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**Transgenic and classically bred apple genotypes: multitrophic
investigation of plant-insect-pathogen interactions**

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¹ Vogler U, Rott AS, Gessler C & Dorn S (revised). Comparison between volatile emissions from transgenic apples and from two representative classically bred apple cultivars

² Vogler U, Rott AS, Gessler C & Dorn S (submitted). Transgenic and classically bred apple genotypes: impact on terpene-mediated parasitoid host location behaviour

³ Vogler U, Rott AS, Gessler C & Dorn S (submitted). How transgenic and classically bred apple genotypes affect non-target organisms on higher trophic levels

1 Summary

Biotechnological methods are used to transform plant genotypes with desired genes without limitation to species compatibility known from classical breeding. Such transgenic plants implicate possible unknown side effects, and prior to releasing transgenic plants into the environment, risk assessments have to evaluate in appropriate comparisons whether transgenic plants are as safe as classically bred plants. Possible unknown side effects of the four apple genotypes (*Malus x domestica*), the apple scab susceptible cultivar 'Gala', the apple scab susceptible transgenic genotype 'Gala-trans0', the apple scab resistant transgenic genotype 'Gala-transVf' and the apple scab resistant cultivar 'Florina', were assessed in a multitrophic system. Multitrophic investigations focused on interactions of these apple genotypes with the fungal pathogen *Venturia inaequalis* (Ascomycotina: Pleosporales) causing apple scab, the apple leafminer *Phyllonorycter blancardella* (Lepidoptera: Gracillariidae) and its parasitoid *Pholetesor circumscriptus* (Hymenoptera: Braconidae).

Plant-derived volatile compounds mediate long-range trophic interactions, and therefore, headspace volatiles of healthy, pathogen inoculated, leafminer infested, or simultaneously pathogen and leafminer infected plants of the four apple genotypes were collected and analysed by thermal desorption coupled with gas chromatography-mass spectrometry (GC-MS). Quantitative differences in headspace volatile emissions between 'Gala-transVf' and the two cultivars 'Gala' and 'Florina' could be detected within healthy and leafminer infested plants, but these differences were in the range of variability of the two cultivars.

Plant-derived non-volatile contact chemicals mediate short-range trophic interactions, and therefore apple leaves of healthy, pathogen inoculated, leafminer infested, or concurrently pathogen and leafminer infected plants of the four apple genotypes were extracted and analysed by GC-MS to quantify leaf content of the triterpene squalene (C₃₀H₅₀). Parasitoids were exposed to leaf extracts or to solvent control and were observed to complement the results of

chemical analyses with information about parasitoids' behaviour. Squalene content in leafminer infested leaf extracts differed between the transgenic apple scab resistant genotype and the apple scab resistant cultivar. Corresponding differences were found in bioassays on extracts from leafminer infested leaves.

Performance of the leafminer *P. blancardella* and its parasitoid *P. circumscriptus* was tested on the four apple genotypes in the absence or presence of *V. inaequalis*. Egg-to-adult development time of *P. blancardella* was similar on the four apple genotypes, but fewer adult moths emerged from 'Florina' than from 'Gala' with or without pathogen inoculation. Egg-to-adult development time of *P. circumscriptus*, number of emerged adult wasps and parasitism success were not significantly affected by the apple genotype neither with nor without pathogen inoculation.

With this fine-tuned multitrophic apple system subtle differences between the two cultivars 'Gala' and 'Florina' could be detected, whereas the transgenic genotypes 'Gala-transV^P' and 'Gala-trans0' were in the range of the variability of the two cultivars, indicating no adverse side effects on multitrophic interactions. In conclusion, the results of these multitrophic investigations reveal that the tested transgenic apple genotypes are as safe as the classically bred apple cultivars.

2 Zusammenfassung

Methoden der Biotechnologie werden angewendet, um Pflanzengenotypen gezielt mit bestimmten Genen zu transformieren, was im Gegensatz zur klassischen Pflanzenzüchtung ohne Einschränkungen der Spezieskompatibilität möglich ist. Transgene Pflanzen können unbekannte Nebeneffekte besitzen, und bevor diese Pflanzen in die Umwelt freigesetzt werden, müssen entsprechende Risikoanalysen durchgeführt werden. Bedingung für eine Freisetzung ist, dass in geeigneten Vergleichsversuchen mit transgenen und klassisch gezüchteten Pflanzen keine unerwünschten Effekte festgestellt werden. In einem multitrophischen System wurden vier Apfelgenotypen (*Malus x domestica*), die Apfelschorf anfällige Sorte ‚Gala‘, der Apfelschorf anfällige transgene Genotyp ‚Gala-trans0‘, der Apfelschorf resistente transgene Genotyp ‚Gala-transVf‘ und die Apfelschorf resistente Sorte ‚Florina‘ miteinander verglichen, um allfällige unbekannte Nebeneffekte feststellen zu können. Der Schwerpunkt der Untersuchungen lag auf dem multitrophischen Zusammenspiel jedes Apfelgenotyps mit dem pilzlichen Erreger des Apfelschorfes *Venturia inaequalis* (Ascomycotina: Pleosporales), der Apfelblattminiermotte *Phyllonorycter blancardella* (Lepidoptera: Gracillariidae) und ihrem Parasitoiden *Pholetesor circumscriptus* (Hymenoptera: Braconidae).

Weiträumige trophische Interaktionen werden mit Hilfe pflanzlicher Duftstoffe vermittelt. Daher wurden Duftstoffe von gesunden, Pathogen inokulierten, Blattminierer infizierten, sowie gleichzeitig mit Pathogen inokulierten und Blattminierer infizierten Pflanzen der vier Apfelgenotypen gesammelt und anschliessend mit thermischer Desorption verbunden mit Gaschromatographie-Massenspektrometrie (GC-MS) analysiert. Unterschiede in der Menge abgegebener Duftstoffe wurden bei gesunden und Blattminierer infizierten Pflanzen des Genotyps ‚Gala-transVf‘ und den beiden Kultursorten ‚Gala‘ und ‚Florina‘ gefunden, allerdings lagen die Unterschiede innerhalb der Variabilität der beiden genannten Sorten.

Trophische Interaktionen mit kurzer Reichweite werden mit Hilfe nicht flüchtiger Kontaktstoffe vermittelt, und daher wurden Apfelblätter von gesunden, Pathogen

inokulierten, Blattminierer infizierten oder gleichzeitig mit Pathogen inokulierten und Blattminierer infizierten Pflanzen extrahiert und anschließend mit GC-MS analysiert, um den Gehalt des verhaltenswirksamen Triterpens Squalen ($C_{30}H_{50}$) festzustellen. Um die Ergebnisse der chemischen Analysen mit verhaltensrelevanten Informationen zu ergänzen, wurden Parasitoiden den Blattextrakten oder der Lösungsmittelkontrolle ausgesetzt und beobachtet. Unterschiede im Squalengehalt konnten in Blattminierer befallenen Blättern der beiden Apfelschorf resistenten Apfelgenotypen gefunden werden. Allerdings haben die Verhaltensbeobachtungen der Parasitoiden Unterschiede zwischen dem transgenen Apfelschorf resistenten Genotyp und der Apfelschorf resistenten Kultursorte ergeben, jedoch nicht zwischen dem Apfelschorf resistenten transgenen Genotyp und seiner isogenen Apfelschorf anfälligen Kultursorte.

Das Entwicklungsverhalten des Blattminierers *P. blancardella* und seines Parasitoiden *P. circumscriptus* wurde mit und ohne Apfelschorfinokulation auf den vier Apfelgenotypen beobachtet. Die Entwicklungszeit vom Ei bis zum Adulten bei *P. blancardella* war auf den vier Apfelgenotypen ähnlich. Allerdings schlüpfen bei ‚Gala‘ mehr Adulte als bei ‚Florina‘, und zwar unabhängig davon, ob die Pflanzen mit dem Pathogen inokuliert waren oder nicht. Die Entwicklungszeit vom Ei bis zum Adulten beim Parasitoiden *P. circumscriptus*, die Anzahl der geschlüpften Adulten und der Parasitierungserfolg wurden nicht deutlich durch den Apfelgenotypen oder durch die Pathogeninokulation beeinflusst.

Mit diesem feinabgestimmten multitrophischen Apfelsystem konnten Unterschiede zwischen den beiden Sorten ‚Gala‘ und ‚Florina‘ bestimmt werden, wohingegen mögliche Nebeneffekte der transgenen Genotypen ‚Gala-transVf‘ und ‚Gala-trans0‘ innerhalb der Variabilität der beiden Kultursorten lagen. Zusammenfassend lässt sich damit zu den Ergebnissen dieser multitrophischen Untersuchungen festhalten, dass die getesteten transgenen Apfelgenotypen ähnlich unbedenklich sind wie entsprechende klassisch gezüchtete Sorten.

3 General Introduction

Risk assessment studies of genetically modified plants are defined as an evaluation of possible direct or indirect, immediate or delayed risks on non-target organisms (Anonymous, 2001). Genetically modified plants are achieved by biotechnological methods, which are used to insert target gene(s) with desirable characteristics into a specific plant genotype (Babu et al., 2003; Collinge et al., 2008). The resulting plants are defined as transgenic, if the inserted target gene(s), promoter and selectable marker gene(s) derive from non-crossable or artificial combinations, or as cisgenic, if the inserted target gene(s), promoter and terminator regions in a sense orientation derive from a crossable donor plant and no foreign gene(s) such as selectable marker gene(s) are present (Schouten et al., 2006).

Plants play a central role in multitrophic interactions of terrestrial ecosystems. For example, insects rely on plant-mediated interactions for habitat and host location (Fritz & Simms, 1992; Hunter & Price, 1992; Price et al., 1980), and many interactions depend on characteristics of the plant *per se* (e.g. resistance, tolerance, susceptibility), which are in turn determined by the plant's genotype. Assessments of fine-tuned plant-mediated interactions were conducted using a multitrophic system based on transgenic and conventionally bred apple plants, which are resistant or susceptible to a fungal pathogen. Two non-target insect organisms complement the studied food web.

Plant resistance

Plants are confronted with various changing conditions within their environment, and are influenced by abiotic (e.g. physical and chemical) and/or biotic (e.g. ecological) factors. Such biotic factors include pathogens or herbivore insects attacking the plant, and on susceptible plant genotypes, these pests are able to complete their life cycle and to reproduce successfully. Since plants are sessile, they have adapted various ways to defend themselves against such attacks. These plant defence mechanisms can be constitutive (related to the plant genotype) or induced (temporarily activated in response to initial damage)

(Gatehouse, 2002; Leitner et al., 2005). Constitutive resistance is of high interest to plant breeders. It can be subdivided into horizontal and vertical resistance. Horizontal or general race unspecific host resistance refers to minor gene resistance controlled by several or single genes, whereas vertical or race specific host resistance describes a major gene resistance controlled by one or few resistance genes (R-genes) (Agrios, 2005; Parlevliet & Zadoks, 1977; Pedigo & Rice, 2009). Single major R-genes are the basis for the principle of the gene-for-gene interaction (Flor, 1971). This principle describes the recognition event of a pathogen avirulence gene by a host plant R-gene (Hammond-Kosack & Jones, 1997). After this recognition event, complex plant defence cascades are triggered (Dangl & Jones, 2001). A signalling network of cross-talking pathways that may be interlinked by specific components is crucial for early defence responses against pathogens and pest insects (Agrios, 2005; Heiser et al., 2005; Maffei et al., 2007). These resistance principles are used in plant breeding programmes in order to obtain new plant genotypes with desired resistance characteristics (Bent, 1996).

Transgenic plants

In 1987, three research groups reported the successful transformation of plants with genes encoding for *Bacillus thuringiensis* (*Bt*) δ -endotoxins (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987). These transgenic plants were developed to obtain insect resistant plants by enabling the plant to synthesise insect order-specific toxins i.e. against lepidopteran or coleopteran pests within their tissues. Nowadays, *Bt*-crops are distributed and planted worldwide (ISAAA, 2007). Still, public acceptance of transgenic crops varies from relatively high to strong opposition (Herdt, 2006). However, plants conveying genes from crossable plant species could be an acceptable alternative combining the knowledge of classical breeding and biotechnological methods.

Risk assessment studies

Transgenic plants contain gene(s), which have been artificially inserted into the plant's genome to achieve improved genotypes with desired characteristics. Possible side effects of these transgenic plants within the ecosystem have to be considered since the gene pool is no longer limited by species compatibility to develop such plants, and because the random insertion site of target gene(s), promoter and selectable marker gene(s) is different from the introgression site. Characteristics of these transgenic plants should be identified and compared to those presented by comparable non-transgenic plants in a scientific and transparent manner. Most available scientific studies evaluated the impact of insect resistant transgenic plants on non-target organisms in terrestrial and aquatic systems (Álvarez-Alfageme et al., 2008; Romeis et al., 2008; Romeis et al., 2006; Rosi-Marshall et al., 2007; Schuler et al., 1998), but still, appropriate comparisons within case-specific risk assessment studies are necessary (Andow & Zwahlen, 2006; Auer, 2008; Poppy & Sutherland, 2004). Therefore, multiple comparisons of transgenic and non-transgenic plant genotypes sharing or differing in a single trait are needed to identify possible risks of biotechnological vs. conventional breeding techniques. To evaluate possible effects within the environment, multiple infections with possible target and non-target organisms have to be included in an appropriate case-by-case study.

The multitrophic apple system

Apple

The common apple cultivars belong to the family of Rosaceae, subfamily Maloideae, genus *Malus* Miller, and are classified as *Malus x domestica* Borkh. (Jackson, 2003; Luby, 2003).

In apple, the fruits are of economic interest and since cultivars are self-incompatible, cross-pollination with compatible pollen donors is required (Brown & Maloney, 2003; Dennis Jr, 2003; Winter et al., 1992). Apple trees planted in orchards are vegetative propagated scions, which are grafted onto compatible

rootstocks (Jackson, 2003; Winter et al., 1992). This results in genetically uniform orchards leading to an increase of possible plant resistance breakdown by pest and pathogen species (Gessler & Patocchi, 2007).

Important arthropod pest species can cause damage to apple buds and flowers (e.g. *Anthonomus pomorum*), apple fruits (e.g. *Cydia pomonella*), and to leaves and shoots because of their sucking (e.g. aphids, spider and rust mites) or chewing feeding activities (e.g. different leafminer species).

Pathogens of apple can damage the apple tree in the field, or the fruits after harvest during storage. The main fungal pathogens are the ascomycetes *Venturia inaequalis*, causing apple scab, and *Podosphaera leucotricha*, causing apple mildew. Further fungal pathogens and Oomycetes are known to cause canker (e.g. *Nectria galligena*), apple rust (*Gymnosporangium juniperi-virginianae*) and rot diseases in the field (*Phytophthora cactorum* (Oomycete)) or during storage (*Alternaria alternata*, *Botrytis cinerea* or *Monilinia fruticola*). Furthermore, bacterial (e.g. *Erwinia amylovora*), viral (e.g. apple mosaic virus) and phytoplasmatic (apple proliferation) pathogens can cause damage to apple trees in the orchards.

One aim of apple breeding is to develop resistant cultivars against *V. inaequalis* (Gessler et al., 2006; Kellerhals et al., 2004). The interaction between apple plants and *V. inaequalis* is determined via minor and major gene resistance factors (Williams & Kuc, 1969). Major apple resistance gene families are *Va*, *Vb*, *Vbj*, *Vf*, *Vm*, and *Vr* (Williams & Kuc, 1969). Within the *Vf* region of the small-fruited *Malus floribunda* 821 a resistance homolog was identified and the members are the so-called “*HcrVf genes*” (homologs to *Cladosporium fulvum* resistance genes of the *Vf* region) (Barbieri et al., 2003; Vinatzer et al., 2001). Successful transformation of the apple cultivar ‘Gala’ with the *HcrVf2* resistance gene confers scab resistance to the scab susceptible cultivar under control of the constitutive promoter CaMV 35S (Cauliflower Mosaic Virus 35S) and the selectable marker gene *nptII* (neomycin phosphotransferase II) (Belfanti et al., 2004). Pyramiding of R-genes based on different molecular mechanisms might improve the degree of plant resistance and therefore may also slow down a

possible breakdown of plant resistance (Charity et al., 2005; Gessler et al., 2006).

Apple scab caused by *V. inaequalis*

Venturia inaequalis (Cooke) Winter (anamorph *Spilocaea pomi* Fr.) (Ascomycotina: Pleosporales) (Brandenburger, 1985) occurs in apple growing regions worldwide (Agrios, 2005; MacHardy, 1996; MacHardy et al., 2001). The fungus overwinters saprophytically in fallen leaves as immature pseudothecia (one sexual cycle per year). In spring, pseudothecia and asci with ascospores mature. Under wet conditions the pseudothecia release the mature ascospores. The ascospores disperse via the wind and germinate on apple buds or young apple leaves under optimal climatic conditions. The fungus grows between the cuticle and the outer cell wall of the epidermis cells, and the mycelium produces conidia (several asexual cycles per year), which can cause new infections on leaves and fruits after they have been washed off or blown away during the growing season. In autumn the mycelium invades the interior of infected fallen leaf and forms in early spring pseudothecia.

Venturia inaequalis causes damage of buds, flowers, leaves, shoots, and fruits in the field, but also in storage. Foliar symptoms occur first as irregular olive-green spots on the leaf overside and later also on the underside developing to black lesions. Infected fruits first develop scab lesions becoming scabby and sometimes crack the fruit and the skin. Heavy infestation causes reduced quality of fruits, reduced shoot growth and defoliation of the apple tree.

Spotted tentiformed apple leafminer *Phyllonorycter blancardella*

The spotted tentiformed apple leafminer *Phyllonorycter blancardella* Fabricius (Lepidoptera: Gracillariidae) is a concealed living insect pest species of apple. The biology of this multivoltine *Phyllonorycter* leafminer species is well described (Kremer, 1963; Pottinger & LeRoux, 1971; Reissig et al., 1982; Weires et al., 1980). After individual eggs are laid on the leaf underside, the larvae hatch and bore straight into the lamina. During the 1st to 3rd larval instars larvae are sap feeders with sucking mouthparts and form U-formed mines, which are only

visible on the leaf underside. After hypermetamorphosis, larvae with chewing mouthparts feed on the palisade mesophyll cells as tissue feeders (4th to 5th larval instars). During this stage white spots occur on the upper side of the leaf. The mines obtain their tentiformed shape, because the larvae bind the sides of the mines together with silky threads.

Parasitoids of the spotted tentiformed apple leafminer

The population of the spotted tentiformed apple leafminer can be controlled under a careful pest-management regime in the field by the parasitoid complex (Balázs, 1997; Dorn et al., 1999). Among them is the solitary koinobiont larval endoparasitoid *Pholetesor circumscriptus* Nees (Hymenoptera: Braconidae). Female wasps are variable in their colour with dark gaster and reddish-yellow tergite, and the basal shape of the tergite is crucial for their identification (Nixon, 1973; Whitfield, 2006). The lifecycle of the parasitoid follows the lifecycle of its host (Delucchi, 1958). Adult wasps are able to parasitize sap and tissue feeding larvae, but sap feeder larvae are preferred (Dutton et al., 2000a). After finishing the larval development in the host larva, the mature parasitoid larva leaves its host to pupate within the leafmine. They spin a white, transparent cocoon, which is suspended by two silky threads between the upper and the lower epidermis (Delucchi, 1958; Van Frankenhuyzen, 1983). The adult wasps leave the mine through a hole in the lower epidermis.

Herbivore – parasitoid interaction

For habitat (long range) and host (short range) location the parasitoids use cues originating from their insect host, the microhabitat and their host plant (Godfray, 1994). These cues can be mediated in different ways, such as visually (Fischer et al., 2004; Lucchetta et al., 2008), mechanosensorially (Kroder et al., 2006; Meyhöfer & Casas, 1999; Meyhöfer et al., 1997) and/or olfactorially (Dutton et al., 2000b; Tumlinson et al., 1993).

The interactions between the spotted tentiformed apple leafminer *P. blancardella* and its parasitoid *P. circumscriptus* are determined in direct and indirect ways by the habits of its concealed living host. Specifically, parasitoids rely on volatiles

deriving from the host-plant complex for host habitat location before landing on the infested plant (Dorn et al., 1999; Lengwiler et al., 1994). After landing on the infested plant, they rely on plant-derived chemical cues, in particular on the contact chemical squalene (Dutton et al., 2002; Dutton et al., 2000b). This triterpene plays a crucial role for successful parasitisation of the leafmining herbivore.

Research questions

The aim of this thesis was to provide a dynamic insight into the behavioural response of a non-target parasitoid of the herbivore insect on possible direct and indirect effects of transgenic apple in a multitrophic context. Emphasis was on comparison of the transgenic scab resistant line ‘Gala-trans*Vf*’ with the transgenic line without the scab resistance gene (‘Gala-trans0’), the isogenic line ‘Gala’ and the conventionally bred cultivar ‘Florina’ containing the *Vf* resistance. The apple plants were subjected to four different infection types (healthy control, inoculation with *V. inaequalis*, infestation with *P. blancardella*, and concurrently infection with *V. inaequalis* and *P. blancardella*) as an indication that plants’ defence mechanisms had been triggered.

(1) The quantitative and qualitative composition of headspace volatiles was investigated to elucidate whether indirect plant-mediated long-range interactions might be impeded.

(2) Since female parasitoids use plant-derived chemicals in short-range interactions, they were exposed to leaf extracts of the four apple genotypes under different infection types to test whether they discriminate between the apple genotypes. Furthermore chemical analyses focusing on a relevant contact chemical were conducted.

(3) The performance of the herbivore *P. blancardella* and its parasitoid *P. circumscriptus* on the four apple genotypes in the absence or presence of *V. inaequalis* was assessed.

4 Comparison between volatile emissions from transgenic apples and from two representative classically bred apple cultivars¹

4.1 Abstract

While most risk assessments contrast a transgenic resistant to its isogenic line, an additional comparison between the transgenic line and a classically bred cultivar with the same resistance gene would be highly desirable. Our approach was to compare headspace volatiles of transgenic scab resistant apple plants with two representative cultivars (the isogenic 'Gala' and the scab resistance gene-containing 'Florina'). As modifications in volatile profiles have been shown to alter plant relationships with non-target insects, we analysed headspace volatiles from apple plants subjected to different infection types by gas chromatography-mass spectrometry. Marked differences were found between healthy and leafminer (*Phyllonorycter blancardella*) infested genotypes, where emissions between the transgenic scab resistant line and the two cultivars differed quantitatively in four terpenes and an aromatic compound. However, these modified odour emissions were in the range of variability of the emissions recorded for the two standard cultivars that proved to be crucial references.

¹ Based on: Vogler U, Rott AS, Gessler C & Dorn S (revised). Comparison between volatile emissions from transgenic apples and from two representative classically bred apple cultivars

4.2 Introduction

Non-target effects of recombinant DNA technology are of major concern in risk assessment studies (Poppy & Sutherland, 2004; Romeis et al., 2008). To date, investigations of the impact of transgenic plants on non-target organisms have largely focused on insect resistant plants expressing *Bacillus thuringiensis* (*Bt*) toxins (Romeis et al., 2006), although there are also transgenic plants conveying resistance to diseases (Collinge et al., 2008). A critical issue in case-specific environmental risk assessments is the inclusion of appropriate comparisons (Andow & Zwahlen, 2006; Marvier et al., 2007). A particular gap refers to direct comparisons between resistant genetically modified and resistant conventionally bred genotypes that were subjected to different infection regimes (Dean & De Moraes, 2006; Turlings et al., 2005).

For example, the abundance of non-target invertebrates in cotton and maize is influenced stronger by insecticide spraying than by the *Bacillus thuringiensis* (*Bt*) toxin expressed by transgenic crops. Furthermore, differences between genetically modified and conventionally bred genotypes need to be identified, particularly considering different health states. In *Bt* crop plants, such comparisons were impossible as there is no conventionally bred cultivar expressing the *Bt* toxin, hence it is unclear whether or not any observed non-target effect of the transgenic plant would also be found in the respective resistant cultivar.

The current study relies on transgenic and classically bred apple genotypes targeting resistance against a key fungal pathogen in orchards worldwide, the apple scab *Venturia inaequalis* (Ascomycotina: Pleosporales) (MacHardy et al., 2001). This target organism is the focus of major resistance breeding programs (Gessler et al., 2006). A gene-for-gene interaction (Flor, 1971) has been found for the *Vf* resistance gene region conveying resistance to this pathogen on apple trees (*Malus x domestica*) (Williams & Kuc, 1969). Scab resistance originating from *Malus floribunda* 821 was introgressed into apple by conventional breeding, yielding the cultivar 'Florina'. Among a set of *HcrVf* genes (homologs to *Cladosporium fulvum* resistance genes of the *Vf* region) (Vinatzer et al., 2001)

from this cultivar, *HcrVf2* was cloned into the scab susceptible cultivar 'Gala' conferring resistance to scab (Belfanti et al., 2004). In the selected transgenic line, this gene is under the control of the CaMV S35 promoter and linked to the selectable marker gene *nptII*. One particular transformed line was lacking the target gene but still carrying the selectable marker construct; it served as control, being as susceptible as the untransformed cultivar 'Gala'. The effect of the introduced *HcrVf2* gene on the target organism is equal to the effect of the *Vf* resistance introgressed by classical breeding yielding 'Florina' (Belfanti et al., 2004). However, nothing is known about the impact of scab resistant transgenic apple plants on non-target organisms associated with the apple tree.

Apart from pathogens, plants interact in their environment with other plants, herbivores, and antagonists of those herbivores. In these interactions, plants use direct and indirect systems to defend themselves (Baldwin et al., 2006; Poppy, 1997). In response to herbivore attack, plants emit volatile compounds derived from different induced defence pathways (Arimura et al., 2005; Fidantsef et al., 1999; Scascighini et al., 2005). These plant volatiles include green leaf volatiles (GLV), terpenes and a wide array of aromatic compounds. The GLV comprise C₅ and C₆ compounds (aldehydes, alcohols and esters) (Connor et al., 2008; Salch et al., 1995) synthesized via the lipoxygenase pathway as breakdown products of membrane lipids (Connor et al., 2008; Paré & Tumlinson, 1999). Terpenes are synthesized via the mevalonate (MVA) or the methylerythritol 4-phosphate (MEP) pathway (Aharoni et al., 2005; Dudareva et al., 2005; Lichtenthaler et al., 1997) using the precursors isopentenyl diphosphate (Dandekar et al., 2004) and dimethylallyl diphosphate (DMAPP). Other compounds like nitriles and aromatics are synthesized via the shikimic acid pathway (Bennett & Wallsgrave, 1994). Qualitative and/or quantitative changes in the volatile blends might impede tri-trophic interactions with insects and therefore, potential risks have to be assessed.

Leafminers such as *Phyllonorycter blancardella* (Lepidoptera: Gracillariidae) are an excellent model as their epidemic outbreak leads to leaf fall and to yield decrease over consecutive seasons (Reissig et al., 1982), but such dynamics can be prevented successfully if parasitoid populations are conserved (Dorn et

al., 1999). Alterations in the volatile profile of healthy or scab infested transgenic apple plants could influence useful tri-trophic interaction between plant, herbivore and natural antagonist. Here we compared the composition of headspace volatiles emitted by apple plants from four different genotypes subjected to four different infection types, as volatile blends emitted by differently induced plants could vary. The plants were (A) healthy, (B) inoculated with apple scab conidia, (C) infested with apple leafminer larvae, or (D) pathogen inoculated and leafminer larvae infested in combination. Our approach was to compare headspace volatiles of (1) transgenic scab resistant apple plants ('Gala-transVf') with two representative classically bred cultivars, namely (2) the isogenic line 'Gala' and (3) the scab resistant 'Florina' that contains the same *HcrVf2* resistance gene as the transgenic resistant line. To evaluate a possible influence of the transgenesis *per se* we included (4) an additional transgenic genotype containing only the selective gene and the promoter (Table 4.1.).

4.3 Materials and Methods

Plant material

The experiments were conducted with four different apple genotypes (Table 4.1.): a scab susceptible cultivar 'Gala', a scab susceptible transgenic line 'Gala-trans0' (consisting only of the selectable marker gene *nptII* under the control of the S35 promoter), a scab resistant transgenic line 'Gala-transVf' (consisting of the selectable marker gene *nptII* under the control of the S35 promoter and the *HcrVf2* resistance gene with 35S as promoter), and a scab resistant cultivar 'Florina'. Two-years old grafted apple plants of the cultivars 'Gala', 'Gala-trans0', 'Gala-transVf', and 'Florina' were planted in pots containing 1.5 L substrate-perlite-sand mixture (mix ratio 6:1:1). The plants were regularly pruned 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 growing stage. A fully equipped closed greenhouse chamber with internal air circulation was used to grow the plants and an equal separate

greenhouse chamber was used to conduct the experiments with all four apple genotypes in parallel. The controlled climatic conditions were: day temperature at 22 ± 2 °C and night temperature at 18 ± 2 °C, 60 ± 5 % relative humidity and a photoperiod of L16:D8. Assimilation lighting was used to complete the daylight when necessary.

Pest management strategies to protect the plants against herbivores and pathogens were applied when necessary during cultivation. Against herbivory of spider mites bromopropylate (bridged diphenyl acaricide, Spomil, EC 250 g/L, 15 mL/10 L) was sprayed and to keep them under continuous control a predatory mite population (*Phytoseiulus persimilis*, Andermatt Biocontrol AG, Grossdietwil, Switzerland) was established. Plants were sprayed with penconazol (triazol, Topaz Vino, EC 100 g/L, 50 mL/100 L) against the apple powdery mildew (*Podosphaera leucotricha*) when necessary. The latest time of pesticide application was 14 days prior to the start of the experiment with different infection types hence the plants were not treated with pesticides during the experiments, i.e. after inoculation and herbivore infestation.

Table 4.1. Specifications and purpose of the four apple lines in this study

Apple genotype	Specification	Purpose
'Gala'	Conventionally bred; scab susceptible	Key reference
'Gala-trans0'	Transgenic; scab susceptible; with S35 promoter and marker <i>nptII</i>	Control 1 (for the key test line regarding transgenic aspects)
'Gala-transVf'	Transgenic; scab resistant; with <i>HcrVf2</i> , S35 promoter and marker <i>nptII</i>	Key test line
'Florina'	Conventionally bred; scab resistant with <i>HcrVf2</i>	Control 2 (for the key test line regarding scab resistance)

Pathogen and pathogen inoculation

Conidia of *Venturia inaequalis* Cooke Winter (Ascomycotina: Pleosporales) for scab inoculation were obtained from sporulating scab lesions on apple leaves collected at the Swiss Federal Research Station Agroscope Changins-Wädenswil ACW (Switzerland). Conidia were washed off from the leaves with water and their concentration was determined microscopically using a Neubauer counting chamber (Laboroptik GmbH, Friedrichsdorf, Germany). The conidia material was stored at -20 °C for later use.

Actively growing apple shoots with at least six fully developed leaves per plant were sprayed with conidia suspension (10^5 conidia per mL) to inoculate the whole leaf area. For the first 48 hours the inoculated plants were kept in a plastic tent at 18 °C, darkness and a relative humidity of 100 % (Gessler & Stumm, 1984). Afterwards the plants were brought to a separate chamber in the greenhouse. Sporulating scab lesions on the inoculated shoots and chlorotic and necrotic lesions were visible after 24 ± 2 days on leaves of the two scab susceptible genotypes ('Gala' and 'Gala-trans0') but not on the scab resistant genotypes ('Gala-transVf' and 'Florina'). genotypes ('Gala' and 'Gala-trans0') but not on the scab resistant genotypes ('Gala-transVf' and 'Florina'). This coincides with a previous paper that classified 'Florina' and 'Gala-transVf' (= Ga2:21) similarly regarding the level of scab resistance (Belfanti et al., 2004).

Herbivores and herbivore infestation

The colony of the herbivore *Phyllonorycter blancardella* (Lepidoptera: Gracillariidae) originated from apple orchards in South Tyrol (northern Italy). Adults were allowed to infest two-month-old potted apple seedlings (*Malus x domestica* Golden Delicious open pollinated seedlings) in Plexiglas cages, covered with a glass plate and gauze on the sides. Protection of young shoots against apple powdery mildew (*Podosphaera leucotricha*) was achieved with penconazol (triazol, Topaz Vino, EC 100 g/L, 50 mL/100 L). The conditions in the insectaries were 22 ± 2 °C, 50 ± 5 % relative humidity and a photoperiod of L16:D8.

Six two- to three-days old adults were allowed to oviposit on actively growing apple shoots with at least six fully developed leaves per plant for three days using gauze bags (14 cm x 23 cm) closed with hook-and-loop fasteners. The successfully infested plants were kept in a separate greenhouse chamber. The feeding habits of sap-feeding leafminer larvae (1st to 3rd larval instar) resulted in leafmines visible on the leaf underside. After the 3rd instar hypermetamorphosis occurs, and the typically spotted tentiformed mine is formed by tissue-feeders (4th and 5th larval instar).

Combined pathogen inoculation and herbivore infestation

Actively growing apple shoots with six fully developed leaves per plant were inoculated with scab conidia suspension (10^5 conidia per mL) and kept under controlled conditions as described above for scab inoculation. Afterwards the plants were moved to a separate greenhouse chamber with controlled conditions (described above), and 55 ± 1 hours after the scab inoculation event, they were infested with six two- to three-days old adults for three days as described for herbivore infestation. After 24 ± 2 days showed the two scab susceptible genotypes ('Gala' and 'Gala-trans0') scab sporulating foliar lesions on inoculated leaves whereas no symptoms were visible on the scab resistant genotypes ('Gala-transVf' and 'Florina') and the larvae achieved the third instar. Infestation and/or inoculation levels used in the combined as well as in the single infection types were within the range of natural infestations, mimicking high levels of herbivore and/or pathogen attack.

Control

Healthy apple plants of the same size and age as the damaged plants were used as healthy control.

Headspace volatile collection and chemical analysis

Headspace volatiles of intact apple shoots with 12 to 15 leaves were collected on different sampling dates over a period of eight months. They were analysed to identify differences in the volatile composition between the four genotypes

under different infection types (healthy, leafminer infestation, scab inoculation, or combined scab inoculation and leafminer infestation) at a single, consistent time interval of 24 ± 2 days from the infestation and/or inoculation. Ten plants for each of the 16 genotype-treatment combinations were considered. We always tested the four genotypes within one infection type in parallel, yielding four samples per date.

Headspace volatiles were collected in a climate chamber (Conviron, Controlled Environment Ltd., Winnipeg, Canada) under controlled conditions at 22 ± 2 °C, 60 % relative humidity and a L16:D8 light regime for two hours from apple plants, which were acclimatized for 30 minutes, between 10 am and 3 pm (i.e. starting 6.5 ± 2.5 hours after onset of photophase). Each plant was packed in a polyester bag construct (Toppits® Brat-Schlauch, Melitta GmbH, Egerkingen, Switzerland) and wrapped at the shoot with cotton wool and Teflon tape (Alltech Socochim SA, Lausanne, Switzerland). The polyester bag was provided with an attached glass funnel and continuous circulation (60 mL/min) of charcoal filtered air (Supelcarb HC filter, Supelco, Bellefonte PA, USA) was maintained during dynamic headspace volatile collection. On the opposite side of the glass funnel, a split Teflon plate (diameter 13.5 cm) was placed with a volatile trap in the middle. The volatile trap was filled with 250 g Supelco Tenax TA (mesh size 60-80, Sigma Aldrich, Buchs, Switzerland) and sealed with Pyrex glass wool at both ends. Prior to use, the tubes were conditioned for four hours at 300 °C under a continuous stream (50 mL/min) of helium 4.6 (purity 99.96 %) (Hern & Dorn, 2004). The conditioned tube was airtight closed with Teflon front ferrules (Swagelok, Supelco, Bellefonte, PA, USA) until it was used for headspace volatile collection. The tube was tightly secured with cotton wool and Stretch-it PTFE Tape (Alltech Socochim SA, Lausanne, Switzerland) to the Teflon plate and fixed together with the polyester bag using a clasp (diameter 13.5 cm). The single parts were preconditioned at 120 °C for two hours in an oven and after mounting, the whole construct was conditioned again at 120 °C for two hours in the oven before using (Stewart-Jones & Poppy, 2006).

The chemical analyses and identifications are based on the method by Vallat et al. (2005), with some modifications as described below. Prior to analyses the

internal standard octylbenzene (~ 99.8 %, Fluka, Buchs, Switzerland) diluted in hexane [100 ng/ μ L] was added to each sample by injecting directly onto the polymer. Collected volatiles were analysed using thermal desorption (Unity and Ultra, Markes Int. Ltd, Llantrisant, UK) coupled with gas chromatography-mass spectrometry (HP 6890 Series GC-System and HP 5973, Hewlett Packard Company, Atlanta, GA, USA). Thermal desorption of absorbed volatiles from Tenax TA was conducted under the use of helium 5.0 (purity 99.99 %) with a prepurge time of 3 min (split on), followed by increasing temperature up to 300 °C within 5 min (split off). Afterwards the volatiles were transferred to a cold trap (-10 °C during tube desorption process), which subsequently heated up to 300 °C at a rate of 60 °C/min. The thermal desorption process was linked to the gas chromatograph-mass spectrometry system (GC-MS) via a fused silica transfer line (150 °C). An HP-retention-gap with deactivated fused silica, 5 m length and 0.25 mm inside diameter (Hewlett Packard Company, Atlanta, GA, USA) and a nonpolar Econo-Cap EC™-5 column with 30 m length, 0.25 mm inside diameter and 0.25 μ m film (Alltech Socochim SA, Lausanne, Switzerland) were used in the GC. The oven program was held at 50 °C for 5 min, then increased to 280 °C at a rate of 5 °C/min, and held at 280 °C for 5 min with a post run at 300 °C for 15 min. Quadrupole and source temperature of the MS were 150 °C and 230 °C, using full scan method. The carrier gas was helium 5.0 (purity 99.99 %) at a constant pressure of 108 kPa. For the identification of the mass spectrometry-data ChemStation software (MSD Productivity Chem Station software, Agilent Technologies Inc., Santa Clara, CA, USA) was used linked to the NIST98 library and our own library of phytochemical compounds. In addition, the retention times of all compounds presented were compared with standard compounds purchased from chemical suppliers or obtained from other laboratories.

The chemicals used as standards in thermal desorption coupled with gas chromatography-mass spectrometry comprised (-)- α -pinene (purity \geq 99.0 %, Fluka, Buchs, Switzerland), R(+)-limonene (\geq 99.0 %, Fluka, Buchs, Switzerland), β -ocimene (~ 70 %, Fluka, Buchs, Switzerland), (\pm)-linalool (\geq 95.0 %, Fluka, Buchs, Switzerland), β -caryophyllene (~ 99 %, Fluka, Buchs, Switzerland), α -

humulene (> 98 %, Fluka, Buchs, Switzerland), (*Z,E*)- α -farnesene (Givaudan-Roure, Dübendorf, Switzerland), isomeric mixture of (*E,E*)- α -farnesene and (*Z,E*)- α -farnesene (77 % (*E,E*)- α -farnesene, 20.7 % (*Z,E*)- α -farnesene, the remainder being impurities, Givaudan-Roure, Dübendorf, Switzerland), benzaldehyde (\geq 99.0 %, Fluka, Buchs, Switzerland), nonanal (\geq 95 %, Fluka, Buchs, Switzerland), decanal (94.1 %, Supelco, Bellefonte, PA, USA), acetophenone (\geq 99.0 %, Fluka, Buchs, Switzerland), geranylacetone (96 %, ABCR GmbH & Co, Karlsruhe, Germany/ Avocado Research Chemicals Ltd, Heysham, UK), (*Z*)-3-hexenyl acetate (99 %, ABCR, Karlsruhe, Germany), methyl salicylate (minimum \geq 99 %, Sigma, Buchs, Switzerland), (*Z*)-3-hexenyl benzoate (Kosher, \geq 97 %, Aldrich, Buchs, Switzerland), benzonitrile (\geq 99.0 %, Fluka, Buchs, Switzerland), and phenylacetonitrile (\geq 98.0 %, Fluka, Buchs, Switzerland).

Data analysis

The statistical analyses were performed using JMP 7.0.1 (SAS Institute, Cary, NC, USA) and SPSS 16.0.0 for Mac (SPSS Inc., Chicago, IL, USA). Amounts of the eighteen definitively identified compounds were calculated in [ng/L] (Trabue et al., 2006) in relation to the internal standard octylbenzene [100 ng/ μ L] and the experimental set up including continuous airflow [mL/min] and duration [min] of the headspace volatile collection. Mean values and standard errors were calculated for each compound analysed.

Data of all samples were included in the test for normal distribution (Shapiro-Wilk W Test) and homogeneity of variance (Levene Test). To fulfil the assumption of normal distribution, data were log 10 transformed (Cuéllar, 1991) and the constant 0.0001 was added to all values (Steiger et al., 2007; Steiner et al., 2007) to apply the log 10 transformation prior to the analyses.

To assess whether the sampling date that was always at a single distinct time interval from infestation and/or inoculation showed any effects on collected headspace volatiles, regression analyses were performed for each genotype-infection type combination separately to verify the primary interest of relationship between the variables.

Multivariate analysis of variance (MANOVA) was performed to identify significant differences between volatiles (dependent variables) emitted by the apple genotypes, subjected to the different infection types. Genotype, infection type and their interaction were the main effects in the model. MANOVA analyses within one infection type across genotypes were conducted, because the interaction could overlay effects between genotypes. If the MANOVA model was significant ($P < 0.05$), one-way analysis of variance (ANOVA) and Tukey HSD post hoc test were conducted to test for differences for individual compounds.

4.4 Results

Volatile blends and effects of infection types

Eighteen definitively identified compounds were found in the blends collected from transgenic apple lines as well as from the isogenic line 'Gala' and the resistant standard cultivar 'Florina' under different infection types. These headspace volatiles comprise eight terpenes, three aldehydes, two ketones, three esters, and two nitriles (Table 4.2.). They coincide with compounds previously reported in association with apple (Casado et al., 2006; Ebel et al., 1995; Hern & Dorn, 2002; Vallat et al., 2005). No significant effect of the sampling date (always 24 ± 2 days from infestation and/or inoculation) on the volatile emission was observed (all $R < 0.39$; all $P > 0.05$).

Apple genotype, infection type (healthy control, scab inoculation, leafminer infestation or a combined infection of scab and leafminer) and the genotype by infection type interaction significantly affected the volatile emission (MANOVA, Pillai's Trace, $P < 0.05$ for all effects; Table 4.3. a). Separate analyses of emitted volatile profiles within one infection type across genotypes detected significant differences for healthy (MANOVA, Pillai's Trace $P < 0.05$), scab inoculated (MANOVA, Pillai's Trace $P < 0.05$) and leafminer infested (MANOVA, Pillai's Trace $P < 0.05$) plants, but a combined infection of scab and leafminer nullified any detected differences between the genotypes (Table 4.3. b).

Table 4.2. Volatile compounds detected in the headspace of four apple genotypes (columns). Comparisons within a row indicate effects of genotypes within a distinct infection type: **A** healthy plants; **B** scab inoculated plants; **C** leafminer infested plants; **D** plants subject to combined scab inoculation and leafminer infestation.

Compound	R.T.		'Gala'	N°	'Gala-trans0'	N°	'Gala-transVf'	N°	'Florina'	N°
<i>Terpenes</i>										
α -Pinene	5.34	A	0.36 ± 0.18	10	0.58 ± 0.22	7	0.15 ± 0.11	4	0.25 ± 0.17	2
		B	0.04 ± 0.02	5	0.21 ± 0.21	1	0.56 ± 0.55	2	0.25 ± 0.14	4
		C	0.10 ± 0.06	4	0.28 ± 0.14	7	0.24 ± 0.13	6	0.48 ± 0.26	6
		D	0.10 ± 0.04	5	0.10 ± 0.02	9	0.08 ± 0.06	4	0.14 ± 0.07	4
Limonene	8.69	A	0.45 ± 0.20	10	1.32 ± 0.33	10	0.15 ± 0.07	7	0.53 ± 0.16	9
		B	0.10 ± 0.03	9	1.08 ± 0.43	7	0.40 ± 0.24	9	0.53 ± 0.24	9
		C	0.21 ± 0.10	7	0.33 ± 0.15	8	0.54 ± 0.30	10	0.61 ± 0.24	10
		D	0.28 ± 0.13	7	1.00 ± 0.33	10	0.85 ± 0.41	8	0.41 ± 0.19	9
β -Ocimene	8.92	A	0.20 ± 0.13	6	1.39 ± 0.77	6	0.64 ± 0.33	8	12.89 ± 5.58	10
		B	0.53 ± 0.25	10	1.59 ± 1.06	7	2.20 ± 1.76	10	17.17 ± 7.97	10
		C	1.01 ± 0.43	10	1.37 ± 0.82	6	0.26 ± 0.18	8	20.14 ± 8.26	10
		D	1.08 ± 0.42	10	0.36 ± 0.19	7	3.70 ± 2.47	9	11.90 ± 3.13	10
Linalool	11.26	A	2.96 ± 1.35	10	2.54 ± 1.41	10	1.39 ± 0.54	10	3.94 ± 2.03	10
		B	1.16 ± 0.61	10	2.26 ± 1.15	9	2.10 ± 1.52	10	1.97 ± 0.76	10
		C	1.19 ± 0.48	10	1.06 ± 0.55	10	0.53 ± 0.35	10	2.27 ± 1.18	9
		D	1.78 ± 0.59	10	0.38 ± 0.15	8	0.97 ± 0.45	10	0.96 ± 0.26	10
β -Caryophyllene	20.42	A	1.67 ± 0.80	10	3.21 ± 1.77	10	2.11 ± 0.85	10	17.40 ± 7.74	10
		B	2.64 ± 1.64	10	3.76 ± 1.84	9	5.22 ± 3.79	10	9.94 ± 4.21	10
		C	2.41 ± 1.06	10	1.26 ± 0.71	10	2.38 ± 1.94	10	9.60 ± 4.43	10
		D	4.33 ± 2.59	10	5.36 ± 2.43	10	4.23 ± 1.86	10	4.53 ± 1.41	10
α -Humulene	21.21	A	0.26 ± 0.25	5	0.19 ± 0.12	3	0.08 ± 0.04	6	0.98 ± 0.44	9
		B	0.11 ± 0.07	10	0.49 ± 0.24	7	0.96 ± 0.82	8	0.46 ± 0.23	8
		C	0.13 ± 0.06	8	0.05 ± 0.03	4	0.09 ± 0.08	2	0.46 ± 0.19	9
		D	0.17 ± 0.09	8	0.04 ± 0.04	2	0.14 ± 0.07	8	0.19 ± 0.06	8
<i>(Z,E)</i> - α -Farnesene	22.54	A	0.31 ± 0.20	7	1.77 ± 0.93	8	0.67 ± 0.29	10	2.21 ± 0.93	6
		B	0.80 ± 0.35	9	3.66 ± 2.18	9	1.63 ± 0.96	10	1.19 ± 0.33	10
		C	0.60 ± 0.23	10	1.30 ± 0.75	9	0.17 ± 0.08	8	1.41 ± 0.43	10
		D	1.13 ± 0.42	10	0.31 ± 0.10	6	1.18 ± 0.52	10	1.15 ± 0.51	9

Compound	R.T.		'Gala'	N°	'Gala-trans0'	N°	'Gala-transVf'	N°	'Florina'	N°
<i>(E,E)</i> - α -Farnesene	23.02	A	1.60 \pm 0.88	7	11.44 \pm 5.24	10	9.49 \pm 4.79	10	18.30 \pm 6.29	10
		B	6.59 \pm 3.16	10	20.73 \pm 8.32	9	9.54 \pm 6.01	10	14.96 \pm 5.27	10
		C	7.50 \pm 3.41	10	21.86 \pm 12.74	10	2.16 \pm 0.98	10	18.64 \pm 7.12	10
		D	13.47 \pm 5.12	10	3.84 \pm 1.08	10	11.10 \pm 5.52	10	12.35 \pm 4.85	10
<i>Aldehydes</i>										
Benzaldehyde	6.95	A	1.65 \pm 1.15	10	1.47 \pm 0.50	10	0.64 \pm 0.31	6	2.24 \pm 1.15	10
		B	0.21 \pm 0.09	10	5.87 \pm 2.26	10	0.78 \pm 0.48	6	0.67 \pm 0.16	10
		C	0.44 \pm 0.25	8	1.27 \pm 0.43	10	0.67 \pm 0.23	3	1.73 \pm 0.99	10
		D	0.48 \pm 0.16	8	0.54 \pm 0.07	10	4.20 \pm 3.59	9	0.94 \pm 0.35	10
Nonanal	11.19	A	2.17 \pm 0.92	10	3.98 \pm 1.33	10	2.04 \pm 0.85	10	7.21 \pm 1.99	10
		B	0.92 \pm 0.47	10	4.16 \pm 1.54	10	3.44 \pm 2.17	10	2.79 \pm 1.00	10
		C	1.38 \pm 0.42	10	1.73 \pm 0.74	10	1.68 \pm 0.85	10	2.97 \pm 1.06	10
		D	2.32 \pm 0.98	10	0.52 \pm 0.10	10	1.85 \pm 0.76	10	2.33 \pm 1.04	9
Decanal	14.44	A	1.23 \pm 0.52	10	3.53 \pm 1.07	10	1.77 \pm 0.71	10	5.76 \pm 1.61	10
		B	0.79 \pm 0.42	10	5.14 \pm 1.99	10	3.97 \pm 2.66	10	2.35 \pm 0.86	10
		C	1.16 \pm 0.32	10	1.92 \pm 0.90	10	1.44 \pm 0.71	10	1.86 \pm 0.73	10
		D	1.72 \pm 0.72	10	0.34 \pm 0.07	10	1.41 \pm 0.62	10	2.04 \pm 0.85	10
<i>Ketone</i>										
Acetophenone	9.81	A	2.39 \pm 1.46	4	0.63 \pm 0.34	4	0.74 \pm 0.64	2	3.11 \pm 1.78	5
		B	0.35 \pm 0.16	4	1.55 \pm 0.61	8	0.60 \pm 0.42	2	0.20 \pm 0.08	5
		C	1.59 \pm 0.97	6	0.38 \pm 0.15	5	0.08 \pm 0.04	4	2.05 \pm 1.74	4
		D	0.16 \pm 0.09	3	0.48 \pm 0.10	8	0.55 \pm 0.33	4	0.18 \pm 0.09	4
Geranylacetone	20.75	A	0.54 \pm 0.31	5	0.58 \pm 0.22	8	0.88 \pm 0.36	9	2.26 \pm 0.80	9
		B	0.47 \pm 0.31	8	1.90 \pm 0.74	10	1.37 \pm 0.91	10	1.04 \pm 0.44	9
		C	0.60 \pm 0.23	10	0.45 \pm 0.25	9	0.39 \pm 0.27	9	0.91 \pm 0.30	9
		D	0.89 \pm 0.44	7	0.05 \pm 0.03	4	0.69 \pm 0.30	9	0.28 \pm 0.12	7

Compound	R.T.		'Gala'	N°	'Gala-trans0'	N°	'Gala-transVf'	N°	'Florina'	N°
<i>Esters</i>										
(Z)-3-Hexenyl acetate	7.94	A	90.52 ± 50.34	10	32.20 ± 7.10	10	56.74 ± 18.98	10	40.92 ± 10.58	9
		B	19.28 ± 4.45	10	53.56 ± 14.58	10	27.37 ± 6.36	10	39.39 ± 13.03	10
		C	61.52 ± 18.15	10	42.70 ± 14.52	10	21.14 ± 10.50	10	30.01 ± 13.82	10
		D	33.61 ± 7.50	10	14.73 ± 2.43	10	54.81 ± 19.95	10	32.40 ± 13.42	10
Methyl salicylate	14.01	A	0.29 ± 0.15	5	3.50 ± 2.87	4	0.96 ± 0.58	5	1.76 ± 0.70	8
		B	1.00 ± 0.51	7	7.71 ± 4.09	7	1.59 ± 1.01	7	4.61 ± 3.15	8
		C	1.06 ± 0.80	5	9.33 ± 6.22	6	0.63 ± 0.35	5	2.72 ± 1.19	7
		D	1.50 ± 0.82	8	0.15 ± 0.10	3	1.06 ± 0.64	5	3.30 ± 2.16	8
(Z)-3-Hexenyl benzoate	23.93	A	0.42 ± 0.39	2	0.87 ± 0.73	2	0.73 ± 0.41	5	2.29 ± 0.99	5
		B	0.74 ± 0.39	6	2.18 ± 1.24	6	1.63 ± 1.26	7	1.25 ± 0.56	9
		C	1.10 ± 0.48	5	1.63 ± 1.04	4	0.31 ± 0.26	3	1.56 ± 0.71	6
		D	0.78 ± 0.40	7	0.11 ± 0.08	3	0.49 ± 0.28	7	0.73 ± 0.27	8
<i>Nitriles</i>										
Benzonitrile	7.12	A	0.13 ± 0.04	7	0.10 ± 0.07	3	0.01 ± 0.01	1	0.43 ± 0.21	5
		B	0.07 ± 0.03	8	0.47 ± 0.38	4	0.19 ± 0.13	5	0.07 ± 0.06	3
		C	0.22 ± 0.12	6	0.06 ± 0.03	4	0.02 ± 0.01	3	0.34 ± 0.15	7
		D	0.02 ± 0.01	3	0.07 ± 0.04	4	0.09 ± 0.09	3	0.06 ± 0.04	5
Phenylacetonitrile	12.41	A	0.21 ± 0.12	10	0.42 ± 0.18	8	0.05 ± 0.04	7	0.34 ± 0.11	10
		B	0.06 ± 0.02	10	0.41 ± 0.18	10	1.67 ± 1.57	10	0.13 ± 0.06	6
		C	0.16 ± 0.07	9	0.60 ± 0.52	10	0.13 ± 0.09	9	0.14 ± 0.07	8
		D	0.04 ± 0.01	6	0.04 ± 0.02	3	0.08 ± 0.04	6	0.13 ± 0.07	8

Mean ± SE [ng/L] calculated for each compound analysed; n.d. = not detected; R.T. = Retention Time [Min]; N° = frequency of detection of the compounds in a total of 10 samples

Individual compounds and effect of genotype

For most of the individual compounds (Table 4.2.), amounts released were not significantly different between the transgenic lines and the isogenic line within the same infection type. Figure 4.1 presents the compounds (a-e) for which significant quantitative differences were noted between genotypes. Only major compounds were considered, i.e. compounds that were positively identified in at least seven out of ten samples analysed (Table 4.2 indicates frequency N° of detection of the compounds in a total of 10 samples).

Healthy plants of the transgenic line 'Gala-transVf' emitted significantly higher quantities of the terpene (*E,E*)- α -farnesene than the isogenic line 'Gala' (Fig. 4.1. d). A similar relationship applies to 'Gala-trans0'. However, these amounts were not significantly different from those found in the headspace of the classically bred scab resistant cultivar 'Florina'. There were also major quantitative differences in terpene release between the two cultivars. The ten-fold amount of the sesquiterpene (*E,E*)- α -farnesene was emitted by 'Florina' compared to 'Gala', and similar relationships apply to the emissions of the monoterpene β -ocimene and the sesquiterpene β -caryophyllene (Fig. 4.1. a-b, d; Table 4.4.). Furthermore, healthy plants of 'Gala-transVf' released significantly lower quantities of the aromatic compound phenylacetonitrile than 'Florina', while there was no significant difference between amounts released by 'Gala-transVf' and 'Gala' (Fig. 4.1. e; Table 4.4.).

In contrast to scab inoculated plants, leafminer infested plants showed major quantitative differences in headspace volatiles between genotypes. While there was a significant difference in the headspace amounts of the terpenes β -ocimene, β -caryophyllene, and (*Z,E*)- α -farnesene between 'Gala-transVf' and 'Florina', no significant difference in the release of these compounds was detected when comparing the transgenic 'Gala-transVf' to the isogenic 'Gala' (Fig. 4.1. a-c; Table 4.4.).

Table 4.3. (a) Interaction effects of apple genotype (G), infection type (I), and genotype by infection type interaction (G*I) on the emitted volatile profile of apple plants; (b) effects of apple genotype within one infection type (A healthy plants; B scab inoculated plants; C leafminer infested plants; D plants subject to combined scab inoculation and leafminer infestation) on the emitted volatile profile of apple plants

(a) Interaction effects					
Effect	Hypothesis	df	Error df	F-value	P-value
G	[Genotype]	54	387	3.410	0.001
I	[Infection]	54	387	2.526	0.001
G*I	[Genotype*Infection]	162	1215	1.507	0.001
(b) Effects of apple genotype within one infection type					
Infection type		df	Error df	F-value	P-value
A	[healthy]	54	63	3.046	0.001
B	[scab]	54	63	1.623	0.032
C	[leafminer]	54	63	1.748	0.017
D	[scab+leafminer]	54	63	1.320	0.144

MANOVA using the 18 emitted volatile compounds α -pinene, limonene, β -ocimene, linalool, β -caryophyllene, α -humulene, (Z,E)- α -farnesene, (E,E)- α -farnesene, benzaldehyde, nonanal, decanal, acetophenone, geranylacetone, (Z)-3-hexenyl acetate, methyl salicylate, (Z)-3-hexenyl benzoate, benzonitrile, and phenylacetone. Number of replicates = 10, number of genotypes = 4, number of infection types = 4.

Bold P-values indicate a significant difference. F-value for Pillai's Trace

Table 4.4. Effects of genotype within one infection type (A healthy plants; C leafminer infested plants) on volatile quantities of compounds positively identified in at least seven of ten samples

Compound	Infection type	df	Mean square	F - value	P - value
		[genotype,error]			
β -Ocimene	A [healthy]	3,36	12.064	4.428	0.009
	C [leafminer]	3,36	12.713	6.797	0.001
β -Caryophyllene	A [healthy]	3,36	1.536	3.399	0.028
	C [leafminer]	3,36	1.717	4.067	0.014
(Z,E)- α -Farnesene	C [leafminer]	3,36	3.320	3.184	0.035
(E,E)- α -Farnesene	A [healthy]	3,36	7.462	4.745	0.007
Phenylacetone	A [healthy]	3,36	3.927	3.217	0.034

One-way ANOVA for between-subject effects. Number of replicates = 10, number of genotypes = 4. Bold P-values indicate a significant difference.

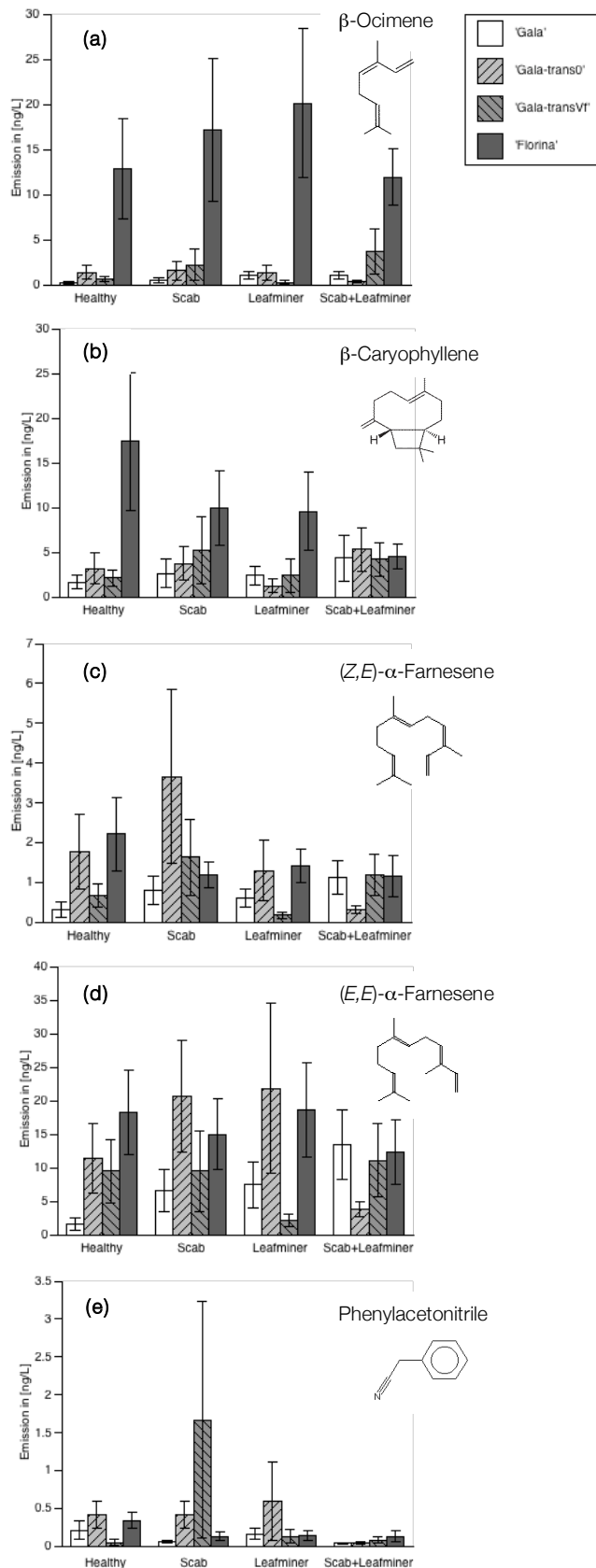


Figure 4.1. Collected headspace volatiles of **(a)** β -ocimene, **(b)** β -caryophyllene, **(c)** (Z,E) - α -farnesene **(d)** (E,E) - α -farnesene, and **(e)** phenylacetonitrile from intact apple shoots of 'Gala', 'Gala-trans0', 'Gala-transVf', and 'Florina' under different infection types: healthy, scab inoculation, leafminer infestation and a combination of scab inoculation plus leafminer infestation (mean \pm SE [ng/L] calculated for each compound analysed. Number of replicates = 10).

4.5 Discussion

The transgenic scab resistant apple genotype 'Gala-transV β ' emitted a quantitatively, but not qualitatively different volatile blend under certain infection types compared to either the isogenic line 'Gala', or the scab resistant classically bred cultivar 'Florina'. Such differences were found only in two of four infection types, and in five of eighteen compounds examined. Our approach was to compare traits of transgenic apple plants with representative classically bred varieties. Most remarkably, headspace volatile amounts of the transgenic scab resistant genotype 'Gala-transV β ' laid within the frame given by 'Gala' and 'Florina', indicating no significant differences of this new line compared to two cultivars that are commonly planted in commercial orchards. Differences in volatile emissions among different cultivars have been reported previously from maize subject to a single infection type, and it was concluded that these maize genotypes likely vary in their attractiveness to natural antagonists of herbivores (Gouinguéné et al., 2001). In the apple ecosystem, both antagonists and herbivores have been found to behaviourally respond to volatiles from healthy apple plants (Rott et al., 2005; Vallat & Dorn, 2005). Furthermore, herbivores of the same or different species may be repelled by (or attracted to) volatiles from infested trees (Hern & Dorn, 2002).

In the current study, volatile profiles differed between genotypes for healthy plants and for leafminer infested plants. Little differences were noted for scab inoculated plants, and none for the plants that were subjected to a combined infection of scab and leafminer. While several studies have addressed volatile emissions of one single plant genotype across different infection types (Kishimoto et al., 2008; Rostás et al., 2006), this study is to our knowledge the first comparing the volatile emissions of several genotypes in response to insect, fungal and combined infections. Our findings suggest that marked differences in volatile profiles found between various healthy or insect infested genotypes are diminished by prior scab infection. Future time course experiments, as they have been carried out for herbivore infested apple (Hern & Dorn, 2001), might help to understand underlying mechanisms.

Quantitative differences between the transgenic scab resistant line and the commercial cultivars were assessed for four terpenes and an aromatic compound. Bioactivity in the apple ecosystem has been reported for several of them. The two sesquiterpene isomers (*E,E*)- α -farnesene and (*Z,E*)- α -farnesene have a similar behavioural effect on mated females of the codling moth *Cydia pomonella*, with low dosages being attractive and high dosages repellent (Hern & Dorn, 1999), emphasizing the importance of emitted amounts. (*E,E*)- α -Farnesene also stimulates oviposition and larval movement of the codling moth (Bradley & Suckling, 1995; Landolt et al., 2000; Wearing & Hutchins, 1973). The sesquiterpene β -caryophyllene attracted codling moth females at a dosage measured in the headspace of the apple cultivar 'Golden Delicious' under field conditions (Vallat & Dorn, 2005). Both β -caryophyllene and the monoterpene β -ocimene elicited electroantennogram responses in the antennae of codling moth males, suggesting a behavioural effect of these terpenes on this species (Casado et al., 2008). Several aromatics including benzaldehyde and benzonitrile have been reported to contribute to insect attraction of fruit trees (Piñero & Dorn, 2007; Piñero et al., 2008; Piñero & Prokopy, 2003), while phenylacetonitrile was behaviourally ineffective in such trials (Piñero & Dorn, 2007). Each of these four terpenes and phenylacetonitrile are emitted by the transgenic apple line 'Gala-transVf' in lower or higher amounts than either by the isogenic line 'Gala' or by the scab resistant cultivar 'Florina' under distinct infection types. Hence, we cannot exclude that these changes have behavioural consequences on insects associated with the apple tree. In fact, antagonists of the apple leafminer such as parasitoids rely on volatile emission of infested apple trees to locate their concealed living host at the stage suitable for parasitism (Dorn et al., 1999; Dutton et al., 2000a). Most importantly, however, the current study provides direct evidence that these modified odour emissions are in the range of variability of the emissions recorded for the two most relevant standard apple cultivars. Differences in chemical composition between 'Gala' and 'Florina' are not restricted to individual headspace volatiles and non-volatile fruit constituents (Kindt et al., 2007). Ratios of individual compounds are also deviating, what applies to the transgenic line 'Gala-transVf' as well. Such differences may further

contribute to differential sensitivity of apple genotypes to insect herbivores (Qubbaj et al., 2005; Stoeckli et al., 2008) or attraction of their antagonists (Boevé et al., 1996), but we have no indication for an increased risk conferred by the genotype 'Gala-transVf' compared to representative classically bred genotypes. Many studies on risk assessment of transgenic plants are limited to pair-wise comparisons between the transgenic and the isogenic lines (Álvarez-Alfageme et al., 2008; Chen et al., 2008; Schuler et al., 2003; Turlings et al., 2005). The current investigation is, to our knowledge, the first to compare the studied trait of transgenic plants with a representative non-transgenic control cultivar that contains the same resistance gene as the transgenic plant. Our findings clearly document the significance of this approach.

5 Transgenic and classically bred apple genotypes: impact on terpene-mediated parasitoid host location behaviour²

5.1 Abstract

We investigated terpene-mediated interactions between transgenic or classically bred apple genotypes and associated insects. 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. Squalene contents of 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. Behavioural bioassays with parasitoid females also reflected these findings. We conclude that alterations in leaf chemistry and corresponding responses of the parasitoid are apparent among classically bred cultivars, rather than among genetically modified lines.

² Based on: Vogler U, Rott AS, Gessler C & Dorn S (submitted). Transgenic and classically bred apple genotypes: impact on terpene-mediated parasitoid host location behaviour

5.2 Introduction

Phytochemicals mediate host location behaviour in numerous parasitoid wasp species, both prior (Degenhardt et al., 2003; Tumlinson et al., 1993) and after landing on a host-infested plant (Muratori et al., 2006). In many plant-insect systems, plant terpenes play a crucial role as semiochemicals, as was shown for monoterpenes (Hern & Dorn, 2002), sesquiterpenes (Vallat & Dorn, 2005), homoterpenes (Turlings et al., 1990), diterpenes (Miller et al., 2005) and in one single case for a triterpene (Dutton et al., 2002). Damaged plants release typically higher quantities of phytochemicals and such herbivore-induced compounds guide natural antagonists, particularly parasitoids, to the site of damage (Connor et al., 2008).

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* (Turlings et al., 2005). Subtle chemical changes caused by transgenesis may have effects on non-target organisms including parasitoids and are therefore of high interest in risk assessment studies (Marvier et al., 2007; Romeis et al., 2008). So far, investigations on chemically mediated interactions involving transgenic crops have largely focused on induced volatile chemicals influencing parasitoid behaviour prior to landing on a host-infested patch (Halitschke et al., 2008; Himanen et al., 2009; Turlings et al., 2005). By contrast, virtually nothing is known on chemically mediated host location after landing of the parasitoid on transgenic vs. isogenic plants (Beale et al., 2006).

Apple genotypes vary in their chemical composition (Hern & Dorn, 2003; Kindt et al., 2007), and different cultivars release different amounts of volatile terpenes upon herbivory (Boevé et al., 1996). However, it is yet unknown whether or to which degree different apple genotypes vary in their contents of bioactive non-volatile terpenes. Upon infestation by the apple leafminer *Phyllonorycter* sp. (Lepidoptera: Gracillariidae) that damages the apple foliage by feeding within the leaf tissue and generates a tentiform mine, squalene contents of the leafmine increase drastically (Dutton et al., 2002; Dutton et al., 2000b). Remarkably, this

single triterpene guides the parasitoid *Pholetesor sp.* (Hymenoptera: Braconidae) to its larval host concealed in the plant tissue (Dutton et al., 2002). Parasitoid females inspect the leaf surface with their antennae and finally insert their ovipositor into the mine to parasitize a young leafminer larva (Dutton et al., 2000a), a behavioural pattern that can also be observed by using simply hexane leaf extracts or synthetic squalene presented on filter paper (Dutton et al., 2002; Dutton et al., 2000b). In the orchard ecosystem, *Pholetesor* parasitoids substantially contribute to regulate populations of the potentially devastating apple leafminer (Dorn et al., 1999). Hence the tritrophic system composed of apple plant, leafminer and its parasitoid is highly suitable and meaningful for studying potential chemically mediated non-target effects of transgenic plants. Different defence responses by a plant may be triggered (Turlings et al., 1998) pending on whether such plant is infected with a herbivore, a pathogen, or concurrently with both organisms, therefore we included inoculations by *Venturia inaequalis* into our study as well.

In apple orchards, the worldwide distributed fungal pathogen *V. inaequalis* causes apple scab, thereby leading to major economic losses or requiring frequent fungicide applications (MacHardy et al., 2001). Resistant apple genotypes are of great promise, pending on a positive outcome of risk assessment studies. We investigated four different apple genotypes. The scab resistant transgenic apple line 'Gala-transVf' 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 (Belfanti et al., 2004; Vinatzer et al., 2001). We further included '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 non-transgenic, scab resistant control. Until now, studies of transgenic resistant plants neither included a transgenic control solely containing the promoter and the selectable marker gene, nor a conventionally bred resistant control, thus limiting interpretations on risk assessments.

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

5.3 Materials and Methods

Insects 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 16L:8D photoperiod), on two-months-old potted apple seedlings (*Malus x domestica* 'Golden Delicious' open pollinated seedlings), in Plexiglas rearing cages (45.7 cm x 25 cm x 27 cm) (Casas & Meyhöfer, 1994). Adult parasitoids were kept in a separate climatic chamber (Conviron, Controlled Environment Ltd., Winnipeg, Canada) in Plexiglas rearing cages (25 cm x 25 cm x 25 cm) at 21 ± 2 °C, 60 ± 10 % relative humidity and a 16L:8D 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 (Dutton et al., 2000a). 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-transVf' and 'Florina' were used for the experiments. Two-years old plants of each genotype were potted in cylindrical pots containing 1.5 L 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 daytime and 18 ± 2 °C during night time, 60 ± 5 % relative humidity and a photoperiod of L16:D8. Assimilation lighting was used to complement daylight conditions when necessary (lighting level 5000 lux; 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. 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. The five youngest leaves per shoot were labelled and subjected to one of the four infection types: (1) inoculation with *V. inaequalis* conidia suspension (10^5 conidia per mL) 24 \pm 2 days prior extraction; (2) infestation for three days with six two- to three-days old leafminer adults 24 \pm 2 days prior extraction; and (3) inoculation with *V. inaequalis* conidia suspension (10^5 conidia per mL) and subsequent infestation (55 \pm 1 hours after inoculation) with six two- to three-days old leafminer adults, 24 \pm 2 days prior to extraction;

and (4) no inoculation nor 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-transVf' 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 Corporation, Tokyo, Japan) together with a reference area of 1 cm², in order to calculate the total leaf area using 'Adobe Photoshop 8.0' (Adobe Systems Inc., San Jose, CA, USA) (Mody et al., 2009). For chemical analysis, the labelled leaves from one shoot of each of the five plants per treatment were pooled together, yielding a total of 80 (5 x 16) samples. For the bioassays, two labelled leaves (second and fourth position) 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 ($\geq 99.0\%$, Fluka, Buchs, Switzerland) per five leaves, or accordingly, 100 mL per ten leaves. After 24 hours 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, \varnothing 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 Corporation, Billerica, MA, USA) packed into a 13 mm-syringe Swinney Filter Holder (Swinney Stainless, Millipore Corporation, Billerica, MA, USA) 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 behavioural testing.

Chemical analysis

To quantify squalene contents in the above mentioned leaf extracts, we used a gas chromatograph-mass spectrometer system (HP 6890 Series GC-System and HP 5973, Hewlett Packard Company, Atlanta, GA, USA) with a split/splitless injector in splitless mode. The GC-MS was fitted with a deactivated fused silica precolumn (5 m x 0.25 mm; Agilent Technologies, Basel, Switzerland) and a nonpolar Econo-Cap EC™-5 column (30 m x 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 °C 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) (Dutton et al., 2000b). For the identification of the mass spectrometry-data, ChemStation software (MSD Productivity Chem Station software, Agilent Technologies Inc., Santa Clara, CA, USA) 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.

Behavioural bioassays

We carried out single choice contact bioassays to test and quantify host searching behaviour of parasitoid females exposed to apple leaf extracts of 16 treatments and an additional solvent control. All bioassays were carried out with naïve two-to-four days 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 0900 h and 1800 h in Petri dish arenas (9 cm diameter) equipped with a filter paper (1.3 cm diameter; corresponding to a fully developed mine (Dutton et al., 2000b)) 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 (Dutton et al., 2000b). Laboratory conditions were 24 ± 2 °C, 50 ± 10 % relative humidity and 1200 ± 250 lux. 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 behavioural observations were recorded on a personal computer using the software 'The Observer 3.0' (Noldus Information Technology 1991, Wageningen, 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*, *abdominal preening*, *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 behaviour were performed using JMP 7.0.2 (SAS Institute, Cary, NC, USA) and SPSS 16.0 for Mac (SPSS Inc., Chicago, IL, USA).

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 HSD post-hoc test.

Parasitoid behaviour data was 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 the 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 behavioural states with genotype or infection type as main effects. If the MANOVA model showed significant differences, one-way ANOVA followed by Tukey 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 behavioural states, a Principle 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 behaviour. Subsequently, the three dissected variables *antennal preening*, *abdominal preening* and *walking*, which explained most of the variance (74 %), were analyzed with a MANOVA.

5.4 Results

Chemical analysis of leaf extracts for squalene contents

Squalene quantities in the leaf extracts of each of the three genotypes of the 'Gala' lines did not change significantly within infection type (Table 5.1.). Lower mean amounts of squalene were found in leafminer infested leaves compared to healthy leaves from 'Gala', 'Gala-trans0' and 'Gala-transVf', but these differences were not significant. In contrast, among the 'Florina' samples, we detected significantly more squalene in extracts from leafminer infested than from healthy leaves. This increase in squalene content from healthy to leafminer infested leaves was more than four-fold. While 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 5.1.).

A comparison across the apple genotypes revealed no significant effect of the three infection types (i.e. healthy, scab and combined scab and leafminer infection) on squalene contents of the extracts. However, in the leafminer treatments, the squalene value measured for the genotype ‘Florina’ was significantly higher than for ‘Gala-transVf’ (Table 5.1.).

Squalene contents in the separately prepared apple leaf extracts used for the bioassays (below) were in the range of those presented in Table 5.1.

Table 5.1. Squalene quantity per leaf surface unit (ng ± SE per cm²). Analysis of four apple genotypes subjected to four different infection types

	Squalene (ng/cm ²) ^{a b}			
	‘Gala’	‘Gala-trans0’	‘Gala-transVf’	‘Florina’
healthy	9.98 ± 4.01 ^{A a}	8.27 ± 4.65 ^{A a}	4.46 ± 1.47 ^{A a}	3.51 ± 1.45 ^{B a}
scab	5.24 ± 1.20 ^{A a}	5.92 ± 3.46 ^{A a}	8.95 ± 2.37 ^{A a}	2.68 ± 0.63 ^{B a}
leafminer	5.80 ± 0.30 ^{A a}	6.50 ± 1.29 ^{A a}	2.62 ± 0.92 ^{A b}	16.41 ± 3.90 ^{A a}
scab+leafminer	3.79 ± 0.46 ^{A a}	7.87 ± 3.29 ^{A a}	5.25 ± 1.11 ^{A a}	7.06 ± 1.13 ^{ABa}

One-way ANOVA of log (x + 0.5) transformed data and Tukey HSD post-hoc test.

^a 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-transVf’: $n = 5$; $F = 3.1866$; $df = 3,16$; $P = 0.0523$; ‘Florina’: $n = 5$; $F = 9.7933$; $df = 3,16$; $P < 0.001$).

^b 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: $n = 5$; $F = 0.5174$; $df = 3,16$; $P = 0.6763$).

Table 5.2. Effects on parasitoid behavior by apple genotype (**G**), infection type (**I**), and genotype by infection type interaction (**G*I**)

Effect		Hypothesis df	Error df	F-value	P-value
G	[Genotype]	18	1470	2.526	< 0.001
I	[Infection]	18	1470	4.337	< 0.001
G*I	[Genotype*Infection]	54	2958	1.271	0.089

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.

Parasitoid behaviour 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, while there was no significant genotype by infection type interaction (MANOVA; Pillai's Trace $P < 0.05$) (Table 5.2.).

The hexane solvent control elicited minimal *antennation* and no *ovipositional probing*, while *antennal preening*, *abdominal preening*, *standing* and *walking* were observed, but still, this response was significantly different from that triggered by healthy leaf extracts (Table 5.3.). Among the healthy genotypes, parasitoid response did not differ significantly, allowing for a general comparison of the solvent control tested 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 HSD post-hoc test), *antennal preening* (one-way ANOVA; $F = 6.190$; $df = 4,145$; $P < 0.001$; Tukey HSD post-hoc test), and *abdominal preening* (one-way ANOVA; $F = 4.967$; $df = 4,145$; $P < 0.05$; Tukey HSD post-hoc test). This comparison indicates that the solvent alone is ineffective regarding *antennation* and *ovipositional probing*, and also elicits low response in *antennal preening* and *abdominal preening* (Table 5.3.).

Table 5.3. Effects of leaf extracts on parasitoid behavior: **(a)** apple genotypes and solvent control within the healthy leaf extracts; **(b)** apple genotypes within one infection type; and **(c)** infection types within apple genotype

(a) Effect of solvent control (analyzed in parallel to healthy leaf extracts) compared with healthy leaf extracts ^a				
	DF	Error df	F-value	P-value
	24	572	2.753	< 0.001
(b) Effects of apple genotypes within one infection type ^b				
Infection type	DF	Error df	F-value	P-value
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 ^c				
Apple genotype	DF	Error df	F-value	P-value
'Gala'	18	339	1.676	< 0.05
'Gala-trans0'	18	339	1.578	0.063
'Gala-transVf'	18	339	2.235	< 0.05
'Florina'	18	339	2.401	< 0.05

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.

^a Number of apple genotypes plus solvent control = 5.

^b Number of apple genotypes = 4.

^c Number of infection types = 4.

Between genotypes within one infection type, no differences in the behavioural response of the parasitoid were found 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 apple genotypes (Table 5.3.). Interestingly, female parasitoids spent significantly more time displaying *ovipositional probing* behaviour on extracts from leafminer infested 'Florina' than from all three leafminer infested 'Gala' lines (one-way ANOVA; $F = 4.549$; $df = 3,116$; $P < 0.05$; Tukey HSD post-hoc test). Total time spent on *ovipositional probing* on 'Florina' exceeded that on 'Gala', 'Gala-trans0' or 'Gala-transVf' by a factor of more than three (Table 5.4.). The time spent on the further behavioural states observed did not differ significantly among the four apple genotypes.

Between infection types within one genotype, significant differences in the behavioural response of the parasitoid were found, except within 'Gala-trans0' where differences fell short of significance at $P = 0.063$ (Table 5.3.). 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 among the genotypes 'Gala' and 'Gala-transVf'. Parasitoids spent more time on *abdominal preening* on extracts from combined infected 'Gala' leaves than on extracts from leaves infested with leafminer (one-way ANOVA; $F = 3.114$; $df = 3,116$; $P < 0.05$; Tukey HSD post-hoc test; Table 5.4.). 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 (one-way ANOVA; $F = 6.999$; $df = 3,116$; $P < 0.001$; Tukey HSD post-hoc test; Table 5.4.). Duration of *ovipositional probing* on extracts from leafminer infested leaves was almost three-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 (*antennal preening*: one-way ANOVA; $F = 2.792$; $df = 3,116$; $P < 0.05$; Tukey HSD post-hoc test; *abdominal preening*: one-way ANOVA; $F = 3.477$; $df = 3,116$; $P < 0.05$; Tukey HSD post-hoc test; Table 5.4.).

Table 5.4. Duration (in seconds) of recorded parasitoid behavioral states on filter papers treated with different leaf extracts (**A** healthy, **B** scab inoculated, **C** leafminer infested, or **D** concurrently inoculated with scab and infested with leafminer) of ‘Gala’, ‘Gala-trans0’, ‘Gala-transVf’ and ‘Florina’. Duration (in seconds) of recorded parasitoid states on hexane treated filter papers is given in brackets (c = solvent control). Means \pm SE are presented. Number of replicates tested on the same extract = 30; number of infection types = 4

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

5.5 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 (Dutton et al., 2002; Dutton et al., 2000b). 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 (Alaphilippe et al., 2008; Müller & Riederer, 2005; Rostás et al., 2008). Changes in the plant genotype may thus be associated with the

risk of impeded or disrupted host location behaviour of the parasitoid. Our chemical leaf analyses as well as our bioassays testify this effect in an amazing way for leafminer infested leaves of the 'Gala' lines compared to those of the cultivar 'Florina'. First, a comparison between the two scab resistant apple genotypes 'Gala-transVf' and 'Florina' indicates that squalene content of leafminer infested leaves is much lower in the transgenic genotype and the parasitoid spent less time on *oviposition probing* on the transgenic 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-transVf', '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 behaviours, in particular of *ovipositional probing*. Hence, transgenesis 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 non-target insects associated with plant genotype.

Our multitrophic apple system demonstrated in chemical analyses and behavioural 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 (Dutton et al., 2000b). Squalene is an intermediate metabolite in the biosynthesis of other terpenoids and of sterol, but it is also known to be an essential compound regulating plant growth (Schaller, 2004; Simmen & Gisi, 1995; Yates et al., 1991). Other studies have attributed photo-protective function to this compound of the epicuticular structures (Guil-Guerrero et al., 2000). The level of squalene is critical for the parasitoid's response, as both marginal and very high levels are behaviourally ineffective (Dutton et al., 2002). 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 behaviour 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 these leaf extracts, even when they were gained from leafminer infested leaves, females exhibited little *ovipositional probing* behaviour. Together with the findings by Dutton et al. (2002) we conclude that squalene concentration on these leaves was too low to stimulate insertion of the ovipositor. Contrary to our expectation, the classically bred 'Gala' genotype failed to increase squalene content upon leafminer herbivory. However, in the genotype 'Florina', a four-fold increase in squalene content was assessed, and the time spent by the parasitoid on *ovipositional probing* increased according to expectation. Thus, our chemical analyses and bioassays carried out on 'Florina' extracts are fully in line with previous conclusions gained for 'Golden Delicious' seedlings (Dutton et al., 2002), verifying the behavioural efficacy of increased levels of squalene. Here we report, for the first time, that apple genotypes, even conventionally bred cultivars, may differ in herbivore-induced contents of this triterpene with profound consequences on a natural antagonist of the leafminer. The finding that parasitoids behaved differently on the two tested apple cultivars, while 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 transgenesis consisting of the selectable marker *nptII* under control of the S35 promoter, affected the behaviour of the parasitoid confronted with the apple leaf extracts.

Parasitoid response to different infection types

Beyond *ovipositor probing* that could be related to substrate content in squalene, the parasitoid exhibited further behaviours in response to distinct 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. Further,

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 non-target herbivore (Dorn et al., 2003).

Risk assessment

Adequate risk assessment of transgenic plants for non-target organisms is extremely important, especially since transgenic plants with resistance to diseases or pests have been developed for commercial use in agriculture (Babu et al., 2003; Collinge et al., 2008). The current investigation is, to our knowledge, the first to compare the studied trait of transgenic plants with a representative non-transgenic control cultivar that contains the same resistance gene as the transgenic plant, and the findings underline the significance of this approach.

6 How transgenic and classically bred apple genotypes affect non-target organisms on higher trophic levels³

6.1 Abstract

Current plant breeding comprises classical techniques as well as biotechnological methods suitable to insert target genes into the plant genome. Potential non-target effects of these newly developed plants have to be evaluated in appropriate risk assessment studies. We investigated non-target effects of four apple genotypes (*Malus x domestica* L. Borkh., Rosaceae) that were either susceptible or resistant to the fungal phytopathogen causing apple scab, *Venturia inaequalis* (Cke.) Wint. (Ascomycotina: Pleosporales), and that were either of transgenic origin or classically bred, differing in or sharing a single trait each. Experiments on insect performance were carried out with two non-target species, namely the apple leafminer *Phyllonorycter blancardella* Fabricius (Lepidoptera: Gracillariidae) and its parasitoid *Pholetesor circumscriptus* Ness (Hymenoptera: Braconidae). Development time of the apple leafminer was not affected by the four apple genotypes. In contrast, the number of emerged adult moths significantly differed between the two classically bred cultivars, while no effect of the two transgenic apple genotypes was detected. Paralleling the observations of the apple leafminer development, no impact of the four apple genotypes on parasitoid development time and parasitism success could be detected. Although mean numbers of emerged parasitoids were lower on the cultivars with lower number of emerged apple leafminers, these differences were not significant. Herbivore and parasitoid performance was not altered in the presence of pathogen inoculation.

³ Based on: Vogler U, Rott AS, Gessler C & Dorn S (submitted). How transgenic and classically bred apple genotypes affect non-target organisms on higher trophic levels

In conclusion, host plant suitability was found to be more determined by variability among classically bred apple cultivars than by the transgenic genotypes tested. To elucidate possible effects of transgenic plants within multitrophic interactions, an appropriate risk assessment incorporating the relevant controls is crucial.

6.2 Introduction

Terrestrial ecosystems are complex nets of multitrophic interactions, in which plants play a central role (Hunter & Price, 1992). They interact directly and indirectly with higher trophic levels namely phytopathogens, insect herbivores and even their antagonists (Poppy, 1997). These interactions depend to some extent on the characteristics of the plant *per se*, including resistance traits, which are in turn determined by the plant's genotype.

New plant genotypes are gained by classical breeding and by biotechnological methods. The latter are used to produce transgenic plants with desirable characteristics by inserting target genes into the plant's genome (Collinge et al., 2008). Since the gene pool is no longer limited by species compatibility, plant foreign DNA can be transferred into the plant genome. While possible non-target effects of these transgenic plants within their ecosystem were not considered initially, recent concerns about their potential environmental impact led to an increasing number of risk assessment studies aimed at quantifying potential adverse effects (Romeis et al., 2006). Most available studies of this kind, however, have only focused on evaluating the direct effects of insect resistant transgenic plants on non-target terrestrial and aquatic organisms (Romeis et al., 2008; Rosi-Marshall et al., 2007). New transgenic plants, including pathogen resistant plants, have been recently achieved, but very few studies have investigated effects of these plants on their respective environments. One of these investigations identified the effect of a pear plant line (*Pyrus communis*) transformed with the lytic peptide gene *D5C1* conferring resistance to the bacterial disease fireblight caused by *Erwinia amylovora* on the reproduction of the pear psylla *Cacopsylla pyricola* Foerster (Homoptera: Psyllidae) (Puterka et

al., 2002). In short-term choice and no-choice experiments, pear psylla adults significantly preferred the transgenic leaves for oviposition over non-transgenic plants, whereas in long-term studies transgenic leaves bore fewer offspring than non-transgenic leaves (Puterka et al., 2002). However, implications of transgenic plants infested with pathogens on non-target organisms is another aspect that needs research (Dean & De Moraes, 2006; Turlings et al., 2005).

This study concerns the impact of four different apple genotypes (*Malus x domestica*, Rosaceae) in the absence and presence of the fungal pathogen *Venturia inaequalis* (Ascomycotina: Pleosporales) on the development of the apple leafminer *Phyllonorycter blancardella* Fabricius (Lepidoptera: Gracillariidae) and its parasitoid *Pholetesor circumscriptus* Nees (Hymenoptera: Braconidae). Previous research described fine-tuned interactions between apple plants, the *Phyllonorycter* herbivore and its parasitoid (Dutton et al., 2002; Dutton et al., 2000a, b). As both leafminer and its endophytic koinobiont parasitoid complete all preimaginal stages within the plant tissue, their performance may respond to subtle changes in their biotic environment, in particular to changes in plant genotype and infection state.

Venturia inaequalis, one of the most serious diseases affecting apple worldwide, causes apple scab that currently can be prevented by ten to 15 fungicide treatments per season (Gessler & Patocchi, 2007). Planting scab resistant genotypes, either classically bred such as the cultivar 'Florina', or achieved by transformation such as the genotype 'Gala-transVf', could minimise the use of fungicides and render agroecosystems more sustainable (Brun et al., 2008; Gessler et al., 2006). The *HcrVf2* scab resistance gene that is shared by both genotypes derives from the wild apple species *Malus floribunda* 821 (Vinatzer et al., 2001). In addition to these two scab resistant genotypes, we included two scab susceptible genotypes, the cultivar (isoline) 'Gala', and the control genotype 'Gala-trans0' that was achieved by transforming 'Gala' with the 35S promoter and the selectable marker gene *nptII* (Belfanti et al., 2004).

Our goal was to compare these genotypes, which differ in or share a single trait, in their effects on the two mentioned non-target insect species, both in the absence and presence of *V. inaequalis*. Inoculation with this phytopathogen was

used as an indicator that the defence mechanism of the plant has been triggered.

The specific objectives were (1) to detect possible direct effects of the four different genotypes on the performance of the leafminer (development time and number of emerged adult moths), as well as possible indirect effects on the performance of its parasitoid (development time, number of emerged wasps and parasitism success), and (2) to evaluate whether the absence or presence of *V. inaequalis* inoculation influences leafminer and parasitoid performance.

6.3 Materials and Methods

Study organisms

This study is based on a multitrophic apple system comprising different apple genotypes, the fungal phytopathogen *V. inaequalis*, the leafmining herbivore *P. blancardella*, and its parasitoid *P. circumscriptus*.

Four different grafted apple genotypes were used in the experiments: (1) the cultivar 'Gala', (2) the transgenic genotype 'Gala-trans0', (3) the transgenic genotype 'Gala-transV^r', and (4) the cultivar 'Florina'. Two-years old plants were grown in cylindrical pots containing 1.5 l substrate-perlite-sand mixture at a ratio of 6:1:1. All plants were kept in closed greenhouse chambers with controlled climatic conditions (L16:D8, 22 ± 2 °C (day)/ 18 ± 2 °C (night), 60 ± 5% r.h.). Assimilation light was used to complement daylight when necessary. 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 an active vegetative growing stage. For protection against spider mite damage, bromopropylate applications (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. Experiments were carried out with leafminer infested plants in the absence or presence of inoculation with the fungal pathogen *V. inaequalis*.

To obtain conidia of *V. inaequalis*, apple leaves with sporulating scab lesions were collected at the Swiss Federal Research Station Agroscope Changins-Wädenswil ACW (Switzerland). Conidia were washed off from the leaves with water, and their concentration was determined microscopically using a Neubauer counting chamber (Laboroptik GmbH, Friedrichsdorf, Germany). The conidia material was stored at -20 °C until further use.

The colony of the spotted tentiform leafminer *P. blancardella* originated from South Tyrol (Italy). It was refreshed on a yearly basis with new individuals collected in apple orchards in the same region. Adult leafminers were allowed to infest two-month-old potted apple seedlings (*M. x domestica* 'Golden Delicious' open pollinated seedlings) in Plexiglas cages with openings at all sides covered with gauze, and a removable glass plate at the top. These Plexiglas cages were placed in an insectary with L16:D8, at 22 ± 2 °C and $50 \pm 5\%$ r.h.. The larvae developed through five larval instars consisting of a sap-feeding stage (1st to 3rd larval instar) and a tissue-feeding stage (4th to 5th larval instar) before they pupated in the mines (Rott & Godfray, 2000). Infested leaves containing last instar larvae were removed from the apple seedlings and placed in a closed transparent plastic box with moist paper until adult emergence.

The colony of the parasitoid *P. circumscriptus* was started from individuals collected in apple orchards in South Tyrol (Italy). Bioassays were carried out after rearing the parasitoid for 10 ± 5 generations. This solitary parasitoid that develops within its concealed living host remains invisible until emergence, when the adult wasp leaves the mine through a hole in the lower epidermis (Dorn et al., 1999; Rott & Godfray, 2000). Rearing took place in a climatic chamber (Conviron, Controlled Environment Ltd., Winnipeg, Canada) under controlled conditions (L16:D8, 21 ± 2 °C, $60 \pm 10\%$ r.h.). Up to ten adult parasitoids comprising both sexes were introduced for four days into a Plexiglas cage with openings on two opposite sides, one of them covered with fixed gauze and the other with a closable gauze sleeve. The cage contained honey and water as

food source for adults as well as sap-feeding leafminer larvae on apple seedlings for oviposition (Dutton et al., 2000a, b). Two and a half weeks later, when parasitoids had pupated, leafmines were dissected and parasitoid cocoons were removed and placed into a transparent plastic box (9.5 x 9 x 8 cm) with moist paper until adult emergence. This rearing protocol (i.e., maintenance of apple seedlings, potted plants, apple leafminers and parasitoids cultures) required some 20 hours of maintenance per week, and yielded sufficient material to carry out parallel experiments with all four plant genotypes under one infection regime.

Herbivore performance

Experiments on performance of *P. blaucardella* were conducted in a fully equipped separate greenhouse chamber with internal air circulation. The controlled climatic conditions were as described above.

For the treatment without *V. inaequalis* inoculation on the four apple genotypes, six two-days-old female moths reared on 'Golden Delicious' seedlings were released into a gauze bag (14 x 23 cm; mesh size: 230 μm^2) fixed with hook-and-loop fastener around a shoot with six mature developed leaves. Observations indicated that the females started to oviposit only at the second day, after which they were removed together with the gauze bag. All exposed leaves were individually labelled. Five weeks later, when the leafminer larvae had reached their last larval instar, labelled leaves from an individual shoot were removed and placed together in a transparent plastic box (9.5 x 9 x 8 cm) for observation of adult moth emergence. To keep the leaves under humid conditions, a moist paper was added and replaced daily. All leaves stayed undamaged and without mould until emergence of adult moths. The transparent boxes were checked daily to count emerged leafminer moths. They were removed and used again for rearing purposes. Development time of the leafminer was calculated from the day of oviposition to the day of adult emergence.

For the treatment with *V. inaequalis* inoculation on the four apple genotypes, shoots with six mature developed leaves were sprayed with conidia suspension

(10^5 conidia per ml) to inoculate the whole leaf area. For the first 48 hours, plants were kept in a plastic tent at 18 °C in darkness at a r.h. approaching 100% (Gessler & Stumm, 1984). Subsequently, plants were brought to the greenhouse chamber, and 55 ± 1 hours after inoculation they were infested with leafminers as described above. At the time of transfer of the leaves containing last instar larvae into the transparent plastic boxes, scab lesions were visible on the youngest inoculated leaves of the scab susceptible genotypes 'Gala' and 'Gala-trans0', but not on the scab resistant genotypes 'Gala-transVf' and 'Florina'. Trial design focused on within-treatment effects among the different apple genotypes that were all tested in parallel, while parallel testing of treatments with and without *V. inaequalis* inoculation was not feasible for logistic reasons. Ten plants were used per genotype with one leafminer infested shoot each, either in the absence or presence of *V. inaequalis* inoculation.

Parasitoid performance

Experiments on performance of *P. circumscriptus* were undertaken on the four apple genotypes in the absence or presence of *V. inaequalis* inoculation under the same conditions as described above for its leafmining host. Leafminer infested shoots of the potted plants were exposed to the parasitoid when they contained leafminer larvae in the sap-feeding stage optimal for parasitism (Dutton et al., 2000a). Gauze sleeve cages (20 x 60 cm; mesh size: 230 μm^2) were placed around the shoots with leaves labelled as described under 'herbivore performance'. A two-days-old parasitoid female was released together with a two-days-old parasitoid male into the cage without adding honey and water. Parasitoids were active for a maximum of a day, and they were removed afterwards, together with the gauze sleeve cage. Two and a half weeks later, labelled leaves from an individual shoot were removed and placed together in a transparent plastic box (9.5 x 9 x 8 cm) for observation of adult wasp or adult moth emergence. To keep the leaves under humid conditions, a moist paper was added and replaced daily. All leaves stayed undamaged and without mould until emergence of adult insects. The transparent boxes were checked daily to count emerged parasitoid wasps as well as leafminer moths.

Adult insects were removed and used again for rearing purposes. Development time of parasitoids was calculated as the number of days between the day of oviposition and adult emergence. The trial design focused on within-treatment effects among the different apple genotypes that were all tested in parallel, while parallel testing of treatments with and without *V. inaequalis* inoculation was not feasible for logistic reasons. Parasitism success was determined on the four different apple genotypes either in the absence or presence of *V. inaequalis* inoculation, using ten plants per genotype with one leafminer infested shoot each.

Data analysis

All analyses were performed using JMP 7.0.2 (SAS Institute, Cary, NC, USA). Means and standard deviation (SD) of development time and of number of emerged insects were calculated separately for each of the ten plants per genotype either with or without *V. inaequalis* inoculation. First, data were log transformed (Cuéllar, 1991) to fulfil the assumption of homogeneity of variance (Levene test) and normal distribution (Shapiro Wilk W test). Subsequently, log transformed data were analysed using one-way ANOVA (analysis of variance) followed by Tukey HSD post hoc test, to evaluate the influence of plant genotype on development time and number of emerged insects in the absence or presence of *V. inaequalis* inoculation.

Percentage of parasitism success was calculated separately for each of the ten plants per genotype (either in the absence or presence of *V. inaequalis* inoculation) as the ratio of the number of emerged parasitoid wasps to the total number of emerged insects (leafminer moths and parasitoid wasps). The data set was tested for homogeneity of variance (Levene Test) and normal distribution (Shapiro Wilk W Test), and differences were analysed using one-way ANOVA followed by Tukey HSD post hoc test.

As experiments in the absence or presence of *V. inaequalis* inoculation could not be carried out in parallel, we refrained from direct statistical comparisons.

6.4 Results

Herbivore performance

In the absence of *V. inaequalis* inoculation, mean development time (egg to adult) of the apple leafminer *P. blancardella* did not differ significantly between the classically bred cultivars ‘Gala’ and ‘Florina’ (One-way ANOVA $F = 2.3742$; $df = 3,36$; $P = 0.0863$; Table 6.1.), nor between the cultivar ‘Gala’ and the two transgenic genotypes ‘Gala-trans0’ and ‘Gala-transVf’ (One-way ANOVA $F = 1.9883$; $df = 2,27$; $P = 0.1565$). In contrast, significantly more adults emerged from plants of the cultivar ‘Gala’ than from ‘Florina’, while there was no difference in number of emerged adults between the two transgenic lines and its classically bred isoline ‘Gala’ (Fig. 6.1.a; One-way ANOVA $F = 3.1057$; $df = 3,36$; $P = 0.0385$; Tukey HSD post hoc test).

In the presence of *V. inaequalis* inoculation, mean development time of the apple leafminer did again not differ significantly between the classically bred cultivars ‘Gala’ and ‘Florina’ (One-way ANOVA $F = 0.2544$; $df = 3,36$; $P = 0.8577$; Table 6.1.), nor between the classically bred cultivar ‘Gala’ and the two transgenic genotypes (One-way ANOVA $F = 0.0459$; $df = 2,27$; $P = 0.9552$). The number of emerged moths differed again between the four apple genotypes, and significantly more adults emerged from pathogen inoculated plants of the cultivar ‘Gala’ than from ‘Florina’, while there was no difference between the two transgenic genotypes and their isoline ‘Gala’ (Fig. 6.1.b; One-way ANOVA $F = 7.0378$; $df = 3,36$; $P = 0.0008$; Tukey HSD post hoc test).

Table 6.1. Egg-to-adult development time of the leafminer *P. blancardella* on four different apple genotypes in the absence or presence of *V. inaequalis* inoculation (mean \pm SD calculated from ten independent replications)

Apple genotype	Development time	
	<i>V. inaequalis</i> absent	<i>V. inaequalis</i> present
‘Gala’	47.3 \pm 5.6	39.5 \pm 2.0
‘Gala-trans0’	44.4 \pm 3.1	39.3 \pm 1.5
‘Gala-transVf’	43.2 \pm 5.2	39.5 \pm 1.7
‘Florina’	42.5 \pm 2.3	39.0 \pm 1.2

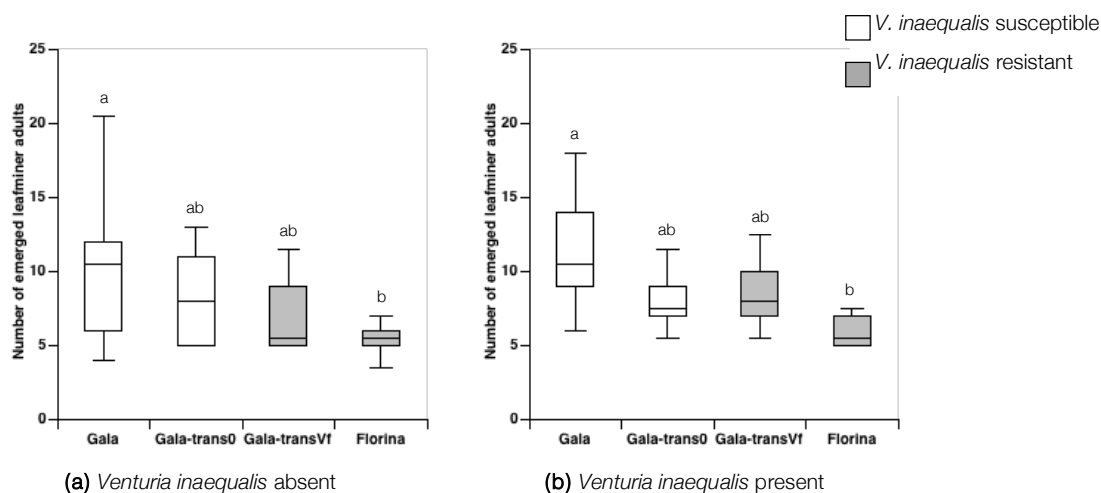


Figure 6.1. Number of emerged adults of the leafminer *P. blaucardella* (means \pm SD of non-transformed data are presented and calculated from ten independent replications) on the four apple genotypes 'Gala', 'Gala-trans0', 'Gala-transVf' and 'Florina' in the (a) absence and (b) presence of *Venturia inaequalis* inoculation. Different letters indicate significant differences (One-way ANOVA of log transformed data followed by Tukey Post hoc test).

Table 6.2. Performance of the parasitoid *P. circumscriptus*: (a) Egg-to-adult development time, (b) number of emerged offspring, and (c) parasitism success on four different apple genotypes in the absence or presence of *V. inaequalis* inoculation (mean \pm SD calculated from ten independent replications)

(a) Development time		
Apple genotype	<i>V. inaequalis</i> absent	<i>V. inaequalis</i> present
'Gala'	24.3 \pm 1.7	26.2 \pm 2.1
'Gala-trans0'	23.7 \pm 1.1	27.2 \pm 1.8
'Gala-transVf'	24.8 \pm 1.3	24.6 \pm 1.5
'Florina'	24.7 \pm 1.2	24.8 \pm 1.7
(b) Number of emerged wasps		
Apple genotype	<i>V. inaequalis</i> absent	<i>V. inaequalis</i> present
'Gala'	11.3 \pm 7.9	9.2 \pm 3.9
'Gala-trans0'	11.9 \pm 7.8	6.8 \pm 4.3
'Gala-transVf'	8.3 \pm 4.0	10.7 \pm 5.5
'Florina'	5.9 \pm 3.4	7.7 \pm 5.1
(c) Parasitism success in %		
Apple genotype	<i>V. inaequalis</i> absent	<i>V. inaequalis</i> present
'Gala'	67.5 \pm 26.5	60.1 \pm 28.2
'Gala-trans0'	76.7 \pm 15.6	56.7 \pm 28.4
'Gala-transVf'	67.6 \pm 29.9	71.7 \pm 27.7
'Florina'	58.0 \pm 21.4	42.4 \pm 28.3

Parasitoid performance

In the absence of pathogen inoculation, mean development time of the parasitoid *P. circumscriptus* from egg to adult did not differ between the four apple genotypes (One-way ANOVA $F = 0.2544$; $df = 3,36$; $P = 0.8577$; Table 6.2.a). Mean numbers of emerged parasitoid wasps on the scab susceptible genotypes 'Gala' and 'Gala-trans0' exceeded mean numbers on the scab resistant genotypes 'Gala-transVF' and 'Florina', but this difference was not statistically significant, and no effect of the apple genotype was evident (One-way ANOVA $F = 1.2684$; $df = 3,36$; $P = 0.2998$; Table 6.2.b).

In the presence of pathogen inoculation, there was again no indication of any indirect effect of the plant genotype on mean development time of the parasitoid (One-way ANOVA $F = 0.2544$; $df = 3,36$; $P = 0.8577$; Table 6.2.a). Further, the number of emerged parasitoids did not differ between the apple genotypes on *V. inaequalis* inoculated leaves (One-way ANOVA $F = 0.8469$; $df = 3,36$; $P = 0.4774$; Table 6.2.b).

Mean values for parasitism success were consistently lowest for the cultivar 'Florina' compared to the other genotypes both in the absence and presence of pathogen inoculation. However, standard deviations in this experiment were relatively high, and differences in parasitism success among the different genotypes were not significant, irrespective of whether or not the apple plants were inoculated with *V. inaequalis* (without pathogen: One-way ANOVA $F = 1.0188$; $df = 3,36$; $P = 0.3957$; with pathogen: One-way ANOVA $F = 1.8342$; $df = 3,36$; $P = 0.1584$; Table 6.2.c).

6.5 Discussion

Multitrophic webs of terrestrial ecosystems are influenced by host plant characteristics determined by plant genotype (Johnson, 2008), which may influence life history traits of herbivores as well as their interactions with natural antagonists (Bottrell et al., 1998; Kahuthia-Gathu et al., 2008; Velten et al., 2008). In particular, a transgenic plant and its isogenic line may differ in their

effects on multitrophic webs, and knowledge of such critical interactions is crucial for risk assessments (Romeis et al., 2006).

The current study focusing on performance of the apple leafminer *P. blancardella* and its parasitoid *P. circumscriptus* used, as a novelty, several plant genotypes that differed in or shared a single trait. They were either susceptible ('Gala' and 'Gala-trans0') or resistant ('Gala-transVf') to apple scab, whereby this latter genotype shared the apple scab resistance gene *HcrVf2* with the cultivar 'Florina'. Our trial design was suitable to disentangle effects of transgenesis and effects of the resistance gene *per se*. An effect of transgenesis will be apparent by comparing 'Gala-transVf' or 'Gala-trans0' with their classically bred isoline 'Gala', and an effect of the apple scab resistance gene by comparing 'Gala' with 'Gala-transVf' and 'Florina'. Our findings document clear genotype effects on leafminer adults egressing from the plant tissue, while all other interactions assessed did not reveal significant differences among genotypes.

Development times of herbivores and parasitoids

The Slow Growth - High Mortality hypothesis predicts that development time is increased for herbivores feeding on plants of suboptimal nutritional composition or with higher contents of defence chemicals (Awmack & Leather, 2002), and that this extension in development time increases the window of exposure to larval parasitoids (Clancy & Price, 1987). Our results document that herbivore development time was not altered by either of these factors, and consistently, parasitoid development time did not differ on hosts feeding on the four plant genotypes. A number of deviating multitrophic effects have been reported from other systems. First, the endophytic herbivore *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae), which damages bean seeds post-harvest, suffers a delay in development on beans containing the natural storage protein arcelin compared to its development on arcelin free beans. However, this plant resistance factor did not exhibit any direct effect on development of the parasitoid *Dinarmus basalis* Rondani (Hymenoptera: Pteromalidae) that is feeding on host haemolymph (Velten et al., 2007), as arcelin remains in the

digestive tract of the host without infiltrating its haemolymph (Paes et al., 2000). Second, parasitoids may respond to slowly developing hosts by an extension of their own development time (Vinson & Iwantsch, 1980), an effect classified as disadvantageous as these parasitoids are longer exposed to secondary parasitoids or predators (Price et al., 1980). Third, faster development of the herbivore holds the potential to increase the number of its generations per year with subsequent increase of herbivory; further, it shortens the window of parasitism opportunity, likely resulting in reduced parasitism success (Bottrell et al., 1998). In the current study, the lacking genotype effect on development times of associated insects rules out the presence of sublethal toxicants to the tested non-target organisms developing in the foliage.

The patterns of insect development times observed in the current study did not differ irrespective of the absence or presence of inoculation with the fungal pathogen *V. inaequalis*. In other systems, fungal infections caused changes in leaf chemistry of infected plants (Johnson et al., 2003; Rostás et al., 2002), with indirect effects on concurrently feeding herbivores (Hatcher, 1995; Omacini et al., 2001; Rostás et al., 2003). A shortening of larval development of the European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) was found as a consequence of an infection with the fungal pathogens *Fusarium graminearum* (Chiang & Wilcoxson, 1961) or *Colletotrichum graminicola* (Carruthers et al., 1986). Furthermore, development time of the grape berry moth *Lobesia botrana* Denis & Schiffermueller (Lepidoptera: Tortricidae) is not only influenced by the grape cultivar (*Vitis vinifera*) (Moreau et al., 2006), but also by the simultaneous presence of the fungal pathogen *Botrytis cinerea* (Mondy & Corio-Costet, 2000, 2004). Development time of the grape berry moth was also shortened when larval diet contained mycelium of *B. cinerea*, but pupal weight was not different from that in cohorts reared without the pathogen (Mondy & Corio-Costet, 2004).

Number of emerged leafminers

The number of emerged *P. blancardella* moths was significantly influenced by the apple genotype, independent of the absence or presence of *V. inaequalis* inoculation. While no evidence was found for an effect of transgenosis or of the resistance gene *per se* on this parameter, an effect of the genotype 'Florina' was apparent, as fewer moths egressed from leafminer infested plants. The cultivar 'Florina' shares the scab resistance gene *HcrVf2* with one of the genotypes tested, 'Gala-transVf', while several other traits may differ from the 'Gala' genotypes. Plant characteristics are expected to be of high relevance for endophytic species such as leafminers that are intimately associated with the plant (Auerbach & Simberloff, 1989). Besides chemical plant constituents, physical plant surface properties may affect herbivore performance, particularly in endophytic species that have to penetrate plant tissue at the beginning and at the end of their development (Velten et al., 2008). In the current system, increased hardness of leaf surface potentially contributes to protect foliage from leafminer damage, as penetration by insects requires stronger forces. Apple plants grown in the greenhouse suffered from higher damage by rosy apple aphid *Dysaphis plantaginea* Passerini (Hemiptera: Aphididae) than those cultivated under field conditions, and such physical effect was proposed to underlie the observed phenomenon (Miñarro & Dapena, 2007). Aphids have to penetrate the leaf tissue with their stylet to reach the phloem for sucking. Interestingly, population dynamics of these aphids significantly differed between the two cultivars 'Florina' and 'Gala', and coincidentally with our results on leafminers, aphid abundance was much lower on 'Florina' compared to 'Gala' after a three weeks observation period (Miñarro & Dapena, 2007). The Tritrophic Level Concept predicts that interactions between two trophic levels will have effects on the third trophic level (Fritz, 1995; Price et al., 1980), and in the case of our system, consequences on number of parasitoids that emerged from the leafminer are of particular interest.

Number of emerged parasitoids and parasitism success

While availability of *Phyllonorycter* hosts of suitable quality is crucial for parasitism success of *Pholetesor* parasitoids (Dutton et al., 2000a; Hagley & Barber, 1986), plant genotypes did not significantly influence the number of emerged parasitoids or parasitism success in the present study. However, irrespective of the absence or presence of inoculation with the pathogen *V. inaequalis*, mean values of parasitism success were consistently lowest on the cultivar 'Florina'. Similar to the herbivore, the parasitoid has to penetrate the leaf surface with the ovipositor, and after completion of its development, as an adult insect again. In analogy to the herbivore, the parasitoid may be impeded by physical characteristics of the leaf surface, although this effect was not significant in the current case.

Neither transgenesis nor the resistance gene *per se* exhibited a measurable effect on number of emerged parasitoids or parasitism success. In another study with *Phyllonorycter* leafminers, plant genotypes of the willow *Salix lasiolepis* affected leafminer survival as well as *Pholetesor* parasitism (Fritz, 1995). Such multitrophic effects can be chemically mediated, as quality and/or quantity of secondary metabolites influencing herbivore performance can also alter higher trophic levels, including survival of certain parasitoid species (Gols et al., 2008).

In conclusion, our findings clearly reveal that the performance of the apple leafminer *P. blancardella* and its parasitoid *P. circumscriptus* are not directly or indirectly affected by the transgenesis, as demonstrated by comparing the two transgenic 'Gala' genotypes to the two classically bred apple cultivars 'Gala' and 'Florina'. Further, the performance patterns of the insects on scab susceptible compared to scab resistant 'Gala' genotype(s) were not altered after plant defence was triggered by *V. inaequalis* inoculation. No increased risks of transgenic plants were recorded in this study.

7 General Discussion

Diversity of host plant genotypes can affect the distribution and abundance of insect herbivores (McIntyre & Whitham, 2003; Stiling & Rossi, 1996), since chemically-mediated trophic interactions are linked with plant-derived volatile compounds. These volatile compounds appear to be relevant for herbivores as attractants or deterrents (Bruce et al., 2005; De Moraes et al., 2001; Hern & Dorn, 2002; Visser, 1986), and function as herbivore-induced indirect plant defence to attract antagonists (Arimura et al., 2005; Dicke & Van Loon, 2000; Mumm et al., 2008; Turlings et al., 1990).

The present study evaluated direct and indirect plant-insect interactions of transgenic apple genotypes ('Gala-transVf', 'Gala-trans0') compared with their isogenic cultivar 'Gala' and the cultivar 'Florina' in a multitrophic apple system. Overall, no negative effects of the transgenic apple genotypes tested could be detected regarding influence on plant-insect interactions in the absence or presence of the fungal pathogen *V. inaequalis*, however, significant differences between the two classically bred cultivars were revealed.

Transgenic plants

Disease and pest resistant (Ammann, 2008; Bale et al., 2008), herbicide tolerant (Devos et al., 2008; Graef, 2009), as well as stress tolerant (Lal et al., 2008; Wu et al., 2009) transgenic plants have been developed in the past. To avoid unwanted side effects on non-target organisms, administrative instructions and regulations claim that products from transgenic plants have to be as safe as products from conventionally bred plants (Kier & Petrick, 2008). Within the European Community, for example, the release of transgenic plants is defined and regulated (Anonymous, 2001) based on risk assessments (Anonymous, 2006). Suitable case-by-case studies, which are in accordance with environmental risk assessments of conventional pesticides, were developed for *Bt*-plants by an international initiative (Romeis et al., 2008), particularly because *Bt*-plants express δ -endotoxins, which are also used as active agents in *Bt*-insecticides. Furthermore, cultivation of *Bt*-plants provides evidence of a

reduction in environmentally undesirable side effects on arthropod abundance in contrast to application of broad-spectrum insecticides (Marvier et al., 2007; Rauschen et al., 2008).

Plant transformation with target gene(s) using a constitutive promoter and a selectable marker gene is not restricted to natural limitations given in classical breeding, although genetic resources can also be found within crossable gene-donors, i.e. in wild species.

For apple, the centre of genetic diversity is in Asia (Büttner et al., 2004; Phipps et al., 1990; Zhou, 1999) and wild apple species are screened for their traits to identify potential compatible donors of resistance to biotic or abiotic factors (Büttner et al., 2004). A gene source for apple scab resistance was found in the wild species *Malus floribunda* 821. A crossing process of *M. floribunda* 821 and 'Rome Beauty' (*Malus x domestica*) at the 'Institut National de Recherche Agronomique' (INRA, Angers, France) resulted in the scab resistant apple cultivar 'Florina' (Crosby et al., 1992). The popular apple cultivar 'Gala' derives from a crossing process of 'Kidd's Orange Red' (*M. x domestica*) and 'Golden Delicious' (*M. x domestica*), and is known for its susceptibility to apple scab. One aim would be to combine high consumer acceptance of a well-known apple cultivar with resistance to an important, worldwide occurring fungal pathogen, *V. inaequalis*. This could be achieved by using biotechnological methods, i.e. improving the susceptible cultivar 'Gala' with an apple-derived target gene conferring apple scab resistance (Belfanti et al., 2004; Szankowski et al., 2009). Such a transgenic scab resistant 'Gala' genotype was successfully achieved with 'Gala-transVf'. Whether, and to which degree this genotype poses an effect to non-target organisms was evaluated in a multitrophic apple system, involving in particular, the leafminer *P. blancardella* and its parasitoid *P. circumscriptus*. Relevant additional plant genotypes differing in, or sharing a single trait were included for comparison.

The multitrophic apple system

Herbivores and their natural antagonists use plant-derived chemicals before landing to locate their suitable host (Dorn et al., 1999; Hern & Dorn, 2004; Vallat & Dorn, 2005). Modification of the host plant genotype might lead to altered plant volatile emission and to an alteration of plant-insect relationship. Therefore, the composition of headspace volatiles of healthy, *V. inaequalis* inoculated, *P. blancardella* infested, and simultaneously *V. inaequalis* inoculated and *P. blancardella* infested apple shoots of the four apple genotypes was chemically analysed and quantified. Differences in the emission of four terpenes and an aromatic compound between the apple genotypes for healthy and *P. blancardella* infested apple shoots were detected. The transgenic scab resistant genotype 'Gala-transVf' emitted quantitatively lower amounts than the two control cultivars 'Gala' and 'Florina', though the emitted volatiles were in the range of the two control cultivars. Moreover, quantitative differences in volatile emission between 'Gala' and 'Florina' were found, which is concurrent with results previously and similar results are reported for phenolic contents of apple leaves of these two cultivars (Kindt et al., 2007).

After successful host habitat location and landing on the leafminer infested apple plant, antagonists of concealed living hosts such as *Pholetesor* parasitoids rely on the leafminer-induced contact chemical squalene to find their host larvae for successful oviposition (Dutton et al., 2000b). Chemical analyses of extracted apple leaves emphasised the difference between the two cultivars 'Gala' and 'Florina'. Higher squalene contents in leafminer infested 'Florina' leaf extracts elicited a more intensive ovipositional probing behaviour of female parasitoids than leafminer infested leaf extracts with lower squalene contents of the 'Gala' lines. This indicates that 'Florina' provides more chemical information for the parasitoid to search and locate its host. However, parasitoids were able to parasitize and develop successfully in all four apple genotypes under the conditions of the trial. In fact, the parasitoids were in a no choice situation with caged plants of one genotype each, leaving no alternative option for females ready to oviposit. The results suggest either a random search by the parasitoids

on the three 'Gala' genotypes, or the use of cues other than squalene after landing. These cues can be of chemical, visual and/or mechanosensory origin (Godfray, 1994). Time parasitoids spent with host location is unknown, and it cannot be excluded that the parasitoids used more time to locate their host on genotypes with low squalene contents in the mine. Searching time may be critical to parasitoids under field conditions, particularly when they are time-limited.

Irrespective of the absence or presence of *V. inaequalis* under controlled conditions, concurrent inoculation and infestation of plants could lead to faster development of insect-herbivores (Carruthers et al., 1986; Chiang & Wilcoxson, 1961; Mondy & Corio-Costet, 2000), because of changes in nutrient availability and accumulation of secondary metabolites (Treutter, 2005, 2006). These changes could result in a stimulus for faster herbivore development and therefore reduce larval mortality.

Tritrophic systems and risk assessment

When dealing with transgenic plants it is important to prevent possible direct and indirect, immediate or delayed risks to the ecosystem. Therefore suitable risk assessment evaluations have to be conducted (Romeis et al., 2008). Since possibilities to reproduce ecosystems under laboratory conditions are limited, adequate and appropriate model systems have to be used. Ideally, these systems should be composed of at least three levels, following the Tritrophic Level Concept, which predicts that interactions between two trophic levels will have effects on the third trophic level (Fritz, 1995; Hare, 1992; Price et al., 1980). For example, alterations in herbivory on *Bt*-plants result in lower inducible emissions of volatiles, which are used by non-target natural antagonists of these herbivores (Dean & De Moraes, 2006). To understand possible alterations in plant volatile emissions, the underlying biochemical and molecular mechanisms of plants' response to herbivory have to be elucidated as well as parasitoids' response to individual volatile compounds (Girling et al., 2008). The triterpene squalene was identified to play a crucial role in host location behaviour of *Pholetesor* parasitoids (Dutton et al., 2002; Dutton et al., 2000b), and contents

of this contact chemical were increased in apple leaves of 'Golden Delicious' seedlings infested with *Phyllonorycter* leafminers (Dutton et al., 2002; Dutton et al., 2000b) as well as in apple leaves of the cultivar 'Florina' infested with *Phyllonorycter* leafminers. Combined chemical analyses and behavioural observations in the multitrophic apple system emphasised the importance of squalene to elicit a specific response in parasitoid behaviour, and furthermore these findings reveal chemical differences with impact on higher trophic levels between the two conventionally bred apple cultivars 'Gala' and 'Florina', whereas no differences between these two cultivars and the two transgenic apple genotypes 'Gala-transVf' and 'Gala-trans0' could be detected.

Conclusions and outlook

In conclusion, results show that the tested apple genotypes vary in their chemical composition in the active vegetative growing stage when they are healthy, subjected to pathogen inoculation or herbivore infestation, with implications for herbivore performance and higher trophic organisms, i.e. natural antagonists of the herbivore. To identify possible subtle side effects of transgenic host plant genotypes on non-target organisms it is highly desirable to include all available relevant controls into risk assessment studies. The four apple genotypes tested differed in or shared a single trait, and therefore possible implications of a single trait in a multitrophic context were identifiable. Remarkably, no differences were detected between the transgenic genotypes 'Gala-transVf' and 'Gala-trans0' and the classically bred cultivars 'Gala' and 'Florina', but between the two cultivars 'Gala' and 'Florina'.

These two apple cultivars differ in many aspects. Well-known is their difference in the apple scab resistance gene *HcrVf2* that is lacking in 'Gala'. The current study demonstrates that they differ also chemically in their emission of terpenes. Since terpenes are known to be crucial for plant-insect interactions, the question arises about the relationship between pathogen resistance and terpene emission. To clarify this question, transgenic apple plants with inhibited terpene biosynthesis have to be developed in order to conduct appropriate experiments. In a first step, development of a fungal pathogen on transgenic apple plants

without terpene emission and appropriate non-transgenic control apple plants with terpene emission should be recorded to gain insight into the importance of terpenes in plant-pathogen interactions. Further, preference of herbivore insects and their parasitoids for either transgenic apple plants without terpene emission or for control apple plants with terpene emission in the absence or presence of pathogen inoculation could be determined. Additional chemical analyses of emitted headspace volatiles and of leaf surface extracts would complete the investigation to be made on higher trophic levels.

Since 'Gala' and 'Florina' are widely planted in the field, existing differences between these two cultivars could be studied under natural conditions. Plant-derived cues, which are influenced by abiotic and biotic factors, could affect plant-herbivore interactions. However, since leafminer larvae of *P. blancardella* cannot change their host plant, they rely on their mothers' preference for egg deposition, and they must have adapted to overcome or to resist plant defence mechanisms to complete their larval development inside the plant tissue. Influences on herbivore performance are also directly linked to parasitoid abundance. The two insect organisms, the apple leafminer *P. blancardella* and its parasitoid *P. circumscriptus*, are evidently suitable insect species to reveal even slight differences in the characteristics of their apple host plant.

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