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Cotton Research and Development Corporation

Report on overseas travel

Karl H.J. Gordon, CSIRO Division of Entomology

REASON FOR TRAVEL

The aims of this trip were to initiate research on HaSV RNA designed to improve our ability to express insect viral genes in transgenic cotton, and to attend two important conferences relevant to the long-term biological control of major cotton pests like *Helicoverpa armigera*: the International Workshop on the Molecular Biology and Genetics of Lepidoptera (Crete) and the International Colloquium on Invertebrate Pathology and Microbial Control (Montpellier).

SUMMARY

(I) WORK ON HASV RNA STRUCTURES AT LEIDEN UNIVERSITY

Preliminary experiments confirmed the existence of special RNA structures resembling tRNAs on the HaSV genomic RNAs. These structures are likely to be critical for virus replication, so that an understandmg of their structure and function is important for engineering of the virus in order to exploit it for pest control.

This work also confirmed that the ribozyme built into our HaSV RNA expression constructs functioned as expected to generate correctly terminated HaSV genomic RNAs, but with unexpectedly low efficiency. Improvement of this efficiency may be important for achieving better pest control in transgenic plants.

(II) MEETINGS

The International Workshop on the Molecular Biology and Genetics of Lepidoptera in Crete (August 21-26)

Work reported at the meeting covered several key areas of insect molecular biology relevant to our nse of insect viruses for pest control. The major areas discnssed at the meeting were:
(i) insect physiology and the regulation of gene expression
(ii) molecular studies on lepidopteran genomes
(iii) progress in research on transformation systems.

The International Colloquium on Invertebrate Pathology and Microbial Control in Montpellier (August 28 - September 2)

This meeting, organised by the Society for Invertebrate Pathology, covered progress with a wide range of microbial pest control agents. Of especial significance for the nse of viral insecticides were results from the first field trial of a recombinant baculovirus insecticide expressing a toxin; novel strategies for design of attenuated or contained recombinant baculovirus insecticides; and recent findings on how baculoviruses spread in infected larvae.

(I) WORK ON HASV RNA STRUCTURES AT LEIDEN UNIVERSITY

Introduction

The success of our CRDC funded project CSE33C entitled "*Helicoverpa armigera* stunt virus (HaSV) as a source of insecticidal genes for engineering into cotton" will rest on our ability to achieve optimal and reliable expression of insect virus genes in cotton. Experience with other insect control genes such as Bt has made this clear. In order to achieve good expression, it is essential to identify gene control signals present on the HaSV genome and to learn how they can be manipulated as required for expression in plant cells.

The RNAs of HaSV are unusual in that they end in tRNA-like structures similar to those found in some plant viruses rather than in the poly(A) tails normally found for cellular and viral RNAs. In plant viruses, these structures are important for viral replication. The same is likely to be true of the HaSV structures, and this has significant implications for manipnlation of the cloned virus in transgenic insect-resistant plants. However the HaSV structures differ significantly in their theoretical folding (they lack a pseudoknot in the aminoacyl stem), making it necessary to confirm their existence experimentally. This can be achieved nsing enzymes like RNase P and tRNA nucleotidyl transferase whicli specifically recognise tRNA-like structures as substrates.

Biochemical studies on expression signals of RNA viruses requires specialised knowledge about RNA structures and biochemistry. Professor C. W. A. Pleij's lab in the Dept. of Biochemistry, University of Leiden, Netherlands, is a leading centre for the experimental analysis of viral RNAs. I therefore spent three weeks working with Prof. Pleij in his laboratory to study the interaction between the above enzymes and specific short RNA fragments corresponding to the HaSV tRNA-like structures. This work provided preliminary evidence that the putative replication signals on HaSV RNAs are real and will provide a solid basis for the exploitation of HaSV for heliothis control. We also were able to confirm that the ribozyme used to generate infections HaSV genomic RNAs in plant cells worked as predicted, albeit at very low efficiency: these observation suggest one strategy for improving the efficiency of HaSV prodnction in transgenic plants.

Results

Synthesis of RNAs for biochemical analysis

In vitro synthesis of RNAs for this work requires construction of template plasmids carrying DNA copies of tlie sequence of interest. HaSV RNA 2 seqnences corresponding to the proposed tRNA-like structure were cloned into the DNA plasmid vector pTZ18U, downstream of the T7 promoter which determines where the T7 RNA polymerase commences synthesis of RNA. The resulting recombinant plasmids were then linearized with restriction endonuclease BstNI in order to provide a defined stop point for RNA synthesis by the polymerase. Transcription by the T7 RNA polymerase yielded large amounts of a specific short RNA molecule corresponding to the terminal 160 residues of RNA 2. The RNA product could be radioactively labelled during synthesis by including radioactive ATP in the transcription reaction.

Sequences corresponding to the HaSV RNA 1 structure were also cloned as above. However, the RNA 1 sequences were followed by sequences corresponding to the hairpin cassette ribozynie rather than a BstNI restriction site for plasmids

linearization. (This ribozyme was used in engineering the HaSV genes into plant expression plasmids - see CSE33C.) Transcription of the RNA 1 sequences terminates after the ribozyme, with self-cleavage generating RNA molecules terminating at the correct position. The *in vitro* activity of the hairpin cassette ribozyme was verified by monitoring self-cleavage during the transcription reaction (4 h). The sizes of the cleavage products confirmed cleavage at the expected site, but with low efficiency - under 5 %.

Cleavage with RNase P

One simple test for a true tRNA-like structure is to ask whether the radioactively labelled RNAs are cut specifically by RNase P to yield two distinct fragments. Mapping of precise RNase P cleavage site would then offer the most direct approach to confirm the proposed folding, which predicts cleavage at a site over 100 residues from the 3'-end of the RNA. In contrast, RNase P cleaves pseudoknot structures within the pseudoknot about 20 residues from the 3' end.

Our initial experiment showed that the HaSV RNA 2 structure is cut into two fragments by RNase P. The site of RNase P cleavage is at about residue 115 from the 3' end, consistent with the absence of a pseudoknot. This initial result provides direct evidence for the existence of tRNA-like structures lacking a pseudoknot.

Labelling using tRNA nucleotidyl transferase

Nucleotidyl transferase, or CCAse, is an enzyme which specifically recognises tRNA and repairs the CCA ends required for their function. This activity means that the enzyme can be used to attach a labelled A residue to the 3' end of the viral tRNA-like structures. To test whether this enzyme also recognises the HaSV RNA 2 tRNA-like structure, an unlabelled *in vitro* transcript was incubated in its presence with radioactive ATP. Analysis of the products showed the RNA had been specifically and efficiently labelled.

Conclusions

(1) The ribozyme

This work has demonstrated that the ribozyme cleaves at the expected site and provided a basis for experiments to improve the efficacy of the ribozyme *in vitro*. This will provide a basis for evaluating the effect of ribozyme modifications on production of HaSV RNAs in protoplasts and transgenic plants.

(2) The tRNA-like structures.

Our initial evidence supports the existence of these structures. We have also shown (in CSE33C) that HaSV RNAs generated in plant cells from cloned DNA copies of the viral genome require a self-cleaving ribozyme for infectivity; the site of self cleavage is directly next to the 3´-tRNA-like structures. Together, these observations suggest that these structures are critical for virus production in plant cells. This information forms the basis for manipulating the structures in the construction of HaSV genes designed to engineer cotton for heliothis control.

(3) Further scientific significance of the HaSV structures

It has been suggested that the tRNA-like structures on plant virus RNAs may be molecular fossils derived from genomic tags involved in primordial RNA world replication, but the presence of the pseudoknots has cast doubt on their antiquity.

The absence of a pseudoknot from the HaSV structure means that this could be a genuine molecular fossil dating back to the primordial RNA world, rather than a more recent molecular reincarnation as the plant virus structures are thought to be. Our work on the HaSV structures is already attracting international attention because of its implications for our understanding of the RNA world and RNA virus evolution.

(4) Future work

Establishing the nature of the 3' end structure on HaSV RNAs has important implications for design of expression strategies in plants in view of its likely significance for the replication of HaSV genomic RNAs. However the above experiments have not yet enabled us to conclusively answer this question. This is because the *in vitro* transcripts used included sequences transcribed from the vector which may interfere with proper structural folding in these short RNAs and lead to artefactual results. We aim to avoid this by testing transcripts from new plasmids engineered to remove or mutate any non-HaSV sequences able to interact with the authentic 3'-terminal sequences. These constructs have been made and are now being tested as part of our ongoing collaboration.

(II) MEETINGS

The International Workshop on the Molecular Biology and Genetics of Lepidoptera in Crete (August 21-26)

Significance of this meeting

This meeting was the third in a valuable series focussing on the molecular genetics and biology of the insect order which includes major pests like heliothis. A special feature of these workshops is that they make the fundamental work on lepidoptera being done iu universities in the northern hemisphere accessible to researchers whose primary concern is with development of new biological control strategies; conversely, the meeting provided an opportunity to confront academic researchers with the economic significance of major lepidopteran pests. An especial irony of lepidopteran molecular biology is that most work has been done with the silkworm, whose considerable economic importance is as a production system rather than as a pest.

Work reported at the meeting shed useful light on the biology underlying the insect response (in terms of immunity and resistance) to virus infection, an area critical to our use of insect viruses for pest control. Viral pathogenesis is however rooted in the biology of the lepidopteran midgut and how this is disturbed by virus infection: an understanding of this requires greater knowledge of insect midgut cell biology and molecular biology, a very under explored area at present. My presentation on the interference of a simple pathogen with just three genes with the normally well regulated process of midgut growth and regeneration drew the attention of those present to a simple system for studying the molecular biology of this important area. One key outcome of the meeting was the agreement to include a prominent session on viruses as biological control agents. The main areas discnssed were

(i) insect physiology and the regulation of gene expression

(ii) molecular studies on lepidopteran genomes

(iii) progress in research on transformation systems.

Insect physiology and the regulation of gene expression

Gene expression in lepidopteran larvae underlies complex and subtle control mechanisms which are subject to developmental and hormonal control. The possibility of nsing biological control agents which exert insecticidal effects by interfering with these mechanisms has made their study very relevant to the insect control community. Several groups reported work on the paths by which hormones affect growth and on the genes that are regulated by hormones, eg during diapause (Palli, Sault St.Marie; Sato, Nagoya).

Iatrou (Calgary) gave an overview of the regulation of chorion gene function during development of the silkworm egg. This is one of the best characterised systems involving hormonal control of an insect gene family. He also described recent work in cloning the ecdysone receptor, which has allowed him to study its central role in ovarian follicle maturation and egg development.

Genomic maps of lepidopterans

The advent of new technologies like the use of Randomly Amplified Polymorphic DNA (RAPD) markers has brought the construction of detailed genetic and molecular linkage maps of major pests like heliothis within our reach. Such maps would be

valuable for pest control strategies by making the cloning of valuable genes like those causing resistance or otherwise interacting with biological control agents readily feasible. Moreover, molecular DNA markers would greatly facilitate pest population studies e.g. for gene flow, migration and the spread of resistance or sensitivity to control agents.

Marian Goldsmith (University of Rhode Island) reported on progress towards constructing a molecular linkage map of the silkworm. This project (in collaboration with T.Tamura (TISES, Japan) and T. Shimada (Tokyo University)) aims to construct a map collecting the hundreds of mutants known in the silkworm together with molecular data obtained using FLPs (DNA Fragment Length Polymorphism), cDNA and genomic sequences and RAPDs. The silkworm is the lepidopteran species whose genome is best characterised, but international collaboration to integrate data on other lepidopteran species like heliothis would greatly aid genome mapping and population genetic analysis of such major pests. Many silkworm molecular probes are likely to be directly useable on heliothis. Of lasting significance was the meeting's recognition that the lepidopteran genomics network should seek to combine information from pests like heliothis with that based on silkworm. Such knowledge of lepidopteran molecular biology and the availability of probes and primers for genome mapping will be valuable in mapping heliothis genes and exploiting the population genetics of this pest for resistance management and biological control.

Transformation of lepidopteran species

The genetic engineering of lepidopteran insects by stable introduction of foreign genes into their chromosomes remains a major goal of insect molecular biologists. This would enable modern techniques of gene isolation to be applied to pests like heliothis and allow detailed studies into insect physiology which would open up new avenues for biological control. Possible control strategies would be based on engineering pests to carry age- and tissue-specific genes which weaken them in the field or render them sensitive to defined agents in agricultural areas. Moreover, the ability to genetically mark pests and to monitor the presence of specific genes would be invaluable for the study of population genetics and gene flow as part of resistance evaluation and management.

One factor hampering lepidopteran transformation has been the lack of suitable vectors comparable to the mobile genetic elements widely used for Drosophila transformation. Those elements that have been isolated from eg the silkworm belong to the more complex group of retrotransposons (described by T. Eickbush, Rochester) and are furthermore not readily applicable for use as vectors. (However it is likely that an intensive research program to explore the retrotransposons of major pests like heliothis would reveal mobile elements capable of being used for transformation as some mammalian retroviruses have become the vectors of choice for gene transfer into vertebrates - including humans.) The above difficulties have forced the use of other approaches and several gronps reported on their progress. While the following approaches have proven of value as research tools for the study of gene expression *in vivo* or protein function in cells, none have yielded transgenic insects.

Transgenic cell lines have been produced

(a) via transfection of plasmids carrying strong silkworm promoters (Iatrou, Calgary) resulted in stably transformed lines capable of continuons high-level production of recombinant protein.

(b) using the yeast 2μ sequence (Thomas, Couble et al., Lyons); this work aims to construct a transgenic line carrying a yeast sequence which can later be targeted for insertion of foreign genes, thereby making the engineering of cells a predictable and straightforward matter; this work is still in its early stages.

Gene transfer into whole animals/organs has been explored by

(a) injection of plasmids into eggs and biolistic bombardment of gamete precursors in larvae/pupae followed by re implantation (Thomas, Couble et al., Lyons) have yielded evidence for transient expression of marker genes, with some evidence for independent replication of the construct, which does not appear however to become integrated into the insect chromosome.

(b) biolistic bombardment of excised silk glands followed by re-implantation (Horard, Couble and Prudhomme, Lyons) has allowed dissection of gene expression under *in vivo* conditions. The implanted glands survive for several days within larvae.

The International Colloquium on Invertebrate Pathology and Microbial Control in Montpellier (August 28 - September 2)

This meeting was a valuable opportunity to obtain information on a insect pathogens and new pest control strategies based on insect viruses; further to present our work in the form of a paper to other insect virologists for discussion.

Significant progress reported on baculoviruses

Spread of baculovirus infection in larvae

Baculoviruses continue to figure prominently as possible agents for pest control. However their slow kill speed (compared to chemical insecticides) means that considerable effort is going into genetic modification to improve their efficacy. In part their slow kill speed is due to their growth cycle in infected larvae. Although the larval midgut is the primary target for these orally acting viruses, it is not a site of pathogenesis - the baculoviruses effectively pass through this organ to infect internal tissues and allow the midgut to recover from the infection withiu about 24 h (this is quite unlike the midgut response to infection by some RNA viruses like HaSV).

Several speakers discussed these early stages of viral infection and the routes taken by the virus in establishing secondary infections. L. Volkman (UC Berkeley) described results suggesting that the tracheae, which are in direct contact with the midgut, were the major conduit for virus spread from the midgut; Brian Federici (UC Riverside) disagreed with this interpretation and stated that these are only secondary sites after infection of the hemolymph. This discussion largely hinges on whether budded virus can cross the basal membrane surrounding the midgut, and different experimental approaches appear to emphasise differing aspects of this question. It is important because our understanding of these early stages of baculovirus infection following the initial infection of the midgut forms the basis for novel genetic modification and toxin strategies.

Recombinant baculoviral insecticides

Results from the first field trial of a recombinant baculovirus carrying a toxin gene were reported by J. Cory (NERC, Oxford). This virus resulted in less damage by larvae to the crop, with the larvae being killed about 1.5 days faster than by wild type virus. The recombinant virus produced an epizootic earlier than did the wild type

virus (at 7-11 days compared to 11-16 days) but infected larvae produced less virus and there were fewer secondary infections of larvae as a result.

Containment strategies.

Concern about the dangers of releasing genetically modified viral insecticides carrying toxin genes has led to great interest in the design and implementation of strategies for the containment of such viral control agents upon release, so that they do not persist in the environment. Such strategies are viewed by many workers in the field as being critical for public and regulatory acceptance of viruses.

Current interest has focussed on strategies involving the deletion of genes essential for horizontal spread of the virus. Since the initial infection of the target insect pest requires the virus to carry the products of these genes, the modified viruses are grown in insect cells either in combination with a complete wild type virus that itself lacks the toxin gene (the co-occlusion strategy) or alone to give, if the polyhedrin gene is absent, virus equivalent to that derived from polyhedra (the pre-occluded virus strategy). In either case, the modified virus is initially infectious. However its deficient genome means that progeny virus cannot persist in the environment.

A. Wood (Boyce Thompson Institute) reported on field trials testing these two strategies. Co-occlusion appears to prevent persistence of the recombinant virus in the virus population, but wild type polyhedra from the co-occluded virus are still found in the soil after several years. Field trials of the pre-occluded virus were still not complete.

A more recent approach is to delete a gene for a protein (P74) required in only small amounts for primary infection and grow the virus in cells which have been engineered to make sufficient of this protein. Progress towards testing this P74 strategy was described by several groups (P. Faulkner (Queen's Uni., Ontario); L. Volkman; A. Wood). Its feasibility has been shown in the laboratory, but no field trials have yet been performed.

Other viruses

Recent progress in the manipulation of densoviruses (DNVs) and entomopoxviruses (EPVs) was reported from Bergoin's group (Montepellier). The DNVs are of great interest because of their potential value as gene vectors for engineering lepidoptera and the EPVs as control agents (once engineered) and expression vectors. One highlight was the construction of a recombinant AmEPV using a high-level promoter from another virus (MmEPV). Contacts established with Prof. Bergoin resulted in a very useful recent visit by a member of our laboratory (Ms Michelle Lincoln) to his laboratory, during which she learnt a range of DNV techniques which will enable us to pursue our observations of a suspected DNV from heliothis.

The first complete sequence of an insect picornavirus, the infectious flacherie virus (IFV) of silkworm, was reported by Bando and colleagues (Hokkaido University.) IFV is only the second insect small RNA virus infecting an economically important species to have been completely sequenced (after our own work on HaSV). Analysis of the IFV sequence will be invaluable in our efforts to clone and exploit for pest control a picornavirus which we tentatively identified in heliothis.