EFFECTS OF EPPONPV INFECTION ON GENE EXPRESSION IN *EPIPHYAS POSTVITTANA* LARVAE

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ABSTRACT

The nucleopolyhedrovirus EppoNPV can control larvae of the leafroller *Epiphyas postvittana* in the field, but has varying effects on New Zealand's other leafroller pests. An understanding of the infection process at the genetic/molecular level within the leafroller complex may lead to strategies to enhance the effectiveness of the virus. Microarray technology was used to examine the effects of viral infection on gene expression levels in *E. postvittana* gut tissue. The results show that a 5 day infection with EppoNPV caused a significant down-regulation of several *E. postvittana* genes, many of which were consistent with a response to injury or stress, or with the triggering of apoptosis (such as components of the oxidative phosphorylation pathway). The majority of the expressed viral sequences detected are structural genes and genes associated with late gene expression. These data provide a basis from which to compare EppoNPV infection in other less susceptible leafroller species.
Keywords: *Epiphyas postvittana*, tortricid, gene expression, EppoNPV, viral infection, microarray, nucleopolyhedrovirus, baculovirus.

INTRODUCTION

Epiphyas postvittana (Walker) (Lepidoptera: Tortricidae), commonly called the lightbrown apple moth, is a widespread horticultural pest attacking a large range of plants including many orchard crops (Thomas 1984). It is one member of a complex of leafroller pests in New Zealand. The larvae feed on foliage, buds, shoots and, with most economic impact, fruits (Wearing et al. 1991). They also construct protective webbing shelters within rolled leaves, between leaves or between leaves and fruit, causing calluses. The presence of leafroller larvae or even the superficial damage they cause to the fruit, is a serious quarantine issue for export apples, especially in the Japanese and USA markets.

Control has typically been achieved using broad spectrum chemical pesticides. The build up of resistance to insecticides (Suckling et al. 1988), along with growing requirements to reduce pesticide use, led to the development of an Integrated Fruit Production (IFP) programme in New Zealand. The programme began in 1996 and by 2001, 100% of export fruit was being produced under IFP. Implementation of IFP reduced organophosphate insecticide use by 95% and overall insecticide use by 50% (Wiltshire 2003). However, the search for novel, non-chemical controls for insect pests to complement and improve on existing methods is still ongoing.

One alternative strategy could be the use of a naturally occurring baculovirus, *E. postvittana nucleopolyhedrovirus* (EppoNPV). EppoNPV infects *E. postvittana* larvae

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through the midgut epithelial cells following ingestion of its polyhedral inclusion bodies. EppoNPV was first found in infected *E. postvittana* larvae in the 1960s, both in New Zealand (Thomas 1989) and Australia (MacCollum & Reed 1971). In New Zealand, effective control of *E. postvittana* larvae in the field was demonstrated when EppoNPV was applied in dilute spray form to apple trees in the Nelson area between 1972 and 1975 (Thomas 1989). Although EppoNPV is very effective against *E. postvittana*, it is less so against the other species that make up the complex of leafroller pests in New Zealand. Markwick et al. (2002) demonstrated that EppoNPV is capable of both entering and replicating in the midgut epithelial cells of all members of the complex (except possibly *Ctenopseustis obliquana*), but the reason why the leafroller hosts range from fully permissive to completely resistant is unclear.

Substantial genetic analysis of both EppoNPV and *E. postvittana* has been carried out over recent years. The genome of a New Zealand isolate of EppoNPV has been mapped and completely sequenced, revealing the smallest group 1 nucleopolyhedrovirus sequenced to date (Hyink et al. 1998, 2002). Expressed Sequence Tag (EST) libraries have been produced from *E. postvittana* midgut and antennal tissues and have been annotated (R.M. Simpson, unpubl. data).

Upon entering a cell baculoviruses very rapidly have a number of their Open Reading Frames (ORFs), the viral early and immediate early genes, transcribed into messenger RNA (mRNA) by the normal cellular transcription machinery of the host. However, to sucessfully establish an infection, a number of blocks and hurdles must be overcome, such as inhibiting apoptosis (Clarke & Clem 2003), and these require the regulation of a number of host and viral genes. Later in the infection process the host's cellular transcription machinery is shut down and the virus uses its own RNA polymerase, which is active for the late and very late genes, and replication of the virus occurs. Again this involves gene regulation of both host and virus.

An understanding of the infection process at the genetic/molecular level within the leafroller complex, and in particular which genes are regulated by the host-virus interaction, may lead to strategies to enhance the effectiveness of the virus. The research described in this paper used microarray technology to examine the interactions between the virus and *E. postvittana*. Microarray technology allows the expression level of numerous genes in healthy and virus-infected tissues to be examined. Up- or down-regulation of certain genes (i.e. more or less mRNA produced by these genes respectively) will help to reveal which processes are being affected by the infection. Determining which *E. postvittana* genes are affected by EppoNPV infection may help to explain why other members of the leafroller complex are less susceptible to EppoNPV infection. In this paper, data from a small microarray of *E. postvittana* ESTs and EppoNPV ORFs, 5 days post-infection, are described.

METHOD

Infection

Epiphyas postvittana larvae were obtained from laboratory colonies at the insect rearing facility at HortResearch, Mt Albert Research Centre, Auckland. EppoNPV was isolated, propagated and prepared as described previously (Markwick et al. 2002). Third instar larvae were starved overnight and then offered a 1 mm cube of diet dosed with 1 µl of EppoNPV (containing $1x10^5$ polyhedral inclusion bodies) or control solution (sterile water). Only those larvae that had consumed the entire cube within 24 h and were still third instars were used in the experiments. They were then transferred to general purpose diet (Singh 1983) and maintained at 21±1ºC for 5 days to allow the infection to develop in virus-treated larvae. After 5 days the larvae were dissected to excise the whole gut, which was immediately ground in Trizol® (Invitrogen; 60 guts pooled per 2 ml Trizol) and stored at 4°C. Three replicates used a total of 180 virus-infected and 180 control larvae.

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RNA preparation

Total RNA was extracted from the gut material ground in Trizol, according to the manufacturer's recommendations, and was resuspended in diethyl pyrocarbonate-treated sterile water and quantified with Ribogreen (Molecular Probes). mRNA was then isolated from this total RNA, using Oligo $(dT)_{25}$ Dynabeads (Dynal) according to the manufacturer's recommendations, and ethanol precipitated using standard procedures. The quality and quantity of the mRNA was determined using a Bioanalyser 2100 (Agilent).

Array production and hybridisation

A small PCR-product microarray was produced comprising 152 *E. postvittana* ESTs, *E. postvittana* actin cDNA, and all 136 ORFs in the genome of EppoNPV. Some genes were represented on the array by PCR products to several distinct regions. For example, the NADH dehydrogenase subunit V and cytochrome c oxidase subunit I genes were represented by PCR products to two and three distinct regions, respectively. PCR products were obtained using plasmid preparations as templates, and amplifying from M13 forward and reverse primers using Platinum *Taq* HiFi (Invitrogen), according to the manufacturer's recommendations, cycling as follows: 94ºC 30 s; 30 cycles of 94ºC 15 s, 55ºC 15 s and 68ºC 3 min; followed by 68ºC 5 min. Products were gel purified, quantified using Picogreen (Molecular Probes), then prepared at 10 µM in 150 mM sodium phosphate pH 8.5 containing 0.00001% SDS. This was used to print on epoxy array slides (Corning) using a MicroGrid TAS arrayer (Genomic Solutions).

Array Use

The mRNA samples prepared from healthy and virus-infected larval guts were resuspended in water to a final concentration of 125 ng/µl. Hybridisations were carried out as described by Schaffer et al. (2007). Briefly, first strand cDNA was prepared by reverse transcription (RT) of the mRNA, the cDNA was ethanol precipitated, resuspended in 5 µl 100 mM Na_2CO_3 (pH 9.0), labelled with Cy3 or Cy5 (Amersham Biosciences) and hybridised to the microarray slides. A dye swap pair of arrays was performed for each biological replicate, i.e. healthy and virus infected material was labelled with Cy3 and Cy5 respectively and used to probe one microarray slide, and labelled with Cy5 and Cy3 respectively for a second slide. Data were captured using Genepix Pro 4.0 software (Molecular Devices Corp.) with manual checking of spot alignment.

Analysis

The fluorescence intensity of each of the spots was measured at 635 nm (Cy5) and 532 nm (Cy3). Spots flagged as bad (denoting no data found, empty spot or a damaged section of slide) were removed from the analysis. Median data were taken for each remaining spot and analysed using Microsoft® Excel. Because of the small size of the array, global normalisation methods could not be used, so data were normalised to the *E. postvittana* actin gene. The effect of this normalisation on the dataset as a whole was checked using diagnostic MA plots (ratio versus intensity in log (base 2) space). If normalisation skewed the data away from a ratio of 1 (0 in $\log_2$ space), then the un-normalised dataset was used for further analysis. Un-normalised data were used for only one of the six datasets (three replicates with two dye swaps for each).

Expression ratios, i.e. the ratio of the fluorescence signals normalised as above, for biological replicates and dye swaps were compared and those showing a consistent difference between virus-infected and controls in four or more of the six datasets were noted. For each gene, the average expression ratio and standard error across the datasets were calculated. Only expression ratios of at least two, after subtracting the standard error, were taken to represent real changes in expression. In this way, genes were selected that consistently showed a significant change in expression above a two-fold cut off.

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RESULTS

Analysis of the microarray data showed that a 5 day infection with EppoNPV resulted in a down-regulation of eight of the 152 *E. postvittana* ESTs represented on the microarray, and an up-regulation of four of the 136 EppoNPV ORFs (Table 1). Seventeen of the insect ESTs and 89 of the viral ORFs were removed from the analysis because they were flagged as bad in three or more of the six datasets. None of the other genes examined showed change in expression above the two-fold cut off.

Table 1 shows data for all the genes consistently showing an average expression ratio of two or more regardless of standard error. Also included in Table 1 are data from four *E. postvittana* ESTs that have lower expression levels but are included because of their relevance to the discussion.

Viral genes

Twenty-five viral ORFs showed signs of up-regulation, but only four (pk1, ODV-E27, lef-9, and ORF3, a hypothetical protein) exceeded the two-fold cut off. The remaining 22 viral ORFs analysed showed no change in expression.

Insect genes

Four cytochrome c oxidase gene subunits were represented on the array. Of these, three were encoded by mitochondrial DNA (subunits I, II and III), and the fourth by nuclear DNA (subunit Vb). The mitochondrial-encoded cytochrome c oxidase subunits all showed a down-regulation of expression, although one of the five subunits failed to meet the two-fold cut off. In contrast, the nuclear-encoded cytochrome c oxidase, subunit Vb, showed a slight up-regulation in expression.

Five NADH dehydrogenase gene subunits were represented on the array, all encoded by mitochondrial DNA. Of these, only three show down-regulation, with subunits IV and V(a) being down-regulated by more than two-fold. However, subunit V(b) showed very little change in expression. Subunit VI showed a slight down-regulation and subunits II and III showed a slight up-regulation in expression.

The only other mitochondrial-encoded gene represented on the array, the ATP synthase subunit VI gene, was also down-regulated by more than two-fold.

Of the nuclear-encoded genes represented on the array, only two, glutathione peroxidase and cathepsin L, showed a down-regulation of expression by more than two-fold. While six of the nuclear-encoded genes on the array showed signs of up-regulation, none met the cut off criterion.

DISCUSSION

A small microarray was used to examine the interactions between EppoNPV and *E. postvittana*. Determining which genes are up- or down-regulated by viral infection may help to reveal which processes are affected by infection and explain why other members of the leafroller complex are less susceptible to EppoNPV infection.

Viral gene regulation

Almost two thirds of the viral genes failed to yield data usable for analysis. The majority of this data was flagged as bad because the fluorescence levels were too close to background. High levels of viral gene expression might have been expected in the insect gut after 5 days of infection. Clearly this was not so. It could be that expression of viral genes has peaked before this time. Alternatively, the majority of infection might occur beyond the gut, leaving only a small proportion of the gut cells infected with virus. If this was the case, then the relative proportion of viral mRNA to insect mRNA in the virus-infected tissues would be low and hence few viral transcripts would show hybridisation above background.

No expression of viral genes would be expected in the healthy larvae, so "up-regulation" in the virus-infected tissues demonstrates active expression during the infection. Of the 47 viral genes analysed, 25 showed signs of up-regulation from background levels, with four showing more than two-fold upregulation (i.e. strong expression in virus-infected tissue).

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TABLE 1: Up- and down-regulation of gene expression levels in virus-infected compared with control *Epiphyas postvittana* guts. Up- and down-regulation is given as average ratio ± standard error (SE). An expression ratio of two, after subtracting the standard error, represents a two-fold difference in expression. N = number of datasets in analysis for this gene.

		Up-regulation	Down-regulation	N
Insect genes				
cytochrome c oxidase subunit I (a)			3.61 ± 0.54	6
cytochrome c oxidase subunit I (b)			2.27 ± 0.12	6
cytochrome c oxidase subunit I (c)			2.45 ± 0.82	6
cytochrome c oxidase subunit II			3.35 ± 0.46	6
cytochrome c oxidase subunit III			3.81 ± 0.57	6
cytochrome c oxidase subunit Vb		1.44 ± 0.29		6
NADH dehydrogenase subunit II		1.87 ± 0.57		4
NADH dehydrogenase subunit III		1.12 ± 0.13		6
NADH dehydrogenase subunit IV			2.23 ± 0.19	6
NADH dehydrogenase subunit V (a)			3.23 ± 0.42	6
NADH dehydrogenase subunit V (b)		1.08 ± 0.08		6
NADH dehydrogenase subunit VI			1.50 ± 0.10	6
ATP synthase subunit VI			3.21 ± 0.20	6
glutathione peroxidase			3.42 ± 0.58	6
cysteine protease (cathepsin L)			2.61 ± 0.32	6
general odorant binding protein (1)		2.10 ± 0.58		6
transcriptional coactivator (putative)		2.00 ± 0.36		6
proteasome regulatory atpase subunit		2.52 ± 1.07		4
antenna binding protein		2.38 ± 0.87		4
glutamate decarboxylase		2.16 ± 0.72		4
general odorant binding protein (2)		2.15 ± 0.69		4
Viral genes				
ORF 135	pk1	3.21 ± 1.00		6
ORF 126	ODV-E27	3.00 ± 0.80		6
ORF 56	lef-9	3.05 ± 0.88		5
ORF 3	hypothetical protein	2.82 ± 0.79		5
ORF 22	pp31	2.30 ± 0.38		5
ORF 120	p10	3.10 ± 1.21		5
ORF 7	protein tyrosine phosphatase	2.66 ± 0.84		5
ORF 123	Ie-0	2.17 ± 0.35		6
ORF 1	polyhedrin	2.03 ± 0.27		6
ORF 64	telokin	2.59 ± 0.86		4
ORF 14	hypothetical 23.7kd protein	2.24 ± 0.55		5
ORF 74	Telokin (Hypothetical)	2.09 ± 0.40		4
ORF 134	hypothetical protein	3.18 ± 1.50		4
ORF 19	hypothetical protein	2.02 ± 0.35		6
ORF 91	p87	2.09 ± 0.44		4
ORF 11	lef-1	3.14 ± 1.49		4
ORF 2	hypothetical protein	2.07 ± 0.45		6
ORF 8	hypothetical 37.2kd protein	2.25 ± 0.63		5
ORF 111	cathepsin	2.43 ± 0.84		4
ORF 15	hypothetical 40.3kd protein	2.08 ± 0.49		6
ORF 13	DA26	2.00 ± 0.46		6
ORF 28	hypothetical protein	2.08 ± 0.54		6
ORF 87	p6.9	2.47 ± 0.94		5
ORF 42	ODV-E66	2.03 ± 0.55		5
ORF 45	lef-8	2.59 ± 1.13		5

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ODV-E27 is an occlusion derived viral (ODV) structural protein that is thought to act as a multifuntional cyclin. It may be involved with the arrest of the host cell cycle at a stage where viral gene transcription is optimised at the expense of host gene transcription, and also with the regulation of viral and/or host DNA replication (Belyavskyi et al. 1998). Pk1 is a late gene transcription factor and might also be be involved in virus-induced cell cycle arrest or viral DNA replication. The late expression factors 8 (lef-8) and 9 (lef-9) have been shown to be components of the viral RNA polymerase, active for the transcription of viral genes late in the infection process when the host's cellular transcription machinery has been shut down (Iorio et al. 1998). Expression of pp31 was detected in virus-infected tissue, although it did not meet the cut off criteria. pp31 is a late gene transcription factor, which has been shown to enhance, but not be essential for, productive viral replication or late gene transcription (Yamagishi et al. 2007). Another notable gene detected in the infected tissue was the viral cathepsin. This is a very late gene that is thought to be important in polyhedral release from infected cells (Hawtin et al. 1997).The majority of the expressed viral sequences detected are structural genes (such as ODV-E27 and p87) and genes associated with late gene expression (such as pk1, lef-9, pp31 and p10). As expected at this stage of infection, the assay was clearly biased towards the later genes.

Impact of infection on E. postvittana gene regulation

The effect on the 12 mitochondrial-encoded genes on the array was notable. Down-regulation of the mitochondrial-encoded cytochrome c oxidase subunits was particularly striking, although it is unclear why the three subunit I targets showed different levels of down-regulation. It is interesting that while most of the mitochondrial-encoded genes examined were down-regulated, three of the six NADH dehydrogenase targets were not significantly changed by viral infection. Further investigation is needed to determine whether this represents a general down-regulation of all mitochondrial-encoded genes, a general down-regulation of the oxidative phosphorylation pathway, a more specific down-regulation of only certain of these genes, or merely a reduction in the number of mitochondria.

The observed down-regulation of a number of mitochondrial-encoded genes is in contrast to the work of Okano et al. (2001) who found that some mitochondrial-encoded genes, including cytochrome c oxidase subunits I and III and NADH dehydrogenase subunit I (referred to as NADH-ubiquinone oxidoreductase chain1), were up-regulated in *Bombyx mori* during NPV infection. However, this work focused on the initial 24 h of infection and was in a cell line. Further investigation is needed to determine whether the down-regulation observed in the present experiments in whole larvae during later infection also occurs in the early stages of infection.

Down-regulation of mitochondrial encoded components of the oxidative phosphorylation pathway (including subunits of the cytochrome c oxidase, ATP synthase and NADH dehydrogenase complexes) is consistent with injury or stress (Dimopoulos et al. 2002; Sanchez-Alcazar et al. 2003), or with the triggering of apoptosis (Ragno et al. 1998). Down-regulation of glutathione peroxidase is also consistent with injury and stress that results in the production of reactive oxygen species and the triggering of apoptosis (Fujii & Taniguchi 1999).

Apoptosis, or programmed cell death, is a known antiviral response in insects (Clem 2005). A key early step in apoptosis is the alteration of mitochondrial membrane permeability, resulting in the release of apoptosis-specific protease activators and the subsequent activation of caspases (Green & Reed 1998; Mignotte & Vayssiere 1998). As cytochrome c oxidase, NADH dehydrogenase and ATP synthase are all embedded in the inner mitochondrial membrane, it is debatable whether down-regulation of these genes represents an early apoptotic signalling event or a downstream consequence of apoptotic signalling.

Numerous stimuli and signalling pathways converge to trigger the disruption of the mitochondrial membrane. These include, among others, perturbations of redox and energy metabolism. Antioxidant enzymes, such as glutathione peroxidase, play a protective

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role, regulating reactive oxygen species (Krishnan & Kodrick 2006; Krishnan & Sehnal 2006). Down-regulation of glutathione peroxidase, and thus the loss of control of these free radical species, again, may represent either an early apoptotic signalling event or a downstream consequence of apoptotic signalling.

Baculoviruses, such as EppoNPV, have been shown to express inhibitors of apoptosis (Maguire et al. 2000; Clem 2005). These act to inhibit caspases, the key effectors of cell death. Viral DNA replication as well as viral gene expression can trigger apoptosis in insect cells (Clarke & Clem 2003). Blocking the apoptosis cascade prevents the cell from destroying the virus along with itself, allowing the virus time and resources to replicate inside the insect cell. Interestingly, viral inhibition of apoptosis occurs downstream of the changes in the mitochondrial membrane, which may be why a number of gene expression changes that are consistent with early apoptotic events were still observed in the present experiment when the insects had been infected for 5 days.

Cathepsin L is thought to play a role in the immune response of insects, being up-regulated upon infection with bacteria and fungi (Irving et al. 2001). It is possible, therefore, that the down-regulation observed here is a specific effect of baculoviral infection. As discussed above, expression of the EppoNPV cathepsin gene was detected in virus-infected tissue. This gene is expressed late in the infection cycle and the down-regulation of host cell cathepsin L may be required to prevent the effects of this enzyme early in viral replication. The virus itself then produces this proteolytic activity late in the infection cycle, allowing release of polyhedra from infected cells.

It is possible to speculate that stress may be an indication of successful infection and that a non-permissive (resistant) leafroller species (e.g. *C. obliquana* or *Planotortrix excessana*) may not show a strong stress response to viral challenge. It may also be speculated that apoptosis could be an indication of unsuccessful infection and that non-permissive leafroller species may show more signs of late apoptosis than permissive (susceptible) species.

The present experiments have demonstrated some of the effects of viral infection on gene expression in the susceptible host *E. postvittana*. This provides a basis from which to compare infection in other hosts of varying susceptibility. To examine the possible effects of EppoNPV on, among other things, stress and apoptosis, future research should include some of the less permissive members of New Zealand's leafroller complex.

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