Paramutation phenomena in non-vertebrate animals

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ABSTRACT

Paramutation was initially described in maize and was defined 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 [1,2]. Thus it implies that the paramutated allele conserves its new properties on the long term over generations even in the absence of the paramutagenic allele and that it turns paramutagenic itself, without undergoing any changes in the DNA sequence. Some epigenetic interactions have been described in two non-vertebrate animal models, which appear to exhibit similar properties. Both systems are linked to *trans*-generational transmission of non-coding small RNAs. In *Drosophila melanogaster*, paramutation is correlated with transmission of PIWI-Interacting RNAs (piRNAs), a class of small non-coding RNAs that repress mobile DNA in the germline. A tandem repeated transgenic locus producing abundant ovarian piRNAs can activate piRNA production and associated homology-dependent silencing at a locus that was previously stably devoid of such capacities. The newly converted locus is then perfectly stable in absence of the inducer locus (>100 generations) and becomes fully paramutagenic. In *Caenorhabditis elegans*, paramutation is correlated with transmission of siRNAs, which are produced by transgenes targeted by piRNAs in the germline. Indeed, a transgenic locus, targeted by the piRNA machinery, produces siRNAs that can induce silencing of homologous transgenes, which can be further transmitted in a repressed state over generations despite the absence of the inducer transgenic locus. As in fly, the paramutated locus can become fully paramutagenic, and paramutation can be mediated by cytoplasmic inheritance without transmission of the paramutagenic locus itself. Nevertheless, in contrast to flies where the induction is only maternally inherited, both parents can transmit it in worms. In addition, a reciprocal phenomenon – (from *off* toward *on*) – appears to be also possible in worms as some activated transgenes can reactivate silent transgenes in the germline, and this modification can also be transmitted to next generations, even so it appears to be only partially stable. Thus, in a given system, opposite paramutation-like phenomena could exist, mediated by antagonist active pathways. As in plants, paramutation in flies and worms correlates with chromatin structure modification of the paramutated locus. In flies, inheritance of small RNAs from one generation to the next transmits a memory mainly targeting loci for repression whereas in worms, small RNAs can target loci either for repression or expression. Nevertheless, in the two species, paramutation can play an important role in the epigenome establishment.

1- Introduction

Various strategies are used to recognize and repress mobile or foreign DNAs in genomes and to epigenetically transfer silencing memory over generations. In fly, transgenerational information seems to ensure predominantly gene repression. Indeed, systems based on the principle of genomic traps have been established in order to identify and further repress Transposable Elements (TEs). The *Drosophila melanogaster* genome contains about 140 discrete loci composed of TE fragments which undergo non-canonical transcription. This results in the inactivation of the transcript of the locus itself and of all the homologous transcripts produced by the genome [3-6]. Therefore, this system based on the PIWI-Interacting RNAs (piRNAs) results in repression of all TE copies in the genome if at least one copy has inserted into a piRNA-producing locus. Euchromatic TE repression occurs at both the transcriptional and post-transcriptional levels [7-10]. Thus, when a TE copy is active, it will move and insert into a piRNA-producing locus and this "piRNA locus" copy will progressively establish repression of the entire TE family: the family gets trapped. An important functional aspect is that production of piRNAs by piRNA loci requires maternal transmission of piRNAs both for repressing TE activity in the embryo germline and for stimulating piRNA production by the piRNA loci in the adult gonads [11,12]. Thus, in flies, a catalog of potentially dangerous sequences is transmitted from one generation to the other *via* cytoplasmic inheritance of piRNAs within the embryo. Worms also have a piRNA machinery but the strategy of transgenerational inheritance appears to be different. Indeed, two large clusters of piRNA loci produce thousands of different piRNAs that associate with the PIWI protein PRG-1 and target many sequences, as mismatches between piRNAs and their targets are tolerated [13,14]. When a sequence is targeted by piRNAs, the signal is amplified by an RNA-dependent RNA Polymerase (RdRP) and further relayed to a small interfering RNA pathway based on double strand RNA slicing performed by WAGO proteins [14-18]. Once this silencing is established, the piRNA machinery is no longer necessary and the information can be maintained in further generations solely by the WAGO protein pathway. The piRNA machinery has thus a crucial role for the establishment of silencing but not for its maintenance. To avoid too extensive, unspecific attack of transcripts by piRNAs, an active pathway exists, linked to production of small RNAs associated with a protein called CSR-1, which counteracts the effect of the piRNAs and consequently licenses transcripts for expression [16,19]. This CSR-1-associated RNAs are also transmitted from one generation to the other and allow transgenerational information of transcripts that have to be protected from degradation by piRNAs loaded onto PRG-1 [19].

It appears that in fly, one generation epigenetically transmits to the next a list of sequences to be repressed, whereas worms transmit information about both, correct repression or expression [20]. In both species, these systems were investigated using transgenes, and transgenic loci can exist at a given locus in opposite epigenetic states. In fly, clusters of transgenes can be stably maintained over generations, in either a quiescent or active state for production of abundant amounts of transgene-homologous piRNAs that establish *trans*-silencing of homologous transgene in the germline [21,22]. In worm, the same transgene can exist in an active or repressed state for their expression depending on the line [16]. The key point is that in both species, epigenetic conversion processes can occur in the germline between repressed and active loci, which can further be transmitted to the next generations in the absence of the converting locus. It results in a paramutation like-phenomenon, resembling this classical epigenetic conversion as observed in maize [1,2,23,24]. This review will describe properties of these epigenetic conversion processes and highlight the parallels and differences between the two models.

2- *Drosophila* paramutation-like conversion

The piRNA pathway was discovered in fly in 2006-2007 [3,4,25] and functionally validated with the use of genomic sites capable of transposon repression [11]. Indeed, two TEs invaded the genome of natural *D. melanogaster* populations in the 20th century, the *P* element (a DNA transposon) [26] and the *I* factor (a LINE retroelement) [27]. For the two TEs, it was shown that the cross of females devoid of TE copies (from lines collected before the invasion) with males carrying numerous copies of the TE (from lines collected after the invasion) produces progeny showing a syndrome of genetic abnormalities in the germline called hybrid dysgenesis (high mutation rate, chromosomal breakages, thermo-sensitive sterility) [28,29]. Cytoplasmic inheritance was shown to play a key role as reciprocal crosses (TE-bearing females x TE-devoid males) produced progeny without dysgenesis [29-32]. Thus cytoplasm from females devoid of *P* or *I* elements was therefore missing "something" [33-35], later shown to be *P*- or *I*-homologous piRNAs [11,36]. For the *P* element, a master locus for repressing hybrid dysgenesis was identified at the telomere of the *X* chromosome [37-40], within subtelomeric heterochromatin called Telomeric Associated Sequences (TAS) [41]. Such telomeric heterochromatin was shown to produce abundant ovarian piRNAs [3]. In addition, *P* copies inserted in TAS were also shown to produce abundant ovarian piRNAs [11,21,42]. A transgenic system based on *P*-derived sequences located in TAS was developed and used to study the phenotypic and genetic properties of piRNA-mediated repression in the germline.

In this system, called *Trans*-Silencing Effect (TSE), a *P*-transgene located in TAS can repress a homologous transgene in the female germline [12,43,44]. Inheritance of this repressive capacity shows both a maternal effect and a partial persistence of the maternal effect over up to 6 generations [12]. TSE studies and a mutant approach allowed to confirm that all the piRNA genes tested were necessary for the *trans*-silencing capacities of telomeric *P* insertions [12,43,45]. Finally, the use of TSE also allowed to discover that some other structures in the genome can establish piRNA-mediated repression [22]. Such studies revealed puzzling situations as the two lines *T-1* and *BX2* carrying a similar cluster of *P-lacZ-white* transgenes exhibit different properties. Indeed the *T-1* induces a strong TSE whereas *BX2* showed no repression capacity [22]. These *P-lacZ-white* clusters show stochastic *on-off* repression of the *white* marker in the eye (called variegation). This variegation results from Repeat Induced Gene Silencing (RIGS) [46,47] and is associated with the local binding of Heterochromatin Protein 1 (HP1) at the cluster, as tested by immuno-staining of salivary gland polytene chromosomes [48]. *T-1* has the same cluster than *BX2* but carries chromosomal inversions and translocations induced by X-ray treatment. The *trans*-silencing capacities of *T-1* and *BX2* appeared stable over more than one decade for the two lines. This allowed investigating possible factors that could activate piRNA production by the quiescent *BX2* locus.

It was then tested if maternal inheritance of *P-lacZ-white* homologous piRNAs could *de novo* activate the production of piRNAs from an initially quiescent *BX2* locus [21,49]. *T-1* heterozygous females were crossed with *BX2* males. Female progeny having inherited the *BX2* locus and a *T-1* cytoplasm, but not the *T-1* locus, produced ovarian *P-lacZ-white* piRNAs and induced a complete *trans*-silencing (**Figure 1**). The phenomenon showed complete penetrance in each experiment. We called *BX2** these paramutated *BX2* females by contrast to the initial "*BX2* naïve" (non-paramutated) flies producing no *P-lacZ-white* piRNAs. *BX2** lines were established and further studied for their capacity to induce TSE and produce piRNAs. Again all the tested lines (more than 20) exhibited stable repression. One line was investigated for very long terms: it still induced complete TSE after more than 120 generations, and ovarian *P-lacZ-white* piRNA production was confirmed at G_{42} and G_{83}. It was also tested if the *BX2** line is paramutagenic by crossing *BX2** females at G_{42} with *BX2* naive males. The progeny (called *BX2***) having inherited a paternal - but not maternal - *BX2* locus showed complete TSE capacity and produced abundant *P-lacZ-white* piRNAs in ovaries (Figure 1). *BX2** females are thus fully paramutagenic. Similarly, *BX2*** females are also completely paramutagenic. Therefore, epigenetic conversion occurs between *BX2**

and *BX2* naive loci; the change is heritable, and the process can be repeated. This epigenetic conversion presents the properties of a paramutation which is mediated *via* cytoplasmic inheritance: this excludes any role of pairing between the loci in the paramutation process. As control, *T-1* males were crossed to *BX2* females, and the progeny having inherited the *BX2* locus did not show any *trans*-silencing capacities. Resulting flies carrying the *BX2* loci failed to show silencing capacities in further generations (our lab, unpublished). Further, the paramutation process can be erased by crossing *BX2** males with females that do not carry any *P-lacZ-white* transgene. This clearance is stable for over than 50 generations (our lab, unpublished). To exclude the hypothesis that an autoreproductive cytoplasmic component would be sufficient to induce paramutation, we also have performed a "passage à vide" (empty step) experiment [49]: *BX2** females were crossed with males devoid of *P-lacZ-white* transgene and G_1 females which did not inherit the *BX2** cluster were crossed with *BX2* naïve males in order to test if their cytoplasm can induce paramutation. G_2 females, whose grand-mother produced *P-lacZ-white* piRNAs, but whose mother were unable to maintain such production due to the lack of *P-lacZ-white* transgene, showed no repression capacities confirming that this paramutation does not result from an autoreplicative cytoplasmic component.

*BX2** females produce not only abundant *P-lacZ-white* 23-28nt piRNAs but also abundant 21nt RNAs [21]. We have tested the effect of a homozygous mutation of *Dicer-2*, involved in the siRNA pathway on *BX2** properties. The *trans*-silencing capacities were unaffected by a *Dicer-2* loss of function, even after 50 generations in a homozygous mutant context. We have also tested the effect of a heteroallelic mutant context for the PIWI family gene *aubergine* on *BX2** females: silencing capacities were completely abolished in such mutant females, showing that paramutation requires piRNAs but not siRNAs.

What is the molecular mechanism of paramutation linked to piRNAs in *Drosophila*? It can be assumed that maternally transmitted piRNAs are incorporated in Primordial Germ Cells (PGCs) in embryos and will potentiate piRNA production by homologous loci [49,50]. One possibility is that piRNAs associated with the Piwi protein reach the PGC nuclei and interact with the nascent transcripts on the piRNA locus to promote the binding of the Rhino protein, a member of the Heterochromatin Protein 1 (HP1) subfamily of chromo box proteins [51], which was shown to have a causal role on piRNA production [6]. Indeed, PIWI loaded with a piRNA has the capacity to enter the nucleus. Such hypothesis is supported by chromatin immunoprecipitation (ChIP) experiments performed on *BX2** *vs BX2* naive ovaries showing an enrichment of posttranslational methylation of

histone H3 (H3K9me3) on the cluster when the locus is paramutated [50]. H3K9me3 residues can indeed be targeted by the chromodomain of Rhino, resulting in Rhino-associated protein complex stabilization on the cluster. Alternatively, piRNAs loaded on the PIWI proteins Aubergine and Ago3 can also be transmitted to PGC in embryos where they could stimulate the amplification step of the piRNA biogenesis, which occurs in the nuage, a peri-nuclear diffuse structure surrounding the germline cell nucleus and enriched in PIWI proteins. In both models, paramutation would be induced by a diffusible cytoplasmic product, the piRNAs.

3- Worm paramutation-like conversions

Transgenesis in worms showed early on that not all single copy transgenes were expressed in the germline and that failure of expression appeared to be linked to the length of foreign DNA present in the transgene construct. Repression of the transgene occurred at both the post-transcriptional and transcriptional levels and is associated with H3K9me3 enrichment present on the silenced transgene [16]. The cross of lines carrying expressed copies with lines carrying repressed ones surprisingly showed that, in a number of cases, the repressed state dominates in the progeny **(Figure 2)**. For example, the cross of hermaphrodites carrying a *gfp::csr-1* repressed transgene with males carrying an expressed *gfp::csr-1* transgene produces progeny in which both transgenes are repressed in the germline (**Figure 2-A**) [16]. The analysis of succeeding generations further revealed that the repressed state of both transgenes is maintained. The same dominance and conversion process was observed between repressed and active *gfp::csr-1* transgenes located on different chromosomes (**Figure 2-B**). Furthermore, the newly repressed transgene conserved its properties over generations, in absence of the initial silencer transgene. Such conversion phenomenon can be mediated by passage of the silenced copy through both parents but it is less efficient *via* male transmission [16]. In these experiments, the initial repressed transgene behaved as a paramutagenic locus and the newly repressed transgene, appearing stable for at least 10 generations in absence of the inducer locus, behaved as a paramutated locus. This silencing conversion was termed *RNA-induced epigenetic Silencing* (**RNAe**) [15,16]. The repressed loci were termed *gfp::csr-1(RNAe)* and the active loci *gfp::csr-1(+).* The capacity of the paramutated locus to be paramutagenic was not tested, but it would probably work since *gfp*-transgene silencing mediated by *gfp* dsRNA in presence of a transgene sensitive to RNAe is stable over generations [16]. Like in *Drosophila*, pairing between the two interacting loci during RNAe conversion is not likely to be necessary as conversion can occur when the transgenes are located on different chromosomes. This type of conversion was

observed with different transgene structures (carrying *cdk-1* instead of *csr-1* with the *gpf* sequence). The presence of an RNAe transgene in the genome does not appear to induce co-suppression of homologous endogenous genes, as tested by western-blot [16].

Strikingly, not all transgenes undergo RNAe. Indeed, the *gfp::wrm-1* and *oma-1::gfp* not only are resistant to the RNAe transgene-mediated repression but are able to switch some of them into an active state ([16], **Figure 2 C and D**). The activation process, called RNA-induced epigenetic gene activation (**RNAa**), is only progressive and not as fast as RNAe since several generations of coexistence of the two transgenes in the same genome are necessary to induce full activation of the repressed transgene or to confer it stability in next generations, after out-cross with the wild-type removing the initial active transgene [19]. For example, the combination of *oma-1::gfp (RNAa)* with *gfp-cdk-1(RNAe)* results in activation of the latter transgene. Segregation after only one generation of contact between the two transgenes results in immediate reversion of the *gfp-cdk-1* transgene to the repressed state. Segregation after ten generations of contact between the two transgenes provides stability for one generation to *gfp-cdk-1*, and 30 generations of contact provide a stability to the activated transgene for 8-10 generations after segregation away from the activating locus (Figure 2 C and D). Some transgenes, however are resistant to RNAa since a *gfp::csr-1 (RNAe)* transgene is insensitive to the presence of the *oma-1::gfp* transgene: in that case, the two transgenes keep their own properties in a double transgenic line and in succeeding generations after cross to wild-type [16,19].

More recently, a case of "cascade" RNAe paramutation was described in *Caenorhabditis elegans* using a different combination of transgenes [52]. The first step involves on the one hand a "*piRNA sensor*" transgene carrying a *gfp* gene and a piRNA-targeted locus (21UR-1), and on the other hand an "*operon*" transgene which expresses *mCherry* and *gfp* mRNAs from a single primary transcript (**Figure 3**). *mCherry* is located upstream of *gfp* within this operon. The strain carrying the *piRNA sensor* transgene exhibits no *gfp* expression in the germline due to the targeting of the 21UR-1 sequence by piRNAs [14,17]. In contrast, the strain carrying the *operon* transgene shows strong *gfp* and *mCherry* expression [52]. Combining the *piRNA sensor* transgene with the *operon* transgene by crossing produces progeny in which the *operon* transgene is repressed for both *gfp* and *mCherry* expression in the germline. Therefore, the *operon* transgene is *trans*-silenced by the *piRNA sensor* transgene, and silencing propagates from the *gfp* sequence to the *mCherry* sequence in the *operon*. Out-crossing this progeny with worms devoid of any transgene generates lines carrying only the *operon* transgene that remains completely repressed over at least 12 generations ("*operon* (OFF)"

lines). In a second step, crossing the *operon* (OFF) worms with worms carrying an active *mcherry::H2A* transgene, but no *gfp* sequence, produces progeny in which *mCherry* expression of both the *operon* and *mcherry::H2A* transgenes is completely repressed. Further, removal of the *operon* silencer transgene by out-crossing results in lines carrying the targeted *mcherry::H2A* transgene alone, and this remains repressed at least for 10 generations. Thus, the *operon* (OFF) transgene, which was paramutated by the *piRNA sensor* transgene, is stably repressed over generations and appears strongly paramutagenic since it can transfer inheritable silencing capacities to the *mcherry::H2A* transgene. Finally, this repressed *mcherry::H2A* transgene, which is a "second-order" paramutated locus, was also shown to be able to *trans*-inactivate other transgenes carrying the *mcherry* sequence. This indicates that the epigenetic conversion process can be recurrent. The conversion processes described here exhibit the properties of a *bona fide* paramutation. In these experiments, it was shown that paramutation can be performed by both hermaphrodite and paternal transmission of the paramutagenic locus but the effect appears stronger *via* hermaphrodite inheritance (Nic Lehrbach and Eric Miska, personal communication). Further, crossing hermaphrodites carrying an *operon* (OFF) transgene at the heterozygous state with males carrying a *mcherry::H2A* transgene generated individuals which did not inherit the *operon* (OFF) transgene but carried a repressed *mcherry::H2A* transgene with properties stably transmitted over generations [52]. This indicates that, like in fly, paramutation can be mediated by cytoplasmic components without inheritance of the paramutagenic locus itself, excluding again of the need for pairing between the loci in the process. Importantly, this combination of structurally different transgenes shows that silencing propagation within a transgene, from the targeted transgene part (*gfp*) to the distal sequence (*mCherry*), is accompanied by production of paramutagenic signal from the distal sequence. Indeed, *operon* (OFF) worms produce siRNAs homologous to the *mCherry* sequence which are called tertiary siRNAs [52], by contrast to siRNAs produced by sequences located near the piRNA target site which are called secondary siRNAs [14]. These tertiary siRNAs can transfer heritable silencing capacities in *trans* and are thus paramutagenic.

Altogether, the different studies show that complete paramutation exists in worms and transgenes can occur in three different epigenetic states [15-17,19,52]: RNAe and RNAa transgenes and neutral transgenes that are expressed, but can be sensitive to the presence of RNAe transgenes [16,19]. However, these neutral alleles by themselves do not have the capacity to modify the expression of other homologous transgenes present in the same genome. What can be the mechanism of RNAe- and RNAa-linked epigenetic conversions? It appears that a complex balance

of antagonistic systems determines the properties of a transgene and its capacity to affect expression of other homologous transgenes in *trans*. The establishment of the RNAe requires the PIWI protein PRG-1, and its maintenance depends on HPL-2, a HP1 ortholog, and several putative histone methyltransferases [15,17]. In addition, maintenance of the RNAe state requires proteins involved in the WAGO-mediated RNAi pathway such as RED3, MUT7, WAGO-1 or WAGO-9 [16]. The establishment of RNAa by contrast requires the Argonaute protein CSR-1 [19]. A dose effect is observed for CSR-1 since heterozygous individuals show impaired RNAa. CSR-1 engages siRNAs homologous to most of the germline-expressed genes [53,54] but CSR-1-interacting small RNAs (22G-RNAs) do not appear to induce silencing [53]. What could be the roles of these antagonistic systems at the genome level? Worms appear to have an opposite strategy to flies. In flies, the piRNA loci represent a repertoire of sequences that have to be repressed and are mostly homologous to mobile elements [3]. Therefore, piRNAs mainly target mobile sequences although they can also target some gene transcripts containing TE fragments [55]. By contrast, in worms, the two piRNA loci located on chromosome *4* produce around 15,000 different piRNAs, which can target transcripts despite incomplete homology (up to 4nt out of 21nt) [13,14,18,54]. Consequently, a very high number of gene transcripts can potentially be targeted by piRNAs, and a protecting system is thus required to ensure gene expression. This system appears to be mediated by the 22G RNAs loaded on the CSR-1 protein, which might protect homologous transcript from the effect of 21-U PRG-1 RNAs. Antagonist 22G CSR-1 RNAs and 21-U PRG-1 RNAs would be therefore able to exert opposite effects on their common homologous transcript targets. If the effect of PRG-1 dominates, the transcript binds an RdRP that produces double-strand RNAs further targeted by the WAGO-RNAi machinery, leading to production of 22-G WAGO RNAs. These WAGO-RNAs can then induce both PTGS of homologous transcripts and TGS of homologous loci [54,56]. If the effect of CSR-1 dominates, there is no double-strand RNA production by RdRP and the siRNA machinery is not activated. So, the piRNA machinery is required for the silencing establishment but not for its maintenance over generations, which relies on siRNA activation [15,17,57]. Cytoplasmic *trans*-generational transmission includes information carried in both WAGO interacting RNAs as a memory of silenced targets and CSR-1 interacting RNAs as a memory of targets licensed for expression. The PRG-1 system is involved in the foreign DNA detection step (which was not previously licensed) and the WAGO machinery is required for amplification and maintenance of the information and repression itself.

It appears that maintenance of the properties of epigenetically repressed RNAe or activated RNAa transgenes, each alone in a genome, involves not only cytoplasmic RNA molecules associated with WAGO (silencing) or CSR-1 (activation), but also chromatin marks on the locus deposited *via* either a WAGO RNAs or potentially a CSR-1 RNA-dependent mechanism for silencing or activation, respectively [16,19,52,58]. Two opposite loops between small RNAs and chromatin could exist for RNAe and RNAa transgenes, and the maintenance of the loop from one generation to the other would be established by cytoplasmic inheritance of WAGO RNAs for RNAe and CSR-1 RNAs for RNAa. When the two types of transgenes are present in the same genome, different small RNAs might compete both at the cytoplasmic and nuclear level. In the cytoplasm, a given transcript could be confronted with 21-U PRG-1 RNAs (and 22G WAGO RNAs) in one hand and with antagonist CSR-1 RNAs on the other hand. For RNAe conversion process, a strong RNAe transgene would generate a high quantity of 22G WAGO RNAs, which could potentially counteract a previously established presence of CSR-1 RNAs. By contrast, for an RNAa conversion process, a strong RNAa allele would generate a high quantity of CSR-1 RNAs that could counteract the effects of previously established RNAe alleles. In the nucleus, the 22G RNAs brought by RNAe or RNAa alleles could displace chromatin tags on the antagonist transgenes. For example, 22G WAGO RNAs could potentially trigger removal of CSR-1 from the chromatin of an RNAa allele and thus break a positive loop in which CSR-1 present on the locus favors the production of 22G CSR-1 RNAs. Reciprocally, 22G CSR-1 RNAs could displace chromatin tags on RNAe alleles similarly involved in a positive loop. In any case, this system implies that for a gene to be expressed, it is necessary that it produces sufficient quantity of RNA for first, establishing a population of CSR-1 RNAs to counteract silencing machinery and second, for being translated. Neutral transgenes alone in a genome would escape RNAe silencing and could be thus expressed. However, in the presence of strong RNAe, neutral alleles would be switched towards RNAe at the cytoplasmic and /or chromatin levels. When the RNAe-inducing allele is removed by crosses to wild-type, repression loops now associated to the repressed targeted transgenes could be maintained by themselves over at least some generations. It appears that the RNAe-induced conversion is relatively stable. This is probably due to the strength of the 22G-RNAs WAGO production based on RdRP activity. Finally, “adoption” of foreign sequences can be observed linked to *cis*-spreading of CSR-1 on a transgene which contains adjacent endogenous and foreign sequences [19].

Conclusion

Flies and worms can undergo epigenetic conversions showing paramutation-like properties. In fly, the conversion is unidirectional, corresponding to the transfer of a homology-dependent silencing capacity from one locus to the next, which reflects the capacity to produce piRNAs [21,49]. The conversion acts on the piRNA producing locus itself. It includes a change on the locus since the chromatin of the new piRNA-producing locus is associated with enriched H3K9 histone methylation marks [50]. In worm, the conversion can occur in two directions, probably reflecting the competition of two antagonist regulation loops whose strength can vary. Apparently, it does not involve the piRNA loci themselves but rather their targeted sequences. It appears, however, that the change toward the repressed state is more stable over generations than the opposite change. This RNAe conversion presents the stability of a true paramutation and can be recurrent. By contrast, the RNAa process appears not only less stable but the activated alleles are also not able to trigger further RNAa conversion [19]. So RNAa conversion does not exhibit all the properties of a complete paramutation. In fly, conversion is mediated only *via* maternal cytoplasmic transmission, in contrast to worms in which RNAe conversion can be mediated by transmission from both parents, possibly because of different proportions of paternal cytoplasmic transmission in the two species. A key point for future work will be to determine if some antagonistic forces may also exist in fly to counteract the action of piRNAs. Furthermore, in both species it will be essential to characterize the developmental events involved in the transgenerational transmission of these conversion processes.

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Figure 1: Paramutation linked to piRNAs in fly.

A tandemly repeated *P-lacZ-white* transgenic locus, called *T-1*, which produces abundant piRNAs in ovaries and induces complete silencing of a homologous transgenic target in the germline was used as a paramutagenic locus. A similar transgenic locus, called *BX2*, located at the same genomic site, and stably devoid of piRNA production and *trans*-silencing capacities was used as paramutable locus. The mating scheme is shown with the number of generations indicated on the left. *CyRoi* and *Cy* are balancer chromosomes carrying distinct dominant phenotypic makers. Orange color indicates cytoplasm inheritance. Histograms show the length distributions (19-29 nt) of ovarian small RNAs matching *P-lacZ-white*. Positive (red) and negative (blue) bars correspond to sense and antisense reads, respectively. *Trans*-silencing capacities were measured by crossing tested females with males carrying a single *P-lacZ* transgene expressed in the female germline and by counting the percentage of egg chambers showing target transgene repression (*n* = total number of egg chambers counted). G_1 females having paternally inherited the *BX2* locus and having maternally inherited a *T-1* cytoplasm, but not the *T-1* locus itself were generated. Lines established from G_1 individuals, called *BX2** (first order paramutation), were further analyzed. *BX2** females produce abundant transgene-homologous piRNAs and induce a complete *trans*-silencing in the germline as tested in G_2 and G_{83}. Finally, females from *BX2** lines (at G_{42}) were crossed with males of a *BX2* line quiescent for piRNA production and *trans*-silencing. This generated *BX2*** females, having paternally inherited the *BX2* locus and maternally inherited a *BX2** cytoplasm - but not the locus -, which produce abundant ovarian piRNAs and induce strong silencing. *BX2*** lines (second order paramutation) stably maintain these properties over generations. This recurrent conversion process has been repeated five times “en cascade”, generating *BX2**^{5} lines showing a complete *trans*-silencing capacity [21]. Thus, this epigenetic conversion process presents functional properties of paramutation. This process does not require the transmission of the paramutagenic locus but requires cytoplasm inheritance.

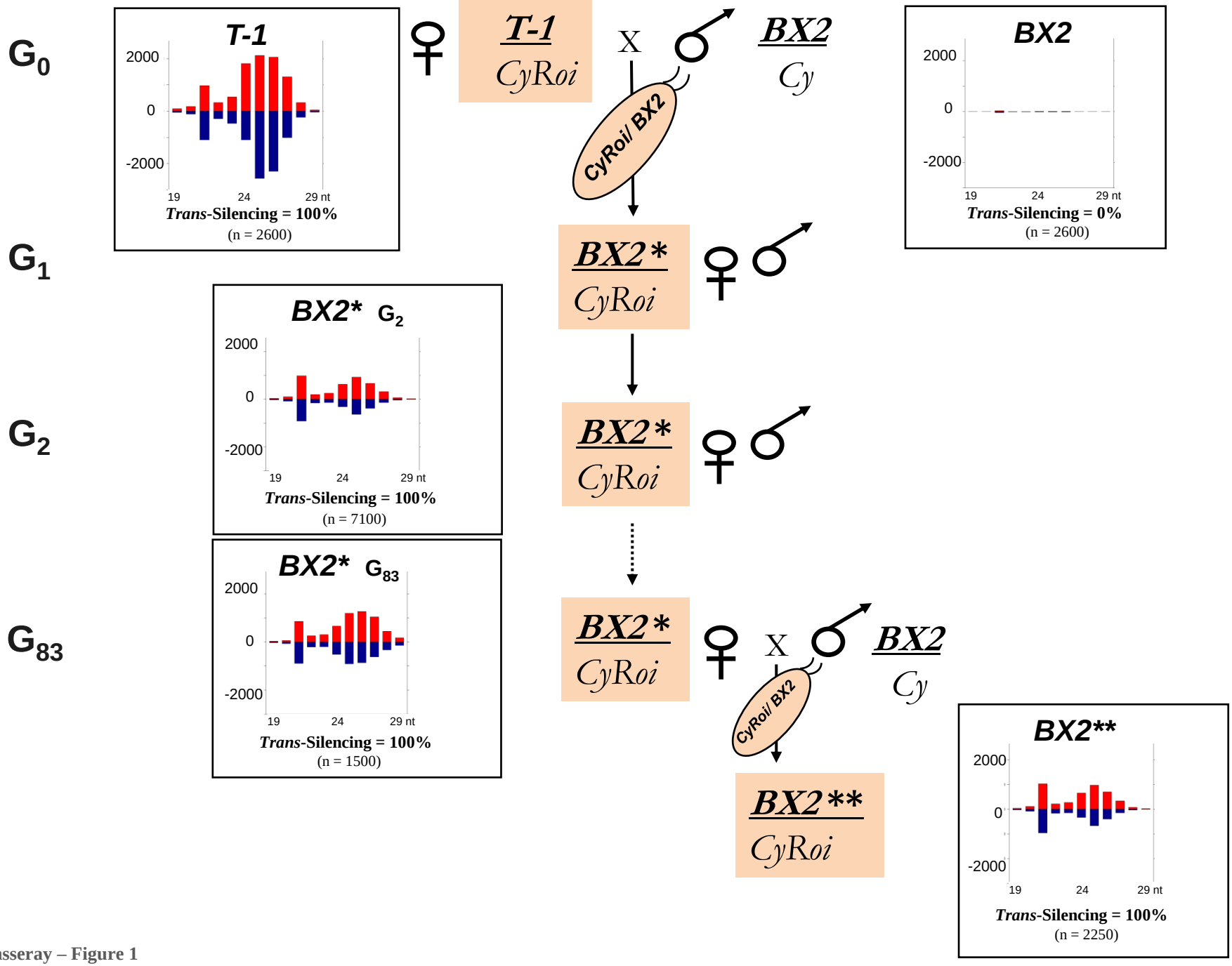
Figure 2: Reciprocal epigenetic conversions in worms.

Single copies of the *gfp::csr-1* transgene can exhibit *gfp* expression or repression depending on PRG-1-associated piRNAs targeting. At the same genomic location, the two states can exist and appear stable over several generations. **RNAe** (RNA-induced epigenetic silencing): **(A)** The cross of hermaphrodites worms carrying a *gfp::csr-1(RNAe)* repressed transgene (grey *gfp* box on the transgene map) with males carrying an expressed *gfp::csr-1(+)* transgene (green *gfp* box), both located on chromosome *2* (LGII), results in a progeny in which the two transgenes are repressed in the germline. Propagation of the derived lines shows that this repressed state is stable over generations at least until G_{10}. **(B)** Similar silencing conversion can occur between transgenes located on different chromosomes. The cross of hermaphrodites carrying a *gfp::csr-1(RNAe)* repressed transgene located on chromosome *4* (LGIV) with males carrying an expressed *gfp::csr-1(+)* transgene located on chromosome *2,* also results in progeny in which the two transgenes are repressed. The repressed state of the transgene located on chromosome *2* is maintained in successive generations after the removal of the silencer transgene located on chromosome *4* by crosses. Such RNAe conversion can occur during inheritance from both parents. **RNAa** (RNA-induced epigenetic gene activation): **(C)** Some active transgenes appear to be resistant to the effect of RNAe transgenes and show opposite properties. The cross of hermaphrodites carrying an active *oma1::gfp(RNAa)* transgene (chromosome *4*) with males carrying a repressed *gfp::cdk-1(RNAe)* transgene (chromosome *2*) results in progeny showing *gfp* expression of both transgenes. If the two transgenes are separated after one generation by out-crossing to wild-type, the *gfp::cdk-1* transgene is immediately re-silenced [19]. Out-crossing after ten generations of double transgenic strains allows persistence of the *gfp::cdk-1* active state for one generation, but then the effect is lost later. **(D)** Propagation of the double transgenic strain for 30 generations allows maintaining of a *gfp::cdk-1* fully active state for 8 generations after out-crossing, before transgene activation decreases. By contrast to RNAe, which can be stably established in one generation, RNAa needs a longer exposure and only results in a transitory active state. (Adapted from [16,19]).

Figure 3: Cascade of RNAe paramutations in worms.

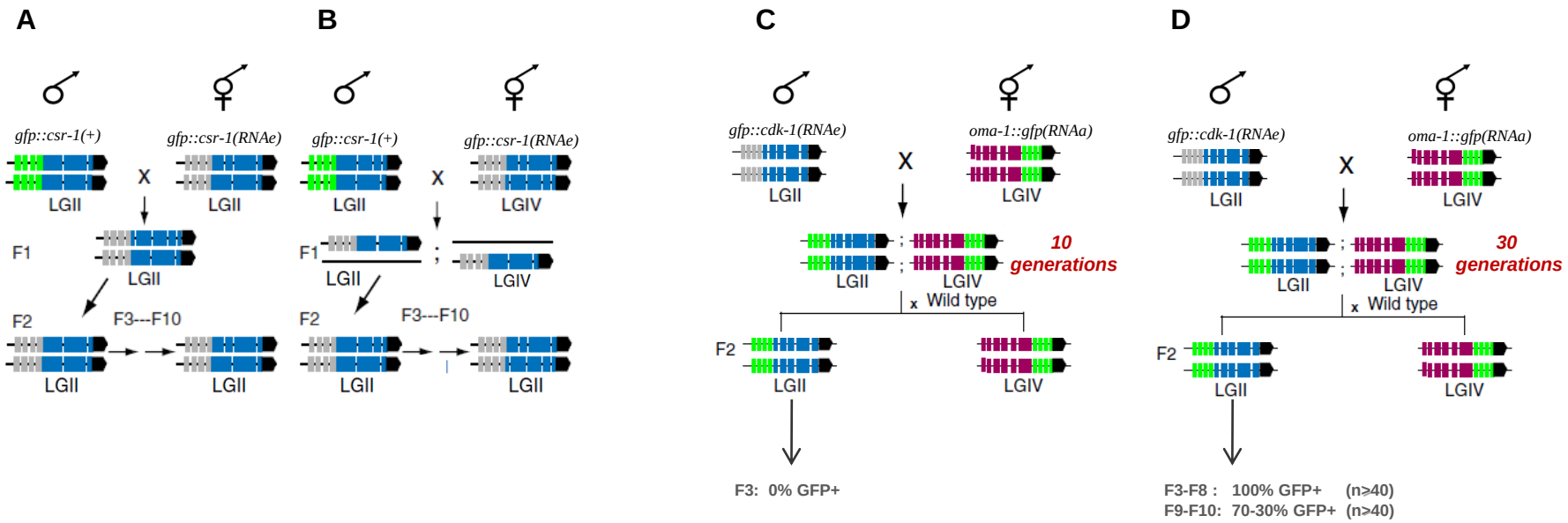
The *piRNA sensor* transgene is targeted by an endogenous piRNA (21UR-1) specific for the "21U" site and shows no *gfp* expression. This repression correlates with production of siRNAs by the *gfp* sequence. Crossing worms carrying a *piRNA sensor* repressed transgene with worms carrying an *operon* expressed transgene generates individuals in which the *operon* transgene is repressed for both *gfp* and *mCherry* expression in the germline. This repression correlates with *cis*-propagation of the capacity to produce siRNAs from the *gfp* until the *mCherry* sequence. This "*operon* (OFF)" transgene remains repressed in the absence of *piRNA sensor* transgene after outcrossing. Crossing worms carrying an *operon* (OFF) transgene with worms carrying a *mcherry::H2A* expressed transgene produces individuals in which both *mCherry* transgenes are repressed. As before, the *mcherry::H2A* transgene remains repressed in the absence of *operon* (OFF) transgene after outcrossing. Finally, crossing individuals carrying a repressed *mcherry::H2A* (OFF) transgene with individuals carrying a *mcherry* expressed transgene produces progeny in which both transgenes exhibit *mcherry* repression. The *operon* (OFF) transgene is thus paramutated by the piRNA sensor transgene (first order paramutation) and can paramutate a *mcherry* transgene (second order paramutation). (Adapted from [52]).

Figures 1-3

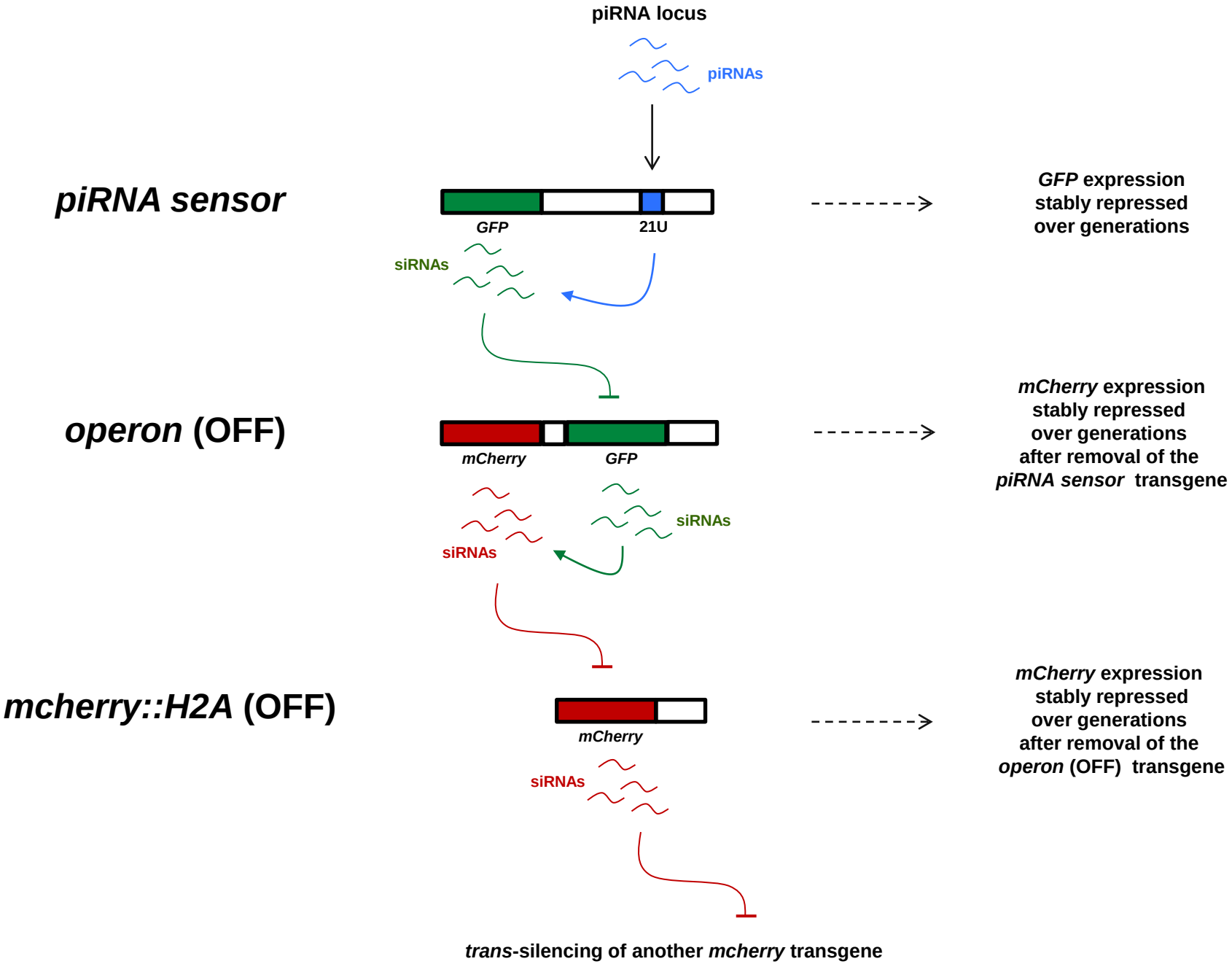

G0
T-1
2000
0
-2000
19
24
29 nt
Trans-Silencing = 100%
(n = 2600)
T-1
CyRoi
X
BX2
Cy
CyRoi/ BX2
BX2
2000
0
-2000
19
24
29 nt
Trans-Silencing = 0%
(n = 2600)
G1
BX2*
CyRoi
G2
BX2* G2
2000
0
-2000
19
24
29 nt
Trans-Silencing = 100%
(n = 7100)
BX2*
CyRoi
G83
BX2* G83
2000
0
-2000
19
24
29 nt
Trans-Silencing = 100%
(n = 1500)
BX2*
CyRoi
X
BX2
Cy
CyRoi/ BX2
BX2**
CyRoi
BX2**
2000
0
-2000
19
24
29 nt
Trans-Silencing = 100%
(n = 2250)

Silencing conversion (RNAe)

Activating conversion (RNAa)



Ronsseray – Figure 2



Ronsseray – Figure 3