Report

Distinct Dynamics of HISTONE3 Variants between the Two Fertilization Products in Plants

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Summary

Sexual reproduction involves epigenetic reprogramming [1] comprising DNA methylation [2] and histone modifications [3–6]. In addition, dynamics of HISTONE3 (H3) variant H3.3 upon fertilization are conserved in animals, suggesting an essential role [7–9]. In contrast to H3, H3.3 marks actively transcribed regions of the genome and can be deposited in a replication-independent manner [10, 11]. Although H3 variants are conserved in plants, their dynamics during fertilization have remained unexplored. We overcame technical limitations to live imaging of the fertilization process in *Arabidopsis thaliana* and studied dynamics of the male-gamete-specific H3.3 [12] and the centromeric Histone Three Related 12 (HTR12) [13]. The double-fertilization process in plants produces the zygote and the embryo-nourishing endosperm [14]. We show that the zygote is characterized by replication-independent removal of paternal H3.3 and homogeneous incorporation of parental chromatin complements. In the endosperm, the paternal H3.3 is passively diluted by replication while the paternal chromatin remains segregated apart from the maternal chromatin (gonomery). Hence epigenetic regulations distinguish the two products of fertilization in plants. H3.3-replication-independent dynamics and gonomery also mark the first zygotic divisions in animal species [5, 15]. We thus propose the convergent selection of parental

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epigenetic imbalance involving H3 variants in sexually reproducing organisms.

Results and Discussion

H3 Variants Mark the Male Germline in Plants

The *Arabidopsis* genome contains nine genes encoding Histone 3 (H3) variants (http://www.chromdb.org/). These comprise the ubiquitous centromeric histone HTR12 [13] and eight predicted H3.3 variants characterized by noncanonical modifications relative to H3.3 identified in animals [10–12]. The H3.3 variant *At1g19890/HTR10/AtMGH3*, hereafter referred to as *H3.3*, is expressed only in male gametes [12]. Immunolocalization studies and live-imaging analyses have shown that HTR12 accumulates at heterochromatin around centromeres [13, 16, 17]. During male gametogenesis, HTR12-GFP was first expressed in the nucleus of unicellular haploid microspores produced by meiosis (Figures 1A and 1D). Each microspore divides asymmetrically in a large vegetative cell and a smaller generative cell [18]. HTR12-GFP remained expressed in the generative cell while it became undetectable in the vegetative cell (Figures 1B and 1E). The generative cell further divides into two sperm cells marked by strong accumulation of HTR12-GFP at five dots corresponding to the five centromeres (Figures 1C and 1F).

In contrast to HTR12, the male-gamete-specific H3.3-mRFP1 was not detected in microspores (Figures 1G and 1J). After the first asymmetric pollen division, H3.3-mRFP1 expression was confined to the generative cell (Figures 1H and 1K) and accumulated in the chromatin of sperm-cell nuclei (Figures 1I and 1L).

In conclusion, in plants the male germline represented by the generative cell and the two sperm cells is marked by specific accumulation of H3 variants. A comparable link between the germline and accumulation of H3.3 was reported in the ciliate *Tetrahymena thermophila* [19]. During animal spermatogenesis, the extreme compaction of the male chromatin depends on modifications of H3 and H4 [3, 6] and is followed by replacement of core histones with basic proteins [20]. However, the centromeric H3 variant is retained during spermatogenesis in *Drosophila* and mammals [21, 22]. Similarly, H3.3 is detected in mature sperm in humans and in *C. elegans* [7, 23], albeit not in *Drosophila* [9]. All together, these data support the idea that accumulation of H3 variants is a conserved feature of male gametogenesis in both plants and animals.

H3.3 Dynamics during Double Fertilization

The double fertilization is a hallmark of reproduction of flowering plants [14]. From meiotic products, flowering plants produce haploid gametophytes where two female gametes differentiate, the egg cell and the central cell. The zygote produced by fertilization of the egg cell develops into the embryo, and the fertilized central cell generates an embryo-supporting tissue, the endosperm
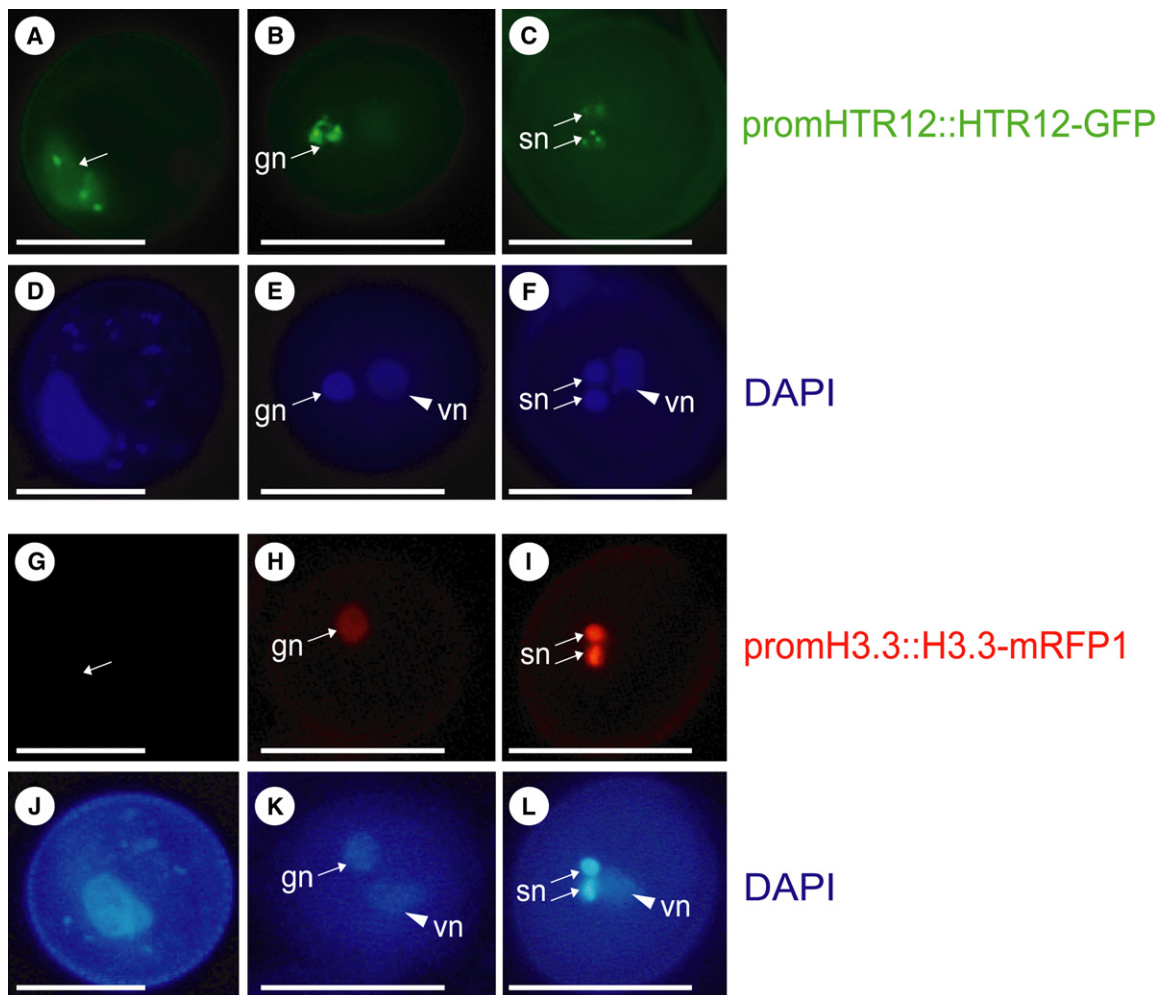
Figure 1. Expression of histone3 Variants during Male Germline Specification

(A–C) Progressive restriction to the germline of the centromeric histone3 variant HTR12-GFP (green) protein fusion under the control of the *HTR12* promoter. (A) Expression of *promHTR12::HTR12-GFP* in wild-type pollen is detected in uninucleate microspores. (B) After the first mitosis, HTR12-GFP expression becomes rapidly undetectable in the vegetative cell and is progressively confined to the nucleus of the generative cell. (C) After the second mitosis HTR12-GFP accumulates strictly in the nucleus of each sperm cell.

(D), (E), and (F) show DAPI-stained nuclei of microspores and developing pollen corresponding to (A), (B), and (C), respectively.

(G–I) Germline-specific expression of the histone3 variant *H3.3-mRFP1* protein fusion (red) under the control of the *H3.3* promoter. (G) H3.3-mRFP1 is absent in uninucleate microspores. (H) H3.3-mRFP1 is first detected in bicellular pollen in the nucleus of the generative cell. (I) After the second mitosis, H3.3-mRFP1 accumulates in the nucleus of each sperm cell.

(J), (K), and (L) show DAPI-stained nuclei of microspores and developing pollen corresponding to (G), (H), and (I), respectively.

Arrowheads indicate the vegetative nucleus. Arrows indicate the generative-cell nucleus and the sperm-cell nuclei.

The following abbreviations are used: gn, generative nucleus; sn, sperm nucleus; and vn, vegetative nucleus. Scale bars represent 25 μm.

[24]. We monitored in vivo the fate of the paternal chromatin upon double fertilization in crosses between wild-type ovules and pollen with sperm cells marked by H3.3-mRFP1 (Figure 2) and HTR12-GFP (Figure 3). These fluorescent markers allowed us to overcome technical problems, which had limited observations to in vitro systems [25–28], and enabled the dynamic observation of the double-fertilization process.

We observed the transport of the male gametes identified by H3.3-mRFP1 fluorescence by the pollen tube emanating from the vegetative cell to the ovule (Figure 2A). After being discharged (Movie S1 in the Supplemental Data available online) into the ovule, sperm cells migrated toward the female gametes (Figure 2B). Subsequently, one sperm cell fused with the egg cell and the other one with the central cell (Figure 2C and Movie S1). Karyogamy resulting from the fusion between the male and female gamete nuclei took place between 3 and 4 hr after release of the gametes (Figure 2D). The timing of these events supports earlier findings based on fixed material [29]. These dynamic observations show clearly that although plant male gametes are not motile, they migrate actively toward the female gametes. This migration may involve the actin cytoskeleton as suggested by immunolocalizations on fixed tissues [30, 31].

In the fertilized egg cell, paternal H3.3-mRFP1 became barely detectable within a few hours after karyogamy (Figures 2E and 2G). This swift displacement of paternal H3.3-mRFP1 variants occurred in the elongated
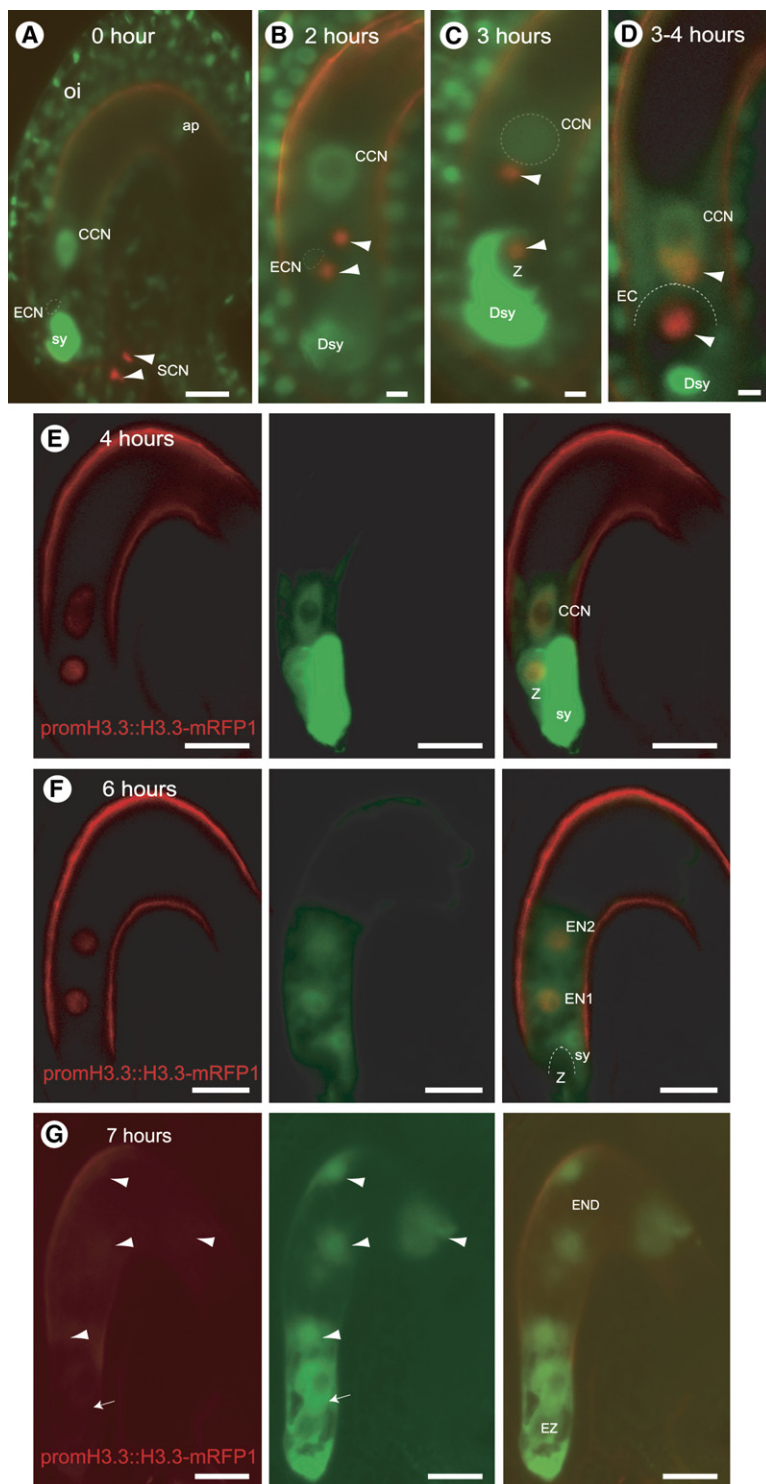
Figure 2. Removal of Paternally Provided H3.3-mRFP1 upon Fertilization

Expression of paternally provided H3.3-mRFP1 (red) was monitored upon fertilization. The reference time (0 hr) corresponds to the discharge of the male gametes.

(A) H3.3-mRFP1 (red) localized to the sperm-cell nuclei (scn, arrowheads) prior to the discharge of the male gametes into one synergid (sy) of the embryo sac.

(B) After discharge, the sperm cells migrate toward the female gametes, the egg cell (ECN) and the central cell (CCN).

(C) Karyogamy is initiated and the male and female chromatins merge.

(D) Male chromatin decondensation occurs in both fertilization products.

(E) Male chromatin decondensation is complete, and paternally provided H3.3-mRFP1 has spread to the entire nucleus of the fertilized central cell (CCN) and of the zygote (Z).

(F) Removal of paternal H3.3-mRFP1 (left panel) occurs after karyogamy in the zygote (Z). In contrast, H3.3 persists in endosperm nuclei (EN) after the first two divisions. Two nuclei out of four are visible in this confocal plane and show uniform distribution of paternal H3.3-mRFP1.

(G) H3.3-mRFP1 is no longer detected in the elongated zygote (EZ), where no division has occurred yet. The arrow shows the zygote nucleus marked by FIE-GFP. In the proliferating endosperm, H3.3-mRFP1 is hardly detectable after the third nuclei division (arrowheads show four out of eight endosperm nuclei).

(A–G) Positions of the zygote and the endosperm are visualized with the FIE-GFP marker (E–G) [46]. The middle panels show FIE-GFP expression, and the right panels show the overlap of green (FIE-GFP) and red fluorescence (H3.3-mRFP1). The following abbreviations are used: ap, antipodals; CCN, central-cell nucleus; Dsy, degenerate synergid; EC, egg cell; ECN, egg-cell nucleus; EN, endosperm nucleus; END, endosperm; oi, ovule integuments; SCN, sperm-cell nucleus, sy, synergids; and Z, zygote. Scale bars represent 5 μm.

zygote before the first zygotic division within 6 hr after discharge of the gametes (Figure 2F). In contrast, the paternally provided centromeric HTR12-GFP was retained in the zygote nucleus at least until 12 hr (Figures 3C and 3E). The persistence of the H3 variant HTR12 at centromeres of the paternal chromosomes suggested that removal of the H3.3 variant in the zygote resulted from a specific mechanism. In order to establish whether DNA replication would not be responsible for removal of H3.3, we investigated the stage of the cell cycle of the zygote by monitoring expression of the Retinoblastoma-Related protein 1 (RBR1), which marks the transition from G1 to S phase [32, 33]. The egg cell did not express RBR1-mRFP1 in contrast to the central cell (Figure 4A). After fertilization, RBR1-mRFP1 expression in the zygote was initiated at 8 hr and increased until 12 hr while the zygote became elongated (Figures 4B and 4C). We conclude that the initiation of the S phase takes
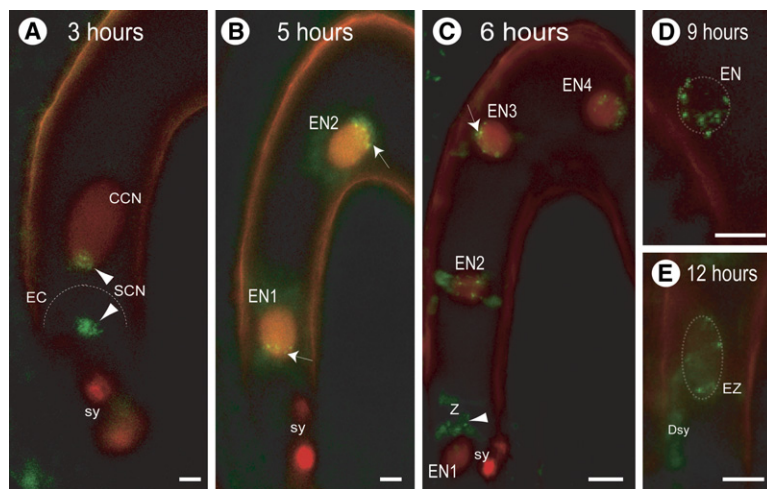
Figure 3. Distribution of the Paternal Chromatin Marked by HTR12-GFP during the Initial Development of the Fertilization Products

Expression of paternally provided HTR12-GFP (green) was monitored upon fertilization. (A–C) Positions of the endosperm nuclei are visualized with the H2B-mRFP1 marker (red). The reference time (0 hr) corresponds to the discharge of the male gametes. (A) HTR12-GFP remains accumulated at the centromeres of the paternal chromatin (SCN, arrowheads) during karyogamy. (B and C) After the first two syncytial mitoses, HTR12-GFP does not spread to maternal centromeres, and the paternal centromeric chromatin remains segregated to one pole of each endosperm nucleus (EN, arrows).

(D) After the third division, the paternal centromeric chromatin marked by HTR12-GFP has spread to the nucleus surface. One nucleus is shown from an endosperm at the 8-nuclei stage.

(E) Before the first division of the elongated zygote, the nucleus adopts an elongated shape (see also Figure 4C) and shows the paternal centromeric chromatin marked by HTR12-GFP equally distributed over the nucleus surface (marked by a doted line).

The following abbreviations are used: CCN, central-cell nucleus; Dsy, degenerate synergid; EC, egg cell; EN1-2-3-4, endosperm nucleus; EZ, elongated zygote; SCN, sperm-cell nucleus; sy, synergids; and Z, zygote. Scale bars represent 5 μm in (A)–(C) and 3 μm in (D) and (E).

place later than 8 hr, after eviction of the paternal H3.3 from the zygotic nucleus, which occurs between 4 and 6 hr. Hence paternally provided H3.3 is removed actively from the zygote in the absence of DNA replication.

Replication-independent removal of H3.3 in the *Arabidopsis* zygote is highly reminiscent of H3.3 dynamics during fertilization in *C. elegans* [7]. In *Drosophila*, mouse, and *C. elegans*, the paternal pronucleus becomes loaded with H3.3 from maternal origin in a replication-independent manner [7–9]. Postfertilization deposition of maternal H3.3 histones in the zygotic nucleus may also occur in plants because unfertilized egg cells express high levels of H3.3 variants [34]. In *Drosophila*, incorporation of H3.3 by the chaperone HIRA into the paternal pronucleus is essential for proper decondensation of the paternal chromatin and its incorporation in the zygote nucleus [9]. However, the role of H3.3 at fertilization in animals remains unclear. The *Arabidopsis* loss-of-function mutant for the male-gamete-specific H3.3 does not display any obvious phenotype, a result

that may stem from a redundant function with one or several of the seven other H3.3-encoding genes [12]. Thus the function of the *Arabidopsis* male-gamete-specific H3.3 remains unknown. Nevertheless, H3.3 probably plays an essential role in the male germline and chromatin remodeling upon fertilization in all sexually reproducing organisms studied to date. Conservation of the roles of H3.3 in sexual reproduction would be surprising because plants separated from animals around 1.1 million yr ago [35] from ancestors that were probably producing isomorphic gametes [36]. Positive selection toward the production of highly dimorphic gametes has taken place independently among plants and animals, leading to specific fertilization mechanisms [37, 38]. This assumption is supported by the lack of conservation in animals of the gamete-specific surface protein GCS1, which is conserved from algae to flowering plants [39]. Hence the similarity of H3.3 dynamics in sexual reproduction between plants and animals probably does not result from conservation but
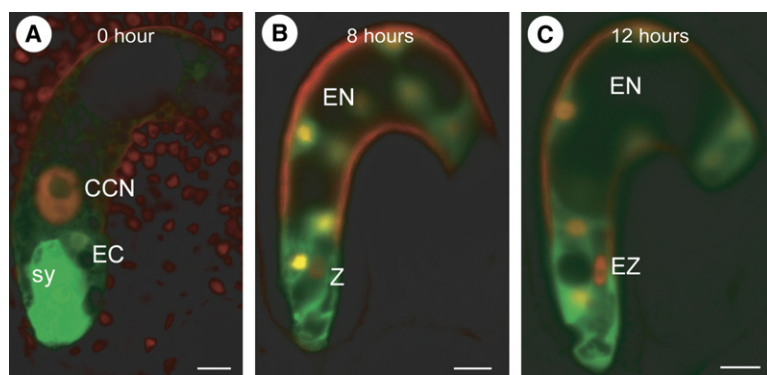

Figure 4. Expression of RBR1 upon Double Fertilization

(A) In the mature ovule, *promRBR1::RBR1-mRFP1* (red) is expressed exclusively in the central-cell nucleus (CCN). No expression is detected in the egg cell (EC), nor in the synergids (sy). The egg apparatus and the central cell of the mature gametophyte are outlined by the expression of *promMYB98::GFP* mostly expressed in synergids (green) [47].

(B) Expression of *promRBR1::RBR1-mRFP1* provided paternally is initiated in the elongated zygote (EZ) at 8 hr (red signal is barely detectable) while it is continuously expressed in endosperm (EN) that has just divided. The endosperm is selectively marked by the expression of *promFWA::FWA-GFP* (green) [48].

(C) Expression of *promRBR1::RBR1-mRFP1* provided paternally is strong in the elongated zygote (EZ) nucleus, presumably marking the S phase at least 6 hr prior to the first zygotic division [29]. The endosperm is selectively marked by the expression of *promFWA::FWA-GFP* (green).

The reference time (0 hr) corresponds to the discharge of the male gametes. The following abbreviations are used: CCN, central-cell nucleus; EC, egg cell; sy, synergids; EN, endosperm; and EZ, elongated zygote. Scale bars represent 4 μm in (A) and 9 μm in (B) and (C).

H3-Variant Dynamics in the Endosperm

In contrast to the replication-independent eviction of H3.3 from the zygotic nucleus, paternal H3.3-mRFP1 remained present after karyogamy in the fertilized central cell (Figure 2E). Mitosis is rapidly initiated in endosperm within 1 to 2 hr after karyogamy [29]. Mitoses are not followed by cell division, and this lack of cell division leads to a syncytial-endosperm development [24]. The signal of H3.3-mRFP1 persisted until the third series of mitoses, although it decreased steadily (Figures 2F and 2G). After the third syncytial division in endosperm, H3.3-mRFP1 became hardly detectable (Figure 2G) and was no longer detected after the fourth division (data not shown). We conclude that paternal H3.3 is passively diluted in the endosperm by successive rounds of replication.

Unlike H3.3, paternal HTR12-GFP was not removed from the endosperm nuclei during the first three syncytial divisions (Figure 3). The fertilization of the homodiploid central cell by the haploid sperm cell assembles five paternal and ten maternal chromosomes. The triploid endosperm nuclei thus display 15 centromeres marked by HTR12 [40]. Surprisingly, the ten centromeres of the maternal chromosomes remained unmarked by paternal HTR12-GFP during the first three syncytial divisions (Figures 3B and 3C). The absence of HTR12-GFP on maternal chromosome implies the immobilized nature of HTR12-GFP at paternal centromeres where it was deposited and the absence of de novo expression of HTR12-GFP during the first endosperm divisions. The persistence of HTR12-GFP on the five paternal centromeres also suggests that HTR12-GFP is inherited in a semiconservative manner by centromeres deriving from the replication of paternal chromosomes.

Surprisingly the chromatin marked by HTR12-GFP remains distinctly segregated from the female chromatin up to the four-nuclei stage (Figure 3C). Such segregation was gradually lost after the third syncytial division (Figure 3D). A similar segregation could not be observed for paternally provided H3.3 that is uniformly distributed at the end of karyogamy, probably as a result of diffusion (Figure 2E). We thus propose that HTR12-GFP strongly tethered to the centromeres reflects the segregation of the paternal chromatin apart from the maternal chromatin during the first mitoses in the endosperm. In Drosophila [15] and in mammals [4, 5], a similar spatial partition of chromatin according to its parental origin has been observed during the first zygotic division (a process known as gonomery). This suggests persistence of structural and epigenetic differences between the paternal and maternal chromatin after fertilization.

In the zygote, HTR12-GFP marks persisted on the five paternal centromeres up to at least 12 hr (Figure 3E). However, the five centromeres marked with HTR12-GFP were dispersed over the surface of the elongated zygote nucleus typical of that stage (compare Figures 3E and 4C). The zygote thus does not display the gonomeric segregation observed in endosperm (Figures 3A and 3B). Hence in the zygote, the chromatin complements from each parent do not remain segregated as in endosperm. This epigenetic distinction between the two fertilization products might be linked to the endosperm-specific paternal silencing of the genome reported at numerous loci [41, 42] in contrast to the widespread biparental mode of expression in the zygote [43].

Conclusions

The male germ lineage is marked by H3 variants in *Arabidopsis*. Our observations show that the paternal chromatin undergoes a distinct remodeling in each product of double fertilization, the zygote and the endosperm. The replication-independent replacement of paternal H3.3 in the zygote has probably evolved in a convergent manner between plants and animals. In contrast to the zygote, the other fertilization event leading to endosperm development is characterized by replication-coupled removal of paternal H3.3 and the segregation of the paternal chromatin from the maternal chromatin. These differences argue against the evolution of endosperm from a second embryonic fertilization [44] and may prefigure parental genomic imprinting, a unique feature of endosperm in plants [45].

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