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https://doi.org/10.5109/25203

出版情報：九州大学大学院農学研究院紀要. 57 (2), pp.441-445, 2012-09-20. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **57** (2), 441–445 (2012)

# RNA Interference Induction by Long Hairpin dsRNAs Expressed from Chromosomal DNA of *Bombyx mori* Cells

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(*Received April 27, 2012 and accepted May 10, 2012*)

RNA interference (RNAi) triggered by long double–stranded RNA (dsRNA) transcribed from chromosomal DNA has a lot of advantages in high–throughput analyses of gene function. In this report, we have constructed Gateway®–based RNAi induction vectors, by which we efficiently integrated expression cassettes for long hairpin dsRNA into chromosomal DNA of *Bombyx mori* cells. RNAi induced by the hairpin dsRNAs using our constructs decreased a reporter gene activity by approximately 70–80% in cultured *B. mori* cells.

**Keywords**: Bombyx mori, dsRNA, Reporter expression, RNA interference, siRNA

## INTRODUCTION

In insect, exogenously introduced long double–stranded RNA (dsRNA) efficiently induce RNA intereference (RNAi) (Lopez–Martinez *et al*., 2012) like plants (Garcia–Perez *et al*., 2004) and nematodes (Parrish *et al*., 2000). This is marked differenceis to mammalian cells in which long dsRNAs trigger sequence non–specific gene silencing by activating the PKR and RNase L pathways (Gil and Esteban, 2000). RNAi can be successfully induced in cultured silkworm cells (Fujita *et al*., 2009; Tomimoto *et al*., 2012) or embryos, but there are a few reports on gene silencing at the larval stage (Quan *et al*., 2002; Tabunoki *et al*., 2004; Huang *et al*., 2007; Hossain *et al*., 2008; Liu *et al*., 2008; Masumoto *et al*., 2009; Pan *et al*., 2009). Furthermore, it is really difficult to achieve a successful phenotype caused by RNAi in some tissues of *Bombyx mori* (Terenius *et al*., 2011). A possible reason for this inefficient RNAi induction by the injection of dsRNA into larvae is due to the difficulty of RNA delivery into silkworm organs or tissues.

One of the most popular method to archieve efficient RNAi induction in many organisms is to express hairpin dsRNA from chromosome (Lee *et al*., 2003; Miki *et al*., 2004; Szulc *et al*., 2006). To date, characteristic vectors have been constructed with an inverted repeat (IR) sequence encoding hairpin dsRNA. However, these take a long time to construct and meet with difficulties due to their structural instability in host cells. In the post–genome era, high–throughput vectors for efficient gene silencing are keenly needed; these would develop efficient RNAi system exploiting long dsRNA transcribed from chromosomes. The required features for these RNAi systems are (1) high efficiency of gene silencing, (2) the convenience for constructing tools, and (3) the constancy of inducing RNAi.

Here, we have constructed a silkworm gene knockdown system using a Gateway®–based plasmid that expresses long hairpin dsRNA. The long hairpin dsRNA expressed from silkworm chromosome decreased a reporter gene activity by approximately 70–80% in BmN4 cells.

## MATERIALS AND METHODS

### Cell culture

*B. mori* BmN4 cells (from a stock of laboratory of Insect Pathology and Microbial Control, Faculty of Agriculture, Kyushu University Graduate School) was maintained in IPL–41 insect medium (Sigma Chemical) supplemented with 10% fetal bovine serum (GIBCO Invitrogen) at 27°C. Before use for transfection, the medium was replaced by Sf–900 Ⅱ serum free medium (Invitrogen).

### Construction of mediators for dsRNA expression vectors

The organization of dsRNA expression constructs (named pAladdinGate, abbreviated to pAG) used in this study is shown in Figure 1A. The plasmid pPigBase1.4 (DDBJ AB254131), which contains *piggyBac* transposase sequence (*piggyBac* L/R fragment) and the multiple cloning site (MCS) sequences, was used as a basic plasmid for the construction of pAG vectors.

Synthetic GFP–Zeocin resistance fusion gene was amplified from pIZT/V5–His plasmid (Invitrogen) by PCR with the primers GFP–Zeocin–5 and GFP–Zeocin–3 (Table 1), and introduced into a *Sca*I site of pPigbase1.4. The resulting plasmid was named pPig1.4Zeo. To construct the Gateway®–based dsRNA expression cassettes, the destination plasmid pBmHSC70–4DEST (Lee *et al*., 2008), which contain insect promoters and *att*R1–ccdB–*att*R2 cassettes, were used as forward oriented

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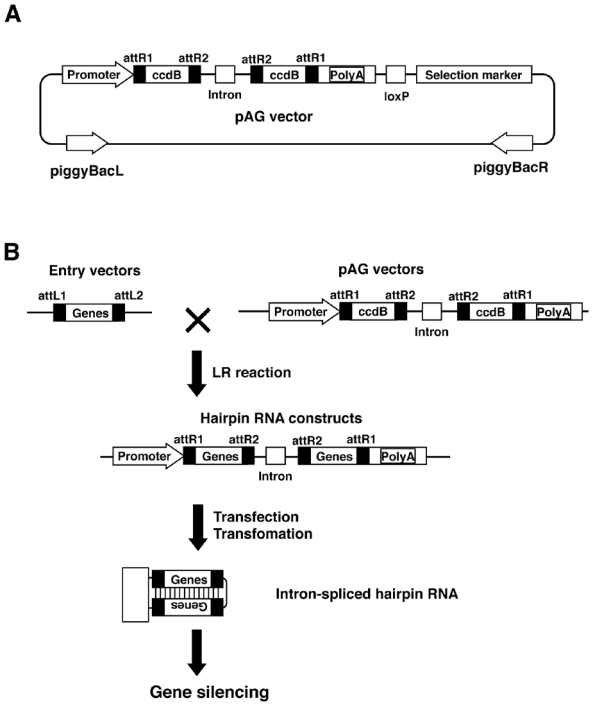


**Fig. 1.** Construction of the hairpin dsRNA expression plasmids, pAG. (A) Molecular structure of Gateway®–based pAG vectors. (B) Construction strategy for dsRNA expression plasmid using the Gateway® system.

DEST cassettes. The pZhscinIDEST, which contains the first intron of the *BmHSC70–4* gene (Lee *et al*., 2008) and reverse orientated DEST cassette (with *att*R2–ccdB–*att*R1 cassettes), were digested with *Xba*I and *Bam*HI, followed by blunting and cloning into the *Xho*I and *Xba*I blunt–ended site of the pBmHSC70–4DEST plasmid. The resulting dsRNA expression cassettes, pBmHSC70–4IRDEST, was digested with *Apa*I and *Sac*I, blunt–ended, and cloned into a *Not*I blunt–ended site of pPig1.4Zeo. Thus formed construct (pAG) was subjected to the Gateway® LR reaction with entry vectors (Fig. 1B) using LR Clonase™ Enzyme Mix (Invitrogen) according to the protocols recommended in manufacturer's manual.

**Construction of entry clones**

All entry clones were constructed using pENTR™11 from Invitrogen. To obtain the partial luciferase fragments, a full luciferase cDNA of pSK8Fb–Luc vector (kindly provided by Professor Hisanori Bando of Hokkaido University Graduate School) was roughly divided into 3 regions: 593 bp of 5'–fragment (F–Luc; 1 to 593), 721 bp of middle–fragment (M–Luc; 588 to 1,308), and 360 bp of 3'–fragment (L–Luc; 1,251 to 1,610). The F–Luc fragment was amplified using the primers F–Luc–5 and F–Luc–3–EcoRI (Table 1). After *Eco*RI digestion, the resulting fragment was inserted into an *Eco*RI and *Eco*RV site of pENTR™11 (pENTR–F–Luc). The M–Luc fragment was amplified using the primers M–Luc–5–EcoRI and M–Luc–3 (Table 1), and inserted into an *Eco*RI and *Eco*RV site of pENTR™11 (pENTR–M–Luc). The L–Luc fragment was amplified using the primers L–Luc–5 and L–Luc–3 (Table 1), and inserted into *Xmn*I and *Eco*RV site of pENTR™11 (pENTR–L–Luc). The entry clones pENTER–F–Luc, pENTER–M–Luc, pENTER–L–Luc, and the destination vectors (pAGs) prepared as described above were subjected to the Gateway reaction according to the protocols recommended in manufacturer's manual (Invitrogen). The final forms will be called pAG–F–Luc, pAG–M–Luc, and pAG–L–Luc, respectively.

**Table 1.** List of primers used for this study

| Primer Nam | Primer Sequence ( 5'–3' ) |
|---|---|
| F–Luc–5 | ATGGAAGACGCCAAAAACATAAAGAAA |
| F–Luc–3–EcoRI | GAGGAATTCATTATCAGTGCAATTGTTTTG |
| M–Luc–5–EcoRI | TAATGAATTCCTCTGGATCTACTGGGTTAC |
| M–Luc–3 | TCAACTATGAAGAAGTGTTCGTCTTCGTC |
| L–Luc–5 | GCTACATTCTGGAGACATAGCTTACTGGGA |
| L–Luc–3 | CTCTGATTTTTCTACGTACGAGTTTTCCGG |
| XDH1–5 | GGATTATTGAATGCTGAGGAAGATCCG |
| XDH1–3–XhoI | TCTCGAGGAAAAATTGGCTCTTGACTAG |
| pBS–T7 | TGAGCGCGCGTAATACGACTCACTATAGGG |
| DMC1–3 | TAATACGACTCACTATAGGGAACAAAAGCT |

Partial *XDH1* (Xanthine dehydrogenase; GenBank Accession Number: AB060285) fragment used as a negative control sequence was amplified by PCR using the primers XDH1–5 and XDH1–3–XhoI (Table 1) using cDNA from ovary of silkworm w051 strain. The PCR fragment was inserted into a *Xho*I and *Xmn*I site of pENTR™11 (pENTR–control). Partial GFP fragment, amplified by PCR using the primers GFP–5 and GFP–3 (Table 1), was inserted into a *Xmn*I and *Eco*RV site of pENTR™11 (pENTR–GFP9900). The final vectors derived from the above constructs are named pAG–XDH and pAG–GFP, respectively.

Final pPig1.4ZeoDEST–Luc plasmid for stable expression of luciferase was generated by Gateway reaction between the pENTR–Luc (Lee *et al*., 2008) and destination vector pPig1.4ZeoDEST. The pPig1.4ZeoDEST was generated by inserting an *Apa*I and *Sac*I/blunt fragment of the pBmHSC70–4DEST (BmHSC70–4 promoter, *att*R1–ccdB–*att*R2 cassettes and polyadenylation sequence) (Lee *et al*., 2008) into a *Not*I blunt–ended site of pPig1.4Zeo.

**Preparation of double–stranded RNA**

Templates for transcription of dsRNA, used for transfection, were synthesized by two–step PCR from silkworm *DMC1* sequence (Mon *et al*., 2004; GenBank Accession Number: NM_00104487) in pBluescript SK (+), using the primers pBS–T7 and DMC1–3 (Table 1) for the first step PCR. For the second PCR, the single primer pBS–T7 was used. The resulting PCR fragment possesses inverted terminal T7 promoters. Templates for the firefly luciferase dsRNA was amplified by the similar two–step PCR method (Sakashita *et al*., 2009). After phenol/chloroform extraction the DNA template was incubated in transcription reaction buffer (40 mM Tris–HCl buffer, pH7.5, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, 2 mM dNTPs, 8 U of RNase inhibitor, and 20 U of T7 RNA

polymerase) for 2 h at 37˚C. The RNA product (dsLuc and dsDmc 1) were purified, dissolved in 100 mM HEPES, pH7.0, heat denatured for 5 min at 94˚C, and allowed to stand at room temperature for 30 min for annealing.

**Transient expression of reporter genes and transformation of cells**

BmN4 cells, or other cells indicated, at a concentration of $1\times10^5$ per well were transfected with appropriate materials by a lipofection method using the CellFECTIN reagent (Invitrogen) for 6 h. The vectors pBmTCTP–Luc, pBmHSC70–4–Luc, and pOpIE–2–$\beta$–gal (Lee *et al*., 2008) were used for the transient expression of luciferase and $\beta$–galactosidase, respectively, as reporters. All constructs were purified using a Plasmid Mini kit (Qiagen) beforehand. The transfected cells were washed once with PBS and lastly incubated in IPL–41 medium supplemented with 10% FBS. Appropriate times after incubation, the luciferase activity was measured by PicaGene® kit (Toyo Ink) using 1,253 Luminometer (Bio–Orbit). The cell extract was also used for measuring $\beta$–galactosidase activity essentially according to the method described previously (Ausubel *et al*., 1988).

To select the transformed cells constitutively expressing partial luciferase dsRNA (named as L16) and full luciferase gene (named as N4–Luc), BmN4 cells ($1\times10^5$ per well) were seeded and co–transfected with 1 $\mu$g of the transformation plasmid pAG–L–Luc or pPig1.4ZeoDEST–Luc and 1 $\mu$g of transposase expressing *piggy Bac*–helper plasmid pA3helper (Cary *et al*., 1989), using CellFECTIN. After 96 h of transfection, the cells were split at a ratio of 1 : 5 and selected for Zeocin–resistance in the medium containing 100 $\mu$g/ml Zeocin (Invitrogen) (Pfeifer *et al*., 1997; Yamamoto *et al*., 2004).

## RESULTS

**RNAi with dsRNA in cultured BmN4 cells**

To investigate the RNAi efficiency in silkworm cells, a 1.6 kbp dsRNA for firefly luciferase (dsLuc) was transfected into BmN4 cells with the luciferase and $\beta$–galactosidase expression plasmids (pBmTCTP–Luc and pOpIE–2–$\beta$–gal, respectively). As shown in Figure 2A, the cells gave no luciferase activity, when the dsRNA and reporter plasmid was co–transfected into BmN4 cells. However, when we transfected the dsLuc into the BmN4 cells stably expressing luciferase mRNA (N4–Luc), the level of the activity was suppressed only >70% in contrast to the control (Fig. 2B, showing the patterns for dsRNA). These observations imply that the transfection efficiency of cultured silkworm cells was considerably low, and for this reason we developed a following new method for RNAi.

**Efficiency of hairpin dsRNA in BmN4 cells**

A series of vectors expressing hairpin dsRNAs were constructed with the pAG plasmids, which had two Gateway® recombination sites for introducing an arbitrary DNA fragment as IR. Upstream of this IR cassette, BmHsc70–4 promoter was located to express dsRNAs in

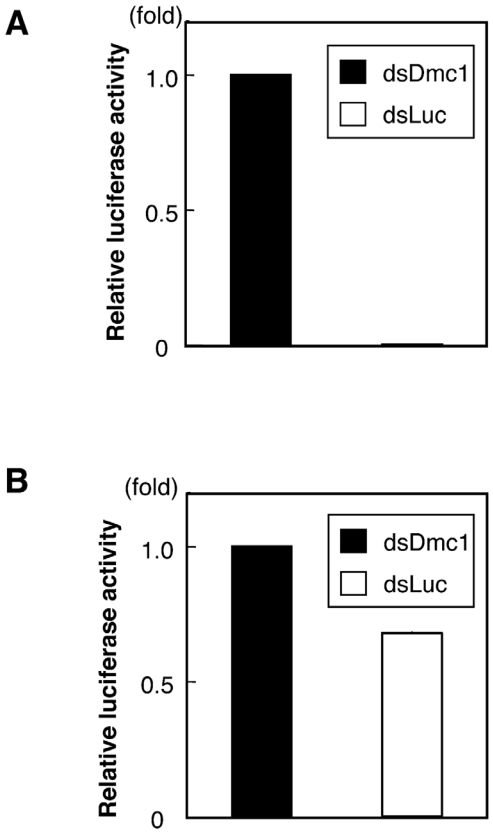


**Fig. 2.** Quantification of the efficiency of RNAi in cultured silkworm cells. Cells were seeded in 24–well plates ($0.7\times10^5$ cells per well) and co–transfected with each 250 ng/well of luciferase– and $\beta$–galactosidase–expressing vectors (pBmTCTP–Luc and pOpIE–2–$\beta$–gal, respectively) as reporters, together with 100 ng/well of dsRNA encoding *DMC1* (dsDmc1 as a control) or luciferase (dsLuc). Four days after transfection, the cells were determined for the reporter activities. Each luciferase activity normalized by $\beta$–galactosidase activity was illustrated as a relative valu of the control (=1.0). Average of three independent experiments with SD was indicated. (A) BmN4 cells. (B) Transformed BmN4 cells stably expressing luciferase (N4–Luc).

*B. mori* cells. BmHsc70–4 intron was set at internal loops, since it was expected that the presence of an intron might increase the genomic stability of IR transgene (Leach, 1994; Bi and Liu, 1996; Brown *et al*., 2003).

The rate of RNAi induction was previously noted to vary depending on the nucleotide sequence of trigger dsRNA (Parrish *et al*., 2000). To investigate this issue, pAG–F–Luc, pAG–M–Luc, and pAG–L–Luc were constructed to express hairpin dsRNA from the three partial luciferase sequences (forward, middle, and late), respectively. The resulting plasmids, as well as reporter vectors encoding luciferase and $\beta$–galactosidase (pBmTCTP–Luc and pOpIE–2–$\beta$–gal, respectively) as reporters, were transfected into BmN4 cells, which were then measured for reporter activities. As shown in Fig. 3A, the RNAi activity induced by pAG–L–Luc was slightly higher than that induced by pAG–F–Luc or pAG–M–Luc. To a maximum degree, the luciferase activity was suppressed by >70%. These findings indicated that a luci-

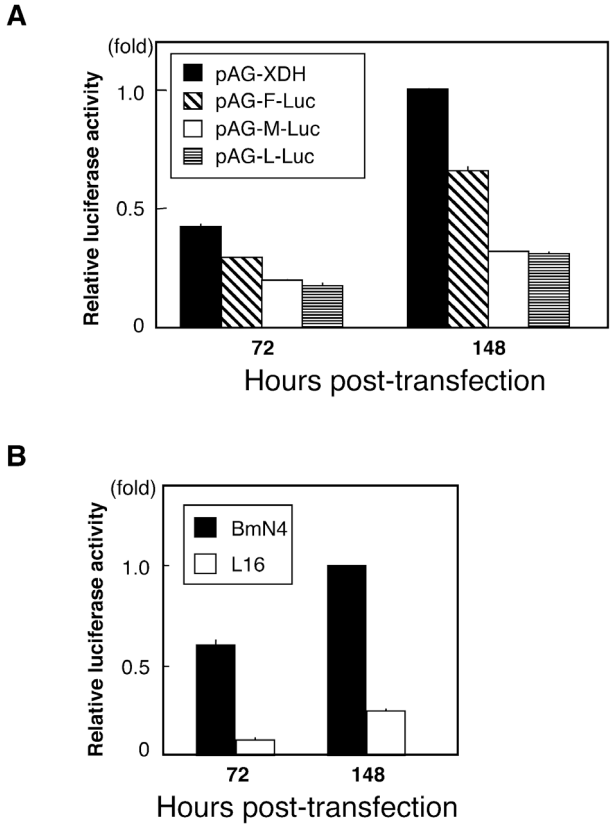


**Fig. 3.** Hairpin dsRNA is potent inducer of RNAi in cultured silkworm cells. (A) Luciferase gene (1.6 kbp) was divided into 3 parts, F–Luc, M–Luc, and L–Luc, and each was inserted into pAG vector as detailed in MATERIALS AND METHODS. Each 250 ng of the resulting pAG plasmids was co–transfected with 250 ng of pBmTCTP–Luc and 30 ng of pOpIE–2–$\beta$–gal into BmN4 cells. pAG–XDH was used as a control. At 72 and 148 h after transfection, the cells were measured for the luciferase activity. Each luciferase activity normalized by $\beta$–galactosidase activity was illustrated as a relative valu of the control at 148 h (=1.0). Average of three independent experiments with SD was indicated. (B) L16 cells (transformed BmN4 cells to express stably L–Luc dsRNA, see text for details) and original BmN4 cells (control) were seeded as above, co–transfected with 250 ng of pBmTCTP–Luc and 30 ng of pOpIE–2–$\beta$–gal, and assayed for reporter activities. Other details are as those described in (A).

ferase cDNA sequence at the L–Luc region 1,251 to 1,610 was the most effective for RNAi under our experimantal condition using pAG.

When IR was inserted into the BmN4 genome, its unstable structure may reduce the RNAi efficiency. This possibility was examined using the BmN4 cells established to express constitutively the hairpin dsRNA from the partial luciferase region corresponding to L–Luc; this newly transformed cell line will be tentatively called L16 in what follows. When transfected with vectors for reporter enzymes, L16 cells gave markedly reduced relative activity compared to the original BmN4 cells (Fig. 3B), indicating the occurrence of efficient RNAi. Although the relative luciferase activity in L16 cells was decreased by about 70% on day 7 after transfection, this RNAi efficiency was maintained even after about 100–days from setting the newly transformed cells (data not shown). On the basis of these findings, we concluded that pAG could stably induce RNAi from the BmN4 genome.

## DISCUSSION

The BmN4 cell line was known as one of cultured cells with low transfection efficiency, like other silkworm–derived cell lines. Therefore, BmN4 cells are difficult to induce efficient RNAi in with dsRNA or siRNA by silencing a target gene function in a nearly the whole cell population of transfected cells. However, the pAG vector has *piggyBac* recognition sequences to transpose IR transgene into a chromosome of the cell, and the resulting cells possessing the integrated copy of the IR structure can be selected by antibiotics.

Under the our experimental condition, the hairpin dsRNA transcribed from IR transgene on chromosomal DNA decreased a reporter luciferase activity by approximately 70–80%. In many organisms, a complete knockdown is not required to produce a phenotype similar to its null–mutation. Therefore, our result indicated that simple use of pAG vectors facilely induced RNAi in the silkworm cells, so that a number of genes can be knocked down by this system. Although other studies have reported that expression plasmids encoding for long hairpin dsRNAs could be used in many organisms (Fujita *et al.*, 2009; Tomimoto *et al.*, 2012), knowledge concerning *B. mori* cells are of special importance, as this species provides the excellent biofactory that enables large–scale production of recombinant proteins in commercial scale. Also for the scientific research on the silkworm genetics, the presently established pAG knockdown system must be useful tools.

## ACKNOWLEDGEMENTS

This work was supported in part 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). We thank Dr. C. Aoki (Kyushu University Graduate School) for providing the BmN4 cell line.

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