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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 09 April 2009
Final form accepted: 24 November 2009
Published online: 27 November 2009

Volume 15 (2010) pp 118-133
DOI: 10.2478/s11658-009-0038-9

Research article

THE TELOMERE-SPECIFIC NON-LTR RETROTRANSPOSONS SART1 AND TRAS1 ARE SUPPRESSED BY PIWI SUBFAMILY PROTEINS IN THE SILKWORM, *Bombyx mori*

TSUNEYUKI TATSUKE[1,2], KOSUKE SAKASHITA[1], YUKI MASAKI[1], JAE MAN LEE[1], YUTAKA KAWAGUCHI[1] and TAKAHIRO KUSAKABE[1]*
[1]Laboratory of Silkworm Science, Kyushu University Graduate School of Bioresource and Bioenvironmental Sciences, Hakozaki 6-10-1, Fukuoka 812-8581, Japan, [2]Research Fellow of the Japan Society for the Promotion of Science

Abstract: The telomere structures in *Bombyx mori* are thought to be maintained mainly by the transposition of the specialized telomeric retroelements SART and TRAS. The silkworm genome has telomeric TTAGG repeats and telomerase, but this telomerase displays little or no activity. Here, we report that the transcription of the telomeric retroelements SART1 and TRAS1 is suppressed by the silkworm Piwi subfamily proteins BmAgo3 and Siwi. The silkworm Piwi subfamily was found to be expressed predominantly in the gonads and early embryo, as in other model organisms, but in BmN4 cultured cells, these proteins formed granules that were separate from the nuage, which is a different behaviour pattern. The expression of TRAS1 was increased in BmN4 cells when BmAgo3 or Siwi were silenced by RNAi. Our results suggest that *B. mori* Piwi proteins are involved in regulating the transposition of telomeric retroelements, and that the functional piRNA pathway is conserved in BmN4 cultured cells.

Key words: *Bombyx mori*, SART1, TRAS1, Telomere, Piwi

INTRODUCTION

The members of the Argonaute family, defined by the presence of the PAZ and PIWI domains, are known to be essential components of pathways such as RNA interference (RNAi), translational repression and heterochromatin formation, all of which are commonly referred to as RNA-silencing pathways [1]. *Drosophila*

* Author for correspondence. e-mail: kusakabe@agr.kyushu-u.ac.jp; tel./fax: +81-92-642-2842

Abbreviations used: Ago – argonaute; miRNA – microRNA; piRNA – Piwi-interacting RNA; siRNA – small interfering RNA

melanogaster has five genes belonging to the Argonaute family: AGO1, AGO2, AGO3, Aubergine and Piwi [1]. AGO1 and AGO2, the members of Argonaute subfamily, are expressed ubiquitously and constitutively, and function in gene silencing events through specific binding to miRNA or siRNA. By contrast, AGO3, Aubergine and Piwi, the members of the Piwi subfamily, are expressed only in germline linkage and are believed to participate in heterochromatin formation and the silencing of endogenous selfish genetic elements such as retrotransposons, although their detailed molecular mechanism of activity remains unknown [2, 3]. In *D. melanogaster*, Piwi proteins regulate the telomere-specific retroelements *HeT-A*, *TART* and *TAHRE*, thereby maintaining the fly genome's telomere length irrespective of short DNA repeat and telomerase activity [4, 5]. It has been demonstrated that the DmPiwi protein binds piRNAs (piwi-interacting RNA), which are small RNAs (24-31 nt) processed from single-strand RNA precursors transcribed from retroelements and heterochromatic regions [2, 6]. It is common to assume that piRNAs are generated through the ping-pong model, a reciprocal cleavage mechanism directed by two distinct populations of piRNAs: the piRNAs associated with DmAgo3 have 'A' rich in 10 nt from the 5' end; and those bound by DmAub and DmPiwi have 'U' rich in the first nucleotide from the 5' end [6, 7]. In *B. mori*, 38,493 kinds of small RNA have been identified in the ovaries [8]. The majority of these small RNAs are homologous to retroelements, mainly non-LTR retrotransposons, but their relationship to the Piwi proteins remains unclear. Recently, the presence of Ago3 or Siwi complexes with PIWI-interacting RNA in BmN4, a *B. mori* ovary-derived cultured cell line, was reported [9].

In this study, we found that the silkworm, *Bombyx mori*, has four Argonaute family proteins: Ago1, Ago2, Ago3 and Siwi. In our previous study, BmAgo2 was found to inhibit extrachromosomal recombination repairs such as homologous recombination induced by DNA double-strand breaks (DSBs), and the invasion of exogenous DNAs into the genome [10], whereas the function of the other three proteins is unknown. The telomere in *B. mori* contains telomeric short DNA TTAGG repeats and two retroelements, SART and TRAS [11]. However, no telomerase activity has been detected in any tissues despite the telomerase homolog in their genome [12, 13]. Here, we report that the *B. mori* telomere-specific retroelements SART1 and TRAS1 are suppressed by the silkworm Piwi subfamily proteins BmAgo3 and Siwi in BmN4 cells.

MATERIALS AND METHODS

Molecular cloning of *B. mori* Argonaute and VLG genes

The entry clones for BmAgo1, BmAgo3, Siwi and BmVLG were constructed on a pENTR™11 from Invitrogen. To obtain the entry clone for these genes, the total RNA was isolated from testes of the silkworm strain p50, stocked in our university. This total RNA was reverse-transcribed with oligo (dT) primer and

amplified with the three primer sets: BmAgo1ATG and BmAgo1StopXho; BmAgo3ATG and BmAgo3StopXho; and SiwiATG and SiwiStopSal or BmVLGATG and BmVLGStopXho. The primer sets were designed from the registered *B. mori* sequence data. The amplified BmAgo1, BmAgo3, Siwi and BmVLG cDNA were digested with *Xho*I or *Sal*I and subcloned in between the blunt-ended-*Nco*I site and the *Xho*I or *Sal*I site of the pENTR™11. The resulting plasmids were named BmAgo1-pENTR11, BmAgo3-pENTR11, Siwi-pENTR11 and BmVLG-pENTR11, and their nucleotide sequences were determined using a ThermoSequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Bioscience). N-terminal fusion DEST vectors, pie2HW, pie2GW or pie2RW, which respectively contain HA peptide, GFP or DsRed-tags, were transcribed by the IE-2 promoter from the Orgyia pseudotsugata nuclear polyhedrosis virus (OpNPV; the promoter is abbreviated as OpIE2). The Gateway® LR reaction between the entry vectors and the DEST vectors was performed using LR Clonase™ Enzyme Mix (Invitrogen) according to the manufacturer's protocols [Yamashita *et al.*, in preparation, 14]. The resulting plasmids were pie2-HA-Ago3, pie2-HA-Siwi, pie2-HA-VLG, pie2-R-Ago3 and pie2-G-Siwi.

Cell culture

The *B. mori* BmN4 cells used were taken from the stock of the laboratory of Insect Pathology and Microbial Control (Faculty of Agriculture, Kyushu University Graduate School). The cells were maintained in IPL-41 medium (Gibco) with 10% fetal bovine serum at 27ºC. These cells were split at a ratio of 1:7 every 4 to 5 days, seeded in 24-well plates at a density of 5×10^4 cells/well, and, one day after seeding, overlaid with a lipid-RNA complex (200 μl/well) in order to undergo transient transfection of dsRNA. Shortly before the transfection, the lipid-RNA complex was prepared by mixing 25 ng of dsRNA against each Argonaute or pI:C with 5 μl of PDD111 solution [15] in a final volume of 30 μl, followed by incubation at room temperature for 20 min and supplementation with 170 μl of Sf-900 II SFM (Gibco). The cells were incubated for 6 h, maintained for another 72 h in 500 μl of IPL-41, and then collected for RNA extraction.

Preparation of double-stranded RNA

Double-stranded RNA (dsRNA) for *in vivo* RNAi reactions was prepared using the following procedures. For the dsRNA template, BmAgo1-541, BmAgo2-578, BmAgo3-545 and Siwi-587 fragments were respectively amplified using the 4 primer pairs, BmAgo1RT5 and BmAgo1RT3, BmAgo2RT5 and BmAgo2RT3, BmAgo3RT5 and BmAgo3RT3, and SiwiRT5 and SiwiRT3, and were inserted into a blunt-ended *Bst*BI-*Xba*I site of the pENTR™11 (Tab. 1). The templates for *in vitro* transcription were synthesized via two-step PCR on the templates containing the BmAgo1-541, BmAgo2-578, BmAgo3-545 or Siwi-587 fragments using the primers attL1toT7 and attL2toT7 for the first PCR,

and the T7 primer for the second PCR (Tab. 1). The thus-amplified DNA templates were extracted with phenol/chloroform, precipitated with ethanol, and dissolved in H_2O. Bi-directional transcription reactions were performed in the buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, 2 mM NTPs, 8 units of RNase inhibitor, and 20 units of T7 RNA polymerase), and incubated for 2 h at 37ºC. The RNA products were purified and dissolved in 100 mM HEPES, pH 7.0, incubated for 5 min at 94ºC, and left to stand at room temperature for 30 min to allow the annealing of the two RNA strands.

Expression analysis of the Piwi genes

Expression profiles of *BmAGO3* and *SIWI* mRNA were respectively analyzed in 40 and 32 cycles of RT-PCR against the cDNA reverse-transcribed from each tissue and embryo of the p50 strain. The respective primers used were BmAgo3RT5 and BmAgo3RT3 for *BmAGO3* mRNAs and SiwiRT5 and SiwiRT3 for *SIWI* mRNAs (Tab. 1). As a control, actin mRNAs were amplified in 30 cycles of RT-PCR with the primers BmActRT5 and BmActRT3 (Tab.1). The amplified products were separated by electrophoresis through a 1% agarose gel in TAE buffer and stained with ethidium bromide.

Strand-specific RT-PCR

To detect the sense and antisense transcription of SART1 and TRAS1 individually, the total RNA was isolated from the BmN4 cells, and reverse-transcribed with the strand-specific primers SART1SS, SART1AS, TRAS1SS or TRAS1AS. The expression profiles of the sense and antisense transcripts were analyzed via 30 cycles of RT-PCR against the cDNA reverse-transcribed from BmN4 cells using SART1RT5 and SART1RT3 or TRAS1RT5 and TRAS1RT3.

Immunofluorescence

Immunostainings were performed using standard procedures. Anti-BmAgo3 IgG and Anti-Siwi IgG (from rabbit) were used at a 1:100 dilution. Cy3-conjugated anti-rabbit IgG (from goat, GE Healthcare) was used as the secondary antibody at a 1:300 dilution. All the images were collected using a KEYENCE BZ-8000.

Immunoprecipitation

BmN4 cells were suspended in the immunoprecipitation buffer, which contained 30 mM HEPES-KOH (pH 7.3), 150 mM KOAc, 2 mM MgOAc, 5 mM DTT, 0.1% NP40, and 50 μg/ml Leupeptin, and the lysates were left to stand for 5 min on ice. The lysates passed several times through a 25 gauge needle, and were clarified by centrifugation for 20 min at 14,000 g. The supernatants were immunoprecipitated with the protein G Sepharose, pre-incubated with the anti-Ago3 or anti-Siwi antibodies as appropriate. The mixture was incubated at 4ºC for 1 h, and washed eight times with immunoprecipitation buffer. The proteins bound to the beads were separated on an 8% SDS-PAGE and transferred onto a PVDF membrane, which was blocked with 5% skimmed milk in Tris-buffered

Tab. 1. The list of primers used in this study.

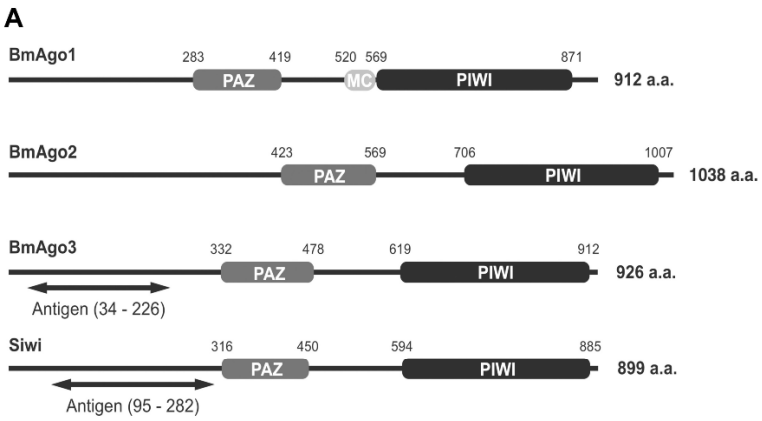
Primer name	Primer sequence (5'-3')
BmAgo1ATG	TACCCCGTTGGTCAACCTCCAGCGGGG
BmAgo1StopXho	GGCTCGAGTTAGGCGAAGTACATGACCTTCTTGGTGACGG
BmAgo3ATG	GCAGACCCAGGCAAAGGCCGGGGGCGAAGC
BmAgo3StopXho	CCCTCGAGCTACAAAAAGAACAGCTTATCGACTAAAACG
SiwiATG	TCAGAACCGAGAGGTAGAGGACGAGCTCG
SiwiStopSal	GTCGACTTAGAGGAAATATAAAGTTTC
BmVLGATG	GATGATGACTGGGATGATTCCTGTGAAGC
BmVLGStopXho	CGAGCTCGAGCTACCATTCTTCTTCAGGTTCC
BmAgo1RT5	CTCTGGATGTAGTGATGAGACATTTACC
BmAgo1RT3	AGAGGTAGGTATGTGTGTTTGTGTTCC
BmAgo2RT5	CTTAATGCACCAACTGACACCATAC
BmAgo2RT3	AGTTCCATCGGTAGATAGATGTTCTTG
BmAgo3RT5	TCTTCCAAGAGAAGGGTCAAGAAATAG
BmAgo3RT3	CTGTCCCACTAGATACGAGAGTTTGTG
SiwiRT5	CCTGAGTTGATATATCTAGTGCCAGAAC
SiwiRT3	TCATACCTATCAGCATAATTCCTAGCC
SART1SS	CTCTTTCGCCTGAGTGCAACTGAGTTTGGG
SART1AS	ATGTCCAGTTATAAAGAAGAATTACCCCAG
SART1RT5	TATTGTGAAGGTTGGGGAAATAAAAATGGG
SART1RT3	TTGAGGTTGCCCTGTAGTATATGATAAGGG
TRAS1SS	TTTATTTTCAAGCTCAAACCTCTGCTTCCC
TRAS1AS	AAACTCTCCTCCGAAAAACGCAGATCATGC
TRAS1RT5	ACCTGAAACAAAAAGAAAAATATGAGTCGG
TRAS1RT3	ATCTATGCTCTAAGCACTTATTGATTAGGC
BmActRT5	GACCCAGATCATGTTCGAAACATTCAACAC
BmActRT3	CCAGGGTACATGGTGGTACCACCGGACA
attL1toT7	TAATACGACTCACTATAGGGTTTGTACAAAAAAGCAGGC
attL2toT7	TAATACGACTCACTATAGGGACTTTGTACAAGAAAGCTGG
T7	CAGTGAATTGTAATACGACTCACTATAGGG

saline (TBS; 10 mM Tris and 150 mM NaCI, pH 7.6) containing 0.1% Tween-20 (TBS-T). The membrane was reacted with anti-HA F7 antibodies (Santa Cruz Biotechnology) and then with rabbit anti-mouse IgG (Sigma) coupled to alkaline phosphatase. After intensive washes, the co-immunoprecipitated proteins were visualized using the CDP-Star chemiluminescent system (Tropix).

RESULTS AND DISSCUSSION

Molecular cloning of *Bombyx mori* Argonaute genes

Four argonaute cDNAs, *AGO1*, *AGO2*, *AGO3* and *SIWI*, were isolated from the silkworm, *B. mori* (Fig. 1). *AGO1* and *AGO2* are members of the Argonaute subfamily and *AGO3* and *SIWI* are members of the Piwi subfamily, as assessed by their amino acid sequence homology. All of them have a PAZ domain and a PIWI domain. The PAZ domain recognizes the single-stranded 3' end of the small RNA. The PIWI domain has an RNase H fold and a 5' binding pocket.



B

	N-Terminal	PAZ	Mid	PIWI
BmAgo1/DmAgo1	64.9 / 69.6	96.4 / 98.5	83.8 / 88.8	94.8 / 98.0
BmAgo2/DmAgo2	13.7 / 24.5	32.5 / 48.3	22.1 / 37.8	42.8 / 59.6
BmAgo3/DmAgo3	27.1 / 43.6	34.9 / 59.7	27.0 / 53.2	52.2 / 64.8
Siwi/DmPiwi	33.1 / 48.3	41.5 / 70.4	30.3 / 53.8	46.6 / 71.4
Siwi/DmAubergine	30.8 / 47.6	39.0 / 64.5	32.9 / 50.3	52.2 / 68.4

Identity / Similarity (%)

Fig. 1. The molecular structures of *BmAGO3* and *SIWI*. A – Schematic stuructures of the *B. mori* Argonaute family proteins. The indicated regions were used as antigens. 193 amino acids at the N terminus of BmAgo3 and 188 amino acids at the N terminus of Siwi fused with 6 x His-tag were used as the antigen to immunize a rabbit. The cDNA sequences of *BmAGO1*, *BmAGO2*, *BmAGO3* and *SIWI* have been submitted to GenBank under the respective accession nos. AB332314, AB206986, AB332312 and AB332313. B – The levels of identity and similarity of each of the domains of the silkworm Argonaute proteins with the corresponding regions from fly Argonaute proteins are shown. N-Terminal: N-Terminal domain; PAZ: PAZ domain; Mid: Middle domain; PIWI: PIWI domain.

The D-D-H motif, which is a catalytic triad within the PIWI domain and is required for their slicer activity, was conserved in their amino acid sequences. Therefore, these four Argonaute proteins are expected to have the ability to cleave the target RNA. Only Ago1 has an MC domain that is similar to the m^7G cap-binding domain of eIF4E, as previously reported [16].

BmAGO3 and *SIWI* are expressed predominantly in the gonads and early embryo

D. melanogaster has five Argonaute genes: *ago1*, *ago2*, *ago3*, *aubergine* and *piwi*. The Argonaute subfamily proteins DmAGO1 and AGO2 are localized to the cytoplasm and expressed constitutively and ubiquitously. The Piwi subfamily proteins DmAGO3 and Aubergine exist in the nuage (the "cloud"), in granules containing putative piRNA processing-related proteins such as Vasa, Spindle-E and Maelstrom, and their expression is limited to germ-line cells [6, 17]. Only Piwi is localized to the nucleus in germ cells, and it interacts with heterochromatin protein 1a [6].

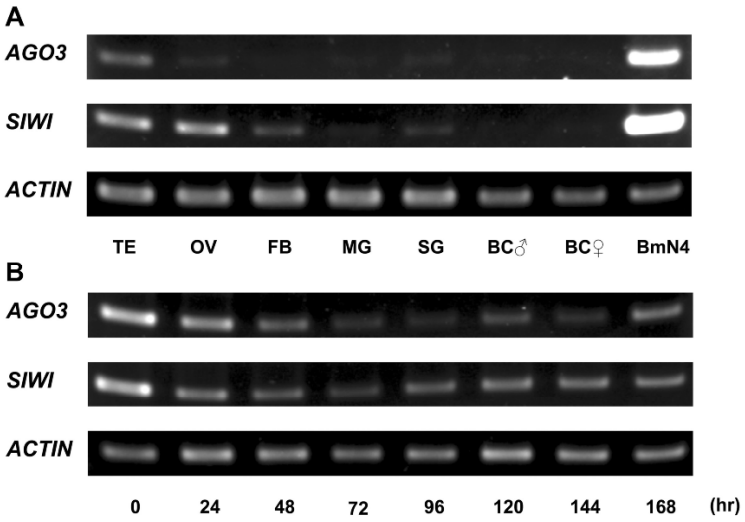


Fig. 2. The expression pattern of the *B. mori* Piwi subfamily proteins, *BmAGO3* and *SIWI*. A – The expression pattern of *BmAGO3* and *SIWI* in various tissues of *B. mori* day 3 fifth instar larvae. Semi-quantitative RT-PCR analyses were performed using the cDNA library from the testes (TE), ovaries (OV), fat bodies (FB), midgut (MG), silk gland (SG), male and female blood cells (BC♂ and BC♀) and *B. mori* cultured cells BmN4. B – The expression patterns of *BmAGO3* and *SIWI* through embryogenesis. Semi-quantitative RT-PCR analyses were performed using the cDNA library from embryos (0 to 168 h).

To examine the *BmAGO3* and *SIWI* expression patterns, we performed semi-quantitative RT-PCR analyses on various tissues from *B. mori* larvae on day 3 of the fifth instar. *BmAGO3* and *SIWI* were expressed predominantly in the gonads

and BmN4 cultured cells (Fig. 2A). In *D. melanogaster*, the Piwi subfamily genes are strongly expressed in the testes and ovaries [2, 18]. The expression patterns of the *B. mori* Piwi subfamily genes appear to be similar to those of *D. melanogaster* and *Mus musculus*. This result indicates the possibility that BmAgo3 and Siwi participate in germ line cell maintenance, like the *D. melanogaster* Piwi subfamily and others [19, 20].

Next, to explore the role of the *B. mori* Piwi subfamily in embryogenesis, we examined the expression pattern of *BmAGO3* and *SIWI* through embryogenesis. *BmAGO3* and *SIWI* were strongly expressed in the earlier embryonic stages, but the level of *BmAGO3* decreased synchronously as development proceeded, while *SIWI* seemed to be upregulated from 72 to 96 h after oviposition (Fig. 2B). This expression pattern is similar to that of the *Drosophila* Piwi subfamily through embryogenesis. These results appear to indicate that *BmAGO3* and *SIWI* are involved in germ line cell generation or embryogenesis.

The expression of silkworm Piwi proteins is detected in the embryo, gonad and BmN4 cells

To investigate the molecular function of the *B. mori* Piwi subfamily proteins in gene silencing, we produced polyclonal antibodies against BmAgo3 and Siwi. As an antigen, we used the N-terminal region of BmAgo3 and Siwi, because there is much less similarity to the other members of the *B. mori* Argonaute family proteins (Fig. 1). Western blotting using a BmN4 cell lysate shows the specific binding of the anti-BmAgo3 and anti-Siwi antibody to the target proteins (Fig. 3A). To confirm this result, we performed the immuno-precipitation of HA-Ago3 or HA-Siwi from the lysates of BmN4 cells transformed with HA-Ago3 or HA-Siwi expression vectors (Fig. 3B). As expected, the respective precipitations of HA-Ago3 and HA-Siwi by the anti-Ago3 or Siwi antibodies were detected using anti-HA antibody. These results confirmed that these antibodies specifically recognize BmAgo3 and Siwi. Western blotting using BmAgo3- or Siwi-specific antibodies revealed that BmAgo3 and Siwi proteins are expressed in the testes and ovaries (Fig. 3C). We could not detect the expression of the BmAgo3 and Siwi proteins in other tissues (data not shown). In agreement with the results obtained via RT-PCR analysis, BmAgo3 was expressed in the early embryo, but its expression decreased in the later stages of development, while Siwi was stably expressed throughout embryogenesis.

***BmAGO3* and *SIWI* are localized to the cytoplasm and form granules that are different from the nuage in BmN4 cells**

To show the subcellular localization of BmAgo3 and Siwi in BmN4 cultured cells, immunofluorescent staining was carried out using the polyclonal antibody against BmAgo3 or Siwi. Unlike DmPiwi, which exhibits a nuclear localization [21], immunofluorescent staining showed that BmAgo3 and Siwi primarily localized in the cytoplasm and formed granules (Fig. 4A). Interestingly, the

granules observed were not the nuage, a germline-specific organelle including Vasa, Spindle-E and Maelstrom, because these granules were not co-localized with the *B. mori* Vasa-like gene (BmVLG), which is a marker gene of the nuage localized around the nucleus (Fig. 4B, [17, 22]).

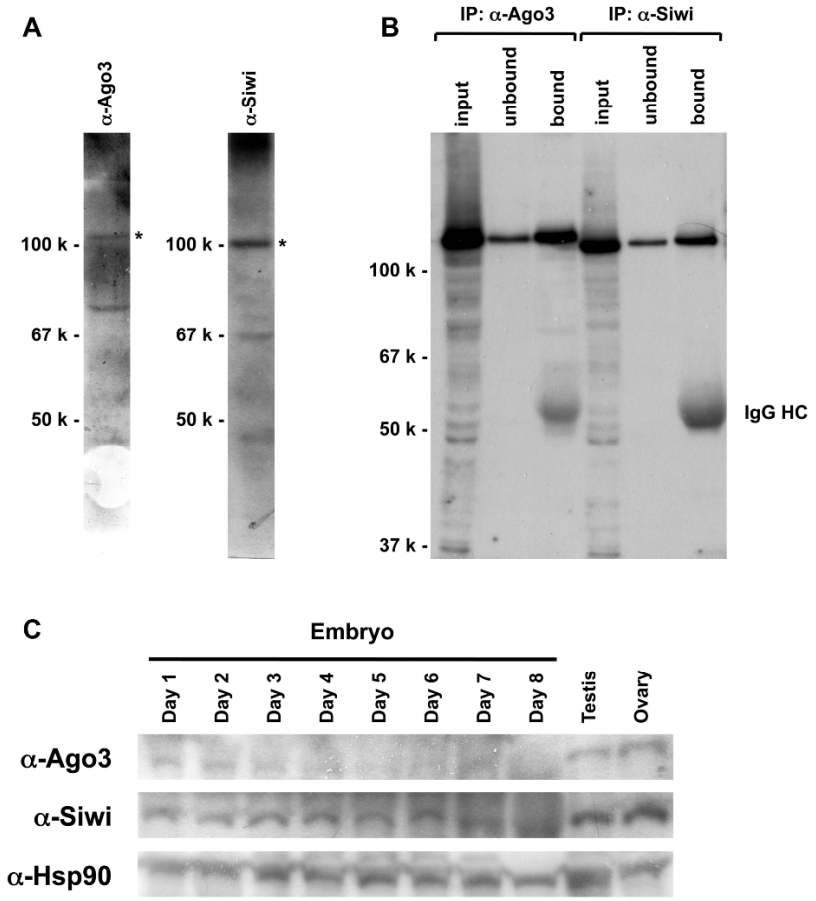


Fig. 3. Western blot analysis of BmAgo3 and Siwi expression. A – Western blotting was performed on a lysate of BmN4 cells using antibodies against BmAgo3 and Siwi (1:1000 dilution). The asterisks indicates the positions of the BmAgo3 or Siwi protein. B – Western blotting was performed using the immunoprecipitates yielded by anti-BmAgo3 or Siwi antibodies from the extracts of BmN4 cells expressing HA-Ago3 or HA-Siwi, and visualized using anti-HA antibody. C – The silkworm Piwi subfamily proteins were detected in the embryo, testes and ovaries. Western blotting was performed on lysates of silkworm embryos (days 1 to 8 after the acid treatment), testes and ovaries using an antibody against BmAgo3, Siwi or Hsp90 (1:1000 dilution). The polyclonal anti-Hsp90 antibody from our laboratory stock was used. A polyclonal antibody against *E. coli*-expressed recombinant His-BmHsp90 protein was generated. Each lane with the label "embryo" was loaded with equal amounts of protein.

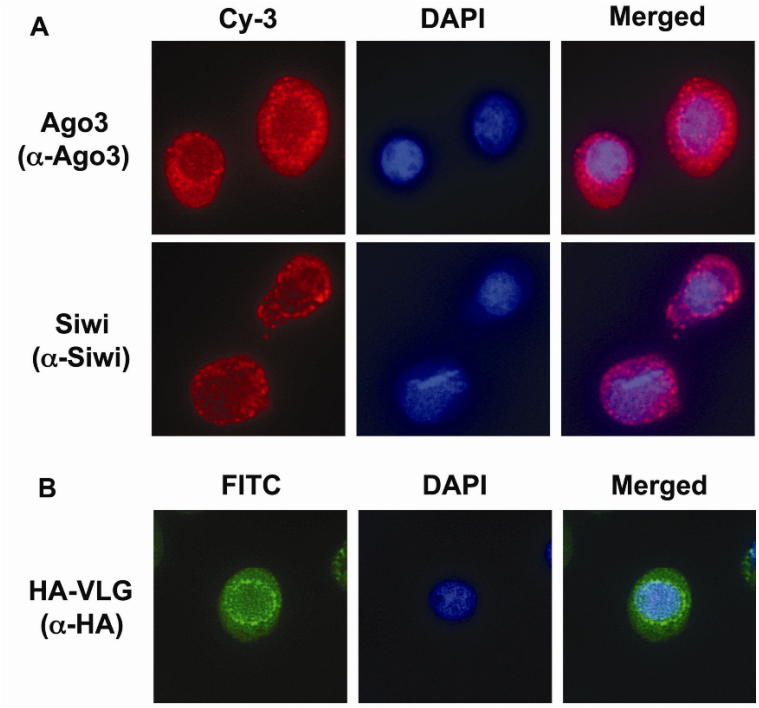


Fig. 4. The subcellular localization of BmAgo3, Siwi and BmVLG in BmN4 cells. A – Immunofluorescent staining of the *B. mori* Piwi subfamily proteins in BmN4 cells using polyclonal antibodies against BmAgo3 or Siwi. B – Immunofluorescent staining of HA-tag fused BmVLG proteins in BmN4 cells using the monoclonal antibody against HA. FITC-conjugated anti-mouse IgG (from rabbit, Sigma) was used as the secondary antibody.

Several studies reported on the differences in the numbers of Piwi subfamily genes and their localizations in different species. In the case of the red flour beetle, *Tribolium castaneum*, there are two Piwi subfamily genes [23]. The differences in the number and localization of Piwi subfamily genes between *D. melanogaster* and other insect species may suggest that the *D. melanogaster* Piwi gene is inherent to the Dipteran, and Siwi may have properties intermediate between Aubergine and Piwi, to some extent retain the features of the ancestral insect Piwi protein. Mammals, for example *M. musculus*, have three or four Piwi genes, which are localized to the cytoplasm but related to heterochromatin formation mediated by *de novo* DNA methylation [24]. MIWI2, one of the mouse Piwi proteins, may shuttle between the nucleus and cytoplasm [25]. In mice, each of the Piwi proteins MIWI, MIWI2 and MILI are expressed in different stages of embryogenesis. The differentiation of mammalian Piwi homolog proteins is considered to be temporal, but in *Drosophila* it is spatial.

The existence of small RNAs derived from the sense and antisense transcripts of SART1 and TRAS1

In *Drosophila*, the telomere-specific transposons *HeT-A*, *TART* and *TAHRE* are regulated by the Piwi subfamily proteins [5]. Therefore, we assumed that the silkworm Piwi subfamily proteins participate in telomere-specific retroelement silencing. The *B. mori* telomere structure is described in Fig. 5A. *B. mori* has two telomere-specific retroelements, SART and TRAS, and telomeric TTAGG repeats in their telomere (Fig. 5B), predicting that the *B. mori* telomere is maintained by a combination of the self-sacrificial transposition of these transposons and the elongation of the telomeric repeats. However, *B. mori* telomerase activity was not detected in any stage of development of the various tissues or in any of the cell lines [12]. Thus, the *B. mori* telomere is suggested to be regulated mainly by the telomere-specific retroelements SART and TRAS. Since SART1 and TRAS1 are well-studied transposons of *B. mori* in terms of the molecular mechanisms of their transcription and transposition [26-30], we selected these transposons as candidates for the target model for Piwi subfamily-mediated silencing.

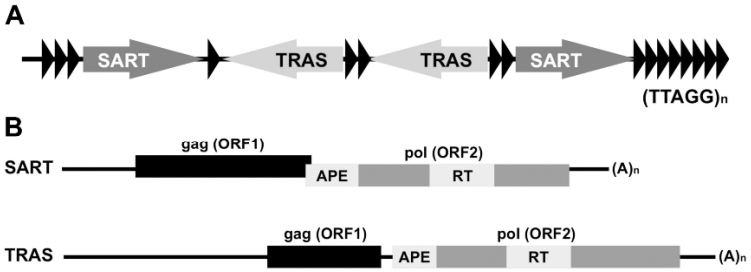


Fig. 5. The typical *B. mori* telomere structure and telomere-specific non-LTR retrotransposons. A – A schematic diagram of the *B. mori* telomere structure. The black triangles represent the telomeric repeats (TTAGG). B – Schematic structures of the *B. mori* telomere-specific non-LTR retrotransposons. APE: AP endonuclease domain; RT: reverse transcriptase domain; $(A)_n$: poly(A) tail.

First, we examined whether the SART1 and TRAS1 regions are transcribed in a bidirectional manner in BmN4 cells. As shown in Fig. 6A, the bidirectional transcription of the SART1 and TRAS1 regions was demonstrated, although the transcriptional start site of the antisense transcripts was unknown. Since the ping-pong model required the presence of long sense and antisense transcripts, it is also approved in silkworm germ cells.

Next, we searched for small RNA derived from TRAS1 using the *B. mori* small RNA library sequences reported on previously [8, 9]. When the database search for TRAS1 as a query sequence was carried out against small RNA databases constructed from silkworm ovary and BmN4 cells (AB386191–AB424683, AHAAC0000001-AHAAC1704525, AHAAD0000001-AHAAD2196220) using NCBI BLAST under the loose search conditions taking into consideration the

presence of many variant forms, the equilibrium distributions of the small RNAs were detected throughout the TRAS1 regions (Fig. 6B). The distribution of small RNA against SART1 was reported on previously [8, 9].

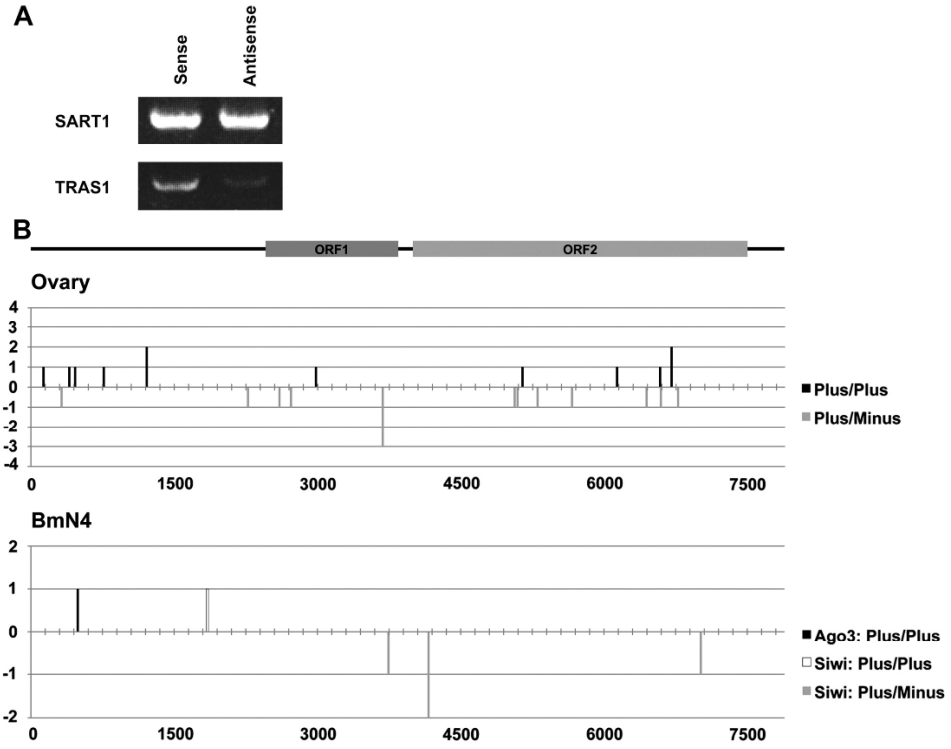


Fig. 6. Small RNAs generated in silkworm ovaries and cultured cells. A – Sense and antisense transcripts from SART1 and TRAS1. B – The distribution of small RNA against TRAS1 in the ovaries or BmN4 cells. The type of small RNA against TRAS1 is shown (E-value > 0.0001). The TRAS1 nucleotide position is indicated along the x-axis. The number of small RNA types is indicated along the y-axis.

The Piwi subfamily-mediated suppression of SART1 and TRAS1

On the basis of the existence of small RNAs against SART1 and TRAS1 in the ovaries and BmN4 cells, it is postulated that their transcription is silenced by Argonaute proteins. At first, we verified the efficiency of dsRNA knockdown using the BmN4 cells co-transfected with the DsRed-Ago3 and GFP-Siwi expression vectors. In the presence of dsRNA, DsRed-Ago3 or GFP-Siwi expression was downregulated specifically depending on the dsRNA species introduced (Fig. 7A). In order to study the above possibility, we performed semi-quantitative RT-PCR using cDNA from BmN4 cells under the conditions in which one of four Argonaute genes is knocked down. As expected, the SART1 and TRAS1 expression levels were increased in the BmAgo3- or Siwi-silenced

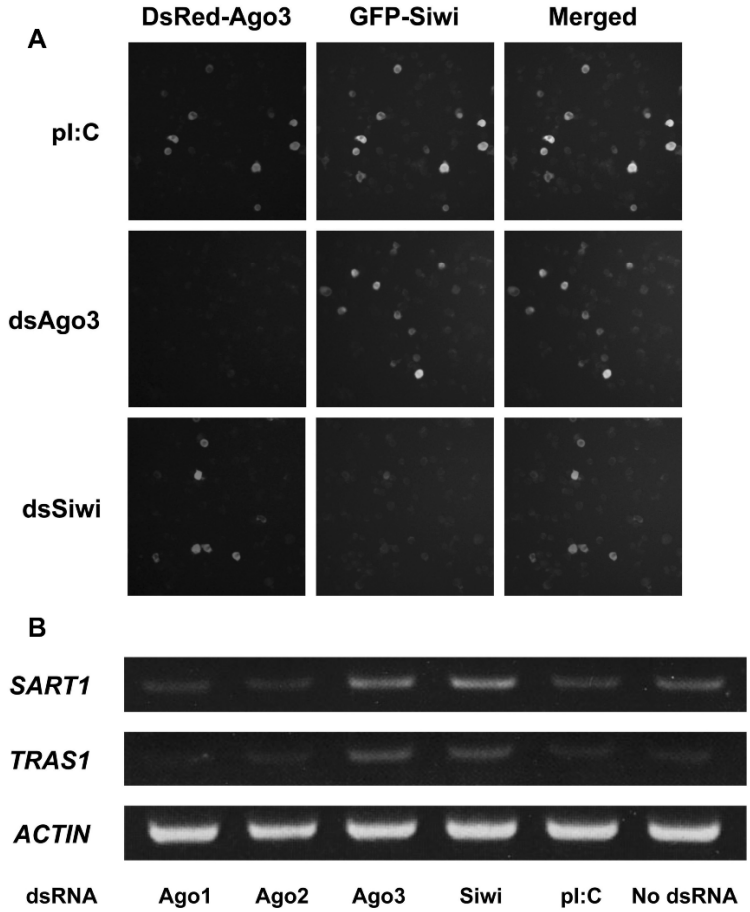


Fig. 7. SART1 and TRAS1 expression in BmN4 cells under the silencing of Argonaute genes using RNAi. A – The RNAi efficiency was verified using the BmN4 cells co-transfected with DsRed-Ago3 and GFP-Siwi expression vectors and the dsRNAs for Ago3 and Siwi. B – Semi-quantitative RT-PCR analyses were performed using the cDNA library from each Argonaute gene knocked-down BmN4 cells.

BmN4 cells (Fig. 7B), suggesting that a functional piRNA pathway in BmN4 cells would play an important role in repressing the transposable elements. Combining the recent report [9] and the results obtained in this study, we can see that BmN4 cells are useful in studying the Piwi subfamily proteins, the piRNA pathway and their manner of silencing.

In spite of the Piwi protein-mediated suppression of TRAS1, there are fewer types of small RNA derived from TRAS1 than from SART1. In agreement with the result obtained from *in silico* small RNA analysis, the transcription from TRAS1 is less active in both the sense and antisense strands than SART1, i.e. the amount of small RNAs is correlated with the transcription levels of their precursor RNAs (Fig. 6, [8]). Previous reports describing small RNA-related

gene silencing in *D. melanogaster* shows that transgenes are silenced post-transcriptionally in a copy number-dependent manner with a certain threshold at which the synthesis of small RNAs against the transgenes are initiated [31]. From this point of view, the transcriptional level of the SART1 region may greatly exceed the threshold level and initiate the synthesis of the small RNA against SART1, while the synthesis of that against TRAS1 remains modest.

Acknowledgements. This study was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Integrated research project for plant, insect and animal using genome technology INSECT-1201) and by Japan Society for the Promotion of Science (JSPS).

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