Close off Report on CSE36C

Project Title: DNA Probes for Key Insecticide Resistance Genes - Maintaining a Sustainable Resistance Management Strategy

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Summary and Conclusions

The project was terminated by CRDC after two years and therefore could not meet its final objectives. Good technical progress was made and the resources and information that were obtained remain available for the future.

One particular benefit of the project was seeu by the CRDC review. held in January 1994. as being that the knowledge gained would assist in resistance management for Bt. In an unexpected way it did this by highlighting the need for us to create a genetic map of *Heliothis armigera*. In the case of CSE36C. the pre-existence of a genetic map of the related American species. *Heliothis virescens*. allowed us rapidly and cost-effectively to decide whether endosulfan resistance was due to a mutation in the GABA gene. Our ability to use information from the American species relied on the happy and unusual coincidence that endosulfan resistance is sex-linked (i.e. its chromosomal location in *H. armigera* is known). This is a rare. if not unique. situation and almost certainly we will not have this advantage when we are faced with *H. armigera* resistant to Bt cotton. Creating a genetic map in *H. armigera* before Bt resistance appears in the field would eliminate reliance on luck and greatly facilitate characterisation and detection of resistance genes when they appear. It would do this by providing markers which could be used to determine the chromosomal location of any resistance genes. Even at the crudest level this would be a very valuable tool. It is therefore one of the most cost-effective steps that the cotton industry can take as insurance against Bt resistance.

From my discussions with CRDC on the reasons for the termination of the project it is clear that the industry believes that managing insecticide resistance for existing chemical insecticides is a lost cause. One of the major problems facing the industry is the lack of a range of good alternative modes of action on which to build a best-case management strategy. Whilst development of Bt cotton may assist with pest control generally. it does not of itself address this situation. since it relies on a mode of action that is already in use. Genetically engineered NPVs will add a new mode of action as would stunt virus-cotton. Redirection of some molecular genetic resources previously devoted to research on insecticide resistance to the new technologies of receptor-based high throughput screening for new chemical insecticides is appropriate and the Division is pursuing this option with other parts of CSIRO and potential commercial partners. The likely commercial partners are likely to be multinational companies with little direct interest in Australian markets of *H. armigera per se*. CRDC may therefore wish to consider whether it should invest in this type of research with *H. armigera* and/or *H. punctigera* as target species.

Background

Controlling heliothis is an ongoing challenge in cotton *Helicoverpa armigera* is a particular problem because it is multiply resistant to chemical insecticides and has the propensity to

develop resistance to new compounds as their use increases. The rationale behind this project was that to manage resistance to insecticides optimally, one needs to rotate a range of compounds with different modes of action. This needs to be guided by accurate data on the changing nature, severity and frequency of any resistance alleles present in field populations and modify control regimes accordingly.

At the start of this project a rough and ready method of determining the frequency of resistant *Helicoverpa* on a field by field basis, was available to growers. The method relies on determining species composition using the LepTon™ Test Kit and combining this information with knowledge of average insecticide resistance frequencies present during the last season as summarised in the "Stop-Go" charts developed by Forrester's group. (Gunning's group has also developed a test which is claimed to discriminate pyrethroid resistant from susceptible *Helicoverpa armigera* but, as far as I am aware, independent field validation data has not yet been published).There is no ability currently to monitor changes in frequencies of different alleles or mechanisms.

Aim

The aim of this project was to develop rapid laboratory procedures that could identify and quantify the most threatening insecticide resistance mechanisms in *H. armigera* and to train research and extension staff in use of the procedures. In essence this would involve developing specific DNA markers for particular resistance alleles. The markers would then be incorporated into the most convenient available screening method. This would provide much more detailed information than is currently available to guide setting of resistance management options.

Changed Environment

Over the first two years of the project, external conditions changed considerably in the cotton industry. One set of factors had to do with the decreasing options for chemical control. These included the developing perception in the cotton industry that pyrethroids have been "lost" to resistance and that, while resistance to endosulfan may be managed successfully, the chemical may be deregistered anyway. Coupled with this are the fact that resistance to OPs (which are unattractive for other reasons) is present and resistance to carbamates is expected to develop quickly the more they are used. The final straw was the furore over chlorfluazuron residues in meat, which where all the more alarming where no direct direct route of contamination could be established. Given all these constraints the industry seems to feel that it will have no viable option for controlling *H. armigera* before the year 2000, other than transgenic cotton. It therefore wishes to concentrate all its work on resistance management into this area.

The other set of factors related to the prolonged drought and severe economic effects this has had on cotton growers, and therefore also CRDC.

Accordingly, CRDC decided not to fund the third year of this project.

Summary of Results

Endosulfan resistance
Endosulfan exerts its effects on insects by binding to and "blocking" the GABA receptor, which controls opening of chloride ion channels in the insect's nervous system. These chloride channels normally damp down activity in the nervous system, so when they are blocked the insect goes into a hyperexcited state and eventually dies. Resistance to insecticides

of the endosulfan class is very common and in a number of overseas examples has been shown to be due to a mutation in the $GABA_A$ receptor, so that the insecticide no longer binds to it.

Our working hypothesis was that resistance to endosulfan in Australian occurs by the same mechanism observed in other species. If true this would have allowed us to develop DNA based markers rapidly. To test this hypothesis we used the polymerase chain reaction (PCR) to clone a small portion of the $GABA_A$ receptor gene spanning the expected site of the resistance mutation from both endosulfan- resistant and -susceptible strains. To our surprise, and against all precedents, it turned out that there was no difference between resistant and susceptible strains of *H. armigera* in this region. Furthermore the type of amino-acid present at the expected site of mutation was different from all the other insect species where it has been examined. Although overseas workers have sequenced this region of the gene from several different insect species, we are the first researchers to examine a lepidopteran. So one possible explanation is that lepidopteran insects have different $GABA_A$ receptors from many other insects. [One of the reasons for our surprise was that Neil Forrester's group has shown that one of the strains we sequenced has the "classical" pattern of cross-resistance to endosulfan and related cyclodiene insecticides.]

To develop a diagnostic test we need to know the site and nature of the resistance mutation(s). We divided the possibilities into two groups. It could be that the mutation that causes resistance occurs somewhere else in this $GABA_A$ gene. Alternatively the mutation could be in a completely different gene, perhaps one encoding another subunit of the same protein or possibly in an enzyme that breaks down endosulfan, or even a regulatory gene. We needed to distinguish between these two possible explanations quickly. If the mutation was elsewhere in the same gene we would have a decent chance of finding the mutation quickly and developing a diagnostic test. On other other hand if the mutation was somewhere else it would become a very large undertaking to locate it, one well beyond the aims and resources of this project.

To make this distinction, we collaborated with Dr Joanne Daly of this Division and Dr David Heckel of Clemson University U.S.A. We also relied heavily on existing work of both Dr Heckel and Dr Daly. From Joanne's work we knew that endosulfan resistance in *H. armigera* is sex-linked, i.e. the mutation occurs in a gene on the X-chromosome. David Heckel had expended considerable effort in building up a genetic map of *Heliothis virescens*, a closely related American species. We provided our amplicon from the $GABA_A$ gene of *H. armigera* and David tested it against his markers to determine whether it also occurred on the X chromosome. In fact the $GABA_A$ gene was actually found to lie on a different chromosome. This indicates that the $GABA_A$ receptor gene that we cloned plays no part in endosulfan resistance in Australian *H. armigera* (There is a remote possibility that we are wrong, if there has been a chromosomal translocation to or from the X-chromosomes in one of these species since they diverged evolutionarily. However, evidence obtained by Dr Heckel from more distantly related Lepidoptera indicates this is unlikely).

This is a novel and exciting scientific finding which had important practical implications too. The first one being that obtaining a diagnostic for endosulfan resistance was far more difficult than originally envisaged and would require a large and unwarranted extra investment. Other implications are that susceptibility and cross-resistance of *H. armigera* to alternative insecticides that bind at this site (e.g. fipronil, avermectins) may not be predicted well by reference to other insect species. In fact this may be good news because although some cyclodiene-resistance flies show high levels of cross-resistance to fipronil this need not necessarily be so in *H. armigera*.

Because of the results described above, work on this area was phased out after 18 months of the project. Instead, as described below, we concentrated on developing diagnostic DNA probes for metabolic resistance to pyrethroids. However, now we have shown that the structure of this species' $GABA_A$ gene differs from those of other insects, it is clear that it could provide a valuable resource for insecticide discovery programs. We therefore decided to clone some larger fragments of the $GABA_A$ gene of *H. armigera* as a stepping stone to that end.

Pyrethroid resistance

There is still much debate on the current mechanisms of resistance to pyrethroids in Australian *H. armigerra*. In my view, it is still unclear whether esterases have any significant role. On the other hand there is a very large body of data based on synergism studies (mainly Neil Forrester's work) implicating cytochrome P450s and oxidative metabolism of pyrethroids and this is consistent with work done overseas.

Despite synergism studies, published attempts to measure enhanced pyrethroid metabolism have used indirect methods (e.g. Hobbs*et al.* NADH-linked assays or Gunning*et al.* aldrin epoxidase assays) and either struggled to measure metabolism in certain tissues or show only marginal changes in the resistant line. The problem is exacerbated by the fact that we know that organisms such as insects probably have several hundred cytochrome P450 genes and that only one or a few may change to confer resistance. The change, is according to the most recent evidence (Feyereisen*et al.*, 1995), due to mutations in regulatory genes that regulate the level of expression of the structural cytochrome P450 genes.

In order to construct DNA probes for resistance mutations we need to determine which cytochrome P-450s are modified and in what way. To establish that constitutive overexpression of a particular cytochrome P-450 leads to a resistance phenotype, it is necessary to establish that there is tight (absolute) linkage between overexpression of a particular cytochrome P-450 gene and the resistance phenotype. Demonstration that a particular gene is induced by pyrethroids, is neither necessary or sufficient to show that it is involved in resistance (although it is not incompatible with it being responsible for resistance). For example, phenobarbital induces a wide variety of cytochrome P-450s in many susceptible organisms.

To overcome the problems of measuring enhanced metabolism of pyrethroids directly, we decided instead to look for overexpression of particular cytochrome P-450 genes. The strategy we adopted was to screen a third instar cDNA library, made from the resistant strain, for cytochrome P-450 genes, to classify the frequency with which particular clones were isolated and to assess the expression of the more abundant genes by Northern analysis of RNA from the resistant and susceptible lines. The actual research proceeded in three parallel strands:

The first was to establish and verify resistant and susceptible colonies in the laboratory. These strains are essential resources for identifying resistance genes. The undertaking was a more substantial one than originally envisaged, since at the start of the project no other laboratory in Australia could provide us with a susceptible colony. When a colony was obtained via Neil Forrester, it took several months to establish it securely under our laboratory conditions.

The second problem was the need to obtain direct and unequivocal evidence for enhanced metabolism of pyrethroids in whole*H. armigera*. A thin layer chromatographic assay was developed, based on that used by McCaffery's group (Reading, U.K.), using radioactively labelled^{14}C-Cypermethrin. The assay was piloted on resistant insects since the susceptible colony only became available after termination of the project.

The third strand was to isolate candidate cytochrome P-450 genes. First we needed to obtain probes that could be used to screen our cDNA library. We were advised by Dr. René Feyereisen (Centre for Insect Science, Univ of Arizona, pers. comm.), whose lab has done the best work in this area, that isolation of probes directly from*H. armigera*by degenerate PCR was likely to be more effective than use of heterologous probes. This is because, outside a few discrete regions of high conservation, diversity of insect cytochrome P-450 genes is quite high. Accordingly we designed a range of PCR primers to amplify members of all the known insect classes of cytochrome P-450. (A new class, class 9, has subsequently been discovered.) Since a resistance-conferring gene might come from any class this was seen as important to avoid excluding relevant genes. Using this approach we were able to clone and sequence a

≈ 500 base pair portion of a class 4 cytochrome p-450 cDNA. To our knowledge this was the first isolation of a lepidopteran class 4 cytochrome P-450. A number of other amplicons were under investigation at the termination of the project. In addition, in March 1995 the sequence of a full-length *H. armigera* class 6 cytochrome P-450 cDNA (Wang & Hobbs, unpublished) became available on the GenBank database. With these independent probes we were therefore in a good position to start the next step, screening a cDNA library with the probes.

ACS Special Conference VII. Molecular Genetics and Ecology of Pesticide Resistance. Montana June 1995.
As part of the Project Leader's involvement in research on resistance management, the Division funded his attendance at the above conference. Apart from the specific scientific content of the conference a number of broader issues were raised:

Threats to agriculture from international trade in infested plants (and resistant weeds).
A recurring theme was the biochemical and molecular genetic evidence that insecticide-resistant strains of insects (blowfly, mosquito, whitefly and aphid) have been and continue to be transported readily around the world. The term "Pest tourism" was coined. The international trade in ornamental plants, which have been heavily sprayed, is believed to be a major source of these infestations. One speaker indicated that a survey showed that 5% of all plants ("even from the Netherlands") travelling across international borders have insects on them. "A real zoo". Dr. Matt Cahill (Rothamsted) reported the detection of resistance to buprofezin, an IGR insecticide, in UK populations of sucking pests even before buprofezin was registered in the UK. The traffic in resistant insects therefore threatens resistance management throughout the world. The recent introduction of the *B. tabaci* B biotype into Australia is just one example of the trend. It was pointed out that, despite the potential risks, current quarantine protocols pay little or no attention to the insecticide resistance status of insect contaminants.

The value of molecular genetic studies for real world management of pesticide resistance
Several of the workshop discussions considered this issue. The conclusions were that molecular genetics had contributed relatively little to date, but that this was to be expected given the relative youth of the field.

In the future it was felt that molecular genetic studies would allow better detection of resistance, provide better markers and information to field ecologists, and allow directed screening for resistance breaking compounds and compounds with negative cross-resistance. (In particular the example was given of "negative cross-resistance" between different herbicide compounds which is seen as the ideal situation for resistance management). The ability to predict field resistance mechanisms through mutagenesis and selection (now validated for the herbicide chlorsulfuron and the insecticide dieldrin) and the new technique of sexual PCR were seen as powerful tools for predicting and understanding likely resistance problems before they arise.

Finally the ability of molecular genetics to identify and clone new targets was seen as critical for successful resistance management. This is particularly relevant to insecticides (rather than herbicides and fungicides) given the continued reliance on an alarmingly small number of insecticidal modes of action which has negative implications for resistance management.

Resistance management strategies for transgenic plants-avish praise was heaped on "The Australian Model" of insecticide resistance management from many quarters. Dr. Randy Deeton of Monsanto was keen that this voluntary model should be the one adopted for resistance management with Bt cotton and other crops in the U.S. A. Dr. Neil Forrester pointed out that, even in Australia, legislation might be the only way of preventing (or controlling) the introduction of the same Bt gene into every summer crop.