Arming cotton plants with an insect virus to beat the bollworm

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INTRODUCTION

Controlling heliothine caterpillars presents an ever greater challenge for the Australian cotton industry. The bollworm, *Helicoverpa armigera*, creates especial problems by evolving resistance to most major chemical insecticides. Chemical insecticides are furthermore associated with environmental dangers and increased production costs due to the need to overcome resistance.

One particularly attractive solution to this problem is to use techniques developed for genetic engineering of cotton (Llewellyn et al., 1992) to insert genes for orally acting biological control factors that confer protection against the bollworm. Until now, the only genes available have been those encoding the insecticidal proteins from *Bacillus thuringiensis* (Bt). After extensive trials, Bt-cotton is planned to be the basis of heliothis control strategies in the Australian cotton industry (Llewellyn et al., 1994). Several lepidopteran pests, including heliothis, have, however, already been found to develop resistance to Bt (Gould, 1991, 1994; Moar et al., 1994; Müller-Cohn et al., 1994). The problem of resistance may be exacerbated by the increase in the usage of Bt-cotton (Edge, 1994) and mixtures of Bt toxins may be unable to slow resistance development (McGaughey and Johnson, 1992). Moreover, there are recent indications that the efficacy of protection by Bt-plants declines as they age (Fitt et al., 1994). These concerns make clear that the long-term viability of the industry requires alternative, orally-acting insert genes offering stable protection throughout the growing season.

We have developed an alternative strategy for engineering pest-resistant plants which produce an insect-specific virus offering protection against the bollworm. The recently discovered *Helicoverpa armigera* stunt virus (HaSV) is lethal for young heliothis larvae, which upon infection cease feeding, become stunted and die (Fig. 1). The simplicity of HaSV allowed us to engineer the first plants (tobacco and white clover) expressing an insect virus for protection against pests: their efficacy is now being tested and early results are promising.

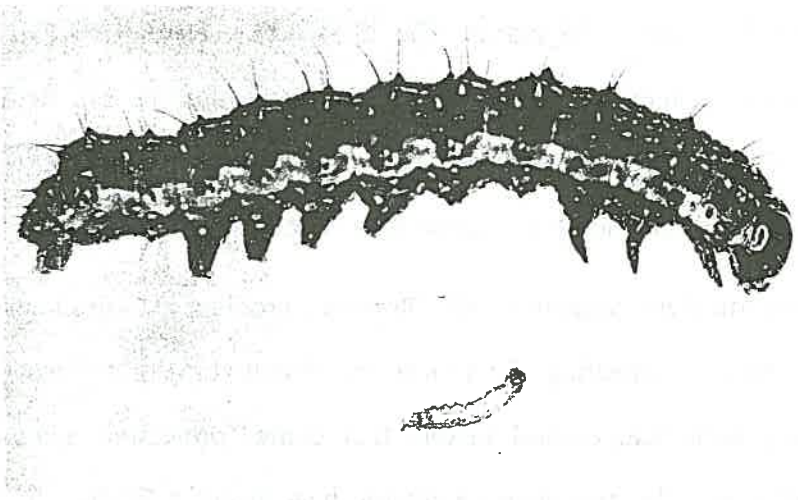

Figure 1. The stunting effect of HaSV. Both larvae shown are 5 days old. The lower one was infected with HaSV shortly after hatching. The upper one is an uninfected control.

THE STUNT VIRUS OF *HELICOVERPA ARMIGERA*

The stunt virus belongs to the tetravirus family of insect viruses. Tetraviruses infect only insects from the order Lepidoptera which contains the moths and butterflies. Most of the 10 confirmed and 8 possible tetraviruses known are restricted to a few families of moths. Although some have been found in the past to control insect pests, only three have been extensively studied. Recent molecular analysis of the complete genetic information of two tetraviruses, including HaSV, has shown that they are only very remotely related to viruses of plants or vertebrates (Hanzlik and Gordon, 1997).

HaSV is specific for heliothine moths and their clos relatives and harmless to beneficial insects and the environment. Tetraviruses show another striking form of specificity, in that they are naturally restricted to the midgut tissue of their hosts. Moreover, since the discovery of the first tetravirus from the emperor gum moth by Tom Grace of the Division of Entomology in 1965, it has not been possible for any tetravirus to be grown in cultured insect cells. These restrictions on their host range and target tissue, coupled with their lack of close relatives outside the moths, make exploitation of their natural insecticidal activity for pest control both attractive and feasible.

HaSV was discovered at the Division of Entomology in Canberra by Peter Christian in 1988. The characteristic particles of 36-38 nm in diameter (Fig. 2) were purified and shown to be the cause of high mortality in laboratory heliothis colonies (Hanzlik et al., 1993).

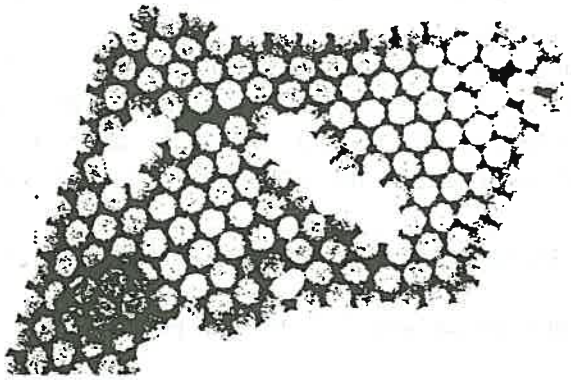

Figure 2. Purified HaSV particles as seen under the electron microscope.

The complete HaSV genome consists of only three genes, carried on two separate RNA molecules (Hanzlik et al., 1995; Gordon et al., 1995), as shown in Fig. 3. The larger genomic RNA encodes the enzyme responsible for replication of the viral RNA in infected cells. The other RNA encodes the virus coat protein and another smaller protein which appears to control virus growth.

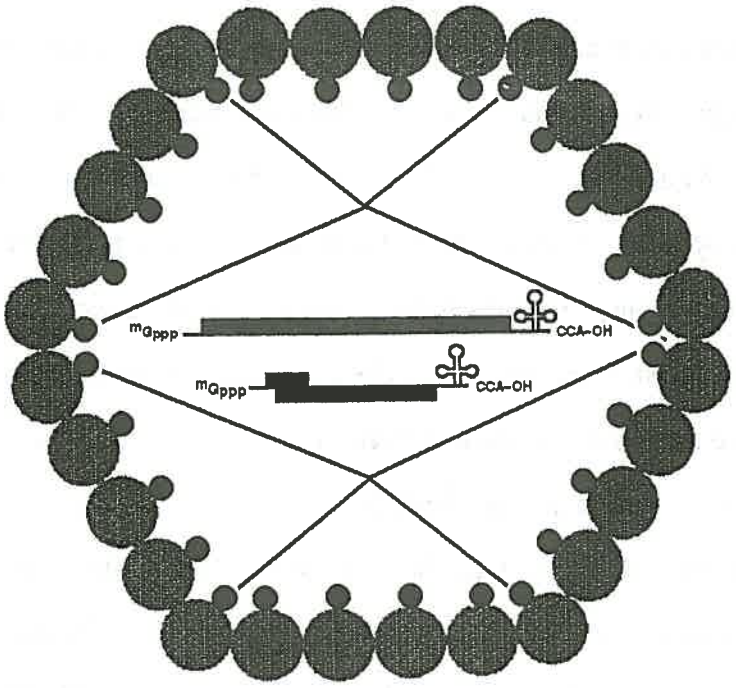

Figure 3. Diagram of HaSV particle showing the two genomic RNA molecules which carry the viral genes and are found within the particle. The discs on the outside represent the virus coat proteins; each particle is formed by 240 copies each of a large and a small protein that result from processing or cleaving the precursor protein translated from the long gene on the shorter RNA. The smaller protein remains within the particle. The protein product of the other (shorter) gene on this genomic RNA is not found in the virus particle.

ENGINEERING TRANSGENIC HaSV-PLANTS

The new protection strategy is to genetically modify the plant so that the tissues which normally serve as food for the heliothis larvae accumulate HaSV particles. These are assembled from the RNA and protein components of the virus, made by the plant from the introduced genes. Larvae feeding on these virally armed plants will stunt and die.

In order for plants to be able to make HaSV components, HaSV's genetic information must be inserted as DNA into the plant chromosomes. Full length DNA copies of the two viral genomic RNAs were assembled and cloned into plasmids under the control of plant gene control signals (Fig. 4). Expression of

these genes in plant protoplasts was found to yield virus particles containing fully infectious genomic RNA: larvae fed protoplast extracts became infected with HaSV. Other experiments have failed to show any evidence for HaSV replication in plant cells, satisfying a further concern about the safety of this new technology.

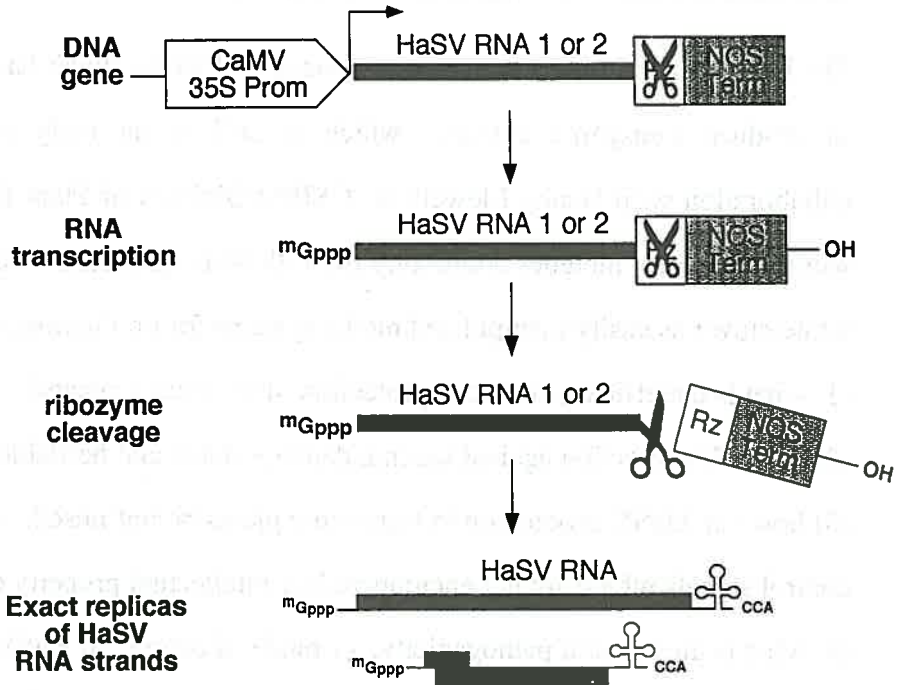

Figure 4. Diagram showing how infectious HaSV RNAs are made from artificial plant genes. At the top, the DNA copy of the HaSV RNAs is shown located between the plant promoter (at left) and terminator (at right). Transcription of this gene in the plant cell nucleus yields RNA molecules whose start is identical to that of natural HaSV RNAs, but which carry a tail derived from the terminator. A specially designed self-cutting RNA sequence or ribozyme "RZ") inserted between the HaSV copy and the terminator then removes this tail (third from top), resulting in RNA molecules which are exact copies of natural HaSV RNAs and able to infect bollworm larvae.

These DNA copies of the viral RNAs were then assembled into a large DNA plasmid molecule, or binary vector (Llewellyn et al., 1992), for engineering transgenic plants carrying the complete HaSV genome. Initial experiments were performed using tobacco and white clover, which can be rapidly transformed. To ask whether infectious HaSV particle had been produced in the transgenic plants,

leaf material from many primary transformants was fed directly to larvae. The HaSV-tobacco leaf shown on the right in Fig. 5 was significantly protected against feeding damage, in contrast to the control leaf. Larvae feeding on the protected leaf became stunted and were found to be infected with HaSV. Similar results have been obtained with engineered white clover.

The binary plasmid used to engineer tobacco and white clover has also been used to produce transgenic cotton, which is still at an early callus stage (in collaboration with Danny Llewellyn, CSIRO Division of Plant Industry). Other work in progress includes addressing the following questions, using tobacco and white clover as easily manipulated model systems for production of HaSV:

(1) what is the efficacy of HaSV protection of transgenic plants?

(2) is HaSV protection against feeding damage stable and heritable?

(3) how can HaSV production in transgenic plants be optimised, using plant gene control signals which are not encumbered by intellectual property constraints?

(4) what is the basis of pathogenicity, or mode of action, of HaSV?

(5) can an attenuated virus which is unable to persist in the environment, thereby addressing safety concerns, be produced in transgenic plants?

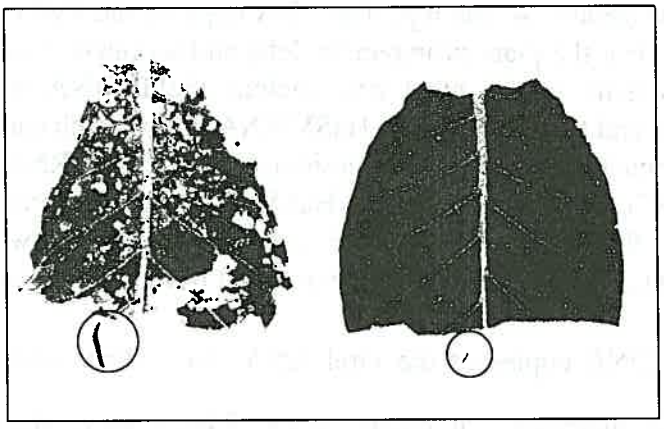

Figure 5. Protection of HaSV-plants against heliothis feeding damage. The unengineered control tobacco leaf on the left has suffered severe feeding damage by larvae, which grow normally (circled at bottom), whereas the HaSV-tobacco leaf on the right was protected. The larvae from the HaSV plant are stunted (circled at bottom) and die.

ARE PLANTS ARMED WITH AN INSECT VIRUS SAFE?

This novel approach for engineering pest-resistant plants raises new regulatory questions which must be addressed before the technology can reach the farmer´s field. We consider the technology is safe and does not present novel risks which might threaten its use. The approach is based on making a natural biological control agent in a new way and does not involve release of a modified or altered virus. The virus used is safe and specific for its target pest. HaSV does not grow in plants and has not shown any effect on the health or viability of the plants transformed. Furthermore HaSV´s adaptation to infecting a particular type of tissue in a particular type of insect make it difficult for the virus to infect other animals. Indeed, its lack of relatedness to plant or animal viruses makes it difficult to conceive of any way in which the virus could acquire the ability to infect new hosts by being assembled in plants. One issue which still needs to be addressed concerns the natural distribution of the virus and the extent to which it is already present in heliothis populations in cotton growing areas and elesewhere.

CONCLUSIONS

The cotton industry urgently needs genes for biological control agents which can be used to engineer crops resistant to major world-wide pests like heliothis caterpillars. We have identified a new pest control strategy based on environmentally safe small insect-specific viruses lethal to caterpillars like the bollworm. Preliminary data has shown that an infectious insect virus, HaSV, can be produced in transgenic plants and confer protection against feeding damage. Further work seeks to optimise HaSV genes for crop protection by elucidating the basis of its action. This will also allow us to address possible regulatory concerns by producing an attenuated subvirus unable to persist in the environment. With its different mode of action, HaSV promises to be an ideal complement to Bt in strategies designed to minimise and overcome resistance and will therefore help

protect the investment in this, the only agent currently available. Should resistance to one form of HaSV be encountered, we anticipate that we will be able to identify resistance-breaking isolates of the virus by challenging the resistant larvae with wild-type virus. Viruses like HaSV therefore represent valuable insurance in case insecticide resistance becomes insurmountable.

ACKNOWLEDGEMENTS

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