Transgenic Research **11:** 185–198, 2002.

Avidin expressed in transgenic tobacco leaves confers resistance to two noctuid pests, *Helicoverpa armigera* and *Spodoptera litura*

Elisabeth P.J. Burgess[1,*], Louise A. Malone[1], John T. Christeller[2], Melissa T. Lester[2], Colleen Murray[2], Bruce A. Philip[1], Margaret M. Phung[2] & Emma L. Tregidga[1]

[1]*The Horticulture and Food Research Institute of New Zealand Limited, Mt Albert Research Centre, Private Bag 92169, Auckland, New Zealand*

[2]*The Horticulture and Food Research Institute of New Zealand Limited, Palmerston North Research Centre, Private Bag 11030, Palmerston North, New Zealand*

Received 23 October 2000; revised 2 April 2001 & 27 June 2001; accepted 18 July 2001

Key words: Cry1Ba Bt toxin, *Helicoverpa armigera*, insect pest-resistant transgenic tobacco, *Nicotiana tabacum*, *Spodoptera litura*, vacuole-targeted avidin

Abstract

Fertile transgenic tobacco plants with leaves expressing avidin in the vacuole have been produced and shown to halt growth and cause mortality in larvae of two noctuid lepidopterans, *Helicoverpa armigera* and *Spodoptera litura*. Late first instar *H. armigera* larvae and neonate (<12-h-old) *S. litura* larvae placed on leaves excised from T_0 tobacco expressing avidin at 3.1–4.6 μM (μmoles/kg of fresh leaf tissue) had very poor growth over their first 8 days on the leaves, significant numbers had died by days 11 or 12 and all were dead by day 22 (*H. armigera*) or day 25 (*S. litura*). Similar results were obtained when late first instar *H. armigera* larvae were placed on leaves from T_1 plants expressing avidin at six different average concentrations, ranging from 3.7 to 17.3 μM. Two larvae on the lowest expressing leaves survived to pupation, but there was total mortality among the other groups and no relationship between avidin concentration and the effects on the larvae. Synergistic effects between avidin-expressing tobacco plants and a purified Bt toxin, Cry1Ba, were demonstrated. Late instar *H. armigera* larvae fed with leaves from T_2 plants expressing avidin at average concentrations of either <5.3 or >12.9 μM, and painted with Cry1Ba protein at a rate equivalent to an expression level of 0.5% of total leaf protein, died significantly faster than larvae given either of the two treatments alone. Larvae fed with avidin-expressing leaves painted with the protease inhibitor, aprotinin, at a rate equivalent to 1% of total leaf protein had mortality similar to those given avidin-leaves alone. There was no evidence of antagonism between these two proteins.

Introduction

Larvae of *Helicoverpa armigera* and *Spodoptera litura* (Lepidoptera, Noctuidae) are serious polyphagous pests of major crops in many countries. Cotton is a particular target for both insects in the United States, China, Egypt, Australia and India, and other countries (Hill, 1975; Li & Rahmann, 1997). *H. armigera* (American or cotton bollworm) is also an economically important pest of a range of other crops including tomatoes, pigeonpeas, groundnuts and glasshouse crops (Kumble & Reed, 1981; Anon, 1985; Marek & Navratilova, 1995; Shanower et al., 1999). *S. litura* (cotton leafworm or tropical armyworm) is a pest of rice, sunflowers, brassicas, groundnuts, tobacco and other crops (Ayyanna et al., 1978; Zaz & Kushwaha, 1984; Sahayaraj & Paulraj, 1998). Resistance to chemical insecticides and *Bacillus thuringiensis* (Bt)-based biopesticides poses significant problems for the control of both species (Reynolds & Armes, 1994; Sekhar et al., 1995; Guilin & Yunxi, 1996; Armes et al., 1997). Transgenic cotton expressing a Bt toxin has been commercially developed as an alternative to insecticide use for control of both insects (Forrester,

*Author for correspondence: *E-mail:* eburgess@hort.cri.nz

1997), but concerns about the evolution of resistance to Bt-transgenic crops (Fitt et al., 1994; Whalon & Norris, 1997) have ensured that the search for alternative genes for noctuid control continues.

Avidin is a water-soluble tetrameric glycoprotein isolated originally from raw chicken egg white which binds strongly to the vitamin, biotin. Biotin is an essential compound for many organisms, including plants and pest insects (Dadd, 1985; Wurtele & Nikolau, 1990). Plants and micro-organisms (with the exception of some fungi) synthesise the biotin they require, while higher organisms obtain it from gut microflora or from dietary sources. Biotin is cofactor of major carboxylases involved in gluconeogenesis, lipogenesis, fatty acid and amino acid catabolism (Wood & Barden, 1977; Knowles, 1989). It is therefore understandable that avidin causes significant mortality when mixed into artificial diets and fed to a wide range of insect pests (Bruins et al., 1991; Morgan et al., 1993; Markwick et al., 2001). Furthermore, avidin expressed in the seeds of transgenic maize has recently been shown to reduce the growth and survival of a range of stored products pests (Kramer et al., 2000).

As plants have a requirement for biotin, the avidin gene cannot be successfully introduced into transgenic plants using conventional gene constructs. Rather, it must be targeted to and sequestered in a sub-cellular space within the plant, only to be released once the insect begins feeding. In the case of transgenic avidin-maize kernels, this has been achieved via the use of gene sequences which target expression to intercellular spaces, particularly in embryonic tissue (Hood et al., 1997). Such maize plants are also male-sterile (Hood et al., 1997). Recently, fertile tobacco plants expressing high levels of avidin in the leaves have been produced using a gene construct which incorporates a vacuole-targeting sequence from a potato proteinase inhibitor gene (Christeller et al., 2000; Murray et al., 2001). This development opens up the possibility of effectively using avidin for pest control in transgenic crop plants that are attacked by leaf-chewing insect pests.

Here we report on the insecticidal effects of feeding transgenic tobacco leaves expressing vacuole-targeted avidin to larvae of *H. armigera* and *S. litura*. We also demonstrate reductions in the growth and survival of *H. armigera* larvae on second and third generation avidin-tobacco plants and on plants expressing avidin at a range of concentrations. The compatibility of such plants with two other pest-resistance proteins, a proteinase inhibitor (aprotinin or bovine pancreatic trypsin inhibitor, BPTI) and a Bt toxin (Cry1Ba), is demonstrated via an experiment in which avidin-tobacco leaves painted with the purified proteins were fed to *H. armigera* larvae.

Materials and methods

Insects and plants

S. litura were obtained from a laboratory colony originally established from moths field-collected in Queensland, Australia, and *H. armigera* from a laboratory colony established from moths collected in Christchurch, New Zealand. Both colonies were reared on artificial diet as described in McManus and Burgess (1995). For experiments, neonate *S. litura* larvae, that is less than 12 h from emergence, were used. In contrast, late first instar larvae of *H. armigera* were used, since this is the earliest developmental stage of this species able to establish and survive well on tobacco.

Tobacco (*Nicotiana tabacum* cv Samsun) plants were transformed with plasmid (pPLA2) using a standard *Agrobacterium tumefaciens*-mediated protocol as previously described (Christeller et al., 2000). Briefly, the transformation plasmid was composed of the binary vector pART27 (Gleave, 1992) expressing a chimeric polypeptide consisting of the 31 amino acid N-terminal sequence from a potato proteinase inhibitor I (PPI-I) cDNA that encodes a vacuolar targeting sequence (Beuning et al., 1994) followed by two amino acids novel to both PPI-I and avidin and completed by a 128 amino acid sequence encoded by an avidin cDNA (Gope et al., 1987). The resulting PPI-I/avidin fusion protein has a total of 161 amino acids: MESKFAHIIV FFLLATPFET LLARKESDGP EIPARKCSLT GKWTNDLGSN MTIGAVNSRG EFTGTYITAV TATSNEIKES PLHGTQNTIN KRTQPTFGFT VNWKFSESTT VFTGQCFIDR NGKEVLKTMW LLRSSVNDIG DDWKATRVGI NIFTRLRTQK E. Expression was driven by a CaMV 35S promoter.

In order to determine whether insects would be affected by feeding on transformed tobacco not expressing the polypeptide, four types of control plants were grown and compared in the first experiment (below). These controls were: (i) four non-transformed plants, (ii) seven plants transformed with the pART27 vector only, (iii) four plants transformed with pART27 vector and the *GUS* gene (uid), and (iv) eight non-expressing plants transformed with a different vector and gene, pRD400 (Raju et al., 1992) and

pumpkin fruit chymotrypsin inhibitor (PFCI) cDNA (Murray & Christeller, 1995). Only non-transformed plants were used as controls for the second and third experiments. All plants were grown in a containment glasshouse at approximately 28°C and used in insect trials once they had formed 12 leaves. Second and third generation plants were produced by self-fertilisation.

pPLA2-transformed regenerant tobacco plants were initially characterised for transformation and retention of the avidin construct by PCR (Figure 1(a)) and for qualitative expression of avidin protein by western blotting (Figure 1(b)) using the methods of Murray et al. (2001). Avidin expression levels in tobacco leaves were subsequently determined using the ELISA method previously described (Christeller et al., 2000; Murray et al., 2001). Briefly, plant tissue was ground in four volumes of sodium carbonate/bicarbonate buffer, pH 9.8, centrifuged and samples incubated overnight in microtitre plates in the same buffer. Standard curves were constructed in equal volumes of control plant extracts using egg white avidin (Sigma Chemicals, St Louis, MO). Plates were developed using rabbit anti-avidin antibodies and goat anti-rabbit alkaline phosphatase-linked antibodies and the appropriate blocking and washing procedures. The level of avidin was determined from initial enzyme rates using sodium p-nitrophenyl phosphate as substrate.

As avidin monomers bind to biotin on an equimolar basis, in order to allow meaningful comparison of avidin expression levels with plant biotin levels we have presented avidin levels in this paper as μM (μmoles/kg of fresh leaf tissue). This calculation is made on the basis that 1 kg of leaf material is equivalent to 1 litre of solution, and therefore provides a molar value on the basis of leaf weight. To enable comparison with other studies where expression levels are reported as a percentage of leaf protein, and if we assume tobacco leaves contain protein as 2% of fresh weight, then avidin expressed at 13 μM is equivalent to 1% of total leaf protein.

Experiment 1: Toxicity of avidin-tobacco leaves to H. armigera *and* S. litura *larvae*

Six lines of avidin-expressing tobacco plants were derived from six separate transformation events. Four clonal plants were grown from each line, that is 24 avidin-expressing plants in total.

In order to determine whether the target insects would be affected by the processes of plant transformation and regeneration or by components of the inserted gene construct other than avidin, four different types of control plant were used. The plasmid vector used in the avidin gene construct, both with and without the *GUS* marker gene, and an alternative pest resistance gene construct, were inserted separately into tobacco to identify any impact of these on the insects. The four control groups were: (i) four non-transformed plants (no foreign DNA inserted), (ii) seven pART27 plants (plants transformed with the same vector as the avidin plants, but without the avidin gene), (iii) four pART27-GUS plants (transformed with the same vector as the avidin plants and additional foreign DNA in the form of the *GUS* marker gene, but without the avidin gene), (iv) eight pRD400-PFCI non-expressing plants (plants transformed with foreign DNA in the form of the vector pRD400 and a gene encoding pumpkin fruit trypsin inhibitor, but not expressing the protein). All transformed control plants were independent transformants, with the exception of two of the pRD400-PFCI plants, which were clonal. In total, 23 control plants were used.

On every plant, the uppermost (youngest) leaf which was 15 cm or more in length from leaf tip to leaf base (the point at which the leaf joined the petiole) was designated as Leaf 1. The leaves below were assigned numbers consecutively down the plant. Leaves 1 and 2 were used for *H. armigera* as previous experiments had shown that these larvae grow best on the youngest leaves (Christeller et al., this issue), while Leaves 4 and 5 were used for *S. litura* as these larvae grew just as well on the older leaves (McManus et al., 1999). Avidin expression levels were measured in two leaf samples (8 cm^2) taken from Leaf 4 of all plants used in the trial. One sample was taken just before larvae were initially placed on the leaf, and the other a few days later, following the transfer of the larvae to a fresh leaf.

For experiments, leaves were cut from plants and each petiole placed immediately into a setting solution of 0.4% agar in a 30 ml plastic 'Coulter' cup. Twelve late first instar *H. armigera* larvae, which had been kept on artificial diet for 48 h from emergence, were placed on the undersides of Leaves 1 and 2 from plants representing each of the six treatment and six control groups. These were placed in separate plastic storage boxes ($300 \times 210 \times 80$ mm) and kept at 24.5 ± 1°C and 60% relative humidity, with a 16 : 8h light:dark cycle. Thus 24 boxes and 288 *H. armigera* larvae

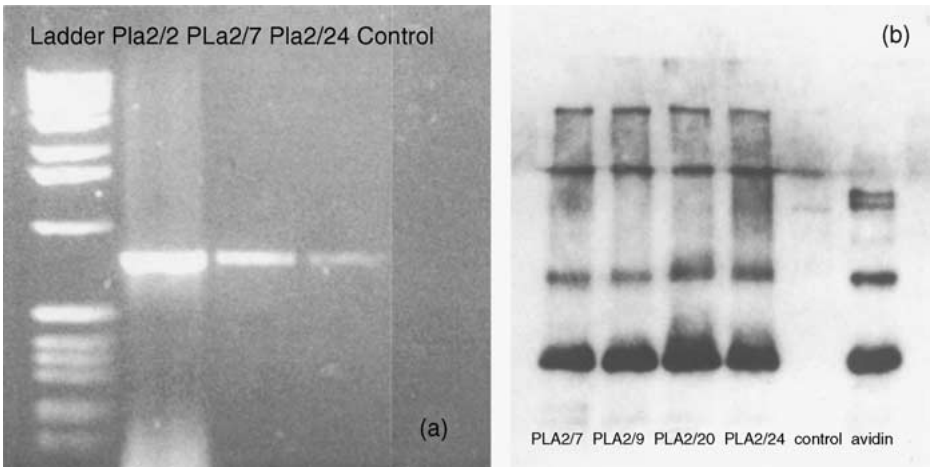


Figure 1. Molecular and gene expression characterisation of avidin-tobacco plants. (a) PCR analysis of three avidin-tobacco plants using primers to the 35S CaMV promoter and the OCS terminator of pART27. A PCR product consistent with the expected size of 840 bp was identified by agarose electrophoresis. The control tobacco lane was run on the same gel. The marker series was the DNA extension ladder (Gibco-BRL). (b) Western blot analysis of four avidin-tobacco plants and one control tobacco plant. SDS-PAGE blots were incubated with rabbit anti-avidin antibodies and developed using goat anti-rabbit IgG-alkaline phosphatase and CDP-Star Chemiluminescence Reagent (NEN Life Sciences). Heating the samples has caused avidin to migrate largely as monomeric peptides but small amounts of dimer and tetramer are visible in both plant and standard protein preparations.

were set up in total: 12 larvae $\times$ 2 leaves $\times$ 12 plant groups. The initial weight of *H. armigera* larvae was determined as the mean of the individual weights of a randomly chosen sample of 48 larvae weighed at the beginning of the experiment.

For *S. litura*, 15 neonate larvae were placed on the upper surfaces of Leaves 4 and 5, and these were set up as described for *H. armigera* above. Thus 24 boxes and 360 *S. litura* larvae were set up in total: 15 larvae $\times$ 2 leaves $\times$ 12 plant groups. *S. litura* initial larval weight was estimated from the mean weight of three samples of 100 larvae.

When necessary, leaves were replaced with new ones from fresh plants. Larvae on avidin plants were re-fed with leaves from within the same clonal line and those on control plants given leaves from the same genetic plant type. When necessary, leaves of the equivalent physiological age from previously used plants were utilised. Larvae were weighed and survivors were counted at regular intervals throughout the experiment until death or until pupation had begun in a treatment.

For all experiments, mean larval weights and biomass (the sum of the weights of live larvae on a given treatment) were calculated and compared using Genstat analysis of variance (ANOVA) (Payne et al., 1993). Survival data were analysed using S-Plus survival analysis (Venables & Ripley, 1997). Survival curves were compared using log-rank analysis (Kalbfleisch & Prentice, 1980). Median survival times were analysed with a chi-square test, and the proportion of larvae alive was determined by ANOVA of arcsine square root-transformed data.

Experiment 2: Toxicity of avidin expressed at a range of concentration levels in tobacco leaf to larval H. armigera

Forty eight plants, expressing avidin at a range of concentrations, were selected from 72 plants representing three different T_1 lines (24 plants of each) grown from seeds collected from self-fertilised clonal plants from three of the original transformant (T_0) lines (see above). Avidin expression levels had been determined by ELISA of a 50–60 cm^2 leaf sample from the tip of Leaf 4 of each plant. Plants were ranked according to expression level and divided into six groups of eight plants representing six non-overlapping ranges of expression level, from 'high' to 'low' (mean avidin concentrations given in Results section below). Each of the six groups contained plants from a mixture of the three different T_1 lines. For controls, 48 non-transformed control plants were grown from seeds produced by self-fertilised non-transformed plants from Experiment 1.

To determine whether an early diet of plant material, instead of the artificial diet used in Experiment 1, would alter the response to ingestion

of avidin-expressing tobacco, neonate *H. armigera* larvae were placed on the leguminous host plant *Lotus corniculatus*. Larvae were kept at 18°C for 3–4 days prior to this experiment. Late first instar larvae were then transferred to control or avidin-expressing tobacco leaves and kept in plastic boxes as described for the Experiment 1. In addition to the six avidin treatments, two control groups, both using leaves from non-transformed plants, were set up. Initial larval weight was determined as the mean of the individual weights of a randomly selected sample of 48 larvae weighed at the beginning of the experiment. Larvae were then weighed on days 8, 11, 13, 14 and 15, and surviving larvae were transferred to fresh Leaves 1 and 2 from the next highest expressing plant in each treatment group on days 6, 8, 11 and 16. To ensure that larvae could feed *ad libitum*, if necessary, additional leaves were cut from positions adjacent to Leaves 1 and 2 on the same plants as in each box, and provided to larvae. Control larvae required many more leaf additions than all other treatments, and thus were given additional leaves from a range of control plants and leaf positions.

Experiment 3: Toxicity of avidin-tobacco leaves painted with either a protease inhibitor or a Bt toxin to larval H. armigera

Three hundred and twenty three T_2 avidin-expressing plants were grown for this experiment, the offspring of self-fertilised T_1 plants used in Experiment 2. Leaves 1, 2 and 3 were used in this experiment. For controls, 241 non-transformed plants were grown as described for Experiment 2.

Before commencing the experiment, ELISA measurements of avidin expression were made for samples comprising a leaf of at least 8 cm in length taken from each of the 263 out of 323 avidin-expressing plants, representing three lines, which had grown the best over an 8 week period. These plants were tested and ranked according to avidin expression level and those with the highest levels were then used in treatments requiring 'high' expressors and those with the lowest levels used where 'low' expressors were required.

Foliage was painted with one of two purified insecticidal proteins. Activated Cry1Ba toxin was obtained from a large-scale fermentation of *B. thuringiensis* Bt4412, purified and cleaved according to the method described by Simpson et al. (1997). The protease inhibitor, aprotinin, was obtained from Intergen® Company, Canada/USA (Product no. 7105, Lot no. NT59808).

Neonate *H. armigera* larvae were placed on artificial diet for 48 h following emergence from eggs. Initial larval weight was determined as the mean of the individual weights of a randomly chosen sample of 54 of the larvae used in the experiment. These late first instar larvae were then subjected to nine different treatments to test the effects of avidin, aprotinin and Cry1Ba separately and in two-way combinations. Each tobacco leaf was weighed before painting, and all solutions were applied at a rate of 100 µl solution/g of fresh leaf. All test solutions were made up in a 0.1% (v : v) aqueous solution of the wetting, spreading and sticking agent, BondXtra®. The treatments were as follows:

1. Control tobacco leaves painted with a control solution of BondXtra® 0.1% (v : v).
2. Control tobacco leaves painted with a 0.2% (w : v) solution of aprotinin in BondXtra® at the same rate as above. If tobacco leaves are about 2% protein, then this solution of aprotinin painted on at this rate (100 µg/g leaf) will approximate a leaf expressing aprotinin as 1% of total soluble protein.
3. Control tobacco leaves painted with a 0.1% (w : v) solution of Cry1Ba in BondXtra® at the same rate as above. This approximates a leaf expressing Cry1Ba as 0.5% of total soluble protein.
4. Tobacco leaves expressing 2.1–5.3 µM avidin ('low') and painted with BondXtra® . Tobacco leaves expressing 2.6–5.3 µM avidin ('low') and painted with a 0.2% (w : v) solution of aprotinin in BondXtra®.
5. Tobacco leaves expressing 3.6–5.2 µM avidin ('low') level and painted with a 0.1% solution of Cry1Ba in BondXtra®.
6. Tobacco leaves expressing 13.0–21.3 µM avidin ('high') and painted with BondXtra®.
7. Tobacco leaves expressing 12.9–21.0 µM avidin ('high') and painted with a 0.2% solution of aprotinin in BondXtra®.
8. Tobacco leaves expressing 14.2–18.1 µM avidin ('high') and painted with a 0.1% solution of Cry1Ba in BondXtra®.

Ten larvae were placed on the underside of each treated leaf and set up as described in the earlier experiments. Three replicate boxes were set up for each treatment, that is 27 boxes in total, 30 larvae per treatment (two of the treatments were inadvertently given 31 larvae). Larvae and leaves were checked daily,

and leaves were replaced with identically treated fresh leaves from similar plants as necessary for larvae to feed *ad libitum*.

Larval deaths were recorded on day 2 and daily thereafter for 14 days or until all had died if this occurred earlier. Larvae were weighed on days 3, 6, 8, 10 and 12. Larvae in a treatment were no longer weighed if any of their number had begun to pupate.

Results

Experiment 1: Toxicity of avidin-tobacco leaves to H. armigera *and* S. litura *larvae*

Avidin was expressed in the leaves of the six plant lines used in this experiment at the following mean levels (± standard error of the mean): Line 1, $3.1 \pm 0.4\,\mu$M; Line 2, $3.3 \pm 0.2\,\mu$M; Line 3, $4.4 \pm 0.5\,\mu$M; Line 4, $3.4 \pm 0.4\,\mu$M; Line 5, $4.6 \pm 0.3\,\mu$M; Line 6, $4.1 \pm 0.2\,\mu$M. Avidin was not detected in any of the control leaf samples.

Both *H. armigera* and *S. litura* exhibited extremely low levels of mortaility as well as exponential growth and biomass accumulation on all four control plant lines. There were no significant differences among the control groups in any of these parameters, demonstrating that no mortality or growth effect resulted from plant transformation or regeneration, or from the presence of foreign DNA other than avidin. Thus the different control plant groups were shown to be essentially equivalent as non-avidin controls and results from the four different groups were pooled for each insect species. As avidin expression levels were similar in each of the six lines, and larval mortality, growth and survival did not differ significantly among the lines, results from all these lines were also pooled in this experiment.

H. armigera larvae fed avidin-expressing leaves were significantly smaller than those fed control leaves by the first weighing on day 8 (control plants: N = 130, mean weight = 0.0909g, s.e. = 0.0031; avidin-leaves: N = 130, mean weight = 0.0375g, s.e. = 0.0013; $p < 0.001$, ANOVA) (Figure 2(a)). Three days later, control larvae had continued to grow well, while avidin-fed larvae were starting to die (Figure 2(b)), and differences in total live biomass between the two treatments were extreme ($p < 0.001$) (Figure 2(c)) (see also Figure 3(a)). The impact of avidin expression on biomass was reflected in dramatic differences in damage sustained by control and avidin-expressing leaves (Figure 3(b)), although leaf consumption was not measured in this trial. No further control measurements were made after day 11 as larvae had begun to pupate. By day 22, all larvae on avidin-expressing leaves had died. Comparisons between survival of larvae fed these leaves and control leaves showed that avidin expression had clearly caused mortality ($p < 0.001$, log-rank test of survival curves; $p < 0.001$ chi-square test of median survival times; $p < 0.05$, ANOVA of arcsine square root-transformed proportion of larvae alive throughout the experiment).

S. litura larvae fed avidin-expressing leaves were significantly smaller than those fed control leaves by day 8 (control leaves: N = 153, mean weight = 0.0304 g, s.e. = 0.0014; avidin-leaves: $N = 160$, mean weight = 0.0151g, s.e. = 0.0007; $p < 0.001$; ANOVA) (Figure 4(a)), but there were no differences in survival at that time. By day 12, larvae eating avidin-expressing leaves had begun to die (Figure 4(b)), and there were clear differences in mean weight and total live biomass present on the two treatments ($p < 0.001$) (Figure 4(c)). By day 15, these differences were even more pronounced (Figure 5(a)), and were reflected in the damage sustained by control and avidin-expressing leaves (Figure 5(b)). After this time control larvae had pupated, so no further control measurements were taken. Larvae on avidin-expressing leaves steadily diminished in numbers and total biomass, and by day 25 all had died. Analysis of survival curves showed that larvae feeding on avidin-expressing leaves had significantly reduced survival ($p < 0.001$, log-rank test; $p < 0.001$, chi-square test of median survival times; $p < 0.01$, ANOVA of arcsine square root-transformed proportion of larvae alive throughout the experiment).

We observed, during the experiment, that both *H. armigera* and *S. litura* larvae feeding on avidin-expressing leaves were unable to successfully complete the process of moulting from one instar to the next. Larvae appeared to stop feeding during ecdysis, and to then turn black and die while still attached to a partially shed larval skin.

Experiment 2: Toxicity of avidin expressed at a range of concentration levels in tobacco leaf to larval H. armigera

Mean avidin expression levels for leaves in the six treatment groups were as follows: Treatment 1, $17.3 \pm 0.4\,\mu$M; Treatment 2, $14.2 \pm 0.1\,\mu$M; Treatment 3, $10.9 \pm 0.1\,\mu$M; Treatment 4, $8.7 \pm 0.1\,\mu$M; Treatment 5, $6.4 \pm 0.1\,\mu$M; Treatment 6, $3.7 \pm 0.1\,\mu$M.

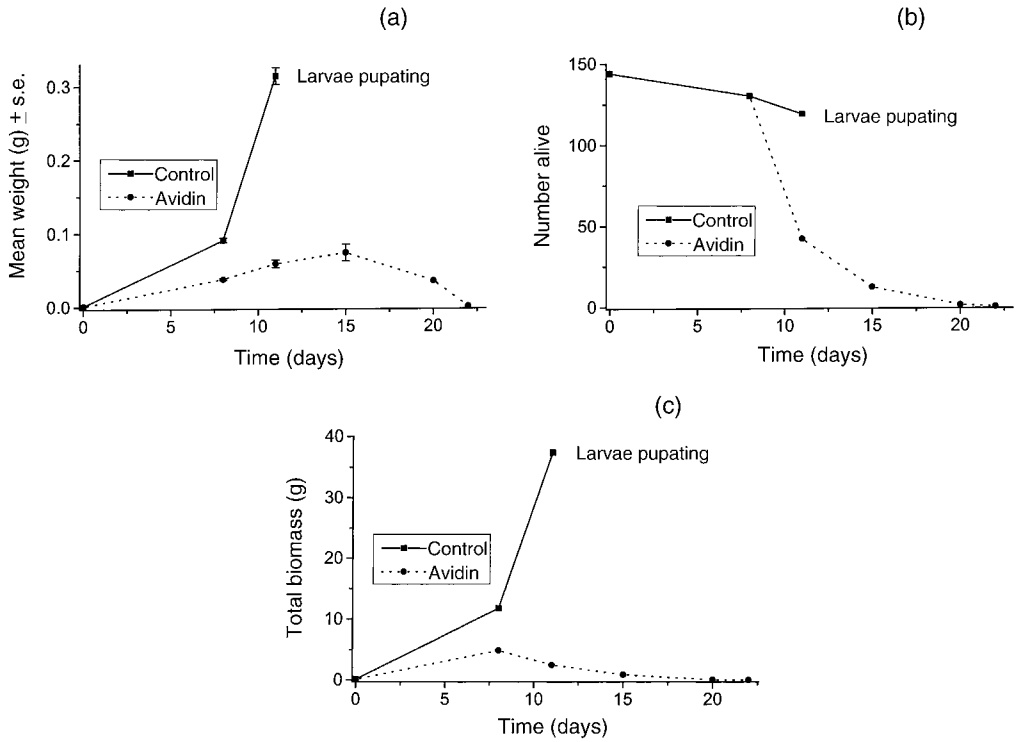


Figure 2. H. armigera larvae fed on T_0 transgenic tobacco leaves expressing avidin or on control tobacco leaves (a) growth, (b) survival, (c) biomass.

Avidin was not detected in any of the control leaf samples.

As there were no significant differences between larval growth, survival and biomass on the two control treatments, these results were combined before comparison with those from the six different avidin-leaf treatments.

By the time larvae were first weighed on day 8 of the experiment, control larvae had grown larger than those in all other treatments (Figure 6(a)) ($p < 0.05$). These differences increased with time. Comparison of larval survival curves showed that survival on all avidin-expressing lines was significantly lower than control survival ($p < 0.001$, log-rank test). There were no significant differences among the survival curves of larvae on the six lines expressing avidin at different levels (log-rank test, chi-square test, ANOVA).

All the larvae fed plants expressing avidin at levels between 6.4 and 17.3 μM failed to achieve substantial growth, and died without pupating (Figure 6(b)). Twenty two of the 24 larvae on the lowest expressing avidin treatment died, but the other two pupated, although they were smaller than control larvae. One of these pupae emerged as a moth. On the control treatments, 31 of 48 larvae successfully pupated and 19 of these emerged as moths. The number of larvae successfully pupating in the control treatments was reduced by cannibalism of prepupae by voracious late instar larvae. This effect may also have reduced the rate of emergence of moths from pupae in the controls. No such effect occurred in the avidin treatments because of the extremely high larval death rate caused by the ingestion of avidin-expressing leaf material.

Accumulation of biomass on the avidin-expressing lines was negligible compared to that on the control lines ($p < 0.001$, ANOVA) (Figure 6(c)). Larvae ingesting avidin-expressing tobacco in this trial had similar growth, biomass and mortality responses to those in Experiment 1. This suggests the effects of avidin were not altered by an early diet of lotus.

Experiment 3: Toxicity of avidin-tobacco leaves painted with either a protease inhibitor or a Bt toxin to larval H. armigera

Survival curves for *H. armigera* in the nine different treatment groups are shown in Figure 7(a). The

(a)

(b)

Figure 3. H. armigera larvae on day 14 of Experiment 1. (a) Larvae fed control tobacco are pictured on the left and those fed avidin-expressing tobacco on the right. (b) A typical control treatment with large larvae and stripped leaves is shown on the left, and a typical avidin-fed treatment with small dead larvae and minimally damaged leaves on the right.

only treatment which did not reduce survival compared with the controls was that with aprotinin-painted control leaves ($p < 0.001$ for all other comparisons, log-rank test).

The four treatments which used leaves expressing avidin at both high and low levels, with and without aprotinin painted on them, killed all larvae within 13 days. Survival on all these treatments was significantly reduced when compared with survival on control leaves with and without aprotinin ($p < 0.001$, log-rank test). Median survival times on these four avidin-expressing treatments did not differ significantly from each other (chi-squared test). Thus the effect on median larval survival of the combination

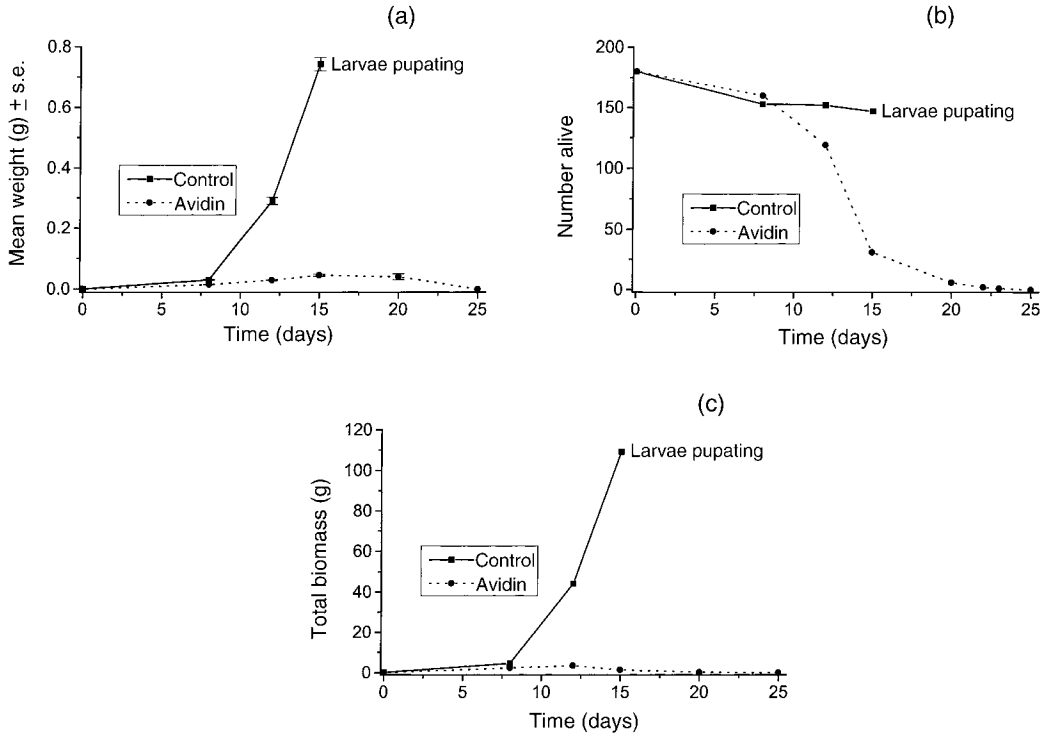


Figure 4. S. litura larvae fed on T_0 transgenic tobacco leaves expressing avidin or on control tobacco leaves (a) growth, (b) survival, (c) biomass.

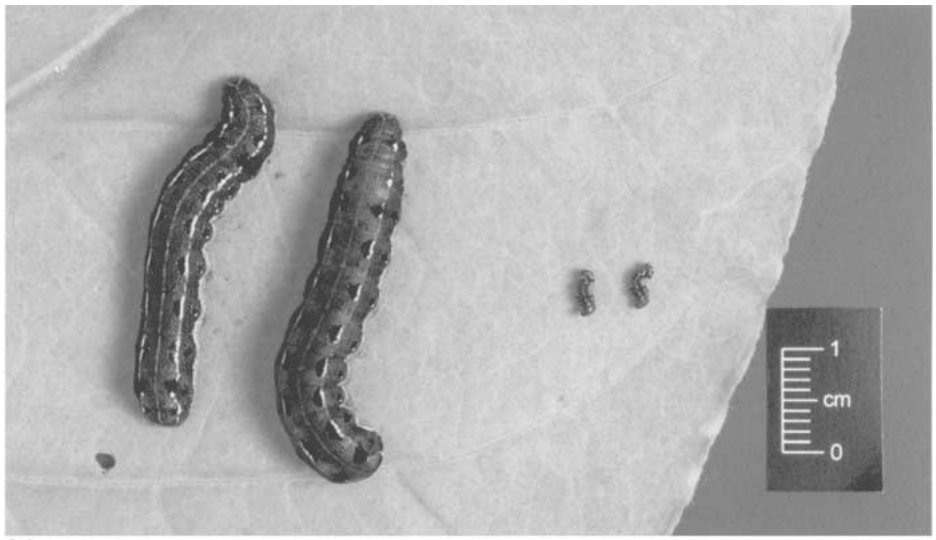
of avidin expression and aprotinin was equivalent to the effect of avidin expression alone. However, closer examination of the survival curves for the 'low avidin' and the 'low avidin with aprotinin' revealed that they diverged between days 8 and 12. The proportion of larvae alive on the 'low avidin with aprotinin' treatment was significantly lower on days 9, 10 and 11 ($p < 0.01$, ANOVA of arcsine square root-transformed data). This suggests that avidin could be combined with a protease inhibitor to produce a more toxic effect on larvae, even though the effect of the protease inhibitor alone may be subtle. Additionally, there was no suggestion of antagonism between the two types of resistance protein.

The three treatments in which Cry1Ba was painted onto the leaves killed all larvae within 4 days. Larvae feeding on high and low avidin-expressing leaves painted with Cry1Ba died significantly faster than those feeding on Cry1Ba-painted control leaves ($p < 0.001$, log-rank test). By day 2, the proportions of larvae dead on the high avidin/Cry1Ba and the low avidin/Cry1Ba treatments were 0.57 and 0.77 respectively. These were both greater than the sum of the proportions dead on the high or low avidin alone (0.03 for both) and the Cry1Ba alone (0.23) (ANOVA of arcsine square root-transformed data, $p < 0.001$). As the effects of combining the avidin and Cry1Ba treatments were greater than the sums of the effects of these treatments alone, synergistic effects on larval mortality were demonstrated when avidin-expressing leaves were painted with Cry1Ba.

Growth rates and biomass were plotted for larvae on all but the treatments involving Cry1Ba. Larvae feeding on control plants painted with the control solution or the aprotinin solution grew and accumulated biomass exponentially, while those on all treatments expressing avidin at high or low levels failed to grow or accumulate substantial biomass (Figures 5(b) and 5(c)). Because of the powerful effects of the avidin alone, it was not possible to measure any more subtle effects that the combination with aprotinin may have had on these two parameters.

Discussion

Transgenic plants expressing avidin in their leaves, via a gene construct which directs expression to the



(a)

(b)

Figure 5. S. litura larvae on day 15 of Experiment 1. (a) Larvae fed control tobacco are pictured on the left and those fed avidin-expressing tobacco on the right. (b) A typical control treatment with large larvae and stripped leaves is shown on the left, and a typical avidin-fed treatment with small dead larvae and minimally damaged leaves on the right.

vacuoles (Murray et al., 2001), were found to be extremely insecticidal to two noctuid pest species, *H. armigera* and *S. litura*. Plants in Experiment 1 with the lowest average expression level tested (3.1–4.6 μM avidin) killed all *H. armigera* and *S. litura* larvae, while 92% insect mortality occurred on plants expressing 3.7 μM avidin in Experiment 2. Plants expressing higher concentrations of avidin caused complete mortality of *H. armigera*. These results agree with those obtained by Kramer et al. (2000) who found that some kernels of maize transformed with a gene construct which directs avidin expression to the intercellular spaces were resistant to a range of stored products pest insects. However, leaves from these transgenic

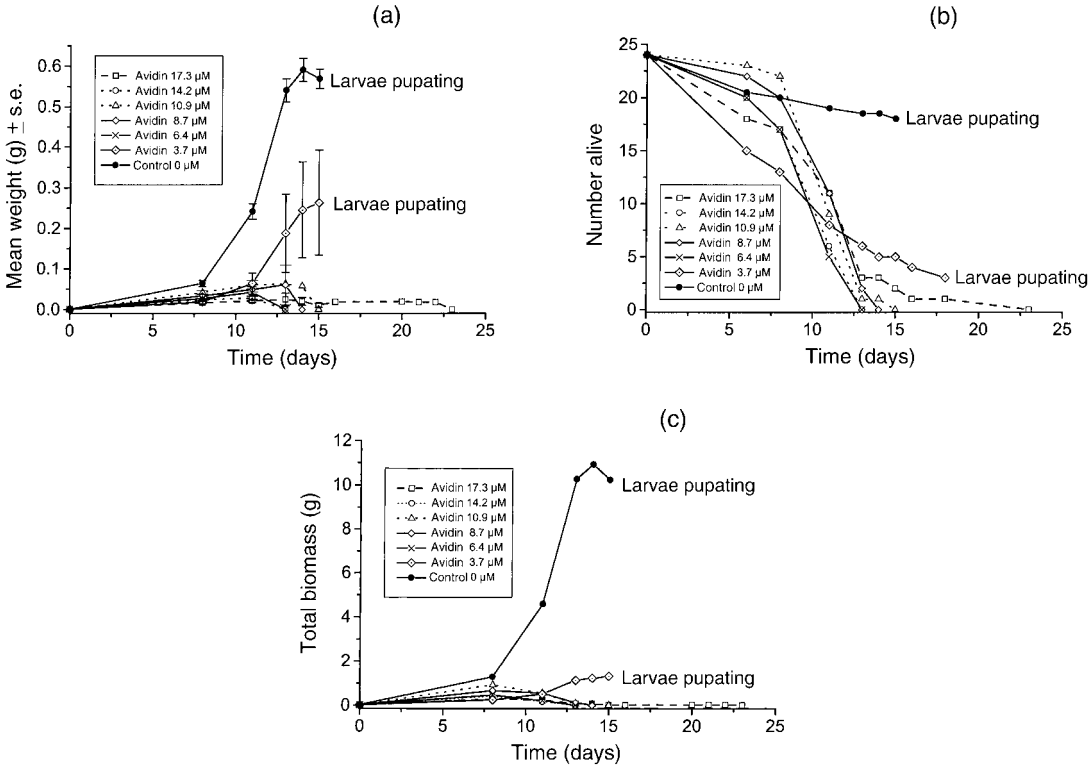


Figure 6. H. armigera larvae fed on T_1 transgenic tobacco leaves expressing avidin at six different concentrations or on control tobacco leaves (a) growth, (b) survival, (c) biomass.

maize plants were not tested and only the kernels expressing avidin at 300 ppm or greater produced 100% insect mortality, whereas in the present study, tobacco leaves expressing 6.4 µM avidin, which is equivalent to 100 ppm, killed 100% of *H. armigera*.

Since we did not observe a dose-dependent effect on *H. armigera*, either the levels tested here were too high for this or avidin's action on insects is better described by a 'threshold' or 'plateau' model, whereby concentrations above a certain level will cause significant mortality and those below will not. Results from experiments in which insects were fed various concentrations of avidin in diet suggest that the threshold/plateau concept may be valid (Morgan et al., 1993; Markwick et al., 2001).

The tobacco plants transformed with the avidin gene used in this study were fertile and the gene was stably inherited, since two successive generations bred from the original transformants expressed insecticidal quantities of avidin in their leaves. This contrasts with the transgenic avidin-maize plants described by Hood et al. (1997) and Kramer et al. (2000) which were male-sterile. This suggests that vacuole-targeting of avidin may avoid the male sterility previously observed in association with the expression of avidin in the intercellular spaces of the seed.

Synergistic toxic effects on *H. armigera* larvae were observed with combinations of avidin-expressing tobacco leaf and the Bt toxin, Cry1Ba. This suggests strongly that plants containing chimeric genes and expressing both avidin and Bt will be highly effective in protecting the plants from pest attack. Plants expressing avidin may enhance the effectiveness of Bt sprays. Furthermore, avidin combined with aprotinin was as effective as avidin expression alone in killing larvae and preventing growth and biomass accumulation. The synergy observed with the Bt toxin and the absence of any antagonistic effects between the biotin binding protein and the protease inhibitor shows the compatibility of these different types of resistance factor. Previous studies have demonstrated compatibility between some Bt toxins and proteinase inhibitors (Mackintosh et al., 1990; Zhang et al., 2000). Our results provide two further examples of gene combinations which may be useful for a gene pyramiding strategy in transgenic plants. It is likely that plants

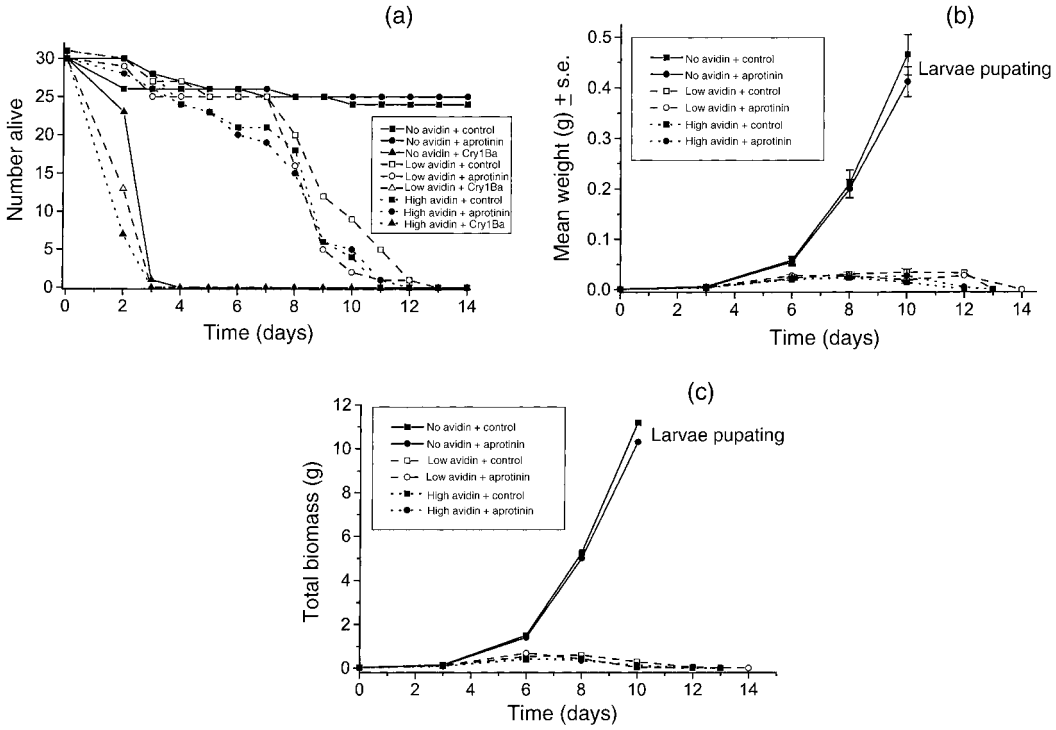


Figure 7. H. armigera larvae fed T_2 transgenic tobacco leaves expressing avidin at two different concentrations, combined with painted-on aprotinin or Cry1Ba, or fed on control tobacco leaves (a) survival, (b) growth (excluding Cry1Ba treatments), (c) biomass (excluding Cry1Ba treatments).

expressing avidin together with a second effective insecticidal protein employing a different mode of action will not only have greater toxicity, but also more durable resistance to pest attack than plants expressing or containing a Bt toxin, a protease inhibitor or another type of pest resistance factor on its own.

The present study has demonstrated that expression of avidin in the vacuoles of transgenic plants may effectively protect them from damage by two leaf-chewing noctuid species. Other studies have shown that avidin is toxic to a wide range of insects representing several different families and orders, and to mites (Levinson et al., 1992). Susceptible insects include other Lepidoptera (Morgan et al., 1993; Du & Nickerson, 1995; Kramer et al., 2000; Markwick et al., 2001), Diptera (Levinson & Bergmann, 1959; Tsiropoulos, 1985; Bruins et al., 1991), Coleoptera (Levinson et al., 1967; Kramer et al., 2000) and Orthoptera (Christeller et al., 2000). This suggests that expression of avidin directed to the vacuoles may be an effective strategy for producing fertile transgenic plants with stable resistance to a wide range of insect pests. This will offer considerable advantages over currently used genes for crops attacked by pests from more than one insect order. For example, transgenic maize and cotton crops expressing lepidopteran-specific Bt toxins are effective against lepidopteran pests, but must sometimes also be sprayed with conventional insecticides to control coleopteran rootworms or hemipteran stinkbugs (Pilcher & Rice, 1998; Bell et al., 1999).

Avidin's broad toxicity may raise ecotoxicological questions which have not yet been addressed for other, more host-specific resistance proteins. New methods may need to be devised to answer these, since avidin acts not as a conventional toxin, but as an antivitamin. The effects of avidin on an organism will be influenced by its biotin metabolism, the turnover of protein-bound biotin, the catabolism and excretion of the cofactor and its metabolites, and by levels of available dietary biotin and its bioavailability, which varies between foodstuffs (Baker, 1995; van den Berg, 1997). Higher organisms contain gut microflora which are capable of producing biotin. Again the amount and bioavailability of this source of biotin will vary but could be considerable: it appears to be low in the monogastric pig (Kopinski et al., 1989) but the situation in ruminants is unknown. These differences may explain why it takes many weeks before biotin

deficiency symptoms can be detected in humans (Velázquez et al., 1990), whereas insects (this paper and others quoted) seem extremely susceptible to biotin deficiency. Additionally, in contrast to the effects of conventional toxins, those of avidin can be readily reversed by supplying additional biotin (Olson, 1999; Kramer et al., 2000). There are some preliminary reports of the impacts of avidin on non-target organisms relevant to crop production. Newly-emerged adult honey bees fed with a pollen-candy mix containing 6.7 or 20 μM avidin consumed this food readily and had similar longevity to control bees (Malone & Pham-Delègue, 2001). Mice were unharmed after being fed for 21 days on a sole diet of avidin-maize kernels (Kramer et al., 2000). Further studies to assess impacts on soil biota and other beneficial organisms are under way.

Provided that regulatory requirements for release can be met, transgenic plants expressing avidin in the vacuoles should provide a potent new means for controlling a wide range of phytophagous pests of major world crops.

Acknowledgements

The authors wish to thank Dr Anne Gunson, of The Horticulture and Food Research Institute of New Zealand Ltd (HortResearch), for statistical analyses and Dr Ngaire Markwick, of the same institute, for helpful discussions. The research comprises part of the work described in International Patent Application no. WO 00/04049, *Chimeric polypeptides allowing expression of plant-noxious proteins*, and was supported by HortResearch.

References

Anon (1985) Heliothis armigera *Hubner, Noctuidae, Tomato noctuid*. Fiches Techniques Sur les Ravageurs des Cultures Vivrieres Tropicales IRATCV, Montpellier, France.

Armes NJ, Wightman JA, Jadhav DR and Roa GVR (1997) Status of insecticide resistance in *Spodoptera litura* in Andhra Pradesh, India. *Pesticide Sci* **50**: 240–248.

Ayyanna T, Subbaratnam GV and Dharmaraju E (1978) Pest complex on sunflower, *Helianthus annus* Lin in Andhra Pradesh. *Indian J Entomol* **40**: 353–356.

Baker DH (1995) Vitamin bioavailability. In: Ammerman CB, Baker DH and Lewis AJ (eds), Bioavailability of Nutrients for Animals: Amino Acids, Minerals Vitamins. (pp. 399–431) Academic Press, San Diego.

Bell DE, Slaughter G, Roberts PM, Ellis CE, Willingham L, Cary T et al. (1999) The effects of over sprays on bt cotton. Proc Beltwide Cotton Conf. Orlando, Florida, USA, 3–7 January, 1999, Vol. 2, pp. 956–958.

Beuning LL, Spriggs TW and Christeller JT (1994) Evolution of the proteinase inhibitor I family and apparent lack of hypervariability in the proteinase contact loop. *J Mol Evol* **39**: 644–654.

Bruins BG, Scharloo W and Thorig GEW (1991) The harmful effect of light on *Drosophila* is diet-dependent. *Insect Biochem* **21**: 535–539.

Christeller JT, Burgess EPJ, Mett V, Gatehouse HS, Markwick NP, Murray C et al. The expression of a mammalian proteinase inhibitor, bovine spleen trypsin inhibitor (SI), in tobacco and effects on *Helicoverpa armigera* larvae. *Transgenic Res* **11**: 163–176.

Christeller J, Sutherland P, Murray C, Markwick N, Phung M, Burgess E et al. (2000) Chimeric polypeptides allowing expression of plant-noxious proteins. International Patent No. WO 00/04049. Viewable at http://www.delphion.com/details?&pn=WO00004049A1&abl=fr#ABST

Dadd RH (1985) Nutrition: organisms. In: Kerkut GA and Gilbert LL (eds), Comprehensive Insect Physiology, Biochemistry and Pharmacology. (pp. 313–390) Pergamon Press, New York.

Du C and Nickerson KW (1995) Insecticidal activity of avidin. *Abstr 1995 Mtg Soc Invertebr Pathol* p. 70.

Fitt GP, Mares CL, Llewellyn DJ, Hokkanen HMT and Deacon J (1994) Field evaluation and potential ecological impact of transgenic cottons *Gossypium hirsutum* in Australia. *Biocontrol Sci Technol* **4:** 535–548.

Forrester NW (1997) The benefits of insect-resistant cotton. In: McLean GD, Waterhouse PM, Evans G and Gibbs MJ (eds), Commercialisation of Transgenic Crops: Risk, Benefit and Trade Considerations, Department of Primary Industries and Energy Bureau of Resource Sciences, Proceedings of a workshop held in Canberra, pp. 239–242.

Gleave AP (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol Biol* **20**: 1203–1207.

Gope ML, Keinänen RA, Kristo PA, Conneely OM, Beattie WG, Zarucki-Schulz T et al. (1987) Molecular cloning of the chicken avidin cDNA. *Nucl Acids Res* **15**: 3595–3606.

Guilin C and Yunxi L (1996) Cotton bollworm resistance and its development in northern cotton region of China 1984–1995. *Resistant Pest Management* **8**: 32–33.

Hill D (1975) Agricultural Insect Pests of the Tropics and their Control. Cambridge University Press, London.

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M et al. (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Mol Breed* **3**: 291–306.

Kalbfleisch JD and Prentice RL (1980) Statistical Analysis of Failure Time Data. Wiley, New York.

Knowles JR (1989) The mechanism of biotin-dependent enzymes. *Ann Rev Biochem* **58**: 195–211.

Kopinski JS, Leibholz J and Love RJ (1989) Biotin studies with pigs. 5. The post-ileal absorption of biotin. *Br J Nutr* **62**: 781–789.

Kramer KJ, Morgan TD, Throne JE, Dowell FE, Bailey M and Howard JA (2000) Transgenic avidin maize is resistant to storage insect pests. *Nature Biotech* **18**: 670–674.

Kumble V and Reed W (1981) *Heliothis*: a global problem. Proc Int Workshop on Heliothis Management, ICRISAT Center, Patancheru, India, pp. 9–14.

Levinson HZ and Bergmann ED (1959) Vitamin deficiencies in the housefly produced by antivitamins. *J Insect Physiol* **3**: 293–305.

Levinson HZ, Barelkovsky J and Bar Ilan AR (1967) Nutritional effects of vitamin omission and antivitamin administration on development and longevity of the hide beetle *Dermestes maculatus* Deg (Coleoptera, Dermestidae). *J Stored Prod Res* **3**: 345–352.

Levinson HZ, Levinson AR and Offenberger M (1992) Effect of dietary antagonists and corresponding nutrients on growth and reproduction of the flour mite (*Acarus siro* L). *Experientia* **48**: 721–729.

Li SQ and Rahmann H (1997) Cotton pest management in China. I: Insect pests. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz* **104**: 611–621.

MacIntosh SC, Kishore GM, Perlak FJ, Marrone PG, Stone TB, Sims SR et al. (1990) Potentiation of *Bacillus thuringirnsis* insecticidal activity by serine protease inhibitors. *J Agric Food Chem* **38**: 1145–1152.

Malone LA and Pham-Delègue MH (2001) Effects of transgene products on honey bees and bumblebees. *Apidologie* **32**: 287–304.

Marek J and Navratilova M (1995) A new glasshouse pest, *Helicoverpa armigera* (*Noctuidae, Lepidoptera*). *Ochrana Rostlin* **31**: 143–147.

Markwick NP, Christeller JT, Docherty LC and Lilley CM (2001) Insecticidal activity of avidin and streptavidin against four species of pest Lepidoptera. *Entomol Exp Appl* **98**: 59–66.

McManus MT and Burgess EPJ (1995) Effects of the soybean (Kunitz) trypsin inhibitor on growth and digestive proteases of larvae of *Spodoptera litura. J Insect Physiol* **41**: 731–738.

McManus MT, Burgess EPJ, Philip B, Watson L, Laing WA, Voisey CR et al. (1999) Expression of the soybean (Kunitz) trypsin inhibitor in transgenic tobacco: effects on larval development of *Spodoptera litura*. *Transgenic Res* **8**: 383–395.

Morgan TD, Oppert B, Czapla TH and Kramer KJ (1993) Avidin and streptavidin as insecticidal and growth inhibiting dietary proteins. *Entomol Exp Appl* **69**: 97–108.

Murray C and Christeller JT (1995) Purification of a trypsin inhibitor (PFTI) from pumpkin fruit phloem exudate and isolation of putative trypsin and chymotrypsin inhibitor cDNA clones. *Biol Chem Hoppe-Seyler* **376**: 281–287.

Murray C, Sutherland PW, Phung MM, Lester MT, Marshall RK and Christeller JT (2001) Expression of biotin-binding proteins, avidin and streptavidin, in plant tissues using plant vacuolar targeting sequences. *Transgenic Res* **11**: 201–216.

Olson RI (1999) Vitamin deficiency, dependency, and toxicity. In: Beers MH and Berkow R (eds), The Merck Manual of Diagnosis and Therapy. The Centennial edn, Whitehouse Station, New York, pp. 35–51.

Payne RW, Lane PW, Digby PGN, Harding SA, Leech PK, Morgan GW et al. (1993) Genstat 5 Release 3 Reference Manual. Lawes Agricultural Trust, Clarendon Press, Oxford.

Pilcher CD and Rice ME (1998) Management of European corn borer (Lepidoptera: Crambidae) and corn rootworms (Coleoptera: Chrysomelidae) with transgenic corn: a survey of farmer perceptions. *Am Entomol* **44**: 36–44.

Raju SSD, Hammerlindl JK, Panchuk B, Pelcher LE and Keller W (1992) Modified binary plant transformation vectors with the wild-type gene encoding NPTII. *Gene* **122**: 383–384.

Reynolds D and Armes N (1994) When insecticides fail: the case of the cotton bollworm in India. In: Cartwright A (ed.), World Agriculture London. (pp. 39–42) Sterling Publications, London.

Sahayaraj K and Paulraj MG (1998) Screening the relative toxicity of some plant extracts to *Spodoptera litura* Fab. (*Insecta: Lepidoptera: Noctuidae*) of groundnut. *Fresenius Environ Bull* **7**: 9–10; 557–560.

Sekhar PR, Roa NV, Rao BR and Venkataiah M (1995) Current status of synthetic pyrethroid resistance in *Helicoverpa armigera* populations on groundnut. *Int Arachis Newslett* **15**: 62–63.

Shanower TG, Romeis J and Minja EM (1999) Insect pests of pigeonpea and their management. *Ann Rev Entomol* **44**: 77–96.

Simpson RM, Burgess EPJ and Markwick NP (1997) *Bacillus thuringiensis* δ-endotoxin binding sites in two Lepidoptera, *Wiseana* spp. and *Epiphyas postvittana. J Invertebr Pathol* **70**: 136–142.

Tsiropoulos GR (1985) Dietary administration of antivitamins affected the survival and reproduction of *Dacus oleae. Z Ang Entomol* **100**: 35–39.

Van den Berg H (1997) Bioavailability of biotin. *Eur J Clin Nutr* **51**: S60–S61.

Velázquez A, Zamudio S, Báez A, Murguía-Corral R, Rangel-Peniche and Carrasco A (1990) Indicators of biotin status: a study of patients on prolonged total parenteral nutrition. *Eur J Clin Nutr* **44**: 11–16.

Venables WN and Ripley BD (1997) Modern Applied Statistics with S-Plus. 2nd edn, Springer-Verlag, New York.

Whalon ME and Norris D (1997) *Bacillus thuringiensis* transgenic plants: Will resistance kill the promise? In: McLean GD, Waterhouse PM, Evans G and Gibbs MJ (eds), Commercialisation of Transgenic Crops: Risk, Benefit and Trade Considerations, Department of Primary Industries and Energy Bureau of Resource Sciences, Proceedings of a workshop held in Canberra, pp. 243–258.

Wood HG and Barden RE (1977) Biotin enzymes. *Ann Rev Biochem* **46**: 385–413.

Wurtele ES and Nikolau BJ (1990) Plants contain multiple biotin enzymes: discovery of 3-methylcrotonyl-CoA-carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase in the plant kingdom. *Arch Biochem Biophys* **278**: 179–186.

Zaz GM and Kushwaha KS (1984) Efficacy of *Bacillus cereus* Frankland and Frankland against different instars of *Spodoptera litura* (Fabricius) fed on treated cauliflower leaves. *J Entomol Res* **8**: 216–219.

Zhang JH, Wang CZ and Qin JD (2000) The interactions between soybean trypsin inhibitor and delta-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera* larvae. *J Invertebr Pathol* **75**: 259–266.