Bombyx mori nucleopolyhedrovirus actin rearrangement-inducing factor 1 enhances systemic infection in B. mori larvae

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The actin rearrangement-inducing factor 1 (arif-1) gene is a baculoviral early gene conserved in most alphabaculoviruses. Previous studies reported that Autographa californica nucleopolyhedrovirus ARIF-1 protein induces filamentous actin concentration on the plasma membrane during the early stage of infection in Trichoplusia ni TN-368 cells, but its role in larval infection remains unknown. In this study, we performed behavioural screening using Bombyx mori larvae infected with Bombyx mori nucleopolyhedrovirus (BmNPV) mutants and found that larvae infected with arif-1-mutated BmNPVs did not show locomotor hyperactivity that was normally observed in BmNPV-infected larvae. arif-1-deficient BmNPVs also showed reduced pathogenicity and total viral propagation in B. mori larvae, whereas viral propagation of arif-1-deficient viruses was comparable with that of control viruses in B. mori cultured cells. An arif-1-defective BmNPV expressing the GFP gene (gfp) was used to monitor the progression of infection in B. mori larvae. GFP expression and quantitative reverse transcription-PCR analyses revealed that infection by the arif-1-disrupted virus was significantly delayed in trachea, fat body, suboesophageal ganglion and brain. These results indicated that BmNPV ARIF-1 enhanced systemic infection in B. mori larvae.

INTRODUCTION

The family Baculoviridae of entomopathogenic viruses is composed of four genera: Alphabaculovirus (lepidopteran-specific nucleopolyhedroviruses), Betabaculovirus (lepidopteran-specific granuloviruses), Gammabaculovirus (hymenopteran-specific nucleopolyhedroviruses) and Deltabaculovirus (dipteran-specific nucleopolyhedroviruses) (Jehle et al., 2006). Baculoviruses have a large (80 to >160 kbp), circular, supercoiled and dsDNA genome (Rohrmann, 2013), which generally encodes >100 protein-coding genes (Rohrmann, 2013). During their infection cycle, baculoviruses produce two types of virions to replicate efficiently in the host larvae and spread their progeny amongst insects. Budded virions (BVs) are required for cell-to-cell infection and are involved in the spread of the virus within an infected host. Occlusion-derived viruses (ODVs) are occluded within an occlusion body (OB) that protects and transmits ODVs from insect to insect via oral infection (Granados & Lawler, 1981; Keddie et al., 1989). At the late stage of infection, infected larvae show enhanced locomotory activity (ELA); infected larvae vigorously move about and finally climb to the upper plant foliage (Goulson, 1997; Kamita et al., 2005; Hoover et al., 2011), where their cadavers are degraded by the viral proteinase and chitinase (Slack et al., 1995; Hawtin et al., 1997).

The actin rearrangement-inducing factor 1 (arif-1) gene is a baculoviral early gene conserved in most alphabaculoviruses (Roncarati & Knebel-Mörsdorf, 1997; Rohrmann, 2013). The ARIF-1 protein has three transmembrane domains at the N terminus and a proline-rich region at the C terminus (Roncarati & Knebel-Mörsdorf, 1997). Previous studies reported that Autographa californica multiple nucleopolyhedrovirus (AcMNPV) ARIF-1 alters the localization of cytoplasmic filamentous (F)-actin during the...
early stage of infection in *Trichoplusia ni* TN-368 cells (Roncarati & Knebel-Mörsdorf, 1997; Dreschers et al., 2001). AcMNPV ARIF-1 is colocalized with F-actin at the plasma membrane, but the loss of the C terminus in AcMNPV ARIF-1 abolishes their localization to the plasma membrane (Dreschers et al., 2001). ARIF-1 is not a component of BVs in AcMNPV (Dreschers et al., 2001), which is consistent with the results of proteomic studies on the BVs of AcMNPV and *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) (Wang et al., 2010; Braconi et al., 2014). *arf*1 disruption does not affect viral propagation in cultured cells (Dreschers et al., 2001; Taka et al., 2013) and the importance of ARIF-1-induced actin rearrangement in baculovirus infection is unknown. During behavioural screening using *Bombyx mori* nucleopolyhedrovirus (BmNPV) and larvae of its host *B. mori*, we found that *arf*1-disrupted BmNPVs did not induce ELA. *arf*1 disruption also caused reduced pathogenicity and total viral propagation in larval infection. Further experiments revealed that ARIF-1 was required for efficient systemic infection in *B. mori* larvae.

**RESULTS**

**Disruption of *arf*1 causes a less virulent phenotype in *B. mori* larvae**

During our behavioural screening of BmNPV mutants in our laboratory, we noticed that *B. mori* larvae infected with a mutant virus (Bmpolh-proD; Fig. S1a, available in the online Supplementary Material) showed significantly reduced ELA (Fig. S1b). Further analyses revealed that Bmpolh-proD had a frameshift mutation in the *arf*1 gene that caused reduced ELA in infected larvae (Fig. S1 and Supplementary Results). *arf*1 was identified as the gene that was involved in the rearrangement of actin during the early stage of AcMNPV infection in *T. ni* TN-368 cells (Roncarati & Knebel-Mörsdorf, 1997). However, the function of *arf*1 in larval infection is unknown. We generated two additional WT-based *arf*1-defective BmNPVs, BmArifD (an *arf*1 deletion virus) and BmArif-FS (a mutant virus possessing the same frameshift mutation as Bmpolh-proD in the *arf*1 gene), and a revertant virus of BmArifD (BmArifDR) (Fig. 1a). Locomotion assays were performed using these three mutants and the results confirmed that *arf*1-defective BmNPVs showed reduced ELA (Figs 1b and S2). To investigate whether *arf*1 was involved in viral virulence in *B. mori* larvae, we compared the survival curves of fourth-instar larvae infected with WT and *arf*1 mutants. As shown in Fig. 1(c), larvae infected with BmArifD and BmArif-FS died 18 h later than larvae infected with WT and BmArifDR. This result indicated that *arf*1 disruption extended the survival time of BmNPV-infected *B. mori* larvae.

**arf*1-disrupted BmNPVs show reduced OB and BV production in *B. mori* larvae, but not in BmN cells**

Next, we examined OB and BV production in the haemolymph of fifth-instar *B. mori* larvae infected with WT and *arf*1 mutants. In larvae infected with *arf*1 mutants, production of both OBs and BVs at 96 h post-infection (p.i.) was significantly lower than those of WT and BmAriFD (Fig. 2a). At 120 h p.i., OB and BV production in *arf*1 mutant-infected larvae increased to 25 (BmAriFD) or 50% (BmAriFS) of those of WT and BmAriFD at 96 h p.i. (Fig. 2a). Combined with the result of the larval bioassay (Fig. 1c), these results indicated that disruption of *arf*1 caused the delay of viral propagation in BmNPV-infected larvae, resulting in decreased pathogenicity. In contrast, there were no significant differences in OB and BV production in BmN (BmN-4) cells infected with WT or *arf*1 mutants (Fig. 2b), which was consistent with prior studies (Dreschers et al., 2001; Taka et al., 2013).

**Disruption of *arf*1 results in delayed infection in larval tissues**

To address the function of ARIF-1 in *B. mori* larval infection, we generated an additional mutant, BmhsGFP-ARIFD, which expressed the GFP gene (*gfp*) under the control of the *Drosophila melanogaster* heat-shock protein 70 (*hsp70*) promoter and lacked a functional *arf*1. GFP fluorescence as an indicator of virus-infected cells was examined in five tissues (haemocytes, fat body, trachea, suboesophageal ganglion and brain) of *B. mori* larvae intra-haemocoeically infected with BmhsGFP (control virus) or BmhsGFP-ARIFD at 24, 48, 72, 96 and 120 h p.i. There were no apparent differences at 24, 48 and 72 h p.i. between BmhsGFP- and BmhsGFP-ARIFD-infected haemocytes, which were the primary target for the virus administered through this infection route (Figs 3a, b and S3a). At later stages of larval infection, GFP fluorescence was saturated in larval haemolymph infected with BmhsGFP at 96 h p.i. and with BmhsGFP-ARIFD at 120 h p.i. due to GFP release from dead tissues (Fig. S3a). The percentage of GFP-positive haemocytes was comparable between the two viruses at 24 and 48 h p.i. (Fig. 4), suggesting that virus can successfully infect in the haemocytes during the early stage of larval infection regardless of the presence of the *arf*1 gene. However, the fat body, trachea, suboesophageal ganglion and brain tissues from the BmhsGFP-ARIFD-infected larvae showed less GFP fluorescence compared with the same tissues from the BmhsGFP-infected larvae at the same time point (Figs 3c–j and S3b–e). In BmhsGFP-infected fat body and trachea, the area of GFP-positive cells started expanding at 48 h p.i., and expanded to most parts of the tissues at 72 and 96 h p.i. (Figs 3c, e and S3b, c). In contrast, GFP-positive cells were observed in the restricted area of the fat body and trachea of the BmhsGFP-ARIFD-infected larvae at 72 h p.i., and spread to the whole tissues by
120 h p.i. (Figs 3d, f and S3b, c). Similarly, in the suboesophageal ganglion and brain, BmhsGFP infection started at 72 h p.i. and spread to the large part of the tissues by 96 h p.i. (Figs 3g, i and S3d, e), whereas viral spread of BmhspGFP-ARIFD showed a 24 h delay compared with BmhspGFP (Figs 3h, j and S3d, e). These results suggested that ARIF-1 contributed to efficient viral spread, at least in some tissues.

Next, we performed quantitative reverse transcription-PCR (qRT-PCR) experiments using total RNA prepared from three tissues (haemocytes, trachea and brain) infected with BmhspGFP or BmhsGFP-ARIFD. qRT-PCR analyses of the viral ie1 gene revealed that the relative expression levels of ie1 were significantly lower in trachea at 48, 72 and 96 h p.i. and in brain at 48 and 72 h p.i. from the BmhspGFP-ARIFD-infected larvae compared with those from BmhsGFP-infected larvae (Fig. 5b, c). Combined with the results of GFP expression (Figs 3c–j and S3b–e), these results suggested that arif-1 disruption resulted in the delay of infection in these tissues. However, no significant differences were observed between haemocytes from the BmhsGFP- and BmhsGFP-ARIFD-infected larvae at 24 and 48 h p.i. The ie1 expression of BmhsGFP-

**Fig. 1.** Effect of arif-1 mutation on virus-induced ELA and viral pathogenicity in B. mori larvae. (a) Schematic representation of the arif-1 loci in WT and arif-1 mutants. In BmARIFD, an hsp70−lacZ gene cassette was inserted at nt +530 in the arif-1 gene. BmARIFDR was a repair mutant in which the hsp70−lacZ gene cassette of BmARIFD was replaced with the original BmNPV sequence. BmARIF-FS contains the same single-nucleotide deletion as Bmpolh-proD at nt +1037 in the arif-1 gene that causes a frameshift mutation. (b) Induction of ELA in fourth-instar B. mori larvae infected with WT or arif-1-mutated BmNPVs. Distances travelled at 84 h p.i. are shown in box-and-whisker diagrams (n=24). The boxes represent the median and 25–75 percentile ranges of the distance travelled. The whiskers indicate the most extreme data points, which were ≤1.5 times the interquartile range from the boxes. *P<0.05, Kruskal–Wallis analysis with Dunn’s post test in comparison with the value obtained from WT. (c) Survival curves of fourth-instar B. mori larvae infected with WT and arif-1-mutated BmNPVs. The median lethal times (LT50s) of WT, BmARIFD, BmARIFDR and BmARIF-FS were 102, 120, 102 and 120 h, respectively. *P<0.05, log-rank (Mantel–Cox) test with Bonferroni’s correction compared with WT.
ARIFD-infected haemocytes continued increasing until 96 h p.i., whilst that of the control virus started to decrease at 72 h p.i. (Fig. 5a). We obtained similar results by qRT-PCR analyses of \textit{gfp} (data not shown), indicating that \textit{arif-1} disruption caused the delay of systemic infection without aberrant expression of the viral early gene in specific tissues. Most of the haemocytes infected with \textit{BmhsGFP} were broken at 96 h p.i. by circulating viral cathepsin and chitinase, whereas haemocytes of \textit{BmhsGFP-ARIFD}-infected larvae were intact even at this time point, presumably due to the reduced secretion of viral cathepsin and chitinase from other infected tissues (Fig. S3a). Collectively, these findings strongly suggested that the ARIF-1 protein enhanced efficient systemic infection in larval tissues except haemocytes, which is a primary target of infection by intrahaemocoelically inoculated BVs, and thereby contributed to virulence and presumably to ELA induction in \textit{BmNPV}-infected \textit{B. mori} larvae.

**DISCUSSION**

\textit{arif-1} is a baculoviral early gene that was reported as the inducer of actin rearrangement during the early stage of \textit{AcMNPV} infection in TN-368 cells (Roncarati & Knebel-Mörsdorf, 1997). The ARIF-1 protein is a membrane-
bound protein that has three putative transmembrane domains at the N terminus and a proline-rich region at the C terminus (Roncarati & Knebel-Mörsdorf, 1997; Dreschers et al., 2001). Although arif-1 disruption in the AcMNPV or BmNPV genome did not affect viral propagation in cultured cells (Dreschers et al., 2001; Takahashi et al., 2013), the role of ARIF-1 in larval infection is unknown. Our present study revealed that arif-1 disruption caused delayed systemic infection of BmNPV in some tissues of B. mori larvae (Figs 3c–j, 5b, c and S3b–e), which led to reduced total viral propagation in larvae, pathogenicity and ELA (Figs 1, 2 and S2). We also found that the arif-1 frameshift mutant exhibited the same phenotype as arif-1 deletion mutants (Figs 1, 2 and S2). Due to this frameshift mutation, the ARIF-1 protein lacked a large part of the C terminus proline-rich region (Fig. 1a). A previous study showed that an AcMNPV derivative expressing C-terminal-truncated ARIF-1 did not induce actin rearrangement (Dreschers et al., 2001), suggesting that the proline-rich region in the C terminus of ARIF-1 might be essential for its function in both cultured cells and insect larvae.

Baculoviruses are known to modify the host’s actin cytoskeleton during different stages of their infection cycle. At the initial stage of infection, viral nucleocapsids released into the cytoplasm move intracellularly and transit to the

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**Fig. 3.** GFP expression in tissues of fifth-instar B. mori larvae intrahaemocoelically infected with BmhspGFP or BmhspGFP-ARIFD. Tissues of the BmhspGFP- or BmhspGFP-ARIFD-infected larvae were excised, washed and mounted on Petri dishes filled with PBS at 24, 48, 72, 96 and 120 h p.i. The samples (haemocytes (HE; a, b), fat body (FB; c, d), trachea (TR; e, f), suboesophageal ganglion (SOG; g, h) and brain (BR; i, j)) were observed under a fluorescence microscope. The left panels show bright-field images and the right panels show GFP expression. Bar, 100 μm.

**Fig. 4.** Ratio of GFP-positive haemocytes in fifth-instar B. mori larvae intrahaemocoelically infected with BmhspGFP or BmhspGFP-ARIFD. The haemolymph of BmhspGFP- or BmhspGFP-ARIFD-infected larvae was collected at 24 and 48 h p.i., and the number of total and GFP-positive haemocytes was counted using fluorescent microscopy. The ratio of GFP-positive cells is shown as mean ± SD (n=6). *P<0.05, Student’s t-test with Welch’s correction.
nucleus by polymerizing actin monomers (Ohkawa et al., 2010). This actin-based motility is mediated by the viral capsid protein P78/83, which contains domains conserved in Wiskott–Aldrich syndrome proteins and activates actin polymerization by the host Arp2/3 complex (Goley et al., 2006; Ohkawa et al., 2010). After the onset of viral early gene expression, actin-based motility is required for accumulation of a subpopulation of nucleocapsids at the cell periphery, which presumably contributes to virion budding during the early phase of infection in midgut epithelial cells (Ohkawa et al., 2010). In addition, P78/83 translocates the host Arp2/3 complex into the nucleus, leading to nuclear actin polymerization that is essential for viral progeny formation during the late stage of infection (Goley et al., 2006). Additionally, it is also known that F-actin on the plasma membrane becomes concentrated by ARIF-1 during the early stage of infection (Roncarati & Knebel-Mörsdorf, 1997), but the role of this actin rearrangement is unknown. The present study revealed that BmNPV ARIF-1 enhanced systemic infection in some tissues of B. mori larva (Figs 3c–j, 5b, c and S3b–e). Confocal microscopy revealed that the arif-1 frameshift mutant of BmNPV induced the same actin rearrangement as WT during the early stage of infection in cultured BmN cells (Fig. S4). These observations raise three possibilities. One possibility is that ARIF-1 is required for actin rearrangement only in the specific cell type(s) of B. mori larval tissues required for efficient systemic infection. Another possibility is that the far C terminus of ARIF-1 is sufficient to induce actin rearrangement, but insufficient to enhance systemic infection. The third possibility is that ARIF-1 is not an inducer for actin rearrangement in BmNPV infection. BmNPV arif-1 has an additional proline-rich sequence of 22 aa in the C-terminal region compared with that in other baculoviruses (Xu et al., 2010), which implies that the function of BmNPV ARIF-1 might be different from the ARIF-1 proteins in other baculoviruses. In this case, BmNPV ARIF-1 may enhance systemic infection by a mechanism other than actin rearrangement.

Insect tissues are fundamentally surrounded by the basal lamina, which is presumed to be the virus-impenetrable extracellular layer considering its small pore size (Passarelli, 2011). However, baculoviruses can escape this barrier by infecting tracheoblasts that exist at the ends of tracheal branches and penetrate the basal lamina into insect tissues (Engelhard et al., 1994). After tracheoblast infection, infected cells release progeny BVs within host tissues or into haemolymph, and the infection spreads to the whole body. In this study, we found that arif-1 disruption caused a delay of BmNPV infection in fat body, trachea, suboesophageal ganglion and brain tissue of intrahaemocoelically infected B. mori larvae (Figs 3c–j, 5b, c and S3b–e), whereas no significant delay of infection was observed in haemocytes, which are not surrounded by the basal lamina (Figs 3a, b, 5a and S3a). We also confirmed that arif-1 disruption did not affect viral propagation in BmN cells (Fig. 2b). Considering the absence of the ARIF-1 protein in BV of AcMNPV and AgMNPV (Dreschers et al., 2001; Wang et al., 2010; Braconi et al., 2014), our results suggest that ARIF-1 is not a component of BV and enhances systemic infection by an unknown
mechanism that is presumably involved in viral entry, spread or propagation in specific tissues.

In conclusion, we have reported for the first time, to our knowledge, the functional characterization of the baculovirus arif-1 gene in larval infection. We found that arif-1 disruption resulted in reduced ELA, viral virulence and pathogenicity in BmNPV-infected B. mori larvae. Further analysis revealed that viral spread is significantly delayed in larval tissues, suggesting that BmNPV ARIF-1 protein is an enhancer of systemic infection in B. mori larvae. To clarify the detailed mechanism by which ARIF-1 enhances systemic infection, we are currently analysing the biochemical characteristics of ARIF-1 using different types of arif-1-mutated BmNPV derivatives.

**METHODS**

**Insects, cell lines and viruses.** B. mori larvae (F1 hybrid Kinsha × Showa) were reared as described previously (Katsuma et al., 2012b). BmN cells were cultured at 26 °C in a TC-100 insect medium containing 10 % FBS (Nakanishi et al., 2010). The T3 strain (Maeda et al., 1985) from BmNPV was used as WT. A polh deletion mutant (BmNPV-abb) (Kang et al., 1998) was used as the parental virus for the recombinant viruses. A mutant BmNPV expressing gfp under the D. melanogaster hsp70 promoter (BmhsGFP) was confirmed by PCR and DNA sequencing.

**Generation of recombinant BmNPVs lacking polh promoter activity.** To generate the polh promoter-deficient mutant Bmpolh-proD (Fig. S1a), the transfer vector pBhEPS1 (carrying the polh gene and the flanking regions) (Kang et al., 1998) was used as a template to introduce a mutation in the polh promoter. The mutation was introduced in the viral very late promoter motif (from ATAGC to ATACA) (Rankin et al., 1988) that led to a loss of either polh transcription or OB production in BmNPV (Shimada et al., 1994; Katsuma et al., 1999). This mutagenesis in the polh promoter was performed by an overlapping PCR (Nakanishi et al., 2010) and confirmed by DNA sequencing. The resultant plasmid was transfected into BmNPV cells using Lipofectin reagent (Invitrogen) (Katsuma et al., 2004). Five days after the transfection, the medium was collected and stored at 4 °C until use. A recombinant BmNPV (Bmpolh-proD) was plaque-purified by identifying the plaques that were OB-negative. The mutation in the polh promoter of Bmpolh-proD was confirmed by DNA sequencing of PCR products amplified from the genomic DNA of a candidate recombinant. To construct Bmpolh-proDR, a revertant virus of the polh promoter (Fig. S1a), the pBhEPS1 plasmid was cotransfected with Bmpolh-proD genomic DNA into BmN cells using Cellfectin II reagent (Invitrogen) with genomic DNA of BmNPVs. A deletion of arif-1 was confirmed by PCR using the primers in Table S1.

**Locomotion assays.** Locomotion assays were performed according to a previously described method with minor modifications (Kokusho et al., 2011). In brief, fourth-instar B. mori larvae were injected with 25 μl viral suspension containing 5 μg kanamycin ml−1 and 1 × 10^7 p.Eu. using a syringe fitted with 30-gauge needle (Nipro) within 18 h after moulting. Larvae were reared with an artificial diet at 25 °C. Infected larvae were photographed at 6 h intervals from 72 h p.i. until death. At each 6 h interval, the 24 larvae were separated into four groups (n=6 larvae per group) and were placed on the centre of a piece of paper. Photographs were taken from a fixed point with a digital camera at 30 s intervals until 3 min after release. The coordinates of each larva, at the midpoint of the third and fourth abdominal segments, were determined at each time point after release using ImageJ software (http://imagej.nih.gov/ij/). The distance travelled during each 30 s interval was determined and summed up to calculate the total distance travelled in the 3 min. The distance travelled by dead larvae was designated zero.

**RNA sequencing of WT and Bmpolh-proD.** Fifth-instar B. mori larvae were injected with 50 μl viral suspension of WT or Bmpolh-proD containing 1 × 10^7 p.Eu. and returned to the artificial diet at 25 °C. At 72 and 96 h p.i., brains from the infected larvae (34–36 larvae per sample) were collected. The total RNA of each sample was prepared using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Using 1 μg total RNA, a RNA sequencing library was constructed using Illumina GA Sample Preparation Kit (Illumina) according to the manufacturer’s instructions, as previously described (Kawaoka et al., 2013). Illumina GA sequencing adapters were ligated to the cDNA ends. cDNAs were amplified by 15 cycles of PCR, using Phusion DNA Polymerase (Finnzymes). Single-end-read RNA sequencing tags of 36 bp generated using an Illumina GA sequencer according to the standard protocol (Kawaoka et al., 2013). Deep-sequencing data obtained in this study are available under GenBank accession number DRA002538.

The RNA sequencing data from WT- or Bmpolh-proD-infected brains were mapped to the genome sequence of BmNPV isolate T3 (GenBank accession number L33180) by Bowtie (Langmead et al., 2009) within two mismatches. Visualization of mapping data was performed by Tablet (Milne et al., 2013), verifying that almost complete genome sequences of WT and Bmpolh-proD were successfully obtained from the RNA sequencing data. By comparing the genome sequences of WT and Bmpolh-proD, 13 nt substitutions were identified except for the polh promoter region. These substitutions
were confirmed by PCR and DNA sequencing (3130xl Genetic Analyzer; Applied Biosystems) using the WT, Bmpolh-proD and BmNPV-abb genomes.

**Larval bioassay.** Fourth-instar *B. mori* larvae were intraheumocoe-lickally infected with a viral suspension (25 µl TC-100 medium containing BVs at 1 × 10^6 pfu and 5 mg kanamycin ml^-1) using a syringe fitted with a 30-gauge needle (Nipro) to determine the median lethal time (LT_{50}). Larvae were inoculated within 18 h after molting (Katsuma & Shimada, 2009) and 24 larvae were used for each virus in this experiment.

**OB and BV production in *B. mori* larvae and in BmN cells.** Fifth-instar *B. mori* larvae were starved for several hours, injected with 50 µl viral suspension containing BVs at 1 × 10^6 pfu and 5 mg kanamycin ml^-1 using a syringe fitted with a 30-gauge needle (Nipro) and returned to the artificial diet at 25 °C. Larvae were inoculated within 18 h after molting (Katsuma & Shimada, 2009). At 96 h p.i., the haemolymph from each infected larva was collected and the released OBs were counted using a haemocytometer as described previously (Katsuma et al., 2009). Haemolymph BV titre was determined by plaque assay on BmN cells (Nakanishi et al., 2010). BmN cells were infected with WT or recombinant BmNPVs at m.o.i. 5. After 1 h incubation, the culture medium containing the virus was removed and fresh medium was added. This medium change was designated 0 h p.i. At 72 h p.i., 1/300 volume of the culture medium was har-vested and used in the plaque assay to determine the BV production. BmNPV-infected BmN cells were gently scraped with a rubber policeman and the total OB production was calculated as described previously (Katsuma et al., 2009).

**Assays for GFP expression in *B. mori* larvae.** Fifth-instar *B. mori* larvae used for GFP expression assays were infected with BmhsGFP or BmhsGFP-ARIFD and reared as described above. Tissues of the infected larvae were excised, washed in PBS and mounted in Petri dishes filled with PBS. Haemolymph was collected from the injected larvae and examined using a haemocytometer. Each tissue was observed under a fluorescence microscope (FLoid Cell Imaging Station; Life Technologies). The number of total and GFP-positive haemocytes was counted (n=6, > 30 haemocytes per sample), and the ratio of GFP-positive haemocytes was calculated.

**qRT-PCR.** Haemocytes, trachea and brain were dissected from fifth-instar larvae (n=5 or 6). TRizol (Invitrogen) was used to isolate the total RNA from these tissues of each larva. Total RNA (13, 250 and 25 ng) from each samples of haemocytes, trachea and brain, respectively, was reverse transcribed into cDNA. qRT-PCR was performed using a KAPA SYBR FAST qPCR kit (Kapa Biosystems) with previously described primers (Katsuma & Shimada, 2009; Table S1) and the StepOnePlus Real-Time PCR system (Applied Biosystems).

**Fluorescence microscopy.** Cultured BmN cells were mock-infected or infected with WT or BmARIF-FS at m.o.i. 5. At 12 h p.i., the supernatant was removed and the cells were washed twice with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min. The fixed cells were washed three times in PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed three more times in PBS and incubated for 20 min with 1% BSA/PBS containing TRITC-conjugated phala-loidin at 10 µM (Molecular Probes). The cells were then washed three times in PBS, sealed in 10 µl ProLong Gold reagent (Molecular Probes) and examined using a confocal laser scanning biological microscope (FV10i-DOC; Olympus).

**Statistical analyses.** Statistical analyses were performed using Prism 5 software (GraphPad). One-way ANOVA was performed with Dunnett’s post tests comparing each of the treatment group means with the mean of the control group. Locomotion assay data were subjected to Kruskal–Wallis analysis with post hoc Dunn’s tests. Student’s t-test with Welch’s correction was used to compare the ratio of GFP-positive haemocytes and the values obtained in the qRT-PCR experiments. A log-rank (Mantel–Cox) test with Bonferroni’s cor-rec-tion was used to compare the survival curves of each mutant with WT.

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