WHAT'S HAPPENING TO THE EXPRESSION OF THE INSECT PROTECTION IN FIELD-GROWN INGARD® COTTON?

E. J. Finnegan[1], D. J. Llewellyn[1, 2], and G. P. Fitt[2, 3]

[1] CSIRO, Plant Industry, PO Box 1600, Canberra, ACT 2601; [2] CRC for Sustainable Cotton Production; [3] CSIRO, Entomology, PO Box 59 Narrabri, NSW 2390

Introduction

Transgenic plants are rapidly dominating World agriculture and already the produce from millions of acres of transgenic insect and herbicide tolerant cotton, corn, soybean and canola being traded around the World, predominantly originating from the U.S.. By the end of the decade there will be few broadacre crops whose management would not have been changed unalterably by this new technology. The experiences from these early transgenic crops are in general good, providing good value to the farmer, but sporadic reports of poor performance of the transgenic traits and of variable performance of transgenic plants between different regions, suggest that we do not yet know enough about how genes function plants to perfectly predict the behaviour of transgenes under field conditions.

At a purely research level, there have been many reports in the literature that novel traits, created through the introduction and expression of foreign DNA in transgenic plants, can be unstable following propagation of the transgenic lines, leading to the loss of the newly acquired characteristics. The introduced genes are not lost or mutated, but are somehow switched off or inactivated. To understand this you should remember that when a gene is active there are a complex set of enzymes that decode the sequence of the gene from the DNA into a closely related information molecule RNA. This RNA is then transported out of the nucleus of the cell where it is produced and then decoded or translated into proteins (the functional products of genes) by another complex set of proteins in the cytoplasm of the cell. In one mechanism of transgene silencing or switch off, the gene sequence is chemically modified or methylated and this prevents the decoding of the DNA into RNA in the nucleus. In the second mechanism the gene remains active, but the RNA produced from the gene is specifically degraded and never accumulates in the cytoplasm to be turned into proteins. Both

mechanisms (reviewed in Matzke and Matzke, 1995; Finnegan and McElroy, 1996; Stam et al., 1997) have the same overall result; no transgene RNA can be detected in the cytoplasm of the cell and no protein is made. The variabile performance of some commercial transgenic traits in the field, particularly with INGARD® cotton in Australia, suggests that some form of transgene inactivation may be occurring, so we have started to look at a molecular level at just what is happening to the transgenes in INGARD® cotton when it fails to adequately control *Helicoverpa* species.

INGARD® Cotton

As a species, *Bacillus thuringiensis* contains a wide array of insecticidal crystal proteins (Cry proteins) each of which is toxic to larvae of a specific set of insects (Schnepf, 1995). Those of the CryIA(c) sub-class are potent insecticides against *Heliothis* and *Helicoverpa* species of Lepidoptera that are important economic pests on many annual crops, including cotton. Ingestion of the CryIA(c) protein results in rapid death of young susceptible caterpillars through disruption of the gut after the toxic protein is activated and bound to a specific protein receptor on the gut lining. Transgenic plants, such as cotton, corn and potatoes, that express different *Cry* genes have been produced and these plants are protected against attack by insects susceptible to the particular protein expressed in the plant tissues (Jouanin et al., 1998). Transgenic cotton, for example, carrying a synthetic, codon-modified *CryIAc* gene (the Bollgard® or INGARD® genes) have been generated by Monsanto (Perlak et al., 1991) and are toxic to a number of Heliothine species of significance to the cotton industry, such as *H. armigera* and *H. punctigera*. The INGARD® gene has been introduced into several Australian and American cotton cultivars in an extensive back-crossing breeding programme to enhance the control of insect pests. Six different INGARD® varieties were released by Cotton Seed Distributors and Deltapine Aust. for commercial cultivation in the 1996/97 (30,000 ha) and 1997/98 (60,000 ha) seasons. The same gene in elite American cultivars was also released by Deltapine in the U.S. in 1996 on a much larger scale. Over one million hectares of Bollgard® cotton was grown in the U.S. in the last season. Bollgard® varieties have performed extremely well in controlling the main American cotton pest *Heliothis virescens*, but in a very small percentage of the area where *Helicoverpa zea*, a more tolerant pest, has been abundant, control was less effective. Very large reductions in the usage of chemical pesticides were recorded and in general growers were very satisfied with the transgenic plants. In contrast, during both seasons of commercial production in Australia the

performance of INGARD® cotton has been variable. Although extensive reductions in pesticide usage have been observed, in most regions the survival of caterpillars increased as the season progressed, but the time at which declining efficacy occurred was highly variable across the season, across regions and even across farms. The loss of efficacy was associated with a reduction of INGARD® protein, as measured in quantitative bio-assays (J. Daly, pers. comm.) and by antibody detection using an ELISA assay (H. Holt, pers. comm.). These patterns of variable performance appear to have been more widespread and agronomically significant in Australia, perhaps because both *Helicoverpa* pests here are relatively more tolerant of the Cry IA(c) protein expressed in INGARD® cotton (they are about as tolerant as *H. zea* in the U.S. (N. Forrester pers. comm)).

Is this loss of insect control in Australia due to the silencing of the INGARD® gene or due to the protein becoming ineffective for some reason? We have started some experiments to look at the molecular level at what is happening to the expression of the INGARD® gene in field collected material of a number of different varieties of INGARD® cotton that has also been bioassayed for its ability to kill *H. armigera*. The results are preliminary, but at this stage it appears that the decline in efficacy is due to lower levels of insecticidal protein and this is because there is a decrease in the level of INGARD® RNA in plants that are no longer killing insects.

Materials

Leaves were harvested from 20-50 cotton plants of each genotype at different times throughout the season; leaves were frozen in liquid nitrogen and ground to a fine powder before freeze drying. RNA and DNA were extracted from the freeze-dried powder as described in Jacobsen-Lyon, (1995) and Paterson et al., (1993), respectively. Southern and Northern blots were prepared as described in Sambrook et al., (1989) and probed with a DNA probe encoding the *CryIAc* gene, 35S promoter or *NptII* gene labelled with $dCTP^{32}$ incorporated by random priming.

Results and Discussion

The level of INGARD® RNAs decline early in the season in transgenic cotton that is failing to control insect pests

As a first step to determine the cause for the loss in efficay of some fields of INGARD® cotton we have monitored INGARD® RNA expression by Northern blots of total RNA isolated from pooled plant material harvested at different times during the season from individual fields. This technique allows us to determine the amounts of INGARD® RNA that is getting into the cytoplasm of the cells of the cotton leaves and buds. INGARD® gene RNAs were readily detected in samples harvested early in the season, around the time of first flower, from three commercial lines of transgenic cotton (Sicala V-2i, Siokra V-15i and NuCotn) when the plants were killing *H.armigera* larvae in the field and by *in vitro* bioassay in the laboratory. No transcripts were detected in samples harvested later in the season for two lines that showed loss of bio-active INGARD® protein. A third line, which retained some INGARD® protein as determined by bio-assay, also showed a reduced level of INGARD® RNAs.

INGARD® cotton also contains another gene, *Npt*II which was used for the selection of the transgenic plants in tissue culture. The INGARD® gene and *NptII* genes are each under the control of separate copies of the 35S promoter, so we can also look in the plants to see what is happening to this other gene. We have compared the levels of *Npt*II and INGARD® RNAs in one of the lines (Sicala V-2i) throughout the season. The level of *Npt*II RNA was consistently high in the samples harvested early (November and December), but in samples harvested in January through to March, the level of RNA was lower, but still readily detectable in Northern blots. In contrast, the level of INGARD® RNA in the same samples declined dramatically over a three week period in November and by January was undetectable. In this line, a reduction of bioefficacy in feeding trials was observed in January, indicating that the decline in RNA levels preceded the loss of efficacy in insect control as would be expected as it takes time for existing INGARD® protein to breakdown in the cell once the gene switches off. These observations suggest that the activity of the 35S promoter driving both transgenes is affected in plants that are losing insect control efficacy and decreases in all transgenic plants as they aged. Other factors, possibly gene silencing, may cause the premature decline in the level of INGARD® RNA in plants from some fields for reasons as yet unknown.

Is the switch off of INGARD® due to gene inactivation by DNA methylation?

A number of techniques are available to look at whether a particular gene is being chemically modified by methylation and hence silenced or switched off. We have used one of these to examine the general level of methylation of the INGARD® gene throughout the season. DNA isolated from pooled leaf material, harvested early or late in the growing season, has been digested with sequence specific restriction enzymes whose activity is either inhibited or unaffected if the DNA is heavily methylated at its cuttting site. Southern blots of DNA cleaved with these methylation sensitive and insensitive enzymes were probed with the INGARD® gene to examine the methylation status of this gene. Preliminary data from these assays showed that the transgene in DNA isolated from leaves harvested early in the season was not methylated, but late in the season there was a low level of methylation of the transgene. However, the majority of copies of the transgene were cleaved completely by these methylation sensitive enzymes indicating that the transgene DNA was not heavily methylated. In other species gene silencing by methylation is usually accompanied by a high level of methylation of the whole gene. Many sites within the INGARD® transgene remain unmethylated so this may rule out methylation as a cause for the loss of efficacy of some INGARD® cotton plants, however more extensive studies are still needed.

Conclusions

We have shown that the activity of the 35S promoter driving the expression of two genes, including INGARD® decreases in field grown plants as they age and this correlates well with previous observations of survival of larvae late in the season on INGARD® cotton. However, in fields where insect control has been lost the level of INGARD® RNAs declined very early (at least 4 - 6 weeks before the decrease in RNAs of the adjacent *Npt*II gene driven by the same promoter). This differential expression may just mean that the INGARD® RNAs are more unstable once both genes are switched off or that the INGARD® gene is more prone to being turned off. Preliminary experiments suggests that the reduction in INGARD® RNA levels was not associated with heavy methylation of the gene and that the switch off of the gene is therefore not primarily due to methylation. Obviously these observations are still very tentative and much more extensive analyses will need to be done to pinpoint the cause of the premature decline in INGARD® efficacy in some fields and not others. These experiments will be carried out over the next year using samples collected in the 1997/98 season and will

include: comparisons of single plants of a number of INGARD® varieties at different locations monitored throughout the season for efficacy and RNA levels of both the INGARD® and NptII genes; assays on the rate at which the INGARD® gene is being decoded in the nucleus (run-on transcription assays) to see if the RNAs are still being made, but are rapidly broken down when they are transported out into the cytoplasm to be turned into INGARD® protein; and genomic sequencing to examine directly the methylation status of all of the bases in the promoter of the INGARD® gene that could possibly be methylated resulting in loss of expression. Combined with controlled experiments being carried out in the glasshouse on INGARD® plants subjected to various environmental extremes we hope by the next conference to have a better idea of why INGARD® performance has been variable and to have developed strategies to remove the variability.

References

Finnegan, E. J. and McElroy, D. (1994). Transgene inactivation: plants fight back! Bio/Technology *12*, 883-888.

Fitt, G., Mares, C.L., and Llewellyn, D.J. (1994) Field evaluation and potential ecological impacts of transgenic cottons (*Gossypium hirsutum*) in Australia. Biocont. Sci. Technol. *4* 535-548.

Jacobsen-Lyon, K., Jensen, E. O., Jorgensen, J. E., Marcker, K. A., Peacock, W. J. and Dennis, E. S. (1995) Symbiotic and nonsymbiotic hemoglobin genes of *Casuarina glauca* Plant cell *7* 213-223.

Jouanin,L., Bonadebottino, M., Girard, C., Morrot, G., and Giband, M. (1998) Transgenic plants for insect resistance. Plant Sci. *131*: 1-11.

Matzke, M. A. and Matzke, A. J. M. (1995). How and why do plants inactivate homologous (*Trans*)genes. Plant Physiol. *107*, 679-685.

Paterson, A. H., Brubaker, C. L. and Wendel, J. F. (1993) A rapid method for extraction of cotton (*Gossypium* spp) genomic DNA suitable for RFLP or PCR analysis. Plant Molec. Biol. Rep. *11* 122-127.

Perlak, F.J., Fuchs, R.L., Dean, D.A., McPherson, S.L., and Fischoff, D.A. (1991) Modifications of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. U.S.A. *88*: 3324-3328.

Sambrook, J., Fritch, E. F. and Maniatis, T. (1989) Molecular cloning. A laboratory manual Cold Spring Harbor Press. Cold Spring Harbor

Schnepf, H. E. (1995) *Bacillus thuringiensis* toxins: regulation, activities and structural diversity. Curr. Op. Biotech. *6*: 305-312.

Stam, M., Mol, J. N. M. and Kooter, J. M. (1997). The silence of genes in transgenic plants. Annal Bot. *79*, 3-12.