the expense of chromocenters, while it was found only in 10% of 2C and 30% of 4C seed nuclei (Figure 5G). Therefore, while redistribution of H3K27me2 occurs in all seed nuclei, redistribution of H3K9me1 is specific to endosperm nuclei. Importantly, H3K9me1 was normally distributed in embryonic nuclei (Figure 5H).

Since endosperm nuclei contain a novel type of heterochromatic foci interspersed in euchromatin, we examined the distribution pattern of H3K9me1 more closely: H3K9me1 enrichment within euchromatin colocalized mainly with ESI heterochromatic foci (Figure 6A). H3K9me1 foci, however, did not always colocalize to detectable heterochromatic foci (Figure 6B). On the one hand, immunolabeling procedures seem to partially destabilize ESI heterochromatin since it appears less distinct than in nuclei from whole-mount stained seeds. On the other hand, it may be that H3K9me1 marks additional loci, the physical size of which may not allow the formation of microscopically detectable, compact DNA foci. In addition, redistribution of H3K9me1 did not

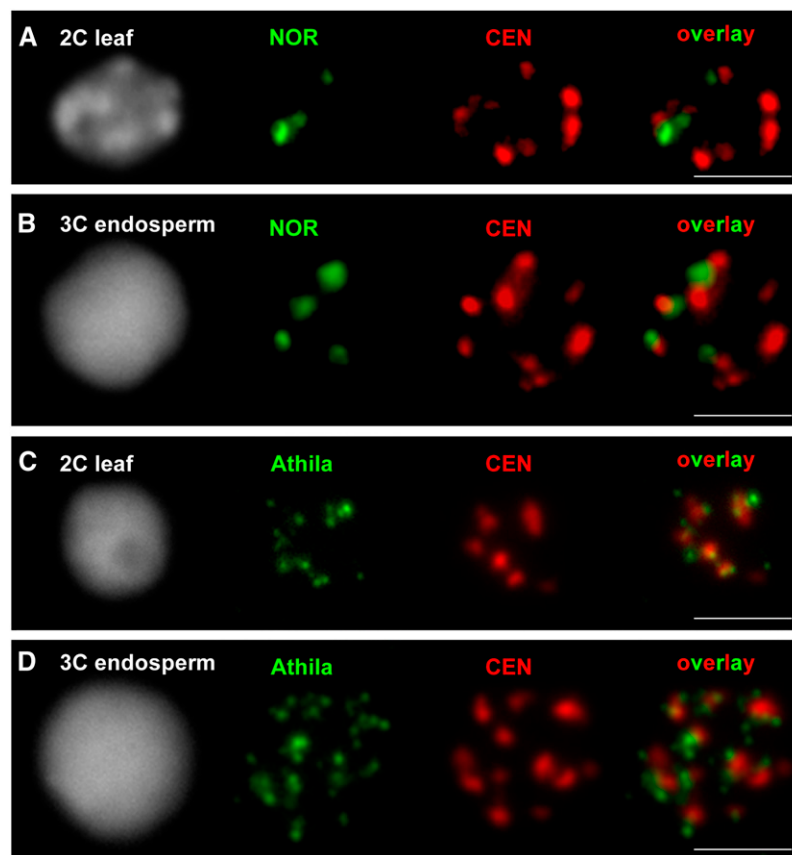
impair the deposition of euchromatin-specific marks, such as H4K16Ac, H3K4me2, H3K4me3, and H3K27me3 (Fuchs et al., 2006) (Figures 6C to 6F). This was confirmed by the simultaneous detection of H3K27me3 and H3K9me1 (Figure 6G).

Taken together, these data show that euchromatin-heterochromatin boundaries in endosperm nuclei are defined by the same histone modifications as in somatic nuclei, except for H3K27me2, which appears redistributed in a seed-specific manner, and H3K9me1, which is redistributed specifically in the endosperm. H3K9me1 seems to define ESI heterochromatin of endosperm nuclei and maybe additional, microscopically not detectable, heterochromatic loci. Redistribution of H3K9me1 suggests that this histone modification marks a specific type of heterochromatin, which is distinct from centromeric and pericentromeric heterochromatin. Because H3K9me1 in embryos is distributed as in somatic nuclei, H3K9me1 redistribution in the endosperm chromatin may be a consequence of the homo-diploid maternal genome provided by the central cell.

## DISCUSSION

### Studying the Nuclear Phenotype of *Arabidopsis* Endosperm Nuclei Is Feasible by Adapted Cytogenetic Methods

Because embryo and endosperm are deeply embedded in maternal organs and are therefore rather inaccessible, the phenotype of their nuclei is largely unknown. Recently, the three-dimensional nuclear architecture of embryo and endosperm has been described for a monocot plant system using a thick section of wheat (*Triticum aestivum*) seeds (Wegel et al., 2005; Wegel and Shaw, 2005a). Our procedure for nondenaturing nuclear staining in whole-mount fixed and semicleared *Arabidopsis* seeds is robust and allows high-resolution imaging of endosperm (this work) and embryo nuclei (data not shown). Nuclei from the maternal tissues show the typical heterochromatin-euchromatin organization and a nucleolus devoid of DNA stain as described for freshly fixed and isolated leaf nuclei (Lysak et al., 2006), demonstrating that our method yields comparable results. From



**Figure 4.** Centromeric, *Athila*, and 45S rDNA Repeats Are Localized at Chromocenters of Endosperm and Leaf Nuclei.

FISH against centromeric repeats (CEN, red) and 45S rDNA ([A] and [B]; nucleolus-organizing regions [NOR], green) or *Athila* ([C] and [D]; green) in 2C leaf ([A] and [C]) and 3C endosperm nuclei from seeds with globular embryos (stages VIII to IX) ([B] and [D]), isolated by flow cytometry according to their ploidy. Left: 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei (gray). Bars = 2  $\mu$ m.

(A) and (B) CEN and rDNA label discrete and compact heterochromatic regions in endosperm nuclei as in leaf nuclei.

(C) and (D) *Athila* repeats colocalize mostly but not entirely with CEN repeats in endosperm nuclei.

the globular embryo seed stage onwards, the seed coat needs to be dissected away for three-dimensional imaging.

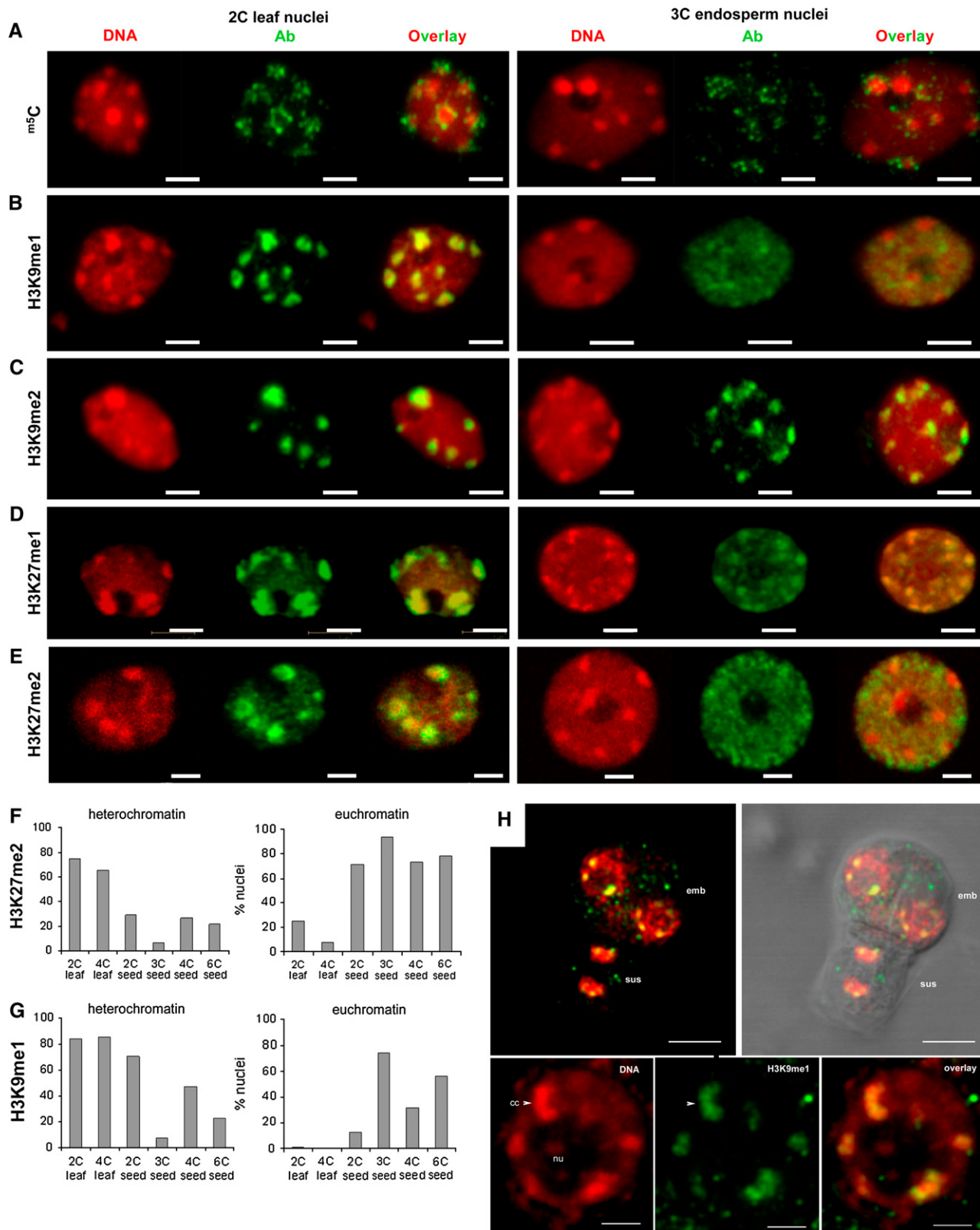
Immunostaining and FISH was not possible with whole-mount seeds despite prolonged enzyme digestion of cell walls and mechanical tearing. Therefore, we used flow-sorted endosperm nuclei for these procedures. This approach could be applied also to embryonic nuclei in combination with an embryo-specific fluorescent marker to distinguish embryonic from seed coat nuclei. The combination of both complementary methods enabled the three-dimensional analysis of chromatin organization in *Arabidopsis* endosperm nuclei.

#### The Large Size of Endosperm Nuclei May Be Attributed to a Low Level of Genome Condensation

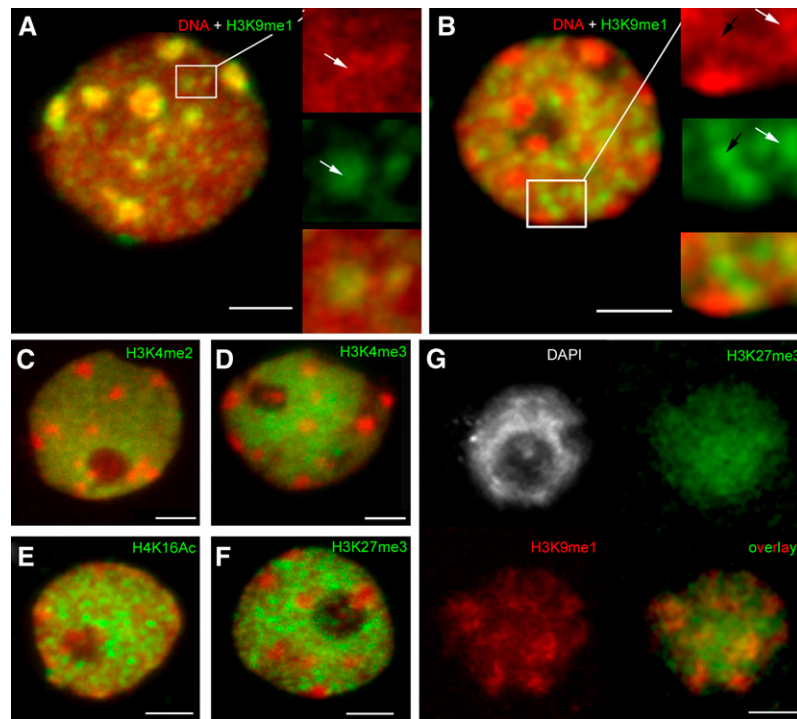
Maize endosperm nuclei were early recognized by their large size (up to 1000 times the volume of diploid nuclei; Duncan and Ross, 1950). Due to a high endoreduplication rate in maize endosperm (up to 690C; Lopes and Larkins, 1993), it is difficult to distinguish between the effects of endoreduplication and chromatin decon-

densation on nuclear size. Young *Arabidopsis* seeds showed a major population of 3C and only a minority of 6C nuclei of endosperm before cellularization. Our measurements showed that 3C nuclei, especially during the first growth stages (II to IV), are 2 to 4 times larger than differentiated or meristematic 2C nuclei. Apparently, a low interphase condensation is a specific feature of the endosperm genome, an observation corroborated by comparative tracing of chromosome territories via FISH signals in 2C and 3C nuclei. Size estimation of the large *Arabidopsis* endosperm nuclei were already reported (Mansfield and Briarty, 1990), but our data provide precise nuclear and nucleolar volume measurements and a quantitative and qualitative picture of genome compaction in endosperm nuclei.

The large *Arabidopsis* endosperm nuclei with their large nucleoli persist until stage IV (eight endosperm nuclei) and may represent a status of high metabolic activity that requires genome reorganization and reprogramming. Soon after differentiation of the three endosperm domains, following stage IV, endosperm nuclei become reduced in size but remain larger than other nuclei, indicating that a change in nuclear phenotype occurred concomitantly



**Figure 5.** Distribution of Heterochromatin Marks in 2C Leaf and 3C Endosperm Nuclei and Specific Redistribution of H3K9me1 in Endosperm Euchromatin.



**Figure 6.** H3K9me1 Is Redistributed toward ESI Heterochromatin without Impairing Deposition of Other Euchromatin Marks.

Immunostaining and PI [(A) to (F)] or DAPI (G) counterstaining of 3C seed nuclei (stages VIII to IX) isolated by flow cytometry according to their ploidy. Bars = 2  $\mu$ m.

(A) and (B) H3K9me1 is present as discrete foci embedded in endosperm euchromatin and colocalizes with ESI heterochromatin mostly (white arrows) but not always (black arrow).

(C) to (F) The euchromatic marks H3K4me2, H3K4me3, H3K27me3, and H4K16Ac are normally distributed in endosperm nuclei. A full data set in comparison to leaf nuclei is shown in Supplemental Figure 3 online.

(G) Simultaneous detection of H3K27me3 and H3K9me1.

with differentiation. Compared with differentiated somatic, but also with meristematic nuclei, endosperm nuclei are larger and have a less condensed genome, a feature that may be functionally related to genome reprogramming and/or epigenetic regulation of the three parental genomes, during endosperm development.

#### Endosperm-Specific Chromatin Organization May Reflect a Relaxed Chromocenter-Loop Architecture

While recent reports describe and interpret the nuclear organization in differentiated leaf and root cells of the dicot *Arabidopsis*

(Fransz et al., 2002; Pecinka et al., 2004; Fuchs et al., 2006) and of the monocots barley (*Hordeum vulgare*), wheat, and maize (Shaw et al., 2002; Fuchs et al., 2006), only few studies report dynamic changes connected with nuclear differentiation (Nagl, 1976; Tessadori et al., 2004; Wegel and Shaw, 2005b; Fransz et al., 2006). In *Arabidopsis* endosperm nuclei, chromocenters are reduced in size and additional heterochromatic foci are formed within the euchromatin, which we named ESI heterochromatin. While centromeric and pericentromeric repeats are retained in chromocenters of endosperm nuclei, a partial repositioning of pericentromeric transposons, such as *Athila*, and

**Figure 5.** (continued).

(A) to (E) Indirect immunostaining of heterochromatin marks (green) and PI counterstaining (red) in 2C leaf nuclei and 3C endosperm nuclei from seeds with globular embryos (stages VIII to IX) isolated by flow cytometry according to their ploidy. H3K9me1 (B) and H3K27me2 (E) show redistribution into endosperm euchromatin compared with leaf nuclei.

(F) and (G) Quantification of H3K27me2 (F) and H3K9me1 (G) immunostaining patterns in leaf and seed nuclei (stages VIII to IX) isolated by flow cytometry according to their ploidy in heterochromatin (left graph) or euchromatin (right graph). H3K27me2 redistribution toward euchromatin is found in all seed nuclei of different ploidy, while H3K9me1 redistribution is specific to endosperm nuclei (3C and 6C) ( $n > 100$ ).

(H) In embryos, H3K9me1 is localized to heterochromatic chromocenters. Top panels, partial projection of a quadrant-stage embryo (confocal imaging) stained with PI (red) and immunostained for H3K9me1 (green), overlaid with a picture in transmission light (right). Bottom panels, detail of an embryonic nucleus: left, DNA (PI) staining; middle, H3K9me1 immunostaining; right, overlay. emb, embryo proper; sus, suspensor; cc, chromocenters; nu, nucleolus. Bars = 2  $\mu$ m.

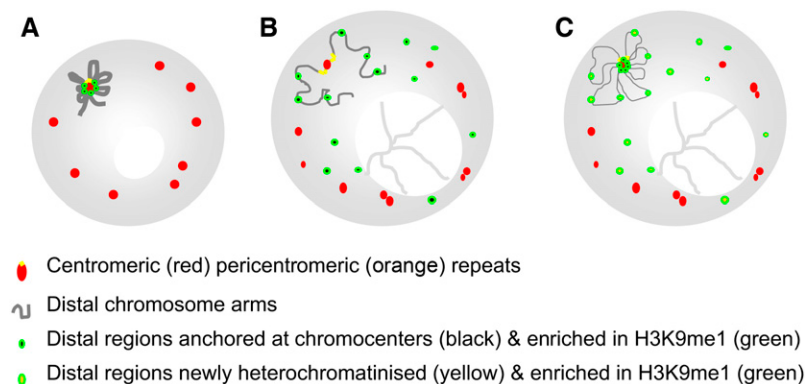
low-copy sequences cannot be excluded. However, in nuclei of the DNA hypomethylation mutants *ddm1* and *met1*, such a repositioning is associated with a severe loss of DNA methylation and H3K9me2 at the chromocenters (Soppe et al., 2002; Probst et al., 2003), which is not observed in endosperm nuclei. The gene-rich euchromatic chromosome arms of *Arabidopsis* are considered to be organized as chromatin loops emanating from the chromocenters, and regions distal to the centromere may associate with chromocenters at the basis of each loop (Fransz et al., 2002; Figure 7A). Because the endosperm genome is globally decondensed, we propose that in endosperm nuclei, the chromocenter-loop architecture is relaxed and some heterochromatinized regions normally forming the loop basis are no longer associated with chromocenters (Figure 7B). Such regions may instead form the ESI heterochromatic foci. Alternatively, the chromocenter-loop architecture may not be altered in endosperm nuclei, but the loops themselves may be more decondensed, with local condensation forming ESI heterochromatin (Figure 7C). We cannot exclude, however, the (dynamic) coexistence of both configurations.

Local condensation along relaxed or detached loops may provide an explanation for H3K9me1 redistribution outside chromocenters, which occurs simultaneously with a loss of this modification in endosperm chromocenters in 70% of the nuclei. This suggests that in somatic nuclei, H3K9me1 may be associated with interstitial, and probably silent, chromosome arm regions anchoring chromatin loops at chromocenters. This is in contrast with other heterochromatin marks, such as DNA methylation, H3K9me2 and H3K27me1, which remain associated with the chromocenters and probably mark (peri)centromeric repeats, and reveals a differential role for mono- and dimethylation of H3K9 within heterochromatin domains. Similarly in maize, H3K9me1 and H3K9me2 mark two distinct domains along the interphase chromosome arms, the heterochromatic chromomeres and the interspersed euchromatin, respectively (Shi and

Dawe, 2006). H3K9me1 in *Arabidopsis* may mark a specific type of heterochromatin, which does not consist of (peri)centromeric repeats and is not constitutive.

### ESI Heterochromatin Seems to Be Linked with Genome Dosage Regulation in Endosperm Nuclei

The presence of ESI heterochromatin and the redistribution of H3K9me1 are specifically associated with the triploid genome of endosperm nuclei and not with higher nuclear ploidy levels in nonendosperm cells. Both phenomena are also not linked to fertilization because embryonic nuclei show a similar nuclear architecture as somatic nuclei. This suggests that ESI heterochromatin and H3K9me1 redistribution may originate from the homodiploid maternal contribution coming from the central cell. Although the *Arabidopsis* ESI heterochromatin is contributed to by both maternal and paternal genomes, its formation seems to be linked to the maternal genome because an increase of the maternal but not the paternal genome induces additional ESI heterochromatin. This further suggests that the atypical heterochromatic features of endosperm nuclei may be linked to dosage regulation of the parental genomes, which are contributed unequally. Maternal genome doubling in endosperm most likely evolved as a result of parental conflicts over resource allocation to the progeny (Haig and Westoby, 1989). Maternal regulatory factors, which are quantitatively dominant in the endosperm, may favor a maternal control of progeny growth. However, doubling of the entire maternal transcriptome may not be beneficial. Aneuploids and polyploids are known to compensate genomic imbalance by partial silencing of sequences if stoichiometric expression of those sequences would be functionally not relevant or detrimental (Birchler et al., 2001; Adams and Wendel, 2005). This may hold true also for parts of the two maternal genomes in endosperm nuclei. In this context, heterochromatinization of



**Figure 7.** A Relaxed Chromocenter-Loop Model for Genome Organization in Endosperm Nuclei.

Usually, heterochromatin in *Arabidopsis* nuclei is confined to chromocenters that harbor centromeric (red), pericentromeric (orange), and 45S rDNA repeats.

(A) In somatic nuclei, euchromatic loops emanate from chromocenters (Fransz et al., 2002).

(B) The basic loop regions normally associated with the chromocenters (black) and enriched in H3K9me1 (green) are no longer anchored at chromocenters in endosperm nuclei and may form heterochromatic, H3K9me1-rich, ESI foci.

(C) Alternatively, the loops in endosperm nuclei may represent strongly decondensed chromatin with locally more condensed regions (yellow) that form ESI heterochromatin and gain H3K9me1 (green). In that case, H3K9me1 should remain associated with the chromocenters.

certain maternal loci and/or their enrichment in H3K9me1 could provide a mechanism of dosage compensation and could explain the predominantly maternal origin of ESI heterochromatin.

## METHODS

### Whole-Mount Seed DNA Staining

We established a protocol of fixation, permeabilization, and semiclearing of *Arabidopsis thaliana* seeds followed by DNA staining using PI. The procedure is nondestructive, and robust staining of endosperm nuclei was achieved, enabling CLSM analysis of whole-mount seeds until the early globular embryo stage and, after removal of the seed coat, also for later stages. Whole-mount seed staining and CLSM imaging combined with three-dimensional reconstructions of entire or dissected seeds allowed measurements of nuclear and nucleolar size in relation to the stages of endosperm growth. All stages of fixation, chlorophyll extraction, permeabilization, and PI staining were performed in Eppendorf tubes with 1 mL of solution on a rocking plate at room temperature, unless indicated otherwise. Three to four 10-d-old seedlings and three to six 4- to 8-mm siliques (opened through a longitudinal cut) were incubated 30 min in freshly prepared fixation buffer (1% formaldehyde, 10% DMSO, 2 mM EGTA, pH 7.5, and 0.1% Tween 20 in 1× PBS), followed by three washes in PBT (PBS and 0.1% Tween 20), 2× 2 min incubation in methanol, 2× 2 min in ethanol, 30 min in xylene:ethanol (1:1, v/v), and 2× 10 min in ethanol. Samples were stored at −20°C in ethanol until used for staining and analysis. Samples were rehydrated in a series of ethanol dilutions (90, 70, 50, and 30%, 5 min each) and 2× 5 min in PBT before 30 min (seedlings and young siliques) to 2 h (older siliques) of enzymatic digestion at 37°C (0.5% driselase, 0.5% cellulase, 0.5% pectolyase [all Sigma-Aldrich] in 50 mM PIPES, 5 mM MgCl<sub>2</sub>, and 5 mM EGTA, pH 7.5). This was followed by two washes in PBT (10 min each), 90 to 120 min of RNase treatment at 37°C (100 µg/mL RNase A [Roche] in PBS with 1% Tween 20), and two washes in PBT. Samples were postfixed for 15 min with 1% formaldehyde in PBT, rinsed two times in PBT, incubated for 15 min with 5 µg/mL of PI (Molecular Probes) diluted in PBS, rinsed two times in PBS and mounted in 0.2 mg/mL of PI in Vectashield (Vector Laboratories). The samples were stored at 4°C until microscopic preparation. Seeds were dissected out of the siliques, and the endosperm was squeezed out by gentle pressure on the partially digested seed coat.

### Isolation of Seed and Leaf Nuclei and Flow Sorting According to Ploidy

To analyze the distribution of chromatin modification in endosperm chromatin, we flow sorted DAPI-stained endosperm nuclei on slides according to their ploidy level. Seed extracts of globular to transition embryo stages, during which the endosperm is not cellularized, were prepared and used for flow sorting of nuclei with a 3C DNA content (1C = haploid unreplicated nuclear genome; for histograms, see Supplemental Figure 4 online). As controls, nuclei of 2C and 4C DNA content were sorted from seed and from leaf extracts. Seed nuclei were prepared following the protocol described by Lysak et al. (2006) with some modifications. Seeds were dissected out from 6- to 10-mm siliques and transferred into Tris buffer on ice (10 mM Tris, 10 mM EDTA, and 100 mM NaCl, pH 7.5), fixed 30 min at room temperature on a rocking plate in freshly prepared 4% formaldehyde in Tris buffer, rinsed three times for 10 min in Tris buffer, and gently squashed with a pestle in 20 µL of nuclei extraction buffer (15 mM Tris, 2 mM EDTA, 0.5 mM spermin, 80 mM KCl, 20 mM NaCl, 15 mM β-mercaptoethanol, and 0.1% Triton X-100). The pestle was rinsed with 400 µL extraction buffer, seed debris were left to settle, and the 420-µL supernatant was filtered through a 35-µm mesh. Suspended nuclei were stained with DAPI and flow sorted using a

FACStar flow cytometer (Becton-Dickinson) according to their ploidy as described (Pecinka et al., 2004). A gate was defined at the position of 3C nuclei, which yielded a bell-shaped peak in the histogram, between 2C and 4C peaks in preparations from seeds (see Supplemental Figure 4 online). Typically, 4 to 10 slides each containing 1000 nuclei sorted from the 3C fraction could be prepared from one seed extract of 12 to 15 siliques.

### FISH and Immunostaining of Modified DNA or Histones

FISH against centromeric and 45S rDNA repeats, chromosome painting, and immunostaining of modified DNA and histones have been performed as described (Baroux et al., 2004; Pecinka et al., 2004; Lysak et al., 2006) with the following minor modifications: prior to FISH, isolated endosperm nuclei were treated for 2 to 5 min at 37°C with 50 µg/mL of pepsin (in 0.01 N HCl), and post-hybridization washes were done at 45°C. For immunostaining, preblocking was performed in 5% BSA (Sigma-Aldrich), and immunostained nuclei were mounted in 1.5 µg/mL of PI in Vectashield. The following primary rabbit antibodies raised against modified histones (Upstate) have been diluted 1/100 in the detection buffer: H3K9me1 (07-450), H3K9me2 (07-212; 1/50), H3K27me1 (07-448), H3K27me2 (07-452), H3K27me3 (07-449), H3K4me2 (07-030), H3K4me3 (07-473), and H4K16ac (07-329). For immunostaining of methylated DNA, a mouse anti-5-methylcytosine IgG (diluted 1/50, MMS-900S-B; Eurogentec) was used. For double labeling of H3K9me1 and H3K27me3, a monoclonal mouse antibody against H3K27me3 (diluted 1/100; Abcam Ab6002) was used.

For immunostaining of whole-mount embryos, seeds were fixed 30 min in freshly prepared 4% paraformaldehyde and 0.1% Tween 20 in PBS, on ice, rinsed twice in PBS and 0.1% Tween 20, transferred with a needle on a microscope slide previously coated with 0.65% agarose (dipped for 2 s in warm 0.65% agarose solution, cooled for 10 min at 4°C, and dried for 30 min at 80°C), covered with 70 µL of 0.7% agarose and a cover slip, and gently squashed. After 10 min at 4°C, the cover slip was removed, slides were dried 30 min at 37°C before enzymatic digestion and RNase treatment as above, and blocking (8 to 12 h with 5% BSA and 0.1% Tween in PBS at 4°C) followed by immunodetection as described above, but with 1/100 primary and secondary antibody, and 8 to 12 h incubation at 4°C. DNA staining was performed in a coplin jar with 5 µg/mL of PI in PBS prior to two washes in PBS and mounting in 1.5 µg/mL of PI in Vectashield.

### Microscopy Analysis and Image Acquisition

Whole-mount DNA staining in seeds and seedlings was recorded with a TCS SP2 confocal laser scanning microscope (Leica) using a ×63 (1.3 numerical aperture) APO objective (glycerol immersion) and a 543/600- to 700-nm excitation/emission range for detection of PI. The scanning resolution for all figures was ~150-, 150-, and 122-nm (x, y, and z, respectively) voxel size.

Immunostained and FISH-treated isolated nuclei were analyzed under an epifluorescence microscope (Axioplan; Zeiss) equipped with filters for detection of DAPI, FITC/Alexa 488, and TRITC/Texas Red fluorescence, and pictures were taken with an AltaU32 cooled CCD camera (Apogee) (Figures 2 and 4) or under confocal microscopy (Figure 5) with 488/500- to 550-nm excitation/emission range for detection of Alexa Fluor 488.

### Analysis and Reconstruction of Three-Dimensional Image Stacks

Images stacks were deconvolved using AutoDeblur Gold CF (Autoquant X 1.3.5), and three-dimensional reconstruction was done with Imaris 4.2.0 (Bitplane). For quantification of heterochromatin, two-dimensional projections in the z axis were created in Imaris 4.2.0 for each individual nucleus. The fluorescence intensity (or integrated density, product of the area and mean of intensity) was measured using Image J 1.35 (National Institutes of Health) for each of the chromocenters (CC) and ESI foci. The distinction between CC and ESI foci was based on a mean intensity

threshold for ESI foci fixed at 80% of the maximum intensity of CCs and validated by a *t* test between the two groups of foci per nucleus ( $P < 0.001$ ).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Nuclear Architecture and Genome Compaction during Endosperm Growth and in Comparison with Differentiated and Meristematic Nuclei.

**Supplemental Figure 2.** ESI Heterochromatin Is Present during the Entire Growth Phase of the Endosperm.

**Supplemental Figure 3.** Distribution of Euchromatin Marks in 2C Leaf and 3C Endosperm Nuclei.

**Supplemental Figure 4.** Ploidy Histograms of Isolated Leaf and Seed Nuclei from *Arabidopsis*.

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