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# Functional analysis of the RNAi response in ovary-derived silkmoth Bm5 cells

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ABSTRACT

Experiments of dsRNA-mediated gene silencing in lepidopteran insects *in vivo* are characterized by high variability although lepidopteran cell cultures have shown an efficient response to RNAi in transfection experiments. In order to identify the core RNAi factors that regulate the RNAi response of Lepidoptera, we employed the silkmoth ovary-derived Bm5 cells as a test system since this cell line is known to respond potently in silencing after dsRNA transfection. Two parallel approaches were used; involving knock-down of the core RNAi genes or over-expression of the main siRNA pathway factors, in order to study possible inhibition or stimulation of the RNAi silencing response, respectively. Components from all three main small RNA pathways (BmAgo-1 for miRNA, BmAgo-2/BmDcr-2 for siRNA, and BmAgo-3 for piRNA) were found to be involved in the RNAi response that is triggered by dsRNA. Since BmAgo-3, a factor in the piRNA pathway that functions independent of Dicer in *Drosophila*, was identified as a limiting factor in the RNAi response, sense and antisense ssRNA was also tested to induce gene silencing but proved to be ineffective, suggesting a dsRNA-dependent role for BmAgo-3 in *Bombyx mori*. After efficient over-expression of the main siRNA factors, immunofluorescence staining revealed a predominant cytoplasmic localization in Bm5 cells. This is the first study in Lepidoptera to provide evidence for possible overlapping of all three known small RNA pathways in the regulation of the dsRNA-mediated silencing response using transfected *B. mori*-derived Bm5 cells as experimental system.



## 1. Introduction

Gene silencing initiated by double-stranded RNA (dsRNA) molecules is an experimental approach that has been well established during the past 15 years and is now termed RNA interference (RNAi). Since its discovery (Fire et al., 1998), a new field on biological research has started to develop, as RNAi is considered a promising tool of reverse genetics with valuable applications including insect pest control (Burand and Hunter, 2012; Swevers and Smagghe, 2012). However, the success of experiments involving RNAi is not always guaranteed. As for insects, much information has been collected concerning the variability of the efficiency of RNAi that depends on species-, tissue- and gene-specific factors (Belles, 2010). Especially for Lepidoptera, while efficiency after transfection of dsRNA or shRNA expression plasmids in cell culture is high (Kim et al., 2012; Swevers et al., 2011; Tanaka et al., 2009), experiments involving *in vivo* silencing present high inconsistency (Swevers and Smagghe, 2012; Terenius et al., 2011).

Among insects, the RNAi machinery has been well analyzed in *Drosophila*, where three pathways have been uncovered. A basic characteristic of the RNAi process is the generation of small RNA molecules (~20–30 nucleotides), which are classified as microRNAs (miRNAs), short-interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs), depending on their sequence, structure, biogenesis and mechanism of action. These small RNAs are able to act with high specificity by identifying and silencing target genes that possess complementary sequences (Obbard et al., 2009). Both miRNA and siRNA pathways are triggered by precursor molecules with dsRNA structures that constitute substrates of ribonuclease-III-type enzymes (dsRNA-specific nucleases), called Dicers (Jinek and Doudna, 2009). Fragmentation of longer dsRNA molecules caused by Dicers associated with dsRNA-binding proteins (Drosha/Pasha and Dcr-1/Loqs for miRNAs; Dcr-2/R2D2 for siRNAs) results in the production of small RNAs (Siomi and Siomi, 2009; Tomari et al., 2007). Differences in the intrinsic structure of the miRNA and siRNA duplexes are critical for the sorting to the respective Argonaute-containing RNA-induced silencing complex

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(Ago-1/RISC or Ago-2/RISC, respectively) (Tomari et al., 2007). Subsequently, the transcriptome is screened by Ago-RISC in order to detect target sequences (Siomi and Siomi, 2009) and efficiently silence them by deadenylation leading to translational repression (miRNA pathway) or by direct degradation (siRNA pathway) (Jinek and Doudna, 2009). Regarding the piRNA pathway, for which Dicer enzymes are dispensable, it has been reported to act as a guard in the germline against transposons (Vagin et al., 2006). In this case, the precursor molecules have been identified as single-stranded transcripts that are processed to "primary" piRNAs in a Dicer-independent manner by an unknown mechanism. In the germline, piRNAs loaded on Argonaute proteins of the PIWI clade (i.e. PIWI, Aubergine and Argonaute-3 proteins in *Drosophila*) can function to cleave sense and antisense transcripts at complementary sites to generate "secondary" piRNAs and to amplify the RNAi response ("ping-pong" model) (Ghildiyal and Zamore, 2009; Jinek and Doudna, 2009).

Apart from the variation in their biogenesis, in *Drosophila* the miRNA and siRNA pathways also seem to differentiate in terms of their functional roles. Recently, it was shown that Ago-2, Dcr-2 and R2D2 belong to the top 3% of fastest evolving genes, in sharp contrast to the miRNA pathway genes (Obbard et al., 2006). Moreover, viral suppressors of RNAi (VSRs) have been identified that inhibit efficiently the siRNA, but not the miRNA pathway (Berry et al., 2009; Li et al., 2002; van Rij et al., 2006; Wang et al., 2006). *Drosophila* siRNA pathway mutants are highly susceptible to viral infections that result in high viral titers compared with wild-type as well as in the detection of viral siRNAs (Kemp and Imler, 2009). All the above add evidence to the importance of the siRNA pathway for the defense against invading nucleic acids with dsRNA structure (Ding and Voinnet, 2007). MiRNAs, on the other hand, are produced from endogenous genes and are involved in the regulation of physiological and developmental processes through translational inhibition of target mRNAs with complementary sites. Regarding the piRNA pathway, while its role in silencing of transposons in the germline is well established (Vagin et al., 2006), recent studies have also identified virus-derived small RNAs with piRNA size in insects, thus implicating a possible role of this pathway too in the defense against viral attackers (Brackney et al., 2010; Chotkowski et al., 2008; Hess et al., 2011; Scott et al., 2010; Vodovar et al., 2012; Wu et al., 2010).

In contrast to *Drosophila*, no detailed characterization of the mechanism of dsRNA-mediated gene silencing has been carried out in other insects. Nevertheless, from the literature it is clear that great differences can exist among insects regarding RNAi efficiency, at least in *in vivo* experiments. In coleopteran insects, injection of dsRNA to induce gene silencing has proven to be very efficient (Tan and Palli, 2008a, b; Zhu et al., 2011), while in Lepidoptera, such as the silkmoth *Bombyx mori*, the RNAi response is considered much less robust (Terenius et al., 2011). This raises the question regarding the limiting factors that contribute to the sensitivity/resistance of insects to RNAi. Limiting factors could act at several levels, such as dsRNA uptake and premature degradation, but of relevance might also be differences in the function of the intracellular RNAi machinery among insects. Recently, it was shown that the mRNAs of all core genes involved in the siRNA and the miRNA pathway are expressed in larval and pupal tissues of *B. mori*, with the exception of *BmR2D2* mRNA, which encodes a dsRNA-binding protein (Swevers et al., 2011). This finding raised questions, since in *Drosophila*, *r2d2* was identified as an essential gene for RNAi (cofactor of Dicer-2) as well as for the immune defense against RNA virus (Liu et al., 2003). However, despite the complete deficiency of silkworm ovary-derived Bm5 cells in *BmR2D2* expression, cell culture experiments that involved dsRNA-mediated gene silencing by transfection were very successful, possibly implying that there might exist an alternative silencing mechanism that does not require the expression of BmR2D2 (Swevers et al., 2011). This example illustrates that differences can occur in the composition of the intracellular RNAi machinery among insects, some of which may have relevance to the question of RNAi efficiency in *in vivo* gene silencing experiments.

In order to elucidate the key factors that regulate the RNAi response in *B. mori*, an adequate study system should be selected. The silkworm ovary-derived Bm5 cell line (Grace, 1967) can be efficiently transfected and transformed (Swevers et al., 2004); moreover, as already mentioned, Bm5 cells present the advantage to respond potently through the RNAi mechanism after transfection of dsRNA, and to express the majority of the mRNAs of the core genes involved in the miRNA and the siRNA pathways (Swevers et al., 2011), as well as the piRNA pathway (*BmAgo-3* and *BmSiwi*; Suppl. Fig. 1).

In the present study, first we performed an "RNAi-of-the-RNAi" assay for the core RNAi factors of *B. mori*, which revealed the involvement of four RNAi genes (*BmDcr-2*, *BmAgo-1*, *BmAgo-2* and *BmAgo-3*) belonging to all three small RNA pathways. The unexpected finding of *BmAgo-3* being involved in the RNAi response was further investigated by attempting to silence the reporter gene via employment of single-stranded sense or antisense specific RNAs. Because of the low response to ssRNA, the experiments point to a possible dsRNA-dependent function of BmAgo-3 in *B. mori*. In a reciprocal experimental approach, in which the main siRNA proteins were over-expressed, stimulating effects on dsRNA-mediated gene silencing were observed for BmDcr-2 and BmAgo-2. The three main siRNA factors, as well as the auxiliary factor BmTranslin-2, were efficiently over-expressed as recombinant proteins and were knocked-down only by gene-specific dsRNAs, confirming thus the efficiency and specificity of the RNAi assays. Finally, immunostaining of the over-expressed RNAi proteins confirmed the cytoplasmic role of all siRNA proteins in Bm5 cells.

## 2. Materials and methods

### 2.1. RNA extraction and reverse-transcription (RT-PCR)

Total RNA was extracted from silkworm (*B. mori*) tissues by use of TRI Reagent (Ambion), according to the manufacturer's instructions, and the quality/quantity of the RNA was estimated with a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Then, 1 μg of RNA was used for first-strand complementary DNA (cDNA) synthesis using RevertAid reverse transcriptase (Fermentas) and oligo-[dT] primer, according to standard protocol (Drevet et al., 1995).

### 2.2. Cloning of fragments of RNAi genes in pLitmus vector

For dsRNA synthesis, fragments of genes of the core RNAi machinery were cloned in pLitmus vector (New England Biolabs; 28i or 38i) in which the polylinker region is flanked by T7 polymerase promoter sites for RNA synthesis (Evans et al., 1995). cDNAs from silkmoth tissues served as templates for amplification of fragments representing genes of the RNAi machinery by PCR (Table 1). Gene-specific primers, spanning at least one intron, for amplification of *BmDcr-1*, *BmLoqs*, *BmAgo-1*, *BmDcr-2*, *BmR2D2*, *BmAgo-2*, *BmTranslin* and *BmTrax-B* are described in Swevers et al. (2011) (used in that work to check expression levels of the genes in various tissues). Primer pairs for amplification of *BmAgo-3* and *BmAub* were 5′-CGAAACTGCCAAGAGTCGTC-3′ (forward)/5′-TAATGGCCGCGTATCTGTCG-3′ (reverse) and 5′-AATACCCTGCAGGAGACACC-3′ (forward)/5′-GCTACTCATGTTACGAGCGC-3′ (reverse), respectively. An overview of the amplified fragments used for cloning in pLitmus vectors

**Table 1**
The fragments belonging to *Bombyx mori* RNAi genes that were cloned in pLitmus vector.

| Gene | Accession number | Small RNA pathway | pLitmus vector | Insert size (bp) | Position in relation to ATG start codon |
|---|---|---|---|---|---|
| *BmAgo-1* | AB332314 | miRNA | 38i | 444 | 1508–1951 |
| *BmAgo-2* | NP_001036995 | siRNA | 38i | 370 | 1150–1519 |
| *BmAgo-3* | NM_001104597.2 | piRNA | 28i | 425 | 1485–1909 |
| *BmAub* | NM_001104596.2 | piRNA | 38i | 383 | 1514–1896 |
| *BmDcr-1* | Sequence not annotated[a] | miRNA | 38i | 266 | – |
| *BmDcr-2* | NM_001193614 | siRNA | 38i | 449 | 3903–4351 |
| *BmLoqs* | NM_001195079 | miRNA | 38i | 500 | 238–735 |
| *BmR2D2* | NM_001195078 | siRNA | 28i | 497 | −4–493 |
| *BmTranslin* | NM_001046817 | auxiliary factor | 38i | 397 | 262–652 |
| *BmTrax-B* | Sequence not annotated[a] | auxiliary factor | 38i | 362 | – |

[a] These inserts were selected after alignment of the respective *Drosophila* genes with Kaikoblast silkworm database (Swevers et al., 2011), since BmDcr-1 and BmTrax-B have not yet been annotated in GenBank.

is presented in Table 1. PCR reactions were carried out using *Taq* DNA polymerase (HyTest) using the following program: template cDNA denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 30 s. The final elongation step took place at 72 °C for 7 min. For *BmR2D2* amplification, the above mentioned program was modified by using 55 °C as annealing temperature and the application of 40 cycles. All PCR products were sequenced to verify their identity (Suppl. Data here, and in Swevers et al. (2011)).

For cloning in pLitmus 38i, the PCR products were purified and phosphorylated by T4 polynucleotide kinase (Fermentas) for 1 h at 37 °C, blunted by T4 DNA polymerase (Fermentas) and used for ligation into the *Eco*RV restriction site of the plasmid. For cloning in pLitmus 28i, the PCR products were first cloned in pGEM-T Easy vector (Promega). After digestion at the two *Eco*RI sites of the vector, the fragments with two *Eco*RI ends were excised, purified and subcloned in the respective site of pLitmus 28i vector. The pLitmus 28i vector for production of dsRNA specific for the maltose-binding protein of *Escherichia coli* (*mal*E; used as negative control in the experiments) (Zhu et al., 2011) was obtained after subcloning of a 753 bp *Bgl*II-*Sac*I fragment from the pMAL-2C vector (NEB).

### 2.3. Expression constructs

In order to create expression constructs for BmAgo-2 and BmR2D2, the ORFs of *BmAgo-2* and *BmR2D2* were amplified by PCR from silkmoth epidermis cDNA and pENTR11-R2D2 vector (kindly provided by Professor T. Kusakabe, Kyosho University, Fukuoka, Japan), respectively. For *BmAgo-2*, the forward primer 5′-AGTA**AGATCT***CACC*ATGGCTAGAGGAAAAAACAAAGG-3′ and the reverse primer 5′-TATA**AGATCT**GACGAAGAACATACGGCTCTGTTTC-3′ were used for amplification and cloning in pEA-MycHis lepidopteran expression vector (Douris et al., 2006). *BmR2D2* was cloned in the same vector, after amplification with the forward primer 5′-CCA-T**AGATCT***CAAC*ATGAAAACTCCCATAACAGTACTG-3′ and the reverse primer 5′-CCAT**AGATCT**CAGAGCGGCGGCGGCGGA-3′. All the above mentioned primers contain a *Bgl*II cloning site (bold). Moreover, each forward primer contains a Kozak initiation sequence (italics; Kozak, 1987) and an ATG start codon (underlined), while each reverse primer is appropriately designed for in-frame cloning with the C-terminal MycHis tag of the pEA-MycHis vector (to generate pEA-BmAgo-2-MycHis and pEA-BmR2D2-MycHis expression vectors). Both vectors containing the ORFs of *BmAgo-2* and *BmR2D2* were verified by sequencing. The construction of pEA-BmTranslin-2-MycHis has previously been described in detail (Swevers et al., 2011), while pEA-Myc-BmDcr-2 was constructed after subcloning of the BmDcr-2 ORF from the pEA-Flag-Dcr-2 vector (Liu et al., 2012).

### 2.4. Synthesis of dsRNA

Each pLitmus construct (Table 1) containing an ORF fragment (266-500 bp) of the RNAi genes or *mal*E control was subjected to linearization in two separate reactions using different restriction enzymes that cut at either side of the insert. Thus, 38i-BmAgo-1, 38i-BmAub, 38i-BmDcr-1, 38i-BmDcr-2, 38i-BmLoqs, 38i-BmTranslin, 38i-BmTrax-B and 28i-BmR2D2, were linearized with *Bam*HI or *Hind*III, while 38i-BmAgo-2 was digested with *Bam*HI or *Mfe*I. Moreover, 28i-BmAgo-3 and 28i-MalE were digested with *Bgl*II or *Stu*I. Each linearized vector was used as template for single stranded RNA (ssRNA) synthesis by use of T7 RNA polymerase (Fermentas). After checking of the quality/quantity of RNA synthesis by 1% agarose gel electrophoresis and digestion of the template DNA with RNase-free DNase I (Fermentas), the synthesized ssRNAs were subjected to phenol/chloroform extraction and 70% ethanol precipitation. Finally, the ssRNAs were resuspended in annealing buffer (150 mM NaCl, 1 mM EDTA), mixed and each complementary pair was allowed to anneal by heating at 95 °C for 2 min followed by slowly cooling down to room temperature. The final dsRNA concentration was assessed by 1% agarose gel electrophoresis and ethidium bromide staining by comparing to known amounts of λ DNA marker (*Hinc*II/*Hind*III digest).

### 2.5. Cell culture and transient transfection experiments

The ovary-derived Bm5 cell line (Grace, 1967) was maintained at 28 °C in IPL-41 insect cell culture medium (Gibco) supplemented with 10% fetal bovine serum (Sigma–Aldrich) and subcultured weekly as previously reported (Douris et al., 2006). Bm5 cells were transfected according to established protocols (Johnson et al., 1992) using Escort IV (Sigma). After transfection the medium was replaced by fresh medium containing gentamycin (50 μg/ml).

### 2.6. RNAi luciferase functional assays

The general protocol for RNAi functional assays was carried out as reported earlier (Swevers et al., 2011) (see outline in Fig. 1). Briefly, for RNAi inhibition experiments, Bm5 cells were transfected with 3.3 μg/ml of ecdysone-responsive luminescence reporter pEcRE.bA.luc (Soin et al., 2008), 0.17 μg/ml of ecdysone-responsive fluorescence reporter pEcRE.bA.gfp (Swevers et al., 2004), as well as with 1.3 μg/ml of RNAi gene-specific dsRNA. DsMalE, which is specific for maltose binding protein mRNA of *E. coli* (Zhu et al., 2011), was used as non-specific control dsRNA. Moreover, all samples, except control dsMalE- (Fig. 2a and b), were transfected with 0.33 μg/ml of luciferase specific dsRNA (dsLuc) (Swevers et al., 2011).

For antisense inhibition luciferase functional assay, the concentrations of nucleic acids used were modified as follows: 1.7 μg/ml of pEcRE.bA.luc, 0.17 μg/ml of pEcRE.bA.gfp, as well as a total of 3.3 μg/ml of ssRNA or dsRNA. DsLuc was applied at 0.17 μg/ml.

Similarly, for siRNA pathway stimulation experiments (see outline in Fig. 1), 0.9 μg/ml of pEcRE.bA.luc, 0.2 μg/ml of pEcRE.-bA.gfp and 0.9 μg/ml of pEA vector (pEA-BmAgo-2-MycHis, pEA-Myc-BmDcr-2, pEA-BmR2D2-MycHis or pEA-pac, that contains the ORF of puromycin resistance gene and was used as negative control) were applied. Moreover, dsLuc was applied at increasing concentrations from 3.125 to 50 ng/ml, where total dsRNA concentration (balanced by dsMalE) was always 50 ng/ml.

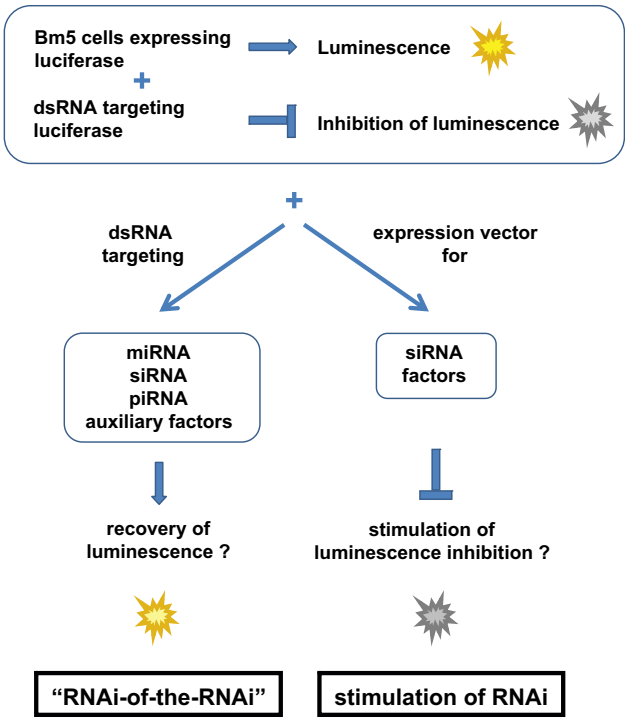


**Fig. 1.** Schematic overview of the RNAi inhibition ("RNAi-of-the-RNAi") and stimulation experiments. Luminescence production by Bm5 cells transfected with luciferase expression vector is suppressed by dsRNA specific for luciferase. For "RNAi of the RNAi" experiments (left), dsRNAs targeting each one of the RNAi factors were co-introduced by transfection, and luminescence was measured. Recovery of luminescence (reflecting inhibition of luciferase silencing) would reveal that the RNAi mechanism had been inhibited due to the dsRNA-mediated targeting of a core RNAi factor. For siRNA pathway stimulation experiments (right), constructs expressing each one of the main siRNA factors were co-introduced by transfection, and luminescence was measured. Decrease of luminescence levels (reflecting further increase of luciferase silencing) would reveal that the RNAi mechanism had been stimulated due to the enhanced expression of a core RNAi factor.

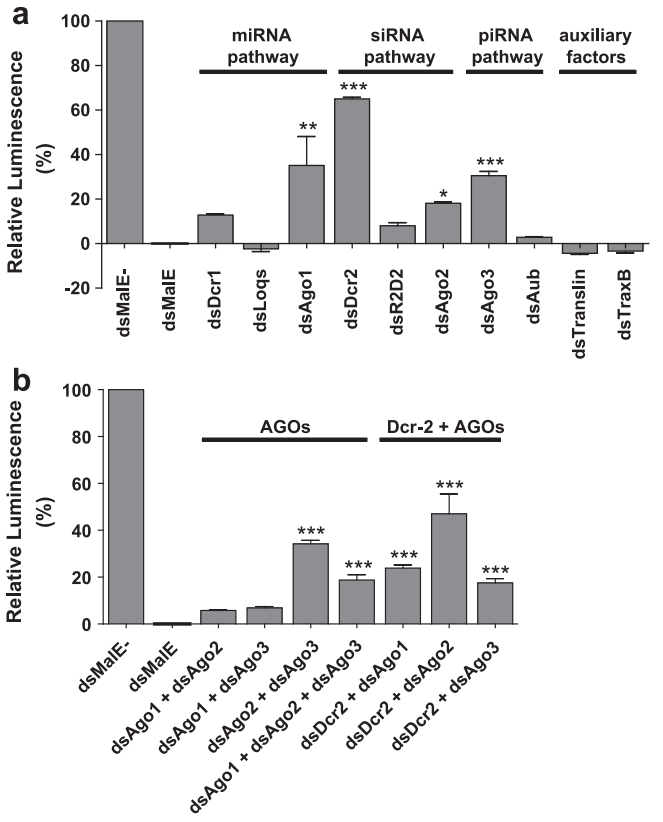


**Fig. 2.** "RNAi of the RNAi" luciferase functional assay targeting individually key components of the miRNA, siRNA and piRNA pathways, as well as auxiliary factors (a), and combinations of the top-four RNAi factors identified in the single gene targeting experiments (b). The graphs depict mean values of the % relative luminescence produced from three biological replications (+SE) after transfection with dsRNA. Each bar is presented as % ratio of the difference between the negative control for dsLuc (dsMalE-; set to 100%) and the positive control (dsMalE; set to 0%). Statistical analysis refers to the comparison of the average value ($n = 3$) of each treatment with the average of the positive control ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

For any of the three types of luciferase assay, 48 h post-transfection, the ecdysone agonist RH-5992 (Rohm and Haas Co; Swevers and Iatrou, 1999) was added to a final concentration of 500 nM to induce the ecdysone reporter element (EcRE) of luciferase and gfp reporters, and cells were harvested 24 h later. To obtain the soluble cellular extracts of transfected cells, the samples were processed as described before (Swevers et al., 2011) and placed in a 96-well plate for measurement first of fluorescence and then, after addition of Steady-Glo Luciferase substrate (Promega), of luminescence, carried out by an Infinite M200 fluoro-/luminometer (Tecan). Then, luminescence and fluorescence values obtained for control cells (i.e. untransfected) were subtracted from the respective values of the treated cells, thus yielding absolute luminescence and fluorescence values. Afterward, the normalized relative luminescence was calculated by division of the absolute luminescence by absolute fluorescence. GraphPad Prism 4 software was employed for the One Way ANOVA analysis for the samples of RNAi and antisense inhibition assays (with Tukey's Multiple Comparison Test as post-hoc test), and for the Two Way ANOVA analysis for the siRNA pathway stimulation assay (with Bonferroni's Multiple Comparison Test as post-hoc test).

### 2.7. Protein extracts preparation and immunoblottings

For protein expression studies, 2 μg/ml of pEA-BmAgo-2-MycHis, pEA-Myc-BmDicer-2, pEA-BmR2D2-MycHis or pEA-BmTranslin-2-MycHis expression vectors together with 0.55 μg/ml of pBmIE1 helper plasmid encoding the *ie-1* gene for *B. mori* nuclear polyhedrosis virus (BmNPV) (Lu et al., 1997) were used in transfection. For the dose-dependence experiments increasing concentrations of specific dsRNA from 0.55 to 1.65 μg/ml were additionally transfected, where total dsRNA concentration (balanced by dsMalE) was maintained at 1.65 μg/ml, while in the experiments that checked for dsRNA specificity 1.3 μg/ml of dsRNA was applied (same as in RNAi inhibition assay described in § 2.6). For the above experiments, 2.5 to 3 × $10^5$ cells were seeded on 24-well plates, transfected and harvested 48 h post-transfection. Cells expressing BmAgo-2, BmDcr-2, BmR2D2 or BmTranslin-2 were centrifuged at 3,000 g for 5 min and cell pellets were resuspended in 100 μl of phosphate-buffered saline (PBS) and frozen at −70 °C for 30 min. For the separation of the soluble protein fraction from the insoluble pellet, samples were subsequently centrifuged at 12,000 g for 10 min. Total protein extracts or soluble/insoluble fractions were diluted in 100 μl of cracking buffer (0.125 M Tris–HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS, 4M urea) (Georgomanolis et al., 2009) and boiled at 95 °C (5 min for soluble, 15 min for insoluble fractions and 10 min for total protein extracts). After addition of 6× SDS/PAGE loading buffer to 25 μl of each sample, all protein fractions were electrophoresed on 10% polyacrylamide gels and subjected to Western blot analysis as described (Georgomanolis et al., 2009). Proteins were transferred to Hybond ECL membranes (Amersham) and the membrane was blocked with 10% non-fat milk. Western blot analysis was carried out using mouse anti-Myc (Cell Signaling;

at 1:1000) as primary antibody and HRP-conjugated anti-mouse antibody (Chemicon; at 1:2000). For loading control, alpha tubulin was detected with rat anti-alpha Tubulin HRP-conjugated antibody (AbD Serotec; at 1:800). ECL chemiluminescent substrate (Amersham) was used for chemiluminescence detection.

### 2.8. Immunofluorescence assay

Bm5 cells were stained using immunofluorescence protocols described previously (Labropoulou et al., 2008). Briefly, transfection of pEA and pBmIE1 vectors took place as described in § 2.7. Then, 48 h after transfection cells were washed with basal IPL-41 (Invitrogen) and incubated for 2 h at room temperature on poly-L-lysine coated slides (Thermo Scientific). Phosphate buffered saline (PBS) was used to wash the samples following each step of immunostaining process. Cell membranes were stained with 5 μg/ml of wheat germ agglutinin (WGA) Texas Red-X conjugate (Molecular Probes) at 28 °C for 10 min. After fixation in 3.7% v/v formaldehyde-PBS solution for 10 min and cellular membrane permeabilization in 0.1% v/v Triton X 100-PBS, blocking was carried out with 3% w/v BSA-PBS for 1 h. Subsequently, the cells were incubated first with anti-Myc (Cell Signaling) for 16 h at 4 °C and then with anti-mouse FITC antibody (Sigma–Aldrich) for 1 h at room temperature in darkness, both diluted at 1:200 in 1% w/v BSA-PBS. Nuclei were stained with DAPI for 5 min (Sigma–Aldrich; at 0.5 μg/ml in PBS), and finally samples were mounted in Mowiol 4-88 (Sigma). Samples were examined under a BIORAD MRC 1024 laser scanning confocal microscope equipped with a Laser Sharp Version 3.2 Bio-Rad software. All images were analyzed with ImageJ software (Abramoff et al., 2004).

## 3. Results

### 3.1. Involvement of all three main small RNA pathways in the dsRNA-mediated silencing mechanism of Bm5 cells

To gain insight into the identity of the main factors that constitute the RNAi machinery in *B. mori*, genes from all three known small RNA pathways were screened using the "RNAi of the RNAi" approach (Dorner et al., 2006; Hoa et al., 2003; Saleh et al., 2006). In this approach, dsRNAs are employed that specifically target each one of the RNAi genes under investigation, in combination with the silencing of the transfected luciferase reporter (carried out by co-transfected dsLuc; Fig. 1). The targets included the basic genes from the miRNA, siRNA and piRNA pathways, such as Dicer and Argonaute proteins, as well as dsRNA-binding proteins and other auxiliary factors (Table 1). Thus, it was hypothesized that after knocking-down a key gene of the *Bombyx* RNAi machinery, the silencing of luciferase gene would also be inhibited and this effect could be assessed by changes in the luminescence produced.

It was observed that blocking of several key genes that belong to all three main small RNA pathways was able to significantly inhibit the silencing process. The major inhibition of the dsRNA-mediated gene silencing was caused after inhibition of *BmDicer-2* from the siRNA pathway (65.1%, Fig. 2a, Table 2a). Lower but statistically significant rates of inhibition of the RNAi mechanism were observed for three Argonaute proteins belonging to miRNA, siRNA and piRNA pathways (*BmAgo-1*, *BmAgo-2* and *BmAgo-3*, respectively, with rates varying from 18.1 to 35.2%; Fig. 2a, Table 2a). In contrast to the other Argonautes, *BmAub* (homologue of *Drosophila* Aubergine; also known as *BmSiwi* (Kawaoka et al., 2009), piRNA pathway) was the only Argonaute gene for which inhibition of expression did not cause inhibition of gene silencing. Targeting of *BmR2D2* did not cause any inhibitory effect to the dsRNA-mediated silencing, an observation that was expected since it has been found that *BmR2D2* is not expressed in Bm5 cells (Swevers et al., 2011).

**Table 2**
Normalized % relative luminescence of "RNAi of the RNAi" luciferase functional assays of Bm5 cells presented in Fig. 2. Mean values (±SE) of the % relative luminescence (normalized against fluorescence) produced from three biological replications after transfection with dsRNA are shown. Each value is presented as % ratio of the difference between the % relative luminescence of the negative control for dsLuc (dsMalE-; set to 100%) and the positive control (dsMalE; set to 0%).

| | dsRNA | Normalized RL (%) |
|---|---|---|
| a | | |
| miRNA | Dcr-1 | 12.83 ± 0.48 |
| | Loqs | −2.33 ± 1.21 |
| | Ago-1 | 35.16 ± 12.96 |
| siRNA | Dcr-2 | 65.06 ± 0.73 |
| | R2D2 | 8.03 ± 1.46 |
| | Ago-2 | 18.14 ± 0.49 |
| piRNA | Ago-3 | 30.54 ± 1.88 |
| | Aub | 2.84 ± 0.35 |
| Auxiliary factors | Translin | −4.39 ± 0.48 |
| | Trax-B | −3.37 ± 0.86 |
| b | | |
| AGOs | Ago-1 + Ago-2 | 5.81 ± 0.20 |
| | Ago-1 + Ago-3 | 6.89 ± 0.54 |
| | Ago-2 + Ago-3 | 34.20 ± 1.54 |
| | Ago-1 + Ago-2 + Ago-3 | 18.82 ± 2.23 |
| Dcr2+ AGOS | Dcr-2 + Ago-1 | 23.90 ± 1.34 |
| | Dcr-2 + Ago-2 | 47.00 ± 8.57 |
| | Dcr-2 + Ago-3 | 17.57 ± 1.77 |

Subsequently, to see whether inhibition of key genes in different small RNA pathways could lead to synergistic effects, Bm5 cells were also transfected with various combinations of the four RNAi-specific dsRNAs that were previously found to have caused significant inhibition of the silencing process (i.e. *BmDcr-2*, *BmAgo-1*, *BmAgo-2* or *BmAgo-3*). Interestingly, the highest inhibitory effect of combined RNAi machinery silencing was observed when *BmDcr-2* and *BmAgo-2* (corresponding to the siRNA pathway) were concomitantly targeted (47%, Fig. 2b, Table 2b).

Results showed that transfection with dsRNAs specific for these key four genes in pairs or triads, caused generally lower inhibiting effect on the luciferase silencing compared to the inhibition caused when applied alone (Fig. 2b, Table 2b). This may be explained by the fact that the amount of the transfected dsRNA became limiting for individual genes when combinations of genes were assayed (2–3 fold less than the amount in single targeting experiments). It is noted that such inhibition of RNAi efficiency after administration of multiple dsRNAs was also observed in gene silencing experiments employing other insects, such as the cockroach (Mané-Padrós et al., 2008).

### 3.2. Efficiency and specificity of dsRNA-mediated knock-down of RNAi factors in Bm5 cells

A major issue regarding the RNAi-of-the-RNAi experiments described above relates to the efficiency and specificity of the knock-down of the targeted RNAi factors. In most studies, RNAi efficiency is usually determined by quantification of target mRNA levels by quantitative real-time RT-PCR after treatment, while specificity is assessed by employment of an unrelated dsRNA, for instance dsGFP (as an example of this approach: Wynant et al. (2012)). While this approach is feasible in experiments were dsRNA is taken up efficiently by tissues, it is not a good choice in transfection experiments that are characterized by limitations in transfection efficiency (approximately 30% in Bm5 cells; unpublished results). Even if complete knock-down would be observed in all transfected cells, this would represent only a rather small

number of cells in the population and therefore knock-down in extracts of the whole population would be difficult to verify. To assess the efficiency and specificity of the knock-down in our experiments, another strategy was therefore used that is based on the inhibition of transfected expression constructs by co-transfected dsRNA.

Expression vectors for four key RNAi factors together with dsRNAs targeting all RNAi-related genes under study were introduced by transfection into Bm5 cells. In particular, BmDcr-2, BmR2D2, BmAgo-2 and BmTranslin-2 were expressed as C-terminal myc-his- or N-terminal myc-tagged recombinant proteins. First, to prove functionality of gene-specific dsRNAs, gradually increasing quantities of specific dsRNA, targeting the RNAi factor in the expression vector, were applied, which resulted in efficient knock-down of protein accumulation in a dose-dependent way (Suppl. Fig. 2). Second, to demonstrate that each dsRNA used was able to silence only the protein for which it had been specifically designed, Bm5 cells expressing the above mentioned RNAi factors were co-transfected separately with each one of the dsRNAs that target the other RNAi-related factors. Western analysis proved that knock-down was only achieved for the dsRNA specific for the over-expressed RNAi factor while all other dsRNAs did not influence protein expression (Fig. 3). For instance, dsAgo1 and dsAgo3 could not silence over-expressed BmAgo-2, while dsDcr1 did not affect the expression of BmDcr-2. This observation is important as it excludes the possibility that relevant genes (Argonautes, Dicers) might have been cross-silenced by dsRNAs of the same family in the RNAi-of the-RNAi experiments.

### 3.3. Single-stranded RNA does not cause an efficient intracellular RNAi response in Bm5 cells

It is well established that in the model insect *Drosophila*, the piRNA pathway functions independently of Dicer activity and that the primary response is initiated by ssRNA transcripts. In the germline, the initial trigger is then amplified by the "ping-pong" mechanism, in which different Argonaute proteins of the PIWI class associate with piRNAs of sense (Ago-3) or antisense (Aubergine, Piwi) polarity to generate piRNAs of the opposite polarity (Jinek and Doudna, 2009; Vodovar et al., 2012).

The finding that BmAgo-3 may play a role in the RNAi response of Bm5 cells could implicate that silencing can be accomplished not only by dsRNA but also by single-stranded sense and antisense RNA molecules (sRNAs and asRNAs). It is noted that Bm5 cells contain dsRNA-unwinding activity (Skeiky and Iatrou, 1991) that could expose single-stranded regions of transfected dsRNA for possible (Dicer-independent) processing and loading into BmAgo-3-containing RISCs. For this reason, the possibility of knock-down effect of antisense and sense RNA (asLuc and sLuc), that targeted exactly the same region as dsLuc, was investigated. The amounts of the ssRNA used were 20-fold higher than dsLuc, to compensate for its greater propensity to intracellular degradation than dsRNA.

As shown in Fig. 4, no significant effect on luciferase reporter activity was observed after application of high concentrations of asLuc or sLuc (3.3 μg/ml). On the other hand, dsLuc was active even at very low concentrations (0.17 μg/ml). These results show that the RNAi response in Bm5 cells cannot be triggered by single-stranded asRNA or sRNA, at least using as high concentrations as the ones applied in our experimental set-up. Thus, the involvement of BmAgo-3 in the silencing response seems to be triggered by dsRNA and not by single-stranded RNA.

### 3.4. Mild stimulation of dsRNA-mediated gene silencing after over-expression of BmDcr-2 or BmAgo-2

The "RNAi-of-the-RNAi" experiments indicated that factors of the siRNA pathway (BmDcr-2, BmAgo-2) are important for RNAi efficiency (Fig. 2a and b). Previously, it has also been reported that Bm5 cells are deficient regarding *BmR2D2* expression, an essential factor in the siRNA pathway in *Drosophila* (Swevers et al., 2011). It is therefore of interest to investigate whether the efficiency of the cells' RNAi mechanism could be stimulated after over-expression of BmDcr-2, BmAgo-2 or BmR2D2. Thus, Bm5 cells were co-transfected with expression vectors of the siRNA factors together with the reporter plasmids as well as with dsLuc from limiting to gradually increasing concentrations (Fig. 1).

Interestingly, it was noted that at very low concentrations of dsLuc (3.125–6.250 ng/ml), increased expression of BmDcr-2 and BmAgo-2 was capable of enhancing the activity of dsLuc to silence reporter gene expression to lower levels compared to the control (Fig. 5; by 14.6 or 16.9% for BmDcr-2, and by 25.3 or 15.4% for BmAgo-2). On the other hand, expression of BmR2D2 presented no statistically significant effect on the RNAi response in Bm5 cells.

### 3.5. Subcellular localization of the main siRNA pathway proteins by immunostaining of Bm5 cells

In order to obtain more information regarding the main siRNA factors' subcellular localization, and thus correlate it with their function in the cellular environment, immunostaining of Bm5 cells

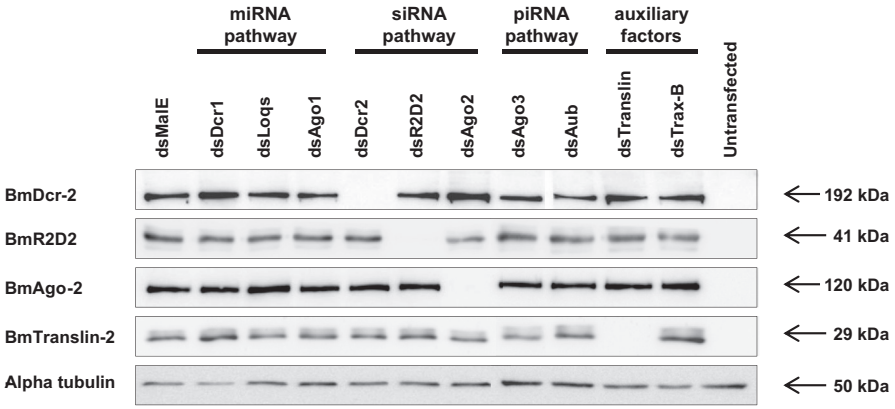


**Fig. 3.** Efficient expression and dsRNA-mediated gene-specific knock-down of four myc-tagged RNAi factors (BmDcr-2, BmR2D2, BmAgo-2 and BmTranslin-2) in transfected Bm5 cells, as analyzed by Western blot. Indicated are the identities of the dsRNAs that were co-transfected with the expression vector. Alpha-tubulin served as a loading control (a representative western blot of alpha-tubulin is shown in the lower panel). Molecular weights are indicated at right.

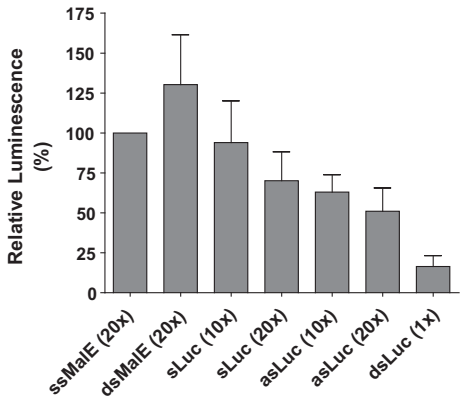


**Fig. 4.** Single-stranded RNA luciferase functional assay. The graph depicts mean values of the % relative luminescence produced from three biological replications (+SE) after transfection with ssRNA or dsRNA. Each bar is presented as % ratio of the negative control (ssMalE; set to 100%).

transfected with expression constructs for myc-tagged BmDcr-2, BmR2D2 and BmAgo-2 was performed. Confocal microscopy revealed that BmDcr-2 is localized in the cytoplasm with a preference for the cellular membrane (Fig. 6). The cytoplasmic localization of BmDcr-2 can be justified according to its suggested role of processing long dsRNAs in the siRNA pathway. A similar pattern of cytoplasmic localization has been observed for Flag-tagged BmDcr-2 when over-expressed in another lepidopteran cell line, *Trichoplusia ni* Hi5 cells (Liu et al., 2012). In contrast to S2 and Hi5 cells where Dcr-2 has been reported to be expressed also in the nucleus (Cernilogar et al., 2011; Liu et al., 2012), BmDcr-2 detection in the nuclei of Bm5 cells was exceptionally rare (data not shown).

BmR2D2 staining revealed that it is also localized mostly on the cellular membrane when over-expressed in Bm5 cells (Fig. 6). As might be expected for Dcr-2/R2D2 complex formation (which is documented in *Drosophila* S2 cells; Liu et al. (2003)), it therefore colocalizes with BmDcr-2. On the other hand, BmR2D2 subcellular localization is different from the one reported for *Tribolium* R2D2 in Hi5 cells where a spot-like pattern is observed (Swevers et al., 2011). Since the homology between *Tribolium* and *Bombyx* is very low, differences in staining pattern may reflect differences in their functional properties such as the absence of recruitment of *Tribolium* R2D2 by lepidopteran Dcr-2 (discussed in Swevers et al. (2011)).

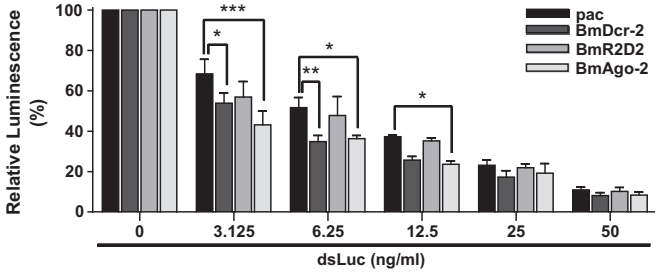


**Fig. 5.** Luciferase RNAi functional assay after co-expression of the main siRNA factors. Expression vectors for BmDcr-2, BmR2D2 and BmAgo-2 were tested. The pac expression vector was used as negative control. Additionally, the cells were transfected with gradually increasing concentrations of Luc-specific dsRNA (dsLuc). The graph depicts mean values of the % relative luminescence produced from three biological replications (+SE) after transfection with dsRNA. Each bar is presented as % ratio of the negative control (dsLuc = 0 ng/ml; set to 100%). Statistical analysis results refer to the comparison of the average value ($n = 3$) of each over-expressed protein treatment with the average of the control pac (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

As for BmAgo-2, immunofluorescence shows that it is localized mostly in the cell periphery and the cytoplasm (Fig. 6). In our experiments BmAgo-2 never appeared in the nucleus, although its homologue in *Drosophila* has also been found to be associated with chromatin in the nucleus during the heat-shock stress response acting probably as transcriptional regulator (Cernilogar et al., 2011).

## 4. Discussion

In this study we have investigated the core RNAi machinery components of the silkmoth *B. mori* that are involved in the silencing response triggered by artificially introduced dsRNA stimuli into the intracellular environment (i.e. by transfection). Our interest in this mechanism is motivated by the search for limiting factors that could explain the high inconsistency in RNAi response that is observed in *in vivo* silencing experiments in lepidopteran insects (Terenius et al., 2011). Based on the data established for *Drosophila*, an RNAi screen of homologues of RNAi-related factors in *B. mori* was performed to identify the genes that encode the core RNAi machinery of the silkmoth.

A major finding was that all three main small RNA pathways were shown to be involved in the dsRNA-mediated gene silencing in Bm5 cells, since knocking-down of *BmAgo-1* (miRNA pathway), *BmAgo-2*/*Bm-Dcr-2* (siRNA pathway) and *BmAgo-3* (piRNA pathway) caused considerable abrogation of RNAi-mediated silencing of luciferase activity (Fig. 2a, Table 2a). With the exception of *Ago-1*, this finding is highly consistent with a similar study on a hemocyte cell line of *Anopheles gambiae*, where *Ago-2*, *Ago-3* and *Dcr-2* genes were found to be indispensable for RNAi (Hoa et al., 2003). Moreover, the up-regulation of *Ago-2* and *Dcr-2* expression upon a dsRNA trigger as an *in vivo* response in the lepidopteran *Manduca sexta* was reported recently (Garbutt and Reynolds, 2012). Nevertheless, it should be noted that according to statistical analysis that revealed the important RNAi components in Bm5 cells, luciferase activity recovery never reached levels higher than 65% of maximum luminescence (Fig. 2a and b; Table 2a and b). It has been commented before that there might exist technical limitations that prevent the complete silencing of a gene whose expression is necessary for gene down-regulation in the first place (Ulvila et al., 2006).

Several observations establish that RNAi-mediated knock-down is an efficient and specific process in Bm5 cells. First, concentrations of co-transfected dsRNA of 0.55 μg/ml completely suppressed the expression of BmDicer-2 and BmAgo-2 from expression vectors, while 2–3 fold higher concentrations limited BmR2D2 and BmTranslin-2 protein production to <10% (Suppl. Fig. 2). Another experiment revealed suppression of a luciferase reporter by approximately 50% using only 6.25 ng/ml of dsLuc in the transfection mixture (Fig. 5). Regarding specificity, it was observed in transient expression experiments that, for four genes, the specific dsRNA caused complete knock-down of expression, while no obvious effect was observed for the remaining 9 non-specific dsRNAs that target other RNAi-related factors (Fig. 3). This strengthens our observation that members of all three RNAi pathways can be involved in the silencing mechanism; for instance, and of relevance to the discussion below, the inhibition of silencing by dsAgo3 cannot be explained by cross-silencing of the siRNA factors BmDcr-2 and BmAgo-2.

Following the model established for *Drosophila*, the involvement of *Dcr-2* and *Ago-2* in the silencing assay was expected, since these two genes encode the RNAi core factors that activate silencing in response to exogenous dsRNA (siRNA pathway; Siomi and Siomi (2009)). Evidence showing significant, but less potent, involvement of BmAgo-1 can be interpreted as partial overlapping of siRNA

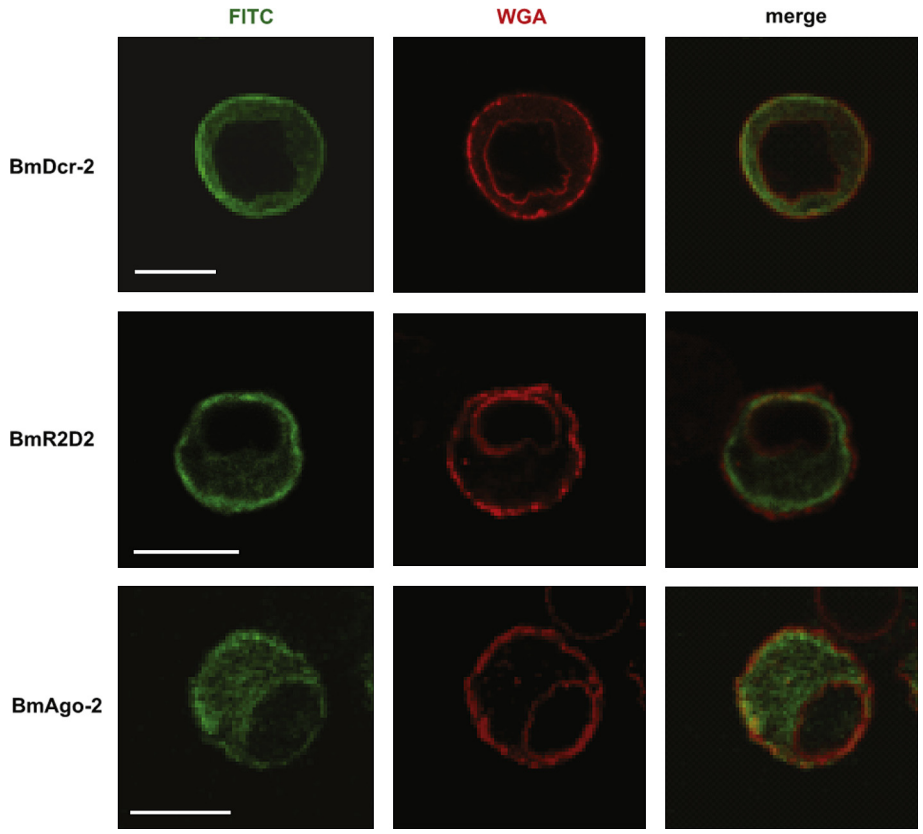


**Fig. 6.** Subcellular localization of siRNA pathway proteins analyzed with confocal microscopy. Bm5 cells were transfected with expression vectors for myc-tagged BmDcr-2, BmR2D2 or BmAgo-2, and processed for immunostaining 48 h after transfection. The cell membrane was stained with WGA-Texas Red-X (red fluorescence), and primary anti-myc and secondary anti-mouse-FITC antibodies (green fluorescence) were used to detect the myc-tagged BmDcr-2, BmR2D2 and BmAgo-2 proteins. Scale bars; 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and miRNA pathways at RISC level, as is also observed in *Drosophila* (Tomari et al., 2007).

The lack of evidence for a dsRNA-binding protein involvement (BmLoqs, BmR2D2) in the RNAi response after the screening of all major RNAi components was unexpected, since the presence of a dsRNA-binding protein is required for both miRNA and siRNA pathways (Liu et al., 2003; Tomari et al., 2007). While *BmR2D2* is not expressed in Bm5 cells (Swevers et al., 2011), one can speculate that knock-down of *BmLoqs* by dsRNA may not be efficient enough to cause phenotypic effects. Although the dsRNA preparations were proven to be highly specific and efficient (Fig. 3, Suppl. Fig. 2), it is possible that BmLoqs protein is much more stable than Dicers or Argonautes or that the latter are present in the cells in more limiting amounts.

On the other hand, this finding could also be explained by two alternative mechanisms that may well act in parallel: (a) another unknown dsRNA-binding protein can carry out the function of BmR2D2 or BmLoqs in Bm5 cells, or, (b) an alternative small RNA silencing pathway exists, that acts in parallel with the siRNA pathway and that requires no dsRNA-binding protein. The first hypothesis is consistent with the observation that ectopic expression of BmR2D2 does not stimulate the silencing process triggered by dsRNA (Fig. 5). Over-expression of BmDicer-2 or BmAgo-2 also results in limited effects on RNAi efficiency, observed only at low concentrations of dsLuc, indicating that BmDcr-2 and BmAgo-2 proteins are close to their optimal levels of expression for efficient RNAi. To gain further insight in the RNAi machinery in Bm5 cells, further biochemical analysis is required, based on the isolation and characterization of RISC complexes from *Bombyx* cells as has been carried out for *Drosophila* embryo extracts and S2 cells (Sontheimer, 2005; Tomari et al., 2004). The second hypothesis may fit with the finding that knocking-down *BmAgo-3*, an Argonaute protein of the Dicer-independent piRNA pathway, caused significant recovery of the luciferase activity (30.5%; Fig. 2, Table 2a).

The identification of *BmAgo-3* in the silencing response was unexpected because it belongs to the Piwi-class of Argonaute proteins that were originally identified for their involvement in transposon silencing of the germline. The primary triggers for the piRNA pathway are thought to be single-stranded transcripts that are transcribed from transposable elements or piRNA loci in the genome (Siomi et al., 2011). In the present study, BmAgo-3 seems to be involved in dsRNA-mediated gene silencing in the absence of single-stranded RNA (Fig. 2a and b). Because of the inability of high doses of single-stranded RNA to provoke efficient silencing of luciferase in Bm5 cells (Fig. 4), it is suggested that BmAgo-3 may function independently from the established piRNA pathway in Bm5 cells that is triggered by single-stranded transcripts. Instead, its function seems to be triggered by dsRNA, similarly to the siRNA pathway. This speculation is reinforced by the absence of any significant effect of Aub-specific dsRNA (Fig. 2a) on the RNAi response, ruling out the participation of the ping-pong mechanism in the observed silencing process. Interestingly, it has been reported that in BmN4 cells, a *Bombyx* embryonic cell line where *BmAgo-3* and *BmAub* are endogenously expressed, both primary and secondary piRNA biogenesis pathways are functional (Kawaoka et al., 2009). Future studies that involve cloning and deep sequencing of Bm5 cells' small RNAs could reveal the relative contribution of siRNA and piRNA pathway components in gene silencing in BmN4 and Bm5 cells.

Lately, it has also been suggested that RNAi efficiency may vary depending on the relative timing between the administration of the

dsRNA trigger and the initiation of the target gene mRNA production, and pretreatment with specific-dsRNA was recommended to increase the silencing effect (Terenius et al., 2011). Thus, in order to enhance the sensitivity, a special feature of our study system was the employment of ecdysone responsive reporters. The reporter activity was triggered by ecdysone agonist (tebufenozide) only 48 h after the transfection with dsRNA, thus ensuring that during this time window dsRNAs had already been processed to small RNAs and had silenced their targets (RNAi factors). This procedure was expected to increase the sensitivity for knock-down of components of the RNAi machinery. Although the involvement of BmDcr-2 in the RNAi response was confirmed employing the non-inducible (constitutive) pActin-Luc vector as reporter (Suppl. Fig. 3), it is clear that the use of a constitutive reporter results in decreased sensitivity since no involvement of BmAgo-1 and BmAgo-2 was uncovered in this set of experiments.

Concluding, to our knowledge this is the first attempt to determine the factors that constitute the core RNAi machinery in *B. mori*. After an RNAi screening, we identified *Ago-1*, *Ago-2*, *Ago-3* and *Dcr-2* genes of the silkmoth as candidate components of the RNAi response in Bm5 cells. BmR2D2, a dsRNA-binding protein in the siRNA pathway that acts as co-factor of Dcr-2 in *Drosophila*, was found to be dispensable for effective dsRNA-mediated gene silencing in Bm5 cells. It must be stressed, however, that all experiments were carried out by transfection which is not a natural process to introduce nucleic acids. Bm5 cells do not respond to soaking of dsRNA in the culture medium but seem to be competent to take up dsRNA without triggering an RNAi response (Swevers and Smagghe, 2012). Because of its close localization at the plasma membrane (Fig. 6), we cannot exclude a role for BmR2D2 in dsRNA-mediated gene silencing in processes where dsRNA is taken up by endocytosis. Additionally, our data suggest the involvement of BmAgo-3 in the dsRNA-mediated silencing of Bm5 cells, which may occur independently of its involvement in the piRNA pathway. This involvement can also be further investigated by investigating possible stimulation of the RNAi response after BmAgo-3 overexpression.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.05.001.

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