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Non-target Host Immune Gene Modulation in Transgenic Silkworm *Bombyx mori* Endowed with RNAi Silence *Bm*NPV Genes

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Authors' contributions

This work was carried out in collaboration between all authors. Author BV conducted the experiments and statistical analyses. Author AKA secured the funding and conceived the research. Author ANRP conceived the research, analyzed the data statistically and wrote the manuscript. Authors VS and KMP contributed material and wrote the manuscript. Authors VS, KMP and RKM wrote the manuscript. All authors read and approved the manuscript.

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ABSTRACT

Aim: To reveal differential expression of host- response genes activated after nuclear polyhedro virus infection in transgenic silkworm *Bombyx mori* larva and to show an influence of '*Bm*NPV transgenes' on expression pattern of host- response genes.
Study Design: Relative expression profile of immune genes was analysed after *Bm*NPV infection in transgenic and non-transgenic larvae by real-time PCR.
Place and Duration of Study: Genomics Division, Seribiotech Research Laboratory, Bangalore, India; 2014 January – 2016 December.
Methodology: Expression of immune genes encoding components of Toll and melanisation

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pathways was analysed in third instar larvae of transgenic *B. mori* line mff118B by quantitative PCR at 0, 6, 9 and 24 h after *Bm*NPV infection and compared with infected non-transgenic larvae. A significant difference in relative expression was analyzed by Students'*t* – test or ANOVA and correlation in expression pattern, by linear regression in the probability distribution of Y as a function of X, at significance level $P < 0.05$.

Results: In transgenic larvae, survival rate after NPV infection was up to 70% compared to 30% in non-transgenic larvae. Immune genes encoding NF-kappa B inhibition factor, *Cactus*, NF kappa B transcription factors, *Dorsal* and *Relish*, Toll- activating cytokine *Spätzle*, melanization pathway components, *prophenol oxidase activating enzyme* and *prophenol oxidase1* showed significantly lower expression in *Bm*NPV infected- transgenic larva whereas enhanced expression in infected non-transgenic larvae. In infected transgenic larvae, selected gene pairs Cactus – Dorsal, Cactus – Relish, Spatzle - Dorsal, Spatzle - Cactus, Relish – Dorsal showed positively correlated expression whereas the correlation derailed in infected non-transgenic larvae.

Conclusion: RNA interference-mediated inhibition of *Bm*NPV multiplication was engineered previously in *B. mori*. In infected transgenic silkworm, NPV multiplication rate is low and host-response genes showed low expression level. Under the influence of transgenes, host response genes showed correlated expression thus transgenes preserve specific host- gene interactions after NPV infection. Notably influence of '*Bm*NPV transgenes' on expression of host response gene is a crucial revelation in the field of transgenesis to develop better antiviral resistance in silkworms.

Keywords: Transgenic silkworm against NPV; host immune genes; non-target host gene expression; expression modulation; Bombyx mori.

1. INTRODUCTION

Even though RNA interference (RNAi) reveals functions of different genes by knockout experiments, it is a major post-transcriptional mechanism that interferes with virus multiplication in mammals, insects, worms and plants [1,2] however viruses can inhibit RNAi defense in insects [3]. The economically important silkworm *Bombyx mori* acquires infection by different pathogens viz., viruses including nucleopolyhedro virus (*Bm*NPV), bacteria, microsporidians and fungus. The host-specific *Bm*NPV causes approximately 15 – 25% economic loss in sericulture. The infectious cycle of *Bm*NPV comprised of biphasic forms, budded viruses (BVs) and occlusion-derived viruses (ODVs), the later are encapsulated in polyhedral inclusion bodies (PIBs) [4]. In the host cell nuclei, BV multiplied and spread infection and encapsulated forming ODVs [5]. On entry into *B. mori* larval midgut during infection, polyhedra dissolve in alkaline gut juice and release the ODVs which infect midgut epithelium of the silkworm larvae.

In order to protect *B. mori* larvae from the massive infection of *Bm*NPV, conventional silkworm breeding techniques were employed to synthesize breeds with *Bm*NPV tolerance, however, this has not been achieved over several generations. Therefore it is proposed to genetically engineer NPV tolerance into susceptible high silk yielding breeds for obtaining improved productivity even under undesired environmental conditions. Tolerance is controlled by the interaction of different genes as part of adaptation since host and pathogen could have co-evolved and developed complementary resistance – virulence system [6]. To develop better NPV tolerance, lines of transgenic *B. mori* of Nistari strain tolerant to *Bm*NPV were developed through RNAi approach [7,8]. Further transgenic lines, engineered with vector, carries multiple transgenes (*Bm*NPV transgenes) that express double-stranded RNA to silence four NPV genes, were developed [9]. The RNAi was developed against four NPV genes viz., *immediate early* (*ie 1), late expression factors (lef 1* and *lef 3)* and *per os infectivity factor* (*p74*) which sequentially express to assist NPV multiplication. The RNA silencing abridged duplication of NPV in the infected larvae [9]. Along with the NPV transgenes, a reporter gene encoding red fluorescent protein (RFP) is also introduced in the vector which is expressed in the ommatidia of silkmoths (Fig. 1) [9,10].

In *B. mori*, host- responses are elicited after recognition of invaded pathogen initially through pathogen-associated molecular patterns (PAMPs). This activates immune mechanisms including Toll pathway, IMD pathway

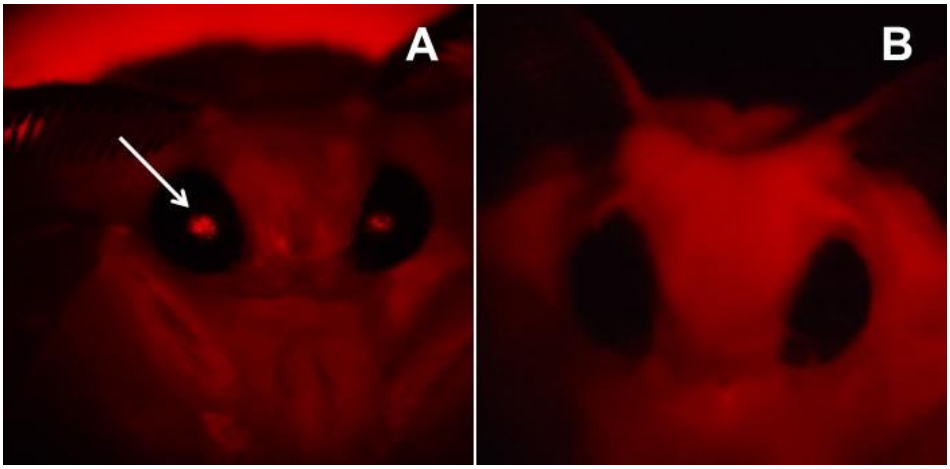

Fig. 1. Transgenic Nistari race of *B. mori* (SBRL stock) showed (arrow) expression of red fluorescent protein (RFP) in the ommatidia of the silkmoth (A) compared to its absence in non-transgenic control (B)

and melanisation pathway [11-13]. However, the transgenic *Anopheles spp.* that carries transgenes to protect hosts from multiplication of parasites, showed lower fitness than non-transformed ones [14,15]. The fitness is associated with immune responses of the host larvae. We hypothesized that in the presence of *Bm*NPV transgenes, expression profile of host immune genes is modulated in the NPV-infected transgenic *B. mori* larva.

2. MATERIALS AND METHODS

2.1 Insects

Multivoltine race of *B. mori* larvae, *Nistari* (non-transgenic control) and transgenic Nistari (line: MFF 118B; [9]) were reared on mulberry leaf (*Morus* spp.) at 25 ± 2°C and 70% relative humidity under natural photo regime (13L: 11D). Immediately after second moult, day 0 third instar larvae were separated and infected (*n* = 100 each) with *Bm*NPV by feeding mulberry leaves smeared with wild-type *Bm*NPV polyhedra of previously determined dose (6000 occlusion body/larva; [9]). The transgenic- and non-transgenic Nistari larvae were observed for symptoms of NPV infection and mortality. Individual larvae were collected at 0, 6, 9 and 24 hpi (hours post infection) from *Bm*NPV- infected transgenic and non-transgenic lines. The transgenic moths emit red fluorescence in the ommatidia which was captured using a stereo zoom fluorescence microscope (Olympus) (Fig. 1 A).

2.1.1 Sample collection, RNA extraction and cDNA synthesis

Since the size of the 3rd instar larvae was small, total RNA was extracted from individual larva using Trizol reagent (Invitrogen). The total RNA was quantified with Nanodrop spectrophotometer (Thermo) and treated with RNAse free-DNAse I (Takara) to remove DNA contamination. First strand cDNA was synthesized following the manufactures' protocol from 1 µg total RNA by using cDNA synthesis kit (PrimeScript- first strand cDNA synthesis kit, Takara, Cat #6110A) in 20 µl reaction mixture containing 50 µM Oligo dT Primer (1 µl), dNTP Mixture (10 mM each; 1 µl), 5X PrimeScript buffer (4 µl), RNase Inhibitor (40 U/µl; 0.5 µl) and PrimeScript RTase (1 µl of 200 U/µl of M-MLV- derived reverse transcriptase).

Two microliters of cDNA were used for each 20µl Real-Time PCR reaction, and qPCR was performed using gene-specific primers of *PPO1* (Table 1) and other genes designed (Primer- 3 software) from the mRNA sequences obtained from NCBI database [16]. Quantitative PCR (qPCR) was performed on Agilent Stratagene Mx3005P qPCR system using DyNAmo Flash SYBR GREEN qPCR Master Mix (Thermo; F-416L) using 50 x ROX as reference dye (0.2 µl) to normalize non-PCR-related fluorescence signal variation. A 20 µl reaction mixture contained 2 µl cDNA template, 2 µl each of the forward and reversed primers (0.5 µM each), and 10 µl SYBR Green qPCR master mix (2 X) containing 2.5 mM $MgCl_2$. The thermal program

Table 1. Key to the primers for the prophenol oxidase gene (PPO 1) used in the study

S. no.	Primer	Primer sequence-Forward/Reverse (5'-3')	Amplicon size (bp)	Melting point (°C)
1	Forward	5'ggtgtccagcgttgtacctt 3';	747bp	57°C
2	Reverse	5' aggtggaaaatgtcgtcgat 3';		

was 94°C for 10 min, followed by 40 cycles of 94°C for 30 seconds and primer-specific annealing temperature for 30 seconds. The PCR products were electrophoresed on agarose gel to confirm the target-specific amplification.

2.2 Statistical Analyses

The fluorescent signals yielded by the Real-Time PCR were detected and Ct value was calculated. The relative expression was performed to check the mRNA transcripts and *β* actin was used as internal control for normalization. A non-template control (NTC) sample was kept to detect any contamination. Comparative Ct values were standardized by Ct values for the house-keeping gene, *β* actin. Ct values were standardized relative to the individual value for the control, yielding the delta Ct value, and these values were standardized to make the average control value '1' (ΔΔ Ct values; [17]). Fold change in gene expression relative to the calibrator was calculated, which allowed displaying the down regulated relative quantities as negative values.

All data were presented as mean ± SD. Single factor Analysis of Variance (ANOVA) as given in MS-Excel and Student's *t*- test were performed for test of significance. Expression level between gene pairs was analyzed by Pearson correlation analysis.

3. RESULTS AND DISCUSSION

Infection of day 0 third instar larvae of *B. mori* Nistari with *Bm*NPV induced 'grasserie' disease symptoms after eight days of infection in non-transgenic larvae. The infected, non-transgenic larvae became sluggish, showed overlapping segments and wandering movement. Further milky white fluid containing numerous polyhedra oozed out from the larvae which succumbed. Upon NPV infection, transgenic larvae showed survival rate of 60 – 70% which was significantly ($P < 0.04$) higher than that in non-transgenic larvae (30 – 40%). In the infected transgenic larvae, the symptoms were initially observed after 10 days of infection due to slow multiplication rate of the virus. Similar observations during NPV multiplication were observed in different insects including *B. mori* [18–21], *Helicoverpa sp.* [22,23] and *Spodoptera exigua* [24]. In the transgenic line, pupa weight increased significantly ($P < 0.03$) whereas larval weight and silk (cocoon shell) weight did not vary significantly ($P > 0.05$) between transgenic- and non-transgenic larvae (Fig. 2) revealed that the non-target fitness trait, pupa weight is not distressed by NPV infection in transgenic larvae.

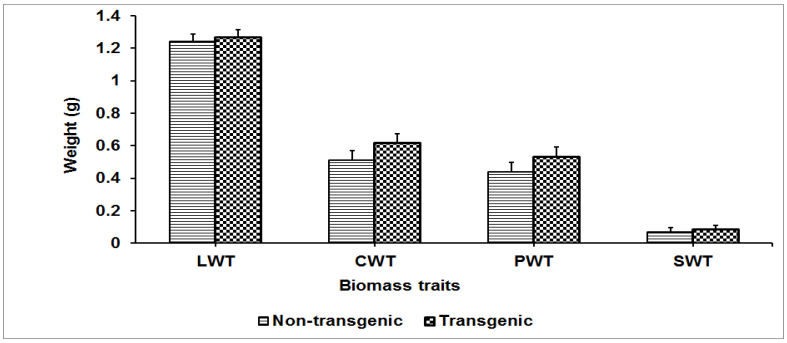

Fig. 2. Variation in quantitative traits in NPV infected transgenic and non-transgenic Nistari race of *B. mori* showed significant increase in cocoon weight and pupa weight indicating that fitness trait (pupa weight) is conserved in the transgenic line. LWT- maximum larval weight attained in final instar; CWT- cocoon weight; PWT- pupa weight; SWT- cocoon shell (silk) weight

Variations in the adaptive traits, larval weight and silk weight were influenced equally by exogenous factors like climatic conditions and feed intake in both transgenic and non-transgenic larvae. In the transgenic *B. mori* larva that over expresses lipase to increase NPV- tolerance no variation in cocoon quality was observed [25]. On the other hand, fitness traits such as fecundity and longevity are weakly affected by the transgene expression in the immune-enhanced transgenic mosquito, *Anopheles* sp against *P. falciparum* [26]. Similar observations were reported after silencing of gene encoding the leucine-rich repeat immune protein 1 which induced increase in oocyst number in the *Plasmodium* [27]. Knockdown of thioester containing protein (TEP1) gene abolished refractoriness towards *Plasmodium berghei* in *Anopheles gambiae* and increased the number of developing oocysts in a susceptible strain [28], showed different non-target organismal effects of RNA silencing.

3.1 Immune Gene Expression in NPV-Infected Transgenic and Non-transgenic Larvae

In the NPV- infected transgenic silkworm, expression of NPV gene was examined earlier [9] however expression pattern of non- target host- response genes was not analyzed. In the NPV- infected transgenic larvae, signal genes showed significantly (P < 0.0003; ANOVA) low expression. Quantitative expression of NF-*kappa* B transcription factors and melanization pathway genes (Table 2) did not vary significantly (P > 0.05) at 0 hpi, in both the lines.

However genes encoding NF-*kappa* B transcription factors, *Dorsal* and *Relish* and inhibitor of NF-*kappa* B transcription factors, *Cactus,* showed differential expression in non-transgenic and transgenic larvae at later stages of infection. In non-transgenic larvae, *Cactus* showed enhanced relative expression (Fig. 3) with 1.6 fold increase at 6 hpi (Fig. 4) whereas in transgenic larvae, it was significantly (P<0.0002) lower. In both lines, *Dorsal* expression remained at lower level at 6 hpi and 9 hpi. In transgenic larvae, *Dorsal* expression significantly (P < 0.002) enhanced at 24 hpi (Fig. 3).

Relative expression of the IMD component *Relish* significantly (P < 0.001; ANOVA) enhanced in non-transgenic larvae at 6, 9 and 24 hpi (Fig. 3) whereas in the transgenic larvae, it was down regulated (Fig. 4). Cyclic expression profile of *Relish* indicates transient activation of the IMD pathway after NPV infection in non-transgenic larva. Immune gene expression in NPV – infected transgenic lines is at lower level than that in NPV- infected non-transgenic lines. This indicated higher NPV resistance of transgenic larva, reduction in NPV multiplication rate and concomitant low host (immune) responses against NPV infection.

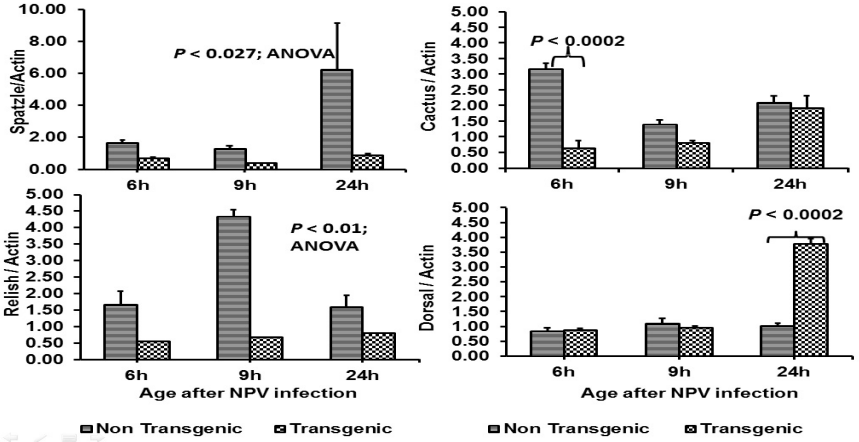

Fig. 3. qPCR showed differential expression of Toll / IMD pathway- associated genes in transgenic and non-transgenic larva of *B. mori* collected at different time points after infection with NPV on day 0 third instar larva. Quantitation of gene expression in the NPV infected- larva relative to the calibrator (defined as 1.0) was calculated using Mx3500P qPCR software (Agilent). Average Ct value of transcript expression was normalized with the house-keeping gene, β-actin

Table 2. Status of reported immune genes that showed significant variation in expression between transgenic and non- transgenic larvae of *B. mori* Nistari strain after infection by *Bm*NPV

Gene	Accession	Immune function of the encoding protein	Reference
Cactus	AB499895	NF kappa B factor inhibitor retains Dorsal in cytoplasm	[29] Lindsay and Wasserman 2014
Dorsal	NM_001043431	NF kappa B transcription factor regulating immune gene expression	[30] Li and Verma 2002
Relish	NM_001102465	NF kappa B transcription factor regulating immune gene expression	[31] Silverman et al., 2000
Spatzle	NM_001114594.1	Activate the toll receptor by binding with the receptor	[32] Valanne et al., 2011
PPO1	NM_001043870.1	Activates in humoral melanisation and is involved in the rapid delivery of phenoloxidase activity	[33] Binggeli et al., 2014
PPAE	AY061936	Serine proteinase catalyzing conversion of prophenoloxidase to active phenoloxidase	[34] Wang et al., 2001

Table 3. Variations in correlation between relative expression of immune genes at different time points in NPV infected transgenic and non-transgenic larvae of *B. mori* Nistari analyzed by Real – Time PCR showing strength of the relationship between the genes

Gene pair	Transgenic larvae		Non- Transgenic larvae	
	Regression equation	Correlation coefficient (R^2)	Regression equation	Correlation coefficient (R^2)
Cactus – Dorsal	y = 1.9681x - 0.3285	0.8367 (+)	y = -0.0927x + 1.1564	0.3401 (-)*
Cactus - Relish	y = 0.1282x + 0.5209	0.6002 (+)	y = -1.1034x + 4.9185	0.4086 (-)
Spatzle - Dorsal	y = 5.0829x - 1.4553	0.6268 (+)	y = -0.0055x + 1.0039	0.0080 (-)
Spatzle - Cactus	y = 2.0328x - 0.2191	0.4038 (+)	y = -0.0277x + 2.4226	0.0117 (-)
Relish - Dorsal	y = 0.0639x + 0.5555	0.6994 (+)	y = 2.2764x + 0.2909	0.0886 (+)
PPAE – PPO1	y = -1.1986x + 3.9687	0.0053 (-)	y = -3.1413x + 15.527	0.7763 (-)

**'(+)' indicates positive correlation and '(-)' for negative correlation; Note the low R^2 value in non-transgenic larvae showing decrease in strength of relationship between the genes*

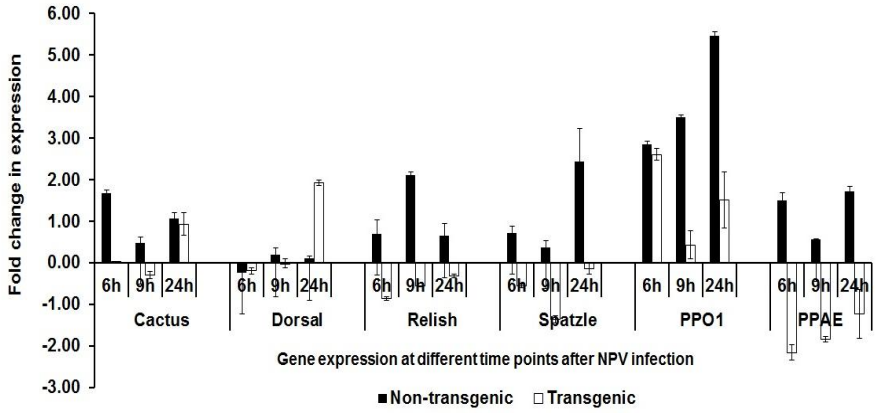

Fig. 4. Fold change in expression of Toll / IMD / melanization pathway associated genes in transgenic and non-transgenic larva of *B. mori* collected at different time points after infection with NPV on day 0 third instar larva

Co-expression pattern of the immune genes after NPV infection was analyzed by Pearson correlation analysis with significance level at $P < 0.05$ (Table 3). In the transgenic larva, *Cactus* expression was positively correlated with *Dorsal* expression ($R^2 = 0.84$). Moreover in transgenic larvae, *Relish* and *Dorsal* showed correlated ($R^2 = 0.699$) increase in expression (Table 3). Similar gene interactions were reported in different model systems including *Drosophila* Hedgehog pathway [34], plasmodium - mosquito interactions [35] and parasitoid- *B. mori* larva interaction [16]. Under normal conditions, cactus binds with the NF Kappa B transcription factors to inhibit expression of antimicrobial proteins (AMPs). In response to microbial infection, cactus undergoes degradation and released the NF kappa B factors to induce transcription of AMPs displaying the regulation of immune signaling [36,37]. Correlation in expression between the genes encoding the NF – kappa B factors/ inhibitor suggests that the regulation of *B. mori* – NPV interaction involved feedback action as noticed earlier [38]. Enhanced immune gene expression in NPV – infected non-transgenic larvae is induced by the enhanced multiplication rate of NPV. However, under low NPV multiplication rate in transgenic larvae, expression level of immune genes also was at lower level showing the influence of infection on gene regulation in transgenic larvae.

Simultaneous activation or suppression of *Dorsal, Relish* and *Cactus* after NPV infection in transgenic larva indicated an interrelated influence of Toll and IMD pathways to induce antimicrobial responses as noticed in *Drosophila* [39]. A recent study showed that co-expression of *Dorsal* and *Relish* suppressed promoters of antimicrobial proteins in *Manduca sexta*. *Ms*Dorsal interacts with *Ms*Rel2 forming heterodimer which negatively regulates the AMP production [40]. In *B. mori*, lipases and serine proteases were activated after NPV infection showing its influential role in NPV infection and multiplication [25,41]. Further, transcriptome and microarray analyses showed differential expression of several genes including those encoding Toll and IMD pathway components as well as cytoskeletal and cytoplasmic proteins in response to NPV infection in *B. mori* [12,15,42] indicating multigene influence on NPV tolerance and susceptibility.

In the NPV- infected non-transgenic larvae, gene encoding the cytokine, spatzle showed gradual increase in expression over time with significantly ($P < 0.0002$) larger expression (2.5 fold increase) at 24hpi. However, spätzle expression was marginally down regulated (-1.37 fold) at all time points in the transgenic silkworms (Figs. 3 & 4). In order to elicit the Toll pathway, spatzle binds with Toll followed by activation of Dorsal to mediate production of AMPs in *Drosophila* [43]. Both *Dorsal* and *Spatzle* expression was down regulated in NPV-infected transgenic larvae in a correlated ($R^2 = 0.626$) manner, whereas no correlation was observed in the NPV-infected non-transgenic larvae indicating variation in strength of relationship between the genes in the transgenic and non-transgenic lines. The transgenes thus defend specific host- gene interactions, in NPV infected- transgenic larvae, probably through reducing the NPV infection.

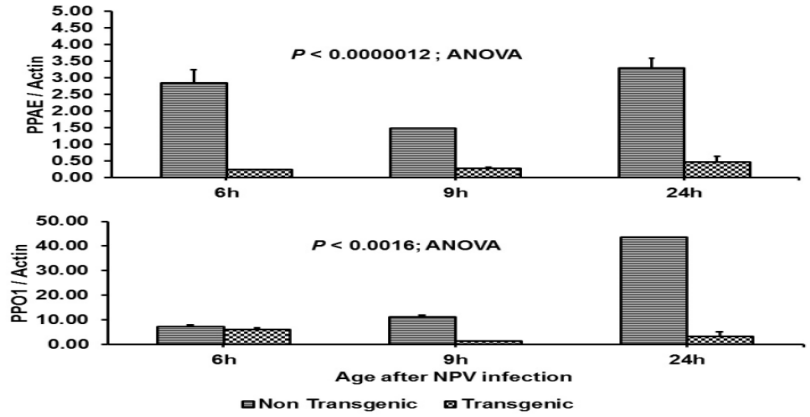

Fig. 5. qPCR showed differential expression of melanization pathway associated genes in transgenic and non-transgenic larva of *B. mori* collected at different time points after infection with NPV on day 0 third instar larva. Details of qPCR analysis are as given in legend of Fig. 3

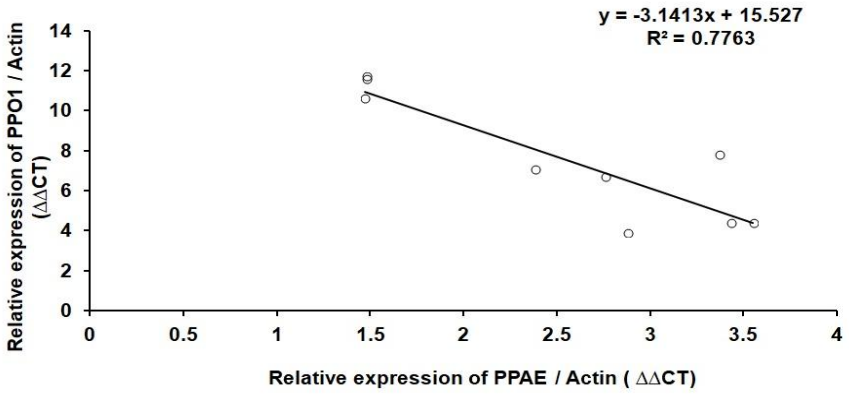

Fig. 6. Relative expression of the gene encoding PPO1 showed linear negative correlation with expression of prophenol oxidase activating enzyme (*PPAE*) after NPV infection of non-transgenic day 0 third instar larva of *B. mori*. Linear regression equation and correlation coefficient are given as inset

Variations in correlation in expression of genes and host- responses after parasitic infection were reported in *B. mori* [44] and few other model systems [45,46].

Genes encoding the melanization component, prophenol oxidase activating enzyme (PPAE) showed enhanced expression at 6, 9 and 24 hpi in the NPV-infected non-transgenic larvae whereas PPO1 expression was enhanced at 24 hpi (Fig. 5).

In insects, PPO1 is activated by PPAE to induce melanization and rapid delivery of phenoloxidase activity [32]. Correlation between expression patterns of these genes was examined by regression of PPO1 expression on PPAE expression (Fig. 6). In the NPV- infected non-transgenic larvae, strong (R^2 = 0.7) negative correlation was observed. In NPV- infected transgenic larvae, expression of both PPAE and PPO1 was down regulated and correlation was not observed, displaying modulation of expression of host- melanization genes (Fig. 5).

As a whole, decreased gene expression was observed in the NPV- infected transgenic larva in comparison to non-transgenic larva. Non-target effects of RNA silencing that affected expression of other host genes are reported in honey bees and mosquitoes [47,48]. Similar observation on non-specific down regulation of genes that encode proteins involved in general physiological functions, induced by dsRNA, was reported in

the transgenic honey bee, *Apis mellifera* [49]. In the transgenic fish, rainbow trout carrying *cecropin* gene, expression of immune genes of the host fish is modulated by the transgene [50].

4. CONCLUSION

Though most of the reports were on transgenes meant for overexpression or suppression of specific host genes, effects of the transgenes on non-target host genes were also accompanied. In the transgenic *B. mori* larvae, *Bm*NPV transgenes prevent or extend the viral multiplication upon NPV infection. Under the influence of transgenes, host response genes showed correlated expression thus transgenes preserve specific host- gene interactions after NPV infection. Notably influence of '*Bm*NPV transgenes' on expression of host response gene is a crucial revelation in the field of transgenesis to develop better antiviral resistance in silkworms and other beneficial insects.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Wilkins C, Dishongh R, Moore SC, Whitt, MA. RNA interference is an antiviral defence mechanism in *Caenorhabditis elegans*. Nature. 2005;436:1044–1047.
2. Alhoot MA, Wang SM, Sekaran SD. Inhibition of dengue virus entry and multiplication into monocytes using RNA interference. PLoS Neglected Tropical Diseases. 2011;5(11):e1410.
DOI: 10.1371/journal.pntd.0001410
3. Gammon DB, Mello CC. RNA interference-mediated antiviral defense in insects. Current Opinion in Insect Science. 2015;8: 111-120.
Available:http://dx.doi.org/10.1016/j.cois.2015.01.006
4. Minion FC, Coons LB, Broome JR. Characterization of the polyhedral envelope of the nuclear polyhedrosis virus of *Heliothis virescens*. Journal of Invertebrate Pathology. 1979;34:303–307.
5. Rahman MM, Gopinathan KP. Systemic and *in vitro* infection process of *Bombyx mori* nucleopolyhedrovirus. Virus Research. 2004;101:109–118.
PMID: 15041178
DOI: 10.1016/j.virus res.2003.12.027
6. Lea HZ. Principles and techniques of silkworm breeding. ESCAP, United Nations, New York; 1993.
7. Kanginakudru S, Royer C, Edupalli SV, Jalabert A, Mauchamp B, Chandrashekaraiah, et al. Targeting *ie-1* gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in transgenic silkworms. Insect Molecular Biology. 2007;16:635-644.
DOI: 10.1111/j.1365-2583.2007.00753.x
8. Zhang P, Wang J, Lu Y, Hu Y, Xue R, Cao G, et al. Resistance of transgenic silkworm to BmNPV could be improved by silencing *ie-1* and *lef-1* genes. Gene Therapy. 2014;21:81-88.
DOI: 10.1038/gt.2013.60
9. Subbaiah EV, Royer C, Kanginakudru S, Satyavathi VV, Babu AS, Sivaprasad V, et al. Engineering silkworms for resistance to baculovirus through multigene RNA interference. Genetics. 2013;193: 63-75.
DOI: 10.1534/genetics.112.144402
10. Uchino K, Imamura M, Sezutsu H, Kobayashi I, Kojima K, Kanda T, et al. Evaluating promoter sequences for trapping an enhancer activity in the silkworm *Bombyx mori*. Journal of Insect Biotechnology and Sericology. 2006;75: 89–97.
Available:http://doi.org/10.11416/jibs.75.89
11. De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. Toll and IMD pathways are the major regulators of the immune response in *Drosophila*. EMBO Journal. 2002;21:2568–2579.
DOI: 10.1093/emboj/21.11.2568
12. Bao YY, Tang XD, Lv ZY, Wang XY, Tian C-H, Xu YP, et al. Gene expression profiling of resistant and susceptible *Bombyx mori* strains reveals nucleopolyhedrovirus-associated variations

in host gene transcript levels. Genomics. 2009;94:138-145.
DOI: 10.1016/j.ygeno.2009.04.003

13. Lekha G, Gupta T, Awasthi AK, Murthy GN, Trivedy K, Ponnuvel KM. Genome wide microarray based expression profiles associated with *Bm*NPV resistance and susceptibility in Indian silkworm races of *Bombyx mori.* Genomics. 2015;106:393-403.
DOI: 10.1016/j.ygeno.2015.09.002
14. Catteruccia F, Godfray HC, Crisanti A. Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. Science. 2003;299:1225-1227.
DOI: 10.1126/science.1081453
15. Moreira LA, Wang J, Collins FH, Lorena MJ. Fitness of Anopheline mosquitoes expressing transgenes that inhibit *Plasmodium* development. Genetics. 2004;166:1337–1341.
PMCID: PMC1470781
16. Pradeep AR, Anitha J, Awasthi AK, Babu MA, Geetha MN, Arun HK, et al. Activation of autophagic programmed cell death and innate immune gene expression reveals immuno-competence of integumental epithelium in *Bombyx mori* infected by a dipteran parasitoid. Cell & Tissue Research. 2012;352:371-385.
DOI: 10.1007/s00441-012-1520-7
17. Gerardo NM, Altincicek B, Anselme C, Atamian H, Barribeau SM, de Vos M, et al. Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. Genome Biology. 2010;11:R21.
DOI: 10.1186/gb-2010-11-2-r21
18. Zhou Y, Wu J, Lin F, Chen N, Yuan S, Ding L, et al. Rapid detection of Bombyx mori nucleopolyhedrovirus (BmNPV) by loop-mediated isothermal amplification assay combined with a lateral flow dipstick method. Molecular and Cellular Probes. 2015;29:389-395.
19. Bebitha B, Mohanraj P, Manimegalai S, Mahalingam CA. Silkworm disease diagnosis through molecular approach and their management. International Journal of Plant Protection. 2016;9:343-352.
20. Shobahah J, Xue S, Hu D, Zhao C, Wei M, Quan Y, Yu W. Quantitative phosphoproteome on the silkworm (*Bombyx mori*) cells infected with baculovirus. Virol Journal. 2017;14:117.
21. Wang X, Yu H, Xu J, Zhang S, Yu D, Liu M, Wang L. Comparative subcellular proteomics analysis of susceptible and near-isogenic resistant *Bombyx mori* (Lepidoptera) larval midgut response to *Bm*NPV infection. Scientific Reports. 2017;7:45690.
DOI: 10.1038/srep45690
22. Gupta RK, Raina JC, Arora RK, Bali K. Selection and field effectiveness of nucleopolyhedro virus isolates against *Helicoverpa armigera* (Hubner). International Journal of Virology. 2007;3: 45-59.
23. Nguyen Q, Chan LC, Nielsen LK, Reid S. Genome scale analysis of differential mRNA expression of *Helicoverpa zea* insect cells infected with a *H. armigera* baculovirus. Virology. 2013;444:158–170.
24. Muñoz D, Murillo R, Krell PJ, Vlak JM, Caballero P. Four genotypic variants of a *Spodoptera exigua* nucleopolyhedrovirus (Se-SP2) are distinguishable by a hypervariable genomic region. Virus Research. 1999;59:61-74.
25. Jiang L, Cheng TC, Zhao P, Yang Q, Wang G, Jin S, et al. Resistance to BmNPV via overexpression of an exogenous gene controlled by an inducible promoter and enhancer in transgenic silkworm, *Bombyx mori*. PloS One. 2012;7(8):e41838.
DOI: 10.1371/journal.pone.0041838
26. Dong Y, Das S, Cirimotich C, Souza-Neto JA, McLean KJ, Dimopoulos G. Engineered *Anopheles* immunity to *Plasmodium* infection. PLoS Pathogen. 2011;7:e1002458.
DOI: 10.1371/journal.ppat.1002458
27. Osta MA, Christophides GK, Vlachou D, Kafatos FC. Innate immunity in the malaria vector *Anopheles gambiae*: Comparative and functional genomics. Journal of Experimental Biology. 2004;207:2551-2563.
DOI: 10.1242/jeb.01066
28. Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, et al. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. Cell. 2004;116:661-670.
DOI:http://dx.doi.org/10.1016/S0092-8674(04)00173-4
29. Lindsay SA, Wasserman SA. Conventional and non-conventional *Drosophila* Toll signalling. Developmental & Comparative Immunology. 2014;42:16–24.
DOI: 10.1016/j.dci.2013.04.011

30. Li Q, Verma IM. NF-kappaB regulation in the immune system. Nature Reviews Immunology. 2002;2:725–734.
DOI: 10.1038/nri910

31. Silverman N, Zhou R, Stöven S, Pandey N, Hultmark D, Maniatis T. A *Drosophila* IκB kinase complex required for Relish cleavage and antibacterial immunity. Genes & Development. 2000;14:2461- 2471.
DOI: 10.1101/gad.817800

32. Valanne S, Wang JH, Rämet M. The Drosophila Toll signalling pathway. Journal of Immunology. 2011;186:649-656.
DOI: 10.4049/jimmunol.1002302

33. Binggeli O, Neyen C, Poidevin M, Lemaitre B. Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*. PLoS Pathogen. 2014;10: e1004067.
DOI: 10.1371/journal.ppat.1004067

34. Wang R, Lee SY, Cerenius L, Soderhall K. Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. European Journal of Biochemistry. 2001;268:895–902.
DOI: 10.1046/j.1432-1327.2001.01945.x

35. Reid AJ, Berriman M. Genes involved in host–parasite interactions can be revealed by their correlated expression. Nucleic Acids Research. 2013;41:1508–1518.
DOI: 10.1093/nar/gks1340

36. Casso DJ, Biehs B, Kornberg TB. A novel interaction between hedgehog and notch promotes proliferation at the anterior–posterior organizer of the *Drosophila* wing. Genetics. 2011;187:485–499.
DOI: 10.1534/genetics.110.125138

37. Tanaka H, Yamamoto M, Moriyama Y, Yamao M, Furukawa S, Sagisaka A, et al. A novel Rel protein and shortened isoform that differentially regulate antibacterial protein genes in the silkworm, *Bombyx mori*. Biochimica et Biophysica Acta. 2005;1730:10–21.
DOI: 10.1016/j.bbaexp.2005.05.007

38. Jiang J, Cai H, Zhou Q, Levine M. Conversion of a dorsal dependent silencer into an enhancer: Evidence for dorsal corepressors. EMBO Journal. 1993;12: 3201–3209.

39. Tanji T, Hu X, Weber ANR, Ip YT. Toll and IMD pathways synergistically activate an innate immune response in *Drosophila melanogaster*. Molecular and Cellular Biology. 2007;27:4578–4588.
DOI: 10.1128/MCB.01814-06

40. Zhong X, Rao XJ, Yi HY, Lin XY, Huang XH, Yu XQ. Co-expression of Dorsal and Rel2 negatively regulate antimicrobial peptide expression in the tobacco hornworm *Manduca sexta*. Scientific Reports. 2016;6:20654.
DOI: 10.1038/srep20654

41. Ponnuvel KM, Nakazawa H, Furukawa S, Asaoka A, Ishibashi J, Tanaka H, et al. A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. Journal of Virology. 2003;77:10725-10729.
DOI: 10.1128/JVI.77.19.10725-10729.2003

42. Wang XY, Yu HZ, Geng L, Xu JP, Yu D, Zhang SZ, et al. Comparative transcriptome analysis of *Bombyx mori* (Lepidoptera) larval midgut response to *Bm*NPV in susceptible and near isogenic resistant strains. PLoS ONE. 2016;11(5): e0155341,
DOI: 10.1371/journal.pone.0155341

43. Lewis M, Arnot CJ, Beeston H, McCoy A, Ashcroft AE, Gay NJ, et al. Cytokine Spatzle binds to the Drosophila immunoreceptor Toll with a neurotrophin-like specificity and couples receptor activation. Proceedings of National Academy of Sciences USA. 2013;110: 20461-20466.
DOI: 10.1073/pnas.1317002110

44. Anitha J, Pradeep AR, Awasthi AK, Murthy GN, Ponnuvel KM, Sasibhushan S, et al. Coregulation of host–response genes in integument: Switchover of gene expression correlation pattern and impaired immune responses induced by dipteran parasite infection in the silkworm, *Bombyx mori.* Journal of Applied Genetics. 2013;55:209-221.
DOI: 10.1007/s13353-013-0183-8

45. LeBlanc PM, Saleh M. Caspases in inflammation and immunity. eLS, Wiley Online Library; 2009.
DOI: 10.1002/9780470015902.a0021990

46. Arvas M, Pakula T, Smit B, Rautio J, Koivistoinen H, Jouhten P, et al. Correlation of gene expression and protein production rate - a system wide study. BMC Genomics. 2011;12:616.
DOI: 10.1186/1471-2164-12-616

47. Flenniken L, Andino R. Non-specific dsRNA- mediated antiviral response in the honey bee. PloS One. 2013;8:e77263.

Available:http://dx.doi.org/10.1371/journal.pone.0077263
48. Nunes FMF, Aleixo AC, Barchuk AR, Bomtorin AD, Grozinger CM, Simões ZLP. Non-target effects of green fluorescent protein (GFP) - derived double-Stranded RNA (dsRNA-GFP) used in honey bee RNA interference (RNAi) assays. Insects. 2013;4:90–103.
DOI: 10.3390/insects4010090
49. Jarosch A, Moritz R. RNA interference in honeybees: Off-target effects caused by dsRNA. Apidologie. 2012;43:128-138.
DOI: 10.1007/s13592-011-0092-y
50. Lo JH, Lin CM, Chen MJ, Chen TT. Altered gene expression patterns of innate and adaptive immunity pathways in transgenic rainbow trout harboring Cecropin P1 transgene. BMC Genomics. 2014;15:887.
DOI: 10.1186/1471-2164-15-887

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