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FINAL REPORT TO

COTTON RESEARCH AND DEVELOPMENT CORPORATION

For the Project

"GENETIC ENGINEERING OF THE *HELIOTHIS* NUCLEAR POLYHEDROSIS VIRUS "

CSE8C

Commonwealth Scientific and Industrial Research Organisation

Division of Entomology

PROJECT DETAILS

Project Number	CSE8C
Project Title	GENETIC ENGINEERING OF THE *HELIOTHIS* NUCLEAR POLYHEDROSIS VIRUS
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Division/Branch	Entomology
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Commencement Date	01/07/1989
Completion Date	30/06/1992

Aims: The aim of the project was to produce more virulent strains of a naturally occuring entomopathogenic nuclear polyhedrosis virus of *Heliothis* species.

Industry Significance: The novel strains of virus produced in this project will provide new biological control agents for use against *Heliothis*, a major pest of cotton. Creation of such novel forms of the virus will be achieved by genetically manipulating the genes of the virus and inserting foreign genes whose products have insecticidal activity.

SUMMARY OF EXPENDITURE

Budget

	Salary $	Travel $	Operating $	Capital $	Total $
Approved 1989/90	39739	600	4000	-	44339
Approved 1990/91	42701	2040	5300	-	50041
Approved 1991/92	51772	600	6400	-	58772

The project employed three staff over its duration; two research scientists were employed, one under the current grant (Dr P. Christian) and one under an agreement with ICI Australia (Dr P. Simpson). A half-time experimental scientist, Ms Carol Fernon, was employed under a grant from the Grains Research and Development Corporation (Oilseeds Research Council).

The operating budget from this grant CSE8C was utilised in the molecular characterisation of the HaNPV genome.

FINAL REPORT

PROGRAM OVERVIEW

The Division of Entomology initiated its program "Genetic engineering of Heliothis NPVs" in January 1988. The program aims to modify the genome of *Heliothis* NPVs to generate more rapidly acting and/or more virulent virus strains. These viruses will then be available as biological control agents against *Heliothis* species. Towards these ends the program can be divided into three distinct phases with timescales shown in parentheses:

1) Research and development of recombinant viruses (1988-1992)

2) Pre-commercial development of recombinant viruses (1992-1995)

3) Commercialisation of recombinant viruses (1995-1999)

The initial research and development phase of the program ended in March 1992. It comprises a core project that aims to develop the techniques for manipulating the genomes of *Heliothis armigera* NPVs (HaNPVs) and a cluster of associated projects on genes that express insecticidal proteins (see Figure 2). Genes isolated in these associated projects will be inserted into the HaNPV genome to produce novel viruses with increased insecticidal activity. The core project and associated projects (with start dates in parentheses) have been:

Core Project

Genetic engineering of Heliothis NPVs (1988)

Associated Projects

Isolation of insect toxin genes (1989)

Isolation of the gene for juvenile hormone esterase (1989)

Isolation of the gene(s) for juvenile hormone receptor (1989)

It is the core project to which funds from CSE8C have contributed.

RESEARCH PROPOSAL SUMMARY

Insect pests are one of the major causes of production losses in most field crops throughout the world. Cotton in Australia are no exception, and are affected by a wide range of insect pests. Their major pests are the two budworms *Heliothis armigera* and *H. punctigera*. In the past outbreaks of these pest species have been controlled by the

conventional use of chemical insecticides. Recently, however, there have been repeated occurences of resistance to chemical insecticides arising in populations of *H. armigera*. Therefore, for the cotton industry to maintain its current levels of production and profitability it is necessary that new ways to control *Heliothis* species be found. One such form of control is biological control. Biological control relies on the use of natural predators, parasites and pathogens to control the pest species.

Biological control has three major advantages over the use of conventional insecticides as control agents. Firstly, it can be highly specific against the target pest species while remaining harmless to beneficial species. Secondly, biological control agents are less likely to engender resistance in the way as chemical insecticides; and finally, they leave no environmental residues toxic to non-target species. In addition, they can have a large degree of persistence and remain within a pest population from one generation to the next.

One such biological control agent is the Nuclear Polyhedrosis Virus (NPV) of *Heliothis*. Although commercial preparations of the virus have been used as biological control agents of *Heliothis*, they are unreliable and inefficient. This is because the virus is relatively unstable in existing commercial formulations and infected larvae continue to feed for a number of days after the initial exposure. Such a delay between exposure and effect allows crop damage to continue, thus reducing the yield. Furthermore, the virus in these commercial preparations is ineffective against medium to large larvae.

The current project, i.e. the core project within the overall program, aimed to genetically engineer an NPV of *Heliothis* (HaNPV) to increase both its speed of action and its ability to control medium/large larvae. The approach we have used aimed to modify the polyhedrin gene of HaNPV to allow insertion of foreign genes. The polyhedrin gene has been selected because it is expressed at high levels during the viral replicative cycle. In addition, precedents already exist for the manipulation of the polyhedrin gene and the promoter sequences that control its expression. The genes from the related NPVs of the lepidopterans *Autographa californica* and *Bombyx mori* have been cloned, analysed and modified to express the genes for numerous mammalian, bacterial and viral proteins (Luckow and Summers, 1988; Maatsura *et al.*, 1987; Maeda *et al.*, 1985). In addition, it has also been shown that a duplicate copy of the promoter can be inserted into baculovirus genomes with no detrimental effect on the expression of either the polyhedrin gene or the gene linked to the second copy of the promoter (Takehara *et al.*, 1988).

A number of workers have been attempting to increase the insecticidal activity of AcMNPV and BmNPV by the insertion of foreign genes. Until recently however, most of these attempts had given only slight improvements in speed of action. The first reports of viruses that give significantly improved levels of control have now been published. In both instances genes encoding insect toxins were introduced into the baculovirus genome; one was a neurotoxin from the mite *Pyemotes tritici* (Tomalski and Miller, 1991), and another derived from the venom of the North African scorpion, *Androctonus australis* (Stewart et al., 1991). In both cases, expression of the toxin from the foreign gene contained in the baculovirus genome reduced in the time taken to paralyse or kill the insect host.

Over the course of the last three years several projects have been initiated in the Division's Molecular Biology and Physiology Section that will produce genes which, after insertion into HaNPV, are likely to increase its insecticidal activity (see Figure 2). In addition, further toxin genes have been made available to the project through our collaboration with ICI (Australia) and ICI Agrochemicals (UK).

Therefore, the contribution of the current (core) project to the overall program was to:

(1) to characterise the HaNPV genome by molecular cloning, to isolate the polyhedrin promoter and to insert it into a suitable region of the genome to allow expression of foreign genes,

(2) to establish an insect cell culture system that will support the growth of HaNPV and to develop a system whereby recombinant viruses that express foreign genes can be generated and selected,

(3) to insert genes encoding insecticidal proteins into the HaNPV genome alongside a second copy of the polyhedrin promoter. To isolate the recombinant viruses containing these genes and assess their pathogenicity against *Heliothis* species. Isolation of these genes will be carried out by other projects.

The major milestones in the core project are summarised in Figure 3.

SUMMARY OF PROJECT RESULTS AND PROGRESS

Overall the program has made good progress over the last three years. All of the Major Milestones in the core project (see Figure 1) were reached by, or close to, the originally predicted dates. The only delays that have occurred have been in the isolation of suitable insert genes and these delays are largely explained by their later starting date. We are still on target to have one and probably multiple insert genes available to the program in the near future.

In March 1992 the program entered its second, pre-commercial development phase. Funding for this phase of the program has been secured from the Cotton Research and Development Corporation (CSE29C - "Genetic Engineering of Heliothis Nuclear Polyhedrosis Viruses: Pre-Commercial Research"), our commercial partners, ICI (Australia) and ICI (Agrochemicals) and the Grains Research and Development Corporation. Due to the wider scope of the research to be conducted in this phase of the project (see Appendix 1), the number of staff on the core project has been increased from 2.5 to 3.5. Dr Christian continues to supervise the core project.

Establishment of Facilities and Staff

During the initial six month's of the project the establishment of a dedicated cell culture facility was completed in the Division. Dr Christian travelled to Queensland to acquire

virus stocks from Dr R. Teakle (Queensland Dept. Primary Industry, Indooroopilly), and while in the USA obtained cell culture material from Dr A. McIntosh (USDA, Columbia).

By the end of 1988, all facilities and materials necessary to commence the project had been obtained. Dr P. Simpson was appointed to the project in March 1989 and Ms Carol Fernon in January 1989.

Progress of the Project

1.1 Cloning and Mapping of HaNPV

Several naturally occurring isolates of HaNPV were obtained from Dr Teakle and passaged through *H. armigera*. These viruses were tested for their pathogenicity against *Heliothis* larvae. One isolate (A44 - originally isolated from *H. armigera* collected on cotton at Brookstead, Queensland in 1974), was selected for further genetic characterisation as it had a pathogenicity broadly comparable with ELCAR (the *H. zea* NPV previously marketed in Australia for control of *Heliothis*).

Restriction profile analysis revealed that A44 was composed of several viral strains and representatives of these strains were subsequently purified by limiting-dilution assay in *H. armigera* larvae. All strains isolated from A44 have been compared by restriction profile analysis with ELCAR and were found to be distinct. The two most common strains (termed A44EA and A44EB) were tested for pathogenicity and virus production *in vivo.* As A44EB proved to be slightly better with respect to the above parameters it was selected for further characterisation.

All *EcoR*I fragments from the genome of A44EB were cloned into bacterial plasmids for restriction mapping of the genome. All sites for the restriction enzymes *Eco* RI, *Bam* HI, *Xho* I and *Hind* III were mapped.

1.2. Developing Cell Culture Systems

The prerequisites for this part of the core project were to:

- develop a cell culture system in which HaNPVs can replicate and the virus growth be quantified and,

- develop a system for the generation and isolation of recombinant viruses.

Cell lines derived from *H. armigera, H. zea* and *H. virescens* were obtained from Dr A. McIntosh (Biological Control of Insects Research Laboratory, USDA, Columbia, Mo.) and established in the Division's cell culture facility.

Each of these lines were screened for their ability to replicate ELCAR (used as a positive control), the original A44 isolate (A44WT), and subsequently A44EA and A44EB. It was

found that only the *H. zea* line is capable of replicating these viruses. In addition, A44EB grew much more rapidly and to higher titres in these cells than A44EA. We also found that the *H. zea* cell line could readily be transfected with DNA from A44EB and viable virus recovered.

Once the growth of HaNPVs of interest had been established *in vitro*, assay systems to quantitate the amount of virus in any given preparation needed to be developed. Unfortunately, the *H. zea* cell line did not produce monolayers upon which it was possible to develop a plaque assay. To obviate this problem, a number of clonal cell lines were derived from the parental *H. zea* line in the expectation that one would provide a more amenable plaquing system. Several of those cloned lines proved to be suitable for plaque assays. In addition, techniques for measuring tissue culture infectious dose ($TCID_{50}$) were also developed.

To test the utility of our reporter gene (esterase-6 from *Drosophila melanogaster*) we used the *A. californica* expression system to generate recombinant viruses carrying it. Analysis of esterase-6 expressed in this system formed a base-line against which our HaNPV recombinants expressing esterase-6 could be gauged (see 1.4 below).

Using the *A. californica* expression system, the relative merits of selecting recombinants by plaque hybridisation (Smith and Summers, 1987) or limiting end-point dilution (Fung *et al.*, 1988) were also assessed. We found that the latter of these methods was much more efficient, and subsequently employed it in the isolation of recombinant HaNPVs (see Section 1.4 below).

More recently the emphasis in this part of the project has been changed to investigate large-scale production of HaNPV in *in vitro* systems. Considerable progress has also been made in the selection of serum-free media for the growth of *Heliothis* cells in culture and cells have also been adapted to growth in suspension culture. Studies are currently underway to assess the production of virus in these systems.

1.3 Isolating the Polyhedrin Gene

To identify and isolate the polyhedrin gene from HaNPV, a clone of the polyhedrin gene from *A. californica* was used to select clones from plasmid libraries constructed from A44EA and A44EB genomic DNAs. Once identified, restriction fragments containing the 5' coding sequence and promoter region of the A44EA and A44EB polyhedrin genes were subcloned into the phagemid pTZ and their nucleotide sequences determined.

The nucleotide sequences of the A44EA and A44EB polyhedrin genes and promoters were found to be very closely related to that of ELCAR, i.e less than 1% sequence diversity. This relationship was much closer than we had expected from the restriction profile analysis. After consultation with our commercial partner (ICI Australia), it was decided that further investigation of the genetic relatedness of HaNPV isolates from various localities was required to establish the levels of variation that were present in field populations of *H. armigera*. Polyhedrin genes from viral isolates from South Africa, China

and India were sequenced and, despite the wide geographical origin of these isolates, little nucleotide variation in the polyhedrin gene and promoter was found among them.

Discussions with patent lawyers at ICI (UK) have led to the conclusion that despite the high degree of genetic relatedness between the HaNPV isolates, patent restrictions on the use of Australian HaNPVs will probably only involve those associated with the broad claims made in the original Smith and Summers (1988) patent on the use of baculoviruses as expression vectors. Having taken account of these discussions, further work on the development of the HaNPV expression system was based around the clonal isolate A44EB (see also Section 1.1).

1.4 Characterisation of the polyhedrin promoter

To characterise the activity of the polyhedrin promoter, plasmid constructs were made using standard *in vitro* manipulation techniques, i.e. restriction fragment sub-cloning and site-directed mutagenesis, to provide plasmids into which foreign genes can be inserted. These constructs were made using the 8kb *Xho* I fragment from A44EB that contains the whole of the polyhedrin gene and approximately 7kb of flanking sequences. Plasmid constructs were initially produced in which either:

(a) the polyhedrin gene itself is "deleted" (polyhedrin is not expressed due to mutation of the ATG) and a unique *Bam* HI insertion site generated 3' of the polyhedrin promoter, or

(b) a second copy of the polyhedrin promoter has been introduced 5' of the endogenous polyhedrin promoter such that foreign genes can be inserted under the control of this second promoter, independently of the endogenous promoter.

The deletion constructs from a), hereinafter referred to as pol- constructs, can be used to generate recombinants which would not persist in the field environment. The duplication constructs from b), hereinafter referred to as pol+ constructs, would generate recombinants that are able to persist in the field.

Prior to the insertion into the HaNPV genome of genes whose products may have insecticidal effects, it was necessary to establish the efficacy of the polyhedrin promoter systems. Using the pol- constructs recombinant viruses were generated that express either esterase-6 or beta-galactosidase from *E. coli*. Quantification of expression levels from these recombinant viruses demonstrated that the HaNPV polyhedrin promoter system was comparable with the *A. californica* system.

PROGRESS IN ASSOCIATED PROJECTS

Central to the ability to generate recombinant HaNPVs with increased insecticidal activity is the availability of cloned genes that produce proteins with insecticidal activity. At the

start of the current program we identified two particular classes of genes that produce proteins that may have these characteristics:

- insect-specific neurotoxins,

- proteins that modify the levels of hormones critical in insect development.

With respect to the neurotoxins we were able to identify one toxin in particular that had many of the properties required, namely the toxin from the parasitic wasp *Bracon hebetor*. In the second class of genes we have identified two proteins that modify the level of juvenile hormone, namely juvenile hormone esterase (JHE) and juvenile hormone receptor (JHR).

The strategy for isolating both classes of genes is essentially the same:

i) purification of the protein and production of antisera against isolated protein (2.2)
ii) N-terminal sequencing of the purified protein and synthesis of homologous oligonucleotides (2.3)
iii) screening of cDNA/genomic libraries and isolation of gene encoding insecticidal protein (2.4)

(Numbers in brackets indicate activities summarised in Figure 2).

Work on projects to isolate the *Bracon* toxin, juvenile hormone esterase (JHE) and juvenile hormone receptor (JHR) all began in early 1989. All projects have progressed relatively well and all have reached the stage of screening cDNA libraries with oligonucleotides and/or antibodies. Current predictions suggest that the gene encoding the *Bracon* toxin and JHE will be ready for insertion into the HaNPV vector system by mid 1992.

Furthermore, two additional genes with the appropriate characteristics have been made available to the program by ICI (Agrochemicals). The vector system as presently designed allows for the rapid insertion of these genes into the HaNPV genome, and work is current;y underway to insert these genes into the HaNPV genome.

PUBLICATIONS ARISING FROM CURRENT PROJECT

Christian,P.D. and Oakeshott,J.G. (1988). The potential of genetically engineered nuclear polyhedrosis viruses in the control of *Heliothis*. Proceedings of the Fourth Australian Cotton Conference, pp 127-131.

Christian,P.D. and Oakeshott,J.G. (1989). The potential for genetically engineered NPV in *Heliothis* control. The Australian Cotton Grower, Feb-Apr. pp 77-78.

Christian,P.D. and Oakeshott,J.G. (1989). The potential for genetically engineered baculoviruses for insect pest control. Aust. J. Biotech. **3**, 264-266.

Sriskantha,A. and Christian,P.D. (1990) Physical map and nucleotide sequence of the polyhedrin gene of an Australian isolate of a *Helicoverpa armigera* single nucleocapsid nuclear polyhedrosis virus. (Paper withheld from publication for commercial reasons).

Christian,P.D. and Oakeshott,J.G. (1991) A new generation of biological pesticides for heliothis control. Australian Cotton Grower, Nov-Dec 1991, 60-63.

Teakle,R.E., Byrne,V.S. and Christian,P.D. (1992) Potencies of three *Heliothis* nuclear polyhedrosis viruses for *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). J. Aust Ent. Soc. (in press).

REFERENCES

Fung,M.-C., Chiu, K.Y.M., Weber,T., Chang, T.-W. and Chang, N.T. (1988). Journal of Virological Methods **19**, 33-42.

Luckow,V.A. and Summers,M.D. (1988). Bio-technology **6**, 47-55.

Maatsura,Y., Possee,R.D., Overton,H.A. and Bishop,D.H.L. (1987). Journal of General Virology **68**, 1233-1250.

Maeda,S., Kawai,T., Obinata,M., Fujiwara,H., Horiuchi,T., Saeki,Y., Sato,Y., and Furusawa,M. (1985). Nature (Lond.) **315**, 592-594.

Smith,G.E. and Summers,M.D. (1988). United States Patent No. 4,745,051.

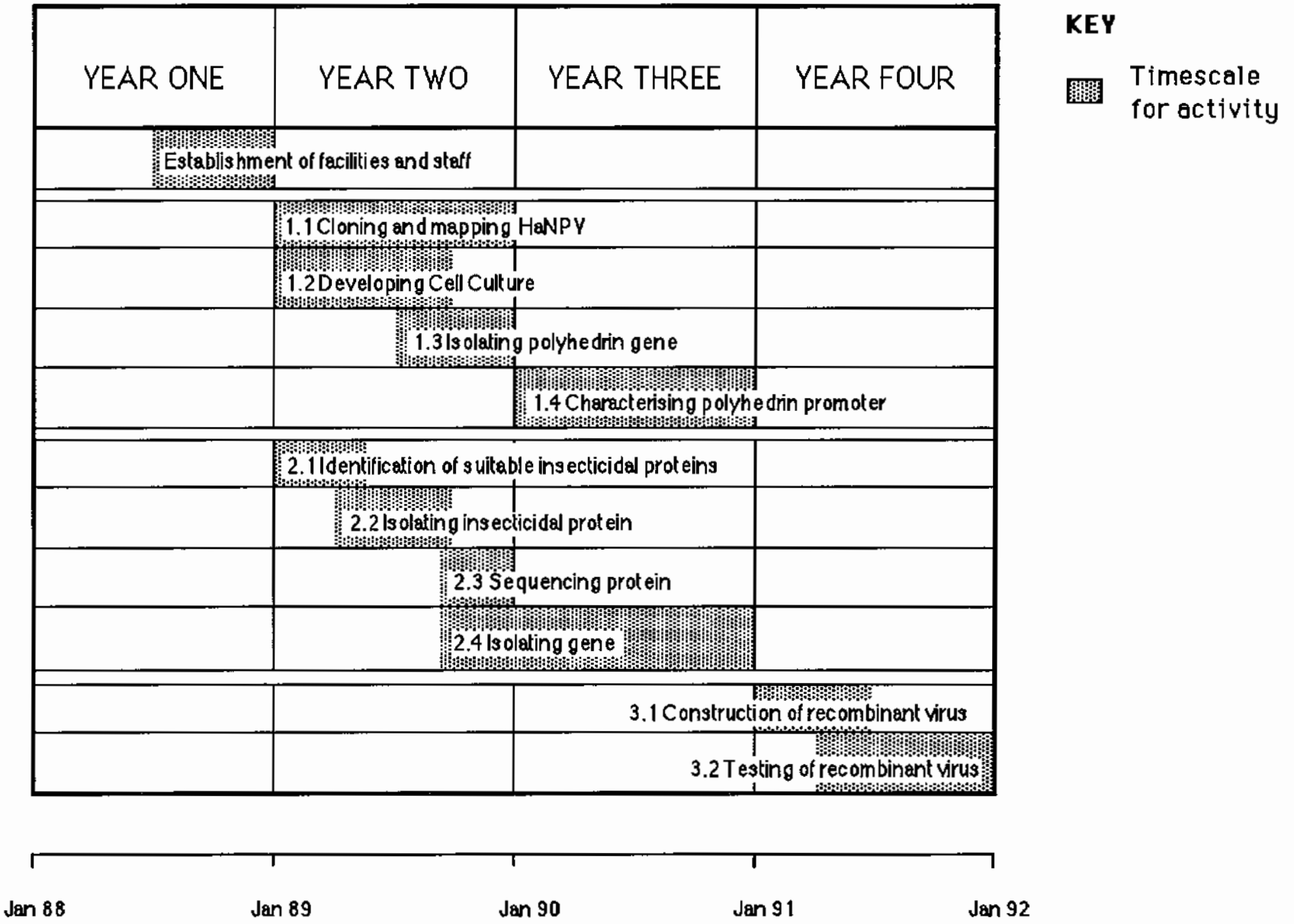
Stewart,L.M.D., Hirst,M., Lopez Ferber,M., Merryweather,A.T., Cayley,P.J. and Possee,R.D. (1991). *Nature (London)*, 352, 85-88.

Summers,M.D. and Smith,G.E. (1987). Texas Agricultural Station Experimental Bulletin No. 1555.

Takehara,K. Ireland,D. and Bishop.D.H.L. (1999). Journal of general Virology **69**, 2763-2777.

Tomalski,M.D. and Miller,L.K. (1991). *Nature (London)*, 352, 82-85.

Figure 1. GANNT chart showing critical research path & major milestones for project CSE8C.



Appendix 1

DETAILED RESEARCH PROPOSAL

Project Number	CSE29C
Project Title	GENETIC ENGINEERING OF THE *HELIOTHIS* NUCLEAR POLYHEDROSIS VIRUS: PRE-COMMERCIAL DEVELOPMENT
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Division/Branch	Entomology
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Administrative Contact	Mr M. Hardwick Telephone: (06) 46 4008
Commencement Date	01/07/1992
Completion Date	30/06/1995

AIMS

To optimise the design of recombinant *Heliothis* viruses with increased insecticidal activity; to develop suitable large-scale production systems, and; to assess the potential impact these viruses may have upon the environment.

BACKGROUND

Heliothis armigera and *H. punctigera* are major pests of cotton crops in Australia. In the past outbreaks of these pest species have been controlled by the conventional use of chemical insecticides. Over the last decade, however, resistance to a number of chemical insecticides has arisen in populations of *H. armigera* and there are currently no chemicals available which do not face a resistance problem. Therefore, it is necessary to find new ways to augment and complement the existing methods of *Heliothis* control. One such form of control is based on biological insecticides.

Several biological options have been suggested for the development of insecticides. In one instance, the entomopathogenic bacterium, *Bacillus thuringiensis* (Bt), a fair degree of commercial success has already been achieved. However, recent demonstrations of field resistance to Bt in other species of lepidopterans has highlighted the fact that biological insecticides may engender resistance and that reliance on one particular agent is not a sustainable strategy.

We have been working on another potential biological insecticide at CSIRO Division of Entomology since 1988, namely *Heliothis* Nuclear Polyhedrosis Virus (HaNPV). A closely related *Heliothis* virus was previously used under the commercial name of ELCAR^{TM}, but it suffered from a short half-life under field conditions, and a slow rate of kill. These factors made it unsuitable for use on many crops and led to its eventual removal as an option in all *Heliothis* control programs.

The overall program "Genetic engineering of *Heliothis* NPVs" that we initiated in 1988 aims to modify the genome of *Heliothis* NPVs to generate more rapidly acting and/or more virulent virus strains. These viruses will then be available as biological control agents against *Heliothis* species. Our research program can be divided into three distinct phases; 1) Research and development of recombinant viruses (1988-92), 2) Pre-commercial research and development of recombinant viruses (1992-95), and 3) Commercialisation of recombinant viruses (1995-99). The first phase of the program finishes in April 1992. The proposed project forms part of the second phase of the overall program.

The potential of new control agents such as the recombinant virus proposed above has recently been demonstrated in two case studies. In both instances genes encoding insect-specific toxins were used; one was a neurotoxin from the mite *Pyemotes tritici* (Tomalski and Miller, 1991), and another derived from the venom of the North African scorpion, *Androctonus australis* (Stewart et al., 1991). In both cases, expression of the toxin-gene in the baculovirus genome was directed by the polyhedrin promoter and reduced the time taken to paralyse or kill the insect host.

Both case studies above involved the NPV of *Autographa californica* (AcMNPV). The first phase of our program has developed an efficient vector system based on a *Heliothis* specific NPV, which now allows us to produce a new generation of effective biological insecticides for the control of *Heliothis*.

INDUSTRY SIGNIFICANCE

Our overall research program aims to provide novel strains of viruses for use as biological insecticides against *Heliothis* spp. Creation of such novel viruses will be achieved by inserting foreign genes, whose products have insecticidal activity, into the genome of *Heliothis* nuclear polyhedrosis viruses, in order to produce strains with increased insecticidal activity and field control potential. Such viruses will offer biological options to the cotton industry compatible with existing chemical control agents and practices.

The benefits to the Australian cotton industry will accrue through the availability of alternatives to the currently used chemical insecticides. Such alternatives will not only have value in thier own right but will also assist in the further refinement of *Heliothis* resistance management programs, thereby extending the useful life of chemicals such as pyrethroids.

COMMERCIALISATION OF RESEARCH PRODUCTS

The program is well placed to generate a commercial product as the Division has a strong commercial partner with complementary skills in ICI. The second phase of the program is a collaboration between CSIRO and ICI that aims to ensure the most commercially viable recombinant viruses are produced. It is anticipated that CSIRO's involvement in the program will be reduced after the completion of the second phase.

During the third phase of the program (1995 onwards) ICI will aim to commercialise recombinant viruses generated during the first and second phases of the program. It is expected that a commercial product will be available to the cotton industry around the turn of the century.

STAFFING AND QUALIFICATIONS

CSIRO

Dr Peter Christian took up a research scientist position provided by the Cotton Research Council in January 1988 and has been funded by the Cotton Research and Development Corporation since 1989 (Project CSE8C). Dr Christian will have responsibility for the day to day management of the CSIRO component of the program during its second phase. He is an insect virologist with eight years experience in the molecular and biochemical characterisation of insect viruses.

Dr John Oakeshott (Head, Molecular Biology Section) has overall administrative responsibility for the program. Dr Oakeshott spends approximately 30% of his time on this program and all of his costs are met by CSIRO appropriation. Dr Oakeshott has fifteen years experience in the molecular and biochemical genetics of insects.

Dr Paul Simpson is an Experimental Scientist funded by ICI who has worked with Dr Christian in the overall program since March 1989. He is a molecular biologist with ten years experience with insects and will spend 100% of his time on the second phase of the program.

Ms Janelle Scown is a Technical Assistant funded by ICI who has worked on an associated project in the overall program since March 1989. Ms Scown will work full-time under ICI funds on the project proposed here from April 1992. Another technician will also be appointed to the project proposed here under the recently renewed funding from ICI.

Ms Fernon joined the program in early 1989 under funding from the Oilseeds and Grain Legumes Research Councils. She is the Division's most accomplished cell culturist and her abilities are demonstrated by her recent promotion from Technical Officer to Experimental Scientist. An application has been made to the Grains Research and Development Corporation to support Ms Fernon's position during the second phase of the program.

ICI

During the second phase of the program ICI Agrochemicals (U.K.) will make a considerable in-house commitment to the overall program by way of commercially developing the enabling technologies and products generated by CSIRO. They will have eight experienced staff involved with the program in areas of molecular biology, large-scale cell culture and biological testing of recombinant viruses. In addition, ICI have undertaken to contract certain parts of the overall program to their in-house formulation and toxicology laboratories. Staff of ICI (Australia) Crop Care will be involved in the field components of the program. They have a large number of experienced personnel in this area.

RESEARCH PLAN AND METHODOLOGY

A research plan for the overall program during its second, pre-commercial development phase is presented below. As the program is a collaboration between CSIRO and ICI, many of the activities involve both partners. Down-stream activities that are the responsibility of ICI alone are indicated. A GANTT chart showing the research critical pathway and milestones is provided in Figure 1.

1. OPTIMISATION OF VECTOR/PROMOTER SYSTEMS

The vector system developed in the first phase of the current program is based around the polyhedrin promoter. As this is a very late promoter (not expressed until 18-24 post-infection) there are possibilities for increasing the control potential of recombinant viruses further by using earlier promoters. This activity will aim to identify and clone such

promoters from HaNPV in order to provide alternatives for optimisation of the control potential of HaNPV recombinants (see activity 2.2).

2. OPTIMISATION OF TOXIN INSERTS

2.1 Isolation of Suitable Insert Genes

Several on-going projects within the Division are isolating genes that may be suitable for insertion into the HaNPV genome (see Attachment 3 - Progress Report). The most promising of these aims to isolate the toxin gene from the parasitic wasp *Bracon hebetor.* It is anticipated that this gene will be made available to the program in the latter half of 1992. In addition, ICI Agrochemicals are able to provide another two toxin genes to the project that are suitable for insertion into HaNPV. These genes will be inserted into HaNPV in the next few months and their insecticidal potential then assessed (see activities 2.2 and 2.3).

2.2 Generation of Recombinants

This activity will involve the construction of transfer vectors and the generation and isolation of recombinant viruses containing suitable toxin-genes, as and when they become available.

2.3 Testing and Optimisation of Recombinants

Recombinants generated in activity 2.2 will be assessed for increased rate of kill of *H. armigera* larvae. Initial constructs will use a transfer vector in which the foreign (toxin) gene is linked to a second copy of the polyhedrin promoter.

The recombinants will be optimised for reduced speed of kill using the alternative promoters isolated in activity 1 and assessed by bioassay in *H. armigera.* The most promising recombinants will then be passed on to ICI for further assessment and development.

The construct/recombinant showing the greatest *Heliothis* control potential will be selected for further detailed biological and toxicological studies (activity 3). It is anticipated that a decision about the most suitable recombinant with which to proceed will be made in late 1993/early 1994.

3. TOXICOLOGY (ICI)

Toxicological data necessary to apply for the first planned release (activity 5.6) of the recombinant virus selected in activity 2.3 will be generated by ICI Agrochemicals. Toxicology will be performed on both the recombinant virus and the toxin that it is capable of generating. Toxicological data generated in this activity will also form the baseline data necessary for eventual registration of the selected recombinant as an insecticide.

4. DEVELOPMENT OF VIRUS PRODUCTION SYSTEMS

4.1 Development of Small-scale Systems

The parental *Heliothis* cell line we are using in research is able to grow in two commercially available serum-free media. However, these media are very expensive (approx $40 per litre) and the recovery of virus is very low (see activity 4.2). This activity will aim to formulate our own serum-free media and to adapt available cell lines to growth in suspension culture.

4.2 Optimisation of Small-scale Production Systems

Several factors are known to attenuate the *in vivo* infectivity of baculoviruses that are produced *in vitro*. The most important of those factors identified to date are caused by genetic instability of the virus (Kool *et al.*, 1991) and the lipid content of the cell culture media (Tomkins *et al.*, 1991). This activity will assess *in vivo* infectivity of HaNPVs produced in systems developed in activity 4.1 in order to optimise the recovery of infectious virus in small-scale serum-free production systems (up to 100ml).

4.3 Development of Scale-up Procedures

Two major systems are currently used for the large-scale production of cells in suspension culture, stirring and air-lift fermentation. The latter is used only when the cells to be produced are particularly sensitive to shearing.

This activity will assess the relative performance of selected cell lines in scale-up fermentation procedures up to the 1 litre stage. Initial data suggest that *Heliothis* cells are not particularly sensitive to shear stress and will perform well in stirred suspension systems. Initially, we will therefore look at the performance of our selected *Heliothis* lines in a continuous stirring system and assess them primarily for cell growth . Only in the event that growth is severly impaired in this system, will an air-lift fermentation system be investigated.

Further optimisation of the systems developed in this activity will be undertaken by ICI Agrochemicals in conjunction with CSIRO.

5. ENVIRONMENTAL IMPACT STUDIES

Activities in this area of the program will increase throughout the second half of the program and will aim to generate sufficient data for the application and subsequent release of a toxin-producing HaNPV in the growing season of 1994/95.

The primary aim of the proposed field trips will be to identify the natural HaNPVs that are present in *Heliothis* populations, both within cotton crops and in nearby crops; to assess the distribution of baculoviruses in species closely related to *H.armigera/punctigera* in

these areas, and to determine the ability of these viruses to replicate in *H.armigera/punctigera*.

Experimental priorities and time-frames may alter within this area of the program depending on the requirements of the relevant regulatory bodies.

5.1 Development of Monitoring Protocols

As a pre-requisite to the field trials that we will carry out as part of the proposed project it will be necessary to develop sensitive and convenient assays for monitoring the distribution and dispersal of viruses during the trials. The assay is likely to involve the polymerase chain reaction (PCR), which can be used to amplify specific fragments of DNA from very small amounts of starting material.

5.2 Contained Glasshouse Studies

Once a suitable recombinant is decided upon, small-scale glasshouse trials will be carried out to assess the control potential under more natural conditions and the rate at which the virus persists in insect cadavers, on the plant and in the soil.

5.3 Field Studies

The field study component of the project will be carried out during 1993-95 and will include surveys of NPVs in *Heliothis* and other lepidopterans in and around cotton growing areas berfore, during and after the normal growing season. Surveying will primarily aim to identify *Heliothis* overtly infected with NPVs but will also include a random sampling of insects, vegetation and soil.

Once isolated from field samples NPVs will be purified by an end-point dilution procedure and then assessed for genetic homogeneity by restriction profile analysis of their genomic DNA. Homegenous genotypes isolated in this way will then be compared by restriction fragment length polymorphism (RFLP) analysis against reference material already available to the project.

5.4 Laboratory Host Range Studies

In addition to the field studies detailed above, laboratory studies will also be conducted to assess two parameters critical to our subsequent GMAC application for field trials of recombinant HaNPVs. The first parameter concerns the infectivity of both wild-type (primarily the strain around which the genetic manipulation work has been based), and engineered HaNPVs for species of insects that are found in cotton crops alongside *Heliothis*. The second parameter concerns the ability of our HaNPV to undego recombination either with other strains of HaNPVs or other NPVs found in insects susceptible to infection with our HaNPV.

5.5 Planned Release of Recombinant HaNPVs

The data gathered in activities 5.2, 5.3 and 5.4, will be used to make an application to GMAC for a field trial with the engineered toxin-containing recombinant for the 1994/95 field season.

6. FORMULATION RESEARCH (ICI)

Once a decision has been made as to which recombinant to develop as a commercial product, ICI will begin formulation research to ensure that the viral insecticide will be stable both to u/v exposure and to the conditions on the cotton leaf surface.

OTHER FUNDS FOR THIS PROJECT

CSIRO: CSIRO Appropriation provides Dr Oakeshott's salary and all of the overheads for himself and the other five staff working in the overall project.

ICI: ICI (Australia) and ICI Agrochemicals will make a direct contribution of approximately $190,000 p.a. to CSIRO research in this project. In addition, ICI Agrochemicals will have eight staff working on downstream aspects of the project at Jealott's Hill in England, and staff of ICI (Australia) will assist in the field trial components of the project.

Grains Research and Development Corporation: The GRDC have agreed to fund Ms Fernon's position in the proposed project. With operating costs, this amounts to approximately $33,000 p.a.

DISSEMINATION OF RESULTS

Due to the commercially sensitive nature of much of the research in the overall program it will not be possible to publish or disseminate many of the results of the research within normal time-spans. However, wherever possible summaries of the research results will be published in industry journals, and eventually in scientific journals.

Obviously requirements from GMAC to make results of the research publicly available during the course of the project will be met.

REFERENCES

Kool,M., Voncken,J.W., van Lier,F.L.J., Tramper,J. and Vlak,J.M. (1992) Virology, (in press)

Stewart,L.M.D., Hirst,M., Lopez Ferber,M., Merryweather,A.T., Cayley,P.J. and Possee,R.D. (1991). *Nature (London)*, 352, 85-88.

Tomalski,M.D. and Miller,L.K. (1991). *Nature (London)*, 352, 82-85

Tompkins,G.J., Dougherty,E.M., Goodwin,R.H. and Adams,J.R. (1991). J. Econ. Entomol. **84**, 445-449.

FIGURE 1 GANNT chart showing the critical research path and major milestones in the project, "Genetic Engineering of Heliothis Nuclear Polyhedrosis Virus: Pre-commercial Research". Activities that are the sole responsibility if ICI are indicated (ICI).