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ARTICLE

Terpene-Mediated Parasitoid Host Location Behavior on Transgenic and Classically Bred Apple Genotypes

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Terpene-mediated interactions between transgenic or classically bred apple genotypes and associated insects were investigated. Apple genotypes were either resistant or susceptible to *Venturia inaequalis* that causes apple scab. They were subjected to infestation by *Phyllonorycter* leafminers and/or inoculation with *V. inaequalis*. Apple leaf extracts were analyzed by gas chromatography−mass spectrometry to quantify squalene, a triterpene known to mediate host location by *Pholetesor* parasitoids that are specialized on leafminers. Squalene contents in leafminer-infested leaves differed between the transgenic apple scab resistant line and a classically bred cultivar sharing the same resistance gene. This resistant cultivar showed an increase in squalene contents from healthy to leafminer-infested leaves. This was not the case in the transgenic resistant line. However, there was also no increase in the susceptible isogenic cultivar. Behavioral bioassays with parasitoid females also reflected these findings. Hence, alterations in leaf chemistry and corresponding responses of the parasitoid are apparent among classically bred cultivars, rather than in the genetically modified resistant line.

KEYWORDS: Semiochemical; GC-MS; apple; transgenic; nontargets; leafminer; *Phyllonorycter* sp.; parasitoid; *Pholetesor* sp.; behavior; oviposition; pathogen

INTRODUCTION

Phytochemicals mediate host location behavior in numerous parasitoid wasp species, both prior to (*1*, *2*) and after landing on a host-infested plant (*3*). In many plant−insect systems, plant terpenes play a crucial role as semiochemicals, as was shown for monoterpenes (*4*), sesquiterpenes (*5*), homoterpenes (*6*), diterpenes (*7*), and in a single case for a triterpene (*8*). Damaged plants release typically higher quantities of phytochemicals, and such herbivore-induced compounds guide natural antagonists, particularly parasitoids, to the site of damage (*9*).

Transgenic plants may differ from their isogenic counterpart by altered emissions of induced semiochemicals, as was shown for quantitatively different volatile emissions from maize plants that were genetically modified with *Bacillus thuringiensis* (*10*). Subtle chemical changes caused by transgenosis may have effects on nontarget organisms including parasitoids and are therefore of high interest in risk assessment studies (*11*, *12*). So far, investigations on chemically mediated interactions involving transgenic crops have largely focused on induced volatile chemicals influencing parasitoid behavior prior to landing on a host-infested patch (*10*, *13*, *14*). By contrast, virtually nothing is known of chemically mediated host location after landing of the parasitoid on transgenic versus isogenic plants (*15*).

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Apple genotypes vary in their chemical composition (*16*, *17*), and different cultivars release different amounts of volatile terpenes upon herbivory (*18*). However, it is yet unknown whether or to what degree different apple genotypes vary in their contents of bioactive nonvolatile terpenes. Upon infestation by the apple leafminer *Phyllonorycter* sp. (Lepidoptera: Gracillariidae), which damages the apple foliage by feeding within the leaf tissue and generates a tentiform mine, squalene contents of the leafmine increase drastically (*8*, *19*). Remarkably, this single triterpene guides the parasitoid *Pholetesor* sp. (Hymenoptera: Braconidae) to its larval host concealed in the plant tissue (*8*). Parasitoid females inspect the leaf surface with their antennae and finally insert their ovipositor into the mine to parasitize a young leafminer larva (*20*), a behavioral pattern that can also be observed by using simply hexane leaf extracts or synthetic squalene presented on filter paper (*8*, *19*). In the orchard ecosystem, *Pholetesor* parasitoids substantially contribute to population regulation of the potentially devastating apple leafminer (*21*). Hence, the tritrophic system composed of apple plant, leafminer, and its parasitoid is highly suitable and meaningful for studying potential chemically mediated nontarget effects of transgenic plants. Different defense responses by a plant may be triggered (*22*) depending on whether such plant is infected with a pathogen or a herbivore or concurrently with both organisms; therefore, inoculations by *Venturia inaequalis* were included in the study as well.

In apple orchards, the worldwide distributed fungal pathogen *V. inaequalis* causes apple scab, thereby leading to major

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economic losses or requiring frequent fungicide applications (*23*). Resistant apple genotypes hold great promise, provided they do not pose any particular risk to nontarget organisms. Four different apple genotypes were investigated. The scab-resistant transgenic apple line 'Gala-trans*Vf*' was tested in comparison to its isogenic line 'Gala' that is susceptible to *V. inaequalis*. This transgenic genotype contains, in addition to the scab resistance gene *HcrVf2*, the selectable marker gene *nptII* under the control of the S35 promoter (*24*, *25*). Further genotypes included in this study comprise 'Gala-trans0', the corresponding apple scab susceptible transgenic genotype devoid of the resistance gene, and 'Florina', a widely used classically bred cultivar that also contains the *Vf* scab resistance and is thus suitable as a nontransgenic scab-resistant control. Until now, no comparative study on nontarget effects of transgenic resistant plants known to the authors included the conventionally bred resistant genotype, the wild type, the transgenic resistant genotype as well as the transgenic control genotype solely containing the promoter and the selectable marker gene, thus limiting interpretations on risk assessments.

The purpose of this study was to elucidate possible effects of transgenic versus classically bred apple cultivars on the nontarget parasitoid *Pholetesor circumscriptus* Nees in multitrophic systems subjected to different infection regimens with the fungal pathogen *V. inaequalis* and/or the insect herbivore *Phyllonorycter blancardella* Fabricius. Investigations comprised chemical analyses as well as observation of parasitoid behavioral responses.

MATERIALS AND METHODS

Insect Rearing. The initial colony of the leafminer *P. blancardella* originated from individuals collected in commercial apple orchards in South Tyrol (Italy). The colony was refreshed on a yearly basis by introducing new individuals collected from the same area. The colony of its parasitoid *P. circumscriptus* originated from individuals collected from the same area in South Tyrol (Italy). The apple leafminer *P. blancardella* was reared under controlled conditions (22 ± 2 °C, 50 ± 5% relative humidity, and 16 h L/8 h D photoperiod), on 2-month-old potted apple seedlings (*Malus* × *domestica* 'Golden Delicious' open pollinated seedlings), in Plexiglas rearing cages (45.7 cm × 25 cm × 27 cm) (*26*). Adult parasitoids were kept in a separate climatic chamber (Conviron, Controlled Environment Ltd., Winnipeg, Canada) in Plexiglas rearing cages (25 cm × 25 cm × 25 cm) at 21 ± 2 °C, 60 ± 10% relative humidity, and a 16 h L/8 h D photoperiod. The adult parasitoids were provided with honey and water, and females were allowed to oviposit on apple seedlings infested with leafminer larvae at the sap-feeding stage (*20*). After parasitoids egressed from the hosts to pupate, their cocoons were removed from the mines and placed in separate plastic boxes with moist tissue paper until adult emergence, after which the parasitoids were transferred into a new Plexiglas cage and were provided only with honey and water.

Apple Plants. Apple plants of the four genotypes 'Gala', 'Gala-trans0', 'Gala-trans*Vf*', and 'Florina' were used for the experiments. As documented in a previous study comprising all of these genotypes, a similar level of scab resistance was found for the two apple genotypes 'Florina' (a commercial scab-resistant cultivar with the resistance gene introgressed from *Malus floribunda* 821) and 'Gala-trans*Vf*' (= Ga2:21) (*24*). Two-year-old plants of each genotype were potted in cylindrical pots containing 1.5 L of a substrate−perlite−sand mixture at a ratio of 6:1:1 and grown in a fully equipped and closed greenhouse chamber with internal air circulation at 22 ± 2 °C during the day and 18 ± 2 °C during the night, 60 ± 5% relative humidity, and a photoperiod of 16 h L/8 h D. Assimilation lighting was used to complement daylight conditions when necessary (lighting level, 5000 lx; sodium vapor lamps, 400 W). The plants were pruned regularly and fertilized weekly (Wuxal liquid fertilizer; concentration 0.2%; N/P/K 10:10:7.5; Maag Syngenta Agro, Dielsdorf, Switzerland) to keep them in a vegetative active growing stage, with two branches each of 40 ± 10 cm in length. For protection against herbivory of spider mites, sprays of bromopropylate (diphenyl acaricide, Spomil, EC 250 g/L, 15 mL/10 L) were used particularly in the initial growth phase and, later, predatory mites (*Phytoseiulus persimilis*; Andermatt Biocontrol AG Grossdietwil, Switzerland) were released. The latest timing of pesticide application or predatory mite release was 14 days prior to the start of the experiment. Plants were examined when they were selected for the subsequent experiments and found to be devoid of mites.

Apple Leaf Extracts. Apple leaf extracts were prepared for chemical analyses as well as for bioassays, using leaves of similar age and size from each of the four genotypes subjected to one of the four different infection types (specified below), yielding a total of 16 treatments. Within a genotype, only a single treatment was induced on a particular tree. The five youngest leaves per shoot were labeled and subjected to one of the four infection types (*27*): (1) inoculation with *V. inaequalis* conidia suspension (10^5 conidia/mL) carried out in a separate inoculation tent (*28*) 24 ± 2 days prior to extraction; (2) infestation for 3 days with six 2−3-day-old leafminer adults 24 ± 2 days prior to extraction (*27*); (3) inoculation with *V. inaequalis* conidia suspension (10^5 conidia/mL) in a separate inoculation tent (*28*) and subsequent infestation (55 ± 1 h after inoculation) with six 2−3-day-old leafminer adults, 24 ± 2 days prior to extraction; and (4) no inoculation or infestation (healthy control). All scab-inoculated plants of 'Gala' and 'Gala-trans0' showed scab symptoms on the youngest inoculated leaves, whereas no symptoms were visible on inoculated leaves of scab-resistant 'Gala-trans*Vf*' and 'Florina'. All leafminer-infested leaves still contained the sap-feeding larvae at the time of extraction.

Leaf samples of each treatment for extraction were first photographed with a digital camera (Nikon Coolpix 990, Nikon Corp., Tokyo, Japan), together with a reference area of 1 cm^2, in order to calculate the total leaf area using Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose, CA) (*29*). For chemical analysis, the labeled leaves from one shoot of each of the five plants per treatment were pooled together, yielding a total of 80 (5 × 16) samples. For the bioassays, two labeled leaves (second and fourth positions) from each of the five plants per treatment were pooled and extracted, resulting in a total of 16 extracts, each of which was tested using 30 female parasitoids, yielding a total of 480 trials with extracts. The extraction of the leaf samples was carried out using 50 mL of hexane as solvent (≥99.0%, Fluka, Buchs, Switzerland) per five leaves, or accordingly, 100 mL per 10 leaves. After 24 h of soaking time at 4 °C in the darkness, the extracts were separated from the plant material by filtration through filter paper (Rundfilter Nr LS 14, ∅ 70 mm, Schleicher & Schuell GmbH, Dassel, Germany). Samples were subsequently concentrated with a rotary evaporator (Laborata 4000 efficient, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at a speed of 60 rpm and a temperature of 32 ± 2 °C. The concentrated extracts were then filtered with Durapore membrane filters (HVLPO 1300, Millipore Corp., Billerica, MA) packed into a 13 mm syringe Swinney filter holder (Swinney Stainless, Millipore Corp., Billerica, MA) at room temperature. The extracts were finally concentrated under a continuous stream of nitrogen (purity = 99.96%) to a final volume of 2 mL and stored at −60 °C for later chemical analyses and behavioral testing.

Chemical Analysis. To quantify squalene contents in the above-mentioned leaf extracts, a gas chromatograph−mass spectrometer system (HP 6890 series GC-System and HP 5973, Hewlett-Packard Co., Atlanta, GA) with a split/splitless injector in splitless mode was used. The GC-MS was fitted with a deactivated fused silica precolumn (5 m × 0.25 mm; Agilent Technologies, Basel, Switzerland) and a nonpolar Econo-Cap EC-5 column (30 m × 0.25 mm, 0.25 μm film thickness; Alltech Socochim SA, Lausanne, Switzerland). The oven temperature was programmed starting at 50 °C for 5 min, then increased to 300 °C at a rate of 5 °C/min, and finally held at 300 °C for 15 min. The carrier gas was helium used at a constant pressure. Quadrupole and ion source temperatures were 150 and 230 °C, using the full-scan method.

The injected samples contained 20 ng of the internal standard octylbenzene (Fluka, Buchs, Switzerland) dissolved in hexane (100 ng/μL) (*19*). For the identification of the mass spectrometry data, ChemStation software (MSD Productivity Chem Station software, Agilent Technologies Inc., Santa Clara, CA) linked to the NIST98 library was used. Squalene (Fluka, Buchs, Switzerland) dissolved in hexane was injected as a reference for definitive identification of this compound in leaf samples.

Behavioral Bioassays. Single-choice contact bioassays were carried out to test and quantify host searching behavior of parasitoid females exposed to apple leaf extracts of 16 treatments and an additional solvent control. All bioassays were carried out with naïve 2−4-day-old mated parasitoid females from the 10th to 15th generation of laboratory rearing.

All females had no previous oviposition experience and/or contact with leafminers or its host plant. Bioassays were carried out between 9:00 a.m. and 6:00 p.m. in Petri dish arenas (9 cm diameter) equipped with a filter paper (1.3 cm diameter; corresponding to a fully developed mine (*19*)) treated with 10 μL of the apple leaf extract. The filter paper treated with the extract was raised at a 45° angle with respect to the surface of the Petri dish with a rolled piece of Teflon (*19*). Laboratory conditions were 24 ± 2 °C, 50 ± 10% relative humidity, and 1200 ± 250 lx. Female parasitoids were allowed to acclimatize for at least 30 min prior to the bioassay, then placed singly in a glass vial (1.1 cm diameter, 3.9 cm in height), and released into the Petri dish with the open end of the vial facing the extract-treated filter paper. Petri dishes, filter papers, and parasitoids were used once only. A total of 30 parasitoids were observed per treatment, and a total of 30 parasitoids were tested on the hexane solvent control. For each parasitoid continuous behavioral observations were recorded on a personal computer using the software 'The Observer 3.0' (Noldus Information Technology 1991, Wageningen, The Netherlands). Recordings were carried out for a period of 20 min starting as soon as the parasitoid was released into the Petri dish. The recorded states of all female parasitoids comprised *ovipositional probing* (touching the substrate with the ovipositor), *antennation* (touching the substrate with the antennae), *antennal preening* (cleaning of antennae with the fore legs), *abdominal preening* (cleaning of abdomen with the hind legs), *standing*, and *walking*. To avoid any day-to-day bias, testing always comprised all four apple genotypes within one infection type as well as the solvent control at the same day, and testing of all 30 parasitoids per treatment was expanded over several days each.

Data Analysis. Statistical analyses of squalene concentration in leaf extracts and parasitoid behavior were performed using JMP 7.0.2 (SAS Institute, Cary, NC) and SPSS 16.0 for Mac (SPSS Inc., Chicago, IL).

The concentration of squalene per leaf extract was calculated in relation to the concentration of the internal standard octylbenzene. Data were log transformed to ensure normal distribution (Shapiro Wilk W test) and homogeneity of variance (Levene test). The differences in the quantity of squalene across all apple leaf extracts were tested using one-way ANOVA followed by Tukey's HSD post hoc test.

Parasitoid behavior data were tested for normality (Shapiro Wilk W test) and homogeneity of variance (Levene test). To ensure normal distribution a constant value of 0.5 was added to all data prior to log transformation. Multivariate analysis of variance (MANOVA) was carried out to test for effects on recorded states of the parasitoids (dependent variables) across the different extracts and the solvent control tested. Apple genotype, infection type, and the interaction between apple genotype and infection type were the main effects. Subsequent MANOVAs were conducted to exclude possible overlaying effects in the duration of behavioral states with genotype or infection type as main effects. If the MANOVA model showed significant differences, one-way ANOVA followed by Tukey's HSD post hoc test was then conducted to test for differences between the different recorded states.

As healthy leaf extracts showed significant differences in the multivariate test of the model, and the tests of between-subject effects yielded no significant P value for the individual behavioral states, a principal component analysis (PCA) was carried out to reduce the data set to the relevant states and to analyze significant differences in genotype and infection type of parasitoid behavior. Subsequently, the three dissected variables *antennal preening*, *abdominal preening*, and *walking*, which explained most of the variance (74%), were analyzed with a MANOVA.

RESULTS

Chemical Analysis of Leaf Extracts for Squalene Contents. Squalene quantities in the leaf extracts across the apple genotypes did not change significantly in three of the four infection types (i.e., healthy, scab, and combined scab and leafminer infection). However, in the leafminer treatments, the squalene value measured for the genotype 'Florina' was significantly higher than that for 'Gala-trans*Vf*' (**Table 1**; comparisons within rows).

A comparison across the infection types of the 'Gala' lines did not reveal any significant differences (**Table 1**; comparisons within columns). Lower mean amounts of squalene were found in leafminer-infested leaves compared with healthy leaves from 'Gala', 'Gala-trans0', and 'Gala-trans*Vf*', but these differences were not significant. In contrast, among the 'Florina' samples, significantly more squalene was detected in extracts from leafminer-infested than from healthy leaves. This increase in squalene content from healthy to leafminer-infested leaves was >4-fold. Whereas mean values of squalene were still higher in samples from combined scab and leafminer infection, the difference to healthy leaf extracts was no longer significant, and scab inoculation alone did not alter squalene content compared to healthy leaves (**Table 1**; comparison within the column 'Florina').

Table 1. Squalene Quantity per Leaf Surface Unit[a] of Four Apple Genotypes Subjected to Four Different Infection Types

	squalene (mean ± SE ng/cm^2)			
	'Gala'	'Gala-trans0'	'Gala-trans*Vf*'	'Florina'
healthy	9.98 ± 4.01 aA	8.27 ± 4.65 aA	4.46 ± 1.47 aA	3.51 ± 1.45 aB
scab	5.24 ± 1.20 aA	5.92 ± 3.46 aA	8.95 ± 2.37 aA	2.68 ± 0.63 aB
leafminer	5.80 ± 0.30 aA	6.50 ± 1.29 aA	2.62 ± 0.92 bA	16.41 ± 3.90 aA
scab + leafminer	3.79 ± 0.46 aA	7.87 ± 3.29 aA	5.25 ± 1.11 aA	7.06 ± 1.13 aAB

[a] One-way ANOVA of log (x + 0.5) transformed data and Tukey's HSD post hoc test. For comparisons within a row, lower case letters indicate significant differences (P < 0.05) between apple genotypes within one infection type (healthy, n = 5, F = 0.7131, df = 3,16; P = 0.5584; scab, n = 5, F = 2.3223, df = 3,16; P = 0.1139; leafminer, n = 5, F = 10.6351, df = 3,16, P < 0.001; scab + leafminer, n = 5, F = 0.5174, df = 3,16, P = 0.6763). For comparisons within a column, capital letters indicate significant differences (P < 0.05) between infection types within one apple genotype ('Gala', n = 5, F = 0.7, df = 3,16, P = 0.5657; 'Gala-trans0', n = 5, F = 0.1688, df = 3,16, P = 0.9159; 'Gala-trans*Vf*', n = 5, F = 3.1866, df = 3,16, P = 0.0523; 'Florina', n = 5, F = 9.7933, df = 3,16, P < 0.001).

Table 2. Effects on Parasitoid Behavior by Apple Genotype (G), Infection Type (I), and Genotype × Infection Type Interaction (G × I)[a]

effect	hypothesis df	error df	F value	P value
G	18	1470	2.526	**<0.001**
I	18	1470	4.337	**<0.001**
G × I	54	2958	1.271	0.089

[a] MANOVA of log (x + 0.5) transformed data using the six recorded states of parasitoid behavior *substrate antennation*, *ovipositional probing*, *antennal preening*, *abdominal preening*, *standing*, and *walking*. Number of replicates = 30, number of apple genotypes plus solvent control = 5, number of infection types = 4. Bold P values indicate a significant difference. F value for Pillai's trace.

Squalene contents in the separately prepared apple leaf extracts used for the bioassays (below) were in the range of those presented in **Table 1**.

Parasitoid Behavior on the Different Leaf Extracts. In the contact bioassay, responses of parasitoid females to the extracts from the different apple genotypes as well as to those from the different infection types were significantly different, whereas there was no significant genotype × infection type interaction (MANOVA; Pillai's trace P < 0.05) (**Table 2**).

To assess whether or not the hexane solvent control was ineffective with regard to parasitoid behavioral responses, the duration of the behavioral states was quantified and directly compared to the parasitoids' response to healthy leaf extracts. The hexane solvent control elicited minimal *antennation* (0.2 ± 0.2 s) and no *ovipositional probing*, whereas *antennal preening* and *abdominal preening* lasted 16.6 ± 3.4 and 37.9 ± 7.8 s, and *standing* and *walking* amounted to 638.8 ± 87.9 and 505.9 ± 91.8 s, respectively (mean ± SE; n = 30). This response to the hexane solvent control was significantly different from that triggered by healthy leaf extracts (**Tables 3** and **4**). Among the healthy genotypes, parasitoid response did not differ significantly, allowing for a general comparison of the solvent control tested

Table 3. Effects of Leaf Extracts on Parasitoid Behavior[a]

	df	error df	*F* value	*P* value
(**A**) Effect of Solvent Control (Analyzed in Parallel to Healthy Leaf Extracts) Compared with Healthy Leaf Extracts[b]				
	24	572	2.753	**<0.001**
(**B**) Effects of Apple Genotypes within One Infection Type [c]				
infection type				
healthy	9	348	1.556	0.127
scab	18	339	1.507	0.085
leafminer	18	339	1.980	**<0.05**
scab + leafminer	18	339	1.233	0.232
(**C**) Effects of Infection Types within One Apple Genotype[d]				
apple genotype				
'Gala'	18	339	1.676	**<0.05**
'Gala-trans0'	18	339	1.578	0.063
'Gala-trans*Vf*'	18	339	2.235	**<0.05**
'Florina'	18	339	2.401	**<0.05**

[a] MANOVA of $\log(x + 0.5)$ transformed data using six states of parasitoid behavior: (a) *substrate antennation*, (b) *ovipositional probing*, (c) *antennal preening*, (d) *abdominal preening*, (e) *standing*, and (f) *walking* (for infection type healthy: MANOVA using the three recorded states of parasitoid behavior (c, d, f) selected by PCA, see Material and Methods). Number of replicates = 30. Bold *P* values indicate a significant difference. *F* value for Pillai's trace. [b] Number of apple genotypes plus solvent control = 5. [c] Number of apple genotypes = 4. [d] Number of infection types = 4.

in parallel to the healthy leaf extracts. In contrast to the solvent control, healthy leaf extracts elicited significantly more *antennation* (one-way ANOVA; $F = 3.983$; df = 4,145; $P < 0.05$; Tukey's HSD post hoc test), *antennal preening* (one-way ANOVA; $F = 6.190$; df = 4,145; $P < 0.001$; Tukey's HSD post hoc test), and *abdominal preening* (one-way ANOVA; $F = 4.967$; df = 4,145; $P < 0.05$; Tukey's HSD post hoc test). This comparison indicates that the solvent alone was ineffective regarding *antennation* and *ovipositional probing* and also elicited low response in *antennal preening* and *abdominal preening* (**Tables 3** and **4**).

A comparison across the apple genotypes within one infection type revealed no differences in the behavioral response of the parasitoid for the three infection types healthy, scab, and combination of scab and leafminer. In contrast, within the extracts from leafminer-infested plants, there were significant differences between the parasitoid responses across the genotypes (**Table 3**). **Table 4** presents detailed results of the duration of the behavioral states. Interestingly, female parasitoids spent significantly more time displaying *ovipositional probing* behavior on extracts from leafminer-infested 'Florina' than from all three leafminer-infested 'Gala' lines (**Table 4**; comparisons within the row Lm; one-way ANOVA; $F = 4.549$; df = 3,116; $P < 0.05$; Tukey's HSD post hoc test). Total time spent on *ovipositional probing* on 'Florina' exceeded that on 'Gala', 'Gala-trans0', or 'Gala-trans*Vf*' by a factor of > 3. Time spent on further behavioral states observed did not differ significantly among the four apple genotypes.

A comparison across the infection types within one genotype revealed significant differences in the behavioral response of the parasitoid, except within 'Gala-trans0', for which differences fell short of significance at $P = 0.063$ (**Table 3**). In the three 'Gala' lines, mean time spent on *antennation* on extracts from leafminer-infested leaves exceeded that spent on healthy leaves, and these differences were significant within each of the genotypes 'Gala' and 'Gala-trans*Vf*' (**Table 4**; comparisons within columns; 'Gala' one-way ANOVA; $F = 6.784$; df = 3,116; $P < 0.05$; Tukey's HSD post hoc test; 'Gala-trans*Vf*' one-way ANOVA; $F = 5.216$; df = 3,116; $P < 0.05$; Tukey's HSD post hoc test). Furthermore,

Table 4. Duration (in Seconds) of Recorded Parasitoid Behavioral States on Filter Papers Treated with Different Leaf Extracts (He, Healthy; Sc, Scab Inoculated; Lm, Leafminer Infested, or LS, Concurrently Inoculated with Scab and Infested with Leafminer) of 'Gala', 'Gala-trans0', 'Gala-trans*Vf*', and 'Florina'[a]

behavioral state (*c*, solvent control)		'Gala'	'Gala-trans0'	'Gala-trans*Vf*'	'Florina'
antennation	He	1.9 ± 0.8	3.5 ± 1.1	3.7 ± 1.6	2.0 ± 0.9
(*c*: 0.2 ± 0.2)	Sc	26.7 ± 23.0	2.0 ± 0.6	1.5 ± 0.4	3.0 ± 0.8
	Lm	12.4 ± 2.8	9.1 ± 2.9	9.4 ± 2.3	10.6 ± 2.5
	LS	8.2 ± 3.3	7.3 ± 2.0	7.3 ± 2.5	5.3 ± 1.7
ovipositional probing	He	0.7 ± 0.4	0.6 ± 0.4	0.7 ± 0.3	0.9 ± 0.5
(*c*: 0.0 ± 0.0)	Sc	0.8 ± 0.5	1.4 ± 0.6	0.6 ± 0.4	0.9 ± 0.4
	Lm	0.5 ± 0.3	0.8 ± 0.4	0.6 ± 0.2	2.4 ± 0.7
	LS	1.4 ± 0.6	0.2 ± 0.1	1.5 ± 0.6	1.7 ± 0.9
antennal preening	He	31.7 ± 3.6	24.2 ± 3.9	30.7 ± 3.9	23.9 ± 2.9
(*c*: 16.6 ± 3.4)	Sc	34.2 ± 5.4	31.7 ± 4.7	30.6 ± 4.7	33.4 ± 4.4
	Lm	29.9 ± 5.1	27.7 ± 4.1	25.0 ± 4.1	20.8 ± 3.1
	LS	48.8 ± 8.9	32.0 ± 3.3	36.0 ± 5.3	22.6 ± 2.5
abdominal preening	He	79.1 ± 19.3	57.7 ± 9.3	71.0 ± 12.0	72.1 ± 10.7
(*c*: 37.9 ± 7.8)	Sc	79.9 ± 11.8	77.0 ± 16.4	95.3 ± 19.4	102.7 ± 15.0
	Lm	64.7 ± 10.1	54.5 ± 13.8	48.0 ± 5.6	47.3 ± 9.0
	LS	117.2 ± 17.7	104.9 ± 15.9	77.6 ± 14.2	89.3 ± 17.4
standing	He	643.5 ± 73.6	721.7 ± 71.8	673.7 ± 76.6	498.2 ± 79.9
(*c*: 638.8 ± 87.9)	Sc	563.5 ± 74.9	652.2 ± 76.1	475.9 ± 74.3	535.9 ± 70.3
	Lm	498.0 ± 77.5	567.0 ± 81.5	616.6 ± 70.9	451.6 ± 73.3
	LS	648.1 ± 70.5	603.1 ± 69.8	542.1 ± 77.0	589.8 ± 76.6
walking	He	442.8 ± 79.3	391.9 ± 73.3	419.6 ± 82.0	602.7 ± 83.3
(*c*: 505.9 ± 91.8)	Sc	530.0 ± 85.9	435.3 ± 81.4	595.9 ± 78.5	523.7 ± 77.5
	Lm	596.4 ± 81.8	540.4 ± 86.8	500.0 ± 69.4	666.8 ± 78.1
	LS	385.2 ± 78.3	452.2 ± 70.7	535.4 ± 80.2	491.1 ± 81.6

[a] Duration (in seconds) of recorded parasitoid states on hexane-treated filter papers is given in parentheses (*c* = solvent control). Means ± SE are presented. Number of replicates tested on the same extract = 30; number of infection types = 4.

parasitoids spent more time on *abdominal preening* on extracts from combined infected 'Gala' leaves than on extracts from leaves infested with leafminer (**Table 4**; comparison within the column 'Gala'; one-way ANOVA; $F = 3.114$; df = 3,116; $P < 0.05$; Tukey's HSD post hoc test). In the genotype 'Florina', values for *antennation* were significantly higher on extracts from leafminer-infested than on extracts from healthy, scab, and combined infected leaves (**Table 4**; comparison within the column 'Florina'; one-way ANOVA; $F = 6.999$; df = 3,116; $P < 0.001$; Tukey's HSD post hoc test). Duration of *ovipositional probing* on extracts from leafminer-infested leaves was almost 3 times longer than on extracts from healthy leaves, but this difference was not significant. Time spent on *preening* behaviors on leafminer-infested leaves was shorter than on extracts from scab-inoculated leaves (**Table 4**; comparison within the column 'Florina'; *antennal preening*, one-way ANOVA; $F = 2.792$; df = 3,116; $P < 0.05$; Tukey's HSD post hoc test; *abdominal preening*, one-way ANOVA; $F = 3.477$; df = 3,116; $P < 0.05$; Tukey's HSD post hoc test).

DISCUSSION

Relationship between Squalene Contents and Plant Genotype on Host Location by the Parasitoid. Parasitoids of the apple leafminer use plant-derived chemical cues to locate their concealed living host (*8*, *19*). Chemical properties of the leaf surface, however, are determined by plant genotype and can be modified by plant development stage as well as by biotic and/or abiotic environmental factors (*30*−*32*). Changes in the plant genotype may thus

be associated with the risk of impeded or disrupted host location behavior of the parasitoid. The presented chemical leaf analyses as well as the bioassays testify to this effect in an amazing way. First, a comparison between the two scab-resistant apple genotypes 'Gala-trans*Vf*' and 'Florina' indicates that the squalene content of leafminer-infested leaves is much lower in the transgenic genotype. The parasitoid spent less time on *ovipositional probing* on this genotype than on the conventionally bred genotype. However, this result may be misleading without considering the two subsequent findings. Second, within the 'Gala' lines, including 'Gala-trans*Vf*', 'Gala-trans0', and 'Gala', no increased levels of squalene were found in leafminer-infested leaves compared to healthy leaves, and no differences were observed in the duration of the relevant parasitoid behaviors, in particular of *ovipositional probing*. Hence, transgenosis was not responsible for the differences noted above between the two scab-resistant genotypes. Surprisingly, the third finding showed that there was a major contrast between the two classically bred cultivars as only 'Florina', but not 'Gala', had the chemical leaf properties favoring intense *antennation* and *ovipositional probing* by the parasitoid. This result highlights the multifaceted complex interactions and the potential risk arising for nontarget insects associated with plant genotype.

The multitrophic apple system studied demonstrated in chemical analyses as well as in behavioral experiments its high sensitivity to detect differences between leaf extracts of various apple genotypes and infection types. In a previous study, the amount of the triterpene squalene was found to be increased on the mine surface of apple seedlings of 'Golden Delicious', triggering ovipositor insertion of the parasitoid (*19*). Given that squalene cannot be synthesized by arthropods (*33*), its presence in infested leaves was considered to be of plant origin (*8*). Squalene is an intermediate metabolite in the biosynthesis of other terpenoids and of sterols, but it is also known to be an essential compound regulating plant growth (*34*−*36*). Other studies have attributed photoprotective function to this compound of the epicuticular structures (*37*). The level of squalene is critical for the parasitoid's response, as both marginal and very high levels are behaviorally ineffective (*8*). In fact, female parasitoids in the current study showed a similar behavior pattern on all healthy apple genotypes, which all contained a low amount of squalene. The same holds true for parasitoid behavior on leaf extracts from the three 'Gala' genotypes, irrespective of the infection type, and again, these extracts all contained low amounts of squalene. On all of these leaf extracts, even when they were gained from leafminer-infested leaves, females exhibited little *ovipositional probing* behavior. Together with the findings by Dutton et al. (*8*), there are strong indications that the concentration of squalene on these leaves was too low to stimulate insertion of the ovipositor. Contrary to expectation, the classically bred 'Gala' genotype failed to increase squalene content upon leafminer herbivory. However, in the genotype 'Florina', a 4-fold increase in squalene content was assessed, and the time spent by the parasitoid on *ovipositional probing* increased according to expectation. Thus, chemical analyses and bioassays carried out on 'Florina' extracts are fully in line with previous conclusions gained for 'Golden Delicious' seedlings (*8*), suggesting a behavioral efficacy of increased levels of squalene also in the current study. In fact, the *Pholetesor* parasitoid species used in the current study (*Pholetesor circumscriptus*) and in the previous study (*Pholetesor bicolor* synonym *pedias*) (*8*) are described explicitly as "extremely closely related" (*38*). All observations on biology and behavior of these two representatives of the so-called *circumscriptus*-group (*39*) are completely coinciding (see ref *8* and this study). The current study reports, for the first time, that apple genotypes, even conventionally bred cultivars, may differ in herbivore-induced contents of this triterpene, which is reflected in the behavioral response of a natural antagonist of the leafminer. The finding that parasitoids behaved differently on the two tested apple cultivars, whereas no differences were noted between the three genotypes of the 'Gala' line, indicates that classical breeding may alter plant traits that remain unchanged in genetically modified plants. Neither scab resistance conferred by *HcrVf2* nor the transgenosis consisting of the selectable marker *nptII* under the control of the S35 promoter affected the behavior of the parasitoid confronted with the apple leaf extracts.

Parasitoid Response to Different Infection Types. Intensified *antennation* was noted on extracts from leafminer-infested leaves compared to healthy leaves, suggesting the presence of additional herbivore-induced bioactive constituents in these leaves. Furthermore, extracts from inoculated leaves compared to extracts from healthy leaves triggered increased *preening* activities, and respective differences were significant in the genotype 'Florina'. This effect likely indicates that the parasitoid is disturbed by chemical modifications elicited by scab on the leaf. Increased *preening* activities of parasitoids have also been observed on leaves infested by mixed species comprising the target and a nontarget herbivore (*40*).

Risk Assessment. No quantified risk is imposed by a transgenic plant if measured differences between this genotype and the wild-type plant are not larger than between classically bred cultivars. On the basis of this criterion, risk assessments should be made for pest- or pathogen-resistant transgenic plants that are under development for commercial use in agriculture (*41*, *42*). The current paper is likely the first to report a multitrophic investigation to compare the studied trait of transgenic plants with a representative nontransgenic control cultivar that contains the same resistance gene as the transgenic plant, and the findings emphasize the significance of this approach.

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