Fitness of Bt-resistant cabbage loopers on Bt cotton plants

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Introduction

Biotech crops engineered to express Bacillus thuringiensis (Bt) toxins have been widely used for insect pest control in agriculture since 1996 (James, 2015). In the past two decades, Bt crops have produced significant environmental and economic benefits (Carpenter, 2010; Cattaneo et al., 2009; Lenormand and Raymond, 2010; Lu et al., 2012). However, development of resistance to Bt toxins in insects threatens the sustainable use of Bt crops (Tabashnik et al., 2008). Since the wide adoption of Bt crops, cases of resistance to Bt crops have been reported in several insect pests (Tabashnik and Carriere, 2015). Studies of Bt-resistant insects have indicated that resistance is often associated with fitness cost when insects are feeding on non-Bt host plants (Gassmann et al., 2009; Wang et al., 2016). The fitness of Bt-resistant insects on host plants, particularly on Bt plants, is a critically important factor determining the speed of resistance development in insect populations (Carriere et al., 2011; Gassmann et al., 2009; Lenormand and Raymond, 1998; Raymond et al., 2005, 2007; Williams et al., 2011).

For delaying the development of insect resistance to Bt crops, a biotechnological strategy currently used is to engineer Bt crops to produce two or more Bt toxins to provide ‘redundant killing’ of target insect pests, known as the ‘pyramid strategy’ (Brévau et al., 2013; Carrière et al., 2015; Tabashnik et al., 2008; Zhao et al., 2003). The widely adopted resistance management strategy in the field is to preserve susceptible alleles in pest populations by planting non-Bt crops as refuge when Bt crops are planted, known as the ‘refuge strategy’ (Gould, 1998; Tabashnik, 2008). Since 2004, pyramided Bt crops have been widely adopted and have now become the majority of transgenic Bt crops cultivated in the USA, Australia and India (Tabashnik et al., 2013). Although no resistance to pyramided Bt crops has been reported in the field, high level of insect resistance to two Bt toxins has been selected in the laboratory and the resistant insects can survive on the dual Bt toxin plants (Kain et al., 2015). In the field where both Bt and non-Bt host plants are present, the reduced performance of resistant insects on non-Bt plants (known as fitness cost) and on Bt crops (known as incomplete resistance) affects the selection of resistant alleles in insect populations (Garcia et al., 2015; Gassmann et al., 2009). The fitness of insects on plants can be affected by various factors, including the insect genetic backgrounds, the specific gene mutations that confer resistance to Bt toxins and the host plants that the insects feed on, in addition to the Bt toxins in the plants (Carrière et al., 2004, 2005; Raymond et al., 2005, 2007, 2011). Therefore, the fitness of Bt-resistant insects on Bt crops impacts the efficacy of the toxin-pyramiding and refuge strategies.

The cabbage looper, Trichoplusia ni, is a generalist herbivore with a highly diverse range of host plants, accounting for 160 plant species from 36 different families (Sutherland and Greene, 1984). T. ni has also developed resistance to Bt toxins in an agricultural environment (Janmaat and Myers, 2003). From the Bt-resistant T. ni population, two genetically independent resistance traits, Cry1Ac resistance and Cry2Ab resistance, have been isolated and introgressed into an inbred laboratory T. ni strain (Song et al., 2015; Wang et al., 2007). The Cry1Ac-resistant T. ni can survive on broccoli and cotton plants engineered to produce the toxin Cry1Ac (Kain et al., 2015; Wang et al., 2007), and the

Summary

Development of resistance to the insecticidal toxins from Bacillus thuringiensis (Bt) in insects is the major threat to the continued success of transgenic Bt crops in agriculture. The fitness of Bt-resistant insects on Bt and non-Bt plants is a key parameter that determines the development of Bt resistance in insect populations. In this study, a comprehensive analysis of the fitness of Bt-resistant Trichoplusia ni strains on Bt cotton leaves was conducted. The Bt-resistant T. ni strains carried two genetically independent mechanisms of resistance to Bt toxins Cry1Ac and Cry2Ab. The effects of the two resistance mechanisms, individually and in combination, on the fitness of the T. ni strains on conventional non-Bt cotton and on transgenic Bt cotton leaves expressing a single-toxin Cry1Ac (Bollgard I) or two Bt toxins Cry1Ac and Cry2Ab (Bollgard II) were examined. The presence of Bt toxins in plants reduced the fitness of resistant insects, indicated by decreased net reproductive rate ($R_0$) and intrinsic rate of increase (r). The reduction in fitness in resistant T. ni on Bollgard II leaves was greater than that on Bollgard I leaves. A 12.4-day asynchrony of adult emergence between the susceptible T. ni grown on non-Bt cotton leaves and the dual-toxin-resistant T. ni on Bollgard II leaves was observed. Therefore, multitoxin Bt plants not only reduce the probability for T. ni to develop resistance but also strongly reduce the fitness of resistant insects feeding on the plants.
resistance has been mapped to an ABCC gene locus (Baxter et al., 2011), a Cry1Ac resistance gene locus known in several other lepidopterans (Baxter et al., 2011; Gahan et al., 2010; Heckel, 2012). The resistance of T. ni to Cry2Ab is a monogenic trait independent of Cry1Ac resistance (Song et al., 2015). The combination of the Cry1Ac and Cry2Ab resistance mechanisms in T. ni enables the insect to survive on the pyramided Bt cotton plants producing both Cry1Ac and Cry2Ab (Kain et al., 2015; Song et al., 2015). A recent study on Bt resistance in T. ni examined the importance of two major factors that affect the fitness cost in Bt-resistant T. ni – the host plants and the specific mechanisms of resistance (Wang et al., 2016). They found that the insects feeding on secondary host plants (i.e. host plants other than their preferred primary host plants) showed a greater fitness cost with higher mortality, slower development and lower fecundity (Wang et al., 2016). Similar observations have also been reported in other Bt-resistant lepidopteran pests on non-Bt crop plants (Bird and Akhurst, 2007; Carriere et al., 2005; Janmaat and Myers, 2007; Raymond et al., 2007). Moreover, fitness cost associated with the resistance to Cry2Ab in T. ni was higher than the fitness cost associated with the resistance to Cry1Ac on nontransgenic plants (Wang et al., 2016).

Most studies investigating fitness costs associated with Bt resistance have been conducted on nontransgenic plants (i.e. without exposure of the insects to Bt toxins; Gassmann et al., 2009; Wang et al., 2016). Although there have been several studies reporting the fitness of insects on Bt crops producing a single toxin (Bird and Akhurst, 2004, 2005; Mahon and Olsen, 2009a,b), no studies have been reported on fitness of multitoxin-resistant insects on Bt crops producing multiple Cry toxins. In this study, the reduction in fitness associated with resistance to a single Bt toxin and dual Bt toxins was determined using near-isogenic T. ni strains grown on leaves of three cotton varieties: conventional non-Bt cotton, the single-toxin (Cry1Ac) Bt cotton Bollgard I and the dual-toxin (Cry1Ac and Cry2Ab) Bt cotton Bollgard II. The near-isogenic susceptible and Bt-resistant T. ni strains and the commercial Bt cotton varieties with a similar genetic background provided a unique insect-Bt plant system to analyse the fitness of resistant and susceptible insects on Bt crop plants and their impact on insect resistance development and resistance management.

Results

Population growth rate of T. ni decreased when the number of toxins in plants increased

The Bt-susceptible Cornell strain (Kain et al., 2015) and the three near-isogenic T. ni strains resistant to Cry1Ac [strain GLEN-Cry1Ac-BCS (Wang et al., 2007)], to Cry2Ab [strain GLEN-Cry2Ab-BCS (Song et al., 2015)] and to both Cry1Ac and Cry2Ab [strain GLEN-Cry1Ac+Cry2Ab-BCS (Kain et al., 2015)] all survived and completed their life cycles on leaves of the conventional non-Bt cotton plants. The GLEN-Cry1Ac-BCS and GLEN-Cry1Ac+Cry2Ab-BCS strains could survive on Bollgard I leaves, which contain Cry1Ac toxin, while GLEN-Cry1Ac+Cry2Ab-BCS could survive on Bollgard II leaves, which contain both Cry1Ac and Cry2Ab toxins. The net reproductive rate (NRR) $R_0$ was above the critical value of 1 (ranging from 22.8 to 102.1) on all the cotton plants tested for the T. ni strains that survived on the respective cotton plants (Figure 1a). Similarly, the intrinsic rate of increase ($r_o$) $r$ was also above the critical value 0 (ranging from 0.076 to 0.159) on all the host plants tested for the T. ni strains that survived on them (Figure 1b). Both the $R_0$ and $r$ values indicated that, for the T. ni strains that could survive on the specific cotton plants, the strains could not only survive but their populations could also grow on the Bt cotton plants. The $R_0$ and $r$ of the susceptible strain were significantly greater than those of the resistant strains on non-Bt cotton leaves (Figure 1), indicating the fitness cost associated with the resistant traits in the T. ni
The three resistant strains exhibited a decrease of 7.7%–13% in survival rate on leaves of non-Bt cotton as compared to the susceptible strain (Figure 2). A significant 33%–53% decrease in survival was observed for GLEN-Cry1Ac-BCS and GLEN-Cry1Ac+Cry2Ab-BCS strains on Bollgard I (Figure 3). For the GLEN-Cry1Ac+Cry2Ab-BCS strain, the larvae on Bollgard II leaves started to show mortality in neonates and the mortality increased with the increasing instar. In contrast, larval mortality was not observed till 3rd instar when the larvae were fed on non-Bt cotton leaves. Even though its mortality within the larval stage of 5th instar was relatively lower on Bollgard II leaves, the mortality increased significantly in the immediate subsequent stage (prepupal stage; Figure 3). Cumulatively, the larvae on Bollgard I and Bollgard II leaves had higher mortality, compared to the same strain on non-Bt cotton leaves (Figure 2). Mortality was not affected by the sex of T. ni, as the sex ratio of survivors did not vary significantly on cotton leaves with or without Bt toxins (Table 1).
Resistant *T. ni* developed more slowly when feeding on cotton leaves with Bt toxins

When reared on non-Bt cotton leaves, the susceptible and the three resistant strains of *T. ni* developed from neonates to adult emergence in 22.2–23.7 days on average without statistically significant difference among the *T. ni* strains (Figure 4). However, the developmental duration of the GLEN-Cry1Ac-Cry2Ab-BCS strain was 1.5 days longer on Bollgard I leaves (one-way ANOVA, *P* = 0.019) and 10.9 days longer on Bollgard II leaves (*P* = 0.0001), compared with its development time on non-Bt cotton leaves (Figure 4). The GLEN-Cry1Ac-Cry2Ab-BCS strain grown on Bollgard II leaves reached the adult stage 12.4 days later than the susceptible strain grown on non-Bt cotton leaves (Figure 4).

The effect of Bt cotton leaves on the duration of larval development varied, depending on the instars, the toxins in plants and the specific resistance mechanisms in *T. ni*. In the Cry1Ac-resistant GLEN-Cry1Ac-BCS strain, the duration of larval development was not significantly slowed on Bollgard I (Figure 5a). In the dual-toxin-resistant GLEN-Cry1Ac+Cry2Ab-BCS strain, the larval development durations were not different in all instars between feeding on non-Bt and Bollgard I cotton leaves (Figure 5b), which is similar to what was observed in GLEN-Cry1Ac-BCS larvae on Bollgard I leaves (Figure 5a). However, the larval developmental duration of the GLEN-Cry1Ac+Cry2Ab-BCS strain was significantly increased at all larval instars when feeding on Bollgard II, as compared to feeding on non-Bt cotton (Figure 5b). In spite of the delayed larval development on Bollgard II leaves, no significant difference in the durations of prepupal nor in pupal stages was observed between those feeding on non-Bt and on Bt cotton leaves in the GLEN-Cry1Ac+Cry2Ab-BCS strain (Figure 4). The extended development time of *T. ni* on Bt cotton leaves did not result in a significant change of pupal weight nor in the rate of emergence of healthy adults (Table 1).

**Feeding on dual-toxin cotton (Bollgard II) leaves affected the kinetics of egg laying in *T. ni***

Feeding on Bollgard I leaves did not significantly affect the egg-laying kinetics of both GLEN-Cry1Ac-BCS (two-sample two-sided Kolmogorov–Smirnov analysis, *D* = 4.98%, *P* = 0.58; Figure 6a) and GLEN-Cry1Ac+Cry2Ab-BCS (*D* = 5.59%, *P* = 0.64) strains (Figure 6b), as compared to these strains feeding on non-Bt cotton leaves. However, significantly different patterns of egg laying were observed in the GLEN-Cry1Ac+Cry2Ab-BCS strain when reared on Bollgard II leaves as compared to the strain on conventional cotton (*D* = 16.1%, *P* = 0.025) and to Bollgard I leaves (*D* = 17.5%, *P* = 0.013; Figure 6b). The significantly different egg-laying pattern of the GLEN-Cry1Ac+Cry2Ab-BCS strain reared on Bollgard II leaves showed slower egg laying of this strain on Bollgard II plant leaves.

The total number of eggs laid per female and the rate of egg hatching on average were lower in *T. ni* fed with leaves from Bt cotton plants than those on non-Bt plants (Table 1). However, the decrease in both the total number of eggs laid and the rate of egg hatching was not statistically significant (Table 1).

**Discussion**

Bt resistance in insects is often associated with a fitness cost, and the level of fitness reduction is affected by the specific resistance-conferring mutations and also by physiological stresses that the insects encounter from feeding on host plants with different nutritional factors and various allelochemicals (Bird and Akhurst, 2004, 2005; Janmaat and Myers, 2005, 2007; Mahon and Olsen, 2009a; Raymond et al., 2005, 2007, 2011; Wang et al., 2016;
Figure 4  Development durations of the susceptible, GLEN-Cry1Ac-BCS (Cry1Ac-R), GLEN-Cry2Ab-BCS (Cry2Ab-R) and GLEN-Cry1Ac+Cry2Ab-BCS (Cry1Ac-Cry2Ab-R) strains on non-Bt (conventional), Bollgard I and Bollgard II cottons. Average development durations of larval instars are indicated in alternate solid dark and light grey, and those for prepupal and pupal stages are indicated with hatched lines. A vertical dashed line indicates the total development duration of susceptible individuals on conventional cotton from neonates to adult emergence. Significant difference (by one-way ANOVA) of the total development duration, from egg hatching to adult emergence, in the same T. ni strain when feeding Bollgard I and Bollgard II, compared to feeding on non-Bt cotton leaves is indicated by a *' (P < 0.05) or ***' (P < 0.001). Values are represented as mean ± SE.

Figure 5 Durations of individual developmental stages of (a) GLEN-Cry1Ac-BCS and (b) GLEN-Cry1Ac+Cry2Ab-BCS on leaves from conventional cotton (white), Bollgard I (grey) and Bollgard II (black) plants. For each strains, the statistical significance of difference between the T. ni on non-Bt cotton leaves and on leaves from Bollgard I and Bollgard II plants were analysed by one-way ANOVA, and the level of statistical significance of the differences are indicated by *' (P < 0.05), ***' (P < 0.01) and ****' (P < 0.001). Values are represented as mean ± SE.

Figure 6 Kinetics of egg laying of females from (a) GLEN-Cry1Ac-BCS and (b) GLEN-Cry1Ac+Cry2Ab-BCS strains reared on conventional cotton (white squares, discontinuous line), on Bollgard I (grey circles and line) and on Bollgard II (black triangles and line). Numbers of cumulative egg laying are indicated as mean ± SE. Values of the Kolmogorov-Smirnov test for each pairwise comparisons performed are indicated.
Williams et al., 2011). The Bt-resistant *T. ni* strains exhibit a higher fitness cost when feeding on an alternative host plant, comparing with feeding on well-adapted preferred host plants (Janmaat and Myers, 2005, 2007; Wang et al., 2016). When feeding on Bt plants, the insects experience physiological stress from the toxicity of Bt toxins, even though the insects may have developed resistance to the Bt toxins, and also from plant allelochemicals. The unique Bt-susceptible and resistant near-isogenic strains of *T. ni* provided an opportunity for analysis of the effects of Bt toxins Cry1Ac and Cry2Ab in transgenic cotton plants on the fitness of Bt-resistant *T. ni* strains with two specific genetically independent Bt resistance mechanisms.

Based on the net reproductive rate $R_0$ and the intrinsic rate of increase $r$ of the *T. ni* strains grown on non-Bt cotton leaves, all Bt-resistant *T. ni* strains showed reduced fitness on cotton leaves. In other words, there is a fitness cost associated with Bt resistance in the *T. ni* strains feeding on non-Bt cotton plants, as previously reported by Wang et al. (2016). Between the *T. ni* strains with the Cry1Ac and Cry2Ab resistance, the fitness of Cry2Ab-resistant individuals was lower than that of the Cry1Ac-resistant individuals. This is consistent with the previous report in which the very same parameters were also found lower in Cry2Ab-resistant strains when *T. ni* was reared on artificial diet, cabbage, tobacco and tomato leaves (Wang et al., 2016). The presence of a single toxin in cotton plants (i.e. Cry1Ac in Bollgard I) further reduced the fitness of the Cry1Ac-resistant *T. ni* strains when grown on Bollgard I leaves by a moderate decrease in larval survival and rate of larval development. The relative fitness of Cry1Ac-resistant *T. ni* on Bollgard I determined in this study (0.50–0.57 by $R_0$ and 0.81–0.83 by $r$, see Figure 1) was higher than the relative fitness of a Cry1Ac-resistant strain of *Helicoverpa armigera* on Bollgard I plants (Bird and Akhurst, 2004, 2005). In the Cry1Ac-resistant *H. armigera* strain, the intrinsic rate of increase $r$ was reduced >50% with a significant decrease in survival rate and a 7-day delay of development when grown on Bollgard I plants (Bird and Akhurst, 2004, 2005). As the genetic mechanism of the Cry1Ac resistance in that *H. armigera* strain is not known, whether the difference between *T. ni* and *H. armigera* in reduction in fitness by the toxin Cry1Ac in cotton plants is due to different resistance mechanisms or due to intrinsic physiological and metabolic difference between the two species is unknown.

Neither the GLEN-Cry1Ac-BCS strain nor the GLEN-Cry2Ab-BCS strain could survive on Bollgard II leaves, which was expected as there is no significant cross-resistance between the Cry1Ac and Cry2Ab resistance in *T. ni* (Kain et al., 2015; Song et al., 2015; Wang et al., 2007). The presence of the dual toxins, Cry1Ac and Cry2Ab, in cotton plants more greatly reduced the fitness of the *T. ni* strain GLEN-Cry1Ac+Cry2Ab-BCS when feeding on Bollgard II leaves. The size of GLEN-Cry1Ac+Cry2Ab-BCS population could grow on Bollgard II leaves but exhibited an extended larval developmental time and a reduced survival rate. The further reduction in fitness of the GLEN-Cry1Ac+Cry2Ab-BCS strain on Bollgard II leaves can be attributed to both the fitness cost associated with the Cry1Ac resistance and Cry2Ab resistance (Wang et al., 2016) and also the low toxicity of the two toxins to the GLEN-Cry1Ac+Cry2Ab-BCS strain (Kain et al., 2015; Song et al., 2015; Wang et al., 2007).

The negative effect of the toxins in Bollgard II leaves on *T. ni* was mainly in the larval stage, shown as reduced survival and slower development, as larval stage is the developmental stage in which the insects are directly exposed to the Bt toxins and plant chemicals. However, the slower larval development on Bollgard II leaves did not lead to a significant change in pupal weight, emergence of healthy adults and the sex ratio. The 1-day delay of the peak of egg laying of GLEN-Cry1Ac+Cry2Ab-BCS strain grown on Bollgard II leaves, compared to the same strain on non-Bt cotton leaves, was the only observed physiological consequence on the fecundity of adults from the larvae grown on Bollgard II leaves.

It is evident that the presence of Cry toxins in plants reduced the overall fitness of *T. ni* feeding on the plant leaves, exhibiting incomplete resistance. The GLEN-Cry1Ac+Cry2Ab-BCS strain showed a lower fitness than the GLEN-Cry1Ac-BCS strain when grown on non-Bt cotton leaves (Wang et al., 2016). Interestingly, the relative fitness (i.e. the fitness on Bt plants relative to the fitness of the same strain on non-Bt plants) of the two Cry1Ac-resistant strains on Bollgard I leaves was similar, regardless the presence of only Cry1Ac resistance mechanism in the GLEN-Cry1Ac-BCS strain or the presence of the Cry1Ac resistance mechanism and an additional Cry2Ab resistance mechanism in the GLEN-Cry1Ac+Cry2Ab-BCS strain. Both $R_0$ and $r$ values for the resistant *T. ni* strains indicate that the resistant *T. ni* populations could survive and grow on the Bt plants. However, presence of a Bt toxin in plants reduces the fitness of the insects, and presence of multiple Bt toxins in plants more significantly reduces the fitness of the insects feeding on the plants. The results from this study showed that pyramiding of Bt toxins in plants not only decreases the probability for insects to develop resistance which requires multiple mechanisms of resistance to all toxins (Zhao et al., 2003), but also reduces the fitness of resistant insects on the Bt plants to decrease their population growth, further delaying the selection of resistance in the insect populations (Wei et al., 2015). With the observations on the decline of relative fitness of resistant insects on Bt plants, it is expected that with more insecticidal genes stacked in a transgenic plant, the relative fitness of resistant *T. ni* on multitoxin plants will be further decreasing.

An asynchrony between adult stages of susceptible and resistant insects has been reported when the insects were grown on secondary host plants or on plants expressing a single Cry toxin (Bird and Akhurst, 2004; Gryspeirt and Gregoire, 2012; Wang et al., 2016). In this study, the developmental delay in the GLEN-Cry1Ac+Cry2Ab-BCS on Bollgard I leaves was observed to be 1.5 days. The GLEN-Cry1Ac+Cry2Ab-BCS strain could survive on Bollgard II leaves but had a much slower larval development, which resulted in a 12.4-day asynchrony between the adults of the dual-toxin-resistant strain grown on Bollgard II leaves and the susceptible adults on non-Bt cotton leaves. The refuge strategy relies on an assumption that the mating of insects in the field is panmictic among the adults from susceptible and resistant populations (Gould, 1998; Tabashnik, 2008). However, with the 12.4-day developmental delay, the active adult stage of the resistant *T. ni* from the Bt cotton leaves will be out of phase with the active susceptible adults from non-Bt cotton leaves. In *T. ni*, females generally mate within the first 3–5 days after emergence (Shorey, 1964). Therefore, a >12-day asynchrony between the susceptible and resistant populations could potentially lead to temporal separation of the susceptible and resistant populations, resulting in resistant adults predominantly mating within the resistant population, rather than random mating between the resistant and susceptible individuals. Such assortative mating can reduce the efficacy of refuge strategy for delaying resistance development (Liu et al., 1999; Peck et al., 1999).
The series of studies on the fitness cost associated with resistance to Bt toxins Cry1Ac and Cry2Ab in T. ni on various host plants (Wang et al., 2016) and the effects of the Bt toxins in transgenic cotton plants on the fitness of T. ni described above indicate that selection of Bt resistance in insect populations can be affected by multiple factors related to fitness of the insects in the field. First of all, successful resistance of insects to Bt plants relies on the Bt resistance mechanisms that confer a sufficiently high level of resistance to the Bt toxins. However, resistance-conferring gene mutations are often associated with a fitness cost, and the fitness cost in an insect is affected by the host plants on which the insect is grown (Carriere et al., 2005). The fitness is generally higher when the insect is grown on a well-adapted preferred host plant, but is lower on alternative host plants (Janmaat and Myers, 2005, 2007; Wang et al., 2016). The Bt toxins in Bt plants will further impact the fitness of the resistant insects when feeding on Bt plants. Furthermore, the results from this study on Bt resistance in T. ni also demonstrated that development of Bt resistance in an insect may be associated with not only a reduced population growth rate, but also slower larval development, leading to asynchrony of the susceptible and resistant populations. Such an asynchrony between the susceptible and resistant populations could be more profound when the resistant populations are grown on Bt plants. Therefore, development of insect resistance to Bt toxins in the field is complex. The fitness of resistant insects in the field depends on various factors including the host plants that the insects feed on, the mechanisms of resistance and the Bt toxins produced in the plants.

**Experimental procedure**

**Insect strains**

Four T. ni strains were used to examine their performance on foliage of Bt and non-Bt cotton plants. The Cornell strain (Wang et al., 2007), a highly inbred laboratory strain, was used as the Bt-susceptible control strain. Bt-resistant strains used in this study included a Cry1Ac-resistant strain, GLEN-Cry1Ac-BCS (Wang et al., 2007), a Cry2Ab-resistant strain, GLEN-Cry2Ab-BCS (Song et al., 2015), and a strain resistant to both Cry1Ac and Cry2Ab, GLEN-Cry1Ac+Cry2Ab-BCS (Kain et al., 2015). These three resistant strains were near-isogenic to the Cornell strain by introgression of the Cry resistance traits into the Cornell strain (Song et al., 2015; Wang et al., 2007). All T. ni strains were maintained on artificial diet without exposure to Bt toxins as previously described (Kain et al., 2004).

**Plants**

Cotton plants of the non-Bt cotton variety Stoneville 474 and the Cry1Ac-producing cotton Bollgard I (event 531) and the dual-toxin (Cry1Ac + Cry2Ab)-producing cotton Bollgard II (event 15 985) were prepared as previously described by Kain et al. (2015). All plants were grown in formulated potting soil, the Cornell Mix (Boodley and Sheldrake, 1982), in 6-L plastic pots in the greenhouse. Eight- to ten-week-old plants were used in this study.

**Examination of fitness costs**

To record the development and mortality of T. ni of the susceptible and resistant strains, the larvae were reared on detached cotton leaves in 8.0 cm × 6.5 cm (D × H) paper cups. Ten neonates from each strain were placed in a cup, and 30 cups were used for each strain (300 larvae in total per T. ni strain). The cups were kept in an insect rearing room at 25 ± 1 °C, 50 ± 10% relative humidity and 16:8-h photoperiod. Cotton leaves were replaced every 48 h before the larvae reached 4th instar and were replaced every 24 h for larvae in 4th instar. When the larvae were in 5th instar, cotton leaves were replaced daily or more frequently as necessary. Larval development and mortality were recorded in an interval of 12 h till all larvae were pupated. Pupae were collected daily, and their weights were measured and their sex was visually determined as previously described (Wang et al., 2016).

For examination of fecundity, one female adult and two male adults from the same strain reared on the same cotton variety were placed in a metal wire cage (ca. 12 cm in diameter and 11 cm in height) and were provided with 10% sugar solution. Thirty ‘1-female + 2-males’ cages were used as replications for each treatment. The cage was wrapped with wax paper for egg collection, and the wax paper was replaced daily. Eggs on the wax paper were counted, and egg hatching was examined daily afterwards.

The net reproductive rate (R0) and the intrinsic rate of increase (r) were calculated for each T. ni strain on different cotton varieties using the formula described by Carey (1993). For each of the 30 biological replicates from each condition (strain × cotton plant), R0 was calculated as $R_0 = N_{t+1}/N_t$, where $N_t$ is the population size of the parent generation and $N_{t+1}$ is that of its next generation. For $R_0$ calculation, the mean sex ratio was applied to each replicate to calculate the number of females from the next generation, while all other parameters (total number of adults, number of eggs laid and percentage of hatching) were measured data for each replicate. $R_0 > 1$ indicates that the number of offspring females is greater than that of the parental females. $r$ was calculated as $r = \ln(R_0)/(x + I_o * m_o * v_0) * R_0$, where $x$ is the females age in days, $I_o$ the age-specific survival, $m_o$ the age-specific fecundity and $R_0$ the net reproductive rate. $r > 0$ indicates that the population size is increased, while $r < 0$ indicates collapsing of the population. The relative fitness ($w$) of the resistant strains on transgenic plants was calculated as the ratio of $R_0$ or $r$ of the strain on transgenic cotton to $R_0$ or $r$ of the same strain on non-Bt cotton foliage.

**Statistical analysis**

The individual cups were treated as independent units in the analysis, using the mean of data from 10 larvae in each cup or from a ‘1-female + 2-males’ for each mating group. A Shapiro–Wilk test was performed for verifying the normality of the data. An ANOVA followed by multiple pairwise comparisons of means (Tukey’s HSD test) was performed to test the statistical significance of the differences in observed life parameters measured between resistant and susceptible strains on the same plant or for the same strains between transgenic and conventional cottons. Pairwise comparisons of egg-laying kinetics were performed with a two-sample two-sided Kolmogorov–Smirnov test. All statistical analyses were performed using the software R 3.0.2 (R Development Core Team, 2011).

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Conflict of interest

The authors declare no competing financial interests.

Author Contributions

R.W. and P.W. designed the experiments. R.W. performed the experiments. G.T. performed the statistical analyses. G.T. and P.W. analysed the data and wrote the article. All authors contributed to manuscript revision and editing.

References

Baxter, S.W., Badenes-Perez, F.R., Morrison, A., Vogel, H., Crickmore, N., Kain, W., Wang, P. et al. (2011) Parallel evolution of Bacillus thuringiensis toxin resistance in Lepidoptera. Genetics, 189, 675–679.

Bird, L.J. and Akhurst, R.J. (2004) Relative fitness of Cry1A-resistant and -susceptible Helicoverpa armigera (Lepidoptera: Noctuidae) on conventional and transgenic cotton. J. Econ. Entomol. 97, 1699–1709.

Bird, L.J. and Akhurst, R.J. (2005) Fitness of Cry1A-resistant and -susceptible Helicoverpa armigera (Lepidoptera: Noctuidae) on Transgenic cotton with reduced levels of Cry1Ac. J. Econ. Entomol. 98, 1311–1319.

Bird, L.J. and Akhurst, R.J. (2007) Effects of host plant species on fitness costs of Bt resistance in Helicoverpa armigera (Lepidoptera: Noctuidae). Biol. Control, 40, 196–203.

Boodley, J.W. and Sheldrake, R. (1982) Cornell peat-lite mixes for commercial Bird, L.J. and Akhurst, R.J. (2004) Relative fitness of Cry1A-resistant and -susceptible Helicoverpa armigera (Lepidoptera: Noctuidae) on conventional and transgenic cotton. J. Econ. Entomol. 97, 1699–1709.

Brixvaut, T., Heuberger, S., Zhang, M., Ellers-Kirk, C., Ni, X., Masson, L., Li, X. et al. (2013) Potential shortfall of pyramided transgenic cotton for insect resistance management. Proc. Natl Acad. Sci. USA, 110, 5806–5811.

Carey, J.R. (1993) Applied Demography for Biologists with Special Emphasis on Insects. New York: Oxford University Press.

Carpenter, J.E. (2010) Peer-reviewed surveys indicate positive impact of commercialized GM crops. Nat. Biotechnol. 28, 319–321.

Carriere, Y., Ellers-Kirk, C., Biggs, R., Higginson, D.M., Dennehy, T.J. and Tabashnik, B.E. (2015) Optimizing pyramided transgenic Bt crops for sustainable pest management. Nat. Biotechnol. 33, 161–168.

Capitaino, M.G., Yafuoso, C., Schmidt, C., Huang, C.Y., Rahman, M., Olson, C., Ellers-Kirk, C. et al. (2006) Farm-scale evaluation of the impacts of transgenic Bt crops on biodiversity, pesticide use, and yield. Proc. Natl Acad. Sci. USA, 103, 7571–7576.

Gahan, L.J., Pauchet, Y., Vogel, H. and Heckel, D.G. (2010) An ABC transporter mutation is correlated with insect resistance to Bacillus thuringiensis Cry1Ac toxin. PLoS Genet. 6, e1001248.

Garcia, M., Ortego, F., Hernandez-Crespo, P., Farinós, G.P. and Castanera, P. (2015) Inheritance, fitness costs, incomplete resistance and feeding preferences in a laboratory-selected MON810-resistant strain of the true armyworm Mythimna unipuncta. Pest Manag. Sci. 71, 1631–1639.

Gassmann, A.J., Carriere, Y. and Tabashnik, B.E. (2009) Fitness costs of insect resistance to Bacillus thuringiensis. Annu. Rev. Entomol. 54, 147–163.

Gould, F. (1998) Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu. Rev. Entomol. 43, 701–726.

Gryspert, A. and Gregoire, J.-C. (2012) Lengthening of insect development on Bt zone results in adult emergence asynchrony: does it influence the effectiveness of the high dose/refuge zone strategy? Toxins, 4, 1323–1342.

Heckel, D.G. (2012) Learning the ABCs of Bt: ABC transporters and insect resistance to Bacillus thuringiensis provide clues to a crucial step in toxin mode of action. Pestic. Biochem. Physiol. 104, 103–110.

Hutchinson, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M. et al. (2010) Aneawide suppression of European Corn Borer with Bt maize reaps savings to non-Bt maize growers. Science, 330, 222–225.

James, C. (2015) 20th anniversary (1996 to 2015) of the global commercialization of biotech crops and biotech crop highlights in 2015. ISAAA Brief No. 51. ISAAA: Ithaca, NY.

Jannaaat, A.F. and Myers, J. (2003) Rapid evolution and the cost of resistance to Bacillus thuringiensis in greenhouse populations of cabbage loopers, Trichoplusia ni. Proc. R. Soc. Lond. Ser. B Biol. Sci. 270, 2263–2270.

Jannaaat, A.F. and Myers, J.H. (2005) The cost of resistance to Bacillus thuringiensis varies with the host plant of Trichoplusia ni. Proc. R. Soc. B Biol. Sci. 272, 1031–1038.

Jannaaat, A.F. and Myers, J.H. (2007) Host-plant effects the expression of resistance to Bacillus thuringiensis kurstaki in Trichoplusia ni (Hubner): an important factor in resistance evolution. J. Evol. Biol. 20, 62–69.

Kain, W.C., Zhao, J.Z., Jannaaat, A.F., Myers, J., Shelton, A.M. and Wang, P. (2004) Inheritance of resistance to Bacillus thuringiensis Cry1Ac toxin in a greenhouse-derived strain of cabbage looper (Lepidoptera: Noctuidae). J. Econ. Entomol. 97, 2073–2078.

Kain, W., Song, X., Jannaaat, A.F., Zhao, J.Z., Myers, J., Shelton, A.M. and Wang, P. (2015) Resistance of Trichoplusia ni populations selected by Bacillus thuringiensis toxins to cotton plants expressing pyramided Bacillus thuringiensis toxins Cry1Ac and Cry2Ab. Appl. Environ. Microbiol. 81, 1884–1890.

Lenormand, T. and Raymond, M. (1998) Resistance management: the stable zone strategy. Proc. R. Soc. Lond. Ser. B Biol. Sci. 265, 1985–1990.

Liu, Y.B., Tabashnik, B.E., Dennehy, T.J., Patin, A.L. and Bartlett, A.C. (1999) Development time and resistance to Bt crops. Nature, 400, 519.

Lu, Y., Wu, K., Jiang, Y., Guo, Y. and Desneux, N. (2012) Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. Nature, 487, 362–365.

Mahon, R.J. and Olsen, K.M. (2009a) Fitness of Cry1A-resistant and -susceptible Helicoverpa armigera (Lepidoptera: Noctuidae) on transgenic cotton with reduced levels of Cry1Ac. J. Econ. Entomol. 102, 708–716.

Mahon, R.J. and Olsen, K.M. (2009b) Limited survival of a Cry2Ab-resistant strain of Helicoverpa armigera (Lepidoptera: Noctuidae) on Bollgard II. J. Econ. Entomol. 102, 708–716.

Peck, S.L., Gould, F. and Ellner, S.P. (1999) Spread of resistance in spatially extended regions of transgenic cotton: implications for management of Heliothis virescens (Lepidoptera: Noctuidae). J. Econ. Entomol. 92, 1–16.

R Development Core Team. (2011) R: A Language and Environment for Statistical Computing. (Computing, T.R.F.I.S. ed.). Vienna, Austria. https://www.r-project.org.

Raymond, B., Sayed, A.H. and Wright, D.J. (2005) Genes and environment interact to determine the fitness costs of resistance to Bacillus thuringiensis. Proc. R. Soc. B Biol. Sci. 272, 1519–1524.

Raymond, B., Sayed, A.H. and Wright, D.J. (2007) Host plant and population determine the fitness costs of resistance to Bacillus thuringiensis. Biol. Lett. 3, 82–85.

Raymond, B., Wright, D.J. and Bonsall, M.B. (2011) Effects of host plant and genetic background on the fitness costs of resistance to Bacillus thuringiensis. Heredity, 106, 281–288.

Shorey, H.H. (1964) Sex pheromones of Noctuid moths. II. Mating behavior of Trichoplusia ni (Lepidoptera: Noctuidae) with special reference to the role of the sex pheromone. Ann. Entomol. Soc. Am. 57, 371–377.
Song, X., Kain, W., Cassidy, D. and Wang, P. (2015) Resistance to Bacillus thuringiensis toxin Cry2Ab in Trichoplusia ni is conferred by a novel genetic mechanism. Appl. Environ. Microbiol. 81, 5184–5195.

Sutherland, D.W.S. and Greene, G.L. (1984) Cultivated and wild host plants. In Suppression and Management of Cabbage Looper Populations (Lingren, P.D. and Greene, G.L., eds), pp. 1–13. United States Department of Agriculture.

Tabashnik, B.E. (2008) Delaying insect resistance to transgenic crops. Proc. Natl Acad. Sci. USA, 105, 19029–19030.

Tabashnik, B.E. and Carriere, Y. (2015) Successes and failures of transgenic Bt crops: global patterns of field-evolved resistance. In Bt Resistance: Characterization and Strategies for GM Crops Producing Bacillus thuringiensis Toxins (Soberon, M., Gao, Y. and Bravo, A., eds), pp. 1–13. New York: CABI Press.

Tabashnik, B.E., Gassmann, A.J., Crowder, D.W. and Carriere, Y. (2008) Insect resistance to Bt crops: evidence versus theory. Nat. Biotechnol. 26, 199–202.

Tabashnik, B.E., Brevault, T. and Carriere, Y. (2013) Insect resistance to Bt crops: lessons from the first billion acres. Nat. Biotechnol. 31, 510–521.

Wang, P., Zhao, J.Z., Rodrigo-Simon, A., Kain, W., Janmaat, A.F., Shelton, A.M., Ferré, J. et al. (2007) Mechanism of resistance to Bacillus thuringiensis toxin Cry1Ac in a greenhouse population of the cabbage looper, Trichoplusia ni. Appl. Environ. Microbiol. 73, 1199–1207.

Wang, R., Tetreau, G. and Wang, P. (2016) Effect of crop plants on fitness costs associated with resistance to Bacillus thuringiensis toxins Cry1Ac and Cry2Ab in cabbage loopers. Sci. Rep. 6, 20959.

Wei, J., Guo, Y., Liang, G., Wu, K., Zhang, J., Tabashnik, B.E. and Li, X. (2015) Cross-resistance and interactions between Bt toxins Cry1Ac and Cry2Ab against the cotton bollworm. Sci. Rep. 5, 7714.

Williams, J.L., Ellers-Kirk, C., Orth, R.G., Gassmann, A.J., Head, G., Tabashnik, B.E. and Carriere, Y. (2011) Fitness cost of resistance to Bt cotton linked with increased gossypol content in pink bollworm larvae. PloS ONE, 6, e21863.

Zhao, J.Z., Cao, J., Li, Y.X., Collins, H.L., Roush, R.T., Earle, E.D. and Shelton, A.M. (2003) Transgenic plants expressing two Bacillus thuringiensis toxins delay insect resistance evolution. Nat. Biotechnol. 21, 1493–1497.