7/89 to 6/92 FINAL REPORT CSP13C

GENETIC TRANSFORMATION OF COTTON: INTRODUCTION OF NOVEL GENES INTO COTTON AND THE DEVELOPMENT OF NOVEL SOURCES OF INSECT RESISTANCE FOR COTTON

This project's major goal was the development of tissue culture regeneration and transformation protocols for Australian cottons and to use such protocols to demonstrate the potential benefits to the industry of producing new cultivars by genetic engineering. These objectives have clearly been met with the production of cotton plants that are either tolerant to a herbicide or to insect pests. Specific details are are outlined below.

Regeneration and Transformation of Australian Cottons.

When this project began in 1989 we were able to take explants of young cotton seedlings, culture them on tissue culture medium, and eventually regenerate some fertile plants. The process was however, very genotype dependent, and even with our best cultivar, Siokra1-3, very inefficient. Over the time of the project we have improved the regeneration capacity of Siokra 1-3 from about 20% to over 90%. We have identified two other cultivar that have some regenerability, both current commercial cultivars (Siokra 1-4 and Siokra S324). As yet, no Sicala varieties have shown any capacity for regeneration.

Over the last year and a half we have adapted the tissue culture regeneration system to use with the *Agrobacterium* gene transfer system and used it to introduce a novel gene into cotton. The key to this success was the use of highly regenerable genotypes such as Siokra 1-3 (or the American variety Coker 315), the identification of an *Agrobacterium* strain that was more efficient in transferring genes into cotton, and an optimised selection regime. The genes first intorduced into Siokra cotton were a selectable maker gene that allows the selection of transformed cells and an easily assayed marker gene the bacterial GUS gene that allows us to stain tissues for the presence of the introduced gene. The first fertile regenerated and transformed Siokra plants set seed and the introduced gene was inherited in its progeny just like any normal gene. The transformation system for Siokra 1-3 was recently published in the Australian Journal of Plant Physiology Vol 18 (1991). The transgenic plants looked normal and expressed the marker gene in all the tissues expected.

Recently we produced our first transgenic plants of Siokra S324, but as yet we do not know whether they are fertile. New cultivars will be tested as they arise and as our expertise in selecting the right tissues for regeneration improve.

Herbicide Tolerant Cotton Varieties Produced Through Genetic Engineering.

CSD and CRDC have jointly funded the development of a cotton variety that would be tolerant to accidental damage from spray drift of the broadleaf herbicide 2,4-D. The herbicide is rapidly broken down in the soil by micro-organisms, so we looked to these organisms to provide us with a gene that would degrade or detoxify 2,4-D if introduced into plants. The bacterium *Alcaligenes eutrophus* provide us with the gene for the 2,4-D mono-oxygenase that carries out the first step in the complex pathway for the degradation of 2,4-D. In the first year of the project (then funded under the one year CRC grant CS61L) the gene was isolated from the bacterium, modified to function in plants and introduced into tobacco as a model system. The transgenic plants proved to be about 50 times more tolerant to 2,4-D than normal tobacco plants. Having

demonstrated the efficacy of the gene, we turned our attention to introducing it into cotton as part of the last two years of this grant. All our initial attempts at transforming Siokra 1-3 and the optimisation of the transformation procedure were carried out with an *Agrobacterium* strain carrying the 2,4-D resistance gene as well as the easily assayed GUS gene. Concurrently with our Siokra work were were also attempting to duplicate overseas success with the American cultivar Coker 315. This cultivar proved easier to transform than Siokra and we were able to produce several transgenic Coker cottons containing the 2,4-D resistance. Four of our fertile plants have produced progeny and we have analysed their tolerance to 2,4-D. All lines appear to be tolerant to about 300 ppm 2,4-D but show damage at higher levels (Figure 1.). We estimate that these plants are 100 times more tolerant to 2,4-D than normal control plants. The normal field rate for 2,4-D is approximately equivalent to 280 ppm so while these Coker transgenics are tolerant to normal field rates, there would be insufficient safety margin to use 2,4-D directly on the cotton. We currently have many transgenic Siokra plants in the pipeline and we expect that they will show similar tolerance levels to the transgenic Coker plants.

CSD has agreed to continue funding this project at the same level (part salary of a Post-doctoral scientist) and we propose to continue by completing our studies on the first generation gene construct in both Coker and Siokra transgenics; carry out field testing on these plants to get a better estimate of how their tolerance relates to field rates; and continue the development of new generation genes which should give much better tolerance levels. We intend to use two strategies, one is to express more of the 2,4-D degrading enzyme in the shoot tips (the major site of damage) by linking the gene to shoot-tip specific gene control sequences, and secondly by cloning the next genes in the 2,4-D degradation pathway and expressing those in plants. This latter step will remove the product of the first breakdown step, which is itself still slightly toxic to plants, but about 100 fold less toxic than 2,4-D.

In the end we hope to be able to produce a cotton plant that is completely tolerant to 2,4-D so that risks of damage caused by spray drift are negligible and perhaps allow 2,4-D to be used directly to control weeds in cotton crops.

Insect Tolerant Cotton Plants

This project is the one that has the most potential to make a major impact on the Australian cotton Industry. The crop is constantly under attack by insect pests, mostly the two *Heliothis* species, and current control measures are costly, potentially environmentally damaging and becoming less effective as each year progresses. Genetic engineering has the potential to markedly decrease the usage of chemical pesticides if the plants natural tolerance to insect pests could be increased by the introduction of novel insecticidal genes using genetic engineering.

At the end of the second year of this project we were successful in signing a research agreement with the American Monsanto Company to access their technology for introducing BT-toxin genes into Australian cotton cultivars. BT-toxins are insecticidal proteins produced by the bacterium *Bacillus thuringiensis* and are the active components of the biological insecticide DIPEL. Monsanto has developed a BT-gene construct that is highly expressed in transgenic plants and have produced Coker cotton varieties that contain sufficient of this toxin to protect plants from attack by *Heliothis zea*. We have begun to introduce the BT gene into Australian varieties by both direct transformation (into Siokra 1-3 and S324) and by crossing it into the Australian varieties from the transgenic Coker cottons produced by Monsanto. Over the last year we carried out an extensive back-cross program from four transgenic Coker cottons into six Australian cultivars. Because of Australia's quarantine regulations this had to be done under quarantine glasshouse conditions at Plant Industry in Canberra. We have

'ested BC1 progeny plants with *Heliothis armigera* larvae (ANo2 strain), and atleast in the laboratory have been able to demonstrate complete toxicity to *Heliothis*. How this will translate to field resistance will be the subject of our next CRDC proposal. The back-crossing program has moved to Narrabri under the direction of Peter Reid, but we will continue to be involved in the screening of plants for the presence of the BT toxins and in conducting field trials with Dr Gary Fitt. The direct transformation of Siokra takes about one year and we have already started to produce transgenic Siokra 1-3 and S324 plants that again show high laboratory resistance to strain ANo2 larvae. As yet we don't know whether these first plants are fertile, but many more are in the pipeline. The advantage of direct transformation is that the plants produced are already in well adapted genotypes and need only be seed increased before they are ready for commercial use.

So although the BT-cotton program is off to a promising start, we still have much to do to produce plants that would be commercially useful and the further analysis of these plants will form much of the basis of our next CRDC proposal on the genetic engineering of cotton.

One of the controversies that still reigns over the use of BT-toxin genes in transgenic plants is that it is still uncertain whether their use will hasten the development of resistance by the insect pests it is used to control. BT-sprays have lasted for many years and could be potentially used on a large scale in the cotton industry. However resistance is starting to develop overseas and it seem probable that it will happen here if usage escalates without some thought of resistance management. BT-plants are an ideal solution for the farmer but there will be continuous exposure to the high levels of toxin produced by the plant. Will this select for rapid resistance development? While intuitively it might seem that it should, there is growing evidence that resistance development could be managed by controlling the ratios of transgenic and non-transgenic plants in the field. These sorts of management strategies will have to be worked out before the plants are released commercially and will form part of the CRDC proposal being submitted by Dr Gary Fitt. We have opted to take the conservative approach and will try to limit the chances of resistance development to plant-based BT-toxins by producing transgenic plants with more than one type of insecticidal resistance gene.

As part of the current project we have been examining the use of other BT toxins, besides the one developed by Monsanto. We screened a number of new isolates gathered by Dr Richard Milner and colleagues for toxins which were highly active against *H. armigera*, but of the best from over 300 new isolates all appeared to be only as effective as the type used by Monsanto and all appeared at the DNA level to belong to the same class of toxins as the Monsanto BT gene. We have now turned our attention to two other distinct classes of BT-toxins which are known to be active against the larvae of moths. These are the Cry IB and Cry IC toxins (as opposed to the Cry IA type used by Monsanto). Genes for these have now been isolated and are being modifed for expression in plants. At the protein level they appear to be less toxic to *Heliothis* but they are known to act at different receptor sites in the insect mid-gut and hence are unlikely to give cross-resistance if the insects develop resistance to the Monsato BT-toxin in our transgenic cotton. There will still be a number of molecular manipulations required to express these alternate BT-toxin genes at high levels in plants, but the early studies can be carried out in transgenic tobacco as a model system.

The location of the toxin in the transgenic plants may also play a role in in delaying resistance development. The Monsanto gene is expressed in all parts of the plant so no matter where the insects feed they are exposed to the toxin. On the one hand this seems an advantage of the transgenic plant approach, but on the other it doesn't allow for any easing of the selection pressure (except when mixtures of transgenics and non-transgenics are used). If the toxin could

just be expressed in the tissues of the plant that are most vulnerable to attack (for cotton this is the young buds and squares) then it could feed on older leaf tissues and provide "refugia" on the same plant. To this end we have isolated bud specific genes from tobacco ,(in collaboration with Dr. Ry Meeks-Wagner, University of Oregon) and will use these to drive the expression of BT-toxin genes in cotton. The same gene promoters will be useful for our 2,4-D resistant cotton work. Completion of the cloning and characterisation of these promoters will be covered in our next CRDC proposal.

BT toxin genes are not the only insecticidal proteins, and we have been investigating the use of trypsin inhibitors to control *Heliothis* species. Trypsin inhibitors are proteins thought to be produced by plants as a defense against insects and other pathogens. Dr Clarence Ryan of the University of Washington has shown protective effects of tomato and potato trypsin inhibitors when introduced into transgenic tobacco. However when we tested some of his transgenic plants against *Heliothis armigera*, we found very little reduction in larval growth. A screening program was begun to identify trypsin inhibitors that might be toxic to *Heliothis* and we have identified one, the Giant Taro trypsin inhibitor, that showed a respectable reduction in the growth rate of larvae fed on a synthetic diet supplemented with this inhibitor. A two year grant was obtained from special CSIRO funds to appoint a post-doc (Dr Anne Mathews) to characterise and clone the gene for the inhibitor and test it in transgenic tobacco. HPLC has been used to purify the protein and antibodies have been raised in rabbits. The proetin sequence was known previously and has been used to design DNA primers which have been used with PCR amplification to isolate a fragment of the coding region of the inhibitor. This fragment will be used to isolate the gene from a cDNA library prepared from giant taro corms flown into Australia from Samoa. The antibody will be used to quantitate inhibitor levels in transgenic plants. The gene will be introduced into cotton once we have demonstrated some efficacy in transgenic tobacco.

Other insecticidal proteins have also been identified in our insect bioassays, including a number of plant hydrolases and these became the subject of a separate CRDC grant (CSP25C) to clone and test them in transgenic plants. We have also shown that partially purified Jack bean urease and glucose oxidase ,are toxic in synthetic diets. A urease clone has been obtained from CSIRO Division of Tropical Animal Production in Queensland and is being engineered for expression in plants. It will be tested in transgenic tobacco over the next year. The glucose oxidase is produced by a fungus and its isolation forms the topic of a PhD project funded by CSD. The gene is being isolated as a potential Verticillium wilt resistance gene (the enzyme is toxic to Vert.) but we will also use it against insects, in which case it has to be expressed in a different way in the transgenic plants..

Conclusion:

In conclusion we have clearly demonstrated our capacity to produce transgenic cotton plants and develop gene constructs that will be useful for the agronomic improvement of cotton. This project represents the beginning of a new age in cotton breeding that will allow us to produce plants with novel characteristics that could not be achieved in any other way. We look to the industry to continue supporting genetic engineering so that the products of our research can find their way into the fields of Australian cotton farmers.

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Llewellyn,D., Cousins,Y., Mathews,A., Hartweck,L., and Lyons,B. (1992) Expression of *Bacillus thuringiensis* insecticidal protein genes in transgenic crop plants. Agric. Ecosystems Environ. (In Press)

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26.10.92

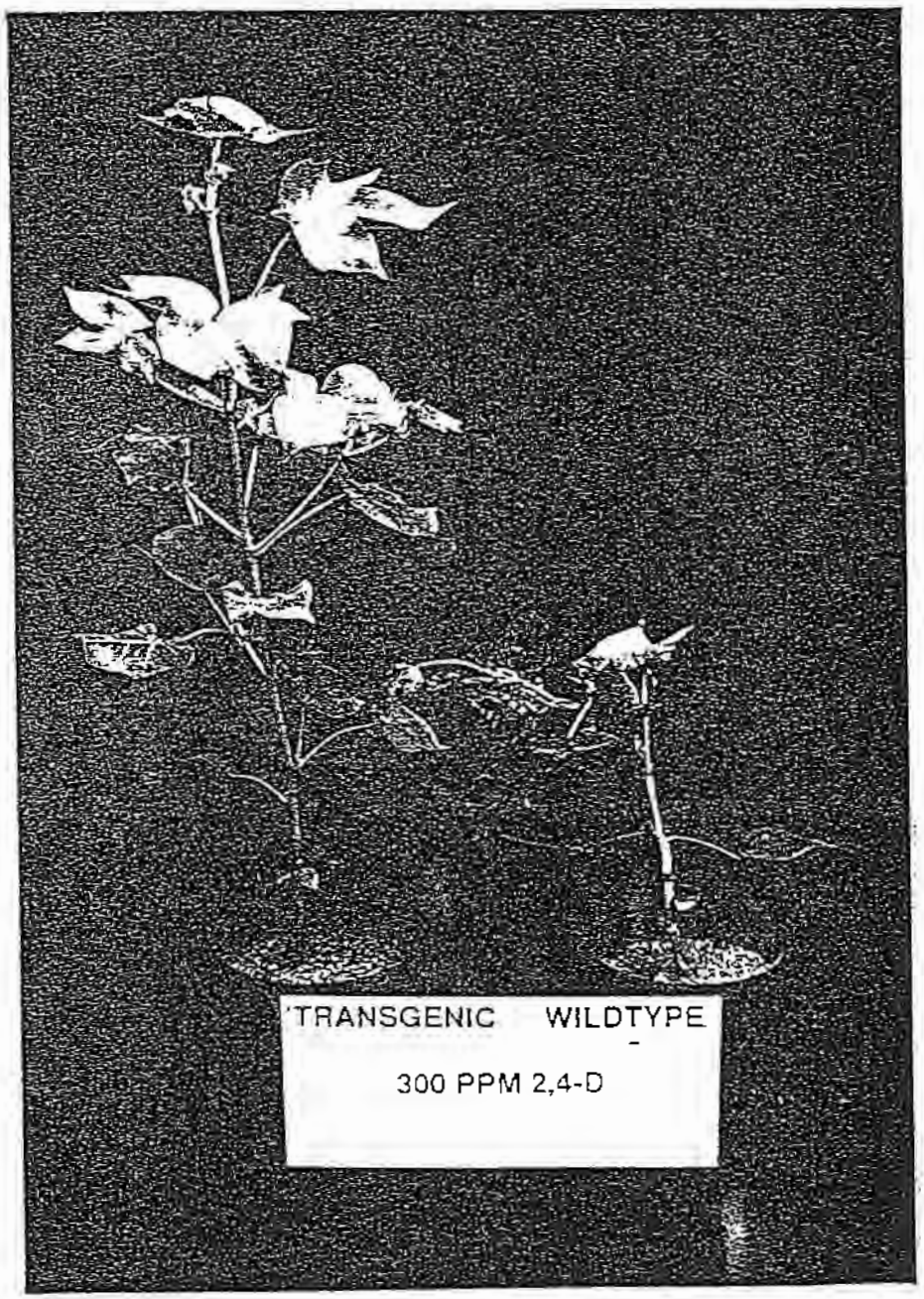

Fig. 1.
Transgenic coker (left) and wild type (right) cottons sprayed with 2,4-D.