HELICOVERPA ARMIGERA STUNT VIRUS AS A SOURCE OF INSECTICIDAL INSERT GENES FOR ENGINEERING INTO COTTON FOR BOLLWORM CONTROL

Karl H. J. Gordon, Damian Triffett, Michelle R. Lincoln, Anthony Millgate[1], Phil J. Larkin[1] and Terry N. Hanzlik

CSIRO Entomology, GPO Box 1700, Canberra ACT 2601, and [1]CSIRO Plant Industry, GPO Box 1600, Canberra ACT 2601.

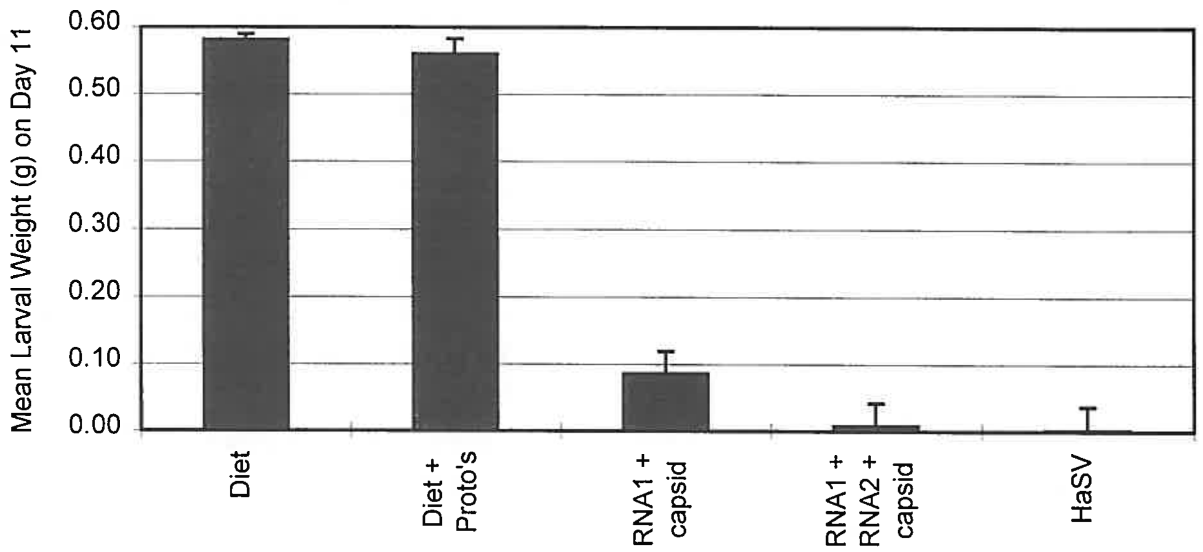
The Australian cotton industry continues to face a major challenge in controlling heliothine caterpillars. As the bollworm, *Helicoverpa armigera*, has evolved resistance to most major chemical insecticides, interest in biological control agents has increased. Such agents would avoid the environmental dangers and costs associated with chemical insecticides.

An important approach to using orally acting biological control agents that confer protection against the bollworm is to genetically engineer cotton by inserting genes for the agents (Llewellyn et al., 1992). The first genes available for use in this approach were those encoding the insecticidal proteins from *Bacillus thuringiensis* (Bt). INGARD-cotton carrying a Bt gene is now being grown commercially in Australia and will form the basis of heliothis control strategies (Llewellyn et al., 1994). However, this current dependence on a single control gene means that an alternative form of resistance to heliothis would be of great interest. This would allow the careful management of the Bt gene now used in INGARD-cotton in order to retard the development of resistance and provide an insurance policy should resistance to Bt become insurmountable.

Viruses like *Helicoverpa armigera* stunt virus (HaSV) are natural experts at killing insects and have significant potential for the control of pests like the cotton bollworm. However, small RNA viruses of insects, such as HaSV, have been little used as pest control agents (Hanzlik and Gordon, 1997). HaSV is a simple virus that rapidly stops young heliothis larvae from feeding. They remain stunted until they die. The virus, which is found naturally in Australia and was discovered at CSIRO Entomology in Canberra, is specific for heliothis pests and harmless to beneficial insects and the environment. Like Bt, the virus is active upon feeding and targets the insect midgut, but its mode of action in killing caterpillars is very different. In the midgut, the virus binds to the goblet cells, the less abundant of the major cell types forming the gut wall. It multiplies rapidly upon entering midgut cells. Virus-infected tissue is rejected from the midgut wall which falls apart as the virus spreads rapidly from one cell to another. In contrast to HaSV, Bt binds the most abundant type of midgut cells, the columnar cells, so that these two control agents appear to bind to different receptor molecules on the midgut surface. These properties make the stunt virus an ideal alternative to Bt, since any insect mutations in the respective receptors, which could confer resistance to either pathogen, are unlikely to reduce the efficacy of the other.

The simplicity of the stunt virus, which has only three genes carried on two RNA molecules, makes it feasible to consider engineering plants to make their own insecticidal virus for heliothis control (Hanzlik et al., 1996). An important feature of this new approach for heliothis control is that the plants will make an insecticide that is capable of amplifying itself in the gut of the caterpillars after it has been taken up through feeding. To establish this new approach, we engineered stunt virus gene constructs in which the stunt virus genes were placed under the control of plant-specific promoters such as that derived from cauliflower mosaic virus. When these constructs were introduced into isolated plant cells (protoplasts), the genes were expressed, resulting in production of stunt virus components in the plant cells. These components self-assembled into infectious HaSV that could be transmitted to caterpillars feeding on the plant cells. The caterpillars became stunted and died.

Figure 1. Bioassay of HaSV expressed in protoplasts.



The combinations of plasmids shown were PEG transformed into protoplasts of *Nicotiana plumbaginifolia*. After growth for 5 days, the protoplasts were collected by centrifugation and added to diet. Aliquots were fed to neonate larvae for one day and the larvae transferred to fresh diet for 11 days. Average weights recorded for larvae at the end of the bioassay are indicated by histograms, together with error bars showing the standard deviation.

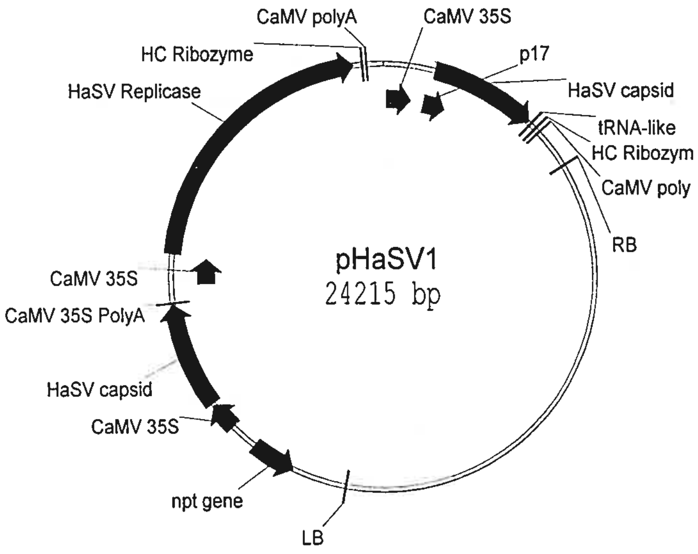
As shown in Fig. 1, greatest efficacy was achieved using a combination of individual plasmids expressing HaSV RNA 1, RNA 2 and the capsid gene separately. This mixture was almost as effective as native virus purified from infected larvae (far right of the figure).

Significant stunting was also achieved with a combination of two individual plasmids expressing only HaSV RNA 1 and the capsid gene separately. While this combination is able to stunt larvae, it results in a form of the virus that is unable to spread in the midgut, and so is less efficacious than the complete virus assembled when all three plasmids were combined. Control larvae fed either diet only or diet mixed with protoplasts to which no HaSV constructs had been added showed no stunting.

The presence of virus in the stunted larvae fed on protoplasts such as those from the experiment shown in Fig. 1 was confirmed by extraction of RNA and analysis using specific portions of the HaSV genome as probes. It was also confirmed that the virus detected in larvae was actually derived from the virus produced in the plant cells, and not through accidental infection with HaSV that is naturally present at low levels in some populations of the insect colony from which these larvae were derived. This was done by genetically marking the virus produced in protoplasts through the introduction of specific mutations designed not to affect the virus' ability to replicate; the same mutations were detected in virus recovered from the larvae infected with modified virus.

Although the virus is very simple, the need to engineer several genes into plants at the same time is a challenging undertaking. We are using tobacco as a model plant system in which to develop the technology. This is because cotton, the primary target for the technology, has a number of disadvantages for this stage of the research. These are its inefficiency of transformation and the long time required to generate transgenic plants and produce progeny; moreover the HaSV constructs have proven difficult to introduce into transgenic cotton (D. Llewellyn, pers. comm.). Tobacco is also a host for heliothis, is easily transformed and can produce seed within a couple of months.

Figure 2. Binary vector constructed for simultaneous transfer of HaSV genes into plants.



The map shows insert genes located in the T-DNA, between the left (LB) and right (RB) borders, for transfer to the plant genome. The genomic RNA 1 (indicated by the HaSV replicase gene) and RNA 2 (indicated by the p17 and HaSV capsid genes) are shown, as is the separate copy of the capsid gene alone. All are driven by the CaMV 35S promoter. The selectable marker gene used was npt for kanamycin resistance.

The above DNA copies of the viral RNAs were assembled into a large DNA plasmid molecule, or binary vector (Llewellyn et al., 1992), for engineering transgenic plants carrying the complete HaSV genome. This initial plasmid carrying the HaSV genes was called pHaSV1 (Figure 2). An initial experiment generated a transgenic tobacco plant that showed very good stunting of heliothis larvae and a few others that showed some stunting but to a lesser degree. Little damage was caused to the plant, and the larvae contained large quantities of stunt virus. However further analysis showed that the stunting ability was lost during continued culture and was not passed to this plant's progeny. Moreover, analysis of the HaSV genes inserted into this plant's genome showed that a deletion in the HaSV genes had occurred, probably during the transfer process, so that the source of the infection initially detected in the larvae was probably HaSV that is occasionally naturally present at low levels in the insect colony from which these larvae were derived. The HaSV transgenes still present in this plant were also found not to show stable expression of virus components. The reason for this instability of HaSV gene expression in some transgenic plants is still unclear and we are currently analysing a large number of further transgenic plants. These plants carry either individual HaSV genes incapable of generating infectious virus, or all the genes present in pHaSV1.

Experiments in protoplasts have proven that it is possible to assemble infectious HaSV in plant cells, and demonstrated the potential of this technology. However, further research and development work will be required to get this technology to the stage where it can be applied to cotton. In addition, we are investigating approaches based on the use of virus-like particles made with the HaSV capsid protein to deliver non-viral control agents to insects (Hanzlik and Gordon, 1998). The number of novel transgenic approaches to crop protection against insects are few and far between and any potential new technologies need to be exhaustively investigated to ensure the long-term protection of our agricultural crops. Our current work is therefore aimed primarily at fine tuning the stunt virus technology in a model plant species (tobacco), to a point where it can routinely generate insect tolerant plants, before transferring the technology to the CSIRO Plant Industry cotton biotechnology program for introduction into cotton.

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