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by Expression of Synergistic Anti-Insect Scorpion Toxins

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Publication Summary (numbers)

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Refereed (published, in press, accepted)	1	7	2	10
Submitted, in review, in preparation	1			1
Invited review papers	1		1	2
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Books				
Master theses			1	1
Ph.D. theses			2	2
Abstracts				
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Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings		N.C.		1
Sabbaticals				
Postdoctorates		3		3

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

The cooperation between the two partners was synergistic since we exchanged technology ,expertise and facilities. _____

- ABSTRACT

Title: Enhancement of Baculoviruses Insecticidal Potency by Expression of Synergistic Anti-Insect Scorpion Toxins

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The extensive use of non-specific, hazardous, chemical insecticides demands the development of "healthier" alternative means for pest control. Insect-specific, baculoviruses expressing anti-insect toxin genes (from mites or scorpions) demonstrated in laboratory assays and field trials enhanced insecticidal activity and provided some protection from lepidopterous larvae to agricultural plantations. To utilize recombinant baculoviruses as commercial biopesticides in row crop agriculture, further increase in their speed of kill should be achieved and the reduction in crop damage should be comparable to the levels obtained with organic insecticides (the problem).

In this project we developed strategies to improve further the efficacy of recombinant baculoviruses which included:

I) Synergism among baculoviruses expressing different anti-insect toxins:

a) Synergism among two complementary anti-insect scorpion neurotoxins each expressed by a separate recombinant baculovirus, both regulated by the same or a different viral promoter.

b) Synergism among two complementary anti-insect scorpion neurotoxins expressed by the same recombinant virus, both regulated by the same or a different viral promoter respectively.

The above included two classes of pharmacologically complementary toxins: i) toxins with strictly anti-insect selectivity (excitatory and depressant); ii) toxins with preferential anti-insect activity (anti-insect alpha toxins).

c) Synergism among wild type viruses, recombinant baculoviruses and chemicals (insecticides and phytochemicals)

II) Identification of more potent toxins against lepidopterous pests for their expression by baculoviruses.

Our approach was based on the synergistic effect displayed by the combined application of pairs of anti-insect toxins to blowfly and lepidopterous larvae that resulted in 5 fold increase in their insecticidal activity without apparent increase in their anti-mammal toxicity (toxins LqhIT2 and Lqh α IT, LqhIT2 and AaIT, and Lqh α IT and AaIT (1).

Thus, we developed new concepts and produced a "second generation" of recombinant baculoviruses with enhanced potencies and speeds of kill comparable to classical insecticides. These achievements contribute to make these biopesticides a viable alternative to minimize the use of hazardous chemicals in pest control. Also, our project contributed new tools and model systems to advance the study of insect sodium channels.

-ACHIEVEMENTS

Significance

The significance of this proposal follows from the increasing demand for environmentally clean, diversified, bioinsecticides of fast and effective action against agricultural pests. The successful completion of our research goals brings the efficacy of the "second generation" of recombinant baculoviruses to potencies and speeds of kill comparable with classical insecticides, thus making these non-polluting biopesticides a viable alternative to minimize the use of hazardous chemicals in pest control.

The above includes:

(a) Novel strategies to implement recombinant baculoviruses as bioinsecticides with the following advantages:

- (1) Efficacy - which follows from a combined action of two complementary neurotoxins which affect, through different mechanism the insect most critical neurophysiological process namely sodium sodium conductance.
 - (2) Potency-which follows from the reduction in chemicals required by their co-application with recombinant or wild type baculovirus.
 - (3) Safety - which follows from the strict and well established insect selectivity of the specific toxins and the respective viruses.
 - (4) Long lasting - due to a simultaneous-combined presence of two toxins that strongly reduce the probability of a simultaneous acquired resistance.
- (b) Our approach of constructing single and double baculovirus recombinants encourages the future employment of a "cocktail" mixture of recombinant viruses affecting more than one target in the insect body, and the combined application of recombinant or wild type baculoviruses with chemical insecticides.
- (c) The present project stimulates the development of various insect model systems to study the relationship between the structure of the insect sodium channels and its function in the recognition of the insect selective neurotoxins. Such approach is of a high applicative as well as academic value.

Potential for application

The first generation of baculoviruses, AcMNPV expressing mainly the scorpion excitatory anti-insect toxin AaIT and depressant anti-insect toxin LqhIT2 showed improved

performance in the laboratory and were tested in field trials approved by EPA (2,3). Thus, the first field trial performed with a recombinant baculovirus expressing the excitatory toxin AaIT, showed about 30 % improvement in protection of a cabbage plantation from *Trichoplusia ni* larvae relative to the wild type virus treatment (4).

The achievements of this project respond to the need for fast-acting baculoviruses. We believe that the strategies of this study that improve the efficacy of the baculoviruses will be adopted by other research groups and industry providing new means for clean pest control (without harming other beneficial insect species and not posing dangers to human health and the environment).

Estimated economic benefits

Lepidopteran insects from the species of *Heliothis* and *Spodoptera* constitute major world pests due to their polyphagic nature. The world pesticide market is mature with a growth of 1-2 % per year from an estimated base of \$ 31 billion. From this market biologicals contribute about \$ 250 million with 60 % being *Bacillus thuringiensis*-based products. However, a variety of drivers are leading to a massive rate of increase of 25 % per year in biologicals. One of these drivers is transgenic crops which underwent a 450 % increase in acreage in a single year (6, and publications 5, 11). In many case crops need to be supplemented by other pest control strategies and microbials could be used rather than broader spectrum synthetic compounds.

Our highly specific recombinant baculoviruses fit perfectly with these economic trends.

Other Spin off benefits

As conceived, the mode of propagation of the recombinant baculoviruses is vertical rather than horizontal: The yield of orally infective recombinant virions (polyhedra yield) in the body of the virus-infected noctuidae larvae is much lower compared to the yield obtained with wild type baculovirus-infected larvae (7). Recombinant virus yields are negatively affected due to rapid desiccation of the insect by the toxin-induced spasms (7). In this respect the development of new recombinant baculoviruses of quicker action is expected to contribute additional higher safety levels (also see publication 9 and significance).

Details of cooperation

This proposal was based on a combined effort of American and Israeli laboratories, guided by the common objective to develop baculovirus-based insecticides to control American and

Israeli major noctuid agricultural pests. Our collaboration included the construction of different transplacement plasmids to transfer the toxin cDNAs to the genome of the baculovirus AcMNPV that infects the American pests *Heliothis virescens* and *Spodoptera exigua* and the Israeli pests *Heliothis armigera* and *Spodoptera littoralis* respectively. This goal required the interchange of:

- (I) Baculovirus-based transplacement plasmids, scorpion toxin cDNAs and synthetically constructed genes.
- (II) Technology-based on the experience of the American and Israeli groups with the AcMNPV expression system and the mode of action of the scorpion toxins.
- (III) Data - obtained from previous experience and on going experimentation on the expression of insect-selective excitatory, depressant and alpha scorpion toxins using single baculovirus expression vectors.
- (IV) Materials, as the American and Israeli isolated different AcMNPV recombinants for the assay of lepidopterous larvae.

The division of tasks will essentially consisted of:

- 1- Adaptation of the cDNA of the toxins LqhIT1, LqhIT2 and Lqh α T for cloning in the different baculovirus-based transplacement plasmids that were performed in Israel and of the synthetically constructed genes of the toxins in the US .
- 2- Expression of the toxin genes in AcMNPV (US and Israel).
- 3- Functional expression of the recombinant toxins tested in both laboratories.
- 4- Potency of recombinant viruses determined in the US on *T. ni* and *H. virescens* and in Israel on *H. armigera* and *S. littoralis*.
- 5- Promoter-toxin expression studies with LqhIT1 (Israel).
- 6- Synergistic effects among scorpion anti-insect toxins expressed by different baculoviruses (required exchange of different recombinant baculoviruses prepared by each side).
- 7- Synergism between baculoviruses and organic insecticides (the American side).

To achieve a coordinated evolution of the project the collaborators met during the second year of operation in Davis and performed joint testing of the double-toxin virus recombinants on *H. virescens*.

SECTION II

- BACKGROUND, SCIENTIFIC AND AGRICULTURAL RELEVANCE OF THE PROJECT

Lepidopterous species of the genera of *Heliothis* and *Spodoptera* constitute major agricultural pests. Public concern over the risks posed to human health and the environment by the extensive use of non-specific chemical insecticides against insect pests led to renewed efforts to develop alternative means for pest control based on natural insecticidal substances and microorganisms (6). Baculoviruses, strictly pathogenic to lepidopteran species, emerged as promising friendly control agents (6). However, a major obstacle for the implementation of baculoviruses in agriculture is their slow speed of kill of the target insect compared to chemical pesticides. Recombinant baculoviruses bearing "foreign genes" coding for insect hormones, anti-insect neurotoxins or genetically engineered baculoviruses with viral gene deletions, increased the speed of kill of lepidopterous larvae (6, 8). Special attention deserved baculoviruses that expressed anti-insect toxins derived from scorpion venoms, due to the selective paralytic activity of the toxins toward insects (reviewed in 4). The "first generation" of recombinant baculoviruses expressing excitatory (AaIT, LqhIT3) and alpha scorpion anti-insect (Lqh α IT) neurotoxin genes respectively, showed about a 30 % reduction in the time of kill of lepidopterous larvae and a reduction of 60 % in crop damage (6, 9, 10). However the implementation of recombinant baculoviruses as commercial biopesticides in row crop agriculture demand further increase in their speed of kill and in reduction in crop damage, comparable to the levels obtained with organic insecticides (the problem).

In this project we developed strategies to improve further the potency of recombinant baculoviruses which included:

I) Synergism among baculoviruses expressing different anti-insect toxins:

a) Synergism among two complementary anti-insect scorpion neurotoxins each expressed by a separate recombinant baculovirus, both regulated by the same or a different viral promoter.

b) Synergism among two complementary anti-insect scorpion neurotoxins expressed by the same recombinant virus, both regulated by the same or a different viral promoter respectively.

The above included two classes of pharmacologically complementary toxins: i) toxins with strictly anti-insect selectivity (excitatory and depressant); ii) toxins with preferential anti-insect activity (anti-insect alpha toxins).

c) Synergism among wild type viruses, recombinant baculoviruses and chemicals (insecticides and phytochemicals).

Our approach was based on the synergistic effect displayed by the combined application of pairs of anti-insect toxins to blowfly and lepidopterous larvae that resulted in 5 fold increase in their insecticidal activity without apparent increase in their anti-mammal toxicity (toxins LqhIT2 and Lqh α IT, LqhIT2 and AaIT, and Lqh α IT and AaIT (1).

II) Identification of more potent toxins against lepidopterous pests, for their expression by baculoviruses.

Thus, we aimed to produce a "second generation" of recombinant baculoviruses with potencies and speeds of kill comparable to classical insecticides, to make these biopesticides a viable alternative to minimize the use of hazardous chemicals in pest control.

-METHODOLOGIES AND MATERIALS

Our methodological approach was based on isolation of different recombinant baculoviruses through the application of recombinant DNA techniques, molecular virology, protein chemistry, pharmacology and the integration of a series of bioassays which combined recombinant baculoviruses and chemicals (see also accompanying papers).

The anti-insect toxins of choice were the excitatory AaIT and LqhIT1, the depressant LqhIT2 and LqhIT5 and the alpha Lqh α IT.

The baculovirus of wide spectrum against Lepidoptera, AcMNPV, was engineered to express one or a pair of anti-insect toxins.

-RESULTS

Do different promoters affect the potency of recombinant baculoviruses?

We constructed recombinant AcMNPV's in which the expression of the anti-insect toxin was directed by one of the following viral promoters that regulate the expression of the toxin gene in a temporal fashion through the viral infection:

a) An immediate early viral promoter, *ie1*, which also possesses a late regulatory element (11).

b) An early-late viral promoter transcription unit the P_{35} promoter (publication 2).

c) A very late viral promoter, P_{10} or polyhedrin (publications 1, 2).

The efficacy of two recombinant baculoviruses each expressing LqhIT1 under the control of the early $p35$ and very late $p10$ baculovirus promoters, respectively, was compared. ET_{50} (time to paralysis) values to first instar *H. armigera* larvae were 73 and 66 h, respectively (complete results are provided in publication 2). Thus, though the early expression of the toxin did not result in improved potency over the very late promoter-driven-expression, these experiments indicated that a minimal effective concentration of the toxin, required for insect-paralysis, was achieved by utilizing the $p35$ promoter (publication 2, *ibid.*)

We extended our studies by monitoring the effect of *cis* acting sequences on toxin expression directed by the immediate early promoter *iel*. Insecticidity of the viruses constructed (ET_{50}) was evaluated in *S. littoralis* (intra-haemocoelic injection, Fig. 1, appendix). Though all the recombinant viruses were more efficient than wild type AcMNPV, the *iel* promoter driven-AaIT expression did not improve the insecticidal activity compared to the $p10$ promoter-AaIT virus. Also, the modulation of the *iel* promoter activity by the *S. littoralis* NPV enhancer SR1 showed some improvement in efficiency compared to the *iel* promoter-AaIT alone.

Can the input of insecticide be reduced either by combined application of recombinant baculoviruses or wild type viruses and classical insecticides?

Recombinant or wild type baculoviruses were simultaneously applied with of chemical insecticides to *H. virescens* larvae and the time-to-kill of the larvae was monitored. For example, for neonate larvae, were recorded LT_{50} values of 73.3 and 51.4 h after application of endosulfan or endosulfan plus AcAaIT, respectively (publication 1). Application of methomyl or methomyl plus wild type AcMNPV resulted in LT_{50} values of 358 and 34.3 h, respectively. The LT_{50} values for the pyrethroid cypermethrin could be reduced from 124 h to 31.5 and 65.8 h by combination with AcAaIT or AcMNPV, respectively. More details are presented in publication 1 (including an pyrethrod-resistant Tobacco Budworm study).

Does the combination of two synergistic toxins (expressed by combined application of two baculoviruses expressing each a single toxin) increase the speed of kill compared to the application of one baculovirus expressing each a single toxin?

To test the effect of concomitant expression of synergistic toxins on the insecticidal activity of baculoviruses we co-infected *H. virescens* larvae with equal doses of vAcLIT1 and vAcLIT2 (bearing the excitatory LqhIT1 and the depressant LqhIT2 toxins), or vAcLIT2 and vAcL α IT (bearing the depressant LqhIT2 and the alpha Lqh α IT toxins, respectively). Co-infection of *H. virescens* with the pair vAcLIT1 and vAcLIT2 (previously designed AcLIT1.p10 and AcLIT2.pol, publication 2) resulted in an advance of the time to paralysis compared to the effect obtained with infection using each of the viruses (ET₅₀ values of 50.31 vs. 59.09 and 62.17 h, Table I and publication 13). A similar trend was observed when the combination of vAcLIT2 and vAcL α IT (previously AcL α 22, ref. 9) was used for infection (Table I). Taken together these and previous results obtained from co-injection of larvae with pairs of the above purified toxins (1) suggested that double-toxin viruses, engineered to express both toxin genes, may display improved insecticidal activity compared to their single-toxin-virus counterpart.

Does the combination of two or more synergistic toxins expressed by the same baculovirus increase the speed of kill when compared to the combined application of two viruses expressing each one a single toxin?

To study this effect we engineered the AcMNPV to express pairs of pharmacologically complementary anti-insect toxins obtaining vAcLIT1-IT2 and vAcL α -IT2. In vAcLIT1-IT2 the expression of the excitatory (LqhIT1) and the depressant (LqhIT2) toxin genes was directed by the very late p10 and polyhedrin baculoviral promoters respectively (publication 13, Fig. 1). In vAcL α -IT2 the expression of both the alpha (Lqh α IT) and the depressant (LqhIT2) toxin genes was directed by the viral polyhedrin promoter (ibid Fig. 1). Infection of insect cells with the above viruses resulted in steady-state levels of LqhIT1 and LqhIT2 or Lqh α IT similar to the levels of expression observed with the correspondent single-toxin viruses (publication 13, Fig. 2).

The double-toxin viruses vAcLIT1-IT2 and vAcL α -IT2 were utilized to orally infect *H. virescens* larvae. vAcLIT1-IT2 displayed improved ET₅₀ values over vAcIT1 and vAcIT2 (48.71 vs. 59.09 and 62.17 h, respectively, publication 13, Table I). Infection *H. virescens* larvae with vAcL α -IT2 showed intermediate ET₅₀ values respect to the single toxin-expressing viruses vAcLIT2 and vAcL α IT (55.39 vs. 62.17 and 47.69 h, respectively, publication 13, Table I).

To determine the generality of this enhanced efficiency we infected the semi-permissive host *H. armigera* and the non-permissive host *S. littoralis* (susceptible to BV infection via intrahaemocoelic injection, 9) with the above viruses.

Oral infection of *H. armigera* with vAcLIT1-IT2 resulted in a minor improvement respect to the single toxin viruses counterparts vAcLIT1 and vAcLIT2 (publication 13, Table II). vAcL α -IT2 α displayed ET₅₀ values of 64.21 vs. 69.52 and 77.67 h for vAcL α IT and vAcLIT2, respectively (ibid. , Table II). Those results were confirmed by injection of *H. armigera* larvae with budded viruses (publication 13, Fig. 3). A remarkable advance in the time to paralysis was observed when 3rd instar *S. littoralis* larvae were infected with either vAcLIT1-IT2 or vAcL α -IT2 as compared to the single toxin expressing viruses vAcLIT1, vAcLIT2 and vAcL α IT, respectively (publication 13, Fig. 4 A and B).

We concluded that that co-expression of two synergistic anti-insect toxins (mainly excitatory and depressant) improve significantly the efficacy of baculoviruses.

The effects of the plants

A limitation of the use of baculoviruses for pest control is that their efficacy is affected by host-plant chemicals such as of phenolic substrates and two foliar oxidative enzymes: peroxidase and polyphenol oxidase. Modifying these chemicals and enzymes during administration of baculoviruses can increase the efficacy of the viruses (see publications 3, 7 and 8). These studies provide the background to consider adverse effect of the plants to maximize effectivity of baculovirus application.

Identification of novel, more potent lepidopterous selective toxins

We purified a new insect selective toxin LqhIT5, that is more potent to lepidopterous larvae than other known insect selective toxins. The purification and peculiar properties of this toxin are described in publication 4.

-DISCUSSION

Minor differences in efficacy were reported in the response of Lepidopteran hosts infected with baculoviruses co-expressing genes from spider toxins (12).

Simultaneous production of the synergistic toxins LqhIT1 and LqhIT2 in AcMNPV-infected *H. virescens* larvae, yielded about 16-20 % reduction in effective time to paralysis compared to larvae infected with single-toxin vAcMNPVs. Moreover, our recombinant double-toxin virus vAcLIT1-IT2 and vAcL α -IT2 showed a remarkable advance in time to paralysis respect to wild type AcMNPV: about 50 and 40 % in *H. virescens* and , 30 and 35 % in *H. armigera* larvae, respectively.

Taken together our results support the concept that co-expression of two synergistic anti-insect scorpion toxins (mainly excitatory and depressant) improve significantly the efficacy of baculoviruses. The utilization of alpha toxin viruses provide further support for the concept, but they are not suitable for practical application due to the low toxicity to mammals displayed by this toxin (9). However, a good candidate is the recently isolated toxin LqhIT5 (publication 4).

We have shown that improved ET₅₀ values could be obtained by combined application of baculoviruses and low amounts of insecticides (including pyrethroids and Acetyl choline esterase inhibitors, publication 1).

Our results further contribute to promote the utilization of baculoviruses in non-polluting control of insects, reducing the amount of chemical pesticides. Furthermore, they suggest that combinations of other synergistic anti-insect toxins may result in further improvement in the efficacy of baculoviruses. Finally, though we utilized mainly very late baculoviral promoters in this study, construction of baculoviruses that could produce earlier in infection the above toxins (or analogous) by using early baculovirus promoters, constitutive promoters or either combinations of promoters may yield additional benefit enhancing the efficiency of these bioinsecticides.

The present project stimulates the development of various insect model systems to study the relationship between the structure of the insect sodium channels and its function in the recognition of the insect selective neurotoxins (publications 6 and 10). Such approach is of a high applicative as well as academic value.

-SCIENTIFIC IMPLICATIONS

During this project several categories of synergism were discovered:

1. Synergism between scorpion anti-insect toxin-gene products expressed by separate or the same baculoviruses
2. Synergism among chemical pesticides and baculoviruses.

The synergism is exerted at the level of the insect sodium channels and it implies that different combinations of toxins and chemicals may modulate differently the biological activity of the channel. These may contribute tools to elucidate the molecular mechanism of channel activity.

The discovery of novel toxins more active towards Lepidoptera contribute to provide new tools for the study of the structure and variability of insect sodium channels and for the understanding the relationship between the structure of the toxin and its specificity.

The interaction of baculoviruses with plant phytochemicals may promote further basic research to understand what are the viral components sensible to plant chemicals and how will be possible to manipulate it to obtain further improvement in insecticidal activity.

This knowledge may be projected on plant interactions with other insect pathogens as well. Finally, the present project stimulates the development of various insect model systems to study the relationship between the structure of the insect sodium channels and its function in the recognition of the insect selective neurotoxins. Such approach is of high applicative and academic value.

-POTENTIAL IMPACT ON AGRICULTURE

The significance of this proposal follows from the increasing demand for environmentally clean, diversified, bioinsecticides of fast and effective action against agricultural pests. A successful completion of our research goals will bring the efficacy of the "second generation" of recombinant baculoviruses to potencies and speeds of kill comparable with classical insecticides, thus making these environmentally friendly biopesticides a viable alternative to minimize the use of hazardous chemicals in pest control.

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APPENDICES

Fig. 2. Efficiency of recombinant baculoviruses expressing the AaIT toxin under the control of various promoters. The AaIT gene was placed under the control of the early promoter iel (with or without the enhancer element SR from *S. littoralis* NPV) or the very late promoter p10. Paralysis and mortality of infected *H. virescens* larvae were recorded as described before.

Abbreviations

AaIT- Anti-insect selective excitatory toxin from the scorpion *Androctonus australis* Hector.

AcNPV = AcMNPV-*Autographa californica* multiple nuclear polyhedrosis virus = *A. californica* M nucleopolyhedrovirus.

BTI-TN-5B1-4- *Trichoplusia ni* insect cell line.

CPU- contractile paralysis unit.

ED- effective dose.

FPU- flaccid paralysis unit.

LC- lethal concentration.

LD- lethal dose.

LT- lethal time.

Lqh α IT- alpha anti-insect toxin from the scorpion *Leiurus quinquestriatus hebraeus*.

LqhIT1, anti-insect selective excitatory toxin from the scorpion *Leiurus quinquestriatus hebraeus*.

LqhIT2- anti-insect selective depressant toxin from the scorpion *Leiurus quinquestriatus hebraeus*.

AaIT- anti-insect selective toxin from the scorpion *Androctonus australis Hector*.

PCR- polymerase chain amplification-based method.

PT- paralysis time.

PU- paralytic unit.