



PI3K-Akt pathway mediated antiviral mechanism in silkworm *Antheraea mylitta*

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Received: 14 November 2019 / Accepted: 17 March 2020 / Published online: 1 April 2020
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Abstract *Bombyx mori* nucleopolyhedrosis virus (BmNPV) is a highly pathogenic virus to domestic silkworm *Bombyx mori*, often causing severe economic losses to silk industry. Interestingly, there are no documented reports of NPV infection in the wild silkworm *Antheraea mylitta*. Analysis of gene expression datasets and comparative genomic analysis of sequence datasets from *B. mori* and *A. mylitta* was undertaken to unravel the potential immune-related proteins and immune pathways involved. The *B. mori* silkworm races Sarupat and CSR-2 which are resistant and susceptible to BmNPV respectively were selected and challenged with virus to study BmNPV resistance related genes and their expression profile. The genes were filtered to isolate membrane proteins, their sequences were retrieved from UniProt and were compared against *A. mylitta* using BLASTp to search for similarity. Major proteins were putative defence proteins. Further, KEGG database was used to check for the presence of differentially regulated proteins in certain metabolic pathways. The analysis of the pathways was carried out using cytoscape software based on betweenness centrality and stress. The PI3K-Akt pathway was found to be the hub of all signals triggered during the course of NPV infection. We analyzed how the NPV infection modulates PI3K-Akt signaling by gene expression studies of the key regulator of the pathway i.e. Akt using real-time PCR in *A. mylitta*. A significant upregulation of Akt expression from 72 h post

infection reaching its peak with a 2.30 fold change at 120 h pi clearly indicates an enhanced level of immune response in host towards the viral infection.

Keywords *Antheraea mylitta* · *Bombyx mori* · PI3K-Akt pathway · Antiviral mechanism · Nucleopolyhedrosis virus (NPV)

Introduction

The silkworm, *Bombyx mori* is one of the economically important insect which is being reared for the last 5000 years for the production of raw silk. Sericulture industry has a potential in generating both direct and indirect employment to various stakeholders and provides sustainable income at regular interval with minimal capital investment with sufficient returns.

India is the second largest producer of silk in the world and also the largest consumer of silk in the world [1]. Sericulture faces biological challenges from pathogenic bacteria, fungi and viruses which causes loss of almost 20–30% of the potential cocoon production every year [2]. Among the various silkworm diseases viral diseases alone accounts for 80% of total cocoon loss of which *B. mori* nucleopolyhedrovirus (BmNPV) is the most serious one which calls for intensive research efforts towards this disease control. Till date, no effective treatment measures are available to combat BmNPV infection [3]. Also traditional strain breeding has been tried to improve BmNPV resistance in silkworms, but it takes considerable time of over years to achieve the same and also results in low cocoon production which is not economically feasible. Therefore, improving silkworm's resistance to NPV by an effective and simple method is the need of hour.

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The silkworm relies on different innate immune responses to act against invading pathogens. Some proteins from silkworm have shown various levels of anti BmNPV activity. The proteins purified from midgut juice of NPV resistant silkworms, such as serine protease [4], Bm-lipase [5] or a NADH- oxidoreductase like protein named BmNOX [6] have shown to impair the virulence of NPV, suggesting these protein's antiviral activity. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway is an evolutionarily conserved innate immune pathway in the insect immune response mechanism [7, 8]. After the infection of *Autographa californica* nucleopolyhedrovirus (AcMNPV) in *Spodoptera frugiperda* Sf9 cells, the key gene stat in the JAK/STAT signalling pathway is activated to mediate the immune response against AcMNPV [9].

Though the domestic silkworm *B. mori* and wild silkworm *A. mylitta* both belong to the order Lepidoptera and being sericigenous insects of economic importance they still differ in their susceptibilities to viral infections. The NPV infection and its crop loss is well established in *B. mori* whereas, there are no reports of NPV infection in *A. mylitta* [10]. The microarray datasets of the *B. mori* silkworm race CSR-2 and sarupat which are susceptible and resistant to BmNPV respectively were selected for this study [11]. To find out the BmNPV resistance related proteins/pathways involved in resistant silkworm, the differentially expressed membrane proteins in the midgut of sarupat and CSR-2 were considered as it is the first site of virus host interaction. The differential regulation of genes in resistant race after BmNPV infection would indicate their possible role in antiviral immune response.

In the present study in order to unravel the potential immune-related genes and pathways we naturally challenged *A. mylitta* with BmNPV to check for the validation of the data driven hypothesis which would prove a step ahead of the TiO₂ nanoparticles induced immunity in silkworms. Immune signalling pathway analysis revealed that TiO₂ nanoparticles inhibited the proliferation of silkworm BmNPV, while promoting the expression of BmAkt to improve immunity reporting the role of PI3K-Akt signalling pathway in *B. mori* in response to BmNPV infection [12]. Our results may provide some reliable mechanism associated with the interaction of virus and the host cell by using *A. mylitta* as the invertebrate insect model for the first time.

Materials and methods

In-silico studies

Analysis of gene expression datasets and comparative genomic analysis of sequence datasets from *B. mori* and *A. mylitta* to identify the key proteins/signalling pathway involved was carried out. Datasets which consisted of microarray data of two sets of experiments, SET 2A (sarupat resistant race) and SET 2B (CSR-2 susceptible race) of *B. mori* were downloaded which consisted of supplementary data in the form of excel sheets. It gave a list of all the upregulated and downregulated proteins along with their UniProt accession numbers, names, fold change, etc. The data was processed for the identification of significantly modulated in their expression specially belonging to membrane proteins i.e. receptors, transmembrane proteins, transporter proteins, exchange proteins, recognition proteins and co-transporters since the focus was on the host-pathogen interactions. The sequence of the filtered proteins was downloaded from Uniprot in FASTA format.

Comparison against *Antheraea mylitta*

Sequence alignment of the sequences was carried out using BLASTp server. All the protein sequences were compared against the genome of *A. mylitta* and the percentage similarity of the sequences was noted down.

KEGG database

The KEGG database was exploited to search for involvement of the proteins in certain pathways [13]. This was done by using the links provided on the UniProt database for the specific proteins [14]. The database was also used to create pathway network of the following pathways: mTOR signaling pathway, FOXO signaling pathway and Longevity regulating pathway.

Pathway reconstruction and cytoscape analysis

From the results obtained for the KEGG database analysis, three pathways were found whose analysis was carried out using cytoscape based on betweenness centrality and stress while treating the network as directed [15].

In-vivo studies: relative gene expression analysis of Akt gene

Fourth instar silkworm *A. mylitta* larvae were procured from Mudimiyal farm, Chevalla dist., Telangana. Indoor rearing of the same on *Terminalia arjuna* branches at

25 ± 1 °C and $75 \pm 2\%$ relative humidity was carried out in our laboratory. Fifth instar 1st day larvae of the experimental zone (30 larvae) were fed with leaves containing BmNPV (4.5×10^8 polyhedra/ml) and the control zone ones (30 larvae) with leaves smeared with sterile water. All treated leaves were air dried before feeding. Silkworms in both control zone and experimental zone were dissected to isolate midgut once every 24 h, flash frozen in liquid nitrogen and stored at -80 °C till further use.

PCR amplification and sequencing of novel gene (Akt)

As the Akt gene in *A. mylitta* was not reported before and also the unavailability of the sequence for further studies drove us to check for its presence in the genome using PCR. Primers were designed for Akt gene using the conserved region from the Clustal W alignment of the genes in different silkworm species. The PCR amplicon product was further sequenced. PCR amplification was carried out whose conditions were 94 °C for 5 min, 35 cycles of 94 °C for 5 s, 55 °C for 10 s, 72 °C for 10 s and 72 °C for 5 min. Primers used for the Akt gene are: 5'-GACATGG-CACGGCTCGGCACCAGC-3' (Forward) and 5'-CTTCAATATCTTCATAGCGTACA-3' (Reverse).

Real time expression analysis of Akt gene

Primers were designed and synthesized for the house-keeping tubulin gene (5'-TCGTCGAGCCCTACAACCTCT-3' (Forward) 5'-ACTCGGTGAGGTCCACATTC-3' (Reverse) and for the Akt gene as above.

Total RNA was extracted from *A. mylitta* midgut samples using Trizol reagent (Invitrogen), reverse transcribed into cDNA using oligo dT primer (Chromous kit). PCR was standardized to obtain amplification for the gene. A Real-time run was taken on ABI Step-one Real Time PCR machine. The PCR conditions were 94 °C for 5 min, 40 cycles of 94 °C for 5 s, 55 °C for 10 s, 72 °C for 10 s and 72 °C for 5 min. Results were calculated using $-\Delta\Delta C_t$ method.

Results and discussion

Genome wide expression profiles associated with BmNPV resistance and susceptibility

The microarray data of differentially regulated genes in response to BmNPV infection showed a total of 1324 genes of which 735 were upregulated and 589 were downregulated in the resistant Sarupat race. Whereas in case of susceptible CSR-2 race out of 4298 genes 2183 were upregulated and 2115 were downregulated.

The disparity of differentially regulated proteins seen between the two races of *B. mori* is evident as shown in Table 1. The number of upregulated receptor proteins in CSR-2 susceptible race was almost thrice than that found in the resistant Sarupat race. Similarly, there was a fivefold increase in the number of transmembrane proteins from resistant to susceptible race. Likewise, the number of downregulated receptors doubled in the susceptible CSR-2 race from the resistant Sarupat race and although there was no significant increase in the number of transmembrane proteins, the transporters showed a quintuple rise.

Comparison against *Antheraea mylitta*

The differentially regulated genes compared with *A. mylitta* genome showed the following ortholog proteins (Tables 2, 3, 4, 5) Since the availability of either the genomic sequence source or expressed sequence tag data is limited for *A. mylitta*, the differential gene expression analysis was done with resistant and susceptible strains of *B. mori*. However, the differentially regulated genes were searched for their presence in *A. mylitta* through reciprocal blast search which confirm the presence of orthologous proteins in *A. mylitta*, genome. The reciprocal blast resulted in identification of 47 orthologs out of 227 differentially expressed genes. For which the UNIPROT IDs were mined and their details are given in Tables 2 and 4 which consists of upregulated proteins found in two different races, resistant Sarupat and susceptible CSR-2 race of *B. mori* that have orthologous proteins present in *A. mylitta*. The table distinctly shows the reduction in number of upregulated proteins found in both the races from the result obtained upon plain filtering of the raw data. Out of 33 upregulated membrane proteins, only 11 had orthologs in *A. mylitta* in the resistant race whereas in the susceptible race, the number reduced to a mere 15 proteins from 82. Even though the number of upregulated proteins in both the races appears almost closer to each other, upon further examination it can be seen that with the exception of 3 proteins, the rest are completely different. This indicates that there is an upregulation of completely different set of proteins in the resistant race as compared to susceptible race.

Out of the three proteins that were similarly upregulated in both the races, accession numbers, Q9U5A8, B7FF34 and A0SY07, only Q9U5A8 which was Tyrosine protein kinase receptor was found to have a role in FOXO signaling pathway. The protein when checked against KEGG database showed that it was in fact involved in not just FOXO signaling pathway, but also in Longevity regulating pathway and mTOR signaling pathway. Tables 3 and 5 consist of downregulated proteins present in the resistant Sarupat race and susceptible CSR-2 race of *B. mori*. As

Table 1 Number of proteins obtained after filtering

	Receptors	Transmembrane	Transporter	Co-transporter	Total
<i>SET2A</i>					
UR	20	1	8	4	33
DR	15	3	8	0	26
<i>SET2B</i>					
UR	63	5	11	3	82
DR	30	4	40	7	81

SET 2A is the resistant sample (Sarupat race) and SET 2B is the susceptible sample (CSR-2 race) of *B. mori*
UR upregulated; *DR* downregulated

Table 2 Upregulated proteins in resistant Sarupat race of *B. mori* (SET 2A)

S. no	Accession number	% similarity	<i>Antheraea mylitta</i> counterpart	Annotation
1.	Q9U5A8	53	Q0Q028.1	Tyrosine-protein kinase receptor
2.	B3XXN6	47	B0JFB8.1	Neuropeptide receptor A23
3.	C5H7S1	58	POC963.1	Guanylatecyclase
4.	B0XBB5	50	Q0Q028.1	Scavenger receptor cysteine-rich protein precursor
5.	B7FF34	37	POC963.1	Gustatory receptor
6.	A0SY07	58	Q0Q028.1	Adiponectin receptor
7.	Q17LB9	44	Q0Q027.1	Guanine nucleotide exchange factor
8.	E2BGX2	42	B0JFB8.1	Putative inorganic phosphate cotransporter
9.	B0XDI9	42	Q0Q027.1	Zinc transporter
10.	Q7YZB6	42	B0JFB8.1	Transporter
11.	B0WLM4	39	Q0Q029.1	Sugar transporter

Table 3 Down regulated proteins in resistant Sarupat race of *B. mori* (SET 2A)

S. no	Accession number	% similarity	<i>Antheraea mylitta</i> counterpart	Annotation
1.	Q9SX31	53	Q0Q028.1	Proline-rich receptor-like protein kinase PERK9
2.	E2BBZ4	75	Q7SID7.1	Gustatory receptor trehalose 1
3.	Q17HY6	36	Q7SID7.1	Glutamate receptor
4.	E0X906	83	Q0Q027.1	Sugar transporter protein 3

with the upregulated proteins, it can be seen that the number of proteins present drastically decreased when compared to that of the filtered proteins. It shows that not all the proteins present in *B. mori* had orthologs present in *A. mylitta*. Out of 26 membrane proteins in the resistant race, only 5 showed similarity with *A. mylitta* whereas in the susceptible race, the number declined down from 81 membrane proteins to 17.

However, unlike the upregulated proteins, there was a significant difference in the number of downregulated proteins between the two races. The susceptible race had almost thrice the number of proteins as compared to the resistant race. Yet, at the same time, the set of proteins

differentially regulated in the resistant variety was different than those in susceptible except for two further supporting the conclusion derived from the analysis of the upregulated proteins that proteins differentially regulated in the susceptible and resistant races during NPV infection are completely different. These results indicate that, it is not just a different set of proteins that is differentially regulated during the infection, there is a difference in the entire biological mechanism that takes place during infection.

Table 4 Upregulated proteins in susceptible CSR-2 race of *B. mori* (SET 2B)

S. no	Accession number	% similarity	<i>Antheraea mylitta</i> counterpart	Annotation
1.	C4B7Z7	75	Q0Q029.1	Olfactory receptor
2.	Q2PGW0	67	P0C963.1	Lipophorin receptor
3.	Q9U5A8	53	Q0Q028.1	Tyrosine-protein kinase receptor
4.	E2BKM9	39	P0C963.1	Tyrosine-protein phosphatase non-receptor type 4
5.	Q86S10	60	Q0Q027.1	Leucine-rich repeat G protein-coupled receptor
6.	C4B7W0	64	B0JFB8.1	Odorant receptor
7.	E2B682	44	Q0Q028.1	Low-density lipoprotein receptor-related protein 1
8.	B7FF34	37	P0C963.1	Gustatory receptor
9.	A0SY07	58	Q0Q028.1	Adiponectin receptor
10.	B3XXN9	37	Q0Q027.1	Neuropeptide receptor A26
11.	C4B7Z4	35	Q0Q027.1	neuropeptide signaling pathway
12.	A8CGK0	83	Q0Q029.1	Nicotinic acetylcholine receptor beta 2 subunit
13.	B0WV85	67	Q0Q027.1	Amino acid transporter, putative
14.	B0WPL1	45	P0C963.1	Sugar transporter
15.	D3VW60	44	P0C963.1	Origin recognition complex subunit 5

Table 5 Downregulated proteins in susceptible CSR-2 race of *B. mori* (SET 2B)

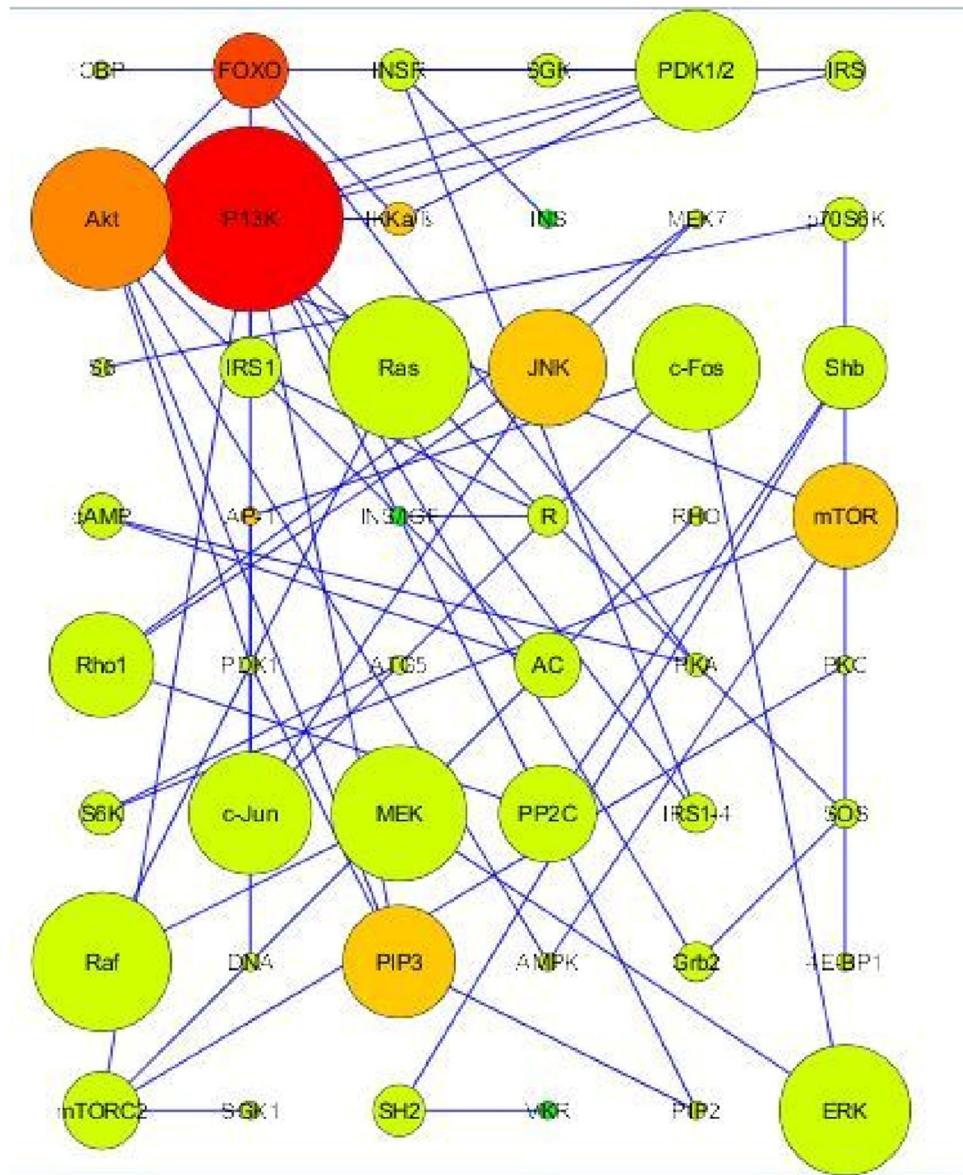
S. no	Accession number	% similarity	<i>Antheraea mylitta</i> counterpart	Annotation
1	P35464	37	B0JFB8.1	Diuretic hormone receptor
2	Q2F5V9	50	Q0Q028.1	Translocon-associated protein subunit beta
3	D9YT55	39	Q0Q029.1	Phosphatidylserine receptor-like protein
4	Q9SX31	53	Q0Q028.1	Proline-rich receptor-like protein kinase PERK9
5	B3XXP9	50	Q0Q028.1	Neuropeptide receptor B1
6	Q2F5L0	40	Q7SID7.1	Signal sequence receptor
7	B3XXM3	57	P0C963.1	Neuropeptide receptor A10
8	B3GTD7	63	Q0Q029.1	Gustatory receptor
9	Q0N2R5	43	Q0Q028.1	Signal recognition particle receptor alpha subunit
10	Q1HPN9	60	P0C963.1	Copper transporter
11	Q1HRQ8	83	P0C963.1	Translocating chain-associated membrane protein
12	E0X906	83	Q0Q027.1	Sugar transporter protein 3
13	F2YDQ6	71	P0C963.1	ATP-binding cassette transporter subfamily G member Bm3
14	B6CY69	64	Q0Q029.1	
15	E0X904	53	P0C963.1	Inorganic phosphate co-transporter
16	D3TM83	55	B0JFB8.1	Signal recognition particle subunit SRP72
17	Q0N2R7	37	P0C963.1	Signal recognition particle subunit SRP68

Cytoscape analysis

Cytoscape analysis of pathways FOXO signaling, Longevity regulating, mTOR signaling, showed that PI3K played a major role connecting all the three pathways (Fig. 1), getting affected even due to a slight change, thereby affecting all three pathways, the receptor of which turned out to be Tyrosine protein Kinase receptor. This was

also shown in one of the previous reports which determined the involvement of PI3K-Akt signaling pathway in the event of BmNPV infection [12].

Fig. 1 Cytoscape analysis of FOXO signalling pathway, longevity regulating pathway and mTOR signalling pathway



Expression characteristics of key gene Akt in PI3K-Akt signaling pathway

The resistance of silkworms against BmNPV is not only associated with resistance genes but also immune signalling pathways [12]. Gene Akt amplified from *A. mylitta* midgut (Fig. 2) was sequenced and deposited in NCBI with Gene accession no. MK263735. In this study, the transcription levels of a key gene in the PI3K-Akt pathway was measured. Time course analysis of gene expression of Akt gene by real-time PCR clearly shows an upregulation of Akt gene in BmNPV infected midgut samples. As shown (Fig. 3) the transcription levels of PI3K-Akt pathway marker gene Akt showed significant increase in Akt transcription level from 72 h pi with a fold change of 1.47 followed by an increase of 2.24 fold change at 96 hpi

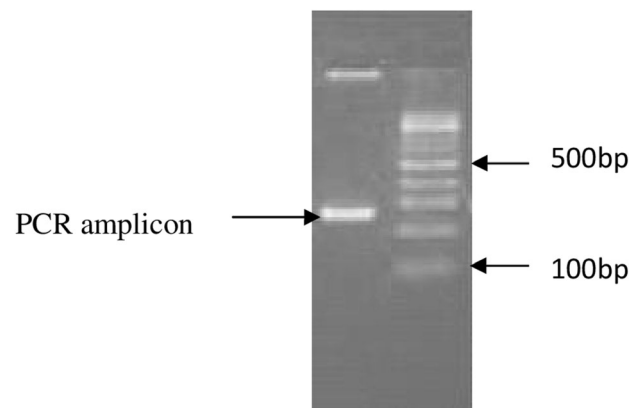
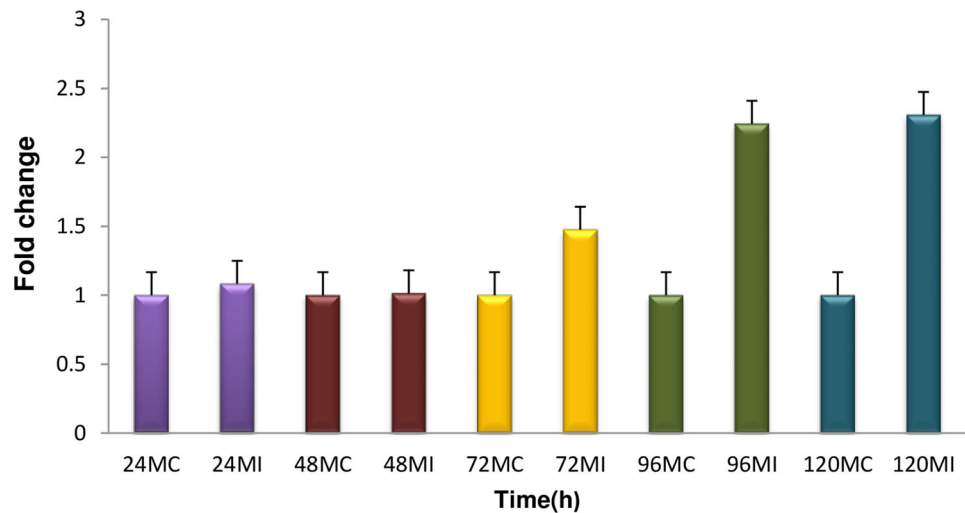


Fig. 2 Agarose gel electrophoresis of PCR product (Akt gene) on 2% agarose gel. Lane 1 = Gene Akt; Lane 2 = DNA ladder

Fig. 3 Relative copies of Akt gene in *A. mylitta* midgut samples at different time intervals post infection (6 h, 24 h, 48 h, 72 h, 96 h and 120 h). *MC* midgut control, *MI* midgut infected



which reached a peak value thereafter with a fold change of 2.30 at 120 h pi indicating that the upregulation of Akt was induced by BmNPV infection aiding in increased host immune response. So, overall Akt gene expression was upregulated 0.47 times, 1.24 times and 1.30 times respectively at 72 h pi, 96 h pi and 120 h pi. Relative expression of Akt gene among the control and infected group at different time intervals was found to be statistically insignificant ($P < 0.05$) upon ANOVA analysis. These data suggest that activation of the PI3K-Akt pathway may be at least partially responsible for the antiviral response in *A. mylitta* upon BmNPV infection restricting a productive infection in a non-permissive host.

Immune response in insects is quite dynamic and different effector genes are likely expressed at variable time points during infection, contributing efficiently to the ability of the insects to ward off infections in spite of the absence of adaptive immunity. Wild silkworms have hardly been the subject of detailed scientific investigations delinquent largely to non-availability of molecular and genetic data on these species. As a first step in the present study we explored the differentially regulated membrane proteins during virus host negotiation in *A. mylitta* upon NPV infection by retrieving the gene expression data for susceptible and resistant variety of *B. mori* as complete genomic data for *A. mylitta* is not available. Doing this we also propose a new strategy for further comparative gene expression studies in other organisms whose genomic data is not available.

This report focuses on combining functional and subtractive genomic approach with special reference to the membrane proteins that were differentially regulated during BmNPV infection which has not been previously done according to the accessible information. Similarly, in spite of countless microarray assay reports being available for antiviral proteins, to this end no reports deal with the

ability of membrane proteins to play an important role in BmNPV infection. All the accessed previous studies dealt with the situation that takes place upon the entry of the virus into the cell, like the differential regulation of the proteins, production of certain enzymes, etc. Shifting the focus from differential regulation of extracellular or intracellular proteins during a viral infection to membrane proteins is important as the first interaction of the virus takes place with that of the membrane proteins. Thus, any differential regulation in the membrane proteins determines the ability of the organism to fend off the virus since it is the site of virus host negotiation. Moreover, many membrane proteins play a significant role in various signaling mechanisms to communicate the external stimulus. Thus, any up or downregulation of membrane proteins is bound to affect the entire transcriptome and in turn the cellular behavior and response.

In silico analysis data has shown the involvement of several membrane proteins which do modulate differentially in a race dependent manner. Many proteins show conserved orthologs in *A. mylitta* further suggesting PI3K-Akt signaling pathway to be the hub of all the signals that is triggered during the NPV infection and can be exploited to understand the immunological consequences. On one hand, PI3K-Akt signalling is critical for many viruses to complete their life cycle, including cellular entry, replication and egress. On the other hand, the same PI3K-Akt pathway also induces antiviral immunity suggesting that PI3K-Akt pathway represents a two-faced player in interactions between the virus and cell [16]. However, the exact role (favourable or unfavourable for virus infection) of the PI3K-Akt pathway seems to be virus-specific. Time course analysis of gene expression of Akt by real-time PCR clearly shows an upregulation of Akt gene in infected midgut samples from 72 h onwards reaching a peak at 120 h pi. This study is the first one to reveal the activation

of Akt in PI3K-Akt pathway that lead to improved resistance of wild silkworm *A. mylitta* to NPV infection. Furthermore, the observed bivalent action of the PI3K-Akt module represents a perfect example of a signalling pathway that is part of the antiviral response, however, is misused by the virus to support its own replication.

However, the biological reasons for NPV activating PI3K-Akt signalling pathway remain unclear, from both a virus and a host perspective which need further investigation. A better understanding of immune signalling pathways is required that could be activated by NPV infection to further delineate the cellular signalling pathways involved in baculovirus infection and finding novel therapeutic targets for nucleopolyhedrosis virus (NPV).

Acknowledgements We thank University Grants Commission of National Fellowship for Higher Education with Award letter number: F1-17.1/2015-16/NFST-2015-17-ST-KAR-1674/(SA-III/Website) Dated: April, 2016 for awarding the fellowship enabling to carry out the present research work.

Funding This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest.

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