Temporal expression profiling of novel Spodoptera litura nucleopolyhedrovirus-encoded microRNAs upon infection of Sf21 cells

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Baculoviruses are arthropod-specific pathogens, and find extensive applications in pest control strategies and recombinant protein expression. Spodoptera litura nucleopolyhedrovirus (SpltNPV) infects the tropical armyworm Spodoptera litura, which is an important polyphagous crop pest widely distributed in regions of Asia and Oceania. Using next-generation sequencing, we report stage-specific profiling of SpltNPV-encoded microRNAs (miRNAs) at different time intervals post-infection (p.i.) of Sf21 cells. Sequence length distribution analysis of the small RNA libraries revealed a significant increase in 20 nt reads and a reduction of other size fractions during late phases of infection. In silico miRNA prediction tools identified 48 novel SpltNPV-encoded miRNAs, of which 10 were validated experimentally in Sf21 cells using Northern blot analysis and TaqMan quantitative real-time (qRT)-PCR. The viral miRNAs were also found to be expressed in fat-body and mid-gut tissues of infected fifth-instar S. litura larva. qRT-PCR analysis confirmed that expression of most viral miRNAs was triggered 12 h p.i. and continued thereafter. Gene Ontology and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway annotation of computationally predicted targets of the reported miRNAs suggested a major impact of these miRNAs on cell signalling, protein translation and metabolic processes.

INTRODUCTION

MicroRNAs (miRNAs) are a well-known family of small non-coding RNAs that regulate spatio-temporal gene expression by sequence-specific binding. Their influence on a myriad of physiological processes, including differentiation, growth, apoptosis and host–pathogen interactions, is indubitable (Ambros, 2011; Blahna & Hata, 2013; Subramanian & Steer, 2010; Zhou & Rana, 2013). Although miRNAs occupy negligible genomic space, they can modulate diverse biological functions. Consequently, after the discovery of the first viral miRNA in 2004 (Pfeffer et al., 2004), several other viruses were found to encode multiple miRNAs from their genome (Ghosh et al., 2009; Kincaid & Sullivan, 2012; Pfeffer et al., 2005; Sullivan et al., 2005). miRBase release 20 housed nearly 500 viral miRNAs, with the majority being encoded by DNA viruses (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008). Recently, there have been reports of miRNAs encoded by members of the family Baculoviridae (Singh et al., 2010, 2014; Zhu et al., 2013), which are known to infect a wide range of insect species.

Baculoviruses are arthropod-specific, 80–180 kb large dsDNA viruses (Adams & McClintock, 1991; Fauquet et al., 2005). These viruses exhibit biphasic morphology in the form of occluded and budded viruses pre-ordained for primary larval infection and horizontal spread within the host, respectively. These have been employed extensively as protein expression vectors (Contreras-Gómez et al., 2014; Hitchman et al., 2011; Kost et al., 2005; Murphy et al., 2004) and as biopesticide agents against major lepidopteran crop pests (Heimpel et al., 1973; Summers, 2006; Sun & Peng, 2007). Spodoptera litura nucleopolyhedrovirus (SpltNPV) has been exploited as a commercially viable alternative to obliterate the polyphagous insect pest Spodoptera litura, which infects more than 120 plant species spanning several regions of Asia and Oceania (Nathan & Kalaivani, 2005; Okada, 1977; Zhang, 1994). Owing to practical limitations of using WT baculovirus as a pesticide, strategies are being employed to introduce robust recombinant baculovirus variants or insecticidal proteins to suppress major crop pests (Bonning et al., 1992; McCutchen et al., 1991). Advances in our understanding of the molecular biology of baculoviral pathogenesis would thus help in formulating effective pest control strategies.
The baculovirus replication cycle is complex, with viral genes being expressed as a cascade in early, late and very late phases (Friesen & Miller, 1986). The early infection phase (0–6 h post-infection (p.i.)) is characterized by host RNA polymerase-driven transcription of genes that are primarily involved in viral replication. This is followed by viral DNA replication, viral RNA polymerase-mediated transcription and production of budded virions in the late phase (~6–24 h p.i.). In the very late or occlusion phase (>24 h p.i.), occluded virions accumulate in the nucleus of the infected cell and are released from the cell upon lysis (Blissard & Rohrmann, 1990). Numerous studies have outlined the transcriptional and proteomic changes associated with these phases (Crawford & Miller, 1988; Friesen & Miller, 1985; Rapp et al., 1998). However, the expression of baculovirus-encoded small RNAs and their subsequent role during these phases has not been investigated extensively. Recent insights into the function of baculoviral miRNAs have revealed interesting revelations about the stratagem of these viruses. *Bombyx mori* nucleopolyhedrovirus-encoded bmn-pv-mir-1 was found to downregulate the expression of host nuclear protein Ran involved in exportin 5-mediated small RNA transport machinery (Singh et al., 2012), whilst bmn-pv-mir-3 was found to be expressed during the early phase of infection and decrease the expression of baculoviral DNA-binding protein P6.9 (Singh et al., 2014). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV)-encoded AcMNPV-mir-1 was found to target viral gene ODV-E25, favouring the generation of occlusion-derived virions (Zhu et al., 2013). Recently, Mehrabadi et al. (2013) reported differential regulation of host miRNAs upon baculoviral infection. Clearly, the non-coding small RNAome in baculovirus infection needs to be decoded further to develop an insight into the functioning of these viruses.

In the present study, we sequenced seven small RNA populations from SpltNPV-infected Sf21 cells at different time intervals p.i. Using computational tools, Northern blotting and TaqMan quantitative real-time (qRT)-PCR analysis, we identified and validated the expression of novel miRNAs encoded by SpltNPV. The temporal expression pattern of the baculoviral miRNAs was analysed at different stages of infection using next-generation sequencing and TaqMan qRT-PCR. Furthermore, *in silico* tools were used to predict the probable viral and cellular targets for the validated miRNAs. The identification and profiling of baculovirus-encoded miRNAs and the subsequent elaboration of their role in pathogenesis will highlight the events critical to virus-inflicted insecticidal activity at progressive stages of infection.

**RESULTS AND DISCUSSION**

**Deep sequencing**

Replication of baculoviruses in host cells is synchronized in early, late and very late phases, which are characterized by explicit events and concomitant transcription of specific viral proteins. To understand the corresponding changes in small RNA profiles during these phases, we sequenced seven small RNA libraries generated from uninfected Sf21 cells and SpltNPV-infected Sf21 cells at 0, 6, 12, 24, 48 and 72 h p.i. Sf21 cells were infected with WT SpltNPV at a low m.o.i. to permit viral establishment as well as to avoid considerable cell lysis. Viral infection of Sf21 cells was confirmed by observed arrest of cell growth, enlargement of cells and nuclei, and appearance of occlusion bodies in nuclei 48 h p.i. Sf21 cells were found to be fully permissive for SpltNPV infection as observed and reported previously (Maeda et al., 1990). Illumina HiScan, a massively parallel next-generation sequencing technology, was used to generate 149,694,981 reads from all seven libraries. After removal of low-quality reads and clipping of adaptor sequences, nearly 145 million reads (97.10 %) remained; of these, 133 million reads (91.46 %) were in the size range 18–30 bp, accounting for a total of 9,860,788 unique sequences in all the libraries. Deep-sequencing results for individual libraries are summarized in Table 1.

**Size distribution analysis**

The size distribution pattern of reads originating from individual libraries was examined and found to be altered considerably upon baculovirus infection. Two major populations were observed in all the libraries, comprising 20–22 and 26–28 nt reads, representing the miRNA/small interfering RNA (siRNA) and PIWI-associated RNA (piRNA) populations, respectively, as also described in other insect species (Chen et al., 2012; Wei et al., 2009). In uninfected Sf21 cells, the piRNA population was preponderant and represented 40.19 % of the total reads. As the Sf21 cell line is derived from ovarian tissue of *Spodoptera frugiperda*, the piRNA population in these cells is expected to be enriched (Girard et al., 2006; Lau et al., 2009). However, the piRNA population was found to be significantly attenuated by 24 h p.i., which declined further until 72 h p.i. On the contrary, 20–22 nt long reads increased from 29 % in uninfected cells to 65 % in infected cells at 72 h p.i., with a sharp increase seen specifically in 20 nt reads (Fig. 1). Normalized read count data across the samples revealed a fourfold increase in the 20 nt fraction, but a nearly threefold decrease in the 26–28 nt fraction at 72 h p.i. The shift in size distribution appeared to be initiated between 12 and 24 h p.i. A similar pattern was also reported for AcMNPV-infected Sf9 cells (Mehrabadi et al., 2013). Expression of novel baculovirus-encoded miRNA might be a contributing factor towards this increase as most baculoviral miRNAs were found to be expressed at 12 h p.i., as described below. The determinants and molecular consequences of perturbations in the piRNA population are not clear at present and need further investigation. The most characterized role of piRNA has been silencing of transposons in the germline. In addition, piRNAs have now been implicated in epigenetic modifications (Peng & Lin, 2013; Yin & Lin, 2007), germ cell
2011; Léger
bp were found on scanning the SpltNPV genome with identification of SpltNPV miRNAs and profiling novel baculovirus-encoded miRNA. The current study has focussed primarily on identification investigation on this subject has not been attempted, and this might have diverse outcomes. However, further analyse the significance of a reduced piRNA population as Houwing et al.

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SRNALOOP and 56 predicted precursor miRNAs were Cox et al.

and antiviral defence (Hess et al., 2013). It would thus be interesting to analyse the significance of a reduced piRNA population as this might have diverse outcomes. However, further investigation on this subject has not been attempted, and the current study has focussed primarily on identification and profiling novel baculovirus-encoded miRNA.

**In silico identification of SpltNPV miRNAs**

In total, 500 potential hairpin like sequences of 60–200 bp were found on scanning the SpltNPV genome with SRNALOOP and 56 predicted precursor miRNAs were collected from the Vir-Mir database. Validation of all these sequences using MiPred yielded 42 sequences, of which 34 belonged to the Vir-Mir dataset, whilst another eight were predicted by our analysis. Nomenclature provided by the Vir-Mir database was retained for convenience. Sequences of the probable precursor miRNAs along with their stem-loop structures and minimum free energy (MFE) are given in Fig. S1 (available in the online Supplementary Material). The description of the genomic co-ordinates of the predicted viral precursors is included in Table S1(a). Merged reads from all seven libraries were aligned to SilkDB and the Sf21 genome to remove the reads originating from the host (Duan et al., 2010; Kakumani et al., 2014; Wang et al., 2005); 2.8 and 60.4 % of the reads mapped onto the respective datasets, which confirmed the specificity of the data. Next, the unaligned reads were mapped onto the precursor sequences to predict miRNA duplexes from each precursor. This resulted in 48 candidate miRNAs from the viral genome, out of which 40 existed as duplex pairs. The majority of the viral miRNAs were 20 nt long, which partly explained the expansion of 20 nt reads observed in the size distribution analysis of baculovirus-infected cells. There seemed to be no specific arm preference for SpltNPV miRNAs because predominant miRNAs were found to originate from both arms: 13 from the 3’ arm and 16 from the 5’ arm. None of the predicted viral miRNAs were found to be significantly homologous to known miRNAs in miRBase release 20 (Griffiths-Jones, 2006), suggesting that these were novel miRNAs. The sequences of all the predicted miRNAs along with their absolute and normalized expression counts in individual libraries are given in Table S2.

All the predicted miRNAs were further checked for their absence in the uninfected Sf21 library to ensure that the sequences were not derived from the host. As expected, all but one of the candidate miRNAs (11684_3p) were absent in the uninfected Sf21 library. Although the reads aligning to the host genome had been removed, this sequence was not detected in the reported draft assembly of the Sf21 genome (Kakumani et al., 2014). Nonetheless, 11684_3p has been retained as a probable viral miRNA candidate because the viral genome contains a validated miRNA precursor sequence that can encode this miRNA sequence. The read count of 11684_3p increased tremendously from 98 reads per million (RPM) in the mock-infected library to 292 231 RPM at 12 h p.i. Moreover, miRNA originating from the other arm of the same viral precursor, i.e. 11684_5p, was also identified in our analysis to be present only in the infected libraries, but to be absent in the uninfected library. There is, however, a possibility that 11684_3p is simultaneously expressed from both the host and the virus, which according to our knowledge has not been reported previously. Fig. 2 depicts the relative expression of putative miRNAs in all seven libraries using a heat map. The majority of the sequences started appearing by 12–24 h p.i. and continued expressing until 72 h p.i. Another notable feature of the study is that the candidate miRNAs were found to be expressed consistently across multiple sequencing libraries. Most of the candidate miRNAs were absent in the 0 and 6 h p.i. libraries, but were consistently present in the 12, 24, 48 and 72 h p.i. libraries, emphasizing their viral origin and adding to the credibility of the data.

**Validation of miRNA expression using Northern blotting**

Putative viral miRNAs showing high expression values in deep-sequencing data were validated for their presence in infected Sf21 cells using Northern blot analysis. As the majority of miRNAs were expressed optimally at 24–72 h p.i. in the deep-sequencing results, small RNA was isolated from uninfected and SpltNPV-infected Sf21 cells at 72 h

<table>
<thead>
<tr>
<th>Library</th>
<th>Total reads (n)</th>
<th>High-quality trimmed reads [n (%)]</th>
<th>Unique tags (n)</th>
</tr>
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<tbody>
<tr>
<td>Mock-infected</td>
<td>25 091 079</td>
<td>24 508 996 (97.68)</td>
<td>2 734 365</td>
</tr>
<tr>
<td>0 h p.i.</td>
<td>24 555 807</td>
<td>23 391 353 (95.26)</td>
<td>2 820 113</td>
</tr>
<tr>
<td>6 h p.i.</td>
<td>20 555 930</td>
<td>19 751 860 (96.08)</td>
<td>2 435 673</td>
</tr>
<tr>
<td>12 h p.i.</td>
<td>10 289 827</td>
<td>9 869 039 (95.91)</td>
<td>1 486 812</td>
</tr>
<tr>
<td>24 h p.i.</td>
<td>26 380 451</td>
<td>25 970 497 (98.45)</td>
<td>3 114 094</td>
</tr>
<tr>
<td>48 h p.i.</td>
<td>22 607 499</td>
<td>22 067 059 (97.61)</td>
<td>2 821 353</td>
</tr>
<tr>
<td>72 h p.i.</td>
<td>20 214 388</td>
<td>19 796 121 (97.93)</td>
<td>2 523 755</td>
</tr>
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</table>
Fig. 1. Trimmed read length size distribution analysis of the small RNA populations of all seven Sf21 libraries: mock-infected Sf21 cells (a), and SpIiNPV-infected Sf21 cells at 0 (b), 6 (c), 12 (d), 24 (e), 48 (f) and 72 (g) h p.i. The small RNA population of Sf21 cells reveals dual-size distribution: 20–22 and 26–28 nt reads. After 24 h p.i., the distribution becomes highly skewed towards the smaller fraction.
and checked for the presence of selected miRNAs. Eight novel miRNAs (11684_3p, 11684_5p, 11672_5p, 11672_3p, 11701_3p, 11698_3p, 11699_3p and 11660_3p) were identified in the infected samples and are shown in Fig. 3. These included two miRNA duplex pairs (11684_3p/11684_5p and 11672_5p/11672_3p). All baculoviral miRNAs were observed in the size range 20–22 nt. 11684_3p, 11684_5p, 11672_5p, 11701_3p and 11698_3p were highly abundant in deep-sequencing data and were identified easily in Northern blot analysis using unmodified \( \gamma \)-\( ^{32} \)P-labelled DNA probes, whilst \( \gamma \)-\( ^{32} \)P-labelled RNA probes were used to identify the remainder in order to increase the sensitivity of detection. As both 11684_3p and 11684_5p were predicted from viral precursor miRNA sequences, and have been validated by Northern blot analysis, this emphasizes the fact that 11684_3p has a viral origin as well. Moreover, the 11684_5p sequence was not present either in the uninfected Sf21 library or upon Northern blot analysis of uninfected Sf21 cells (data not shown).

**Expression validation of miRNAs in insect tissues**

The identification of chosen miRNAs was carried out in mid-gut and fat-body tissues of infected *S. litura* larvae using custom-designed TaqMan qRT-PCR assays. The presence of the selected miRNAs in insect tissues was depicted by monitoring the fold change in expression of the selected viral miRNAs with respect to uninfected mid-gut and fat-body tissue samples. For the sake of comparison, expression of chosen miRNAs in uninfected tissues was considered as a minimum value. All previously validated miRNAs showed significant expression in both the infected tissues, whilst 11660_3p was seen only in infected fat-body tissue and was absent in mid-gut tissue. The restricted distribution of 11660_3p might be indicative of a specific function relevant to the tissue. Such tissue specificity for viral miRNA expression and occurrence has been reported previously for other viruses, e.g. rat cytomegalovirus and Epstein–Barr virus (Kim *et al.*, 2007; Meyer *et al.*, 2011). Two other miRNAs (11673_5p and
11701_5p were also checked for their presence in infected tissues. 11701_5p was the proposed duplex partner of the abundantly expressed 11701_3p miRNA and was hence verified for its presence, whilst 11673 _5p showed good expression in deep-sequencing studies. Indeed, the two miRNAs were detected in both infected fat-body and mid-gut tissues. Fig. 4 depicts the expression of SpltNPV miRNAs in infected and uninfected fat-body and mid-gut tissues of S. litura larvae. bmo-miR-2766-3p was used for normalization across infected and mock-infected samples. Three host miRNAs (bmo-miR-2766-3p, bmo-bantam-3p and dya-bantam) were chosen for normalization of miRNA expression across infected and mock-infected tissues due to their consistent levels in our sequencing analysis. All three are conserved miRNAs and their presence has been predicted in Sf9 cells previously (Mehrabadi et al., 2013). Out of these three, bmo-miR-2766-3p proved to be the most appropriate normalization control as it showed the highest expression without much variation upon infection.

**Expression profiling of SpltNPV miRNAs at different time intervals in Sf21 cells**

Expression profiling of selected miRNAs was also performed using TaqMan-based qRT-PCR assays to correlate with the deep-sequencing data. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative change in expression of the miRNAs in uninfected and infected Sf21 cells at 6, 12, 24, 48 and 72 h p.i. Again, expression of miRNA in uninfected cells was considered minimal for comparison. Expression of all the tested miRNAs began at 12–24 h p.i. and continued to rise until 72 h p.i. The results of deep-sequencing and TaqMan qRT-PCR assays are thus in good agreement with one another. However, some minor aberrations were also seen, e.g. 11699_3p was found to have a higher expression at 72 h p.i. in TaqMan qRT-PCR assays, with a lower expression in deep-sequencing analysis. Nevertheless, there was a high correlation in both sets of data and such anomalies have been reported previously for deep-sequencing studies. bmo-miR-2766-3p was used as a normalization control among the samples. Relative expression of individual miRNAs at different time intervals is shown in Fig. 5.

**Target prediction**

Viruses are known to encode miRNAs to alter both the host and viral proteome to facilitate their survival and spread. For our study, we used RNAhybrid v2.0 software to predict the probable viral and cellular targets of the validated SpltNPV miRNAs. Viral mRNA sequences were collected from RefSeq (http://www.ncbi.nlm.nih.gov/refseq/). Major viral targets of the validated SpltNPV miRNAs are depicted in Fig. 6, and a list of the targets in uninfected and infected Sf21 cells at 6, 12, 24, 48 and 72 h p.i. Again, expression of miRNA in uninfected cells was considered minimal for comparison. Expression of all the tested miRNAs began at 12–24 h p.i. and continued to rise until 72 h p.i. The results of deep-sequencing and TaqMan qRT-PCR assays are thus in good agreement with one another. However, some minor aberrations were also seen, e.g. 11699_3p was found to have a higher expression at 72 h p.i. in TaqMan qRT-PCR assays, with a lower expression in deep-sequencing analysis. Nevertheless, there was a high correlation in both sets of data and such anomalies have been reported previously for deep-sequencing studies. bmo-miR-2766-3p was used as a normalization control among the samples. Relative expression of individual miRNAs at different time intervals is shown in Fig. 5.

**Fig. 3.** Northern blot hybridization of selected SpltNPV miRNAs in infected Sf21 cells. The figure demonstrates the Northern blot analysis of selected SpltNPV miRNAs at 72 h p.i. using $\gamma^{32P}$-labelled probes. Small RNAs of infected Sf21 cells were run on a denaturing PAGE gel alongside a NEB small RNA ladder ranging from 25 to 17 nt. A specific band was obtained at 20–22 nt for baculoviral miRNAs.

**Fig. 4.** Detection of selected SpltNPV miRNAs in fat-body and mid-gut tissues of infected fifth-instar S. litura larvae. Relative expression of selected baculoviral miRNAs in SpltNPV-infected fifth-instar S. litura larvae with respect to mock-infected larvae using TaqMan qRT-PCR. bmo-miR-2766-3p was used as a normalization control.
along with MFEs and P values of prediction are presented in Table S3. Many viral structural proteins and nucleocapsid-associated proteins, e.g. polyhedrin, ODV-EC27, VP1054, VP80 and VP91, emerged as probable targets of several miRNAs. It is worthwhile noting that AcMNPV-miR-1 has been found to target viral gene ODV-E25, which likely resulted in the reduction of budded virions and enhances the generation of occlusion-derived virions (Zhu et al., 2013). Targeting of structural proteins by the virus might be a mechanism to regulate its own production or act as a switch between the production of budded and occluded virions, as described by Zhu et al. (2013). Our analysis proposes that baculoviruses might encode other miRNAs such as AcMNPV-miR-1 to regulate the production of their structural proteins. In addition, it has been reported that Heliothis virescens ascovirus (HvAV) downregulates the expression of its DNA polymerase I using HvAV-miR-1 at late stages of infection (Hussain et al., 2008). In our study, miRNA 11698_3p is proposed to target both viral DNA polymerase and helicase. Thus, baculoviruses might employ a mechanism similar to ascoviruses to regulate viral replication. Experimental validation of these miRNA targets might help in our understanding of the role of baculoviral miRNAs in the regulation of various viral processes.

For prediction of host targets, analysis was carried in two systems: B. mori and Sf21. As the Sf21 genome has been sequenced recently, its gene annotation is relatively incomplete and hence there was a need to use another system in parallel. B. mori was reported to have the closest homology among the available genomes and was hence used in our

Fig. 5. Expression profiling of selected SpltNPV miRNAs in mock-infected and SpltNPV-infected Sf21 cells at 6, 12, 24, 48 and 72 h p.i. Relative expression of selected SpltNPV miRNAs is shown at different time intervals after SpltNPV infection with respect to mock-infected Sf21 cells using the $2^{-\Delta\Delta C_t}$ method. bmo-miR-2766-3p was used as a normalization control.
analysis. The dataset for *B. mori* included all RefSeq mRNA sequences as well as 3' and 5' untranslated regions (UTR) sequences reported in the UTR database. For Sf21, all of the reported 11,595 Sf21 mRNA sequences were used. The 3' and 5' UTR sequences of the available genes were fetched from the draft genome and were also used for the target prediction. Out of the available 15,055 and 11,595 unique RefSeq/mRNA sequences for *B. mori* and Sf21, respectively, 1,170 and 1,287 sequences were found to have binding sites in their mRNA, 3' or 5' UTR. Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway annotation of these target genes were performed, and it was observed that 144 GOs and 77 biological pathways were commonly targeted in both the organisms. Conservation of targets between homologous species enhances the relevance of this study. In addition, 78 *B. mori*-specific and 8 Sf21-specific pathways were also targeted. Annotated target genes in the host along with MFE and *P* values for prediction are included in Table S3. The GOElite tool was used to identify statistically significant GOs and KEGG pathways targeted by the miRNA by normalizing the over-represented genes. It was observed that the enriched targets in Sf21 were a subset of
B. mori. Fig. 7 presents pie-chart distributions of the enriched GO categories and KEGG pathways targeted by viral miRNAs in B. mori.

GO analysis indicated that the molecular function of nearly 60% of the targeted genes was in stimulus response. KEGG pathway annotation indicated that baculoviral miRNA might play an important role in manipulating major host cell signalling pathways. Target genes for several miRNAs were associated with the Forkhead factor FoxO, mammalian target of rapamycin (mTOR), Hippo and Hedgehog signalling pathways. Baculoviruses had been reported to have an effect on host signalling pathways in previous

**Fig. 7.** Pie-chart distribution for statistically significant GOs (a) and KEGG pathways (b) targeted by SpltNPV miRNAs in B. mori. Viral miRNAs targeting the specific GO/KEGG pathway have been mentioned along with the total number of targets observed in each category.
It was observed that knockdown of ERK (extracellular signal-regulated kinase)- and JNK (c-Jun N-terminal kinase)-dependent signalling pathways leads to a reduction in budded and occluded virion formation in BmNPV infection, and that these signalling pathways are in turn activated during the late phase of baculovirus infection (Katsuma et al., 2007). Xiao et al. (2009) found increased phosphorylation of cellular Akt upon baculovirus infection. FoxO signalling is involved in the response against oxidative stress and DNA damage, apoptosis, and cell cycle control (Alvarez et al., 2001; Dijkers et al., 2000; Kops et al., 2002; Tran et al., 2002). The mTOR signalling pathway has a major role in protein translation and regulation of cellular growth (Fingar et al., 2004; Gingras et al., 2001). The Hippo signalling pathway is responsible for the control of organ size through regulation of cell proliferation and apoptosis (Badouel & McNeill, 2011) whilst Hedgehog signalling has been implicated in the regulation of the morphogenesis of various tissues and organs (Ingham et al., 2011). There have been various studies reporting perturbations in cell cycle and apoptotic mechanisms after baculovirus infection. In one study, AcMNPV was observed to arrest the Sf9 cell cycle at the G2/M stage (Braunagel et al., 1998) whilst in another study, synchronized Sf9 cells when infected at the G1 or S phase were arrested in the S phase, whilst those infected at the G2/M stage were blocked at the G2/M stage (Ikeda & Kobayashi, 1999). In yet another study, Helicoverpa armigera nucleopolyhedrovirus was found to arrest cell growth at the G2/M stage (Zhou et al., 2004). Clem (2001) reported that apoptosis induction in baculovirus-infected cells was associated with some late viral protein or an event during early-to-late phase transition. In addition, baculoviral proteins are known to block apoptosis for optimal infection (Clem et al., 1991; Crook et al., 1993). However, to date, baculoviral miRNAs have not been known to regulate cellular apoptosis, growth and development. Our report indicates the probability of the existence of a regulatory link between these events and baculoviral miRNAs. Further validation of reported miRNA targets might thus help in our understanding of the underlying mechanisms behind the physiological changes observed during baculoviral infection.

Furthermore, it is known that baculoviral infection is associated with global shutdown of host protein synthesis at 12–18 h p.i., the mechanism of which is still unknown (Nobiron et al., 2003; Ooi & Miller, 1988; Tjia et al., 1979). It is interesting to note that most of the baculoviral miRNAs in our deep-sequencing analysis also start to appear by 12 h p.i. The target genes of some of these miRNAs are linked to our deep-sequencing analysis also start to appear by 12 h p.i. Thus, there seems to be a high correlation between these studies, and the role of baculoviral miRNAs in translational control should be investigated.

In summary, this study reported the identification of miRNAs from SpltNPV using deep-sequencing. Northern blotting and TaqMan qRT-PCR analysis. Their stage-specific expression was examined in Sf21 cells at different time intervals. In silico prediction of both cellular and viral targets of these miRNAs was undertaken to understand the effect of SpltNPV miRNAs on baculoviral–host interactions.

**METHODS**

**Infection of Sf21 cells with SpltNPV.** Neonate *S. litura* larvae were obtained from NBAII (Bangaluru, India) and maintained on a semi-synthetic diet at 25 °C with a 16 h light/8 h dark photoperiod. Third-instar larvae were administered with ~10^5 SpltNPV occlusion bodies that had been partially purified using deionized water (Hoover et al., 1995). Haemolymph was extracted from infected fifth-instar larvae on ice and diluted in a 1:2 ratio with ice-cold PBS containing 20 mM reduced glutathione to prevent melanization. Sf21 cells were maintained as a monolayer in serum-free TNM-FH insect medium at 27 °C. The diluted haemolymph was passed through a 0.22 μm Millipore filter and used to infect Sf21 cells for 1 h. Serum containing TNM-FH medium was replaced after infection. Cells were monitored for signs of infection after 48 h and the supernatant from infected cells was used to titrate the virus by the plaque assay method (Brown & Faulkner, 1977). Sf21 cells infected at m.o.i. 2 were used for subsequent studies.

**Small RNA library preparation and sequencing.** Total RNA was extracted from pooled samples of mock-infected and SpltNPV-infected Sf21 cells at 0, 6, 12, 24, 48 and 72 h p.i. Total RNA was confirmed for integrity using an Agilent 2100 Bioanalyzer. The samples were size-fractionated on a 15% PAGE gel and the 16–30 nt fraction was collected. The proprietary 5' RNA adaptor (5'-GUUCAGAGUUCCAGUCGGCGACGAUC-3') was ligated to the RNA pool with T4 RNA ligase. Ligated RNA was size-fractionated on a 15% agarose gel and the 40–60 nt fraction excised. The 3' RNA adaptor (5'-pUCGUAUCGCCGCUUCUGCUUGidT-3'; p, phosphate; idT, inverted deoxythymidine) was subsequently ligated to precipitated RNA using T4 RNA ligase. Ligated RNA was size-fractionated on a 10% agarose gel and the 70–90 nt fraction (small RNA plus adaptors) excised. Small RNAs ligated with adaptors were subjected to reverse transcription-PCR (SuperScript II reverse transcriptase, 15 cycles of amplification) to produce sequencing libraries. PCR products were purified. Prepared small RNA libraries were further sequenced using Illumina HighScan, a massively parallel sequencing technology, as per Illumina protocols.

**Prediction of viral miRNA candidates.** Fifty-six predicted SpltNPV precursor miRNA sequences were collected from the Vir-Mir database (http://alk.ibms.sinica.edu.tw/) (Li et al., 2006, 2008). In addition, other possible miRNA precursor sequences were identified by screening the SpltNPV genome (RefSeq NC_003102.1) using RNAlooper with length ≤95 and score ≥17. Newly identified precursors along with the predicted precursors from the Vir-Mir database were subjected to further validation using MiPred. High-quality reads in the size range 18–30 nt were merged from all the libraries and mapped onto the latest version of the Silkworm Genome Database: SilkDB (http://silkworm.genomics.org.cn/) (Duan et al., 2010; Wang et al., 2005) and the Sf21 genome (Kakumani et al., 2014) to remove host miRNA and other sequences originating from the host. Unaligned sequences were mapped onto the MiPred-validated precursor sequences with zero mismatch/gap to predict miRNA duplexes from each precursor (Jiang et al., 2007). The most abundant
reads mapping onto the precursor arm and forming a probable miRNA duplex were considered as putative miRNA candidates. In the absence of duplex sequences, the most abundant read mapping onto one of the arms of a predicted precursor was considered as a candidate. Sequences having a count of <10 reads in all the stages of infection were discarded.

Validation of mature miRNAs using Northern blotting. Small RNA was extracted from mock-infected and 72 h infected Sf21 cells using a mirVana miRNA isolation kit (Ambion). Small RNA (40 μg) of each sample was resolved on 15% denaturing PAGE gel alongside a NEB small RNA ladder and blotted onto a Hybond-N nylon membrane. The membrane was UV cross-linked and the presence of selected miRNAs detected using [γ-32P]ATP-labelled DNA or RNA probes depending on the sensitivity of detection for each miRNA using mirVana guidelines. The NER ladder probe sequence was also labelled using [γ-32P]ATP and used for size determination. Nucleotide probes used for this study are listed in Table S1(b).

Expression validation of miRNAs in insect tissues. Total RNA was extracted from mid-gut and fat-body tissues of mock-infected and SpltNPV-infected fifth-instar larvae using TRIzol reagent (Invitrogen) followed by DNase I digestion (Invitrogen) to remove genomic DNA contamination. Reverse transcription was carried out using a TaqMan miRNA reverse transcription kit (Applied Biosystems) with miRNA-specific reverse transcription primers contained in TaqMan MicroRNA Assays and 10 ng RNA template as described by the manufacturer. qRT-PCR was performed in triplicate using TaqMan 2x Universal PCR Master Mix without AmpErase UNG (Applied Biosystems) in an ABI StepOnePlus Real-Time PCR system with the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative expression of selected viral miRNA in infected and mock-infected tissues was normalized against the levels of host miRNA bmo-miR-2766-3p using the 2-ΔΔCt method. (Livak & Schmittgen,

Expression profiling of selected viral miRNAs. Total RNA was extracted from mock-infected and SpltNPV-infected (6, 12, 24, 48 and 72 h p.i.) Sf21 cells using TRIzol reagent (Invitrogen) followed by DNase I treatment (Invitrogen). Reverse transcription and TaqMan qRT-PCR were performed using custom-designed TaqMan miRNA assays as described above. bmo-miR-2766-3p was used for normalization among the samples.

Target prediction. In total, 142 cDNA sequences of SpltNPV and 14,594 cDNA sequences of B. mori were obtained from the_REFSEQ database (RefSeq NC_003102.1 and Taxonomy ID 7091, respectively). The 5' and 3' UTR sequences of B. mori were obtained from the UTR database. (http://utrdb.bio.ith.cnir.ir/) (Grillo et al., 2010). In total, 11,595 cDNA sequences of Sf21 reported by Kakumani et al. (2014) were obtained, and their 5' and 3' UTR sequences were fetched from the reported genome assembly NCBI GenBank Accession ID JQCY00000000 (Kakumani et al., 2014). RNAhybrid (v2.0) target prediction software was used to predict viral and host target sites from the mentioned datasets (Rehmsmeier et al., 2004). The RNAcalibrate option was used to generate 5000 random sequences with a Gaussian distribution for accurate P value calculation whilst obtaining the normalized MFE. Targets with P<0.05 were considered as true positive for downstream significant biological analysis. All the cDNA sequences obtained for B. mori, Sf21 and SpltNPV were annotated against the UniProt database specific for insect proteomes by tBLASTx with an E value cut-off of 0.01. GO and KEGG pathway annotation of genes was obtained based on the UniProt ID. GOElite (http://www.genmapp.org/go_elite) was used to identify statistically significant GOs and KEGG pathways enriched by the targets of the miRNA. A custom database for B. mori and Sf21 was

created in GOElite with GO and KEGG pathway annotation. Genes targeted by selected miRNAs were used as Query, and all the annotated genes in B. mori and Sf21 were used as the database for the statistical analysis. ORA (Over Representation Analysis) of the miRNA targets was performed using GOElite, and enriched GO categories and KEGG pathways that harboured the targets were identified for B. mori and Sf21 separately. ORA analysis results of B. mori and Sf21 were integrated to identify specific GO categories and KEGG pathways that were targeted by the differentially expressed miRNA by means of harbouring the target genes.

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