Cotton produces insecticidal terpenoids that are induced by tissue-feeding herbivores. Damage by Heliothis virescens caterpillars increases the terpenoid content, which reduces the abundance of aphids. This effect is not evident in Bt-transgenic cotton, which is resistant to H. virescens. We determined whether induction of terpenoids by caterpillars influences the host quality of Aphis gossypii for the parasitoid Lysiphlebus testaceipes and whether this interaction is influenced by Bt cotton. The exposure of parasitoids to terpenoids was determined by quantifying terpenoids in the aphids. We detected several terpenoids in aphids and found a positive relationship between their concentrations in plants and aphids. When L. testaceipes was allowed to parasitize aphids on Bt and non-Bt cotton that was infested or uninfested with H. virescens, fewer parasitoid mummies were found on infested non-Bt than on Bt cotton. Important parasitoid life-table parameters, however, were not influenced by induced resistance following H. virescens infestation, or the Bt trait. Our study provides an example of a tritrophic indirect interaction web, where organisms are indirectly linked through changes in plant metabolites.

1. Introduction

Cotton possesses a range of insect-resistance mechanisms, including the production of a set of terpenoids, e.g. gossypol, hemigossypolone and the heliocides 1–4 [1,2]. Production of these compounds is systemically induced by tissue-feeding herbivores [1,2], but not by aphids and other phloem-feeding herbivores [3]. Phloem-feeders are, however, affected by cotton terpenoids [2]. Cotton thus provides an excellent model to study indirect interaction webs that include non-trophic, indirect links among organisms that contribute to the structure of multi-trophic food webs [4].

We previously reported that the induction of terpenoids by the fruit- and leaf-feeding caterpillars of Heliothis virescens reduced the population growth of the cotton aphid Aphis gossypii, a case of indirect plant-mediated competition between herbivores [5]. Bt-transgenic cotton is highly resistant against lepidopteran larvae owing to the expression of Cry-toxins derived from the bacterium Bacillus thuringiensis. Bt cotton is thus only marginally damaged by caterpillars, thereby reducing the terpenoid concentration and improving host plant suitability for aphids [5]. Here, we assess the effects of caterpillar-induced resistance in Bt and non-Bt cotton on higher trophic levels, represented by the aphid parasitoid Lysiphlebus testaceipes. Aphis gossypii is causing economic losses in cotton in the USA [2] and L. testaceipes is one of the principal parasitoids associated with this pest [6].
2. Material and methods

(a) Plants and insects

Two varieties of transgenic cotton, provided by Monsanto Company, were used. Bt cotton (Deltapine DPL143B2 RF, event: MON15985 × MON88913) produces Cry1Ac and Cry2Ab, insecticidal proteins derived from the bacterium *B. thuringiensis* with known toxicity against Lepidoptera, and a herbicide-tolerance trait. Non-Bt cotton (Deltapine DPL147 RF, MON88913) has a similar genetic background, but contains only the herbicide-tolerance trait. Plants were grown in the glasshouse as described [5].

*Aphis gossypii* was reared on DPL147 plants, and third instars of *H. virescens* were obtained from Syngenta (Stein, Switzerland). Mummies of *L. testaceipes* were obtained from Katz Biotech AG (Baruth, Germany).

Insects were reared, and experiments were conducted at 25 ± 5°C, RH 70 ± 10% and 16 : 8 h long-day conditions in a glasshouse.

(b) Parasitoid performance (experiment 1)

Bt and non-Bt cotton plants were used when the fourth leaf was fully expanded. This corresponds with the growth stage at which aphids start to colonize cotton in the southern USA [2]. Plants were infested with one third instar of *H. virescens* or were left uninfested. The larva was enclosed in a gauze bag that surrounded the youngest leaf. After 7 days, larvae were removed, and their weight was recorded. Non-Bt cotton on which the larvae had died were excluded from the experiment.

Following caterpillar removal, 100 *A. gossypii* (mixed stages) were transferred to the youngest leaf of each plant, enclosed in a clip-cage. After 48 h, the cage was removed. After 7 days, three freshly emerged, mated, female *L. testaceipes* were enclosed for 4 h in a gauze bag surrounding the aphid colony. Aphids were checked daily for pupae of aphid parasitoids (mummies), which were individually transferred to 0.5 ml reaction tubes. Parasitoid emergence rate, development time and sex ratio were recorded. Mummy diameter (width behind the coxa of the hind legs) was measured for 10 mummies per plant.

The longevity of the first five emerging female parasitoids was determined. Females were kept individually in glass vials (60 × 12 mm) that contained wet cotton wool. A water–sucrose solution (1 M; 0.5 μl) was provided and changed every 2 days. Survival of parasitoids was checked twice each day. Once the parasitoid had died, its hind tibia length was measured.

The data obtained for the mummies and parasitoids from each plant were pooled for analyses. Per treatment, 33–37 plants were used.

After the experiment, leaves exposed to *H. virescens* were photographed, and damage was quantified using IMAGEJ v. 1.46. The youngest expanded leaf of each plant was collected for terpenoid quantification.

(c) Terpenoid concentration in leaves and aphids

(experiment 2)

Initial procedures for experiment 2 were identical to those in experiment 1. Seven days after the aphids were released, the aphids and the youngest expanded leaf on each plant were carefully collected using a brush and scissors, respectively, and stored at −80°C. Terpenoid content (gossypol, hemigossypolone, heliocides 1 + 4, heliocides 2 + 3) was analysed by HPLC using the protocol established in Hagenbucher et al. [5]. The heliocides 1 and 4 as well as the heliocides 2 and 3 are stereoisomers of each other and could not be analysed separately [2]. Sample size for plant material was 8–10 mg (dry weight) per plant, whereas for aphids, it ranged from 4 to 12 mg, which represented the aphid population of a single plant. Lyophilized plant and insect material was used for the analyses. For leaf material 1 ml of the extraction mixture was used, but only 0.5 ml for aphids. This was done in order to adjust for the lower biomass and expected lower terpenoid content of the aphid samples.

(d) Statistical analyses

Damage by *H. virescens* was analysed using Welch’s *t*-test. Terpenoids were analysed using one-way analysis of variance (ANOVA) followed by Tukey’s HSD test. Total terpenoid and gossypol data were log-transformed to meet the ANOVA assumptions. Remaining datasets were analysed by generalized linear models (GLMs) using the glm() function of R. Plant treatment (uninfested Bt, infested Bt, uninfested non-Bt and infested non-Bt) was used as fixed factor (called treatment from here on) and the recorded parameter as dependent variable. For the analysis of the percentage of plants with successful parasitization (number of mummies) and the percentage of plants from which parasitoids emerged (number of adults), a binomial error
distribution was used. Plants without mummies were excluded from calculations of adult emergence. As the model detected a significant main effect for the number of mummies, the number of infested non-Bt plants was subsequently compared with the other three treatments. False discovery rate control was applied for multiple tests following linear models [7]. For sex ratio, a binomial error distribution was used, whereas for emergence rate, a quasi-binomial distribution was used to account for overdispersion. Poisson distribution was assumed for count data (number of mummies; number of adults); in both cases, however, a quasi-Poisson distribution was used to account for overdispersion of the model. Gamma distribution was used for the remaining datasets. In all cases, the default link function given by R was used. All statistical analyses were conducted using the R v. 3.0.1 statistical software (http://www.r-project.org/).

3. Results

(a) Performance of Heliothis virescens (experiment 1)

Bt plants remained nearly free from H. virescens damage (leaf area consumed ± s.e.: 0.3 ± 0.06 cm²; n = 34) compared with non-Bt plants (44.6 ± 3.45 cm²; n = 37; t-test: t = 13.1, p < 0.0001). No larva was found alive on Bt cotton, whereas all larvae on non-Bt cotton survived and showed a significant weight gain (weight gain ± s.e.: 247 ± 20.9 mg; n = 37).

(b) Terpenoid concentration in leaves and aphids (experiment 2)

The concentration of total terpenoids and of each terpenoid class in the youngest leaf did not differ between uninfested Bt and uninfested non-Bt cotton (figure 1a and the electronic supplementary material, table S1). Caterpillar damage induced terpenoid production in non-Bt cotton, but not in Bt cotton (figure 1a and the electronic supplementary material, table S1; F3,28 = 6.9, p < 0.001). The increase was significant for hemigossypolone (F3,28 = 4.4, p = 0.011), gossypol (F3,28 = 7.7, p < 0.001) and heliociodes 1 + 4 (F3,28 = 4.3, p = 0.013) but not for heliociodes 2 + 3 (F3,28 = 2.5, p = 0.080); the electronic supplementary material, table S1). In leaves that were collected when experiment 1 was terminated (approx. four weeks after caterpillar damage occurred), the induction of cotton terpenoids was no longer detectable (F3,105 = 0.26, p = 0.85).

Aphids contained gossypol, hemigossypolone and heliocides 1 + 4, but not heliocides 2 + 3 (the electronic supplementary material, table S2). The concentrations were less than 10% of those found in leaves (figure 1b). Aphids from infested non-Bt plants contained higher amounts of terpenoids than those from the remaining treatments (figure 1b and the electronic supplementary material, table S2; F3,31 = 3.0, p = 0.045). On non-Bt plants, terpenoid levels in aphids from the infested treatment were almost triple those from the uninfested plants, whereas no increase was seen in the corresponding treatments on Bt plants. Increased concentrations were found for the terpenoids gossypol (F3,31 = 4.1, p = 0.014) and heliociodes 1 + 4 (F3,31 = 4.0, p = 0.016), but not for hemigossypolone (F3,31 = 2.7, p = 0.064; the electronic supplementary material, table S2).

(c) Parasitoid performance (experiment 1)

The percentage of plants that supported the development of aphid mummies did not differ between uninfested Bt and uninfested non-Bt plants or between uninfested and infested Bt plants (table 1). However, fewer caterpillar-infested non-Bt cotton plants carried mummies when compared with uninfested non-Bt plants or Bt plants that were infested or uninfested. This indicates that caterpillar-induced cotton plants are less likely to carry mummies than the remaining plant types.

For the plants that carried aphid mummies, the percentage of plants that supported the emergence of adult parasitoids did not differ between any of the four plant treatments (table 1). Similarly, no differences among treatments were detected for the number of mummies formed, the number of adults emerged, mummy size, development time, sex ratio, hind tibia length or survival of females (table 2).

4. Discussion

We here demonstrate, for the first time to our knowledge, that A. gossypii ingest terpenoids when feeding on cotton. We detected hemigossypolone, gossypol and heliociodes 1 + 4. Terpenoid concentration increased about threefold, reaching 803 ng mg⁻¹ or 0.085% of aphid dry weight, when the cotton had been infested with H. virescens, mirroring the terpenoid increase in the plants. While terpenoid concentration in the

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<tr>
<td>mummies</td>
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<td>χ² (3,137) = 9.14; p = 0.027</td>
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<td>% plants with mummies</td>
<td>χ² (3,79) = 7.53; p = 0.067</td>
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<tr>
<td>adults</td>
<td>total no. plants</td>
<td>χ² (3,137) = 9.14; p = 0.027</td>
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<tr>
<td></td>
<td>% plants with adults</td>
<td>χ² (3,79) = 7.53; p = 0.067</td>
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<td>Non-Bt uninfested</td>
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<td>non-Bt infested</td>
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* Significant different (p < 0.03) from the other three treatments according to GLM with false discovery rate control [7].
aphids was relatively low, i.e. less than 10% of that in the host plants, altered aphid terpenoid levels could affect higher trophic organisms and thus food-web dynamics. Uptake of other plant-derived toxins by Sternorrhyncha has been established in a few studies [8], and sequestrating aphids were found to accumulate plant compounds to levels up to 2% of their body weight [9].

The percentage of plants on which parasitoid mummies formed was lower for the caterpillar-infested non-Bt plants than for the uninfested non-Bt or the protected Bt plants. However, neither plant genotype nor infestation affected mummy size or performance of emerging parasitoids. The observed effect is a case of reduction in mummy formation is most likely explained by mummy size or performance of emerging parasitoids. The effect is a case of an indirect multi-trophic interaction, where damage by non-host caterpillars affects the natural enemy of another herbivore, i.e. the aphids. The fact that cotton terpenoids affect links in indirect interaction webs has previously been reported [5,10–12]. Previous root herbivory by larvae of Agrilus lineatus reduced food consumption and the relative growth rates of Spodoptera exigua larvae by more than 50% in comparison with larvae feeding on the undamaged cotton plants [10]. The omnivorous thrips Frankliniella occidentalis also exhibits a different feeding behaviour on damaged and undamaged cotton plants. On plants previously damaged by spider mites, thrips adults reduced their uptake of (terpenoid-rich) plant material by 50% in favour of the consumption of (low terpenoid) spider mites [11]. In another case, the biocontrol fungus Trichoderma virens mediates resistance against the seedling disease Rhizoctonia solani mainly via an increase of hemigossypol and desoxyhemigossypol [12].

Our findings indicate that developing L. testaceipes are not affected directly by the insecticidal trait in Bt cotton, but there are some indications that it is indirectly affected through changes in plant terpenoid levels as a response to caterpillar damage.

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Data accessibility. Data are available from the figshare Data Repository (http://dx.doi.org/10.6084/m9.figshare.899882).

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