Cape Peninsula
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The characterisation of a nucleopolyhedrovirus infecting the insect *Trichoplusia ni*

by

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DECLARATION

I, **Michael Tobin**, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

Background: Baculoviruses have great potential as alternatives to conventional chemical insecticides. The large scale adoption of such agents has however been hampered by the slow killing times exhibited by these bio-insecticides, limitation to single target insect and difficulty of large scale production of these preparations. *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV), initially identified in the Eastern Cape region of South Africa, has potential as a biocontrol agent as it possesses a higher speed of kill compared to other baculoviruses.

Aims and methods: The main objective of this study was the identification, molecular characterisation and cloning of a structural core gene (polyhedrin) and three auxiliary genes, the inhibitor of apoptosis (*iap2* and *iap3*) and the *ecdysteroid* UDP-*glucosyltransferase* (*egt*) genes, from TnSNPV in order to delineate its phylogenetic relationship to a Canadian isolate of the same virus and to other baculoviruses. In addition, the genes were expressed in an *Escherichia coli* (*E. coli)* based system as a prelude to genetic modification to increase the pesticidal property of the virus.

Results: The genome size of the South African strain of TnSNPV was estimated at 160 kb and is significantly larger than the Canadian isolate of TnSNPV and may reflect genetic variation as the two strains have adapted to varying environmental conditions. Occlusion bodies of the South African strain of TnSNPV were visualised by Transmission Electron Microscopy and consisted of rod shaped single virions composed of a single enveloped nucleocapsid. Insect bioassays showed that the median lethal time (LT_{50}) of the virus strain averaged 1.8 days which is significantly faster than other baculoviruses. The South African and Canadian strains of TnSNPV share nucleotide similarities greater than 95% for the genes analysed in this study, which indicates that they are closely related. From this analysis, the South African strain of TnSNPV identifies as a Group II NPV with the closest relatives being the Canadian strain of TnSNPV and ChchNPV. The topology of the tree for the polyhedrin protein was better resolved than that of the IAP2, IAP3 and EGT proteins and was comparable to the tree inferred from a concatenated data set consisting of complete polyhedrin/granulin, LEF8, and LEF9 proteins of 48 completely sequenced genomes. For the IAP2, IAP3 and EGT proteins, the separation of the lepidopteran and hymenopteran specific baculoviruses was not evident while the separation of Group I and II *Alphabaculoviruses* diverged from that observed from the baculovirus core gene polyhedrin as well as the tree inferred from complete polyhedrin/granulin, LEF8, and LEF9 proteins. Five distinct groups relating to IAP-1, 2, 3, 4 and 5 could be distinguished from the tree inferred from all IAP proteins from 48 fully sequenced baculoviruses. From this analysis, the IAP protein from the South African isolate of TnSNPV can be designated as an IAP3 due to sequence homology to other IAP3 proteins. Similarly, the IAP2 can be confirmed as an IAP2 protein as it clusters with other IAP2 proteins.

RNA transcripts of the four genes were detected by RT-PCR at one hr after induction with L-arabinose in BL21-A1 *E. coli* and persisted until four hrs post induction. Antisera directed against the C-terminal 6X His tag was able to detect the recombinant proteins at two hours after induction confirming the rapid rise in expression of the proteins which persisted at high levels until four hrs after induction. The discrepancy observed with the predicted molecular mass of the EGT protein and the migration on SDS-PAGE may be due to the absence of post-translational modification in the *E. coli* expression system and the hydrophobic residues present in the N-terminal signal sequence.

Conclusion:

Sequence and phylogenetic analysis suggest that the two isolates of TnSNPV have been exposed to similar evolutionary pressures and evolved at similar rates and represent closely related but distinct variants of the same virus. The difference in genome size between the two strains is likely to reflect actual genetic differences as the strains have adapted to their local environments and hosts and the extent of the differences will only be apparent as more sequencing results become available. Phylogenetic analysis of the IAP and EGT proteins yields a tree that varies from the phylogenetic reconstruction observed for the polyhedrin gene as well as the concatenated data set consisting of complete polh/gran, LEF8, and LEF9 proteins and highlights the risks inherent in inferring phylogenetic relationships based on single gene sequences. The tree inferred from the concatenated data set of polh/gran, LEF8, and LEF9 proteins was a quick and reliable method of identification particularly, when whole genome data is unavailable and mirrors the accepted lineage of baculoviruses. Expression of the recombinant IAP2, IAP3, EGT and polyhedrin was confirmed by RT-PCR and immunoblot analysis and rose rapidly after induction and persisted at high levels. It is as yet unclear if the expressed proteins are functional particularly as post translation modifications are lacking in this system.

KEYWORDS

Trichoplusia ni single nucleopolyhedrovirus (TnSNPV), phylogeny, polyhedrin, inhibitor of apoptosis (*iap2* and *iap3*), *egt*, phylogeny, heterologous expression

DEDICATION

To my father, James Frank Tobin

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GLOSSARY

Terms/Acronyms/Abbreviations	Definition/Explanation
NPV	Nucleopolyhedrovirus
NC	Nucleocapsids
BV	Budded Virus
ODV	Occlusion Derived Virus
SNPV	Single Nucleopolyhedrovirus
MNPV	Multiple Nucleopolyhedrovirus
OB	Occlusion Body
GV	Granulovirus
ORF	Open Reading Frame
PIFs	Per Os Infectivity Factors
PM	Peritrophic Membrane
JHE	Juvenile Hormone Esterase
egt	*ecdysteroid* UDP-*glucosyltransferase*
lef	late expression factor
iap	inhibitor of apoptosis
BIR	baculovirus *iap* repeat
RING	Rather Interesting New Gene
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
LB	Luria-Bertani
nt	nucleotide
RBS	Ribosome Binding Site or Shine Delgarno sequence
bp	base pair
hrs, min and sec	hours, minutes and seconds
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Chapter 1

Introduction & Literature Review

Sections of this chapter have been published in:
Tobin, M., Abrahams-Fredericks, R., Khan, W. and Khan, S. 2017. Comparison of a South African and Canadian isolate of the nucleopolyhedrosis virus infecting the insect *Trichoplusia ni*. African Entomology, 25:341-360.

1.1 Introduction

Baculoviruses have been utilised for the control of insect pests as early as the 1900's with the introduction of a nucleopolyhedrovirus (NPV) to control *Lymantria monacha* (nun moth) in conifer forests in Germany (Granados and Federici, 1986). Since then, baculoviruses have been frequently utilised to control insect pests, with varying degree of success. This includes the first field trials in the USA with the NPV of *Lymantria dispar* (LdNPV) in 1913 (Cunningham, 1982) and the first commercially registered viral insecticide (Elcar™, by Sandoz) targeting insect pests in the *Heliothis* genus in 1975 (Szewczyk et al., 2011).

Baculovirus based insecticides offer distinct advantages over traditional chemical agents since they are characterised by a limited host range and exhibit a good safety profile towards non-target organisms (Gröner, 1986). However, factors that includes slow killing time, selectivity (often to single or closely related species), the difficulties and high cost of *in vivo* and *in vitro* production, the effect of prevailing weather conditions as well as the negative attitude of many farmers towards biological pest control, have limited the commercial development of baculovirus based insecticides (Lacey et al., 2015). The increase of insect resistance to conventional chemical insecticides, the prohibition of these chemical agents in some countries and the general demand for safer and ecologically less toxic agents, has nevertheless triggered a renewed interest in industry (Knox et al., 2015) and the general scientific community in the development of baculoviruses as viable biopesticides (Haase et al., 2015). Thus, while the market for biopesticides (including bio-insecticides, acaricides, fungicides, herbicides, bacteriocides, algaecides and stimulants) has seen little expansion over the past decades (Arthurs and Dara, 2018), it is predicted that the market in the United States is likely to expand at more than 5 times than that of conventional insecticides by 2025 (Markets and Markets, 2016), which could lead to the increased utilization of such agents globally.

In addition to their potential as biopesticides, baculovirus expression vectors provide a reliable and versatile system for the expression of recombinant heterologous proteins in cultured insect cells and insect larvae (Hitchman et al., 2009; Van Oers et al., 2015). While many such systems have been developed, it is the prototype baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), that is the most widely utilised. In most systems the polyhedrin gene, a gene not essential for virus replication, is replaced by foreign genes (Berger and Poterszman, 2015), which allows for expression at high yields under the regulation of the highly efficient polyhedrin promoter.

Baculoviruses also have potential as gene delivery vehicles in mammalian systems (Mansouri and Berger, 2018). Genetically stable recombinant baculoviruses with inserts of up to 38 kilobases (kb) have been generated and propagated (Cheshenko et al., 2001). These expression vectors are able to deliver their inserts to a wide range of mammalian cell types *in vitro* and *in vivo* (Kost and Condreay, 2002) with no evidence of cytopathic effects, while being inherently incapable of replication in mammalian cells (Mudgal et al., 2013). The inability to

replicate within mammalian cells while being able to deliver genes to a variety of cell types make these agents ideal for gene therapy though their long term safety has yet to be determined (Kenoutis et al., 2006; Liu et al., 2017).

The World Summit on Sustainable Development held in Johannesburg, South Africa in September 2002, reported on how the global environment is under threat from a variety of factors. The increasing loss of biodiversity, depletion of fish stocks, the adverse effects of climate change, natural disasters and the effects of air, water and marine pollution, threatens the global environment (United Nations, 2002). The World Summit affirmed that all societies "have a responsibility to advance and strengthen the interdependent and mutually reinforcing pillars of sustainable development, economic development, social development and environmental protection" at all levels of society (United Nations, 2002). This sentiment was confirmed at the 4th International Conference On Sustainable Development that focused on the reconciling of the need for economic development and environmental sustainability for societies today and in the future on both a local and global level (Magnini, 2016). Baculovirus-based insecticides have the potential to contribute to this ideal of a sustainable future for the 21st century and with continued scientific endeavour will contribute to the achievement of the goals established at this conference.

A South African isolate of the nucleopolyhedrovirus infecting the highly polyphagous insect pest *Trichoplusia ni* exhibits potential as a baculovirus based insecticide due to its faster killing time when compared to other baculoviruses. However, the relative paucity of information on the molecular characteristics of this isolate hinders the proper assessment of the pesticidal potential of the virus. With continued research and development efforts aimed at broadening the application of environmentally-benign baculovirus based biocontrol agents, the potential of a sustainable future may be realised.

1.2 *Trichoplusia ni*, the insect pest

Trichoplusia ni, (Hübner) (Insecta: Lepidoptera: Noctuidae) (*T. ni*), the cabbage looper, (**Fig. 1.1**), is a global polyphagous pest of a large variety of cultivated plants and weeds and a significant pest of greenhouse vegetables. The cabbage looper larvae feeds readily on crucifers including broccoli, cabbage, and cauliflower as well other vegetable crops such as beet, lettuce, tomatoes and squash. A variety of ornamental plants from the *Antirrhinum* and the *Alcea* genera such as snapdragon and hollyhocks, respectively, as well as crops including tobacco and cotton, which are grown on a commercial scale, are also consumed. Not all plants are suitable for development of the larvae with less than a third of all hosts able to support larval development (Soo Hoo et al., 1994).

Cabbage loopers feed readily on leaves and may consume up to three times their dry weight daily (McEwan and Hervey, 1960). Third instar larvae initially restrict their feeding to the ventral leaf surface, while fourth and fifth instar larvae produce large holes distal to the leaf

margins. The development time (from egg to adult) at temperatures of 21°C to 32°C is usually 18-25 days (Toba et al., 1973).

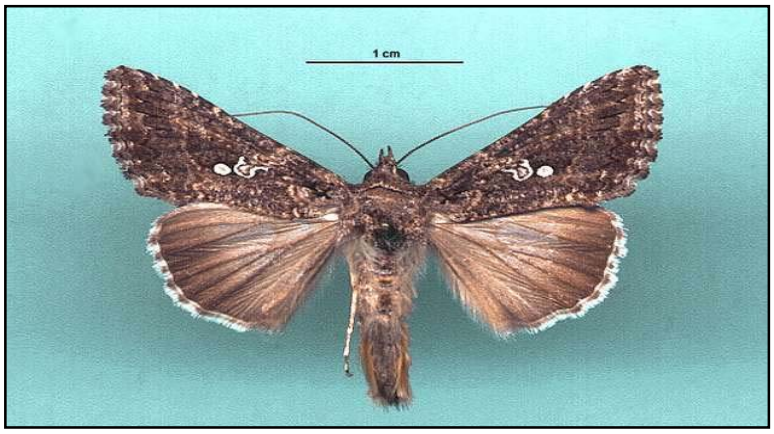

Figure 1.1 Adult *Trichoplusia ni* (cabbage looper) (Lafontaine et al., 1991).

Larvae are initially white in colour becoming pale green as feeding commences (**Fig. 1.2**). Three pairs of "true" legs are located behind the head on thoracic segments T1, T2 and T3, with a further two pairs of vestigial prolegs located on abdominal segments three and four (A3 and A4) - if the segment with the last pair of true legs is considered as the first segment (Gilligan and Passoa, 2014). The lack of legs in the middle section results in the characteristic looping crawling motion. Dorsally, the larvae display several white stripes gathered in two broad bands. Length is usually 3-4 cm. The cabbage looper larvae may be distinguished from other larvae by the presence of small vestigial prolegs found on the third and fourth abdominal segments, which is absent in other loopers (Capinera, 2017).

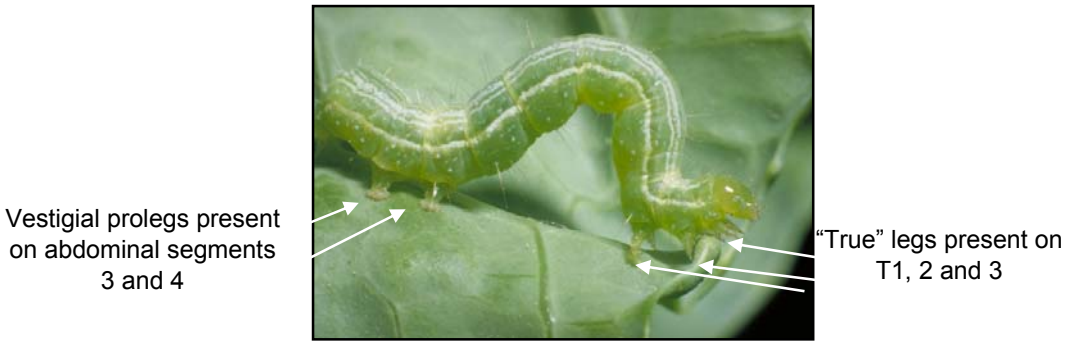

Figure 1.2 *Trichoplusia ni* larva showing characteristic looping crawling motion (Sparks, 2018).

The eggs of the cabbage looper (**Fig. 1.3**) are hemispherical and are deposited individually or in clusters of six or seven on both sides of the leaf surface. The colour of the eggs ranges from yellowish-white to green, are ridged and are 0.6 and 0.4 mm in diameter and length, respectively. The time taken to hatch is temperature dependent since at 32°C it takes two days to hatch while hatching may be delayed up to ten days at 15°C (Jackson et al., 1969).

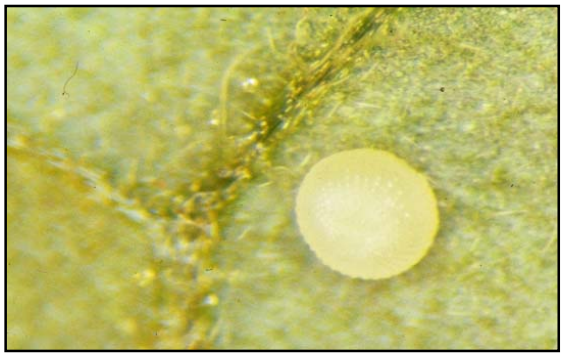

Figure 1.3 A cabbage looper egg (Cranshaw, 2005).

At pupation, a thin white cocoon (approximately two cm in length) is formed on the abaxial leaf surface, in plant debris and in soil. The duration of the pupal stage is similarly temperature dependent and varies from four days at 32°C to 13 days at 20°C. The forewings of the adult moth are mottled grey-brown in colour while the hind wings are a lighter brown at the base of the wing while the distal portions are darker brown (**Fig. 1.1**). The cabbage looper moth can be distinguished from other crop-feeding moths of the Noctuidae family by the silvery white spots on the forewing composed of a u-shape and circle that may be joined. Wingspan ranges from 33 to 38 mm and during the adult stage, averaging 10 to 12 days, the female may produce 300 to 600 eggs (Capinera, 2017). The adults are semi nocturnal. Blacklight and pheromone traps can capture a large number of moths, but is unable to prevent crop damage (Debolt et al., 1979).

Control of the pest is currently achieved by integrated pest management (IPM) programmes which may include the egg parasite such as *Trichogramma pretiosum*, larval parasites including *Hyposoter exiguae, Microplitis brassicae* and *Copidosoma truncatellum*, and the parasitic fly *Voria ruralis* as well as preparations based on *Bacillus thuringiensis* (Janmaat and Myers, 2006; Capinera, 2017).

1.3 Baculovirus Structure and Taxonomy

Baculoviruses consist of large circular supercoiled double stranded (ds) DNA viruses varying in size from 80 to 180 kb. Members of this group are obligate, parasitic pathogens exclusive to arthropods with the majority infecting over 600 insect species in the order Lepidoptera (butterflies and moths). Baculoviruses have been identified in Hymenoptera (sawflies), Diptera (flies, mosquitoes), Coleoptera (beetles), Neuroptera (net-winged insects), Trichoptera (caddis flies), and Thysanura (bristletails) as well as in the order Crustaceae (Ikeda et al., 2015).

Baculovirus virions consists of rod-shaped nucleocapsids (NC) approximately 30-70 and 200-400 nm in in diameter and length, respectively enclosed by a lipid bilayer (Wang et al., 2016) (**Fig. 1.4**). The infection cycle of baculoviruses is characterised by a replication cycle

consisting of two phases that results in the production of two types of virion phenotypes. Budded viruses (BV), which are present in the insect haemocoel, initiates secondary infections and dissemination of the infection in the insect, while occlusion derived viruses (ODVs) initiate primary infection of the midgut epithelial cells (**Fig. 1.4**). Polyhedral occlusion bodies of NPVs are known as polyhedra, ODVs or polyhedral inclusion bodies (PIBs) and may contain one nucleocapsid- the single nucleopolyhedroviruses (SNPVs) or multiple nucleocapsids- the multiple nucleopolyhedroviruses (MNPVs) (Rohrmann, 2013).

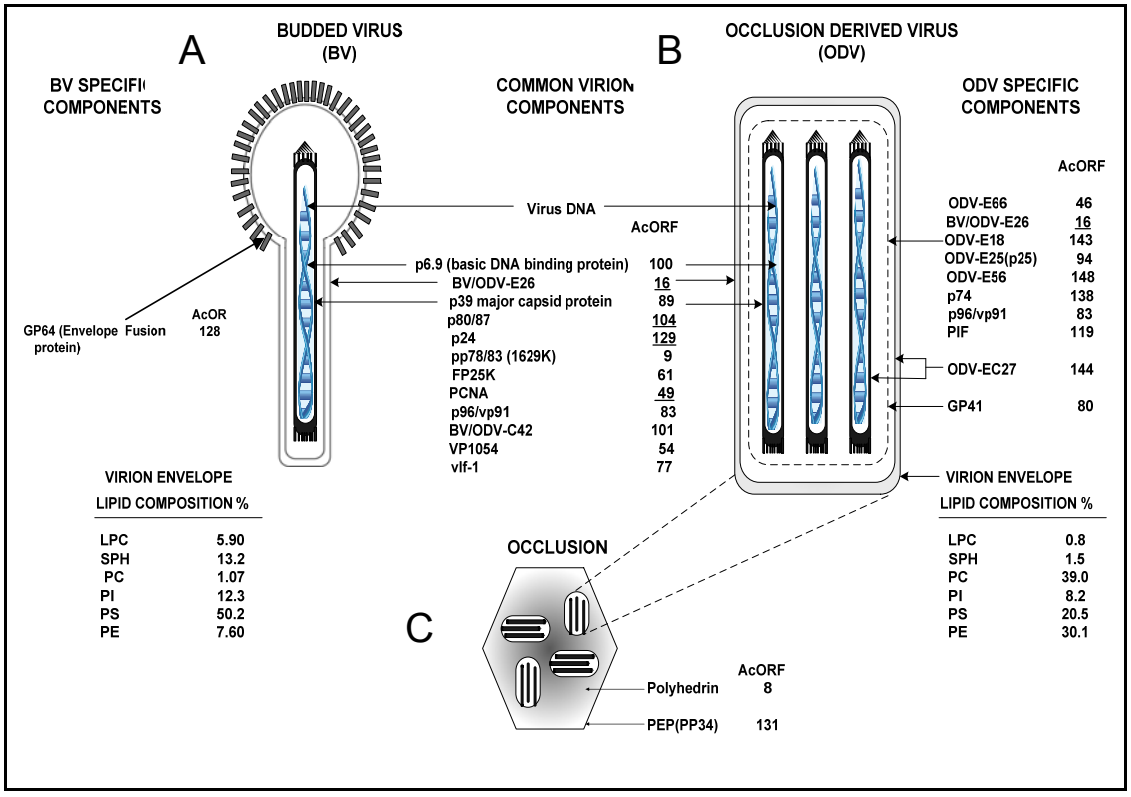

Figure 1.4 Structural composition of the budded virus (BV) and occlusion derived virus (ODV). The ODV structure in B and C represents the multiple nucleopolyhedrovirus group. Specific proteins for BV (A) and ODV (B) are indicated. Proteins common to both phenotypes are shown. The corresponding AcMNPV ORF are indicated (Ayres et al., 1994), those underlined are not baculovirus conserved genes (Herniou et al., 2003). Lipid composition of the ODVS and BVs are derived from AcMNPV-infected Sf9 cells (Braunagel and Summers, 1994). LPC (lysophosphatidlycholine), SPH (sphingomyelin), PC (phosphatidylcholine), PS (phosphatidylserine), PE (phosphatidylethanolamine). Adopted from Blissard (1996) and Braunagel et al. (2003).

Several criteria were used to classify baculoviruses into two genera including occlusion body (OB) morphology and the mechanism of nucleocapsid envelopment in infected cells (Blissard et al., 2000). The genera, *Nucleopolyhedrovirus* (NPVs) have virions that are fixed in a crystalline protein matrix composed of polyhedrin which protects the virion from environmental

degradation. The second genera, *Granulovirus* (GVs) have one virion fixed in a crystalline matrix of granulin (Ikeda et al., 2015).

The lepidopteran-specific NPVs are further classified into the group I NPVs and group II NPVs (Herniou et al., 2003), which corresponds to the presence of two envelope fusion proteins required by the BV to gain entry into the cell. Group I NPVs contain a GP64-like major envelope glycoprotein while Group II NPVs contain a functionally similar major envelope glycoprotein F (Westenberg et al., 2007). Analysis of the complete genome sequences of 13 baculoviruses also supported this division (Herniou et al., 2003). While the phylogenetic analysis of Group I and Group II NPVs previously revealed that both groups were monophyletic (consisting of one clade) (Herniou et al., 2003; Jehle et al., 2006b), recent evidence suggests that the Group II NPVs consists of multiple clades and may represent different baculovirus lineages (Harrison et al., 2017).

A new classification system based on analysis of 29 completely sequenced baculovirus genomes has replaced the traditional morphologically based classification (**Fig. 1.5**). This classification shows that baculovirus phylogeny follows the classification of the insect host's more closely than the morphological traits used as the basis for the previous classification system. With this classification system, the order Baculoviridae will consist of four genera with all lepidopteran-specific nucleopolyhedroviruses forming the *Alphabaculovirus* group, the previous *Granulovirus* genus would comprise the *Betabaculovirus* genus, the hymenopteran-specific nucleopolyhedroviruses forms the *Gammabaculovirus* genus while *Culex nigripalpus* nucleopolyhedrovirus (NPV) and possibly other Dipteran-specific baculoviruses comprise the *Deltabaculovirus* genus (Jehle et al., 2006a). This new classification was submitted to the International Committee for the Taxonomy of Viruses (ICTV) for consideration and comment from the scientific community and has been officially ratified by the ICTV (Carstens, 2010).

While more than 80 baculovirus genomes have been fully sequenced (**Table 1.1**), this probably represents only a fraction of the baculovirus diversity present in nature. The lepidopteran-specific *Alphabaculoviruses* and *Betaculoviruses* are most closely related to each other while the *Gamma*-and *Deltabaculoviruses* are more divergent. The *Deltabaculoviruses,* with the sole member of the *Deltabaculoviruses*, CuniNPV, is the most divergent (Clem, 2015). With the increasing availability of more sequence data, the phylogenetic relationship of the baculoviruses is likely to be further refined.

1.4 Baculovirus Life Cycle

Once the larvae ingest the occluded nucleopolyhedroviruses (ODVs) on the surfaces of plants and in the soil, the protective polyhedral coat is dissolved in the highly alkaline environment of the midgut of the infected larvae (**Fig. 1.6**). The now liberated virions target and enter the columnar epithelium cells of the anterior midgut (Javed et al., 2016) by way of the peritrophic membrane and fusing to the microvilli of the cells before release into the cytoplasm by direct

membrane fusion (Passarelli, 2011). Fusion and entry of the ODV is mediated by ODV derived envelope proteins - per os infectivity factors (PIFs). The PIFs are required for oral infectivity and initial infection of the larvae.

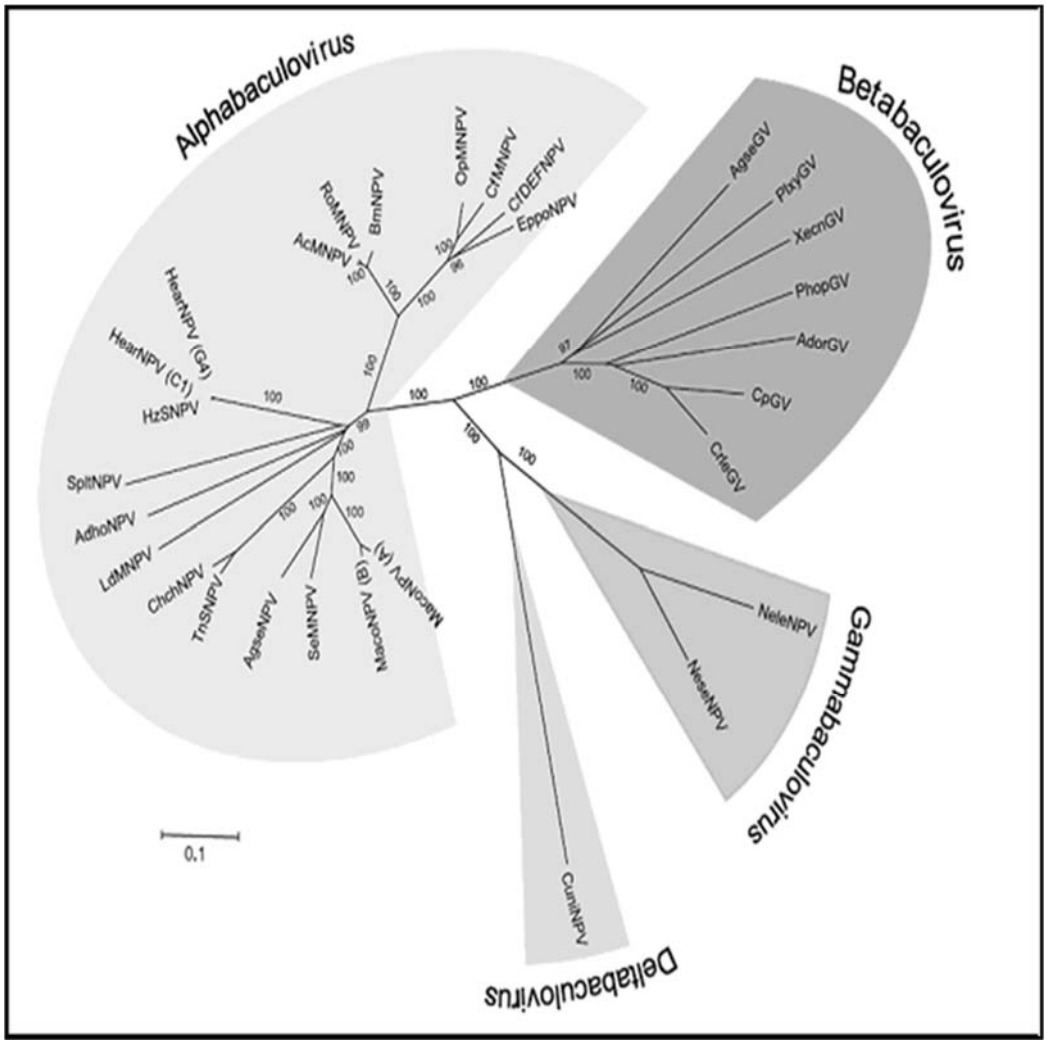

Figure 1.5 Neighbour-joining tree of the amino acid alignment of 29 baculovirus core genes of 29 sequenced baculovirus genomes. The alignment comprised 16349 positions. All branches have bootstrap values exceeding 50%. Bootstrap values >95% are given along the branches. Adopted from Jehle et al. (2006b).

The PIF proteins associate with the ODV envelope to form the core of a protein complex [PIF1 (ac119), PIF2 (ac22), PIF3 (ac115), PIF4 (ac96) and P95 (ac83)], which also includes additional associated proteins [(PIF0 (ac138, P74), PIF5 (ac148, ODV-E56) and PIF6 (ac68)] (Wang et al., 2017). The protein core complex and associated proteins are responsible for binding to an as yet unidentified ligand located in the host midgut cell brush border facilitating entry of the ODV into the midgut cell (Javed et al., 2016).

Table 1.1 List of 80 baculovirus genomes sequenced (National Center for Biotechnology Information, 2018).

Alphabaculovirus	*Adoxophyes honmai* NPV	AdhoNPV	NC_004690	*Helicoverpa armigera NPV G4*	HaNPV G4	NC_002654
	Adoxophyes orana NPV*	AdorNPV	NC_011423	*Hemileuca* sp. NPV*		NC_021923
	Agrotis ipsilon MNPV	AgipMNPV	NC_011345	*Hyphantria cunea* NPV	HcNPV	NC_007767
	Agrotis segetum MNPV A	*AgseNPV*	*NC_007921*	*Lambdina fiscellaria NPV*	*LafiNPV*	*NC_026922*
	Agrotis segetum MNPV B	AgseNPV-B	NC_025960	*Leucania separata* NPV	LsNPV	NC_008348
	Antheraea pernyi MNPV	AnpeMNPV	NC_008035	*Lymantria dispar* MNPV	LdMNPV	NC_001973
	Anticarsia gemmatalis NPV*	AgMNPV 2D	NC_008520	*Lymantria xylina NPV*	LyxyMNPV	NC_013953
	Anticarsia gemmatalis MNPV	AgMNPV	NC_031761	*Mamestra brassicae* MNPV	MbMNPV	NC_023681
	Apocheima cinerarium NPV*	ApciNPV	NC_018504	*Mamestra configurata* NPV	MacoNPV A	NC_003529
	Autographa californica MNPV	AcMNPV	NC_ 001623	*Mamestra configurata* NPV	MacoNPV B	NC_004117
	Bombyx mori NPV	BmNPV	NC_001962	*Maruca vitrata* MNPV	MaviMNPV	NC_008725
	Buzura supressaria NPV	BusuNPV	NC_02344	*Orgyia leucostigma* NPV	OlNPV	NC_010276
	Catopsilia pomona NPV	CapoNPV	NC_0302402	*Orgyia pseudosugata* MNPV	OpMNPV	NC_001875
	Choristneura fumiferana MNPV	CfMNPV	NC_004778	*Peridroma* Alphabaculovirus*		NC_024625
	Choristoneura fumiferana DEFMNPV	CfDEFMNP	NC_005137	*Pseudoplusia includens* SNPV IE	PsinSNPV-IE	NC_026268
	Choristoneura murinana NPV*	ChmuNPV	NC_023177	*Spodoptera exigua* MNPV	SeMNPV	NC_002169
	Choristoneura rosaceana NPV	ChroNPV	NC_021924	*Spodoptera frugiperda* MNPV	SfMNPV	NC_009011
	Chrysodeixis chalcites NPV	ChchNPV	NC_007151	*Spodoptera litura* MNPV	SpltMNPV	NC_003102
	Clanis bilineata NPV	ClbiNPV	NC_008293	*Spodoptera litura* NPV II*	SpltNPV II	NC_011616
	Condylorrhiza vestigialis MNPV*	CoveMNPV	NC_026430	*Sucra jujuba* NPV	SujuNPV	NC_028636
	Ectropis obliqua NPV	EcobNPV	NC_008586	*Thysanoplusia orichalcea* NPV	ThorMNPV	NC_019945
	Epiphyas postvittana NPV	EppoNPV	NC_003083	*Trichoplusia ni* SNPV	TnSNPV	NC_007383
	Euproctis pseudoconspersa NPV	EupsNPV	NC_012639	*Wiseana signata* NPV	WisiNPV	NC_038370
	Helicoverpa armigera NPV C1	HaSNPV C1	NC_003094			

Betabaculovirus	*Adoxophhyes orana* GV	AdorGV	NC-005038	*Harrisina brillians GV*	HabrGV	NC_038372
	Agrotis segetum GV	AgseGV	NC_005839	*Helicoverpa armigera* GV	HaGV	NC_010240
	Artogeia rapae GV*	PrGV	NC_013797	*Lacanobia oleracea* GV	LoGV	NC_038868
	Choristoneura fumiferana GV	ChufuGV	NC_008168	*Mocis latipes* GV*	MolaGV	NC_029996
	Clostera anachoreta GV	ClanGV	NC_015398	*Mythimna unipuncta* GV*	MyunGV	NC_033780
	Clostera anastomosis GV*Henan	ClasGV-HBHN	NC_022646	*Phthorimaea operculella* GV	PhopGV	NC_004062
	Clostera anastomosis GV B	ClasGV B	NC_038371	*Plodia interpunctella* GV	PiGV	NC_032255
	Cnaphalocrocis medinalis GV	CnmeGV	NC_029304	*Plutella xylostella* GV	PlxyGV	NC_002593
	Cryptophlebia leuoctreta GV	CrleGV	NC_005068	*Pseudaletia unipuncta* GV	PsunGV	NC_013772
	Cydia pomonella GV	CpGV	NC_002816	*Spodoptera frugiperda* GV	SfGV	NC_026511
	Diatraea saccharalis GV	DsGV	NC_028491	*Spodoptera litura* GV	SpltGV	NC_009503
	Epinotia aporema GV	EpapGV	NC_018875	*Trichoplusia ni* GV LBIV-12	TniGV	NC_038375
	Erinnyis ello GV	ErelGV	NC_025257	*Xestia c-nigrum* GV	XecnGV	NC_002331
Gamma	*Neodiprion lecontii* NPV	NeleNPV	NC_005906	*Neodiprion abietis* NPV*	NeabNPV	NC_008252
	Neodiprion sertifer NPV	NeseNPV	NC_005905			
Delta	*Culex nigripalpus* NPV	CuniNPV	NC_003084			
****UNCLASSIFIED***	*Perigonia lusca* SNPV	PeluSNPV	NC_027923	*Urbanus proteus* NPV*	UrprNPV	NC_029997

*Unclassified/unassigned names

The NCs enter the nucleus by way of the nuclear pore (Fay and Panté, 2015) where they are uncoated. Transcription of viral genes, replication of the genome and assembly of the NCs occurs in the nucleus.

The initial round of viral replication produces the budded virus (BV) which buds through the basal cell membrane and acquires a loose fitting plasma membrane which is modified by

the action of viral proteins (Wang et al., 2016) (**Fig. 1.6**). Infection of other larval tissue by the BVs occurs via the haemolymph and tracheal system (Rohrmann, 2013). Entry into the cells occurs via endocytosis, mediated by a viral encoded glycoprotein, GP64, unique to the BVs (Wang et al., 2016). Progeny nucleocapsids acquire an envelope in the nucleus and are occluded within polyhedrin late in the infection cycle.

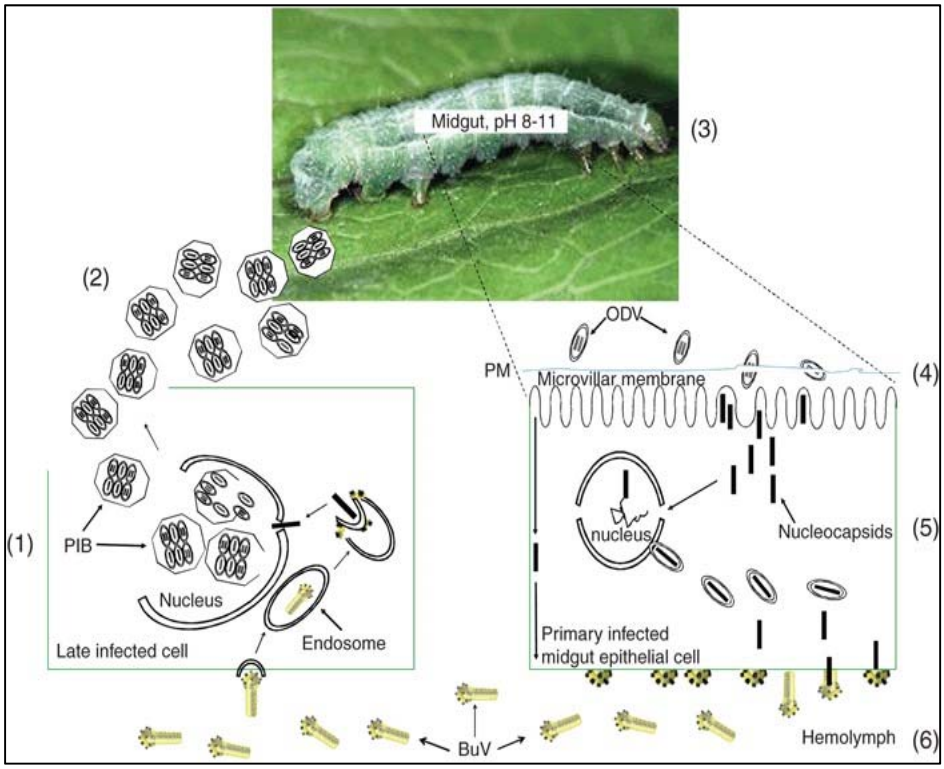

Figure 1.6 Life cycle of baculoviruses. Infection between hosts is mediated by progeny nucleocapsids produced late in the infection cycle (1). Larvae ingest the occluded nucleopolyhedroviruses (ODVs) present on plant surfaces and in the soil (2). The polyhedra dissolve in the midgut (3), releasing virions that target and enter the columnar epithelium cells of the anterior midgut by passing through the peritrophic membrane (PM) (4). The viral DNA replicates in the nucleus (5) and progeny virus buds through the cytoplasmic membrane to disseminate the infection (6) through the tracheal system and haemolymph. After the initial round of infection, nucleocapsids produced in the nucleus are enclosed with polyhedrin. When the insect dies, the polyhedra are released from the lysed cell. BV, budded virus; PIB, polyhedral inclusion body. Adapted from Airenne et al. (2013).

Upon rupturing of the cell and nuclear membranes, the mature polyhedra are liberated into the environment. The ODVs are enveloped in a carbohydrate rich polyhedrin envelope or calyx (Rohrmann, 2013) composed of 28.8 kDa polyhedrin subunit as well as a 35.4 kDa PEP/calyx protein that forms a surface on the ODV (Sajjan and Hinchigeri, 2016). There is evidence that the baculovirus p10 protein, expressed late in the infection cycle, plays a role in the formation of the polyhedrin envelope (Sajjan and Hinchigeri, 2016). Other proteins which

may form part of the ODVs include metalloprotease enhancins (Rohrmann, 2013). Proteins of non-baculovirus origins such as alkaline proteases (Sajjan and Hinchigeri, 2016) have also been reported.

During the later stages of the infection, the larvae become sluggish and there is a cessation of feeding. Prior to death, the infected larvae ascend the plant and suspends from this elevated position. This enables the propagation of the virus as the larvae decomposes (**Fig. 1.7**). Liquefaction of the cadaver promotes the dispersal of the ODVs and is mediated by the baculovirus proteins cathepsin (Ohkawa et al., 1994) and chitinase (Hawtin et al., 1995).

Figure 1.7 *Trichoplusia ni* larvae. The uppermost larvae shows evidence of baculovirus infection (Zungoli, 2003).

1.5 Baculoviruses as Pesticides

While a number of baculovirus preparations have been utilised for the control of insect pests (**Table 1.2**), the most successful application of baculovirus to control insect pests has been in Brazil where the NPV of the soybean caterpillar (*Anticarsia gemmatalis*) (AgMNPV) has been successfully applied to approximately one million hectares of soybean annually (Haase et al., 2015). Many factors which have contributed to the success of this large-scale application includes the high virulence of the host larvae to low viral dose, efficient transmission of the baculovirus preparation within the host by predators and abiotic factors (Boucias et al., 1987; Moscardi, 1989; Fuxa and Richter, 1993). Additionally, the lack of other key insects present on the crop (Moscardi, 1999) and the tolerance of the soya bean plant to defoliation without a reduction in the yield of soybean crop (Kogan and Turnipseed, 1987) have contributed to the success in this application. The establishment of integrated pest control (IPM) programmes in Brazil since the mid-1970s (Moscardi et al., 2011) has also contributed to farmer acceptance of AgMNPV.

Despite the availability of several baculoviruses with relatively broad host ranges, such as the NPVs of *Autographa californica*, *Anagrapha falcifera* and *Mamestra brassicae*, and recent scientific and technical advances, baculoviruses are still not widely employed as microbial pesticides. Baculovirus-based insecticides are limited by their slow killing time, narrow host range and the technical difficulties for *in vitro* commercial production (Lacey et al., 2015). In

addition, commercial and small scale farmers regard such agents as ineffective because of the slow killing time when compared to conventional insecticides (Lacey et al., 2015). With the successful use of a baculovirus based insecticide in Brazil and other locations, it is hoped that the widespread uptake of these agents will occur. The use and success of such agents may also precipitate the gradual acceptance of the improvement through genetic modification and utilisation of these biological pesticides.

The focus of genetic modification of baculoviruses has been to reduce the killing time and decrease the feeding capacity of the insect host (Bonning and Hammock, 1996; Haase et al., 2013). Strategies include the expression of foreign genes such as insect-specific toxins (arthropod venom, *Bacillus thuringiensis* toxins), insect-derived hormones such as eclosion, diuretic, and prothoracic hormones, juvenile hormone esterase (JHE), that is able to catalyse the hydrolysis of juvenile hormone thus interrupting insect metamorphosis (Szewczyk et al., 2006); proteases (Harrison and Bonning, 2001) as well as the deletion of the *ecdysteroid* UDP-*glucosyltransferase* gene (*egt*) from the baculoviral genome (O'Reilly and Miller, 1991).

Initial results for the toxin derived from the scorpion *Androctonus australis* (AaIT) (Szewczyk et al., 2006), the mite toxin from the predatory mite, *Pyemotis tritici* (TP1) (Tomalski and Miller, 1991; Popham et al., 1997) and *egt* gene deletion (Treacy et al., 1997), have been promising. These recombinant viruses exhibit increased short term effects but because of the lower yield of ODVs (up to 20-60% less) when compared to wild type virus, they would require frequent field applications (Cory et al., 1996). While this is potentially lucrative for commercial companies, the farmer will need to acquire such agents at considerable cost and necessitate frequent application. Field trials with these agents have been conducted (Cory et al., 1996) but to date no genetically modified baculoviruses has been registered commercially or utilised for large-scale applications (Lacey et al., 2015). With the continued public distrust towards genetically modified products, it seems unlikely that use of such agents is likely to happen in the near future (Glare et al., 2012).

Additives, such as boric acid (Shapiro and Bell, 1992), chitinase (Shapiro et al., 1987), extracts of the neem tree (*Azidarachta indica*) (Cook et al., 1996) and optical brighteners of the stilbene group (Farrar Jr et al., 2003), have all been reported to enhance baculovirus activity. These additives together with viral enhancing factor or enhancin have been shown to increase the virulence of AcMNPV and TnGV by rupturing the peritrophic membrane of the midgut epithelial cells (Tanada, 1985). Optical brighteners enhance viral activity at low doses, reduces killing time and provides UV protection from sunlight thus enhancing environmental persistence (Farrar Jr et al., 2003). While these formulations and other additives have good potential for use in recombinant baculovirus preparations, they have not been widely adopted due to a number of factors including high cost, phytotoxicity and difficulties associated with application (Lacey et al., 2015). The attendant use of baculovirus preparations with insecticides shows no adverse

effects on the baculovirus (Moscardi and Sosa-Gómez, 1992) and is used predominantly in Brazil, to reduce the damaging effects of insecticides.

Table 1.2 Baculoviruses developed as microbial insecticides to control Lepidoptera. Adapted from Moscardi (1999), Haase et al. (2015) and Lacey et al. (2015).

Host insect	Baculovirus[1]	Crops	Commercial name	Producers/Country
Adoxophyes orana	AdorGV	Apple	Capex 2	Andermatt (Switzerland)
Adoxophyes sp.	AdhoGV	Tea		Japan
Agrotis segetum	AgseGV	-		China
Anagrapha falcifera[2]	AfMNPV	Cotton, vegetables		USA
Antcarsia gemmatalis	AgMNPV	Soybean	Baculoviron Baculovirus Nitral Coopervirus, Protégé Baculo-soja Baculovirus Nitral Coopervirus SC 3, Multigen	Coodetec. CNP So, Nova Era Biotechnologica Agricola, Nitral Urbana Laboratorios, Coop Central Milenio Agro Ciencias (Brazil)
Autographa californica[2]	AcMNPV	Cabbage, cotton Ornamentals	VPN80	Guatemala
Autographa californica[2] *Trichoplusia ni Pseudoplusia includens Heliothis virescens Spodoptera exigua Estigmene acrea Plutella xylostella*	AcMNPV + *Spodoptera albula NPV*	Alfalfa, vegetable crops	VPN-ULTRA	Guatemala
Spodoptera spp.	*Spodoptera sunia* NPV	Vegetables	VPN 82	Gautamela
Buzura suppressaria	BusuNPV	Tea, tung oil tree Apples, pears	- Carpovirusine	China France
Cydia pomonella, Cydia pomonella, Grapholita molesta	CpGV	Apple, pear, Walnut, peach	CYD-X, Granusal Virin_GyAp Carpovirus Plus Madex Carpovirusine Madex Twin	Chile Germany Russia Uruguay Certis (USA) BioTepp (Canada) Arysta Lifescience (France), Andermatt (Switzerland) Hoerst (Germany) BioBest (Belgium) Arysta Life Science (France) Agro Roca (Argentinia)
Erinnyis ello	*Erinnyis ello* GV	Cassava Rubber Trees	- Baculovirus erinnyis	Brazil Venezuela Colombia
Heliothis and *Helicoverpa* spp.	*Heliothis zea* NPV	Cotton	Elcar Gemstar	USA USA

		Maize, tomato and tobacco	HzNPV CCAB	Mexico Brazil Certis (USA)
Heliothis and Helicoverpa spp.	*Helicoverpa armigera* NPV	Tomato, sweet pepper, maize, soybean, tobacco, vegetable crops	Diplomata 13 Helicovex	Brazil
Heliothis viriscens	NPV	Cotton	Elcar Gemstar	USA USA
Helicoverpa armigera	NPV	Cotton, tomato	- Virin-HS	China Russia Andermatt, (Switzerland) AgBioTech (Australia) Jiyuan Baiyun Industry Company Ltd. (China) BioControl Research Labs (India) Kenya Biologics (Kenya) plus other producers in India, China
Homona magnanima	GV	Tea	-	Japan
Hyhpantrea cunea	NPV	Forest, mulberry	Virin-ABB	Russia
Lymantria dispar	NPV	Forests	Gypcheck Disparvairus Virin-ENSH	USDA (USA) Sylvar Technology (Canada) Russia China Andermatt (Switzerland)
Mamestra brassicae	NPV	Cabbage	Mamestrin Virin-EKS	France Russia
Orgyia pseudosugata	NPV	Forests	TM Biocontrol 1 Virtuss Baculovirus Corpoica PTM baculovirus	USA Canadian Forest Service Colombia Peru Costa Rica
Phthorimaea operculella *Tecia solanivora*	*Phthormaea operculella* GV	Field and stored potatoes	PTM baculovirus	Peru Egypt Tunisia
Phtorimaea operculella *Tecia solanivora* *Symmetrischema tangolias*	*Phthorimaea operculella* GV + *Bacillus thuringiensis*	Potatoe	Matapol Plus Bacu-Turin	Bolivia Ecuador Centro Internacional de la Papa (Peru), Proinpa (Bolivia)
Pieris rapae	GV	Cabbage		China
Plodia interpunctella	GV	Stored almonds and raisins	-	USA
Plutella xylostella	GV	Cabbage		China Jiyuan Baiyun Industry Company Ltd. (China)
Spodoptera exigua	NPV	Ornamentals & vegetables Shallot, peas, grape, Chinese kale Flowers, ornamentals	SPOD-X SPOD-X LC	USA Thailand Netherlands Mexico Andermatt, (Switzerland)

		Tomato, chili, eggplant		Certis (USA) Jiyuan Baiyun Industry Company Ltd.,(China) BioTech (Thailand)
Spodoptera frugiperda	*Spodoptera frugiperda* NPV	Maize Maize, sorghum		Brazil
Spodoptera littoralis	NPV	Cotton		Africa Andermatt, (Switzerland)
Spodoptera litura	NPV	Vegetables, cotton, rice peanuts		China Biocontrol Research Lab, Ajay Biotech, Bassarass Biocontrol, Biotech International, BioControl Research Labs (India) Jiyuan Baiyun Industry Company Ltd. (China)
Spodoptera sunia	NPV	Vegetables	VPN 82	Agricola el Sol (Guatemala)
Neodiprion lecontei	NPV	Pine trees		Sylvar Technology (Canada)
Neodiprion abietis	NPV	Forests		Sylvar Technology (Canada)
Cryptophlebia leucotreta	GV	Citrus		Andermatt (Switzerland) River Bioscience (South Africa)
Homona magnanima	GV			Arysta life science (Japan)
Trichoplusia ni	NPV	Vegetables		Andermatt (Switzerland)
Ectropis obliqua	NPV	Tea		Small scale commercial production China

[1]GV, Granulovirus; NPV, Nucleopoyhedrovirus

[2]These NPVs have been developed primarily for use against Lepidopteran species other than their original hosts because of their wide host range

1.6 Gene Expression Within Baculoviruses

During infection, expression of the viral genes and DNA replication within the insect cell occurs in a co-ordinated sequential manner with each phase dependant on the previous phase (Friesen and Miller, 1986). Gene expression may be categorised into three phases namely: early, late and very late (**Fig. 1.8**) (Ikeda et al., 2015). Early viral genes are transcribed by utilising the insect cellular machinery with no expression of viral genes during this stage (Berretta et al., 2013). Late and very late genes require early gene expression and DNA replication and is characterised by a shift from the host DNA polymerase to a viral α-amanitin resistant DNA polymerase (Rohrmann, 2013).

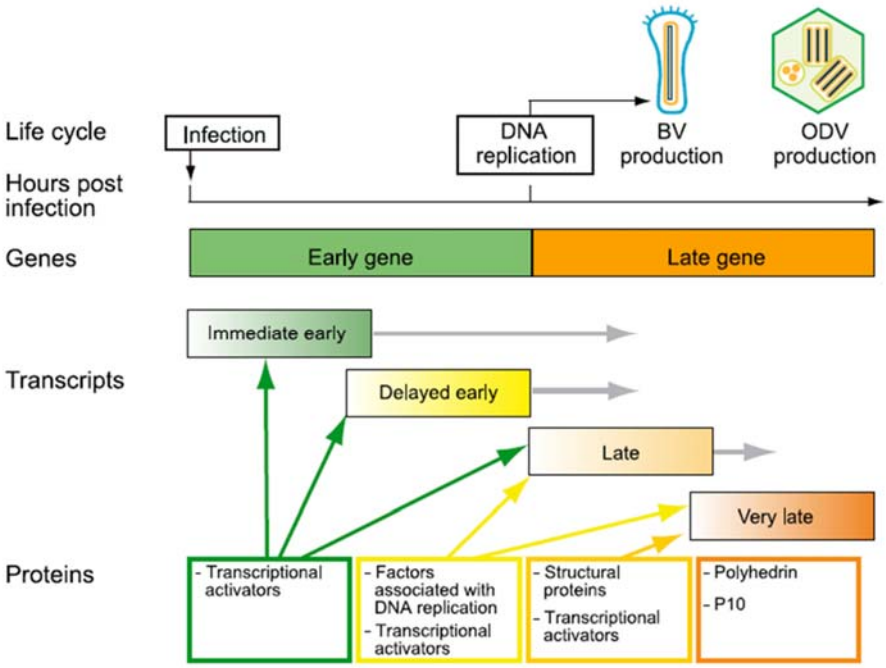

Figure 1.8 Regulation of baculovirus gene expression. Baculovirus genes may be categorised into four classes (immediate early, delayed early, late and very late) which are expressed in a co-ordinated sequential manner and are primarily regulated at the level of transcription. Adopted from Ikeda et al. (2015).

Early gene expression may be further categorised into immediate early genes and delayed early genes. Immediate early genes can be transcribed by insect cells prior to viral infection and do not require viral factors for their expression whereas delayed early genes require viral gene factors for expression. Early genes that are transcribed by the RNA polymerase II complex of the host cell (Huh and Weaver, 1996) such as the immediate early transactivator *ie1*, are necessary for the transactivation of delayed early and late gene expression. The gene products which results from delayed early and late gene expression is required for viral DNA replication and for late gene expression (Ikeda et al., 2015). Late and very late genes are transcribed by a RNA polymerase of viral origin (Ikeda et al., 2015) that is resistant to α-amanitin (a selective RNA polymerase I and II inhibitor) with a distinctive subunit composition (Yang et al., 1991).

Late gene expression is closely linked to DNA replication and if viral replication is inhibited, the late genes will not be expressed (Rohrmann, 2013). The late genes code for proteins that form part of the structure of the virus particle. This includes the basic protein (Wilson et al., 1987), the 39 kDa capsid protein (Thiem and Miller, 1989), and the virus membrane glycoprotein *gp67* (Whitford et al., 1989). Hyper-expressed late genes are transcribed when the virus is assembling occlusion bodies in the nucleus of the infected cell. They do not contribute to the formation of infectious virus particles and if deleted do not affect virion production (Vlak et al., 1988).

Baculovirus gene expression (early and late) is regulated at the level of transcription. Baculovirus early gene promoters closely resemble RNA-polymerase II-responsive promoters of the insect host, principally the initiator (INR) tetranucleotide CAGT sequence and the TATA box element which precedes it (Berretta et al., 2013). Transactivation by repetitive DNA elements known as homologous replication sequences (*hrs*) also functions as potent early transcriptional enhancers (Guarino and Summers, 1986; Rodems and Friesen, 1995) as well as possible origins of DNA replication (Pearson et al., 1992; Leisy and Rohrmann, 1993).

Late and very late gene expression coincides with the formation of BVs and ODVs respectively and is transcribed by a α-amanitin resistant RNA polymerase which is likely to be wholly or partially encoded by the viral genome (Rohrmann, 2013). Late promoters initiate from the sequence (A/G/T)TAAG with a 18 bp sequence with an internal TAAG motif being essential for late promoter activity (Miller, 1997).

Eighteen late expression factors (*lefs*) that are essential for gene expression from late promoters, have been identified by transient expression assays (Lu and Miller, 1995; Todd et al., 1996). In this method, a plasmid containing a reporter gene under control of a baculovirus late promoter is co- transfected with overlapping subclone segments of the AcMNPV genome. The *lefs* were identified by their ability to support expression of the reporter gene. A number of these genes (the immediate early gene, *ie-1*, the late expression factors, *lef-2*, *lef-1*, *lef-3*, and *p143* and *p35)* are implicated in DNA replication (Lu and Miller, 1995). Analyses of amino acid sequence motifs conserved in both prokaryotic and eukaryotic RNA polymerases (Lu and Miller, 1994; Passarelli et al., 1994), identified four genes (*lef-8*, *lef-9, lef-4*, *p47*) predicted to be components of a viral encoded RNA polymerase and has been confirmed by protein sequencing, peptide fingerprinting, and immunochemical analyses. Very late gene expression which is required for occlusion body formation also requires the function of the very late expression factor 1 gene (*vlf-1*) which has motifs typical of a family of integrases/resolvases (McLachlin et al., 2001).

1.7 The *ecdysteroid* UDP-*glucosyltransferase* (*egt*) gene

The *ecdysteroid* UDP-*glucosyltransferase* (*egt*) gene is one of the main targets of genetic modification to enhance the pesticidal properties of baculoviruses. First identified in the prototype virus AcMNPV, it encodes an *ecdysteroid* UDP-*glucosyltransferase* (UGT), an enzyme which is required for catalytic conjugation of ecdysone and UDP-glucose (O'Reilly, 1995). Consequently identified in the two baculovirus genera that infect Lepidoptera, *Alphabaculovirus* and *Betabaculovirus* (Ahn et al., 2012), the EGT protein inactivates the steroid moulting hormone, ecdysone, with consequent delay in normal lepidopteran larval moulting, with no weight loss of the larvae prior to pupation and earlier mortality (O'Reilly and Miller, 1991). The virus-infected larvae are able to feed longer thus causing greater foliar damage and are able to produce a higher yield of progeny virus. Disruption of the *egt* gene sequence increases

the virulence of the baculovirus without reducing infectivity. Insects infected with the AcMNPV *egt* mutants feed less and die quicker than those infected with wild type AcMNPV (O'Reilly and Miller, 1991). These modifications in the expression of *egt* have been shown to improve the pesticidal properties of the baculoviruses. In addition to the inactivation of host ecdysteroid hormones by glycosylation, the EGT protein is able to modify host behaviour. The European gypsy moth, *Lymantria dispar*, infected by LdMNPV will climb to the top of trees to die, liquefy and thus disseminate the virus on the foliage below to infect new hosts (Han et al., 2015; Ros et al., 2015). The *egt* deletion also occurs in naturally occurring fast killing genotypes of SfMNPV (Simón et al., 2005; Harrison, 2009) and wild type SeMNPV (Muñoz et al., 1999) isolates where if confers a fast killing phenotype with reduced occlusion body formation. It is as yet unclear why the deletion mutants occur with slower killing non-deletion variants but they may play a role in shaping the pathogenicity, virulence and productivity of the population (Simón et al., 2012).

The *egt* gene is highly conserved in baculovirus genomes which suggests that it has some evolutionary advantage that is common to all the baculoviruses. There is considerable homology throughout the length of the EGT protein. The protein contains 5 conserved regions with the largest and most highly conserved region found at position 237-268 [according to *Christoneura fumiferana* NPV (CfMNPV) alignment] (Barrett et al., 1995). This region is 100% identical in CfMNPV, CfMNPV DEF and OpMNPV; 90% identical in AcMNPV and 77% identical in LdMNPV and may play a role in baculovirus specific activities such as substrate recognition (Riegel et al., 1994).

The AcMNPV-*egt* is secreted from the virus infected cell and contains a N-terminal signal sequence that consists of 18 amino acids which is later cleaved from the mature enzyme (O'Reilly, 1995). The alignment of these proteins may indicate that all these proteins are secreted from the virus infected cell since they all have hydrophobic sequences at the N-termini that resemble signal peptides at the N-terminus. The sequence homology increases immediately after the proposed cleavage site suggesting that the signal sequence is not related to the actual gene sequence. The EGT protein does not contain the polar sequence at the C-terminus of mammalian UDP-*glucuronosyltransferase* that serves as a membrane anchor (O'Reilly, 1995) further confirming the secretory pathway of the EGT proteins.

Phylogenies based on the *egt* gene (Barrett et al., 1995), the polyhedrin gene (Zanotto et al., 1993) and phylogenies based on complete baculovirus genome sequences (Herniou et al., 2001; Jehle et al., 2006b) have been shown to be broadly similar. This suggests that the *egt* gene was present in the ancestral baculovirus genome before they diverged. It is also likely to be present in all baculoviruses. Baculoviruses *egt* genes bear a striking similarity to the cellular hosts (Hughes and Friedman, 2003) suggesting that they may have been acquired by horizontal gene transfer (Hughes, 2013). Due to the increased availability of sequences of UGTs from lepidopteran hosts, it has been shown that the closest relative of baculovirus EGT is the UGT33 and UGT34 families of lepidopteran UDP-glycosyltransferases. The analysis further suggests

that the baculovirus EGTs were most likely acquired by ancient horizontal gene transfer originating via a single transfer event after the formation of the order Lepidoptera (Hughes, 2013).

1.8 Apoptosis

A number of stimuli and conditions, both physiological and pathologic, which may originate from within the cell or from external sources, may trigger apoptosis. Infection by many viruses elicits the onset of apoptosis in an attempt to limit viral replication and this process is mediated by a family of cysteine proteases known as caspases (Kannourakis and Hay, 2002). Many viruses have however evolved mechanisms to counter the attempts by the host cell to limit viral replication. This strategy can enhance viral multiplication, or establish viral persistence and latency and thus contributes to viral survival (Clem et al., 1991; Crook et al., 1993).

Caspases (from Cysteine proteases that cleave after ASPartate residue) are a conserved family of proteins that are the central mediators of apoptosis. While the first caspase, [Interleukin-1β - converting enzyme (ICE)], was identified in humans (Cerretti et al., 1992; Thornberry et al., 1992), the central role of caspases in apoptosis was described in the nematode worm, *Caenorhabditis elegans* (*C. elegans*). Caspases have been identified in species extending from *C. elegans* to the dipteran *Drosophila melanogaster* (Kumar and Doumanis, 2000) and the yeast *Sachromyces cerevisiae* (Madeo et al., 2002). At least 14 mammalian caspases, including 11 in humans, have been identified (Shi, 2002).

Caspase proteins are produced as catalytically inactive zymogens that require proteolytic cleavage and contain a conserved active site consisting of five amino acids glutamine, alanine, cysteine, one of either arginine, glutamine or aspartic acid and finally glycine or “QACXG” (where X can be R, Q or D). The N-terminal of the pro-domain in procaspases contains a highly diverse structure of variable length that is required for caspase activation. All caspases have the ability of auto-activation as well as activating other caspases resulting in in a heterodimer consisting of a small and large subunit. Two heterodimers are required for assembly of the enzymatically active heterotetramer (Fan et al., 2005) often referred to as the “homodimers of heterodimer” structure (Wilson et al., 1994).

Caspases consists of two groups: the initiator caspases are characterised by a N-terminal domain consisting of > 90 amino acids; while the N-terminal prodomain sequence of effector caspases is shorter and consists of 20-30 residues. While initiator caspases are capable of autoactivation, the effector caspases are activated by initiator caspases. The activation of initiator caspases elicits a downstream caspase activation cascade that is highly regulated culminating in the proteolytic cleavage of a wide variety of targets and results in cell death (Stennicke et al., 2002; Fan et al., 2005). The caspase gene family consists of two major subfamilies related to either the inflammatory group (ICE; caspase-1; inflammation group) which are restricted to mammals and higher eukaryotes or to the apoptosis group consisting of the

mammalian counterparts of the *C. elegans* cell-death gene *ced*-3. Members of the two groups differ in their substrate specificities and amino acid sequence while members of the same subfamily are similar in amino acid sequence, structure, and substrate specificity (Abraham and Shaham, 2004; Fan et al., 2005).

Caspase activation and inactivation is regulated at a variety of levels. This can be at a transcriptional level, by post-translational modification (Earnshaw et al., 1999) as well as by ions and other factors, such as the inhibitor of apoptosis genes (IAPs), B-cell leukaemia/lymphoma 2 family proteins (Bcl-2), calpain, Ca2+, Gran B and cytokine response modifier A (Crm A) (Shi, 2002).

1.8.1 Baculoviruses and apoptosis

Four types of anti-apoptotic genes are found in baculoviruses: *p35*, the functionally identical *p49*, the novel *apsup* and inhibitor of apoptosis genes (*iap*), which can all mediate the cellular apoptotic response thus preventing premature insect cell death and promote virus multiplication (Clem and Miller, 1994; Clem and Duckett, 1997; Yamada et al., 2011). Generally, each baculovirus will contain only one functional apoptosis inhibitor, either the inhibitor of apoptosis protein or substrate inhibitor protein such as P35 (Clem and Miller, 1994; Ikeda et al., 2004; Guy and Friesen, 2008). It is unclear why non-functional anti-apoptotic genes are retained in the baculovirus genome.

While first identified in the baculoviruses CpGV and OpNPV by their ability to prevent apoptosis induced by infection by the prototype baculovirus AcMNPV lacking a functional *p35* gene in *Spodoptera frugiperda* (SF-21) cells (Crook et al., 1993; Birnbaum et al., 1994), many IAPs have subsequently been identified in genomes, including diverse organisms such yeasts, nematodes, insects and humans. These genes play a role in a variety of cellular functions, including regulation of apoptosis in both insects and mammals as well as the process of cell division as a result of mitosis and meiosis in mammals, yeast and nematodes (Duckett et al., 1996). The IAPS have been identified in eight virus families including Ascoviridae, Asfarviridae, Baculoviridae, Hytrosaviridae, Iridoviridae, Malacoherpesviridae, Nudiviridae, and Poxviridae (subfamily Entomopoxvirinae). Members of these families consist of large DNA viruses except for the kelp fly virus (Hartley et al., 2005) and mud crab dicistrovirus that consist of single stranded RNA (Clem, 2015). All of these viruses infect arthropods or are present in arthropods during a part of their replication cycle.

While the genes of the *p35* family are only found in some baculoviruses, all lepidopteran baculovirus sequenced to date, with the sole exception of *Culex nigripalpus* NPV, the singular member of the distantly related *Deltabaculoviridae*, have contained IAPs. The *Alphabaculoviruses* contain combinations of IAP1, 2, 3 and 5 while the *Betabaculoviruses* contain combinations of IAP3 and 5. An unusual IAP homolog present in only 4 members of the

Betabaculovirus consisting of a single BIR and RING domain may represent an additional class of IAP homologs as suggested by Clem (2015).

The IAPs may be classified into five members (IAP1 – IAP5) based on amino acid sequence homology (Clem, 2015; Tobin et al., 2017). This classification based on homology groups is more informative than naming the IAPs in the order in which they have been identified. Phylogenetic analysis further suggests that baculovirus IAPs have been derived from their insect hosts by gene capture events (Hughes, 2002).

Proteins in the IAP family are defined by the occurrence of one to three tandem copies of a baculovirus *iap* repeat (BIR) motif at their amino terminal (Clem and Duckett, 1997) with some also containing a Rather Interesting New Gene (RING) finger, also known as C3HC4 motifs, at their C-terminus. Each BIR contains approximately 70 amino acid residues with six absolutely conserved residues including a C2HC motif (Miller et al., 1998). The RING domain consist of six to seven cysteine residues in addition to two histidine residues buried deep in the domain core where they interact with two zinc atoms thus stabilising the structure (Oberemok and Skorokhod, 2014). The BIR domains are zinc finger-like structures that are able to chelate zinc ions and bind to the caspase surface. This allows the amino acid sequences between BIR domains to block the catalysing grooves of caspases. Thus apoptosis is prevented by inhibiting caspase activity (Fan et al., 2005). Mutation of the conserved amino acid residues within the BIR motifs is sufficient to prevent the inhibition of apoptosis (Wright et al., 2005). However, while the BIR motifs are essential for anti-apoptotic activity, not all IAPs, including some baculovirus IAPs possess such activity. The majority of the IAP-3 homologues, that closely resembles the cellular IAPs of their hosts (Cerio et al., 2010; Byers et al., 2016) possess the highest anti-apoptotic activity. The anti-apoptotic activity of IAP1 and IAP2 homologues is variable while no anti-apoptotic activity has as yet been demonstrated for IAP4 and IAP5 (Clem, 2015). It seems likely that some IAPs may act on different pathways (or at least different stages) or on different tissues of the host insect or are involved in other signal transduction pathways (Hozak et al., 2000; Ito et al., 2014). All of the baculovirus IAPs with the exception of IAP4 contain two well-conserved BIR domains and a RING finger. The IAP4 homologues generally consist of a RING domain and truncated BIR domain with the conserved C2HC residue replaced by a CX2C motif. The BIR domains also lack two zinc-binding domains present in the other IAPs (Clem, 2015).

The RING domain of IAPs can also function as E3 ubiquitin protein ligases (Green et al., 2004; Kamada, 2013). This modification is a key mechanism in many cellular processes, including signal transduction, cell cycle control, and apoptosis (Ciechanover, 1998). The three enzymes required for this post translational sequential activation cascade are E1 (ubiquitin activating enzyme); E2 (ubiquitin conjugating enzyme); and E3 (ubiquitin protein ligase) (Green et al., 2004). The ubiquitin protein E3 ligase enzyme recognises and binds to specific substrate proteins so that the transfer of ubiquitin from the E2 enzyme to the target protein may occur. This confers specificity in the ubiquitination process (Deshaies and Joazeiro, 2009). The RING

domain of several IAPs have been shown capable of auto-ubiquitination targeting these molecules for proteasomal degradation and may thus play a role in regulating the apoptosis process. A number of IAPs including three mammalian IAPs - cIAP1, cIAP2, and XIAP, as well as *Drosophila* DIAP1 are capable of auto-ubiquitination and degradation. In addition, IAPs have also been shown to target a variety of pro-apoptotic molecules such as caspases and Reaper, HID, and Grim for proteasomal degradation (Yang et al., 2000; Suzuki et al., 2001; Olson et al., 2003) while ubiquitin ligase activity *in vitro* has also been demonstrated (Imai et al., 2003).

In addition to the BIR motifs, sequences within or adjacent to the BIR domains are required for the binding of IAP molecules with pro-apoptotic proteins. These include apoptotic mediators such as Grim, Reaper and HID from *Drosophila melanogaster* and Smac/DIABLO from mammals (Green et al., 2004). The IAP proteins may also be involved in signal transduction by interacting with proteins in the signal transduction pathway. This interaction is mediated by the BIR domains (Salvesen and Duckett, 2002). Several IAP proteins are able to bind and directly inhibit caspases and are mediated by the BIR domains or sequences adjacent to the BIR domains (Shi, 2002).

The viral IAPs are able to interact and stabilise cellular IAPs thus promoting their anti-apoptotic activity (Byers et al., 2016). Viral IAPs closely resemble the host cellular IAPs from which they most likely evolved but lack the N-terminal leader degron sequence (Cerio et al., 2010; Vandergaast et al., 2015) which results in signal induced degradative pathways including auto ubiquination, ensuring their stability in infected cells (Byers et al., 2016). This persistence when compared to the cellular IAPs allows it to interact with other anti-apoptotic co-factors and exerts its anti-apoptotic activity. By directly binding to the host cellular IAP, the life span and activity of the host IAP is increased by protecting it from death signal-induced depletion. The cellular IAPs are then able to exert their anti-apoptotic effect.

Unlike proteins of the IAP family, the baculovirus *p35* gene is a general suppressor of programmed cell death able to suppress apoptosis in different organisms. Baculovirus *p35,* an immediate early gene*,* is able to suppress baculovirus-induced apoptosis and restore virus replication (Clem and Miller, 1993) and is required to protect Sf-21 cells from apoptosis induced by infection with AcMNPV lacking a functional P35 protein (Hershberger et al., 1994). It is also able to prevent developmental programmed cell death in *C. elegans* (Sugimoto et al., 1994) and *Drosophila melanogaster* (Hay et al., 1994), blocks neuronal cell death (Rabizadeh et al., 1993), and suppresses tumor necrosis factor- and Fas-induced death of mammalian cells (Beidler et al., 1995). This general activity suggests that P35 must act at a highly conserved step in the cell death pathway. The P35 protein of AcMNPV is able to inhibit a variety of caspases including caspase-1, -2, -3,-4, -7, -8 and -10 when purified proteins are used (Zhou et al., 1998). Because of this characteristic, the protein is commonly referred to as a general or pan-caspase inhibitor.

The P35 protein inhibits apoptosis by blocking caspase activity, the central mediators of apoptosis. The P35 protein is a caspase substrate and binds to and forms a stable complex with

the caspase protease, inhibiting its activity (Zhou et al., 1998). The protein is cleaved at the Asp^{87} residue found in the unique reactive site loop (RSL) by the target caspase into a 10 and 25 kDa fragment (Riedl et al., 2001). The cleavage of the caspase recognition site causes a change in the conformation of the protein that positions the N terminus of the protein into the caspase active site. This conformational change prevents hydrolysis of the peptide and results in the formation of a stable inhibitory complex (Shi, 2002). The inhibitory complex is composed of the caspase homodimer with each of the two active sites occupied by a separate monomer of P35 (Shi, 2002; Guy and Friesen, 2008). The larger 25 kDa fragment binds to the active site of the caspase such that they are unable to interact and activate downstream effector caspase. Apoptosis is thus inhibited (Bertin et al., 1996).

It has recently been shown that the P35 protein is also capable of modulating the antiviral small interfering RNA (siRNA) response that is triggered upon viral infection. This anti-viral response results in the formation of virus derived siRNA by the action of Dicer-2 and cleavage of the target viral RNA by the RNA-induced silencing complex (RISC) (Siomi and Siomi, 2009). To counteract this anti -viral response, many viruses have evolved virus-encoded proteins, called viral suppressors of RNAi (VSRs), which can circumvent this response. The P35 protein is able to suppress this anti-viral response in both insect and mammalian cells (Mehrabadi et al., 2015) and suggests an additional function in the anti-viral response of viruses.

Identified by its ability to inhibit insect and human effector caspases, the *p49* gene of *Spodoptera littoralis* (Spli) MNPV, represents a third class of baculovirus genes able to mediate the host apoptotic response (Rohrman, 1986). Unlike the P35 protein which is unable to inactivate initiator caspases (Zoog et al., 2002), P49 is able to inactivate both effector and initiator caspases (Zoog et al., 2002; Guy and Friesen, 2008). In order to form an inhibitory complex with the target caspase, the P49 protein has to be cleaved at Asp^{94} within the caspase recognition site $TVTD^{94}G$ (Jabbour et al., 2002). The P35 and P49 proteins share approximately 50% amino acid sequence similarity that suggests that the structure of P49 resembles that of P35 including the unique RSL that contains an aspartate residue (A^{94}) required for cleavage (Pei et al., 2002; Guy and Friesen, 2008). Replacement of the P49 TVTD recognition site by the P35 DQMD motif impaired the ability of P49 to inhibit initiator caspases while replacement of the P35 motif did not alter that protein's specificity for effector caspases indicating that additional components other than the recognition sites are involved in target selection (Guy and Friesen, 2008).

Homologues of a novel apoptosis suppressor gene initially identified in LdMNPV by its ability to inhibit apoptosis induced by AcMNPV infection lacking a functional P35 protein has been identified in nine NPVs, four granuloviruses, and one poxvirus (Yamada et al., 2011). The suppressor gene, *apsup*, is also able to inhibit apoptosis induced by actinomycin D and UV exposure by preventing the proteolytic catalysis of the initiator caspase Dronc (Yamada et al.,

2013). It is unclear if this novel gene represents a fourth class of baculovirus genes able to mediate the anti-apoptotic response.

Caspases have also been identified in insects. In *Drosphila*, the most widely studied model system for apoptosis, seven caspases have been identified (Hay and Guo, 2006). The Sf-9 and Sf-21 cell lines derived from the fall armyworm, *Spodoptera frugiperda* (Order: Lepidoptera), has also been employed as a model system for apoptosis. These cell lines are well suited for such studies as they are sensitive to a large variety of apoptotic stimuli, including baculovirus infection, chemical agents such as actinomycin D, and overexpression of *Drosophila* death genes such as *reaper* or *hid* and UV radiation (Manji and Friesen, 2001).

In SF-9 cells, the principal effector caspase is Sf- Caspase 1 (Ahmad et al., 1997; LaCount et al., 2000). The activation process involves the initiator caspase (Sf-caspase-X) which cleaves pro-Sf-caspase-1 at D^{195} to produce a small and intermediate subunit, p12 and p25, respectively. The small prodomain from the p25 fragment is consequently cleaved at D^{28}, producing prodomains p6 and the large subunit p19 (Manji and Friesen, 2001). An alternative activation method in which pro-Sf-caspase-1 is first cleaved at D^{28} and then D^{195}, has also been proposed (Liu et al., 2006). This alternative method also results in in active Sf-caspase-1.

Baculovirus anti-apoptotic proteins act at different stages in the apoptotic cascade. Members of the IAP family, block apoptosis upstream of the effector caspase, Sf-caspase-1. This may be due to the ability of the IAPs to prevent the activation of Sf-caspase-X or other pro-apoptotic proteins (Birnbaum et al., 1994; Vucic et al., 1997; Zoog et al., 2002). P35, the pan caspase inhibitor, blocks cleavage of the prodomain of the caspases (Bertin et al., 1996; Manji et al., 1997; LaCount et al., 2000), while P49 from *Spodoptera littoralis* nucleopolyhedrovirus (SlNPV) prevents cleavage of caspases into the large and small subunits (Zoog et al., 2002).

Outside of these two model systems, there are few reports on other insect caspases other than those in *Spodoptera littoralis* (Pei et al., 2002; Liu et al., 2005) and very recently *Trichoplusia ni* (Hebert et al., 2009).

1.9 Outline of the thesis

Trichoplusia ni single enveloped nucleopolyhedrovirus (TnSNPV) was first isolated from diseased *T. ni* larvae found in the Eastern Cape Province of South Africa in 1990. The virus was propagated in third instar *T. ni* larvae and initial characterisation showed that the virus exhibited a killing time (LT_{50}) of approximately 2 days. This is in sharp contrast to most wild-type baculoviruses where killing times range from days to weeks (Kadir, 1990; Kumar et al., 2011; Zhu et al., 2016) thus there is considerable potential for this virus to be developed into a fast acting bio-insecticide as it addresses one of the key limitations of all baculovirus-based insecticides i.e. slow killing time.

The main objective of this thesis was the identification, molecular characterisation and cloning of a structural gene and three auxiliary genes from TnSNPV in order to delineate its

phylogenetic relationship to a Canadian isolate of the same virus and to other baculoviruses. Furthermore, as a prelude to genetic modification to increase the pesticidal property of the virus, the genes were expressed in an *Escherichia coli* (*E. coli)* based Gateway cloning system. This fundamental insight into the molecular characteristics of TnSNPV is essential for the successful and bio-safe genetic modification of this virus in order to design efficient preparations and to reduce costs.

Chapter One is a description of the pertinent literature applicable to this study and provides context to the research chapters which follow. **Chapter Two** details the materials and methods employed during this study. The results and discussion are presented in **Chapter Three**. Both Chapters Two and Three are divided into three sections viz. phenotypic characterisation of *T. ni*; phylogenetic characterisation of *T. ni* utilising the highly conserved structural polyhedrin (*pol*) gene, the auxiliary *ecdysteroid* UDP-*glucosyltransferase* (*egt*) as well as the anti-apoptotic *iap*2 and *iap*3 genes and; heterologous expression of the POL, EGT, IAP2 and IAP3 recombinant proteins.

In **Chapter Four**, the concluding remarks are provided in the context of recent literature data and current insights on baculovirus taxonomy and expression.

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Chapter 2

Materials & Methods

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2.1 Phenotypic characterisation of TnSNPV

2.1.1 Virus isolation

The virus, TnSNPV-SA, was isolated from diseased *Trichoplusia ni* (*T. ni*) larvae collected from the town of Hankey in the Eastern Cape Province of South Africa in July 1998 (GPS coordinates 33° 49′ 53″ S, 24° 52′ 51″ E). The larvae and portions of the host potato plant (*Solanum tuberosum L.*) were transported back to Cape Town where they were maintained at 27°C with 90% humidity in a 14:10 light:dark photoperiod in a growth chamber. The virus was purified from the field-collected material by continuous and discontinuous sucrose density gradient centrifugation using a modified protocol described by O'Reilly et al. (1994). In brief, diseased insect cadavers were collected and stored in 0.1% sodium dodecyl sulphate (SDS) at 4°C before being homogenised in a mortar and pestle and filtered through cheesecloth. Cellular debris was removed by low speed centrifugation of 5000 × *g* for 10 min. To prepare the sucrose gradients, varying concentrations of sucrose (10 ml each of a 40%, 50% and 60% sucrose solution prepared with 0.1% SDS), were gently overlaid in a 40 ml centrifuge tube. The supernatant containing the virus particles and polyhedra was overlaid on the 40-60% (w/w) linear sucrose gradient and was centrifuged at 8000 × *g* for 90 min using a Beckman Coulter SW28 swing-out rotor. The virus containing band was collected and the virus particles were centrifuged at 72000 × *g* for 30 min to form a pellet. The pellet was then re-suspended in sterile distilled water and layered on a 20-60% (w/w) linear sucrose gradient (6 ml each of a 20%, 30%, 40%, 50% and 60% sucrose solution prepared with 0.1% SDS) and centrifuged at 96000 × *g* for 90 min at 4°C in a Beckman Coulter SW28 swing-out rotor. The bands containing virus particles (white hue) were collected, washed twice with sterile distilled water before being concentrated at 55000 × *g* for 60 min at 4°C using a Beckman Coulter SW28 rotor. The resulting pellet was washed twice in sterile distilled water to remove any traces of sucrose and SDS before being resuspended overnight in sterile distilled water or TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) and stored at 4°C.

2.1.2 Baculovirus propagation in *T. ni* larvae

Formalin sterilised *T. ni* eggs (Entopath, USA) were allowed to hatch and feed on a modified artificial diet originally described by McMorran (1965). This diet has been extensively modified by Adkisson et al. (1960), Vanderzant et al. (1962), Berger (1963) and Grisdale (1973) and recently reviewed by Hervet et al. (2016) . The feed contained 1.8% agar, 3.5% casein, 3.5% sucrose, 3% wheat germ, 1% Wesson's salt, 20 mM KOH, 0.5% linseed oil, 0.4% ascorbic acid, 0.1% choline chloride and 0.05% of a 37% formalin solution. The sterilised feed was supplemented with a 1% Vanderzant vitamin solution (Sigma-Aldrich, USA) before being allowed to set in covered glass containers and stored at 4°C. Third instar *T. ni* larvae were isolated in 6 well culture plates and were infected per os with a 0.5 cm^3 plug of feed that was

saturated with 50 µl of the purified polyhedral suspension and monitored at 27°C. Diseased larvae were collected and stored in 0.1% SDS at 4°C until all the larvae had either pupated or died. Three insect bioassays each consisting of 30 larvae were conducted in parallel over a one-week period. Data was analysed with Statsdirect software version 3 (StatsDirect Ltd, UK) using the probit analysis function to determine the LT_{50} values with 95% confidence intervals. Dead larvae showing no sign of baculoviral infection was excluded from the analysis (Paramasivam and Selvi, 2017).

2.1.3 Extraction of haemolymph from *T. ni* larvae

Two days after infection with the purified virus, the larvae were inactivated by immersion in ice water. The larvae were removed from the ice water, dried on a paper towel and the larval body bowed with the legs on the outside. One of the forelegs was severed by incision with an ethanol dipped and flame sterilised scissors and the green haemolymph was collected in a microcentrifuge tube and supplemented with 5 mM phenylthiocarbamide (Sigma-Aldrich, USA) to inhibit prophenoloxidase activity. The haemolymph was sterilised through a 0.45 µm syringe filter and stored at -80°C until required.

2.1.4 Viral infection of High Five cells

The filtered haemolymph was used to infect High Five (BTI-TN-5B1-4) cells (Invitrogen, USA) maintained in Express 5 medium (Thermo Fisher Scientific, USA) supplemented with 18 mM L-glutamine. The initial round of infection was conducted in 35 mm sterile petri dishes at a density of 1×10^6 cells/ml using 0.5 ml of the purified haemolymph before being supplemented with 1.5 ml of complete Express 5 medium (Thermo Fisher Scientific, USA) containing the L-glutamine supplement. The infection was allowed to proceed for two days and the supernatant containing the budded virus (BV) was collected by low speed centrifugation at 1000 × *g* for 5 min as described by O'Reilly et al. (1994). The BV was used to initiate a second round of infection conducted in 25 cm^2 flasks that was allowed to proceed for four days prior to viral DNA extraction.

2.1.5 Viral DNA extraction from budded virus

Viral DNA was extracted according to O'Reilly et al. (1994). Briefly, the BV was pooled and centrifuged at 72000 × g using a 25% sucrose cushion. The pellet was carefully resuspended in viral disruption buffer (10 mM Tris-HCl, pH 7.6; 10 mM EDTA; 0.25% SDS) supplemented with 500 µg/ml Proteinase K (Sigma-Aldrich, USA) and incubated at 37°C until clear or was left to incubate overnight. The DNA was purified by the addition of an equal volume of phenol: chloroform: isoamyl alcohol (PCI) in the ratio 25:24:1 (v/v) and was subjected to high speed centrifugation at 16000 × *g* for 15 min at 4°C. The upper aqueous phase was carefully transferred to a new microcentrifuge tube and was supplemented with 1/10 volume of sodium

acetate (3 M, pH 5.2) and two volumes of absolute ethanol and was incubated at -20°C for a minimum of 30 min or left overnight at -20°C to precipitate the genomic DNA. The solution was centrifuged at 14000 × *g* for 30 min at room temperature to pellet the genomic DNA before being washed twice with 1 ml of 70% ethanol. The pellet was allowed to dry at room temperature for a maximum of 15 min to allow residual ethanol to evaporate and was carefully resuspended in 100 µl of TE buffer and stored at 4°C.

2.1.6 Viral DNA extraction from insect larvae and haemolymph

Viral DNA was also extracted using the Nucleospin Tissue kit (Machery-Nagel, Germany) according to the manufacturer's instructions from insect larvae and haemolymph. Larvae were homogenised in a mortar and pestle, filtered through cheese cloth and subjected to low speed centrifugation at 5000 × *g* for 10 min to pellet cellular debris. Individual larvae were homogenised directly in a microcentrifuge tube using a micropestle before being subjected to a low speed spin at 5000 × *g* to pellet cellular debris. The supernatant was removed and centrifuged at 16000 × *g* for 30 sec and the pellet resuspended in 180 µl Buffer T1 and 25 µl Proteinase K (Sigma-Aldrich, USA) and vortexed to pre-lyse the samples. Haemolymph samples were centrifuged at 16000 × *g* for 30 sec and the pellet was carefully resuspended in 180 µl Buffer T1 and 25 µl Proteinase K (Sigma-Aldrich, USA). The samples were pre-lysed at 56°C for a minimum of 3 hrs or left overnight at 56°C until complete lysis was achieved before the addition of 200 µl Buffer B3 and incubation at 70°C for 10 min. After addition of 210 µl absolute ethanol, the sample was loaded onto the supplied minicolumn and was centrifuged at 11000 × *g* for 1 min and the column was placed in a new collection tube. The membrane was washed twice with 500 µl Buffer BW and 600 µl Buffer B5, respectively and was centrifuged for 11000 × *g* for 1 min after each addition followed by a dry spin at 11000 × *g* for 1 min to remove residual ethanol. Viral DNA was eluted into a clean sterile microcentrifuge tube with the addition of 100 µl Buffer BE, incubation at room temperature for 1 min, followed by centrifugation at 11000 × *g* for 1 min and was stored at 4°C.

2.1.7 Transmission Electron Microscopy

For Transmission Electron Microscopy (TEM), the virus suspension was centrifuged at 16000 × g for 5 min and the pellet fixed in 2.5% electron microscopy grade glutaraldehyde (Sigma-Aldrich, USA) in 0.1 M phosphate buffer (PB) (pH 7.4). Fixation was allowed to proceed for 2 hrs before post fixation with 1% aqueous osmium tetroxide (Sigma-Aldrich, USA) in 0.1 M phosphate buffer for a further 60 min. The sample was subjected to five washes in distilled water before dehydration in ethanol. The dehydration schedule included exposure to 50% ethanol for 15 min; 70% ethanol for 15 min; 95% ethanol for 15 min and 100% ethanol for 2 × 15 min. The sample was embedded in a 2:1 mix of propylene oxide:resin and was allowed to polymerize overnight at 60°C. Ultra-thin 50-70 nm sections, prepared using a diamond knife,

were sectioned onto grids before staining. The staining schedule included 5% uranyl acetate for 15 min, a rinse with distilled water followed by staining with 1% lead citrate for 5 min before a second rinse with distilled water. The samples were viewed on a FEI Tecnai T20 TEM at the University of Cape Town's Centre for Imaging and Analysis (Cape Town, South Africa).

2.2 Phylogenetic characterisation of TnSNPV

2.2.1 Cloning of the ecdysteroid UDP–glucosyltransferase (*egt*) gene

2.2.1.1 Identification of the *egt* gene

The TnSNPV-SA fragment containing the *egt* gene was initially identified by Southern hybridisation to a *Helicoverpa armigera* single nucleopolyhedrosis (HaSNPV) *egt* probe as outlined in Khan et al. (2003). Briefly, the HaSNPV *egt* gene was labelled with digoxygenin using the DIG-Nick Translation Mix (Roche, Germany) and was allowed to hybridise with TnSNPV DNA digested with the restriction enzyme *Hin*dIII. The viral DNA was allowed to separate overnight on a 0.8% agarose gel at 40 V before transfer to Hybond N+ nylon membrane (Amersham, GE Healthcare, USA). The labelled probe was allowed to hybridise overnight and the blot was subjected to low stringency washes with 2X saline and sodium citrate (SSC) buffer (0.3 M NaCl, 0.03 M trisodium citrate) supplemented with 0.1% SDS at room temperature before being visualised using NBT/BCIP chromogenic reagent (Roche, Germany) following the manufacturer's instructions.

2.2.1.2 PCR amplification of the *egt* gene from TnSNPV

To amplify the complete ORF of the *egt* gene from the 7 kb *Hin*dIII fragment identified in section 2.2.1.1, the primer pair EGT-REV-F and EGT-REV-R (**Table 2.1**) was utilised in a polymerase chain reaction (PCR) conducted in a final volume of 100 µl containing 10 µl of 10X *ExTaq* DNA polymerase buffer (Takara Bio, Japan), 100 ng of plasmid DNA, 1 µM of each of the primers, 2.5 mM of each of the supplied dNTPs (Takara Bio, Japan) and 2.5 U of TaKaRa *ExTaq*™ DNA polymerase (Takara Bio, Japan). The PCR was conducted in a Biorad T100 thermal cycler using the cycling parameters detailed in **Table 2.1**. The resulting 2.1 kb amplicon was visualised on a 1% agarose gel prepared with 40 mM Tris, 20 mM acetic acid and 1 mM EDTA (TAE) buffer containing 0.4 mg/ml ethidium bromide (Promega, USA).

Table 2.1. PCR primers and cycling parameters for the amplification of the *egt* gene.

Primer sequence (5′-3′)	Primer name	Target gene	Product size	PCR cycling conditions
AGTATTCTGTCGCCGTCGC	EGT-REV-F	*egt*	2083 bp	98°C for 10 sec 48°C for 30 sec 72°C for 2 min 30 sec (X30)
TTGCAATATCGCGATGGC	EGT-REV-R			

2.2.1.3 Ligation of the *egt* gene into the vector pGEM-T Easy

The PCR amplicons were purified using the Wizard SV Gel and PCR Clean-up system (Promega, USA) according to the manufacturer's instructions. DNA concentration and purity was assessed using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) prior to ligation into the vector pGEM-T Easy (Promega, USA) to form pGEM-T-EGT.

The ligation reaction was prepared with a 3:1 molar ratio of insert (100 ng) to vector (50 ng) as recommended by the manufacturer (**Table 2.2**) and was allowed to ligate overnight at 4°C before being transformed into JM109 *Escherichia coli* (*E. coli*) competent cells (Promega, USA). Positive and negative control reactions that contained and lacked control insert DNA, respectively, were also included (**Table 2.2**).

Table 2.2 Ligation reaction for cloning of the *egt* gene into pGEM-T Easy.

Component	Experimental Reaction (µl)	Positive control (µl)	Negative control (µl)
2X Rapid Ligation Buffer, T4 DNA Ligase	5	5	5
pGEM-T Easy Vector (50 ng)	1	1	1
PCR product (72 ng/µl)	1.4	-	-
Control Insert DNA	-	2	-
T4 DNA Ligase (3 Weiss units/µl)	1	1	1
Nuclease-free water	1.6	1	3
TOTAL VOLUME	10	10	10

2.2.1.4 Screening for *egt* recombinants

Successful transformants were confirmed with restriction enzyme analysis with *Eco*RI (Thermo Fisher Scientific, USA). For the restriction digests, 200 ng of the plasmid DNA was digested in a total volume of 30 µl containing 3 µl of 10X *Eco*RI buffer (Thermo Fisher Scientific, USA) and 5 U of the enzyme and the digest was allowed to proceed for 90 min in a 37°C water bath. The digest was visualised on a 1% agarose gel prepared with 1X TAE buffer containing 0.4 mg/ml of ethidium bromide. Successful recombinant clones were sequenced at the Stellenbosch University Central Analytical Sequencing Facility and/or Inqaba Biotec (Pretoria, South Africa) and sequence data was compiled and analysed using the CAP3 Sequence Assembly Program (Huang and Madan, 1999) and DNAMAN v4.13 (Lynnon BioSoft). Completed sequence data was submitted to Genbank and accession numbers were assigned (**Table 2.3**).

Table 2.3 *Baculoviridae* - 48 completely sequenced genomes with Genbank accession numbers used for the phylogenetic analyses. Accession numbers of additional sequences are also indicated (National Center for Biotechnology Information, 2018).

	NAME	ABBREVIATION	ACCESSION NUMBER	NAME	ABBREVIATION	ACCESSION NUMBER
ALPHABACULOVIRUSES	*Adoxophyes honmai* NPV	AdhoNPV	NC_004690	*Helicoverpa armigera* SNPV G4	HaSNPV-G4	NC_002654
	Adoxophyes orana NPV	AdorNPV	NC_011423	*Helicoverpa zea* SNPV	HzSNPV	NC_003349
	Agrotis ipsilon MNPV	AgipMNPV	NC_011345	*Hyphantria cunea* NPV	HcNPV	NC_007767
	Agrotis segetum MNPV	AgseNPV	NC_007921	*Leucania separata* NPV	LsNPV	NC_008348
	Antheraea pernyi MNPV	AnpeMNPV	NC_008035	*Lymantria dispar* MNPV	LdMNPV	NC_001973
	Anticarsia gemmatalis NPV	AgNPV	NC_008520	*Mamestra configurata* NPV	MacoNPV A	NC_003529
	Autographa californica MNPV	AcMNPV	NC_ 001623	*Mamestra configurata* NPV	MacoNPV B	NC_004117
	Bombyx mori NPV	BmNPV	NC_001962	*Maruca vitrata* MNPV	MaviMNPV	NC_008725
	Choristneura fumiferana MNPV	CfMNPV	NC_004778	*Orgyia leucostigma* NPV	OlNPV	NC_010276
	Choristoneura fumiferana DEFMNPV	CfDEFMNPV	NC_005137	*Orgyia pseudosugata* MNPV	OpMNPV	NC_001875
	Chrysodeixis chalcites NPV	ChchNPV	NC_007151	*Plutella xylostella* MNPV CL3	PlxyMNPV-CL3	NC_008349
	Clanis bilineata NPV	ClbiNPV	NC_008293	*Rachiplusia ou* MNPV	RoMNPV	NC_004323
	Ectropis obliqua NPV	EcobNPV	NC_008586	*Spodoptera exigua* MNPV	SeMNPV	NC_002169
	Epiphyas postvittana NPV	EppoNPV	NC_003083	*Spodoptera frugiperda* MNPV	SfMNPV	NC_009011
	Helicoverpa armigera SNPV NNg1	HaSNPV-NNg1	NC_011354	*Spodoptera litura* NPV II	SpltNPV II	NC_011616
	Helicoverpa armigera NPV C1	HaSNPV C1	NC_003094	*Spodoptera litura* MNPV	SpltMNPV	NC_003102
	Helicoverpa armigera MNPV	HaMNPV	NC_011615	*Trichoplusia ni* SNPV	TnSNPV	NC_007383
BETA BACULOVIRUSES	*Adoxophyes orana* GV	AdorGV	NC_005038	*Helicoverpa armigera* GV	HaGV	NC_010240
	Agrotis segetum GV	AgseGV	NC_005839	*Plutella xylostella* GV	PlxyGV	NC_002593
	Choristoneura occidentalis GV	ChocGV	NC_008168	*Spodoptera litura* GV	SpltGV	NC_009503
	Cryptophlebia leuoctreta GV	CrleGV	NC_005068	*Phthorimaea operculella* GV	PhopGV	NC_004062
	Cydia pomonella GV	CpGV	NC_002816	*Xestia c-nigrum* GV	XecnGV	NC_002331
DELTA	*Neodiprion lecontii* NPV	NeleNPV	NC_005906	*Neodiprion abietis* NPV	NeabNPV	NC_008252
	Neodiprion sertifer NPV	NeseNPV	NC_005905			
GAMMA	*Culex nigripalpus* NPV	CuniNPV	NC_003084			
Additional sequences	*Trichoplusia ni* NPV-SA *iap3*	TnSNPV-SA	EU708967	*Trichoplusia ni* NPV-SA pol	TnSNPV-SA	JX503102
	Trichoplusia ni NPV-SA *egt*	TnSNPV-SA	JX576797.1			

2.2.2 Cloning of the polyhedrin (pol) gene

2.2.2.1 PCR amplification of the polyhedrin gene

To identify the polyhedrin gene, the primer pair TNF and TNR (**Table 2.4**), based on 10 conserved polyhedrin gene sequences, which were aligned using CLUSTAL-W (Thompson et al., 1994), was used in a PCR to amplify a 1.4 kb fragment from TnSNPV genomic DNA. The PCR was conducted in a 100 µl final volume containing 10 µl of 10X *ExTaq* buffer (Takara Bio., Japan), 1 µM of each primer, 2.5 mM each of the supplied dNTPs and 2.5 U of *ExTaq* DNA polymerase (Takara Bio., Japan). The PCR was conducted in a Biorad T100 thermal cycler utilising the cycling parameters outlined in **Table 2.4**. The PCR amplicon was visualised on a 1% agarose gel prepared with 1X TAE containing 0.4 mg/ml of ethidium bromide (Promega, USA) before being purified using the Wizard SV Gel and PCR Clean-up system (Promega, USA). The purified PCR amplicon was visualised on 1% agarose gel prepared with 1X TAE containing 0.4 mg/ml of ethidium bromide (Promega, USA) and the DNA concentration was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) prior to ligation into the vector pGEM-T Easy (Promega, USA) to form pGEM-T-POL.

Table 2.4 PCR primers and cycling parameters for the amplification of the polyhedrin gene from TnSNPV.

Primer name	Primer sequence (5′-3′)	Target gene	Product size	PCR cycling conditions
TNF	CGTCCTCGGTTTTGGCATG	polyhedrin	1400 bp	30 cycles consisting of: 95°C for 30 sec
TNR	GAATAGCGGAAACGAAAACG			46°C for 30 sec 72°C for 90 sec

2.2.2.2 Ligation of the polyhedrin gene into the vector pGEM-T Easy

A total of 50 ng of the pGEM-T Easy vector (Promega, USA) and ~70 ng of the purified DNA was combined with 2X Rapid ligation buffer and 1 µl of T4 DNA ligase supplied with the kit (**Table 2.5**) and were allowed to ligate overnight at 4°C before being transformed into JM109 *E. coli* competent cells (Promega, USA).

Table 2.5 Ligation protocol for cloning of the polyhedrin gene into pGEM-T Easy.

Component	Experimental Reaction	Positive control	Negative control
2X Rapid Ligation Buffer, T4 DNA Ligase	5	5	5
pGEM-T Easy Vector (50ng)	1	1	1

PCR product	3		-
Control Insert DNA	-	2	-
T4 DNA Ligase (3 Weiss units/µl)	1	1	1
Sterile distilled water	0	1	3
TOTAL VOLUME	10	10	10

2.2.2.3 Screening for polyhedrin recombinants

Recombinant clones containing the insert were confirmed with restriction enzyme digestion with *Eco*RI (Thermo Fisher Scientific, USA) and/or PCR analysis using the primer pair TNF and TNR (**Table 2.4**) before being sequenced at the University of Stellenbosch's Central Analytical Sequencing Facility and/or Inqaba Biotec (Pretoria, South Africa). The restriction digests were conducted in a final volume of 30 µl containing ~500 ng of plasmid DNA, 3 µl of 10X *Eco*RI buffer (Thermo Fisher Scientific, USA) and 5 U of the enzyme. The digests were allowed to incubate at 37°C for 90 min before visualisation on a 1% TAE agarose gel containing 0.4 mg/ml ethidium bromide. PCR analysis was conducted in a total of 30 µl containing 6 µl of the 5X Green GoTaq® buffer (Promega, USA), 0.2 mM dNTP mix (Thermo Fisher Scientific, USA), 100 ng of plasmid DNA and 1.25 U of GoTaq® DNA Polymerase (Promega, USA) and was conducted in a Biorad T100 thermocycler before being visualised on a 1% TAE agarose gel containing 0.4 mg/ml ethidium bromide (Promega, USA).

2.2.3 Cloning of the *iap2* and *iap3* genes

2.2.3.1 PCR amplification of the *iap2* and *iap3* genes

The primer pair EK-IAP2-FOR and EK-IAP2-STOP (**Table 2.6**) was used to amplify the complete *iap2* gene from a *Hin*dIII fragment of the TnSNPV clone library before the 900 bp amplicon was visualised on a 1% agarose gel prepared with 1X TAE containing 1X Pronasafe Nucleic Acid Stain (Conda Laboratories, Spain). For the amplification of the *iap3* gene, the primer pair EK-IAP3-FOR and EK-IAP3-STOP (**Table 2.6**) was used to amplify the complete ORF from a 7 kb *Pst*I fragment of the TnSNPV clone library initially identified by random sequencing of TnSNPV library clones and the 828 bp amplicon was visualised on a 1% agarose gel prepared in the same manner described in section 2.2.2.3. Both PCR reactions were conducted in a final volume of 50 µl containing 100 ng of template genomic DNA, 25 µl of 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, South Africa) and 0.3 µM of each primer and were conducted in a Biorad T100 thermal cycler utilising the cycling parameters indicated in **Table 2.6**. Amplicons were purified using the Wizard SV Gel and PCR Clean-up system (Promega, USA) according to the manufacturer's instructions and DNA concentration of the purified amplicons was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Table 2.6 PCR primers and cycling conditions for the amplification of the *iap2* and *iap3* genes.

Primer name	Primer sequence (5′-3′)	Target gene	Product size	PCR cycling conditions
EK-IAP2-FOR	GACGACGACAAGATGGATTATTATTCGAATATCATG	*iap2*	900 bp	30 cycles consisting of: 98°C for 20 sec 50°C for 15 sec 72°C for 60 sec
EK-IAP2-STOP	GAGGAGAAGCCCGGTTACTGTAAATAAACTTC			
EK-IAP3-FOR	GACGACGACAAGATGGAAAGTTACGAGCATTCC	*iap3*	828 bp	30 cycles consisting of: 98°C for 20 sec 50°C for 15 sec 72°C for 60 sec
EK-IAP3-STOP	GAGGAGAAGCCCGGTTAACCAAAATAAATCTTG			

*The 5′ LIC extension required for ligation-independent cloning is underlined.

2.2.3.2 Treatment of the *iap* inserts and annealing into the vector pIEX1 Ek/LIC

To generate compatible overhangs for ligation into the pIEX1 Ek/LIC vector, the purified fragments were diluted in TlowE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) prior to treatment with T4 DNA Polymerase included in the Ek/LIC Cloning Kit (Novagen, USA). Once all the components of the reaction were assembled (**Table 2.7**), the reaction was initiated by the addition of the T4 DNA polymerase and allowed to incubate for 30 min at 22°C before the enzyme was heat inactivated at 75°C for 20 min.

Table 2.7 T4 DNA Polymerase treatment of the *iap* inserts.

Component	*iap2* (µl)	*iap3* (µl)	Positive control (µl)	Negative control (µl)
0.2 pmol purified PCR product	4	4	-	-
Ek/LIC β-gal Control Insert	-	-	4	-
10X T4 DNA Polymerase Buffer	2	2	2	2
25 mM dATP	2	2	2	2
100 mM DTT	1	1	1	1
Nuclease-free water	10.6	10.6	10.6	14.6
2.5 U/ml T4 DNA Polymerase	0.4	0.4	0.4	0.4
TOTAL VOLUME	20	20	20	20

The pIEX1 Ek/LIC vector and the treated insert were allowed to anneal (**Table 2.8**) at 22°C for 5 min before addition of 25 mM EDTA and further incubation at 22°C for 5 min.

Table 2.8 Annealing the pIEX1 Ek/LIC vector and Ek/LIC inserts.

Component	Experimental sample (µl)	Positive control (µl)	Negative control (µl)
pIEX1 Ek/LIC Vector	1	1	1
T4 DNA Polymerase treated Ek/LIC insert (0.02 pmol)	2	2	2
Incubate at 22°C for 5 min			
25 mM EDTA	1	1	1
TOTAL VOLUME	4	4	4
Incubate at 22°C for 5 min			

2.2.3.3 Transformation of the annealing reaction

Vials of the NovaBlue GigaSingles competent cells (Novagen, USA) were allowed to thaw on ice before addition of 1 µl of each annealing reaction followed by incubation on ice for 5 min. The reaction mixture was heat shocked at 42°C for 30 sec, incubated on ice for 2 min before recovery with 250 µl room temperature Super Optimal broth with Catabolite repression (S.O.C) medium (Sigma-Aldrich, USA). The transformation mixture was incubated at 37°C with shaking (200 rpm) before 50 and 100 µl aliquots were plated on pre-warmed Luria-Bertani (LB) agar plates containing 50 µg/ml ampicillin, which were incubated overnight at 37°C.

Several colonies from each set of transformants were inoculated into 5 ml of fresh LB broth containing 50 µg/ml ampicillin (Sigma-Aldrich, USA) and were incubated overnight at 37°C before being subjected to plasmid DNA extraction utilising the Pureyield Plasmid Miniprep System (Promega, USA). Screening for the insert was accomplished by PCR using vector specific primers (**Table 2.9**). The PCR reactions were conducted in a final volume of 30 µl and contained 50 ng plasmid DNA, 15 µl of Ampliqon *Taq* 2X Master Mix (Ampliqon, Denmark) and 0.2 µM of each primer. Following confirmation of the correctly sized insert, the purified plasmid DNA was submitted for sequencing at the University of Stellenbosch's Central Analytical Sequencing Facility (Stellenbosch, South Africa).

Table 2.9 PCR primers and cycling parameters using vector specific primers to screen for *iap2* and *iap3* recombinants.

Primer name	Primer sequence (5'-3')	Target gene	Product size	PCR cycling conditions
IE1-PROM	TGGATATTGTTTCAGTTGCAAG	*iap2*	1400 bp	35 cycles consisting of:
				95°C for 30 sec
IE1-TERMINATOR	CAACAACGGCCCCTCGATA			50°C for 30 sec
				72°C for 90 sec

IE1-PROM	TGGATATTGTTTCAGTTGCAAG	*iap3*	1325 bp	35 cycles consisting of: 95°C for 30 sec
IE1-TERMINATOR	CAACAACGGCCCCTCGATA			50°C for 30 sec 72°C for 90 sec

2.2.4 Phylogenetic analysis

Alignment of all sequences at the amino acid level was performed using CLUSTAL-X (Thompson et al., 1994), with default parameters and the BLOSUM protein matrix. Sequences were analysed for protein domains using Prosite (Hulo et al., 2007). The deduced amino acid sequences of polyhedrin, *lef*-8, and *lef*-9 genes of 48 completely sequenced baculoviruses (**Table 2.3**) were accessed from the NCBI online database (National Center for Biotechnology Information, 2018), aligned and concatenated to a single data set using BioEdit (Hall, 1999). The South African isolate of TnSNPV was not included in this data set due to lack of complete sequence data. The amino acid sequences of polyhedrin, IAP2, IAP3 and EGT proteins were aligned as described above. The polyhedrin gene of a South African isolate of TnSNPV (accession number AF093405) was also included in the analysis. Phylogenetic trees were reconstructed from deduced amino acid sequence by the Neighbour joining method with 1000 bootstrap replicates using MEGA 4.1 (Kumar et al., 2004) and viewed with Treeview (Page, 1996). Postulated gaps in the sequence alignments was considered as missing data and were excluded.

2.3 Heterologous Expression in *E. coli*

2.3.1 PCR amplification of the *iap2*, *iap3*, *egt* and polyhedrin genes

For prokaryote expression in *E. coli*, the *iap2*, *iap3, egt* and polyhedrin genes were amplified using primers (**Table 2.10**) containing a 5′-CACC overhang required for directional cloning into the Gateway entry vector pENTR/SD/D-TOPO (Thermo Fisher Scientific, USA). Blunt-ended PCR products were generated using a proofreading *Taq* polymerase in a 50 µl reaction containing 25 µl of 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, South Africa), 10 ng of DNA and 0.3 µM of each primer set. The PCRs were conducted in a Biorad T100 thermocycler utilising the cycling parameters indicated in **Table 2.10**. The amplicons were visualised on a 1% agarose gel prepared with 1X TAE containing 0.4 mg/ml of ethidium bromide (Promega, USA). The control PCR reaction was also conducted in a final volume of 50 µl containing 0.1 µg/µl of each control primer, 100 ng of control template DNA and 25 µl of 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, South Africa) and the 750 bp product was used to assess cloning efficiency of the TOPO cloning reaction. PCR cycling conditions for the control reaction are indicated in **Table 2.10**.

Table 2.10 PCR primers and cycling parameters for Gateway cloning into the vector pENTR/SD/D-TOPO.

Primer name	Primer sequence (5′-3′)	Target gene	Product size	PCR cycling conditions
*pENTR/SD/ TOPO/POL-F	**CACC**ATGTATACACGTTA CAGC	pol	738 bp	35 cycles consisting of: 98°C for 20 sec
pENTR/SD/ TOPO/POL-R[1]	TTAGTATGCTGGGCCTGA ATACAG[1]			50°C for 15 sec 72°C for 60 sec
*pENTR/SD/ TOPO/EGT-F	**CACC**ATGACGTCCATTAT AGTATT GTC	*egt*	1572 bp	35 cycles consisting of: 98°C for 20 sec
pENTR/SD/TOPO/ EGT-R[1]	AGCACCTTAAAATAAATTT ATTCTCAA CAATTGACGC[1]			50°C for 15 sec 72°C for 2 min
*pENTR/SD/ TOPO/IAP2-F	**CACC**ATGGATTATTATTCG AATATC	*iap2*	900 bp	35 cycles consisting of: 98°C for 20 sec
pENTR/SD/ TOPO/IAP2-R[1]	TTACTGTAAATAAACTTCT AATCGCTC[1]			50°C for 15 sec 72°C for 60 sec

*The 5′ CACC overhang required for directional cloning into the Gateway entry vector is in bold.
[1]Primers required for gene specific cDNA synthesis.

2.3.2 The TOPO cloning reaction

*pENTR/SD/ TOPO/IAP3-F pENTR/SD/ TOPO/IAP3-R[1]	**CACC**ATGGAAAGTTACGAGCATTC TCAACCAAAATAAATCTTGATCACATTAG[1]	*iap3*	828 bp	35 cycles consisting of: 98°C for 20 sec 50°C for 15 sec 72°C for 60 sec
Control reaction primers (Provided by manufacture)	Not specified by manufacturer	Not specified by manufacturer	750 bp	35 cycles consisting of: 98°C for 20 sec 55°C for 15 sec 72°C for 60 sec

For the TOPO cloning reaction, a 0.75:1 molar ratio of PCR product to entry vector (pENTR/SD/D-TOPO) was employed. The TOPO cloning reaction (**Table 2.11**) included 100 ng of purified PCR product and 1 µl each of the included salt solution and pENTR/SD/D-TOPO vector and was incubated at room temperature for 30 min. Where necessary, dilutions of the purified PCR product in sterile distilled water were conducted for the cloning reaction.

Table 2.11 The TOPO cloning reaction.

Component	**Volume (µl) Experimental samples**				**Volume (µl) "Vector Only" Control**	**Volume (µl) Vector + PCR Insert" Control**
	pol	***egt***	***iap2***	***iap3***		
PCR product	1	0.7	1	1	0	4
Salt Solution	1	1	1	1	1	1
Sterile Water	3	3.3	3	3	4	0
pENTR/SD/D-TOPO	1	1	1	1	1	1

For the transformation reaction, 2 µl of each transformation mixture was added to a vial of Mach1-T1 competent cells (Thermo Fisher Scientific, USA) and was incubated on ice for 30 min. The mixture was heat shocked at 42°C for 30 sec and immediately transferred to ice before recovery with 250 µl of room temperature S.O.C medium (Sigma-Aldrich, USA). The mixture was incubated with horizontal shaking at 200 rpm at 37°C for 60 min before 20 and 100 µl aliquots were plated onto pre-warmed LB agar plates containing 50 µg/ml of kanamycin (Sigma-Aldrich, USA) and were allowed to incubate overnight at 37°C. Several colonies from each set of plates were inoculated into 5 ml LB broth supplemented with 50 µg/ml of kanamycin (Sigma-Aldrich, USA). They were allowed to incubate at 37°C in a shaking incubator overnight, whereafter the broth was used directly in a colony PCR using gene specific primers (**Table 2.10**).

For the colony PCR, 5 µl of the overnight culture was included in the PCR. The reaction included an additional initial denaturation step at 95°C for 5 min to ensure lysis of the bacterial

cells after which a conventional PCR was conducted. The reactions were conducted in a total volume of 30 µl using 15 µl of *Taq* 2X Master Mix (Ampliqon, Denmark) and 0.2 µM of each primer and were visualised on a 1% agarose gel prepared with 1X TAE containing 1X Pronasafe Nucleic Acid Stain (Conda Laboratories, Spain). PCR primers and cycling conditions are indicated in **Table 2.10**.

2.3.3 TOPO LR recombination

For the recombination reaction (**Table 2.12**), 100 ng each of the entry recombinant vectors, pENTR/SD/D-TOPO-IAP2/IAP3/EGT/POL were combined with 150 ng of pDEST17 (Thermo Fisher Scientific, USA) and TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8) to make up a total of 8 µl. This was supplemented with 2 µl of the LR Clonase enzyme mix (Thermo Fisher Scientific, USA) and was incubated at 25°C for 60 min before addition of 1 µl of Proteinase K solution, whereafter an additional incubation step at 37°C for 10 min was conducted.

Table 2.12 Components of the LR recombination reaction.

Component	Sample				Control
	pol	***iap2***	***iap3***	***egt***	**Positive control**
Entry vector pENTR/SD/D-TOPO (100 ng/reaction)	1	1	1	1	-
Destination vector pDEST17 (150 ng/µl)	1	1	1	1	1
pENTR-gus (50 ng/µl)	-	-	-	-	1
TE Buffer, pH 8.0	6	6	6	6	5
LR Clonase II Enzyme Mix	2	2	2	2	2
TOTAL VOLUME	10	10	10	10	10

The recombination mixture was used to transform DH5α *E. coli* competent cells (Thermo Fisher Scientific, USA) which was plated on LB agar plates containing 100 µg/ml ampicillin. In brief, 1 µl of the recombination reaction was added to 50 µl of Library Efficiency DH5α competent *E. coli* (Thermo Fisher Scientific, USA) and was allowed to incubate on ice for 30 min before being heat shocked at 42°C for 30 sec. The cells were recovered by the addition of 450 µl of room temperature S.O.C medium and incubated at 37°C for 60 min with horizontal shaking before 20 and 100 µl aliquots were plated on pre-warmed LB plates containing 100 µg/ml ampicillin and were incubated overnight at 37°C.

Transformants were cultured in 10 ml of LB broth containing 100 µg/ml ampicillin and plasmid DNA was extracted using the Pureyield Plasmid Miniprep System (Promega, USA). Positive transformants were confirmed by PCR using gene specific primers (**Table 2.10**) as well as restriction enzyme analysis with *Not*I (Roche, Switzerland). Restriction analysis was conducted in a final volume of 30 µl using 500 ng of plasmid DNA, 5 U of enzyme, and 3 µl of

10X restriction buffer. To ensure complete digestion, an additional 5 U of *Not*I (Thermo Fisher Scientific, USA) was added after 60 min and the reaction was allowed to proceed for an additional 60 min in a 37°C water bath before being visualised on a 1% agarose gel prepared with 1X TAE containing 1X Pronasafe Nucleic Acid Stain (Conda Laboratories, Spain). The constructs were confirmed by sequencing at the University of Stellenbosch Central Analytical Sequencing Facility (Stellenbosch, South Africa).

The expression plasmids pDEST17-IAP2, pDEST17-IAP3, pDEST17-EGT and pDEST17-POL were used to transform BL21-A1 One Shot cells (Thermo Fisher Scientific, USA). In brief, approximately 100 ng of each construct was added to a vial of the competent cells which had been allowed to thaw on ice. This was incubated on ice for 30 min, heat-shocked at 42°C for 30 sec and returned to ice. The cells were recovered by the addition of 250 µl of room temperature S.O.C medium (Sigma-Aldrich, USA) and were incubated at 37°C for 30 min with shaking at 200 rpm before 20 and 100 µl aliquots were plated on pre-warmed LB agar plates containing 100 µg/ml ampicillin. The plates were incubated overnight at 37°C. Several transformants were selected from the agar plates and were inoculated into 5 ml fresh LB broth containing 100 µg/ml ampicillin (Sigma-Aldrich, USA) and were incubated overnight at 37°C with shaking. For long term storage, glycerol stocks containing 700 µl of culture and 300 µl of sterile molecular biology grade glycerol was prepared and stored at -80°C.

2.3.4 Pilot expression in *E. coli*

Glycerol stocks of BL21-A1 *E. coli* cells (Thermo Fisher Scientific, USA) containing the expression plasmids pDEST17-IAP2, pDEST17-IAP3, pDEST17-EGT and pDEST17-POL were streaked onto LB agar plates containing 100 µg/ml ampicillin and were incubated overnight at 37°C. Colonies were inoculated into 5 ml LB broth containing 100 µg/ml ampicillin (Sigma-Aldrich, USA) and were grown at 37°C with shaking until the OD_{600} reached 0.6-1.0 when measured using a PG Instruments Limited T60V spectrophotometer. An aliquot of this culture, representing a ~1/20 dilution, was diluted in fresh LB media containing 100 µg/ml ampicillin until an OD_{600} of 0.01-0.5 was reached and the culture was then incubated at 37°C with shaking until the OD_{600} reached ~0.4. The culture was divided into two aliquots with one of the fractions induced by supplementation with 0.2% arabinose (Thermo Fisher Scientific, USA) to induce expression from the T7 RNA polymerase before the incubation was continued. The second aliquot served as the non-induced sample reference.

2.3.5. RNA extraction

At various points (0, 1, 2, 3 and 4 hrs), 500 µl of the culture media was removed and the cells were pelleted by centrifugation at 11000 × *g* for 30 sec before being washed twice with ice cold sterile 1X PBS (Lonza, Switzerland). RNA extraction was conducted using the NucleoSpin RNA kit (Machery-Nagel, Germany) according to the manufacturer's recommendation. The cells were resuspended in 100 µl TE buffer containing 1 mg/ml lysozyme (Sigma-Aldrich, USA), vigorously vortexed and were allowed to incubate in a water bath at 37°C for 10 min to digest or weaken the bacterial cell wall. The cells were lysed by the addition of 350 µl Buffer RA1. If multiple samples were collected, they were stored at -70°C before further processing. Once all the samples were collected, they were allowed to thaw on ice before the addition of 3.5 µl ß-mercaptoethanol (Sigma-Aldrich, USA). The samples were mixed by vigorous vortexing and were applied to the supplied spin column and centrifuged at 11000 × *g* for 1 min to reduce the viscosity of the lysate. The lysate was supplemented with 350 µl of 70% ethanol, vortexed and applied to the column and centrifuged at 11000 × *g* for 30 sec. The filter was placed in a new collection tube before the addition of 350 µl of membrane desalting buffer and centrifuged at 11000 × *g* for 60 sec. To remove contaminating DNA from the mixture, 95 µl of the DNase reaction mixture containing 10 µl reconstituted rDNase and 90 µl reaction buffer, was directly applied to the column and allowed to incubate for 15 min at room temperature. The column was subjected to a series of three washes consisting of 200 µl Buffer RAW2 to inactivate the DNase, 600 µl Buffer RA3 and 250 µl Buffer RA3. The column was centrifuged at 11000 × *g* for 30 sec after addition of the first two washes before a final centrifugation step at 11000 × *g* for 2 min. The RNA was eluted in 50 µl nuclease free water and centrifuged at 11000 × *g* for 1 min. Nucleic acid concentration was determined using the Nanodrop 2000 (Thermo Fisher Scientific, USA) before 10 µl aliquots were stored at -80°C.

2.3.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The ImProm-II Reverse Transcription System (Promega, USA) was employed for first strand complementary DNA (cDNA) synthesis using gene specific primers (**Table 2.10**) according to the manufacturer's instructions. For each reaction, approximately 100 ng of total RNA was combined with 10 pmol of gene specific primer and sterile distilled nuclease free water. The 5 µl volume containing the primer and RNA template was denatured at 70°C, immediately cooled on ice and subjected to a short pulse spin to collect the condensate. The reverse transcription reaction (**Table 2.13**) was assembled on ice and the template/primer reaction was added to this mixture, and was allowed to anneal at 25°C for 5 min and then incubated at 42°C for 60 min. This was followed by incubation at 70°C for 15 min to inactivate the reverse transcriptase enzyme. A control reaction consisting of 1 µg of the 1.2 kb Kanamycin Positive Control RNA and oligo $(dT)_{15}$ primer, included with the kit, was also included. Two reactions which did not include a RNA sample and which lacked the RT enzyme were included as controls.

Table 2.13 Components included in the reverse transcription reaction.

Reagent	Final concentration	Volume (µl)
ImProm-II 5X Reaction Buffer	1X	4
$MgCl_2$ (25mM)	4 mM	3.2
dNTP Mix (10mM)	0.5 mM	1
Recombinant RNasin Ribonuclease Inhibitor	20 U	0.5
ImProm-II Reverse Transcriptase		1
Nuclease free water		5.3
TOTAL		15

Two microliters of the cDNA from the reverse transcription reaction was employed as the template in the PCR reactions. The PCR's were conducted in sterile ultrapure water in a 30 µl final volume containing 15 µl of *Taq* 2X Master Mix (Ampliqon, Denmark) and 0.2 µM of each primer. The PCRs were conducted in a Biorad T100 thermal cycler utilising the PCR primers and cycling parameters indicated in **Table 2.14**. The fragments were analysed on a 1% agarose gel prepared with 1X TAE buffer containing 0.4 mg/ml ethidium bromide and visualised on the Alliance 2.7 system (Uvitec, UK). In order to assess DNA contamination of the RNA sample, samples were subjected to PCR analysis using the same PCR primers and cycling conditions indicated in **Table 2.14**.

Table 2.14 PCR cycling conditions for Ampliqon 2X Master Mix.

PCR stage	Polyhedrin	*egt*	*iap3*	*iap2*	Cycles
Denaturation	95°C for 30 sec	95°C for 30 sec	95°C for 30 sec	95°C for 30 sec	35 cycles
Annealing	50°C for 30 sec	50°C for 30 sec	50°C for 30 sec	50°C for 30 sec	
Extension	72°C for 60 sec	72°C for 2 min	72°C for 60 sec	72°C for 60 sec	
Primer sets	pENTR/SD/ TOPO/POL-F & -POL-R*	pENTR/SD/ TOPO/EGT-F & -EGT-R*	pENTR/SD/ TOPO/IAP3-F & -IAP3-R*	pENTR/SD/ TOPO/IAP2-F & -IAP2-R*	

*For primer sequences, see **Table 2.10**.

2.3.7 Protein concentration determination

The protein concentration was determined using the 2-D Quant kit (GE Healthcare, USA) according to the manufacturer's instructions. Briefly, a working stock of the colour reagent was prepared by mixing 100 parts of colour reagent A with 1 part colour reagent B. A standard curve was prepared using the provided bovine serum albumin (BSA) standard solution

(2 mg/ml) over a range of 0–50 µg. An aliquot (5 µl) of each sample, including the standards, was supplemented with 500 µl each of precipitant and co-precipitant. The mixtures were briefly vortexed after each addition with the inclusion of a 2-3 min incubation period at room temperature after addition of the precipitant. The samples were subjected to centrifugation at 10000 × *g* for 5 min and the supernatant was carefully removed. Care was taken to ensure that the protein pellet was not disturbed. The protein pellet was re-suspended in 100 µl of copper solution and 400 µl of sterile distilled water and was thoroughly mixed to dissolve the protein pellet, before being supplemented with 1 ml of the working colour reagent and was immediately mixed. The samples and standards were incubated for 15-20 min at room temperature before the A_{480} was determined using water as the reference blank.

2.3.8 Sample preparation

Aliquots of the *E. coli* culture (500 µl) was pelleted by centrifugation at 16000 × *g* for 30 sec. Both the supernatant and cell pellet were stored at -20°C. Once all the samples were collected, the pellet was resuspended in 80 µl of 1X SDS-PAGE buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 6.8), boiled for 10 min and fractionated on a 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) prepared using the TGX FastCast Acrylamide kit (Biorad, USA) or 40% Acrylamide-Bis (37.5:1) stock solution (Promega, USA) according to the protocol outlined by Roland (2007).

2.3.9 SDS PAGE and immunoblot analysis

The solutions required for casting the 0.75 mm polyacrylamide gels was prepared according to the manufacturer's instructions (**Table 2.15**) and cast using the Mini Protean®3 system (Biorad, USA). To prepare the gels using the 40% Acrylamide-Bis stock solution, the components were assembled according to **Table 2.16** and the gels were cast and run using the Mini Protean®3 system (Biorad, USA) according to the manufacturer's instructions.

Table 2.15 Casting volumes for 12% TGX FastCast polyacrylamide gels.

Preparation of 0.75 mm Bio-Rad SDS PAGE gels (n = number of gels)		
Component	**Stacker**	**Resolver**
Resolver A	_	2 ml × n
Resolver B	_	2 ml × n
Stacker A	1 ml × n	_
Stacker B	1 ml × n	_
Total Volume	2 ml × n	4 ml × n
TEMED	2 µl × n	2 µl × n
10% APS	10 µl × n	20 l × n

Cell lysates (20 µg of total protein) were fractionated on the 12% PAGE gels at 100-150 V before being equilibrated in transfer buffer [5 mM Tris-HCl (pH 7.6), 192 mM glycine and 20% methanol] for 10-15 min to remove residual SDS. The polyvinylidene fluoride (PVDF) membrane (Roche, Switzerland) was activated according to the manufacturers recommendations and transfer was completed at 300 mA in transfer buffer with an ice pack and magnetic stirrer for 60 min using standard methods (Sambrook and Russell, 2001).

Table 2.16 Casting volumes for 12% SDS PAGE polyacrylamide gels using 40% Acrylamide-Bis (37.5:1) stock solution (Roland, 2007).

Preparation of 2 × 0.75 mm Bio-Rad Glass Plates		
Component	**6% Stacker**	**12% Resolver**
Distilled water	2.9 ml	3.4 ml
40% Acrylamide-Bis (37.5:1)	0.75 ml	2.4 ml
1.5 M Tris pH 8.8	-	2 ml
10% SDS	50 µl	80 µl
TEMED	5 µl	8 µl
10% APS	50 µl	80 µl
0.5 M Tris pH 6.8	1.25 ml	-
TOTAL VOLUME	5	8

Upon completion of the run, protein loading on the membrane was assessed using Ponceau staining (Sigma-Aldrich, USA) by immersion in the reagent for 5 min followed by washing in distilled water to visualise the protein bands. Once efficient transfer was confirmed, the membrane was rinsed in distilled water to remove the Ponceau reagent completely. The membrane was blocked in blocking buffer [1% bovine serum albumin (BSA) (Roche, Switzerland) dissolved in 1X Tris-buffered saline (TBS)] for one hour at room temperature prior to exposure to monoclonal anti His-6 antibody (Roche, Switzerland) diluted to a final concentration of 0.2 µg/ml in blocking buffer for one hour. The membrane was washed in TBST [Tris-buffered saline with 0.1% Tween-20 (Merck, USA)] for a total of five consecutive 5 min washes before immunostaining with horseradish peroxidase (HRP)-conjugated secondary antibody (R & D Systems, USA) diluted 1:5000 in blocking buffer for 60 min. The five washes were repeated, and the membrane was developed using Clarity Western ECL substrate (Biorad, USA) and visualised using the Alliance 2.7 Chemiluminescence system (UVITEC, UK) or the MyECL Imager (Thermo Fisher Scientific, USA).

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Chapter 3

Results & Discussion

Section 3.2 of this chapter has been published in:

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3.1 Phenotypic characterisation of TnSNPV

In order to purify the baculovirus occlusion bodies from the crude virus suspension, the virus preparation was subjected to isopycnic centrifugation in a linear sucrose media. The virus particles were harvested from a thick white band corresponding to the position of the ~50% sucrose gradient before being layered on a 20-60% gradient (**Fig. 3.1**). Pelleted insect debris could be observed at the bottom of the centrifuge tube after the initial sucrose gradient. The polyhedra were harvested at a position corresponding to the 54-56% sucrose layer and this step served to separate the insect debris, which has a similar sedimentation rate to the polyhedra (King and Possee, 1992).

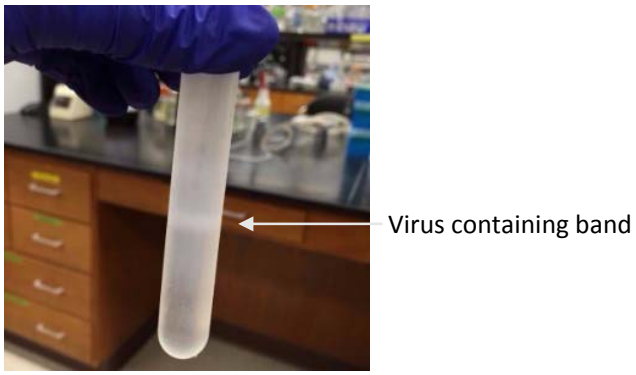

Figure 3.1 Linear sucrose gradients showing thick white virus containing band (Wilhelm, 2017).

The density gradient centrifugation employed in this analysis, remains the standard for the initial isolation of most viruses and has been successfully applied to isolate viruses from at least 39 virus families (Bondoc Jr, 2000). While a variety of differential centrifugation methods have been employed, isopycnic centrifugation in which viruses are separated solely on the basis of density, remains the most common. Virus particles are allowed to migrate until the density of the particle (ranging from 1.1-1.2 g/cm^3) is the same as that of the surrounding medium. Due to its low cost, ready availability, stability and non-reactivity to most biological materials, sucrose is the most popular gradient medium (Lawrence and Steward, 2010) though other media such as caesium chloride (CsCl) have also been employed (Bruce et al., 1991; Huhti et al., 2010). However, since sucrose has high osmotic activity, it may result in the loss of surface glycoproteins from enveloped viruses (Palker, 1990) and may thus not be suitable for sensitive viruses such as human respiratory syncytial virus (hRSV) (Gias et al., 2008) and human T-cell leukaemia virus type 1 (Palker, 1990). It is further estimated that sucrose gradient ultracentrifugation can only recover up to 50% of infectious particles (Van Noi et al., 2016) which limits its use for the isolation of particularly sensitive viruses. Alternative media such as glycerol, sorbitol and OptiPrep™ amongst others, could be considered in such cases and the chosen media should be optimised for the particular virus to be isolated, particularly if biologically active and highly purified virus particles are required (Harrap and Longworth, 1974;

Lawrence and Steward, 2010). Similarly, the gradient shape and type of rotor should be determined empirically to maximise yield, to preserve stability of the virus and be cost efficient (Hutornojs et al., 2012). Despite these limitations, as indicated sucrose was selected as the appropriate ultracentrifugation medium due to its availability and low cost. Furthermore, this technique has been successfully employed in many laboratories (Khan et al., 2004; Wang et al., 2004; Brito et al., 2018) and remains the gold standard for baculovirus isolation (O'Reilly et al., 1994; Rohrmann, 2013).

Viral DNA was extracted from various samples (BV, haemolymph and diseased larvae) using a method described by O'Reilly et al. (1994) and a silica column-based kit, the Nucleospin Tissue kit (Machery-Nagel, Germany). Both methods produced DNA of high quality at concentrations suitable for downstream application such as restriction enzyme analysis (results not shown) and PCR. DNA concentrations ranged from 453 ng/µl to 1.73 µg/µl and in general, the method described by O'Reilly et al. (1994) produced DNA of higher concentration. However, this method was more time and labour intensive than the column-based kit. The size of TnSNPV genome was also estimated at ~160 kb (Fielding et al., 2002) based on restriction endonuclease (REN) profiles which falls within the range for baculoviruses at 80 to 180 kb (Rohrmann, 2013). When compared to the Canadian isolate (Willis et al., 2005), the estimated size of the South African isolate was larger (~160 kb vs 134.4 kb). The size difference may reflect genetic variation as the two strains have adapted to varying environmental conditions as observed with strains of *Lymantria dispar* MNPV (Martemyanov et al., 2017) and *Spodoptera frugiperda* NPV (Wolff et al., 2008; Simón et al., 2011).

Occlusion bodies (OBs) of the TnSNPV sample visualised by transmission electron microscopy (TEM), displayed the elongated oval shape that is characteristic of the occlusion bodies in the *Alphabaculovirus* genus (**Fig. 3.2**). Each OB contained a rod-shaped single virion composed of a single enveloped nucleocapsid. Nucleocapsid size corresponded to 30-70 nm and 200-400 nm in in diameter and length respectively, expected for *Alphabaculovirus* OBs (Wang et al., 2016). Occlusion body morphology and other criteria had previously been used to classify baculoviruses into the *Nucleopolyhedrovirus* and *Granulovirus* genera (Jehle et al., 2006). The rapid accumulation DNA sequence results has however shown that the division of baculoviruses into MNPV and SNPV phenotypes did not correspond to the phylogeny of the viruses and resulted in the adoption of a new classification system based on baculovirus phylogeny rather than morphological characteristics (Rohrmann, 2013). It is also difficult to attribute characteristics to a virus, based on SNPV or MNPV phenotype, due to the large differences in genetic content of these viruses. This nomenclature system however persists largely due to its historical use and because it is still useful when distinguishing different viruses that are pathogenic to the same host (Rohrmann, 2013).

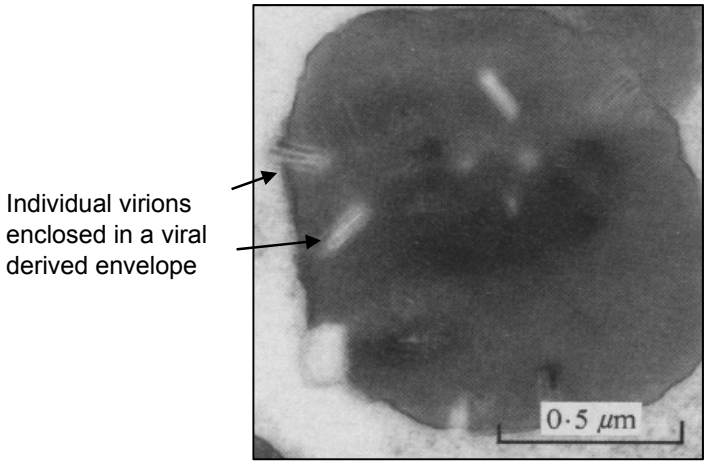

Figure 3.2 Occlusion of virus particles of TnSNPV.

For the insect bioassays, thirty *T. ni* larvae were individually isolated in six well culture plates and infected per os with the polyhedral suspension and monitored at 27°C. To exclude the possible effect of the host plant and the presence of parasites which may be present on the host plant as observed in a study by Cory and Myers (2004), artificial media was employed in the bioassay. Dead larvae were collected each day until all the larvae had either pupated or died. A total of three biological replicates over a period of three weeks were completed. Larvae typically stopped feeding within 24 hrs after introduction of the spiked feed plug and became lethargic within 6-12 hrs post infection. A colour change from the typical green of healthy 2nd instar larvae to brown (indicative of baculovirus infection) was observed 24 hrs after consumption due to melanisation of the larval cuticle (Harrison et al., 2012). In the late stages of infection, complete rupturing of the cuticle and liquefaction of the internal organs of the larvae was apparent.

Speed of kill for baculoviruses is a useful criterion to evaluate the potential of baculoviruses as biological pesticides. The median lethal time (LT_{50}) (with 95% confidence interval) (**Fig. 3.3**) required to kill 50% of the *T. ni* larvae ranged from 1.7 (Replicates one and two) to 1.9 days (Replicate three) with an average of 1.8 days for the three replicates (**Table 3.1**). Mortality of 2nd instar larvae averaged 78% after adjustment for fatalities not due to baculovirus infection. The LT_{50} of other baculoviruses ranged from 2.5 to 4.3 for *Galleria mellonella* NPV and 4.9 to 5.4 for *Plutella xylostella* GV (Kadir, 1990) and from 5.11 to 9.91 days for a variety of baculoviruses including *Helicoverpa armigera* NPV, *Spodoptera litura* NPV and *Amsacta albistriga* NPV (Kumar et al., 2011). Furthermore, the LT_{50} for wild type AcMNPV, the prototype baculovirus, varies from 4.2 days (Gilbert et al., 2005; Zhu et al., 2016) to 5.5 days (Tuan et al., 2005). As AcMNPV is able to infect a wide variety of insect hosts, the speed of kill is dependent on the insect that is targeted. Mortality generally increases with the concentration of the infective dose and is affected by larval development with older larvae less susceptible than younger larvae (Magholi et al., 2014). The LT_{50} of the South African isolate of

TnSNPV observed in this analysis confirms the potential of the virus as a biological pesticide since the average LT_{50} of 1.8 days is significantly lower than that recorded for other NPVs.

Table 3.1 Infection of *T. ni* larvae. Second instar larvae were infected per os with a 0.5 cm^3 plug of feed that was saturated with 50 µl of the purified polyhedral suspension and monitored at 27°C.

Dead larvae by Replicate number		Day 1	Day 2	Day 3	Day 4	*Mortality rate % (*n* = 30)	*LT_{50}
Number of Dead Larvae	Replicate 1	4	8	6	4	73	1.7
	Replicate 2	5	8	4	6	77	1.7
	Replicate 3	4	7	8	6	83	1.9
Average		4.3	7.7	6		78	1.8

*Mortality rate and LT_{50} of insects killed by baculovirus infection

In a study by Harrison et al. (2012), North American isolates of TnSNPV displayed varying kill rates with LT_{50} ranging from 3.3 to 3.84 days which suggests that geographically separated isolates have adapted to different environmental conditions. Similar differences were observed for the NPV of *Lymantria dispar* (LdMNPV), with a North American strain being more virulent than an Asian strain, while local North American strains of LdMNPV did not exhibit any differences in virulence (Martemyanov et al., 2017). The differences in the virulence of the two LdMNPV strains was attributed to differences in the viral genetics of the two strains suggesting that spatially separated baculoviruses have adapted to varying environmental conditions. The differing environmental conditions and adaptation to the host population of the two isolates of TnSNPV may also have driven genetic diversity in the two strains and sequence comparison and phylogenetic reconstruction is likely to reveal the extent of the genetic differences between the two strains.

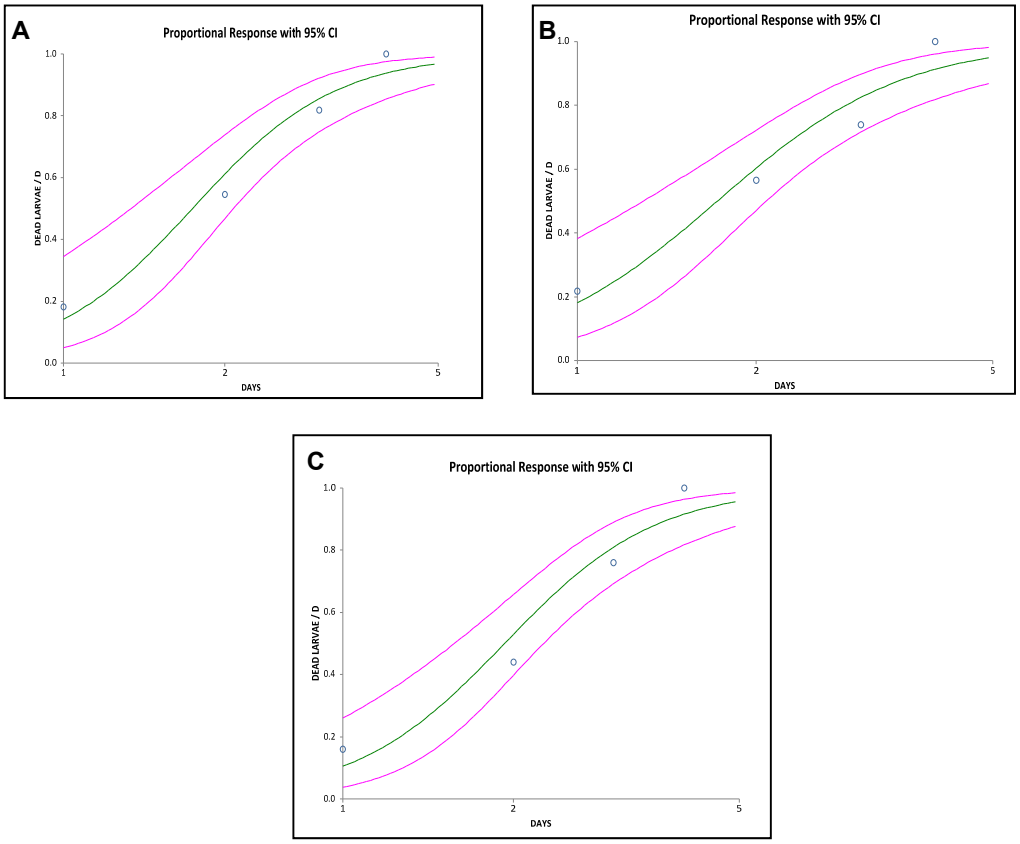

Figure 3.3 Statistical mortality analysis of *T. ni* larvae. Data was analysed using Statsdirect statistical software to determine the median lethal time (LT_{50}) required to kill 50% of the *T. ni* larvae. A, B and C represents three biological replicates one, two and three (indicated in green), respectively. Confidence intervals (95%) are indicated in pink.

3.2 Phylogenetic characterisation of TnSNPV

3.2.1 Identification, cloning and sequence analysis of the *egt* gene

First identified in the prototype virus AcMNPV, the *egt* gene encodes an ecdysteroid UDP-glucosyltransferase which catalyses the conjugation of ecdysone and UDP-glucose (O'Reilly and Miller, 1989; O'Reilly and Miller, 1991; O'Reilly, 1995). The EGT protein inactivates the steroid moulting hormone, ecdysone, with consequent delay in normal lepidopteran larval moulting, with no weight loss of the larvae prior to pupation and earlier mortality (O'Reilly and Miller, 1991). Disruption of the *egt* coding region increases the virulence of the baculovirus without reducing infectivity (O'Reilly and Miller, 1991). This modification has been shown to improve the pesticidal properties of the baculoviruses and is one of the main targets of genetic modification to improve the pesticidal properties of baculoviruses.

Analysis of the sequenced region revealed an ORF of 1572 nt with a predicted protein of 524 amino acids with a molecular mass of 60.03 kDa. The TnSNPV EGT protein shares sequence identity of 98% and 89% with EGT proteins of a Canadian isolate of TnSNPV and ChchNPV, respectively (**Table 3.2**). In common with other EGT proteins, the non-coding region of the gene has various control elements for early transcription (**Appendix 1**). Four putative TATA boxes were identified at 297, 94, 45 and 35 nt upstream of the ATG translational start codon. The consensus poly-adenylation sequence AATAAA is located 506 nt downstream of the termination codon. Transcription initiator (INR) motifs are found at 67 nt (CATA), and 117 nt (CAGT) upstream of the translational start codon. Two additional early transcription initiator motifs are found at 67 (CATA), and 117 (CAGT) nt upstream of the start codon. For TnSNPV-Can, three putative TATA boxes at 40, 50 and 391 nt upstream were identified. In addition, INR motifs (CATA) were located at 71, 101 and 173 nt upstream while the CAGT motif was located 91, 109 and 184 nt upstream of the start codon. The TATA box is one of the core elements in baculovirus early promoters and has been found in all baculovirus *egt* genes sequenced (Chen et al., 1997). No other transcription motifs (such as the late transcription motif, TAAG) were identified in both isolates confirming that *egt* is an early gene transcribed by the host RNA polymerase II using one of the TATA boxes identified. The consensus poly-adenylation sequence AATAAA was identified at 1576 nt and 506 nt downstream of the termination codon for TnSNPV-SA and TnSNPV-Can, respectively. A hydrophobic N-terminus indicative of a putative signal peptide sequence consisting of 23 amino acid residues was identified in both isolates which may indicate that EGT is secreted from the infected cell (Chen et al., 1997).

The phylogenetic tree inferred from EGT proteins (**Fig. 3.4**) was less well resolved when compared to the tree inferred from baculovirus core gene polyhedrin (**Fig. 3.5**). Three groups corresponding to Group I and II NPVs of the *Alphabaculoviruses*, *Betabaculoviruses* and LdMNPV may be distinguished for the tree inferred from EGT proteins. However, the topologies are not well resolved and the clear separation of the *Alphabaculoviruses* and

Betabaculoviruses is not observed. The *Betabaculoviruses* represent a separate clade but shares ancestry with certain members of the Group II *Alphabaculoviruses* and differs from the accepted natural evolution of baculoviruses observed in the trees inferred from polyhedrin, as well as the trees inferred from complete polyhedrin/granulin, LEF8, and LEF9 sequences (**Fig. 3.6**).

Table 3.2 Sequence analysis of the *iap*2, *iap*3, *egt* and polyhedrin genes of a South African isolate of TnSNPV.

Gene	ORF (bp)	Amino acids	Predicted molecular mass (kDa)	Sequence similarities to TnSNPV-Can	
				Nucleotide sequence identity (%)	Amino acid sequence identity (%)
polyhedrin	738	246	22.9	98	98
iap2	900	299	34.4	97	97
iap3	828	276	32.2	95	95
egt	1572	524	60.0	98	98

3.2.2 Identification, cloning and sequence analysis of the polyhedrin gene

When compared to other baculovirus polyhedrin genes, homologies were greater than 80% and confirms that the polyhedrin/granulin gene is one of the most conserved structural proteins with greater than 46% amino acid identity observed between members of the lepidopteran and hymenopteran NPVs and GVs (Okano et al., 2006). This high level of conservation is also exhibited by the closely related species of HaSNPV-NNg1 and HaSNPV-G4, HzSNPV and HaSNPV-G4 and SfMNPV with nucleotide and amino acid identities of 99% and above for the polyhedrin gene.

Similar to other baculovirus polyhedrin genes, the untranslated leader sequences contained promoter elements necessary for very late transcription (**Appendix 2**). The core late transcription element TAAG was identified 41 nt upstream from the ATG translational start codon (Rohrman, 1986). This is separated from the start codon by two cis-acting transcriptional enhancer elements, TTCGTA and TTGTGA, at 23 and 12 nt respectively, upstream from the start codon. A similar pattern is observed with TnSNPV-Can with the core late transcription element TAAG located 46 nt upstream from the start codon, with the sole enhancer element TTCGTA, located 30 nt from the start codon. As suggested by Kozak (1983), an adenine residue was identified 3 nt upstream from the start codon in both isolates. In common with other Group II NPVs, the polyhedrin genes of both isolates contains an N-terminal peptide sequence MYT(R/P)YS and lack a poly (A) tail with the length of the protein at 246 amino acids as suggested by Zanotto et al. (1992).

When compared to TnSNPV-Can, a number of nucleotide and amino acid substitutions were apparent (**Tables 3.3 & 3.4**). Despite the highest frequency of nucleotide substitutions for the polyhedrin gene at 10.9%, the protein shows a higher level of conservation when compared to IAP3.

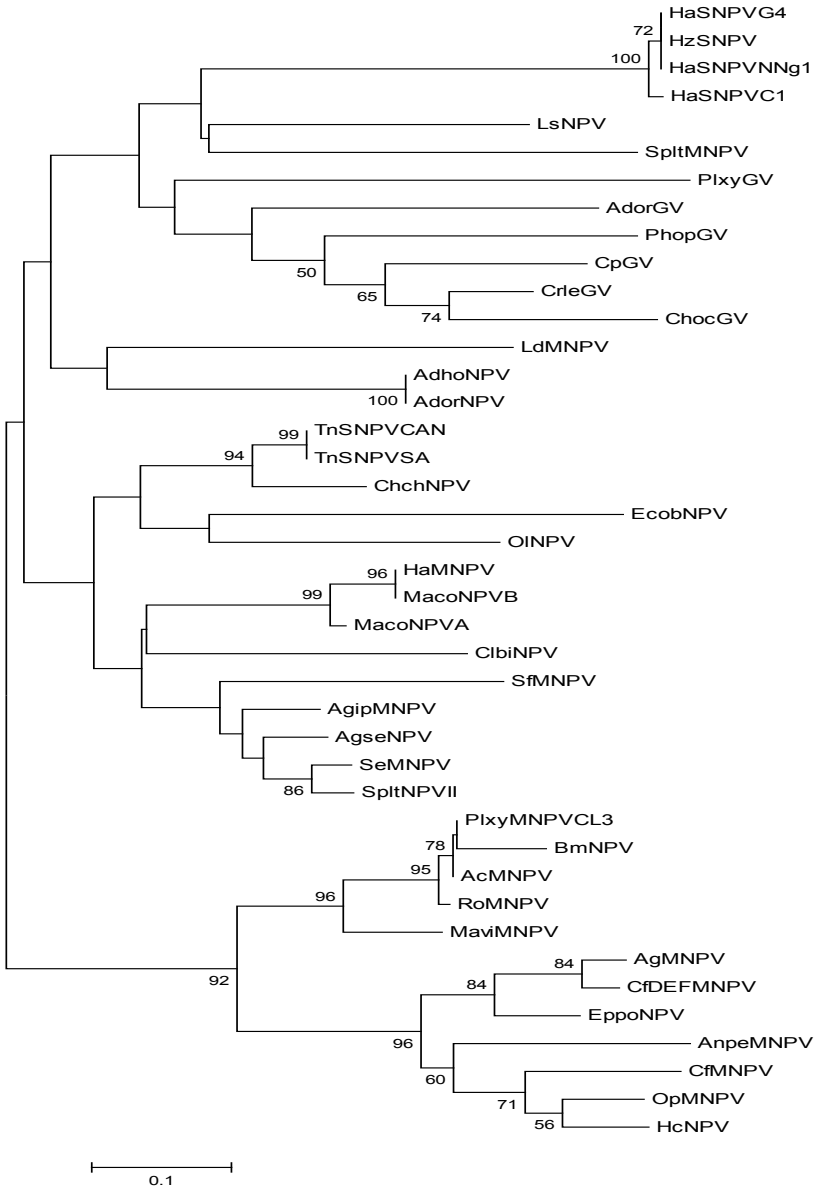

Figure 3.4 Neighbour joining distance trees based on amino acid sequences of EGT proteins. Numbers at the nodes indicate the bootstrap values from Neighbour joining (NJ) distance. Only boot strap values >50% are shown. For accession numbers of sequences used, see **Table 2.3**.

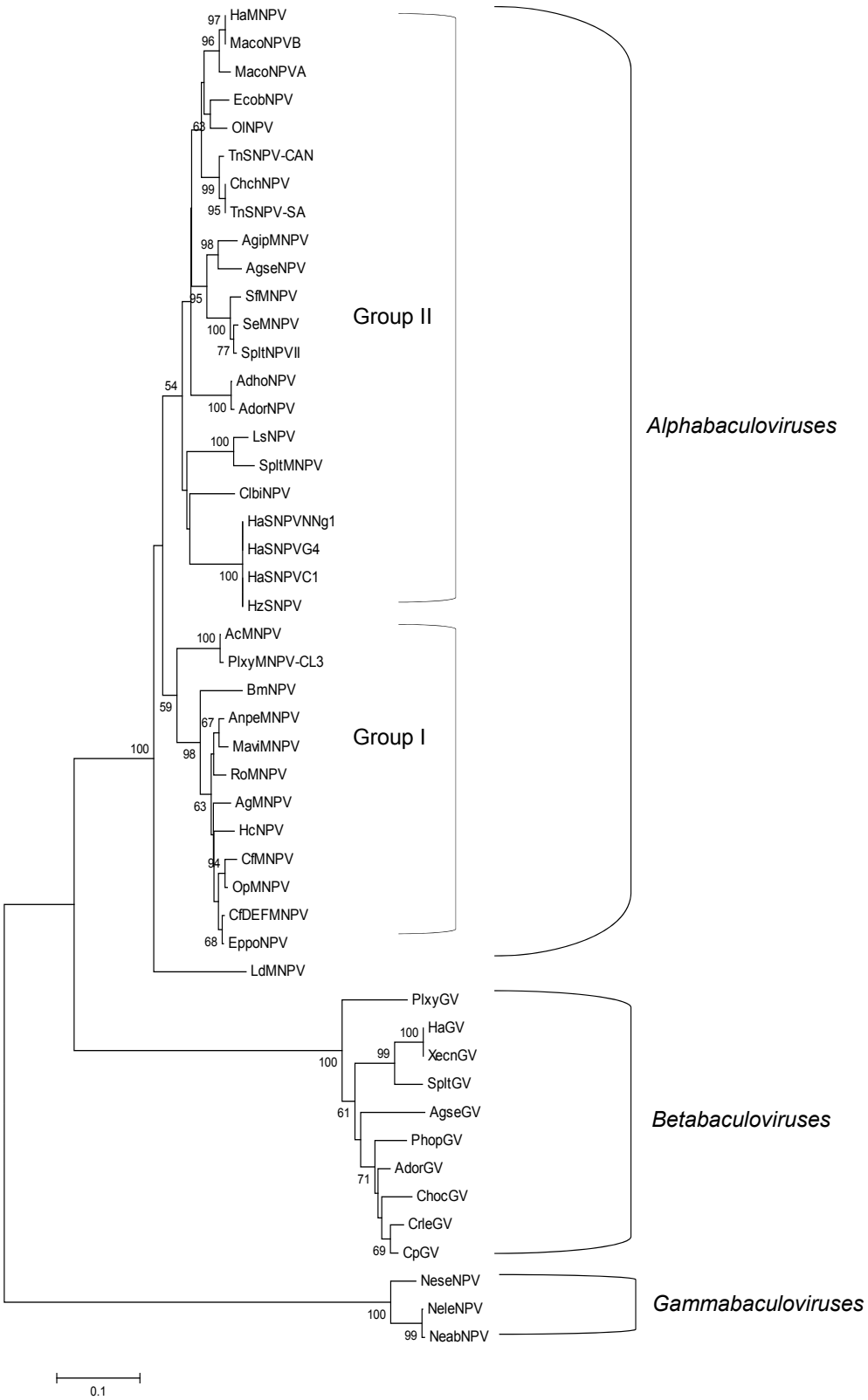

Figure 3.5 Neighbour joining distance trees based on amino acid sequences of polyhedrin/granulin genes. Numbers at the nodes indicate the bootstrap values from Neighbour joining (NJ) distance. Only boot strap values >50% are shown. For accession numbers of sequences used, see **Table 2.3**.

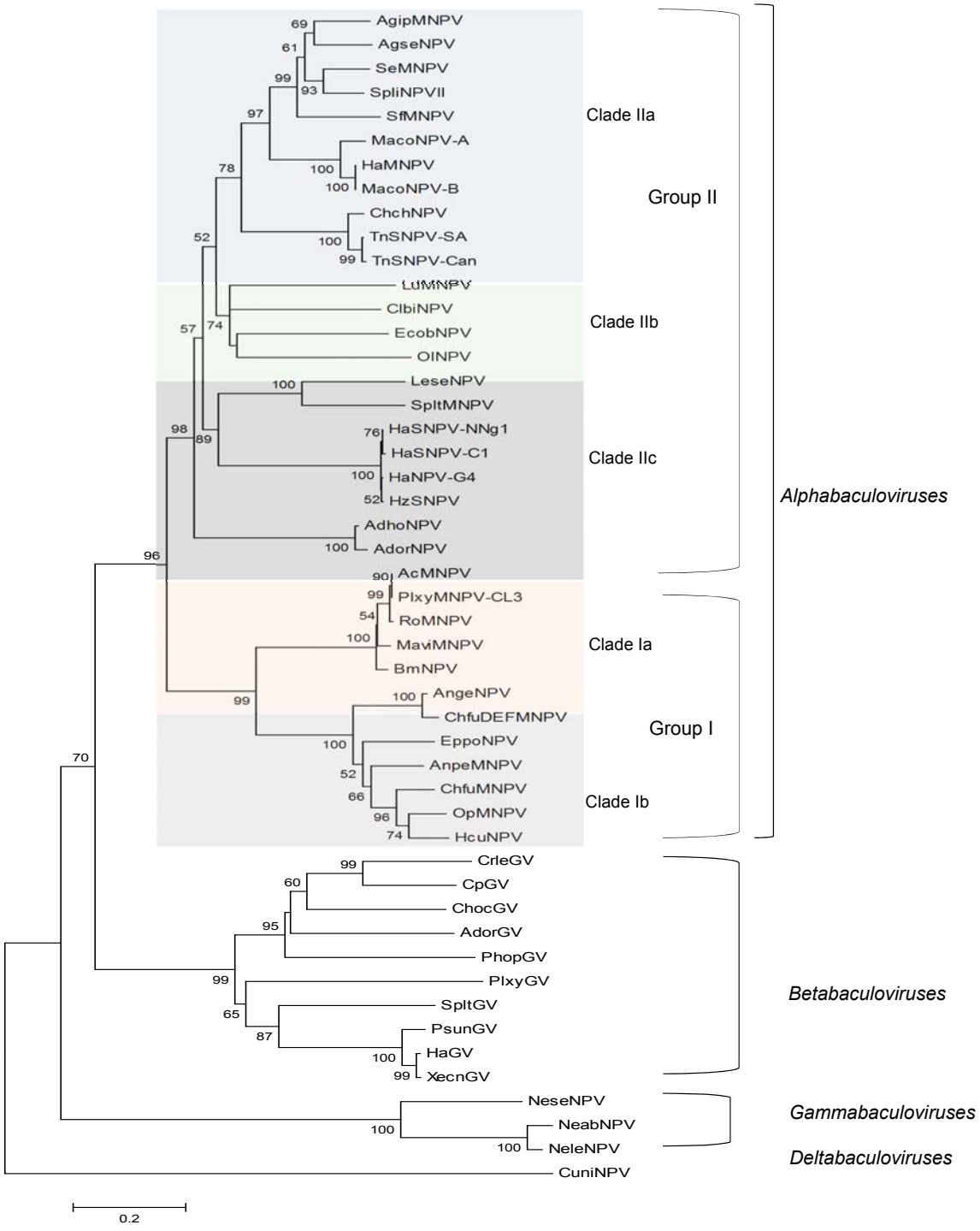

Figure 3.6 Neighbour joining distance tree based on concatenated amino acid sequences of complete polh/gran, *lef*-8, and *lef*-9 genes of the 48 fully sequenced baculovirus genomes (for accession numbers, see **Table 2.3**). Numbers at the nodes indicate the bootstrap values from Neighbour joining (NJ) distance. Only boot strap values >50% are shown. CuniNPV, the sole member of the *Deltabaculoviruses*, was used as an outgroup.

Table 3.3 Nucleotide substitutions between the polyhedrin, *iap2*, *iap3*, *egt* and polyhedrin genes of the South African and Canadian isolate of TnSNPV.

	Nucleotide Substitutions											
	A-T	A-G	A-C	T-A	T-G	T-C	C-G	C-A	C-T	G-A	G-C	G-T
polyhedrin	3	8	5	3	1	18		7	20	16	1	3
egt		2	1		2	6	2	2	17	3	2	1
iap3	1	7	4		3	10			7	6	2	
iap2		6	1			7		1	2	4	1	

Table 3.4 Amino acid substitutions between the IAP2, IAP3, polyhedrin and EGT proteins of the Canadian and South African isolates of TnSNPV. Numbers indicate the position of the amino acid substitution. Residues in bold are that of TnSNPV-Can. For single letter amino acids refer to Merck KGaA (2018).

TnSNPV IAP2			TnSNPV IAP3			TnSNPV polyhedrin			TnSNPV EGT		
22	**N**	D	66	**E**	D	8	**S**	N	8	**L**	S
32	**T**	A	92	**C**	Y	46	**A**	K	9	**S**	P
50	**G**	S	94	**L**	R	164	**S**	N	11	**T**	M
103	**A**	T	184	**R**		197	**N**	A	19	**P**	S
147	**V**	A	199	**S**	P				21	**G**	V
196	**P**	Q	203	**L**	P				327	**T**	L
220	**D**	N	204	**D**	A						
			208	**V**	A						
			219	**D**	A						

Phylogenetic analyses show that while the two isolates are closely related, they are distinct and represent two distinct clades (**Fig. 3.5 & 3.6**). Bootstrap analysis of the data show that the tree topology is well supported. The separation of the hymenopteran and lepidopteran-specific baculoviruses is apparent while within the lepidopteran specific baculoviruses two clusters with high bootstrap values were identified relating to the NPVs (*Alphabaculoviruses*) and GVs (*Betabaculoviruses*). The division of the *Alphabaculoviruses* into groups I and II has less support as evidenced by the lower bootstrap values for the basis nodes of the tree. From this analysis, TnSNPV-SA is placed as a Group II NPV with the closest relatives being TnSNPV-Can and ChchNPV. Two distinct clades were identified in the taxa of group I NPVs while the group II NPVs exhibited lower bootstrap support, and conflicting tree topologies when compared to **Fig. 3.6**.

3.2.3 Identification, cloning and sequence analysis of the *iap2* and *iap3* genes

The putative IAP2 and IAP3 proteins of TnSNPV-SA (**Appendix 3 & 4**) are highly homologous to the equivalent genes of TnSNPV-Can with a greater than 95% sequence identity at both the nucleotide and amino acid level (**Table 3.2**). A similar pattern is observed in the closely related but distinct species of HaSNPV-NNg1 and HaSNPV-G4, HzSNPV and HaSNPV-G4 as well as the geographically isolated species of SfMNPV with nucleotide and amino acid identities greater than 96% for all of the genes surveyed in this study (Chen et al., 2002). The high level of sequence conservation observed in these isolates suggests the evolutionary importance of these genes.

Analysis of TnSNPV-SA IAP proteins using Prosite (Hulo et al., 2007) revealed both BIR motifs at the amino terminal with a RING finger domain with the characteristic C3HC4 motif (Birnbaum et al., 1994) present at the carboxy-terminal (**Fig. 3.7**). Similar motifs located in similar positions were identified in TnSNPV-Can (**Fig. 3.7**). The BIR and RING motifs are characteristic of the IAP protein family with at least one IAP found in all lepidopteran baculoviruses sequenced to date (Clem, 2015).

When compared to the Canadian isolate, a number of nucleotide (**Table 3.3**) and amino acid substitutions (**Table 3.4**) were apparent. A nucleotide substitution frequency of 4.8% and 2.4% were identified for *iap3* and *iap2*, respectively. Similarly, amino acid substitutions were detected at nine positions for IAP3 and seven positions for IAP2. The majority of amino acid substitutions were located in the region between the second BIR domain and RING domain of the IAP3 protein with one substitution occurring within the first BIR domain. For the IAP2 protein, the majority of the substitutions flanked the BIR and RING domains. The relative paucity of substitutions within the BIR and RING domains suggests its relative importance to the normal functioning of these proteins (Clem, 2015). The BIR domains are zinc finger-like structures that can bind to the surface of caspases enabling the amino acid sequences between BIR domains to block the catalysing grooves of caspases. Consequently, IAPs inhibit apoptosis by inhibiting the activity of caspases (Fan et al., 2005). While the BIR motifs are essential for anti-apoptotic activity (Deveraux et al., 1998; Vucic et al., 1998; Wright et al., 2005), other sequences within or adjacent to the BIR domains are also necessary for the association of IAP molecules with various pro-apoptotic proteins (Green et al., 2004). The amino acid substitutions in the BIR and adjacent regions observed may thus translate to differences in activity and specificity for the two proteins.

Baculoviruses may contain multiple IAPs that are classified into five groups, IAP1–5, based on sequence homology (Luque et al., 2001; Clem, 2015). In most baculoviruses, IAP3 has anti-apoptotic activity (Crook et al., 1993; Birnbaum et al., 1994; Ikeda et al., 2004; Carpes et al., 2005) although exceptions have been described. For EppoMNPV and AnpeNPV, only IAP2 and IAP1 respectively, have the ability to suppress apoptosis (Maguire et al., 2000; Yan et al., 2010). The exact roles of baculovirus IAPs, other than IAP3, in virus infection and

multiplication are as yet unclear. While EppoMNPV IAP1 and *Spodoptera littoralis* MNPV IAP4 cannot suppress apoptosis, it is able to delay the onset of apoptosis induced by a p35 mutant of the prototype *Autographa californica* MNPV and other apoptotic stimuli while *Cydia pomonella* GV IAP5 has the ability to enhance the anti-apoptotic function of CpGV IAP3 (Vilaplana and O'Reilly, 2003). The knockout of the *iap1* and *iap2* genes of AcMNPV can occur without any loss of viral replication capacity (Griffiths et al., 1999) and AcMNPV lacking an active *iap1* gene carries a replication advantage over *iap1*-containing wild-type AcMNPV in TN368 cells co-infected with these viruses. The reason for this replication advantage remains unclear (McLachlin et al., 2001). Further analysis is required to elucidate the exact roles of the *iap*s in virus infection and multiplication.

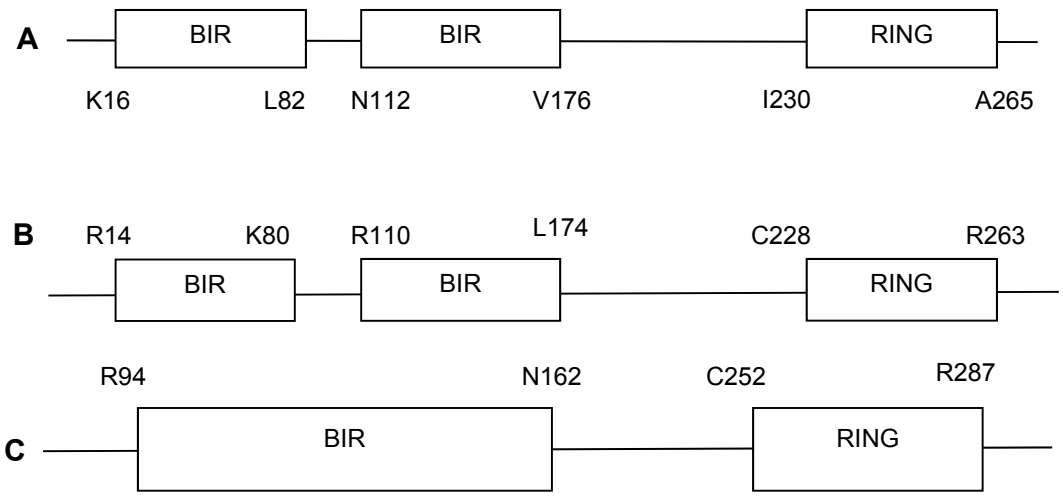

Figure 3.7 Diagrammatic representation of the BIR and RING domains in IAP proteins for the two isolates of TnSNPV. (A = IAP3 of TnSNPV-SA, B = IAP3 of TnSNPV-Can, C = IAP2 from both isolates of TnSNPV).

For the phylogenetic analysis of IAP proteins, both the IAP4 and IAP5 proteins have been included though none of the proteins have been shown to be active. From this analysis, the classification of the IAP proteins into five distinct groups (**Fig. 3.8**) can be confirmed. The IAP1 protein of AgseGV, IAP2 protein of OlNPV, IAP3 protein of XecnGV, IAP3 protein of SpltMNPV II, IAP3 protein of LdMNPV and the IAP3 protein of ClblNPV did not cluster with other members of their groups, which may indicate that they have been named in order of occurrence in the viral genome or in the order in which they were first identified rather than sequence homology. Similarly, the undesignated IAP proteins of CrleGV and CpGV; PlxyGV and SpltMNPV 2/3 can be designated as IAP1, IAP5 and IAP2 proteins respectively, as they cluster with these proteins. The truncated IAP4 protein of CfDEFMNPV was excluded from this

analysis because it lacked the characteristic BIR and RING domains typical of IAP proteins. The criteria used to classify this protein as an IAP protein may need to be revisited.

From this evidence, the IAP protein from TnSNPV-SA can be designated as an IAP3 due to sequence homology to other IAP3 proteins with its closest relatives TnSNPV-Can (designated as an IAP protein) and ChchNPV. Similarly, the IAP2 protein from TnSNPV-SA can be confirmed as an IAP2 due to its sequence homology with other IAP2 proteins (**Fig. 3.8**). The tree inferred from IAP protein sequences is not well resolved and the clear separation of the *Alphabaculoviruses* and *Betabaculoviruses* is not observed with group II NPVs of EcobNPV and SpltNPVII forming a clade that is distinct from the rest of the GVs and NPVs. The reason for this conflict is unclear but may reflect the conflicts observed when inferring phylogeny from single gene data sets. A variety of factors have been proposed to account for such discrepancies and include unequal rates of evolution, outgroup choice and the number of variable sites within the protein sequence (Herniou et al., 2001; Rokas et al., 2003). However, there is no reliable identifiable criterion that can account for or predict the performance of phylogenies inferred from single genes.

3.2.4 Phylogenetic analysis

The phylogenetic tree inferred from the amino acid sequence of the baculovirus core gene polyhedrin (**Fig. 3.5**) was well resolved and comparable to the tree inferred from the concatenated amino acid sequences of complete polyhedrin/granulin, LEF8, and LEF9 proteins of the 48 fully sequenced baculovirus genomes (**Fig. 3.6**). This is supported by the findings of Lange et al. (2004) which showed that the trees inferred from partial polyhedrin/granulin, LEF8, and LEF9 of 20 baculovirus mirrored the phylogeny based on 30 core genes. This approach was shown to be particularly useful where whole genome sequences are not available and represents a quick and easy method of identification for baculoviruses. The division of the lepidopteran NPVs is also in accordance with the hypothesis presented by Zanotto et al. (1993) and Bulach et al. (1999) and supported by the topologies presented by Lange et al. (2004), Herniou et al. (2001) and Thézé et al. (2018). All of these trees clearly showed the separation of the hymenopteran and lepidopteran specific baculoviruses as well as the Group I and II *Alphabaculoviruses*.

The trees inferred from the auxiliary proteins EGT (**Fig. 3.4**) and IAPs (**Appendix 5 & 6**) were not well resolved with lower bootstrap values and showed numerous conflicts with the tree inferred from complete polyhedrin/granulin, *lef-8*, and *lef-9* genes of the 48 fully sequenced baculovirus genomes. The separation of the lepidopteran and hymenopteran specific baculoviruses was not evident while the separation of Group I and II *Alphabaculoviruses* diverged from that observed from the baculovirus core gene polyhedrin as well as the tree inferred from complete polyhedrin/granulin, LEF8, and LEF9 proteins.

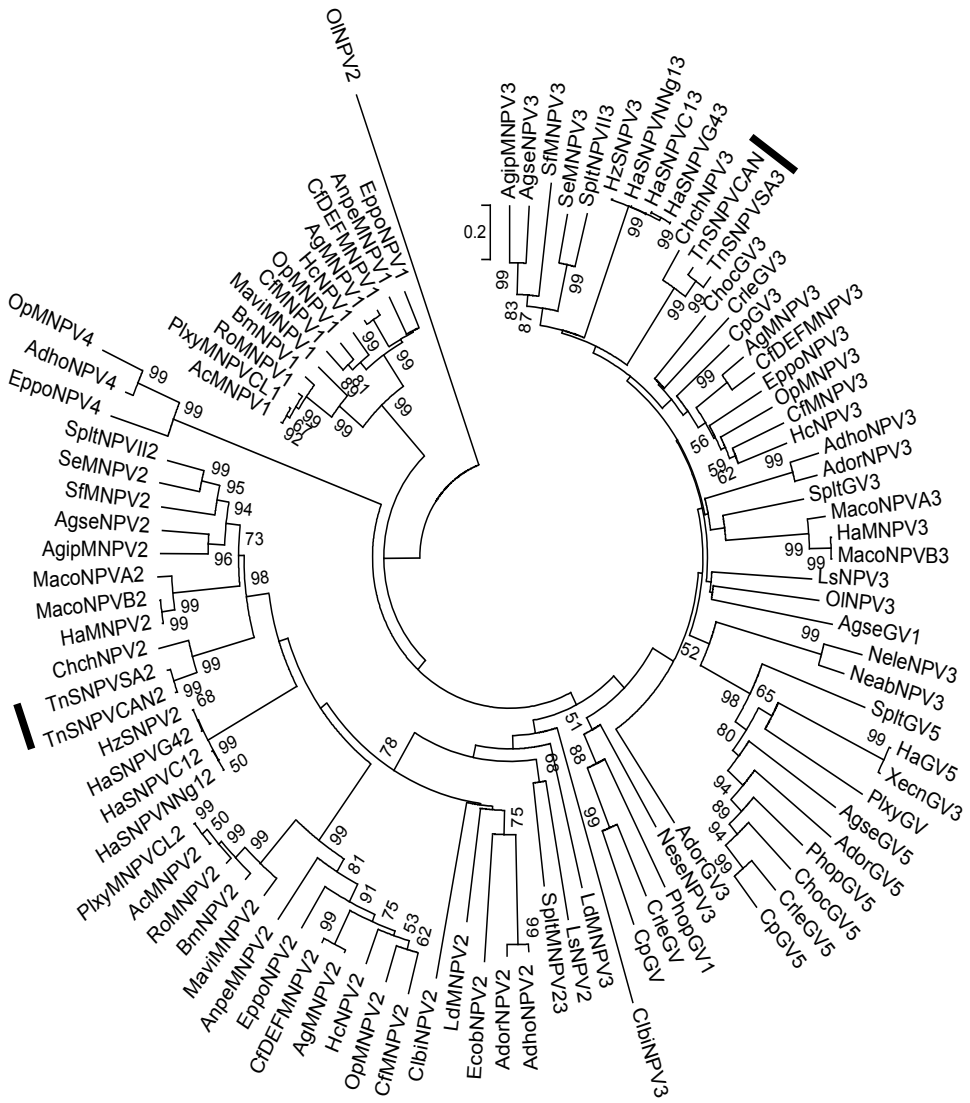

Figure 3.8 Neighbour joining distance trees based on amino acid sequences of all baculovirus IAP proteins from 48 fully sequenced baculovirus genomes. Numbers at the nodes indicate the bootstrap values from Neighbour joining (NJ) distance analyses. Only boot strap values >50% are shown. For accession numbers of sequences used, see **Table 2.3**. Numbers after names indicate IAP designation according to GenBank submission. The location of the South African and Canadian isolates of TnSNPV is indicated.

The two isolates of TnSNPV share nucleotide similarities greater than 95% for the genes in this study which indicates that they are closely related. While many of the baculovirus genomes have diverged quite extensively from each other, a number of genomes exhibits comparable structural similarity and nucleotide similarities of greater than 95% (Harrison, 2009). The genomes of HzSNPV and HaSNPV-G1 share nucleotide sequence identity of 97% (Chen et al., 2002) with the variation due to a novel 334 bp insert in HzSNPV while the Nicaraguan (-NIC), USA (-3AP2) and Brazilian (-19) isolates of SfMNPV share 99.35% sequence identity with each other (Wolff et al., 2008; Simón et al., 2011).

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a migratory pest native to tropical areas distributed from the United States to Argentina (Simón et al., 2011). This migration pattern may explain the high levels of gene conservation observed among the strains of this region despite the geographic isolation. The variations in these genomes and others are due to substitutions, insertions and deletions and are generally concentrated round the baculovirus repeat ORF genes (*bro*) and the homologous repeat regions (*hrs*). Extensively catalogued in baculovirus and other double stranded DNA virus families (Bideshi et al., 2003), a number of functions have been attributed to the *bro* genes but the exact role of the *bro* gene family in the baculovirus life cycle is as yet undetermined (Kang et al., 1999; Zemskov et al., 2000; Kang et al., 2006).

The intergenic *hrs* sequences consist of repeated units of approximately 70 bp with an imperfect 30 bp palindrome near their centre (Guarino and Summers, 1986a; Rohrmann, 2013) and can act as cis-acting transcriptional enhancers of gene transcription (Guarino and Summers, 1986b) and origins of viral DNA replication in transient replication assays (Pearson et al., 1992; Hilton and Winstanley, 2007). The *hrs* and *bro* gene sequences often flank regions of internal rearrangement and major insertions and deletions, which may indicate involvement in baculovirus gene loss and acquisition and contribute to strain heterogeneity (Hayakawa et al., 1999; Li et al., 2002; de Jong et al., 2005).

Initial sequence results suggest that the two isolates of TnSNPV have been exposed to similar evolutionary pressures and evolved at similar rates and represent variants of the same virus. This pattern is also observed with the closely related but distinct species of SfMNPV (Simón et al., 2011) and HzSNPV and HaSNPV-G1 (Chen et al., 2002). These viruses exhibit nucleotide and amino acid identities of greater than 96% with very similar but distinct structural and biological properties, morphotype, host range and virulence. Further investigation including assessment of biological activity as well as sequence data will be required to further elucidate the relationship between the two isolates.

3.3 Heterologous expression of recombinant proteins in *E. coli*

3.3.1 PCR amplification of the *iap2*, *iap3*, *egt* and polyhedrin genes

Escherichia coli expression based systems have been extensively utilised for the expression of many proteins and offers many advantages when compared to other systems. Chief among them is the speed of producing strains capable of overexpressing proteins and the relatively low cost of large scale production. Under optimal environmental conditions and in glucose-salts media, the doubling time of *E. coli* is about 20 min (Sezonov et al., 2007; Rosano and Ceccarelli, 2014), which means that even diluted, overnight cultures can reach stationary phase in a few hours. Many systems have been optimised for the construction of efficient expression vectors capable of high level expression in *E. coli* and the rapid and relatively simple transformation of the appropriate cell line. This system may not be suitable for the expression of all proteins, particularly if eukaryotic post-translational modifications such as glycosylation is necessary for the proper functioning of the protein. In such cases, aternative expression in the yeast, *Pichia pastoris*, a baculovirus expression system and mammalian cell lines should be considered.

The Gateway system utilised in this analysis offers many advantages over other expression systems. This technology allows for the insertion of DNA fragment(s) into an entry vector which can then be transferred to a variety of destination vectors using a site-specific recombinase, the Integrase enzyme, to produce the expression clone (Chee and Chin, 2015). Cloning does not require the use of restriction enzymes and is instead facilitated by a Vaccinia virus derived topoisomerase I enzyme covalently bound to each 3′ phosphate in the linearised entry vector pENTR/SD/D-TOPO (**Appendix 7**). The biological role of this enzyme is to catalyse the cleavage and ligation of DNA during replication and has been adapted to function as both a restriction enzyme and as a ligase in the cloning reaction (Koehn and Hunt, 2009). This system allows for high throughput and as a variety of reading frames, promoters, terminators and fusion tags are available, it is very versatile. However, the use and relatively high cost of the proprietary enzyme mixes required for the recombination reaction implies that this sytem is more costly than traditional restriction enzyme cloning (Chee and Chin, 2015).

To facilitate cloning into the entry vector, pENTR/SD/D-TOPO, the forward PCR primer included a 5′ CACC overhang (**Table 2.10**) which facilitates base pairing with the overhang sequence, GTGG, in the entry vector. PCR primers were designed with the overhang so that the initiation codon of the gene (ATG) falls within the Kozak consensus sequence CRCCaugG (where R is a purine, A or G). This allows the ribosome to identify the initiation codon and initiate translation of the gene (Acevedo et al., 2018). The spacing of the initiation codon with the CACC overhang allows for the proper distance from the Shine Delgarno sequence (ribosome binding site or RBS), which is located ~16 bp upstream in this vector (**Appendix 8**). The reverse primer in each primer set should not be complementary to the CACC overhang found in the forward primer as this would reduce directional cloning efficiency of the insert. A

one base pair mismatch is estimated to reduce directional cloning efficiency from 90% to 50% increasing the likelihood of the insert cloning in the reverse orientation (Thermo Fisher Scientific, 2012).

The *iap2*, *iap3*, *egt* and polyhedrin genes were amplified successfully from genomic TnSNPV DNA by PCR using a proof reading high fidelity DNA polymerase. The resulting blunt ending PCR products viz. 1572 bp for *egt* (**Fig. 3.9**, Lane 2), 900 bp for *iap2* (**Fig. 3.9**, Lane 4), 828 bp for *iap3* (**Fig. 3.9**, Lanes 6), 738 bp for polyhedrin (**Fig. 3.9**, Lanes 8), were purified prior to the annealing reaction with the TOPO vector, pENTR/SD/D-TOPO and transformation into *E. coli*. Negative controls were included for each of the PCR's (**Fig. 3.9**, Lanes 3, 5, 7 & 9) and the lack of any amplification products indicates the no contamination was present in the reactions.

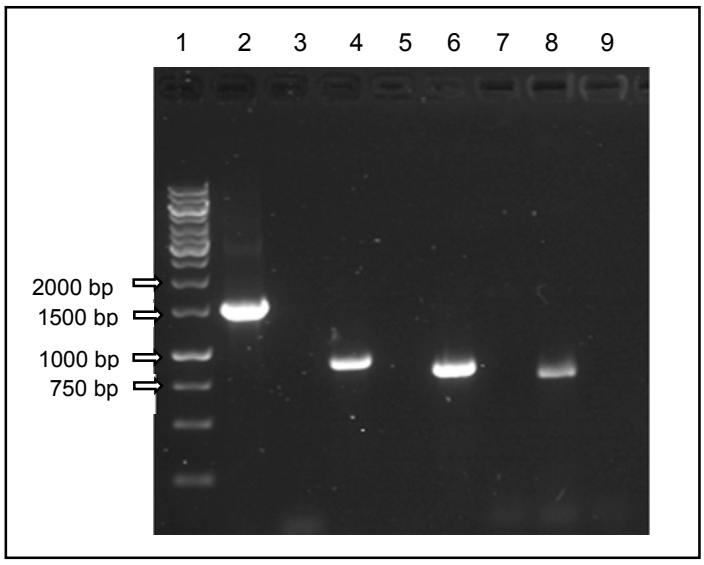

Figure 3.9 PCR amplification of the *egt*, *iap2*, *iap3* and polyhedrin genes from TnSNPV genomic DNA. Lane 1, Generuler 1kb DNA ladder; Lane 2, *egt*; Lane 3, no template control; Lane 4, *iap2*; Lane 5, no template control; Lane 6, *iap3*; Lane 7, no template control; Lane 8, polyhedrin; Lane 9, no template control.

The recombination reaction was facilitated by the Gateway® LR Clonase® II enzyme mix (Thermo Fisher Scientific, USA). The enzyme mix contains a mixture of Int (Integrase), IHF (Integration Host Factor) and Xis (Excisionase) enzymes that catalyses the recombination of the entry vector (containing the *iap2*, *iap3*, *egt* and polyhedrin gene flanked by attL sites) and pDEST^{TM}17 (containing the attR sites) (**Appendix 9 & 10**) to generate pDEST17-IAP2, pDEST17-IAP3, pDEST17-EGT and pDEST17-POL. All constructs were confirmed by sequencing prior to expression analysis. The control plasmid pENTR-gus containing the gene encoding *β-glucuronidase* (*gus*) was included as a positive control for the LR recombination reaction.

3.3.2 Expression of recombinant proteins in BL21-A1 *E. coli*

The expression vector constructs pDEST17-IAP2, pDEST17-IAP3, pDEST17-EGT and pDEST17-POL were transformed into BL21-A1 *E. coli* competent cells (Thermo Fisher Scientific, USA) and the expression of the proteins were examined. For pilot expression, overnight cultures of BL21-A1 *E. coli* were allowed to grow until the OD_{600} reached 0.6-1 which ensured that the culture remained in the exponential phase before being diluted in fresh media. Dilution of the overnight culture ensures that the cells rapidly returned to the exponential phase to maximise protein expression (Tate et al., 2003; Rosano and Ceccarelli, 2014). The LB broth utilised for cell growth remains the most common media for protein expression as it is nutrient rich and the optimal osmolarity allows for growth at the early log phase. These attributes compensate for the inability of the media to support high cell densities as it lacks carbohydrates and divalent cations necessary for such growth. While supplementation with yeast extract and peptone can improve growth and support high cell densities (Sezonov et al., 2007), it was not required for this analysis.

The *egt* gene is a viral protein capable of arresting larval development in the insect host to increase production of viral progeny. The insect is able to feed longer with enhanced weight gain, causes greater foliar damage and increased virus production (Evans and O'Reilly, 1999). It has also been shown to modify larval behaviour with larvae infected with *Spodoptera exigua* MNPV (SeMNPV) (Han et al., 2015) and *Lymantria dispar* MNPV (LdMNPV) (Hoover et al., 2011), climbing to elevated positions thus enhancing viral dispersal from these elevated positions.

Expression of EGT from pDEST17-EGT was monitored by RT-PCR and immunoblot analysis at 0, 1, 2, 3 and 4 hrs after induction with L-arabinose. Transcripts of the *egt* gene using gene specific primers (**Table 2.10**) were detected at one hour after induction (**Fig. 3.10A**, Lane 3) and persisted at high levels until four hrs post induction (**Fig. 3.10A**, Lane 4-6). The 1572 bp amplicon was detected soon after induction confirming the rapid rise of gene transcripts driven by the *ara*BAD promoter (PBAD), which is positively and negatively regulated by the product of the *ara*C gene located upstream of the L-arabinose operon. A positive control consisting of TnSNPV genomic DNA was included (**Fig. 3.10A**, Lane 8) and yielded an amplification product of 1572 bp. No contamination of the PCR was apparent (**Fig. 3.10A**, Lane 9) as evidenced by the lack of amplification products in that lane.

Many viruses have developed mechanisms to subvert the apoptosis induced by cells in response to viral infection (Clem, 2015; Ikeda et al., 2015). In baculoviruses, this process is mediated by four types of anti-apoptotic genes: *p35*, the functionally identical *p49*, the novel *apsup* and inhibitor of apoptosis genes (*iap*s). The IAP family is characterised by the presence of one to three tandem copies of a BIR motif at their amino terminal (Clem and Duckett, 1997) with some also containing a RING finger, at their C-terminus (Miller et al., 1998) (**Fig. 3.7**) and

are classified into five members (IAP1 – IAP5) (**Fig. 3.8**) based on amino acid sequence homology (Ikeda et al., 2004; Clem, 2015; Tobin et al., 2017).

Two vector constructs, pDEST17-IAP2 and –IAP3 were used to analyse expression of the IAP2 and IAP3 proteins in BL21-A1 *E. coli*. Expression of the two proteins were monitored by RT-PCR and immunoblot analysis at 0,1, 2, 3 and 4 hrs post induction with L-arabinose. RNA transcripts of 900 and 828 bp representing the *iap2* and *iap3* genes respectively, were detected from one hour and persisted until four hrs post induction (**Fig. 3.10B & C**, Lanes 3-6). TnSNPV genomic DNA was included as positive control and yielded amplification products of 900 and 828 bp for *iap2* and *iap3* respectively (**Fig. 3.10B & C**, Lane 8). No amplification products were observed in the negative control (**Fig. 3.10B & C**, Lane 9).

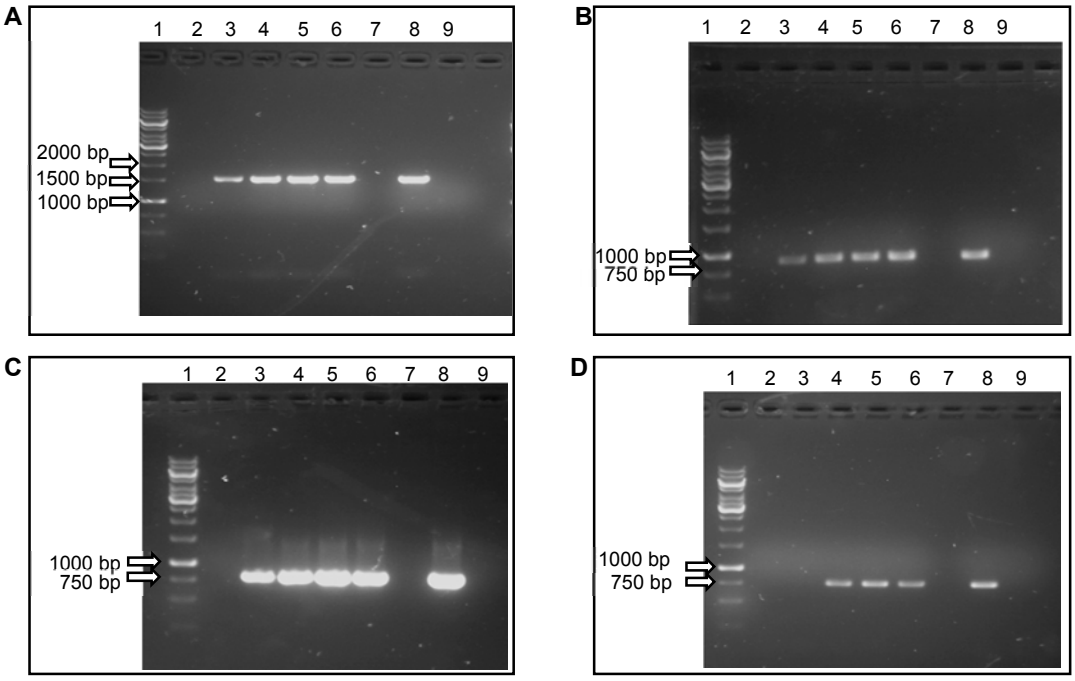

Figure 3.10 Transcript expression profile of EGT, IAP2, IAP3 and polyhedrin proteins with RT-PCR using gene specific primers. **A**: Lane 1, Generuler 1kb DNA ladder; Lanes 2-6, *egt* at 0, 1, 2, 3 and 4 hrs post induction; Lane 8, *egt* positive control; Lane 9, no template control. **B**: Lane 1, Generuler 1kb DNA ladder; Lanes 2-6, *iap2* at 0, 1, 2, 3 and 4 hrs post induction; Lane 8, *iap2* positive control; Lane 9, no template control. **C**: Lane 1, Generuler 1kb DNA ladder; Lanes 2-6, *iap3* at 0, 1, 2, 3 and 4 hrs post induction; Lane 8, *iap3* positive control; Lane 9, no template control. **D**: Lane 1, Generuler 1kb DNA ladder; Lanes 2-6, polyhedrin at 0, 1, 2, 3 and 4 hrs post induction; Lane 8, polyhedrin positive control; Lane 9, no template control.

In the late stages of baculovirus infection, large proteinaceous inclusion bodies primarily composed of the protein polyhedrin are formed within the cytoplasm of infected cells which serves to protect the infectious virion from environmental degradation (Rohrmann,

2013). This structural baculovirus core gene is absolutely conserved in all baculovirus sequenced to date emphasising its importance to baculovirus survival. To examine the expression of the polyhedrin protein, RNA transcripts and immunoblot analysis at 0, 1, 2, 3 and 4 hrs post induction was analysed. Transcripts of 738 bp in size were detected at one hour and continued at high levels until 4 hrs after induction (**Fig. 3.10D,** Lanes 3-6). The positive control sample consisting of genomic TnSNPV DNA yielded an amplicon of 738 bp (**Fig. 3.10D,** Lane 8) as expected and no amplification products were present in the negative control (**Fig. 3.10D,** Lanes 9) indicating the absence of contamination in the PCR.

Potential genomic DNA (gDNA) contamination of the RNA extracts was assessed by direct PCR of the RNA extracts using gene specific primers (**Table 2.10**) and was visualised by agarose gel electrophoresis (**Fig. 3.11**). The on-column deoxyribonuclease (DNAse) treatment included in the RNA extraction kit efficiently removed all contaminating gDNA prior to RT-PCR as evidenced by the lack of any amplification products for all four genes at the various time points (**Fig. 3.11A-D**). For the *egt* gene, no amplification was present at the various time points (**Fig. 3.11A**, Lanes 2-6) indicating the complete removal of contaminating gDNA. A positive control sample consisting of TnSNPV genomic DNA yielded a 1572 bp amplicon as expected (**Fig. 3.11A**, Lane 7) and no amplification products could be observed in the negative control. Similarly, no gDNA contamination of total RNA extractions was apparent as evidenced by the lack of amplification products in the PCR of the RNA extracts using gene specific primers for *iap2* (**Fig. 3.11B,** Lanes 2-6) and *iap3* (**Fig. 3.11C,** Lanes 2-6). The positive control samples yielded amplicons of 900 bp (**Fig. 3.11B**, Lane 7) and 828 bp (**Fig. 3.11C**, Lane 7) for *iap2* and *iap3* respectively, and no contamination was observed in the negative control samples (**Fig. 3.11B and C**, Lanes 8). No contamination of the RNA extracts by TnSNPV gDNA was detected as indicated by the lack of any amplification products on the agarose gel (**Fig. 3.11D,** Lanes 2-6) confirming that the DNase treatment included in the RNA extraction kit was effective at removing contaminating genomic DNA. The positive control sample yielded a band of 738 bp as expected for polyhedrin (**Fig. 3.11D,** Lane 7) and no contamination of the PCR was evident (**Fig. 3.11D,** Lanes 8).

Removal of contaminating gDNA from RNA preparations is vital as it can result in the inaccurate measurement of gene expression (Padhi et al., 2016) as the PCR cannot differentiate between the cDNA synthesised by reverse transcriptase and contaminating gDNA and forms part of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines which aims to promote better experimental practice and transparent scientific reporting (Bustin et al., 2009). The guidelines also recommend that RNA be quantified using fluorescent RNA-binding dyes due to the high sensitivity of such assays and that if DNase treatment was required, the assay conditions be reported. For this analysis, RNA concentration was determined spectrophotometrically as fluorescent RNA-binding dyes was not readily available while the on-column DNase treatment included in the Nucleospin

RNA kit (Machery-Nagel, Germany) efficiently removed contaminating TnSNPV gDNA as indicated by the by the lack of any amplification products in the PCR of RNA extracts using gene specific primers (**Fig. 3.11 A-D**).

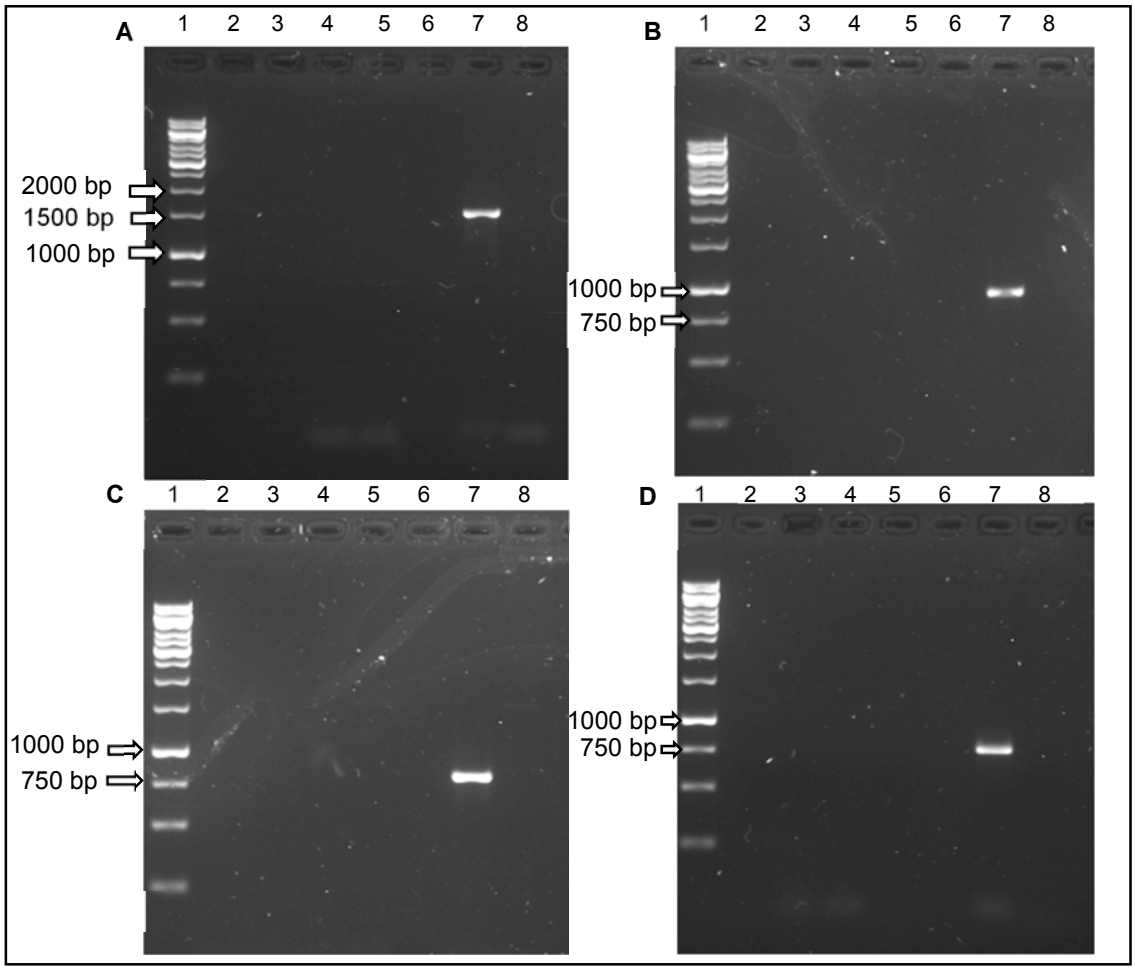

Figure 3.11 Genomic DNA contamination of RNA extracts. RNA extracts were subjected to PCR using gene specific primers before analysis on a 1% agarose gel. **A**: Lane 1, Generuler 1kb DNA ladder; Lanes 2-6, *egt* at 0, 1, 2, 3, and 4 hrs post induction; Lane 7, *egt* positive control; Lane 8, no template control. **B**: Lane 1, Generuler 1kb DNA ladder; Lanes 2-6, *iap2* at 0, 1, 2, 3, and 4 hrs post induction; Lane 7, *iap2* positive control; Lane 8, no template control. **C**: Lane 1, Generuler 1kb DNA ladder; Lanes 2-6, *iap3* at 0, 1, 2, 3 and 4 hrs post induction; Lane 7, *iap3* positive control; Lane 8, no template control. **D**: Lane 1, Generuler 1kb DNA ladder; Lanes 2-6, polyhedrin at 0, 1, 2, 3 and 4 hrs post induction; Lane 7, polyhedrin positive control; Lane 8, no template control.

For immunoblot analysis, cell lysates were harvested post induction and monitored with monoclonal antisera directed against the C-terminal 6X His fusion tag located on the recombinant protein. To ensure that the same amount of protein was loaded into each well of the SDS-PAGE, the total protein concentration of the cell extracts was determined using the 2-D Quant kit (GE Healthcare, USA)**.** This ensured that a similar amount of protein was

resolved and detected by immunoblot analysis. Total protein concentration of cell extracts was compared to a standard curve consisting of varying BSA standards and ranged from 1.1 to 6.2 µg/µl. The measurement is unaffected by substances such as SDS, EDTA and Tris which are components of the sample lysis buffer and buffers utilised downstream during the running and transfer of the SDS-PAGE. These substances have been shown to affect the results of certain assays such as the Bradford assay which is commonly employed to determine total protein concentrations (Bio-Rad Laboratories Inc., 2018).

SDS-PAGE analysis showed that the EGT fusion protein migrated at a molecular weight of approximately 51 kDa and was visible from 2 hrs post induction (**Fig. 3.12A,** Lane 2) indicating that expression of the EGT protein rose rapidly after induction of BL21-A1 *E. coli*. The discrepancy or "gel shifting" between the protein size of the EGT protein on SDS-PAGE and the predicted size of the protein at 60 kDa may be attributed to a variety of factors. EGT monomers have been shown to be post-translationally modified by the addition of sugar residues that increase its apparent molecular mass by approximately 11 kDa (Evans and O'Reilly, 1999). However, the *E. coli* based expression system utilised here lacks the ability to attach a variety of a biochemical groups after translation (Rosano and Ceccarelli, 2014) though recent evidence suggests that a number of modifications such as lysine acetylation, serine/threonine phosphorylation, tyrosine phosphorylation and ubiquitination-like protein ligation thought to be exclusive to eukaryotes, is more common in prokaryotes that initially thought (Brown et al., 2017). A number of predicted translation/post translation modification sites are present within the *egt* gene ORF including three N-linked glycosylation sites located at amino acid residues 77-81, 303-306 and 489-4925, nine casein kinase II phosphorylation sites and six N-myristoylation sites which could contribute significantly to the molecular mass of the protein. While N-linked glycosylation of the EGT protein is not absolutely required for activity, it may be necessary for other aspects of the enzyme's function, such as stability (Evans and O'Reilly, 1999). The lack of these modifications in *E. coli* system utilised in this analysis may thus affect the proper functioning of the expressed protein.

The N- or C-terminal fusion tags expressed as part of the recombinant protein will also increase the size of the protein and result in altered mobility on SDS-PAGE due to the increase in molecular weight. Fusion tags such as the N-terminal or C-terminal glutathione S-transferase (GST) tags in pDEST™15 and pDEST™24 respectively, contributes significantly to the size of the recombinant protein (~27 kDa) while the smaller C-terminal 6X His tag found on the pDEST™17 vector accounting for 2.6 kDa (Thermo Fisher Scientific, 2012).

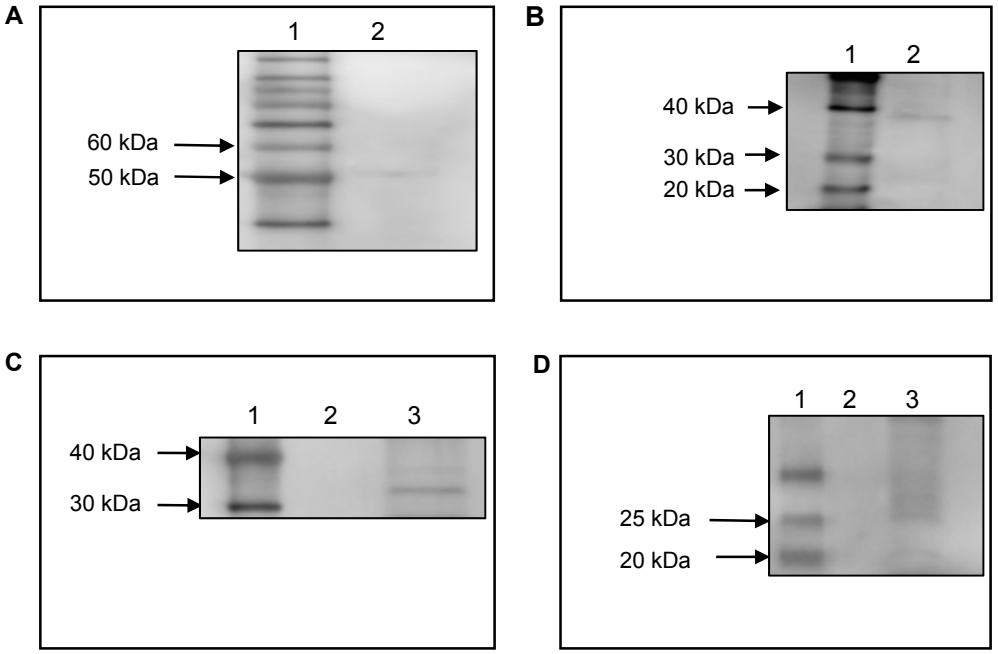

Figure 3.12 Expression of recombinant proteins in BL21-A1 *E. coli*. Proteins were resolved by separation on 12% SDS-PAGE prior to immunoblot analysis with anti His_6 to test expression of *E. coli* transformants. Expression could be observed at two hrs after each transformant was induced by 0.2% arabinose. **A**: Lane 1, BenchMark™ Protein Ladder; Lane 2, EGT. **B**: Lane 1, BenchMark™ Protein Ladder; Lane 2, IAP2. **C**: Lane 1, BenchMark™ Protein Ladder; Lane 2, blank; Lane 3, IAP3. **D**: Lane 1, BenchMark™ Protein Ladder; Lane 2, blank; Lane 3, POL.

The physiochemical properties of the protein itself can also effect mobility on the SDS-PAGE with an estimated 40% of proteins surveyed in a study by Shirai et al. (2008) not migrating to their predicted positions. This altered mobility has been largely attributed to the hydrophobicity of the protein (Shirai et al., 2008). Altered mobility of helical membrane proteins has also been attributed to the degree of hairpin helicity and the altered SDS binding by the hairpin structures and can alter mobility by up to 30% when compared to predicted molecular weights (Rath et al., 2009). The high proportion of hydrophobic residues in the secretion peptide sequence of the EGT protein which directs the protein to the extracellular compartment (Evans and O'Reilly, 1999) (**Appendix 1**), may affect the mobility of the EGT protein on SDS-PAGE.

Truncation of recombinant proteins and altered mobility on SDS-PAGE could also be due to proteolysis of recombinant proteins in *E. coli*. The BL21-A1 *E. coli* cell line however, lacks two proteases genes*, lon* (cytoplasmic protease), able to degrade many foreign proteins and *ompT* (periplasmic protease), which is able to degrade extracellular proteins (Ryan and Henehan, 2013) and is not likely to be a contributing factor in this analysis.

Following immunoblot analysis, bands of 38 and 32 kDa for IAP2 and IAP3 respectively, were detected from 2 hrs post induction (**Fig. 3.12B,** Lane 2 & **Fig. 3.12C,** Lane 3) indicating

the rapid rise of expression of the proteins in BL21-A1 *E. coli*. The observed sizes on SDS-PAGE were in accordance with the predicted sizes of 44 and 32.2 kDa for IAP2 and IAP3 respectively. The IAP3 protein size corresponds to the IAP3 protein detected for CpGV (Miller et al., 2002), OpMNPV (Byers et al., 2016), LdMNPV (Yamada et al., 2012) and is slightly larger than the IAP3 protein from HycuNPV (Ikeda et al., 2004). There was greater size variation observed with the IAP2 protein with sizes ranging from 22 kDa for *Antheraea pernyi* NPV (Yan et al., 2010), 30 kDa for BmNPV (Ito et al., 2014) and LsMNPV (Kim et al., 2007) and 29 kDa for AcMNPV (Zeng et al., 2009).

Expression of the polyhedrin protein rose rapidly after induction and was initially detected at 2 hrs (**Fig. 3.12D,** Lane 3) post induction. The size of the protein on SDS-PAGE corresponded to the size predicted from the primary amino acid sequence at 23 kDa.

Expression from the expression constructs pDEST17-IAP2, pDEST17-IAP3, pDEST17-EGT and pDEST17-POL rose rapidly after induction of BL21-A1 *E. coli* with L-arabinose. RNA gene transcripts were detected at 1 hr after induction and persisted at high levels until 4 hrs after induction. Expression of the recombinant proteins was detected at 2 hrs after induction and remained at high levels (results not shown).

The *E. coli* expression system is widely utilised for the production of recombinant proteins due to its speed, relative simplicity and low cost. This system does however face some challenges as it may be difficult to produce correctly folded proteins due to the lack of post translational modifications as well as producing proteins at high levels in soluble form (Steinmetz and Auldridge, 2017). The inability to attach a variety of a biochemical groups after translation may result in altered activity and may thus translate into altered functionality for the IAP, EGT and polyhedrin proteins surveyed here. While not required for enzyme activity, N-linked glycosylation of the EGT protein is necessary for enzyme stability (Evans and O'Reilly, 1999)and its absence may thus have a direct effect on enzyme activity and warrants further investigation.

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Chapter 4

Conclusion

This thesis describes the identification, molecular characterisation and cloning of a baculovirus core gene and three auxiliary genes from TnSNPV in order to delineate its phylogenetic relationship to a Canadian isolate of TnSNPV and to other baculoviruses. In addition, recombinant IAP2, IAP3, EGT and polyhedrin proteins were expressed in *Escherichia coli* (*E. coli)* as a lead up to genetic modification of the virus.

In order to accomplish this, virions were purified from *T. ni* larvae by sucrose gradient centrifugation (Harrap and Longworth, 1974; O'Reilly et al., 1994) before being propagated in the homologous host, *T. ni*, maintained on artificial media (Hervet et al., 2016). The analysis revealed that the median killing time, to kill 50% of the larvae (LT_{50}), averaged 1.8 days which is significantly faster than for most other baculoviruses and confirms that this virus has promise as a microbial insecticide against the cabbage looper, *T ni*, and the closely related cabbage semi-looper. Transmission electron microscopy of the purified innoculum revealed the presence of occlusion bodies containing a single enveloped virion with size typical for members of the *Alphabaculoviruses* genus.

The BV from the infected insects was introduced and maintained in High Five (BTI-TN-5B1-4) cells (Invitrogen, USA) prior to DNA extraction using a method described by O'Reilly et al. (1994) as well as a silica column-based method. The genome size of TnSNPV was estimated at 160 kb which falls within the range expected for baculoviruses of 81 to 160 kb (Blissard and Rohrman, 1990; Rohrmann, 2013) but is significantly larger than the Canadian isolate of TnSNPV (Willis et al., 2005). The increased genome size of the South African isolate could reflect genetic variation as the isolates have adapted to varying environmental conditions and insect hosts as observed in strains of LdMNPV (Martemyanov et al., 2017) and SfMNPV (Wolff et al., 2008; Simón et al., 2011).

Phylogenetic analyses of the *iap2*, *iap3*, *egt* and polyhedrin genes and a concatenated data set consisting of complete polyhedrin, *lef*-8, and *lef*-9 genes of 48 completely sequenced baculoviruses show that while the two isolates are closely related, they are distinct and represent two variants of the same virus. TnSNPV-SA was identified as a Group II NPV with the closest relatives being TnSNPV-Can and ChchNPV. Analysis of the polyhedrin gene and the concatenated data set revealed the clear separation of the hymenopteran and lepidopteran-specific baculoviruses. Within the lepidopteran specific baculoviruses two clusters with high bootstrap values were identified relating to the *Alphabaculoviruses* and *Betabaculoviruses*. The division of the *Alphabaculoviruses* into groups I and II had less support as evidenced by the lower bootstrap values. Only the phylogenetic analysis inferred from the baculovirus core gene polyhedrin was able to resolve the accepted lineage of baculoviruses and was comparable to the tree inferred from the concatenated data set and corresponded to the findings by Lange et al.,(2004) and Thézé et al., (2018). The auxiliary genes, *iap2*, *iap3* and *egt* produced trees with conflicting topologies and poor bootstrap support. This approach highlights the pitfalls experienced when utilising single genes to infer phylogeny. A variety of

factors have been proposed to account for such discrepancies (Herniou et al., 2001; Rokas et al., 2003) but there is no reliable identifiable criterion that can account for or predict the performance of phylogenies inferred from single genes.

Five distinct groups relating to IAP-1, 2, 3, 4 and 5 could be distinguished from the tree inferred from all IAP proteins from 48 fully sequenced baculoviruses. From this analysis, the IAP protein from the South African isolate of TnSNPV is identified as an IAP3 due to sequence homology to other IAP3 proteins. Similarly, the IAP2 can be confirmed as an IAP2 protein as it clusters with other IAP2 proteins.

Expression of the expression constructs pDEST17-IAP2, pDEST17-IAP3, pDEST17-EGT and pDEST17-POL in BL21-A1 *E. coli* was monitored by RT-PCR and immunoblot analysis. Expression of the recombinant proteins rose rapidly and transcripts were detected at one-hour post induction and persisted at high levels until 4 hrs after induction. No genomic DNA contamination of the RNA extracts was evident. Production of recombinant proteins was monitored with monoclonal antisera directed against the C-terminal 6X His fusion tag present on the recombinant protein. Proteins were detected at 2 hrs post induction. Mobility of the recombinant proteins on SDS-PAGE was in accordance with the predicted molecular mass. The EGT protein at 51 kDa was however significantly smaller than the predicted molecular mass and may be due to the lack of post translational modifications in the *E. coli* based expression system utilised in this analysis (Evans and O'Reilly, 1999; Rosano and Ceccarelli, 2014) or the presence of hydrophobic residues in the secretion signal (Shirai et al., 2008). While post translation modification is not absolutely required for activity of the EGT enzyme (Evans and O'Reilly, 1999), it may be required for other aspects of the enzyme's function and thus may have an impact on the proper functioning of the enzyme.

Baculoviruses may contain multiple copies of the *iap* gene (Rohrmann, 2013) though generally only one may be functional (Ikeda et al., 2004; Clem, 2015). Homologues of IAP3 exhibits the highest anti-apoptotic activity apoptotic activity while the anti-apoptotic activity of IAP1 and IAP2 homologues is variable. Further investigation to determine which of the two *iaps* identified in this analysis possess anti-apoptotic activity is warranted.

The development of baculoviruses as viable biopesticides is likely to proceed along two parallel approaches. In countries where genetically modified organisms are either limited or banned, developments will focus on the formulation of improved baculovirus preparations, the monitoring of baculovirus infection and on the development of *in vitro* cultures of naturally occurring baculoviruses (Szewczyk et al., 2006). Detection of the absolutely conserved gene polyhedrin by PCR and other genomic based molecular detection methods will be useful in order to monitor baculovirus infection particularly in the early stages of infection. In countries where genetic modification is possible, the focus will be on the modification of host physiology by the deletion of baculovirus genes and insertion of foreign genes to improve the pesticidal properties of baculoviruses (Moscardi et al., 2011; Szewczyk et al., 2011). This includes the

deletion of the *ecdysteroid* UDP-*glucosyltransferase (egt*) gene (O'Reilly and Miller, 1991) as well as genes which determine host range such as the inhibitor of apoptosis (*iaps*) and late expression factors (*lefs*) (Thiem, 2009*)*. The insights from fundamental and applied research in this study and others will contribute to the development of baculovirus based biopesticides and fulfil the enormous potential of baculoviral pesticides globally.

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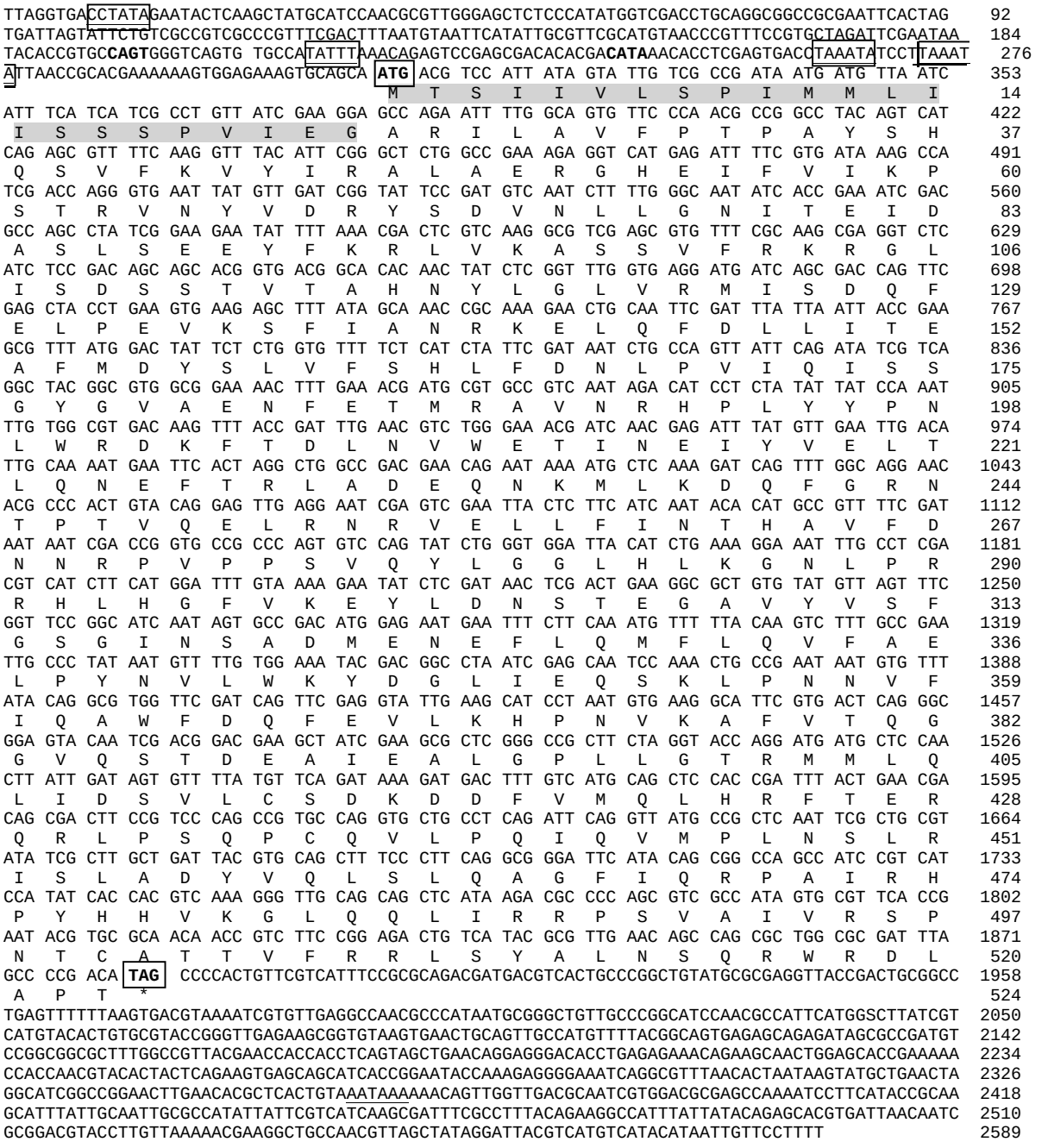
Appendices



Appendix 1 Nucleotide and predicted amino acid sequence of the *egt* gene and flanking sequence from the South African isolate of TnSNPV. Numbers on the right indicate the nucleotide position and the amino acid position in the putative TnSNPV EGT protein (the dark grey shaded N-terminal sequence indicates the putative signal sequence). The initiation and termination codons are boxed and in bold. The TATA boxes upstream of the translation initiation site are boxed and underlined. The putative early gene promoters (CAGT and CATA) are in bold and the consensus polyadenylation sequence is underlined.

```
ACATTAGCACTGTATTCTTCAAACTCATCCTCGTGTATATTGTCATAGATTCCGCAAAAAATTTTATCGGCAATATAGTTAAAGTCGAGTTA    92

TTCTTTATTTTTCTTCAAGTCACCACTTCGAGTATAAAGATAAGTTTTGATGTAGTCCATATTTACTTTGACCGACTCTCGGCCGTTCCTCA   184
AAATCATTTGATATGAAGAAAACATTTTAAAATCACTATACAAAAGTCACGACTCGGTGTCAAGGTGTATGTAGTAATAATAAAACACATTT   276
GATTGTCGAGTATATTTATATCATTTAATCGCTGAATGATGCTCGTCGGATTGAGAAAGAGATAAAAAATTCTCATATTAGATGCGTTTGCC   368
GTAATCATGTTTCGACCAAAAATTTTTCTATTGATCATGCACAACAAAATAAGTATTTTTCTCCTTTCGTAAAAGTTTGTGAAAAACAAATA   460

TA ATG TAT ACA CGT TAC AGC TAT AAC CCT TCT CTG GGC CGC ACC TAC GTT TAC GAC AAC AAA TAC TAC      528
    M   Y   T   R   Y   S   Y   N   P   S   L   G   R   T   Y   V   Y   D   N   K   Y   Y       22

AAA AAT TTG GGT GCC GTA ATC AAG AAT GCC AAG CGT AAG AAG CAC TAC GCC GAA CAT GAA TTA GAA GAA     597
 K   N   L   G   A   V   I   K   N   A   K   R   K   K   H   Y   A   E   H   E   L   E   E      45

AAA ACC CTT GAT CCC CTA GAC AAC TAC TTG GTA GCT GAA GAT CCT TTC CTG GGA CCC GGT AAG AAC CAA     666
 K   T   L   D   P   L   D   N   Y   L   V   A   E   D   P   F   L   G   P   G   K   N   Q      68
 23+23
AAA CTC ACT TTG TTT AAA GAG ATC CGT AAT GTA AAG CCC GAT ACC ATG AAG CTT GTC GTT AAC TGG AGC     735
 K   L   T   L   F   K   E   I   R   N   V   K   P   D   +   M   K   L   V   V   N   W   S      91

GGC AAA GAG TTT CTC AGG GAA ACT TGG ACC CGC TTC ATG GAG GAC AGC TTC CCC ATC GTT AAC GAC CAA     804
 G   K   E   F   L   R   E   T   W   T   R   F   M   E   D   S   F   P   I   V   N   D   Q     114
 23+23
GAA ATC ATG GAC GTA TTT CTT GTG GTT AAC ATG CGC CCG ACA AGA CCC AAT CGT TGC TTC AAA TTC TTA     873
 E   I   M   D   V   F   L   V   V   N   M   R   P   T   R   P   N   R   C   F   K   F   L     137

GCC CAA CAC GCT TTA CGT TGC GAC CCC GAT TAT GTT CCT CAC GAG GTG ATT AGA ATC GTA GAG CCG TCT     942
 A   Q   H   A   L   R   C   D   P   D   Y   V   P   H   E   V   I   R   I   V   E   P   S     160

TGG GTA GGC AAC AAC AAC GAA TAC AGA ATT AGT CTG GCC AAG AAA GGC GGT GGC TGT CCC ATC ATG AAC    1011
 W   V   G   N   N   N   E   Y   R   I   S   L   A   K   K   G   G   G   C   P   I   M   N     183

CTT CAC TCT GAG TAC ACC AAC TCG TTT GAA GAG TTT ATT GCT CGC GTG ATC TGG GAA AAC TTC TAC AAA    1080
 L   H   S   E   Y   T   N   S   F   E   E   F   I   A   R   V   I   W   E   N   F   Y   K     206

CCC ATA GTT TAC GTA GGA ACC GAT TCC GCC GAG GAA GAG GAG ATT CTT CTT GAA GTG TCT TTA GTC TTT    1149
 P   I   V   Y   V   G   T   D   S   A   E   E   E   E   I   L   L   E   V   S   L   V   F     229

AAA ATT AAG GAA TTC GCT CCC GAC GCG CCT CTG TAT TCA GGC CCA GCA TAC TAA  GCCGTAGATTCGTTGTCGA    1222
 K   I   K   E   F   A   P   D   A   P   L   Y   S   G   P   A   Y   *                        247

CTTTTTCGTTAATTTCTGTTAAAAGTCTTTCTGCTTGTTTAATGTCTTGTCTGCTAACGTMYTRYSYNPSLGRTYVYDNKYYKNLGAVIKNA   1314
KRKKHYAEHELEEKTLDPLDNYLVAEDPFLGPGKNQKLTLFKEIRNVKPDTMKLVVNWSGKEFLRETWTRFMEDSFPIVNDQEIMDVFLVVN   1406
MRPTRPNRCFKFLAQHALRCDPDYVPHEVIRIVEPSWVGNNNEYRISLAKKGGGCPIMNLHSEYTNSFEEFIARVIWENFYKPIVYVGTDSA   1498
EEEEILLEVSLVFKIKEFAPDAPLYSGPAY                                                                  1528
```

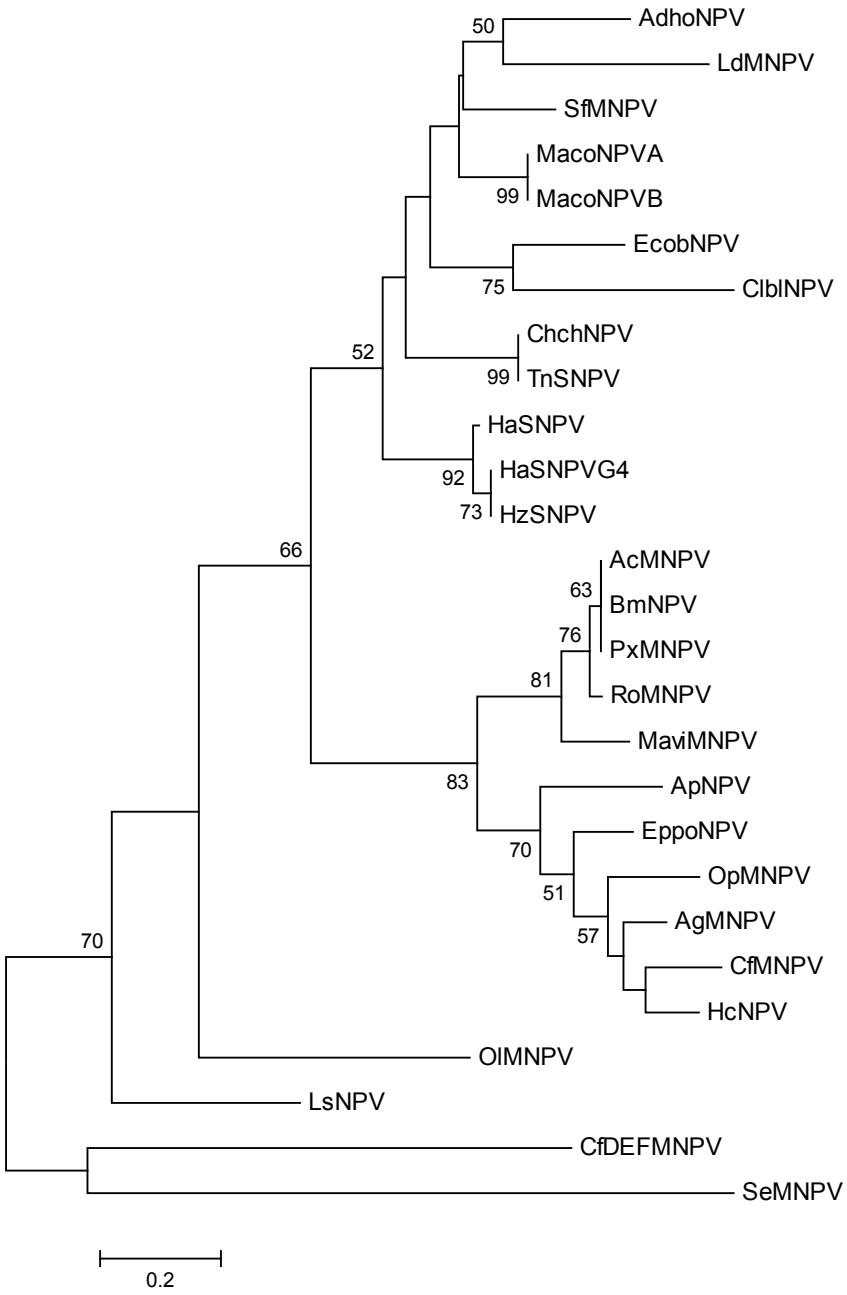
Appendix 2 Nucleotide and predicted amino acid sequence of the polyhedrin gene with its flanking sequence from the South African isolate of TnSNPV. Numbers on the right indicate the nucleotide position and the amino acid position in the putative TnSNPV polyhedrin protein. The translation initiation and termination codons are indicated in bold. The putative late translation initiation motif-core (TAAG) is underlined and in bold. No N-terminal polyadenylation site was identified. The N-terminal peptide sequence is shaded while promoter enhancer elements are boxed.

```
  1 M  D  Y  Y  S  N  I  M  N  S  R  L  A  P  P  S  F  Y  H  E
  1 ATGGATTATTATTCGAATATCATGAACTCTCGACTCGCGCCGCCGTCGTTTTACCACGAA

 21 Y  D  N  R  I  E  S  F  A  G  S  A  L  T  R  E  Y  K  R  N
 61 TACGACAATCGAATCGAAAGTTTCGCCGGCAGTGCGCTCACCAGAGAATACAAACGGAAT

 41 L  A  K  F  G  I  Y  Y  E  S  G  N  G  V  Y  K  C  A  F  C
121 TTAGCAAAGTTTGGTATATACTACGAAAGCGGCAATGGCGTTTATAAATGTGCTTTTTGT

 61 P  L  V  L  V  R  L  D  M  R  T  L  K  Y  H  T  F  S  T  C
181 CCTCTAGTATTGGTCAGATTGGACATGAGAACACTGAAATATCACACGTTTTCGACGTGT

 81 S  M  A  T  T  I  L  A  T  N  E  T  L  R  K  E  S  F  R  K
241 TCGATGGCGACGACTATACTCGCCACAAACGAAACTCTCAGAAAAGAATCGTTTCGCAAG

101 F  K  T  G  R  R  I  F  K  E  N  G  N  F  L  A  V  N  G  F
301 TTTAAGACCGGTCGACGCATCTTCAAAGAAAACGGCAACTTTTTGGCTGTAAACGGATTC

121 Y  Y  Y  G  K  S  I  E  I  R  C  A  G  C  R  L  T  I  V  K
361 TATTATTACGGAAAAAGTATCGAAATTCGATGCGCCGGTTGTCGTCTCACAATCGTCAAA

141 L  N  R  T  D  R  A  E  D  I  H  R  K  Y  S  P  E  C  Q  F
421 TTAAATCGAACCGATCGTGCCGAGGATATTCATCGAAAATATTCACCCGAATGCCAATTC

161 N  N  K  P  S  A  P  P  A  S  D  S  D  T  E  I  D  A  L  H
481 AATAACAAACCATCGGCTCCGCCCGCCAGCGACAGCGACACTGAAATTGATGCCCTACAC

181 V  N  I  E  E  N  D  C  D  D  S  D  R  V  T  Q  K  I  Y  P
541 GTCAATATTGAGGAAAACGACTGCGACGACTCGGATCGTGTTACTCAAAAAATTTATCCT

201 V  L  D  L  V  K  N  N  N  V  S  V  D  D  K  N  D  N  N  N
601 GTATTGGATTTGGTTAAAAATAATAATGTCAGCGTCGACGACAAAAACGATAATAATAAT

221 D  V  N  S  F  F  G  S  N  R  V  A  D  S  N  K  Y  T  Q  S
661 GATGTCAACTCTTTTTTCGGATCGAACCGTGTCGCCGACAGTAATAAATATACGCAATCG

241 V  S  T  A  V  G  E  D  D  R  F  C  K  I  C  F  E  N  E  R
721 GTCTCGACGGCTGTCGGAGAGGACGACCGTTTTTGTAAAATATGCTTTGAGAACGAGAGA

261 N  T  C  F  L  P  C  K  H  V  S  T  C  A  D  C  A  R  K  C
781 AATACGTGCTTTTTGCCTTGTAAACACGTCAGTACATGTGCCGATTGTGCTCGCAAATGT

281 K  V  C  C  I  C  R  M  K  I  K  E  R  L  E  V  Y  L  Q  *
841 AAAGTTTGTTGTATATGTAGAATGAAGATAAAAGAGCGATTAGAAGTTTATTTACAGTAA
```

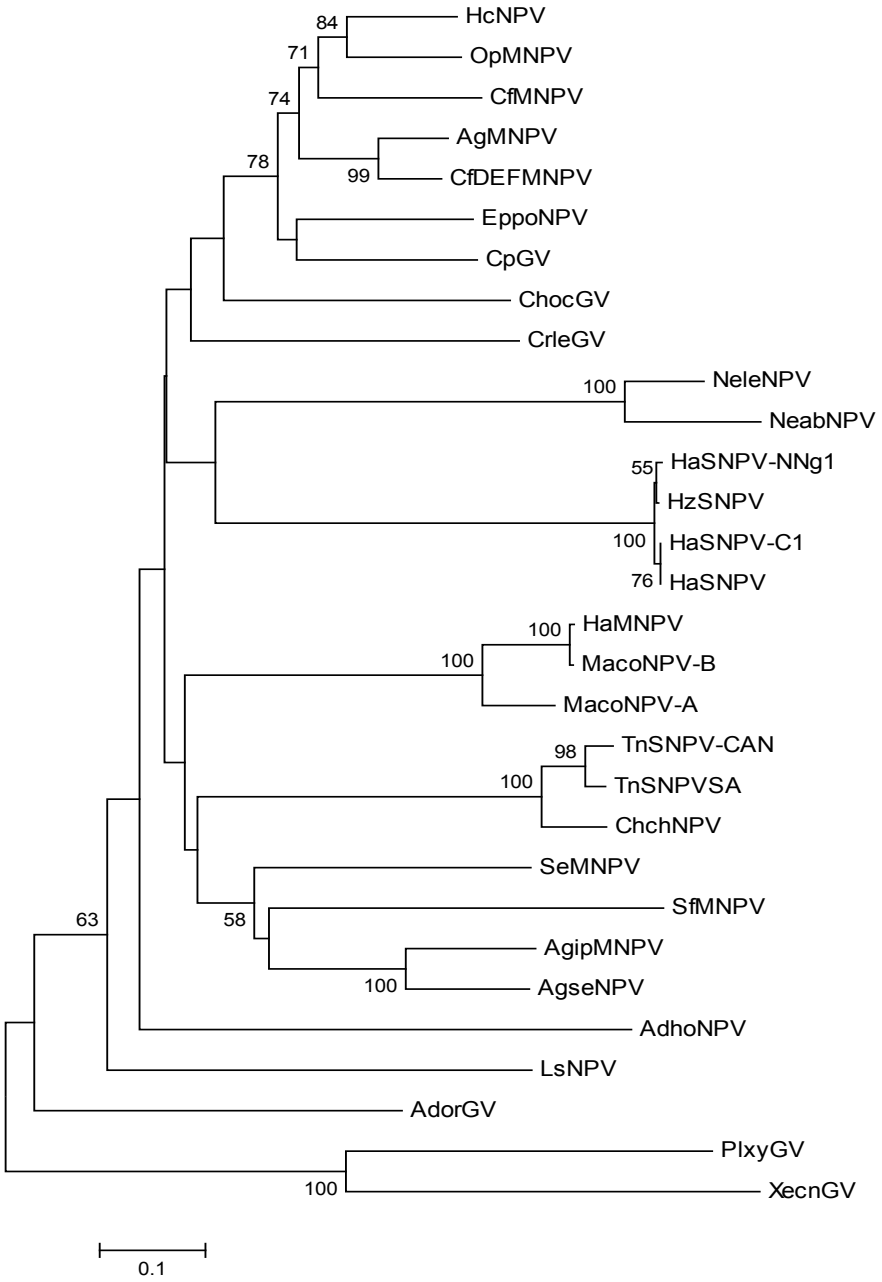
Appendix 3 Nucleotide and predicted amino acid sequence of the *iap2* gene from TnSNPV-SA. The amino acids of the single N-terminal BIR and the C-terminal RING finger motifs are underlined. The initiation and termination codons are in bold. Numbers on the left indicate nucleotide position and the amino acid position in the TnSNPV IAP2 protein.

```
ATG GAA AGT TAC GAG CAT TCC ATG CAA CGC TTC AGT AAC AGA CTT AAA ACT TTT GAC AGT AAA GAA TGG    69
 M   E   S   Y   E   H   S   M   Q   R   F   S   N   R   L   K   T   F   D   S   K   E   W    23

ATC AAT CCC TAT GTT CTA CCC ATA GAG TTG GCA CTG AAT GGA TTC TAT TAT TTG GGT ACT CGG GAT CAA   138
 I   N   P   Y   V   L   P   I   E   L   A   L   N   G   F   Y   Y   L   G   T   R   D   Q    46

GTC CGA TGT GCC TAT TGT AAA GTT GAG ATT TGC AAT TGG CAA CAG GAG GAC GTG GTG GAC CGC GAT CAC   207
 V   R   C   A   Y   C   K   V   E   I   C   N   W   Q   Q   E   D   V   V   D   R   D   H    69

AGG CAC TAT GCC CCT CAG TGT CCG TTC ATA AAA AAA CTG GAT GAG GAA AAA AAT AAG CAC GAT ACA TAT   276
 R   H   Y   A   P   Q   C   P   F   I   K   K   L   D   E   E   K   N   K   H   D   T   Y    92

GAA CGA AAT AAG TTT ATT TTC AAA TAT CCT AAT TTC GAT AAT GTA GTT AAG CGA ATC AAC AGC TTT CGT   345
 E   R   N   K   F   I   F   K   Y   P   N   F   D   N   V   V   K   R   I   N   S   F   R   115

AAC TGG CCT CGC GAT CGC ACC GAC TAC ATA GAT TTG GCG GAA GCG GGC TTC TTT TAC ACC GGC CTC GGC   414
 N   W   P   R   D   R   T   D   Y   I   D   L   A   E   A   G   F   F   Y   T   G   L   G   138

GAC AGG GTG AAA TGT TTC TAC GAA GGC TGT ACT CTG TCC GAT TGG AGT TGC GAT CGA GTA CCA TGG CAA   483
 D   R   V   K   C   F   Y   E   G   C   T   L   S   D   W   S   C   D   R   V   P   W   Q   161

CAG CAC GCT AGA TGG TTT CCC AAC TGT CGA TAC GTG CTG TTC GTC AAA GGC CGC GAC TAC GTG CAA CAG   552
 Q   H   A   R   W   F   P   N   C   R   Y   V   L   F   V   K   G   R   D   Y   V   Q   Q   184

GTT ATC TCT GAA TCG TGC GTT ATA CCA GCG CCG AAA CCC GAT CCT ATA CCG AAA CCA GCG GCG CCG ATT   621
 V   I   S   E   S   C   V   I   P   A   P   K   P   D   P   I   P   K   P   A   A   P   I   207

GCT CAA GAA ACA GCC AGC ACA TCT TCC CTA GAG GCT ACT CAA GAG TTA TCT AAA TCC GAA TGT AAA ATT   690
 A   Q   E   T   A   S   T   S   S   L   E   A   T   Q   E   L   S   K   S   E   C   K   I   230

TGT TTT AGC AAA GAG ATT AAC GCT TGC TAT ATA CCT TGC GGT CAT GTC GTG GCT TGT ATC GAG TGC GCT   759
 C   F   S   K   E   I   N   A   C   Y   I   P   C   G   H   V   V   A   C   I   E   C   A   253

TGG AGT ATT CCC GAC TGT CCA ATA TGT AGA AAA GCT TTT ACT AAT GTG ATC AAG ATT TAT TTT GGT TGA   828
 W   S   I   P   D   C   P   I   C   R   K   A   F   T   N   V   I   K   I   Y   F   G   *   276
```

Appendix 4 Nucleotide and predicted amino acid sequence of the *iap3* gene from TnSNPV-SA. The amino acids of the two N-terminal BIR and the C-terminal RING finger motifs are underlined. The initiation and termination codons are in bold. Numbers on the right indicate nucleotide position and the amino acid position in the TnSNPV IAP3 protein.



Appendix 5 Neighbour joining distance trees based on amino acid sequences of baculovirus IAP2 proteins. Numbers at the nodes indicate the bootstrap values from Neighbour joining (NJ) distance. Only boot strap values >50% are shown. For accession numbers of sequences used, see **Table 2.3**.

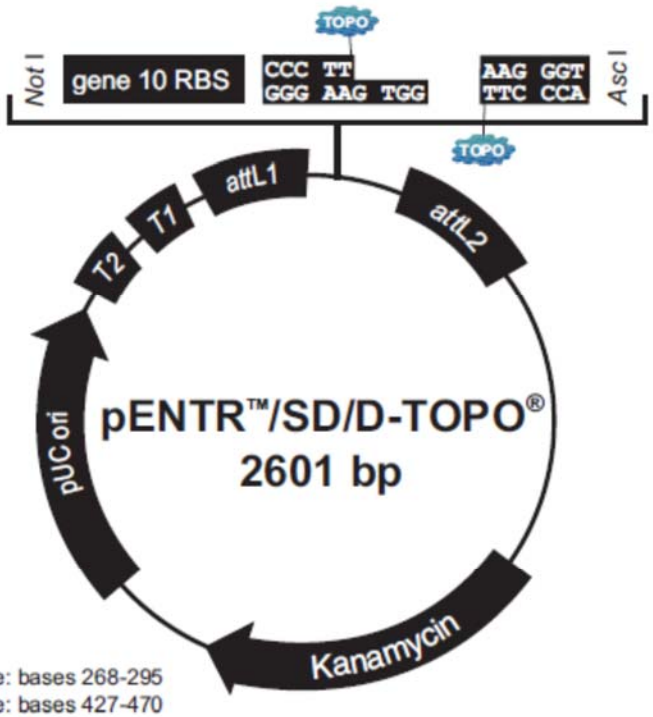


Appendix 6 Neighbour joining distance trees based on amino acid sequences of baculovirus IAP3 proteins with 2 BIR and 1 RING domain. Numbers at the nodes indicate the bootstrap values from Neighbour joining (NJ) distance. Only boot strap values >50% are shown. For accession numbers of sequences used, see **Table 2.3.**

Map and Features of pENTR™/SD/D-TOPO®

pENTR™/SD/D-TOPO® Map

The figure below shows the features of pENTR™/SD/D-TOPO® vector. **The sequence of pENTR™/SD/D-TOPO® is available from www.lifetechnologies.com/support or by contacting Technical Support (see page 35).**

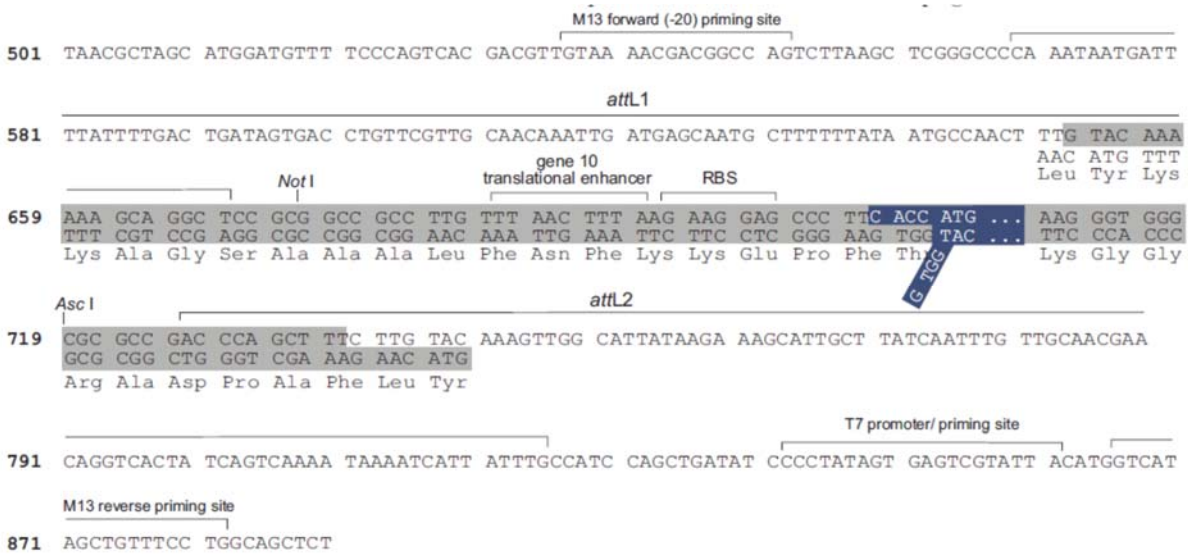


Comments for pENTR™/SD/D-TOPO®
2601 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
T7 gene 10 translational enhancer: bases 684-692
Ribosome binding site: bases 694-700
TOPO® recognition site 1: bases 701-705
Overhang: bases 706-709
TOPO® recognition site 2: bases 710-714
*att*L2: bases 726-825
T7 Promoter/priming site: bases 842-861 (c)
M13 reverse priming site: bases 866-882
Kanamycin resistance gene: bases 995-1804
pUC origin: bases 1925-2598

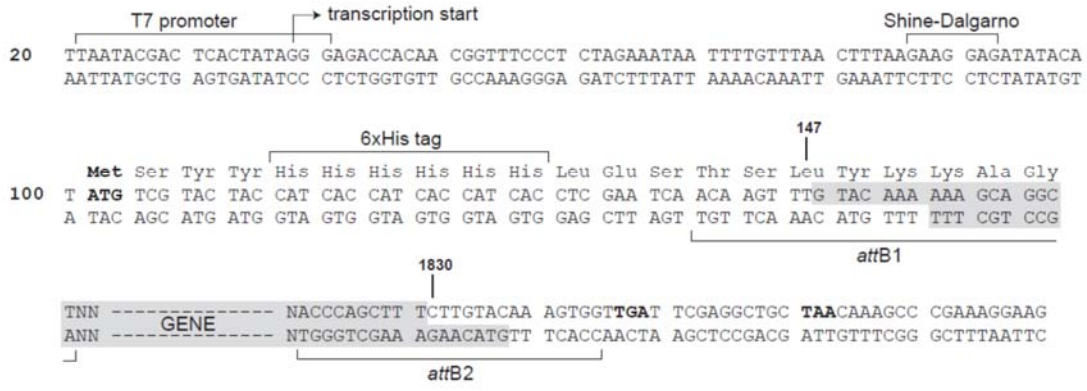
(c) = complementary sequence

Appendix 7 Map and Features of pENTR™/SD/D-TOPO®.



Appendix 8 TOPO® Cloning Site for pENTR™/SD/D-TOPO®.

invitrogen
by Thermo Fisher Scientific

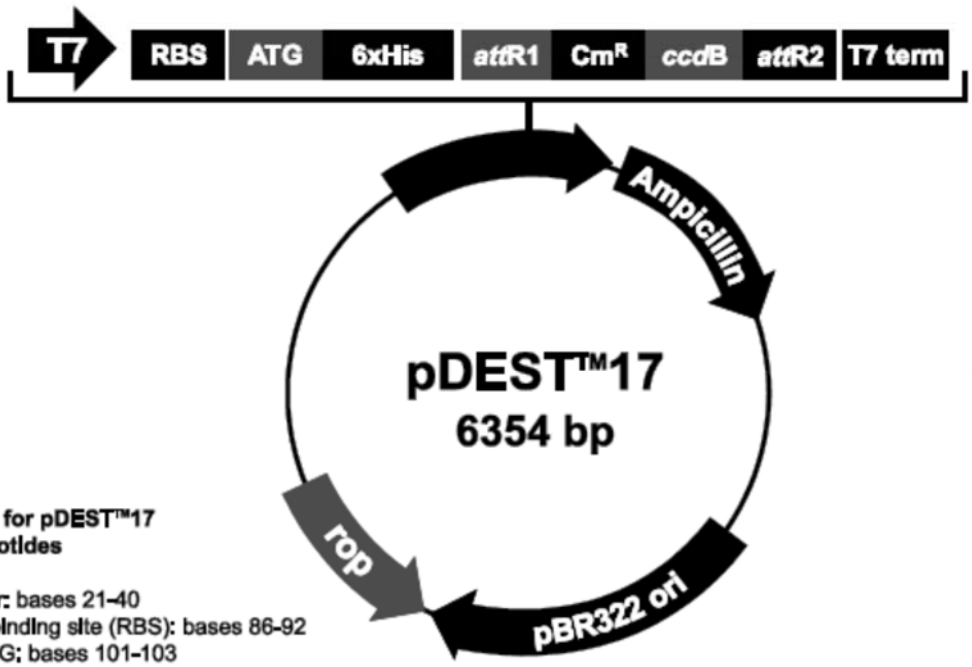


Appendix 9 Recombination Region of pDEST™17.

Map and Features of the pDEST™ Vectors, Continued

pDEST™17 Map

The map below shows the elements of pDEST™17. DNA from the entry clone replaces the region between bases 147 and 1830. The vector sequence for pDEST™17 is available from www.lifetechnologies.com or by contacting Technical Support (see page 31).



Comments for pDEST™17
6354 nucleotides

T7 promoter: bases 21-40
Ribosome binding site (RBS): bases 86-92
Initiation ATG: bases 101-103
6xHis tag: bases 113-130
attR1: bases 140-264
Chloramphenicol resistance gene (Cm^R): bases 373-1032
ccdB gene: bases 1374-1679
attR2: bases 1720-1844
T7 transcription termination region: bases 1855-1983
bla promoter: bases 2471-2569
Ampicillin (*bla*) resistance gene: bases 2570-3430
pBR322 origin: bases 3575-4248
ROP ORF: bases 4619-4810 (C)

C=complementary strand

Appendix 10 Map and features of the pDEST17 vector.