Paramutation in *Drosophila* requires both nuclear and cytoplasmic actors of the piRNA pathway and induces *cis*-spreading of piRNA production.

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Transposable element (TE) activity is repressed in the germline in animals by PIWI-Interacting RNAs (piRNAs), a class of small RNAs produced by genomic loci mostly composed of TE sequences. The mechanism of induction of piRNA production by these loci is still enigmatic. We have shown that, in Drosophila melanogaster, a cluster of tandemly repeated P-lacZ-white transgenes can be activated for piRNA production by maternal inheritance of a cytoplasm containing homologous piRNAs. This activated state is stably transmitted over generations and allows trans-silencing of a homologous transgenic target in the female germline. Such an epigenetic conversion displays the functional characteristics of a paramutation, i.e., a heritable epigenetic modification of one allele by the other. We report here that piRNA production and trans-silencing capacities of the paramutated cluster depend on the function of the rhino, cutoff and zucchini genes involved in primary piRNA biogenesis in the germline, as well as on that of the aubergine gene implicated in the ping-pong piRNA amplification step. The 21nt RNAs which are produced by the paramutated cluster in addition to 23-28nt piRNAs are not necessary for paramutation to occur. Production of these 21nt RNAs requires Dicer-2 but also all the piRNA genes tested. Moreover, cytoplasmic transmission of piRNAs homologous to only a subregion of the transgenic locus can generate a strong paramutated locus which produces piRNAs along the whole length of the transgenes. Finally, we observed that maternally-inherited transgenic small RNAs can also impact transgene expression in the soma. In conclusion, paramutation involves both nuclear (Rhino, Cutoff) and cytoplasmic (Aubergine, Zucchini) actors of the piRNA pathway. In addition, since it is observed between non-fully homologous loci located on different chromosomes, paramutation may play a crucial role in epigenome shaping in Drosophila natural populations.
Introduction

Genomes must confront the presence of a large fraction of mobile DNA whose activity can result in severe deleterious effects on chromosome stability and gametogenesis. In the germline of animals, a system of genomic traps exists into which any transposable element (TEs) can insert, thereby generating loci which contain a catalogue of potentially dangerous sequences that have to be repressed (BRENNECKE et al. 2007; PANÉ et al. 2011; IWASAKI et al. 2015). In the Drosophila melanogaster germline, most of these loci are transcribed in both directions (dual strand clusters) and undergo non-canonical transcription and RNA processing (MOHN et al. 2014; ZHANG et al. 2014). This results in production of non-coding small RNAs having the capacity to target the transcripts of the homologous, potentially active, TE copies scattered throughout the genome. These small RNAs are called PIWI-Interacting RNAs (piRNAs) and repress TE activity at both the transcriptional and post-transcriptional levels (SATO and SIOMI 2013; WEICK and MISKA 2014). piRNA biogenesis in the germline involves a nuclear and a cytoplasmic step. In the nucleus, piRNA-producing cluster transcription requires in most of the cases the presence of the HP1 paralog Rhino associated with Deadlock and Cutoff on the locus, forming the so-called RDC complex (KLATTENHOFF et al. 2009; PANÉ et al. 2011; MOHN et al. 2014; ZHANG et al. 2014). In the cytoplasm, transcripts produced by the piRNA locus are sliced in an optically dense region surrounding the nucleus, called the nuage, and small RNAs (23-28nt) loaded on Piwi or Aubergine proteins are produced (primary piRNAs). Further, piRNAs loaded on Piwi enter the nucleus and target euchromatic TE copies to induce their transcriptional repression via heterochromatin formation which involves HP1 (WANG and ELGIN 2011; SIENSKI et al. 2012; LE THOMAS et al. 2013; ROZHKOV et al. 2013). In contrast, piRNAs loaded on Aubergine remain in the nuage and target homologous transcripts being exported from the nucleus which are produced by both homologous TE copies and the piRNA locus. This produces secondary piRNAs loaded on Aubergine or Ago3, other PIWI proteins (WEICK and MISKA 2014; IWASAKI et al. 2015) and results in a piRNA amplification process called ping-pong amplification (BRENNECKE et al. 2007; GUNAWARDANE et al. 2007). Finally, piRNA loaded on Piwi can also be produced downstream of the ping-pong amplification step by a slicing process which can spread on targeted RNA, increasing both piRNA quantity and diversity (HAN et al. 2015; MOHN et al. 2015; SIOMI and SIOMI 2015). Upstream of this complex machinery, the presence of Rhino on the piRNA-producing locus is particularly important since it appears sufficient to promote processing of transcripts by the piRNA
machinery (KLATTENHOFF et al. 2009; ZHANG et al. 2014). How Rhino is addressed to a piRNA-producing locus is still unclear.

In Drosophila, in contrast to C. elegans (RUBY et al. 2006; BATISTA et al. 2008), the production of piRNAs by a piRNA locus in the germline does not appear to be only genetically determined, i.e. no specific sequence motif or structure has been identified which is sufficient to promote piRNA-production by DNA adjacent to this sequence. Conversely, using piRNA-producing loci which repress P transposable elements (RONSSERAY et al. 1996), it was shown that maternal transmission of piRNAs together with the piRNA locus can stimulate production of piRNAs by paternally-inherited P elements scattered through the genome (BRENNECKE et al. 2008). In addition, analysis of ageing effects on I transposable element repression capacities showed that the amount of I-homologous piRNAs in adult ovaries is correlated to the amount of homologous piRNAs deposited in embryos (GRENTZINGER et al. 2012). Furthermore, it was shown that de novo, long-term activation of a piRNA locus can be achieved in Drosophila by maternal transmission of homologous piRNAs, without transmission of the initial piRNA donor locus. Under these conditions, there is emergence of a new autonomous and stable piRNA locus (DE VANSSAY et al. 2012). Indeed, a transgenic cluster of P-element deriving transgenes (called BX2), inert for repression and piRNA production, can be activated by crossing with females bearing a transgenic cluster (called T-1) that produces abundant piRNAs in both orientations homologous to the BX2 cluster (DE VANSSAY et al. 2012). Once activated, this new piRNA cluster, called BX2*, is stable over generations (n>100) in absence of the inducer T-1 locus and is able to produce abundant transgenic sense and antisense piRNAs. Cytoplasm of oocytes produced by these females can again de novo activate piRNA production by a paternally-inherited inactive BX2 cluster (DE VANSSAY et al. 2012). This recurrent epigenetic conversion process presents all the features of a “paramutation” process, a phenomenon previously described in plants (BRINK 1956; COE 1959), and recently in worms (SHIRAYAMA et al. 2012; SAPETSCHNIG et al. 2015), and described as “an epigenetic interaction between two alleles of a locus, through which one allele induces a heritable modification of the other allele without modifying the DNA sequence” (BRINK 1956; CHANDLER 2007). It was shown further that a BX2* locus has a modified chromatin structure, showing an increase in H3K9 methylation with regard to the non-paramutated BX2 locus (LE THOMAS et al. 2014). This example of paramutation thus results in communication within a genome between homologous clusters allowing one cluster to transfer piRNA production capacity to a previously inert cluster. Paramutation may play an important role in establishment of TE repression following a genetic invasion since master sites of repression could activate piRNA secondary sites thus
reinforcing their repressive capacity. Investigating the mechanism of de novo activation of piRNA-producing loci is therefore important for understanding aspects of epigenome shaping in general.

We have therefore analysed the functional requirements and properties of a BX2* paramutated locus. This locus allows the investigation of the autonomous properties of a piRNA-producing locus, i.e. independently of the influence of homologous sequences present in the genome which could interfere with the process. The BX2* locus was previously shown to produce, not only abundant transgenic piRNAs, but also 21nt RNAs inferred to be small-interfering RNAs (siRNAs), but the precise nature of these 21nt RNAs and their functional role in silencing capacities were not elucidated. Here, we show first that BX2* silencing and piRNA production capacities depend on the rhino and cutoff genes, involved in the non-canonical transcription of piRNA producing loci, and on the aubergine and zucchini genes, involved in piRNA cytoplasmic processing. In addition, transgenic 21nt RNA production depends on Dcr-2 which characterizes them as siRNAs but they are not necessary for paramutation to occur. Surprisingly, production of these siRNAs depends also on the piRNA pathway. Moreover, paramutation can occur between only partially homologous loci within a genome and is accompanied by a rapid spreading of the capacity to produce piRNAs to the non-homologous sequences within the paramutated locus. Finally, an impact of maternally inherited small RNAs linked to paramutation is also observed in somatic cells. These findings allow us to characterize more precisely the molecular mechanism of paramutation in animals and to propose that it is a key factor in epigenome shaping in natural populations.

**Materials and Methods**

**Experimental conditions.**

All crosses were performed at 25°C and involved 3-5 couples in most cases. lacZ expression assays were carried out using X-gal overnight staining as described in Lemaitre et al. 1993 (LEMAITRE et al. 1993), except that ovaries were fixed for 8 min. Trans-silencing was quantified as previously described by determining the percentage of egg chambers with no lacZ expression in the germline (JOSSE et al. 2007). It is referred as the Trans-Silencing Effect percentage (TSE%).
**Dissection and RNA extraction.**

Total RNAs were extracted using TRIzol® (Life Technologies™) as described in the Reagent manual (http://tools.lifetechnologies.com/content/sfs/manuals/trizol_reagent.pdf). For the RNA precipitation step, 100% ethanol was used instead of isopropanol. For ovarian small RNAs, 25 pairs of ovaries were manually dissected for each sample in PBS 1X.

**Pigment dosage.**

Head pigments were quantified as described in (WEILER and WAKIMOTO 2002). For each genotype, three replicates were measured. For each replicate, five heads of 4 to 6 day old females were manually dissected for the dosage. Absorbance was measured at 480nm.

**Transgenes and strains.**

*P-lacZ* fusion enhancer-trap transgenes: *P-1152*, and *BQ16*, contain an in-frame translational fusion of the *E. coli lacZ* gene to the second exon of the *P* transposase gene and a *rosy* transformation marker (O’KANE and GEHRING 1987). The *P-1152* insertion was mapped to the telomere of the *X* chromosome (cytological site 1A) and consists of two *P-lacZ* insertions in the same Telomeric associated sequence unit (TAS, (KARPEN and SPRADLING 1992)) and in the same orientation (JOSSE *et al.* 2007). *P-1152* is homozygous viable and fertile. *BQ16* is located at 64C in euchromatin of the third chromosome and is homozygous viable and fertile. *P-1152* shows no *lacZ* expression in the ovary, *BQ16* is strongly expressed in the nurse cells and in the oocyte (JOSSE *et al.* 2007). *RS3* is a *P-FRT-white* transgene (FBtp0001534). It is inserted in the TAS of the 3R chromosomal arm (cytological site 100E3). It is homozygous viable and fertile (DGRC, Kyoto #123282). *P-1152* and *RS3* induce a strong TSE and produce abundant ovarian transgenic piRNAs (ROCHE and RIO 1998; JOSSE *et al.* 2007; DE VAnsSAY *et al.* 2012; DUFOUTR *et al.* 2014) and this study, (Figure 3-A). The *Lk-P*(1A) line carries two full-length *P* elements inserted in TAS at the X-chromosome telomere (RONSSERAY *et al.* 1991). These telomeric *P*-insertions derive from a chromosome present in a natural population (BIEMONT *et al.* 1990) and induce very strong repression of *P*-element transposition and *P*-induced hybrid dysgenesis (RONSSERAY *et al.* 1991; RONSSERAY *et al.* 1996). *Lk-P*(1A) females produce abundant *P*-homologous ovarian piRNAs (BRENNERCKE *et al.* 2008). The repressive capacity of the *Lk-P*(1A) line was verified immediately prior to the experiments reported in Figure S6 and a very strong repression capacity was found (data not shown).

*P{lacW} clusters.* The BX2 line carries seven *P-lacZ-white* transgenes, including at least one
defective copy, inserted in tandem and in the same orientation at cytological site 50C on the second chromosome. The transgene insertion site is located near the *mRpL53* gene in an *Ago1* intron (De Vanssay et al. 2012). This site is not a piRNA-producing locus, as observed for instance in the deep-sequencing dataset from P-1152 or *w^{1118}* ovaries (data not shown). The *P-lacZ-white* construct contains the *P-lacZ* translational fusion and is marked by the mini-white gene (*P{lacW}, FBtp0000204, 10691bp*). The *T-1* line derives from X-ray treatment of *BX2* (Dorer and Henikoff 1994; Dorer and Henikoff 1997). *T-1* has chromosomal rearrangements including translocations between the second and the third chromosomes. *DX1* was generated during the same *P-lacZ-white* transposase-mediated remobilization at 50C than *BX2* and carries six *P-lacZ-white* transgenes, one of which is in opposite orientation to the others, at the same genomic site as *BX2* (Dorer and Henikoff 1994; Dorer and Henikoff 1997). After overnight staining, weak *lacZ* expression is detected in the follicle cells of *BX2*, *T-1* and *DX1* female ovaries, presumably because of a position effect at 50C, but no staining is observed in the germline (data not shown). The three *P{lacW}* clusters show variegated repression of *white* in the eyes due to Repeat Induced Gene Silencing (RIGS) (Dorer and Henikoff 1994; Dorer and Henikoff 1997). The extent of variegated repression of *white* is different between the clusters (*T-1 >> DX1 > BX2*). The *BX2* cluster was not used to assay the somatic phenotypic impact of piRNA maternal inheritance since RIGS-induced repression is too low in this case.

Lines carrying transgenes have M genetic backgrounds (devoid of *P* transposable elements), as do the multi-marked balancer stocks used in genetic experiments. The *w^{1118}* and Canton*"* lines were used as controls completely devoid of any *P* element or transgene. Crosses performed with *BX2*, *T-1* or *DX1* were performed with females carrying the cluster at the heterozygous state because of the sterility (*BX2*, *DX1*) and lethality (*T-1*) induced by transgene clusters.

Two strong hypomorphic mutant alleles of *aubergine* (*aub*) induced by EMS were used. Both of them are homozygous female sterile and TSE was previously shown to be abolished by a heteroallelic combination of these alleles (Josse et al. 2007). *aub^{QC42}* comes from the Bloomington Stock Center (stock #4968) and has not been characterized at the molecular level (Schupbach and Wieschaus 1991). *aub^{N11}* has a 154bp deletion, resulting in a frameshift which is predicted to add 16 novel amino acids after residue 740. *Dicer-2^{L811fsX} (Dcr-2^{L811fsx})* is a loss of function allele induced by EMS which has a sequence variant at residue 811 resulting in a stop codon (Lee et al. 2004). It is homozygous viable and fertile. *rhino^{02086} (rhi^{2})* results from a *P[PZ] insertion (P-lacZ-
rosy) at nucleotide 267. The \( P\{PZ\} \) transgene is oriented in the opposite direction to that of \( rhi \) transcription (VOLPE et al. 2001) (Bloomington #12226). \( rhi^{KG00910} \) (\( rhi^{KG} \) is due to the insertion of a \( P\{SUPor-P\} \) transgene which carries the \textit{yellow} and \textit{white} transformation markers (Gene disruption project 2001, Bloomington #13161). \textit{cutoff}\(^{WM25} \) (\( \textit{cuff}^{WM25} \) and \( \textit{cuff}^{QQ37} \) alleles were isolated from an EMS screen (SCHUPBACH and WIESCHAUS 1991). \( \textit{cuff}^{WM25} \) corresponds to the replacement of the first methionine by a lysine and \( \textit{cuff}^{QQ37} \) has a T to A substitution at the position 786 that could result in a splicing alteration (data not shown). \textit{zucchini} (\( \textit{zuc} \)) alleles were isolated from an EMS screen (SCHUPBACH and WIESCHAUS 1991). \( \textit{zuc}^{HM27} \) contains a stop codon at residue 5 and \( \textit{zuc}^{SG63} \) a substitution of histidine 169 with a tyrosine in the conserved HKD domain presumably involved in nuclease activity. TSE was previously shown to be abolished by a heteroallelic combination of these alleles (TODESCHINI et al. 2010). The crosses performed to generate hetero-allelic mutant BX2* females are indicated in Table S1. When information was available, the most severe allele was introduced maternally. Lines carrying mutations of \( \textit{zuc} \) and \( \textit{cuff} \) were kindly provided by Attilio Pane and Trudi Schüpbach and \( \textit{aub}^{N11} \) was kindly provided by Paul Macdonald.

All the alleles described above being are located on the second chromosome and are maintained over a \( Cy \) balancer chromosome. Additional information about mutants and stocks are available at flybase: \url{http://flybase.bio.indiana.edu/}

Deep sequencing analyses

A small RNA fraction, from 18nt to 30nt in length, was obtained by separating it on a denaturing polyacrylamide gel from total RNA extracted from dissected ovaries. This fraction was used to generate multiplexed libraries with Illumina TruSeq Small RNA library preparation kits (RS-200-0012, RS200-0024, RS-200-036 or RS-200-048) at Fasteris (http://www.fasteris.com). A house protocol based on TruSeq, which reduces 2S RNA (30nt) contamination in the final library, was performed (except for libraries whose GRH number is below #40). Libraries were sequenced using Illumina HiSeq 2000 and 2500. Sequence reads in fastq format were trimmed from the adapter sequence 5’-CTGTAGGCACCATCAATCGTA-3’ (GRH12, GRH13, GRH14, GRH17) or 5’-TGGAAATTCTCGGGTGCCAAG-3’ (other samples) and matched to the \textit{D. melanogaster} genome release 5.49 using Bowtie (LANGMEAD et al. 2009) and to the sequences of the \( P \)-element constructs \( P\{lacW\} \) (Flybase ID FBtp0000204) as indexed reference. Only 19-29nt reads matching the reference sequences with 0 or 1 mismatch were retained for subsequent analysis. For global
annotation of the libraries (Table S1), we used the release 5.49 of fasta reference files available in Flybase, including transposon sequences (dmel-all-transposon_r5.49.fasta) and the release 20 of miRNA sequences from miRBase (www.mirbase.org).

Sequence length distributions, small RNA mapping and small RNA overlap signatures were generated from bowtie alignments using Python and R (www.r-project.org/) scripts, which were wrapped and run in a Galaxy instance publicly available at http://mississippi.fr. Tools and workflows used in this study may be downloaded from this Galaxy instance. For library comparisons, read counts were normalized (Table S1) to the total number of small RNAs that matched the D. melanogaster genome (release 5.49) and did not correspond to abundant cellular RNAs (tRNAs and miscRNAs). For small RNA mapping (Figures 1-3, S1, S3, S6), we took into account only RNA reads which uniquely aligned to P[lacW] or the 42AB locus.

Distributions of piRNA overlaps (ping-pong signatures, Figure S2, S5) were computed as first described in (KLATTENHOFF et al. 2009) and detailed in (ANTONIEWSKI 2014). Thus, for each sequencing dataset, we collected all the 23-28nt RNA reads matching P[lacW] or the 42AB locus whose 5’ ends overlapped with another 23-28nt RNA read on the opposite strand. Then, for each possible overlap of 1 to 28nt, the number of read pairs was counted. Distributions of siRNA overlaps (Figure S3) were computed using a similar procedure, except that 20-21nt RNA reads were collected instead of the 23-28nt RNA reads. The distributions of piRNA/siRNAs overlaps (Figure S3) were computed by collecting separately the 20-21nt and 23-28nt RNA reads matching P[lacW] or the 42AB locus and counting for each possible overlap of 1 to 21nt the number of read pairs across these two distinct read datasets. To plot the overlap signatures, a z-score was calculated by computing, for each overlap of 1 to i nucleotides, the number O(i) of read pairs and converting it, using the formula z(i) = (O(i)-mean(O))/standard deviation (O).

Results

Effect of mutation affecting the piRNA or siRNA pathway on BX2* small RNA production and silencing capacities.

Paramutated BX2* females were previously shown to produce 23-28nt and 21nt ovarian sense and antisense small RNAs homologous to the cluster of P-lacZ-white transgenes (called P[lacW]) and to induce complete silencing of homologous transgene expression in the female
germline (DE VANSSAY et al. 2012). This homology dependent silencing, which reflects piRNA functionality, is quantified using the Trans-Silencing Effect (TSE) assay (JOSSE et al. 2007) in which the percentage of repressed egg chambers is measured. The 23-28nt, but not 21nt, RNAs presented the molecular signature of the ping-pong amplification step of the piRNA pathway. This strongly suggests that production of the 23-28nt RNAs is dependent on the piRNA pathway, whereas that of the 21nt RNAs would depend on the siRNA pathway. Moreover, the TSE capacities of the BX2* paramutated locus were shown to be completely impaired by loss of function of aubergine (aub) involved in the piRNA ping-pong step, whereas they were insensitive to loss of function of Dicer-2 (Dcr-2) involved in the siRNA pathway (DE VANSSAY et al. 2012). This suggested that the 21nt would not be necessary for BX2* silencing capacities.

We thus performed deep sequencing of ovarian small RNAs of mutant females for aub and Dcr-2 and extended the analysis to rhino (rhi) and cutoff (cuff), which are nuclear actors involved in piRNA loci non-canonical transcription (MOHN et al. 2014; ZHANG et al. 2014), and to zucchini (zuc) which is involved in the cleavage of piRNA precursors and secondary piRNAs in the cytoplasm (IPSARO et al. 2012; NISHIMASU et al. 2012; VOIGT et al. 2012; HAN et al. 2015; MOHN et al. 2015). All these genes are located, as BX2 is, on chromosome 2. For each gene tested, a recombinant chromosome carrying a BX2* locus and a mutated allele was generated. Females heterozygous for this recombinant chromosome were crossed with heterozygous males, carrying one mutant allele of the corresponding tested gene, to generate control heterozygote, loss of function heteroallelic (aub, rhi, cuff, zuc) and homozygote (Dcr-2) females for deep sequencing of ovarian small RNAs (Figure 1, upper box, Table S1). In addition, females heterozygous for recombinant chromosomes were crossed with heterozygous males carrying a corresponding mutated allele and a target P-lacZ transgene expressed in the female germline to investigate the effect of the mutation tested on the trans-silencing capacities of BX2* (Figure 1, upper box). As controls, we analysed small RNAs from ovaries of BX2* females at generation 83 (G83) after the paramutation process and of BX2 non-paramutated females (called “BX2 naive”). BX2* lines maintained at 25°C show a very strong stability through generations, as tested every five generations, showing a complete repression capacities (for example G83: TSE=100%, n= 1500; G115: TSE=100%, n=1150). Figure 1 shows control females together with heterozygous and homozygous females for loss of function mutations for each gene tested, in terms of their capacity for BX2* silencing (TSE%) and for production of ovarian small RNAs homologous to the P{lacW} transgene. As expected, the BX2 naive cluster showed no silencing capacities (TSE= 0%, n=2000) and did not produce
significant amounts of $P\{lacW\}$ homologous 23-28nt or 21nt RNAs (Figure 1-A) whereas the $BX2^*$ paramutated cluster continued to induce complete trans-silencing and to produce high levels of $P\{lacW\}$ homologous 23-28nt and 21nt RNAs 83 generations after the paramutation process (Figure 1-B). $aub$ loss of function resulted in the complete loss of 23-28nt RNAs homologous to the $P\{lacW\}$ transgene (Figure 1-D), when compared to heterozygous $aub$ females (Figure 1-C). Surprisingly, $aub$ loss of function also affected production of the 21nt RNAs by the $P\{lacW\}$ cluster (Figure 1-D). This did not result from a genomic background effect on the siRNA pathway in $aub$ mutant females since the production of endogenous siRNAs at the esi-1 locus was not affected (Figure S4-D). Dcr-2 loss of function resulted in a strong decrease of 21nt RNAs homologous to the $P\{lacW\}$ transgene but the production of corresponding 23-28nt RNAs was not affected, nor their ping-pong signature (Figure 1-F, Figure S2-J). Analysis of mutations affecting rhi, cuff or zuc produced similar results to that of $aub$ mutations. TSE percentages showed that $rhi$, $cuff$ or $zuc$ loss of function resulted in loss of $BX2^*$ silencing capacities (Figure 1-H, J, L), whereas no effect was found with $rhi$, $cuff$ and $zuc$ heterozygotes (Figure 1-G, I, K). $rhi$, $cuff$ and $zuc$ loss of function also resulted in complete loss of ovarian $P\{lacW\}$ piRNA production together with an almost complete loss of 21nt RNAs homologous to the transgene (Figure 1-H, J, L). As expected, these mutants exhibited no effect on the production of endogenous siRNAs at the esi-1 locus (Figure S4) but production of piRNAs by the 42AB typical dual-strand piRNA locus was affected (Figure S1). In conclusion, $BX2^*$ silencing capacities and $P\{lacW\}$ homologous piRNA production depend on all the piRNA pathway genes tested, including nuclear actors, and do not depend on Dcr-2. Interestingly, Dcr-2-dependent 21nt RNAs production by the cluster also depends on all the piRNA pathway genes tested. $BX2^*$ is thus a typical dual strand piRNA cluster, requiring the primary and secondary piRNA pathways, which allows the study of the properties of a piRNA cluster, independently from any influence of endogenous homologous sequences in trans.

**Production of ovarian transgenic siRNAs does not require siRNA maternal inheritance.**

Our previous work (DE VANSSAY et al. 2012), combined with the results in Figure 1-F, shows that $BX2^*$ silencing properties and production of piRNAs homologous to the $P\{lacW\}$ transgene do not require maternal inheritance of siRNAs. Results in Figure 1 show that presence of ovarian $P\{lacW\}$ 21nt siRNAs depends, in addition to Dcr-2, on the function of all piRNA pathway genes tested. We asked next if production of $P\{lacW\}$ ovarian siRNAs requires maternal inheritance of such siRNAs. We thus generated $BX2^*$ females carrying a paternally-inherited Dicer-2 wild type allele and maternally-inherited $P\{lacW\}$ homologous piRNAs but no siRNAs, because their $G_0$
mothers were homozygous for Dcr-2 mutations (Figure 2). As expected (DE VANSSAY et al. 2012), these G₁ Dcr-2 heterozygous females showed complete silencing capacities. Deep sequencing shows that, in addition to ovarian P{lacW} piRNAs, they produce P{lacW} siRNAs. Further, these G₁ females were crossed with Dcr-2 +/+ males and increased ovarian P{lacW} siRNA levels were found in BX2* G₂ females having recovered a Dcr-2 +/+ genotype. Therefore, production of ovarian P{lacW} siRNAs by females having a functional siRNA pathway does not require homologous siRNA inheritance and can be induced by inheritance of solely homologous piRNAs.

*Paramutation of a locus only partially covered by maternally inherited piRNAs can be stable and is accompanied by cis-spreading of piRNA production capacity.*

Targeting of transgenes or natural transposable elements by piRNAs can lead to spreading of piRNA production to sequences adjacent to the targeted elements (MUERDTER et al. 2011; OLOVNIKOV et al. 2013; SHPIZ et al. 2014). We tested if spreading could be observed for a silencer locus induced by paramutation. Telomeric transgenes inserted in subtelomeric heterochromatin can show strong trans-silencing capacities (ROCHE and RIO 1998; RONSSERAY et al. 2003; JOSSE et al. 2007; JOSSE et al. 2008; DUFORT et al. 2014) and produce abundant piRNAs in the germline (MUERDTER et al. 2011; DE VANSSAY et al. 2012). These transgenes have a different structure than the P{lacW} transgene of BX2 but share some common sequences (DE VANSSAY et al. 2012). It was therefore possible to produce females, having paternally inherited a BX2 naive locus and maternally inherited cytoplasm containing piRNAs homologous to only a subset of the P{lacW} transgene structure. Two telomeric transgenic loci were used (Figure 3). The P-1152 line carries two copies of the P-lacZ-rosy transgene (P{lArB}) in the Telomeric Associated Sequences (TAS) of the X-chromosome (JOSSE et al. 2007). The P-1152 telomeric locus was the canonical silencer locus used in previous Trans-Silencing Effect studies (ROCHE and RIO 1998; JOSSE et al. 2007; JOSSE et al. 2008; TODESCINI et al. 2010; PÖYHÖNEN et al. 2012). RS3 carries a P-FRT-white transgene in the TAS of the third chromosome (3R arm). It also induces a strong TSE (DUFORT et al. 2014). Thus, BX2 P{lacW} transgenes shares P- element and lacZ sequences with P-1152 and shares P- element and white sequences with RS3. In both cases, a part of the P{lacW} sequence is missing in the telomeric transgenic construct (see Figure 3-A). In order to transmit the cytoplasm, but not the telomeric transgenes, females heterozygous for these telomeric transgenes were generated. We further crossed BX2 males, from a non-paramutated line, with females heterozygous for each or both telomeric transgenes. G₁ females having inherited the BX2 locus, but not the telomeric
transgenes, were recovered and potentially paramutated BX2 lines were established and analyzed for trans-silencing and ovarian piRNA production capacities over generations. Figure 3-B shows that G1 females that carried a paternally-inherited BX2 cluster and recovered cytoplasm containing piRNAs produced by partially homologous transgenes, can show strong or complete silencing capacities. However, the efficiency was not as strong as that of paramutation experiments in which the P{lacW} transgenes of the BX2 paramutated locus inherit piRNAs homologous to the entire transgene length, which always result in a complete and stable paramutation ((DE VANSSAY et al. 2012), data not shown). For the BX2* lines activated by piRNAs homologous to the P-1152 transgenes (called P^{1152}BX2*), two lines (QA5 and QA6) showed very strong silencing capacities after more than 30-50 generations (QA5 is at 100% at each generation tested), which appeared even more stable that those reported in (DE VANSSAY et al. 2012). One line (BB5) exhibited a period of strong silencing capacity followed by a reduction in silencing and one line showed partial silencing capacities (BB6). For the BX2* lines activated by piRNAs homologous to the P-white transgene (called RS3BX2*), one line (QA8) established a long-term strong level of repression, while the other line (BB7-4) showed diminishing repression over time. For the BX2* lines activated by piRNAs homologous to both P-1152 and RS3 transgenes (called P^{1152} + RS3BX2*), the situation resembled that of lines activated by P-1152 cytoplasm, with one line showing complete repression capacity (QA1) and other lines showing intermediate repression levels. An additive effect was not observed since these P^{1152} + RS3BX2* lines did not appear stronger than the P^{1152}BX2* lines despite the fact that small RNAs covering most of the sequence were maternally transmitted in G0. We conclude that paramutation by partially homologous transgenes is globally less efficient than paramutation by fully homologous transgenes. However, piRNAs produced by partially homologous transgenes can induce stable and strong paramutation we call “partially homologous paramutations”.

For each type of G0 maternal inheritance, lines showing strong silencing capacities were chosen and ovarian small RNA production was analyzed in G3, G5 and G10 by deep sequencing (Figures 3-C and S5). Figure 3-C shows that, as early as generation 3, the three types of G0 maternal inheritance resulted in strong production of small RNAs covering the entire BX2 P{lacW} transgene length. Thus, partially homologous paramutations are associated to rapid and stable spreading of piRNA production by non-homologous sequences of the targets, similarly to what was found for target transgenes (MUERDTER et al. 2011; OLOVNIKOV et al. 2013; HAN et al. 2015; MOHN et al. 2015) or transposable elements (SHIPIZ et al. 2014).

Further, the paramutagenicity of P^{1152}BX2*, RS3BX2* and P^{1152} + RS3BX2* females was tested. Figure 4 shows that maternal transmission of cytoplasm produced by these females, combined to
paternal transmission of a BX2 naive locus, resulted in female progeny showing strong trans-silencing properties. These capacities are transmitted over generations, as tested until G_{36}. Thus, paramutation mediated via maternal inheritance of piRNAs homologous to a subregion of the targeted locus can be strong and stable and can generate strongly paramutagenic loci.

**Maternally transmitted small RNAs can affect an adult somatic phenotype.**

piRNAs are maternally transmitted not only to the posterior pole of *Drosophila* embryos but also to more anterior regions where they can be implicated in gene regulation. Indeed, piRNAs homologous to transposable elements (roo, 412) have been implicated in nanos mRNA decay at the anterior part of the embryo by a mechanism involving mRNA deadenylation (ROUGET *et al.* 2010). Furthermore, a high number of maternal RNAs were shown to undergo piRNA dependent destabilization in the soma during maternal to zygotic transition in embryos (BARCKMANN *et al.* 2015). In addition, piRNAs can have an effect in somatic cells of the gonads. Indeed, in *Drosophila simulans*, expression of the tirant transposable element in ovarian follicle cells was shown to be affected by tirant homologous piRNA maternal transmission (AKKOUCHE *et al.* 2013). Finally, maternal depletion of Piwi in *Drosophila melanogaster* impacted white variegation in adult eyes (GU and ELGIN 2013). Therefore, piRNA maternal deposition can impact, not only the germline of progeny, but also somatic tissues. During the course of experiments performed to study paramutation in the germline using the T-1 line as paramutagenic line (DE VANSSAY *et al.* 2012), we detected impact of *P{lacW}* homologous small RNA maternal inheritance on somatic tissues, which we further investigated.

The T-1 paramutagenic line which carries seven *P{lacW}* copies inserted in tandem (DORER and HENIKOFF 1994; DORER and HENIKOFF 1997) was shown to produce abundant ovarian piRNAs including piRNAs homologous to the white transformation marker (DE VANSSAY *et al.* 2012). The white gene is involved in eye pigment transport and its loss of function results in white eyes instead of red ones (Figure 5-B). The T-1 cluster shows strong variegation for the white marker, i.e. stochastic ON-OFF white repression from cell to cell referred to as Repeat Induced Gene Silencing (RIGS) (DORER and HENIKOFF 1994; DORER and HENIKOFF 1997). RIGS is associated to heterochromatin formation on tandem repeats, which includes de novo binding of HP1 at the locus (FANTI *et al.* 2003). In the T-1 line, RIGS-induced variegation was enhanced by X-ray-induced chromosomal rearrangements. We first investigated the potential somatic effects of *P{lacW}* piRNA maternal inheritance by performing reciprocal crosses between T-1/Cy and white mutant individuals.
from different genetic backgrounds and comparing female progeny eyes from these crosses (eye images and pigment amounts). Figure 5-A shows the very clear difference observed between the three backgrounds tested (w<sup>1118</sup>, w<sup>1118</sup> Cy/Xa, yw<sup>c</sup>). Females having maternally inherited the T-1 cluster presented stronger white repression than females having paternally inherited the cluster. Note that in each case the two types of females have strictly the same genotype. The differences were confirmed by analysis of pigment levels (Figure 5-A).

We further tested the effect of P{lacW} homologous piRNA maternal inheritance on the eye phenotype in flies carrying another P{lacW} cluster called DX1. This cluster has six tandem P{lacW} copies, one of which is in opposite orientation to the others (DORER and HENIKOFF 1994; DORER and HENIKOFF 1997). This cluster shows a low level of variegation at 25°C but variegation is stronger at higher temperatures presenting almost a fully white phenotype at 29°C (data not shown). DX1 has no trans-silencing capacities (RONSSERAY et al. 2001; DE VANSSAY et al. 2012). The two reciprocal crosses were first performed between DX1 and w<sup>1118</sup>; Cy/Xa individuals to test for the existence of a maternal effect of DX1 inheritance on its eye phenotype and no significant difference was observed between the two types of progeny for white expression (Figure 5-B). Further DX1 individuals were crossed with T-1 individuals and female progeny having inherited DX1, but not T-1, were analyzed. Figure 5-B shows that DX1 females having maternally inherited T-1 cytoplasm, i.e. containing transgenic small RNAs, presented eyes with a stronger level of white repression than DX1 females having inherited DX1 cytoplasm. Thus, T-1 maternal cytoplasm induced DX1 repression in adult heads. Moreover, females produced by crossing DX1 females and T-1 males showed significantly stronger white repression than those produced by crossing DX1 females and w<sup>1118</sup>; Cy/Xa males. This likely resulted from genetic background effects since no paternal inheritance from T-1 P{lacW} piRNAs was previously detected (DE VANSSAY et al. 2012).

In contrast, the difference observed between the progeny of reciprocal crosses of DX1 and T-1 individuals can be attributed to piRNA maternal transmission since these two types of female progeny have the same genotype. Consequently, a repressive effect of a T-1 cytoplasm which carries a high level of P{lacW} piRNAs can be observed in progeny adult eyes with both the T-1 and DX1 P{lacW} clusters as a read out. To understand this small RNA somatic impact in progeny, it will be necessary to test it in zygotes which are mutants for small RNA silencing pathways or chromatin based-repression pathways.
Discussion

The present results establish the paramutated BX2* locus as a typical piRNA dual strand cluster whose de novo small RNA production involves nuclear and cytoplasmic actors of the piRNA pathway. Among the small RNAs produced by the BX2* locus, the 23-28nt piRNAs, but not the 21nt RNAs, are required for paramutation. Finally, a stable paramutation can be generated between partially homologous loci which are not allelic.

Implication of the primary piRNA pathway in BX2* capacities.

Mutant analysis shows thus that aubergine, rhino, cutoff and zucchini, are required for a paramutated locus to exhibit piRNA production capacities and trans-silencing. Two main molecular models, one nuclear, one cytoplasmic, have been proposed for the paramutation process (DE VANSSAY et al. 2013; LE THOMAS et al. 2014). Considering a cytoplasmic paramutation model, it can be proposed that a trans-generational nuage to nuage transfer of information can be the only factor which explains the acquisition of silencing capacities by the BX2* paramutated locus. It was previously shown by qRT-PCR that the BX2 naive (non-paramutated) locus is transcribed in both orientations (DE VANSSAY et al. 2012). Thus, during paramutation, piRNAs loaded on Aub or Ago3 in the nuage of T-1 or BX2* female nurse cells could be transferred to the nuage of zygotic primordial germ cells (PGC) having paternally inherited a BX2 naive locus, resulting in de novo targeting of BX2 P{lacW} transcripts by the ping-pong machinery and accounting by itself for the acquisition of silencing capacities by the BX2* paramutated locus. In a nuclear paramutation model, maternally transmitted piRNAs, loaded on Piwi, could enter into PGC nuclei and activate de novo targeting of BX2 naive transcript by the primary piRNA pathway via promoting the binding of the Rhino-Deadlock-Cutoff complex on the P{lacW} cluster. This model is suggested by the fact that deposition of Rhino at several piRNA producing loci depends on Piwi (MOHN et al. 2014). It is also strongly supported by ChIP analysis which showed that a BX2* cluster, as tested in G1 ovaries of the paramutation experiment, exhibited an increase in H3K9 methylation levels when compared to a naive BX2 locus (LE THOMAS et al. 2014). H3K9me3 residues can indeed be targeted by the chromodomain of Rhino, resulting in RDC complex stabilization on the cluster. The mutant analysis reported here provides the functional assay which shows that rhino, cutoff and zucchini are necessary for BX2* trans-silencing and piRNA production capacities (Figure 1). Therefore, paramutation-linked repressive properties do not solely depend on cytoplasmic ping-pong activation resulting from nuage to nuage trans-generational transfer of information. These properties depend
also on the primary piRNA pathway which is linked to the binding of the RDC complex on the locus (MOHN et al. 2014; ZHANG et al. 2014). Analysis focused on the T-1 paramutagenic locus, which produces a high level of ovarian P[lacW] piRNAs, strongly reinforces this model (LE THOMAS et al. 2014). Indeed, ChIP analysis performed on ovaries of G₁ females produced by the two reciprocal crosses between T-1 and wild type (devoid of transgene) individuals showed that maternal inheritance of the T-1 cytoplasm is associated with an increase of Rhino and Cutoff binding on the T-1 P[lacW] cluster in progeny ovaries (LE THOMAS et al. 2014). Paramutation associated repressive properties also depend on the Zuc and on Aub activities (Figure 1) which take place in the nuage. In conclusion, chromatin analyses (LE THOMAS et al. 2014) and functional analyses performed with mutants (Figure 1) complement each other to show that trans-generational induction of piRNA-mediated silencing by maternally inherited piRNAs involves nuclear and cytoplasmic steps of the piRNA pathway, including those involved in the atypic transcription of the piRNA loci. Thus, during paramutation, the paramutated locus is modified and remains so over generations.

**BX2* transgenic 21nt RNAs are actual siRNAs whose production also depends on the piRNA machinery.**

Deep sequencing of ovarian small RNAs associated to both telomeric P-transgene insertions and to the T-1 paramutagenic or BX2* paramutated clusters showed abundant 21nt transgene homologous RNAs, in addition to 23-28nt piRNAs (DE VANSSAY et al. 2012). Both sense and antisense 21nt RNAs were found which contained no ping-pong signatures. These 21nt RNAs were proposed to be Dicer-2 dependent. Here, ovarian small RNAs of BX2*, Dcr-2⁻/⁻ female have been analyzed by deep sequencing. As expected, BX2* 21nt RNAs were almost absent in these Dcr-2 mutant females showing that they are genuine siRNAs (Figure 1). Strikingly, all the piRNA pathway mutations tested also resulted in the loss of the transgene-derived 21nt RNAs (Figure 1). This suggests that P[lacW] siRNAs could be generated downstream of the piRNA pathway. It was shown further that production of these transgene-derived siRNAs by a female requires piRNA, but not siRNA, maternal inheritance, confirming that their appearance is downstream of piRNAs (Figure 2). 21nt small RNA production appears to be a common feature for a number of piRNA-producing loci (CZECH et al. 2008; MALONE et al. 2009; PANE et al. 2011). cuff mutant analysis was shown to strongly affect 21nt RNA production by a number of double-strand piRNA-producing loci including the 42AB locus (PANE et al. 2011; LE THOMAS et al. 2014). What mechanism could
explain the 21nt RNA dependence on the piRNA pathway? P{lacW} homologous siRNA biogenesis depends on all the piRNA actors tested including for example Rhino which is necessary for production of piRNA precursors, Zucchini which is responsible for slicing steps in the cytoplasm and Aubergine which is an actor of the ping-pong amplification of the pool of piRNA. One possibility is that P{lacW} siRNA production results from the fact that both sense and anti-sense RNAs, produced by this dual-strand cluster, are concentrated locally due to the piRNA machinery, for example in the nuage, leading to occurrence of double-strand RNAs which can further be processed by Dcr-2. The piRNA mutants tested could alter the production of the piRNAs associated with Piwi, which are produced downstream of the ping-pong amplification step (HAN et al. 2015; MOHN et al. 2015), and consequently reduce a positive feedback in the nucleus of these piRNAs (MOHN et al. 2014). This would reduce deposition of Rhino at several piRNA producing loci (MOHN et al. 2014) and thus affect dual-strand transcription of the BX2 locus, abolish the sense and anti-sense RNA concentration step, and siRNA production as well. However, a low level of P{lacW} 21nt RNA is still detectable in Dcr-2 homozygous mutant females (Figure 1-F) which present a ping-pong signature (Figure S3-J). If we analyse small RNAs produced by the 42AB locus, which is a typical dual strand cluster piRNA locus, piRNAs are reduced or disappear in all piRNA pathway mutant backgrounds tested (aub−/−, rhi−/−, cuff−/−, zuc−−/− - Figure S1 - D, H, J, L). 21nt RNAs with a ping-pong signature also disappear in these mutant backgrounds for the 42AB locus (Figure S3 - H, P, T, X). An intriguing variation is observed in the zuc−/− background (Figure S1-L), which showed reduction in piRNA levels and a dramatic increase in 21nt RNA levels for the 42AB locus. This suggests that in the absence of Zuc, precursor transcripts coming from double stranded piRNA clusters can be processed into 21nt RNA instead of piRNAs. This could be achieved by Dcr-2 since this enzyme is responsible for the presence of the majority of the 21nt RNAs coming from piRNA clusters (see above) and these 21nt RNAs show no ping-pong signature (Figure S3-X). Thus, for a given locus, two 21nt RNA populations coexist in the Drosophila germline: a high level of Dcr-2 dependent siRNAs showing no ping-pong signature and a low level of Dcr-2 independent 21nt piRNAs showing a ping-pong signature.

**Spreading of the piRNA production capacity within a piRNA-producing cluster can be completed within 3 generations.**

piRNA clusters behave as genomic traps in which new insertions of TEs lead to de novo production of piRNA homologous to the new insertions. The kinetics of appearance of this new
piRNA production is not clear, but some functional evidence has been provided from previous studies concerning the repression capacity inheritance of the I factor and of the P element (Kidwell et al. 1977; Bucheton 1978; Engels W 1979). In both systems, it appears that establishment of repressive capacities, following paternal inheritance of I or P elements carrying genomes in a cytoplasm devoid of I or P homologous piRNAs, is progressive and require several generations to be completed (Engels W 1979; Bregliano et al. 1980; Engels 1989; Finnegan 1989; Rio 2002). For example, experiments were performed with a line (Lk-P(1A)) carrying only two regulatory P elements inserted in a telomeric strong piRNA-producing locus (Telomeric Associated Sequences, TAS) (Coen et al. 1994; Ronsseray et al. 1996). It was shown that paternal transmission of the telomeric P-element locus in a cytoplasm devoid of P-homologous piRNAs, followed by maternal inheritance of the telomeric P elements in successive generations, requires four generations to progressively establish complete P-repression (Coen et al. 1994). Thus, during these generations, a progressive spreading must occur within the TAS locus for piRNA production capacity which finally results in strong production of piRNAs homologous, not only to the TAS, by also to the P-element. A spreading process for piRNA production was also observed for the flamenco piRNA-producing locus which functions in the somatic follicle cells which surround germline cysts (Muerdter et al. 2011). Further, analysis of previously-established transgenic lines which carry an I fragment within a P transgene have shown that piRNA production capacity can spread within transgenes (Olovnikov et al. 2013). The capacity to produce piRNAs invaded the whole I-derived transgene and reached in some cases flanking sequences. A similar spreading process into flanking sequences was also found for natural transposon insertions (Shpiz et al. 2014). It is however possible that spreading processes inside or outside a transgene or a TE involve different mechanisms. However, in these last experiments, the capacity of targeted sequences to maintain piRNA production on their own, in absence of the endogenous homologous piRNA locus which stimulated them, was not tested. In this paper, we show that cis-spreading of piRNA production on BX2* is maintained over generations in absence of the original input, the telomeric transgenic locus. In addition, this spreading is completed within a few generations.

What mechanism could responsible for the spreading process? Very recent studies have shown that piRNAs associated to Piwi can be produced downstream of piRNA generation by ping-pong amplification which can increase piRNA sequence diversity (Han et al. 2015; Mohn et al. 2015). Indeed, piRNAs associated to Ago3 can target complementary RNA and induce production of phased piRNAs (27nt) corresponding to the slicing of the 3’ tail of the cleaved target RNAs. These phased “trailer” piRNA, once loaded on Piwi, can potentially return to the nucleus and
increase both transcriptional repression of homologous targeted TEs or stimulate binding of Rhino on the piRNA-producing locus. One consequence of such an additional mechanism would be the adjustment of the force of repression to the level of transcription of active copies. Another would be the formation of novel piRNAs which are cis to the sequence targeted initially by piRNAs. This phenomenon would explain why a P-lacZ-white transcript targeted by P-lacZ homologous piRNAs is able to produce piRNAs homologous to white. However, some observations remain to be explained. First, according to this model, when telomeric P-insertions are paternally introduced in a lineage and thereupon are maternally transmitted, establishment of repression would be expected to require only one generation to be completed but in fact it was shown to be progressive over generations (COEN et al. 1994). It is possible that nuclear reinforcement mediated by trailer piRNAs loaded on Piwi on the piRNA producing locus is necessary, and this reinforcement involves maternal transmission to be efficient and thus requires more than one generation to be completed. Second, we performed an additional paramutation experiment in which a paternally-inherited BX2 locus was introduced in a maternal cytoplasm produced by Lk-P(1A) females. In that experiment, no paramutation of the paternally-transmitted BX2 cluster was observed (Figure S6) despite maternal inheritance of P-element homologous piRNAs corresponding to 820bp of homology out of the 10691bp of the P{lacW} transgene. It remains however possible that production of such trailer piRNAs is limited by the propagation mechanism itself making it possible to fill a gap of 4 kb (Figure 3) but not a gap of almost 10 kb (Figure S6).

**Role of paramutation in TE repression establishment and stabilization in natural populations.**

The *Drosophila* genome contains more than 140 piRNA-producing loci, most of them composed of truncated versions of TEs (BRENNECKE et al. 2007). How have these loci been activated in natural populations to produce piRNAs? Paramutation may have played a key role in such process. Once activated in a single fly by an unknown factor, which could be environmental (SCHOTT et al. 2014), a first piRNA-producing locus may have progressively replaced the inactive allele within the population by a paramutation process similar to paramutation of a BX2 naive locus by a BX2* locus (DE VANSAY et al. 2012). Further, the first locus activated may have activated other TE-accumulating loci, despite showing only partial homology and being located on a different chromosome, by a paramutation process similar to that of BX2 by P-1152 or RS3 (Figure 3). These secondarily-activated sites could maintain TE repression capacities in individuals from the population which did not inherit the first active piRNA locus if this first locus is not fixed in the population. With time, epigenetic communication between TE-accumulating loci may activate all
“genetically” competent loci (sufficient number of tandem repeats, bidirectional transcription…). Moreover, these partially homologous paramutations could also have played a role during migration processes between natural populations. Indeed, a fly bearing active loci arriving from one population into another one could activate loci present on the chromosomes of the recipient population, despite the existence of potential partial divergences between TE-accumulating loci in these different populations. Therefore, it is possible to propose that paramutation may play an important role in epigenome shaping of the Drosophila germline, especially because it can occur even when interacting loci partially diverge in their sequence.

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Literature cited


**Figure Legends:**

Figure 1. Effect of mutations affecting the piRNA or siRNA pathways on trans-silencing and small RNA production capacities of a BX2* cluster

**Upper box:** Mating scheme used to analyse the effect of mutations (mut) affecting aubergine, Dicer-2, rhino, cutoff and zucchini on BX2* ovarian small RNA production and trans-silencing capacities. As the BX2 cluster, all genes are located on chromosome 2. **(Left)** Heterozygous BX2* aub\(^{−}\), BX2* Dcr-2\(^{−}\), BX2* rhi\(^{−}\), BX2* cuff\(^{−}\) or BX2* zuc\(^{−}\) females were crossed with heterozygous males carrying a mutant allele of the same gene to generate either heterozygous control or loss of function females for deep sequencing of ovarian small RNAs. **(Right)** The same heterozygous females were crossed with heterozygous males carrying a mutant allele of the same gene plus a P-\(lacZ\) target transgene (BQ16) to measure the effect of the mutation tested on BX2* trans-silencing capacities (TSE %). TSE was quantified by determining the percentage of egg chambers with no \(lacZ\) expression in the germline. In the genotypes presented, maternally-inherited chromosomes (chromosomes 2 and 3) are indicated above the bar. Cy is a balancer chromosome carrying a dominant phenotypic marker. **(A-L)** In each case, the genotype tested is indicated, and the percentage of TSE is given below the genotype with the total number of egg chambers assayed in parenthesis. Non-paramutated BX2 ("naive") and BX2* at generation 83 were analyzed as controls. Histograms show the length distributions of ovarian small RNAs matching the \(P\{lacW\}\) locus. Positive and negative values correspond to sense and antisense reads, respectively. Plots show the abundance of 19-29nt small RNAs matching the \(P\{lacW\}\) locus. Analysis of the effect of aub, rhi, cuff and zuc mutations shows that BX2* silencing capacities, ovarian \(P\{lacW\}\) 23-28nt piRNA, but also 21nt RNA, production depend on the piRNA pathway. Dcr-2 loss of function strongly affects production of \(P\{lacW\}\) homologous 21nt RNAs without affecting that of 23-28nt RNAs, nor trans-silencing capacities. \(P\{lacW\}\) 21nt are not necessary for the maintenance of the paramutated state and their biogenesis depends on both Dcr-2 and the piRNA pathway. TSE results for Dcr-2 mutants (E-F) are reprinted from (De Vanssay et al. 2012).
Figure 2. Paramutation by a cytoplasm devoid of siRNAs

(A) BX2* Dcr-2^-/- females were crossed to BX2 Dcr-2^+/+ (naive) males to generate G1 females having paternally inherited a BX2 locus and maternally inherited piRNAs but no 21nt siRNAs homologous to the P{lacW} cluster. In addition these G1 females paternally inherited a Dcr-2^+ allele. These G1 females were crossed with Dcr-2^+ males to establish a BX2* Dcr-2^-/+ Dcr-2^+ line in which no maternal P{lacW} siRNAs have been initially introduced in G0. Cy is a balancer chromosome carrying a dominant phenotypic marker. Maternal chromosomal complement is written above the bar. (B) At generations 0, 1 and 2, deep sequencing of ovarian small RNAs was performed and silencing capacity was controlled in parallel by crossing females with males carrying the BQ16 target transgene and scoring TSE in female progeny. The genotype tested is indicated and TSE percentages and ovarian 19-29nt RNAs are presented as in Figure 1. As expected, G1 and G2 females show complete silencing capacities. The same was true for successive Gn generations (G6, TSE= 100%, n=450; G10, TSE= 100%, n=1100). Despite the lack of maternal P{lacW} siRNA maternal transmission in G0, such ovarian small RNAs appear already in G1 females and increase in G2 in BX2* Dcr-2^-/+ females (dashed arrow). Therefore, maternal inheritance of P{lacW} homologous piRNAs, but no siRNAs, results in a strong and stable paramutated BX2* locus and in immediate production of both P{lacW} piRNAs and siRNAs in females carrying a Dcr2^+ allele.

Figure 3. Paramutation of a BX2 locus by partially homologous piRNAs loci

BX2 males carrying a P{lacW} naive cluster were crossed to females carrying partially homologous P-transgenes inserted in telomeric piRNA producing loci at the heterozygous state. Female progeny were recovered which carry the BX2 locus but did not carry the telomeric transgenes. These G1 females inherited a cytoplasm carrying piRNA homologous to the telomeric transgenes, which therefore cover only partially the BX2 P{lacW} transgene sequence. Lines were established and the trans-silencing capacities of these putative BX2* lines were tested in subsequent generations, as in Figures 1 and 2. In addition, deep sequencing of ovarian small RNAs was performed in G3, G5 and G10. (A-left and middle) Structure of the P-1152 and RS3 telomeric P-transgenes used and of the BX2 P{lacW} transgenes. Colored thick lines below the transgenes indicate the sequences shared by the P{lacW} and telomeric transgenes. The P-1152 line carries two P-lacZ-rosy (P{lArB}) transgenes inserted in the Telomeric Associated Sequences (TAS) of the X-chromosome. RS3 carries a P-FRT-white transgene in the TAS of the 3R chromosomal arm. These two lines induce a
strong TSE. *(A-right)* Ovarian piRNA production in females that carry a maternally-inherited P-1152 or RS3 locus at the heterozygous state (G₁ females from crosses between P-1152 and RS3 females with males devoid of transgene) is mapped on the P{lacW} transgene. *(B)* Three types of replicate BX2* sublines were generated. Sublines which inherited in G₁: 1- a cytoplasm from females heterozygous for the P-1152 locus (P⁻¹¹⁵²BX2 sublines, in orange); 2- a cytoplasm from females heterozygous for the RS3 locus (RS³BX2 sublines, in green); 3- a cytoplasm from females heterozygous for both the P-1152 and RS3 loci (P⁻¹¹⁵²⁺RS³BX2 sublines, in purple). *Trans*-silencing capacities of various BX2* lines are shown with regard to generations (TSE%, n>500 in all assays). Names of replicate sublines are given below the graphs. *(C)* Deep sequencing of ovarian small RNAs in various BX2* sublines at generations 3, 5 and 10. Plots show the abundance of 19-29nt small RNAs matching P{lacW}. TSE assays show that silencing capacities vary among replicate BX2* lines but strong and stable paramutated lines can be recovered with the three types of G₀ cytoplasmic inheritance (B). Ovarian small RNAs were analyzed for a subset of the BX2* lines showing strong silencing capacities. The name of the line is indicated on the left of the graph. Deep sequencing analysis shows that piRNAs corresponding to the whole length of P{lacW} can be produced as early as generation 3 (C), showing that piRNA production by a paramutated locus can rapidly extend to the part of the locus which did not originally receive maternally-inherited piRNAs.

**Figure 4. Paramutagenicity of a partially homologous paramutation**

BX2* lines having, at G₀, maternally inherited piRNA homologous to a part of the BX2 P{lacW} transgene sequence, and showing strong silencing capacities at the 20th generation (Figure 3), were tested for their ability to *de novo* paramutate a naive BX2 locus. *(A)* The name of the line is indicated beside the G₀ female genotype. Maternal chromosomal complement is written above the bar. *Cy* and *CyRoi* are balancer chromosomes devoid of transgenes and carrying different dominant markers. These P⁻¹¹⁵²BX2*, RS³BX2* and P⁻¹¹⁵²⁺RS³BX2* females were crossed with males carrying a BX2 naive cluster and G₁ progeny having paternally, but not maternally, inherited a BX2 cluster were crossed to establish lines. These lines carry a second order paramutated locus. *(B)* These lines were subsequently tested for *trans*-silencing capacities at generations 1, 4 and 36. The TSE percentage is indicated with the number of egg chambers counted in parenthesis. Strong silencing capacities are observed over generations showing that P⁻¹¹⁵²BX2*, RS³BX2* and P⁻¹¹⁵²⁺RS³BX2* are strongly paramutagenic.

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09 October 2015
Figure 5. Somatic impact of maternally transmitted small RNAs

The effect of $P\{lacW\}$ small RNA maternal inheritance on $P\{lacW\}$ cluster expression in adult eyes was tested using two different transgene clusters, T-1 (A) and DX1 (B), as a read-out. T-1, but not DX1, has trans-silencing capacities, correlated with the presence of abundant ovarian piRNAs. The G0 cross is indicated above the picture and was performed at 25°C. Green female symbol indicates cytoplasmic inheritance containing $P\{lacW\}$ piRNAs. The genotype (chromosome 2) of the G1 females analyzed is given below the picture. Cy and Xa are chromosomes devoid of transgenes and carrying dominant markers. The maternally-inherited chromosome is written above the bar. (A and B) Pigment levels (absorbance at 480nm) are shown beside the pictures. Numbers in abscissa indicate the genotype analyzed (reported directly on the head picture). For pigment level dosages, Canton$^\text{y}$ and $w^{1118}$ female heads served as WT and $w$ null mutant controls respectively. Control pictures are boxed in B. Bars indicate the standard error. Comparisons were performed using the Student’s $t$-test: (*) p<0.05; (**) p<0.01; (***) p<0.001. Maternal inheritance of $P\{lacW\}$ small RNAs produced by a T-1 female results in increased silencing of both T-1 and DX1 expression in the eye.
Fig. 1
Fig. 1 continued
A

\[ \begin{align*}
G_0 & \quad \frac{BX2^* \text{ Dcr-2}^-}{\text{Dcr-2}^-} \times \frac{BX2^\text{naive} \text{ Dcr-2}^+}{\text{Cy} \text{ Dcr-2}^+} \\
G_1 & \quad \frac{\text{Dcr-2}^-}{BX2^* \text{ Dcr-2}^+} \times \frac{\text{Cy} \text{ Dcr-2}^+}{\text{Dcr-2}^+} \\
G_2 & \quad \frac{BX2^* \text{ Dcr-2}^+}{\text{Cy} \text{ Dcr-2}^+} \\
G_n & \quad \frac{BX2^* \text{ Dcr-2}^+}{\text{Cy} \text{ Dcr-2}^+}
\end{align*} \]

B

\[ \begin{align*}
BX2^*, \text{ Dcr-2}^-/- \quad (G_0) \\
\text{TSE} = 100\% \quad (n=1150)
\end{align*} \]

\[ \begin{align*}
BX2^*, \text{ Dcr-2}^-/+ \quad (G_1) \\
\text{TSE} = 100\% \quad (n=500)
\end{align*} \]

\[ \begin{align*}
BX2^*, \text{ Dcr-2}^+/+ \quad (G_2) \\
\text{TSE} = 100\% \quad (n=800)
\end{align*} \]

Fig. 2
**A**

**P-1152**
Telomeric transgene (chr. X)

![Diagram](image)

Maternal piRNA transmission

**RS3**
Telomeric transgene (chr. 3)

![Diagram](image)

**BX2**
Transgene cluster (chr. 2)

![Diagram](image)

**B**

![Diagram](image)

**C**

![Diagram](image)

Fig. 3
A

\[
\begin{align*}
G_0 & \quad \text{♀} \quad + \quad \frac{P-1152\text{BX2}^*}{Cy} \quad + \quad (QA5) \\
& \quad \text{♂} \quad + \quad \frac{RS3\text{BX2}^*}{Cy} \quad + \quad (QA8) \\
& \text{♀} \quad + \quad \frac{P-1152+RS3\text{BX2}^*}{Cy} \quad + \quad (QA1)
\end{align*}
\]

\[X \quad \text{♂} \quad \overset{\Rightarrow}{\frac{BX2^\text{naive}}{CyRoi}} \quad +
\]

\[
\begin{align*}
G_1 & \quad \text{♀} \quad + \quad \frac{Cy}{BX2^*} \quad + \\
& \quad \text{♂} \quad + \quad \frac{BX2^*}{Cy} \quad + \\
G_n & \quad \text{♀} \quad + \quad \frac{BX2^*}{Cy} \quad +
\end{align*}
\]

B

<table>
<thead>
<tr>
<th></th>
<th>TSE percentage</th>
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<tbody>
<tr>
<td>(QA5\text{BX2}^*)</td>
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<tr>
<td>(G_1)</td>
<td>100% (n=950)</td>
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<tr>
<td>(G_4)</td>
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<td>(G_{36})</td>
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<td>(QA8\text{BX2}^*)</td>
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<td>(G_1)</td>
<td>100% (n=1025)</td>
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<td>(G_4)</td>
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<td>(G_{36})</td>
<td>95.0% (n=1300)</td>
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<tr>
<td>(QA1\text{BX2}^*)</td>
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<tr>
<td>(G_1)</td>
<td>100% (n=800)</td>
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<tr>
<td>(G_4)</td>
<td>100% (n=1175)</td>
</tr>
<tr>
<td>(G_{36})</td>
<td>92.1% (n=1275)</td>
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</tbody>
</table>

Fig. 4
Fig. 5