Non-target effects of proteinase inhibitors expressed in potato as an anti-nematode defence

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ABSTRACT

The survival and rate of growth of nymphs of *Myzus persicae* and *Macrosiphum euphorbiae* were adversely affected by ingestion of cystatins in artificial diet assays. Prolonged exposure to cystatins in diets produced a reduction in the rate of development, survival time and fecundity of *M. persicae* adults. However, when aphids were caged on transgenic potato expressing chicken egg white cystatin (CEWc) there was no adverse effect on aphid fitness. Similarly, there were no significant differences in field populations of aphids between control and CEWc-expressing potato grown under field conditions, although the levels of expression were sufficient to provide control of potato cyst nematodes. The results highlight that tissue-specific levels of expression are important in determining the effects of cystatins on different invertebrates and it is suggested that aphids feeding on transgenic plants may provide a route via which various natural enemies are exposed to cystatins.

INTRODUCTION

Nematodes cause global crop losses that have been valued at over $100 billion per year. In Britain control of nematodes on potato relies heavily on the use of nematicides, some of which are known to pose a risk to groundwater and drinking water supplies under certain soil types (Gustafson, 1993). Consequently there is a need to develop improved methods of nematode control. Transgenic plants expressing proteinase inhibitors (PIs) have the potential to fulfil this role.

PIs have been shown to affect members of the three principal groups of economic nematodes and may represent a broad-range resistance strategy for control of nematode pests. A modified version of a cysteine proteinase inhibitor from rice, Oc-IΔD86, has been shown to limit the growth of *Heterodera schactii*, beet cyst-nematode and *Meloidogyne incognita* (a root knot nematode) when expressed in *Arabidopsis thaliana* (Urwin *et al.*, 1997).

Transgenic plants expressing serine or cysteine PIs have negative effects on the growth and survival of several insect pests (Hilder *et al.*, 1987; Leple *et al.*, 1995; Xu *et al.*, 1996). As part of our study of the environmental consequences of a PI-based anti-nematode defence we have examined the invertebrate associates of potato crops to establish the range of common beneficial and non-economic insects commonly associated with this crop in UK. Histochemical studies and information from published literature were used to select a subset of insects for further study. Work has concentrated on those species which were identified to contain cysteine proteinases or those which had been shown to be affected by exposure to PIs in previous studies. This paper describes the results of a range of laboratory and field studies

to quantify the effects on exposure to cysteine PIs on two aphid species *Myzus persicae* and *Macrosiphum euphorbiae*.

MATERIAL & METHODS

The *M. persicae* clone was initially introduced in our laboratory as a single individual collected from potato in 1996. A culture of *M. euphorbiae* was obtained from a laboratory clone maintained at IACR-Rothamsted.

Cloned genes for cystatins (Oc-IΔD86, chicken egg white cystatin, maize cystatin, sunflower cystatin) were expressed in *E. coli* using a pQE vector and the QIAexpress™ expression system. Trans-epoxysuccinyl-L-leucylamido-(4-guadino) butane (E64) was purchased from Sigma Chemical Company.

Growth and survival of aphids on artificial diets containing cysteine PIs (cystatins)

Assays were carried out on 0-24h aphids over a 9 day period on artificial diets containing purified cystatin. The diet was a modified version of that described by Dadd & Mittler (1966). Feeding chambers containing 200µl artificial diet were made using UV-sterilised Parafilm stretched over a PVC ring. The diet sachets were replaced every 48 hours. The length and breadth of the nymphs was measured on alternate days using a video camera linked to a Leica Quantimet image analysis package.

The survival and fecundity of *M. persicae* feeding on artificial diets containing cystatins

0-24h old *M. persicae* nymphs were transferred from Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*(Lour.)) plants to individual diet cylinders with artificial diets containing either OcIΔ86 (200ppm) or CEWc (200ppm). The survival of nymphs was recorded daily. From day 8 onwards the number of newly deposited nymphs was also recorded. The nymphs were removed daily and the mortality of the adult aphids monitored. Values for the intrinsic rate of increase (r_m) were estimated by the method of Wyatt and White (1977) using the following equation:

$$r_m = 0.738 . \log_e (M_d)/ d$$

where M_d is the mean number of progeny produced in a time equal to the prereproductive period (d). The parameter provides a theoretical way of comparing the aphids reproductive potential on different diet types. The data were also used to calculate the mean generation time ($T = d/0.738$) and the doubling time ($DT = \log_e (2)/ r_m$).

The survival and fecundity of *M. persicae* feeding on transgenic plants

Transgenic potatoes were generated expressing CEWc constitutively. The level in leaf tissue was 60ppm which is sufficient level to achieve nematode control in roots. *M. persicae* females of known age were placed individually in clip cages on either transformed or control plants. The aphids were caged on the abaxial leaf surface throughout the canopy, avoiding immature or senescing leaves. For each plant line, 3 plants were used and 7 clip cages were

set up per plant. After 24 hours, the adults were removed together with all but 2 first instar nymphs. This day was labelled day 0. The remaining 2 nymphs per clip cage were left undisturbed until adulthood. At the teneral adult stage, one aphid per cage was left so that the daily reproduction of all individually-caged second generation adult aphids could be recorded. The assays were carried out under controlled environment conditions of 20°C, 16h light, 8h dark regime.

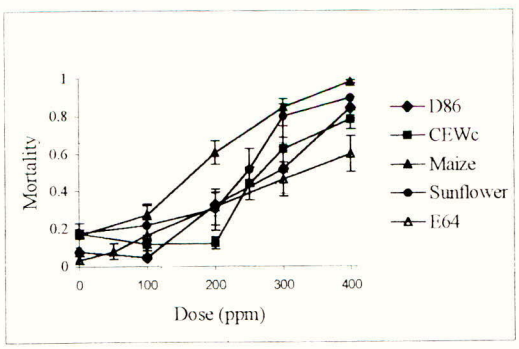
Aphid populations on transgenic plants in the field

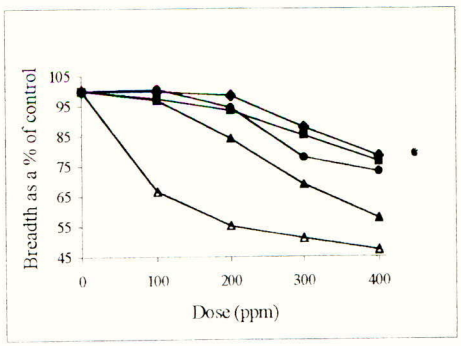
Four CEWc-expressing lines and an ex-tissue culture control line of the same cultivar, cv Desiree were planted in a randomised block design with 4 replicates. Each plot contained nine plants. The number of invertebrates on the above ground parts of five plants per plot were censused at weekly intervals from 1 July to 3 October 1998.

RESULTS

Growth and survival of aphids on artificial diets containing cystatins

Figures 1 and 2 show that both the survival and rate of growth of *M. persicae* nymphs was adversely affected by ingestion of cystatins from artificial diet. The cystatins varied in their toxicity and growth inhibiting properties. Mortality was most pronounced in nymphs reared on maize cystatin. Surviving nymphs reared on E64, a low molecular weight, irreversible inhibitor of papain-like cysteine proteinases, were significantly smaller than those reared on the cystatins from plants or chicken egg white.





Figs. 1 & 2. Survival and growth of *M. persicae* nymphs after 9 days on artificial diets containing cystatins.

Similar effects on growth and survival were obtained with nymphs of *M. euphorbiae*. After 9 days on artificial diet containing CEWc at 400ppm the surviving nymphs were approximately 35% smaller than those on control diets. Survival was also significantly reduced at concentrations greater than 300ppm.

The survival and fecundity of *M. persicae* feeding on artificial diets containing cystatins

Rates of development, survival time and fecundity of *M. persicae* adults were all reduced on diets containing OclΔ86 or CEWc. The mean number of days from birth to reproduction was 8.24 days for control aphids, 10.42 days for aphids reared on OclΔ86 and 11.0 days for aphids reared on CEWc. The survival of aphids on CEWc declined rapidly from day 8 onwards. By day 10, 50% of these aphids were dead. Fifty per cent of the aphids reared on OclΔ86 had died by day 14, some of the remaining adults survived until day 22 of the study. The total number of nymphs produced on control diet was 215 compared to 91 on OclΔ86 diet and 17 on CEWc diet. The number of nymphs produced per female per day peaked on day 11 in all the treatments. The values were 1.45 and 1.6 for the control and OclΔ86 respectively, and 0.63 for CEWc. There were insufficient aphids surviving on CEWc diets to calculate the r_m value but the values for OclΔ86 and control diets are shown in Table 1.

Table 1. Adult performance of *M. persicae* reared on control diet or diet containing OclΔ86

Diet	r_m female/female/ day	prereproductive time (days)	generation time (T)	doubling time (DT)	Total fecundity (nymphs)
Control	0.21	8.2	11.16	3.30	215
OclΔ86	0.14	10.4	14.12	5.02	91

The survival and fecundity of *M. persicae* feeding on transgenic plants

No adverse effects were observed on the rate of development, fecundity or doubling time when aphids were caged on plants expressing CEWc (0.4% total soluble protein). The number of nymphs produced per female per day peaked at 3.93 and 4.09 on control and D6/2 plants, respectively. The reproductive peak occurred 7 days earlier on D6/2 than on control plants. The differences in reproductive dynamics are reflected in the calculated r_m values for aphids on the two plant lines (Table 2).

Table 2. Adult performance of *M. persicae* reared on transgenic or control potatoes.

Plant	r_m female/female/ day	prereproductive time (days)	generation time (T)	doubling time (DT)
Control	0.299	8.2	11.11	2.32
D6/2	0.330	7.6	10.35	2.10

Aphid populations on transgenic plants in the field

Cool, wet weather during the 1998 growing season resulted in low aphid population densities. Consequently, the counts for *M. persicae* and *M. euphorbiae* were pooled for analysis. Fig. 4

summarises the cumulative number of aphids per 5 plants recorded during the period July-October.

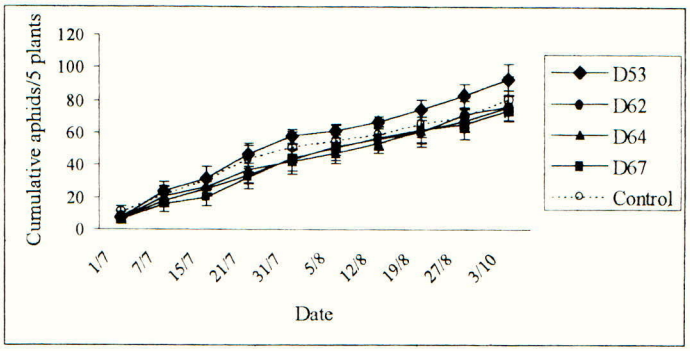


Fig. 4. The cumulative number of aphids on cv Desiree potato plants not expressing (control) or expressing CEWc (D53, D62, D64 & D67). Values were recorded over a 10 week sampling period.

DISCUSSION

Aphids such as *M. persicae* and *M. euphorbiae* represent important non-target insects in the potato-nematode system. Previous studies have shown that although the digestive tract of aphids does not contain detectable amounts of endo-protease activity, the ingestion of some serine PIs can result in reduced growth and survival (Rahbe *et al.*, 1995). Cereal aphids fed PIs from potato also showed reduced survival and fecundity compared to aphids on control diets (Tran *et al.*, 1997). Our studies have shown that cysteine PIs from a range of plant and animal sources also influence aphid growth, survival and fecundity in artificial diet assays. The variation in toxicity between the different cystatins may reflect their different actvity spectra. Maize cystatin has a wider inhibition spectrum than OcI and inhibits cathepsins H and L more efficiently than OcI (Abe *et al.*, 1994).

The observation that the rate of development, fecundity and doubling time of *M. persicae* were not affected when aphids were caged on transgenic plants establishes that tissue specific expression is important in determining effects of PIs on different invertebrates. Levels of expression in the root system of 0.4% total soluble protein were sufficient to provide nematode control (Urwin *et al.*, 1997) but concentrations in the phloem were insufficient to reproduce the adverse effects on aphids shown in the artificial diet assays.

The fact that PI-transformed plants do not stop aphids feeding on the plants immediately has implications for other components of the ecosystem. *M. persicae* and *M. euphorbiae* represent important prey items for several of the predators and parasites that are known to occur in potato crops. Aphid honeydew can also represent an important resource for some predators (Majerus, 1994). Artificial diet studies have shown that PI is present in the honeydew of aphids feeding on diet containing PIs (pers obs.). It is possible that aphid natural enemies may be exposed to cystatins expressed in transgenic plants via either of these two routes. Recent studies have shown that two important predatory insects can be adversely affected by ingestion of cystatins. Walker *et al.* (1998) showed that proteolytic activity of the larval

midguts of *Adalia bipunctata* was almost totally inhibited by E64 or CEWc. Ashouri *et al.* (1998) have shown that fecundity and egg eclosion were effected when a pentatomid predator of Colorado potato beetle larvae ingested oryzacystatin (Oc-I), via contaminated prey. These results highlight the importance of studying the effects of PI expression in transgenic plants at all trophic levels and we are currently examining the interaction between cystatin-expressing potatoes, aphids and their natural enemies.

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Honeybees as vectors of GM oilseed rape pollen

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ABSTRACT

One hive of honey bees, placed 800 m from a GM OSR crop, was studied to determine the extent of mixing of pollen taking place. In a one hour period, ten types of pollen were collected with OSR, rosebay willowherb and clover predominating. A combination of cytological analysis and PCR tests demonstrated that OSR pollen was spread through pollen loads of different types. This was likely to be due to worker bees switching crops between foraging trips, or surface contamination within the hive between worker bees returning from different crops. The presence of GM pollen in largely non-GM *Brassica* pollen loads, and the generation of mixed GM and non-GM progeny following the pollination of male sterile plants with pollen from single bees, demonstrates directly that bees can readily spread OSR pollen around their foraging area.

INTRODUCTION

Honeybees (*Apis mellifera*) are an important insect pollinator of oilseed rape (OSR, *Brassica napus* and *B. rapa*) in the UK, numerically more common than *Bombus* species in most field crops. In addition, oilseed rape provides beekeepers with the single most productive honeybee forage crop, and this mutual importance is exploited by oilseed rape growers and beekeepers alike to ensure both good seed set and good honey crops. Some farmers now pay beekeepers for pollination services, which is particularly important for growing non-restored male sterile hybrid OSR. Furthermore, the importance of managed honeybee colonies to OSR production is increasing as feral populations of honeybees are lost due to the spread of the parasitic mite *Varroa*.

Honeybees are known by beekeepers to forage regularly in a radius of about 2 km from the hive, leading to the 'three feet or three miles' rule when moving hives to avoid losing bees returning over familiar territory to the old site of the hive. OSR has a relatively high concentration of sugars in the nectar, and can attract bees from a longer distance. One professional beekeeper, for example, has reported bees in one colony placed in the Aberdeenshire hills flying to a rape field 5 km away (M. McGregor, pers. com.).

Individual honeybees are often regarded as being faithful to one plant type on a particular foraging trip or over days, although switches between forage species have been documented (Proctor, Yeo and Lack 1996). Although studies have been made on bee pollination in regard to quantifying dye analogue (Cresswell *et al.* 1995) or pollen (Ohsawa & Namai 1988) spread from flower to flower during single foraging trips, we are not aware of studies of the mixing and spread of OSR pollen at the level of the colony. In this paper we present

some observations on the foraging behaviour of one colony placed 600 m from a commercial spring-sown GM OSR trial, describe the apparent inconstancy of individual bee foraging behaviour and discuss this particularly in relation to the mixing of pollen that a colony may achieve over its foraging area.

MATERIALS AND METHODS

In mid-July 1998, a small colony of honey bees with brood on 6 frames, which was building up from a June swarm, was moved to a site 800 m from a GM OSR field of about 11 ha in Perthshire. The GM OSR was carrying a thioesterase gene from *Umbellularia californica* and an *npt*II marker gene conferring kanamycin resistance. A pollen sampler containing a screen with 4.5 mm diameter perforations was placed in the hive entrance and used to dislodge and collect pollen loads from returning worker honey bees. Pollen loads are masses of pollen grains, collected by grooming and mixing with nectar before transferring onto the hind legs where combs of bristles on a flattened segment (the pollen basket) aid the storage of the pollen for transport back to the colony. Pollen loads were sorted into colour classes, weighed, air dried and stored at room temperature. They were carefully dissected to sample five parts of the load from the position nearest the comb on the leg (determined by the puckering of the load at this point) to the most recent and outermost part. Pollen type was determined where possible by comparison with a colour guide to pollen loads (Kirk 1994) and verified by microscopic observation.

Samples were taken of worker bees emerging from the hive by trapping single bees in a clean plastic centrifuge tube on the hive wall as they emerged. This was performed on the same occasions as pollen sampling, within 30 min of the removal of the pollen sampler from the hive. Bees were stored at -20°C, and used for pollinating male sterile plants or for microscopic examination.

DNA was extracted from pollen loads and from the seedling progeny of pollinations made on male sterile plants and subjected to anchored-SSR PCR fingerprinting or PCR using primers to the *Umbellularia californica* thioesterase and *npt*II transgenes (see Thompson *et al*, these proceedings).

Some additional casual observations were made on a large established hive on the institute farm in an area with spring OSR within 1 km and no GM OSR within flying distance.

RESULTS AND DISCUSSION

Fingerprinting of pollen loads determines their origin

Fingerprints of pollen loads using anchored-SSR PCR (Charters *et al.* 1996) were used to determine the origin of pollen collected at an SCRI hive in an area where no GM OSR was grown. In this case, comparing pollen load patterns with field-collected leaf samples clearly shows the origin of the pollen samples as being cv. Ostara (Fig. 1). The nearest field of Ostara was 900 m from the hive. This method will be used in more extensive studies to examine the number of OSR sources which one colony may be working at any one time.

Pollen load samples

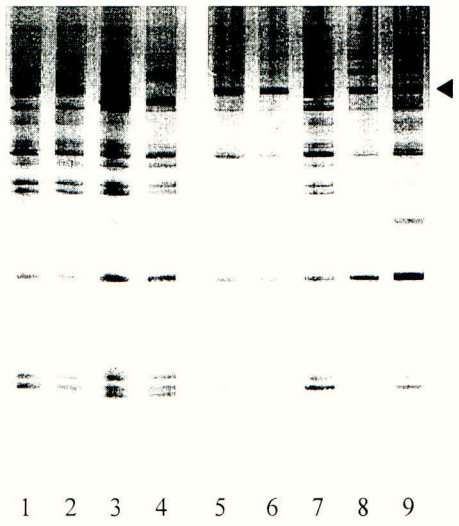

Figure 1. Anchored-SSR PCR fingerprints of pollen loads collected at a hive at SCRI compared to PCR fingerprints of leaf samples from candidate fields. 1-2, leaf samples of cv. Ostara at 900 m from the hive; 3-4, leaf samples of cv. Hyola 401 at 4 km from the hive; 5-9, five separate pollen loads from SCRI hives. An arrowhead marks a diagnostic band.

The data presented in table 1 were collected on 30 July 1998, while the GMO crop was still in full flower, and other OSR crops (the nearest at 500 m distance) were coming out of flower. In one hour, 483 pollen loads were collected from workers returning to the hive placed 800 m from the GMO OSR crop under study. Ten classes were discriminated by the colour of the fresh pollen load (Table 1). *Brassica* pollen accounted for 70% of the pollen loads, with rosebay willowherb (*Chamaenerion angustifolium*) and clover (*Trifolium repens*) making up the majority of the remainder. As OSR was the only *Brassica* type present locally in quantity, we have assumed that all *Brassica* pollen is OSR. Although this represents only one short period of activity of one hive, similar proportions of OSR and non-OSR pollen grains were noted in samples collected earlier in the same month at a hive at SCRI. Close inspection of the pollen loads revealed that although each one is largely composed of one type of pollen grain, many carried a yellow patch where the load had adhered to the comb on the worker's hind leg.

Table 1. Frequency of pollen loads of different types recovered during one hour of sampling.

	Pollen load type									
	1	2	3	4	5	6	7	8	9	10
no.	338	98	37	3	2	2	2	2	2	1
% of total	70	20	8	1	t	t	t	t	t	t
fresh wt (mg)	2308	448	148	19	23	16	7	7	6	8

1 *Brassica* sp., oilseed rape; 2 *Chamaenerion angustifolium*, rosebay willowherb; 3 *Trifolium repens*, clover; 4-10 various *Asteraceae, Rosaceae* and other taxa; t - trace.

Four pollen loads were dissected and examined in more detail to determine the admixture of other pollen (Table 2). Five central portions were taken from each of five sections of the pollen load, numbered from 1 (closest to the original point of attachment to the worker's leg) to 5 (the most recently collected pollen).

Table 2. The percentages of total pollen grains falling into different types in five sections each of 4 pollen loads of different type.

No	1° pollen type	Patch	Sectn.	Percentage of Pollen Grains							
				bra	rbw	clo	urt	cir	com	oth	*n*
1	rbw	N	1	7	93	-	1	-	-	2	150
			2	13	85	-	-	-	-	1	295
			3	9	89	-	1	-	-	1	330
			4	3	95	-	1	-	-	1	466
			5	10	88	-	1	-	-	-	582
2	rbw	Y	1	65	34	-	1	-	-	-	437
			2	36	63	-	1	-	-	-	212
			3	1	99	-	-	-	1	-	140
			4	13	83	-	-	1	1	1	78
			5	16	84	-	-	-	-	-	114
3	clo	N	1	2	-	98	-	-	-	t	273
			2	t	-	99	t	-	-	-	353
			3	2	-	98	-	-	t	-	284
			4	-	-	100	-	-	t	-	241
			5	1	-	99	-	-	1	-	375
4	bra		1	100	-	-	-	-	-	-	407
			2	100	-	-	-	-	-	-	383
			3	100	-	-	-	-	-	-	374
			4	100	-	-	-	-	-	t	366
			5	100	-	-	-	-	-	-	397

Patch – an area of visually different pollen grains at the attachment point, bra – *Brassica* species, rbw – rosebay willowherb *Chamaenerion angustifolius*, clo – clover *Trifolium repens*, urt – nettle *Urtica* sp., cir – thistle *Cirsium* sp., com – other composite *Asteraceae*, oth – other, t - trace.

All four pollen loads carried more than the main pollen type. Trace amounts of pollen of several species were found, including that of *Urtica* (nettle), an anemophilous type. The higher numbers of *Urtica* pollen grains in the rosebay willowherb pollen loads may arise from aerial contamination at the time of collecting due to the frequent proximity of these two plants in fertile waste ground. There was no particular pattern to the distribution of these types of trace admixtures across each pollen load as they were present across all five parts of the loads sampled. *Brassica* pollen was almost ubiqitous. Molecular studies later confirmed that this was OSR pollen. The greatest amount was found in pollen load no. 2 close to the point of attachment to the comb, where 65% of pollen grains scored were OSR. This pollen load was composed predominantly of rosebay willowherb pollen but carried a noticeable yellow patch. The pattern of OSR pollen in this pollen load strongly suggests that the bee

previously visited OSR. The OSR pollen in the oldest part of the pollen load could represent either a portion of the previous load adhering to the comb, or further pollen grains groomed and added to the comb after the main load had been placed in the pollen basket for transport. The spread of OSR pollen grains throughout the pollen load suggests that the bee recovered additional OSR grains still contaminating its body surface while grooming to collect rosebay willowherb grains during a foraging trip. The alternative possibility of bees on foraging trips primarily visiting one flower type but regularly moving to OSR during the trip seems unlikely as no banding was detected in any pollen load. The switching from one crop to another between foraging trips was confirmed by microscopic examination of a sample of 30 workers captured emerging from the hive. Most of the workers had signs of OSR pollen adhering to wings and body hairs, or in caked masses on legs, the face and in the pollen baskets. Pollen grains could be seen at different depths on the baskets, indicating the latest and previous foraging activity. Some had last visited OSR after previous visits to rosebay willowherb. In others the reverse was true, and other possible foraging histories were noted.

Mixing of GM pollen with other pollen

The admixture of OSR pollen in non-OSR pollen loads suggested that mixing was taking place. To investigate the mixing of pollen further, DNA was extracted from several OSR, rosebay willowherb and clover pollen loads. Both pollen load 1 and pollen load 2 (Table 2), predominantly rosebay willowherb but with 8.4% and 26.2% *Brassica* content respectively, gave a positive PCR signal (not shown). Among the OSR pollen loads tested (Fig. 2 lanes 14-19), two of the 6 OSR pollen loads gave strong signals for the thioesterase transgene (Fig. 1) suggesting that these were largely composed of GM pollen. Most of the other OSR pollen loads and the rosebay willowherb (lanes 2-7) and clover (lanes 8-13) pollen loads gave a weak signal for the transgene. In a larger sample of OSR pollen loads, 17/44 did not give any detectable signal for GM OSR. This, together with the cytological determination of OSR pollen contamination throughout the 4 pollen loads examined, demonstrates clearly that the GM pollen was widely dispersed on bees in the colony.

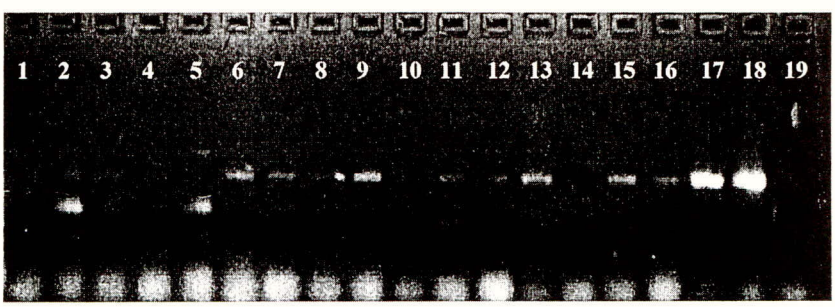

Fig. 2. PCR detection of pollen containing the *Umbellularia californica* thioesterase gene in pollen loads collected from workers returning to the hive. 1, negative control; 2-7, rosebay willowherb pollen loads; 8-13, clover pollen loads; 14-19, oilseed rape pollen loads.

Whether this mixing occurs because of regular switching between foraging on GM rape and other flowers, or by bee to bee spread within the colony was not clear. However one worker returning to an SCRI hive and bearing the typical yellow contamination on the face and elsewhere typical of bees working OSR was found to be carrying 60,000 OSR pollen grains in addition to those in the pollen loads. Workers clean up within the hive and generally emerge free of rape pollen visible to the unaided eye. It seems, therefore, that bee to bee pollen transfer within the hive is inevitable.

Five visually clean bees collected on emergence from the hive near the GM site were used to pollinate flowers on male sterile OSR plants. Two of these failed to induce seed set. The other three all induced fertilisation and the progeny seedlings from each bee contained both transgene carrying and normal individuals. Overall from 62 seedlings screened, 12 carried the transgenes. This demonstrates the mixed nature of the OSR pollen on these bees, and also that this pollen is viable.

The observations presented here illustrate that bees switch from one forage type to another, and that bees carrying many viable OSR pollen grains can be found emerging from a hive. The OSR pollen found on bees leaving a hive can be from mixed sources, indicating either switching between crops and relatively long persistence of the pollen grains on bees, or the picking up of some of the larger numbers of loose pollen grains available on other bees in the close phyical contact in the hive. With most honeybee colonies foraging up to 2 km from the hive, some pollen transfer and fertilisation up to 4 km must be expected. In some circumstances honeybees are capable of much longer foraging trips and these limits will be exceeded.

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