Report Cover Sheet for Annual & Final Reports

The following Reporting Requirements MUST BE MET

All Projects
You must submit an **ANNUAL PROGRESS REPORT** by the first Friday in February 1999, detailing the progress of your research. NOTE: IF you are seeking continuation of funding for 2000–2001 for the project, this report will form the basis for CRDC's consideration of ongoing funding. Please complete the budgetary requirements if this is a continuing project.

Terminating Projects
A **FINAL REPORT** must be submitted within three months of completion of the project. This applies in **ALL** cases including research projects, travel, conference attendances, postgraduate, postdoctoral and funded capital items.

Tick Report Purpose

Annual Progress Report *(Due 1st Fri Feb. to determine continuation of funding)*

Final Report (Due 30 September or 3 months after completion of project) √

Actual start date: ***01/07/97***

Anticipated completion date: ***30/06/99***

OFFICE USE ONLY:
Date of receipt:

Project title (as per original application)

Quantification of *Bacillus thuringiensis* Insecticidal Crystal Proteins for Season-long Monitoring of Transgenic Field Crops

CRDC Project Code CSE66C

CRDC Responsible Director (if known)

Organisation CSIRO Entomology

Administrative contact Mark Hardwick

Phone: 02 6246-4008 E-mail: mark.hardwick@ento.csiro.au Fax: 02 6246-4000

Postal Address CSIRO Entomology
PO Box 1700
Canberra, ACT, 2601

Project supervisor Dr Ray Akhurst

Phone: 02 6246-4123 E-mail: ray.akhurst@ento.csiro.au Fax: 02 6246-4173

Principal Researcher Dr Helen Holt

Phone: 02 6246-4110 E-mail: helen.holt@ento.csiro.au Fax: 02 6246-4173

Final Report

Use the cover sheet format as above.

Please ensure the following criteria are addressed when completing your Final Report

What was the background of the project?

The insect resistance of INGARD™ cotton crops has been variable across sites and throughout the growing season. This variability is of concern to growers individually and to the industry as a whole. The reasons for variability in performance can only be fully investigated when we have a reliable method for measuring the levels of the insecticidal protein (Cry1Ac) produced throughout the season in the different tissues. The sources of variability in efficacy are being investigated through the development of a method to quantify Cry1Ac levels in plants, together with bioassay of field and glasshouse material against the heliothine pests.

INGARD™ cotton contains the full length *cry1Ac* gene and so encodes the protoxin, which is usually proteolytically cleaved and activated in the insect gut. The current literature does not discuss the form in which Cry1Ac is present in plant tissues, whether as the protoxin or as the cleaved toxin. During the conduct of this project (CSE66C) we discovered that at least some of the Cry1Ac is present as the activated toxin. Variation in the extent of activation may be a significant component of the variation in insecticidal activity of the plants.

During an earlier project (CSE46C) we showed that we could reliably extract good quality total protein from cotton tissues and could detect Cry1Ac among the complex mixture of proteins in cotton tissues. We also showed that we could detect changes in the levels of the Cry1Ac extracted by use of Enzyme-Linked Immunosorbent Assay (ELISA). To complete the development of the assay we needed to determine the efficiency of both total protein and Cry1Ac extraction to allow comparison of data between trials and, if necessary, to improve the efficiency of the techniques.

Data obtained from the first project (CSE46C), in collaboration with Dr Gary Fitt's group and Dr Joanne Daly's group, indicated that the decline in Cry1Ac levels is highly correlated with the decline in efficacy of plants in the field as determined by bioassay and observation. This project aimed to investigate the relationship between plant age, Cry1Ac level and the toxicity of the cotton to *H. armigera*.

What were the project objectives and to what extent were these achieved?

The objectives were to provide a reliable method for quantifying the Cry1Ac produced by transgenic cotton plants and to use this to examine the changes in Cry1Ac levels during a field season. We also undertook to train relevant laboratory personnel in Canberra and Narrabri in the use of the techniques for extracting and measuring Cry1Ac levels in plants.

The project has been successful in developing a method for estimating the content of Cry1Ac in cotton tissues of all ages and developmental stages. Statistical analysis of the efficiency of extraction is still being completed and will be reported in the scientific literature once this assessment has been completed.

Approximately 1500 samples from several trials collected during the course of the project were tested to determine the changes in Bt levels in plant tissues across the growing season and under differing environmental constraints. In addition, 1000 samples from field and glasshouse trials established to test the effects of stressors on INGARD™ performance have been analysed for both total protein and Cry1Ac levels. These data are currently being statistically analysed and once this analysis has been completed the data will form the basis for three or four research papers in conjunction with Dr Joanne Daly's and Dr Greg Constable's research groups.

This project raised the issue of the nature of the Cry1Ac protein in INGARD™. We demonstrated that a proportion of Cry1Ac is apparently processed from the protoxin to the toxin form in the plant. This was very important for the measurement of the Cry1Ac content of the plants because we discovered that the antibodies used in these assays detected Cry1Ac toxin far better than the protoxin. Our results indicated that the antibodies detected the activated Cry1Ac toxin protein very well in both Western blotting and ELISA, but by comparison recognized the full length

protoxin poorly in ELISA. As the ELISA showed a range of detectable Cry1Ac levels in transgenic tissues, it appears that at least some of the Cry1Ac is present as the activated toxin. Until samples are tested with antibodies which recognize both toxin and protoxin, we cannot determine whether the most important influence on efficacy is from a change in total Cry1Ac, a change in the toxin/protoxin ratio, or a change in the toxin level only.

The methods for extraction and detection of cotton total protein and Cry1Ac have been taught to technical staff from Canberra and Narrabri and Ms Cheryl Mares has used them to analyse her own samples. A technical paper detailing the standard methods is currently in preparation and is intended for use as a standard laboratory manual for extracting and measuring total protein and Cry1Ac from cotton tissues.

The preliminary findings and details on the potential uses of the methods have been communicated through posters and oral presentations at conferences, including the ACGRA Conference in August 1998 and at the AAERC conference in September 1998. An article detailing the potential uses of the technique was published in the January/February 1998 issue of The Australian Cottongrower magazine.

What Methodology was used, and a justification for the use of this methodology?

The central method for developing a technique for measuring Cry1Ac in transgenic cotton was an ELISA test specific for Cry1Ac. ELISA relies on the recognition of the protein in extracts by an antibody that binds specifically to the protein of interest. It is a very sensitive assay that can detect proteins at nanogram (or lower) levels, making t one of the most sensitive techniques available. The assay is conducted on microplates that can be used to test up to 72 samples and triplicate standards on each plate, making it suitable for processing a large number of samples in a single assay. Also, with suitable antibodies, the assay is very specific, detecting the protein of interest without the complication of false positive results. The assay relies on the development of a colour reaction caused by the reaction of an enzyme attached to the antibody and a developer. Several different enzyme/developer combinations were tested so that we could select the most suitable of these to give consistent, specific and sensitive results for a variety of plant samples.

We also used Western Blotting techniques to confirm that the size of the protein detected by our antibody was consistent with that of Cry1Ac. This method also relies on antibody detection and is useful for confirming the presence of Cry1Ac qualitatively. However, it is not suited to large-scale quantitative trials due to the difficulties involved in providing and measuring standards and the limited number of samples which can be run on each blot. This technique was used for determining the recognition of protoxin and toxin by a range of antibodies recognizing either the protoxin or toxin forms of Cry1Ac.

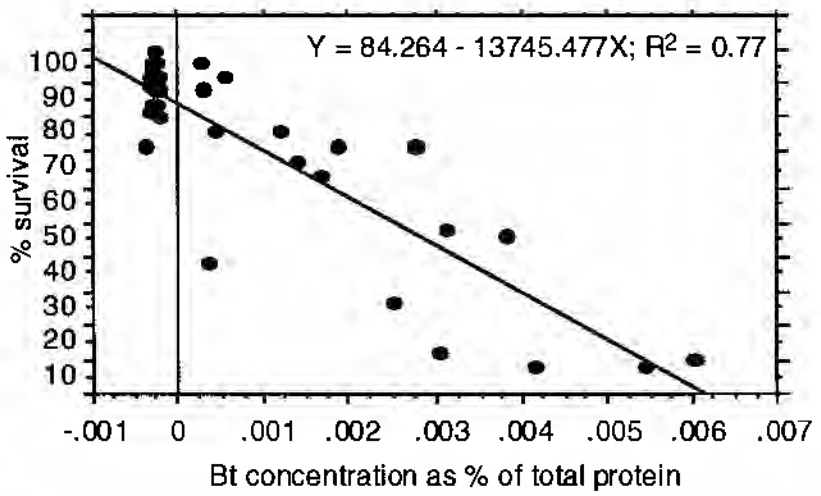
The reliability of the extraction technique was tested by a repeated extraction trial. Individual samples were subjected to serial extractions to determine the percentage efficiency of the initial extraction for both total protein and Cry1Ac. Included in this trial were samples containing known ratios of transgenic and non-transgenic tissues to enable us to determine whether the Cry1Ac detected was proportional to the amount of transgenic material included in the mixture. The data from this trial are currently with the statistician. The efficiency factors for total protein and Cry1Ac extraction will be used to correct for unextracted Cry1Ac in the analysis of data from other trials to give an estimate of total Cry1Ac levels.

Glasshouse trials were conducted using single plant samples, whereas field trials used pooled samples. The single plant measurements were designed to provide information about variation in expression between transgenic cotton plants that will be useful in the development of new plant lines. This type of variation can be more easily and accurately studied in glasshouse trials than in field trials, due to the easier accessibility of glasshouse plants. The use of pooled samples in field trials gives an indication of changes occurring in the crop as a whole. These data are expected to have implications for practical whole-crop management strategies.

Detailed results including statistical analysis of results?

Samples were taken during the 1996/97 growing season in Narrabri were divided. Some sub-samples were bioassayed by Dr Gary Fitt's group and the Cry1Ac levels determined for the others. ELISA showed a decline in Cry1Ac levels across the field season. Dr Fitt's comparison of the ELISA data with his bioassay data showed a significant correlation between bioassay survival and Cry1Ac concentration expressed as a percentage of total protein. (Figure 1). This result shows that the decline in efficacy of INGARD™ cotton is related to changes in the Cry1Ac content of the plants and demonstrates the value of an ELISA test for monitoring plant performance.

Figure 1. Insecticidal activity of INGARD™ cotton is correlated with the Cry1Ac content of the plants.



(Reproduced from Efficacy of INGARD Cotton – Patterns and Consequences. *In* "Proceedings of the Ninth Australian Cotton Conference. Gold Coast, 1998." pp. 233-245.)

Plants from glasshouse trials and a field season trial conducted by Dr Gary Fitt were also used to test the overall efficacy of the extraction and detection techniques. The extraction technique was shown to provide undegraded protein for the detection assays. The protein and Cry1Ac content data are currently with the statistician and firm conclusions cannot be drawn until this statistical analysis has been completed. These analyses will then form the bases for three or four papers.

Tissue samples were collected from a field trial and three glasshouse trials conducted by Dr Greg Constable's group to examine the effects of shading, temperature stress and water stress on INGARD™. Subsets of these samples were bioassayed by Dr Joanne Daly's group and the Cry1Ac content estimated in this project. The data are being statistically analysed to determine the factors that adversely affect Cry1Ac levels.

We also undertook testing of a commercial ELISA kit from the Agdia company which is supplied in Australia through TASAG ELISA and Pathogen Testing Service, Plant and Animal Health Laboratories, Dept of Primary Industries, Water and Environment, Tasmania. We ran the assay with samples from field and glasshouse grown INGARD™ cotton. The tests with the assay kit were run concurrently with our own method, using replicate samples in each. There were some differences in estimates of Cry1Ac content between replicate samples tested with the kit on different days and there were also differences between results obtained from the kit and from our ELISA. However, the kit did consistently recognise transgenic samples without producing false positive or negative results and it was considered to show sufficient reliability to use on a variety of test samples. As with most commercial kits it was expensive to purchase, but bulk kits (50 plates, with 96 test/standard wells per plate) were a viable option where large numbers of samples require testing. This kit was then used to test approximately 1500 samples from both field and glasshouse trials.

Western blotting of extracts from INGARD™ cotton with anti-Cry1Ab antibodies provided by Dr Danny Llewellyn detected a protein band of approximately 65kDa, which is the size of the activated toxin rather than protoxin. Tests on Cry1Ac purified from the HD73 strain of Bt and on trypsin activated-Cry1Ac showed that these antibodies were far more effective in detecting the toxin than the protoxin. As the anti-Cry1Ab antibodies were produced against activated toxin, we then tested antibodies that had been produced with the crystal protoxin of Cry1Ac. The anti-Cry1Ac antibodies recognised both protoxin and toxin, albeit the former elicited stronger recognition. Western blotting of plant extracts with the anti-Cry1Ac antiserum indicated that the bulk of the Cry1Ac in INGARD™ cotton was present as the toxin rather than protoxin.

A discussion of the results, including an analysis of research outcomes compared with the objectives?

We have shown that we are able to measure the Cry1Ac levels across the season using both our own and a commercial ELISA. However, until statistical analysis of our data is completed it is not prudent to draw conclusions on the degree of and basis for changes in Cry1Ac levels throughout the growing season.

The data from trials to investigate possible environmental triggers for changes in the levels of Cry1Ac have yet to be analysed. Once analysed and combined with the bioassay data from the same trials these data will form the basis for determining the relationships between environmental conditions, Cry1Ac levels and plant efficacy in protecting against insect damage.

The use of our own ELISA was limited in the latter part of the project by the difficulties in producing and independently measuring sufficient purified toxin for use as standards in the ELISA. Standards supplied by Dr Danny Llewellyn, CSIRO Plant Industry were from a Monsanto kit. The definition of a suitable standard was complicated by variation between the detection of protoxin and/or toxin. If the quantity of protoxin in INGARD™ cotton is significant, the test may have to be run with two antisera and protoxin and toxin standards.

The range of Cry1Ac levels in transgenic tissues detected with the Cry1Ab anti-toxin antibody indicates that at least some of the Cry1Ac is present as the activated toxin. However, until we have an antibody which detects both forms of Cry1Ac equally well and antibodies that differentiate between protoxin and toxin, we cannot be sure whether plants process all or only a proportion of the total Cry1Ac to the activated form. Since the protoxin is significantly more toxic than the activated Cry1Ac toxin for *H. armigera*, it is not only important to determine the total Cry1Ac content but also the ratio of protoxin to toxin. If differential processing of the Cry1Ac occurs in plant tissues over time there may be varying ratios of the two forms present in plant tissues throughout the growing season. This has very important implications not only for plant efficacy, but also for resistance management strategies. This question could not be fully explored within the limits of this project.

An assessment of the likely impact of the results and conclusions of the Research project for the Cotton industry, and where possible a statement of the costs and potential benefits to the Australian Cotton Industry and future research needs?

The ELISA test developed in CSE46C and tested in this project will facilitate the examination of the effect of environmental factors on the efficacy of INGARD™ cotton. This has important implications for long-term management of transgenic cotton and also for its future development and refinement. The data on Cry1Ac levels may also have implications for resistance management strategies for insect populations. The degree and prevalence of plant-to-plant variation will influence the way in which resistance management strategies could best be implemented to combat field resistance.

The data on plant-plant variation among transgenic crops in the glasshouse trials has implications for breeding programs. The pooled samples of the field trials give an indication of the crop as a whole and have implications for practical whole-crop management strategies in the long run. Further field trials are necessary to provide a good bank of knowledge in this area and to determine if possible what occurs during a "typical" growing season. It is anticipated that this further evaluation will be conducted by Dr Gary Fitt's group, using techniques developed in this project.

A description of the project technology (eg commercially significant developments, patents applied for or granted, licences, etc)

A technical summary of any other information developed as a part of the Research Project including discoveries in methodology, equipment design, etc.

Recommendations on the activities or the steps that may be taken to further develop, disseminate, or exploit the project technology

The question of protoxin/toxin production in plants and presentation to insects in the field must be resolved. We need to be sure that we are not only measuring the correct protein (i.e. cleaved toxin or protoxin), but also that resistance management strategies are based on the correct information about which form is presented to the insect. This can potentially affect how and when resistance may develop in the field and is a vital piece of knowledge in correctly managing, developing and supporting what is potentially a very valuable tool for the cotton industry.

One of the technical difficulties encountered was in developing a standard Cry1Ac for the ELISA method. Although this should have been only a small part of the development of the technique, it proved to be a much larger issue than anticipated. The standard cannot be defined until the extent and significance of activation of the protoxin is established.

A list of publications arising from the research project

Holt, H. (1998) Season-long monitoring of transgenic plants - Development of an assay for quantification of *Bacillus thuringiensis* insecticidal crystal protein. In "Proceedings of the Ninth Australian Cotton Conference. Gold Coast, 1998." pp. 331 - 335.

Holt, H.E. (1998). Season-long quantification of *Bacillus thuringiensis* insecticidal crystal protein in field-grown transgenic cotton. In "Proceedings of the Australian Applied Entomology Conference. Brisbane, 1998. Volume 1" pp. 215-222.

Holt, H. (1997). Measuring toxin levels in Bt cotton. The Australian Cottongrower Volume 18, No. 6, pp. 62-63.

Currently a technical paper detailing the extraction and detection methods is in draft form. This publication is intended as a practical, laboratory guide to using the technique for other lab personnel.

In addition to published papers the information from this project has been disseminated to industry and the scientific community by means of a number of oral and poster presentations. An oral presentation to a meeting of CSD consultants in May in Narrabri outlined the background and development of the ELISA, as well as presenting summarised results and indicating possible future uses for the technique. It also provided a forum for consultants to ask questions concerning the development and use of the technique and its relevance to their work. A brief presentation to cotton agronomists, organised by Adam Kay, CSD, consisted of a very short discussion of the background and anticipated future uses of the ELISA method and associated extraction techniques.

A one page plain English summary of the project outcomes must be submitted, and this may be used in CRDC publications and on our proposed web site.

Transgenic cotton expressing insecticidal proteins offers a great opportunity to Australian growers through a significant reduction in the use of synthetic chemical insecticides and the problems of environmental damage that they pose. However, as with any new technology, we have to learn its limitations and the way in which we can derive the greatest sustainable benefit. A significant limitation detected early in the deployment of Bt cotton has been the variations in plant resistance to insect pests.

This project has developed methods that allow us to measure the amount of the insecticidal protein from cotton tissues of all ages and types. This involves extraction of all the proteins in these tissue and capturing the insecticidal component by using an antibody that binds only to the insecticidal protein. The amount of insecticidal protein bound to the antibody can then be detected by using methods that are now commonplace in medical practice and industry. By collaborating with other researchers we have obtained data on the level of Cry1Ac in INGARD™ cotton throughout the season and under various growing regimes. Other collaborators have conducted bioassays with tissues from the same plants. By combining these data we are improving our understanding of the relationships that exist between plant performance and insecticidal protein levels. We can then start to identify the environmental factors that trigger changes in plant performance and what these changes mean for short- and longer-term management strategies, including resistance management.