Transgenic Research **12:** 229–241, 2003.
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Tritrophic interactions between transgenic potato expressing snowdrop lectin (GNA), an aphid pest (peach–potato aphid; *Myzus persicae* (Sulz.) and a beneficial predator (2-spot ladybird; *Adalia bipunctata* L.)

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Received 14 March 2002; accepted 26 July 2002

Key words: ladybirds, snowdrop lectin (GNA), tissue specific expression, transgenic plants, tritrophic interactions

Abstract

Tritrophic interactions between transgenic potato expressing the insecticidal lectin from snowdrop (*Galanthus nivalis* agglutinin; GNA), an aphid pest, *Myzus persicae* (Sulz.), and a beneficial predator, the 2-spot ladybird (*Adalia bipunctata* L.) were investigated. Clonal plants expressing GNA at 0.1–0.2% total soluble protein in leaves were used. No significant effects on development and survival of ladybird larvae fed on aphids from these transgenic plants were observed, with larval survival in the experimental group being 90% compared to 89% for controls. There were also no effects on subsequent female or male longevity. Female fecundity was also investigated. Although no significant differences ($p > 0.05$) were observed in egg production between control and experimental groups, a 10%, reduction ($p < 0.01$) in egg viability (determined by % hatch) occurred in ladybirds fed aphids reared on transgenic plants. Additional studies were carried out using aphids fed on artificial diet containing GNA, to deliver quantified levels of the protein to ladybird adults. GNA had no deleterious effects upon adult longevity, but resulted in a consistent trend for improved fecundity. Egg production was increased by up to 70% and egg viability also increased significantly. The results suggest that GNA is not deleterious to ladybirds. Results from these studies highlight the need to discriminate between direct and indirect effects when studying tritrophic interactions between plants/pests/natural enemies. Furthermore, it emphasises the importance of demonstrating 'cause and effect'.

Introduction

Use of plant-derived transgenes as a strategy for control of insect pests has received much attention in recent years. Transgenic crops expressing a range of different insecticidal proteins such as lectins and enzyme inhibitors have been generated which show improved resistance to a variety of insect orders, including Lepidoptera (Fitches et al., 1997; Gatehouse et al., 1997; Mochizuki et al., 1999), Coleoptera (Shade et al., 1994; Leple et al., 1995; Ishimoto et al., 1996; Graham et al., 1997; Morton et al., 2000) and Homoptera (Rao et al., 1998; Gatehouse et al., 1999; Stoger et al., 1999). Previous studies using transgenic potato expressing a lectin from snowdrop (*Galanthus nivalis* agglutinin; GNA) demonstrated reduced fecundity in two aphid species, *Aulocorthum solani* (Down et al., 1996) and *Myzus persicae* (Gatehouse et al., 1996).

However, there is now growing concern over potential risks that insect-resistant transgenic crops might pose to non-target beneficial insects (Schuler

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et al., 1999; Sharma & Ortiz, 2000). Many beneficial insects may be exposed to insecticidal proteins expressed in transgenic plants either directly, by consuming parts of the plant (e.g., honeybees; Picard-Nizou et al., 1997; Malone et al., 1999), or indirectly through the target pest by consuming (e.g., predatory beetles; Sims, 1995; Ashouri et al., 1998; Hilbeck et al., 1998; Birch et al., 1999) or parasitising (e.g., parasitoid wasps; Bell et al., 1999, 2001a,b) insects that have been feeding from these plants. Since natural enemies can play an important role in controlling pest populations (Verkerk et al., 1998), it is desirable that crop protection by GM technology is compatible with biological control within an integrated pest management scheme.

Dogan et al. (1996) showed that the convergent ladybird, *Hippodamia convergens*, was unaffected when fed aphids (*Myzus persicae*) colonising transgenic potato expressing *Bacillus thuringiensis* δ-endotoxin (cry 3A). In contrast, Birch et al. (1999) observed that aphids (*M. persicae*) raised on leaves from one line of transgenic potato expressing the snowdrop lectin (GNA) exerted significant detrimental effects on fecundity, egg viability and female longevity of adult 2-spot ladybirds (*Adalia bipunctata*) predating them. The detrimental effects of snowdrop lectin differ greatly amongst insect species, but more recent results suggest that ladybirds are not susceptible to this protein. When GNA was delivered to the aphid, *M. persicae*, via an artificial diet system, no significant detrimental effects were observed on growth or survival of 2-spot ladybird larvae feeding on dosed aphids, although slight delays in development were seen (Down et al., 2000). Any effect of GNA on the ladybird larvae was suggested to be indirect, in that the aphid prey were adversely affected and were therefore a sub-optimal food source for the ladybird larvae.

This paper addresses the issues raised by the work of both Birch et al. (1999) and Down et al. (2000), to establish whether the detrimental effects observed on adult 2-spot ladybirds were due to a direct effect of GNA on the ladybirds, an indirect effect resulting from the ladybirds consuming pests fed on transgenic plants expressing GNA, or due to other, possibly pleiotropic, effects relating to the specific line of transgenic potato plants investigated. Two different types of trial were performed with the 2-spot ladybird: in the first, aphids (*M. persicae*) colonising transgenic potato plants expressing GNA were used as a food source for the ladybirds whilst in the second, aphids dosed GNA via artificial diet were used as the food source. Effects on development, survival and fecundity were monitored.

Materials and methods

Materials

Purified GNA from snowdrop (*Galanthus nivalis*) bulbs (Van Damme et al., 1987) was purchased from Drs W. Peumans and E. van Damme (Catholic University, Leuven, Belgium). Antibodies against GNA were raised in rabbits using standard procedures (supplied by Drs L.N. Gatehouse and R.D.D. Croy, University of Durham). Bradford protein assay reagents and HRP-conjugated goat anti-rabbit antibodies were obtained from Bio-Rad Laboratories plc (Hemel Hempstead, UK). ECL detection kit was supplied by Amersham, Bucks, UK. All other chemicals and reagents were obtained from either Sigma Chemical Company or BDH (Poole, Dorset) and were of analytical grade unless otherwise stated.

Insect cultures

Peach–potato aphids (*Myzus persicae* (Sulz.)) were reared on non-transformed control potato leaves in Blackman boxes (Blackman, 1971) or on Chinese cabbage plants, at a temperature of 21 ± 2°C with a lighting regime of 16L:8D. Adults and eggs of the 2-spot ladybird (*Adalia bipunctata* L.) came from laboratory stocks reared on the pea aphid (*Acyrthosiphon pisum* (Harris)).

Transgenic plant material

Transgenic potato (*Solanum tuberosum* L. cv. Desirée line, PWG6#85) was used in this investigation; details of production and characterisation of this line are described elsewhere (Gatehouse et al., 1997).

Preparation of plant material obtained from tissue culture (Trial 1)

Plantlets of the transgenic potato line PWG6#85 and untransformed control potato were bulked up by clonal propagation in tissue culture and potted up in John Innes No. 3 loam-based compost (Gatehouse et al., 1997). Plants were grown for a month in a controlled environment (21 ± 2°C; 16L:8D), transferred to the glasshouse (temperature fluctuated widely ranging from daytime temperatures >40°C on some days

to ca. 10°C on some nights), and then repotted into 15 cm diameter pots. When the plants were 2.5 months old they were repotted into 25 cm diameter pots; at all times in the glasshouse, plants were contained in porous cellophane bags.

Determination of GNA-expression levels in transgenic plant material (Trial 1)

Expression levels were determined by immunoassay. Leaf samples (comparable age and position) were initially taken from a small sample of plants, when the plants were ca. 2 months old. GNA-expression was determined as described in detail by Gatehouse et al. (1997). Briefly, flash frozen leaf samples were extracted in 50 mM Tris HCl pH 9.5 containing 1% PMSF and centrifuged at 13,000*g* for 20 min. Protein estimations were carried out on the extracts (Bradford, 1976) and 5 µg of protein per sample were immunodot-blotted on to 0.2 µm nitrocellulose (Schleicher & Schuell BA85). A range of standards was prepared from untransformed potato, spiked with known amounts of GNA (ranging from 0 to 2.0%). Blots were detected according to Gatehouse et al. (1997) using polyclonal rabbit antibody raised against GNA and HRP-conjugated goat anti-rabbit as the secondary antibody. ECL (Amersham, UK) was used to visualise GNA in the extracts, following the manufacturer's instructions. Blots were scanned using a densitometer (BIO-RAD GS690) and the software package Molecular Analyst for Apple Macintosh computers (BIO-RAD). Standards were used to construct a calibration curve from which the amount of GNA as % total soluble leaf protein was estimated for each sample. Following initial determination of expression levels, further samples were taken from the test plants (approximate age 2.5 months) and the presence of GNA was detected by SDS-PAGE electrophoresis (5 µg total protein per sample, 15% acrylamide gels; Hames, 1981) followed by semi-dry western blotting (Kyhse-Andersen, 1974). Proteins were visualised and levels of GNA estimated as described above.

Trial 1: Transgenic plants as GNA source

Colonisation of trial plants with aphids

Mature plants (approximately 3 months old), individually confined in porous cellophane bags, were randomly separated into two groups, each containing 25 non-transformed and 25 transgenic (GNA-expressing) plants. Each plant from the first group was inoculated with apterous, newly matured, parthenogenetic *M. persicae* by placing the aphids on the adaxial leaf surfaces; infested leaves were tagged to enable identification and population build-up was assessed for both control and experimental plants.

Similarly, plants from the second group were inoculated with *M. persicae* at later dates during the trial, once the ladybirds had reached adulthood. These were used to feed the ladybirds throughout their adult life.

Feeding of A. bipunctata *through the larval stages*

To ensure sufficient numbers of ladybird larvae survived during the first instar, larvae were 'lab-reared' through this instar. On hatching, clutches were randomly and equally separated into those to be fed a diet of aphids from the non-transformed potatoes and those to be fed on aphids colonising the transgenic plants. Larvae were individually contained (Down et al., 2000) and fed an excess of aphids on a daily basis (uneaten aphids from the previous day were removed), thus ensuring that the maximum length of time that aphids had been away from the plants (and hence the presence of GNA) was 24 h. Each larva was reared on aphids collected from the plant to which it was destined to be transferred. Consumption of aphids, duration of the first instar and weight after moulting to the second instar were recorded. Second instar larvae were transferred to their respective plants (3 per plant) and left undisturbed until pupation.

Egg production and egg hatch of ladybirds from transgenic plants

Ladybird pupae were removed and maintained under controlled-environmental conditions (21 ± 2°C; L16:D8). Adults were weighed and sexed on emergence; in some instances, adult ladybirds were found on the plants, these too were removed, weighed and sexed. Adults were maintained in a controlled-environment (see above) and fed an excess of aphids collected from either non-transformed or transgenic plants accordingly, on a daily basis. After ca. 2.5 weeks of adult life, the ladybirds were divided into mating groups so that each group contained 1 male and 1 or 2 females. The numbers of eggs produced by each group were recorded daily and adults separated from the eggs. Eggs were maintained under the above controlled conditions and the numbers of larvae emerging from each egg batch recorded. Male and female adult survival was also recorded. As deaths occurred, remaining adults were combined ensuring that females always had a male present so that eggs

would be fertilised. Adults were kept until the supply of aphids from the non-transformed and transgenic plants were exhausted (due to death of the plants after approximately 2.5 months following initial infestation).

Trial 2: Artificial diet as GNA source

Delivery of GNA and feeding regime of adult ladybirds

Male and female adult 2-spot ladybirds from 15 families were divided into mating pairs (each pair equal to one replicate) in reciprocal crosses. Twenty replicates were set up for both the control and treatment (those receiving GNA-dosed aphids). Each ladybird pair was confined in an 85 cm diameter petridish, sealed with parafilm punctured with small perforations for ventilation and maintained in a controlled-environment (21 ± 2°C; L16:D8). For the initial 12 days of the trial, adults were fed a diet of aphids which had been dosed with GNA-containing (0.1% w/v) or control artificial diet (Down et al., 2000). Aphids were maintained on this diet for 48 h prior to being offered to the ladybirds. Each ladybird mating pair was given 25 dosed aphids on each of these 12 days. For 5 of these days (chosen randomly) each batch of 25 aphids was weighed prior to being given to the ladybirds, enabling a comparison of weight of food being offered to the control and treatment groups. After this 12-day period, all adult ladybirds (control and experimental) were switched to a diet of aphids which had been reared on non-transformed control potato, grown from tubers. Ladybirds were maintained on this diet until the end of the trial and were fed in excess on a daily basis. Ladybird fecundity, egg viability and egg hatch were monitored during the initial 12-day dosing period and for the subsequent 4 weeks. Adults were removed from the batches of eggs laid every 24 h to keep egg predation to a minimum. Adult survival was recorded on a daily basis.

GNA accumulation studies

Aphids sampled at 24 h intervals for 7 days were flash-frozen in liquid nitrogen and stored at −20°C for future analysis. Neonate ladybird larvae individually reared in chambers (see 'Feeding of *A. bipunctata* through the larval stages') and fed on a daily basis with an excess of aphids colonising either control or transgenic plants were sampled (as above) at the following time points: after moulting to the 2nd, 3rd and 4th instars and 2 days after moulting into the 4th instar. The assay was repeated with a 'chase' aspect introduced, whereby after the above sampling points the larvae fed on aphids from transgenic plants were fed for an additional 48 h on aphids from control potato, before sampling. For each time point, 5 larvae were sampled. Aphids and ladybirds were homogenised in 50 mM Tris HCl pH 9.5 containing 1% PMSF (36 mg/ml in ethanol). Total soluble protein was extracted and the concentrations estimated as above. Samples (10 µg for aphids, 5 µg for ladybirds) were separated by electrophores on 15% SDS-PAGE minigels. Polypeptides were electro-blotted onto nitrocellulose and the presence of GNA was detected as described above.

Statistical analyses

Survival data were analysed using the Kaplan–Meier Logrank χ^2 test. All other data were analysed using either unpaired *t*-tests or non-parametric Mann–Whitney *U*-tests; differences were considered to be significant at the $p < 0.05$ level. Analyses were carried out using Statview v4.5 (Abacus Concepts, Berkeley, CA) software for Apple Macintosh computers.

Results

Trial 1: Transgenic plants used as GNA source

Expression of GNA in transgenic potato plants

Levels of GNA expression in test plants used for bioassay were quantified by immuno-assay. Expression levels were found to range from approximately 0.07 to 0.20% total soluble leaf protein (Figure 1). Western blot analyses demonstrated that the GNA polypeptide was of similar size to GNA purified from snowdrop; as expected, no proteins with cross reactivity to the GNA antibodies were detected in non-transformed control plants (data not shown).

Performance of A. bipunctata *through the first larval instar*

Ladybird larvae individually 'lab-reared' through the first instar were fed aphids from the plant they were destined for on reaching the second instar. The following parameters were investigated: aphid consumption, duration of the first instar, weight on entering the second instar and survival during this period.

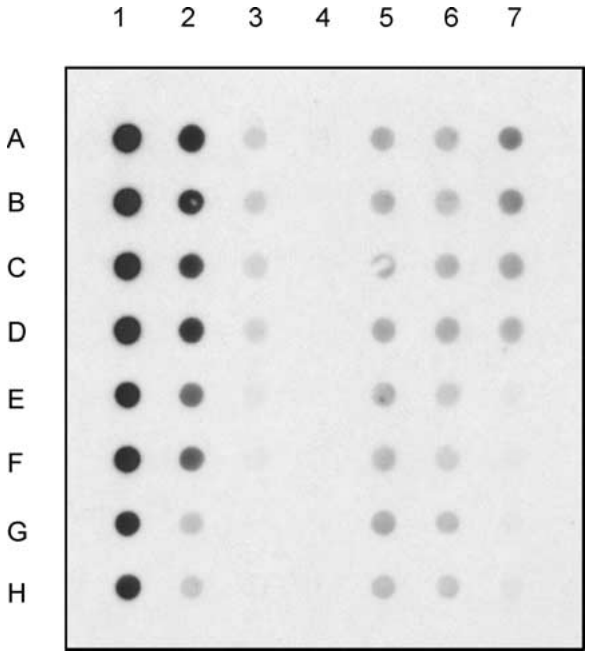

Figure 1. Immuno-blot of GNA expression in potato plants used in Trial 1. Columns 1–3, standards with known amounts of GNA to give % GNA: 1A&B 1.0%; 1C&D 0.7%; 1E&F 0.5%; 1G&H 0.4%; 2A&B 0.3%; 2C&D 0.2%; 2E&F 0.1%; 2G&H 0.07%; 3A&B 0.05%; 3C&D 0.03%; 3E&F 0.00%. Wells 5A–H, 6A–H and 7A–D, 10 experimental plants; each extract is loaded in duplicate vertically. Wells 7E–H, 2 control plants, loaded in duplicate vertically.

Figure 2(A) shows that no significant differences were observed in the mean total number of aphids consumed during the first instar by larvae fed on aphids taken from GNA-expressing potato (17) compared to those fed on aphids from non-transformed potato (16.4; unpaired *t*-test, $p = 0.47$). Duration of the first instar was slightly extended for larvae feeding on aphids from GNA-expressing potatoes, compared to aphids from untransformed potatoes (mean durations of 3.5 and 3.4 days, respectively; Figure 2(B)). However, the difference was not significant (non-parametric Mann–Whitney *U*-test, $p = 0.38$). Mean weights of larvae on entering the second instar are recorded in Figure 2(C). Although this was slightly higher (0.66 ± 0.03 mg) for experimental larvae compared to controls (0.62 ± 0.03 mg), this difference was not significant (unpaired *t*-test, $p = 0.36$). As can be seen from Figure 2D, no significant differences (χ^2 test, $p > 0.99$) were observed between the survival of ladybird larvae during the first instar; ladybirds supplied with aphids colonising GNA-expressing potato showed 90% survival compared to 89% for those consuming aphids from untransformed potato.

A. bipunctata *adult emergence*

Ladybird larvae were transferred to control or transgenic plants from second instar, and allowed to complete their development *in planta*. Numbers of adult *A. bipunctata* retrieved from the plants, both in the pupal stage and as adults, were low on both untransformed control and GNA-expressing transgenic potato. A total of 75 second instar larvae were introduced on to each group of plants but only 27 (36%) were retrieved from the control and 33 (44%) retrieved from the GNA-expressing potatoes; this difference was not found to be significant ($\chi^2 = 0.69$, $p = 0.40$). After pupal emergence, a total of 21 (28%) adults were retrieved alive from the controls compared to 33 (44%) from the GNA-expressing plants; this difference was also not significant ($\chi^2 = 3.5$, $p = 0.06$). No significant differences in the mean weight of adult ladybirds were observed with both groups showing a mean weight of 11.6 mg (unpaired *t*-test, $p = 0.92$).

A. bipunctata *egg production and egg hatch*

Approximately 2.5 weeks after adult emergence, on the first day that egg laying was observed, the adults were divided into mating groups. Each group contained one male with one or two females. Egg production was monitored on a daily basis until the supply of aphids colonising either experimental or control plants was exhausted (after approximately 2 months of egg laying). The numbers of eggs successfully hatching from each batch laid was recorded.

Egg production, shown as mean cumulative eggs produced/female with mate is shown in Figure 3. Both ladybird groups showed very similar trends and rates of egg production. Over the first 40 days, control ladybirds showed a mean of 7.7 eggs/female with mate/day compared to a value of 8.3 for those ladybirds feeding on aphids colonising GNA-expressing plants. Over the following 16 days rates for both groups approximately tripled to 22.8 and 22.0 mean eggs/female/day for the control and treatment groups, respectively. However, over the final 8 days, the rate for the control group increased to 30.2 mean eggs/female/day, whereas the rate for those in the experimental group decreased to approximately half this level (17.6 mean eggs/female/day). Statistical analyses (non-parametric Mann–Whitney *U*-tests) were performed at 7-day intervals over the 63-day egg laying period. No significant differences ($p > 0.05$) were observed, at any of the time points analysed, in the cumulative number of eggs produced per replicate, between control and experimental groups.

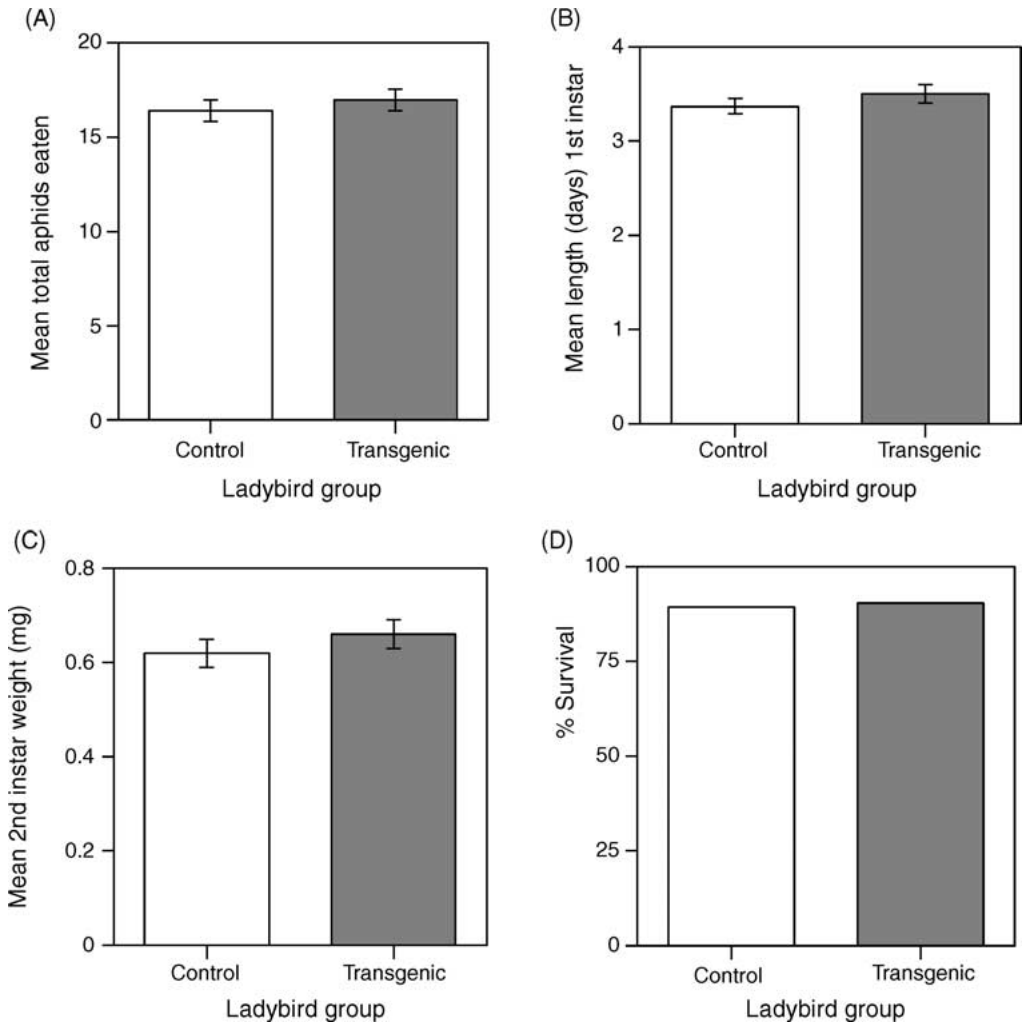

Figure 2. (A) Mean total of aphids consumed; (B) mean duration of the first instar; (C) mean 2nd instar weight; (D) percent survival of ladybird larvae, fed aphids from control or experimental plants, during their first instar. No significant differences were observed (unpaired *t*-tests for (A) and (C); non-parametric Mann–Whitney *U*-test for (B); χ^2 test for (D)). (Error bars represent S.E. of the mean.)

Egg hatch, shown as mean percentage of eggs successfully hatched from each batch laid by a mated female, is shown in Figure 4. The mean percentage of eggs successfully hatching is lower in the batches laid by adults supplied with aphids colonising the GNA-expressing plants (approximately 47%) compared to those laid by adults feeding on control aphids (approximately 57%). This difference in successful egg hatch between the two groups was significant (non-parametric Mann–Whitney *U*-test, $p < 0.01$).

A. bipunctata *survival*

In the 2-week period between ladybird adult emergence and the setting up of the mating pairs, 9.5% of the control adults died compared to 15.2% of adults which were fed on aphids from GNA-expressing potato. This approximate 5% difference was not statistically significant (data not shown; χ^2 test, $p > 0.05$).

Adult ladybird mortality was recorded on a daily basis until termination of the trial. Overall survival (male and female adults combined) during the approximately 60-day period over which egg laying was monitored is presented in Figure 5(A), along with separate survival curves for female (Figure 5(B)) and male (Figure 5(C)) ladybirds. Overall control survival (Figure 5(A)) remained at 100% until day 23 and then decreased in steps to approximately 89% by day 33. However, although survival of the ladybirds in the experimental group dropped very slightly

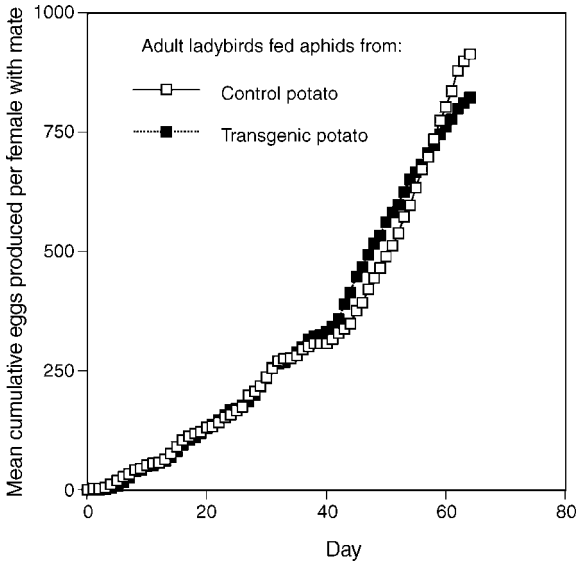

Figure 3. Mean cumulative number of eggs/female with mate, over time, for adult ladybirds fed aphids from control or GNA plants. No significant differences were observed at any time interval tested (every 7 days; non-parametric Mann–Whitney *U*-test).

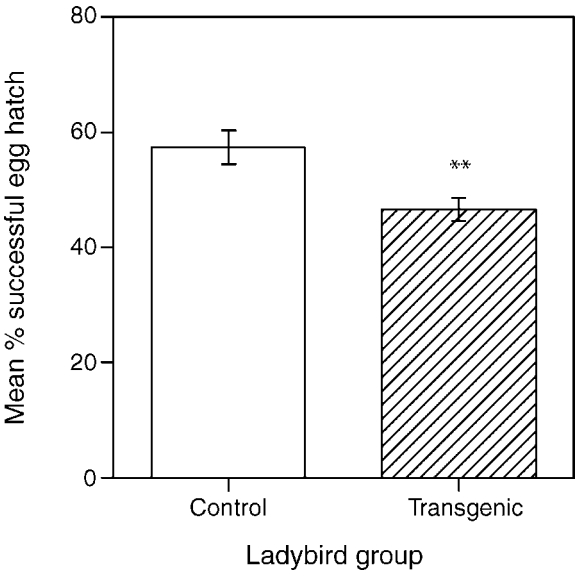

Figure 4. Mean percent successful egg hatch for adult ladybirds fed aphids from control or GNA plants. ** denotes significant difference at the $p < 0.01$ level (non-parametric Mann–Whitney *U*-test). (Error bars represent S.E. of the mean.)

to approximately 96% after 4 days, survival then remained at this level until day 33. Over the next 30-day period, both control and treatment adult ladybirds exhibited a steady decline in survival so that by day 63 control survival was at 58%, whereas survival of those fed on aphids from GNA-expressing plants was approximately 13% higher at 71%. No significant difference was found in the survival between the two groups of ladybirds over this recording period (Logrank χ^2 analysis, $p > 0.05$).

Female survival (Figure 5(B)) showed a similar trend to overall adult survival. Control survival remained at 100% until day 23, dropped to approximately 85% on day 24, showed a further reduction to approximately 71% on day 27 and decreased once again to 43% on day 50. In comparison, although female ladybirds from the experimental group only showed 100% survival for 4 days, survival remained at approximately 94% until day 47. A gradual decline of approximately 20% was seen over the next 10 days so that by the end of the trial female survival of this ladybird group was just over 25% higher (at 71%) than survival of the control females (43%). Survival of female ladybirds from the two groups was not significantly different over this 63-day period (Logrank χ^2 analysis, $p > 0.05$).

Survival of the male ladybirds (Figure 5(C)) remained at 100% until day 33 for both control and treatment groups. Control survival then declined to 75% over the next 5 days and remained at this level until the last day when a further slight decline to 67% was observed. The number of male ladybirds from the experimental group declined to approximately 81% on day 34 and remained at this level until 2 days prior to the end of the trial when an approximately 10% reduction to 73% occurred. By the end of the trial, males fed aphids colonising the GNA-expressing potato showed a very slightly (5%) higher survival than the control group. Survival over the whole of this time course was not significantly different (Logrank χ^2 analysis, $p > 0.05$).

Trial 2: Artificial diet as GNA source

Weights of dosed aphids

On 5 randomly selected days during the 12-day dosing period, each batch of GNA-dosed and control-dosed aphids was weighed prior to being offered to each ladybird pair (replicate), to verify that control and treatment ladybirds were receiving equal amounts of food. On one of these 5 days the mean weight ($\pm$ SE) of the GNA-dosed aphid batches was found to be significantly lower than the control-dosed aphid batches (11.55 ± 0.16 mg compared to 12.05 ± 0.22 mg; unpaired *t*-test, $p < 0.05$), but on all other days tested no significant differences were observed.

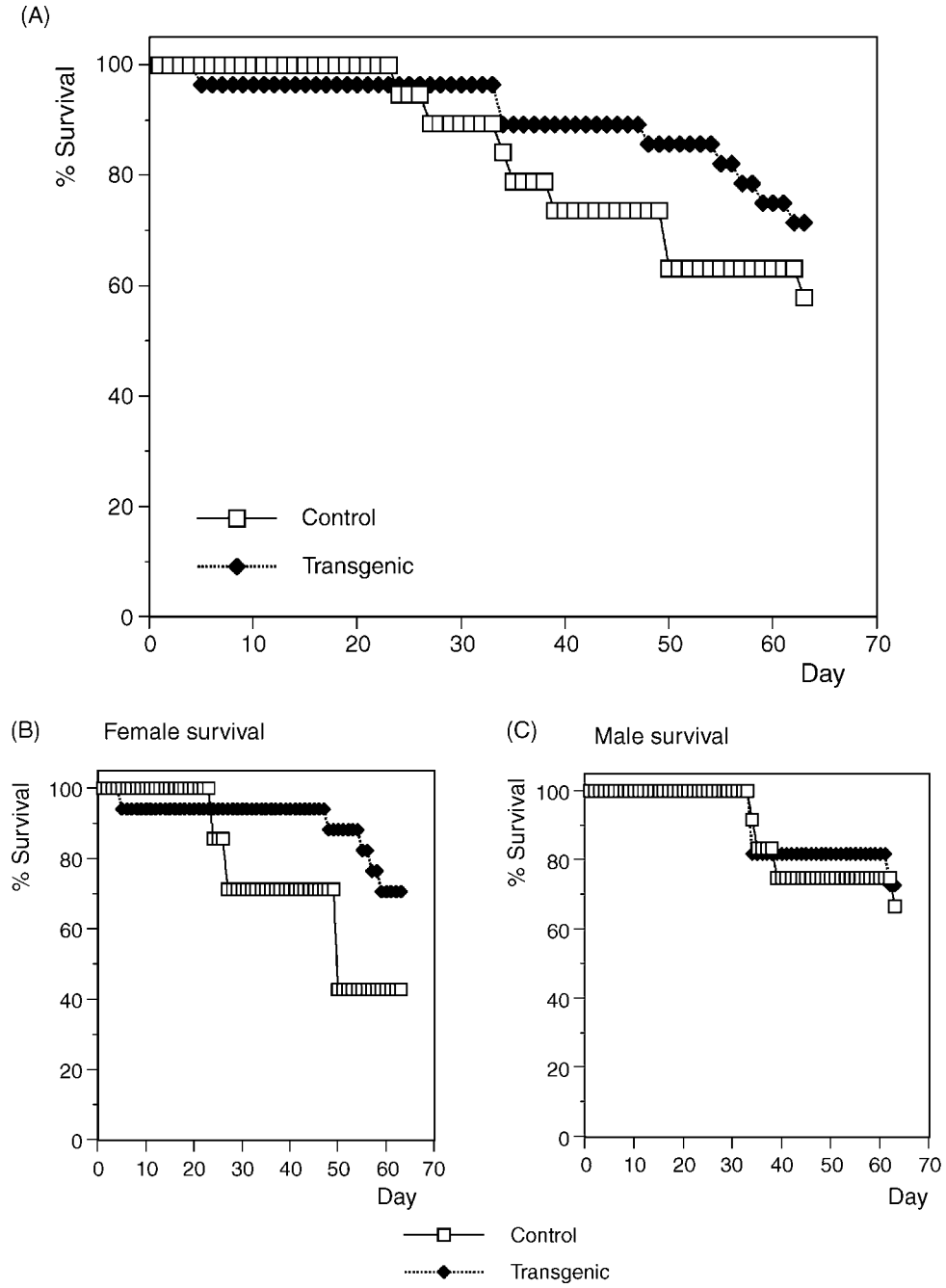

Figure 5. Percent survival for ladybirds fed aphids from control or GNA plants; (A) male and female combined; (B) female; (C) male. No significant differences were observed (Kaplan–Meier, Logrank χ^2 test).

A. bipunctata *egg production, viability and hatch*

The mean cumulative number of eggs laid per mated female was recorded over the initial 12-day dosing period and for the subsequent 4 weeks (Figure 6(A); data excludes the mating pairs which either never produced eggs or never produced any viable eggs). During the initial dosing period, when the treatment group of ladybirds were supplied with aphids fed on a GNA-containing diet, both control and treatment groups showed very similar mean cumulative eggs/mated female (85 ± 14 compared to 82 ± 12). Over the next 4 weeks of egg laying, mean cumulative

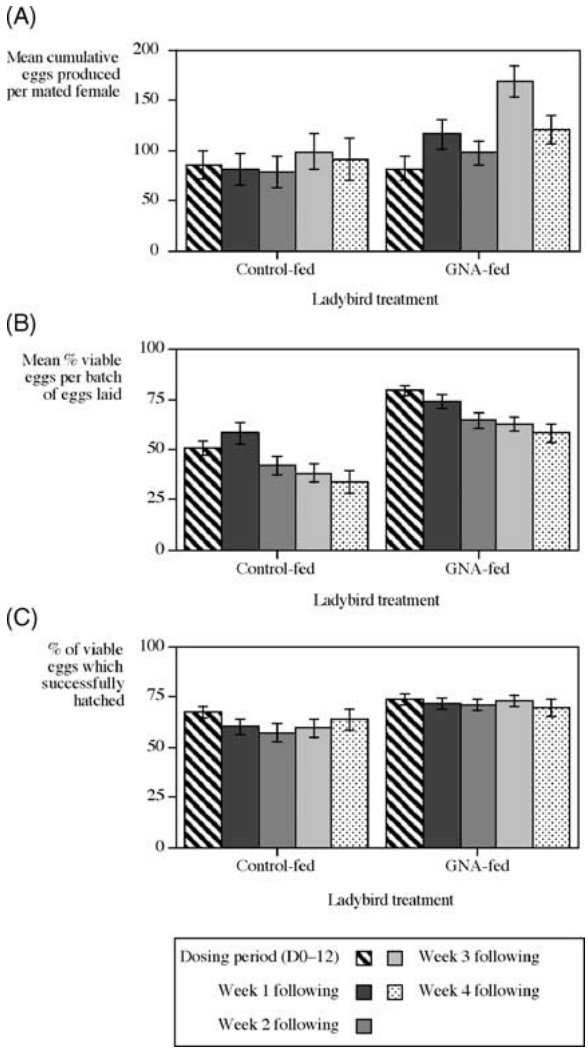

Figure 6. (A) Mean cumulative number of eggs/mated female; (B) Egg viability; mean percent/batch laid; (C) Mean percent viable egg hatch (Error bars represent S.E. of the mean.) For the initial 12 days adults were fed either GNA-dosed aphids or aphids from control diet, thereafter all ladybirds were fed aphids from control plants.

eggs/mated female was consistently higher for adults in the treatment group, by factors ranging from 1.3 to 1.7. On one occasion (third week following the dosing period), cumulative egg production was significantly greater for ladybirds supplied with GNA-dosed aphids (non-parametric Mann–Whitney *U*-test, $p < 0.05$).

The viability of eggs laid is shown in Figure 6(B) (clutches laid by females which never produced any viable eggs during their lifetime were excluded). The experimental group consistently had a higher percentage (10–20%) of viable eggs per batch compared to the control group, with both groups showing a clear decline in egg viability with time. The difference between the two groups was significant during all but one time interval (week 1 following the dosing period; non-parametric Mann–Whitney *U*-test, $p < 0.01$).

Egg hatch, recorded as percentage of viable eggs which successfully hatched, is shown in Figure 6(C). Once again, the experimental group showed a consistently higher percentage of viable eggs which successfully hatched, compared to the control group, although differences were generally less than 10%. The differences between the two groups were statistically significant during all but one time interval (non-parametric Mann–Whitney *U*-test, $p < 0.05$ for the initial 12-day dosing period and weeks 1 and 3 following; $p < 0.01$ for week 2 following).

A. bipunctata *survival*

Male and female ladybird survival was monitored daily over the egg laying period and beyond, for a total of 107 days (Figure 7). Throughout the trial female survival for both control and experimental ladybirds showed very similar survival curves. Up to day 22, both groups showed ≥90% survival. Between days 23 and 53 the maximum difference between the two groups was only 10%; this difference increased slightly to 15% during days 54–60 and then dropped back to 10% from day 61 onwards. By the end of the trial, 4 (20%) GNA-fed females remained alive compared to a total of 2 (10%) for the control group. The survival curves were statistically analysed using the Kaplan–Meier Logrank χ^2 test but no significant differences were found ($p > 0.05$). Male survival appeared to be marginally better than female survival. Up to day 23 both groups showed ≥90% survival. During days 24–84 the maximum difference in survival seen between the two groups was 15% but during days 85–98 this difference increased to 25% (in favour of the controls). By the end of the trial the difference had reduced to 15% with 8 (40%) of the control males still alive compared to 5 (25%) of the GNA-fed males still alive. As with the female data, no significant differences were observed in the survival curves (Kaplan–Meier Logrank χ^2, $p > 0.05$).

GNA accumulation studies

Aphid samples were collected such that a time course for accumulation of GNA for aphids feeding on GNA-expressing potato could be obtained over 1–7

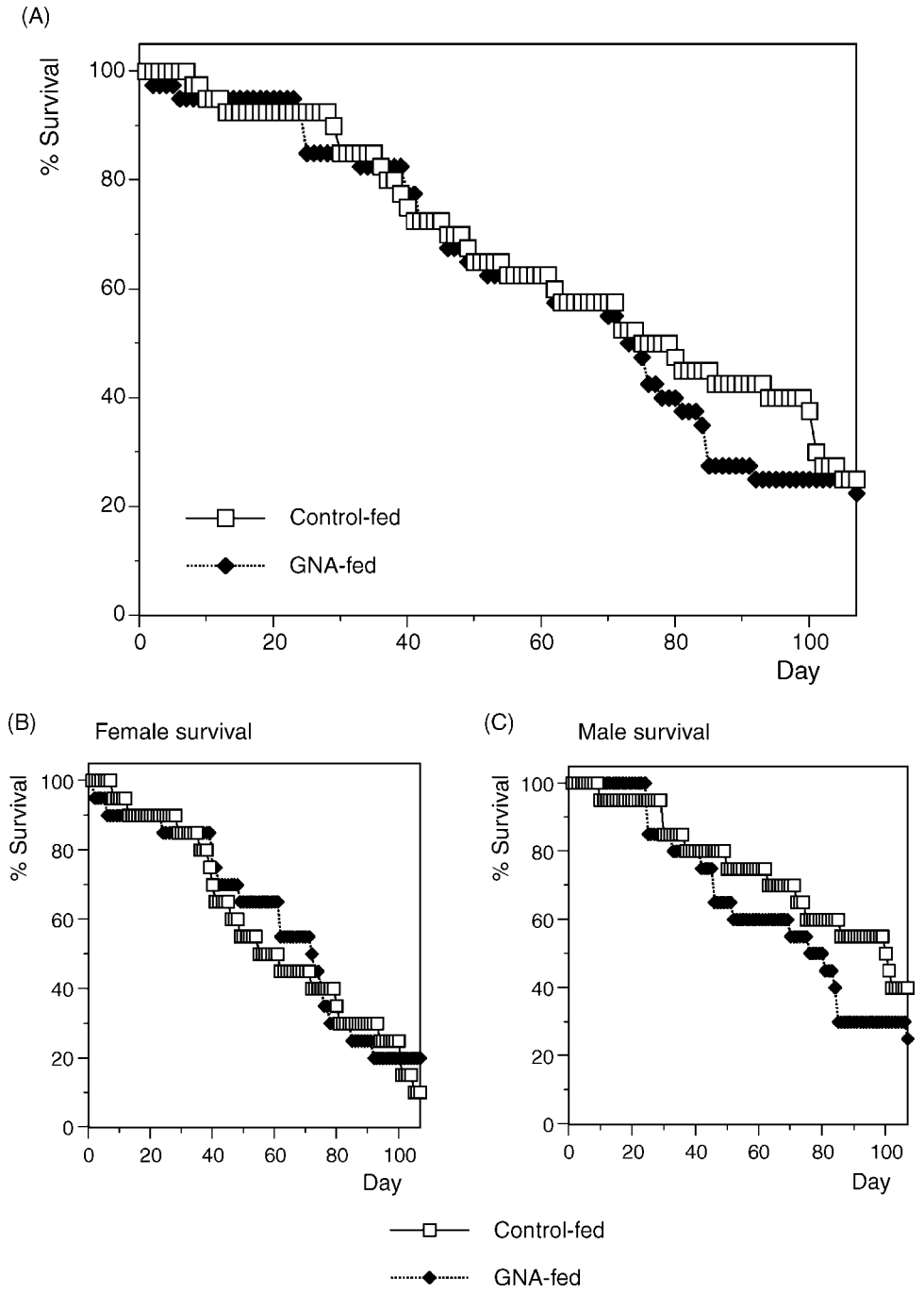

Figure 7. Male and female survival for adult ladybirds fed 12 days on control or GNA-dosed aphids, and for the subsequent 110 days when ladybirds from both groups were fed on aphids colonising non-transformed potato. No significant differences were observed (Kaplan–Meier, Logrank χ^2 test).

days. For 'feed and chase' studies, aphids were transferred from transgenic to control plants for a further 48 h. Analysis of these samples by immuno-assay failed to detect the presence of GNA in any of the aphid samples at any of the time points, despite readily detecting as little a 5 ng of GNA in the standards (data not shown). These results suggest that GNA was absent, or only present in negligible quantities, within

the aphid extracts. Immuno-assays of aphids colonising transgenic plants (in Trial 1) also failed to detect any GNA.

Ladybird larvae, fed aphids colonising GNA-expressing potato, were collected at a range of developmental stages during 'feed-only' and 'feed and chase' investigations. No GNA was detected in any of the ladybird samples (data not shown).

Discussion

Results from this study show that transgenic potato plants expressing snowdrop lectin (GNA) had no acute detrimental consequences for 2-spot ladybirds feeding on aphids raised on these plants, either in terms of larval development, adult fecundity, longevity, or overall survival. However, there was a small deleterious effect on egg viability in terms of percent successful egg hatch. These results represent first tier testing and as such, may not necessarily reflect effects on the population dynamics which might occur in the field. They can be compared to those previously reported by Birch et al. (1999). In that study, GNA-expressing potato plants exerted a significant detrimental effect on the fecundity, egg viability, and female longevity of adult 2-spot ladybirds, although the adverse effects on reproduction could be reversed by providing an optimal diet. It must be noted that a different transgenic potato 'line' (i.e., clonal replicates of a primary transformant) was used in the previous work.

Despite all transgenic plants in the present study expressing GNA at levels of approximately 0.1–0.2% of total soluble leaf protein, GNA could not be detected in aphids colonising those plants, or in ladybird larvae predating the aphids. Since aphids are predominantly phloem feeders, this finding must reflect low levels of GNA in the phloem sap of the transgenic plants. Similarly, Foissac et al. (2000) suggested that GNA was not being efficiently targeted to the phloem sap in transgenic rice. However, the failure to detect GNA is likely to be a consequence of the sensitivity of the immunological assay rather than complete absence, since GNA was detected in aphids (*M. persicae*) feeding on *Arabidopsis thaliana* plants transformed with the same gene construct used to produce the transgenic potato plants (C. Ilett, personal communication); GNA expression levels in *A. thaliana* were found to be significantly higher than in the potato plants used in the present study, thus the differences in the ability to detect GNA in the aphid may reflect differences in transgene expression levels in the host plant. Other transgene products have been shown to be absent from phloem; for example, Raps et al. (2001) could not detect the presence of Cry1Ab in the phloem sap of transgenic corn expressing the appropriate Bt toxin gene. In contrast, the artificial diet system is an efficient method of delivering GNA to aphids and to ladybirds predating those aphids, and previous studies (Down et al., 2000) have shown that GNA accumulates in dosed aphids to levels of approximately 0.08 µg after 4 days and 0.13 µg after 7 days.

The results from Trial 2, in which GNA was supplied through the artificial diet system at a concentration similar to that being expressed *in planta*, confirm the conclusion drawn by Down et al. (2000), that the 2-spot ladybird does not suffer any significant adverse effects from preying on aphids containing the protein. Using this delivery system for GNA, no detrimental effects on any of the insect parameters measured were observed in the present study, whereas work reported previously found that ladybird larvae showed a small reduction in growth (resulting in approximately 10% weight decrease at pupation) and a slightly extended development time (Down et al., 2000). It was suggested that these effects were indirect, possibly as a consequence of feeding limited numbers of aphids, and the aphids themselves being compromised and therefore a less suitable food source. The present study is not directly comparable, since adult ladybirds were used rather than larvae, and aphids were fed to ladybirds *ad libitum* and in excess. However, the absence of any effects of GNA on such sensitive parameters as egg production strongly suggests that the effects observed in the earlier study were indeed indirect. Interestingly, the results from the present study show an increase in ladybird fecundity and egg viability when fed GNA-loaded aphids, and although this effect is only significant for some of the time periods studied, the results taken as a whole can only be interpreted as a positive effect. This conclusion is supported by previous studies carried out by Sauvion et al. (1996) who found that very low levels of GNA delivered via artificial diet to *M. persicae* similarly caused a probiotic effect; at higher levels this protein was shown to be deleterious to the aphid.

It is clear that GNA is not directly responsible for the significant deleterious effects on egg production, egg fertility and adult longevity previously observed when adult ladybirds were fed on aphids raised on transgenic plants expressing the lectin (Birch et al.,

1999). The results of Trial 1, in which ladybird larvae and adults were fed on aphids raised on transgenic plants, also largely contradict the earlier data, in that ladybird adult fecundity, survival and longevity were not significantly different to controls, despite continuous exposure of the adults to transgenic plants by carrying out the assays *in planta.* The earlier data was obtained by feeding ladybird adults with aphids collected from transgenic plants for only a limited period before transferring them to an optimal diet. Further, the transgenic plants were expressing GNA at lower levels than the plants used in this work. In the present study a small decrease in egg viability was observed in Trial 1, in contrast to results obtained in Trial 2 where known amounts of GNA were delivered to the ladybirds via the dosed aphids. Slightly different parameters were used to measure egg viability in the two trials since potential cannibalism was not taken into account in Trial 1; this may explain why egg hatch was low for both groups in Trial 1, but does not provide an explanation as to why egg hatch was slightly lower in the experimental group. This must be considered to be a real effect, and supports the earlier observations by Birch and colleagues.

The data obtained from the present set of assays on transgenic plants indicates deleterious effects on ladybird egg hatch, which, although small, needs to be explained. Both the artificial diet-based studies and the failure to detect GNA in aphids fed on transgenic plants show that the effects are not due to the lectin. They fall under the heading of 'unintended effects' of the transformation process, which were anticipated when the FDA regulations for assessing transgenic crops as potential foodstuffs were drawn up (see Chrispeels & Sadava, 1994). These effects can result from the gene insertion site in the particular line investigated (Schuler et al., 1999), which may result in the disruption of the function of an endogenous gene. A further, possibly more likely, factor is somaclonal variability resulting from the tissue culture process employed in plant transformation. The nature of the transgenic material used in the present study, clonal replicates of primary transformants, means that somoclonal variability has not been removed from the transgenic plants (as would be the case for plants propagated through seeds, which would go through gamete formation, at which stage somaclonal variability would be removed). The difficulty of producing potato through seed made the use of clonally propagated material necessary. Both gene insertion and somaclonal variability effects will differ between transgenic 'lines' in experiments of the type described, since they are clonal replicates of different primary transformations. The apparent discrepancies between trials using different transgenic potato 'lines' must thus be viewed in the knowledge that 'unintended' effects have been anticipated in plant transformation, especially in primary transformant plant material.

The present study highlights the need to screen several plant lines when investigating effects of transgene products on insects, whether they be pest insects or natural enemies of these pest species. Furthermore, it also highlights the need to discriminate between direct and indirect effects within the plant/pest/predator interaction.

Acknowledgements

Financial support from the Scottish Executive (FF818, FF821 and FF831) and European Commission (Project BIO4-CT96-0365) are gratefully acknowledged. The authors would like to thank Drs R.D.D. Croy and L.N. Gatehouse for supplying antibodies. Work involving transgenic plants was carried out under MAFF licence PHF 346B/1459/35.

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