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Transposable element repression using piRNAs, and its relevance to Endogenous Viral Elements (EVEs) and immunity in insects.

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Abstract

The piRNA system controls transposable element (TE) mobility by transcriptional gene silencing and post-transcriptional gene silencing. Dispersed in insect genomes, piRNA clusters contain TE copies, from which they produce piRNAs (specific small RNAs). These piRNAs can both target the nascent transcripts produced by active TE copies and directly repress them by heterochromatinization. They can also target mature transcripts and cleave them following amplification by the so-called “ping-pong” loop mechanism.

Moreover, piRNA clusters contain endogenous viral elements (EVEs), from which they produce piRNAs. The current idea is that these piRNAs could participate in the antiviral response against exogenous viral infection.

In this review, we show that among insects, to date, this antiviral response by the piRNA system appears mainly restricted to mosquitoes, but this could be due to the focus of most studies on arboviruses.

Highlights

- piRNA clusters contain, in addition to transposable elements, endogenous viral elements (EVEs) from which piRNAs are produced.
- The piRNA antiviral response upon viral infection is to date mainly known from mosquitoes.
- The piRNA antiviral response is based on a specific mechanism of piRNA amplification (the “ping-pong” loop).

Introduction

Mobile genetic elements such as transposable elements (TEs) and viruses constantly invade eukaryote genomes. During virus invasion, a viral genome fragment can integrate into the eukaryotic host genome. If this happens in the germline, it can be transmitted vertically to the offspring, mostly under neutral evolutionary pressure. These integrated viral sequences are referred to as endogenous viral elements (EVEs). Remains of retroviruses are the most

described viral sequences inserted in genomes due to their replication strategy involving an RNA reverse transcription step and integration into the DNA of the infected cell. However the analysis of EVE sequences shows that in fact all types of viruses can be found integrated in animal genomes [1–3], especially in arthropod genomes where EVEs are widespread [2,4]. Some of these EVEs have even been conserved and selected over long evolutionary timescales and confer new essential functions to their hosts as described in some parasitoid wasp lineages [this issue and 5,6].

To suppress the movements of TEs, metazoans use a control system based on 23-30 nucleotides long small RNAs, called piRNAs for Piwi interacting RNAs [7]. Some EVEs also produce piRNAs [8] suggesting that the cell might use the same defense system against EVEs as against TEs. In fact, in both cases, the newly integrated sequences potentially bring regulatory and / or coding sequences that the cell might control through the piRNA pathway. As the repression of TEs by piRNAs takes place at the transcriptional and post-transcriptional levels, referred to respectively as transcriptional gene silencing (TGS) and post-transcriptional gene Silencing (PTGS) [9–11], it can be expected that the piRNAs produced by EVEs also have similar actions against viral transcripts upon viral infection. A topic of wide interest is whether piRNAs produced by EVEs could participate in the antiviral action against exogenous viruses.

Here, we summarize, for insects, the state of the art and the latest advances on TE regulation by piRNAs, the potential antiviral role of piRNAs and the links with EVEs located in piRNA clusters. Until now, studies on TE piRNA systems have been carried out mainly in *Drosophila melanogaster* and those on piRNAs involved in antiviral immunity in mosquitoes due to their central role as vectors of arboviruses. Generally, in arthropods, the piRNA system can occur in somatic and germinal tissues, meaning that the proteins of this pathway and piRNAs can be produced in both tissue types. However, the piRNA system in *Drosophila* appears to be mainly restricted to germinal tissues [12]. The primary production of piRNAs comes from the transcription of heterochromatic regions of the genome called piRNA clusters [9,13]. While most piRNA clusters are transcribed from a single strand, the *Drosophila* germinal clusters are generally transcribed on both strands. However, the first studied piRNA cluster called “flamenco” expressed in a particular somatic tissue surrounding the germline (follicle cells) is uni-strand transcribed. Flamenco has also the particularity of having only one origin of transcription, while in other species, uni-strand clusters have several origins [14]. Mosquitoes which harbor a large duplication of genes involved in the piRNA pathway and which as bloodfeeders are more susceptible to encounter viruses, seem to have enhanced piRNA-related immune capacities compared to other insects [12]. When evaluating the involvement of the piRNA system in the antiviral response in other insects, the knowledge acquired in *Drosophila* and mosquitoes should be used cautiously, bearing in mind that these well-studied models may have specific features [15].

Primary piRNA production by TE piRNA clusters

TEs are classified into two classes according to their transposition intermediary (DNA or RNA). Both TE classes are regulated by piRNA pathways. Production of piRNAs occurs from the transcription of particular regions of the genome, so-called piRNA clusters, which are heterochromatic (with the heterochromatic marks H3K9me3 and HP1 present within them)

and rich in TE copies. In *Drosophila*, piRNA clusters are transcribed by RNA polymerase II, which is indirectly recruited by the heterochromatin protein-1 variant Rhino [16]. This particular heterochromatic transcriptional context of the piRNA clusters leads to the primary piRNA production (Figure 1) [17] and appears common to most animals [18]. The primary piRNAs are obtained during the maturation of the pre-piRNAs and possess as a molecular signature a Uridine (U) at their 5' end (1U bias) [15]. In general, the primary piRNAs produced by the clusters are mainly antisense to the TE transcript. The conservation of this orientation of TEs inserted in clusters might have been favored because it allows hybridization of the primary piRNAs with the TE transcripts. These primary piRNAs associated with the Piwi protein repress TE copies at two molecular levels: on the active genomic TE copies by TGS; and on the TE transcripts by PTGS.

piRNA effect on TEs

In brief for TGS, the primary piRNAs associated with Piwi detect in the nucleus the transcription of active TE copies and recruit Panoramix, Nxf2 and Nxt1 proteins on the nascent transcript [19]. This protein complex, in turn, recruits histone modifying enzymes, which will deposit H3K9me3 marks on histones (major histone modification linked to heterochromatin) allowing HP1 (major heterochromatic protein) recruitment to the TE copies, thus completing their transcriptional silencing by heterochromatinization [10].

The primary piRNAs associated with Piwi will also go into the cytoplasm to repress the TE transcript by PTGS (Figure 2). First, the primary antisense piRNAs specifically bind to the TE transcript based on sequence recognition. Then, the TE transcript is cleaved into sense and complementary piRNAs, together forming the so-called secondary piRNAs. This degradation of the TE transcript by primary piRNAs is thus an amplification mechanism of piRNAs called ping-pong [9]. Due to the transcript cleavage, the secondary piRNAs have a characteristic molecular signature. Antisense piRNAs have a U at the 5' end and sense piRNAs have an Adenine (A) in position 10 (10A bias), the two hybridize over an average length of 10 nucleotides (Figure 3). In a pool of sequenced piRNAs, the detection of ping-pong signatures signals the degradation of cellular transcripts by the piRNA system.

The presence of TE copies in the piRNA clusters is at the basis of TE control by the piRNA system. Once a copy of an invading TE becomes integrated into a piRNA cluster, it will induce a piRNA response. A recent work confirmed experimentally that the recent integration of a TE in a piRNA cluster allowed the piRNA system to produce piRNAs against it [20*]. The clusters contain mostly truncated TEs suggesting that during a TE invasion, TE copies integrated in piRNA clusters rapidly erode. It is now accepted that the composition of TE sequences in piRNA clusters provide a record of successive TE invasions over evolutionary time. Moreover, only a portion of TE sequences in piRNA clusters is necessary to induce the TGS or PTGS response against TEs. During TGS, heterochromatinization spreads to adjacent sequences causing their repression, and during PTGS, one transcript cleavage is sufficient to cause its degradation. With their diversified and high concentration in TE sequences, TE copies in piRNA clusters protect the cell against active TEs present in the genome and could also repress a new invading TE if a part of its sequence is present in the clusters.

The piRNA system therefore regulates TEs by keeping TE copies in piRNA clusters. As it was described relatively early on in mosquitoes (2010s) that piRNAs were involved in the

antiviral response against arboviruses [21], one may postulate that viral sequences inserted into piRNA clusters could be source of piRNAs involved in the antiviral response against exogenous viruses. EVEs inserted in the clusters would be the “memory” of past viral infections and the guardians against future viral infections.

However, RNA interference (RNAi) is the primary antiviral mechanism in arthropods [22]. It is therefore of interest first to verify the actual participation of the piRNA system in the antiviral response in insects, and second to study the involvement of EVEs located in piRNA clusters, in this antiviral response.

The antiviral role of piRNAs

It is now clear that the piRNA system is indeed involved in the host antiviral response but seems to be restricted, in the insect world, to a few species, mainly mosquitoes. Since the early 2010s, mosquitoes have been found to harbor piRNAs specific to arboviruses [21,23–26], as well as to other insect viruses, such as the cell-fusing agent virus (CFAV) or the Phasi Charoen-like virus (PCLV) [27]. All correspond to secondary piRNAs displaying typical ping-pong signatures. If, as in the case of TE repression, this ping-pong signature reveals the specific destruction of the corresponding transcript, observation of secondary piRNAs against viral sequences would be an indication of the degradation of the viral transcript. To our knowledge, besides mosquitoes, the hemipteran *Diaphorina citri* is the only other insect species showing a piRNA ping-pong signature upon infection by a densovirus (arthropod virus) [28*].

To demonstrate that the presence of secondary piRNAs is linked to the regulation of viral replication, several studies have focused on disrupting the piRNA system. In *Aedes aegypti*, the knockdown of the genes producing PIWI 4-6 proteins leads to a decrease in piRNA quantities and the increase in virus RNA quantity [27,29,29–31]. Thus in mosquito, the piRNA system indeed appears to be involved in the antiviral response.

In contrast, in *Drosophila*, the antiviral response against different types of viruses does not involve the piRNA system [32]. Likewise, no virus specific piRNAs have been found in a global analysis of *Apis mellifera* [33], in which only siRNA is detected upon infection by the Sacbrood virus [34]. Similarly, no piRNAs are produced during *Bombyx mori* infection by the baculovirus BmNPV [35]. The number of PIWI proteins encoded by the different insect species could indicate whether the piRNA pathway is involved in antiviral response. Indeed, whereas *Drosophila* has three PIWI proteins (Piwi, Aub and Ago3), as most insects *Apis mellifera* two (Piwi/Aub and Ago3) and *Bombyx mori* two (Siwi and BmAgo3), *Aedes aegypti* has no less than seven PIWI proteins (Piwi 1-6 and Ago3) [12]. It is now accepted that this large number of PIWI proteins broadens the targets of the mosquito piRNA system. This hypothesis is sustained by the fact that PIWI proteins interacting with piRNAs against viruses (vpiRNAs) differ from those against TEs [31*].

The conclusion that the involvement of the piRNA response in the antiviral response might be a specific feature of mosquitoes is mainly sustained by the fact that in a global RNA analysis including small RNAs and involving 20 arthropod species, nine species showed an RNAi antiviral response. Among those nine species, five produced primary piRNAs but secondary piRNA ping-pong signatures against viruses were only detected in the *Aedes aegypti* mosquito [12].

Of note, even in mosquito, the involvement of the piRNA system generates a lower antiviral response than the RNAi system. In fact, in Aag2 mosquito cells, the down regulation of the piRNA system protein PIWI4 clearly showed a lower increase in viral replication than when Dicer2, a central protein in the system of interference RNAs was down regulated [29].

Many studies have therefore focused on the search for EVEs in genomes, and the association of EVEs with piRNA clusters and their capacity to produce primary piRNAs. This type of investigation has mainly been carried out in mosquitoes [3,27,31,36, 37*,38], but also in *Ixodes scapularis* ticks [39] and *Ornithodoros* ticks [40]. A general approach carried out in 48 arthropod species summarizes all of this work [8]. For 30 of these species, EVEs (mostly corresponding to unclassified viruses, *Rhabdoviridae* and *Parvoviridae*) have been found in piRNA clusters and the vast majority of them produce primary piRNAs [8].

Implication of piRNA clusters in antiviral immunity

Whether the primary piRNAs produced by the EVEs inserted in piRNA clusters serve to generate the ping-pong type PTGS response against a viral transcript is a key question, however, the answer is not straightforward.

For secondary piRNA PTGS to operate, the sequence of the primary piRNAs, coming from piRNA clusters and initiating the ping-pong cycle, must hybridize almost perfectly with the targeted transcript. Mechanistically, the primary piRNAs produced by the clusters can initiate ping-pong type PTGS against the transcript when the nucleotide identity is perfect. Indeed, the artificial insertion of an EVE sequence (also present in a piRNA cluster and producing primary piRNAs) into a virus was shown to reduce viral replication during the infection of Aag2 cells by this modified virus [31*].

However, as EVEs found in piRNA clusters generally show very weak nucleotide identity to known exogenous viruses, researchers are currently focusing on EVEs presenting the highest nucleotide identity with known viruses. This is the case in the Aag2 mosquito cells, persistently infected with PCLV, that exhibit a PTGS phenotype with a secondary piRNA ping-pong type signature against this virus. Mapping of piRNA libraries to the virus and mosquito genomes showed that secondary sense piRNAs are specific to the viral transcript, while the secondary antisense piRNAs are specific to an EVE inserted in a piRNA cluster, thus suggesting a role of EVEs in the piRNA clusters in the ping-pong type PTGS [27].

Currently, a single study establishes a direct link between the production of primary piRNAs from clusters and PTGS by secondary piRNAs on a virus transcript. In the *Ae aegypti* mosquito, the deletion of an EVE with 96% nucleotide identity to CFAV causes the loss of the secondary piRNA ping-pong signature and leads to higher viral replication [41**].

In contrast, in *Diaphorina citri*, the only model other than mosquitoes with an antiviral ping-pong type PTGS signature upon virus infection, this ping-pong type PTGS response against the densovirus was first thought to depend on the presence of an EVE having a very large nucleotide identity with densovirus and producing primary piRNAs. However, studies in one *D. citri* population that does not possess the EVE but still induces a piRNA response upon densovirus infection showed that the ping-pong type PTGS response is not dependent on EVE presence [28*].

Several ideas emerge from these findings. First, with sufficient nucleotide identity, EVEs in piRNA clusters can repress viral transcripts by a ping-pong type PTGS. As most EVEs in clusters are unclassified and as many insect viruses are yet to be discovered, the antiviral role of EVEs in piRNA clusters could be dedicated to these undescribed viruses. Indeed, although the piRNAs deriving from EVEs are mainly primary, the global analysis detected ping-pong type signatures [8,37]. Secondly, the ping-pong-type PTGS antiviral response could apparently take place without the production of primary piRNAs by the clusters. The study of the interconnection between the RNAi and piRNA systems, represented by the PIWI4 protein in mosquito [29], may allow us one day to understand this intriguing phenomenon.

Finally, we would like to propose a new mechanism by which the piRNA system could impact viral infection. As the piRNA system represses the expression of euchromatic TE copies by TGS, another role of viral piRNAs could be to repress by TGS a fortuitous genomic viral insertion, using piRNAs to target it (Figure 4). Local heterochromatinization would repress the fortuitous genomic viral insertion and protect the genome from potential perturbations induced by inserted regulatory and / or coding viral sequences. These insertions could later become EVEs following erosion of viral sequences. However, to our knowledge, the use of TGS (unlike for PTGS) against viruses in insects has not yet been investigated.

In conclusion, the piRNA system seems so far to participate in the antiviral response but mainly in mosquitoes based on current knowledge. The study of viruses not yet described in insects should make it possible to determine whether the piRNA antiviral response system works in most insects. For future studies, it is also important to bear in mind that piRNAs are involved in multiple other pathways such as the maintenance of germinal stem cells, DNA repair mechanisms, sex determination as well as learning and memory, and that piRNAs are also deregulated in cancer cells, therefore much remains to be discovered in this piRNA world [42–46].

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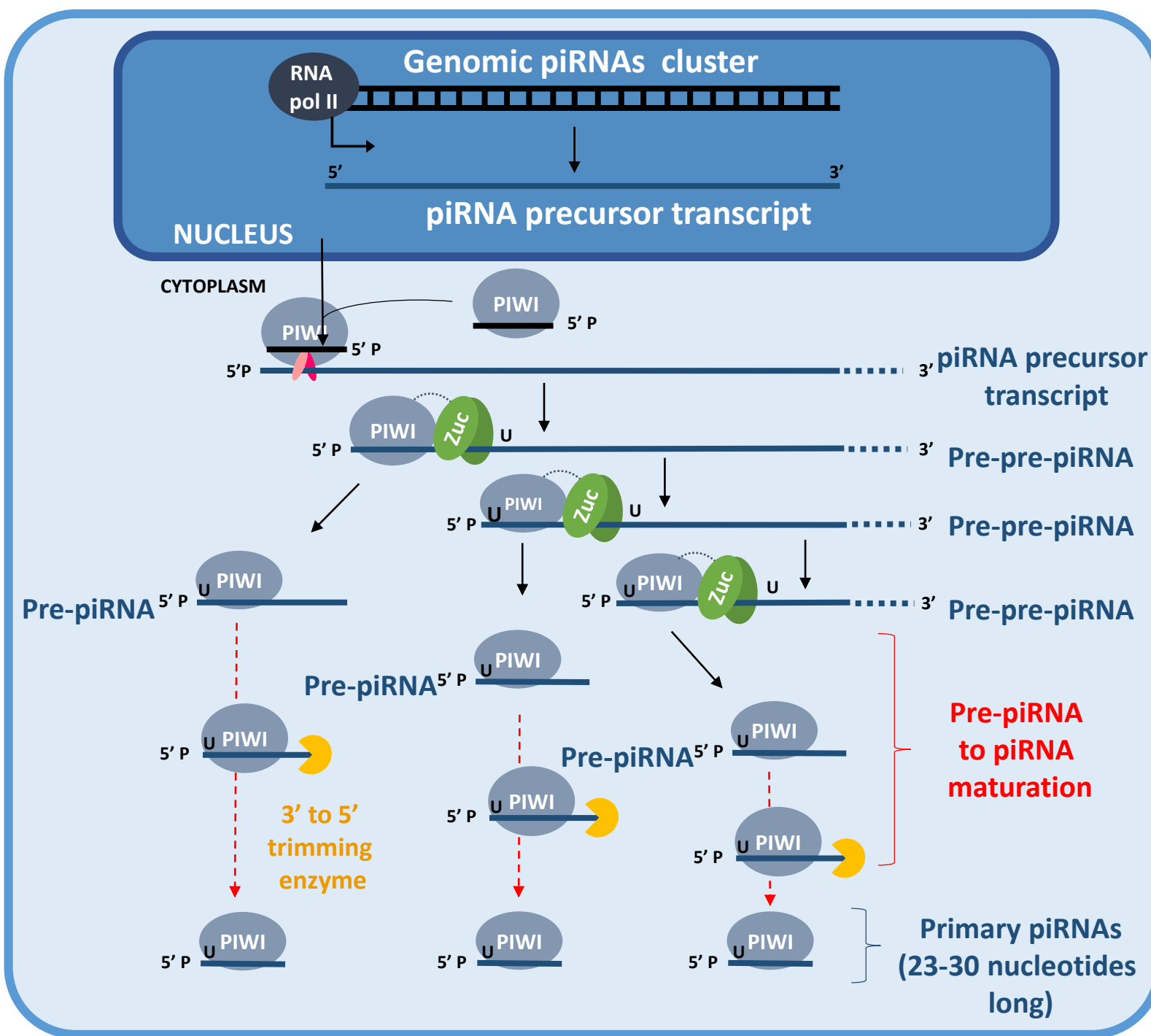
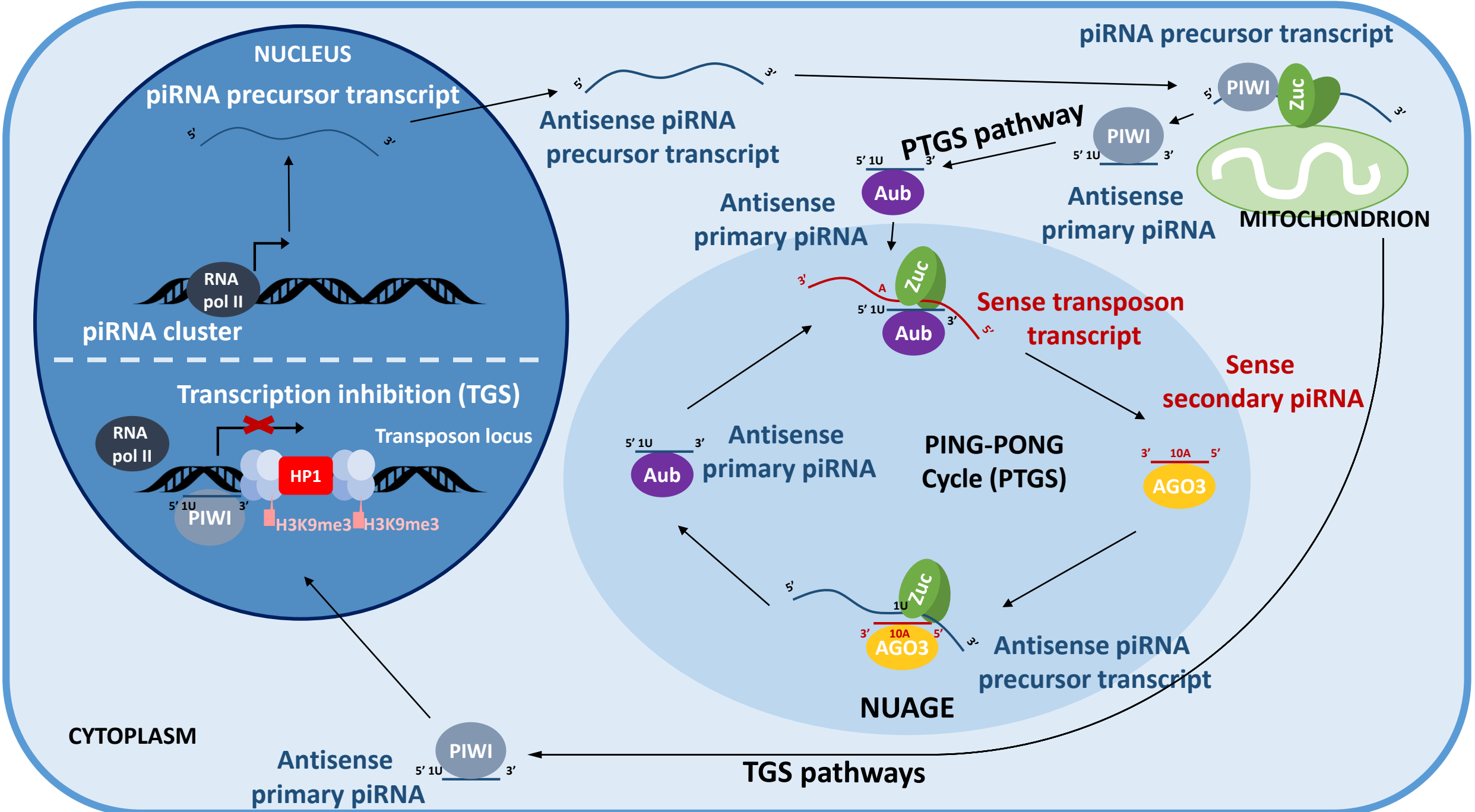


Figure 1 : Primary piRNA production by TE copies inserted in piRNA clusters.
piRNA cluster transcription by RNA polymerase II generates a long transcript (called piRNA precursor transcript) on which the Piwi protein binds at the 5' end. The Piwi protein recognizes the precursor transcript thanks to a sense primary piRNA the origin of which is not well understood. An endonuclease (Zucchini [16]) positions itself at the 3' end adjacent to the Piwi protein and cuts the transcript just before the first accessible Uridine (U). This cleavage generates two fragments: the one linked to PIWI called pre-piRNA that will be matured into piRNA (due to the 3' to 5' exonuclease Trimmer) ; and the remaining fragment, with a 5' U , which is called pre-pre-piRNA. A new Piwi protein binds to the pre-pre-piRNA, next to which the endonuclease (Zucchini) is attached, which cuts in 3' before the first accessible U thus again producing a pre-piRNA (with a U in 5') and a pre-pre-piRNA (with a U in 5') on which a new Piwi protein binds.



NUCLEUS

piRNA precursor transcript

5' 3'

piRNA cluster

Transcription inhibition (TGS)

Transposon locus

5' 1U 3'

PIWI

H3K9me3

H3K9me3

CYTOPLASM

Antisense primary piRNA

5' 1U 3'

Antisense piRNA precursor transcript

5' 3'

Antisense primary piRNA

Aub

5' 1U 3'

PTGS pathway

Antisense primary piRNA

5' 1U 3'

PIWI

piRNA precursor transcript

5' 3'

PIWI

Zuc

MITOCHONDRION

Sense transposon transcript

Sense secondary piRNA

5' 1U 3'

Aub

Antisense primary piRNA

PING-PONG Cycle (PTGS)

3' 10A 5'

AGO3

NUAGE

3' 10A 5'

AGO3

Antisense piRNA precursor transcript

5' 3'

Zuc

1U

TGS pathways

5' 1U 3'

Antisense primary piRNA

5' 1U 3'

Figure 2 : The piRNA system controls TE mobility by TGS and PTGS.

The piRNA pathway presented here corresponds mostly to the one described in *Drosophila*. piRNA precursor transcripts are transcribed from piRNA clusters in the nucleus thanks to the RNA polymerase II. piRNA precursor transcripts are then transported to the cytoplasm and cleaved by Zucchini (Zuc) protein, a protein localized in the outer membrane of mitochondria, to form primary piRNAs which possess a U bias at the first position of the 5' end. For the PTGS pathway, primary piRNAs, translocated from Piwi to Aubergine protein are mostly antisense to TE transcripts. The specific sequence pairing between the primary antisense piRNAs and the TE transcript allows the Aub protein with Zucchini to cleave the TE transcript approximately 10 nucleotides from the 5' end of the piRNA. Therefore, a secondary sense piRNA bound to the AGO3 protein, is produced from the TE transcript. It targets transcripts originating from piRNA clusters in order to generate even more antisense piRNAs thus amplifying the pool of what is called secondary piRNAs. For the TGS pathway, Piwi proteins harbouring an antisense piRNA will enter into the nucleus where they will bind to TE nascent transcripts. The TE active copies are transcriptionally repressed by heterochromatinization and marked by H3K9me3 histone flags and the presence of Heterochromatin Protein 1 (HP1).

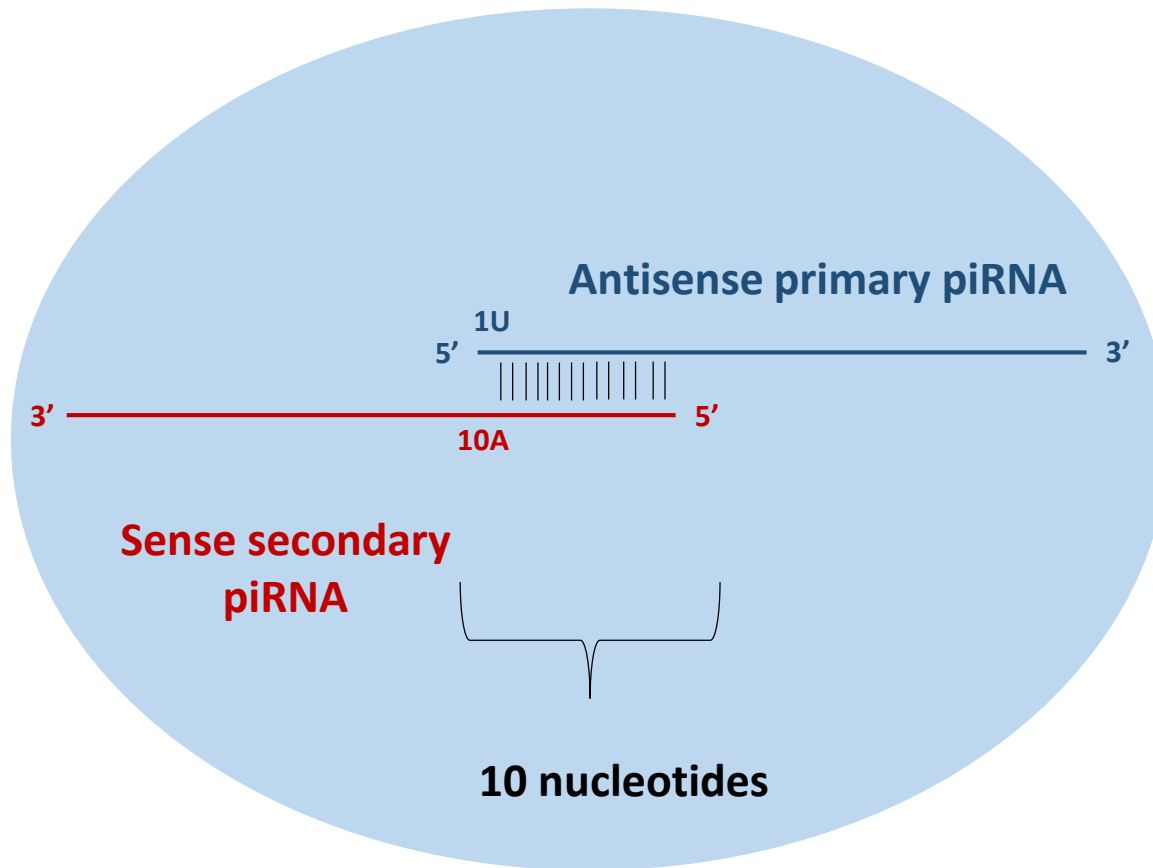
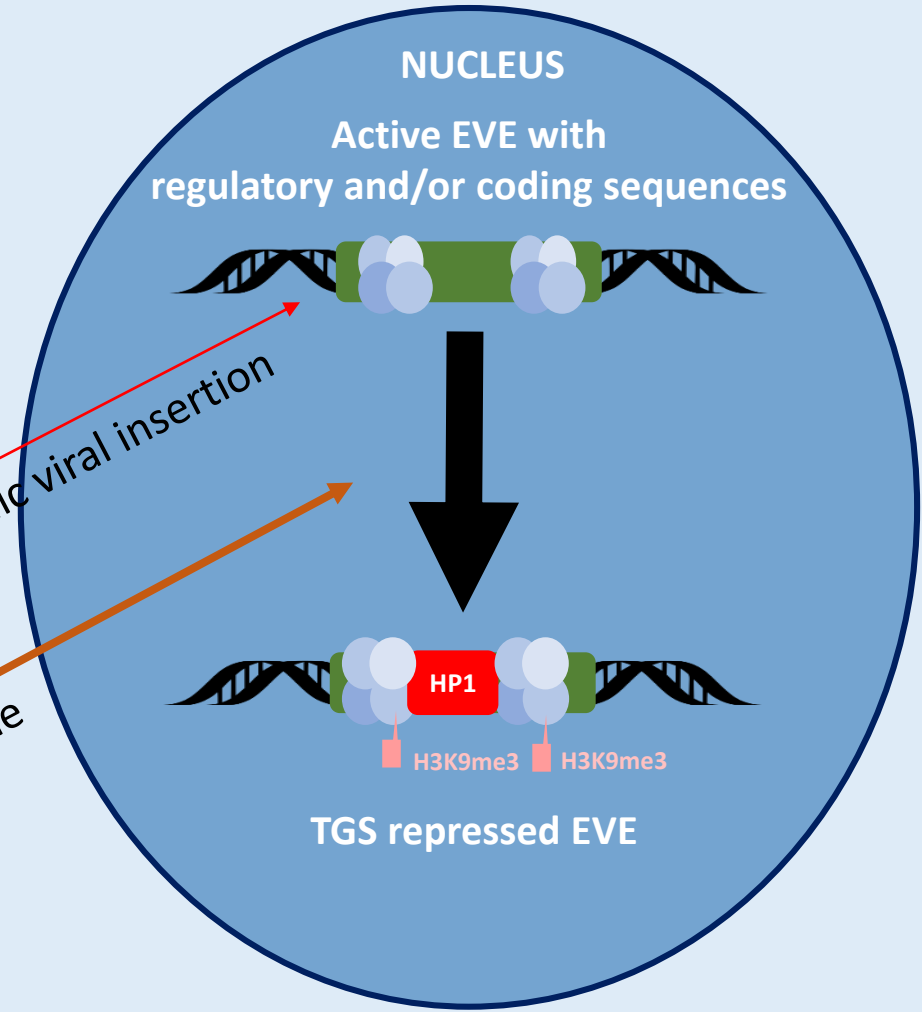
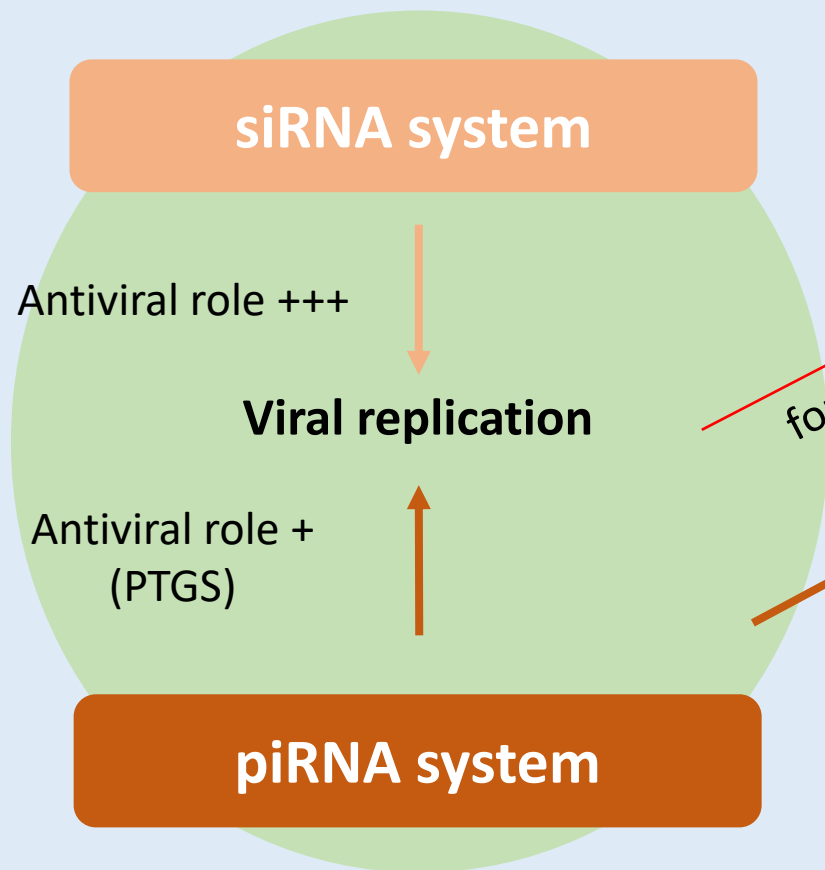


Figure 3 : the secondary piRNA ping-pong signature.



piRNAs TGS role

CYTOPLASM

Figure 4: potential role of the piRNA pathway in transcriptional gene silencing of a recently integrated EVE. Presence of an EVE in a genome must have arisen from an insertion of a viral copy during a viral infection. This newly inserted copy (named here “Active EVE”) potentially provides regulatory and / or coding sequences that could interfere with cell function. We propose here that a role of piRNAs produced by piRNA clusters would be to repress these sequences by TGS as it has been shown for the TEs.