Research Article

Received: 26 September 2014 Revised: 15 December 2014 Accepted article published: 7 January 2015 Published online in Wiley Online Library: 30 January 2015

(wileyonlinelibrary.com) DOI 10.1002/ps.3971

Inheritance, fitness costs, incomplete resistance and feeding preferences in a laboratory-selected MON810-resistant strain of the true armyworm *Mythimna unipuncta*

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Abstract

BACKGROUND: The low efficacy of MON810 maize against *Mythimna unipuncta* represents a scenario of non-compliance with the 'high-dose' strategy, raising concerns about potential resistance development and outbreaks of this secondary pest. The present study offers insight into the different components related to resistance in a laboratory-selected MON810-resistant (MR) strain of *M. unipuncta*.

RESULTS: The resistance in the MR strain is autosomal and inherited as a partially dominant trait. We have found a lack of fitness costs in this strain for essential life history traits, reproductive potential and most of the population growth parameters analysed, the only exception being an increment in the mean generation time. Larvae of the MR strain reared on *Bacillus thuringiensis* (*Bt*) maize took longer to develop, presented a high adult cumulative emergence time and had lower growth rate than those reared on non-*Bt* maize, suggesting the existence of incomplete resistance. Feeding preference assays reveal a low discrimination between *Bt* and conventional maize.

CONCLUSION: Both resistant and heterozygous larvae of *M. unipuncta* survive the Cry1Ab toxin expressed on *Bt* maize, with a weak fitness cost for the homozygous larvae, indicating the potential risk of field-evolved resistance and its relevance to resistance monitoring.

Keywords: true armyworm; Cry1Ab toxin; secondary pest; *Bt* corn; resistance management

1 INTRODUCTION

Transgenic maize engineered to express insecticidal proteins from the bacterium *Bacillus thuringiensis* (*Bt* maize) provides good control over primary pests of maize, such as corn borers, rootworms and some leaf-feeder lepidopterans.[1] However, *Bt* crops do not properly control various secondary pest species that are also directly exposed to the toxin(s) expressed in the plant.[2] Thus, *Bt* maize hybrids expressing the Cry1Ab toxin have shown low efficacy for controlling some lepidopteran secondary pests of maize, such as the western bean cutworm *Striacosta albicosta* (Smith) (Lepidoptera: Noctuidae)[3], the cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and the true armyworm *Mythimna unipuncta* Haworth (Lepidoptera: Noctuidae).[4,5] Widespread planting of *Bt* maize can effectively eliminate competition from primary pests of maize, as suggested by intraguild competition studies with *S. albicosta*, facilitating potential outbreaks of lepidopteran secondary pests by providing a relatively exclusive habitat.[6] Moreover, it has been indicated that *Bt* crops may be exposed to the emergence of secondary pests owing to fewer pesticide applications.[2]

The low efficacy of *Bt* maize hybrids expressing the Cry1Ab toxin against *M. unipuncta* has raised concerns about the consequences that *Bt* maize deployment could have on the assemblage of the lepidopteran community in maize fields.[4] Larvae of *M. unipuncta* can feed from neonates on Cry1Ab-expressing maize plants, and about 1–2% are capable of completing their life cycle.[7] The high rates of toxin elimination and the rapid recovery of the midgut epithelium after *Bt* toxin ingestion are probably involved in the basal tolerance of this species to the Cry1Ab toxin.[5,8] This situation represents a scenario of non-compliance with the 'high-dose' strategy for this species. Although outbreaks of *M. unipuncta* have not been reported in *Bt* maize fields, the populations of this secondary pest are exposed to high selection pressure in hotspot areas (high adoption rate and repeated cultivation of *Bt* maize) in the Ebro Valley (north-east Spain), where the risk of field-evolved resistance increases.[7] Indeed, we have demonstrated that a laboratory-selected MON810-resistant (MR) strain of *M.*

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unipuncta was able to develop resistance to MON810 maize after 5–12 generations of laboratory selection (22–57% of the larvae completed their life cycle on *Bt* maize plants), this resistance being mediated by the alteration of toxin activation by midgut proteases.[7]

In this context, knowledge of the components involved in the development of resistance in *M. unipuncta* is essential for evaluating the risk of resistance in field populations and for the implementation of management strategies that prevent or mitigate resistance. The rate at which resistance evolves is determined by the inheritance of the resistant traits, and it is expected to occur faster when resistance is functionally dominant and encoded by a single gene (monogenic).[9,10] However, in those cases where resistance imposes a fitness cost for resistant individuals, which are less fit than susceptible ones in the absence of *Bt* toxins,[11] the appearance of resistance can be delayed by natural selection against resistant genotypes in refuges and alternative hosts.[12] Incomplete resistance, which occurs when resistant insects suffer a disadvantage on *Bt* crops relative to non-*Bt* crops, is predicted also to delay the selection for resistance owing to fitness depletion when the resistant genotypes grow on *Bt*.[12,13] In addition, the feeding preferences (*Bt* versus non-*Bt* plants) of susceptible and resistant insects will influence the exposure to the toxin, which in turn will determine the selection pressure in field populations.[14,15]

The present study offers insight into all these components related to resistance development by analysing the inheritance of resistance, the fitness costs associated and the existence of incomplete resistance in the MR strain of *M. unipuncta*.[7] We have also investigated the feeding preferences of resistant and susceptible *M. unipuncta* larvae. The significance of these results in relation to the potential of this secondary pest to develop resistance in nature and the implications for resistance monitoring is discussed.

2 MATERIALS AND METHODS

2.1 Insects and plant materials

All experiments were performed with a resistant (MR) and a control (MC) strain of *M. unipuncta* derived from the same field population.[7] In brief, a field population collected in conventional maize in Monzón (Huesca, Ebro Valley, Spain) in 2009 was maintained in the laboratory without exposure to *Bt* maize or Cry toxins for six generations. A subset of this strain (F0) was then selected by feeding larvae on MON810 *Bt* maize leaves during the whole larval stage to obtain the resistant MR strain, whereas another subset was kept on non-*Bt* maize to obtain the MC strain. Owing to high mortality, MR adults of generation F10 were crossed with adults from MC (61% of adults from MR and 39% of adults from MC) and the progeny was selected again with *Bt* maize. The selection of the MR strain increased larval survival from 2% at F0 to 22–57% at generations F5 to F12[7] and to 51–74% at generations F16 to F18. The survival of the MC strain on *Bt* maize remained within 1–2% during this period.

Two maize hybrids were used: *Bt* maize DKC6451YG (event MON810, Cry1Ab toxin) and its near isogenic conventional line DKC6450 (non-*Bt* maize). Plants were grown in plastic pots (25 cm diameter) using Compo Sana® Universal as a substrate and maintained in a controlled greenhouse at 25 ± 5 °C and >60% RH with a 16:8 h (L:D) photoperiod. A laboratory colony of *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) was used, as a positive control, to verify the biological activity of every *Bt* maize plant used in this study.[7]

2.2 Cry1Ab protein

The Cry1Ab toxin used in this study was obtained from *Escherichia coli* cultures, strain XL1-blue {recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F0 proAB lacIqZDM15 Tn10 (Tetr)]}, transformed with the plasmid pBD140, kindly donated by Dr RA de Maagd (Plant Research International B.V., Wageningen, the Netherlands), as reported by González-Cabrera *et al*.[7]

2.3 Susceptibility bioassays

Susceptibility to Cry1Ab of neonate larvae (<24 h) was assessed using a leaf-disc dipping bioassay. Fresh non-*Bt* maize leaf discs (17 mm diameter) were obtained (avoiding mid-rib) from non-*Bt* plants (12–13-leaf stage). All discs were first dipped in a 0.1% Triton X-100 solution as surfactant. After drying in a laminar flow hood, discs were dipped for 10 s in one of eight different concentrations of Cry1Ab, ranging from 2.5 to 2960 μg mL^{-1} in 50 mM sodium carbonate buffer (pH 10.5), or in carbonate buffer (control discs). Discs were air dried again in a laminar flow hood and deposited in a plastic container (2 cm diameter × 2 cm height) coated with a 5 mm layer of 2.5% agar. One larva per disc was used. The bioassays were conducted at 23 ± 0.2 °C and 80 ± 5% RH with a 16:8 h (L:D) photoperiod in a growth chamber (Sanyo MLR-350 H; Sanyo, Tokyo, Japan). For each concentration of Cry1Ab, six replicates of 12 larvae were used. After 7 days, larval mortality was recorded. The concentrations needed to cause 50% mortality (LC_{50}) were obtained by probit analysis using POLO-PC (LeOra Software, Berkeley, CA), which automatically corrected for control mortality. In a parallel experiment, larval mortality when fed on *Bt* maize discs during a 7 day period (LM7 days) was assessed by dipping *Bt* maize discs in carbonate buffer and following the same methodology.

2.4 Inheritance of resistance

Reciprocal crosses were performed by mating ♂MC × ♀MR (F_{1-1}) and ♀MC × ♂MR (F_{1-2}). As no differences were found in their susceptibility, both strains were pooled into F1, and the resulting progeny were reared to adults on non-*Bt* maize. Backcrosses were performed by mating F1 adults with adults from the parental strains: Bc1 (♀MC × ♂F1), Bc2 (♂MC × ♀F1), Bc3 (♀MR × ♂F1) and Bc4 (♂MR × ♀F1). A minimum of 21 adults from each strain were used in each cross and backcross (divided into three batches of seven adults each). Susceptibility bioassays were done as previously described (see Section 2.3) with parental strains, F1 crosses and backcrosses. Sex linkage was determined using a hypothesis test to compare the slopes and intercepts of probit regressions derived from F1 reciprocal crosses. We tested null hypotheses that the lines were parallel or equal using POLO-PC (LeOra Software, 1987).[16]

Dominance of resistance (D_x) was calculated as described by Bourguet *et al*.:[17] $D_x = (x_{RS} - x_{SS})/(x_{RR} - x_{SS})$, where x_{SS}, x_{RS} and x_{RR} are the quantitative values calculated for a trait (x) for susceptible homozygotes, heterozygotes and resistant homozygotes respectively. Values of D_x range from 0, representing completely recessive resistance, to 1, representing completely dominant resistance. When D_x is 0.5, resistance is referred to as semi-dominant or codominant.[17] To calculate the dominance level of Cry1Ab resistance, the trait assessed was log LC_{50}. The effective dominance was estimated by using mortality data from LM7 days (mortality when fed on *Bt* maize discs during a 7 day period, see

Section 2.3) and LMlarval cycle (mortality when fed on *Bt* maize leaf pieces throughout the larval cycle) (life history traits, see Section 2.5).

For direct testing of monogenic inheritance, the observed mortalities in the backcrosses were compared at each concentration tested with the expected mortalities when assuming a monogenic model, as described by Tabashnik *et al.*[18,19] The expected number of deaths was calculated as n_iM_i, where n_i is the number of larvae tested at concentration i, and M_i (estimated response probability under the monogenic model) is $0.5P_i$ (F1) + $0.5P_i$ (parent), where P_i is the mortality probability estimate at concentration i for F1 and parental strains. For statistical comparison between the observed and expected number of deaths in the backcrosses, a χ^2 value was calculated for each concentration, following Preisler *et al.*:[20] $\chi^2 = (r_i - n_iM_i)^2/[n_iM_i(1 - M_i)]$, where r_i is the observed number of deaths at concentration i in backcrosses, and n_iM_i is calculated as described above. The test statistic χ_i^2 was compared with a chi-square distribution with one degree of freedom (df). In addition, a $\Sigma\chi_i^2$ was calculated for each backcross (df = $r - 1$, where r is the number of concentrations tested). The null hypothesis was rejected if these tests indicated $P < 0.05$.

2.5 Life history traits

Five reproductive cages (containing 10 ♀ + 10 ♂ reproductive adults each) of each strain (MC and MR) were utilised. Forty neonate larvae were randomly collected from each reproductive cage and reared individually in plastic vials (4 cm diameter × 2 cm height), where they were fed *ad libitum* on either non-*Bt* maize leaf pieces (20 larvae) or *Bt* maize leaf pieces (20 larvae). Maize leaves were replaced every 2–3 days. All vials were daily checked to assess development time, survival, larval (L_2) and pupal (24–48 h after pupation) weights, emergence of adults and sex ratio. The relative growth rate was calculated using the formula: $RGR = [W_2 - W_1]/T$, where W_1 and W_2 are the L_2 and pupal weights respectively, and T is the time (days) from L_2 to pupal stage.[21] Cumulative adult emergence data were used to estimate the time needed to reach 50% emergence (CE_{50}) by probit analysis using POLO-PC. Each larva was considered to be a replication ($N = 100$) to determine development time, pupal weight and RGR. Survival rate and sex ratio were estimated from the observation of individuals originating from each reproductive cage, which were considered in this case to be replications ($n = 5$). Additionally, three reproductive cages (10 ♀ + 10 ♂ each) were used for each reciprocal cross (F_{1-1} and F_{1-2}) to estimate the effective dominance of resistance. Twenty neonate larvae from each reproductive cage were individually reared on *Bt* maize, and mortality throughout the larval cycle was recorded (LMlarval cycle, see Section 2.4). Each reproductive cage was considered to be a replication ($n = 3$).

Newly emerged virgin adults were paired within groups representing the number of reproductive pairs: 18 for MC on non-*Bt* maize; 14 for MR on non-*Bt* maize and 18 for MR on *Bt* maize. No adults of MC on *Bt* maize were obtained because mortality at the pre-imaginal stage was 99%. Each couple was kept in a plastic vial (9 cm diameter × 7 cm height) containing cotton soaked with a solution of 10% honey in water as food, and a plastic cylinder as oviposition substrate. Eggs were collected and counted every day, and cylinders were replaced. All mating pairs were kept until they died. Once dead, the mating status of females was determined by the presence or absence of a spermatophore in their bursa copulatrix. The percentage of copulation success was estimated by the mean number of spermatophores present.[22] Dissections were performed using a Leica M125 stereomicroscope equipped with a Leica DFC420 digital camera (Leica Microsystems S.A., Barcelona, Spain). We estimated the preoviposition period of copulated females, the total fecundity (as total number of eggs/copulated females), fertility (average number of hatched eggs) and adult longevity. Each reproductive pair was considered to be a replication.

2.6 Life table parameters

For both MC and MR strains, we assumed that the populations had an exponential growth described in the model $N_t = N_0 \cdot e^{r_m \cdot t}$, where N_t is the size of the population at time t, N_0 is the initial size of the population and r_m is a parameter related to the rate of population growth, referred to as the intrinsic rate of increase.[23] From the life history trait data obtained above (Section 2.5.), the following life table parameters were estimated using the methods of Birch[23] and Carey:[24] net reproductive rate or the number of daughters replacing an average female over a course of a generation, $R_0 = \Sigma(l_x \cdot m_x)$, where x is the average parental age since emerging from the egg stage, l_x is the number of individuals alive at time x (age-specific survival) and m_x is the number of female offspring per female at time x (age-specific fertility); mean generation time or average time of one generation, $T = \Sigma(x \cdot l_x \cdot m_x)/\Sigma(l_x \cdot m_x)$; intrinsic rate of population increase (rate of growth of a population), $r_m = \ln(R_0)/T$, where T is the mean generation time; finite rate of increase (number of times the population multiplies in unit time), $\lambda = \ln(r_m)$; doubling time (time a population needs to double its size), $DT = \ln(2)/T$. Calculation was done by the jackknife procedure, using the SAS program developed by Maia *et al.*[25]

2.7 Feeding preference bioassays

The arena for the bioassays consisted of plastic petri dishes (9 cm diameter × 7 cm height) coated on their bottom half with about 20 mL of a 2.5% agar solution. Maize leaf discs were obtained as described in Section 2.3 and fitted into holes punched in the agar layer. Fifth-instar larvae (<24 h) were starved for 3 h and individually placed in each petri dish at 23 ± 0.2 °C and 80 ± 5% RH with a 16:8 h (L:D) photoperiod in a growth chamber (Sanyo MLR-350 H).

No-choice assays were performed by placing six *Bt* maize or six non-*Bt* maize leaf discs in each arena. The assay was terminated after larvae in the control arenas (non-*Bt* maize) had ingested about 75% of the discs (4.5 discs approximately). Choice assays were conducted by arranging alternately three *Bt* maize and three non-*Bt* maize leaf discs within each arena. The assay was terminated when larvae in an external control arena containing six non-*Bt* maize leaf discs consumed about 50% of the discs (three discs approximately). Three replicates of ten arenas each were used in both bioassays.

Consumption of leaf discs was calculated on a dry weight (DW) basis. The fresh weight of the leaf discs used in each replica was measured with an analytical balance (Mettler Toledo AX205; Mettler-Toledo International Inc., Columbus, OH), and their DW was estimated from the ratio of fresh to dry weight, previously calculated using 30 sets (containing three leaf discs each) of each maize type. At the end of the experiment, the uneaten leaf discs were oven dried at 60 °C for 2 days and weighed.

2.8 Statistical analysis

Homogeneity of variances (Levene test) and normal distribution (Kolmogorov–Smirnov test) were tested for all life history traits

Table 1. Susceptibility to Cry1Ab of larvae of *M. unipuncta* from susceptible (MC) and resistant (MR) parental strains, F1 crosses and backcrosses

Strain	n[a]	Slope ± SE	LC_{50}[b](CI 95%)	χ^2	df	LCR_{50}[c](CI 95%) relative to MC	LCR_{50}[c](CI 95%) relative to MR
Parental							
MC	1080	0.7 ± 0.1	103 (62–158)	53.90	76	1	–
MR	864	1.2 ± 0.2	1123 (806–1560)	22.24	58	10.9 (6.1–19.2)*	1
F1 crosses							
F_{1-1} (♂MC × ♀MR)[d]	512	0.4 ± 0.1	492 (226–1644)	6.71	30	4.8 (1.6–14.4)*	2.3 (0.8–6.6)
F_{1-2} (♀MC × ♂MR)[d]	512	0.5 ± 0.1	617 (285–2017)	8.32	30	6.0 (2.0–17.8)*	1.8 (0.6–5.2)
Backcrosses[e]							
Bc1 (♀MC × ♂F1)[f]	432	1.1 ± 0.1	82 (58–114)	22.60	26	0.8 (0.4–1.5)	13.6 (7.8–23.5)*
Bc2 (♂MC × ♀F1)[f]	432	1.0 ± 0.1	113 (79–161)	20.36	26	1.1 (0.6–2.0)	9.9 (6.0–16.2)*
Bc3 (♀MR × ♂F1)[g]	432	0.4 ± 0.1	927 (364–5835)	10.31	26	9.0 (2.0–39.3)*	1.2 (0.3–5.2)
Bc4 (♂MR × ♀F1)[g]	432	0.5 ± 0.1	906 (424–3306)	13.66	26	8.8 (2.8–27.4)*	1.2 (0.4–3.7)

[a] n = number of larvae tested.
[b] Lethal concentrations (LC_{50}) and their confidence intervals (CI 95%) are expressed in μg Cry1Ab mL^{-1}.
[c] Lethal concentration ratio (LCR) with respect to MC and MR strains at LC_{50}.
[d] Slopes and intercepts of the probit regressions of F_{1-1} and F_{1-2} were not significantly different ($\chi^2 = 0.73$; df = 2; $P > 0.05$).
[e] F_{1-1} and F_{1-2} were pooled (F1) for backcrossing.
[f] Slopes and intercepts of the probit regressions of Bc_1 and Bc_2 were not significantly different ($\chi^2 = 0.41$; df = 2; $P > 0.05$).
[g] Slopes and intercepts of the probit regressions of Bc_3 and Bc_4 were not significantly different ($\chi^2 = 0.60$; df = 2; $P > 0.05$).
*LCRs are significantly different ($P < 0.05$) if the 95% confidence interval does not include 1.

and life table parameters analysed. When these requirements were not fulfilled, data were transformed using logarithmic transformation (if variables were continuous) or square root transformation (if variables were counts) to normalise distributions and stabilise variances. Percentage values were arcsine transformed before analysis [arcsin(x/100)]. Differences among groups were analysed by analysis of variance (one-way ANOVA) using the general linear model (GLM) procedure (SAS 9.3; SAS Institute, Cary, NC), followed by Dunnett's test (MR reared on non-*Bt* maize was always used as the control in comparisons). Copulation failure among groups was analysed by the χ^2-test. Lethal concentration ratios (LCRs) with respect to MC and MR strains were estimated at the LC_{50} value and were considered to be significantly different if the LCR 95% fiducial interval did not include 1.[16] Cumulative adult emergence (CE_{50}) values were compared by relative potency, as slopes were equal, and considered to be different if 95% confident limits for CE_{50} did not overlap. Differences in consumption of *Bt* maize versus non-*Bt* maize leaf discs in choice and no-choice bioassays were analysed using a paired and unpaired t-test respectively. Additionally, a t-test was performed to compare the consumption of both maize leaf discs (*Bt* maize + non-*Bt* maize leaf discs) in the choice arenas versus the consumption of non-*Bt* maize leaf discs in an external control arena.

A significance level of $P < 0.05$ was considered for all tests.

3 RESULTS

3.1 Inheritance of resistance

As expected, the parental strains differed in their susceptibility to Cry1Ab, the resistant strain being about tenfold significantly less susceptible to the Cry1Ab toxin ($LCR_{50} = 10.9$) (Table 1). The susceptibility of the F1 progeny (F_{1-1} and F_{1-2}) was also significantly lower than that of the parental susceptible strain ($LCR_{50} = 4.8$ and 6.0 for F_{1-1} and F_{1-2} respectively). On the other hand, when compared with the resistant strain, the F1 progeny was not significantly different. The slopes and intercepts corresponding to the concentration–mortality probit regression lines obtained for each reciprocal cross (F_{1-1} and F_{1-2}) did not differ significantly ($P > 0.05$). Consequently, data obtained on the two reciprocal crosses were pooled (F1) in order to simplify further analysis.

The grade of dominance of resistance estimated from the LC_{50} values $\left(D_{LC_{50}}\right)$ was 0.70 on a scale of 0 to 1. The effective dominance of resistance on *Bt* maize was obtained from larval mortality data in a bioassay where neonate larvae were fed on *Bt* maize leaf discs over a 7 day period (79.2 ± 5.1%, 16.6 ± 2.1% and 30.2 ± 3.9% for MC, MR strains and F1 progeny respectively), the corresponding $D_{LM_{7\ days}}$ value being estimated as 0.78. A further estimation of the effective dominance was obtained from the mortality observed on larvae fed on *Bt* maize leaves during their entire cycle (99.0 ± 1.0%, 25.0 ± 3.9% and 61.7 ± 9.7% for MC, MR strains and F1 progeny respectively), which resulted in a $D_{LM_{larval\ cycle}}$ value of 0.50.

Reciprocal backcrosses were performed in order to evaluate whether the inheritance of resistance was compatible with a monogenic model. The backcrosses with the parental susceptible strain reverted resistance, whereas the backcrosses with the parental resistant strain maintained resistance (see LCR values in Table 1). The analysis of the slopes and intercepts corresponding to the concentration–mortality probit regression lines obtained for the reciprocal backcrosses within each parental line (Bc1 versus Bc2 and Bc3 versus Bc4) did not differ significantly ($P > 0.05$). Therefore, data for backcrosses within each parental line were pooled for direct testing of monogenic inheritance. The backcross of F1 with susceptible parents (MC) showed a significant deviation between the observed and expected mortality at the four highest concentrations evaluated (Table 2). Moreover when $\Sigma\chi^2$ was used, the monogenic hypothesis was rejected ($\chi^2 = 62.2$; df = 6; $P > 0.05$). Likewise, the backcross of F1 with resistant parents (MR) showed a significant deviation between the observed and expected mortality at the first three concentrations evaluated, and when $\Sigma\chi^2$ was used the monogenic hypothesis was also rejected ($\chi^2 = 33.9$; df = 6; $P > 0.05$) (Table 2). Taken together, these

Table 2. Direct test of monogenic inheritance of resistance to Cry1Ab in *M. unipuncta* by comparing expected and observed mortality of the backcrosses

Backcross[a]	Concentration[b]	*n*	Observed number of deaths	Expected number of deaths[c]	χ^2(df = 1)	$\Sigma\chi^2$
MC × F1	7.5	96	13	19.6	2.82	
	18.8	96	22	25.0	0.47	
	46.9	96	30	34.3	0.85	
	117.2	96	57	42.7	8.60*	
	293.0	96	69	51.4	13.03*	
	732.4	96	78	59.6	14.96*	
	1831.1	96	88	67.2	21.54*	
						62.26* (df = 6)
MR × F1	7.5	96	20	9.5	12.87*	
	18.8	96	22	11.2	11.69*	
	46.9	96	26	17.3	5.30*	
	117.2	96	30	24.0	2.00	
	293.0	96	40	33.4	2.03	
	732.4	96	45	44.9	0.002	
	1831.1	96	58	57.3	0.02	
						33.90* (df = 6)

[a] Pooled data from Table 1: MC × F1 = Bc1 (♀MC × ♂F1) + Bc2 (♂MC × ♀F1) and MR × F1 = Bc3 (♀MR × ♂F1) + Bc4 (♂MR × ♀F1).
[b] µg Cry1Ab mL^{-1}.
[c] Expected number of deaths at each concentration was calculated as the average mortality probability of the F1 and the corresponding parental line (monogenic model).
*Indicates significant differences between expected and observed mortality ($P < 0.05$).

results do not allow the acceptance of the single-locus null hypothesis, as significant differences were found between the observed and expected values for some of the Cry1Ab concentrations tested, as well as for the sum of the chi-square values.

3.2 Fitness cost and incomplete resistance

No fitness cost was detected in the study of life history traits comparing MR and MC strains reared on non-*Bt* maize. No significant differences were observed in the development time on the different pre-imaginal stages, MC and MR strains needing around 30 days to complete their immature development (Fig. 1). Other parameters, such as larval survival, pupal weights (males and females), sex ratio, cumulative adult emergence (CE_{50}), preoviposition period, copulation failure, number of spermatophores transferred by males to females, fecundity, fertility and adult longevity, remained unaffected (Table 3). Significant differences were only found in the relative growth rate, which was greater in the MR strain (0.282) than in the MC strain (0.243). Life table parameters (R_0, r_m, λ and DT) also presented similar values between MC and MR fed on non-*Bt* maize, except for the mean generation time (T) of the MR strain, which needed approximately 2 days more to complete a generation (Table 4).

When incomplete resistance was evaluated, the performance of the MR strain on *Bt* maize was lower than on non-*Bt* maize for some of the life history traits analysed. We found a significant delay in the developmental time at L_1, L_2, L_4 and L_5 instars when MR was continuously fed on *Bt* maize, the time needed to complete immature development being lengthened by about 4 days (Fig. 1). The MR strain reared on *Bt* maize also presented a lower relative growth rate, a higher cumulative adult emergence (CE_{50}) and a reduced male and female adult longevity (Table 3). Finally, the

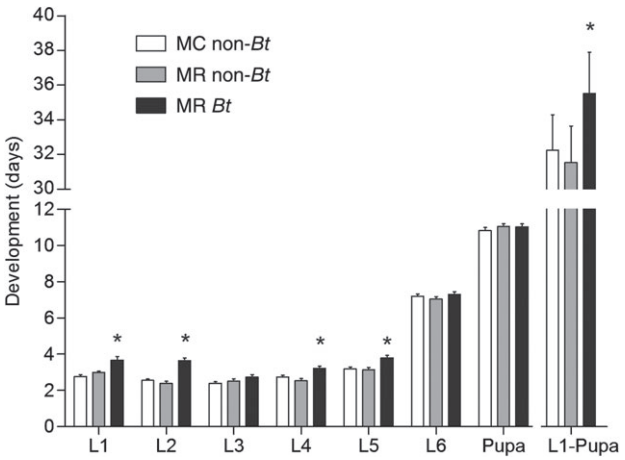

Figure 1. Pre-imaginal development time (L_1 to pupa) in MC reared on non-*Bt* maize and MR reared on non-*Bt* or *Bt* maize. Means were compared by one-way ANOVA, followed by Dunnett's comparison test ($P < 0.05$). * Indicates significant differences with respect to MR reared on non-*Bt* maize used as the control in all comparisons.

mean generation time (T) was significantly increased by 2 days on *Bt* maize (Table 4). The rest of the life history traits and population parameters analysed were unaffected when comparing the two food regimes (Tables 3 and 4).

3.3 Feeding preference bioassays

No-choice assays demonstrate that the consumption of *Bt* maize leaf discs was significantly lower than that of non-*Bt* leaf maize discs for both strains, and was more acute for MC larvae

Table 3. Mean estimates of life history traits of *Bt*-maize-susceptible (MC) and resistant (MR) strains of *M. unipuncta*[a]

Fitness components	MC non-*Bt* maize	MR[b] non-*Bt* maize	MR *Bt* maize
Larval survival (%)	85.0 ± 2.2	78.0 ± 7.5	75.0 ± 3.9
Pupal weight (♀) (mg)	262.9 ± 7.4	271.8 ± 6.5	281.3 ± 5.2
Pupal weight (♂) (mg)	263.4 ± 4.9	279.1 ± 4.0	295.8 ± 6.0
Relative growth rate	0.243 ± 0.003*	0.282 ± 0.003	0.254 ± 0.004*
Sex ratio (males/females)	1.7 ± 0.3	0.9 ± 0.3	1.2 ± 0.4
CE_{50} (95% CL) (days)[c]	31.6 (31.2–32.0)	31.1 (30.7–31.6)	34.9 (34.4–35.3)*
Preoviposition period (days)	5.5 ± 0.6	5.5 ± 1.0	5.1 ± 0.6
Fecundity (number of eggs per female)	960.9 ± 161.7	943.33 ± 247.6	1111.1 ± 163.2
Fertility (%)	78.7 ± 7.4	52.1 ± 11.8	65.3 ± 10.7
Copulation failure (%)	38.9	53.8	28.6
Spermatophores transferred by males	1.8 ± 0.2	1.4 ± 0.4	1.9 ± 0.3
Adult longevity (♀) (days)	15.1 ± 0.9	16.7 ± 0.9	13.6 ± 0.6*
Adult longevity (♂) (days)	18.0 ± 0.7	20.1 ± 1.7	15.4 ± 1.5*

[a] Data are means ± SE. Means were compared by one-way ANOVA, followed by Dunnett's comparison test, except for copulation failure, which was compared by the χ^2-test.
[b] The resistant strain reared on non-*Bt* maize was used as control in all comparisons.
[c] Cumulative adult emergence (CE_{50}).
*Indicates significant differences with respect to MR reared on non-*Bt* maize ($P < 0.05$).

Table 4. Population growth parameters of *Bt*-maize-susceptible (MC) and resistant (MR) strains of *M. unipuncta*[a]

Life table parameters[b]	MC non-*Bt* maize	MR[c] non-*Bt* maize	MR *Bt* maize
R_0	284.5 ± 48.3	202.5 ± 28.5	256.9 ± 54.9
r_m	0.139 ± 0.005	0.124 ± 0.004	0.126 ± 0.006
λ	1.149 ± 0.006	1.132 ± 0.005	1.135 ± 0.007
DT	5.0 ± 0.20	5.6 ± 0.18	5.5 ± 0.25
T	40.87 ± 0.66*	42.90 ± 0.62	44.90 ± 0.30*

[a] Data are means ± SE.
[b] R_0 = net reproductive rate (females female^{-1} generation^{-1}); r_m = intrinsic rate of population increase (day^{-1}); λ = finite rate of increase (day^{-1}); DT = time needed by a population to double its size (days); T = mean generation time (days).
[c] The resistant strain reared on non-*Bt* maize was used as control in all comparisons.
*Indicates significant differences with respect to MR reared on non-*Bt* maize ($P < 0.05$). Means were compared by one-way ANOVA, followed by Dunnett's comparison test.

Table 5. Feeding preference bioassays with *Bt*-maize-susceptible (MC) and resistant (MR) strains of *M. unipuncta*

Bioassay	Treatments	Consumption[a] MC	Consumption[a] MR
No-choice	*Bt*	3.3 ± 0.6*	9.4 ± 0.6*
	Non-*Bt*	13.4 ± 0.4	14.8 ± 0.6
Choice	*Bt*	2.6 ± 0.3*	4.0 ± 0.4
	Non-*Bt*	4.1 ± 0.2	4.9 ± 0.4
	Bt + non-*Bt*	6.7 ± 0.4*	8.9 ± 0.6*
	External control (non-*Bt*)	12.0 ± 0.4	11.0 ± 0.3

[a] Data are means dry weight of leaf discs consumed ± SE (mg).
*Denotes statistical differences between *Bt* (or *Bt* + non-*Bt*) and non-*Bt* treatments ($P < 0.05$). Paired *t*-test for the comparison of *Bt* versus non-*Bt* in the choice bioassay, and *t*-test for the comparison of *Bt* versus non-*Bt* in the no-choice bioassay and *Bt* + non-*Bt* versus external control (non-*Bt*) in the choice bioassay.

(Table 5). However, when given the choice between non-*Bt* and *Bt* maize leaf discs, MC larvae consumed a significantly higher amount of non-*Bt* discs, whereas MR larvae consumed a similar amount of both (Table 5). Moreover, when total consumption (*Bt* maize + non-*Bt* maize leaf discs) in the choice arenas was considered, a significant suppression of feeding was observed for both MC and MR larvae when compared with the consumption in an external control arena containing only non-*Bt* maize leaf discs (Table 5).

4 DISCUSSION

Knowledge of the genetic basis of resistance to *Bt* crops is critical for designing resistance management strategies, a major concern for the sustainability of this control technology. Our data reveal that resistance to Cry1Ab toxin in the on-plant (MON810) selected MR strain of *M. unipuncta* is inherited as an autosomal trait on the basis of analysis of reciprocal crosses and backcrosses. In addition, the analysis of the susceptibility of the F1 generation in comparison with the parental strains shows that resistance is inherited as a partially dominant trait ($D_{LC_{50}} = 0.7$). It is worth noting that semi-dominant to partially dominant values were also estimated for the effective dominance on *Bt* maize ($D_{LM_{larval\ cycle}}$ and $D_{LM_{7\ days}}$ ranged between 0.50 and 0.78), indicating that a significant number of heterozygous larvae would survive on *Bt* plants if the resistant trait were established in the field. In addition, our data reveal lack of fitness costs on essential life history traits, such as larval mortality, development time, pupal weight, growth rate, adult emergence time, adult longevity and reproductive potential (fecundity and fertility), and on most of the population growth parameters analysed (R_0, r_m, λ and DT). A small but significant increment in the mean generation time (T) is the only fitness cost observed in the MR strain. Thus, except for a

slight developmental delay, resistant individuals are not expected to be at a disadvantage with respect to susceptible individuals when not subjected to selection. Increases in the developmental time when feeding on *Bt* crops has also been reported in resistant strains of various pests, such as *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae),[9] *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae)[26] and *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae).[27]

The comparison of the performance of resistant insects when reared on non-*Bt* and *Bt* plants suggests the existence of incomplete resistance. We have found that larvae from MR reared on Cry1Ab-expressing *Bt* maize took longer to develop, presented a high adult cumulative emergence time (needing almost 4 days more to reach the 50% adult emergence) and had a lower growth rate than those reared on non-*Bt* maize. Moreover, longevity of females and males of MR reared on *Bt* maize was significantly lower than when reared on non-*Bt* maize. Similar results have been reported on a resistant strain of *P. gossypiella* fed on Cry1Ac-*Bt* cotton, the resistant larvae exposed to *Bt* cotton having lower survival, delayed development (12 days longer) and lower pupal weight and fecundity.[9,28] Incomplete resistance has also been reported in *O. nubilalis* resistant to Cry1F, the resistant larvae showing lower survival and a reduction in their weight when feeding on *Bt* maize.[29] The incomplete resistance found in the MR strain is expected to reduce the selective advantage of resistant insects on *Bt* maize, as suggested by Tabashnik *et al.*[30] Nevertheless, nutrient utilisation was not impaired in MR when fed on *Bt* maize, as reported by González-Cabrera *et al.*,[7] enabling the pupa to reach the standard weight and leading to adults whose fecundity was not affected.

Understanding whether larvae of *M. unipuncta* have the ability to detect and discriminate between *Bt* and non-*Bt* maize also has implications for potential resistance development. When feeding preferences were evaluated, we found that larvae from the resistant MR strain did not discriminate between non-*Bt* and *Bt* maize leaf discs when given a choice, although a reduction in the consumption occurred when *Bt* maize was the only available food. A significant reduction in the consumption of *Bt* maize by MC larvae under choice conditions was observed, but this was less acute than that obtained under no-choice conditions. Moreover, a significant suppression of feeding was observed for both MC and MR larvae when total consumption (*Bt* maize + non-*Bt* maize leaf discs) under choice conditions was compared with the consumption of non-*Bt* maize leaf discs alone in an external control arena. This suppression effect on choice bioassays was attributed to post-ingestive toxic effects, as larvae are expected to reduce or cease their feeding activity when at least one of the two treatments in the assay evokes a toxic response.[31] It is known that the ingestion of *Bt* toxins can lead to partial midgut paralysis, resulting in a drop in *Bt* leaf consumption after a few hours of ingestion.[32] Taken together, our results suggest a low discrimination between *Bt* and conventional maize for both susceptible and resistant larvae, which will readily feed on *Bt* maize until post-ingestive toxic effects take place. This is especially relevant, as a small percentage of larvae from field populations of this species can survive and complete their development on *Bt* maize, favouring the development of resistance.[4,7] Similarly, a study aimed at determining whether *Bt* resistance influences behaviour found that resistant larvae from *O. nubilalis* do not avoid dietary Cry1Ab.[33] A lack of feeding and/or oviposition preferences between *Bt* and conventional plants has also been reported for *P. gossypiella*,[34,35] *H. zea*[36] and *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae).[37]

Data reported herein are congruent with our previous studies using the same MR strain, which suggest that resistance in this strain is mediated by an alteration of the gut proteases participating in the activation of Cry1Ab.[7] Firstly, genes coding for proteolytic enzymes participating in digestion are expected to be located in autosomes, as occurs with other essential physiological processes.[38] Thus, inheritance of resistance meditated by proteases in the MR strain is not expected to be sex linked. Secondly, in the same previous study, up to three enzymatic activities (trypsin-, chymotrypsin- and elastase-like) are shown to be reduced in the gut of MR larvae compared with susceptible MC larvae, which is compatible with the multilocus resistance found in the analysis of the backcrosses reported herein. Finally, a reduction in the activity of gut proteases in resistant larvae could clearly lead to the weak fitness cost found in the MR strain, or to incomplete resistance. It is worth noting that resistance mediated by an alteration of the toxin activation by proteases has also been associated with a weak fitness cost in a Cry1F-resistant strain of *O. nubilalis*[39] and in a Dipel-resistant strain of *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae).[40]

Altogether, our data reveal the potential risk of the evolution of resistance in *M. unipuncta*. Only five generations of selection on plants were needed to obtain the resistant MR strain from a field-derived population collected in the Ebro Valley,[7] a region in Spain where the adoption rate for *Bt* maize has reached >75% in the last 2 years. Although the frequency of the resistance alleles in the field is still to be determined, the survival on *Bt* maize plants of the larvae from several non-selected strains originating from the Ebro Valley area[4,7] may indicate a rather high frequency of resistant alleles in the field. We have shown that both resistant and heterozygous larvae of *M. unipuncta* survive the Cry1Ab toxin expressed on *Bt* maize, with a weak fitness cost for the homozygous larvae, which would compromise the effectiveness of the refuges if this resistance trait were established in the field.[1] No attempt was made to assess fitness costs in the heterozygous larvae because of the weak fitness costs mentioned for the homozygous larvae. The incomplete resistance found in the MR strain is not expected to reverse resistance evolution, but it can help to delay it.[11] Remarkably, resistance in the maize noctuid pest *Busseola fusca* Fuller (Lepidoptera: Noctuidae), which rapidly evolved in the field and failed to comply with the high-dose strategy, was also inherited as a dominant trait and had no detectable fitness costs associated with resistance.[41,42] Moreover, asynchronies in mean generation time between susceptible and resistant individuals found herein could promote assortative or non-random mating.[43] Especially relevant is the difference of 4 days between resistant individuals reared on *Bt* plants and susceptible individuals reared on non-*Bt* maize. Accordingly, mating between susceptible *M. unipuncta* adults from refuges and resistant moths from *Bt* fields will be limited owing to developmental asynchrony, increasing the risk of selection for resistance.[13] Nevertheless, the exposure of the populations of this species to *Bt* maize is difficult to assess because the outbreak of secondary pests is highly dependent upon cultivation practices, farming systems and regional environmental factors.[44]

Our data provide a better understanding of the different components related to *Bt* maize resistance in *M. unipuncta*. Yet, ongoing studies on the initial frequency of resistance in field populations would be useful for predicting more precisely the risk of resistance in nature. The uncertain but possible risk of

outbreaks in a hotspot area for *Bt* maize in Europe (Ebro Valley, Spain), where selection for resistance might already be operating, raises the question of whether this secondary pest should be considered in the ongoing resistance monitoring programme. The use of pyramided *Bt* crop products expressing multiple toxins, which could provide a better control for this species and help in delaying resistance development, is not currently allowed in the EU. Therefore, monitoring the evolution of resistance in the field will be the only way of implementing resistance management strategies in a timely manner to sustain the effectiveness of this technology.

ACKNOWLEDGEMENTS

This work was supported by a grant (AGL2012-34289) of the Ministerio de Economía y Competitividad. The authors thank Nuria Arranz and Carolina Navas for technical assistance.

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