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RESEARCH ARTICLE

Age-related increase in levels of insecticidal protein in the progenies of transgenic oilseed rape and its efficacy against a susceptible strain of diamondback moth

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Abstract

Many crops transformed with insecticidal genes isolated from *Bacillus thuringiensis* (Bt) show resistance to targeted insect pests. The concentration of Bt endotoxin proteins in plants is very important in transgenic crop efficacy and risk assessment. In the present study, changes in levels of Cry1Ac protein in the leaves of transgenic Bt oilseed rape (*Brassica napus*) carrying a Bt *cry1Ac* gene under the control of the cauliflower mosaic virus 35S promoter were quantified during vegetative growth by enzyme-linked immunosorbent assay. Plants were grown in a glasshouse, sampled at 2, 4, 5 and 6 weeks, and the concentration of Cry1Ac was quantified in basal, top and previous top leaves. The mean concentration differed between sowing dates when Cry1Ac concentration was expressed as ng g^{-1} fresh leaf weight but not when expressed as ng mg^{-1} total soluble protein. It was demonstrated that Cry1Ac concentration increased significantly as the leaf aged, while the total soluble plant protein decreased significantly. Levels of Cry1Ac were therefore higher in leaves at the base of the plants than in leaves close to the growing point. However, even young leaves with very low Cry1Ac concentrations caused high mortality in the larvae of a Cry1Ac-susceptible laboratory strain of the diamondback moth. The feeding area of leaves consumed by larvae *in vivo* and *in situ* was similar. Leaf damage caused by sampling (i.e. artificially) or by feeding of larvae did not affect the levels of Cry1Ac in the leaves under the experimental conditions in this study.

Introduction

Brassica crops are important vegetable and oilseed crops in many parts of the world (Raymer, 2002). Pests such as the diamondback moth (*Plutella xylostella* L.) remain a major problem, despite advances in pest control (Talekar & Shelton, 1993). A number of *Brassica* spp. have been transformed with δ-endotoxin genes from *Bacillus thuringiensis* (Bt) to produce transgenic plants with high levels of resistance to such lepidopteran pests (Metz *et al.*, 1995; Stewart *et al.*, 1996*a*, Jin *et al.*, 2000; Zhao *et al.*, 2000; Kuvshinov *et al.*, 2001), but so far none of these Bt plants have been commercially released.

Oilseed rape (*Brassica napus* L.), also known as canola, was used as a model *Brassica* crop in this study. It is a major arable crop in many countries, including the UK, central Europe, North America and Australia (Sovero, 1993). *B. napus* was successfully transformed by Stewart *et al.* (1996*a*), with a truncated synthetic *cry1Ac* Bt gene under the control of the cauliflower mosaic virus

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promoter (CaMV, 35S). They found that the resulting transgenic plants caused 100% mortality of a susceptible strain of *P. xylostella* larvae (Stewart *et al.*, 1996*a*). Progenies from this transgenic oilseed rape line have been utilised as the first trophic level in several tri-trophic effect studies (e.g. Schuler *et al.*, 1999, 2001, 2004), with a Bt-resistant strain of *P. xylostella* (NO-QA) (Tabashnik *et al.*, 1997) as the second trophic level. However, no data were reported on the detailed synthesis levels of Bt Cry1Ac insecticidal crystal proteins in plants, especially at various developmental stages and organs of the plant, and on its effect on susceptible *P. xylostella* larvae, which is the focus of this study.

The CaMV 35S promoter and its derivatives have been used in the majority of insect-resistant transgenic plants (Schuler *et al.*, 1998). Although not completely constitutive, this promoter produces continuous gene expression in most plant tissues. However, recent studies of Bt cotton in the USA, Australia and China have shown that levels of gene expression in cotton vary depending not only on plant tissue but also on plant age and environmental conditions (Fitt *et al.*, 1994; Holt, 1998; Sachs *et al.*, 1998; Zhang *et al.*, 2001). Reductions in toxin expression have been shown to lower the resistance of Bt cotton to pests such as cotton bollworms (Fitt *et al.*, 1998; Sachs *et al.*, 1998; Zhang *et al.*, 2001). Understanding the expression patterns of Bt toxins in transgenic plants is vital for successful Bt resistance management as well as for predicting the magnitude of any nontarget effects because risk is a function of both the nature of and exposure to the hazard (Poppy, 2004).

The present study investigated the age-related variation in transgenic expression levels in Bt oilseed rape and its efficacy against insect pests to provide important information for the pest management as well as the environmental risk assessment. In contrast to earlier studies with cotton, this study concentrated on the vegetative phase of plant growth, which is, for many *Brassica* spp., the stage at which the plant is most vulnerable to attack by lepidopteran species. The effect of leaf damage on synthesis levels of Cry1Ac in leaves was also investigated.

Materials and methods

Plants

The spring oilseed rape line (*B. napus* cv. Oscar, line O52) used in this study expressed a synthetic Bt *cry1Ac* gene under the control of the CaMV 35S promoter. The plants were transformed with the Bt *cry1Ac* cassette that also carried a hygromycin phosphotransferase gene as a selectable marker, placed in vector pH 602, renamed PH602-Bt (Stewart *et al.*, 1996*a*,*b*). All plants used in this study were the progeny of a single selfed F_3 plant. Untransformed wild-type plants of the parent cv. Oscar were used as control plants. Plants were grown in 13-cm pots in a glasshouse at Rothamsted Research (Hertfordshire, UK) set at 20°C and a minimum day length of 14 h.

Leaf samples were taken from plants at 2, 4, 5 and 6 weeks after sowing. The plants were sampled until just before inflorescences emerged. Leaf samples taken included the first true ('basal') leaf, the youngest expanding ('top') leaf and, after the first sampling, also the leaf that was the youngest expanding leaf at the previous sampling date (previous top leaf). The position of the previous top leaf was labelled 1 week before sampling when it was still the top expanding leaf. Five plants were sampled at each sampling date, and fresh plants were used at each sampling date. The experiment was repeated thrice. The sowing dates for the three experiments were 16 August 2000, 28 November 2000 and 27 January 2001, respectively.

Insects

A Bt-susceptible strain of *P. xylostella*, originally from the Philippines, and a Bt-resistant strain of *P. xylostella* (NO-QA), originally selected with a microbial Bt formulation and resistant to Cry1Ac protein (Tabashnik *et al.*, 1997), were used in this study. Both strains were cultured on the untransformed oilseed rape cv. Falcon at 22 ± 3°C.

Expression assay

For the expression assay, samples of fresh leaf tissue were homogenised in 400 μL of phosphate buffered saline plus 0.05% Tween-20, using micro homogenisers and a cordless drill. Each homogenate was centrifuged at 18.000g for 10 min. Total soluble protein (TP) content was measured using the Bio-Rad protein assay (Bio-Rad, Watford, UK). The total protein content of each leaf sample was measured at two concentrations and the results averaged to obtain an accurate result.

Concentrations of Cry1Ac toxin in leaf samples were quantified with the commercial Btk ELISA PathoScreen kit for *B. thuringiensis kurstaki* Cry1Ac protein expressed in genetically modified plants, following the standard protocol provided by the manufacturer (Agdia, Elkhart, IN, USA) except that milk powder and Tween-20 were only added after the Bio-Rad protein assay. As dilution may affect the quantification of Bt protein, each sample was tested at two concentrations of total protein, and the mean value was taken as the final Cry1Ac. All absorbency

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data from both Bio-Rad protein assay and enzyme-linked immunosorbent assay (ELISA) were recorded by a Labsystems Multiskan RC microplate reader (Helsinki, Finland). The concentrations were expressed as ng mg^{-1} TP and ng g^{-1} fresh leaf weight (FW) or as ng mm^{-2} leaf area (LA) where appropriate.

Leaf consumption

Leaf discs are commonly used for bioassays with transgenic plants in order to standardise the amount of food supplied to the test insects (Kota *et al.*, 1999; Schuler *et al.*, 2003). It was therefore important to assess if the feeding behaviour of larvae on leaf discs is comparable to that of larvae on intact leaves because it is known that there can be considerable chemical changes induced by herbivory (Karban & Baldwin, 1997). Whether the damage to leaves alters the Bt toxin levels in leaves was also investigated. Additional plants of transgenic *B. napus* and nontransgenic *B. napus* cv. Falcon were sown on 13 October 2000 and grown until nearly 6 weeks old. Three bioassays were performed to assess the effects of leaf damage on the level of Cry1Ac in leaves and on the extent of larval feeding damage. Previous top leaves were used as plant material in these three bioassays. The size of the leaf discs used was 3.8 cm in diameter. In the first bioassay, leaf discs were cut from one leaf (of a same age and position) of each of 10 Bt plants. Cry1Ac concentrations were quantified by ELISA. The cutting of discs represented artificial damage to the leaves. Six hours later, Cry1Ac concentrations in the same leaves were quantified to assess the effect of this artificial damage. In the second bioassay, both intact leaves *in situ* and leaf discs cut from leaves of untransformed *B. napus* cv. Falcon were fed to susceptible *P. xylostella* larvae. Leaf discs used *in vivo* were generally cut from the leaves of individual oilseed rape plants at similar positions and with leaf sizes similar to the ones used for the bioassay *in situ*. Two second instar larvae were placed on each intact leaf (n = 10) and on each leaf disc (n = 10). Larvae were allowed to feed for 24 h during which time they did not move off the leaves. The amount of feeding damage (area mm^{-2}) caused by the larvae was measured using a graph paper. In the third bioassay, the Cry1Ac concentrations of leaves on intact Bt plants were quantified before placing two second instar larvae of the resistant *P. xylostella* strain on eight leaves, one from each of eight individual Bt plants, and the Cry1Ac levels of the same leaves were again quantified after 20-h feeding (i.e. after feeding damage). The experiments were carried out at a temperature of 25 ± 2°C and a light : dark cycle of 18:6 h.

Effect of differences in Cry1Ac levels on diamondback moth larvae

Three further bioassays were performed using nineteen 5-week-old transgenic Oil seed rape (OSR) plants. At this age, the basal leaf was old enough to contain a high amount of Bt protein, healthy and not senescent. Following quantification of Cry1Ac levels, leaves with the highest Cry1Ac content [normally the first true (oldest) leaf] and leaves with the lowest content [normally the top (youngest expanding) leaf] of the same plants were collected and immediately tested for efficacy against larvae of the susceptible *P. xylostella* strain. A leaf disc (3.8-cm diameter) cut from either a high Cry1Ac content leaf or a low Cry1Ac content leaf was placed in a Petri dish (9-cm diameter), and 10 second instar *P. xylostella* larvae were added. The first bioassay was replicated 10 times for each Cry1Ac level (i.e. one high Cry1Ac content leaf and one low Cry1Ac content leaf were sampled from each of 10 plants), the second bioassay 4 times and the third bioassay 5 times. It has been previously reported that survival of susceptible second instars of *P. xylostella* on transgenic broccoli leaves expressing Bt Cry1Ac protein declined significantly after 72-h feeding (Tang *et al.*, 1999). Hence, larval mortality in each bioassay was assessed after 3 days at 25 ± 2°C and a light : dark cycle of 18:6 h.

Statistical analysis

The Cry1Ac concentrations of test samples were obtained from their ELISA optical density (OD) values via interpolation from a nonlinear curve fitted to the OD values of Cry1Ac standards using the statistical package Genstat (Genstat 5 Committee, 1997). Analysis of variance (ANOVA) in SAS 8.0 (SAS Institute, 1998), with variation partitioned appropriately, was used to test for treatment effects on Cry1Ac concentrations and TP levels. The raw data were transformed by either logarithmic (base 10) or square root transformation where appropriate to satisfy the assumption of equal variances for ANOVA procedure. The percentage mortality of larvae on basal and top leaves was compared using a binomial test. The effects of artificial and feeding damage on Cry1Ac levels in transgenic *B. napus* leaves were tested using two-tailed paired *t*-tests (bioassays 1 and 3) or a two-tailed standard *t*-test (bioassay 2) in Microsoft Excel.

Results

Levels of Cry1Ac in leaves

After allowing for overall differences among experiments ($F_{2,6}$ = 4.37, P = 0.067, based on mg TP; $F_{2,6}$ = 15.33,

$P = 0.0044$, based on FW), the concentration of Cry1Ac in the basal leaves of transgenic *B. napus* plants increased significantly with plant age when measured based on TP ($F_{3,6} = 44.23$, $P < 0.001$) and when measured based on FW ($F_{3,6} = 29.29$, $P < 0.001$) (Table 1).

The top expanding leaves cannot be recognised at 2 weeks; thus, Cry1Ac concentrations of only three plant ages (4, 5, 6 weeks old) were quantified for top leaves. When comparing top leaves with basal leaves, there was a significant effect of sowing date when Bt Cry1Ac expression was measured as ng g^{-1} FW ($F_{2,4} = 29.32$, $P = 0.0041$) but not when measured as ng mg^{-1} TP scale ($F_{2,4} = 0.61$, $P = 0.59$). Significant effects of age were detected when measured based on FW ($F_{2,4} = 15.91$, $P = 0.0125$) and on TP ($F_{2,4} = 21.75$, $P = 0.0071$). The concentration in basal leaves was always significantly higher than in top leaves ($F_{1,36} = 1262.60$, $P < 0.001$, based on TP; $F_{1,36} = 436.14$, $P < 0.001$, based on FW; Table 2). There were significant interactions between age and position ($F_{2,36} = 61.33$, $P < 0.001$, based on TP; $F_{2,36} = 19.54$, $P < 0.001$, based on FW), that is, the difference between top and basal leaves differed depending on age.

Concentrations differed in top and previous top leaves (Table 3) ($F_{1,48} = 83.03$, $P < 0.001$, based on TP; $F_{1,48} = 34.07$, $P < 0.001$, based on FW), which demonstrated that the Bt levels within top expanding leaves significantly increased after permitting for 1 week's growth. There were only two comparisons in each experiment between Cry1Ac concentration in the newly emerged leaves and that in the previous top leaves 1 week later, as no leaf was sampled later than 6 weeks after sowing, that is, new leaves at 4 and 5 weeks versus previous top leaves at 5 and 6 weeks, respectively. Cry1Ac levels either in top or previous top leaves at same positions did not differ between different sampling periods (i.e. sampling from fourth to fifth week and from fifth to sixth week after sowing, respectively) ($F_{1,2} = 0.68$, $P = 0.41$, based on TP; $F_{1,2} = 6.6$, $P = 0.12$, based on FW), and there was no interaction between the leaf types and the different sampling periods ($F_{1,48} = 0.73$, $P = 0.40$, based on TP; $F_{1,48} = 2.46$, $P = 0.12$, based on FW). The overall Cry1Ac levels were not very different for each sowing date ($F_{2,2} = 10.30$, $P = 0.09$) based on TP, while the difference was significant based on FW ($F_{2,2} = 37.04$, $P = 0.026$).

Total soluble protein in plant leaves

In contrast to Cry1Ac levels, the TP (Table 1) in basal leaves of transgenic OSR, expressed as mg g^{-1} FW, decreased significantly with plant age ($F_{3,6} = 25.75$, $P = 0.0008$) after allowing for significant differences overall amongst the three sowing dates ($F_{2,6} = 10.71$, $P = 0.011$).

The overall level of TP differed amongst sowing dates ($F_{2,4} = 64.60$, $P = 0.0009$) and was significantly greater in top (6.50 mg g^{-1} FW) leaves than in basal (2.75 mg g^{-1} FW) leaves ($F_{1,36} = 516.63$, $P < 0.001$, $n = 45$, SED = 0.286, d.f. = 36, root square transformation). There was no significant effect of age ($F_{2,4} = 1.97$, $P = 0.25$) and no interaction between plant age and leaf position ($F_{2,36} = 0.50$, $P = 0.61$).

Total soluble protein in top leaves was significantly higher than in previous top leaves ($F_{1,48} = 151.17$, $P < 0.001$; Table 4); however, there was also an interaction between this term and the sampling periods ($F_{1,48} = 58.43$, $P < 0.001$). However, there were no overall differences amongst sowing dates ($F_{2,2} = 2.87$, $P = 0.26$) or between the different ages of the whole plants ($F_{1,2} = 0.01$, $P = 0.93$).

Effect of leaf damage on Cry1Ac levels

In the first bioassay, concentrations of total soluble plant protein (Table 5) were not affected by artificial damage $t = -2.33$, $P = 0.064$, d.f. = 9) although the difference bordered on significance. Cry1Ac levels (Table 5) were also not affected when Cry1Ac concentration was expressed as ng mg^{-1} TP ($t = 2.09$, $P = 0.066$, d.f. = 9),

Table 1 Mean (n = 15) Bt Cry1Ac and total soluble protein concentrations in basal leaves of transgenic *Brassica napus* plants of various ages

Number of Weeks After Sowing (plant age)	Bt Cry1Ac Concentration (ng mg^{-1} TP)[a]	Bt Cry1Ac Concentration (ng g^{-1} FW)[b]	Total Soluble Protein Concentration (mg g^{-1} FW)[b]
2	1.24	19.81	4.73
4	1.64	24.18	3.65
5	2.06	26.59	2.50
6	2.45	34.10	2.08
SED (d.f.)	0.112 (6)	1.565 (6)	0.333 (6)

TP, total soluble protein; FW, fresh leaf weight.
[a]Logarithmic transformation (base 10).
[b]Square root transformation.

Table 2 Mean (n = 15) Bt Cry1Ac levels in basal and top leaves of transgenic *Brassica napus* plants of various ages

Leaf Position	Number of Weeks After Sowing (plant age)	Bt Cry1Ac Concentration (ng mg^{-1} TP)[a]	Bt Cry1Ac Concentration (ng g^{-1} FW)[b]
Top	4	0.74	15.98
	5	0.83	15.85
	6	0.54	13.88
Basal	4	1.64	24.18
	5	2.06	26.59
	6	2.45	34.10
SED (d.f.)[c]		0.066 (36)	1.083 (36)
SED (d.f.)[d]		0.069 (12.57)	1.047 (16.13)

TP, total soluble protein; FW, fresh leaf weight.
[a]Logarithmic transformation (base 10).
[b]Square root transformation.
[c]SED for comparing means with the same level of age.
[d]SED for all other comparisons.

although the test here again bordered on significance, or as ng g^{-1} FW (t = 0.85, P = 0.417, d.f. = 9) or as ng mm^{-2} LA (t = 0.40, P = 0.701, d.f. = 9). In the second bioassay, the feeding area was 13.3 mm^2 (SEM 2.42 mm^2, n = 10) when larvae fed on intact leaves compared to 13.5 mm^2 (SEM 4.39 mm^2, n = 10) when fed leaf discs (t = −0.04, P = 0.969, d.f. = 18). Artificial damage, therefore, did not affect the feeding rate of larvae, and it can be assumed that it also did not affect the total Bt protein that larvae digested. In the third bioassay, when Bt-resistant *P. xylostella* larvae were allowed to feed on the leaves for 20 h, the TP was not different before and after damage (Table 5) (t = −1.91, P = 0.098, d.f. = 8). Similarly, Cry1Ac concentrations (Table 5) were similar before and after damage when the Cry1Ac concentration was expressed as either ng mg^{-1} TP, ng g^{-1} FW or ng mm^{-2} LA (t = 1.397, 1.0698 and 1.107, with P = 0.205, 0.320 and 0.305, respectively; d.f. = 8).

Effect of differences in Cry1Ac levels on *Plutella xylostella* larvae

The lowest Cry1Ac concentration in youngest expanding (top) leaves as quantified by ELISA was 1.9 ng mg^{-1} TP (mean = 5.2 ± 1.57 ng mg^{-1}, n = 19), while the highest Cry1Ac concentration in mature (basal) leaves was 228.43 ng mg^{-1} TP (mean = 170.6 ± 23.31 ng mg^{-1} TP, n = 19). Similar results were obtained in each of the three bioassays: 100%, 100% and 94% (SE 3.4%) of larvae, respectively, fed mature leaves died, but the newly emerged leaves only caused 97% (SE 1.7%), 93% (SE 4.2%) and 86% (SE 4.9%) mortality, respectively. On combining results from all three bioassays, there was evidence of a difference [P = 0.011; 95% confidence interval for the difference (1.26%, 9.3%)] in the survivorship of larvae on mature (98%, n = 190) versus newly emerged (93%, n = 190) Bt leaves. Larvae that survived to day 3 had not developed normally, were not much bigger than on the day of treatment and caused very little feeding damage.

Table 3 Mean (n = 30) Bt Cry1Ac levels in top expanding leaves, and these top leaves aged for 1 week of transgenic *Brassica napus*

Leaf Age	Bt Cry1Ac Concentration (ng mg^{-1} TP)[a]	Bt Cry1Ac Concentration (ng g^{-1} FW)[b]
Top	0.78	15.92
Previous Top	1.28	22.81
SED (d.f.)	0.055 (48)	1.180 (48)

TP, total soluble protein; FW, fresh leaf weight.
[a]Logarithmic transformation (base 10).
[b]Square root transformation.

Discussion

The Cry1Ac levels in transgenic oilseed rape were presented on two scales of measurement in this study: as ng mg^{-1} TP in the extract and as ng g^{-1} FW of leaf tissue. The former reflects the magnitude of Cry1Ac expression relative to the level of expression of other soluble protein genes, while the latter reflects the magnitude of Cry1Ac expression independent of the level of expression of other soluble protein genes (Sachs *et al.*, 1998). Higher concentrations of Bt expressed as ng mg^{-1} TP do not necessarily mean a higher concentration expressed as ng g^{-1} FW, which might depend on the TPs recovered from plant tissue. In this study, the Cry1Ac concentration in basal leaves, when expressed as ng mg^{-1} total protein, approximately doubled each week for the first and third sowing dates and increased approximately threefold each week for the second sowing date (data for individual sowing dates not shown here). The Cry1Ac concentration of basal leaves was unusually high 6 weeks after the second sowing date (sown on 28 November 2000 and measured on 10 January 2001) when most of the basal leaves measured had turned yellow. Basal leaves were still green at this time for the other two sowing dates. However, this effect was not apparent when Cry1Ac concentration was measured in ng g^{-1} FW. On this scale, the concentration in basal leaves for the second sowing date was lower than that for the other two sowing dates. The results confirmed that estimates of Cry1Ac expression based on measures of either TP or FW can give rise to different conclusions.

The results of Sachs *et al.* (1998) implied that *cry1A* expression and the synthesis of other soluble proteins were influenced by similar factors, yet they behaved differently. Our results corroborate this as in our study, Bt Cry1Ac concentration increased while that of TP

Table 4 Mean (n = 30) total soluble protein concentrations in top expanding leaves, and these top leaves aged for 1 week of transgenic *Brassica napus*

Leaf Age	Sampling Period	TP Concentration (mg g^{-1} FW)[a]
Top	1[b]	1.64
	2[c]	1.53
Previous Top	1[b]	1.34
	2[c]	1.46
SED (d.f.)[d]		0.015 (48)
SED (d.f.)[e]		0.032 (2.56)

TP, total soluble protein; FW, fresh leaf weight.
[a]Logarithmic transformation (base 10).
[b]Sampled the top and previous top leaves at fourth and fifth week after sowing, respectively.
[c]Sampled the top and previous top leaves at fifth and sixth week after sowing, respectively.
[d]SED for comparing means with the same level of sampling period.
[e]SED for all other comparisons.

decreased as leaves aged (Table 1). Because glasshouse temperatures were relatively stable (25 ± 2°C), the main environmental factor that differed among different dates was probably solar irradiance. However, Cry1Ac concentration expressed as ng mg^{-1} TP did not differ between sowing dates. Differences in total protein reached significance, and the concentration expressed as ng g^{-1} FW differed significantly, which is arguably more biologically relevant than comparisons using TP as a metric.

It was clearly demonstrated that, during the vegetative phase of plant growth, the Cry1Ac concentrations, either in ng mg^{-1} TP or in ng g^{-1} FW, increased as transgenic OSR leaves aged. In contrast, Bt cotton has been reported to demonstrate reduced Cry1Ac expression, as plants age (Benedict *et al.*, 1996; Holt, 1998; Sachs *et al.*, 1998). However, cotton was monitored throughout the whole season and the decrease in expression levels was mainly noticed once the plants started to flower and set fruit (Holt, 1998; Sachs *et al.*, 1998), while the Bt oilseed rape plants in this study were sampled before flowering. In these previous studies, the youngest expanding terminal leaf was generally sampled to measure Bt expression, which was at a similar position to the top expanding leaf we sampled in oilseed rape. However, Bt concentration in this type of leaves in Bt oilseed rape did not differ between 4 and 5 weeks after sowing in this study, neither did the Bt levels in previous leaves of 5- and 6-week-old plants.

Leaf bioassays are an easy, repeatable and highly tractable method. Schuler *et al.* (2003) used similar bioassays and found that transgenic Bt Cry1Ac *B. napus* plants did not have sublethal effects on a Cry1Ac-resistant strain of *P. xylostella* larvae. However, it was important to also establish if leaf assays give similar results to *in vivo*/*in situ* studies and if insect-feeding behaviour is affected in *in vivo* assays. The present results show that the LA consumed by the larvae did not differ significantly between *in vivo* and *in situ* assays. The promoter used to transform the Bt oilseed rape plants, CaMV 35S, is not an inducible promoter (Odell *et al.*, 1985), and it was therefore not surprising that neither artificial nor insect damage affected Cry1Ac levels in leaves. However, damage can induce changes in plant chemical components including an accumulation of defence proteins (Karban & Baldwin, 1997). The accumulation of such proteins would not necessarily affect the total Cry1Ac content in leaf tissues but might dilute Cry1Ac concentration when measured in ng mg^{-1} TP. In contrast, measurements of ng Cry1Ac per unit leaf weight or leaf area would not be affected by changes in other soluble proteins, thus could be a more relevant representation.

Stewart *et al.* (1996*a*) utilised Western blot analysis and reported an average expression level of 586 ng mg^{-1} TP for 2-week-old T_1 plants of the Bt oilseed rape line used in this study (Oscar 52). The plant material used in this study was the F_4 generation derived from a single selfed F_3 plant. In contrast to the results of Stewart *et al.* (1996*a*), the average expression level of Cry1Ac in 2-week-old plants across the three experiments in this study was only 20.2 ± 2.82 ng mg^{-1} TP. (It turned to be 1.24 ng mg^{-1} after a logarithmic transformation, see Table 1.) The difference might be as result

Table 5 Effect of artificial/feeding damage on mean (±SEM) Cry1Ac levels in transgenic *Brassica napus* leaves

	Total Soluble Plant Proteins of Bt Oilseed Rape (mg g^{-1})		Bt Protein Expressed as ng mg^{-1} TP		Bt Protein Expressed as ng g^{-1} FW		Bt Protein Expressed as ng mm^{-2} Feeding Area of Larvae	
	Before	After	Before	After	Before	After	Before	After
Artificial damage (bioassay 1; n = 10)	22.9 ± 1.47	25.1 ± 1.45	32.7 ± 4.78	28.9 ± 4.13	726.9 ± 97.78	701.7 ± 92.64	0.15 ± 0.019	0.14 ± 0.019
Feeding damage (bioassay 3; n = 8)	21.5 ± 1.36	23.3 ± 1.09	29.7 ± 5.41	23.4 ± 1.64	603.6 ± 70.55	538.5 ± 31.54	0.12 ± 0.012	0.10 ± 0.007

TP, total soluble protein; FW, fresh leaf weight.

of the different methods employed in detection of Cry1Ac protein, advanced generations used in this study or environmental differences.

However, plants from the same F_3 seed descent used in the present study have been tested in a series of bioassays over several years, and mature leaves of these plants have always caused 100% mortality of susceptible *P. xylostella* larvae after 5–6 days in laboratory bioassays (Schuler *et al.*, 2004). In the final part of this study, a Cry1Ac level as low as 1.9 ng mg^{-1} in total plant protein was enough to kill most of the susceptible *P. xylostella* larvae, and there was a significant difference in the mortality of larvae between old and young leaves. Tang *et al.* (1999) reported that the effect on the survival of susceptible *P. xylostella* was similar among leaves at various positions on the transgenic broccoli expressing Cry1Ac protein. They used bioassays rather than ELISA to measure variation expression of Bt Cry1Ac in various parts of plants and found that the variation was relatively small compared to the difference between transgenic and nontransgenic plants. However, when we considered exclusively on the difference among leaves within transgenic plants, we were not surprised to find a difference in mortality of diamondback larvae caused by the oldest and the youngest leaves.

In conclusion, this study demonstrated that Cry1Ac concentration in leaves was affected by plant age during vegetative growth stages of transgenic *B. napus*. The results suggested that plant age should be taken into account in efficacy assays as well as for nontarget risk assessment of transgenic crops.

No significant changes of Bt Cry1Ac concentration, expressed as ng mg^{-1} TP, ng g^{-1} FW or ng mm^{-2} LA, were found after artificial or feeding damage. Neither was significant differences found in feeding area when intact leaves or leaf discs were fed to diamondback moth larvae. The results showed that leaf assays utilising leaf discs could give similar results to *in situ* studies.

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