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Current Status of Genetically Modified Baculovirus Insecticide for Pest Control

(Status Terkini Racun Serangga Bakulovirus Terubah Suai Genetik untuk Kawalan Serangga Perosak)

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ABSTRACT

Baculovirus is an insect specific virus which is harmless to human. This feature has made it suitable to be applied as biopesticide. It has been used to control the insect pest particularly in agriculture sector for half a century and several success stories have been shared. Nevertheless, this insecticide still cannot compete with the synthetic pesticides owing to its slow killing speed and deficiency of compatible hosts. Genetically engineered baculovirus has improved pathogenicity against insect by incorporating foreign genes. These foreign genes encode neurotoxin, hormones, enzymes, and antisense DNA. Expression of these genes can enhance the insecticidal activities of the recombinant baculovirus. Nonetheless, the genetically modified baculovirus still has not been commercialised until today. This might be associated with the concern about the release of the genetically modified organism (GMO) into the environment as the environmental impact of the genetically modified virus is not well understood. Furthermore, it has been found to have effect on certain parasitoid. In conclusion, genetic modifications of the baculovirus have successfully improved its insecticidal activities but insufficient knowledge about its safety has limited its use in the field.

Keywords: Baculovirus insecticide; biopesticide; insecticidal gene; neurotoxin

ABSTRAK

Bakulovirus adalah virus khusus terhadap serangga yang tidak berbahaya kepada manusia. Ciri ini menyebabkan ia sesuai digunakan sebagai bio-racun perosak. Ia telah digunakan untuk mengawal serangga perosak terutamanya dalam sektor pertanian selama setengah abad dan beberapa kejayaan telah dikongsikan. Walau bagaimanapun, penggunaan racun serangga ini tidak dapat mengatasi racun serangga sintetik disebabkan kadar pembunuhannya yang perlahan dan kekurangan hos yang bersesuaian. Pengubahsuaian genetik bakulovirus telah menambah baik kepatogenan bakulovirus terhadap serangga dengan memasukkan gen asing ke dalamnya. Gen asing ini mengekodkan neurotoksin, hormon, enzim dan DNA antisens. Pengekspresan gen ini dapat meningkatkan aktiviti insektisid bakulovirus rekombinan. Namun demikian, bakulovirus terubah suai genetik masih tidak dipasarkan sehingga kini. Ini mungkin berpunca daripada kegusaran terhadap pelepasan organisma terubah suai genetik (GMO) ke persekitaran memandangkan kesan virus terubah suai genetik terhadap persekitaran masih belum difahami sepenuhnya. Tambahan pula, ia telah didapati memberi kesan terhadap parasitoid tertentu. Secara kesimpulannya, pengubahsuaian genetik terhadap bakulovirus telah berjaya meningkatkan aktiviti insektisid tetapi kurangnya pengetahuan tentang keselamatan terhadap penggunaannya telah mengehadkan penggunaannya di lapangan.

Kata kunci: Bakulovirus insektisid; Bio-racun perosak; gen insektisid; neurotoksin

Introduction

Insect pest is a real threat to agriculture. The global crop losses specifically caused by the insect pests alone was estimated at 10.8% with highest losses occurred in rice (Cramer 1967; Dhaliwal et al. 2015). Until recently, chemical pesticides remain the most preferred method for the control of the insect pest. This has led to the accumulation of chemicals in the soil and destruction of non-target organisms. The most persistent chemicals in the environment such as organochlorine can be accumulated in soil organisms especially invertebrates and transferred to other animals via the food chain (Iyaniwura 1991). Over

the century, thousands of biopesticides which include microbial insecticides have been discovered to find the substitutes for chemical pesticides. However, farmers still depend on chemical pesticides to eradicate pest in the field. There is growing concern regarding the negative impact of synthetic pesticides on the environment (Haase et al. 2015). At the same time, the public also worried about the effects of chemical pesticides on their health. In fact, non-pesticide applicators would get exposure through aerially applied pesticide. Furthermore, the death caused by chemical pesticides was estimated at hundreds of thousands annually (Kergunteuil et al. 2016). Baculoviruses do not infect plants, human or other vertebrates and even their infectivity is limited to the order of insects that they are isolated from. The use of baculovirus to control agriculturally important pest has been initiated a long time ago and several success stories have been recorded. For example, Nuclear Polyhedrosis Virus (NPV) was successfully controlling the velvet bean caterpillar, *Anticarsia gemmatalis*, in a soybean plantation in Brazil (Moscardi 1999). In China, baculovirus insecticide successfully controlled *Helicoverpa armigera* in cotton farming (Bonning & Nusawardani 2007). Baculovirus insecticide has been commercialised for the first time in the USA in 1975 under the name Elcar™ for the control of cotton bollworm *H. zea* (Bonning & Nusawardani 2007). Now, many novel strains have been isolated and commercialised around the world.

Slow killing speed is the main challenge for baculovirus based biopesticides to compete with synthetic insecticides. Other challenges include high production cost, instability in the field and registration difficulties (Beas-Catena et al. 2014). Efforts have been made to improve the efficiency of the baculovirus insecticides through genetic modifications. This review emphasises on the history and the latest development of the recombinant baculovirus insecticides especially nuclear polyhedrosis virus (NPV) for controlling lepidopteran pests.

GENETIC MODIFICATION OF BACULOVIRUS INSECTICIDAL HORMONES AND ENZYMES

Hormones are very important substances for insect especially for completing the metamorphosis stage in their life cycle. Changes in the hormonal balance will arrest the insect development as well as interfering with other living processes. This has made the insect hormone as a target for overexpression in baculovirus to kill the infected larvae faster or to reduce the damage it causes. The earliest gene coding for hormone inserted into baculovirus genome found in the literature was diuretic hormone of the tobacco hornworm, *Manduca sexta* (Maeda 1989). Interestingly, the recombinant baculovirus killed the tested larvae 20% faster than the wild type virus (Table 1). After the successful trial of using diuretic hormone, another hormone from *M. sexta*, eclosion hormone (EH) was inserted into baculovirus (Eldridge et al. 1992a). Unlike the previous hormone, EH hormone neither improved nor reduced the pathogenicity of the recombinant baculovirus.

Besides hormones, enzymes play an important role in insects for controlling hormone secretion as well as for shedding the old cuticles during ecdysis. The most reported enzyme inserted into baculovirus found in the literature is juvenile hormone esterase (JHE). JHE regulates the juvenile hormone (JH) titer in the larvae during metamorphosis. JH present in hemolymph at a normal level maintains the juvenile stage of the insect (Kamita & Hammock 2010). A drop in JH titer through its metabolism by JHE will give a way for the transformation of larvae into pupa. JHE has been overexpressed in baculovirus to reduce the JH level so that it would interfere with metamorphosis by inducing premature molting which in turn led to feeding cessation and reduced feeding damage (Eldridge et al. 1992b). Nevertheless, the recombinant baculovirus expressing JHE showed no improvement over wild type virus (Bonning et al. 1992). The possibility of increasing the virulence of recombinant baculovirus expressing JHE was studied further. The JHE was expressed under the modified viral promoter XIV (Eldridge et al. 1992b). Additionally, the gene encoding ecdysteroid UDP-glucosyltransferase, a naturally occurring enzyme which functions by blocking the insect molting and so inhibiting the functionality of the JHE, was deleted, or disrupted so that the high level of JHE could greatly reduce the JH level. Despite that, the infectivity of the baculovirus still did not improve. The author speculated that the insects might have responded to the high level of JHE which caused extreme reduction in the JH level by synthesising more JH to offset its loss. The effort was further continued with the use of mutated JHE by replacing lysine residues at position 29 (Lys-29) and 524 (Lys-524) with arginine (Bonning et al. 1997). Lys-29 and Lys-524 are amino acids involved in recognition of JHE by lysosomal protein degradation pathway. Replacement of these lysine residues with arginine might reduce the JHE degradation efficiency by lysosomal targeting. The resultant baculovirus was shown to reduce the larval

feeding damage to 50% compared to the wild type virus. It was reported later that the recombinant baculovirus killed the infected larvae approximately 25% faster than wild type virus (Bonning et al. 1999). The insecticidal activity of mutated JHE was further investigated by replacing lysine residue at position 204 (Lys-204) and arginine residue at position 208 (Arg-208) with histidine (El-Sheikh et al. 2011). These mutations caused the JHE to chelate Ni^{2+} and so its binding with JHE receptor of pericardial cells was blocked, thereby its removal from hemolymph was prevented. The infected larvae exhibited 40 to 50% and 70 to 90% mass reduction compared to control and wild type viruses, respectively.

TABLE 1. Killing speed improvement of the baculoviruses expressing hormones and enzymes with their respective hosts

Virus	Foreign gene	Host	Killing speed improvement[a]	References
BmNPV	Diuretic Hormone (DH)	*Bombyx mori*	20%	Maeda (1989)
AcMNPV	Eclosion Hormone (EH)	*Spodoptera frugiperda*	N.I	Eldridge et al. (1992a)
AcMNPV	Juvenile hormone esterase (JHE)	*Trichoplusia ni*	N.I	Bonning et al. (1992)
AcMNPV	Juvenile hormone esterase (JHE)	*Trichoplusia ni*	N.I	Eldridge et al. (1992b)
AcMNPV	ScathL	*Spodoptera frugiperda*	Third instar larvae, 66% Neonate larvae, 26%	Gramkow et al. (2010)
AcMNPV	Keratinase	*Spodoptera frugiperda*	Third instar larvae, 33% Neonate larvae, 48%	Gramkow et al. (2010)
AcMNPV	cathepsin B-like proteinase	*Helicoverpa armigera*	11%	Shao et al. (2008)
AcMNPV	chitinase	*Haemaphysalis longicornis*	N.C	Assenga et al. (2006)
AcMNPV	pyrimidine dimer-specific glycosylase (cv-PDG)	*Spodoptera frugiperda*	vHSA50L, 48% vHSA50LORF, 41%	Petrik et al. (2003)
AcMNPV	pyrimidine dimer-specific glycosylase (cv-PDG)	*Trichoplusia ni*	N.C	Petrik et al. (2003)

[a] The killing speed improvement is measured as the percentage of decreased LT_{50}/ST_{50} of recombinant virus over the wild type or control virus. N.I = no improvement; N.C = not calculated

Protease is an enzyme involved in the degradation of the cuticles during metamorphosis that has been engineered into baculovirus. Expression of protease genes in recombinant baculovirus were found to significantly reduced the time to kill the infected *Spodoptera frugiperda* larvae by degrading extracellular matrix protein as well as interfering with the phenoloxidase activity of the host (Gramkow et al. 2010). The recombinant baculovirus expressing proteases genes, cathepsin-L (ScathL) and Keratinase, showed median lethal time (LT_{50}) of 47 and 91 h, respectively, for 3rd instar larvae. The insecticidal activity of the baculovirus expressing ScathL against 3rd instar larvae was so far the highest baculovirus insecticidal activity recorded ever (Table 1). In neonate larvae, LT_{50} for baculovirus expressing ScathL and Keratinase genes were 77 and 54 h, respectively. Other study reported that the insertion of *Helicoverpa armigera* cathepsin B-like proteinase (HCB) into *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) reduced the median survival time of *H. armigera* larvae by 12 h compared to control (Shao et al. 2008). It was speculated that the faster killing rate was due to the hydrolysis of the basement

matrix of the tissues during the diffusion of viruses which eased their movement through the basement barrier to develop quicker systemic infection.

Aside from protease, chitinase is also involved in the shedding of the old cuticles during metamorphosis. An engineered baculovirus expressing a chitinase from the hard tick *Haemaphysalis longicornis* possessed insecticidal activity (Assenga et al. 2006). It was further reported that the application of baculovirus pesticide in chitinase solution could kill tick faster than pure chitinase solution or just baculovirus alone.

All the above-mentioned recombinant viruses focused on the direct elevation of the insecticidal activity. There was a study that reported indirect improvement of the insecticidal activity of the engineered baculovirus by the insertion of enzyme for the protection of the virus against UV radiation. The infectivity of baculovirus engineered with an algal virus pyrimidine dimer-specific glycosylase (cv-PDG) was improved by the 3-fold decrease in inactivation of budded virus by UV and the reduction of LC_{50} and LT_{50} values of the *S. frugiperda* larvae compared to the larvae infected with the control virus (Petrik et al. 2003).

TOXIN GENES

The first attempt to increase the virulence of baculovirus by the insertion of insect-specific neurotoxin gene from *Buthus eupeus* (BeIt) was not successful (Table 2) (Carbonell et al. 1988). The insertion of δ-endotoxin gene from *Bacillus thuringiensis* subsp. kurstaki HD-73 into polyhedrin-positive AcMNPV was also found to have no effect on baculovirus insecticidal activities (Merryweather et al. 1990). Moreover, the δ-endotoxin was also introduced into the *Hyphantria cunea* nuclear polyhedrosis virus (HcNPV) genome where it showed insecticidal activity but no comparison with wild type virus was reported (Lee et al. 1998). Later, an attempt to improve baculovirus insecticide using other toxin gene from *B. thuringiensis* (CryIA(b)), in truncated form, was unsuccessful (Martens et al. 1995). BT toxin engineered into baculovirus was shown to exhibit insecticidal activity only when fused into polyhedron (Chang et al. 2003). AcMNPV producing occlusion bodies that contained Cry1Ac crystal protein toxin showed high insecticidal activities compared to the wild type as much as 100-fold LD_{50} reduction and faster median survival time (ST_{50}). This represented the second highest baculovirus insecticidal activity reported (Table 2).

After the first trial of using insect-specific neurotoxin was not successful, the work was continued with the *Androctonus australis* Hector insect toxin (AaIT). The AaIT toxin is an insect specific toxin that could cause paralysis as well as death (Deng et al. 2019). AaIT gene was inserted into recombinant baculovirus under the control of p10 promoter (Stewart et al. 1991). Additionally, envelope surface glycoprotein (gp67) signal peptide sequence was added to the coding region to accommodate the toxin secretion. The insecticidal activity of the resultant virus was improved through the reduction of ST_{50} by 25b% and LD_{50} by 30%. Expression of AaIT gene under the control of baculovirus immediate early gene promoter (ie1) did not increase the insecticidal activity. However, the expression of the toxin gene under ie1 promoter occurred earlier than the expression under p10 promoter (Jarvis et al. 1996). Maeda et al. (1991) incorporated AaIT gene into *Bombyx mori* nuclear polyhedrosis virus (BmNPV) under the control of the polyhedrin gene promoter. The killing speed of the recombinant BmNPV increased by 40% compared to the wild type virus. Another type of insectotoxin from scorpion are the *Leiurus quinquestriatus hebraeus* excitatory toxin (LqhIT1) and depressant toxin (LqhIT2). Recombinant AcMNPV expressing LqhIT2 showed increasing insecticidal activity with effective paralysis time 50% (ET_{50}) higher than the wild type virus (Gershburg et al. 1998). LqhIT2 was also more effective than LqhIT1. Later on, LqhIT1 and LqhIT2 were combined together in an engineered AcMNPV and it exhibited 40% efficacy over the wild type virus and 22% over virus that expressed each toxin individually (Regev et al. 2003).

The AcMNPV expressing LqhIT2 was engineered further by Jinn et al. (2006) by inserting the LqhIT2 gene under the control of a modified early promoter pag (Ppag). Ppag was modified for early expression of the inserted gene in baculovirus by trimming the promoter sequence down to 90 nucleotides to form $Ppag_{90}$. The mortality of the infected larvae was elevated by 50% while their body weight and LT_{50} were significantly reduced.

The venom of the scorpion *Buthus occitanus tunetanus* (BotIT6) was the third scorpion venom incorporated into the baculovirus genome. The AcMNPV producing BotIT6 significantly accelerated the killing speed of the virus compared to the unmodified virus (Bel Haj Rhouma et al. 2005). A year later, another scorpion venom from the South Indian red scorpion, *Mesobuthus tamulus*, was inserted into AcMNPV (Rajendra et al. 2005). This toxin gene named as ButaIT, enhanced the recombinant baculovirus insecticide by reducing the ST_{50} and decreasing feeding damage caused by the *Heliothis virescens* larvae. The most recent insect specific

toxin inserted into baculovirus was *B. martensii* Karsch insect toxin (BmK IT). It was reported that the degree of apoptotic cell death in the midgut tissue of the larvae infected with recombinant virus expressing BmK IT was higher than in the parental AcMNPV at 8 h past infection (Fu et al. 2015).

TABLE 2. Killing speed improvement of the baculoviruses expressing toxin genes with their respective hosts

Virus	Foreign gene	Host	Killing speed improvement [a]	References
AcMNPV	BeIt	*Trichoplusia ni*, *Galleria mellonella* and *Sarcophaga*	N.I	Carbonell et al. (1988)
AcMNPV	δ-endotoxin	*Trichoplusia ni*	N.I	Merryweather et al. (1990)
HcNPV	δ-endotoxin	*Bombyx mori*	N.C	Lee et al. (1998)
AcMNPV	CryIA(b)	*Spodoptera exigua*	N.I	Martens et al. (1995)
AcMNPV	Cry1A(c)	*Plutella xylostella*	63%	Chang et al. (2003)
AcMNPV	AaIT	*Bombyx mori*	25%	Stewart et al. (1991)
AcMNPV	AaIT	*Heliothis virescens*	N.I	Jarvis et al. (1996)
BmNPV	AaIT	*Bombyx mori*	40%	Maeda et al. (1991)
AcMNPV	LqhIT1 & LqhIT2	*Helicoverpa armigera*	N.C	Gershburg et al. (1998)
AcMNPV	LqhIT1 & LqhIT2	*Heliothis virescens*	40%	Regev et al. (2003)
AcMNPV	LqhIT2	*Trichoplusia ni*	50%	Jinn et al. (2006)
AcMNPV	BotIT6	*Spodoptera littoralis*	N.C	Bel Haj Rhouma et al. (2005)
AcMNPV	ButaIT	*Heliothis virescens*	42.8%	Rajendra et al. (2005)
AcMNPV	BmK IT	Sf9	N.C	Fu et al. (2015)
AcMNPV	TxP-I	*Galleria mellonella* and *Trichoplusia ni*	N.C	Tomalski and Miller (1991)
HzNPV	TxP-I	*Helicoverpa zea*	40%	Popham et al. (1997)
AcMNPV	Poneratoxin	*Spodoptera frugiperda*	18%	Szolajska et al. (2004)
AcMNPV	Ba3	Sf21	N.C	Ardisson-Araújo et al. (2013)
BmNPV	Cit1a	*Bombyx mori*	26%	Ali et al. (2015)
AcMNPV	cry1-5 & AaIT	*Plutella xylostella* and *Spodoptera exigua*	*Plutella xylostella*, N.C *Spodoptera exigua*, 50%	Shim et al. (2013)
AcMNPV	cry1-5 & Bi-KTI	*Plutella xylostella* and *Spodoptera exigua*	*Plutella xylostella*, N.C *Spodoptera exigua*, 20% (7d.p.i); 32% (14d.p.i)	Choi et al. (2013)

[a] The killing speed improvement is measured as the percentage of decreased LT_{50}/ST_{50} of recombinant virus over the wild type or control virus. N.I = no improvement; N.C = not calculated

Apart from scorpion, the insect-specific toxin gene from mites was also being tried. The insectotoxin gene from the mite *Pyemotes tritici* is known as Tx-P1. cDNA encoding this gene designated as *tox34* was cloned and expressed in AcMNPV. The modified AcMNPV caused larval paralysis during infection (Tomalski & Miller 1991). Besides AcMNPV, tox34 gene was also engineered into *H. zea* nuclear polyhedrosis virus (HzNPV) (Popham et al. 1997). The expression of tox34 under the control of DA26 increased the virulence of HzNPV as shown by the reduction of ET_{50} by 40% less than wild type HzNPV. Toxin from ant was also subjected to insertion into baculovirus. The Poneratoxin venom is a neuropeptide produced by the ant *Paraponera clavate* (Szolajska et al. 2004). The average survival time of the S. *frugiperda* larvae infected with the genetically modified virus were reduced by 25 h compared to the larvae infected with the unmodified virus.

Venom from spiders is another target for enhancing the baculovirus insecticide. A venom with high insecticidal activity has been identified from the Mexican theraphosid *Brachypelma albicebs* Pocock (Ardisson-Araújo et al. 2013). The genetically enhanced AcMNPV induced 80% cell death at 48 h.p.i. The most recent study reported the use of insect-specific cytoinsectotoxin (Cit1a) from the venom of the central Asian spider *Lachesana tarabaevi* (Ali et al. 2015). The modified BmNPV virus not only caused early death of the infected cells but also significantly reduced the LT_{50} of the silkworm larvae compared to the control.

Two studies reported the insertion of the two insecticidal genes from different organism. The use of BT toxin for improving baculovirus insecticides was extended further through the combination with the AaIT toxin gene (Shim et al. 2013). The recombinant AcMNPV showed a significant increase in insecticidal activity as reported. Later, the combination of BT and the venom of bumblebee *Bombus ignites* in baculovirus was reported (Choi et al. 2013). The insecticidal activity was better than controlling the virus. It was also found that the low dosage was effective against *S. exigua* larvae, but more rapid insecticidal activity was shown in *Plutella xylostela* larvae.

RECOMBINANT POLYHEDRIN

Polyhedrin is a protein that forms baculovirus occlusion bodies. Incorporation of foreign protein or substances into polyhedrin can increase the infectivity of the baculovirus. It was reported that the incorporation of the envelope fusion protein GP64 from AcMNPV improved the insecticidal activity of the baculovirus by accelerating the mortality of the larvae and reducing the LC_{50} by 20% compared to the larvae infected with the control larvae (Table 3) (Shen et al. 2012). Fusion of nano-zinc oxide-binding peptides to the polyhedrin resulted in the recombinant virus more resistant to UV radiation with the infectivity of about 9-fold higher than the control virus (Li et al. 2015). Moreover, the half-life of the recombinant baculovirus binding nano-ZnO particles was also longer. Another study reported the fusion of the truncated *Agrotis segetum* granulovirus enhancin or truncated *Cydia pomonella* granulovirus ORF13 (GP37) to the C-terminal 95 amino acid of the polyhedrin yielding the recombinant viruses expressing the fusion protein embedded into the polyhedrin (Yang et al. 2017). This resulted in more virulent recombinant viruses with the reduction in LD_{50} of these two AcMNPV recombinants by 3- to 5-fold lower than that of the control virus. Recent study reported the use of the truncated enhancin from *Agrotis segetum* granulovirus and GP37 from *Cydia pomonella* granulovirus (Lei et al. 2019). Those proteins were fused to the N-terminal and middle domain of the polyhedrin envelope protein of AcMNPV. The recombinant virus was more infectious as indicated by the reduction of LD_{50} values to 3.9-fold to 7.4-fold lower than those of the wild type virus against the second and fourth instar of *S. exigua* larvae.

TABLE 3. Killing speed improvement of the baculoviruses expressing fusion proteins with their respective hosts

Virus	Fusion protein	Host	Killing speed improvement [a]	References
HearNPV	GP64	*Helicoverpa armigera*	20%	Shen et al. (2012)
AcMNPV	Nano-zinc oxide-binding peptides	*Spodoptera exigua*	N.C	Li et al. (2015)
AcMNPV	GP37	*Spodoptera exigua*	N.C	Yang et al. (2017)
AcMNPV	GP37 and EN4	*Spodoptera exigua*	N.I	Lei et al. (2019)

[a] The killing speed improvement is measured as the percentage of decreased LT_{50}/ST_{50} of recombinant virus over the wild type or control virus. N.I = no improvement; N.C = not calculated

GENE EDITING

Gene editing has been shown to increase the virulence of the modified virus. The deletion of the baculovirus ecdysosteroid UDP-glycosyltransferase (EGT) gene improved the insecticidal activity of the edited virus (Table 4) (O'reilly & Miller 1991). EGT functions in prolonging the larvae feeding time by delaying ecdysis after the infection. The deletion of this gene resulted in early feeding cessation and pupation within 1 to 2 d past infection (p.i) compared to the wild type infected larvae where they continued to feed until shortly before death at maximum 6 d p.i. Generation of baculovirus expressing mutated EGT gene using the clustered regularly interspaced short palindromic repeats-Cas9 system (CRISPR/Cas9) has also been found to increase the baculovirus infectivity. AcMNPV with edited EGT gene (AcMNPV-Δ49egt) showed decreased ST_{50} values to 12 h (Pazmiño-Ibarra et al. 2019). Overall, only gene editing involving EGT gene has been reported and the insecticidal activities recorded was not more than 22% (Table 4).

TABLE 4. Killing speed improvement of the edited baculoviruses with their respective hosts

Virus	Edited gene	Host	Killing speed improvement[a]	References
AcMNPV	EGT	*Spodoptera frugiperda*	22%	O'reilly & Miller (1991)
AcMNPV	EGT	*Spodoptera exigua*	10%	Pazmiño-Ibarra et al. (2019)

[a] The killing speed improvement is measured as the percentage of decreased LT_{50}/ST_{50} of recombinant virus over the wild type or control virus

OTHER SUBSTANCES

Other substances such as mitochondrial protein, antisense gene fragment, miRNA and growth-blocking peptide were also used to create recombinant baculovirus insecticides. The mitochondrial gene T-urfl3 was the only plant gene inserted into baculovirus for improving baculovirus biopesticides (Table 5) (Korth & Levings III 1993). The *T-urfl3* encodes URF13 which interact with T toxin or methomyl to permeabilize the plasma membranes of the insect cells. Interestingly, the recombinant virus expressing URF13 showed enhanced insecticidal activity even with the absence of T toxin or methomyl. *Trichoplusia ni* larvae injected with the recombinant virus dead within 60 h after injection as compared to 106 h for the larvae infected with the wild type virus. An antisense gene fragment *c-myc* codes for antisense transcripts which block the translation of a protein essential for larval growth and development has been inserted into baculovirus (Lee et al. 1997). The resultant virus exhibited enhanced insecticidal activities as characterized by lower feeding rate and survival as compared to the control virus. Wan et al. (2015) reported the use of growth blocking peptide to increase the efficacy of the baculovirus biopesticide (Wan et al. 2015). The engineered virus was found to be significantly reduce the weight of the infected larvae as compared to the larvae infected with the wild type virus. In addition, the recombinant AcMNPV killed the larvae approximately one day earlier compared to larvae infected with the wild type virus. Bantam sponge is another antisense transcript inserted into baculovirus. It contains multiple partial binding sites for bantam miRNA (Ran et al. 2018). Bantam sponge functions to suppress the Bantam level in the infected larvae so that the molting hormone 20-hydroxyecdysone (20E) level in the infected larvae will be increased. The increasing 20E level in the infected larvae will shortened their survival time by increasing its susceptibility to virus infection through inhibition of cellular activities and inducing cell apoptosis. The LC_{50} and LT_{50} of the recombinant virus were 1/40 and 1/2, respectively, of both the control and wild type virus.

SAFETY ASSESSMENT

The earliest safety assessment of the baculovirus biopesticide expressing foreign insecticidal gene on the non-target species was performed on the Social Wasp, *Polistes metricus* Say (McNitt et al. 1995). In this study, the colonies of *P. metricus* were fed with the larvae infected

with the recombinant virus expressing mite toxin (TxP-1). The ingestion of the toxin did not have any effect on the wasp. This was because the toxin was rapidly degraded and inactivated upon ingestion. This feature was highly desirable for the safety of the non-target species that fed on the infected larvae. This study also reported that AaIT was not toxic to the wasp.

TABLE 5. Killing speed improvement of the baculoviruses expressing foreign substances with their respective hosts

Virus	Foreign substances	Host	Killing speed improvement [a]	References
AcMNPV	URF13	*Trichoplusia ni*	43%	Korth and Levings III (1993)
AcMNPV	c-myc antisense	*Spodoptera frugiperda*	N.C	Lee et al. (1997)
AcMNPV	growth blocking peptide	*Spodoptera exigua*	16%	Wan et al. (2015)
AcMNPV	Bantam sponge	*Spodoptera exigua*	50%	Ran et al. (2018)

[a] The killing speed improvement is measured as the percentage of decreased LT_{50}/ST_{50} of recombinant virus over the wild type or control virus. N.C = not calculated

Safety assessment was also conducted in non-target Lepidopteran hosts. AcMNPV expressing neurotoxin gene did not show enhanced killing speed against the less susceptible host *Mamestra brassicae* but the yield of the virus was greater than susceptible host, *T. ni* (Hernandez-Crespo et al. 2001). It was also found that the recombinant AcMNPV did not kill the non-target larvae of *M. brassicae* faster than the wild type virus. Moreover, it was suggested that the wild type virus would outcompete the recombinant virus in the environment given that the yield of the wild type virus was more than the recombinant virus. Furthermore, a high-level recombinant virus was needed to infect *M. barssicae*. This implied that the transmission or amplification of the virus by *M. brassicae* was negligible unless the recombinant virus was present at high density.

The effect of genetically engineered baculovirus and Dimilin growth-regulating insecticide on the microbial communities in aquatic microcosms was also studied (Kreutzweiser et al. 2001). The simulation of aquatic microcosm was designed to consist of natural surface water, bottom substrates, and organic material. The wild type and recombinant baculovirus did not affect the aquatic microcosms unlike Dimilin. The viral DNA of both wild type and recombinant virus, however, were detected by PCR, accumulated on the bottom substrates 3 day after virus inoculations and persisted for 21 days. The author speculated that the DNA was present in the occlusion bodies (OBs) given that the crystalline protein shell of baculovirus OBs protected the virions from harsh environmental conditions which resulted in the persistency of the baculovirus in soil for years (Thompson et al. 1981). The implications of long-term persistence of genetically modified virus OBs in the bottom substrates are still unknown and hence further studies are required. The recombinant baculovirus insecticide expressing protease gene has effect on the survival of the *Cotesia marginiventris* (Cresson) parasitioid that parasitises the infected second instar larvae of *H. virescens* (Nusawardani et al. 2005). The number of survival parasitoid emerged from the host infected with the recombinant virus was significantly lower than the number of survival parasitoid emerged from the host infected with the wild type virus and from the uninfected control. Hence, the use of baculovirus expressing protease specifically AcMLF9. ScathL may pose risk to the parasitoids.

The safety assessment was also carried out with the mammalian cells. The expression of the inserted gene drive by the 39K promoter occurred in insect cells but not in the mammalian cells (Regev et al. 2006). Thus, the recombinant virus seems safe and may comply with biosafety requirement for genetically modified organism (GMO).

FIELD TRIAL

The field release of the genetically engineered baculovirus began in 1986 (Wood & Granados 1991).

The baculovirus has 80-bp insert as genetic marker which contained no start codon and therefore did not express any protein other than the parental virus. The first field trial of the genetically enhanced baculovirus insecticide was done with AcMNPV expressing the AaIT toxin gene (Cory et al. 1994). The virus was sprayed against third instar *T. ni* larvae on cabbage. The damage caused by the infected larvae was significantly lower than the untreated larvae. Additionally, the death of the recombinant infected larvae was accelerated to 10 to 15% earlier than insect infected with wild type virus though the figures were lower than that reported in the lab settings. The mortality peak in the recombinant treatment also occurred earlier than the wild type treatment. It was further reported that the insect infected with the recombinant virus fell onto the ground and did not liquefy as the wild type virus, thus the chances of the recombinant virus transmission to the other larvae may be less. Another field study conducted at the Oxford University Farm was reported 5 years later assessing the impact of recombinant AcMNPV virus to the target species, *T. ni*, and the non-target species, *Mamestra brassicae,* in the field setting (Hernandez-Crespo et al. 1999). The impact, however, could not be easily predicted as the susceptibility of *M. brassicae* recorded in the field was different with the laboratory assays. There are other factors that affect the susceptibility of the host to the viral infection which require further studies. So far this is the last field study of the recombinant baculovirus found in the literature.

Conclusion

The recombinant baculovirus insecticides has been developed for the past 30 years with the aim of increasing the killing speed of baculovirus insecticides and most demonstrated enhanced insecticidal activities. Nevertheless, the genetically engineered baculovirus is still not being commercialised or used in the field although numerous trials were reported. This might be due to the environmental concern associated with the release of genetically modified virus to the non-target species. Even though the impact has been studied, some additional factors that affect the baculovirus infectivity need to be studied further. In addition, the infectivity of the recombinant virus should be tested in more species, especially the native lepidopteran species. Comprehensive studies will provide better understanding on the behavior of the genetically enhanced baculovirus in the environment if it is going to be adopted in the field.

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