RESEARCH ARTICLE

Wolbachia Influences the Maternal Transmission of the *gypsy* Endogenous Retrovirus in *Drosophila melanogaster*

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ABSTRACT **The endosymbiotic bacteria of the genus *Wolbachia* are present in most insects and are maternally transmitted through the germline. Moreover, these intracellular bacteria exert antiviral activity against insect RNA viruses, as in *Drosophila melanogaster*, which could explain the prevalence of *Wolbachia* bacteria in natural populations. *Wolbachia* is maternally transmitted in *D. melanogaster* through a mechanism that involves distribution at the posterior pole of mature oocytes and then incorporation into the pole cells of the embryos. In parallel, maternal transmission of several endogenous retroviruses is well documented in *D. melanogaster*. Notably, *gypsy* retrovirus is expressed in permissive follicle cells and transferred to the oocyte and then to the offspring by integrating into their genomes. Here, we show that the presence of *Wolbachia w*Mel reduces the rate of *gypsy* insertion into the *ovo* gene. However, the presence of *Wolbachia* does not modify the expression levels of *gypsy* RNA and envelope glycoprotein from either permissive or restrictive ovaries. Moreover, *Wolbachia* affects the pattern of distribution of the retroviral particles and the *gypsy* envelope protein in permissive follicle cells. Altogether, our results enlarge the knowledge of the antiviral activity of *Wolbachia* to include reducing the maternal transmission of endogenous retroviruses in *D. melanogaster*.**

IMPORTANCE **Animals have established complex relationships with bacteria and viruses that spread horizontally among individuals or are vertically transmitted, i.e., from parents to offspring. It is well established that members of the genus *Wolbachia*, maternally inherited symbiotic bacteria present mainly in arthropods, reduce the replication of several RNA viruses transmitted horizontally. Here, we demonstrate for the first time that *Wolbachia* diminishes the maternal transmission of *gypsy*, an endogenous retrovirus in *Drosophila melanogaster*. We hypothesize that *gypsy* cannot efficiently integrate into the germ cells of offspring during embryonic development in the presence of *Wolbachia* because both are competitors for localization to the posterior pole of the egg. More generally, it would be of interest to analyze the influence of *Wolbachia* on vertically transmitted exogenous viruses, such as some arboviruses.**

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Prokaryotic organisms are present in many eukaryotic species and can establish symbiotic relationships with their hosts that can range from detrimental to beneficial. In recent years, studies have shown that insect-endosymbiotic bacteria replicate within eukaryotic cells and are maternally transmitted. Among them, the genus *Wolbachia* is present in all insect orders, and its within-species propagation is optimized due to a biased efficient transmission through infected female ovaries. Interestingly, it has been shown that *Wolbachia* confers protection against several RNA viruses in insects (1–3), including arboviruses present in transmission vectors such as *Aedes* mosquitos, and so gives the opportunity to improve arbovirus control in natural populations of vectors (4). The cellular and evolutionary characteristics of *Wolbachia* and its variants (*w*Mel, *w*MelCS, and *w*MelPop) in *Drosophila* species are well documented, thanks to the powerful genetic model *Drosophila melanogaster* (5, 6). One important point concerns the host mechanisms hijacked by *Wolbachia* for its maternal transmission: *Wolbachia* localizes at the posterior pole of mature oocytes through an active mechanism that relies mostly on microtubules and pole plasm (7, 8). This polarized concentration ensures that *Wolbachia* is incorporated into the pole cells of the embryos, in order to be maternally transmitted. *Wolbachia* bacteria from *Drosophila melanogaster* (*w*Mel) also show a strong tropism for the somatic stem-cell niche (SSCN) and are therefore present in the somatic follicle cells covering the germline at the early stage of oogenesis (9). Interestingly, horizontal transmission of *Wolbachia* can also occur within and between *Drosophila* species, and the results of experimental infections of *D. melanogaster* by microinjection of *Wolbachia*-infected hemolymph demonstrate the capacity of *Wolbachia* to enter the SSCN and, later, the follicle cells surrounding the germline (10). Follicle cell-to-oocyte transcytosis is not restricted to bacteria and cellular proteins; it has been shown that several *Drosophila* endogenous retroviruses (ERVs), including the *gypsy* retroelement, are maternally trans-

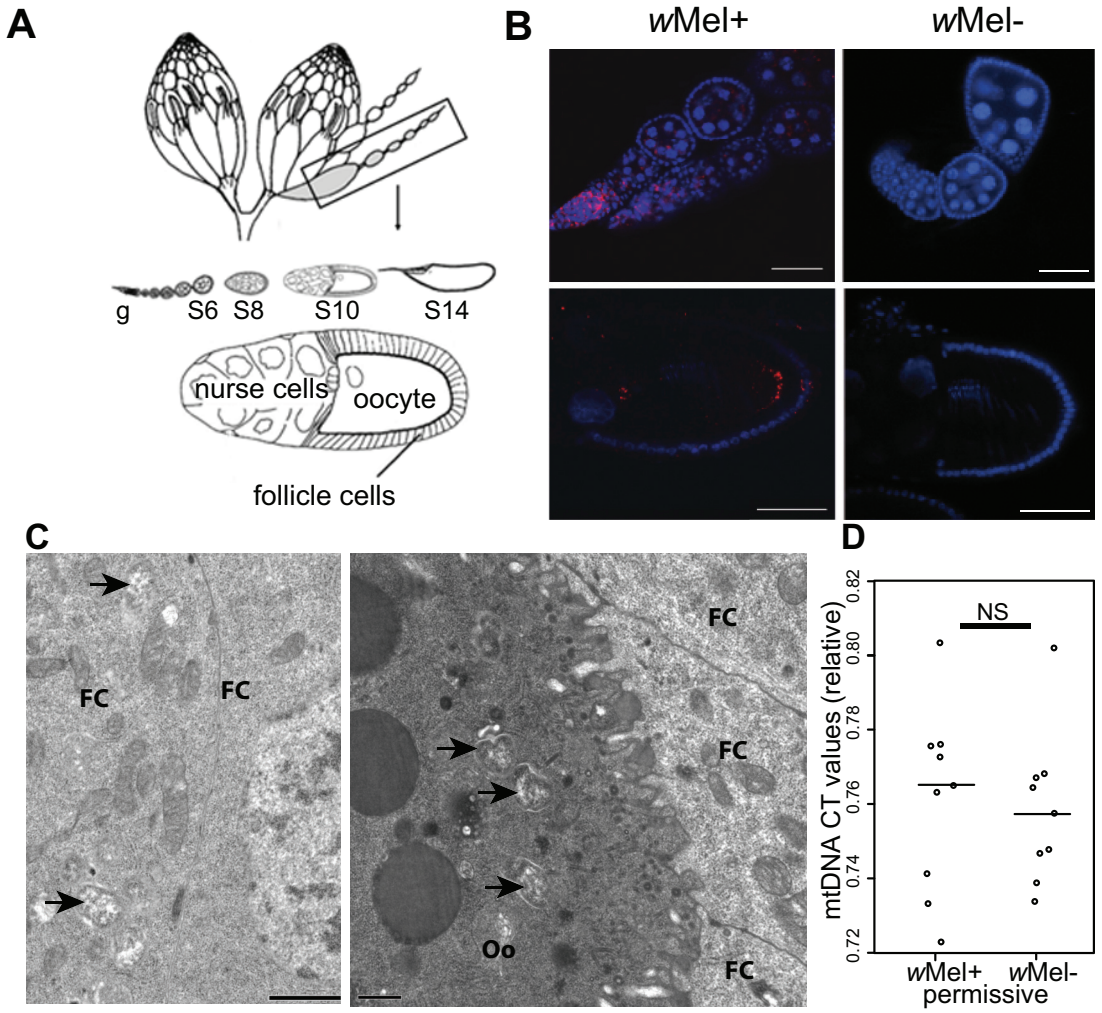

FIG 1 Characterization of *Wolbachia* in the N271 strain. (A) Schematic representation of *Drosophila melanogaster* ovaries, stages of oogenesis (germarium [g] and stages S2 to S14), and enlarged view of S10 egg chamber. (B) Detection of *Wolbachia* by *in situ* hybridization. Blue, DAPI; red, rhodamine-labeled probe against *Wolbachia* 16S DNA. Scale bars = 50 μm. Top, early-stage egg chambers infected with *Wolbachia*; bottom, stage 10 egg chamber. (C) Observation by electron microscopy of *Wolbachia* (black arrows) in the cytoplasm of a follicle cell (FC) (left, scale bar = 1 μm) and at the posterior pole of the oocyte (Oo) surrounded by follicle cells (right, scale bar = 0.5 μm). (D) Relative mtDNA *COI* cycle threshold values (C_T) after normalization with *rp49*. NS, no statistically significant difference; *w*Mel$^-$, tetracycline-treated flies; *w*Mel$^+$, untreated flies. Horizontal bars represent medians.

mitted to the next generation. *gypsy* is an active endogenous retrovirus present in several strains of *Drosophila melanogaster*. Its 7.5-kb genome contains three open reading frames similar to the *gag*, *pol*, and *env* genes present in vertebrate retroviruses. The *gypsy* RNAs and proteins are mainly expressed in the ovaries of permissive females at stages 8 to 10 (11–13). Females are permissive if they are defective for the production of specific P-element-induced wimpy testis (Piwi)-interacting RNAs (piRNAs) that are able to target *gypsy* RNAs (14–16). It was shown that these piRNAs are encoded by the X-linked *flamenco* locus, which has two classes of alleles, *flam*^{P} (permissive) and *flam*^{R} (restrictive) (15, 17). The integration of *gypsy* occurs only into the germline of *flam*^{P}/*flam*^{P} females lacking *gypsy* piRNAs. *gypsy*, like other ERVs, is expressed in the follicular cells of permissive females and integrates into the nuclei of the offspring, suggesting that there is a transfer from follicle cells to oocytes (11, 13, 18, 19). Moreover, it has been demonstrated that the trafficking of the endogenous retrovirus *ZAM* relies on the transport of vitellogenin (20). There is experimental evidence to indicate that the *gypsy* endogenous retrovirus is also horizontally transmitted and then integrates into the chromosomes of the offspring by virtue of a strong tropism to the germline (18). Our results indicate that the frequency of *gypsy* insertion-induced *ovo* mutants is decreased in the presence of *w*Mel, suggesting a new role for this endosymbiont in the control of endogenous retroviruses.

RESULTS

The *w*Mel variant is present in the *gypsy*-rich *Drosophila melanogaster* strain N271. We investigated *Wolbachia*'s distribution pattern in N271 permissive ovaries using fluorescence *in situ* hybridization. *Wolbachia* was observed in the germarium and mainly in the posterior pole of the stage 10 oocyte, as previously described (Fig. 1A and B) (7, 9, 10). Ultrastructural electron microscopy (EM) analysis of permissive ovarian late egg chambers enabled us to identify at the posterior pole of the oocyte several *w*Mel cells showing the typical morphology of *Wolbachia*, i.e., a

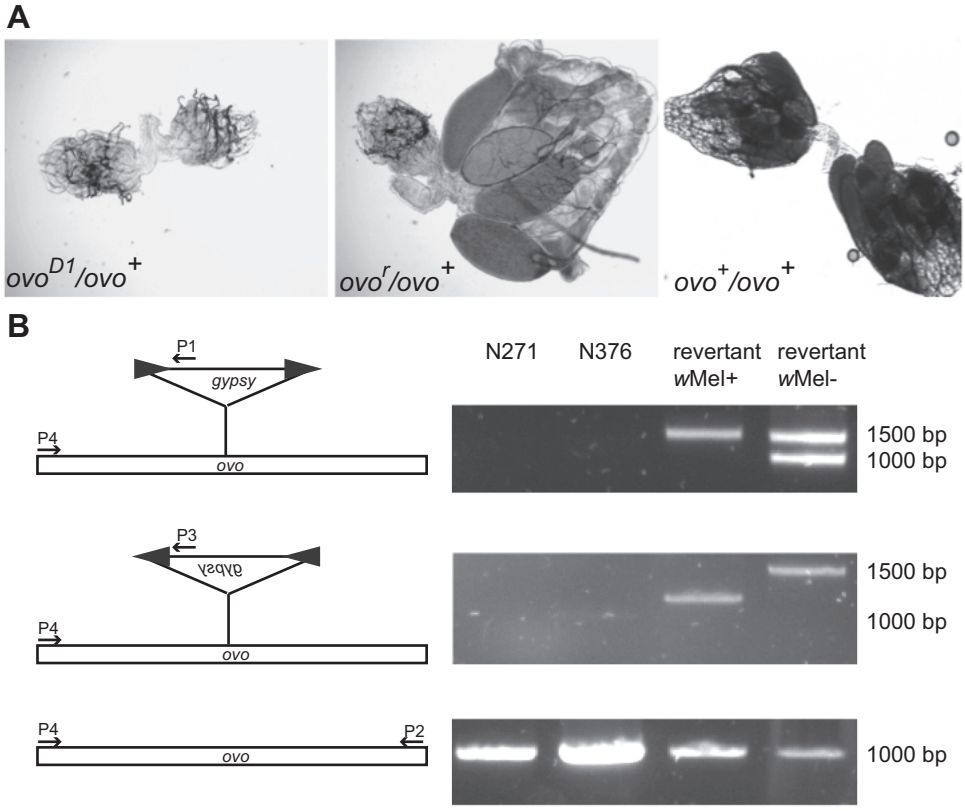

FIG 2 Ovaries of the progeny of an ovo^{D1} reversion test and detection of *gypsy* insertion into *ovo* by PCR. (A) Ovaries from a sterile ovo^{D1}/ovo^{+} female, a revertant ovo^{r}/ovo^{+} female, and a wild-type ovo^{+}/ovo^{+} female shown by phase-contrast microscopy (×50 magnification). (B) PCR detection of *gypsy* insertion into *ovo* in the two parental lines and samples of revertant F1 females. The schematic depicts the primers used (P1, P2, P3, and P4) and their localization in *ovo* and *gypsy*. Large arrows show *gypsy* long terminal repeats (LTR).

three-layered envelope surrounding a matrix of moderate density (Fig. 1C, right). We also distinguished several *Wolbachia* cells within the cytoplasm of follicle cells (Fig. 1C, left). Altogether, our data suggest that the N271 females contain a consistent level of *Wolbachia* variant *w*Mel bacteria in follicle cells and in the oocytes of late-stage egg chambers. In order to compare genetically identical females with or without *Wolbachia* (denoted as *w*Mel^{+} and *w*Mel^{-} females, respectively), we treated N271 individuals with tetracycline as previously described (3). *w*Mel was undetectable either by PCR (see Fig. S1 in the supplemental material) or by *in situ* hybridization in flies treated with tetracycline to be *w*Mel^{-} (Fig. 1B). Moreover, we estimated the levels of mitochondria in *w*Mel^{+} (untreated) and *w*Mel^{-} permissive females, as it was shown that tetracycline increased the mitochondrial DNA (mtDNA) density in flies raised for two generations on standard medium after treatment (21). We performed quantitative PCR (qPCR) on *w*Mel^{+} and *w*Mel^{-} permissive females after tetracycline treatment, using primers specific for the cytochrome-*c* oxidase subunit I (*COI*) gene. Our results indicate that the average relative mtDNA threshold cycle (C_T) values are not different for *w*Mel^{+} and *w*Mel^{-} flies (Fig. 1D).

The *gypsy* insertion rate decreases in the presence of *Wolbachia*. The fact that *Wolbachia* and *gypsy* are both vertically transmitted prompted us to test whether their mechanisms of transmission may interfere with each other. To address this question, we measured the rate of integration of *gypsy* into the genomes of offspring from permissive females in the presence or absence of *w*Mel. The *ovo* gene is a hot spot for *gypsy* integration (22) and can be used as a readout for *gypsy* transposition (11, 13, 14, 22). To estimate the rate of *gypsy* integration into *ovo*, permissive females were crossed with X-linked mutant ovo^{D1} males. As the ovo^{D1} allele is dominant, the ovo^{D1}/ovo^{+} daughters are sterile, because ovarian development does not occur (23). However, several daughters with one functional ovary were observed due to *gypsy* insertion into the ovo^{D1} gene occurring after colonization of the gonads by germ cells (Fig. 2A). Hence, the percentage of daughters with restored fertility was positively related to the *gypsy* insertion rate. The percentages of *ovo* reversions were estimated in daughters from crosses between (i) *w*Mel^{+} permissive females or (ii) *w*Mel^{-} permissive females with ovo^{D1} males (Table 1). The results indicated that the percentage of ovo^{D1} reversion was significantly higher in *w*Mel^{-} than in *w*Mel^{+} females ($P < 0.01$) (Table 1). In order to check that tetracycline-sensitive commensal bacteria were not involved in this phenomenon, we restored the gut microbiota in *w*Mel^{-} permissive females (see Fig. S1 in the supplemental material) and concluded that the gut microbiota had no effect on the percentage of ovo^{D1} reversion. Indeed, the percentage of ovo^{D1} reversion was significantly lower in the progeny of *w*Mel^{+} permissive females than in the progeny of *w*Mel^{-} permissive females and *w*Mel^{-} permissive females with restored microbiota ($P < 0.05$) (Table 1). There was no statistical difference between the percentage of ovo^{D1} reversion in the progeny of *w*Mel^{-} permissive

TABLE 1 Frequency of reversion of ovo^{D1} in the progeny of permissive and restrictive females crossed with ovo^{D1} males[a]

Group	Genotype of females	Treatment	Total no. of F1 females	No. of fertile F1 females	% ovo^{D1} reversion
A	Permissive	*w*Mel$^+$	625	31	4.96
B	Permissive	*w*Mel$^-$	560	52	9.29
C	Permissive	*w*Mel$^-$ plus microbiota	440	33	7.50
D	Restrictive	*w*Mel$^+$	617	3	0.49
E	Restrictive	*w*Mel$^-$	578	2	0.35
F	Restrictive	*w*Mel$^-$ plus microbiota	395	4	1.01

[a] Females that were not treated (*w*Mel$^+$) (groups A and D) or treated with tetracycline (*w*Mel$^-$) (groups B and E) or treated to be *w*Mel$^-$ and then restored with the original N271 microbiota (groups C and F) were crossed with ovo^{D1} males. Percentages in the last column represent fertile females divided by females present in the sample. Three independent ovo^D tests were performed for groups A, B, D, and E, and a single ovo^D test was performed for groups C and F. The nonparametric Kruskall-Wallis test was first applied to test homogeneity within experiments, and the results indicated that the mean values of the three ovo^{D1} reversion tests obtained for group A (4.42, 5.66, and 5.02) were lower than those obtained for group B (9.46, 11.32, and 8.50) ($P < 0.05$). We then pooled the three replicates for each experiment and performed proportion tests in order to increase the statistical power of our test. The percentage of ovo^{D1} reversion was significantly lower in the progeny of *w*Mel$^+$ permissive females than in the progeny of *w*Mel$^-$ permissive females with or without microbiota (group A versus B, $P < 0.01$; group A versus C, $P < 0.05$). There was no statistical difference between the results for the percentage of ovo^{D1} reversion in the progeny of *w*Mel$^-$ permissive females with or without microbiota (group B versus C, $P > 0.05$). There were no statistical differences between the percentages of ovo^{D1} reversion values in the progeny of the groups of restrictive females (group D versus E versus F, Bonferroni correction, $P > 0.05$).

females with or without microbiota ($P > 0.05$) (Table 1). As negative controls, we performed similar crosses using restrictive females in which the presence of fertile daughters might be due to mitotic crossovers generating ovo^+/ovo^+ cells, as previously shown (17). In this case, the presence of bacteria had no influence on the very low percentage of ovo^{D1} reversion estimates ($P > 0.05$) (Table 1). In order to determine whether ovo^{D1} reversion events resulted from *gypsy* insertions (denoted as ovo^r alleles), we performed PCR on DNA samples of a pool of revertant ovaries using primers specific for *gypsy* and *ovo*, respectively, in the two *gypsy* orientations according to the method of Dej et al. (22). Indeed, we obtained several PCR products in the *w*Mel$^+$ and *w*Mel$^-$ revertant ovaries, meaning that multiple independent *gypsy* integrations were responsible for the reversion. No positive signal for *gypsy* insertion into *ovo* was observed in the two parental lines (Fig. 2B). In conclusion, we demonstrated that the presence of *Wolbachia* diminishes the rate of integration of *gypsy*, which indicates for the first time cross talk between an endosymbiont and an ERV in *Drosophila*.

***Wolbachia* does not modify *gypsy* expression levels.** To assess whether the differential *gypsy* insertion rate is due to modulation of its expression induced by *Wolbachia*, we first performed quantitative reverse transcription PCR (qRT-PCR) to compare *gypsy* RNA levels (11) between *w*Mel$^+$ and *w*Mel$^-$ *flamenco* permissive and restrictive ovaries. We found that the relative *gypsy* RNA levels did not differ significantly between *w*Mel$^+$ and *w*Mel$^-$ permissive ovaries (Wilcoxon test, $P = 0.439$) (Fig. 3A), suggesting that *Wolbachia* does not interfere with the RNA transcription machinery. The *gypsy* RNA level was very low in restrictive ovaries, as expected (11). The presence of *Wolbachia* did not significantly modify the *gypsy* RNA level (Wilcoxon test, $P = 0.093$) (Fig. 3A), which suggests that *Wolbachia* does not interfere with the repression mechanism induced by *flamenco*. The *gypsy* Env protein levels were also monitored by Western blotting in permissive and

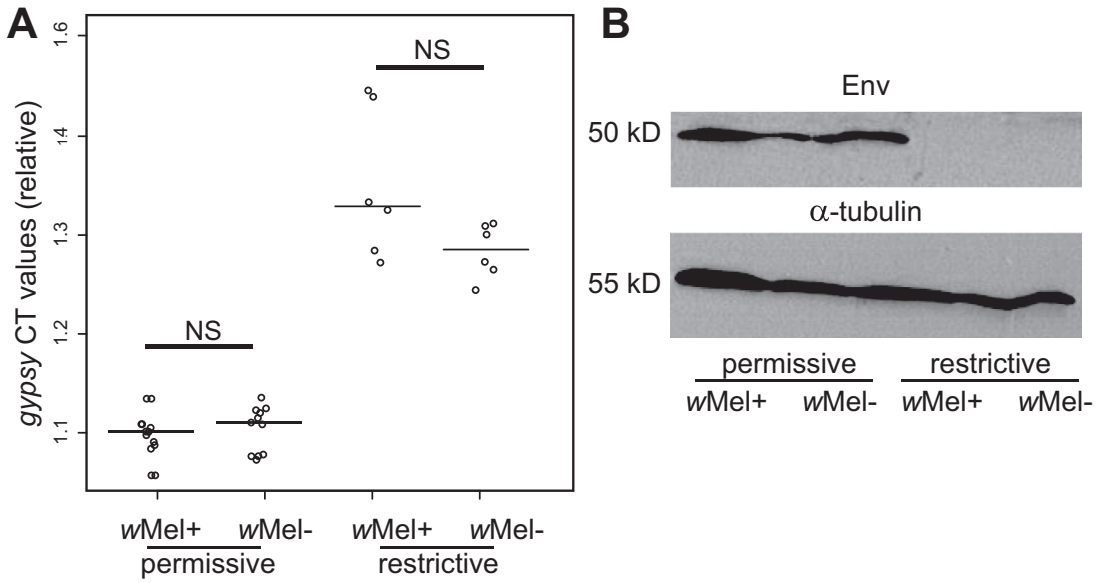

FIG 3 Quantitative RT-PCR analysis and Western blot analysis of *gypsy* expression. (A) Relative C_T values for *gypsy* levels after normalization with *rp49*; NS, no statistically significant difference. Thin horizontal bars represent medians. (B) Expression levels of *gypsy* envelope protein in permissive and restrictive ovaries with the presence or absence of *w*Mel were analyzed using Western blotting. α-Tubulin protein was used as a loading control. The 50-kDa band revealed by the Env antibody corresponds to the full-length envelope glycoprotein. Actual band sizes are indicated at the left.

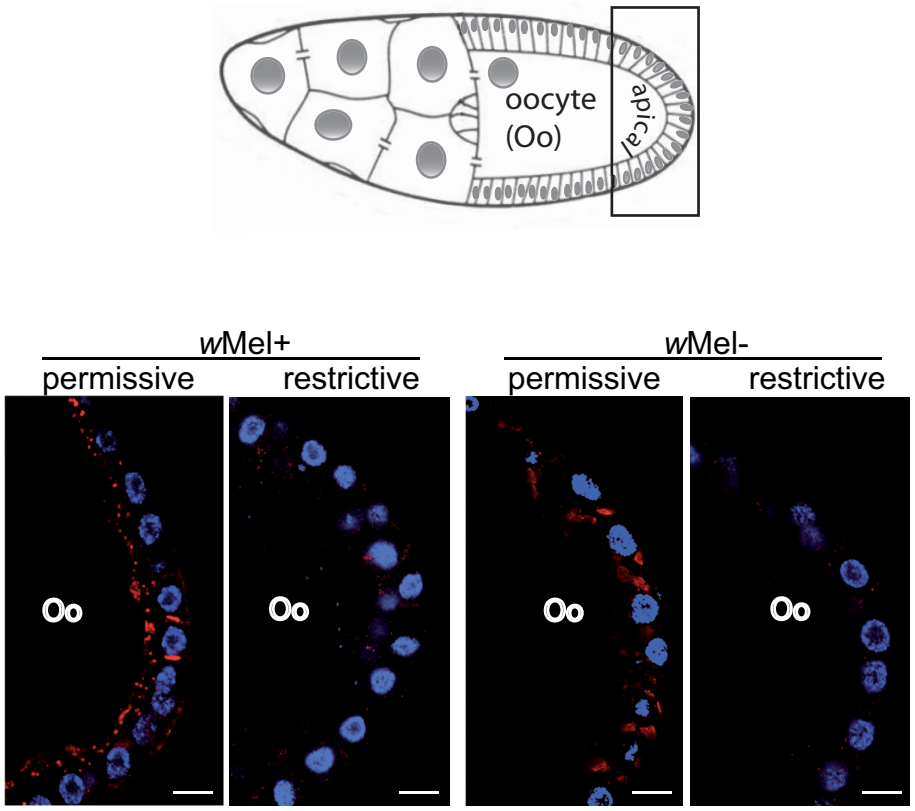

FIG 4 Immunostaining against *gypsy* envelope protein in permissive *w*Mel$^+$ and *w*Mel$^-$ follicle cells and their respective restrictive counterparts (scale bar = 10 μm). Blue, DAPI; red, *gypsy* envelope. Top, schematic representation of a stage 10 egg chamber; the apical pole is boxed and corresponds to the region observed in confocal microscopy. Oo, oocyte. *gypsy* Env patterns differ between permissive *w*Mel$^+$ and permissive *w*Mel$^-$ follicle cells, whereas Env is not detected in restrictive egg chambers whatever the *Wolbachia* status.

restrictive ovaries. The presence of *Wolbachia* did not modify the Env protein level independently of the *flamenco* genotype, as shown by the results in Fig. 3B. Altogether, our results indicate that *Wolbachia* did not affect *gypsy* expression levels.

The *w*Mel variant modifies the *gypsy* envelope distribution pattern. Whole-mount permissive ovaries with the presence (*w*Mel$^+$) or absence (*w*Mel$^-$) of *Wolbachia* were immunostained using an antibody raised against the *gypsy* Env protein (Fig. 4). *gypsy* envelope glycoprotein (Env) is mainly detected in the follicle cells of late egg chambers (stage 8 to 10) (11). In the permissive *w*Mel$^+$ follicle cells surrounding the posterior pole of the oocyte, *gypsy* Env displayed stick-shaped signals, as well as showing dot-shaped signals polarized at the apical pole (Fig. 4, bottom left). The *w*Mel$^-$ permissive ovaries exhibited a different pattern: round *gypsy* Env staining was observed in the cytoplasm in a non-polarized manner (Fig. 4, bottom right). Interestingly, we did not observe any difference between *w*Mel$^+$ and *w*Mel$^-$ restrictive egg chambers, i.e., *gypsy* Env was nearly absent in follicle cells whatever the *Wolbachia* status, meaning that neither tetracycline treatment nor the absence of *w*Mel affected the *flamenco* restriction (Fig. 4).

***Wolbachia* affects the distribution of intracytoplasmic *gypsy* virus-like particles.** As *Wolbachia* and *gypsy* are both maternally transmitted, we hypothesized that *Wolbachia* could interfere with *gypsy* within the oocyte and/or the follicle cells. To test this hypothesis, we investigated the *gypsy* distribution pattern in the presence or absence of *w*Mel. We identified intracytoplasmic particles of about 50 nm that were present in follicle cells of permissive flies but absent in restrictive follicle cells (Fig. 5). We confirmed that these particles corresponded to *gypsy* by immunoelectron microscopy (immuno-EM) using an antibody raised against *gypsy* Env. We observed gold beads localized near the virus-like particles present along the plasma membrane (Fig. 5A). This observation fully agreed with the description of *gypsy* particles obtained previously by Lecher et al. (12) and strongly suggested that the particles were *gypsy* virus-like particles. Then, the distribution patterns of these particles were compared between *w*Mel$^+$ and *w*Mel$^-$ permissive follicle cells. A major difference concerned the distribution of particles at the boundaries between two follicle cells: particles were scattered regularly at both sides of the junction between *w*Mel$^-$ follicle cells (Fig. 5C), whereas they clustered asymmetrically, i.e., they were present in one cell and absent in its neighbor cell, at the junction between *w*Mel$^+$ follicle cells (Fig. 5D). We also noticed that the cell junctions close to the particles were tightly sealed along a straight line in *w*Mel$^-$ follicle cells, which was never observed in *w*Mel$^+$ follicle cells (Fig. 5C). Moreover, we several times observed groups of particles in the cytoplasm of follicle cells, but these groups were systematically surrounded with a double membrane in *w*Mel$^-$ females (Fig. 5E) but not in *w*Mel$^+$ females (Fig. 5F). Altogether, these observations indicate that the presence of *w*Mel modifies the distribution of *gypsy* virus-like particles in follicle cells.

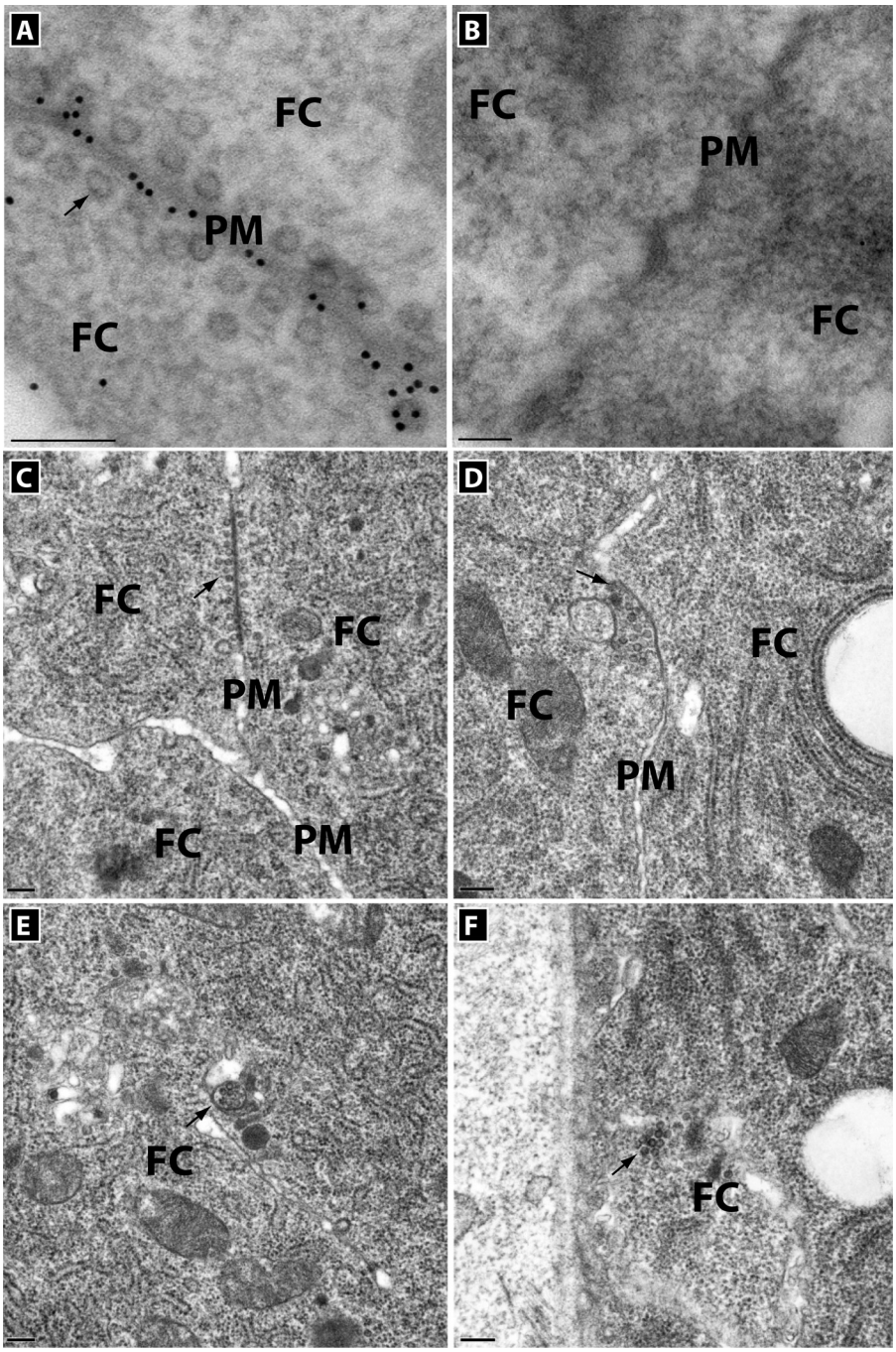

FIG 5 Distribution of *gypsy* virus-like particles in permissive fly stage 10 egg chambers in the presence or absence of *Wolbachia*. Images show immuno-EM labeling (10-nm gold particles [black dots]) (A, B) of the *gypsy* envelope protein in stage 10 egg chambers from *w*Mel$^-$ permissive females (A), which is absent from restrictive females (B), as expected, and electron microscopy observations of cytoplasmic 50-nm particles (black arrows) in permissive *w*Mel$^-$ (A, C, E) and permissive *w*Mel$^+$ (D, F) follicle cells (FC) (scale bars = 0.2 μm). Note the asymmetrical clustering of viral particles at the plasma membrane (PM) of two follicle cells in panel D compared to their distribution in panel C and the double membrane surrounding viral particles in panel E compared to panel F.

DISCUSSION

In this study, we show an effect of *Wolbachia* in reducing the rate of *gypsy* insertion into the *ovo* gene of the offspring of *Drosophila melanogaster* females permissive for *gypsy* expression. While *Wolbachia* is known to affect exogenous RNA virus replication, we show for the first time that it could also affect endogenous retroviruses. *Wolbachia* was horizontally transferred to *D. melanogaster* and then vertically transmitted (25). Like *Wolbachia*, *gypsy* has probably entered the genome of *D. melanogaster* recently, after its divergence from its sibling species, *D. simulans* (26). Colonization of the oocyte via follicle cells is the strategy used by several ERVs (11, 13, 20, 24). Toomey et al. have proposed that *Wolbachia* is delivered to the oocyte directly from the stem cell niches or indirectly through the somatic follicle cells (9). Conversely, it has been

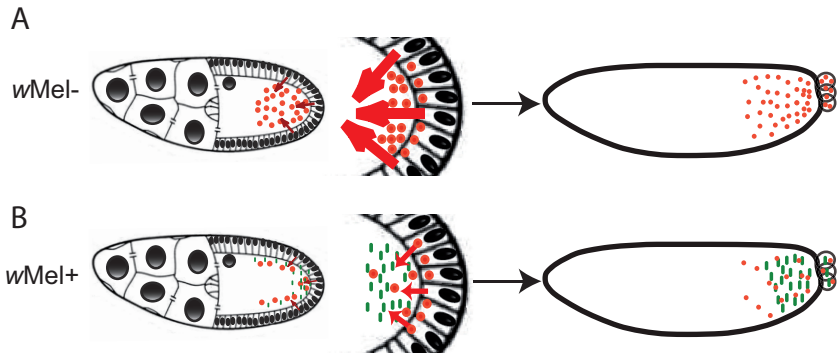

FIG 6 A model for *gypsy* transfer to the germline in the presence (bottom) or absence (top) of *Wolbachia*. (A) *gypsy* viral particles (red dots) assemble in the follicle cells and are transferred to the oocyte of a stage 10 egg chamber. (B) The *gypsy* particles that are localized posteriorly in the embryo are taken into the pole cells when they bud. *Wolbachia* cells (green dots) decrease the rate of *gypsy* transfer at the posterior pole because they occupy the posterior part of the oocyte and/or modify the *gypsy* assembly process, leading to fewer *gypsy* particles in the pole cells.

proposed that *Spiroplasma poulsonii*, a natural endosymbiont of *D. melanogaster*, interacts with the host yolk machinery to pass between follicle cells and enter the oocyte (27). The similarity between the *Wolbachia* and *gypsy* transmission pathways prompted us to ask whether *Wolbachia* could affect the dynamics of *gypsy* transfer from follicle cells to the oocyte. Pélisson et al. (11) and Lécher et al. (12) have previously described distributions of *gypsy* Env and *gypsy* virus-like particles similar to those we observed in this study for the *w*Mel$^-$ ovaries. Our results indicate that the presence of *Wolbachia* in the oocyte and/or follicle cells modifies the *gypsy* Env pattern of distribution and localization of gypsy viral particles in follicle cells, which could ultimately reduce the maternal transmission of *gypsy*. Immunofluorescence images suggest that, in *w*Mel$^+$ permissive egg chambers, *gypsy* Env is "stuck" near the junctions between follicle cells and at the apical domain of the follicle cells. EM images indicate that the presence of *gypsy* viral particles at the tightly sealed junctions between follicle cells vanishes when *w*Mel is present. The mechanism by which *Wolbachia* alters the distribution of *gypsy* in follicle cells is still unknown, and it could be worthwhile to further investigate the interaction between *Wolbachia*, *gypsy*, and cellular proteins involved in septate junctions. Another possibility worth investigating is whether a reciprocal influence of *gypsy* on *Wolbachia* maternal transmission could occur. We obtained data that indicated that the levels of *Wolbachia* maternally deposited in 0- to 2-h embryos are higher when the embryos are laid by permissive females than when they are laid by restrictive females (see Fig. S2 in the supplemental material). This result, which needs to be investigated further, corroborates the presence of interplay between *gypsy* and *Wolbachia* during maternal transmission. Our hypothesis is that *Wolbachia* could modify *gypsy* localization at junctions between follicle cells and at the apical domain, as it has previously been shown that bacteria and viruses can interact with junctions between epithelial cells (28–30). We therefore propose a model which takes into account previously proposed *gypsy* and *Wolbachia* transmission models (8, 9, 13). *Wolbachia* and *gypsy* share the same strategy, which is to localize at the posterior end of the oocyte and be taken up into the pole cells of the embryos when they bud (Fig. 6). In this model, *Wolbachia* exerts a repressive effect on the maternal transmission of *gypsy*: it modifies *gypsy* assembly and slows down follicle cell-to-oocyte transfer because of its presence in follicle cells and at the posterior pole of the oocyte, i.e., where the transfer occurs.

It was shown that *Wolbachia* manipulates a host miRNA in *Aedes aegypti* that decreases the expression of *AdDnmt-2*, a methyltransferase gene that is upregulated by dengue virus (31). In contrast, the small interfering RNA pathway seems not to be involved in the antiviral activity of *Wolbachia* in *Drosophila melanogaster* (32). We show here that *Wolbachia* does not modify *gypsy* RNA and protein expression levels in *flamenco* permissive and restrictive ovaries, suggesting that *Wolbachia* does not interfere with *gypsy* RNA and envelope levels in permissive ovaries or with the Piwi-mediated repression of *gypsy* by *flamenco* acting in restrictive ovaries. While the precise mechanism has not been elucidated, *Wolbachia* confers to the host a protective effect against *gypsy* integration. The antiviral protective effect of *Wolbachia* has been demonstrated for several exogenous viruses (33), and our results enlarge the spectrum of action of *Wolbachia* to include activity against endogenous retroviruses. The potential long-term consequence of a reduction of the endogenous retrovirus integration rate would be to confer a selective advantage to *Wolbachia*, increasing its frequency in natural populations. Furthermore, *Wolbachia* makes *gypsy* less harmful to the host, which may also contribute to the maintenance of *gypsy* and other retroelements that use the same road to the germline in *D. melanogaster*. Finally, it is notable that transovarial transmission has been demonstrated for several arboviruses and parasites (34, 35), and it would be worthwhile to investigate the effect of *Wolbachia* on their rates of maternal transmission.

MATERIALS AND METHODS

***Drosophila* strains.** *Drosophila melanogaster* strain N271 (14) was a gift from A. Pélisson (CNRS, France). It contains several active *gypsy* copies and a permissive *flamenco* allele (*flam*^{P}). This strain segregates homozygous permissive (*flam*^{P}/*flam*^{P}) or restrictive (*flam*^{P}/*FM7*) females for *gypsy* expression. The *ovo*^{D1} strain N376 has been previously described (23). This strain is maintained by crossing females with attached X chromosomes to *ovo*^{D1} males. All flies were reared on standard corn medium at 25°C.

In order to remove *Wolbachia*, the flies were grown during three generations on standard agarose medium containing 0.25 mg/ml tetracycline (Sigma-Aldrich). The strains were screened for the presence of *Wolbachia* using the *Wolbachia* 16S primers F (5′ TTGTAGCCTGCTATGGTAT

AACT 3′) and R (5′ GAATAGGTATGATTTTCATGT 3′). We also used the *Wolbachia wsp* primers F (5′-TGGTCCAATAAGTGATGAAGAAAC 3′) and R (5′ AAAAATTAAACGCTACTCCA 3′) for *Wolbachia* variant determination as described previously (6). *rp49* gene amplification was used as a control for the PCR (36). Some *Wolbachia*-free flies were also maintained on standard medium that was inoculated with commensal bacteria (5). Bacterial universal *16S* primers 27F (5′ AGAGTTTGATCCT GGCTCAG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′) were used as described previously (37) to detect bacteria. Treated flies were maintained on standard medium for at least three generations before experiments were performed. Concerning the ovo^D test (see below), the crossing experiments were performed seven generations after treatment.

Fluorescence microscopy. Ovaries were processed and stained using standard immunofluorescence techniques with antibodies (7). All flies were 1.5 to 2 days old at the time of dissection. The antibodies used were rabbit polyclonal antibody against *gypsy* envelope (anti-E78P antibody) (38) and Alexa Fluor 633 mouse anti-rabbit antibody (Invitrogen). Samples were then rinsed in phosphate-buffered saline (PBS) and mounted on a glass slide with the 4[prime],6-diamidino-2-phenylindole (DAPI)-containing mounting medium Vectashield (Cliniscience). A dozen egg chambers from three different immunostaining experiments were observed for each condition.

For *in situ* hybridization with *Wolbachia* DNA probes, ovaries were fixed for 20 min in 4% formaldehyde and heptane, postfixed for 10 min in 4% formaldehyde, and then washed once with PBS. Samples were incubated for 16 h at 37°C in hybridization buffer [50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 200 mg·liter^{-1} dextran sulfate, 250 mg·ml^{-1} poly(A), 250 mg·ml^{-1} salmon sperm DNA, 250 mg·ml^{-1} tRNA, 0.1 M dithiothreitol (DTT), 0.5× Denhartdt's solution] containing 10 ng of *Wolbachia* 16S DNA probes W2 (5′ CTTCTGT GAGTACCGTCATTATC 3′) and Wol3 (5′ TCCTCTATCCTCTTTCAA TC 3′) that were 5′-end labeled with rhodamine (4). Samples were washed twice in 1× SSC–10 mM DTT and twice in 0.5× SSC–10 mM DTT at 55°C for 15 min. Samples were then rinsed in PBS and mounted on a glass slide with the DAPI-containing mounting medium Vectashield (Cliniscience). All ovaries were analyzed with an SP5 confocal microscope (Leica).

Electron microscopy. Ovaries from 1.5- to 2-day-old flies were dissected in PBS and then fixed in 2% glutaraldehyde for 2 h at 4°C and washed three times in 0.4 M saccharose–0.2 M sodium cacodylate-HCl, pH 7.4, for 1 h at 4°C. Then, pieces were postfixed with 2% OsO4–0.3 M sodium cacodylate-HCl, pH 7.4, for 1 h at 4°C, dehydrated with an increasing ethanol gradient (5 min in 30%, 50%, 70%, and 95%), and treated three times for 10 min in absolute ethanol. Impregnation was performed with equal parts of Epon A and Epon B plus DMP30 (1.7%). Inclusion was obtained by polymerization at 60°C for 72 h.

Ultrathin sections (approximately 70 nm thick) were cut on a Reichert ultracut E (Leica) ultramicrotome, mounted on 200-mesh copper grids coated with 1:1,000 polylysine, stabilized for 1 day at room temperature, and then treated for contrast with uranyl acetate and lead citrate. Sections were examined with a Jeol JEM-1400 (Tokyo, Japan) transmission electron microscope equipped with an Orius 1000 camera and digital micrograph. Immuno-EM was performed as described previously (12).

Quantitative real-time PCR. Total RNA from dissected *Drosophila* ovaries was isolated using Nucleospin RNA (Macherey-Nagel) following the manufacturer's protocol. Then, 1 μg of total RNA was reverse transcribed using the Omniscript reverse transcription kit (Qiagen) with oligo(dT) primers (Invitrogen). Roche Universal Sybr green mix (Roche) and StepOne Plus (Applied biosystem) were used for quantitative RT-PCR to amplify a *gypsy* envelope fragment gene with *gypsy* primers F (5′ G GCTCATTGCCGTTAAACAT 3′) and R (5′ TCTTCCTTCTTTCGCTG AGG 3′). The changes in cycle threshold (ΔC_T) values were calculated within the log-linear phase of the amplification curve with StepOne Plus software, version 2.2.2 (Applied Biosystems). Quantification was normalized to that of the mRNA encoding the endogenous ribosomal protein Rp49, which was amplified using the *rp49* primers F (5′ CGGATCGATA TGCTAAGCTGT 3′) and R (5′ GCGCTTGTTCGATCCGTA 3′). Statistical analyses were performed in R (http://www.R-project.org).

Quantitative PCR. For mitochondrial DNA density quantification, total DNA was extracted from *w*Mel$^-$ and *w*Mel$^+$ permissive N271 ovaries. Quantitative PCRs were performed with 30 ng of total DNA as described previously (21), using the following primers specific for the *Drosophila melanogaster* cytochrome-*c* oxidase subunit I (*COI*) gene: F (5′ G CTCCTGATATAGCATTCCCACGA 3′) and R (5′ CATGAGCAATTCC AGCGGATAAA 3′). Three independent DNA extractions were performed for each condition, and quantitative PCR assays were done in triplicate.

Wolbachia quantitative PCR was performed as previously described (39). DNA from 30 0- to 2-h embryos from *w*Mel$^+$ permissive or restrictive N271 females was extracted as described previously (21). Quantitative PCR was done with 60 ng of total embryonic DNA. Three independent DNA extractions were performed for each condition (permissive/restrictive), and quantitative PCR assays were done in triplicate.

Protein extraction and Western blot analysis. Ovaries from 25 flies were dissected in cold PBS and then squashed in 50 μl of lysis buffer (Thermo) with protease inhibitor (Roche). Protein extracts were mixed with 2× Laemmli buffer (Sigma-Aldrich) and loaded on a 12% acrylamide gel. The same quantity of each sample was loaded twice in the same gel. Protein was transferred to nitrocellulose membranes and used for Western blot analysis as described previously (19). The membrane was cut into two pieces containing exactly the same samples. One piece was incubated with the rabbit polyclonal anti-*gypsy* envelope antibody E78P (38) and the other with rabbit anti-α-tubulin antibody (Ab12546; Abcam), and both were revealed by a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (A6154; Sigma-Aldrich) and SuperSignal (Pierce), following the manufacturer's instructions.

The ovo^D reversion assay. The ovo^D test is a genetic assay for *gypsy* transposition that has been described previously (36). Briefly, the X-linked *ovo* gene is involved in ovarian maturation, and the dominant-negative mutation ovo^{D1} results in sterile females with no functional ovaries. The *ovo* locus is a hot spot for *gypsy* insertion, and the insertion of *gypsy* into the ovo^{D1} allele (denoted as an ovo^r allele) of a heterozygous female prevents the production of the repressor OvoD1 protein. The ovo^r/ovo^+ females are then fertile, and most of them carry only one ovary because *gypsy* integration happens in a late stage of germline development. The ovaries of five revertants were pooled, and the DNA was extracted using the Nucleospin tissue XS kit (Macherey-Nagel). The presence of *gypsy* in *ovo* was checked by PCR as described previously (24), using primers P1 (5′ CAACATGACCGAGGACGGTCATAAAC 3′), P2 (5′ CTCCC GCTCTGCGGGCTTCTCTTT 3′), P3 (5′ CTTTGCCGAAAATATGCAA TG 3′), and P4 (5′ CGGCTTTTTCAGCGGCTAACC 3′) (Fig. 4).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01529-14/-/DCSupplemental.

Figure S1, EPS file, 0.1 MB.

Figure S2, EPS file, 0.1 MB.

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