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Effects of *Galanthus nivalis* agglutinin (GNA) expressed in tomato leaves on larvae of the tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae) and the effect of GNA on the development of the endoparasitoid *Meteorus gyrator* (Hymenoptera: Braconidae)

M.E. Wakefield[1]*, H.A. Bell[1], E.C. Fitches[1], J.P. Edwards[1] and A.M.R. Gatehouse[2]

[1]Central Science Laboratory, Sand Hutton, York, YO41 1LZ, UK: [2]School of Biology, Institute for Research on Environment and Sustainability, Devonshire Building, University of Newcastle, Newcastle upon Tyne, NE1 7RU, UK

Abstract

The effect of ingestion of transgenic tomato leaves expressing the plant lectin *Galanthus nivalis* agglutinin (GNA) on development of larvae of *Lacanobia oleracea* (Linnaeus) was studied under laboratory conditions. When *L. oleracea* larvae were fed on tomato line 14.1H, expressing approximately 2.0% GNA, significant increases in the mean larval weight and in the amount of food consumed were found. This resulted in an overall reduction in the mean development time to the pupal stage of approximately 7 days. A significant increase in the percentage survival to the adult moth was also recorded when newly hatched larvae were reared on transgenic tomato leaves (72%) compared to larvae reared on untransformed leaves (40%). The effects of ingestion of GNA by *L. oleracea* larvae, via artificial diet or the leaves of transgenic tomato or potato plants, on the subsequent development of its solitary endoparasitoid *Meteorus gyrator* (Thunberg) was also studied. No significant effects on the life cycle parameters of *M. gyrator* developing in *L. oleracea* fed on GNA-containing diets were observed. Experiments with transgenic potato plants indicated that the stadium of the host larvae at parasitism had a greater influence on *M. gyrator* development than the presence of GNA. Potential GNA-binding glycoproteins were detected in the gut and body tissues of larval *M. gyrator*. Despite detection in host tissues, GNA could not be detected in adult *M. gyrator* and therefore it is likely that at the time of pupation *M. gyrator* are able to void the GNA in the meconial pellet.

Keywords: *Lacanobia oleracea*, *Galanthus nivalis* agglutinin (GNA), insect-resistant transgenic plants, *Meteorus gyrator*, tritrophic interactions

*Fax: +44(0)1904 462111
E-mail: m.wakefield@csl.gov.uk

Introduction

Transgenic crops targeted at reducing damage by phytophagous insect pests offer an alternative to the use of conventional pesticides that may be harmful to non-target species, the environment and users. To date, only insect-resistant transgenic crops expressing *Bacillus thuringiensis* toxins have been commercialized, although there has been much research into the potential of plants expressing lectins, protease- and α-amylase inhibitors. Snowdrop lectin, *Galanthus nivalis* agglutinin (GNA), when expressed in crops, has resulted in reduced herbivory and increased levels of mortality in a range of pest insect species (Powell *et al*., 1993; Down *et al*., 1996; Gatehouse *et al*., 1996; Fitches *et al*., 1997; Rao *et al*., 1998; Fossiac *et al*., 2000; Sétamou *et al*., 2002a).

A wide range of crops have been transformed to express GNA and the commercial potential of several of these is under investigation. As part of these investigations, the potential impact that the expression of this lectin may have upon the predators and parasitoids of pestiferous insects has been widely researched (Bell *et al*., 1999, 2001a; 2003a; Birch *et al*., 1999; Down *et al*., 2000; Couty *et al*., 2001a,b; Sétamou *et al*., 2002b; Tomov & Bernal, 2003). A range of effects from toxicity to no detectable effects have been reported. These investigations have categorized the effects on various life cycle parameters and fecundity of the parasitoids and predators as either direct (where the insecticidal protein elicits a toxic effect directly) or indirect (where effects are a result of reduction in host/prey quality). More recently, the effect of direct ingestion of GNA on parasitoid species (Romeis *et al*., 2003; Bell *et al*., 2004) and effects on various aspects of parasitoid behaviour such as host location and acceptance have also been considered (Tomov *et al*., 2003). Research into the direct and indirect consequences that these crops may have on both pest and beneficial insects is essential in order to determine their likely efficacy and ecological impact. It is important that laboratory studies, which may form part of a risk assessment process (Dutton *et al*., 2003; Poppy & Sutherland, 2004), identify whether effects are as a direct consequence of the insecticidal protein, or as an indirect result of the reduced quality of the host. However, in many of the studies to date, the transgenic plants (or artificial diets containing the transgene protein) examined have elicited some detrimental effect on the growth/survival of the host/prey making a direct link between transgene protein toxicity and deleterious effects on beneficial insects somewhat difficult to ascertain. Therefore, it is necessary that the ecological impact of transgene proteins, such as GNA, should be evaluated in scenarios where the negative effects on the host are removed, such that the direct effect of the insecticidal protein on the beneficial insect can be determined in the absence of other confounding issues.

Meteorus gyrator (Thunberg) (Hymenoptera: Braconidae) is a solitary koinobiont endoparasitoid of the larvae of several families of Lepidoptera, primarily noctuids. A common host in the UK is the tomato moth, *Lacanobia oleracea* (Linnaeus) (Lepidoptera: Noctuidae) (H. Bell, personal communication) and it has been shown that although all larval stadia are parasitized, there is a preference to parasitize third instar larvae (Bell *et al*., 2000). Previous studies have shown that although ingestion of GNA does not result in an increase in mortality of *L. oleracea*, significant effects on larval development, growth and food consumption have been recorded when feeding on the leaves of transgenic potatoes at expression levels of 0.07–2.0% (Fitches *et al*., 1997; Gatehouse *et al*., 1997; Bell *et al*., 1999). GNA has been shown to accumulate in the gut of *L. oleracea* larvae and to be transported to the haemolymph (Fitches *et al*., 2001). The presence of GNA in the haemolymph of host caterpillars has the potential to deleteriously affect parasitoids that develop on or within *L. oleracea*. The larvae of *M. gyrator* are thought to feed exclusively on the haemolymph during development and there is, therefore, the potential that the presence of the lectin in the host blood may directly affect the developing parasitoid. Furthermore, as koinobiont parasitoids are reliant on the growth of the host following parasitization, any factors that impact on host growth, such as presence of transgene proteins in the food plant, may affect the development and survival of the parasitoid larva indirectly through the generation of a poorer quality host.

The present study investigates the effect of the consumption of transgenic tomato leaves expressing GNA on the survival and life history parameters of *L. oleracea*. A transgenic line of tomatoes was chosen that had been previously indicated to have no effects on the growth and development of the pest noctuid *Chrysodeixis chalcites* (Esper) (unpublished). This line, therefore, provided a candidate for the delivery of GNA to a host insect without causing it significant deleterious effects. The current study was designed to investigate firstly, whether these transgenic plants would have any deleterious effects on *L. oleracea* and, secondly, whether the accumulation of GNA within the larvae would impact on the survival and development of *M. gyrator*. In this way, the direct toxicity of the lectin to the parasitoid in the absence of other factors, such as retarded growth and elevated mortality, could be ascertained. In addition, two other diets were used in this study; an artificial maize-based diet, and transgenic GNA-expressing potato plants, to further investigate the possible influence of host diet quality on the effect of the lectin on the life cycle parameters of *M. gyrator*.

Materials and methods

Insect stocks

Lacanobia oleracea larvae were derived from a standard laboratory culture maintained at 20°C, 70% r.h. under a 16 : 8 L : D photoperiod as described by Corbitt *et al*. (1996). Stages were selected on the basis of head capsule width. *Meteorus gyrator* were reared according to the procedures described by Bell *et al*. (2000) and were kept at 25°C, 70% r.h. under a 16 : 8 L : D photoperiod.

Production of transgenic tomato plants

GNA gene constructs

A GNA coding region, excised as an *Kpn I/XbaI* fragment from p1GNA2 in a pUC19 based plasmid, was sub-cloned into the binary vector pBI121 (from which the β-glucuronidase GUS gene had been removed) between the cauliflower mosiac virus (CaMv) 35S promoter sequence and the nopaline synthase (nos) transcriptional terminator sequence. The expression cassette, which also contained the nos-neo gene encoding neomycin phosphotransferase (conferring

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kanamycin resistance), was mobilized into *Agrobacterium tumefaciens* (strain LBA4404) by electroporation.

Transgenic tomato plant production

Surface sterilized tomato seeds (var. Moneymaker) were sown on 1/2 Murashige and Skoog macro and micronutrients (MSO) (2.2 g l^{-1} MS salts, 30 g l^{-1} sucrose, 1.2 g l^{-1} phytoagar, pH 5.8) containing 1 × Gamborgs B5 vitamins and left to germinate for 7 days at 24°C (16 : 8 h, L : D). Cotyledons were selected for transformation at the stage when there were no true leaves on the seedlings. The top of the cotyledons were removed whilst submerged in Petri dishes containing MSO liquid (4.3 g l^{-1} MS salts, 30 g l^{-1} sucrose pH 5.8 with 1 × Gamborgs B5 vitamins). Cotyledons were cut at both ends and placed (upside down) onto D1 media (4.3 g l^{-1} MS salts, 30 g l^{-1} sucrose, 1.2 g l^{-1} phytoagar pH 5.8 with 1 × Gamborgs B5 vitamins, 1 m g l^{-1} zeatin riboside). Cotyledons were left to pre-callus for 48 h at 24°C under low light conditions.

Agrobacterium suspension, grown for 2 days at 28°C and diluted 1 in 20 with MSO containing acetosyringone to a final concentration of 375 μM, was pipetted onto plates (5 ml suspension per plate) containing pre-callused cotyledons and left for 1 h. Excess fluid was removed and sealed plates were left for 2 days at 24°C under low light conditions. Cotyledons were then transferred to D1 media containing 500 μg ml^{-1} carbenicillin and 100 μg ml^{-1} kanamycin. After 2 weeks, cotyledons were transferred to shoot-inducing media D2 (as for D1 media with 0.1 μg ml^{-1} zeatin riboside). Shootlets were excised and transferred to rooting media (MS media containing 500 μg ml^{-1} carbenicillin, 500 μg ml^{-1} kanamycin and 0.1 μg ml^{-1} α-napthalene acetic acid). Established shootlets were subcultured to produce clonal replicates for further analysis.

Plant propagation

Plantlets were grown in a loam-based compost (John Innes No. 3) at 24°C with a 16 h photoperiod until fruit development. F1 seeds were collected from self-pollinated primary transformant fruits and washed in 10% (w/v) sodium carbonate solution. Seeds were then rinsed in distilled water and air-dried prior to storage at 4°C.

Experimental plant material

Transgenic tomato leaves used in the bioassays were obtained from GNA line 14.1H. The mean GNA expression level in vegetative tissues of this line was determined as 2.0% of total soluble protein. Control tomato leaves were derived from untransformed seed. Seeds were sown in seed trays and plants were transferred to pots containing John Innes No. 3 compost when approximately 5 cm high. Immediately prior to testing, GNA expression of individual plants was confirmed by Western blotting.

Transgenic potato leaves used in the bioassays were obtained from GNA line PWG85 and were produced as described by Fitches *et al*. (1997). The mean GNA expression level of this line was determined as 0.1% of total soluble protein. Control plants were derived from untransformed stock.

All plants were maintained in a plant growth room at 20°C, 16 : 8 L : D. All leaves used in bioassays were young but fully expanded; yellowing or desiccated leaves were not used.

Effect of transgenic tomato plants on development of L. oleracea

Newly hatched (0–24 h old) *L. oleracea* larvae were removed from the culture and placed in tissue-lined 250 ml plastic pots (Cryovac, Poole, UK) in groups of ten. Individual tomato leaves were excised from the plant, and the cut petiole pushed into a water-containing microcentrifuge tube through a hole punched in the lid. A single leaf was placed in each pot containing larvae and this was replaced every second day. After 7 days larvae were randomly chosen by placing all surviving larvae in a single pot and transferring the required number individually to ventilated, tissue-lined plastic pots. The larvae were provided with weighed amounts of tomato leaf every two days. Larvae were weighed on a daily basis and uneaten leaf was collected every second day, dried in an oven at 90°C overnight and weighed. This allowed the consumption of dry matter to be calculated from a standard curve of wet weight vs. dry weight for each plant type. Larvae were kept at 25°C, 70% r.h. throughout the experiment. Plant types tested were control (untransformed) tomato plants and line 14.1H. Twenty five larvae were used for each treatment. Growth, survival and food consumption were compared for larvae reared on each leaf type.

Effect of GNA fed hosts on development of M. gyrator

Transgenic tomato plants

Lacanobia oleracea larvae were fed either on control (untransformed) tomato leaves, or tomato leaves expressing GNA at a level of approximately 2.0% total protein, from 0–24 h old or from the beginning of the fourth larval stadium. Newly hatched (0–24 h old) larvae were kept in batches of ten in plastic pots and provided with leaves from control or line 14.1H plants. On reaching the fourth instar, newly ecdysed larvae were placed in a plastic box (150 × 150 × 75 mm) sealed with a muslin lid. Female *M. gyrator* (< 7 days old), with no previous oviposition experience, were introduced into the box in a ratio of 1 parasitoid to 5 host larvae. Larvae were exposed to the parasitoid overnight and were subsequently placed individually in small ventilated, tissue-lined plastic pots (250 ml) with the appropriate diet. For the experiment commencing with fourth instar larvae, newly ecdysed larvae were removed from the main *L. oleracea* culture and immediately exposed to the parasitoid overnight and subsequently maintained as described above. Host larvae were weighed daily and the day of cocoon formation, cocoon weight, cocoon duration and the longevity of the resulting *M. gyrator* adults was recorded. A food source of aqueous honey solution (50% w/w) was provided for the adult wasps that were maintained individually in the absence of hosts for their lifespan.

Transgenic potato plants

Lacanobia oleracea larvae were exposed to *M. gyrator* overnight as described above from the start of either the third or fourth stadia. Subsequently, larvae were kept in individual plastic pots, weighed daily and the life

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parameters of the resulting *M. gyrator* recorded, as previously described. Larvae were reared on either control (untransformed) potato leaf or potato leaf expressing GNA at a concentration of approximately 0.1% of total soluble protein.

Artificial diet

Purity of GNA (purchased from E. Van Damme, Catholic University of Leuven, Belgium) was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and the protein was shown to be functionally active by haemagglutination assay (Raemaekers *et al*., 1999). The lectin was incorporated into a standard laboratory artificial diet (Poitout & Bues, 1974), which allows full development of *L. oleracea*, at a concentration of 2% of dietary protein. Control diet was supplemented with 2% casein to ensure protein concentrations of the diets were equivalent (Fitches *et al*., 1997; Bell *et al*., 1999). Larvae were fed on either control artificial diet or artificial diet containing GNA from newly hatched (0–24 h old) or from the beginning of the fourth larval stadium. Larvae fed from 0–24 h old were parasitized as newly ecdysed fourth instars. Experimental procedures and parameters recorded were as described for the studies with transgenic plants.

Determination of transgene expression and functionality

Confirmation of transgene expression was obtained by immunoassay (Western blotting) using the enhanced chemiluminesence (ECL) method (Amersham). This was carried out as previously described by Fitches *et al*. (2001). Plant leaves frozen in liquid nitrogen were extracted in 50 mM Tris-HCl pH 9.5 (+1% fresh 36 mg ml^{-1} phenylmethane sulphonyl fluoride (PMSF) in ethanol), prepared for SDS–PAGE on 15% gels and proteins were subsequently transferred to nitrocellulose filters. Blots were probed with primary antibody polyclonal rabbit-anti-GNA serum (1 : 10,000 dilution) and secondary affinity purified goat anti-rabbit IgG horseradish peroxidase conjugate (1 : 10,000 dilution, Biorad). GNA expression levels were estimated visually by comparing GNA levels in known amounts of leaf protein against a range of GNA standards. In addition to determining expression levels in plants, this method was also used to detect the presence of the transgene in host larvae, parasitoid cocoons and adults. For these samples the extraction buffer used was 50 mM Tris-HCl pH 7.5 (+1% fresh 36 mg ml^{-1} PMSF in ethanol).

Functionality of the GNA in transgenic tomato plants was confirmed by determination of binding to a mini mannose-agarose column based on the method of Longstaff *et al*. (1998). A suspension of mannose-agarose (400 µl) was transferred to a microcentrifuge tube and washed with extraction buffer (50 mM Tris-HCl pH 9.5 +1% fresh 36 mg ml^{-1} PMSF in ethanol). The supernatant was removed and the leaf extract solution was applied to the agarose and left for 20 min on a shaker. The contents were spun down and the supernatant, containing non-bound GNA, removed. The agarose was washed with extraction buffer and bound GNA was eluted from the mannose-agarose using 50 mM 1,3-diaminopropane. The presence of GNA in the various supernatants was confirmed by Western blotting, as previously described.

Table 1. The survival and life parameters of *Lacanobia oleracea* reared on control (untransformed) tomato leaf or tomato leaf expressing GNA at approximately 2.0% of total soluble protein.

	Control (*n*)	GNA (*n*)
Survival (day 7 to pupa)(%)	40 ± 9.8 (25)a	72 ± 9.0 (25)b
Survival to adult (%)	40 ± 9.8 (25)a	72 ± 9.0 (25)b
Development to pupa (days)	36.1 ± 1.8 (10)a	29.4 ± 1.3 (18)b
Development pupa to adult (days)	15.9 ± 0.3 (10)a	15.3 ± 0.3 (18)a
Pupal weight (mg)	227.8 ± 14.0 (10)a	241.2 ± 8.0 (18)a

Mean values (± SE) followed by the same letter in each row are not significantly different ($P > 0.05$).

Detection of potential lectin-binding sites in M. gyrator

Second and third instar *M. gyrator* larvae were dissected out of parasitized hosts and their guts removed. Extracts of the parasitoid's gut, larval body minus gut and whole larvae were made in Tris-HCl pH 7.4 (+1% fresh 36 mg ml^{-1} PMSF in ethanol). Samples were centrifuged at 10,000 *g* at 4°C for 10 min and the supernatent removed. Samples were stored at −20°C. Protein content was determined using the method of Bradford with BSA as a standard. Binding of GNA to glycoproteins within the extracts was assessed using the DIG Glycan Differentiation Kit (Roche), utilizing digoxigenin-labelled GNA as per the manufacturer's protocol.

Statistical analysis

Daily larval weight and food consumption for *L. oleracea* reared on control or transgenic tomato leaves were compared by repeated measures analysis of variance (ANOVA). Developmental times and pupal weight for *L. oleracea* were compared for different treatments using Student's t-test. *Meteorus gyrator* developmental times, cocoon weight and longevity were compared for different treatments using Student's t-test for the experiments with tomato plants and artificial diet. Effects of larval stage parasitized and diet were compared using two-way ANOVA followed by Tukey HSD post-hoc tests for the experiment with potato plants. The percentage survival was compared using the chi-square test. Differences between treatments were considered significant at the 5% level.

Results

Effects of transgenic tomato plants on development of L. oleracea

Larvae reared on tomato leaves expressing GNA showed significantly greater percentage survival to both the pupal and adult stages than larvae reared on untransformed tomato leaves (χ^2 test, $P < 0.05$) (table 1). The mean larval weight from day 7 to day 25 was significantly greater for larvae fed on transgenic tomato leaves than for larvae fed on untransformed leaves (repeated measures ANOVA, $F = 37$, $df = 1,48$, $P < 0.001$) (fig. 1). The total amount of food consumed over the course of the experiment was also significantly greater for larvae fed on transgenic tomato leaves (repeated measures ANOVA, $F = 29.5$, $df = 1,48$, $P < 0.001$) (fig. 2). Development was also more rapid for

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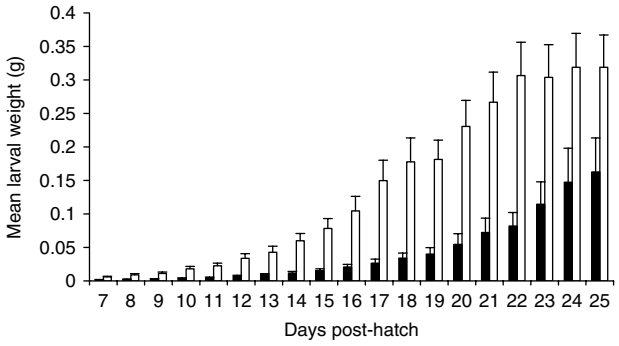

Fig. 1. Mean (±SE) daily larval weight of *Lacanobia oleracea* reared on control (■) (untransformed) tomato leaf or tomato line 14.1H (□) expressing GNA at approximately 2.0% of total soluble protein. The mean larval weight from day 7 to day 25 was significantly greater for larvae fed on transgenic tomato leaves than for larvae fed on untransformed leaves (repeated measures ANOVA, F = 37, df = 1,48, $P < 0.001$; $n = 25$ at day 7 post-hatch).

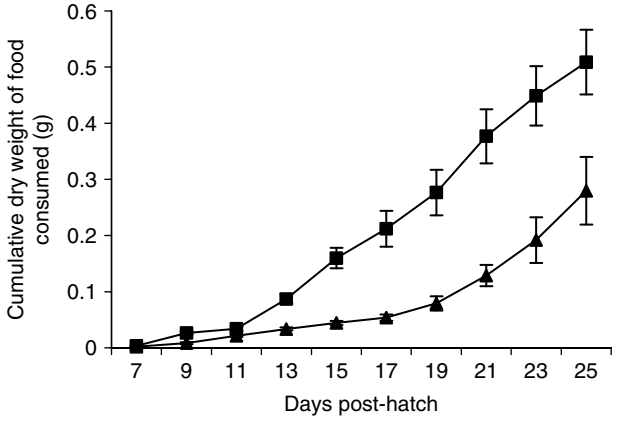

Fig. 2. Cumulative (±SE) dry weight of food consumed by *Lacanobia oleracea* larvae reared on control (▲) (untransformed) tomato leaf or tomato line 14.1H (■) expressing GNA at approximately 2.0% of total soluble protein. The cumulative dry weight of food consumed was significantly greater for larvae fed on transgenic tomato leaves than for larvae fed on untransformed leaves (repeated measures ANOVA, F = 29.5, df = 1,48, $P < 0.001$; $n = 25$ at day 7 post-hatch).

larvae reared on transgenic tomato leaves. The mean time taken to reach the pupal stage was approximately 7 days shorter for the larvae fed GNA-expressing tomato leaf than for the larvae reared on untransformed leaves (table 1).

Effects of GNA fed hosts on development of M. gyrator

Transgenic plants

The development of *M. gyrator* in *L. oleracea* fed untransformed or GNA-expressing tomato leaves, from either newly hatched (0–24 h old) or from the start of the fourth stadium, was investigated. No significant differences were found for survival to egression, or to the adult stage, or for any of the life cycle parameters measured for *M. gyrator*

Table 2. The survival and life parameters of *Meteorus gyrator* developing in hosts parasitized at the start of the fourth instar and subsequently reared on control (untransformed) tomato leaf or tomato leaf expressing GNA at approximately 2.0% of total soluble protein.

	Control (*n*)	GNA (*n*)
Survival to egression (%)‡	90.9 ± 3.5 (66)a	95.6 ± 2.5 (68)a
Survival to adult (%)‡	80.3 ± 4.9 (66)a	85.3 ± 4.3 (68)a
Development to emergence (male) (days)	9.9 ± 0.18 (30)a	9.8 ± 0.16 (38)a
Development to emergence (female) (days)	10.3 ± 0.19 (22)a	10.4 ± 0.20 (19)a
Cocoon period (male) (days)	6.6 ± 0.14 (31)a	6.5 ± 0.12 (40)a
Cocoon period (female) (days)	6.9 ± 0.11 (22)a	7.0 ± 0.15 (19)a
Longevity (male) (days)	42.8 ± 1.91 (27)a	41.4 ± 2.31 (39)a
Longevity (female) (days)	52.1 ± 4.44 (19)a	52.1 ± 5.03 (18)a
Cocoon weight (males) (mg)	9.9 ± 0.27 (31)a	9.7 ± 0.28 (40)a
Cocoon weight (females) (mg)	11.3 ± 0.28 (22)a	11.1 ± 0.38 (19)a

‡ *n* value represents the total number of parasitized hosts.
Mean values (±SE) followed by the same letter in each row are not significantly different ($P > 0.05$).

Table 3. The survival and life parameters of *Meteorus gyrator* developing in hosts reared on control (untransformed) tomato leaf or tomato leaf expressing GNA at approximately 2.0% from neonate and parasitized at the start of the fourth instar.

	Control (*n*)	GNA (*n*)
Survival to egression (%)‡	71.4 ± 8.5 (28)a	79.3 ± 7.5 (29)a
Survival to adult (%)‡	46.4 ± 9.4 (28)a	68.9 ± 8.6 (29)a
Development to emergence (male) (days)	9.3 ± 0.33 (10)a	9.4 ± 0.19 (15)a
Development to emergence (female) (days)	9.3 ± 0.33 (3)a	9.8 ± 0.20 (5)a
Cocoon period (male) (days)	6.3 ± 0.15 (10)a	6.5 ± 0.16 (15)a
Cocoon period (female) (days)	7.0 ± 0 (3)a	6.8 ± 0.20 (5)a
Cocoon weight (males) (mg)	9.6 ± 0.41 (10)a	10.2 ± 0.46 (14)a
Cocoon weight (females) (mg)	12.7 ± 0.26 (3)a	12.8 ± 0.61 (5)a

‡ *n* value represents the total number of parasitized hosts.
Mean values (±SE) followed by the same letter in each row are not significantly different ($P > 0.05$).

that had developed in hosts reared on transgenic tomato leaves ($P > 0.05$) (tables 2 and 3). Survival of parasitized hosts reared on the untransformed leaves was lower when hosts were fed from 0–24 h old than when they were fed from the start of the fourth stadium (χ^2 test, $P < 0.01$) but there was no significant difference for hosts reared on the GNA-expressing tomato leaves (χ^2 test, $P > 0.05$). The total number of host larvae that were parasitized was unaffected by diet type, nor was the percentage parasitized altered as a result of the length of time that the larvae had to consume the diet prior to parasitism (results not shown).

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Table 4. The survival and life parameters of *Meteorus gyrator* developing in hosts parasitized at the start of the third or fourth stadium and subsequently reared on potato expressing GNA at approximately 0.1% of total soluble protein.

	Third instars		Fourth instars	
	Control (*n*)	GNA (*n*)	Control (*n*)	GNA (*n*)
Survival to egression (%)‡	68.5±5.4 (73)a	77.2±4.8 (77)a	78.6±4.7 (75)a	70.4±5.1 (81)a
Survival to adult (%)‡	57.5±5.8 (73)a	64.6±5.4 (77)a	72.0±5.2 (75)a	63.0±5.4 (81)a
Development to emergence (male) (days)	10.7±0.27 (30)a	10.8±0.24 (35)a	10.4±0.22 (34)a	10.7±0.36 (27)a
Development to emergence (female) (days)	12.3±0.35 (13)a	11.9±0.43 (16)ac	10.4 ±0.27 (20)b	11.1±0.24 (21)bc
Cocoon period (male) (days)	6.6±0.13 (29)a	6.3±0.12 (33)a	5.7±0.15 (34)b	5.9±0.15 (27)b
Cocoon period (female) (days)	7.0±0.28 (12)a	7.1±0.22 (15)a	6.5±0.18 (20)a	6.4±0.14 (21)a
Longevity (male) (days)	31.3±3.65 (27)a	27.9±1.87 (30)a	29.1±1.5 (32)a	28.8±1.58 (22)a
Longevity (female) (days)	37.5±3.57 (12)a	43.6±4.8 (13)a	39.1±3.5 (15)a	39.4±3.00 (21)a
Cocoon weight (males) (mg)	7.6±0.31 (30)a	8.0±0.26 (34)ac	9.8±0.17 (34)b	9.0±0.50 (27)bc
Cocoon weight (females) (mg)	9.7±0.28 (13)ab	9.0±0.62 (16)a	11.2±0.24 (20)b	10.3±0.45 (21)ab

‡ *n* value represents the total number of parasitized hosts.
Mean values (±SE) followed by the same letter in each row are not significantly different ($P > 0.05$).

Table 5. The survival and life parameters of *Meteorus gyrator* developing in hosts parasitized at the start of the fourth instar and subsequently reared on artificial diet or artificial diet containing GNA at approxmately 2% of total soluble protein.

	Control (*n*)	GNA (*n*)
Survival to egression (%)‡	88.6±4.8 (44)a	95.6±3.0 (46)a
Survival to adult (%)‡	86.4±5.2 (44)a	95.6±3.0 (46)a
Development to emergence (male) (days)	8.8±0.1 (25)a	9.1±0.13 (22)a
Development to emergence (female) (days)	9.3±0.13 (13)a	9.1±0.07 (22)a
Cocoon period (male) (days)	6.0±0.1 (25)a	6.0±0.08 (21)a
Cocoon period (female) (days)	6.8±0.11 (12)a	7.0±0 (22)a
Longevity (male) (days)	25.4±2.94 (19)a	31.9±2.42 (12)a
Longevity (female) (days)	33.0±8.22 (4)a	38.8±3.6 (5)a
Cocoon weight (males) (mg)	10.1±0.3 (25)a	10.2±0.25 (22)a
Cocoon weight (females) (mg)	11.40±0.32 (12)a	11.2±0.18 (22)a

‡ *n* value represents the total number of parasitized hosts.
Mean values (±SE) followed by the same letter in each row are not significantly different ($P > 0.05$).

Table 6. The survival and life parameters of *Meteorus gyrator* developing in hosts reared on artificial diet or artificial diet containing GNA at approximately 2% of total soluble protein from neonate and parasitized at the start of the fourth instar.

	Control (*n*)	GNA (*n*)
Survival to egression (%)‡	75.7±7.5 (33)a	72.5±7.1 (40)a
Survival to adult (%)‡	57.8±8.6 (33)a	50±7.9 (40)a
Development to emergence (male) (days)	9.3±0.18 (12)a	9.7±0.33 (6)a
Development to emergence (female) (days)	10.0±0.44 (7)a	9.4±0.14 (14)a
Cocoon period (male) (days)	6.0±0 (12)a	6.0±0 (6)a
Cocoon period (female) (days)	6.7±0.28 (7)a	6.8±0.15 (14)a
Longevity (male) (days)	46.2±4.91 (12)a	50.2±7.04 (6)a
Longevity (female) (days)	66.9±3.79 (7)a	55.0±7.2 (13)a
Cocoon weight (males) (mg)	9.0±0.55 (12)a	9.7±0.58 (6)a
Cocoon weight (females) (mg)	11.0±0.67 (7)a	10.1±0.32 (14)a

‡ *n* value represents the total number of parasitized hosts.
Mean values (±SE) followed by the same letter in each row are not significantly different ($P > 0.05$).

In experiments with potato plants, no significant differences for survival to egression, or to the adult stage, were found for *M. gyrator* that had developed in larvae reared on transgenic potato leaves compared to those reared on untransformed leaves from either the third or fourth stadia (χ^2 test, $P > 0.05$). No significant differences were found for any of the measured life cycle parameters irrespective of the diet on which the host was reared. However, the stadium of the host at the time of parasitism did have a significant effect on the wasp's larval development time (female), cocoon weight (males) and cocoon period (male) (ANOVA, $P < 0.05$) (table 4).

Artificial diet

There were no significant differences in any of the measured life cycle parameters for *M. gyrator* developing in *L. oleracea* reared on artificial diet containing GNA from either 0–24 h old and parasitized as fourth instars, or in hosts parasitized at the start of the fourth stadium and subsequently maintained on the respective diets (Student's t-test, $P > 0.05$) (tables 5 and 6). As was observed for experiments with transgenic tomato plants, survival from hosts fed on either the control or GNA-containing diet was lower when hosts were fed from 0–24 h old than from the start of the fourth stadium. The total number of hosts that were parasitized was similar for *L. oleracea* reared on the control and GNA-containing diets from 0–24 h old. This suggests that in a no-choice situation *M. gyrator* will readily parasitize GNA-fed hosts.

Determination of transgene expression and functionality

All plants used for bioassays were shown to express GNA, and functionality was confirmed by Western blotting

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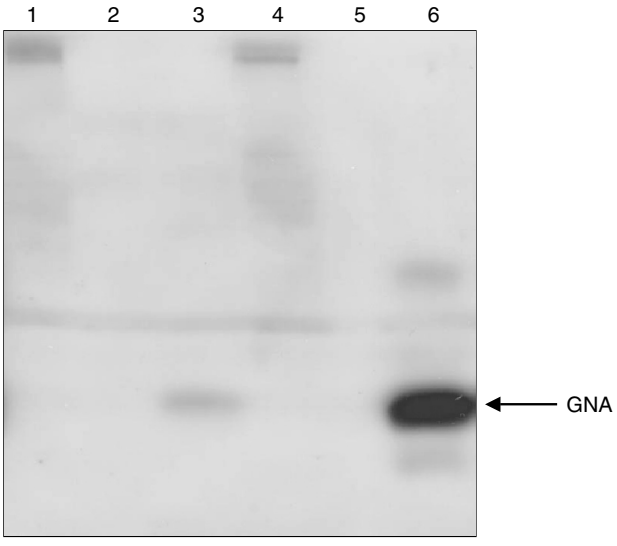

Fig. 3. Immuno-assay by Western blot analysis of *Meteorus gyrator* cocoons and adults reared from hosts fed on artificial diet with or without the inclusion of GNA. Lane 1, *M. gyrator* adult from GNA diet fed host; lane 2, cocoon from control diet fed host; lane 3, cocoon from GNA diet fed host; lane 4, *M. gyrator* adult from control diet host; lane 5, blank; lane 6, GNA standard, 20 ng.

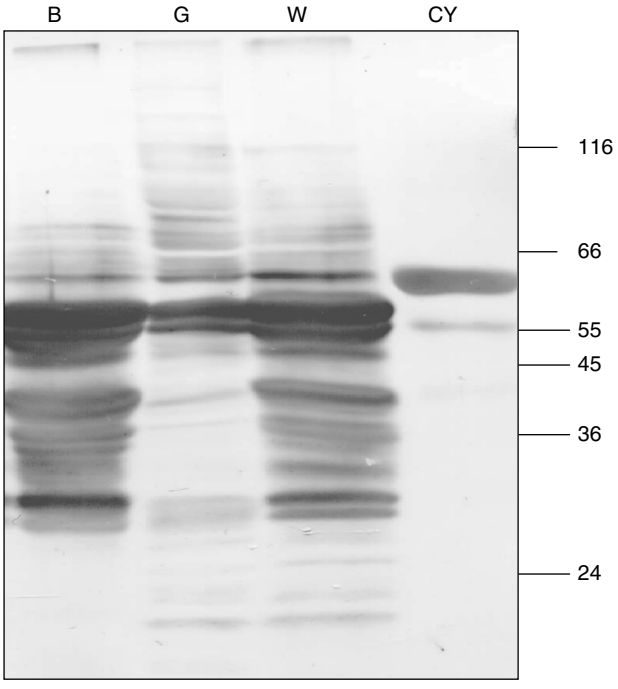

Fig. 4. Binding of GNA to *Meteorus gyrator* body and gut proteins *in vitro*. Denatured protein samples (*c*. 20 μg protein per lane) were analysed by SDS–PAGE (15% gel), blotted onto nitrocellulose and probed with digoxigenin-labelled GNA. B, body with gut removed; G, gut; W, whole body; CY, carboxypeptidase Y (control glycoprotein, 10 μg).

of samples eluted from a mini mannose-agarose column using 1,3-diaminopropane (results not shown). GNA was also present in host larvae that had fed on diet containing the lectin, irrespective of whether it was artificial diet or transgenic plants, and in *M. gyrator* cocoons. However, it was not detected in adult *M. gyrator* from GNA-fed hosts (fig. 3).

Potential for lectin-binding by M. gyrator

Numerous GNA-binding glycoproteins were present both in samples of the gut and the body of *M. gyrator* (fig. 4). Prominent bands were visible at molecular weights of approximately 30,000, 43,000, 55,000 in all samples.

Discussion

Examination of the effects of ingestion of tomato leaves expressing GNA on the development of the tomato moth, *L. oleracea*, showed that larvae grew larger, developed more quickly and consumed more plant material than larvae fed on untransformed tomato leaves. Previous studies with GNA and *L. oleracea* have shown that GNA in artificial diet and when expressed in transgenic potato plants reduced larval weight and food consumption (Fitches *et al*., 1997; Gatehouse *et al*., 1997). However, Bell *et al*. (1999) reported that GNA in a maize-based artificial diet had little or no effect on insect growth and food consumption for this species. The results from the present studies with transgenic tomato plants are surprising given that the concentration of GNA expressed was at least as much as that used in these previous studies with artificial diets and transgenic potato plants. Sétamou *et al*. (2002a) reported that larvae of the sugarcane borer, *Diatraea saccharalis* (Fabricius) (Lepidoptera: Pyralidae), grew larger, and male larvae had a shorter developmental time, when reared on diet containing sugarcane expressing GNA compared to controls. A similar effect has also been observed with the proteinase inhibitor mustard trypsin PI2 (MTI-2) targeted at *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) (de Leo *et al*., 1998) when present at a low concentration. It is unlikely that any potential increase in protein content through expression of GNA in the transgenic tomato plants was responsible for the increased growth, as it has been shown that in *L. oleracea* larvae GNA is resistant to gut proteolysis (Fitches *et al*., 2001). Testing of a different tomato line expressing GNA at a concentration of approximately 0.7% of total soluble protein also showed that *L. oleracea* larvae grew larger and consumed more than insects reared on untransformed tomato leaf (unpublished results), although this difference was not as great as that reported here.

Survival of *L. oleracea* to the adult stage was much reduced on untransformed tomato leaf compared to previously published figures for survival on untransformed potato leaf and artificial diet (Bell *et al*., 2001b). This may have been as a result of secondary plant substances (e.g. alkaloids) naturally present in tomato leaf that may be deleterious to *L. oleracea*. Indeed, Lloyd (1920) reported that *L. oleracea* larvae proved difficult to rear on a diet of tomato foliage alone, although considerable variation between egg batches and individuals was recorded. Larvae were observed to cease feeding and die and it was suggested that this may be due to the presence of 'poisons' or, alternatively, due to deficiency of a key nutrient in the tomato foliage.

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Survival was significantly greater for larvae reared on GNA-expressing tomato leaves compared to those reared on the untransformed tomato leaves. This result combined with the observations that larvae grew larger, developed more quickly and consumed more plant material when fed on the GNA-expressing tomato leaves could suggest an unintended effect of the transformation process, i.e. the transgene had integrated into an endogenous gene thus adversely affecting gene function. Depending upon the gene disrupted, this may affect the phenotype, alter the nutritional composition, or disrupt biochemical pathways in the plant. Some examples of unintended effects found in transgenic plants are listed in Cellini *et al*. (2004). It is possible that the GNA-expressing tomato plants differed due to some nutritional inferiority compared to the untransformed plants leading to a degree of compensatory feeding by the moth larvae. However, this is very unlikely to have resulted in the significantly higher survival and growth rates recorded for larvae developing on the leaves of these plants. The increased levels of feeding do, however, demonstrate that the quantities of GNA ingested by a pest may bear little relationship to the effects observed, a phenomenon similarly observed by Gatehouse *et al*. (1997) with *L. oleracea* fed on transgenic potato leaves.

In a further series of experiments we assessed the effects of ingestion of GNA by the host larvae on the development and survival of the solitary endoparasitoid *M. gyrator*. Life history parameters examined for development of *M. gyrator* in hosts reared on transgenic or nontransgenic diets were not significantly altered, regardless of the time period for which the host was maintained on the diet. Although some significant differences were found in the experiment with transgenic potatoes, these showed no pattern, and were attributable to the age of the larvae at the time of parasitism rather than to the diet on which the host was reared. It has previously been shown that transgenic potatoes expressing GNA reduce larval weight gain and food consumption of *L. oleracea* (Fitches *et al*., 1997; Gatehouse *et al*., 1997). Therefore, it was anticipated that this negative effect on weight gain would indirectly affect the parasitoid development. However, no effects were observed and it is likely that the ability of *M. gyrator* to develop within a very wide range of host sizes (Bell *et al*., 2003b) renders the relatively small impact of GNA within the diet of its host inconsequential.

The maize-based artificial diet containing GNA has previously been shown to elicit little or no effect on survival and growth parameters for *L. oleracea* (Bell *et al*., 1999). Similarly, the transgenic tomato line used in these studies delivered a high level of GNA with no deleterious effects to *L. oleracea*. It was therefore anticipated that any observed effects on the development of *M. gyrator* would have been a direct consequence of the presence of GNA within the host. Thus, the use of the different diets ensured that both direct and indirect effects on the life cycle parameters of *M. gyrator* could be evaluated.

Immunoassay by Western blot analysis confirmed the presence of GNA in *L. oleracea* larvae irrespective of the type of diet that contained the GNA (results not shown). Thus, it is likely that the developing *M. gyrator* larvae would have been exposed to GNA within the host. Other authors have examined the effect of ingestion of GNA-containing diets by hosts on the subsequent development of parasitoids. Significant sub-lethal effects have been recorded for some species. For example, Couty *et al*. (2001a) reported that GNA did not have direct toxic effects on *Aphelinus abdominalis* (Dalman) (Hymenoptera: Aphelinidae) but indirect effects, related to host size, for the sex ratio and the size of parasitoids were found. Sétamou *et al*. (2002b) examined the development of *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) on *D. saccharalis* larvae and showed effects of GNA-containing diet on host suitability, immature stage mortality, sex ratio and adult longevity. Sublethal effects that varied with generation were shown for the development of *Parallorhogas pyralophagus* (Marsh) (Hymenoptera: Braconidae) parasitizing *Eoreuma loftini* (Dyar) (Lepidoptera: Pyralidae) fed a GNA-containing artificial diet (Tomov & Bernal, 2003). However, Bell *et al*. (1999) reported little or no effect of GNA for *Eulophus pennicornis* (Nees) (Hymenoptera: Eulophidae) developing on *L. oleracea* larvae. Thus, it is likely that the susceptibility of different parasitoid species to GNA can differ greatly. Several factors may determine the outcome of exposure of a parasitoid to GNA. In addition to the concentration at which the lectin is present in the host, the wasp's innate susceptibility, and the life history of the parasitoid will also influence the extent to which it may be affected.

Analysis of extracts from the gut and body tissues of second and third instar *M. gyrator* larvae revealed the presence of several glycoproteins with the ability to bind GNA. However, although binding is a prerequisite for toxicity, the degree of binding to glycoproteins within insect tissues does not necessarily reflect the level of toxicity (Fitches *et al*., 2001). GNA-binding glycoproteins have also been found for the parasitoid *E. pennicornis* (Bell *et al*., 2004) and the predator *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) (Bell *et al*., 2003a). Western blot analysis of cocoons, post eclosion of the adult parasitoid, and adult *M. gyrator* showed that although GNA could not be detected in adult *M. gyrator*, it was present in the cocoon. This result indicates that *M. gyrator* larvae are able to void GNA in the meconial pellet prior to pupation, thus removing the lectin from the adult tissues. Less likely, GNA may have remained in the adult wasps but at a level that was below the limit of detection used in these studies. The ability of a parasitoid to excrete GNA in the meconial pellet has also been recorded in the aphid parasitoid, *Aphidius ervi* Haliday (Hymenoptera: Braconidae) (Couty *et al*., 2001b).

The present study has shown that development of *M. gyrator* does not appear to be affected by the presence of GNA in the host at the concentrations used. Feeding of the host on artificial diet enabled studies to be made under conditions where the GNA was delivered in a quantifiable manner and in a diet allowing complete development of the host. Studies *in planta* enabled the consequences of potential, though unidentified, pleiotropic effects of transgene insertion, sub-optimal host diet and potentially variable quantities of GNA, to be observed. The concentrations of GNA delivered to the host in both the artificial diet and tomato plant experiments were high in comparison to many other studies investigating the effects of this transgene product on parasitoids. The results obtained in the present studies suggest that *M. gyrator* is not particularly sensitive to GNA present in the host. Furthermore, susceptibility may be further reduced if the parasitoid is able to excrete the lectin in the meconial pellet prior to pupation leading to the development of an ostensibly normal adult parasitoid.

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