Molecular Ecology (2003) **12**, 493–504

Impact of oilseed rape expressing the insecticidal cysteine protease inhibitor oryzacystatin on the beneficial predator *Harmonia axyridis* (multicoloured Asian ladybeetle)

N. FERRY,* R. J. M. RAEMAEKERS,* M. E. N. MAJERUS,† L. JOUANIN,‡ G. PORT,* J. A. GATEHOUSE§ and A. M. R. GATEHOUSE*

**School of Biology, University of Newcastle Upon Tyne, Newcastle, NE1 7RU, UK, †Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB3 3EH, UK, ‡Laboratoire de Biologie Cellulaire, INRA, Route de Saint Cyr, 78026 Verailles Cedex, France, §Department of Biological Sciences, University of Durham, Durham, DH1 3LE, UK*

Abstract

Insect-resistant transgenic plants have been suggested to have deleterious effects on beneficial predators through transmission of the transgene product by the pest to the predator. To test this hypothesis, effects of oilseed rape expressing the cysteine protease inhibitor oryzacystatin-1 (OC-1) on the predatory ladybird *Harmonia axyridis* were investigated using diamondback moth *Plutella xylostella* as the pest species. As expected, oilseed rape expressing OC-1 had no effects on either development or survival of the pest, which utilizes serine digestive proteases. Immunoassays confirmed accumulation of the transgene product in pest larval tissues at levels of up to 3 ng per gut. Characterization of proteolytic digestive enzymes of *H. axyridis* demonstrated that larvae and adults utilize cysteine and aspartic proteases; the former activity was completely inhibited by oryzacystatin *in vitro*. However, when *H. axyridis* larvae consumed prey reared on OC-1 expressing plants over their entire life cycle, no significant effects upon survival or overall development were observed. The inhibitor initially stimulated development, with a shortening of the developmental period of the second instar by 27% ($P < 0.0001$) accompanied by a 36% increase in weight of second instar larvae ($P = 0.007$). OC-1 had no detrimental effects on reproductive fitness of adult *H. axyridis*. Interestingly there was a significant increase in consumption of OC-1 dosed prey. The results show that prey reared on transgenic plants expressing a protein which inhibited ladybird digestive enzymes *in vitro* had no effects *in vivo*; the ladybird was able to up-regulate digestive proteases in response to the inhibitor.

Keywords: beneficial predators, *Harmonia axyridis*, oryzacystatin (OC-1), *Plutella xylostella*, transgenic oilseed rape, tritrophic interactions

Received 5 July 2002; revision received 14 October 2002; accepted 14 October 2002

Introduction

Genetic engineering of crops for enhanced levels of resistance to insect pests has the potential to offer large benefits to global agriculture, not least in a significant reduction in pesticide application. Insect-resistant transgenic crops expressing *Bacillus thuringiensis* (*Bt*) δ-endotoxins have been commercially grown for many years with minimal impact on nontarget organisms (Dogan *et al*. 1996; Riggin-Bucci & Gould 1997; Lozzia *et al*. 1998; Riddick & Barbosa 1998; Lozzia 1999; Riddick *et al*. 2000, 1998; Hellmich *et al*. 2001; Saxena & Stotzky 2001). However, field durability is predicted to be limited by the development of resistance to *Bt* toxins in pest populations (Gould 1998; Tabashnik *et al*. 2000; Carriere & Tabashnik 2001).

Transgenic plants expressing other insect resistance genes, including plant-derived protease inhibitors, are currently being developed (Jouanin *et al*. 1998; Gatehouse & Gatehouse 1999). Protease inhibitors are divided into four classes, inhibiting serine, cysteine, metallo- or aspartic proteases. They bind specifically to target gut proteases, and thus inhibit the ability of the insect to digest protein (Gatehouse & Gatehouse 1999). The potential of protease inhibitors to

Correspondence: A. M. R. Gatehouse. Fax: 0191 2225228; E-mail: A.M.R.Gatehouse@ncl.ac.uk

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provide resistance to insect pests has been demonstrated for lepidopteran pests (Hilder *et al*. 1987; Duan *et al*. 1996; Xu *et al*. 1996; Gatehouse *et al*. 1997; Graham *et al*. 1997; Bell *et al*. 2001), coleopteran pests, molluscs and nematodes (Leple *et al*. 1995; Vain *et al*. 1998; Walker *et al*. 1999; Urwin *et al*. 2000). The protease inhibitor oryzacystatin-1, isolated from rice, has been shown to inhibit proteases and/or affect growth of several coleopteran species (Bonadé-Bottino *et al*. 1999; Leple *et al*. 1995; Girard *et al*. 1998a, 1998b; Lecardonnel *et al*. 1999) and transgenic oilseed rape lines expressing OC-1 have been produced as part of a programme to develop insect-resistant lines of oilseed rape.

However, for this approach to be successful, and if agriculture is to move towards more sustainable practices, it is important that plant biotechnology is compatible with other pest control strategies, including biological control within integrated pest management (IPM). Protease inhibitors have a broad spectrum of activity and thus have the potential to affect nontarget beneficial arthropods such as predators either via predation of phytophagous pests, or by direct exposure to the plant itself.

Ladybirds are well-known beneficial insects and some, including the multicoloured Asian ladybeetle (*Harmonia axyridis*), are commercially available as biological control agents for use in greenhouses to control aphid pests (Koppert 2001). Most studies to date have demonstrated that crops transformed for enhanced pest resistance have no deleterious effects on beneficial insects (Dogan *et al*. 1996; Picard-Nizou *et al*. 1997; Lozzia *et al*. 1998; Riddick *et al*. 1998, Riddick & Barbosa 1998; Bell *et al*. 1999, 2000; Lozzia 1999; Malone *et al*. 1999; Down *et al*. 2000; Wraight *et al*. 2000; Saxena & Stotzky 2001; Schuler *et al*. 2001). However, a limited number of studies have reported either positive (Johnson *et al*. 1997; Riddick *et al*. 2000) or negative effects (Hilbeck *et al*. 1998, 1999; Birch *et al*. 1999). For example, Ashouri *et al*. (1998) demonstrated that OC-1 affected fitness and feeding of the stinkbug, *Perillus bioculatus* when exposed to the protein via its prey.

The aim of the present study was to characterize the proteolytic digestive enzymes of *H. axyridis* in order to predict potential deleterious effects of transgenic crops expressing cysteine protease inhibitors on this generalist ladybird. Furthermore, it reports the effects of OC-1 expressing oilseed rape on the digestive biochemistry, and on subsequent development, survival and fecundity of this ecologically important predator. Implications for the wide-scale growing of insect-resistant crops on natural enemies, are discussed.

Materials and methods

Reagents

The EnzChek Protease Assay Kit (Molecular Probes) was obtained from Cambridge BioScience and the ECL detection kit was supplied by Amersham International, Bucks, UK. ELISA plates were obtained from Griener Labortechnik. ABTS® solution was purchased from Boehringer Mannheim. Chromatography resins were obtained from Pharmacia. Bovine Serum Albumin (BSA), transepoxysuccinyl-L-leucylamido(4-guanidio)butane (E-64), phenylmethylsulphonyl fluoride (PMSF), pepstatin A, EDTA (ethylenediaminetetra-acetic acid disodium salt) and papain were purchased from Bachem (UK) Ltd. Recombinant oryzacystatin-1 (rOC-1) was purified from *E. coli* by ion-exchange chromatography as previously described (Edmonds *et al*. 1996) and polyclonal antibodies against the protein were raised in rabbits by standard protocols. All other chemicals were from Sigma and were of analytical grade unless otherwise stated.

Plant material

Homozygous transgenic spring oilseed rape seed (*Brassica napus* (L.) cv. Drakkar, line OC-1 Drakkar 4B) expressing the cysteine protease inhibitor oryzacystatin 1 was generated as previously described (Bonadé-Bottino 1993).

Insects

Stocks of diamondback moth (*Plutella xylostella*) from continuous culture over many generations were reared on Chinese cabbage plants in environmentally controlled rooms at 20 ± 2 °C, under a L16:D8 light regime. *Harmonia axyridis* was reared on pea aphids, *Acyrthosiphon pisum*, maintained on *Vicia faba* plants. Eggs were collected daily and maintained at 20 ± 2 °C, L16:D8 until hatch.

H. axyridis *enzyme preparations and protease assays*

Either 4th instar larvae, or adults, were cold anaesthetised and whole guts dissected into chilled 1 mM dithiothrietol (DTT; 1 gut/20 µL). Guts were homogenized on ice, centrifuged at 14 000 *g* for 5 min at 4 °C, and the supernatants were extracted with 3 volumes of chloroform, flash frozen and finally stored at −20 °C for future use. General proteolytic activity was determined using the fluorescent protein substrate BODIPY-FL Casein. Briefly, 2 mL of ladybird gut extract (adult or larval) was incubated with 188 µL of 50 mM buffer at 25 °C and the reaction was initiated by the addition of 10 µL of 10 mM substrate to give a final substrate concentration of 0.5 mM. Fluorescence was monitored in a fluorescence microtitre plate reader at 25 °C, excitation/emission maxima 485/538 nm, every 2 min over a 60-minute period against appropriate controls. Incubations were performed in triplicate. In order to determine the pH optima for larvae and adults, a range of overlapping buffer systems was used: McIlvaines citric-acid phosphate (pH 4.0–5.0), citric-acid phosphate + MES (2-[-morpholino]

ethanesulphonic acid) (pH 5.5), MES (pH 6.0), MES + bis-Tris propane (pH 6.5), bis-Tris propane (pH 7.0–9.5) and CAPS (3-[cyclohexylamino]-1-propanesulphonic acid) (pH 10.0). All buffers contained DTT and Brij35, at final assay concentrations of 5 mM, and 0.1% (v/v), respectively.

Enzyme inhibition assays were carried out at pH 5.0 (larval gut extract) or pH 6.0 (adult gut extract) as above. Inhibitors were preincubated with gut extract at 25 °C for 10 min, prior to addition of the substrate. The class-specific protease inhibitors E-64, Pepstatin A, EDTA and PMSF (final assay concentrations; 10 μM, 1 μM, 10 mM and 1 mM, respectively) were tested, as was rOC-1 (final assay concentrations; 2 μM and 5 μM). Further studies of larval proteolysis were also performed using a concentration range of rOC-1 (10^{-5}–10^{-9} M). All assays were performed in triplicate against appropriate controls.

Determination of transgene expression levels in OSR

Leaf samples were taken at random from both transgenic plants and nontransformed control plants, frozen in liquid nitrogen, ground to a fine powder and extracted in 50 mM Tris-HCl buffer, pH 8.0 (containing 1% PMSF; 36 mg/mL in ethanol) as previously described (Gatehouse *et al*. 1997). Extracts were centrifuged at 10 000 *g* for 15 min and total soluble protein of the supernatants was estimated by Bradford assay, with BSA as a standard. For immuno-assay by Western blotting, samples (containing 30 μg total protein) were separated by electrophoresis on SDS-PAGE (15% acrylamide minigels). Following electrophoresis, the proteins were transferred electrophoretically on to 0.02 μm nitrocellulose. OC-1 was detected by enhanced chemiluminescence (ECL) as previously described (Gatehouse *et al*. 1997), using polyclonal antibodies raised against OC-1 as the primary antibody, with HRP-conjugated goat antirabbit IgG (Bio-Rad Laboratories, Hertfordshire, UK) as the secondary antibody; rOC-1 produced in *E. coli* was used as a positive standard.

Determination of OC-1 accumulation in P. xylostella *(prey) by ELISA*

The midguts from 15 final instar *P. xylostella* larvae, previously fed either transgenic OC-1 expressing plants or nontransformed control plants for a minimum of 7 days, were dissected into 50 mM Tris-HCl, pH 8.0 containing 1% PMSF (1 gut μL^{-5}). Following homogenization, samples were extracted for 1 h at room temperature, and centrifuged at 13 000 *g* for 10 min at 4 °C. Total soluble protein was estimated by Bradford assay and samples diluted in phosphate buffered saline (PBS) to give a final protein concentration of 20 $\mu g/mL^{-1}$.

OC-1 present in insect samples was detected by ELISA. In brief 50 μL of either insect gut extracts, or purified OC-1 standards (to give final concentrations from 1 to 40 ng of rOC-1 in PBS) were incubated for 1.5 h in sealed 96 well microtitre plates. Unbound protein was removed by washing with PBS + 0.01% Tween 20 (v/v) (PBST), and the wells blocked with bovine serum albumin (BSA; 20 mg/mL PBST) for 1.5 h. Wells were again washed and then incubated with primary antibody (anti OC-1 antisera; 1 : 10 000 dilution) for 1 h, re-washed, and then incubated with secondary antibody (peroxidase-coupled antirabbit IgG; 1 : 10 000 dilution) for 1 h. The assay was developed by the addition of 50 μL ABTS® solution and absorbance read at 405 nm in a microtitre plate reader. Levels of OC-1 in insect samples were determined from the OC-1 calibration curve. Assays were performed for three separate samples, five guts per sample, and each sample was assayed in triplicate. All incubations were performed at room temperature.

Bioassay of the effects of OC-1 expressing oilseed rape plants on Harmonia axyridis *through the tritophic interaction*

Effects of OC-1 expressing transgenic oilseed rape on the pest. Whole plant bioassays were conducted to determine the effects of OC-1 expression on development and survival of the pest *P. xylostella*. Mated adult females were allowed to lay eggs on either OC-1 expressing oilseed rape plants or nontransformed control oilseed rape plants. Neonate larvae from these egg batches were isolated and transferred to either transgenic or control plants (plants 6 to 8-weeks-old) and survival and weight gain were recorded on a daily basis until pupation; larval instar duration was also monitored over this period. Six plants were set up for each of the two treatments, with each plant infested with 5 neonates.

Effects of OC-1 expressing transgenic oilseed rape on the predator via the pest. (i) Effects of OC-1 on predator survival and development. Eggs of *H. axyridis* were divided into two groups, designated OC-1 fed and control fed, and placed individually into 3.5 cm Petri dishes with a piece of dry filter paper; lids were sealed with Parafilm. Immediately upon hatching, *H. axyridis* larvae were fed daily on an equal weight (ad libitum) of either OC-1 fed, or control fed, final instar *P. xylostella* larvae; uneaten pest remains were removed (*P. xylostella* larvae used as a food source for ladybirds were fed for a minimum period of 5 days on transgenic or control OSR so as to ensure that the pest/host larvae were sufficiently dosed with OC-1). Survival of *H. axyridis* larvae through to adulthood was monitored on a daily basis. Larval weight (after the moult into each instar), prey consumption (measured via the weight of remaining uneaten prey) and development (time to each instar, time to pupation, time to adult emergence) was recorded throughout development. Thirty replicates were carried out for each of the two treatments.

(ii) Effects of OC-1 on predator fecundity. Upon emergence (see above), the adult ladybirds from each sibling group were sexed and assigned to mating pairs (OC-1 male × OC-1 female and control male × control female) and fed OC-1 fed or control fed prey, respectively, for 14 days. During this period, the number of eggs laid per pair was recorded. Adult female fecundity was estimated by counts of number of eggs produced per individual and egg viability assessed by determining the percentage of eggs that hatched to produce viable second-generation larvae.

Protease activity in H. axyridis *larvae following ingestion of OC-1 dosed prey. H. axyridis* larvae were fed for 7 days on *P. xylostella* that had been reared on either transgenic oilseed rape or nontransformed control oilseed rape, as detailed above. Guts were dissected into chilled 1 mM DTT (1 gut/20 µL) and extractions performed as previously described. Proteolytic activity was determined at pH 5.0, with BODIPY-FL Casein as substrate. The sensitivity of larval gut proteases from OC-1 fed larvae to subsequent inhibition by rOC-1 was determined, as was sensitivity to inhibition by class specific protease inhibitors; ladybird larvae fed *P. xylostella* from nontransformed plants were used as controls.

Production of recombinant oryzacystatin (rOC-1)

OC-1 was purified according to the method of Edmonds *et al*. (1996). Briefly, cells of *E. coli* (DH5α) containing the oryzacystatin-1 expression construct pHEV2 were grown in 1 L of TB (terrific broth) (Ausubel *et al*. 1999) plus 0.5 mM IPTG. Cells were harvested, resuspended in 10 mM Tris-HCl pH 8.0, with 2 mM EDTA and disrupted by sonication. Cell debris was removed by centrifugation (11 000 *g*) and the supernatant heated to 85 °C for 15 min; the resulting precipitate was removed by further centrifugation at 11 000 *g*. Ammonium sulphate precipitation to 65% saturation was carried out, the precipitated proteins were pelleted by centrifugation (11 000 *g*), resuspended in 20 mM Tris-HCl, pH 8.0 and finally dialysed exhaustively against dH_2O. Initial purification was achieved by anion exchange chromatography (Q-Sepharose), using 20 mM Tris-HCl pH 8.0 with a linear gradient from 0 to 1 M NaCl. An additional purification step was then conducted on a Phenyl-Sepharose column, using 20 mM Tris-HCl pH 8.0 with a reverse linear gradient (4 M-0 M NaCl). The pure recombinant oryzacystatin (rOC-1) was dialysed exhaustively against dH_2O and finally lyophilized; biological activity was assayed against papain.

Statistical analysis

All statistical analyses were performed with either Microsoft EXCEL or STATVIEW software on a Power Macintosh computer. Differences between treatments were considered significant at the $P < 0.05$ level. When data were not normally distributed the nonparametric Mann–Whitney *U*-test replaced Unpaired *t*-tests in the analyses.

Results

Production and purification of rOryzacystatin (rOC-1)

Recombinant OC-1 was produced using *E. coli* as the microbial expression system. The recombinant protein was partially purified by anion exchange chromatography (Fig. 1a), and finally purified to homogeneity by reversed phase chromatography on a Phenyl-Sepharose column. This additional step resulted in the removal of contaminating high molecular weight bands to give a single band of rOC-1 at 14 kDa (Fig. 1b). Purity of the recombinant protein was confirmed by immunoassay on Western blot using anti-rOC-1 antibodies (Fig. 2 lanes 1, 2), whilst the biological activity of the inhibitor was

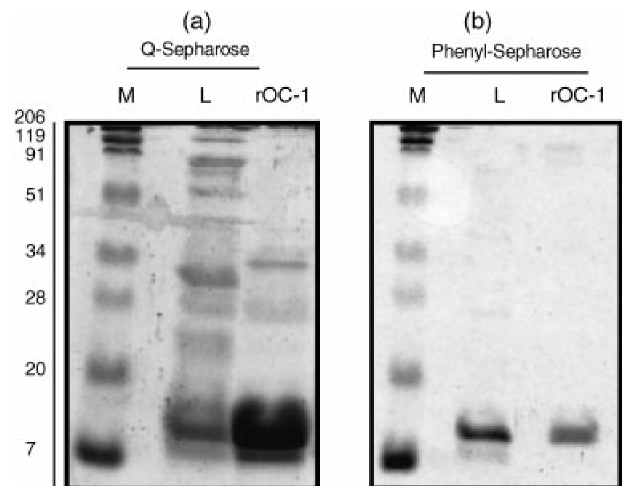

Fig. 1 Purification of rOC-1. SDS-PAGE analysis of fractions following: (a) ion-exchange chromatography (b) reverse phase chromatography; M = molecular weight markers, L = load, and rOC-1 = fraction containing rOC-1.

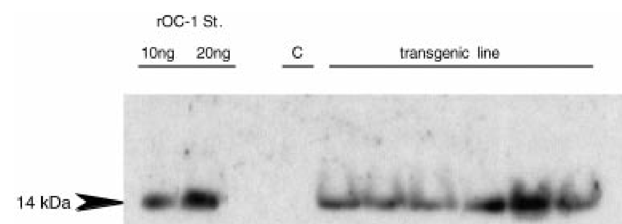

Fig. 2 Western blot showing the presence of OC-1 in leaf tissue of transgenic oilseed rape (OSR). Lane 1, 10 ng purified rOC-1; lane 2, 20 ng rOC-1; lane 4, nontransformed control OSR (30 µg protein); lanes 5–10, transgenic OSR (30 µg protein).

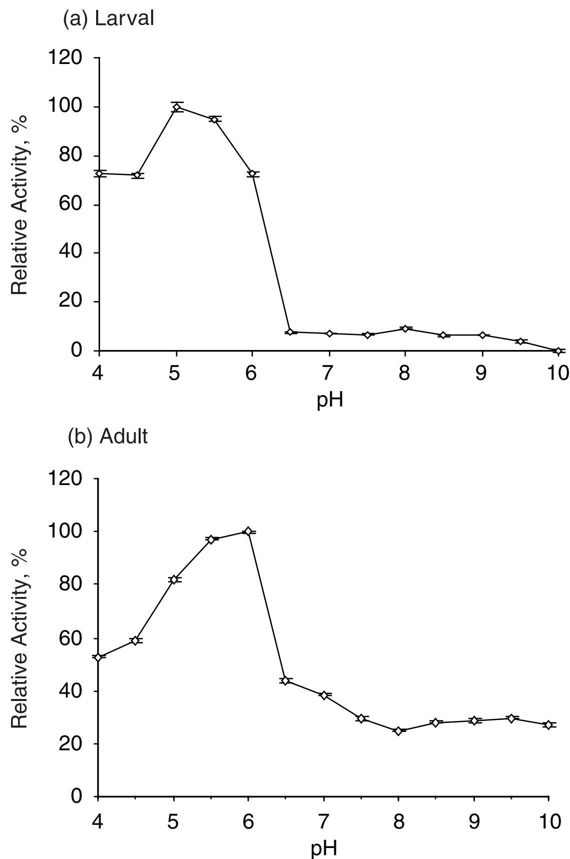

Fig. 3 pH optima of *H. axyridis* (a) larval (4th instar) and (b) adult gut proteolytic activity with BODIPY-FL Casein as substrate. Points and bars represent mean ± SE for triplicated independent determinations.

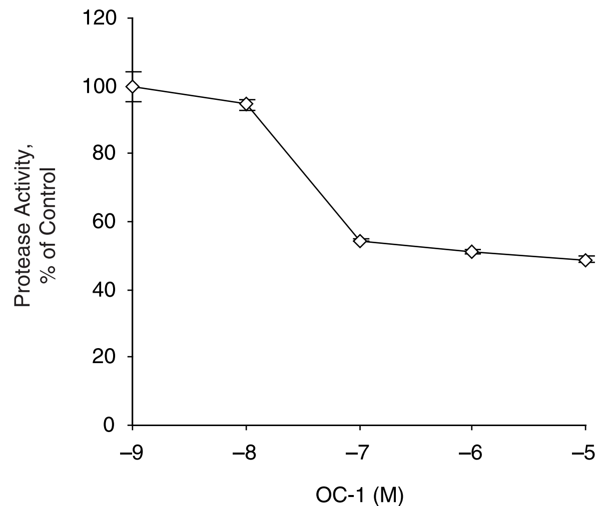

Fig. 4 Inhibition of *H. axyridis* larval (4th instar) gut proteolytic activity by rOC-1, with BODIPY-FL Casein as substrate. Points and bars represent mean ± SE for triplicated independent determinations.

Table 1 Inhibition of *H. axyridis* larval and adult gut extracts

Inhibited class	Inhibitor	Final assay concentration	Residual activity (%) against BODIPY-FL Casein: larvae	adult
Cysteine	E-64	10 μM	39.9	45.6
	rOC-1	2 μM	53.1	47.5
		5 μM	43.1	48.6
Aspartic	Pepstatin A	1 μM	53.1	53.0
Metallo-	EDTA	10 μM	100.0	86.7
Serine	PMSF	1 μM	100.0	100.8

demonstrated by its ability to inhibit (*in vitro*) the cysteine protease, papain (results not shown).

Characterization of digestive proteases in Harmonia axyridis

General proteolytic activity was demonstrated in both larval and adult *H. axyridis* with the fluorogenic protein substrate BODIPY-FL Casein. Using an overlapping buffer system from pH 4.0 to pH 10.0, the optima pH for larval and adult gut proteolysis was shown to be pH 5.0 and pH 6.0, respectively (Fig. 3).

Protease activity in *H. axyridis* larvae was further characterized using a range of diagnostic inhibitors. The synthetic cysteine protease inhibitor E-64 was shown to inhibit activity by up to 60%, whilst pepstatin A, a synthetic aspartic protease inhibitor, caused approximately 47% inhibition. rOC-1 was shown to be equally effective compared to these synthetic inhibitors, resulting in up to 57% inhibition at a final concentration of 10^{-7} M; higher inhibitor concentrations failed to further increase the level of inhibition (Fig. 4). PMSF, a nonspecific serine protease inhibitor, and EDTA, a metallo-protease inhibitor, showed no inhibition of larval proteolytic activity (Table 1).

Studies with adult *H. axyridis* showed similar results to those obtained for larvae, with E-64, pepstatin A and rOC-1 (5 μM) causing approximately 55%, 47% and 51% inhibition, respectively. Similarly, PMSF showed no inhibitory activity, but EDTA was found to cause low levels of inhibition (12–13%). These results suggest that both the larvae and adults of *H. axyridis* rely predominantly on cysteine and aspartic proteases, with minor metallo-protease activity in adults (Table 1).

Expression of OC-1 in leaves of transgenic oilseed rape plants

Semi-quantitative immunoassay by Western blotting demonstrated that OC-1 was being expressed and correctly

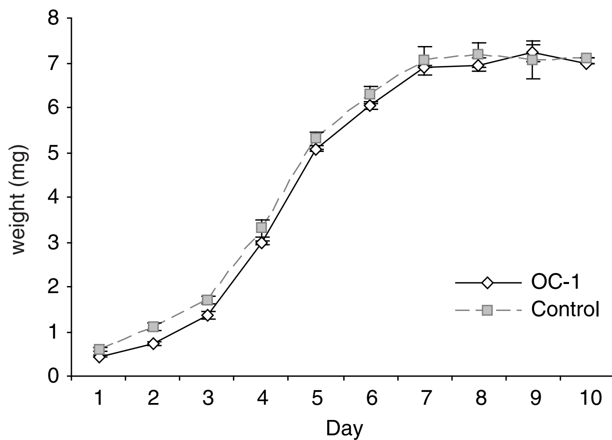

Fig. 5 Effects of OC-1 expressed in vegetative tissues of transgenic OSR on *P. xylostella* larvae. Larval weight (mg) was used as a measure of performance from first to final instar. Weight of fourth instars (age type provided as prey) were compared using Mann–Whitney *U*-tests. Points and bars represent mean ± SE ($N = 30$).

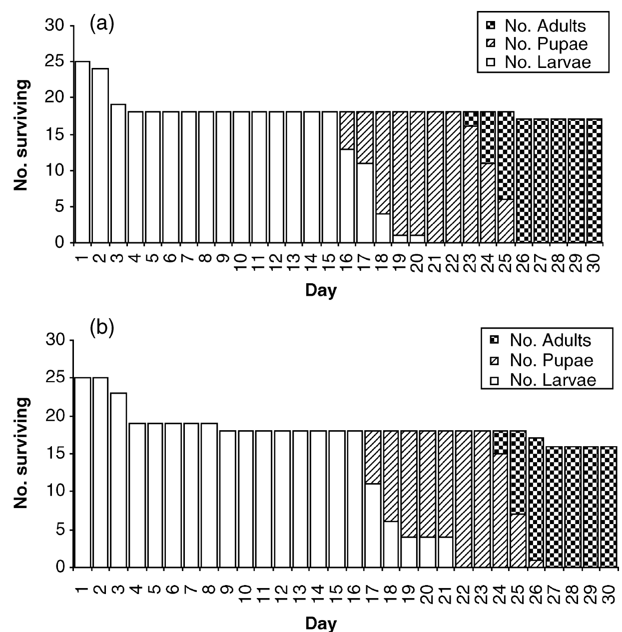

Fig. 6 Effects of OC-1 on survival and development of *H. axyridis* from neonate through to adulthood when fed (a) OC-1 fed prey and (b) control fed prey. Survival was compared by Kaplan-Meir Survival Analysis at two time points (day 21 and day 26).

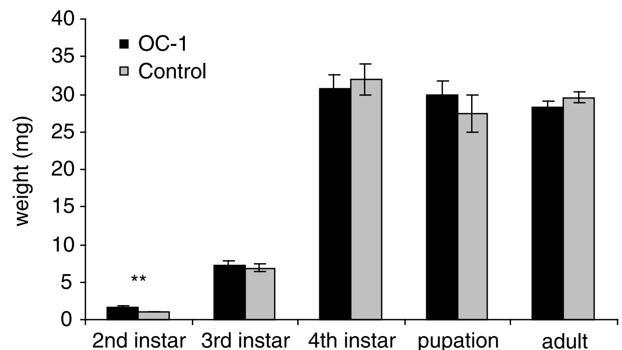

Fig. 7 Effects of OC-1 on mean weight (mg) of *H. axyridis* larvae in each instar (1st instar excluded from analysis), at pupation and of newly emerged adults when fed either OC-1 fed prey or control fed prey. Weights were compared using the Mann–Whitney *U*-test. **Significant difference ($P < 0.01$). Points and bars represent mean ± SE ($N = 30$).

processed in the vegetative tissues of transgenic oilseed rape (Fig. 2, lanes 5–10); samples from nontransformed plants confirmed that the antibodies were highly specific to OC-1, with no cross reactivity occurring (Fig. 2, lane 4). Expression levels for the different plants tested of line OC-1Drakkar 4B, ranged from 0.03 to 0.06% of total soluble protein.

Effect of OC-1 on Plutella xylostella *larvae and accumulation in gut tissue*

As expected, results from plant bioassays clearly demonstrated that expression of OC-1 in the vegetative tissues of transgenic OSR had no significant effects on growth or development of *P. xylostella* larvae (Fig. 5; Mann–Whitney *U*-test, $P = 0.8$). Furthermore, the presence of OC-1 had no significant effect upon insect survival, with survival on transgenic and control plants being 80% and 76%, respectively.

Immunoassay by ELISA readily detected the presence of OC-1 in the gut of *P. xylostella* larvae fed on transgenic OSR, the levels of transgene product accumulation ranging from 0.8 to 2.7 ng OC-1 per gut, with a mean value of 1.6 ng (± SE of 0.001 ng OC-1/gut, $N = 15$). No OC-1 was detected in guts of *P. xylostella* fed nontransformed control plants.

Effects of OC-1 on Harmonia axyridis *larvae*

A large-scale trial was carried out to investigate the effects of transgenic oilseed rape expressing oryzacystatin on *H. axyridis*. The results demonstrated that exposure to OC-1 had no effect on the survival of neonate ladybird larvae through to adulthood (Fig. 6a,b). Statistical analyses were performed at two time points within the trial, at day 21 when all OC-1-fed larvae, but not all control fed larvae, had pupated at day 26; at this stage all adults in the OC-1-fed group had emerged, but again not all of the control group had reached adulthood. Kaplan-Meier Survival Analysis at both these time points showed no significant differences between the two groups (day 21, Logrank (Mantel-Cox) $P = 0.85$; day 26 Logrank (Mantel-Cox) $P = 0.93$).

Ladybird larval weight was monitored from the second instar onwards, with weights being recorded after the moult into each instar. Exposure to OC-1 via prey significantly increased the rate of larval growth during the early stages of development (Fig. 7), with second instar OC-1 fed larvae

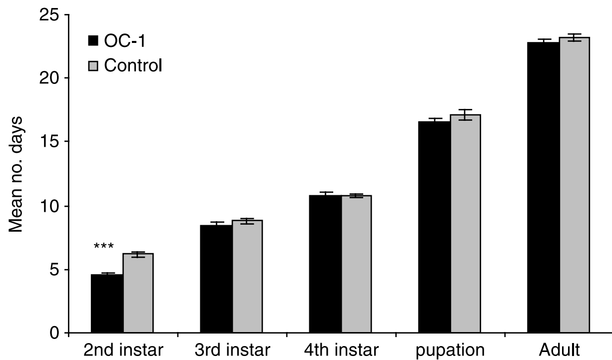

Fig. 8 Effects of OC-1 on rate of development of *H. axyridis*, showing mean number of days taken to develop through the larval instars, time to pupation and time to adult emergence when fed either OC-1 fed prey or control fed prey. Developmental time was compared using the Mann–Whitney *U*-test. ***Significant difference ($P < 0.0001$). Points and bars represent mean ± SE ($N = 30$).

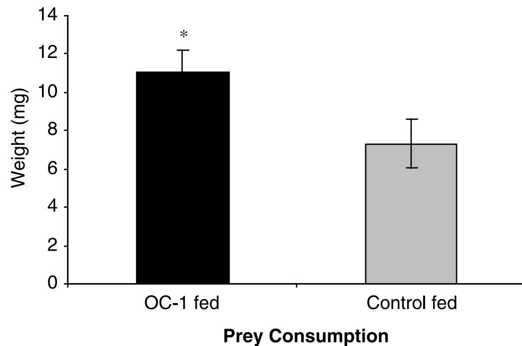

Fig. 9 Effects of OC-1 on mean daily prey consumption (mg) by *H. axyridis* larvae (over entire larval period) of *P. xylostella* larvae fed on either transgenic or control OSR. Weights consumed were compared using the Mann–Whitney U-test. Points and bars represent mean ± SE ($N = 30$).

being significantly heavier (Mann–Whitney *U*-test $P = 0.007$; on average 0.6 mg) than controls. The effect of OC-1 on instar duration was also recorded (Fig. 8) and showed that the mean number of days to the second instar was significantly lower (Mann–Whitney *U*-test; $P < 0.0001$), these being 4.5 and 6.1 days for the experimental and control group, respectively. However, as the trial progressed the differences between the treatments were no longer significant, with the experimental group less than 1 day ahead, both at pupation and adult emergence. Interestingly, there was a significant increase in consumption of prey by the OC-1 fed ladybirds throughout the larval period (Mann–Whitney *U*-test; $P = 0.03$) (Fig. 9), with this group consuming approximately 34% more per day compared to the control group.

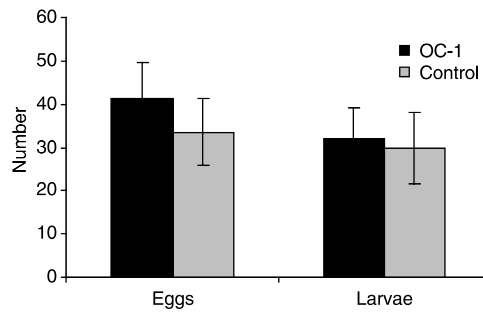

Fig. 10 Effects of OC-1 ingestion on *H. axyridis* fecundity (mean number of eggs laid by a single female *H. axyridis* adult) and egg viability (mean number of larvae successfully emerging from eggs laid) when fed either OC-1-fed or control fed prey. Numbers of eggs laid and numbers of larvae emerging were compared using Unpaired *t*-tests. Points and bars represent mean ± SE [$N = 12$ (control), $N = 10$ (OC-1)].

Effect of OC-1 on fecundity of adult Harmonia axyridis

Adult *H. axyridis* emerging from the feeding trials (see above) were assigned to breeding pairs, and allowed to consume either OC-1-fed or control-fed prey for 14 days. The number of eggs laid per female was used as a measure of relative fecundity and the number of second generation neonate larvae emerging from the eggs was used as a measure of egg viability (Fig. 10). The results showed that OC-1 had no significant effect on mean cumulative ladybird fecundity (Unpaired *t*-test; $P = 0.52$), with OC-1 fed ladybirds laying approximately 7.7 more eggs per female, although it should be noted that the number of eggs laid per female was highly variable within groups. Furthermore, OC-1 had no significant effect on subsequent egg viability (Unpaired *t*-test; $P = 0.8$), with 88.6% of the control group and 77.7% of the experimental group successfully hatching as neonates. (Thirty-three percent of control and 44% of OC-1 matings failed, and were thus excluded from the analysis.)

Effects of OC-1 on endogenous gut proteolytic activity in H. axyridis

The effects of consumption of OC-1 on the endogenous gut proteolytic activity of ladybird larvae following ingestion of OC-1-fed prey was investigated. Larvae that had ingested OC-1-fed prey showed a significant increase (Unpaired *t*-test; $P < 0.01$) in general proteolysis of approximately 31% (expressed per μg of protein) when compared to larvae that had ingested control-fed prey (Fig. 11). However, subsequent sensitivity of the two gut extracts to inhibition by 5 μM OC-1 was identical (Fig. 11), resulting in 68.4% and 68.7% inhibition of proteolytic activity of gut extracts from experimental and control groups, respectively. Further inhibition studies with class

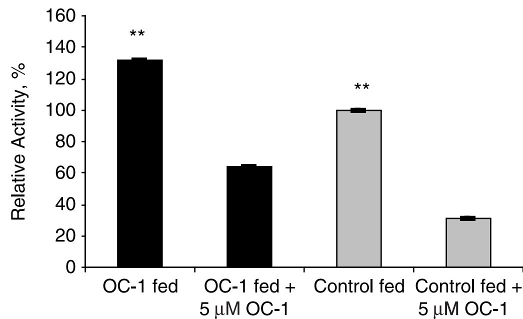

Fig. 11 Response of *H. axyridis* larval proteases to ingestion of either OC-1- fed or control-fed prey and the subsequent sensitivity of *H. axyridis* larval proteases to inhibition with 5 μM rOC-1 (expressed per μg protein) following ingestion of either OC-1 fed or control fed prey. Protease activities were measured with BODIPY-FL Casein as substrate at pH 5.0. Protease activity was compared using the Students *t*-test. **Significant difference for which $P < 0.01$. Points and bars show mean ± SE for triplicated independent determinations.

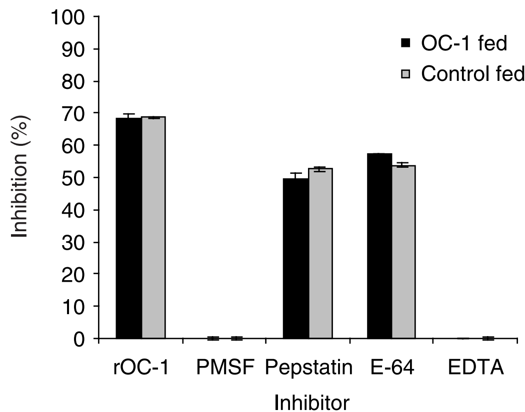

Fig. 12 Inhibition of *H. axyridis* larval proteases with class specific protease inhibitors following ingestion of either OC-1 fed or control fed prey. Protease activities were measured with BODIPY-FL Casein as substrate at pH 5.0. Inhibition was expressed as a percentage of control activity (no inhibitor). Points and bars show mean ± SE for triplicated independent determinations.

specific inhibitors showed that the proteolytic enzyme profile remained the same (Fig. 12), suggesting that the ratio of OC-1 sensitive cysteine protease activity : OC-1 insensitive aspartic activity remained unchanged. The results demonstrated that E-64 caused 58% inhibition of proteoytic activity from the experimental group with Pepstatin A causing 50% inhibition, compared to 54% and 53%, respectively, for the control group; neither PMSF nor EDTA caused any inhibition with either group.

Discussion

To date, the only insect-resistant transgenic crops to have been commercialized are those expressing *Bt* Cry proteins (James 2000). Other strategies being developed include those based on expression of protease inhibitors. Since many members of the Coleoptera rely predominantly on cysteine proteases for protein digestion (Terra *et al.* 1996), expression of cysteine protease inhibitors, such as oryzacystatin, have been suggested as a means of control of pests from this order (Jouanin *et al.* 1998). Characterization of the proteolytic activity in both larvae and adults of the multicoloured Asian ladybeetle, *H. axyridis*, demonstrated that this ladybird utilizes both cysteine and aspartic proteases for protein digestion. This activity was not only sensitive to inhibition by the synthetic cysteine-protease and aspartic-protease inhibitors, E-64 and Pepstatin A, but also to inhibition by rOC-1, with activity in both the larvae and adults being inhibited by up to 50%. These results are in agreement with those previously reported for the 2-spot ladybird, *Adalia bipunctata* (Walker *et al.* 1998). Given that *H. axyridis*, like other members of the Coleoptera, relies upon cysteine proteases, there is thus the potential for transgenic crops expressing inhibitors of such enzymes to have unintentional deleterious effects on beneficial nontarget insects such as ladybirds, including *H. axyridis*, either directly or through the tritrophic interaction.

Studies investigating the potential effects of insect-resistant transgenic plants on ladybirds through the tritrophic interaction (plant/pest/predator) have utilized aphids as the pest species (Dogan *et al.* 1996; Birch *et al.* 1999), despite reports that transgene products are not efficiently trafficked to the phloem sap (Foissac *et al.* 2000; Raps *et al.* 2001); in these earlier studies there is thus little exposure of the predator to the transgene products. In order to overcome this limitation, larvae of the diamondback moth, *P. xylostella*, were used as the prey in the present study. This was justified on the grounds that although ladybirds normally feed upon scale-insects, mites, whitefly and aphids, *H. axyridis* will also predate small lepidopteran larvae in the field (Hodek 1973). One of the aims of plant genetic engineering for pest control is to target expression of insecticidal proteins to the phloem sap for more efficient control of homopteran (phloem-feeding) insects (Rao *et al.* 1998; Sudhakar *et al.* 1998; Gatehouse & Gatehouse 1999). Achievement of this goal would result in aphid predators being exposed to such proteins through their prey, where they might then exert a deleterious effect. Protease inhibitors have been demonstrated to be potential candidates for control of aphids (Rahbe & Febvay 1993).

Transgenic oilseed rape plants used in the present study were shown to both express and process OC-1 correctly, with expression being up to 0.06% total soluble protein. These values are within the ranges reported for expression of other plant-derived insect resistance genes (Leple *et al.* 1995; Gatehouse *et al.* 1997). Immunoassay of *P. xylostella* (pest) larvae clearly demonstrated the presence of the foreign protein (at levels of between 0.86 and 2.7 ng per gut),

thus confirming that predating ladybirds would become exposed to the OC-1. This result is consistent with a recent study (Head *et al*. 2001), which investigated the risk of secondary exposure to *Bt* (*Bacillus thuringiensis*) Cry1Ab toxin via lepidopteran pests feeding on transgenic corn, where the authors found that larvae of all lepidopteran species tested contained *Bt* toxin after consumption of transgenic tissue, although the level of toxin was found to be uniformly low. As expected, OC-1 expressing oilseed rape had no effect on either the development or survival of *P. xylostella*, since this insect relies on serine proteases for protein digestion (Meenakshisundaram & Gujar 1998); such enzymes are not sensitive to inhibition by OC-1. This lack of OC-1 toxicity towards *P. xylostella* makes it an ideal pest to use in the subsequent tritrophic studies, since any effects of the OC-1-fed prey on the ladybird would be due to the inhibitor itself, and not a consequence of differences in prey quality.

Studies to investigate the effects of OC-1 expression on ladybirds through the interaction with *P. xylostella* as the pest, clearly demonstrated that OC-1 had no deleterious effects upon either survival or development of *H. axyridis* from neonate through to adulthood. In fact, *H. axyridis* performed better in that second instar larvae ingesting OC-1 fed prey were not only significantly heavier compared to controls, but the developmental period of this instar was reduced by approximately 27%. These results are thus contrary to what might have been predicted, particularly since *H. axyridis* relies on cysteine proteases for protein digestion. The antinutritive effects of protease inhibitors and their detrimental consequences on growth and development of insect herbivores at the second trophic level have long been recognized (Gatehouse & Boulter 1983; Wolfson & Murdock 1995), but similar growth stimulation of beetle larvae has previously been reported for the coleopteran pest, *Psylliodes chrysocephala* (Girard *et al*. 1998a). When these larvae were reared on transgenic plants expressing OC-1, they too showed an increase in weight gain compared to those reared on controls. The age of larvae appears to be an important factor in their sensitivity to dietary protease inhibitors, since only early instar ladybird larvae were affected by the presence of OC-1, with no overall effects on development or survival. Alternatively, this could reflect the fact that the larvae are able to respond rapidly to the presence of the inhibitor (Bown *et al*. 1997). These findings are consistent with some other studies in that only first or second instar larvae were sensitive, although in these studies the effects upon development were detrimental (McManus & Burgess 1995; Michaud *et al*. 1995).

Results from the present study suggest that *H. axyridis* are able to respond to the presence of the cysteine protease inhibitor in a compensatory manner by significantly increasing the level of digestive proteolytic activity. A trypsin inhibitor from soybean (SBTI) was shown to increase growth rates of grasshoppers and to induce an increase in digestive proteases (Hinks & Hupka 1995). Similarly, *P. chrysocephala* were shown to increase proteolytic activity by up to 2-fold in response to dietary OC-1 (Girard *et al*. 1998a), whilst Colorado potato beetle (*Leptinotarsa decemlineata*) larvae were able to compensate for exposure to OC-1 by both overproduction of digestive enzymes and by increasing consumption of plant material (Cloutier *et al*. 2000); similar results have been reported for Diamond Back moth (Winterer & Bergelson 2001). Interestingly, *H. axyridis* larvae were also observed to increase their consumption of OC-1-fed prey relative to the controls. Several studies have demonstrated that some Lepidoptera (Bown *et al*. 1997; Mazumdar-Leighton & Broadway 2001) and Coleoptera (Bonadé-Bottino *et al*. 1999; Bolter & Jongsma 1995; Jongsma & Bolter 1997; Cloutier *et al*. 1999) are able to circumvent the effects of dietary protease inhibitors by producing novel proteases insensitive to inhibition. This was not found to be the case for ladybirds where there was an increase in native proteases, with the ratio of cysteine to aspartic enzymes remaining the same. Similar results have been reported for the tomato moth where there was a 4-fold increase in native digestive proteases, but no synthesis of novel enzymes (Gatehouse *et al*. 1997).

It can be argued that the more subtle effects that protease inhibitors may have on female fitness and fecundity are more important ecological parameters than their effects on survival per se. Female nutrition is critical during oogenesis, affecting both the level and quality of egg production. Results from the present trials, however, showed that OC-1 did not significantly affect either the fecundity of the ladybird or egg fertility/viability. This result is consistent with studies with female adult Colorado potato beetles which showed that when fed OC-1 expressing plants, sufficient nutrition was provided for female postemergence growth and full reproductive maturation (Cloutier *et al*. 2000). In addition there was no indication of female fitness loss on either egg production or eclosion rates, despite hypertrophic behaviour to compensate for the presence of the inhibitor. In contrast, studies with Two-Spotted Stinkbug, *Perillus bioculatus*, demonstrated that whilst there was no impact of exposure to OC-1 on mortality, which is consistent with the results presented here, there was a 50% reduction in the fertility of females (Ashouri *et al*. 1998). However, these results are not directly comparable with those of the present study, since the stinkbugs were exposed to levels of OC-1 unlikely to be encountered in the field (8–16 µg of OC-1 injected into prey/day compared to an average of 1.6 ng/gut in *P. xylostella*).

In the present study *P. xylostella* was used as a model pest to ensure efficient exposure of the ladybird to the transgene product through the tritrophic interaction. This study thus enabled the potential effects of deploying

insect-resistant transgenic crops on beneficial predators to be evaluated in a meaningful way. Despite the potential for toxicity, in that ladybirds rely on cysteine proteases for protein digestion, expression of OC-1 in transgenic oilseed rape plants did not cause any significant effects either on development or survival of *H. axyridis*, nor on female fecundity. The results further demonstrated that this species of ladybird has the ability to modulate both its food intake and protease activity, and so overcome the effects of exposure to OC-1. As far as the authors are aware this is the first demonstration of a compensatory response at the third trophic level. This study also demonstrated the potential for insecticidal proteins expressed at the first trophic level to have unpredictable effects on nontarget beneficial arthropods at the third trophic level and thus highlights the need for thorough investigations to be carried out when evaluating potential effects of transgenic crops on natural enemies.

Acknowledgements

The authors wish to thank the Yorkshire Agricultural Society, and the University of Newcastle-upon-Tyne, for funding. Technical staff at Close House Field Station are gratefully acknowledged for growing of plants.

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The authors' interests are in the field of plant-insect interactions, with particular reference to the potential impact of transgenic plants on beneficial insect predators. Members of the consortium have differing and complementary expertise ranging from plant and insect molecular biology/biochemistry (NF, AMRG, RJMR, LJ, JAG), insect physiology (NF, AMRG), evolutionary genetics (MENM) to behavioural ecology (GP).