Latex Secondary Metabolites in the Common Dandelion

- Their Composition, Function and Evolution in Root Herbivore Defense -

Dissertation

To Fulfill the Requirements for the Degree of

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Submitted to the Council of the Faculty of Biology and Pharmacy

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Gutachter:

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Tag der öffentlichen Disputation:

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1. INTRODUCTION

As primary producers, plants need to defend their tissues against heterotrophic organisms. The sessile life style of plants restricts the physical escape from attackers, resulting in the evolution of a rich variety of defense mechanisms (Dixon & Strack 2003; Mithöfer & Boland 2012). Plant defenses are categorized into indirect and direct defenses (War *et al.* 2012). Indirect defenses involve natural enemies of the herbivores that consume, parasitize or fend off plant consumers (Kessler & Baldwin 2001; Heil 2008). Direct defenses incorporate physical barriers, such as trichomes, spines, and waxy or lignified surfaces, as well as chemical barriers, such as deterrent or toxic chemicals, that directly reduce feeding damage. These chemicals – also referred to as plant secondary metabolites (PSMs), natural products or specialized metabolites – are usually not directly needed for the immediate growth and development of plants, but mediate the interaction of plants with their abiotic and biotic environment (Kossel 1891; Rosenthal 1982; Rosenthal 1991; Hartmann 2007). To date, more than 200,000 individual PSMs have been identified (Dixon & Strack 2003). Apart from the diversity of chemical structures, expression levels of PSMs also greatly differ between and within species (Agrawal *et al.* 2012a; Bernhardsson *et al.* 2013). The extensive variability in the composition and abundance of PSMs has fascinated ecologists and evolutionary biologists alike, and raised an intriguing question:

Why is there so much variation in plant secondary metabolism?

1.1 Function and evolution of plant secondary metabolites

The elucidation of a metabolite's function is a first step to understand the diversification of plant secondary metabolism. During the last two centuries, various hypotheses for the function and diversity of PSMs have been formulated (Haslam 1994; Jenke-Kodama, Müller & Dittmann 2008):

- **Secondary metabolites are side or waste products of the primary metabolism.** First stated in the late 19th century (Sachs 1873), this hypothesis was among the first to explain the occurrence of PSMs, and it remained popular until the 1970s (Peach 1950). The hypothesis lost support during the second half of the 20th century, when a plethora of research demonstrated that secondary metabolites can serve important ecological functions.
- **PSMs are side products of imperfect multi-product enzymes.** Multi-product enzymes are well-known in many PSM pathways (Kushiro *et al.* 2000). As the biological function of all products is often not clear, it was hypothesized that many PSMs are waste products of imperfect enzymes (Zulak & Bohlmann 2010; Moore *et al.* 2014).
- **PSM diversity arises through evolutionary load** (Davies 1990). This hypothesis states that metabolites once had a function during the evolution of the plant, but that the selective force

driving their production has now vanished. However, the metabolites are still produced, since costs of production are not sufficiently large to eliminate the pathway.

- **Screening hypothesis**. PSM diversity serves as a pre-adaptation to future stress (Jones, Firn & Malcolm 1991). A PSM may not have a function at the moment, but plants producing a wide range of PSMs may respond better to future stress. This hypothesis has been questioned because secondary metabolism, assumed to incur metabolic costs, would not be expected to be evolutionarily stable without a significant function for the end product.
- **Synergism.** Several metabolites together exert a stronger effect than the sum of the individual metabolites (Steppuhn & Baldwin 2007).
- **PSMs serve ecological functions.** Since the middle of the 20th century, a plethora of studies has demonstrated that PSMs are involved in the interaction of plants with their abiotic and biotic environment (Fraenkel 1959; Ehrlich & Raven 1964; Baba & Schneewind 1998).

Although it remains a major challenge to assess the validity of these hypotheses simultaneously, much progress has been achieved in deciphering the ecological functions of certain PSM groups. PSMs are involved in the interaction with the abiotic environment: they can alleviate temperature, UV, salt and water stress, and can facilitate nutrient acquisition (Ramakrishna & Ravishankar 2011). Furthermore, many PSMs mediate biotic interactions including plant-plant signaling (Karban *et al.* 2000; Baldwin *et al.* 2006), mutualistic or commensalistic interactions with pollinators (Kessler, Gase & Baldwin 2008), parasitoids (McCormick *et al.* 2014) and microbes (Bais *et al.* 2006; Mandal, Chakraborty & Dey 2010), as well as antagonistic interactions with competing plants (Bertin, Yang & Weston 2003), pathogens (Sarwar *et al.* 1998; Chitwood 2002) or herbivores (Mithöfer & Boland 2012). These functions are by no means exclusive: metabolites may be involved in interactions with both abiotic and biotic stresses, as was shown for volatile isoprenoids (Vickers *et al.* 2009). Moreover, PSMs can mediate the interaction between different biotic stresses: many defensive PSMs may affect both herbivores and pathogens (Schmidt 1999; Biere, Marak & van Damme 2004; Ahmad *et al.* 2011; Stotz *et al.* 2011). Even metabolites that interfere with specific targets of herbivore physiology such as cardenolides also reduce the performance of pathogens (Jacobsohn & Jacobsohn 1985; Akhtar *et al.* 1992; Agrawal *et al.* 2012b). Due to the multiple functions of individual metabolites, it remains a major challenge to elucidate which environmental stress imposes the strongest selection pressure on a metabolite.

During the last century, a great deal of research investigated the role of PSMs in herbivore defense. Ernst Stahl was among the first who experimentally showed that PSMs are defensive (Stahl 1888). This gave rise to a vast body of evidence demonstrating that many major classes of PSMs deter herbivores and/or exert toxic or anti-nutritive effects on plant feeders (Mithöfer & Boland 2012). Based on the deterrent and toxic properties of these chemicals and the ubiquitous presence of heterotrophic organisms (Gilbert *et al.* 1979; Bernays 1998), it was hypothesized that herbivores are major drivers for the evolution of PSMs (co-evolution hypothesis) (Ehrlich & Raven 1964). Alternatively, it was argued

that while the evolution of herbivores may be greatly affected by PSMs (Dobler *et al.* 2012; Zhen *et al.* 2012), herbivores do not impose a strong selection pressure on plant chemistry. Instead, PSMs may interact with other ecological factors that impose stronger selection pressure than plant feeders. Thus, herbivores would follow the evolution of plant chemicals without exerting a major evolutionary feedback on PSMs (sequential evolution hypothesis) (Jermy 1976; Jermy 1984). The two hypotheses mainly differ in regard to the evolutionary driver for PSMs: in the co-evolution hypothesis, biotic stresses are considered to drive the evolution of plant chemicals, whereas in the sequential evolution hypothesis factors other than biotic stresses shape PSM diversity. Thus, to investigate these two hypotheses, the agent imposing selection on PSMs must be identified. The establishment of a direct link between the strength of selection pressure, trait variation, and plant fitness would greatly facilitate the elucidation of the selective forces.

1.2 Below ground herbivores

In contrast to the phyllosphere, little is known about the ecology and evolution of PSMs in the rhizosphere. The diversity and concentration of below ground chemicals rivals the ones above ground, and many PSMs that are defensive above ground are also found in below ground tissues (Rasmann & Agrawal 2008; van Dam 2009). Due to the distinct abiotic and biotic environments of the phyllo- and rhizosphere, PSMs in shoots and roots likely evolve under different selection regimes. Putative evolutionary forces below ground include nutrient shortages (Ramakrishna & Ravishankar 2011), the presence of competing plants (Bertin, Yang & Weston 2003), symbionts (Bais *et al.* 2006; Mandal, Chakraborty & Dey 2010), soil-borne pathogens (Sarwar *et al.* 1998; Chitwood 2002) and herbivores (Rasmann & Agrawal 2008; van Dam 2009).

Relatively few insect species, from only six out of 26 insect orders for the most part, exhibit below ground feeding habits (Brown & Gange 1990). Despite this restricted phylogenetic occurrence, below ground herbivores are prevalent in many temperate ecosystems and can severely reduce plant fitness in both natural and agricultural ecosystems (Andersen 1987; Blossey & Hunt-Joshi 2003; Saito, Hirai & Way 2005; Morris *et al.* 2007; Gray *et al.* 2009; Johnson *et al.* 2012). As a result of their agricultural importance, the role of PSMs in below ground herbivore defense has been investigated mainly in crop plants (Cole 1987; Schmelz *et al.* 2002; Nutt, O'Shea & Allsopp 2004; Clark, Hartley & Johnson 2011; Johnson *et al.* 2011; Robert *et al.* 2012), and little is known about the ecology and evolution of PSMs in natural systems below ground. One of the few studies that investigated the evolution of below ground chemicals compared root alkaloid concentrations between one mainland and two island *Eschscholzia californica* (Papaveraceae) populations (Watts, Dodson & Reichman 2011), suggesting that the absence of pocket gopher herbivory on the island populations resulted in the evolutionary decline of root alkaloid concentrations of the host plant. Given the massive impact of root herbivores on terrestrial ecosystems and crop production, the role of below ground feeding insects in driving the evolution of PSMs warrants further attention.

Below ground feeding insects differ from above ground herbivores not only in their identity, but also in their distribution, physiology and behavior (Brown & Gange 1990). The surrounding matrix, the soil, differs dramatically in its properties compared to the air. Movements through the soil demand more energy than movements above ground. Temperature and light, two abiotic factors that modulate the behavior of insects, are much more homogenously distributed over the day and over the season below than above ground. Although the soil may protect below ground feeding insects from many predators, the soil is rich in pathogenic bacteria. Apart from environmental differences between the phyllo- and rhizosphere, shoot and roots also differ as a food source (van Dam 2009). Roots are considered as nutritionally inferior to shoots (van Dam 2009), which may have forced many below ground feeding insects to have longer life cycles than above ground herbivores (Brown & Gange 1990). Taken together, these factors highlight that below ground feeding insects may impose different selection pressure on PSMs than above ground herbivores. Thus, below ground systems offer an additional opportunity to evaluate the importance of biotic factors to shape variation in plant chemical defenses.

1.3 Specialized defense reservoirs minimize costs and maximize benefits of plant secondary metabolites

Assuming that PSMs are under positive selection, plants should evolve an unlimited diversity and abundance of PSMs over evolutionary time. As species only contain a small subset of the total diversity of PSMs, and since conspecifics differ in the expression of defensive metabolites, evolution of PSMs appears to be restricted. Among the most popular evolutionary explanation for the emergence, maintenance and disappearance of a trait is the balance between costs and benefits: the expression of a trait is advantageous in certain environments, but disadvantageous in others. Potential disadvantages of PSMs include:

Costs: The production of secondary metabolites is assumed to be costly, although this assumption is highly controversial (Bergelson 1994; Bergelson & Purrington 1996; Almeida-Cortez, Shipley & Arnason 1999; Koricheva 2002; Paul-Victor *et al.* 2010; Züst *et al.* 2011). Costs include direct costs, caused by the production of the metabolite, as well as indirect costs, originating for example from decreased interactions with mutualists (Strauss *et al.* 2002). Both direct and indirect costs can be reduced by

a. Production of the metabolites only when needed, i.e. their induction upon herbivore attack (Bennett & Wallsgrove 1994; Zhao, Davis & Verpoorte 2005).
b. Storage of the metabolites predominantly in cells that come in close contact with the target (Kojima *et al.* 1979; Agrawal & Konno 2009).

Self-intoxication: The metabolites might harm the plant itself (Baldwin & Callahan 1993; Gog *et al.* 2005; Sirikantaramas, Yamazaki & Saito 2008). Self-intoxication can be alleviated by

a. Storage of the metabolites as non-toxic protoxins that are only activated upon attack (Bones & Rossiter 1996; Jones & Vogt 2001).
b. Storage of metabolites in separate intracellular compartments or in specialized cells and tissues (Levin 1973; Dell & McComb 1979; Fahn 1988; Wink 1993; Agrawal & Konno 2009).
c. Evolution of insensitivity to the plant's own PSMs (Rosenthal 1991).
d. Evolution of efficient detoxification systems (Takanashi *et al.* 2008).
e. Targeting molecules that do not occur in plants (Agrawal *et al.* 2012b).

The formation of specialized reservoirs can help plants to overcome both costs and self-intoxication of PSMs. Indeed, many plants produce specialized cells such as glandular trichomes, resin ducts and laticifers, which store high concentrations of secondary metabolites (Fahn 1988).

1.3.1 Laticifers

Laticifers are among the most common defensive reservoirs in plants, being produced by almost 10% of all flowering plants (Metcalfe 1967; Farrell, Dussourd & Mitter 1991; Lewinsohn 1991). Laticifers are defined as elongated cells with a particular anatomy, development and cellular origin (Hagel, Yeung & Facchini 2008). Non-articulated laticifers are extremely elongated, multinuclear, single cells that often branch and spread through the plant tissue, but never reconnect (Dussourd & Denno 1991; Hagel, Yeung & Facchini 2008; Pickard 2008). They are typical laticifers of the milkweeds (*Asclepias* spp.). Articulated laticifers have multicellular origins and often connect with other laticifers by perforations in the cell wall (Hagel, Yeung & Facchini 2008). These interconnected chains of laticifers tend to deliver more latex than non-articulated laticifers. Laticifers of this type are typical for the Asteraceae. More than 40 distantly related families in the monocots and dicots produce laticifers. The large phylogenetic distances between laticifer-producing plants and the different cellular organizations suggest multiple evolutionary origins of these defensive reservoirs (Agrawal & Konno 2009). As laticifer-producing lineages are more species-rich than non-laticifer containing sister clades, it was suggested that laticifers are key innovations that spurred the evolution of Angiosperms (Farrell et al. 1991).

The often milky cytoplasm of laticifers is called latex. Latex typically consists of an emulsion of various highly concentrated secondary metabolites. One of the first experiments showing that latex is important for plant resistance against herbivores was conducted by Kniep (1904). He demonstrated that slugs readily consumed leaves that were drained of latex beforehand, whereas non-treated leaves were rejected. To date, a large body of evidence supports that latex has no function in primary metabolism but is involved in herbivore defense (Farrell, Dussourd & Mitter 1991; Mahlberg 1993; Hunter 1994; Agrawal & Konno 2009). Zalucky and colleagues showed that depressurizing the laticifers of

milkweeds (*Asclepias* spp.) improved the fitness of caterpillars of the specialist herbivore *Danaus plexicppus* (Zalucki & Brower 1992; Zalucki & Malcolm 1999; Zalucki, Brower & Alonso 2001). Furthermore, addition of latex to artificial diet deterred herbivorous insects (Huang, McAuslane & Nuessly 2003; Chow, Akhtar & Isman 2005; Sethi *et al.* 2008). Konno *et al.* (2004) demonstrated that cysteine proteases present in the latex of the papaya tree (*Carica papaya*, Caricaceae) reduced larval growth and protected plants from consumption by three oligophagous and polyphagous lepidopteran larvae. Furthermore, many insect herbivores that have specialized on latex-bearing plants evolved strategies to circumvent exposure to latex by depressurizing and draining laticifer cells (Dillon, Lowrie & McKey 1983; Dussourd & Eisner 1987). Surprisingly however, despite the overwhelming evidence for latex as a defense, the fitness benefits of latex production for plants remain unclear (Agrawal 2005; Agrawal & Konno 2009)**.**

1.4 Study species

1.4.1 Taraxacum officinale

Taraxacum officinale agg. (Flora Helvetica, 5th edition) (Asteraceae) is one of the dominant plant species in European grasslands. It produces latex in almost all of its organs especially its roots. The strong tap root is particularly important for *T. officinale*, as the plant relies heavily on it for re-sprouting in spring. Roots have a bitter taste, and are used as a natural remedy against gastrointestinal ailments. Major constituents in roots include inulin (linear β-2,1 fructane), phenolics such as chicoric acid and flavonoids, sesquiterpenes and triterpenes (Schutz, Carle & Schieber 2006). However, detailed information about the composition and abundance of secondary metabolites in the latex is lacking. It is thus not clear which of the above mentioned compounds primarily occur in the specialized laticifer cells. Furthermore, the ecological role of the latex secondary metabolites in *T. officinale* remains unknown.

Figure 1 Exuding latex from *T. officinale* roots. Picture: M. Huber

Taraxacum officinale is native to Eurasia, but has recently gained cosmopolitan distribution due to human activities. The taxon is described as a species complex with approximately 10% diploids, 90% triploids and <1% tetraploids (Menken, Smit & DenNijs 1995; Verduijn, Van Dijk & Van Damme 2004). Diploids of *T. officinale* are usually self-incompatible (Verduijn, Van Dijk & Van Damme 2004), but self-fertilization exceptionally occurs (Warmke 1944), often due to a break-down of the self-incompatible system due to the sterile pollen of polyploids (Morita, Sterk & Den Nijs 1990; Tas & Van Dijk 1999). Polyploid *T. officinale* are asexual apomicts that form seeds without the influence of a pollen tube (diplosporic gametophytic apomixis of the *Taraxacum* type) (Richards 1973). Thus, the offspring of triploid and tetraploid individuals are identical to the mother plant. Diploids are mainly

distributed in central and southern Europe, as well as in glacial refuge areas. Triploids occur in central and northern Europe, and have recently spread across the globe. A *T. officinale* population usually consists of several clones (Mogie & Ford 1988). For example, in a survey in North America using electrophoretic analysis for three enzyme systems combined with seed color, the number of independent clones per location ranged from 1-13 (Lyman & Ellstrand 1984). In English meadows, 20-30 *T. officinale* clones commonly co-occur (Richards 1986). The different ploidy levels make *T. officinale* an ideal study system to investigate how differences in the reproductive system can affect the evolutionary response to a selection pressure.

1.4.2 Melolontha melolontha

In 1478/79, the Bishop of Lausanne was standing on a field in canton Bern (Switzerland), proclaiming (Keller 1984):

„Du unvernünftig, unvollkommene Kreatur, du Inger! Deines Geschlecht ist nicht gewesen in der Arche Noah. Im Namen meines gnädigen Herren und Bischofs von Lausanne gebeut ich euch, allen und jeden, in den nächsten 6 Tagen zu weichen von allen Orten, an denen wächst und entspringt Nahrung für Mensch und Vieh!“

[„May bug, you unreasonable, defective creature! You as a species were not present in Noah’s ark! On behalf of my merciful Lord and as a bishop of Lausanne, I demand you, each and every one of you, to leave within 6 days all places where food for man and livestock grows.”] [English translation, M. Huber]

Figure 2 Excommunication of *M. melolontha* by the Bishop of Lausanne in 1478/79. Picture from http://homepage.hispeed.ch/bellwald/Lehr/abb3.giff

The creatures the bishop was referring to are the larvae of the common cockchafer (May bug), *Melolontha melolontha* (Coleoptera: Scarabeidae). This insect is native to and widely distributed in Europe, except for the most southern and northern parts of the continent. For three years, *M. melolontha* larvae feed in the top layers of the soil on roots of plants. *Melolontha melolontha* larvae are rather mobile: the mean distance of first instar larvae to their hatching site was found to be 45 cm and that of the second instar larvae 2 m, and third instar larvae can move 10-20 cm a day (Hasler 1986; Keller *et al.* 1986). In the fourth year, the adult beetles emerge from the soil and mate above ground on nearby trees. The female lays 10-20 eggs close to the emergence site (Huiting *et al.* 2006; Pener 2013), resulting in relatively stable geographic distribution patterns. It is not yet clear which factors affect the site of oviposition most: heat emission appears to have strongest effect on female oviposition choice (Hasler 1986), but high densities of *T. officinale* may also attract ovipositing females (Hauss & Schütte 1978).

Figure 3 *M. melololontha* feeding on *T. officinale* roots. Picture from M. Huber.

Melolontha melolontha is commonly referred to as polyphagous, but the larval stages differ in their preferred food source (Hauss & Schütte 1976). In the first instar, the white grubs mainly feed on grasses. These larvae also feed on *T. officinale* when they have no other choice, but they lose weight and have increased mortality after 14 days of feeding (Hauss & Schütte 1976). In the second and especially in the third instar, *M. melolontha* larvae prefer to feed on *T. officinale* (Hauss & Schütte 1976). Due to their polyphagous feeding habit and their high consumption rate, *M. melolontha* has been a major pest for diverse crops. Intensification of agriculture, as well as biological control with entomopathogenic fungi of the genus *Beauveria* (Zimmermann 1992; Keller *et al.* 1997; Enkerli, Widmer & Keller 2004) has resulted in a severe decrease in the abundance of this species. However, *M. melolontha* has remained an important pest in certain regions. During recent decades, the distribution of the species has been monitored closely in central Europe (Heer 1841; Keller *et al.* 1986; Rezbanyai-Reser 1986; Fröschle 1996; Cate 2004; Keller 2004). The presence of detailed distribution maps, the fidelity of *M. melolontha* females to lay their eggs close to the emergence site, and the devastating effect of these larvae on plant performance offer an ideal system to study how variation in root herbivore abundance drives the defensive chemistry of their host plants.

1.5 Thesis outline

Studying the interaction between *T. officinale* and *M. melolontha* holds great promise to elucidate the function and evolution of PSMs below ground. For this doctoral thesis, I aimed to **(1)** synthesize previous knowledge about how root secondary metabolites affect insect performance, **(2)** establish an ecological and evolutionary root-herbivore system to study the effects of below ground herbivory on the evolution of root chemicals, and **(3)** provide an experimental link between the defensive function of a plant secondary metabolite, its environment-dependent fitness costs and benefits, and its herbivore-driven geographic variation in natural populations. These three objectives are addressed in the following chapters:

In **chapter 1** we review the literature about the role of plant primary and secondary metabolites in root herbivore behavior, nutrition and physiology. In this chapter, we aim to specifically identify how below ground feeding insects may have adapted their behavior and physiology to the conditions in the rhizosphere.

In **chapter 2** we describe the composition, quantities, spatiotemporal distribution and genetic variation of the major latex secondary metabolites in *T. officinale*. This chapter describes the basic methods to quantitatively analyze variation in latex secondary metabolites in *T. officinale*.

In **chapter 3** we investigated the function of the latex-derived sesquiterpene lactone taraxinic acid β-D glucopyranosyl ester for below ground defense against *M. melolontha*. This chapter provides an ecological and evolutionary frame work for herbivore-driven natural selection.

In **chapter 4** we tested whether *M. melolontha* shapes variation in latex chemistry in natural *T. officinale* populations. This chapter analyzes the importance of root herbivores for the evolution of defensive chemistry in nature.

In the last section of the thesis I discuss the implications of our findings in the context of plant-herbivore interactions and evolution of PSMs.

2. OVERVIEW OF MANUSCRIPTS

2.1 Manuscript I

The role of primary and secondary plant metabolites in root-herbivore behaviour, nutrition and physiology

Matthias Erb, Meret Huber, Christelle A.M. Robert, Abigail P. Ferrieri, Ricardo A.R. Marchado, Carla C.M. Arce

Advances in Insect Physiology (2013)

Summary

Root feeding insects encounter different environmental and nutritional conditions than above ground feeding insects. Here, we review how root herbivores may have adapted their physiology and behavior to deal with root primary and secondary metabolites. The sensory system of root feeding insects allows these herbivores to respond specifically to various root chemicals. Evidence for direct toxic effects of secondary metabolites remains scarce. Using physiological and ecological relevant conditions to study root herbivory could further improve our understanding for processes below ground.

Author contributions

Conceived project: ME

Wrote the manuscript: ME, MH (30%), CAMR, APF, RARM, CCMA

2.2 Manuscript II

Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (*Taraxacum officinale* agg.)

Meret Huber, Daniella Triebwasser-Freese, Michael Reichelt, Sven Heiling, Christian Paetz, Jima N. Chandra, Stefan Bartram, Bernd Schneider, Jonathan Gershenzon, Matthias Erb

Phytochemistry (2015)

Summary

While the chemical constituents of *T. officinale* roots, leaves and flowers have been studied in detail, it remains unclear which metabolites accumulate in the specialized laticifer cells. We found that the latex of *T. officinale* is dominated by three classes of secondary metabolites: phenolic inositol esters, triterpene acetates and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester. The three metabolite classes occurred at high concentrations, each of them accounting for 5-7% of the latex fresh mass. Latex secondary metabolite concentrations increased with plant age. Highest concentrations were found in the latex of the main roots. Phenolic inositol esters differed both quantitatively and qualitatively between three *T. officinale* genotypes, whereas triterpene acetates and taraxinic acid β-D-glucopyranosyl ester differed only quantitatively. Bioassays with the root herbivore generalist *Diabrotica balteata* suggest a defensive function of these metabolites.

Author contributions

Conceived project: ME, MH

Designed experiments: MH (80%), ME

Conducted experiments: MH (80%), DTF, MR, SH, CP, JNC, SB, BS

Performed data analysis: MH (70%), DTF, MR, SH, CP, BS

Wrote the manuscript: MH (70%), ME

2.3 Manuscript III

A latex metabolite increases plant fitness upon root herbivore attack

Meret Huber, Janina Epping, Christian Schulze Gronover, Julia Fricke, Zohra Aziz, Theo Brillatz, Michael Swyers, Tobias G. Köllner, Heiko Vogel, Almuth Hammerbacher, Daniella Triebwasser-Freese, Christelle A. M. Robert, Koen Verhoeven, Veronica Preite, Jonathan Gershenzon, Matthias Erb

Submitted to Plos Biology (May 2015)

Summary

Plants produce a tremendous diversity of secondary metabolites, which deter or intoxicate phytophagous insects. Although these chemicals are generally assumed to be defensive, it remains a major challenge to demonstrate herbivore-dependent fitness advantages of secondary metabolites, especially below ground. Here, we tested whether latex secondary metabolites of the common dandelion (*Taraxacum officinale*) decrease root herbivore performance and improves plant fitness upon attack of its major native root herbivore, the common cockchafer (*Melolontha melolontha*). Using a combination of phytochemical manipulation, genetic modification and field experiments, we demonstrate that the latex-derived sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester deters *M. melolontha* feeding and thereby directly protects root and improves plant fitness. Taken together, our study demonstrates that a latex secondary metabolite benefits the plant under root herbivore attack, thus providing an evolutionary frame work for herbivore-mediated selection of chemical defenses in plants.

Author contributions

Conceived project: ME, MH

Designed experiments: MH (80%), ME, TGK, CSG

Conducted experiments: MH (70%), JE, CSG, JF, ZA, TB, MS, TGK, HV, AH, DTF, CAMR, KV, VP, JG, ME

Performed data analysis: MH (90%), TGK.

Wrote the manuscript: MH (70%), ME

2.4 Manuscript IV

A below ground herbivore shapes root defensive chemistry in natural plant populations

Meret Huber, Zoe Bont, Julia Fricke, Theo Brillatz, Zohra Aziz, Jonathan Gershenzon, Matthias Erb

In preparation

Summary

Plant roots exhibit extensive intraspecific variation in secondary metabolites, but the evolutionary drivers shaping this diversity remain often unknown. Using the common dandelion (*Taraxacum officinale*) and its major native insect root herbivore, the common cockchafer larvae (*Melolontha melolontha*), we tested whether root herbivores shape variation in root chemical defenses. We found that the major latex secondary metabolites in natural *T. officinale* populations co-vary with present and historic *M. melolontha* abundance. By cultivating offspring of the field populations, we provide evidence that both phenotypic plasticity and heritable variation contribute to the patterns in the field. Furthermore, we found that the latex-derived sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester is under *M. melolontha*-imposed divergent selection. Taken together, this study highlights the role of soil-dwelling insects for the evolution of plant defenses below ground.

Author contributions

Conceived project: MH, ME

Designed experiments: MH (80%), ME

Conducted experiments: MH (70%), ZB, JF, ME

Performed data analysis: MH (100%)

Wrote the manuscript: MH (70%), ME

3. MANUSCRIPTS

3.1 Manuscript I

The role of primary and secondary plant metabolites in root-herbivore behaviour, nutrition and physiology

CHAPTER TWO

The Role of Plant Primary and Secondary Metabolites in Root-Herbivore Behaviour, Nutrition and Physiology

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Contents

Abstract

Many insect herbivores feed on belowground plant tissues. In this chapter, we discuss how they have adapted to deal with root primary and secondary metabolites. It is becoming evident that root herbivores can use root volatiles and exudates for host location and foraging. Their complex sensory apparatus suggests a sophisticated recognition and signal transduction system. Furthermore, endogenous metabolites trigger attractive or repellent responses in root feeders, indicating that they may specifically fine-tune food uptake to meet their dietary needs. Little evidence for direct toxic effects of root secondary metabolites has accumulated so far, indicating high prevalence of tolerance mechanisms. Root herbivores furthermore facilitate the entry of soil microbes into the roots, which may influence root nutritional quality. Investigating the role of plant metabolites in an ecologically and physiologically relevant context will be crucial to refine our current models on root-herbivore physiology and behaviour in the future.

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1. INTRODUCTION

The ability to take up nutrients and at the same time cope with harmful chemicals is essential for all life on Earth, including insect herbivores. Over evolutionary time, they have evolved strategies to exploit specific feeding niches of their host plants. One of the most remarkable environmental adaptations in this context is their ability to feed on belowground plant organs during the larval stage and then switch to the consumption of leaf material as adults. Members of at least seven insect orders, including many important agricultural pests, spend a part of their life cycle as belowground feeders (Blossey and Hunt-Joshi, 2003; Hunter, 2001).

Although feeding on roots comes with advantages, including protection from harsh environmental conditions, predators and competitors, it also poses a number of major challenges. Host finding and foraging, for example, is complicated by the absence of visual cues and the increased energy required for moving through the soil matrix (Fig. 2.1). Growth is constrained by the low nutritional value and unique blend of bioactive secondary compounds of roots (Kaplan et al., 2008a; Vaughan et al., 2013). Finally, there are many microorganisms, including beneficial, opportunistic and pathogenic fungi and bacteria (Burrows et al., 2009; Van Der Heijden et al., 2008), that need to be dealt with within the soil.

For all these reasons, the capacity of insects to develop on roots is expected to depend on a number of specific metabolic and behavioural adaptations. Studying these processes has the potential to facilitate our understanding of insect physiological processes and may also unravel novel ways to control some of the most devastating agricultural pests. Significant progress in understanding belowground plant–herbivore interactions has been made, and patterns are emerging that indicate general mechanisms of root-herbivore adaptation. For instance, while CO_2 has long been seen as the single most important plant volatile that root feeders use to locate host plants, it is now becoming clear that the perception of root volatiles is much more sophisticated and enables root-feeding larvae to make fine-tuned foraging decisions. Furthermore, it is becoming evident that secondary metabolites not only serve defensive purposes but also can be used by root feeders for host recognition and foraging. Specific host manipulation and detoxification strategies are being unravelled, and we are beginning to understand how closely root herbivores interact with the rhizosphere microbiome.

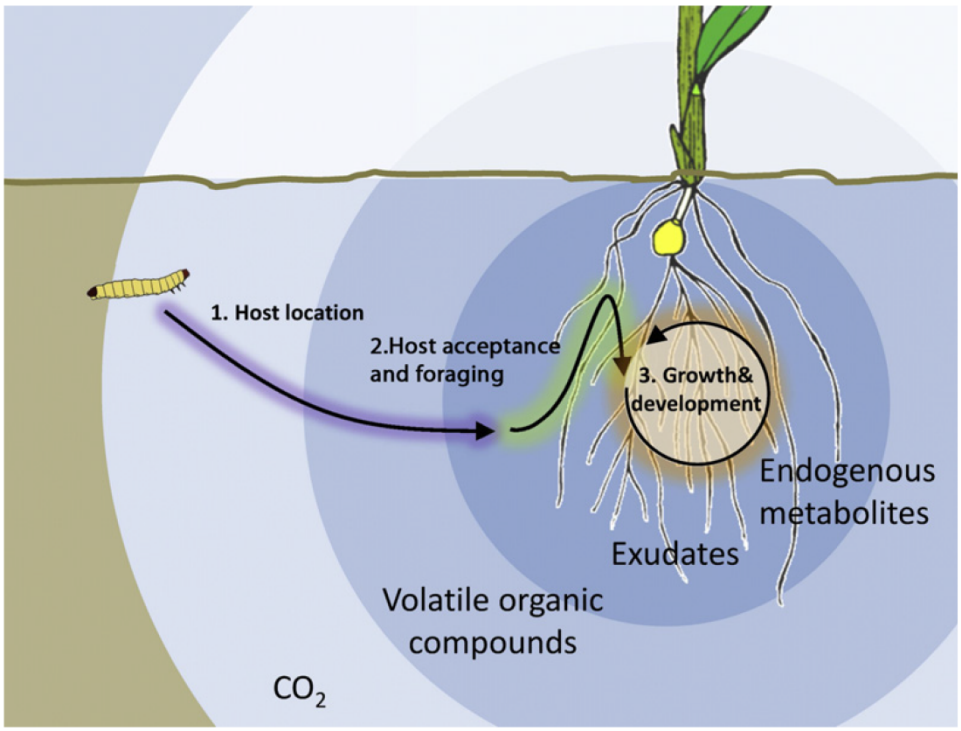

Figure 2.1 *Host location, foraging and development of root herbivores in the soil.* The spatial scale of different plant metabolites is depicted. CO_2 and other small-molecular-weight compound volatiles can diffuse several centimetres in the soil and can be used by root herbivores to locate host plants. Soluble root exudates may also be used for host location and foraging and may be required for host acceptance in direct proximity to the emitting roots. Endogenous primary and secondary metabolites determine the growth and development of the attacking herbivore but may also be used for foraging decisions.

In this chapter, we bring together recent and traditional findings on the role of plant metabolites in root-herbivore interactions. First, we review how larvae can use volatile and nonvolatile cues to find the roots of their host plants. Second, we discuss how primary and secondary metabolites influence host acceptance and foraging. Third, we discuss how the metabolic make-up of the roots determines herbivore growth, development and fitness. Fourth, we review the emerging evidence for an important role of soil microorganisms in determining root-herbivore interactions.

By compiling the literature on these subjects, we hope to complement other recent reviews on belowground herbivory and plant defences (Rasmann and Agrawal, 2008; van Dam, 2009), chemically mediated host plant location by root feeders (Johnson and Gregory, 2006) and root chemistry (Kaplan et al., 2008a). As the current state of the art regarding specific host plant adaptations that shape root-herbivore behaviour at different stages

of the interaction has not been compiled comprehensively, so far, it has remained difficult to identify common motives that point to root-herbivore specific adaptations. Our review therefore contributes to a general view on the evolutionary trajectories that insects may have taken when conquering the soil environment.

2. VOLATILE-MEDIATED HOST LOCATION

In contrast to leaf herbivores, many root-feeding insects have to locate host plants upon hatching, as eggs are deposited by gravid adults into adequate oviposition sites in the soil rather than on the plant surface. In the absence of any visual stimuli, larvae must locate a food source by relying on tactile or chemical cues, and it has been hypothesized that the insects should possess the capacity to locate host plants from a distance, for instance, using volatile or exudate cues. A number of behavioural studies document that root herbivores are indeed capable of sensing the presence of host plants long before physical contact. Using X-ray tomography, Johnson et al. (2004) demonstrated that neonates of the clover root weevil (*Sitona lepidus*) can recognize the presence of plant roots in the soil at a distance of up to 6 cm (Johnson et al., 2004). Similar observations have been made for the western corn rootworm (*Diabrotica virgifera virgifera*) (Robert et al., 2012a) and many other root-feeding insects (Johnson and Gregory, 2006).

One signal that elicits a strong behavioural response in the majority of root-feeding insects is carbon dioxide (CO_2). Recent reviews on the role of root volatiles as host location cues compiled over 20 studies that document a major role of CO_2 in long-distance host location (Johnson and Gregory, 2006; Johnson and Nielsen, 2012). The carrot root fly (*Psila rosae*), for instance, was found to be attracted to CO_2, but not to essential oils released from carrot seeds (Städler, 1971). Similarly, prairie grain wireworms (*Ctenicera destructor*) moved towards a variety of CO_2 sources, an effect that was eliminated by passing the airstream over a KOH solution that eliminated CO_2 from the system (Doane et al., 1975; Vaughan et al., 2013). Bernklau and Bjostad (2008) found that the attractiveness of maize extracts to the western corn rootworm can be matched and even overcome by increasing doses of CO_2.

Despite the clear potential of CO_2 to attract a wide variety of root-feeding insects, doubts have been raised about its reliability as a chemotactic signal. First, it has been argued that the soil environment contains a wide variety of CO_2 sources, including degrading organic matter and patches

of high microbial turnover (Agus et al., 2010). Second, all plant roots release CO_2, including not only host plants but also toxic nonhost species, rendering the signal unreliable to a foraging insect. Third, CO_2 emission from plant roots shows strong diurnal variation (Hansen, 1977) and mostly vertical concentration gradients (Pline and Dusenbery, 1987), which diminishes its importance as a signal for horizontal root location behaviour (Johnson and Gregory, 2006). Fourth, the integration of nonvolatile signals in an ecologically relevant concentration has been found to abolish the relative attractiveness of CO_2 for some herbivores (Reinecke et al., 2008).

An increasing number of studies document that root feeders can use signals other than CO_2 for host plant location from a distance (Fig. 2.2; Chapter 3). The large pine weevil (*Hylobius abietis*), for instance, was found to be attracted to α-pinene (Nordenhem and Nordlander, 1994), a

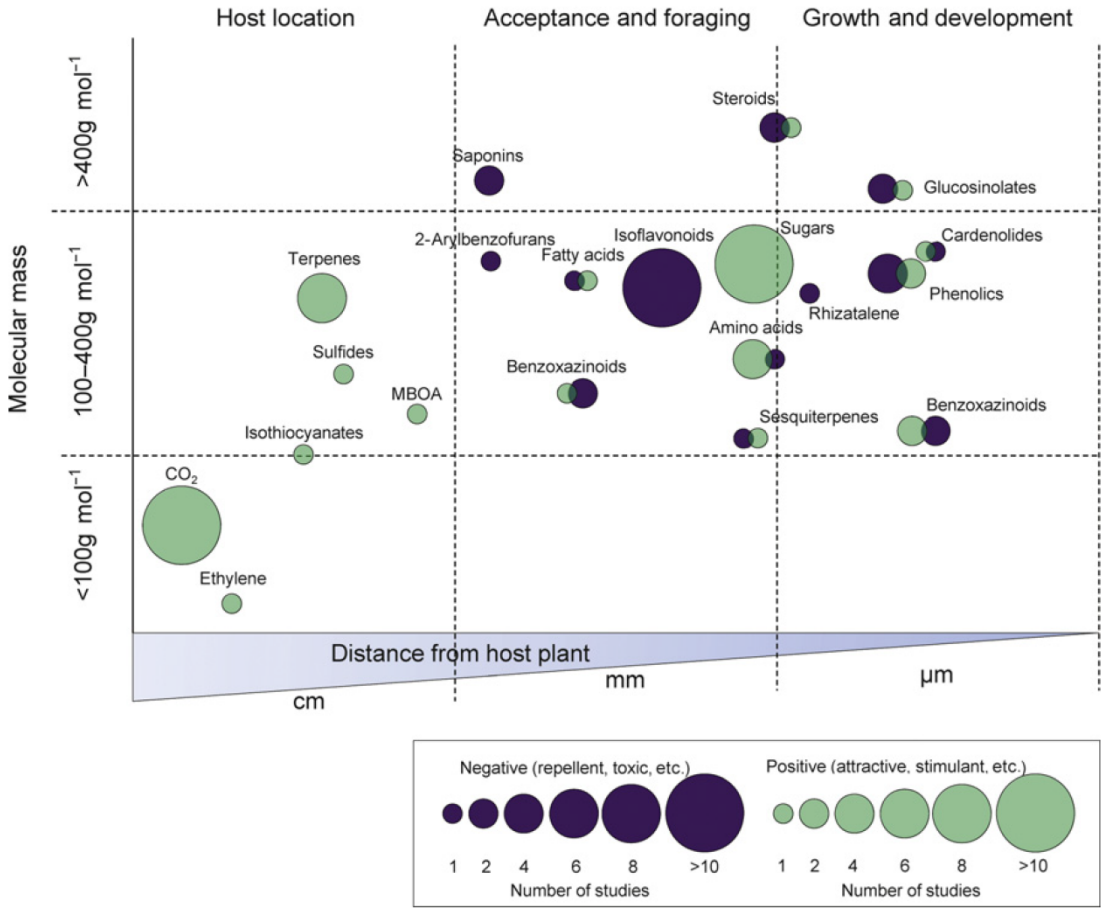

Figure 2.2 *Overview of plant metabolites with demonstrated activity in root-herbivore interactions*. Purple bubbles show negative effects on herbivores and green bubbles stand for positive effects. The size of the bubbles is proportional to the number of studies documenting the effect.

monoterpene emitted by its host plant. The cabbage root fly (*Delia radicum*) is attracted to isothiocyanates, a class of breakdown products of glucosinolates produced by cabbage plants and other crucifers (Koštál, 1992). Equally, the onion root fly (*Delia antiqua*) was found to orient towards sulphides emitted from onion bulbs (Soni and Finch, 1979), and the damage-induced oak volatiles eucalyptol and anisole were found to attract forest cockchafer (*Melolontha hippocastani*) larvae at concentrations below 5 ppb. Also, the western corn rootworm, despite its strong responsiveness to CO_2, has been shown to use other volatile cues. The benzoxazinoid breakdown product 6-methoxy-2-benzoxazolinone (MBOA), for instance, was found to attract western corn rootworm larvae (Bjostad and Hibbard, 1992). The sesquiterpene (*E*)-β-caryophyllene and the hormone ethylene were found to be used by this root feeder as well to distinguish leaf- and root-infested maize plants from uninfested individuals (Robert et al., 2012a). The attractive effect of (*E*)-β-caryophyllene was confirmed *in vivo* in the laboratory by using transgenic plants that overexpress (*E*)-β-caryophyllene (Robert et al., 2013). In this context, it was also shown that the response to (*E*)-β-caryophyllene is species-specific: in contrast to the specialist root feeder, the generalist southern corn rootworm (*Diabrotica undecimpunctata howardi*) was not attracted to the volatile (Robert et al., 2013).

Two recent studies shed light on the capacity of root herbivores to perceive volatile cues by dissecting their sensory apparatus (Eilers et al., 2012; Weissteiner et al., 2012). White grub (*Melolontha melolontha*) larvae possess 17 sensillum types on the antennae, maxillary and labial palps (Eilers et al., 2012). Three of them were found to have the morphological characteristics that are commonly associated with olfactory functions. Small pores and pore tubes connected to branching dendritic segments, for instance, were observed in cryptic antennal pore plates (sensilla placodea). Densities of sensory units (300) and sensory neurons (1000) in the antenna were comparable to those of adults of the common fruit fly (*Drosophila melanogaster*) (Shanbhag et al., 1999), which have a large repertoire of odour recognition and odour-guided behaviour. One basiconic sensillum was found on the tip of the maxillary and labial palps (S10 sensillum) and one on the subapical antennal segments (S7 sensillum).The size (S7, S10) and the position (S7, pore plates) of these olfactory organs prevent any direct contact with the substrate, and they can therefore only be stimulated by volatile compounds. By combining behavioural and electrophysiological assays, the authors could provide evidence that the sensilla placodea of the antennae are involved in the perception of different volatiles, including CO_2. Furthermore, based on

the facts that (i) some volatiles elicited responses in the palps and that (ii) palpographic signals are unlikely to be picked up from a single neuron, the authors suggested that gustatory sensilla may have a dual function in olfaction and taste. In the European forest cockchafer (*M. hippocastani*), the antennal lobe, the first brain centre to process olfactory input, was found to contain about 70 glomeruli (Weissteiner et al., 2012). The high number of glomeruli and the large antennal surface covered by antennal pore plates points to a sophisticated olfactory system in this root herbivore as well. Taken together, these two physiological studies clearly support the notion that root-herbivore olfaction is complex and is likely to go way beyond the simple detection of and response to CO_2. Evolutionary aspects of insect olfaction are discussed in detail by Hiltpold in Chapter 3.

From an ecological perspective, several explanations may help to resolve the dispute on the relative importance of CO_2 and volatile organic compounds as root host location cues. First, it was suggested that specialist herbivores may use host-specific volatile organic compounds, while generalists may be more likely to follow CO_2 as a universal cue. However, while this may be true for certain specialists (Nordenhem and Nordlander, 1994; Soni and Finch, 1979), the current literature contains many examples that go against this general assumption. Cockchafer larvae, for example, are polyphagous, yet able to respond to many cues other than CO_2 (Eilers et al., 2012; Weissteiner et al., 2012). The highly monophagous western corn rootworm on the other hand is strongly attracted to CO_2 (Bernklau and Bjostad, 1998), a finding that is also true for other root feeders with a restricted host range. Therefore, little support exists to support a generalist specialist dichotomy. As an alternative hypothesis, based on the observation that most root feeders are attracted to both CO_2 and volatile organic compounds, an integrated model was proposed by Johnson and Gregory (2006). This model proposes that CO_2 is used primarily as a long-distance cue that elicits searching activity, while VOCs are employed at a shorter range to distinguish host plant species and their different quality. Testing this model will require tight spatial control of volatile concentrations in the soil coupled with detailed behavioural observations.

Here, we propose a third, not mutually exclusive hypothesis that may reconcile CO_2 and VOC-mediated attraction. It is based on the fact that respiration, and hence CO_2 release, is an unavoidable consequence of root metabolic activity and that any emission of root VOCs will invariably be accompanied by an elevated CO_2 background. We propose that CO_2 can be used as a background odour by root feeders, which they integrate with

other volatile cues to optimize their searching behaviour. In the absence of any other cues, which is an unlikely situation in the rhizosphere, root herbivores will use CO_2 as an attractant, while in the absence of CO_2, VOCs would be less likely to trigger searching behaviour. This hypothesis is supported by research on aboveground perception and attraction of insects to plant odours (Schröder and Hilker, 2008), where CO_2 was found to enhance the attraction of the common fruit fly (*D. melanogaster*) to apple cider vinegar (Faucher et al., 2013). Also, many of the earlier-mentioned root-herbivore studies are compatible with the notion that CO_2 acts synergistically with other volatiles to attract belowground insects. The western corn rootworm, for instance, is attracted to the sesquiterpene (*E*)-β-caryophyllene only when an appropriate plant background is provided (Robert et al., 2012a but see Chapter 3). The finding that removing CO_2 from a volatile blend reduces wireworm (*Ctenicera destructor*) attraction also speaks in favour of CO_2 as a necessary background odour source (Doane et al., 1975). Apart from the studies conducted with the western corn rootworm, little information is available that allows testing of the CO_2 background hypothesis (but see Turlings et al., 2012), and more detailed experiments are clearly required to explore volatile-mediated host location belowground in more detail.

3. HOST SELECTION VIA SOLUBLE ROOT EXUDATES

Upon successful localization of a host plant, insects have to make a decision to start feeding or to move on to search for another root system to feed on. As roots are constantly exuding large amounts of organic carbon and other soluble metabolites into the rhizosphere, it can be expected that 'tasting exudates' provides a means for root herbivores to assess host plant susceptibility without having to chew through the root epidermis (Box 2.1).

Although direct evidence for the utilization of soluble exudates in host selection processes is scarce, several studies provide indirect support for this concept. Wireworms (*Agriotes* spp.), for instance, are attracted to malic acid (Thorpe et al., 1947), which is exuded by roots to mobilize phosphate (Hoffland et al., 1992). Western corn rootworm feeding is stimulated by MBOA (Bjostad and Hibbard, 1992), a partially soluble breakdown product of benzoxazinoids that is released by maize roots (Robert et al., 2012c). The western corn rootworm has also been shown to be repelled by the exuded precursor molecule 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one

BOX 2.1 Volatile vs. soluble root exudates: A blurred dichotomy

Contrary to the phyllosphere, the distinction between volatile and soluble exudates is much less clear in the rhizosphere. Many typical volatile compounds, for instance, are likely to diffuse through the soil at the interface between the soluble and vapour phase. The sesquiterpene (*E*)-β-caryophyllene, for instance, diffuses better at 5% soil humidity than lower or higher levels (Hiltpold and Turlings, 2008). On the other hand, soluble compounds may behave like volatiles, as illustrated by the soluble benzoxazinoid breakdown product 6-methoxy-2-benzoxazolinone (MBOA), which can be perceived as a volatile by the western corn rootworm (Bjostad and Hibbard, 1992). The situation with root herbivores is further complicated by the finding that the gustatory sensilla of white grubs (*M. melolontha*) may be able to perceive both gaseous and soluble molecules (Eilers et al., 2012). From a chemical point of view, volatile organic compounds are roughly defined as organic chemicals with high vapour pressure and low boiling points. As, for most investigated chemicals, no information is available on their state of aggregation in the soil, we rely on boiling points and the type of bioassay (i.e. olfactometer vs. contact experiments) to distinguish volatile vs. soluble exudates in this chapter.

(DIMBOA) (Xie et al., 1992a), even though this finding was later contested by the fact that the herbivore actually prefers to feed on maize mutants that produce higher levels of benzoxazinoids (Robert et al., 2012c). Also, formononetin, an isoflavonoid exuded from root nodules (Mathesius, 2001), was found to be attractive to the clover root weevil (*S. lepidus*) at concentrations of 0.01 M (Johnson et al., 2005). A noteworthy interaction between CO_2 and soluble cues was observed in a study involving the European cockchafer (*M. melolontha*) and the common dandelion (*Taraxacum officinale*) (Reinecke et al., 2008): while synthetic CO_2 attracted the root herbivore, adding *T. officinale* or an aqueous rhizosphere extract caused this effect to disappear, suggesting that exudate cues are integrated by the herbivore to yield a specific behavioural response.

As most experiments on the behavioural activity of root exudates have been conducted using root extracts or purified compounds, it remains unclear whether soluble signals are used for short-distance host location or whether they are actively involved in host-acceptance processes and small-scale foraging decisions. Formononetin exudation, for example, may be a reliable indicator of active nodulation and may actively guide foraging decisions by the clover root weevil (*S. lepidus*) (Johnson et al., 2005). Similarly, high benzoxazinoid exudation is associated with crown roots of

maize plants (*Zea mays*), which are more nutritious than other root types (Robert et al., 2012c) and may therefore be used as reliable foraging signals. To conclusively demonstrate the role of phytochemicals in exudate-mediated host selection and foraging, classical pharmacological approaches may be difficult to implement as the simulation of exudation patterns is challenging. A promising alternative research strategy may be the use of mutants or transgenic plants with altered levels of exudate metabolites. This approach has been used in thale cress (*Arabidopsis thaliana*) and maize to demonstrate the role of malic acid and benzoxazinoids in the recruitment of beneficial microbes (Neal et al., 2012; Rudrappa et al., 2008). However, one drawback of this approach is that many exudate metabolites also accumulate in the roots themselves, and changing their biosynthesis may alter other aspects of the root metabolic make-up. A more targeted approach would therefore be to alter the expression of the transport proteins that are responsible for the release of the chemicals from the cells into the apoplast and rhizosphere. The first transporters involved in the exudation of specific plant metabolites, including an ABC transporter that is responsible for the release of strigolactones, have been identified in petunia (*Petunia hybrida*) (Kretzschmar et al., 2012) and thale cress (Badri et al., 2009, 2012) and may provide a starting point for such an approach in root-herbivore model systems.

4. THE ROLE OF ENDOGENOUS PRIMARY METABOLITES IN HOST ACCEPTANCE AND FORAGING

Apart from sensing root exudates, root herbivores can assess the quality of their host plant by biting into the roots and directly tasting their food (Johnson and Gregory, 2006; Johnson et al., 2011; van Dam, 2009; Watts et al., 2011). This ability is essential to any heterotrophic organism that needs to achieve a balance of nutrients for successful development (Dethier, 1973; Schoonhoven, 1974) and may be particularly important for root feeders that feed from low-quality food matrices. Screening compounds for their potential phagostimulatory or deterrent effects on belowground herbivores generally involves measuring biting responses towards or the ingestion of artificial substrate treated with the chemical of interest. This approach was pioneered by Thorpe et al. (1947) and Crombie and Darrah (1947) who pretreated filter paper discs with chemical solutions that stimulated biting responses of the wireworms *Agriotes lineatus*, *A. sputator* and *A. obscurus*. Despite being developed over 50 years ago, similar bioassays are still used

today to understand behavioural aspects of important agricultural pests, including the Pacific coast wireworms (*Limonius canus*) (Horton et al., 2012), turnip root flies (*D. floralis*) (Hopkins et al., 1993) and the western corn rootworm (Bernklau et al., 2011).

Approximately 80% of the primary metabolites analysed with the above bioassay have been classified as 'phagostimulants' due to their ability to increase consumption rates by herbivores when applied to artificial diet. In general, low-molecular-weight compounds, such as alcohols, esters and aldehydes, appear to have attractive properties, while larger hydrocarbons are considered to be repellent. Despite this overarching pattern, other studies suggest that biochemical relatedness may be more important than functional groups in determining attractiveness or repellency (Finch and Skinner, 1974; Mochizuki et al., 1989; Soni and Finch, 1979; Weissteiner and Schütz, 2006). Here, we discuss the effects of three major classes of essential elements, endogenous sugars, amino acids and fatty acids, to illustrate the specific behavioural patterns that primary metabolites induce in root-feeding insects.

Sugars account for nearly half of all phagostimulatory chemicals identified to date (Johnson and Nielsen, 2012) and have been shown to stimulate feeding by a number of root-feeding insects (Johnson and Gregory, 2006). An early survey by Sutherland (1971) found that 10 out of 13 sugars could enhance biting and feeding responses of grass grub larvae (*Costelytra zealandica*) feeding on treated agar-cellulose discs, with sucrose eliciting the strongest response. Similar observations have been made for pruinose scarabs (*Sericesthis geminata*) (Wensler and Dudzinski, 1972), the cane beetle (*Antitrogus parvulus*), the negatoria canegrub (*Lepidiota negatoria*) (Allsopp, 1992) and Japanese beetles (*Popillia japonica*) (Ladd, 1988). Grass grub larvae (*C. zealandica*) feed on a wide variety of plant species (Radcliffe, 1970), which may explain their broad responsiveness to sugars. In general, studies with oligophagous insects find a more restricted range of carbohydrates to be effective phagostimulants (Augustine et al., 1964; Beck, 1957; Gothilf and Beck, 1967; Heron, 1965; Hsiao and Fraenkel, 1968; Ito, 1960; Tsuneyama et al., 2005). An investigation of feeding responses by Japanese beetles (*P. japonica*) found that three disaccharides, sucrose, maltose and trehalose, and two hexoses, fructose and glucose, were strong feeding stimulants for third-instar larvae feeding at concentrations of 0.1 M (Ladd, 1988). All of these sugars except trehalose also elicited feeding responses in adult beetles (Ladd, 1986), suggesting that chemoreception is retained during metamorphosis. Artificial diet experiments by Sutherland and Hillier (1976) on the other hand found that the root-feeding African black beetle

larvae (*Heteronychus arator*) respond most strongly to maltose while adult beetles, which also feed at the base of plants, exhibit positive feeding responses to many different sugars, including maltose, glucose and fructose (Sutherland and Hillier, 1976), indicating that sugar perception may change over the course of insect development as the relation to the part of the plant or host may also vary. These results, and later studies by Sutherland (1983) who found that 0.1 M maltose induces twice as much feeding by black beetle larvae compared to responses elicited by several amino acids and inorganic salt mixtures, strongly suggest that soluble sugars play a prominent role in stimulating feeding by root herbivores.

Sugars have been shown to act synergistically with other metabolites in eliciting feeding behaviour by above (Bartlet et al., 1994; Hsiao and Fraenkel, 1968; Shanks and Doss, 1987)- and belowground phytophagous insects (Crombie and Darrah, 1947). An early study by Crombie and Darrah (1947) found that while individual solutions of 0.5% glucose and 0.126% sucrose failed to elicit significant changes to orientation or biting response of wireworms (*Agriotes* spp.) compared to a control solution, combining these two sugars increased the number of bites taken in 24 h by approximately 70% when compared to the number of bites elicited by each sugar alone. Similar responses have been observed for wireworms (*Agriotes* spp.) presented with mixtures of glucose and the peptone, triolein or tannins (Crombie and Darrah, 1947). Sucrose was also found to act synergistically with L-ascorbic acid to induce feeding responses in grass grubs (Sutherland and Hillier, 1973), more than doubling the number of faecal pellets produced by third-instar larvae during 24 h exposure compared to larvae feeding on sucrose or L-ascorbic acid alone. In contrast, bioassays by Horton et al. (2012) found that presenting larvae of Pacific coast wireworms (*L. canus*) with sucrose in combination with plant essential oils including winter savoury, tea tree and cedar wood may lead to additive, synergistic and antagonistic effects on feeding behaviour, depending on the additive. The addition of a stimulant blend consisting glucose, fructose, sucrose and linoleic acid (Bernklau and Bjostad, 2008) overcame the normally repellent effects of thiamethoxam insecticide against western corn rootworm larvae (Bernklau et al., 2011). In both 30 min and 4 h bioassays, approximately 60% of larvae stayed on discs treated with thiamethoxam and the feeding stimulant blend for every concentration of the insecticide tested, compared to 5–35% of larvae that stayed when the feeding stimulant was absent (Bernklau et al., 2011). Interestingly, the presence of the stimulant blend was also found to improve the efficacy of thiamethoxam against

rootworm larvae; in 4 h bioassays, the concentration of thiamethoxam required for 50% mortality was 2800 pg/mL for the insecticide alone but was reduced by more than 100,000-fold to 0.0075 pg/mL when the feeding stimulant blend was added. The ability of sugars to mask deterrent effects of noxious compounds has also been demonstrated for above ground herbivores (Cocco and Glendinning, 2012; Shields and Mitchell, 1995), highlighting the potential similarities that may exist between chemically mediated host plant foraging above- and belowground.

Proteins and free amino acids constitute major nutrients for insects, as they are needed to supply nitrogen in a digestible form. Furthermore, up to 10 amino acids (arginine, isoleucine, leucine, lysine, histidine, methionine, phenylalanine, threonine, tryptophan and valine) are essential for insects (Dadd, 1977). Therefore, an insect's performance directly depends on its ability to detect and feed on plants that contain these necessary amino acids. Most amino acids in plants occur in the form of proteins. Until today, little evidence has accumulated that insects can detect and taste proteins, with the exception of those that contain terminal sequences that resemble polypeptide neurotransmitters (Chapman, 2003). To our knowledge, no study to date has investigated protein sensing and its effect on root feeders' host acceptance and foraging, but several studies highlight the role of amino acids in this context. Sutherland and Hillier (1973) tested the individual effect of 20 amino acids, at 0.01 and 0.1 M, on the feeding behaviour of the grass grub larvae. After 24 h of starvation, the larvae were fed an agar/cellulose medium containing one of the 20 ubiquitous amino acids. The number of faecal pellets after 24 h of feeding was recorded as a parameter of feeding. Six amino acids induced higher number of faecal pellets at a concentration of 0.1 M: L-alanine, L-serine, L-threonine, L-aspartic acid and L-glutamine (Sutherland and Hillier, 1973). Only L-aspartic acid and L-glutamic acid enhanced the feeding at 0.01 M. Using the same methodology, Sutherland (1983) was also able to demonstrate a stimulating effect for 0.1 M L-alanine, 0.01 M L-aspartic acid and 0.01 M L-glutamic acid on black beetle larvae (*H. arator*) (Sutherland, 1983). Allsopp (1992) tested the effect of 18 amino acids at 0.01 M concentrations in cellulose acetate discs on the foraging of the negatoria canegrub (*L. negatoria*). By assessing the percentage of the disc eaten by the larvae, the author found that L-cysteine, L-glutamic acid and L-histidine monohydrochloride stimulated the feeding of the larvae (Allsopp, 1992). In the same study, the author demonstrated that only L-tyrosine (0.01 M) stimulated feeding by larvae of the cane beetle (Allsopp, 1992). L-alanine as well as two of the essential amino acids,

L-isoleucine and L-leucine, on filter papers stimulated the feeding behaviour of the pruinose scarab at low concentrations, while they inhibited food intake at high concentrations (Wensler and Dudzinski, 1972). Clearly, many root-feeding insects have the ability to perceive and respond to the presence of amino acids. Yet, it remains unclear why only a specific subset of them elicits behavioural activity. We speculate here that in general, the perception of free amino acids may enable root feeders to assess host plant quality or root identity. The western corn rootworm, for instance, develops better on crown than primary roots of maize. Crown roots contain both higher protein and free amino acid levels, and amino acids could therefore be used as a proxy for the nutritional quality of the plants (Robert et al., 2012c).

Fatty acids such as linoleic acid and oleic acid were reported to act as phagostimulants for root herbivores as well. Their role was discovered following the finding that the liquid pressed from germinating maize roots contains phagostimulants for the larvae of the western corn rootworm (Bernklau and Bjostad, 2005). An ethyl acetate and an aqueous fraction of the extract elicited feeding together but not individually, suggesting that both polar and nonpolar compounds are required for stimulation (Bernklau and Bjostad, 2008). Subsequent bioassays with sugars, amino acids, diacids and free fatty acids demonstrated that the stimulatory activity of a sugar mix (glucose/fructose/sucrose; 30:4:4 mg/mL) was strongly enhanced by applying linoleic (3 mg/mL) or oleic acid (0.3 mg/mL) (Bernklau and Bjostad, 2008). It is interesting to note that the same ecologically relevant concentrations of fatty acids lead to 70% of mortality when tested alone (Bernklau and Bjostad, 2008). This is consistent with other studies reporting insecticidal effects of both fatty acids against aboveground insects (Guang et al., 1991; Harada et al., 2000; Ramsewak et al., 2001; Yang et al., 1992). The mechanism behind this *in vitro* phenomenon remains to be determined.

Taken together, the earlier evidence demonstrates that root-feeding herbivores can sense primary metabolites, including sugars, amino acids and fatty acids, as a means of assessing host plant quality. Carbohydrates are shown to act primarily as phagostimulants (Chapter 3). To our knowledge, no sugar has been shown to elicit deterrent effects on root-feeding herbivores. The mechanisms by which sugars are perceived by root feeders and how or why this perception changes over the course of an insect's development warrant further study. It appears from the literature that the affinity for amino acid classes is species-specific. For example, L-alanine, L-isoleucine and L-leucine, which are all amino acids with hydrophobic side chains, were

shown to stimulate feeding by pruinose scarabs (Wensler and Dudzinski, 1972), but not of the negatoria canegrub (Allsopp, 1992). Given that roots of different species differ in their primary metabolome, one might expect that root herbivores use them to distinguish suitable from unsuitable host plants and to differentiate between parts of the root system (e.g. root nodules and root meristems) as they take exploratory bites. While the current bioassays enable measurement of the influence of individual compounds on root-herbivore behaviour, they are not sufficient to simulate natural situations. The fact that physiological levels of fatty acids lead to excessive mortality of the highly specialized western corn rootworm *in vitro*, for instance, illustrates this point (Bernklau and Bjostad, 2008). An alternative approach may be the use of mutants and transgenic plants. The fatty acid dehydrogenase (fad) mutants of thale cress, for instance, have significantly altered linoleic acid levels (Okuley et al., 1994). However, in many cases, this approach is not without problems due to pleiotropic effects associated with perturbing the biosynthesis of primary metabolites (Yang et al., 2006).

5. THE ROLE OF ENDOGENOUS SECONDARY METABOLITES IN HOST ACCEPTANCE AND FORAGING

Plant roots produce a diverse array of endogenous secondary compounds that trigger behavioural responses in root-feeding insects. Sutherland et al. (1980), for instance, found that grass grubs were deterred by isoflavonoids and other phytoalexins that are commonly found in legumes. Artificial diets containing pisatin, phaseolin, medicarpin, maackiain, vestitol, phaseollinisoflavan and 2′-methoxyphaseollinisoflavan were specifically avoided by these larvae. Also, African black beetle (*H. arator*) larvae were deterred by the flavonoids phaseolin, medicarpin, maackiain, vestitol, coumestrol, genistein and biochanin (Sutherland et al., 1980). Phaseolin, phaseollinisoflavan, medicarpin, vestitol, maackiain and 2′-methoxyphaseollinisoflavan have been found in legume roots at concentrations ranging from 1 to 6 μg/g FW (Gaynor et al., 1986; Liu et al., 1995; Russell et al., 1978; Stevenson et al., 1997; Sutherland et al., 1980) and pisatin at concentrations between 40 and 500 μg/g (Cannesan et al., 2011; Christenson and Hadwiger, 1973). Bioassays with artificial diets containing these flavonoids suggest that concentrations commonly found in roots (between 30 ng/g and 55 μg/g) are sufficient to reduce grass grub feeding by 50% (FD50) (Lane et al., 1985, 1987; Russell et al., 1978).

Lane et al. (1987) investigated the biological activity of root-derived isoflavonoids of lupin legumes (*Lupinus angustifolius*). Of the nine isoflavones that were isolated and tested, four showed significant deterrent properties against grass grubs at concentrations of 2 µg/g: licoisoflavone A, licoisoflavone B, 2-hydroxygenistein and luteone.

Apart from flavonoids, a number of other secondary metabolites have been demonstrated to deter root herbivores. Two-arylbenzofurans contained in the roots of the common sainfoin (*Onobrychis viciifolia*), for instance, have been shown to deter grass grubs at a physiological concentration of 2 µg/g (Russell et al., 1984). Similarly, saponins (Sutherland et al., 1982) 20-hydroxyecdysone (20E) (Schmelz et al., 2002) and the benzoxazinoid DIMBOA (Xie et al., 1990) were shown to exhibit a strong antifeedant activity against root herbivores *in vitro*.

In contrast to the classical phytochemical studies that focused on repellent compounds, a number of studies demonstrate that endogenous secondary metabolites may in fact stimulate feeding by belowground herbivores. As discussed in the previous sections, benzoxazinoids, which are highly concentrated in maize roots (up to several mg/g FW), are an attractant and feeding stimulant for the western corn rootworm (Bjostad and Hibbard, 1992; Robert et al., 2012c), and isoflavonoids found in white clover roots stimulate the clover root weevil (*S. lepidus*) (Johnson et al., 2005). Also, feeding by the spotted cucumber beetle was found to be stimulated by bitter and toxic cucurbitacins (De Heer and Tallamy, 1991). Furthermore, as breakdown products of glucosinolates are attractive for the cabbage root fly (Koštál, 1992), it is possible that they are also acting as feeding stimulants, similarly to what has been observed for crucifer specialists aboveground (Nielsen et al., 1979). The currently available studies suggest that feeding stimulation by secondary metabolites occurs in root herbivores with a restricted host range, and it is conceivable that host plant specialization determines the degree of behavioural adaptations to secondary compounds as host recognition signals.

Just as with the investigated primary metabolites, two major issues remain to be resolved in order to understand the role of endogenous secondary metabolites in determining root-herbivore foraging and behaviour. First, the ecological relevance of the behavioural assay needs to be improved. A majority of tests were conducted in artificial substrates, and only few studies have attempted to observe root-herbivore behaviour in a natural soil context. Given the structural and chemical composition of the soil, it is well possible that the full behavioural range that root herbivores display will only

become apparent once they are exposed to the behaviourally active compounds in such an environment. Pioneering work in that respect comes from Johnson et al. (2007). The authors used X-ray tomography to track insect movement in a soil matrix. Although no detailed activity tests with plant metabolites were carried out in this study, it would in theory be possible to adapt this system to test individual root chemicals for behavioural activity. Second, the role of plant metabolites must be assessed *in planta*. Most behavioural assays have been conducted with individual compounds, neglecting the fact that endogenous metabolites occur within a complex plant matrix consisting not only of many different metabolites but also of enzymes that modify the chemical structure of compounds upon tissue disruption. Plant–insect interactions have benefitted tremendously from the availability of molecular and genetic plant resources, including mutants and transgenic plants with an altered metabolism that enable investigation of the importance of phytochemicals in their natural context (Jander and Howe, 2008). Unfortunately, research on root-herbivore interactions has not taken full advantage of these tools yet, possibly because the major molecular plant models are not typically attacked by root-feeding insects. Exceptions include the use of transgenic and mutant maize to investigate the role of root phytochemicals (Degenhardt et al., 2009) as well as studies in the thale cress with the opportunistic root-feeding fungus gnat (*Bradysia* spp.) (Vaughan et al., 2013).

6. FOOD-QUALITY ASPECTS OF ROOT-HERBIVORE INTERACTIONS I: PRIMARY METABOLITES

Apart from its effects on herbivore foraging and host acceptance, the metabolic make-up of the roots directly determines how well the attacking insect will grow and develop. The major dietary factors for insects in general include nitrogen in the form of proteins or amino acids, carbohydrates, lipids, sterols, minerals and vitamins (Awmack and Leather, 2002). As discussed in a previous section, root herbivores respond strongly to many dietary constituents, illustrating how important nutrient foraging is for belowground plant feeders. In this section, we review some of the general nutritional challenges that insects face when feeding on the roots (nitrogen, carbohydrates and fatty acids) and discuss possible adaptations that herbivores may have developed during their transition to root-feeding behaviour.

Traditionally, nitrogen (N) has been regarded as the major growth-limiting factor for insect herbivores. This notion is based on the inherent

imbalance in N concentrations between insects and their host plants (Slansky and Feeny, 1977). Depending on species, developmental and ontogenetic stage, N content per dry mass ranges between 0.03% and 7% for plants and between 7% and 14% for phytophagous insects (Mattson, 1980). Although roots of some plants contain lower N levels than leaves (Hol et al., 2003; Murray et al., 1996), a phenomenon that has been proposed as a general pattern (van Dam, 2009), this does not seem to hold true across plant species. Red oaks (*Quercus rubra*), for instance, have similar N levels in the leaves and roots (2.3%) (Frost and Hunter, 2008), and the spotted knapweed (*Centaurea maculosa*) contains about 4% N in both roots and shoots (Newingham et al., 2007). Interestingly, however, attack of the spotted knapweed (*Centaurea maculosa*) by the root-feeding larvae of the sulphur knapweed moth (*Agapeta zoegana*) leads to a dramatic reduction of root N levels, which again may enforce N limitation for the herbivore (Newingham et al., 2007). That root-feeding insects need to forage actively for N is indicated by behavioural studies (see sections earlier). The western corn rootworm was found to feed preferentially on maize crown roots, which contain higher protein and free amino acid levels (Robert et al., 2012c). And the first-instar clover weevil larva feeds preferentially on nodules (Bigger, 1930; Hackell and Gerard, 2004), which have pronouncedly higher N levels than the rest of the root system (Murray et al., 1996). Western corn rootworm attack was also found to increase free amino acid levels in maize roots (Robert et al., 2012b). Whether root herbivores manipulate the metabolism of their host plant to acquire additional N remains to be determined. Even though N in the form of proteins and free amino acids may be just as limiting for root-herbivore growth as for leaf herbivores, few studies have specifically investigated the role of this element belowground. Way et al. (2006) treated maize plants with different levels of N fertilizer ranging from 34 to 202 kg N/ha and recorded larval and pupal densities in different plots (Way et al., 2006). Overall, higher doses of N increased larval densities of the rice water weevil (*Lissorhoptrus oryzophilus*). However, as N fertilization also increases adult feeding and oviposition (Jiang and Cheng, 2003), it is unclear whether this effect is due to improved larval nutrition or an increase in oviposition by the leaf-feeding adults. Overall, we hypothesize that adaptations of root feeders to N as a limiting factor should mirror the strategies that leaf-feeding insects typically use, including foraging for N-rich tissues (Kimmerer and Potter, 1987), compensatory feeding (Awmack and Leather, 2002) and the acquisition of nutritional symbioses (Douglas, 2003).

As the primary source of energy, carbohydrates are an indispensable part in insect nutrition. In particular, the ratio between proteins and carbohydrates determines insect growth (Lee et al., 2002). Starch, mono- and disaccharides can be expected to play a particularly important role in this context, as they are much easier to break down and digest than more complex molecules like cellulose. Strikingly, the concentrations of accessible carbohydrates are often lower in the roots than the leaves of plants. In the wild tobacco (*Nicotiana attenuata*), for instance, the total concentration of sugars and starch is 50% lower in the roots (Machado et al., 2013). Similar patterns have been observed in bean (*Phaseolus vulgaris*) (Cakmak et al., 1994), maize (Braun et al., 2006; Robert et al., 2012c), thale cress (Zeeman and Rees, 1999) and strawberry (*Fragaria* × *ananassa*) (Eshghi et al., 2007). In contrast, five kiwi species (*Actinidia* spp.) exhibited no consistent allocation pattern, but substantial seasonal variation with a depletion of root carbohydrates towards the middle of the growing season and replenishment at the end (Boldingh et al., 2000). A simple physiological explanation for this pattern may be that carbon is assimilated and primarily stored in the leaves and only transported to the roots as a source of energy for growth, development and storage. In some perennial plants, carbohydrates can be stored in belowground organs for overwintering: Starch concentrations in potato tubers, for instance, can reach 15% fresh weight. While belowground feeders that specialize on these types of plants (Rondon, 2010) are unlikely to be carbohydrate-limited, it is possible that root feeders that live on plants that do not store carbon suffer from low sugar and starch levels of their host tissues. Again, the strong stimulatory effect of many sugars on root feeders (as discussed earlier) may serve as an argument that supports this hypothesis. Unfortunately, while the multidimensional nutritional spaces, including optimal carbon/nitrogen ratios, are well understood for leaf feeders (Joern and Behmer, 1997), very little is known for root herbivores in this respect.

We hypothesize here that root herbivores may have developed several strategies to cope with low starch and sugar levels of their host plants. Firstly, they could efficiently metabolize cellulose either by microbial symbiosis or by the production of endogenous proteins. Secondly, root-feeding insects may actively mix diets and satisfy their carbohydrate needs by feeding on carbon-storage plants temporarily. Thirdly, they may manipulate the plant to increase root carbon allocation. Cellulose digestion is rare in insects having been detected in about 20 families in eight orders (Martin, 1983, 1991). Most cellulose digesters are xylophagous insects,

notably the termites with a digestion efficiency reaching 99% (Esenther and Kirk, 1974), the larvae of siricid wood wasps and anobiid, buprestid and cerambycid beetles (Martin, 1991) and the omnivorous silverfish (*Ctenolepisma lineata*) (Lasker and Giese, 1956), firebrat (*Thermobia domestica*) (Treves and Martin, 1994; Zinkler et al., 1986) and several roaches (Wharton and Wharton, 1965). Interestingly, the root-feeding larvae of the European rhinoceros beetle (*Oryctes nasicornis*) have a cellulose-digesting efficiency reaching 68% (Rössler, 1961) and the Australian pruinose scarab larvae were reported to exhibit cellulase activity (Soo Hoo and Dudzinski, 1967). Notably, evidence for substantial cellulose digestion by leaf-feeding arthropods is lacking (Martin, 1991; Prins and Kreulen, 1991) although weak cellulase activity was detected in the desert locust (*Schistocerca gregaria*) (Evans and Payne, 1964), the migratory locust (*Locusta migratoria migratorioides*) (Morgan, 1975) and the Mexican leaf beetle (*Epilachna varivestis*) (Taylor, 1985). These findings support the hypothesis that insects have evolved digestive adaptations to reduce carbohydrate limitations imposed by their food source and it is possible that these are common among root-feeding insects. A second possibility is that root herbivores may feed on carbon-storage plants temporarily. Diet mixing is well documented for aboveground herbivores. Unfortunately, because observing root herbivores in natural plant communities is difficult, very little is known out of host-switching behaviour and nutrient uptake optimization belowground. A third possible adaptation is that root herbivores may benefit from herbivore-induced carbon allocation to the roots. Two studies have found higher sucrose concentrations in root-herbivore-attacked belowground plant organs (Pierre et al., 2012; Robert et al., 2012b). Furthermore, some root feeders induce water stress in their host plant (Erb et al., 2009; Masters et al., 1993), a condition that is known to increase carbon allocation belowground (Ibrahim et al., 1997). It is tempting to speculate that the induction of mild water stress may help root herbivores gain access to leaf carbohydrates by hijacking a plant reallocation response that is geared towards stabilizing water supply via an increase in root proliferation.

Apart from sugars, fatty acids can also be used to satisfy the energy requirements of an insect's metabolism. Certain fatty acids, including sterols (Friend, 1958; House, 1961) and polyunsaturated fatty acids (PUFAs) (Dadd, 1973), are essential for insect development. Linolenic, linoleic and oleic acid are often the most common PUFAs in plants. There has been a controversy in the past as to whether linolenic and linoleic acid can be used

interchangeably in insect nutrition. Hoppers of the Acrididae family (*Locusta* spp.) had a similar growth and survival rate when fed on either linoleic or linolenic acid at 5 mg/g of diet (Dadd, 1961). However, it became increasingly clear that in some Lepidoptera and Hymenoptera, proper metamorphosis only took place when linolenic acid was added to artificial diets (Dadd, 1973): in the wax moth (*Galleria mellonella*), all emerging adults looked normal when reared with 5 mg linolenic acid per 6 g artificial diet, while proper adult formation dropped to one third when linolenic acid was substituted with linoleic acid. The major sources of PUFAs in the leaves are cell and chloroplast membranes. It is therefore not surprising that PUFA patterns in the roots differ substantially from the aboveground tissues. For instance, in eight out of the nine tested species of Fabaceae (*Astragalus* spp.), the concentration of linoleic acid was 30–80% higher in roots than shoots, while the concentration of linolenic acid was more than twofold lower in below- than aboveground tissue in six of these species (Keskin and Kacar, 2012). This shift from linolenic to linoleic acid was also observed in Solanaceae (*Capsicum* spp. and *Solanum* spp.) (Lyons and Lippert, 1966; Ouariti et al., 1997) and Poaceae (*Agrostis* cultivars) (Larkindale and Huang, 2004). Given that linolenic acid is essential for the development of many insects (Dadd, 1973), it is possible that root herbivores, at least in extreme cases, may be limited by the available root pools. Possible counter adaptations might include (i) a reduced demand of PUFAs as shown for the common fruit fly (Rapport et al., 1984), (ii) an improved exploitation of the available PUFAs, for example, by breaking down cell walls, (iii) endogenous production of PUFAs as shown for linoleic acid in the pea aphid (*Acyrthosiphon pisum*) (De Renobales et al., 1986) or (iv) association with beneficial microbes providing PUFAs. We are unaware of experimental evidence for any of these hypotheses for belowground feeding insects.

From the three examples earlier, it becomes clear that the nutritional composition of roots differs substantially from the leaves. The evolution of root-feeding behaviour was therefore likely accompanied by physiological adaptations that enabled insects to optimize growth and development on their new substrate (see Chapter 3). We propose that an experimental approach that combines evolutionary and physiological aspects may reveal interesting adaptive patterns that may improve our general understanding of insect nutrition and plasticity. In this context, it is noteworthy that many root herbivores only spend their larval stage belowground, while adults feed on foliage and flowers. It can, therefore, be expected that there is a major developmental shift in dietary physiology as the root feeders mature.

Comparing the dietary requirements of larval and adult stages within the same species may prove particularly useful to unravel adaptations associated with a root-feeding habit.

7. FOOD-QUALITY ASPECTS OF ROOT-HERBIVORE INTERACTIONS II: SECONDARY METABOLITES

The diversity of secondary metabolites in roots rivals the diversity in aboveground plant parts (Rasmann and Agrawal, 2008; van Dam, 2009), including many classes of secondary metabolites that reduce plant quality for herbivores such as alkaloids (Dawson, 1941), glucosinolates (Kaplan et al., 2008b), terpenoids (Malcolm, 1991), phenolics and benzoxazinoids (Niemeyer, 2009). It is therefore likely that, apart from influencing herbivore behaviour, these metabolites will also determine the nutritional value of the roots for root feeders, for instance, by reducing the efficacy of digestive enzymes (Houseman et al., 1992) or by interfering with essential functions in the insect body (Dobler et al., 2011). Although in many cases behavioural and nutritional effects are difficult to separate experimentally (Stout, 2013), it remains important to distinguish these two biological dimensions in order to understand plant–insect interactions from a mechanistic and evolutionary point of view. We therefore address behavioural and food-quality effects separately in this chapter. Compared to the situation aboveground, several rhizosphere-specific aspects need to be taken into account when studying root secondary metabolites in a nutritional context:

- Although roots produce the same classes of secondary metabolites as the leaves, their secondary metabolite blends differ both quantitatively and qualitatively. Some secondary compounds, for instance, are exclusively produced in the roots. Given that the activity of a metabolite is directly determined by its specific structure rather than by chemical classification (Mazoir et al., 2008), extrapolating data from leaf-herbivore interaction studies to the roots is problematic.
- The toxicity of secondary metabolites depends in many cases directly on their nutritional context. Nitrogen limitation, for instance, increases the toxicity of tannins (Raubenheimer, 1992). As the primary metabolome of the roots is different from the leaves, secondary metabolites may therefore have a different effect belowground than aboveground.
- Roots are colonized by a multitude of microorganisms that live at the root surface and in the extracellular spaces. Many microorganisms process plant secondary compounds (Neal et al., 2012), and root herbivores

may therefore encounter a cocktail of plant compounds that is partially digested by microorganisms.

- Roots are constantly wounded: Lateral roots and root hairs break through the epidermis of their mother roots and thereby directly destroy cells. Given that many secondary metabolites are only activated upon tissue rupturing, for instance, by deglycosylation (Hopkins et al., 2009; Rask et al., 2000), it can be expected that root feeders will encounter higher concentrations of preactivated secondary compounds.

Few examples from the literature clearly demonstrate negative effects of root secondary metabolites on the food quality of roots for belowground herbivores. Schmelz et al. (1998, 2002) showed that in spinach (*Spinacia oleracea*), the steroid insect moulting hormone homologue 20-hydroxyecdysone (20E) was induced after feeding by the larvae of the dark-winged fungus gnat (*Bradysia impatiens*), wounding and methyl jasmonate (MeJA) application. Apart from a repellent effect of 20E, the authors also found that larval survival dropped and prepupae formation increased dramatically when 20E was added in physiological concentrations to potato-based diets (Schmelz et al., 2002). Given the homology of 20E to insect moulting hormones, it is likely that this compound interferes with insect development directly and reduces the food quality of its host plant beyond its repellent effect. Cucurbitacins, another class of defensive steroids produced by the *Cucurbitaceae,* on the other hand, had no negative effects on the southern corn rootworm (Halaweish et al., 1999). On the contrary, the southern corn rootworm larvae even grew slightly better when feeding on a cucurbitacin-rich squash cultivar (Halaweish et al., 1999). It remains to be determined whether the stimulation in growth is due to feeding stimulation or a nutritional effect.

In a phylogenetically corrected correlation, total root cardenolide content of 18 milkweed species (*Asclepias* spp.) was negatively associated with milkweed beetle (*Tetraopes tetraophthalmus*) larval performance in the greenhouse (Rasmann and Agrawal, 2011). In line with these findings, the red milkweed beetle larvae survival rate was almost 3× higher on *A. syriaca* plants with low cardenolide contents compared to conspecifics with more than double the cardenolide concentration (Rasmann et al., 2011). As cardenolides inhibit the animal sodium–potassium pump that is vital for membrane potential maintenance, these metabolites likely decrease growth by directly interacting with the insect's physiology. In contrast to these results, the number of larvae of the generalist fungus gnats (*Bradysia* spp.), which spontaneously infested plants in the greenhouse, was not correlated to cardenolide content across 14 milkweed species (Vannette and

Rasmann, 2012). It remains to be determined whether the fungus gnats have evolved physiological mechanisms to cope with cardenolides as shown for aboveground herbivores (Dobler et al., 2012; Zhen et al., 2012) or whether specific cardenolides, rather than the total concentration, determines the performance of this opportunistic root herbivore.

In kale (*Brassica oleracea*), rape (*B. napus*) and swede (*B. napus*), Birch et al. (1992) found that the percentage of pupae formation of the turnip root fly differed between both species and genotypes. However, neither total nor the abundance of any specific glucosinolate was correlated with root fly performance. In both broccoli (*Brassica oleracea* subsp. *italica*) and turnip (*Brassica rapa* subsp. *rapa*), aliphatic and aromatic glucosinolates were not changed in roots upon cabbage fly infestation, while indolyl glucosinolate was upregulated two- to fourfold (Pierre et al., 2012). However, these changes in indolyl glucosinolate were not correlated with larval performance: in broccoli, pupation rate on infested plants was similar to control plants, while in turnip, pupae formation increased by approximately 10%. These results indicate that indolic glucosinolates do not affect the herbivore. Recently, van Leur et al. (2008) investigated cabbage fly larval performance on two natural chemotypes of bittercress (*Barbarea vulgaris*), which differed in the ratio of two major glucosinolate species glucobarbarin ((S)-2-hydroxy-2-phenylethyl-glucosinolate) and gluconasturtiin (2-phenylethyl-glucosinolate). In this greenhouse experiment, pupae mass of the cabbage fly larvae was higher on the cultivar with the low gluconasturtiin ratio. Interestingly, the low gluconasturtiin cultivar contained approximately 15% less total free amino acids and total sugars, indicating that the glucosinolate profile rather than the nutritional quality might account for the differences in larval performance. These results are in line with bioassays showing toxic effects of gluconasturtiin against nematodes (Potter et al., 1998, 2000). Apart from affecting insect behaviour, the breakdown products of glucosinolates are thought to react with amino and sulfhydryl groups of proteins (Kawakish et al., 1987), suggesting that some of the negative effects of glucosinolates are due to antibiosis. Taken together, antibiosis of glucosinolates towards root herbivores remains ambiguous and clearly deserves more attention.

The few studies that explored the role of phenolics on root-herbivore performance showed little toxic effects of these metabolites on belowground feeders. In a greenhouse experiment with 15 sugar cane clones (*Saccharum* spp.) differing in their soluble and cell-wall-bound phenolic composition, there was no correlation between the growth of the sugar cane white grub

and the abundance of these compounds (Nutt et al., 2004). Unexpectedly, Johnson et al. (2011) found a positive correlation between growth and survival of the black vine weevil (*Otiorhynchus sulcatus*) and total phenolics in the roots of blackcurrant (*Ribes nigrum*). A similar pattern was observed with black vine weevil larvae (*O. sulcatus*) feeding on raspberry (*Rubus idaeus* cv. Glen Ample) (Clark et al., 2011). It is unclear whether this positive relationship is due to covarying beneficial metabolites or whether phenolics had a positive effect on the insects. Beneficial effects of phenolics were demonstrated in leaf-feeding insects (Bernays and Woodhead, 1982), assumingly by reducing the demand of amino acids in the sclerotized exoskeleton. However, specific phenolic compounds may still be active against root herbivores: for instance, in a comparison of two varieties of sweet potato (*Ipomoea batatas*), fewer larvae of the sweet potato weevil (*Cylas puncticollis*) emerged from a variety with high levels of octadecyl and hexadecyl esters of hydroxycinnamic acids than from a variety with low levels (Stevenson et al., 2009). Incorporation of hydroxycinnamic acids that occur in the latex, hexadecylcaffeic acid and hexadecyl-p-coumaric acid into artificial diet increased mortality of the weevil larvae in a dose-dependent manner, thereby providing indirect evidence for a defensive function of hydroxycinnamic acids against the sweet potato weevil. Cole (1987) reported a positive correlation between plant resistance to the lettuce root aphid (*Pemphigus bursarius*) and the concentration of isochlorogenic acid in lettuce (*Lactuca sativa*). Monitoring of feeding patterns revealed differences in the behaviour of the lettuce root aphid (*Pemphigus bursarius*) on resistant and susceptible lettuce varieties (Cole et al., 1993). In contrast, carrot (*Daucus carota* subsp. *sativus*) seedlings resistant to the carrot root fly (*Psila rosae*) contained lower concentrations of chlorogenic acid than susceptible plants (Cole, 1987). Taken together, the role of phenolics in belowground defence remains ambiguous. Investigations on specific phenolics rather than on total content could help to clarify the situation.

Early field studies in the 1990s suggested a positive correlation between the concentration of benzoxazinoids and resistance against the western corn rootworm in nine maize inbred lines, assumingly mediated by antibiosis (Assabgui et al., 1995). Similarly, there was a negative correlation between root DIMBOA content and the survival and performance of the western corn rootworm in seven inbred maize lines (Xie et al., 1992b). At the beginning of the twenty-first century, however, evidence accumulated that hydroxamic acids were not correlated with western corn rootworm resistance: after infestation of 19 maize lines with the western corn rootworm

in the field, Abel et al. (2000) did not find differences in root damage between lines differing in benzoxazinoid concentration. Direct evidence for tolerance of the western corn rootworm against benzoxazinoids recently came from Robert et al. (2012c) by using a 1,4-benzoxazin-3-one-deficient maize mutant and its parental wild-type line. The authors showed that the western corn rootworm larvae grew equally well over 24 h on the two plant genotypes. In addition, the presence of benzoxazinoids was needed by the larvae to localize the most nutritious roots, which also contained the highest amount of benzoxazinoids. All in all, these studies show no negative effect of a prominent secondary chemical on its specialized root herbivore. It remains an open question whether tolerance of the western corn rootworm to benzoxazinoids is a recently evolved counteradaptation against high benzoxazinoid levels in maize. Benzoxazinoids are known to reduce the nutritional value of plants by interfering with insect digestive enzymes (Houseman et al., 1992), and it is, for instance, possible that the western corn rootworm is able to compensate for these inhibitory effects, either by over-producing digestive enzymes or by detoxifying the secondary compounds.

Terpenoids show contrasting effects on root feeders. Genetically modified maize lines that constitutively emit (*E*)-β-caryophyllene and α-humulene showed similar root damage in the field compared to its parental line (Robert et al., 2013). Accordingly, the western corn rootworm emergence rate in the field was the same in transformed and untransformed plants. Moreover, the beetle larvae grew equally well on these plants under controlled conditions (Robert et al., 2013) and emergence was not affected by addition of (*E*)-β-caryophyllene in the lab (Rasmann et al., 2005). In contrast, the southern corn rootworm had a lower emergence rate from the constitutively emitting maize plants in the field, possibly because of a higher abundance of natural enemies (Robert et al., 2013). Taken together, these results provide no evidence for a direct negative effect of sesquiterpenes on the western corn rootworm. Evidence for a direct defensive function of terpenoids in roots recently came from thale cress (Vaughan et al., 2013): silencing the production of the semivolatile diterpene rhizathalene rendered the plants more susceptible to opportunistic fungus gnats (*Bradysia* spp.). Plant biomass was lower in the silenced plants compared to the parental wild-type and T-DNA insertion control when infested with fungus gnats. The addition of rhizathalene to semiartificial diet reduced diet consumption in a dose-dependent manner, indicating that plant resistance was at least partly mediated by antixenosis. As no information about the prevalence of root herbivores in thale cress in nature is available, the

ecological and evolutionary context of rhizathalene-mediated resistance remains to be determined.

Specialized leaf-feeding insects can sequester plant secondary metabolites to protect themselves against natural enemies (Opitz and Müller, 2009), and there is some evidence that root feeders are able to pursue similar strategies. The southern corn rootworm, for instance, can sequester cucurbitacins (Tallamy et al., 1998). The secondary metabolites protect larvae and eggs against the entomopathogenic fungus *Metarhizium anisopliae* (Tallamy et al., 1998). As discussed later, protection against soil microbes may be one of the most important advantages of toxin sequestration for root-feeding herbivores. How widespread this strategy is belowground remains to be determined.

In conclusion, roots produce a variety of secondary metabolites. Whereas the impact of such chemicals on food quality has been studied in detail in aboveground systems, there is surprisingly little evidence that they reduce the nutritional value of belowground tissues. This might partly be due to the small number of studies that explicitly tested this hypothesis. We found in total four metabolite classes that exhibit negative effects on belowground feeders, namely, phytoecdysteroids (Schmelz et al., 2002), cardenolides (Rasmann and Agrawal, 2011; Rasmann et al., 2011), hydroxycinnamic acids (Stevenson et al., 2009) and the diterpenoid rhizathalene (Vaughan et al., 2013). In contrast, we found no toxic or even beneficial effects of benzoxazinoids (Robert et al., 2012c) and total phenolics (Clark et al., 2011; Johnson et al., 2011). The role of glucosinolates remains ambiguous. Therefore, the long-standing assumption that secondary metabolites reduce food quality belowground remains largely untested and clearly deserves more attention. *In vitro* bioassays with pure compounds are only a first step to investigate the toxic function of a metabolite. As the toxicity of metabolites often depends on the surrounding matrix, an *in vivo* approach is necessary as well. In aboveground studies, two approaches have been widely used: on one hand, the abundance of a secondary metabolite between clones or species can be correlated to herbivore performance. This approach has also been employed belowground. However, many studies suffer from a low number of species or clones, thereby increasing the probability that covarying factors rather than the metabolites of interest are responsible for the observed pattern. On the other hand, genetic manipulation has successfully been employed in aboveground studies to verify the effect of secondary metabolites on herbivore performance. Notably, there have been only three studies using a mutant approach, providing much insight into the

presence (Vaughan et al., 2013) and absence (Robert et al., 2012c, 2013) of toxic effects of secondary metabolites on root feeders. A combination of these two approaches could greatly help to elucidate the role of chemicals for herbivore defence. Furthermore, experimental or observational evidence that belowground herbivores drive the evolution of toxic metabolites in nature, as was recently provided in aboveground systems (Agrawal et al., 2012; Prasad et al., 2012; Züst et al., 2012), is lacking. The lack of such data is also attributable to the fact that most of the studies investigated belowground pest on agricultural plants. Much insight into the importance of secondary metabolites in root-herbivore interactions could be gained by investigating the interaction of natural root-herbivore systems.

8. MICROBIAL INTERACTIONS

One important factor that sets the rhizosphere apart from the phyllosphere is the diversity and abundance of microorganisms: One gram of soil can contain up to 10^{11} individual bacteria, 50,000 bacterial species and 200 m of fungal hyphae (Van Der Heijden et al., 2008). Compared to these high concentrations, atmospheric microbe counts are modest and at least several orders of magnitude lower (10^4 individual bacteria per m^3) (Burrows et al., 2009). In contrast to leaf feeders, root feeders are therefore constantly exposed to a variety of microbes that live around, on and in the roots and that may influence the interaction. In the context of plant metabolites, soil microbes may play an important role in the following ways (Fig. 2.3):

- Microbial colonization of the roots may change the plant primary and secondary metabolism and thereby alter the root nutritional value for insects.
- Soil microbes may be transferred to the plant during feeding and influence the nutritional value of the plants.
- Soil microbes ingested by the root feeders may influence the gut microflora and affect the digestion and uptake of plant metabolites.
- Soil microbes at the root surface may metabolize soluble and volatile root exudates and alter root-herbivore recognition and foraging patterns.

Note that nematodes are sometimes also considered as microbes and can have a strong influence on root-herbivore interactions. However, as a number of recent reviews cover their effects in detail, they will not be discussed here (Hiltpold and Turlings, 2012; Turlings et al., 2012).

Several studies demonstrate that mycorrhizal fungi influence the performance of root-feeding herbivores. Infection of dandelion and strawberry

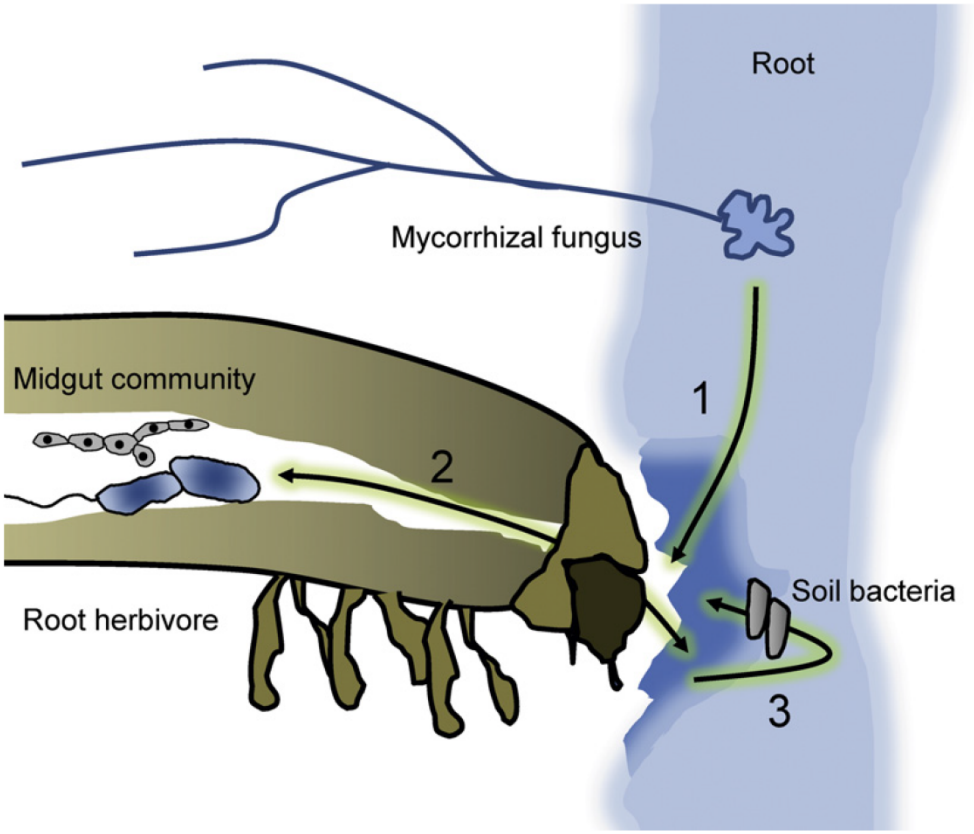

Figure 2.3 *Conceptual overview of the potential role of soil microbes in root-herbivore interactions*. 1. Soil microbes can colonize the plant and influence root herbivores by changing the plants metabolism or via direct interference. 2. Soil microbes in the gut of root herbivores may change the absorption and conversion of plant material. 3. Microbes may invade the wound sites of root-herbivore-attacked plants and thereby influence the interaction.

with the mycorrhizal fungus *Glomus mosseae* reduced the survival of black vine weevil larvae (Gange et al., 1994). Arbuscular mycorrhizal colonization of milkweeds reduced the colonization of the plants by opportunistic fungus gnats (*Bradysia* spp.) (Vannette and Rasmann, 2012). A protective effect of ectomycorrhizae against the black vine weevil larvae was also observed for the Russian larch (*Larix sibirica*) in a 3-year field study (Halldórsson et al., 2000). It remains to be determined whether the protective effect of the fungi is due to changes in the plants metabolism. *Glomus intraradices* colonization, for instance, increased concentrations of hydroxycinnamic acid amides and cyclohexenone derivatives in barley (*Hordeum vulgare*) plants (Peipp et al., 1997), and *G. intraradices* enhanced concentrations of amino and fatty acids as well as isoflavonoids in the roots of the barrel clover (*Medicago truncatula*) (Schliemann et al., 2008). It is, therefore, possible that mycorrhizal fungi

increase plant resistance by inducing the production of secondary compounds. However, in the absence of studies that go beyond the mere observation of herbivore performance, other explanations have to be taken into account as well. It is, for example, possible that fungal mycotoxins directly affect the root feeders or that the fungal hyphae alter root structure, morphology and exudation patterns in a way that influences their foraging behaviour. Interestingly, a recent study found that root herbivory by crane fly (*Tipula paludosa*) larvae increased the colonization of the common bent grass (*Agrostis capillaris*) by *G. mosseae* and *G. intraradices* (Currie et al., 2006), pointing to the intriguing possibility of feedback effects between the plant, the root herbivore and the mycorrhizal fungi.

The importance of tripartite interactions in the soil is further illustrated by series of studies conducted with the western corn rootworm. Kurtz et al. (2010) found that rootworm-attacked maize plants increased root colonization by the plant-pathogenic fungus *Fusarium verticillioides* 50-fold. At the same time, *F. verticillioides* infection slowed down larval development (Kurtz et al., 2010). When *F. verticillioides* spores were applied in low densities before rootworm attack, an increase in larval growth was observed, an effect that was attributed to potential positive effect of fungal colonization on root N levels (Kurtz et al., 2010). Interestingly, a follow-up study identified *Fusarium* spp. as the dominant fungal population in the gut of the western corn rootworm, irrespective of the soil type that was used (Dematheis et al., 2012a). This finding opens up the possibility that the western corn rootworm acts as a vector for the disease and suggests that the presence of *Fusarium* spp. in the system may determine both plant and insect performance. Just as with the mycorrhizal fungi, role of plant primary and secondary metabolites in the interaction remains to be determined. However, given how strongly maize roots respond to colonization by pathogens (Balmer et al., 2013), it is likely that *Fusarium*-induced changes in the primary and secondary plant metabolome will influence the behaviour and performance of the western corn rootworm. That root-herbivore behaviour is altered by the interactions between plants and fungal pathogens is demonstrated by a study on the black vine weevil and the entomopathogenic fungus *M. anisopliae* (Kepler and Bruck, 2006). In a two-choice setup, the vine weevil larvae did not show any preference for pots with or without the fungus. However, when host plants (*Picea abies*) were added to the system, the larvae preferred to orient towards host plants with the fungus (Kepler and Bruck, 2006), because of either an interaction of host orientation cues from the plant and the fungus or a fungus-mediated influence on plant metabolism.

Recent research on soil feedbacks demonstrates yet another route by which soil microorganisms influence root-herbivore interactions. Kostenko et al. (2012) found that wireworm (*A. lineatus*) attack of ragwort (*Jacobaea vulgaris*) led to significant changes in the soil fungal community. A second generation of plants cultivated in the conditioned soils had significantly lower concentrations of pyrrolizidine alkaloids (PAs) in the leaves. As PAs are partially synthesized in the roots, it is likely that the metabolic make-up of belowground tissues was also significantly altered by the soil feedback (Kostenko et al., 2012). Alterations of the fungal and bacterial soil microbial community were also found in the rhizosphere of western corn rootworm-attacked maize plants (Dematheis et al., 2012b). As many root herbivores develop over several years, microbe-mediated soil-legacy effects that influence plant secondary metabolites may be an important determinant for their performance and development, as well as final plant productivity (Sonnemann et al., 2013).

Given (i) their high abundance and (ii) their strong influence on herbivore fitness via plant-mediated effects, we propose here that root herbivores have evolved strategies to manage the soil microbial community to their own benefit. Soil-dwelling leaf-cutter ants, for instance, are known to cultivate fungi as a food source (Quinlan and Cherrett, 1979), and beewolf digger wasps (*Philanthus* spp.) protect their brood in the soil by transmitting streptomycetes that excrete protective antibiotic compounds (Kroiss et al., 2010). Root herbivores may use similar strategies, including the maintenance of a gut microbial flora that favours the digestion of root material and the protection of eggs and larvae with antimicrobial compounds. Until today, very little information is available about these possible 'management tools', and further research is clearly warranted.

9. CONCLUSIONS AND OUTLOOK

The current state of research on the role of plant primary and secondary metabolites in root-herbivore interactions highlights a number of adaptations that may enable root herbivores to survive in the soil environment.

First, an increasing number of studies clearly demonstrate that root herbivores possess an exquisite capacity to sense volatile and nonvolatile plant metabolites and use them to adjust their foraging decisions and feeding patterns. Foraging decisions are made both at a distance using volatile and exudate cues and during the feeding process itself. A major open question in this context is how the different cues are integrated spatially, temporally and

physiologically. Experiments using molecular approaches in relevant soil environments are likely to yield novel and interesting responses on root-herbivore orientation and foraging.

A second conclusion from our review is that root herbivores are likely to have adapted their digestive physiology to the poor quality of their food source. These adaptations include (i) tolerance to otherwise toxic plant secondary metabolites and (ii) digestive strategies that allow growth and development from diets that are poor in soluble carbohydrates and essential PUFAs. A set of relatively straightforward experiments, for example, using geometric analysis, could shed light on possible shifts in nutritional optima that accompanied the evolution of root-feeding behaviour. The knowledge gained in the process could then serve as a starting point for more detailed studies to pinpoint the mechanisms behind root-herbivore physiology.

A third point that is becoming evident from recent work is that soil microbes may influence the interaction between root-feeding insects and their host plants. The systems described so far are highly dynamic, with the herbivores vectoring or facilitating microbial colonization of the plants followed by feedbacks on their own performance. Although the earlier examples illustrate the potential of soil microbes to influence root-herbivore interactions by changing the metabolism of their host plants, current research efforts are barely scratching the surface of this topic. Understanding tripartite interactions should be a priority of future research, especially because they may be a defining feature of root-herbivore interactions *per se*. The results obtained from such studies could clearly point the way to a better grasp on evolutionary and ecological processes in the rhizosphere.

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REFERENCES

Abel, C.A., Berhow, M.A., Wilson, R.L., Binder, B.F., Hibbard, B.E., 2000. Evaluation of conventional resistance to European corn borer (Lepidoptera: Crambidae) and western corn rootworm (Coleoptera: Chrysomelidae) in experimental maize lines developed from a backcross breeding program. J. Econ. Entomol. 93, 1814–1821.

Agrawal, A.A., Hastings, A.P., Johnson, M.T.J., Maron, J.L., Salminen, J.P., 2012. Insect herbivores drive real-time ecological and evolutionary change in plant populations. Science 338, 113–116.

Agus, F., Handayani, E., Van Noordwijk, M., Idris, K., Sabiham, S., 2010. Root respiration interferes with peat CO_2 emission measurement. In: 19th World Congress of Soil Science, Soil Solutions for a Changing World, 2010.

Allsopp, P.G., 1992. Sugars, amino acids, and ascorbic acid as phagostimulants for larvae of *Antitrogus parvulus* and *Lepidiota negatoria* (Coleoptera: Scarabaeidae). J. Econ. Entomol. 85, 106–111.

Assabgui, R.A., Arnason, J.T., Hamilton, R.I., 1995. Field evaluations of hydroxamic acids as antibiosis factors in elite maize inbreds to the western corn-rootworm (Coleoptera, Chrysomelidae). J. Econ. Entomol. 88, 1482–1493.

Augustine, M.G., Fisk, F.W., Davidson, R.H., Lapidus, J.B., Cleary, R.W., 1964. Host-plant selection by the Mexican bean beetle, *Epilachna varivestis*. Ann. Entomol. Soc. Am. 57, 127–134.

Awmack, C.S., Leather, S.R., 2002. Host plant quality and fecundity in herbivorous insects. Annu. Rev. Entomol. 47, 817–844.

Badri, D.V., Quintana, N., El Kassis, E.G., Kim, H.K., Choi, Y.H., Sugiyama, A., Verpoorte, R., Martinoia, E., Manter, D.K., Vivanco, J.M., 2009. An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. Plant Physiol. 151, 2006–2017.

Badri, D.V., Chaparro, J.M., Manter, D.K., Martinoia, E., Vivanco, J.M., 2012. Influence of ATP-binding cassette transporters in root exudation of phytoalexins, signals and in disease resistance. Front. Plant Sci. 3, 149. http://dx.doi.org/10.3389/fpls.2012.00149.

Balmer, D., De Papajewski, D.V., Planchamp, C., Glauser, G., Mauch-Mani, B., 2013. Induced resistance in maize is based on organ-specific defence responses. Plant J. 74, 213–225.

Bartlet, E., Parsons, D., Williams, I.H., Clark, S.J., 1994. The influence of glucosinolates and sugars on feeding by the cabbage stem flea beetle, *Psylliodes chrysocephala*. Entomol. Exp. Appl. 73, 77–83.

Beck, S.D., 1957. The European corn borer, *Pyrausta nubilalis* (Hubn.) and its principal host plant. IV. Larval saccharotrophism and host plant resistance. Ann. Entomol. Soc. Am. 50, 247–250.

Bernays, E.A., Woodhead, S., 1982. Plant phenols utilized as nutrients by a phytophagous insect. Science 216, 201–203.

Bernklau, E.J., Bjostad, L.B., 1998. Reinvestigation of host location by western corn rootworm larvae (Coleoptera: Chrysomelidae): CO_2 is the only volatile attractant. J. Econ. Entomol. 91, 1331–1340.

Bernklau, E.J., Bjostad, L.B., 2005. Insecticide enhancement with feeding stimulants in corn for western corn rootworm larvae (Coleoptera: Chrysomelidae). J. Econ. Entomol. 98, 1150–1156.

Bernklau, E.J., Bjostad, L.B., 2008. Identification of feeding stimulants in corn roots for western corn rootworm (Coleoptera: Chrysomelidae) larvae. J. Econ. Entomol. 101, 341–351.

Bernklau, E.J., Bjostad, L.B., Hibbard, B.E., 2011. Synthetic feeding stimulants enhance insecticide activity against western corn rootworm larvae, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). J. Appl. Entomol. 135, 47–54.

Bigger, J.H., 1930. Notes on the life history of the clover root curculio, *Sitona hispidula* Fab., in Central Illinois. J. Econ. Entomol. 23, 334–342.

Birch, A.N.E., Griffiths, D.W., Hopkins, R.J., Macfarlane Smith, W.H.M., McKinlay, R.G., 1992. Glucosinolate responses of swede, kale, forage and oilseed rape to root damage by turnip root fly (*Delia floralis*) larvae. J. Sci. Food Agr. 60, 1–9.

Bjostad, L.B., Hibbard, B.E., 1992. 6-Methoxy-2-benzoxazolinone—a semiochemical for host location by western corn-rootworm larvae. J. Chem. Ecol. 18, 931–944.

Blossey, B., Hunt-Joshi, T.R., 2003. Belowground herbivory by insects: influence on plants and aboveground herbivores. Annu. Rev. Entomol. 48, 521–547.

Boldingh, H., Smith, G.S., Klages, K., 2000. Seasonal concentrations of non-structural carbohydrates of five *Actinidia* species in fruit, leaf and fine root tissue. Ann. Bot. 85, 469–476.

Braun, D.M., Ma, Y., Inada, N., Muszynski, M.G., Baker, R.F., 2006. Tie-dyed1 regulates carbohydrate accumulation in maize leaves. Plant Physiol. 142, 1511–1522.

Burrows, S., Elbert, W., Lawrence, M., Pöschl, U., 2009. Bacteria in the global atmosphere—part 1: review and synthesis of literature data for different ecosystems. Atmos. Chem. Phys. 9, 9263–9280.

Cakmak, I., Hengeler, C., Marschner, H., 1994. Partitioning of shoot and root dry matter and carbohydrates in bean plants suffering from phosphorus, potassium and magnesium deficiency. J. Exp. Bot. 45, 1245–1250.

Cannesan, M.A., Gangneux, C., Lanoue, A., Giron, D., Laval, K., Hawes, M., Driouich, A., Vicré-Gibouin, M., 2011. Association between border cell responses and localized root infection by pathogenic *Aphanomyces euteiches*. Ann. Bot. 108, 459–469.

Chapman, R.F., 2003. Contact chemoreception in feeding by phytophagous insects. Annu. Rev. Entomol. 48, 455–484.

Christenson, J.A., Hadwiger, L.A., 1973. Induction of pisatin formation in the pea foot region by pathogenic and nonpathogenic clones of *Fusarium solani*. Phytopathology 63, 784–790.

Clark, K.E., Hartley, S.E., Johnson, S.N., 2011. Does mother know best? The preference–performance hypothesis and parent–offspring conflict in aboveground–belowground herbivore life cycles. Ecol. Entomol. 36, 117–124.

Cocco, N., Glendinning, J.I., 2012. Not all sugars are created equal: some mask aversive tastes better than others in an herbivorous insect. J. Econ. Entomol. 215, 1412–1421.

Cole, R.A., 1987. Intensity of radicle fluorescence as related to the resistance of seedlings of lettuce to the lettuce root aphid and carrot to the carrot fly. Ann. Appl. Biol. 111, 629–639.

Cole, R.A., Riggall, W., Morgan, A., 1993. Electronically monitored feeding-behaviour of the lettuce root aphid (*Pemphigus bursarius*) on resistant and susceptible lettuce varieties. Entomol. Exp. Appl. 68, 179–185.

Crombie, A.C., Darrah, J.H., 1947. The chemoreceptors of the wireworm (*Agriotes* spp.) and the relation of activity to chemical constitution. J. Econ. Entomol. 24, 95–109.

Currie, A.F., Murray, P.J., Gange, A.C., 2006. Root herbivory by *Tipula paludosa* larvae increases colonization of *Agrostis capillaris* by arbuscular mycorrhizal fungi. Soil Biol. Biochem. 38, 1994–1997.

Dadd, R.H., 1961. The nutritional requirements of locusts. 5. Observations on essential fatty acids, chlorophyll, nutritional salt mixtures, and the protein or amino acid components of synthetic diets. J. Insect Physiol. 6, 126–145.

Dadd, R.H., 1973. Insect nutrition—current developments and metabolic implications. Annu. Rev. Entomol. 18, 381–420.

Dadd, R.H., 1977. Qualitative requirements and utilization of nutrients: insects. In: Rechcigl, M. (Ed.), Handbook Series in Nutrition and Food. CRC Press, Cleveland, OH, pp. 305–346.

Dawson, R.F., 1941. The localization of the nicotine synthetic mechanism in the tobacco plant. Science 94, 396–397.

Degenhardt, J., Hiltpold, I., Köllner, T.G., Frey, M., Gierl, A., Gershenzon, J., Hibbard, B.E., Ellersieck, M.R., Turlings, T.C.J., 2009. Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. Proc. Natl. Acad. Sci. U. S. A. 106, 13213–13218.

De Heer, C.J., Tallamy, D.W., 1991. Affinity of spotted cucumber beetle (Coleoptera: Chrysomelidae) larvae to cucurbitacins. Environ. Entomol. 20, 1173–1175.

Dematheis, F., Kurtz, B., Vidal, S., Smalla, K., 2012a. Microbial communities associated with the larval gut and eggs of the western corn rootworm. PLoS ONE 7, e44685.

Dematheis, F., Zimmerling, U., Flocco, C., Kurtz, B., Vidal, S., Kropf, S., Smalla, K., 2012b. Multitrophic interaction in the rhizosphere of maize: root feeding of western corn rootworm larvae alters the microbial community composition. PLoS ONE 7, e37288.

De Renobales, M., Ryan, R.O., Heisler, C.R., McLean, D.L., Blomquist, G.J., 1986. Linoleic acid biosynthesis in the pea aphid, *Acyrthosiphon pisum* (Harris). Arch. Insect Biochem. 3, 193–203.

Dethier, V.G., 1973. Electrophysiological studies of gustation in lepidopterous larvae. 2. Taste spectra in relation to food–plant discrimination. J. Comp. Physiol. 82, 103–134.

Doane, J.F., Lee, Y.W., Klingler, J., Westcott, N.D., 1975. The orientation response of *Ctenicera destructor* and other wire worms (Coleoptera: Elateridae) to germinating grain and to carbon dioxide. Can. Entomol. 107, 1233–1252.

Dobler, S., Petschenka, G., Pankoke, H., 2011. Coping with toxic plant compounds—the insect's perspective on iridoid glycosides and cardenolides. Phytochemistry 72, 1593–1604.

Dobler, S., Dalla, S., Wagschal, V., Agrawal, A.A., 2012. Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na, K-ATPase. Proc. Natl. Acad. Sci. U. S. A. 109, 13040–13045.

Douglas, A.E., 2003. The nutritional physiology of aphids. Adv. Insect Physiol. 31, 73–140.

Eilers, E.J., Talarico, G., Hansson, B.S., Hilker, M., Reinecke, A., 2012. Sensing the underground—ultrastructure and function of sensory organs in root-feeding *Melolontha melolontha* (Coleoptera: Scarabaeinae) larvae. PLoS ONE 7, e41357.

Erb, M., Flors, V., Karlen, D., De Lange, E., Planchamp, C., D'Alessandro, M., Turlings, T.C.J., Ton, J., 2009. Signal signature of aboveground-induced resistance upon belowground herbivory in maize. Plant J. 59, 292–302.

Esenther, G.R., Kirk, T.K., 1974. Catabolism of aspen sapwood in *Reticulitermes flavipes* (Isoptera-Rhinotermitidae). Ann. Entomol. Soc. Am. 67, 989–991.

Eshghi, S., Tafazoli, E., Dokhani, S., Rahemi, M., Emam, Y., 2007. Changes in carbohydrate contents in shoot tips, leaves and roots of strawberry (*Fragaria* × *ananassa* Duch.) during flower-bud differentiation. Sci. Horti. 113, 255–260.

Evans, W.A.L., Payne, D.W., 1964. Carbohydrases of the alimentary tract of the desert locust, *Schistocerca gregaria Forsk*. J. Insect Physiol. 10, 657–674.

Faucher, C.P., Hilker, M., De Bruyne, M., 2013. Interactions of carbon dioxide and food odours in Drosophila: olfactory hedonics and sensory neuron properties. PLoS ONE 8, e56361.

Finch, S., Skinner, G., 1974. Studies of the cabbage root fly. Report of the National Vegetable Research Station. National Vegetable Research Station, Wellesbourne.

Friend, W.G., 1958. Nutritional requirements of phytophagous insects. Annu. Rev. Entomol. 3, 57–74.

Frost, C.J., Hunter, M.D., 2008. Herbivore-induced shifts in carbon and nitrogen allocation in red oak seedlings. New Phytol. 178, 835–845.

Gange, A.C., Brown, V.K., Sinclair, G.S., 1994. Reduction of black vine weevil larval growth by vesicular-arbuscular mycorrhizal infection. Entomol. Exp. Appl. 70, 115–119.

Gaynor, D.L., Lane, G.A., Biggs, D.R., Sutherland, O.R.W., 1986. Measurement of grass grub resistance of bean in a controlled environment. New Zeal. J. Exp. Agr. 14, 77–82.

Gothilf, S., Beck, S.D., 1967. Larval feeding behaviour of cabbage looper *Trichoplusia ni*. J. Insect Physiol. 13, 1039–1053.

Guang, Y., Isenhour, D.J., Espelie, K.E., 1991. Activity of maize leaf cuticular lipids in resistance to leaf-feeding by the fall armyworm. Fla. Entomol. 74, 229–236.

Hackell, D.L., Gerard, P.J., 2004. Nodule preference by first instar clover root weevil. New Zeal. Plant Prot. 57, 319–322.
Halaweish, F.T., Tallamy, D.W., Santana, E., 1999. Cucurbitacins: a role in cucumber beetle steroid nutrition? J. Chem. Ecol. 25, 2373–2383.
Halldórsson, G., Sverrisson, H., Eyjólfsdóttir, G.G., Oddsdóttir, E.S., 2000. Ectomycorrhizae reduce damage to Russian Larch by *Otiorhyncus* larvae. Scand. J. Forest Res. 15, 354–358.
Hansen, G.K., 1977. Adaption to photosynthesis and diurnal oscillation of root respiration rates for *Lolium multiflorum*. Physiol. Plant. 39, 275–279.
Harada, K., Suomalainen, M., Uchida, H., Masui, H., Ohmura, K., Kiviranta, J., Niku-Paavola, M.L., Ikemoto, T., 2000. Insecticidal compounds against mosquito larvae from *Oscillatoria agardhii* strain 27. Environ. Toxicol. 15, 114–119.
Heron, R.J., 1965. Role of chemotactic stimuli in feeding behaviour of spruce budworm larvae on white spruce. Can. J. Zool. 43, 247–269.
Hiltpold, I., Turlings, T.C.J., 2008. Belowground chemical signaling in maize: when simplicity rhymes with efficiency. J. Chem. Ecol. 34, 628–635.
Hiltpold, I., Turlings, T.C.J., 2012. Manipulation of chemically mediated interactions in agricultural soils to enhance the control of crop pests and to improve crop yield. J. Chem. Ecol. 38, 641–650.
Hoffland, E., Boogaard, R.V.D., Nelemans, J., Findenegg, G., 1992. Biosynthesis and root exudation of citric and malic acids in phosphate-starved rape plants. New Phytol. 122, 675–680.
Hol, W.H.G., Vrieling, K., Veen, J.A.V., 2003. Nutrients decrease pyrrolizidine alkaloid concentrations in *Senecio jacobaea*. New Phytol. 158, 175–181.
Hopkins, R.J., Griffiths, D.W., Birch, A.N.E., McKinlay, R.G., Hall, J.E., 1993. Relationships between turnip root fly (*Delia floralis*) larval development and the sugar content of swede (*Brassica napus* ssp. *rapifera*) roots. Ann. Appl. Biol. 122, 405–415.
Hopkins, R.J., van Dam, N.M., van Loon, J.J.A., 2009. Role of glucosinolates in insect–plant relationships and multitrophic interactions. Annu. Rev. Entomol. 54, 57–83.
Horton, D., Guedot, C., Landolt, P., 2012. Identification of feeding stimulants for Pacific Coast wireworm by use of a filter paper assay (Coleoptera: Elateridae). J. Entomol. Soc. BC 109, 38–47.
House, H.L., 1961. Insect nutrition. Annu. Rev. Entomol. 6, 13–26.
Houseman, J.G., Campos, F., Thie, N.M.R., Philogene, B.J.R., Atkinson, J., Morand, P., Arnason, J.T., 1992. Effect of the maize-derived compounds DIMBOA and MBOA on growth and digestive processes of European corn-borer (Lepidoptera, Pyralidae). J. Econ. Entomol. 85, 669–674.
Hsiao, T.H., Fraenkel, G., 1968. Influence of nutrient chemicals on feeding behaviour of Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Am. 61, 44–54.
Hunter, M.D., 2001. Out of sight, out of mind: the impacts of root-feeding insects in natural and managed systems. Agr. Forest Entomol. 3, 3–9.
Ibrahim, L., Proe, M., Cameron, A., 1997. Main effects of nitrogen supply and drought stress upon whole-plant carbon allocation in poplar. Can. J. Forest Res. 27, 1413–1419.
Ito, T., 1960. Effect of sugars on feeding of larvae of the silkworm, *Bombyx mori*. J. Insect Physiol. 5, 95–107.
Jander, G., Howe, G., 2008. Plant interactions with arthropod herbivores: state of the field. Plant Physiol. 146, 801–803.
Jiang, M., Cheng, J., 2003. Feeding, oviposition and survival of overwintered rice water weevil (Coleoptera: Curculionidae) adults in response to nitrogen fertilization of rice at seedling stage. Appl. Entomol. Zool. 38, 543–549.

Joern, A., Behmer, S.T., 1997. Importance of dietary nitrogen and carbohydrates to survival, growth, and reproduction in adults of the grasshopper *Ageneotettix deorum* (Orthoptera: Acrididae). Oecologia 112, 201–208.

Johnson, S.N., Gregory, P.J., 2006. Chemically-mediated host-plant location and selection by root-feeding insects. Physiol. Entomol. 31, 1–13.

Johnson, S.N., Nielsen, U.N., 2012. Foraging in the dark—chemically mediated host plant location by belowground insect herbivores. J. Chem. Ecol. 38, 604–614.

Johnson, S.N., Gregory, P.J., Murray, P.J., Zhang, X., Young, I.M., 2004. Host plant recognition by the root feeding clover weevil, *Sitona lepidus* (Coleoptera: Curculionidae). Bull. Entomol. Res. 94, 433–439.

Johnson, S.N., Gregory, P.J., Greenham, J.R., Zhang, X., Murray, P.J., 2005. Attractive properties of an isoflavonoid found in white clover root nodules on the clover root weevil. J. Chem. Ecol. 31, 2223–2229.

Johnson, S.N., Crawford, J.W., Gregory, P.J., Grinev, D.V., Mankin, R.W., Masters, G.J., Murray, P.J., Wall, D.H., Zhang, X., 2007. Non-invasive techniques for investigating and modelling root-feeding insects in managed and natural systems. Agr. Forest Entomol. 9, 39–46.

Johnson, S.N., Barton, A.T., Clark, K.E., Gregory, P.J., McMenemy, L.S., Hancock, R.D., 2011. Elevated atmospheric carbon dioxide impairs the performance of root-feeding vine weevils by modifying root growth and secondary metabolites. Glob. Change Biol. 17, 688–695.

Kaplan, I., Halitschke, R., Kessler, A., Rehill, B.J., Sardanelli, S., Denno, R.F., 2008a. Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. Ecol. Lett. 11, 841–851.

Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S., Denno, R.F., 2008b. Constitutive and induced defenses to herbivory in above- and belowground plant tissues. Ecology 89, 392–406.

Kawakishi, S., Kaneko, T., 1987. Interaction of proteins with allyl isothiocyanate. J. Agric. Food Chem. 35, 85–88.

Kepler, R.M., Bruck, D.J., 2006. Examination of the interaction between the black vine weevil (Coleoptera: Curculionidae) and an entomopathogenic fungus reveals a new tritrophic interaction. Environ. Entomol. 35, 1021–1029.

Keskin, C., Kacar, S., 2012. Fatty acid composition of root and shoot samples of some *Astragalus* L. (Fabaceae) taxa growing in the east and southeast of Turkey. Turk. J. Biol. 37, 122–128.

Kimmerer, T.W., Potter, D.A., 1987. Nutritional quality of specific leaf tissues and selective feeding by a specialist leafminer. Oecologia 71, 548–551.

Koštál, V., 1992. Orientation behaviour of newly hatched larvae of the cabbage maggot, *Delia radicum* (L.) (Diptera: Anthomyiidae), to volatile plant metabolites. J. Insect Behav. 5, 61–70.

Kostenko, O., Van De Voorde, T.F.J., Mulder, P.P.J., Van Der Putten, W.H., Bezemer, T.M., 2012. Legacy effects of aboveground–belowground interactions. Ecol. Lett. 15, 813–821.

Kretzschmar, T., Kohlen, W., Sasse, J., Borghi, L., Schlegel, M., Bachelier, J.B., Reinhardt, D., Bours, R., Bouwmeester, H.J., Martinoia, E., 2012. A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature 483, 341–344.

Kroiss, J., Kaltenpoth, M., Schneider, B., Schwinger, M.G., Hertweck, C., Maddula, R.K., Strohm, E., Svatoš, A., 2010. Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nat. Chem. Biol. 6, 261–263.

Kurtz, B., Karlovsky, P., Vidal, S., 2010. Interaction between western corn rootworm (Coleoptera: Chrysomelidae) larvae and root-infecting Fusarium verticillioides. Environ. Entomol. 39, 1532–1538.

Ladd, T.L., 1986. Influence of sugars on the feeding response of Japanese beetles (Coleoptera: Scarabaeidae). J. Econ. Entomol. 79, 668–671.

Ladd, T.L., 1988. Japanese beetle (Coleoptera: Scarabaeidae)—influence of sugars on feeding response of larvae. J. Econ. Entomol. 81, 1390–1393.

Lane, G.A., Biggs, D.R., Russell, G.B., Sutherland, O.R.W., Williams, E.M., Maindonald, J.H., Donnell, D.J., 1985. Isoflavonoid feeding deterrents for *Costelytra zealandica*: structure–activity relationships. J. Chem. Ecol. 11, 1713–1735.

Lane, G.A., Sutherland, O.R.W., Skipp, R.A., 1987. Isoflavonoids as insect feeding deterrents and antifungal components from root of *Lupinus angustifolius*. J. Chem. Ecol. 13, 771–783.

Larkindale, J., Huang, B.R., 2004. Changes of lipid composition and saturation level in leaves and roots for heat-stressed and heat-acclimated creeping bentgrass (*Agrostis stolonifera*). Environ. Exp. Bot. 51, 57–67.

Lasker, R., Giese, A.C., 1956. Cellulose digestion by the silverfish *Ctenolepisma lineata*. J. Econ. Entomol. 33, 542–553.

Lee, K.P., Behmer, S.T., Simpson, S.J., Raubenheimer, D., 2002. A geometric analysis of nutrient regulation in the generalist caterpillar *Spodoptera littoralis* (Boisduval). J. Insect Physiol. 48, 655–665.

Liu, L., Punja, Z.K., Rahe, J.E., 1995. Effect of *Pythium* spp. and glyphosate on phytoalexin production and exudation by bean (*Phaseolus vulgaris* L.) roots grown in different media. Physiol. Mol. Plant Pathol. 47, 391–405.

Lyons, J.M., Lippert, L.F., 1966. Characterization of fatty acids from root and shoot lipids of *Capsicum* species. Lipids 1, 136–140.

Machado, R.A.R., Ferrieri, A.P., Robert, C.A.M., Glauser, G., Kallenbach, M., Baldwin, I.T., Erb, M., 2013. Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling. New Phytol., http://dx.doi.org/10.1111/nph.12438.

Malcolm, S.B., 1991. Cardenolide-mediated interactions between plants and herbivores. In: Rosenthal, G.A., Berenbaum, M.R. (Eds.), Herbivores: Their Interactions with Secondary Metabolites. Academic Press, San Diego, CA, pp. 251–296.

Martin, M.M., 1983. Cellulose digestion in insects. Comp. Biochem. Physiol. 75, 313–324.

Martin, M.M., 1991. The evolution of cellulose digestion in insects. Philos. Trans. R. Soc. B 333, 281–288.

Masters, G.J., Brown, V.K., Gange, A.C., 1993. Plant mediated interactions between aboveground and belowground insect herbivores. Oikos 66, 148–151.

Mathesius, U., 2001. Flavonoids induced in cells undergoing nodule organogenesis in white clover are regulators of auxin breakdown by peroxidase. J. Exp. Bot. 52, 419–426.

Mattson, W.J., 1980. Herbivory in relation to plant nitrogen content. Annu. Rev. Ecol. Syst. 11, 119–161.

Mazoir, N., Benharref, A., Bailén, M., Reina, M., González-Coloma, A., 2008. Bioactive triterpene derivatives from latex of two Euphorbia species. Phytochemistry 69, 1328–1338.

Mochizuki, A., Ishikawa, Y., Matsumoto, Y., 1989. Olfactory response of the larvae of the onion fly, *Hylemya antiqua* Meigen (Diptera, Anthomyiidae) to volatile compounds. Appl. Entomol. Zool. 24, 29–35.

Morgan, M.R.J., 1975. Qualitative survey of carbohydrases of alimentary-tract of migratory locust, *Locusta migratoria migratorioides*. J. Insect Physiol. 21, 1045–1053.

Murray, P.J., Hatch, D.J., Cliquet, J.B., 1996. Impact of insect root herbivory on the growth and nitrogen and carbon contents of white clover (*Trifolium repens*) seedlings. Can. J. Bot. 74, 1591–1595.

Neal, A.L., Ahmad, S., Gordon-Weeks, R., Ton, J., 2012. Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. PLoS ONE 7, e35498.

Newingham, B., Callaway, R., Bassirirad, H., 2007. Allocating nitrogen away from a herbivore: a novel compensatory response to root herbivory. Oecologia 153, 913–920.

Nielsen, J.K., Dalgaard, L., Larsen, L.M., Sørensen, H., 1979. Host plant selection of the horse-radish flea beetle *Phyllotreta armoraciae* (Coleoptera: Chrysomelidae): feeding responses to glucosinolates from several crucifers. Entomol. Exp. Appl. 25, 227–239.

Niemeyer, H.M., 2009. Hydroxamic acids derived from 2-hydroxy-2H-1,4-benzoxazin-3 (4H)-one: key defense chemicals of cereals. J. Agr. Food Chem. 57, 1677–1696.

Nordenhem, H., Nordlander, G., 1994. Olfactory oriented migration through soil by root-living *Hylobius abietis* (L.) larvae (Col., Curculionidae). J. Appl. Entomol. 117, 457–462.

Nutt, K.A., O'Shea, M.G., Allsopp, P.G., 2004. Feeding by sugarcane whitegrubs induces changes in the types and amounts of phenolics in the roots of sugarcane. Environ. Exp. Bot. 51, 155–165.

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., Browse, J., 1994. Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6, 147–158.

Opitz, S.W., Müller, C., 2009. Plant chemistry and insect sequestration. Chemoecology 19, 117–154.

Ouariti, O., Boussama, N., Zarrouk, M., Cherif, A., Ghorbal, M.H., 1997. Cadmium- and copper-induced changes in tomato membrane lipids. Phytochemistry 45, 1343–1350.

Peipp, H., Maier, W., Schmidt, J., Wray, V., Strack, D., 1997. Arbuscular mycorrhizal fungus-induced changes in the accumulation of secondary compounds in barley roots. Phytochemistry 44, 581–587.

Pierre, P.S., Dugravot, S., Cortesero, A.M., Poinsot, D., Raaijmakers, C.E., Hassan, H.M., Van Dam, N.M., 2012. Broccoli and turnip plants display contrasting responses to belowground induction by *Delia radicum* infestation and phytohormone applications. Phytochemistry 73, 42–50.

Pline, M., Dusenbery, D.B., 1987. Responses of plant-parasitic nematode *Meloidogyne incognita* to carbon dioxide determined by video camera-computer tracking. J. Chem. Ecol. 13, 873–888.

Potter, M.J., Davies, K., Rathjen, A.J., 1998. Suppressive impact of glucosinolates in *Brassica* vegetative tissues on root lesion nematode *Pratylenchus neglectus*. J. Chem. Ecol. 24, 67–80.

Potter, M.J., Vanstone, V.A., Davies, K.A., Rathjen, A.J., 2000. Breeding to increase the concentration of 2-phenylethyl glucosinolate in the roots of *Brassica napus*. J. Chem. Ecol. 26, 1811–1820.

Prasad, K.V.S.K., Song, B.H., Olson-Manning, C., Anderson, J.T., Lee, C.R., Schranz, M.E., Windsor, A.J., Clauss, M.J., Manzaneda, A.J., Naqvi, I., Reichelt, M., Gershenzon, J., Rupasinghe, S.G., Schuler, M.A., Mitchell-Olds, T., 2012. A gain-of-function polymorphism controlling complex traits and fitness in nature. Science 337, 1081–1084.

Prins, R.A., Kreulen, D.A., 1991. Comparative aspects of plant-cell wall digestion in insects. Anim. Feed Sci. Technol. 32, 101–118.

Quinlan, R.J., Cherrett, J.M., 1979. The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). Ecol. Entomol. 4, 151–160.

Radcliffe, J.E., 1970. Some effects of grass grub (*Costelytra zealandica* (White)) larvae on pasture plants. New Zeal. J. Agr. Res. 13, 87–104.

Ramsewak, R.S., Nair, M.G., Murugesan, S., Mattson, W.J., Zasada, J., 2001. Insecticidal fatty acids and triglycerides from *Dirca palustris*. J. Agr. Food Chem. 49, 5852–5856.

Rapport, E.W., Stanley-Samuelson, D., Dadd, R.H., 1984. Ten generations of *Drosophila melanogaster* reared axenically on a fatty-acid-free holidic diet. Arch. Insect Biochem. 1, 243–250.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., Meijer, J., 2000. Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol. Biol. 42, 93–113.

Rasmann, S., Agrawal, A.A., 2008. In defense of roots: a research agenda for studying plant resistance to belowground herbivory. Plant Physiol. 146, 875–880.

Rasmann, S., Agrawal, A.A., 2011. Evolution of specialization: a phylogenetic study of host range in the red milkweed beetle (*Tetraopes tetraophthalmus*). Am. Nat. 177, 728–737.

Rasmann, S., Köllner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J., Turlings, T.C.J., 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434, 732–737.

Rasmann, S., Erwin, A.C., Halitschke, R., Agrawal, A.A., 2011. Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. J. Ecol. 99, 16–25.

Raubenheimer, D., 1992. Tannic acid, protein, and digestible carbohydrate—dietary imbalance and nutritional compensation in locusts. Ecology 73, 1012–1027.

Reinecke, A., Müller, F., Hilker, M., 2008. Attractiveness of CO_2 released by root respiration fades on the background of root exudates. Basic App. Ecol. 9, 568–576.

Robert, C.A.M., Erb, M., Duployer, M., Zwahlen, C., Doyen, G.R., Turlings, T.C.J., 2012a. Herbivore-induced plant volatiles mediate host selection by a root herbivore. New Phytol. 194, 1061–1069.

Robert, C.A.M., Erb, M., Hibbard, B.E., Wade French, B., Zwahlen, C., Turlings, T.C.J., 2012b. A specialist root herbivore reduces plant resistance and uses an induced plant volatile to aggregate in a density-dependent manner. Funct. Ecol. 26, 1429–1440.

Robert, C.A.M., Veyrat, N., Glauser, G., Marti, G., Doyen, G.R., Villard, N., Gaillard, M.D.P., Köllner, T.G., Giron, D., Body, M., Babst, B.A., Ferrieri, R.A., Turlings, T.C.J., Erb, M., 2012c. A specialist root herbivore exploits defensive metabolites to locate nutritious tissues. Ecol. Lett. 15, 55–64.

Robert, C.A.M., Erb, M., Hiltpold, I., Hibbard, B.E., Gaillard, M.D.P., Bilat, J., Degenhardt, J., Cambet-Petit-Jean, X., Turlings, T.C.J., Zwahlen, C., 2013. Genetically engineered maize plants reveal distinct costs and benefits of constitutive volatile emissions in the field. Plant Biotechnol. J. 11, 628–639.

Rondon, S.I., 2010. The potato tuberworm: a literature review of its biology, ecology, and control. Am. J. Potato Res. 87, 149–166.

Rössler, M.E., 1961. Ernährungsphysiologische Untersuchungen an Scarabaeidenlarven (*Oryctes nasicornis* L., *Melolontha melolontha* L.). J. Insect Physiol. 6, 62–74.

Rudrappa, T., Czymmek, K.J., Paré, P.W., Bais, H.P., 2008. Root-secreted malic acid recruits beneficial soil bacteria. Plant Physiol. 148, 1547–1556.

Russell, G.B., Sutherland, O.R.W., Hutchins, R.F.N., Christmas, P.E., 1978. Vestitol: a phytoalexin with insect feeding-deterrent activity. J. Chem. Ecol. 4, 571–579.

Russell, G.B., Shaw, G.J., Christmas, P.E., Yates, M.B., Sutherland, O.R.W., 1984. Two 2-arylbenzofurans as insect feeding deterrents from sainfoin (*Onobrychis viciifolia*). Phytochemistry 23, 1417–1420.

Schliemann, W., Ammer, C., Strack, D., 2008. Metabolite profiling of mycorrhizal roots of *Medicago truncatula*. Phytochemistry 69, 112–146.

Schmelz, E.A., Grebenok, R.J., Galbraith, D.W., Bowers, W.S., 1998. Damage-induced accumulation of phytoecdysteroids in spinach: a rapid root response involving the octadecanoic acid pathway. J. Chem. Ecol. 24, 339–360.

Schmelz, E.A., Grebenok, R.J., Ohnmeiss, T.E., Bowers, W.S., 2002. Interactions between *Spinacia oleracea* and *Bradysia impatiens*: a role for phytoecdysteroids. Arch. Insect Biochem. 51, 204–221.

Schoonhoven, L.M., 1974. What makes a caterpillar eat? The sensory codes underlying feeding behaviour. In: Chapman, R.F., Bernays, E.A., Stoffolano, J.G. (Eds.), Advances in Chemoreception and Behaviour. Springer, New York, pp. 69–97.

Schröder, R., Hilker, M., 2008. The relevance of background odor in resource location by insects: a behavioural approach. Bioscience 58, 308–316.

Shanbhag, S.R., Müller, B., Steinbrecht, R.A., 1999. Atlas of olfactory organs of *Drosophila melanogaster*. 1. Types, external organization, innervation and distribution of olfactory sensilla. Int. J. Insect Morphol. 28, 377–397.

Shanks, C.H., Doss, R.P., 1987. Feeding responses by adults of five species of weevils (Coleoptera: Curculionidae) to sucrose and sterols. Ann. Entomol. Soc. Am. 80, 41–46.

Shields, V.D.C., Mitchell, B.K., 1995. The effects of phagostimulant mixtures on deterrent receptor(s) in two crucifer-feeding lepidopterous species. Philos. Trans. R. Soc. B 347, 459–464.

Slansky, F., Feeny, P., 1977. Stabilization of rate of nitrogen accumulation by larvae of cabbage butterfly on wild and cultivated food plants. Ecol. Monogr. 47, 209–228.

Soni, S., Finch, S., 1979. Laboratory evaluation of sulphur-bearing chemicals as attractants for larvae of the onion fly, *Delia antiqua* (Meigen)(Diptera: Anthomyiidae). Bull. Entomol. Res. 69, 291–298.

Sonnemann, I., Hempel, S., Beutel, M., Hanauer, N., Reidinger, S., Wurst, S., 2013. The root herbivore history of the soil affects the productivity of a grassland plant community and determines plant response to new root herbivore attack. PLoS ONE 8, e56524.

Soo Hoo, C.F., Dudzinski, A., 1967. Digestion by the larva of the pruinose scarab, *Sericesthis geminata*. Entomol. Exp. Appl. 10, 7–15.

Städler, E., 1971. Über die Orientierung und das Wirtswahlverhalten der Möhrenfliege, *Psila rosae* F. (Diptera: Psilidae) I. Larven. Z. Angew. Entomol. 69, 425–438.

Stevenson, P.C., Turner, H.C., Haware, M.P., 1997. Phytoalexin accumulation in the roots of chickpea (*Cicer arietinum* L.) seedlings associated with resistance to fusarium wilt (*Fusarium oxysporum* f.sp. *ciceri*). Physiol. Mol. Plant P 50, 167–178.

Stevenson, P.C., Muyinza, H., Hall, D.R., Porter, E.A., Farman, D.I., Talwana, H., Mwanga, R.O.M., 2009. Chemical basis for resistance in sweetpotato *Ipomoea batatas* to the sweetpotato weevil *Cylas puncticollis*. Pure Appl. Chem. 81, 141–151.

Stout, M.J., 2013. Reevaluating the conceptual framework for applied research on host-plant resistance. Insect Sci. 20, 263–272.

Sutherland, O.R.W., 1971. Feeding behaviour of grass grub *Costelytra zealandica* (White) (Coleoptera: Melolonthinae)—1. Influence of carbohydrates. New Zeal. J. Sci. 14, 18–24.

Sutherland, O.R.W., 1983. The influence of amino-acids and inorganic salts on feeding by larvae of *Heteronychus arator* (Coleoptera: Scarabaeidae). New Zeal. J. Zool. 10, 117–119.

Sutherland, O.R.W., Hillier, J.R., 1973. Feeding behaviour of the grass hub *Costelytra zealandica* (White) (Coleoptera: Melolonthinae). 3: the influence of amino acids, ascorbic acid, and inorganic salts. New Zeal. J. Zool. 1, 211–216.

Sutherland, O.R.W., Hillier, J.R., 1976. The influence of maltose and other carbohydrates on the feeding behaviour of *Heteronychus arator* (Scarabaeidae: Coleoptera). Experientia 32, 701–702.

Sutherland, O.R.W., Russell, G.B., Biggs, D.R., Lane, G.A., 1980. Insect feeding deterrent activity of phytoalexin isoflavonoids. Biochem. Syst. Ecol. 8, 73–75.

Sutherland, O.R.W., Hutchins, R., Greenfield, W., 1982. Effect of lucerne saponins and Lotus condensed tannins on survival of grass grub, *Costelytra zealandica*. New Zeal. J. Zool. 9, 511–514.

Tallamy, D.W., Whittington, D.P., Defurio, F., Fontaine, D.A., Gorski, P.M., Gothro, P.W., 1998. Sequestered cucurbitacins and pathogenicity of *Metarhizium*

anisopliae (Moniliales: Moniliaceae) on spotted cucumber beetle eggs and larvae (Coleoptera: Chrysomelidae). Environ. Entomol. 27, 366–372.

Taylor, E.C., 1985. Cellulose digestion in a leaf eating insect, the Mexican bean beetle, *Epilachna varivestis*. Insect Biochem. 15, 315–320.

Thorpe, W.H., Crombie, A.C., Hill, R., Darrah, J.H., 1947. The behaviour of wireworms in response to chemical stimulation. J. Econ. Entomol. 23, 234–266.

Treves, D.S., Martin, M.M., 1994. Cellulose digestion in primitive hexapods—effect of ingested antibiotics on gut-microbial populations and gut cellulase levels in the firebrat, *Thermobia domestica* (Zygentoma, Lepismatidae). J. Chem. Ecol. 20, 2003–2020.

Tsuneyama, I., Ikejima, S., Iida, N., Tsurui, Y., Tanaka, Y., Ninagi, O., Oshiki, T., 2005. Combined effect of calcium carbonate and sugar on the feeding behaviour of the newly hatched larvae of the silkworm, *Bombyx mori*. J. Seric. Sci. Jpn. 74, 9–14.

Turlings, T.C.J., Hiltpold, I., Rasmann, S., 2012. The importance of root-produced volatiles as foraging cues for entomopathogenic nematodes. Plant Soil 358, 51–60.

Van Dam, N.M., 2009. Belowground herbivory and plant defenses. Annu. Rev. Ecol. Evol. Syst. 40, 373–391.

Van Der Heijden, M.G.A., Bardgett, R.D., Van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Lett. 11, 296–310.

Van Leur, H., Raaijmakers, C.E., Van Dam, N.M., 2008. Reciprocal interactions between the cabbage root fly (*Delia radicum*) and two glucosinolate phenotypes of *Barbarea vulgaris*. Entomol. Exp. Appl. 128, 312–322.

Vannette, R.L., Rasmann, S., 2012. Arbuscular mycorrhizal fungi mediate below-ground plant-herbivore interactions: a phylogenetic study. Funct. Ecol. 26, 1033–1042.

Vaughan, M.M., Wang, Q., Webster, F.X., Kiemle, D., Hong, Y.J., Tantillo, D.J., Coates, R.M., Wray, A.T., Askew, W., O'Donnell, C., Tokuhisa, J.G., Tholl, D., 2013. Formation of the unusual semivolatile diterpene rhizathalene by the *Arabidopsis* class I terpene synthase TPS08 in the root stele is involved in defense against below-ground herbivory. Plant Cell 25, 1108–1125.

Watts, S.M., Dodson, C.D., Reichman, O.J., 2011. The roots of defense: plant resistance and tolerance to belowground herbivory. PLoS ONE 6, e18463.

Way, M.O., Reay-Jones, F.P.F., Stout, M.J., Tarpley, L., 2006. Effects of nitrogen fertilizer applied before permanent flood on the interaction between rice and rice water weevil (Coleoptera: Curculionidae). J. Econ. Entomol. 99, 2030–2037.

Weissteiner, S., Schütz, S., 2006. Are different volatile pattern influencing host plant choice of belowground living insects? M. D. Gesell. Allg. Ange. Entomol. 15, 51–55.

Weissteiner, S., Huetteroth, W., Kollmann, M., Weißbecker, B., Romani, R., Schachtner, J., Schütz, S., 2012. Cockchafer larvae smell host root scents in soil. PLoS ONE 7, e45827.

Wensler, R.J., Dudzinski, A.E., 1972. Gustation of sugars, amino acids and lipids by larvae of the scarabaeid, *Sericesthis geminata* (Coleoptera). Entomol. Exp. Appl. 15, 155–165.

Wharton, D.R.A., Wharton, M.L., 1965. The cellulase content of various species of cock-roaches. J. Insect Physiol. 11, 1401–1405.

Xie, Y.S., Arnason, J.T., Philogene, B.J.R., Lambert, J.D.H., Atkinson, J., Morand, P., 1990. Role of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in the resistance of maize to western corn-rootworm, *Diabrotica virgifera virgifera* (LeConte) (Coleoptera, Chrysomelidae). Can. Entomol. 122, 1177–1186.

Xie, Y.S., Arnason, J.T., Philogene, B.J.R., Atkinson, J., Morand, P., 1992a. Behavioural responses of western corn rootworm larvae to naturally occurring and synthetic hydroxamic acids. J. Chem. Ecol. 18, 945–957.

Xie, Y.S., Arnason, J.T., Philogene, B.J.R., Olechowski, H.T., Hamilton, R.I., 1992b. Variation of hydroxamic acid content in maize roots in relation to geographic origin of maize

germ plasm and resistance to western corn-rootworm (Coleoptera, Chrysomelidae). J. Econ. Entomol. 85, 2478–2485.

Yang, G., Wiseman, B.R., Espelie, K.E., 1992. Cuticular lipids from silks of 7 corn genotypes and their effect on development of corn-earworm larvae *Helicoverpa zea* (Boddie). J. Agr. Food Chem. 40, 1058–1061.

Yang, M., Zheng, G., Zhang, F., Xu, Y., 2006. FAD2-silencing has pleiotropic effect on polar lipids of leaves and varied effect in different organs of transgenic tobacco. Plant Sci. 170, 170–177.

Zeeman, S.C., Rees, T.A., 1999. Changes in carbohydrate metabolism and assimilate export in starch-excess mutants of *Arabidopsis*. Plant Cell Environ. 22, 1445–1453.

Zhen, Y., Aardema, M.L., Medina, E.M., Schumer, M., Andolfatto, P., 2012. Parallel molecular evolution in an herbivore community. Science 337, 1634–1637.

Zinkler, D., Goetze, M., Fabian, K., 1986. Cellulose digestion in primitive insects Apterygota and oribatid mites. Zool. Beitr. 30, 17–28.

Züst, T., Heichinger, C., Grossniklaus, U., Harrington, R., Kliebenstein, D.J., Turnbull, L.A., 2012. Natural enemies drive geographic variation in plant defenses. Science 338, 116–119.

3.2 Manuscript II

Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (*Taraxacum officinale* agg.)

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Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (*Taraxacum officinale* agg.)

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ABSTRACT

The secondary metabolites in the roots, leaves and flowers of the common dandelion (*Taraxacum officinale* agg.) have been studied in detail. However, little is known about the specific constituents of the plant's highly specialized laticifer cells. Using a combination of liquid and gas chromatography, mass spectrometry and nuclear magnetic resonance spectrometry, we identified and quantified the major secondary metabolites in the latex of different organs across different growth stages in three genotypes, and tested the activity of the metabolites against the generalist root herbivore *Diabrotica balteata*. We found that common dandelion latex is dominated by three classes of secondary metabolites: phenolic inositol esters (PIEs), triterpene acetates (TritAc) and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G). Purification and absolute quantification revealed concentrations in the upper mg g^{-1} range for all compound classes with up to 6% PIEs, 5% TritAc and 7% TA-G per gram latex fresh weight. Contrary to typical secondary metabolite patterns, concentrations of all three classes increased with plant age. The highest concentrations were measured in the main root. PIE profiles differed both quantitatively and qualitatively between plant genotypes, whereas TritAc and TA-G differed only quantitatively. Metabolite concentrations were positively correlated within and between the different compound classes, indicating tight biosynthetic co-regulation. Latex metabolite extracts strongly repelled *D. balteata* larvae, suggesting that the latex constituents are biologically active.

1. Introduction

Plants produce numerous secondary metabolites with current estimates exceeding 200,000 individual structures (Dixon and Strack, 2003). Many of these metabolites serve ecological functions, including herbivore resistance (Mithöfer and Boland, 2012). As a prerequisite for their functional analysis, the identification and quantification of secondary metabolites remains a major bottleneck in chemical ecology.

Due to their biological activity, many plant secondary metabolites are produced and/or stored in specialized cells, cellular compartments or organs, including vacuoles, trichomes and resin ducts (Dell and McComb, 1979; Fahn, 1988; Levin, 1973; Wink, 1993). Laticifers are among the most common secondary metabolite reservoirs, being produced by over 10% of all land plants (Farrell et al., 1991; Lewinsohn, 1991; Metcalfe, 1967). The sap that is released from laticifers upon cell rupture is referred to as latex (Farrell et al., 1991; Metcalfe, 1967). Latex typically contains high concentrations of toxic and sometimes sticky metabolites (reviewed in Agrawal and Konno, 2009; Konno, 2011) which exude from wounds made by attacking herbivores. Therefore, latex is widely accepted to be defensive against herbivores (Agrawal, 2005; Agrawal and Konno, 2009).

The common dandelion (*Taraxacum officinale* agg. Flora Helvetica 5th edition) possesses laticifers in almost all of its organs, including the main and side roots, leaves, flower stalk, involucre and pappus. The common dandelion is described as a species complex that consists of diploid outcrossing plants and a large number

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of apomictic clones, which produce clonal seeds (Kirschner and Stepanek, 1994). It is native to Eurasia, but has recently been introduced into new habitats across the globe (Richards, 1973). The plant is used as a natural remedy against gastrointestinal ailments and is of recent interest because of its capacity to produce rubber when hybridized with *Taraxacum koksaghyz* (Post et al., 2012; Schmidt et al., 2010).

The chemical composition of common dandelion leaves, flowers and roots has been studied extensively (Schütz et al., 2006). Prominent constituents in roots include sesquiterpene lactones such as taraxinic acid β-D-glucopyranosyl ester (Hänsel et al., 1980; Kisiel and Barszcz, 2000), triterpenes and their acetate derivatives such as α- and β-amyrin (Akashi et al., 1994; Burrows and Simpson, 1938), several phenolic acids, including chicoric acid and flavonoids (Clifford et al., 1987; Schütz et al., 2005), as well as the recently characterized 4-hydroxyphenylacetate inositol esters (Kenny et al., 2014). However, detailed information about the composition and abundance of secondary metabolites in the latex is lacking. It therefore remains unclear which of the above compounds are produced by non-specialized cells and which ones accumulate specifically in the latex. A detailed phytochemical characterization of common dandelion latex is important to study the ecological role of laticifers and may help to exploit common dandelion as a natural biofactory.

Based on these considerations, we aimed at identifying and quantifying the major secondary metabolites of *T. officinale* latex. We screened latex extracts by LC–MS, LC–UV, GC–MS, and NMR and established an HPLC-UV and GC-FID based quantification method for the three dominant compound classes. We then used these methods to evaluate variation between different organs, growth stages and common dandelion genotypes. Finally, we performed bioassays with latex and latex extracts using root-feeding larvae of the generalist herbivore beetle *Diabrotica balteata* (Coleoptera, Chrysomelidae, LeConte) to test the bioactivity of *T. officinale* latex.

2. Results and discussion

2.1. Structural elucidation and quantification of latex secondary metabolites

To characterize the major latex metabolites, we first analyzed a MeOH extract from latex collected from the main roots of common dandelions with HPLC coupled to a PDA detector and an ESI–ion

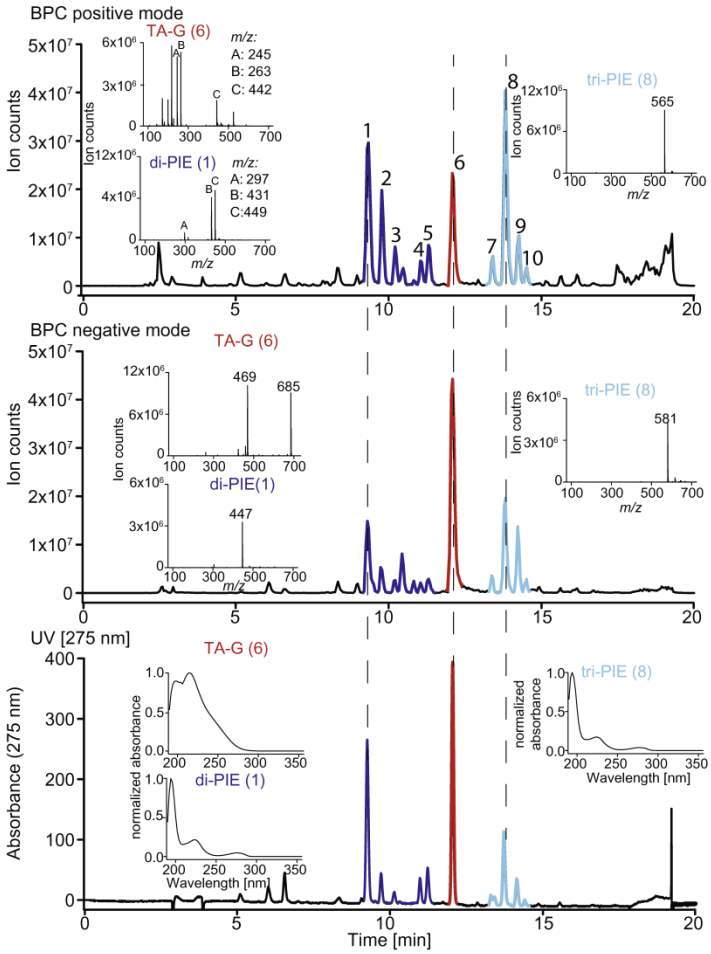

Fig. 1. Base peak chromatograms (BPC) in positive and negative mode and UV trace (275 nm) of a common dandelion latex MeOH extract with mass spectra and corresponding UV spectra of selected metabolites as insets. Two compound classes dominated the latex MeOH profile: phenolic inositol esters (PIE) with two (di-) or three (tri-) 4-hydroxyphenylacetic acid side groups, and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G). Peak numbers correspond to the compound numbers in bold throughout the manuscript.

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trap mass spectrometer. The PDA data revealed the presence of two metabolite classes with diagnostic UV spectra (Fig. 1, Table S1). Using a combination of ion trap-MS/MS spectra, high resolution (Q-TOF) mass spectra and literature comparisons, we identified the two compound classes as di- and tri-4-hydroxyphenylacetate inositol esters (PIEs) and sesquiterpene lactone glycosides (Fig. 1,

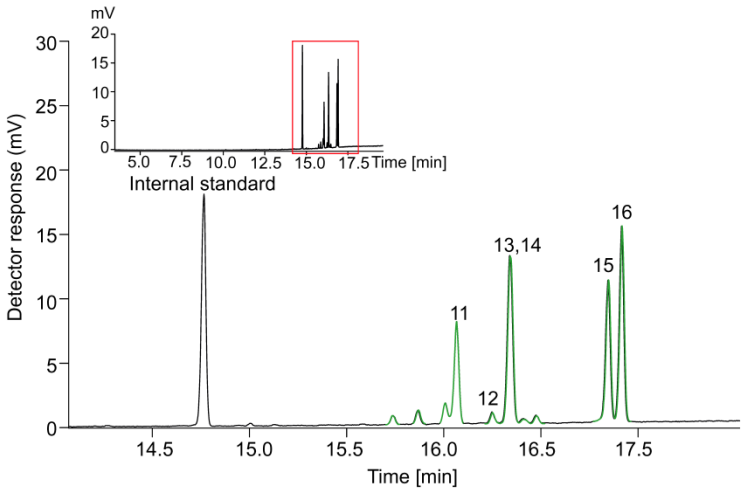

Fig. 2. GC-FID chromatogram of a common dandelion latex hexane extract containing 100 μg ml^{-1} cholesteryl acetate as internal standard. All detected analytes were identified as triterpene acetates. Inset shows an extended chromatogram. Peak numbers correspond to the compound numbers in bold throughout the manuscript.

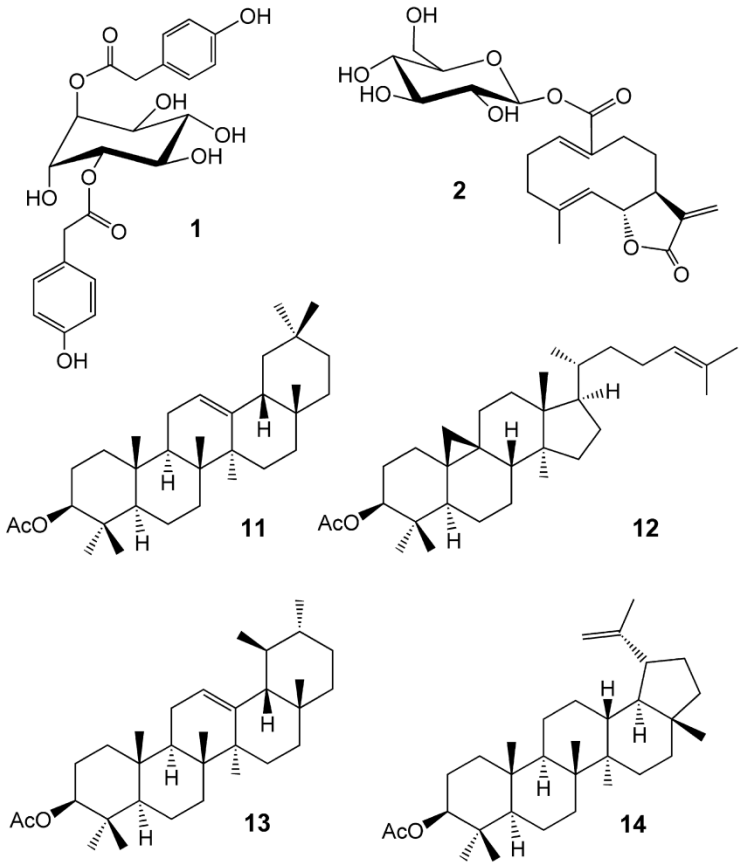

Fig. 3. Chemical structures of latex secondary metabolites from *Taraxacum officinale*. **1** = 1, 5 substituted di-PIE; **2** = taraxinic acid β-D-glucopyranosyl ester; **11** = β-amyrin acetate; **12** = cycloartenol acetate; **13** = α-amyrin acetate; **14** = lupeol acetate.

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Table S1). The major PIE (**1**) was isolated and identified as a 1, 5 substituted di-ester identical to that previously described (Michalska et al., 2010) based on MS, high resolution MS, ^{1}H NMR and UV data (Fig. 3). These are listed along with ^{13}C NMR data (Fig. S1, Tables S1 and S2). The chemical structures of the remaining di- (**2**–**5**) and tri- (**7**–**10**) PIEs were not further determined, but their MS, high resolution MS and UV data and chromatographic retention times were documented (Table S1). The sesquiterpene lactone was identified by MS and NMR as taraxinic acid β-D-glucopyranosyl ester (TA-G, **6**) (Fig. 3, Fig. S2, Tables S1 and S3), a compound previously identified from common dandelion root extracts (Hänsel et al., 1980). While sesquiterpene lactones are frequent across the Asteraceae (Huo et al., 2008; Wu et al., 2002, 2011), PIEs have been exclusively reported from *Taraxacum* and species of the closely related genus *Lactuca* (Kenny et al., 2014; Michalska et al., 2010; Zidorn et al., 1999).

To study the less polar constituents, we extracted *T. officinale* latex with hexane and analyzed it by GC–MS. All detected analytes showed a molecular ion with m/z = 468 and very similar fragmentation patterns. They were identified as triterpene acetates (TritAc) (Fig. 2) by comparisons of retention times and fragmentation patterns with those of standards. Compounds included β-amyrin acetate (**11**) and cycloartenol acetate (**12**), as well as α-amyrin acetate (**13**) and lupeol acetate (**14**), which coeluted (Fig. 3). The two other peaks were identified as TritAc (**15** and **16**), but the structures were not further characterized. The presence of triterpene acetates in common dandelion roots has been described previously (Akashi et al., 1994).

2.2. Quantification of latex secondary metabolites

To quantify PIEs, TA-G and TritAc, standard curves were established using purified PIEs, TA-G and synthetic lupeol acetate. Each of the three compound classes, PIEs, TA-G and TritAc, was found to account for 5–7% of the latex fresh mass. Together, they represented over 18% of the latex fresh mass (Fig. 4a). TA-G was the most abundant single metabolite with concentrations of up to 70 μg mg^{-1} (Fig. 4b). High concentrations of secondary metabolites are typical for specialized defensive organs including laticifers, glandular trichomes and resin ducts. For example, isoquinoline alkaloids account for 20% of the latex fresh mass of *Chelidonium majus* (Papaveraceae) (Tome and Colombo, 1995), and sesquiterpene lactones for 14% of the latex fresh mass of *Lactuca sativa* (Asteraceae) (Sessa et al., 2000). Unlike resin ducts, where metabolites are secreted into extracellular spaces, latex metabolites are stored in the specialized cytoplasm of laticifer cells, which must be adapted to high concentrations of potentially phytotoxic compounds. Mechanisms of adaptation include the compartmentalization of metabolites in vacuoles (Wink, 1993; Yamaki, 1984) or vesicles (Otani et al., 2005). The highly hydrophobic rubber molecules for instance are enclosed in a monolayer membrane in the laticifer cytosol (Cornish et al., 1999; Schmidt et al., 2010; Wood and Cornish, 2000). It remains to be determined if and how secondary metabolites are compartmentalized within *T. officinale* laticifers.

2.3. Organ and age specificity

2.3.1. Abundance, but not composition is affected by plant organ and time

To elucidate the influence of organ, age and plant genotype on latex secondary metabolites, we analyzed extracts from main roots, side roots, petioles, flower stalk and involucre from three randomly selected common dandelion genotypes from a collection of clones originating from a north–south transect (henceforth called 2.8A from Ostbevern in Germany, 4.3A from Mühlheim am Main in Germany and 20.3B from Haernoesand in Sweden) at different growth stages from 5 to 15 weeks. The abundance, but not the composition of the latex metabolites changed with plant organ and age (Fig. 5). Latex from the main roots contained the highest concentrations of secondary metabolites, while the other organs did not significantly differ among each other (Fig. 5). This pattern was more pronounced for TA-G and TritAc than for PIEs. Overall, metabolite concentrations in the latex increased with plant age. Interestingly, the concentration of TA-G and of the major di-PIE (**1**) decreased at the onset of flowering. Furthermore, the abundance of one minor unidentified triterpene acetate (TritAc A, retention time = 17.3 min, Fig. 5b lower panel) decreased with plant age.

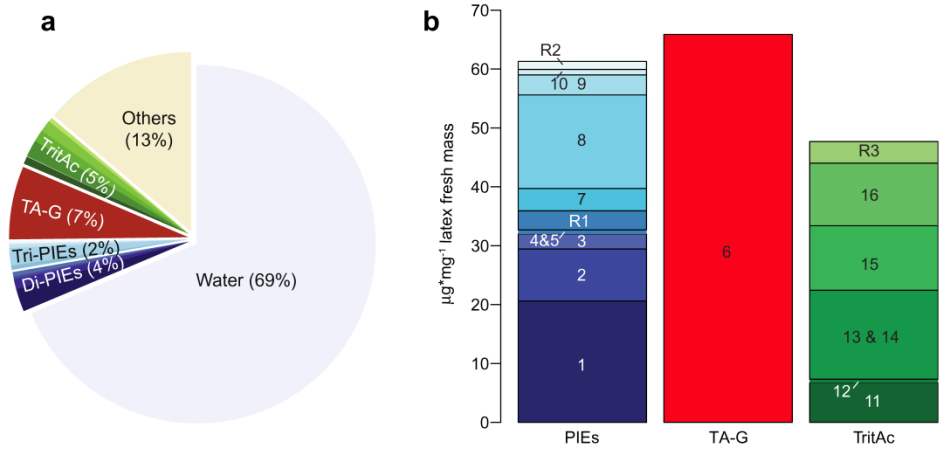

Fig. 4. Quantification of major common dandelion latex secondary metabolites. (a) Pie chart of the relative contribution of the different secondary metabolite classes to latex fresh mass. Each color shade represents one metabolite. Values correspond to the mean of three replicates. (b) Concentrations of individual latex metabolites of each compound class. Numbers refer to compounds depicted in Figs. 1 and 2. Concentrations represent the means of three replicates. PIE = phenolic inositol esters with either two (di-) or three (tri-) 4-hydroxyphenylacetic acid side groups; TA-G = taraxinic acid β-D-glucopyranosyl ester; TritAc = triterpene acetates. R1 = remaining di-PIEs; R2 = remaining tri-PIEs; R3 = remaining TritAc. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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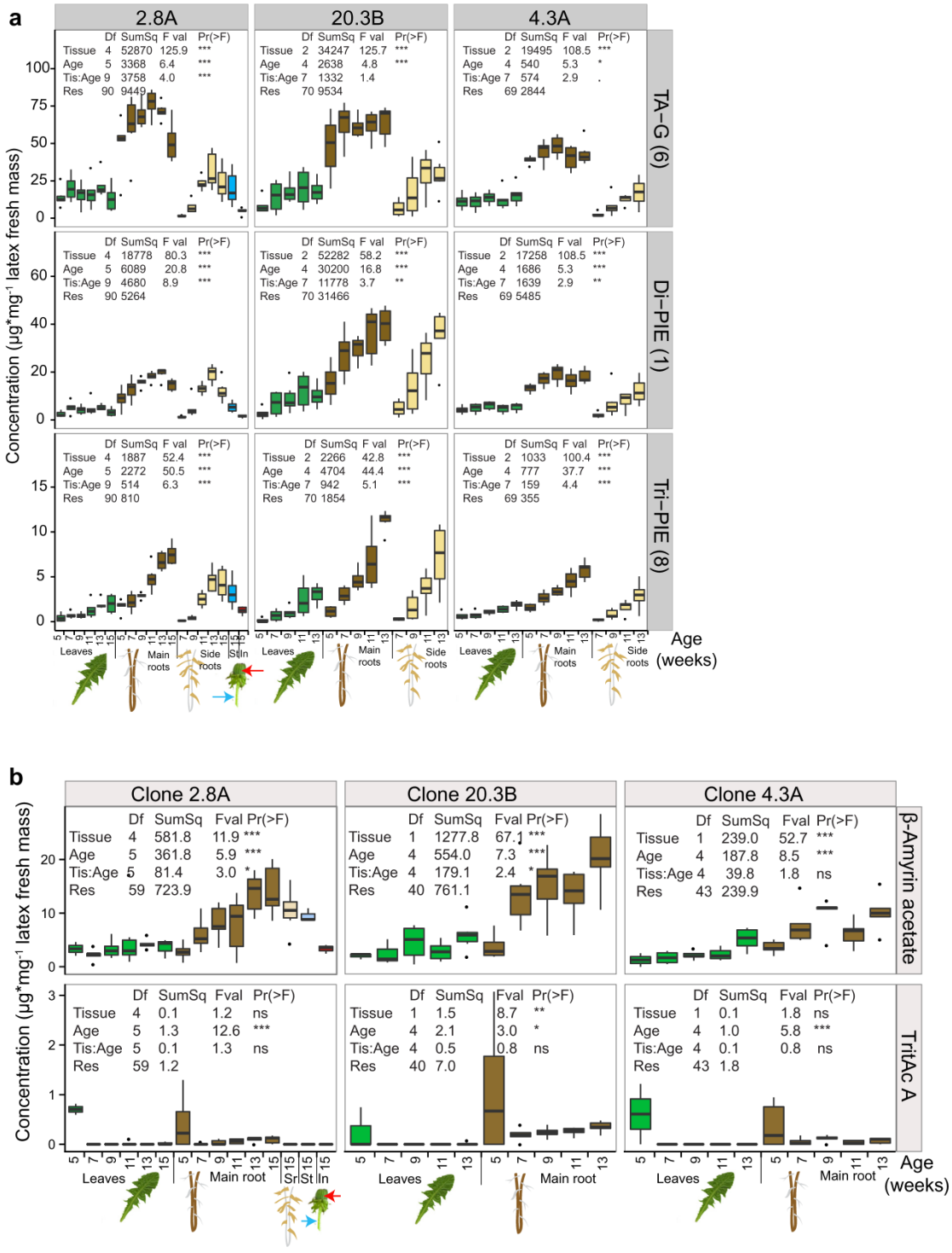

Fig. 5. Concentrations of selected common dandelion latex metabolites according the genotype, organ and plant age. (a) Taraxinic acid β-D-glucopyranosyl ester (TA-G) and phenolic inositol esters (PIEs). Compounds **1** and **8** are depicted as representative members of di-PIEs and tri-PIEs. (b) Triterpene acetate (TritAc) concentrations. β-Amyrin acetate is depicted as a representative member of TritAc. TritAc A is shown separately because of its atypical concentration pattern. Statistics from two-way ANOVAs are included for each metabolite and clone separately. Df = degrees of freedom, SumSq = Sum of squares, *F*-val = *F*-value, Pr (>*F*) = *p*-value of *F*-statistics (***$p < 0.001$, **$p < 0.1$, *$p < 0.05$, $p < 0.1$); RT = retention time [min]; St = flower stalk; In = involucre.

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While age- and organ-specific secondary metabolite patterns are well documented (Brown et al., 2003; Gaia et al., 2014; Lubbe et al., 2013; Tuominen et al., 2013), few studies so far have investigated variation of latex metabolites in this context (Rasmann et al., 2009). Two evolutionary scenarios are commonly discussed to explain variation among organs. First, differences in herbivore and pathogen communities attacking different organs may have selected for differences in secondary metabolite profiles. Second, differences in tissue value among organs may determine defensive investment (Rhoades and Cates, 1976). The latex patterns in common dandelion with highest concentrations in the main root provide support for the second hypothesis: The main root is essential for re-sprouting and flowering in spring and is therefore likely of higher value than side roots or leaves.

2.3.2. PIEs, but not TA-G and TritAc show genotype specific patterns

The three investigated common dandelion clones differed significantly in both the abundance and composition of latex metabolites. The greatest quantitative and qualitative variability was observed for PIEs. For example, several di-PIE isomers were only present in Clone 20.3B, which lacked tri-PIEs found in the other clones. TA-G and all TritAc were detected in all clones and varied only slightly in concentration. All three clones exhibited similar quantitative differences in latex secondary metabolites among organs and developmental stages (Fig. 5).

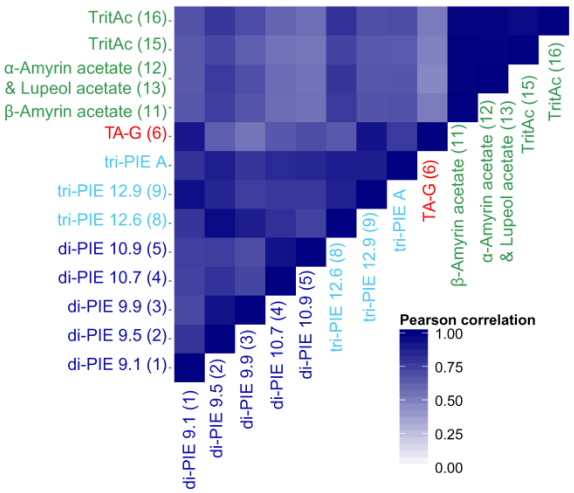

Fig. 6. Heat map of Pearson correlation matrix between major common dandelion latex secondary metabolites across different tissue and time points. Note that only positive correlations were found. TA-G = taraxinic acid β-D-glucopyranosyl ester; PIE = phenolic inositol ester; TritAc = triterpene acetate. Tri-PIE A is an unidentified tri-PIE, which is absent in chromatogram of Fig. 1.

2.3.3. Latex metabolite concentrations are positively correlated with each other

To investigate the co-regulation of latex secondary metabolites, we analyzed correlations between major metabolite concentrations across time and tissue of the genotype 2.8A. The concentrations of all latex secondary metabolites were positively correlated, with r^2 values ranging from 0.16 to 0.97 (Fig. 6). The concentrations of all major triterpene acetates were very tightly correlated, with mean r^2 values greater than 0.95. Triterpene acetates were only weakly correlated to TA-G and PIEs. TA-G showed a relatively weak correlation with most PIEs, except di-PIE **1** and tri-PIE **9**. Overall, PIEs were strongly correlated with each other, but there were substantial differences in the degree of the pairwise correlations. High correlations between secondary metabolites of the same class may indicate that they are formed by the same enzyme. Multi-product cyclase enzymes are well known in triterpene biosynthesis and several cyclases that form products derived from the dammerenyl cation have been reported to produce all of the major triterpenes alcohol moieties represented in the TritAc of *T. officinale* latex (Husselstein-Muller et al., 2001; Kushiro et al., 2000). However, the alcohol moiety of the minor compound cycloartenol acetate likely results from a different cyclase, as cycloartenol is produced through the protosteryl cation (Phillips et al., 2006). In contrast to the triterpene acetates, differences in the degree of correlation among PIEs suggest the presence of both multifunctional and co-regulated enzymes. However, virtually nothing is known about the biosynthetic pathway of PIEs. The fact that the concentrations of the three different classes of latex secondary metabolites were positively correlated with each other suggests tight co-regulation of latex compound formation. Promoter and gene expression analyses will help to identify the regulatory elements that govern latex secondary metabolite accumulation in common dandelion. Laticifer-specific promoters have been identified in *Taraxacum* species, including the polyphenoloxidase ToPPO-1 promoter (Wahler et al., 2009). A combination of DNA sequencing and bioinformatics could be used to determine whether the promoters of putative biosynthetic genes contain common regulatory motifs.

2.4. Bioactivity

To get some insight into the biological activity of *T. officinale* latex, we tested the effect of whole latex and latex MeOH and latex hexane extracts on *D. balteata* larvae (Fig. 7a). *D. balteata* is a generalist herbivore, but is not commonly associated with *T. officinale*. *D. balteata* was strongly deterred by all three mixtures (Fig. 7b). The profile of the MeOH extract is dominated by TA-G and PIEs.

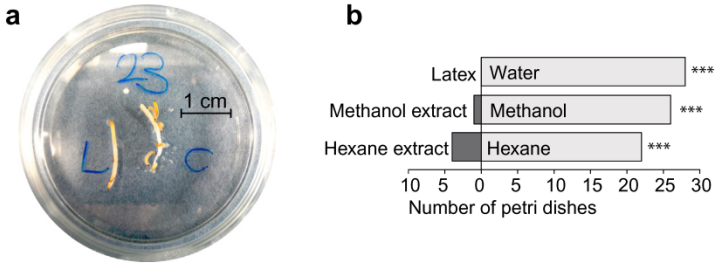

Fig. 7. Choice experiment for *Diabrotica balteata* larvae feeding on latex and latex fractions. (a) Experimental setup. (b) Choice of *D. balteata* between whole latex, latex MeOH and latex hexane extracts with each compared to a solvent control. Asterisk correspond to *p*-values of paired Wilcoxon–Mann–Whitney tests (***$p < 0.001$). For illustrative purpose, an overall choice is shown for each petri dish depending on where the majority of five larvae were feeding.

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Sesquiterpene lactones, such as TA-G, are commonly viewed as major defensive metabolites of the Asteraceae (Picman, 1986; Schmidt, 1999). For example, several sesquiterpene lactones from chicory (*Cichorium intybus* L.) deterred feeding of the desert locust (*Schistocerca gregaria*) (Rees and Harborne, 1985). Ahern and Whitney (2014) showed that the trans-fused lactones – as is the lactone ring of TA-G – deterred the polyphagous grasshopper (*Schistocerca americana*) stronger than cis-fused lactones. Picman (1986) provided a detailed review of the deterrence of sesquiterpene lactones against herbivorous insects, their anti-bacterial, anti-fungal, cytotoxic and anti-tumor activity. In sesquiterpene lactones, the exocyclic methyl group of the α-methylene-γ-lactone moiety is thought to react with nucleophilic targets, especially thiols via Michael addition (Schmidt, 1999). In contrast, we are unaware of any studies investigating the biological activity or mode of action of PIEs. The hexane extract is dominated by TritAc. TritAc have been associated with plant resistance before (reviewed in Gonzalez-Coloma et al., 2011). In previous work, the antifeedant and toxic effects of triterpenes against several herbivorous insects partially depended on the C3 substituent, the site of acetylation in *T. officinale*. Acetylation either increased or decreased toxicity depending on the compound and insect (Gonzalez-Coloma et al., 2011; Mazoir et al., 2008). Furthermore, two latex TritAc, α- and β-amyrin acetate, exhibited anti-inflammatory activity (Okoye et al., 2014), and β-amyrin acetate showed cytotoxic activity (Ding et al., 2010). To determine whether *T. officinale* latex protects plants against root herbivores, more detailed growth and performance bioassays involving native *T. officinale* herbivores would be necessary.

3. Conclusions

In this study, we identified and quantified the major MeOH- and hexane-soluble secondary metabolites of common dandelion (*T. officinale* agg.) latex. Bioassays with larvae of *D. balteata*, a generalist root herbivore, suggest a role for latex secondary metabolites in plant resistance. The detected variability between clones, organs and developmental stages highlight the potential for natural selection in shaping the abundance and composition of latex secondary metabolites in common dandelion. At the same time, the high degree of genetic conservation is indicative of the importance of the latex secondary metabolites for plant performance.

4. Experimental

4.1. Plant growth conditions

Unless stated otherwise, experiments were performed in a climate chamber under the following conditions: day length: 16 h; light: 58 μmol m^{-2} s^{-1} supplied by sodium lamps (NH 360 FLX SUNLUX; ACE, Japan); temperature: day 22 °C; night 20 °C; humidity: day 55%, night 65%. All plants were potted in sand and watered with 0.05–0.01% fertilizer (N:P:K 15:10:15 (Ferty 3, Raselina, Czechoslovakia). Plants were typically harvested 8–12 weeks after germination. As a reference genotype we used the clone A34, a triploid, synthetic apomict created by crossing a sexual diploid mother from France with diploid pollen from a triploid apomict from the Netherlands (Verhoeven et al., 2010). To investigate clonal variability, we used three other randomly selected genotypes from northern Europe (2.8A from Ostbevern, Germany; 4.3A from Mühlheim am Main, Germany; 20.3B from Haernoesand, Sweden). All plants were well watered the day before harvest to ensure that the plant's water status did not influence latex exudation and latex water content.

4.2. Structure elucidation and identification of latex secondary metabolites

To elucidate the composition of latex secondary metabolites, we analyzed *T. officinale* latex from clone A34 by HPLC–MS, HPLC-PDA and GC–MS. The main root of 3 month-old plants was cut 0.5 cm below the tiller and exuding latex was collected onto a pre-weighed pipette tip or glass insert, which was placed into a pre-weighed Eppendorf tube or glass vial, respectively, and immediately frozen in liquid nitrogen. To determine latex mass, Eppendorf tubes and vials were weighed after having placed them at room temperature for four minutes to minimize the effect of condensation water. Latex was then immediately extracted by adding 1 ml MeOH or hexane to the tubes or glass vials respectively. The vessels were then vortexed for 5 min, the tubes centrifuged at room temperature at 17,000*g* for 10 min and the vials at 3030*g* for 15 min and the supernatants stored at −80 °C until analysis.

4.2.1. HPLC–MS and HPLC-PDA

Latex MeOH extracts were analyzed by HPLC 1100 series equipment (Agilent Technologies), coupled to a photodiode array detector (G1315A DAD, Agilent Technologies) and an Esquire 6000 ESI–ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany), operating in alternating mode in the range 60–1400 *m/z* with skimmer voltage 60 V, capillary exit −128.5 V, capillary voltage 4000 V, nebulizer pressure 35 psi; drying gas 11 l min^{-1}; and gas temperature 330 °C. Metabolite separation was accomplished with a Nucleodur Sphinx RP column (250 × 4.6 mm, 5 μm particle size, Macherey–Nagel). The mobile phase consisted of 0.2% formic acid (A) and acetonitrile (B) utilizing a flow of 1 ml min^{-1} with the following gradient: 0 min, 10% B, 15 min: 55% B, 15.1 min: 100% B, 16 min: 100% B, followed by column reconditioning. Chromatograms were analyzed with data analysis and post processing software from Bruker Daltonics.

4.2.2. U(H)PLC/Q-TOF-MS

A latex MeOH extract , prepared as described under Section 4.2., was analyzed using a Dionex UltiMate 3000 Rapid Separation LC System (Thermo Fisher GmbH, Idstein, Germany), combined with a Dionex Acclaim RSLC 120 C18 2.2 μm 120 Å (2.1 × 150 mm) column. Sample elution steps were as follows: 0–3 min at 10% B, 3–30 min 10–50% B, 30–45 min 50–90% B, 40–45 min at 90% B, followed by column reconditioning. The injection volume was 2 μl and the flow rate 0.4 ml min^{-1}. MS was performed using a Bruker microTOF-Q II system (Bruker Daltonics, Bremen, Germany) with an electrospray ionization (ESI) source operating in positive ion mode. ESI conditions – micrOTOF-Q II: end plate offset 500 V, capillary voltage 4500 V, capillary exit 130 V, dry temperature 180 °C, dry gas flow of 10 l min^{-1}. MS data were collected over a range of *m/z* from 100 to 1600. Mass calibration was performed using sodium formate (50 ml isopropanol, 200 μl formic acid and 1 ml 1 M NaOH in water). Data files were calibrated post-run on the average spectrum from this time segment, using the Bruker HPC (high-precision calibration) algorithm. MS^2 experiments were performed using AutoMS/MS runs at different CID voltages (10 eV, 20 eV, 30 eV). Molecular formulae were determined manually by SmartFormula and 3D (Data Analysis 4.1, Bruker Daltonics).

4.2.3. GC–MS

GC–MS analysis of a latex hexane extract was performed with an Agilent series 6890 gas chromatograph with the carrier gas He at 1 ml min^{-1}, splitless injection (injector temperature 280 °C, injection volume 1 μl), a ZB-5MS column (30 m × 0.25 mm × 0.25 μm film, Zebron, Phenomenex, USA) employing a temperature program from 200 °C (3 min hold) at 10 °C min^{-1} to 340 °C (2 min hold). Detection was performed on

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an Agilent series 5973 mass spectrometer, with a quadrupole mass selective detector, transfer line temperature 280 °C, ionization potential 70 eV and a scan range of 50–550. Compounds were identified by comparing retention times and mass spectra to those of authentic standards or synthesized compounds. The triterpenes lupeol acetate, β-amyrin acetate, as well as α-amyrin and cycloartenol were purchased from Extrasynthese (France). Cycloartenol and α-amyrin were acetylated using the method described in Tanaka and Matsunaga (1988). In short, 60 µl pyridine and 60 µl acetic anhydride were added to 1 mg of the triterpenes and stirred for 12 h at room temperature before analysis on GC–MS.

4.2.4. GC-FID

GC-FID analysis was performed using a Varian CP-3800 connected to a ZB-5 ms column (30 m × 0.25 mm, 0.25 µm film thickness, Phenomenex). 1 µl samples were injected by a CP-8400 autoinjector (Varian) onto the column in a splitless mode. The injector was returned to a 1:70 split ratio 2 min after injection until the end of each run. The GC program was set as follows: injector at 280 °C, initial column temperature at 200 °C held for 3 min, then ramped at 10 °C min^{-1} to 340 °C and held for 3 min; Helium carrier gas was used and the column flow set to 1.5 ml min^{-1}. Analytes eluted from the GC column were measured by an FID (300 °C, airflow 300 ml min^{-1}, hydrogen 30 ml min^{-1}, nitrogen make-up gas 5 ml min^{-1}).

4.2.5. NMR

A Bruker Avance 500 NMR spectrometer (Bruker, Karlsruhe, Germany) was used to measure 1D and 2D NMR spectra (^{1}H, ^{1}H-COSY, HSQC, HMBC) of the sesquiterpene lactone glycoside (TA-G, **6**) and the di-PIE (**1**). The spectrometer was equipped with a 5 mm TCI CryoProbe. MeOH-d_4 was used as a solvent and spectra were referenced to the residual solvent signals of MeOH-d_4 at δ_H 3.31 and δ_C 49.05, respectively. Chemical shifts are given in δ values (Tables S2 and S3). Data acquisition as well as processing was accomplished using Bruker Topspin v2.1.

To identify the sesquiterpene lactone, 30 mg of main root latex from genotype 2.8A was collected onto the upper part of a filter paper and freeze-dried. Compounds were partially separated by stepwise elution with 10 ml hexane, ethyl acetate, acetone and MeOH. Solvents were applied onto the top of the filter paper and allowed to run through the matrix before collecting into a glass vial. All solvents were evaporated under nitrogen. The ethyl acetate and acetone extracts were combined and reconstituted with MeOH-d_4 for NMR analysis.

To obtain structural information on the PIEs, latex was harvested as described under Section 4.2. Extracts of all plants were pooled and immediately flash frozen in liquid nitrogen. The pooled sample was extracted with 10 ml MeOH, vortexed for 10 min, and centrifuged at 17,000 g for 10 min. The compounds were concentrated under nitrogen, and the pooled solution diluted to 16% MeOH in H_2O and subfractioned through a C18 SPE cartridge (MeOH:H_2O 16:84 and 40:60) (Macherey–Nagel, 1 g bed weight). The column bed was not allowed to dry before elution of subfraction 2 with 40% MeOH. Both fractions were rotary-evaporated at 30 °C, at 120 mbar and lyophilized. The remaining matrix from the first fraction was dissolved into 100% MeOH, and further separated by semi-preparative HPLC-UV. For semi preparative HPLC, one di-ester isomer (**1**) was isolated using a C18 HPLC column (Supelcosil LC18-DB, 250 × 10 mm, 5 µm). Separation was achieved with a flow rate of 4 ml min^{-1}, mobile phases of water (A) and acetonitrile (B), and a gradient condition of: 0 min: 2.5% B, 10 min: 17.6% B, 14 min: 43.7% B, 15 min 100% B followed by column reconditioning. The injection volume was 20 µl. The fractionated compound was freeze-dried and purity was assessed by HPLC–ESI–ion trap-MS and ^{1}H NMR. The final yield of the di-PIE was 1 mg.

4.3. Quantification of latex secondary metabolites

To quantify the abundance of major latex secondary metabolites, standard curves with isolated TA-G, di-PIEs and authentic lupeol acetate were established as described below.

4.3.1. TA-G and PIE

TA-G was isolated by semi-preparative HPLC. First, crude latex from main roots of 80 *T. officinale* individuals was collected in 2 ml 95 °C water, incubated at this temperature for 10 min to stop enzymatic activity, followed by 10 min centrifugation at 17,000 g at room temperature. The supernatant was fractionated by HPLC-UV coupled to a fraction collector (Advantec SF-2120) using a Nucleodur Sphinx RP column (250 × 4.6 mm, 5 µm particle size, Macherey–Nagel). The mobile phase consisted of water (A) and acetonitrile (B). Flow rate was set to 1 ml min^{-1} with following gradient: 0 min: 25% B, 8 min: 49% B, 8.1 min: 100% B followed by column reconditioning. The elution time of TA-G was 7.0 min, and was visualized with a UV detector at 245 nm wavelength. The fraction containing TA-G was concentrated using rotary-evaporation at 40 °C at 120 mbar and lyophilized. Purified TA-G was analyzed for contamination with HPLC–ESI–IonTrap-MS using the method described in Section 4.2.1. and with ^{1}H NMR, showing >98% purity. A standard curve of TA-G was prepared with the purified compound on HPLC-PDA using the method described in Section 4.2.1. at 245 nm wavelength. A weight based response factor was calculated relative to loganin (Santa Cruz Biotechnology, Dallas, TX, USA) as an internal standard: The weight response factor for TA-G was 1.9.

Similarly, a quantification method was established for the di- and tri-PIEs. To accumulate enough di-ester for standard curve preparation, the di-ester isomers were fractionated as a group on the semi-preparative HPLC. The fraction containing only di-esters was rotor-evaporated at 30 °C and lyophilized. Because each di-ester isomer exhibits identical UV spectra, thus having the same molar absorption at 275 nm, we could determine the mass contribution of each individual di-ester in the standard mix. The individual peak areas, provided by HPLC-PDA at 275 nm, were divided by the total peak area of all di-esters and the ratio applied to the final mass of the standard di-ester mix. We then calculated a weight based response factor for di-PIEs relative to salicin (Sigma–Aldrich) as the reference compound. The weight response factor for di-PIEs was 0.53. Since the UV absorbance of PIEs at 275 nm originates from the phenylacetic acid residues, and as di-esters contain two and tri-esters three of these residues, we assumed a molar based absorption ratio between di- and tri-PIEs of 2:3. Based on this assumption, we then calculated a weight based response factor for tri-PIEs using salicin as a reference compound. The weight response factor for tri-PIEs was 0.46. This form of quantification was necessary given that the PIE composition (isomer presence and abundance) exhibit strong genotypic, ontogenetic and tissue specific patterning.

4.3.2. Triterpene acetates

To establish a quantification method for TritAc, a standard curve of authentic lupeol acetate (Extrasynthese) and of cholesteryl acetate (Sigma–Aldrich) in hexane was constructed. Analytes were separated by Varian CP-3800 GC-FID using the method described in Section 4.2.4. Individual peaks were quantified using MS Work Station Method Builder. A weight based response factor of lupeol acetate relative to cholesteryl acetate was calculated, which was 1.09. Since all latex triterpene acetates had the same molecular mass, the same response factor was applied for each compound.

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4.3.3. Quantification

To quantify the abundance of TA-G, PIEs and TritAc, six A34 plants were cultivated for 14 weeks. The root systems were exposed, washed and the main root cut 0.5 cm below the tiller. Exuding latex was collected into pre-weighed glass vials, and latex mass was determined immediately before flash-freezing in liquid nitrogen. To quantify TA-G and PIEs, three samples were lyophilized, weighed and extracted with 1 ml MeOH containing 10 μg ml^{-1} loganin and 100 μg ml^{-1} salicin as internal standards. To quantify TritAc, the remaining three samples were extracted with hexane containing 100 μg ml^{-1} cholesteryl acetate as internal standard. For both MeOH and hexane extraction, 1 ml solvent was added, vials were vortexed for 5 min, centrifuged at 3030 g for 15 min and supernatant was analyzed immediately on HPLC-PDA and GC-FID as described in Sections 4.2.1 and 4.2.4. For the MeOH extracts, peak area was integrated at 245 nm for TA-G and at 275 nm for PIEs and quantification assessed according to the response factor of the respective internal standard. For the hexane extracts, individual peaks were quantified using MS Work Station Method Builder and standardized to the peak area of cholesteryl acetate for each sample.

4.4. Organ and age specificity

To elucidate organ and age specific distribution patterns of the different metabolites, we collected latex of three common dandelion genotypes (2.8A, 4.3A, 20.3B) every second week from the main roots, side roots and petioles. Latex from the flower stalk and involucre was collected from genotype 2.8A – the only genotype that flowered at this time – during the final harvest. Plant tissue was cut and exuding latex collected into Eppendorf tubes and glass vials, immediately flash-frozen in liquid nitrogen and stored at −80 °C before extraction. 1 ml MeOH or hexane containing 0.1 mg ml^{-1} cholesteryl acetate as internal standard was added to the Eppendorf tubes or glass vials respectively, vortexed for 5 min, centrifuged at room temperature and supernatant was stored at −80 °C until analysis.

Latex MeOH extracts were analyzed by HPLC on an Agilent Technologies HP 1100 Series instrument equipped with a photodiode array detector using the method described under Section 4.2.1. Peak area was integrated at 245 nm for TA-G and at 275 nm for PIEs and quantified with an external standard curve. Analytes of hexane extracts were separated by GC-FID as described in Section 4.2.4. and individual peaks were quantified using MS Work Station Method Builder and Batch Report software (Varian) and normalized to the peak area of cholesteryl acetate in each sample. The major di-PIE (**1**), tri-PIE (**8**) and TA-G, as well as β-amyrin acetate and a minor unidentified triterpene acetate were analyzed with two-way analyses of variance (ANOVAs) for each genotype separately. Correlations among the eight major PIEs, TA-G and four major TritAc were calculated with Pearson correlations. All statistical analysis was performed in R (R Core Team, 2014) using ggplot2 (Wickham, 2009) and gridExtra (Auguie, 2012).

4.5. Bioactivity

Latex, latex MeOH and hexane extracts were tested for deterrence against the generalist root herbivore *D. balteata*. To obtain latex extracts, we collected the main root latex of 2 month-old plants from clone 2.8A and immediately flash-froze the latex in liquid nitrogen. Latex was extracted with either MeOH or hexane. MeOH samples were centrifuged and the supernatant evaporated to almost complete dryness using a rotary-evaporator at 30 °C. 1 ml H_2O was added before freeze-drying. Hexane samples were pooled and evaporated under nitrogen to complete dryness. For all extracts, 1 μl solvent for each mg of fresh latex was added to the pooled, evaporated samples. Control solvents were treated the same way as the extracts. We tested the deterrence of the extracts by arranging roots of 4 day-old maize seedlings pairwise in a petri dish: One maize root was painted with 10 μl extract (equivalent to 10 mg latex fresh mass), the other maize root with 10 μl control solvent. Five L2 and L3 *D. balteata* larvae were placed into the center of the petri dish and the feeding site was recorded 30 min after the start of the experiment. Preference was tested with a paired Wilcoxon–Mann–Whitney-test. An overall choice is shown for each petri dish depending on where the majority of the five larvae were feeding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.01.003.

References

Agrawal, A.A., 2005. Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. Evol. Ecol. Res. 7, 651–667.
Agrawal, A.A., Konno, K., 2009. Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. Annu. Rev. Ecol. Evol. Syst 40, 311–331.
Ahern, J.R., Whitney, K.D., 2014. Stereochemistry affects sesquiterpene lactone bioactivity against an herbivorous grasshopper. Chemoecology 24, 35–39.
Akashi, T., Furuno, T., Takahashi, T., Ayabe, S., 1994. Biosynthesis of triterpenoids in cultured-cells, and regenerated and wild plant organs of *Taraxacum officinale*. Phytochemistry 36, 303–308.
Auguie, B., 2012. gridExtra: functions in Grid graphics.
Brown, P.D., Tokuhisa, J.G., Reichelt, M., Gershenzon, J., 2003. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry 62, 471–481.
Burrows, S., Simpson, J.C.E., 1938. The triterpene group. Part IV. The triterpene alcohols of *Taraxacum* root. J. Chem. Soc., 2042–2047
Clifford, M.N., Shutler, S., Thomas, G.A., Ohiokpehai, O., 1987. The chlorogenic acids content of coffee substitutes. Food Chem 24, 99–107.
Cornish, K., Wood, D.F., Windle, J.J., 1999. Rubber particles from four different species, examined by transmission electron microscopy and electron-paramagnetic-resonance spin labeling, are found to consist of a homogeneous rubber core enclosed by a contiguous, monolayer biomembrane. Planta 210, 85–96.
Dell, B., McComb, A.J., 1979. Plant resins – their formation, secretion and possible functions. In: Woolhouse, H.W. (Ed.), Advances in Botanical Research, vol. 6. Academic Press, pp. 277–316.
Ding, Y., Liang, C., Kim, J.H., Lee, Y.-M., Hyun, J.-H., Kang, H.-K., Kim, J.-A., Min, B.S., Kim, Y.H., 2010. Triterpene compounds isolated from *Acer mandshuricum* and their anti-inflammatory activity. Bioorg. Med. Chem. Lett. 20, 1528–1531.
Dixon, R.A., Strack, D., 2003. Phytochemistry meets genome analysis, and beyond. Phytochemistry 62, 815–816.
Fahn, A., 1988. Secretory-tissues in vascular plants. New Phytol. 108, 229–257.
Farrell, B.D., Dussourd, D.E., Mitter, C., 1991. Escalation of plant defense – do latex and resin canals spur plant diversification. Am Nat. 138, 881–900.
Gaia, A.M., Yamaguchi, L.F., Jeffrey, C.S., Kato, M.J., 2014. Age-dependent changes from allylphenol to prenylated benzoic acid production in *Piper gaudichaudianum* Kunth. Phytochemistry 106, 86–93.
Gonzalez-Coloma, A., Lopez-Balboa, C., Santana, O., Reina, M., Fraga, B.M., 2011. Triterpene-based plant defenses. Phytochem. Rev. 10, 245–260.
Hänsel, R., Kartarahardja, M., Huang, J.T., Bohlmann, F., 1980. Sesquiterpenlacton-β-D-glucopyranoside sowie ein neues eudesmanolid aus *Taraxacum officinale*. Phytochemistry 19, 857–861.
Huo, J., Yang, S.-P., Xie, B.-J., Liao, S.-G., Lin, L.-P., Ding, J., Yue, J.-M., 2008. Cytotoxic sesquiterpenoids from *Vernonia bockiana*. J. Asian Nat. Prod. Res. 10, 571–575.
Husselstein-Muller, T., Schaller, H., Benveniste, P., 2001. Molecular cloning and expression in yeast of 2,3-oxidosqualene–triterpenoid cyclases from *Arabidopsis thaliana*. Plant Mol. Biol. 45, 75–92.

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Kenny, O., Smyth, T.J., Hewage, C.M., Brunton, N.P., McLoughlin, P., 2014. 4-Hydroxyphenylacetic acid derivatives of inositol from dandelion (*Taraxacum officinale*) root characterised using LC–SPE–NMR and LC–MS techniques. Phytochemistry 98, 197–203.
Kirschner, J., Stepanek, J., 1994. Clonality as a part of the evolution process in *Taraxacum*. Folia Geobot. Phytotx. 29, 265–275.
Kisiel, W., Barszcz, B., 2000. Further sesquiterpenoids and phenolics from *Taraxacum officinale*. Fitoterapia 71, 269–273.
Konno, K., 2011. Plant latex and other exudates as plant defense systems: roles of various defense chemicals and proteins contained therein. Phytochemistry 72, 1510–1530.
Kushiro, T., Shibuya, M., Masuda, K., Ebizuka, Y., 2000. A novel multifunctional triterpene synthase from *Arabidopsis thaliana*. Tetrahedron Lett. 41, 7705–7710.
Levin, D.A., 1973. Role of trichomes in plant defense. Q Rev. Biol. 48, 3–15.
Lewinsohn, T.M., 1991. The geographical distribution of plant latex. Chemoecology 2, 64–68.
Lubbe, A., Gude, H., Verpoorte, R., Choi, Y.H., 2013. Seasonal accumulation of major alkaloids in organs of pharmaceutical crop *Narcissus* Carlton. Phytochemistry 88, 43–53.
Mazoir, N., Benharref, A., Bailen, M., Reina, M., Gonzalez-Coloma, A., 2008. Bioactive triterpene derivatives from latex of two *Euphorbia* species. Phytochemistry 69, 1328–1338.
Metcalfe, C.R., 1967. Distribution of latex in the plant kingdom. Econ. Bot. 21, 115–127.
Michalska, K., Zylewski, M., Marciniuk, J., Kisiel, W., 2010. Structural analysis of 1L-chiro-inositol diester from *Taraxacum udum*. Carbohydr. Res. 345, 172–174.
Mithöfer, A., Boland, W., 2012. Plant defense against herbivores: chemical aspects. In: Merchant, S.S. (Ed.), Annual Review of Plant Biology, vol. 63. Annual Reviews, Palo Alto, pp. 431–450.
Okoye, N.N., Ajaghaku, D.L., Okeke, H.N., Ilodigwe, E.E., Nworu, C.S., Okoye, F.B.C., 2014. Beta-Amyrin and alpha-amyrin acetate isolated from the stem bark of *Alstonia boonei* display profound anti-inflammatory activity. Pharm. Biol. 52, 1478–1486.
Otani, M., Shitan, N., Sakai, K., Martinoia, E., Sato, F., Yazaki, K., 2005. Characterization of vacuolar transport of the endogenous alkaloid berberine in *Coptis japonica*. Plant Physiol. 138, 1939–1946.
Phillips, D.R., Rasbery, J.M., Bartel, B., Matsuda, S.P.T., 2006. Biosynthetic diversity in plant triterpene cyclization. Curr. Opin. Plant Biol. 9, 305–314.
Picman, A.K., 1986. Biological activities of sesquiterpene lactones. Biochem. Syst. Ecol. 14, 255–281.
Post, J., van Deenen, N., Fricke, J., Kowalski, N., Wurbs, D., Schaller, H., Eisenreich, W., Huber, C., Twyman, R.M., Prüfer, D., Gronover, C.S., 2012. Laticifer-specific cis-prenyltransferase silencing affects the rubber, triterpene, and inulin content of *Taraxacum brevicorniculatum*. Plant Physiol. 158, 1406–1417.
Rasmann, S., Agrawal, A.A., Cook, S.C., Erwin, A.C., 2009. Cardenolides, induced responses, and interactions between above- and belowground herbivores of milkweed (*Asclepias* spp.). Ecology 90, 2393–2404.
R Core Team, 2014. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rees, S.B., Harborne, J.B., 1985. The role of sesquiterpene lactones and phenolics in the chemical defense of the chicory plant. Phytochemistry 24, 2225–2231.
Rhoades, D., Cates, R., 1976. Toward a general theory of plant antiherbivore chemistry. In: Wallace, J., Mansell, R. (Eds.), Biochemical Interaction Between Plants and Insects, vol. 10. Springer, US, pp. 168–213.
Richards, A.J., 1973. The origin of *Taraxacum* agamospecies. Bot. J. Linn. Soc. 66, 189–211.
Schmidt, T., Lenders, M., Hillebrand, A., van Deenen, N., Munt, O., Reichelt, R., Eisenreich, W., Fischer, R., Prufer, D., Gronover, C.S., 2010. Characterization of rubber particles and rubber chain elongation in *Taraxacum koksaghyz*. BMC Biochem. 11, 10.
Schmidt, T.J., 1999. Toxic activities of sesquiterpene lactones: structural and biochemical aspects. Curr. Org. Chem. 3, 577–608.
Schütz, K., Carle, R., Schieber, A., 2006. *Taraxacum* – a review on its phytochemical and pharmacological profile. J. Ethnopharmacol. 107, 313–323.
Schütz, K., Kammerer, D.R., Carle, R., Schieber, A., 2005. Characterization of phenolic acids and flavonoids in dandelion (Taraxacum officinale WEB. ex WIGG.) root and herb by high-performance liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun. Mass Spectrom. 19, 179–186.
Sessa, R.A., Bennett, M.H., Lewis, M.J., Mansfield, J.W., Beale, M.H., 2000. Metabolite profiling of sesquiterpene lactones from *Lactuca* species – major latex components are novel oxalate and sulfate conjugates of lactucin and its derivatives. J. Biol. Chem. 275, 26877–26884.
Tanaka, R., Matsunaga, S., 1988. Triterpene dienols and other constituents from the bark of *Phyllanthus flexuosus*. Phytochemistry 27, 2273–2277.
Tome, F., Colombo, M.L., 1995. Distribution of alkaloids in *Chelidonium majus* and factors affecting their accumulation. Phytochemistry 40, 37–39.
Tuominen, A., Toivonen, E., Mutikainen, P., Salminen, J.-P., 2013. Defensive strategies in *Geranium sylvaticum*. Part 1: Organ-specific distribution of water-soluble tannins, flavonoids and phenolic acids. Phytochemistry 95, 394–407.
Verhoeven, K.J.F., Van Dijk, P.J., Biere, A., 2010. Changes in genomic methylation patterns during the formation of triploid asexual dandelion lineages. Mol. Ecol. 19, 315–324.
Wahler, D., Gronover, C.S., Richter, C., Foucu, F., Twyman, R.M., Moerschbacher, B.M., Fischer, R., Muth, J., Prüfer, D., 2009. Polyphenoloxidase silencing affects latex coagulation in *Taraxacum* species. Plant Physiol. 151, 334–346.
Wickham, H., 2009. Ggplot2: elegant graphics for data analysis. Springer, New York.
Wink, M., 1993. The plant vacuole – a multifunctional compartment. J. Exp. Bot. 44, 231–246.
Wood, D.F., Cornish, K., 2000. Microstructure of purified rubber particles. Int. J. Plant Sci. 161, 435–445.
Wu, S.-H., Luo, X.-D., Ma, Y.-B., Hao, X.-J., Wu, D.-G., 2002. Anti-gastric ulcer sesquiterpene lactone glycosides from *Crepis napifera*. Yaoxue Xuebao 37, 33–36.
Wu, Z.-J., Xu, X.-K., Zeng, H.-W., Shen, Y.-H., Tian, J.-M., Su, J., Li, H.-L., Shan, L., Liu, R.-H., Zhang, W.-D., 2011. New sesquiterpenoids from *Ainsliaea macrocephala* and their nitric oxide inhibitory activity. Planta Med. 77 (1545–U102).
Yamaki, S., 1984. Isolation of vacuoles from immature apple fruit flesh and compartmentation of sugars, organic acids, phenolic compounds and amino acids. Plant Cell Physiol. 25, 151–166.
Zidorn, C., Ellmerer-Müller, E.P., Stuppner, H., 1999. Eudesmanolides and inositol derivatives from *Taraxacum linearisquameum*. Phytochemistry 51, 991–994.

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3.2.1 Supplemental

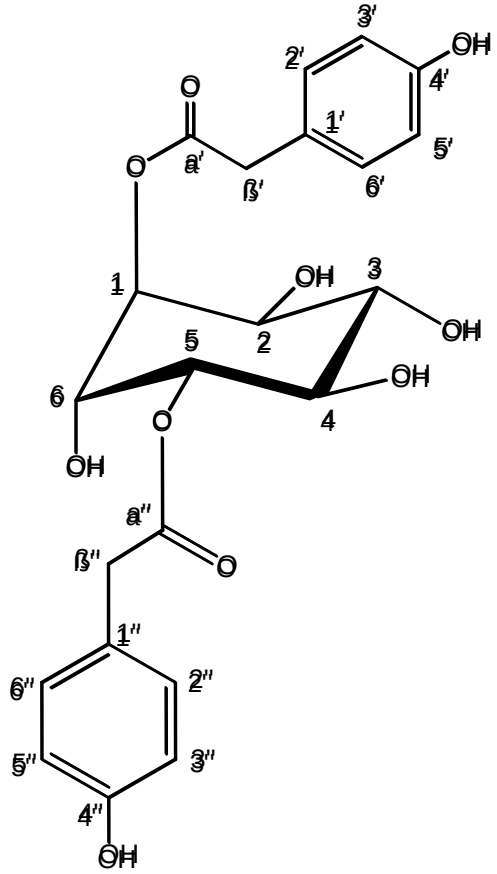

Figure S1: Structure of di-PIE (**1**). ^{1}H- and ^{13}C-NMR chemical shift data are listed in the supplemental table S2.

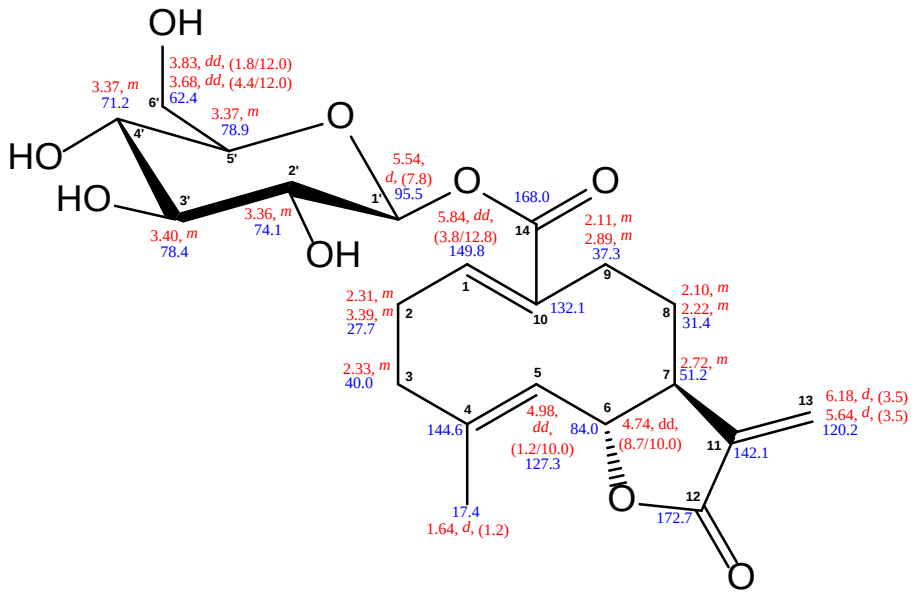

Figure S2: Structure of taraxinic acid β-D-glucopyranosyl ester (**6**) including ^{1}H- and ^{13}C-NMR chemical shift data (as listed in the supplemental table S3)

Table S1: Metabolite ID as depicted in chromatogram of Fig 1; RT, Retention time on Esquire 6000 ESI-Ion Trap mass spectrometer, in minutes; CID voltage for U(H)PLC/ESI-QTOF (MicrOTOF-Q II Bruker Daltonics), voltage used for collision induced dissociation; Mol Form., Molecular Formula of the metabolite; Precursor Ion, m/z feature used for MS/MS experiments; Theo. Mass, theoretical mass calculated for precursor ion; Mean delta (ppm), deviation between the average of found accurate mass and real accurate mass, in ppm; UV absorbtion maxima on an HPLC-PDA (0.2% formic acid : acetonitrile), in nm.

Metabolite ID	RT (min)	CID voltages	MSMS Fragments	Mol Form.	Precursor Ion	Theo. Mass	m/z	Mean (ppm)	UV λ_{max}
1	7.2	10, 20, 30 eV	431, 413, 315, 297, 279, 261, 179, 163, 153, 145, 127	$C_{22}H_{24}O_{10}$	$[M+H]^+$	449.144	449.1441	-0.22	224, 276
2	9.7	10, 20, 30 eV	431, 413, 297, 279, 261, 153, 127	$C_{22}H_{24}O_{10}$	$[M+NH_4]^+$	466.171	466.1702	1.29	222, 275
3	10.2	10, 20, 30 eV	431, 315, 297, 279, 153, 145	$C_{22}H_{24}O_{10}$	$[M+H]^+$	449.144	449.1442	-1.11	222, 276
4	10.6	-	-	$C_{22}H_{24}O_{10}$	$[M+H]^+$	449.144	449.1425	3.785	224, 276
5	11.5	10, 20, 30 eV	431, 315, 297, 279, 261	$C_{22}H_{24}O_{10}$	$[M+H]^+$	449.144	449.1433	2	224, 276
6	14.3	10, 20, 30 eV	263, 245, 227, 217, 199, 181, 175, 171, 145, 143, 131	$C_{21}H_{28}O_9$	$[M+NH_4]^+$	442.207	442.207	0.4523	197, 218
7	16	10, 20, 30 eV	565, 431, 413, 279	$C_{30}H_{28}O_{11}$	$[M+NH_4]^+$	600.208	600.2066	1.5	224, 276
8	16.3	10, 20, 30 eV	565, 449, 431, 413, 297, 261, 213, 179, 153, 127	$C_{30}H_{28}O_{11}$	$[M+NH_4]^+$	600.208	600.2067	1.33	226, 276
9	16.9	10, 20, 30 eV	565, 449, 431, 413, 297, 279	$C_{30}H_{28}O_{11}$	$[M+H]^+$	583.181	583.1801	1.54	225, 276
10	18.2	10, 20, 30 eV	565, 547, 449, 431, 413, 395, 315, 305, 297, 287, 279, 261, 213, 153, 145	$C_{30}H_{28}O_{11}$	$[M+H]^+$	583.181	583.181	0	222, 276

Table S2: ^{1}H (500 MHz) and ^{13}C NMR data (125 MHz) of di-PIE (**1**) in MeOH-d_4.

Pos.	Mult.	δ_C	δ_H	J_{HH} [Hz]
1	CH	75.2	5.13	*dd*, 3.7/3.7
2	CH	70.1	3.90	*dd*, 10.0/3.7
3	CH	74.8	3.55	*dd*, 10.0/10.0
4	CH	71.8	3.81	*dd*, 10.0/10.0
5	CH	75.5	4.81	*dd*, 10.0/3.7
6	CH	68.1	3.97	*dd*, 3.7/3.7
α'	C	172.7	-	-
β'	CH_2	40.8	3.62	*d*, 16.5
1'	C	126.0	-	-
2',6'	CH	131.3	7.10	*d*, 8.4
3',5'	CH	116.1	6.71	*d*, 8.4
4'	C	157.4	-	-
α''	C	173.5	-	-
β''	CH_2	40.7	3.65	*d*, 16.5
1''	C	126.0	-	-
2'',6''	CH	131.3	7.13	*d*, 8.4
3'',5''	CH	116.1	6.73	*d*, 8.4
4''	C	157.4	-	-

Supplemental table S3: ^{1}H- and ^{13}C-NMR data of taraxinic acid β-D-glucopyranosyl ester (**6**) in d_6-acetone.

No.		δ_C	δ_H	J_{HH} [Hz]
1	**CH**	149.8	5.84	*dd*, 3.8/12.8
2a **2b**	**CH_2**	27.7	2.31 3.39	*m* *m*
3a **3b**	**CH_2**	40.0	2.33	*m*
4	**C**	144.6	-	-
5	**CH**	127.3	4.98	*dd*, 1.2/10.0
6	**CH**	84.0	4.74	*dd*, 8.7/10.0
7	**CH**	51.2	2.72	*m*
8a **8b**	**CH_2**	31.4	2.10 2.22	*m* *m*
9a **9b**	**CH_2**	37.3	2.11 2.89	*m* *m*
10	**C**	132.1	-	-
11	**C**	142.1	-	-
12	**C**	172.7	-	-
13a **13b**	**CH_2**	120.2	6.18 5.64	*d*, 3.5 *d*, 3.5
14	**C**	168.0	-	-
1'	**CH**	95.5	5.54	*d*, 7.8
2'	**CH**	74.1	3.36	*m*
3'	**CH**	78.4	3.40	*m*
4'	**CH**	71.2	3.37	*m*
5'	**CH**	78.9	3.37	*m*
6'a **6'b**	**CH_2**	62.4	3.83 3.68	*dd*, 1.8/12.0 *dd*, *4.4/12.0*

3.3 Manuscript III

A latex metabolite increases plant fitness upon root herbivore attack

Manuscript for Plos Biology

A latex metabolite increases plant fitness upon root herbivore attack

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Abstract

Plants produce large amounts of secondary metabolites in their shoots and roots and store them in specialized cells and cell compartments. Although these traits are commonly assumed to have a defensive function, evidence that they increase plant fitness upon herbivore attack is scarce, especially below ground. Here, we tested whether latex secondary metabolites decrease root herbivore performance and improve plant fitness through a combination of genetic modification, chemical complementation and field experiments involving the common dandelion (*Taraxacum officinale* agg.) and its major native insect root herbivore, the larvae of the common cockchafer (*Melolontha melolontha*). Across 17 *T. officinale* genotypes screened by gas and liquid chromatography, the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G) was negatively correlated with *M. melolontha* larval growth. Silencing a newly identified germacrene A synthase (ToGAS1), which catalyzes the first committed step of TA-G biosynthesis, resulted in a 90% reduction of TA-G levels, enhanced attractiveness of the transgenic plants to *M. melolontha* and increased root damage upon *M. melolontha* herbivory. Furthermore, purified TA-G reduced larval feeding on artificial diet at physiological concentrations. In a common garden experiment involving over 2000 plants, TA-G was positively correlated with plant growth and reproductive fitness upon *M. melolontha* infestation. Taken together, our study demonstrates that a latex secondary metabolite directly benefits plants under herbivore attack. Our results set the stage for evolutionary scenarios in which soil dwelling insects contribute to the evolution and variation in the defensive physiology and chemistry of their host plants.

Introduction

Plants produce a tremendous diversity of secondary metabolites (1). Many secondary metabolites have been found to deter or poison insects (2-6), leading to the hypothesis that they originally evolved as defenses against herbivores (7). A number of recent studies demonstrate that herbivore abundance correlates with natural variation in secondary metabolites on a genetic and phenotypic level (8-10). An important underlying assumption of these observations is that plant secondary metabolites improve plant fitness in the presence, but not in the absence of herbivores. However, demonstrating such conditional benefits in the field remains challenging (11-14). Recently, both transgenic and genetic approaches have demonstrated associations between the biosynthesis of plant secondary metabolites and herbivore damage (15) as well as plant reproductive output (14, 16). The significant temporal and spatial fluctuations in these patterns were attributed to differences in herbivore community composition (16) and interactions between biotic and abiotic factors (14). Targeted manipulation of the herbivore community composition may therefore be necessary to clarify the relative contribution of herbivores to the observed patterns (8).

Plant roots are vital for plant development, produce diverse and abundant blends of secondary metabolites (17, 18) and are attacked by highly damaging root herbivores (19). However, because fitness benefits of root secondary metabolites in the context of natural plant-herbivore interactions have not been investigated, it remains elusive whether they may have evolved in response to root feeding insects (20). The rhizosphere differs substantially from the phyllosphere in both biotic and abiotic conditions (19, 21, 22), and root secondary metabolites may serve a variety of functions, including herbivore and pathogen resistance (17, 23), the promotion of symbiotic relationships (24, 25) and the acquisition of minerals and nutrients (26). Below ground systems therefore provide an excellent opportunity to understand how herbivores shape defensive syndromes in complex environments.

Plant secondary metabolites often accumulate in cellular compartments or specialized reservoirs (27, 28). Laticifers are among the most common defensive reservoirs in flowering plants and are produced by 10% of all angiosperms (29-31). Laticifers are elongated individual or interconnected cells whose often milky cytoplasm, also called latex, typically contains high concentrations of toxic secondary metabolites (13, 32, 33). Laticifers are commonly assumed to be defensive, as many phytophagous insects are deterred by their secondary metabolite-rich cytoplasm and avoid contact with the often sticky emulsions (13, 34). Moreover, specialist herbivores display behavioral adaptations to reduce their exposure to latex (34). Surprisingly however, direct evidence that laticifers are defensive, i.e. that they improve plant fitness in the presence but not in the absence of herbivory, is virtually absent (13, 35, 36). A study by Agrawal (35) showed that latex exudation is under positive selection in common milkweed (*Asclepias syriaca*) under ambient insect pressure. However, whether this pattern is herbivore dependent remains to be elucidated.

One of Europe's most prevalent native latex-producing plants is the common dandelion (*Taraxacum officinale* agg.) (Flora Helvetica, 5th edition). *Taraxacum officinale* is a species complex consisting of sexual, outcrossing diploids that are native to central and southern Europe and a multitude of apomictic, clonal triploids that are spreading across the globe (37-39). Similar to many other perennials in temperate ecosystems, the plant relies on its roots for resprouting and flowering in spring. *Taraxacum officinale* produces latex in all major organs, with the highest amounts found in the tap root (40). The latex is dominated by three classes of secondary metabolites: phenolic inositol esters (PIEs), triterpene acetates (TritAcs) and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G). Each compound class accounts for 5-7% of latex fresh mass (40). In its native range, *T. officinale* is frequently attacked by the larva of the common cockchafer (also called May bug), *Melolontha melolontha* (Coleoptera: Scarabaeidae). *Melolontha melolontha* is among Europe's largest and most prevalent native root feeding insects and periodically causes devastating damage to crops and pastures (41-43). Although the larvae are highly polyphagous, they preferentially feed on *T. officinale* in their third and final instar (44, 45).

In this study, we explored the putative defensive function and fitness benefits of *T. officinale* latex secondary metabolites against *M. melolontha* larvae. First, we investigated which latex secondary metabolites are likely to be involved in root herbivore defense using a correlative approach. Second, we decreased the production of the major candidate compound (TA-G) by identifying the gene encoding the first committed biosynthetic step and silencing it by RNA interference (RNAi), which allowed testing the effect of TA-G deficiency on plant and insect performance. Third, we purified TA-G to investigate its impact on *M. melolontha in vitro*. Fourth, we performed a common garden experiment with different *T. officinale* genotypes to determine whether TA-G enhances plant vegetative and reproductive performance under *M. melolontha* attack in the field. Through the above approaches, we demonstrate that TA-G protects the roots and improves plant fitness in the field upon *M. melolontha* attack, suggesting that root herbivores can shape root defensive chemistry in nature.

Results

The concentration of the sesquiterpene lactone TA-G is negatively correlated with *M. melolontha* performance

Three classes of secondary metabolites dominate the latex of *T. officinale*: phenolic inositol esters (PIEs, Fig. 1A, left panel), the sesquiterpene lactone taraxinic acid-β-D-glucopyranosyl ester (TA-G, Fig. 1A, left panel) and triterpene acetates (TritAcs, Fig. 1A, right panel) (40). We measured the concentrations of the major latex secondary metabolites in 40 triploid *T. officinale* genotypes collected across central and northern Europe and selected 17 genotypes that displayed maximal variation in latex traits, but minimal variation in growth (text S1, table S1) to correlate latex secondary metabolites with herbivore performance. *Melolontha melolontha* larval mass gain after 11 days of feeding on the 17 genotypes was negatively correlated with the concentration of TA-G (Fig. 1B, left panel, p = 0.007, linear model). By contrast, larval mass gain was not correlated to the total concentrations of PIEs or TritAcs (Fig. 1B, middle and right panel, p = 0.58 for PIEs; p = 0.53 for TritAcs, linear models).

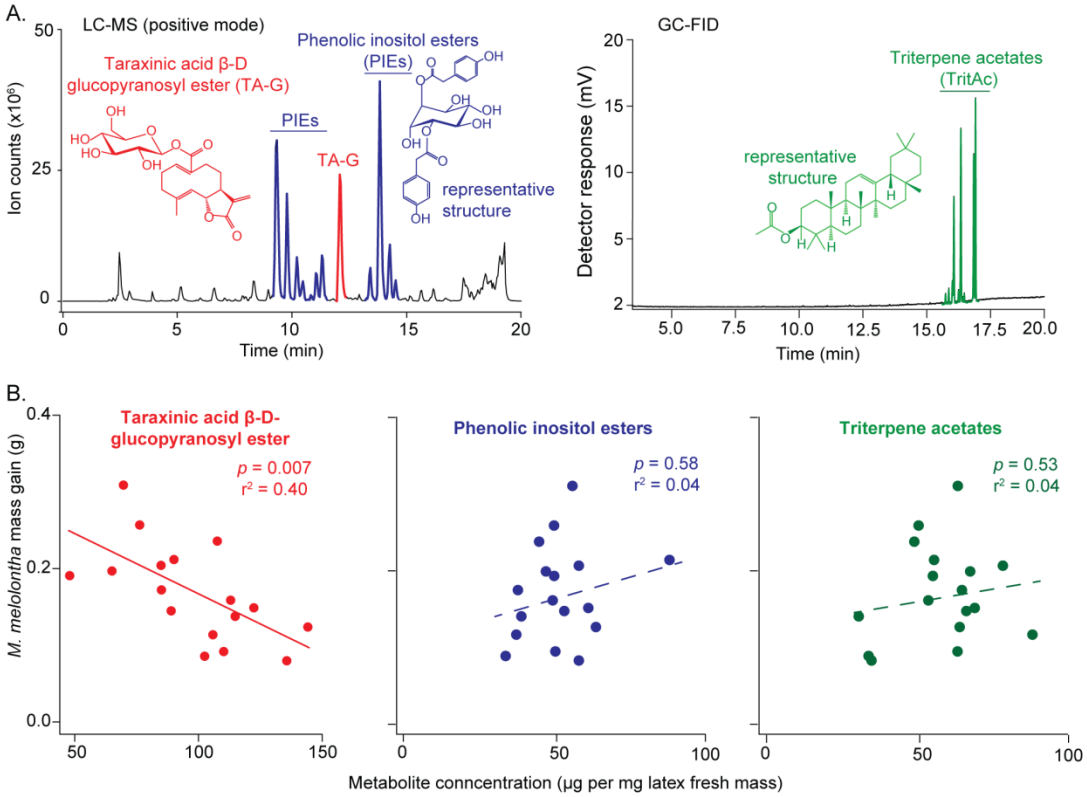

Fig. 1. *Melolontha melolontha* growth correlates negatively with the concentration of the latex metabolite taraxinic acid β-D-glucopyranosyl ester (TA-G). A. Typical LC-MS chromatogram of a latex methanol extract (left panel) and GC-FID chromatogram of a latex hexane extract (right panel) depicting the three major classes of latex secondary metabolites in *T. officinale*. LC = liquid chromatograph. MS = mass spectrometer. GC = gas chromatograph. FID = flame ionization detector. B. After 11 days of feeding, growth of *M. melolontha* larvae on 17 *T. officinale* genotypes was negatively correlated with TA-G concentration in the root latex (linear model, p = 0.007, left panel). *Melolontha melolontha* growth was not correlated with the total concentrations of phenolic inositol esters (middle panel) or triterpene acetates (right panel). Each data point represents the mean *M. melolontha* growth rate of 12 independent replicates per *T. officinale* genotype.

Genetic manipulation shows that TA-G deters *M. melolontha* and improves plant performance

To investigate the effect of TA-G *in planta*, we identified and silenced a gene that encodes for a germacrene A synthase, the enzyme that mediates the first committed step of TA-G biosynthesis, by RNA interference (RNAi) (Fig. 2A). To identify germacrene A candidate genes in *T. officinale*, we sequenced a transcriptome of the main root and of the latex, and constructed a reference transcriptome with the pooled reads. Putative germacrene A synthases were identified based on amino acid sequence similarity with two known germacrene A synthases from chicory (46). Through this approach, we obtained full-length sequences of two putative germacrene A synthase genes, *ToGAS1* and *ToGAS2*, which share 71% identity at the amino acid level. Phylogenetic comparison with other Asteraceae terpene synthases revealed that ToGAS1 belongs to the larger of two germacrene A synthase clusters, while ToGAS2 belonged to the smaller cluster (Fig. 2B). Heterologous expression in *Escherichia coli* showed that both recombinant proteins produced (+)-germacrene A when incubated with the substrate farnesyl diphosphate (Fig. 2C, Fig. S1). To further characterize the two genes, we analyzed their expression in the outer root cortex, latex and the entire main root. As *ToGAS1* was more strongly expressed than *ToGAS2* in both latex and entire main roots (Fig. 2D), we targeted *ToGAS1* through RNA interference by expressing a 191 base pair fragment of this gene under the control of the constitutive *35S* promoter. A reduction of TA-G by over 90% compared to wild type was observed in three independently transformed lines: -1, -12b and -16 ("TA-G-deficient lines"). No reduction in TA-G concentration was found in two other lines, -9 and -15, compared to wild type (all designated as "control lines") (Fig. 2E). *ToGAS1* was suppressed by more than 90% in the TA-G deficient lines compared to control lines, whereas *ToGAS2* expression was not affected (Fig. S2). These results show that ToGAS1 is involved in TA-G biosynthesis in *T. officinale* latex.

To test the function of TA-G *in planta* using the transgenic lines, we first measured the effect of *M. melolontha* attack on 8 week-old TA-G-deficient and control *T. officinale* lines. As non-infested TA-G-deficient and control lines differed in their growth (Fig. S3), we expressed the biomass of herbivore-infested plants relative to the mean biomass of control plants of each genotype. After herbivory, TA-G-deficient lines had lower main and side root mass (Fig. 2F, main roots: $p = 0.04$; side roots: $p = 0.01$, Kruskal-Wallis rank sum test), but not leaf mass (Fig. S4, $p = 0.8$, Kruskal-Wallis rank sum test), expressed relative to non-infested plants of each genotype. To exclude the possibility that the observed effects are due to differences in root growth, we performed a choice experiment with the TA-G-deficient and control lines using 5 week-old plants, which did not show any differences in growth or biomass accumulation (Fig. S5). *Melolontha melolontha* larvae preferred to feed on TA-G-deficient rather than on control lines three hours after start of the experiment (Fig. 2G, top panel, $p = 0.03$, binomial test). Congruently, TA-G-deficient lines lost three times more root mass than control lines (Fig. 2H, $p = 0.04$, paired Student's *t*-test). Additional metabolic profiling revealed that TA-G-deficient and control lines differed in total root protein levels (Figs. S6-8). However, no correlation of this trait with *M. melolontha* behavior was found (Fig. S9). To specifically test the effect of TA-G silencing on

latex bioactivity, we painted 6 week-old carrot seedlings with latex from TA-G-deficient and control plants. *Melolontha melolontha* preferred to feed on carrots painted with latex from the TA-G-deficient lines compared to that from the control lines as measured three hours after start of the experiment (Fig. 2G, lower panel, $p = 0.01$, binomial test).

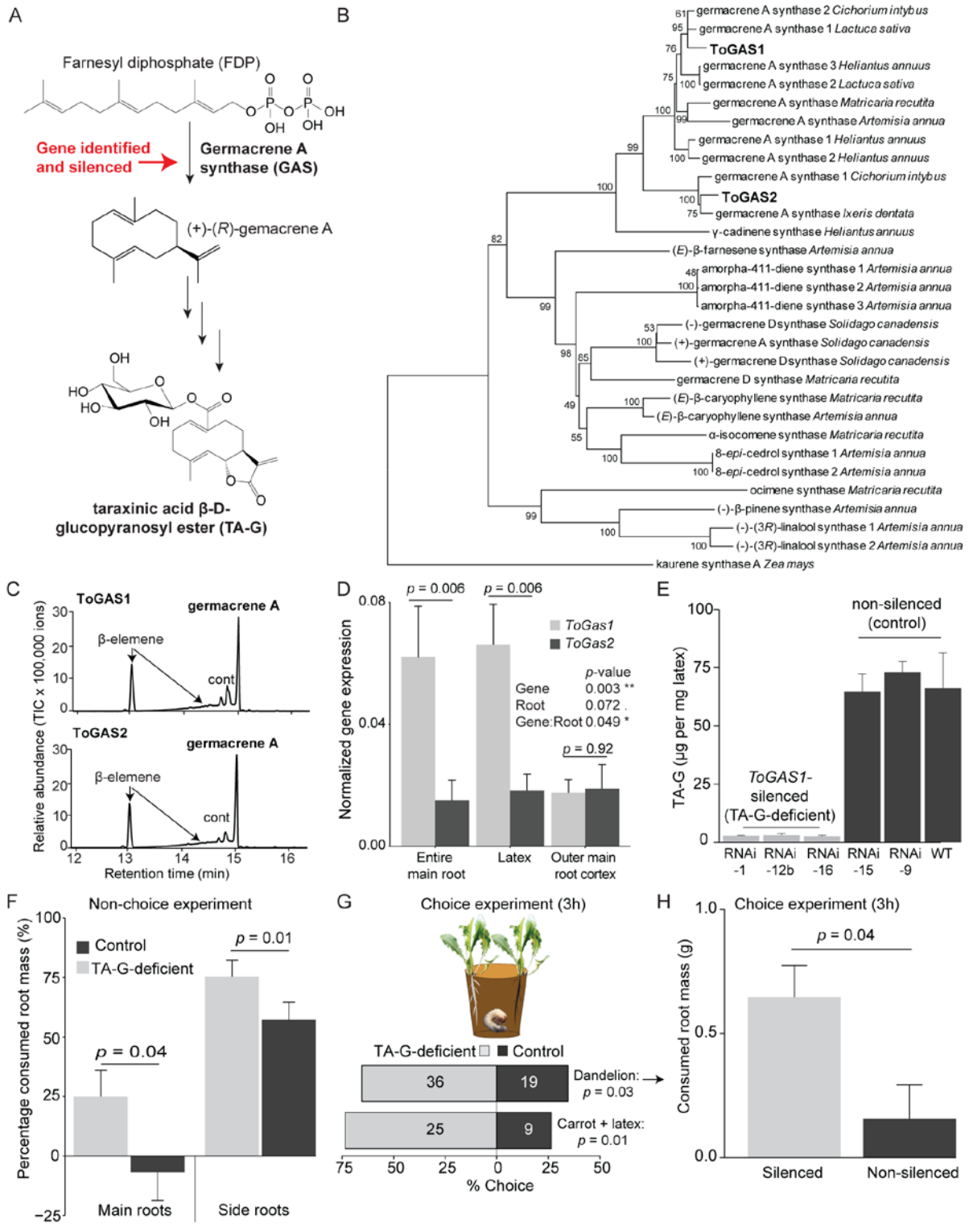

Fig. 2. Silencing of a germacrene A synthase gene reduces TA-G and increases *M. melolontha* feeding. A. Partial biosynthetic pathway of taraxinic acid β-D-glucopyranosyl ester (TA-G). B. Phylogenetic tree of *T. officinale* germacrene A

synthases ToGAS1/2 and known Asteraceae terpene synthases (neighbor-joining method, n=1000 replicates). Bootstrap values are shown next to each node. Accession numbers can be found in table S2. C. GC-MS analysis of enzyme products from recombinant ToGAS1/2 expressed in *Escherichia coli* and incubated with the substrate farnesyl diphosphate. Germacrene A is converted to β-elemene during hot GC injection. cont, contamination. GC-MS = gas chromatograph coupled to a mass spectrometer. D. Expression of *T. officinale* germacrene A synthase genes (*ToGAS1* and *ToGAS2*) in the entire main root, latex and outer main root cortex as determined by RT-qPCR. Statistics of two-way ANOVA and pairwise comparison according to Tukey's post hoc test are shown. Mean Sq = Mean of squares. N = 3. E. Silencing of *ToGAS1* by RNAi generated three independently silenced lines with strongly depleted TA-G concentrations and two transformed lines with similar TA-G concentrations as the parental wild type. N=3. F. In a non-choice experiment, TA-G-deficient lines lost more main and side root mass than control lines after 10 days of feeding by *M. melolontha* relative to undamaged control plants of each accession ("relative root mass") (Kruskal-Wallis rank sum test, n = 36). G. TA-G-deficient *T. officinale* (top bar) and carrot seedlings painted with latex from TA-G-deficient *T. officinale* (lower bar) were preferred by *M. melolontha* over controls after three hours of feeding (binomial test). Diagrams show pooled data of all possible pairwise comparisons of individual TA-G-deficient and control lines. Numbers inside bars refer to number of larvae. H. *M. melolontha* consumed more root mass from TA-G-deficient *T. officinale* seedlings compared to control seedlings in a choice experiment after 4 h (paired Student's *t*-test, n = 81).

Addition of purified TA-G deters *M. melolontha* feeding

Latex profiling revealed that TA-G-deficient lines also had lower PIE levels, suggesting an interaction between the two pathways (Figs. S10-11). To test whether TA-G alone is sufficient to reduce larval consumption, we isolated and purified TA-G by preparative chromatography and performed a feeding experiment with *M. melolontha* larvae feeding on artificial diet containing TA-G. To determine physiologically relevant TA-G concentrations, we first quantified TA-G in different *T. officinale* tissues. Latex contained 75 µg TA-G per mg per fresh mass, and the main roots, side roots and leaves contained 0.2 – 0.7 µg TA-G per mg fresh mass (Fig. 3A). For the artificial diet experiment, we used a concentration of 3 µg TA-G per mg diet to represent a natural situation in which *M. melolontha* feeds on a root that accumulates latex at the site of wounding. Over 24 h, *M. melolontha* larvae consumed 40% less TA-G containing diet than control diet (Fig. 3B, $p = 0.045$, Student's *t*-test).

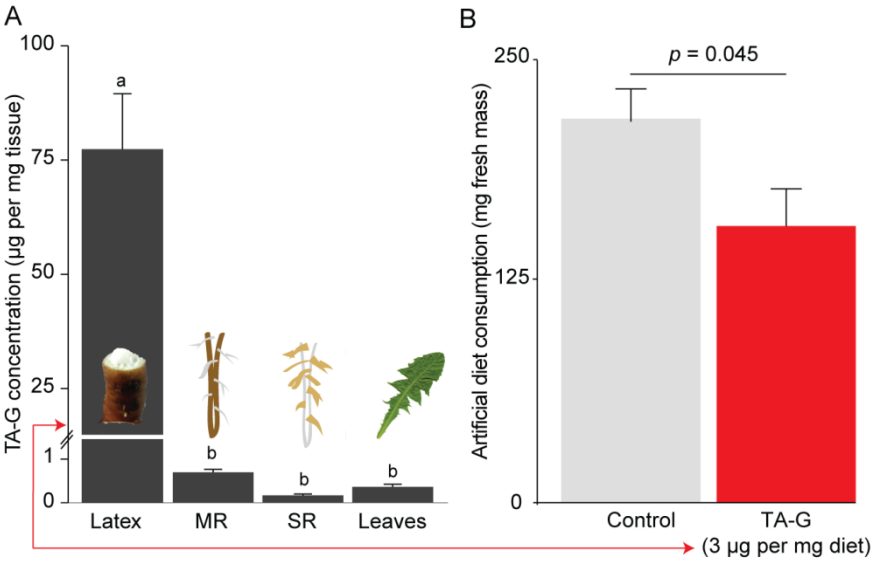

Fig. 3. TA-G reduces larval feeding on artificial diet at physiological relevant concentrations. A. TA-G concentration across tissues. B. *Melolontha melolontha* consumed 40% less TA-G containing diet compared to control diet in a non-choice experiment after 24 h (Student's *t*-test, n = 15).

TA-G improves plant fitness upon *M. melolontha* attack under field conditions

To investigate whether TA-G improves plant performance under *M. melolontha* attack in the field, we grew 2040 *T. officinale* individuals of the experimental population (consisting of the 17 genotypes as described above) in a common garden. We established 20 circular plots, each of them containing 6 individuals of each genotype, and infested half of the plots with 72 *M. melolontha* larvae (23 larvae per m^2) (Fig. S12), a density similar to the damage threshold in pastures (47). In the first year during which most plants did not flower, we measured the length of the longest leaf ("maximal leaf length") – a reliable predictor for leaf and root mass under greenhouse conditions (Fig. S13) – and correlated this parameter with latex secondary metabolite concentrations. To standardize growth rates, we expressed the size increase of the longest leaf of the herbivore-infested plants relative to the size increase of the longest leaf of control plants of the same genotype ("relative leaf growth"). Shortly after infestation of the plants in June, no correlation was observed between relative leaf growth and the concentration of the three latex secondary metabolite classes. During the course of the growing season, a significant positive correlation between relative leaf growth and TA-G concentration emerged (Fig. 4A, p(September) = 0.01, linear model; Fig. S14). No correlation between relative leaf growth and the total concentrations of PIEs or TritAcs was observed throughout the entire growing season (Fig. S15). Leaf length of the herbivore-infested plants was proportional to leaf length of non-infested plants, indicating that plant size did not affect the degree of damage (Fig. S16). To assess whether TA-G also improves plant reproductive fitness, we correlated the number of flowers to latex secondary metabolite concentration in the following year. At the beginning of the flowering season, TA-G was positively correlated with the relative number of flowers (number of flowers of the herbivore-infested plants expressed relative to non-infested plants of each genotype) in the genotypes that flowered at this time point (Fig. 4B, left panel). No correlation between the relative number of flowers and the total concentrations of PIEs and TritAcs were observed (Fig. 4B, middle and right panel). The positive correlation between the relative number of flowers and TA-G disappeared at the end of the flowering period (p = 0.33, Pearson's product-moment correlation), likely because almost all *M. melolontha* larvae had stopped feeding by this time (table S3). Together, these data strongly suggest that TA-G improves plant fitness upon root herbivory.

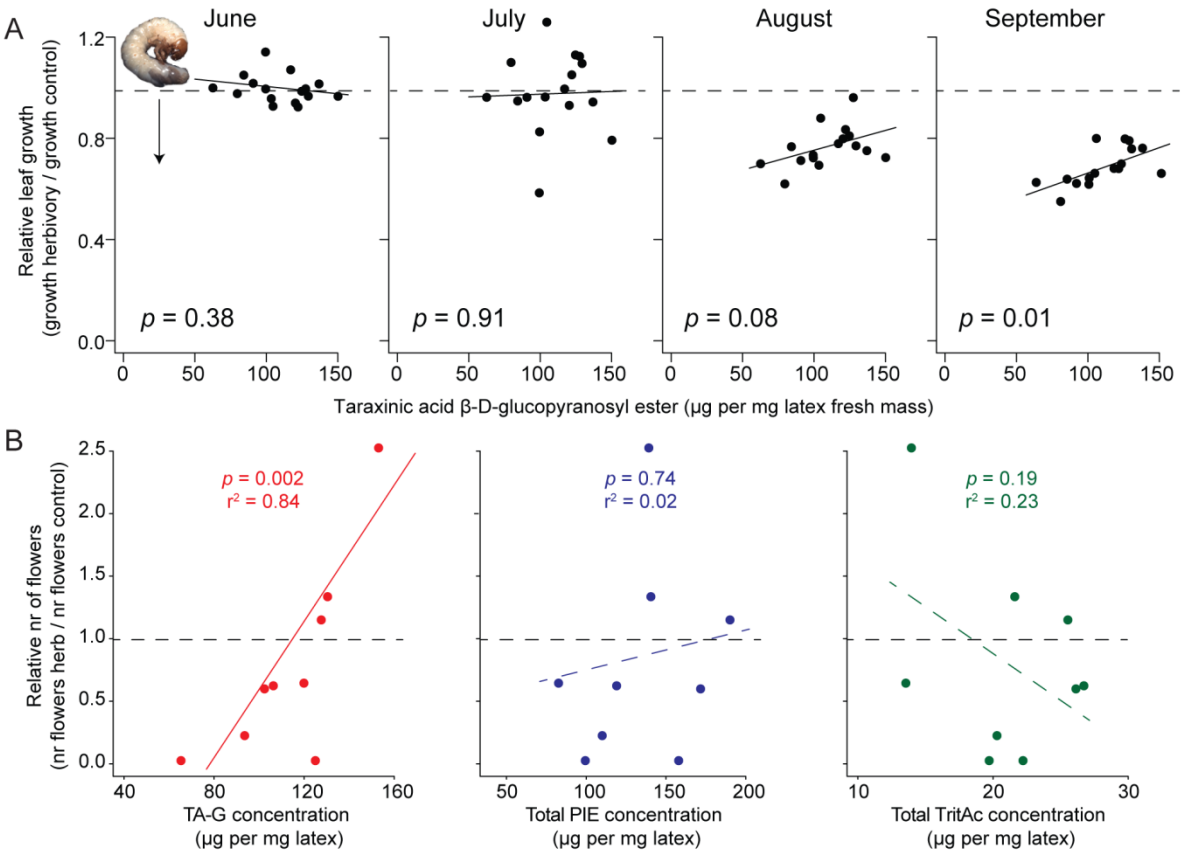

Fig. 4. TA-G improves plant vegetative and reproductive performance upon *M. melolontha* attack in the field. A. TA-G concentration was positively correlated to relative leaf growth across 17 *T. officinale* genotypes in a common garden experiment towards the end of the growing season. Relative leaf growth is the mean leaf growth of herbivore-infested plants of each genotype during the infestation period compared to the mean leaf growth of the control plants of each genotype (leaf growth: increase in maximal leaf length compared to maximal leaf length before infestation). Each data point represents the mean of one genotype. Plants were infested at the end of June. Statistics of linear models based on mean values per genotype are shown. B. The relative number of flowers (number of flowers of the herbivore-infested plants expressed relative to non-infested plants of each genotype) was positively correlated with the concentration of TA-G, but not with the total concentrations of PIEs or TritAcs at the beginning of the flowering season. Only genotypes that flowered at this time point are shown.

Discussion

In this study, we demonstrate that the sesquiterpene lactone TA-G, a major secondary metabolite of *T. officinale*, benefits the plant upon attack by its major native root herbivore *M. melolontha.* TA-G deters *M. melolontha* larvae from feeding and thereby directly protects the roots and improves plant fitness. The observed pattern indicates that root herbivores may exert positive selection pressure on latex secondary metabolites and may thereby drive their evolution..

Our experiments involving natural variation, chemical manipulation and genetic modification provide parallel lines of evidence for a negative effect of TA-G on *M. melolontha* larvae. First, across different *T. officinale* genotypes, TA-G concentration was negatively correlated with *M. melolontha* growth. Second, purified TA-G reduced food consumption *in vitro*. Third, TA-G suppression through *ToGAS1*-silencing increased the attractiveness and consumption of *T. officinale* roots and decreased the deterrent effect of *T. officinale* latex towards *M. melolontha*. Sesquiterpene lactones can have deterrent and toxic effects against a wide range of organisms (48, 49). Their toxicity is attributed to the reactivity of the exocyclic methyl group of the α-methylene-γ-lactone moiety with nucleophilic targets such as cysteine residues of proteins (49). It is likely that TA-G acts via a similar mechanism.

Many studies demonstrate that plant secondary metabolites are toxic to root and leaf herbivores (2-6). Surprisingly however, the benefits for the plant often remain unclear (11-13). Plant secondary metabolites may reduce food quality for the herbivores and may thereby trigger compensatory feeding, leading to higher plant damage (50). In addition, herbivore-imposed loss of biomass can lead to an overcompensation of plant growth and sexual reproduction, which may mask fitness benefits of resistance factors (51-53). Furthermore, secondary metabolites can also reduce plant performance in the field by attracting specialized herbivores that use the chemicals as oviposition (54) and foraging cues (55-57). All these factors may constrain the fitness benefits of bioactive secondary metabolites. Finally, the heterogeneity of natural environments, including varying herbivore communities and abiotic factors can render the detection of fitness benefits difficult (10, 14, 16). We manipulated the abundance of a major root herbivore within artificial populations consisting of plant genotypes that differ substantially in their capacity to produce plant secondary metabolites. This approach allowed us to demonstrate herbivore-dependent fitness benefits under field conditions. Similar experimental designs could be used in combination with transgenic or genetic mapping populations to quantify the contribution of individual herbivore species and herbivore communities to secondary metabolite-dependent fitness benefits in heterogeneous environments (10, 14, 16).

So far, evidence for metabolite-mediated fitness advantage upon insect attack has remained particularly scarce for below ground plant-herbivore interactions. Vaughan, Wang (58) showed that silencing the production of a semi-volatile diterpene increased root damage of *Arabidopsis thaliana* by the opportunistic fungus gnat *Bradysia* spp. Assabgui, Arnason (59) found that inbred maize lines with high root benzoxazinoid concentrations had lower root damage and higher yield than inbred lines with

low benzoxazinoid concentrations. However, follow-up experiments conducted under more controlled conditions failed to confirm this pattern (5, 55). Thus, the importance of root herbivores for shaping the high concentrations and natural variations in root chemistry remains elusive. We show here that high TA-G concentration improves plant vegetative and reproductive performance upon attack of *T. officinale*'s major native root herbivore, *M. melolontha,* using both transgenic and natural variation approaches under controlled and field conditions. TA-G-deficient *T. officinale* lost more root mass than control lines upon feeding by *M. melolontha*. In a common garden experiment, TA-G concentration was positively correlated with leaf growth and flower production across natural *T. officinale* genotypes. Future experiments that correlate TA-G concentration to *M. melolontha* abundance in natural *T. officinale* populations should investigate whether spatial variation in *M. melolontha* abundance can shape variation in TA-G concentration in nature.

Laticifers are commonly assumed to be defensive, as many phytophagous insects are deterred by their secondary metabolite-rich cytoplasm and avoid contact with the often sticky emulsion (13, 34). The defensive features of latex may include both chemical and physical traits. Toxic metabolites or proteins can reduce herbivore performance (32, 36), while the stickiness of latex can trap entire insects or glue their mouth parts (34, 60). Despite the overwhelming evidence that latex reduces herbivore performance, experimental validation that latex improves plant fitness remains scarce (35, 36). We show that a toxic metabolite in the latex benefits the plant in the presence, but not in the absence of an herbivore, and thereby provide an experimental validation of the assumption that micro-evolutionary processes govern intra-specific variation in plant defense traits. These micro-evolutionary processes are consistent with the observed macro-evolutionary patterns in which latex represents a key innovation that has spurred the evolution of the angiosperms (29). Taken together, our results furnish an ecological and evolutionary explanation for the high concentrations of root and latex secondary metabolites and highlight the potential of soil-dwelling insects to shape the chemical defenses of their host plants.

Material and Methods

Plant growth conditions

All indoor experiments were performed in a climate chamber operating under the following conditions: 16 h light 8 h dark; light supplied by a sodium lamp NH 360 FLX SUNLUX ACE Japan; light intensity at plant height: 58 µmol m^2 s^{-1}; temperature: day 22 °C; night 20 °C; humidity: day 55%, night 65% (unless specified otherwise). Plants were potted in 0.7 – 1.2 mm sand and watered with 0.01 – 0.05% fertilizer with N-P-K of 15-10-15 (Ferty 3, Raselina, Czech Republic).

Insects

Melolontha melolontha larvae (Fig. S17) were collected from meadows in Switzerland and Germany. Experiments were performed with larvae in the third larval stage (L3) unless indicated otherwise. Insects were reared individually in 200 ml plastic beakers filled with a mix of potting soil and grated carrots in a phytotron operating under the following conditions: 12 h day 12 h night; temperature: day 13 °C, night 11 ° C; humidity: 70%; lighting: none.

Statistical analyses

All statistical analyses were performed in R version 3.1.1 (61). Pairwise comparison were performed with the agricolae (62) and lsmeans (63) package. Results were displayed using ggplot2 (64) and gridExtra (65). More details on the individual statistical procedures are given in the experimental sections below.

Correlations between latex secondary metabolites and M. melolontha performance

To investigate the effects of latex secondary metabolites on *M. melolontha* performance, we measured growth of *M. melolontha* larvae on 17 *T. officinale* genotypes. To establish an experimental *T. officinale* population, we screened 40 triploid genotypes from central and northern Europe (66) for secondary metabolite concentrations and growth rates. Twenty genotypes were selected based on maximal difference of latex chemistry with minimal variation in plant growth rate using cluster analysis (table S1, text S1). Among these 20 genotypes, three genotypes completely lacked TA-G, but were later found to contain other unidentified sesquiterpene lactone glycosides (Fig. S18). These genotypes were subsequently excluded from analysis. The remaining 17 genotypes were used to correlate larval growth with latex secondary metabolite concentrations. For each genotype, 12 plants were infested at an age of 7 weeks with one pre-weighed *M. melolontha* larva, while 12 plants were left herbivore-free. Eleven days after infestation, *M. melolontha* larvae were recovered and larval mass difference was determined. To measure the concentration of latex secondary metabolites, main roots of control plants were cut and exuding latex collected into Eppendorf tubes and glass vials, immediately flash-frozen in liquid nitrogen and stored at -80 °C until extraction.

For extraction, 1 ml methanol was added to the plastic tubes, and 1 ml hexane containing 0.1 mg*ml^{-1} cholesteryl acetate as internal standard was added to the glass vials. Both types of vessels were vortexed for 5 min, centrifuged, and the supernatant was stored at -80 °C until analysis. Methanol samples were measured on a high pressure liquid chromatograph (HPLC 1100 series equipment, Agilent Technologies), coupled to a photodiode array detector (G1315A DAD, Agilent Technologies) and an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Bremen, Germany). For quantification, peak areas were integrated at 245 nm for TA-G and at 275 nm for PIEs, and quantified using external standard curves. Hexane samples were analyzed on an Agilent series 6890 gas chromatograph coupled to a flame ionization detector (GC-FID). Individual triterpene acetates were quantified based on the internal standard. Methodological details for the analytical procedure have previously been described (40).

Correlations between TA-G, total PIE and total TritAc concentrations and *M. melolontha* mass gain were analyzed using linear models on the mean values of each of the 17 genotypes. The assays were performed in two blocks within two months, and latex for GC analysis was collected from a third batch of plants grown in the same growth chamber under identical conditions.

Transcriptome sequencing

To identify putative germacrene A synthases, we sequenced the transcriptome of *T. officinale* main root and latex using Illumina HiSeq 2500. The main roots of six 10 week-old plants from genotype *A34* were cut, the exuding latex was collected into 100 µl homogenization buffer (67) (4 M guanidine isothiocyanate, 100 mM Tris-HCl, pH 7.0, and 5 mM dithiothreitol) and the latex as well as main root samples were frozen in liquid nitrogen. Main root samples were ground to a fine powder and RNA was extracted from 100 mg tissue with the RNAeasy plant mini kit (Qiagen) following the manufacturer's instructions. For latex RNA extraction, 900 µl QIAzol lysis reagent was added to the latex samples, vortexed and RNA isolated using RNAeasy Plant Lipid Tissue Mini Kit (Qiagen) following the standard procedure. On-column DNA digestion for main root and latex samples was performed using DNase free RNase (Qiagen). The six samples for main root and latex were pooled equimolarly. TruSeq RNA compatible libraries were prepared and PolyA enrichment was performed before sequencing the two transcriptomes on an Illumina HiSeq 2500 with 20 Mio reads per library, 100 base pair, paired end. *De novo* transcriptome assembly on pooled reads from main root and latex sample was performed using Trinity (version Trinityrnaseq_r20131110) (68, 69) running at default settings. Raw reads were deposited in the NCBI Sequence Read Archive (SRA) [number to be inserted at a later stage].

Identification and phylogenetic analysis of T. officinale germacrene A synthases

To identify putative germacrene A synthase genes in the *T. officinale* transcriptome, we performed a BLAST analysis using the amino acid sequences of two known germacrene A synthases

from chicory as templates (46). Two putative germacrene A synthase genes were identified and designated as *ToGAS1* and *ToGAS2*. Sequences were deposited in GenBank with the accession numbers xxx (*ToGAS1*) and yyy (*ToGAS2*). For the estimation of a phylogenetic tree of ToGAS1, ToGAS2 and characterized terpene synthases from other Asteraceae (table S2), we used the MUSCLE algorithm (gap open, -2.9; gap extend, 0; hydrophobicity multiplier, 1.5; clustering method, upgmb) implemented in MEGA5 (70) to compute an amino acid alignment using a neighbor-joining algorithm (Poisson model). All positions with less than 80% site coverage were eliminated. A bootstrap resampling analysis with 1000 replicates was performed to evaluate tree topology.

Cloning and heterologous expression of germacrene A synthases ToGAS1/2

The two putative germacrene A synthases were heterologously expressed in *Escherichia coli* to verify their biochemical function. The complete open reading frames (text S2) encoding putative proteins with 559 amino acids for ToGAS1 and 583 amino acids for ToGAS2 could be amplified from root cDNA using the primers GAS1fwd (ATGGCAGCAGTTGAAGCCAATGGG) and GAS1rev (TTACATGGGCGAAGAACCTACA) for ToGAS1 and the primers GAS2fwd (ATGGCTCTAGTTAGAAACAACAGTAG) and GAS2rev (TCAGTTTTCGAGACTCGGTGGAGGAC) for ToGAS2. The genes were cloned into the vector pET100/D-TOPO® (Invitrogen, Carlsbad, CA, USA) and an *E. coli* strain BL21 Codon Plus (Invitrogen) was used for heterologous expression. Expression was induced by addition of isopropyl-1-thio-D-galactopyranoside to a final concentration of 1 mM. The cells were collected by centrifugation at 4000 g for 6 min, and disrupted by a 4 × 30 sec treatment with a sonicator in chilled extraction buffer (50 mM Mopso, pH 7.0, with 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.5 mM phenylmethanesulfonylfluoride, 5 mM dithiothreitol and 10% v/v glycerol). The cell fragments were removed by centrifugation at 14 000 g, and the supernatant was desalted into assay buffer (10 mM Mopso, pH 7.0, 1 mM dithiothreitol, 10% v/v glycerol) by passage through a Econopac 10DG column (BioRad, Hercules, CA, USA). Enzyme assays were performed in a Teflon®-sealed, screw-capped 1 ml GC glass vial containing 50 µl of the bacterial extract and 50 µl assay buffer with 10 µM (*E*,- *E*)-FPP, 10 mM $MgCl_2$, 0.2 mM $NaWO_4$ and 0.1 mM NaF. An SPME (solid phase micro-extraction) fiber consisting of 100 µm polydimethylsiloxane (SUPELCO, Belafonte, PA, USA) was placed into the headspace of the vial for 60 min incubation at 30 °C and then inserted into the injector of the gas chromatograph for analysis of the adsorbed reaction products. GC-MS analysis was conducted using an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 quadrupole mass selective detector (interface temp, 250 °C; quadrupole temp, 150 °C; source temp, 230 °C; electron energy, 70 eV). The GC was operated with a DB-5MS column (Agilent, Santa Clara, USA, 30 m x 0.25 mm x 0.25 µm). The sample (SPME) was injected without split at an initial oven temperature of 50 °C. The temperature was held for 2 min, then increased to 240 °C with a gradient of 7 °C*min^{-1}, and further increased to 300 °C with a gradient of 60 °C*min^{-1} and a hold of 2 min. For the GC-MS analysis with a cooler injector, the injector temperature was reduced from 220 °C to 150 °C. Chiral GC-MS analysis was performed

using a Rt™-βDEXsm-column (Restek, Bad Homburg, Germany) and a temperature program from 50 °C (2 min hold) at 2 °C*min^{-1} to 220 °C (1 min hold). A (+)-germacrene A synthase (MrTPS3) from chamomile (*Matricaria recutita*) (71) was used to prepare an authentic (+)-germacrene A standard.

Expression analysis of ToGAS1 and ToGAS2

To measure the expression of *ToGAS1* and *ToGAS2*, we harvested latex, main roots and outer cortex cells of 8 week-old *A34* plants. Plants we cultivated in a growth chamber at 18°C and 75% humidity with a 16-h photoperiod (250 µmol m^{-2} s^{-1}) in 50% Ricoterlanderde (RICOTER Erdaufbereitung AG, Aarberg, Switzerland), 40 % sphagnum peat and 10 % sand. Plants were fertilized every week with 0.1% Plantaktiv 16 + 6 + 26 Typ K (Hauert HBG Dünger, Grossaffoltern, Switzerland) according to the manufacturer`s instructions. Total RNA was isolated from roots using the GeneJET Plant RNA Purification Mini Kit (Thermo Scientific) according to the manufacturer's instructions. Total RNA was isolated from latex by dissecting the main root with a razor blade and harvesting 10 µl of expelling latex in 100 µl homogenization buffer (see above). After the addition of 900 µl QIAzol Lysis Reagent, RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. All RNA samples were treated on column with RNase-free DNase I (Qiagen) and the RNA quality and quantity was determined on agarose gels as well as by spectrophotometric analysis using a ND-1000 spectrophotometer (NanoDrop Technologies).

From each sample, 1 µg total RNA was used for reverse transcription using oligo(dT) primers and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA quality was determined by PCR using the primer combination ToActin-fwd (5`-CGTGACATCAAGGAGAAGC-3`) und ToActin-rev (5`-GCTTGGAGATCCACATCTG-3`). Quantitative real-time PCR (qRT-PCR) was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (72). All primer sequences were validated *in silico* (Oligo Proberty Scan, Eurofins MWG, http://www.mwg-biotech.com) and accepted when they yielded single amplicons as it was proven by melt curve analysis, agarose gel electrophoresis and sequencing.

qRT-PCR primers for ToGAS1 (ToGAS1-fwd, 5`-AAATTTCCCTCCTTCAGTATGGGG-3`; ToGAS1-rev, 5`-CTTATTGGAATCCATGGTTGGATCTAC-3`) and ToGAS2 (ToGAS2-fwd, 5`-CTGATACTACCATTGATGCAACCAC-3`; ToGAS2-rev, 5`-CAGCATCAATCTCTTCTGGATAAAG-3`) were designed to anneal at positions of significant sequence divergence between the these two *GAS* genes to yield specific products. The *T. officinale* transcription elongation factor encoding *EF-1α* gene was used as a reference and amplified with the primer combination ToEF1α-fwd (5`-ACTGGTACTTCCCAGGCCGATTGC-3`) and ToEF1α-rev (5`-TTGTTTCACACCAAGGGTGAAGGCG-3`). qRT-PCR experiments were carried out with the LightCycler 96 Real-Time PCR System (Roche Diagnostics International Ltd) using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems) according to the manufacturer's instructions. For each experiment,

three biological replicates were performed with two technical replicates for each biological triplicate. Relative gene expression levels were calculated with the LightCycler 96 Application Software (Version 1.1.0.1320, Roche Diagnostics International Ltd). Expression between different tissues and genes was analyzed with a two-way ANOVA, and pairwise comparison of the expression levels of the two genes performed with Tukey post hoc test.

RNA interference

Based on the transcriptome data, we targeted *ToGAS1* by RNAi. For silencing, we used the triploid genotype *A34* from the above-mentioned 17 *T. officinale* genotype based on transformation compatibility and intermediate levels of TA-G concentration. *A34* is a triploid, synthetic apomict created by crossing a diploid mother from France with diploid pollen from a triploid apomict from the Netherlands (73). For the construction of the germacrene A synthase RNAi vector, a 191-bp germacrene A synthase PCR fragment was amplified from *T. officinale* leaf cDNA using the RNAi-dicer optimized primers ToGermA-RNAi-BamHI_fw (5'-aaaGGATCCGGGATAGAGTACCAGAGATT-3') and ToGermA-RNAi-XhoI_rev (5'-aaaCTCGAGGGCACTAATGTCCCACCTA-3'). This fragment was digested with BamHI and XhoI and inserted into the respective sites of the Gateway vector pENTR4 (Invitrogen). The resulting vector was used for LR recombination (mediated by LR clonase, Invitrogen) with the GW-compatible destination vector pFGC5941 (http://www.chromDB.org), which contains the CaMV 35S promoter and the chalcone synthase intron from *Petunia hybrida*. The integrity of the constructs was verified by sequencing and subsequently used for *Agrobacterium tumefaciens*-mediated stable transformation of the *T. officinale A34* genotype using the same method as described previously (74).

Screening and characterization of transgenic lines

The T1 generation of 13 transformed lines was screened for latex secondary metabolite concentrations using three individuals of each line. Main root latex of 8 week-old *T. officinale* was collected into Eppendorf tubes and frozen in liquid nitrogen. Latex was extracted as described above using 1 ml methanol containing 10 µg*ml^{-1} loganin as internal standard. Samples were analyzed on HPLC-DAD as described above. Five lines were selected for further molecular and phenotypic characterization. First, the transgenic lines were confirmed to be triploid by flow cytometry. Second, the insertion of the transgene was verified by PCR and sanger sequencing on genomic DNA using the primer combination P2 + ToGermA-RNAi-XhoI_rev and P3 + ToGermA-RNAi-XhoI_rev, with 5'-TACCTTCCCACAATTCGTCG-3'f for P2, 5'-CAGGTATTGGATCCTAGGTG-3' for P3 and 5'-AAACTCGAGGGCACTAATGTCCCACCTA-3' for ToGermA-RNAi-XhoI_rev. Third, transcript levels of *ToGAS1* and *ToGAS2* were determined in the T2 generation by qPCR using the primers described above. Four individuals of the TA-G-deficient (RNAi-1, -12b, -16) and control (RNAI -9, -15, WT) lines grown in soil were harvested at an age of 8 weeks. Main root tissue was frozen in liquid nitrogen and ground under liquid nitrogen to a fine powder. RNA was extracted using the GeneJET

Plant RNA Purification Mini Kit (Thermo Scientific) according to the manufacturer's instructions. RT-qPCR was performed for *ToGAS1*, *ToGAS2* and *ToEF1α* as described above (n=4). Relative gene expression levels were calculated with the LightCycler 96 Application Software. Gene expression was analyzed with generalized linear models using a gamma error distribution for *ToGAS1* and a Gaussian error distribution for *ToGAS2*. Forth, we determined latex secondary metabolites, total protein, amino acid and sugar concentrations in the roots. To analyze concentration of TA-G and total PIEs in the transgenic plants, we harvested six individuals of three TA-G-deficient (RNAi-1, -12b, -16) and three control (wild type, RNAi-9, -15) lines in the T2 generation at an age of 8 weeks. Main root latex was collected into Eppendorf tubes, which were flash-frozen in liquid nitrogen. 1 ml methanol containing 10 $\mu g*ml^{-1}$ loganin and 100 $\mu g*ml^{-1}$ salicin as internal standards for TA-G and PIEs, respectively, were added to the Eppendorf tubes. Samples were extracted and analyzed as described above. Differences in the concentration of TA-G and total PIEs between TA-G-deficient and control lines were analyzed with one-way ANOVAs. To determine whether total TritAc concentration was affected by silencing, we collected main root latex from 6 individuals of 9 week-old TA-G-deficient (RNAi-1, -12b, -16) and control lines (wild type, RNAi-9, 15) into glass vials, which were immediately frozen in liquid nitrogen. Samples were extracted with 1 ml hexane containing 100 $\mu g*ml^{-1}$ cholesteryl acetate as internal standard. Samples were processed and analyzed on GC-FID as described above. Differences in the concentration of total TritAcs were analyzed with a one-way ANOVA.

To determine soluble protein, free amino acid and soluble sugar concentrations in the roots of the TA-G-deficient (RNAi-1, -12b, -16) and control (wild type, RNAi-9, -15) lines, we harvested 5 individuals of each line at an age of 12 weeks. Root systems were exposed, washed and main and side roots frozen in liquid nitrogen. Root tissue was ground under liquid nitrogen to a fine powder. For extraction, 1 ml 0.1 M TRIS-HCl, pH=7.0 was added to 100 mg ground tissue, vortexed and centrifuged at room temperature at 17,000 g for 10 min. The supernatant was stored at -20 °C until analysis.

Soluble protein concentration was determined using the Bradford assay and quantified using a standard curve of albumin (75). Differences in soluble protein concentrations between TA-G-deficient and control lines, as well as between root tissues, were analyzed with two-way ANOVAs. To determine free amino acid concentrations, 10 µl of the diluted samples were mixed with 90 µl ^{13}C, ^{15}N labelled amino acid mix (20 μg amino $acids*ml^{-1}$) (Isotec, Miamisburg, USA) and 100 µl borate buffer (0.9 M, pH=10.0). To derivatize amino acids, 22 µl 30 mM fluorenylmethoxy-carbonyl chloride was added and samples were vortexed. After 5 min, 800 µl hexane was added to stop the reaction; the samples were vortexed and placed at room temperature until phases had separated. The lower, aqueous phase was analyzed on an Agilent 1200 HPLC system coupled to an API 5000 tandem mass spectrometer according to (76). To determine soluble sugar concentrations, main root samples were diluted 1:10 and side root samples 1:5 in 0.1 M TRIS-HCl, pH=7.0. Samples were analyzed on an Agilent 1200 HPLC system (Agilent Technologies, Germany) coupled to an API 3200 tandem mass spectrometer (Applied

Biosystems, Germany) equipped with a turbospray ion source operating in negative ionization mode. Injection volume was 1 µl. Metabolite separation was accomplished by an apHera NH_2, 15 cm x 4.6 mm x 3 µm. The mobile phase consisted of water (A) and acetonitrile (B) utilizing a flow of 1 ml*min^{-1} with the following gradient: 0 min: 20% A, 0.5 min: 20% A, 13 min: 45% B, followed by column reconditioning. The column temperature was maintained at 20 °C. The ion spray voltage was maintained at -4.5 keV. The turbo gas temperature was set at 600 °C. Nebulizing gas was set at 50 psi, curtain gas at 20 psi, heating gas at 60 psi and collision gas at 5 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: m/z 178.9 →89 (collision energy (CE) -10 V; declustering potential (DP) -25 V), for glucose; m/z 178.9 →89 (CE -12V; DP -25V) for fructose; m/z 341.03 →58.96 (CE -52V; DP -45V) for sucrose; Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing. All compounds were identified based on comparison of retention times and mass spectra to those of commercial standards. Glucose, fructose and sucrose concentrations were quantified using external standard curves obtained from commercial standards. Differences in the sugar concentrations between TA-G-deficient and control lines, as well as between root tissues, were analyzed with two-way ANOVAs.

No-choice experiment with M. melolontha and transgenic T. officinale

To investigate whether silencing of *ToGAS1* affects plant performance, we measured root and leaf mass of three TA-G-deficient (RNAi-1, -12b, -16) and three control (wild type, RNAi-9, -15) lines. For each line, 24 plants of the T2 generation were cultivated for 8 weeks. Half of the plants were infested with one pre-weighed *M. melolontha* larva. One week after infestation, plants were separated into side roots, main roots and leaves, and plant material was dried for three days at 60 °C before weighing. As TA-G-deficient lines had 50% lower root mass than control lines, resistance was expressed relative to the control plants of each genotype (100*(1-(mass herbivore plant / mean mass control plants of its genotypes))) and analyzed with Kruskal-Wallis rank sum tests.

Choice experiment with M. melolontha and transgenic T. officinale

In order to test *M. melolontha* preference for and plant resistance of TAG-deficient and wild type *T. officinale* plants, three TA-G-deficient (RNAi-1, -12b, -16) and three control (wild type, RNAi-9, -15) lines were tested in a choice experiment with *M. melolontha* larvae. Larvae were starved for three days prior to the experiment. Each larva was placed into a 180 ml plastic beaker, which was filled with 2-3 mm vermiculite. The roots of 5 week-old *T. officinale* seedlings of the T2 generation (grown in soil in seedling trays) were washed, briefly dried with a tissue and the mass of the plants determined. One TA-G-deficient and one control plant was embedded into the vermiculite-filled beaker at opposite edges, with 9 replicates of each possible pairwise combination. Larval feeding site was scored visually after 3 h by inspecting the beakers from the outside. To determine root mass consumption, plants were recovered after 4 h. The plants were separated into shoots and roots and dried for three days at 60 °C.

Fresh mass was calculated from dry mass using a common conversion coefficient based on the fresh/dry mass ratio of five non-manipulated seedlings of each genotype. Root mass consumption was analyzed using paired Student's *t*-tests. To obtain sufficiently large sample sizes for a binomial test, larval preference was analyzed by pooling the data for the three TA-G-deficient and control lines. In order to test whether differences in primary metabolites affected *M. melolontha* choice, we correlated *M. melolontha* preference and root mass consumption to total main root protein concentration as determined from 12 week-old plants as described above. Data were analyzed with Pearson's product-moment correlation.

Choice experiment with carrot seedlings painted with TA-G-deficient and wild type latex

To test whether *M. melolontha* preference for TA-G-deficient *T. officinale* is mediated by latex metabolites, we recorded larval choice among carrot seedlings painted with latex of three TA-G-deficient (RNAi-1, -12b, -16) and three control (RNAi-9, -14, -15) lines. *M. melolontha* larvae were starved for two days. Each larva was placed into the center of a 180 ml plastic beaker, which was filled with 2-3 mm vermiculite. The roots of the 6 week-old carrot seedlings were completely covered with latex of 5 month-old TA-G-deficient and control *T. officinale* of the T1 generation, cultivated in 2 l pots in soil (identical growth conditions as described above, except light source from NH 360 FLX SUNLUX ACE Japan). Seedlings painted with TA-G-deficient and control latex were pairwise arranged on opposite edges of the beaker, resulting in 6-11 replicates of each possible pairwise combination. Larval feeding site was visually scored after three hours. Larval preference was analyzed based on pooled data for the three TA-G-deficient and control lines using a binomial test.

Choice experiments with purified TA-G

To determine physiologically relevant TA-G concentrations for bioassays, we analyzed TA-G concentration from latex, main and side roots and leaves from three wild type *A34* plants. Main root latex of 11 week-old *T. officinale* was collected into Eppendorf tubes, frozen in liquid nitrogen and extracted with 1 ml methanol containing 10 µg*ml^{-1} loganin as an internal standard as described above. Main roots, side roots and leaf tissues were flash-frozen in liquid nitrogen and ground to fine powder. 100 mg tissue was extracted with 1 ml methanol containing 10 µg*ml^{-1} loganin, vortexed, centrifuged and the supernatant transferred to HPLC vials. All samples were analyzed on analyzed by HPLC chromatograph 1100 series equipment (Agilent Technologies, Germany), coupled to a photodiode array detector (G1315A DAD, Agilent Technologies, Germany). Metabolite separation was accomplished with a Nucleodur Sphinx RP column (250 x 4.6 mm, 5 µm particle size, Macherey-Nagel). The mobile phase consisted of 0.1% acetic acid (A) and acetonitrile (B) utilizing a flow of 1 ml*min^{-1} with the following gradient: 0 min: 5% B, 18 min: 43% B, followed by column reconditioning. Peak area was integrated at 245 nm for TA-G and normalized to loganin as internal standard.

To test whether TA-G deters *M. melolontha*, we isolated TA-G from latex and added it to artificial diet at a concentration of 3 µg TA-G*mg^{-1} diet. TA-G was isolated using 300 ml latex methanol extracts obtained from 300 *A34* plants grown in the greenhouse. 10 ml water was added to the methanol extract before methanol was completely evaporated using rotary-evaporation. The aqueous solution was loaded on a Sephadex LH20 (GE-Healthcare, Germany) column with 2.5 cm x 30 cm dimensions. The compounds were eluted from the column using water at a flow speed of 1 ml*min^{-1}. 15 ml fractions were collected and analyzed for TA-G on an Agilent 1200 HPLC system (Agilent Technologies, Germany) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems, Germany) equipped with a turbospray ion source operating in negative ionization mode. Injection volume was 5 µl using flow injection analysis. The mobile phase consisted of 0.05% formic acid (A) and acetonitrile (B) utilizing a flow of 1 ml*min^{-1}. 50% A was maintained for 0.5 min. The column temperature was kept at 20 °C. The ion spray voltage was maintained at -4.5 keV. The turbo gas temperature was set at 600 °C. Nebulizing gas was set at 50 psi, curtain gas at 30 psi, heating gas at 60 psi and collision gas at 5 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: m/z 423 →261 (collision energy (CE) -14 V; declustering potential (DP) -40 V), for TA-G; m/z 447 →151 (CE -26V; DP -100V) for di-PIEs; m/z 581 →151 (CE -38V; DP -140V) for tri-PIEs. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

Pure fractions were pooled and lyophilized using an Alpha 1-4 LD plus freeze dryer (Martin Christ GmbH, Germany). 30 *M. melolontha* larvae were starved for two days before providing them 300 mg artificial diet supplemented with either 30 µl 30 mg*ml^{-1} TA-G or with 30 µl water as solvent control (artificial diet: 25 g bean flower, 2.4 g Agar from Roth, Agar-Agar bacteriologist, 105 ml tap water, 33.3 g cooked and mashed carrots). Larvae were allowed to feed for 24 h inside a 180 ml plastic beaker covered with a moist tissue before the remaining food was weighed. Food consumption was analyzed using Student's *t*-test. Larvae that consumed less than 30 mg diet were considered inactive and were excluded from the analysis.

Common garden experiment

In order to examine the effects of latex secondary metabolites on plant resistance in the field, we cultivated 2400 *T. officinale* from the above-mentioned 20 genotypes in a common garden with and without *M. melolontha* infestation over one year at a field site in Jena, Germany (50°54'34.8"N; 11°34'00.1"E). Seeds were surface sterilized, germinated on moist filter paper in petri dishes in spring 2013, and the emerging seedlings were transferred onto peat balls after 10 days. One month after germination, seedlings were conditioned outside for one week before planting them into the field site. At the field site, the top 50 cm soil layer was removed and a metal mesh installed on the ground to confine vertical *M. melolontha* movement. Experimental units ("plots") were set up using 20 circular plastic tubes (50 cm depth, 2 m diameter), that were placed on the top of the mesh and filled up with the

original soil. One wheelbarrow of peat was mixed with the top 20 cm of soil to facilitate plant growth. In each plot, 6 replicates of all 20 genotypes were placed randomly in a quadratic grid with 10 cm distance between plants. To buffer edge effects, these experimental plants were surrounded with an additional row of *T. officinale* plants, which were excluded from data analysis. Plants were watered as necessary during the first two months after planting and plots weeded monthly. Three weeks after planting, the length of the longest leaf ("maximal leaf length") was measured for each plant. Subsequently, 72 late L2 or early L3 *M. melolontha* larvae were homogenously distributed in half of the plots ("herbivory"), while the remaining plots were not manipulated ("control"). As a non-destructive measurement of plant performance, we measured maximal leaf length - a reliable predictor for above and below ground biomass under greenhouse conditions (Fig. S13) - of each plant every month until the end of the growing season.

For statistical analysis, the length of the longest leaf at the beginning of the experiment was subtracted from the maximal leaf length measured each month to reduce the impact of initial differences in plant size ("leaf growth"). To normalize between genotypes, leaf growth of herbivore-treated plants was expressed relative to control plants of the same genotype ("relative leaf growth").

$$Relative\ leaf\ growth\ (j) = \frac{Mean(Max\ leaf\ length\ H(ij) - Initial\ max\ leaf\ length\ H(ij))}{Mean(Max\ leaf\ length\ C(ij) - \ Initial\ max\ leaf\ length\ C(ij))}$$

with H = herbivore infested plants

C = control plant

i = individual plant

j = genotype

Initial max leaf length = maximal leaf length in June before infestation

Correlations between relative leaf growth and TA-G, total PIEs and total TritAcs were performed based on mean values for each genotype for each month separately with linear models in R. Secondary metabolite concentrations were obtained from the experiment with the 20 genotypes in the greenhouse as described above. Three genotypes lacking TA-G were excluded from the analysis due to the presence of unknown and thus unquantifiable sesquiterpene lactone glycosides.

In order to test whether damage caused by *M. melolontha* in the field is proportional to plant size, we assessed the correlation between leaf length of herbivore-infested individuals and leaf length of non-infested individuals of the 17 genotypes with Pearson's product-moment correlations.

To correlate secondary metabolite concentrations to reproductive plant fitness, the number of flowers was counted every month in the following year. Correlations between relative number of

flowers (number of flowers of the herbivore-infested plants expressed relative to non-infested plants of each genotype) and TA-G, total PIEs and total TritAcs were analyzed with linear models and Pearson product-moment correlations based on the mean value of each of the 17 genotypes.

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References

1. Dixon RA, Strack D. Phytochemistry meets genome analysis, and beyond. Phytochemistry. 2003;62(6):815-6.
2. Dawson RF. The localization of the nicotine synthetic mechanism in the tobacco plant. Science. 1941;94(2443):396-7.
3. Kaplan I, Halitschke R, Kessler A, Sardanelli S, Denno RF. Constitutive and induced defenses to herbivory in above- and belowground plant tissues. Ecology. 2008;89(2):392-406.
4. Malcolm SB. Cardenolide-mediated interactions between plants and herbivores. In: Berenbaum GARR, editor. Herbivores: Their interactions with secondary plant metabolites. Second Edition ed. San Diego: Academic Press; 1991. p. 251-96.
5. Niemeyer HM. Hydroxamic acids derived from 2-hydroxy-2h-1,4-benzoxazin-3(4h)-one: Key defense chemicals of cereals. J Agr Food Chem. 2009;57(5):1677-96.
6. Mithöfer A, Boland W. Plant defense against herbivores: chemical aspects. Annu Rev Plant Biol. 2012;63:431-50.
7. Ehrlich PR, Raven PH. Butterflies and plants - a study in coevolution. Evolution. 1964;18(4):586-608.
8. Züst T, Heichinger C, Grossniklaus U, Harrington R, Kliebenstein DJ, Turnbull LA. Natural enemies drive geographic variation in plant defenses. Science. 2012;338(6103):116-9.
9. Agrawal AA, Hastings AP, Johnson MTJ, Maron JL, Salminen JP. Insect herbivores drive real-time ecological and evolutionary change in plant populations. Science. 2012;338(6103):113-6.

10. Prasad KVSK, Song B-H, Olson-Manning C, Anderson JT, Lee C-R, Schranz ME, et al. A gain-of-function polymorphism controlling complex traits and fitness in nature. Science. 2012;337(6098):1081-4.

11. Kroymann J. Natural diversity and adaptation in plant secondary metabolism. Curr Opin Plant Biol. 2011;14(3):246-51.

12. Adler LS, Irwin RE. Ecological costs and benefits of defenses in nectar. Ecology. 2005;86(11):2968-78.

13. Agrawal AA, Konno K. Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. Annu Rev Ecol Evol S. 2009;40:311-31.

14. Kerwin R, Feusier J, Corwin J, Rubin M, Lin C, Muok A, et al. Natural genetic variation in *Arabidopsis thaliana* defense metabolism genes modulates field fitness. eLife. 2015;4.

15. Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT. Nicotine's defensive function in nature. Plos Biol. 2004;2(8):1074-80.

16. Brachi B, Meyer CG, Villoutreix R, Platt A, Morton TC, Roux F, et al. Coselected genes determine adaptive variation in herbivore resistance throughout the native range of *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences. 2015;112(13):4032-7.

17. Rasmann S, Agrawal AA. In defense of roots: A research agenda for studying plant resistance to belowground herbivory. Plant Physiol. 2008;146(3):875-80.

18. van Dam NM. Belowground herbivory and plant defenses. Annu Rev Ecol Evol S. 2009;40:373-91.

19. Brown VK, Gange AC. Insect herbivory below ground. Advances in Ecological Research. 1990;20:1-58.

20. Watts SM, Dodson CD, Reichman OJ. The roots of defense: Plant resistance and tolerance to belowground herbivory. PLoS One. 2011;6(4).

21. Curtis TP, Sloan WT, Scannell JW. Estimating prokaryotic diversity and its limits. Proceedings of the National Academy of Sciences. 2002;99(16):10494-9.

22. Vorholt JA. Microbial life in the phyllosphere. Nat Rev Micro. 2012;10(12):828-40.

23. Chitwood DJ. Phytochemical based strategies for nematode control. Annu Rev Phytopathol. 2002;40:221-49.

24. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol. 2006;57(1):233-66.

25. Mandal SM, Chakraborty D, Dey S. Phenolic acids act as signaling molecules in plant-microbe symbioses. Plant Signaling & Behavior. 2010;5(4):359-68.

26. Ramakrishna A, Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling & Behavior. 2011;6(11):1720-31.

27. Wink M. Biochemistry of plant secondary metabolism. London: Blackwell; 2010.

28. Matile P. The toxic compartment of plant cells. Naturwissenschaften. 1984;71(1):18-24.

29. Farrell BD, Dussourd DE, Mitter C. Escalation of plant defense - do latex and resin canals spur plant diversification? Am Nat. 1991;138(4):881-900.

30. Lewinsohn TM. The geographical distribution of plant latex. Chemoecology. 1991;2:64-8.

31. Metcalfe CR. Distribution of latex in the plant kingdom. Economic Botany. 1967;21(2):115-27.

32. Konno K. Plant latex and other exudates as plant defense systems: Roles of various defense chemicals and proteins contained therein. Phytochemistry. 2011;72(13):1510-30.

33. Hagel JM, Yeung EC, Facchini PJ. Got milk? The secret life of laticifers. Trends Plant Sci. 2008;13(12):631-9.

34. Dussourd DE, Eisner T. Vein-cutting behavior - insect counterploy to the latex defense of plants. Science. 1987;237(4817):898-901.

35. Agrawal AA. Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. Evol Ecol Res. 2005;7(5):651-67.

36. Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y, Hattori M, et al. Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. Plant J. 2004;37(3):370-8.

37. Kirschner J, Stepanek J. Clonality as a part of the evolution process in *Taraxacum*. Folia Geobot Phytotx. 1994;29(2):265-75.

38. Richards AJ. The origin of *Taraxacum* agamospecies. Bot J Linn Soc. 1973;66(3):189-211.

39. Verduijn MH, Van Dijk PJ, Van Damme JMM. Distribution, phenology and demography of sympatric sexual and asexual dandelions (*Taraxacum officinale* s.l.): geographic parthenogenesis on a small scale. Biol J Linn Soc. 2004;82(2):205-18.

40. Huber M, Triebwasser-Freese D, Reichelt M, Heiling S, Paetz C, Chandran J, et al. Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (*Taraxacum officinale* agg.). Phytochemistry. 2015:In press.

41. Keller E, Keller S, Buchi R, Meier W, Staub A, Wildbolz T. Neuere Erkenntnisse über den Maikäfer. Beiheft zu den Mitteilungen der Thurgauerischen Naturforschenden Gesellschaft. 1986;89:7-95.

42. Heer O. Ueber geographische Verbreitung und periodisches Auftreten der Maikäfer. Verhandlungen der Schweizerischen Naturforschenden Gesellschaft. 1841;26.

43. Hasler T. Abundanz- und Dispersionsdynamik von *Melolontha melolontha (*L.*)* in Intensivobstanlagen: Eidgenössische Technische Hochschule Zürich; 1986.

44. Hauss R. Methoden und erste Ergebnisse zur Bestimmung der Wirtspflanzen des Maikäferengerlings *Melolontha melolontha* L. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin Dahlen. 1975;163:72-7.

45. Hauss R, Schütte F. Experiments on polyphagous habits of white grubs *Melolontha melolontha* on plants of grassland. Anzeiger für Schädlingskunde, Pflanzenschutz, Umweltschutz. 1976;49(9):129-32.

46. Bouwmeester HJ, Kodde J, Verstappen FW, Altug IG, de Kraker JW, Wallaart TE. Isolation and characterization of two germacrene A synthase cDNA clones from chicory. Plant Physiol. 2002;129(1):134-44.

47. Huiting HF, Moraal LG, Griepink FC, Ester A. Biology, control and luring of the cockchafer, *Melolontha melolontha*. Wageningen: Applied Plant Research, 2006.

48. Picman AK. Biological activities of sesquiterpene lactones. Biochem Syst Ecol. 1986;14(3):255-81.

49. Schmidt TJ. Toxic activities of sesquiterpene lactones: Structural and biochemical aspects. Curr Org Chem. 1999;3(6):577-608.

50. Steppuhn A, Baldwin IT. Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. Ecol Lett. 2007;10(6):499-511.

51. Paige KN, Whitham TG. Overcompensation in response to mammalian herbivory - the advantage of being eaten. Am Nat. 1987;129(3):407-16.

52. Lennartsson T, Tuomi J, Nilsson P. Evidence for an evolutionary history of overcompensation in the grassland biennial *Gentianella campestris* (Gentianaceae). Am Nat. 1997;149(6):1147-55.

53. Hendrix S, Trapp EJ. Plant-herbivore interactions: Insect induced changes in host plant sex expression and fecundity. Oecologia. 1981;49(1):119-22.

54. Mewis I, Ulrich C, Schnitzler WH. The role of glucosinolates and their hydrolysis products in oviposition and host-plant finding by cabbage webworm, *Hellula undalis*. Entomol Exp Appl. 2002;105(2):129-39.

55. Robert CAM, Veyrat N, Glauser G, Marti G, Doyen GR, Villard N, et al. A specialist root herbivore exploits defensive metabolites to locate nutritious tissues. Ecol Lett. 2012;15(1):55-64.

56. Miles CI, del Campo ML, Renwick JA. Behavioral and chemosensory responses to a host recognition cue by larvae of *Pieris rapae*. Journal of comparative physiology A, Neuroethology, sensory, neural, and behavioral physiology. 2005;191(2):147-55.

57. Gabrys B, Tjallingii WF. The role of sinigrin in host plant recognition by aphids during initial plant penetration. In: Nielsen J, Kjær C, Schoonhoven L, editors. Proceedings of the 11th International Symposium on Insect-Plant Relationships. Series Entomologica. 57: Springer Netherlands; 2002. p. 89-93.

58. Vaughan MM, Wang Q, Webster FX, Kiemle D, Hong YJ, Tantillo DJ, et al. Formation of the unusual semivolatile diterpene rhizathalene by the *Arabidopsis* class I terpene synthase TPS08 in the root stele is involved in defense against belowground herbivory. Plant Cell. 2013;25(3):1108-25.

59. Assabgui RA, Arnason JT, Hamilton RI. Field evaluations of hydroxamic acids as antibiosis factors in elite maize inbreds to the western corn-rootworm (Coleoptera, Chrysomelidae). J Econ Entomol. 1995;88(5):1482-93.

60. Dussourd DE. Entrapment of aphids and whiteflies in lettuce latex. Annals of the Entomological Society of America. 1995;88(2):163-72.

61. R Core Team. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2014.

62. de Mendiburu F. agricolae: Statistical Procedures for Agricultural Research. R package version 1.2-0 ed2014.

63. Lenth RV, Hervé M. lsmeans: Least-Squares Means. 2015.

64. Wickham H. ggplot2: elegant graphics for data analysis: Springer New York; 2009.

65. Auguie B. gridExtra: functions in Grid graphics. R package version 0.9.1 ed2012.

66. Oplaat C, Verhoeven KJF. Range expansion in asexual dandelions: selection for general-purpose genotypes? J Ecol. 2015;103(1):261-8.

67. Post J, van DN, Fricke J, Kowalski N, Wurbs D, Schaller H, et al. Laticifer-specific cis-prenyltransferase silencing affects the rubber, triterpene, and inulin content of *Taraxacum brevicorniculatum*. Plant Physiol. 2012;158(3):1406-17.

68. Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols. 2013;8(8):1494-512.

69. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology. 2011;29(7):644-U130.

70. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. Mega5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28(10):2731-9.

71. Irmisch S, Krause ST, Kunert G, Gershenzon J, Degenhardt J, Köllner TG. The organ-specific expression of terpene synthase genes contributes to the terpene hydrocarbon composition of chamomile essential oils. Bmc Plant Biol. 2012;12.

72. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009;55(4):611-22.

73. Verhoeven KJF, Van Dijk PJ, Biere A. Changes in genomic methylation patterns during the formation of triploid asexual dandelion lineages. Mol Ecol. 2010;19(2):315-24.

74. Wahler D, Gronover CS, Richter C, Foucu F, Twyman RM, Moerschbacher BM, et al. Polyphenoloxidase silencing affects latex coagulation in *Taraxacum* species. Plant Physiol. 2009;151(1):334-46.

75. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.

76. Pande S, Merker H, Bohl K, Reichelt M, Schuster S, de Figueiredo LF, et al. Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria. Isme Journal. 2014;8(5):953-62.

Supporting Information

S1 text. Selection procedure of 20 *T. officinale* genotypes.

S2 text. Full length sequences of ToGAS1 and ToGAS2.

S1 table. Origin of 20 *T. officinale* genotypes. Genotype A34 is a triploid, synthetic apomict, created by crossing a sexual diploid mother from France with diploid pollen from a triploid apomict from the Netherlands (Verhoeven, Van Dijk & Biere 2010).

S2 table. Accession numbers of protein sequences used for dendrogram analysis of Asteraceae terpene synthases.

S3 table. Density of *M. melolontha* per m^2 in common garden field experiment at the end of the flowering season in the second year.

S1 figure. Heterologous expression of germacrene A synthases (ToGAS1/2) in *Escherichia coli*. a. GC-MS analysis of enzyme products from recombinant ToGAS1 and ToGAS2 incubated with the substrate farnesyl diphosphate (FPP). Germacrene A produced by ToGAS1 and ToGAS2 is converted to β-elemene during hot GC injection. cont, contamination. b. Chiral analysis of recombinant ToGAS1 and ToGAS2 enzyme products. Retention times and mass spectra of ToGAS enzyme products were compared to those of (-)-β-elemene obtained as a thermal rearrangement product of (+)-germacrene A synthesized by MrTPS3 from chamomile (Irmisch *et al.* 2012). c. GC-MS analysis of monoterpene products from recombinant ToGAS1 and ToGAS2 incubated with the substrate geranyl diphosphate (GPP). 1, myrcene; 2, limonene; 3, (*Z*)-β-ocimene; 4, (*E*)-β-ocimene; 5, terpinolene; 6, linalool; 7, α-terpineol. IC = ion count.

S2 figure. Silencing efficiency of *ToGAS1/2*. Expression of *ToGAS1* and *ToGAS2* of the TA-G-deficient (RNAi -1, -12b, 16) and control (RNAi -9, -15, WT) lines normalized to the elongation factor *ToEF1α*. Different letters indicate significant differences in expression of *ToGAS1* (lower case) and *ToGAS2* (upper case) between the different lines in a generalized linear model. N = 4.

S3 figure. Vegetative biomass of TA-G-deficient and control *T. officinale* lines at an age of 8 weeks. X-axis shows individual silenced lines. Statistics show one-way ANOVA. Sum Sq = sum of squares. N = 12.

S4 figure. Relative leaf mass of TA-G-deficient and control *T. officinale* lines upon *M. melolontha* attack. Larvae fed for 7 days on 8 week-old *T. officinale* seedlings. Relative leaf mass is the mass of each herbivore infested plant relative to the mean leaf mass of the control plants of its genotype. Statistics from Kruskal-Wallis rank sum test is shown. N = 12.

S5 figure. Vegetative biomass of TA-G-deficient and control *T. officinale* lines at an age of 5 weeks. X-axis shows individual silenced lines. Statistics show one-way ANOVA. Sum Sq = sum of squares. N = 5.

S6 figure. Concentration of soluble proteins in roots of TA-G-deficient and control *T. officinale*. 8 week-old *T. officinale* were analyzed. X-axis shows individual silenced lines. Statistics of two-way ANOVA is shown. Sum Sq = sum of squares. N = 6.

S7 figure. Concentrations of free amino acids in roots of TA-G-deficient and control *T. officinale*. 8 week-old *T. officinale* were analyzed. Data from the three TA-G-deficient (RNAi-1, -12b, -16) and control lines (wild type, RNAi-9, RNAi-15) were pooled. N = 6.

S8 figure. Concentrations of soluble sugars in roots of TA-G-deficient and control *T. officinale*. 8 week-old *T. officinale* were analyzed. X-axis shows individual silenced lines. Statistics of two-way ANOVA is shown. Sum Sq = sum of squares. N = 6.

S9 figure. Correlations of soluble protein concentration and *M. melolontha* choice across TA-G deficient and control lines. *P*-values from Pearson product-moment correlations are shown.

S10 figure. TA-G and total PIE concentrations in latex of TA-G-deficient and control *T. officinale* lines. Latex of 8 week-old *T. officinale* was analyzed. X-axis shows individual silenced lines. TA-G = taraxinic acid β-D-glucopyranosyl ester; PIE = phenolic inositol ester. Statistics of one-way ANOVA is shown. N = 6.

S11 figure. Total triterpene acetate concentrations in latex of TA-G-deficient and control *T. officinale*. Latex of 8 week-old *T. officinale* was analyzed. X-axis shows individual silenced lines. Statistics of one-way ANOVA is shown. N = 6.

S12 figure. Overview of common garden experiment.

S13 figure. Correlation between leaf length and vegetative biomass. Correlation between leaf length and leaf and root dry mass across three genotypes (*19.31, 2.8A, A34*) over a growth period of 5 weeks. 5 plants per genotype were harvested every week starting with 6 week-old plants cultivated in a growth chamber. Each data point represents the mean of each genotype and time point. Statistics from linear models are shown.

S14 figure. Correlation between TA-G and leaf growth in a common garden experiment with and without *M. melolontha* infestation. TA-G concentration tended to be positively correlated to leaf growth (maximal leaf length of each month – maximal leaf length before infestation) under *M.*

melolontha attack and negatively correlated to leaf growth in the control treatment towards the end of the growing season. Plants were infested in June. Each data point represents the mean of one genotype. *P*-values from linear models based on mean values of each genotype are shown.

S15 figure. Correlations of relative leaf growth with total concentrations of PIEs and TritAcs in a common garden. Relative leaf growth is the size increase of the longest leaf of the herbivore infested plants compared of the size of the longest leaf before infestation, expressed relative to the leaf growth of the control plants of each genotype. Each data point represents the mean relative leaf growth of one *T. officinale* genotype. Plants were infested in June. *P*-values from linear models based on mean values of each genotype are shown.

S16 figure. Correlation between average leaf length of *M. melolontha*-infested plants and mean leaf length of non-infested plants in the common garden experiment in September. Herbivore-damage was proportional to plant size. The *P*-value of a Pearson product-moment correlation is shown. One data point represents the mean of one genotype.

S17 figure. Picture of *M. melolontha* feeding on *T. officinale* roots.

S18 figure. In-source fragmentation pattern of TA-G and putative sesquiterpene lactone glycosides. A. In-source fragmentation pattern of taraxinic acid β-D-glucopyranosyl ester (TA-G), obtained from a latex methanol extract of genotype *A34*. B-D. Putative sesquiterpene lactone glycosides. A latex methanol extract from genotype 17.20A was screened for fragmentation patterns resembling TA-G. All samples were analyzed on an Esquire 6000 ESI-Ion Trap mass spectrometer in positive ionization mode (40).

Author contributions

M.E. and M.H. conceived the project. M.H., M.E., T.G.K and C.S.G. designed experiments. M.H., J. P., J.F., Z.A., T.B., M. S., T.G.K., H.V., A.H., D.F.-T., C.A.M.R., M.E., K.V. and V.P. performed experiments. M.H. and T.G.K. analyzed data. M.H. and M.E. wrote the manuscript. J.G. and M.E. provided reagents/materials and analysis tools. All authors contributed to editing an earlier version of the manuscript.

3.3.1 Supplemental

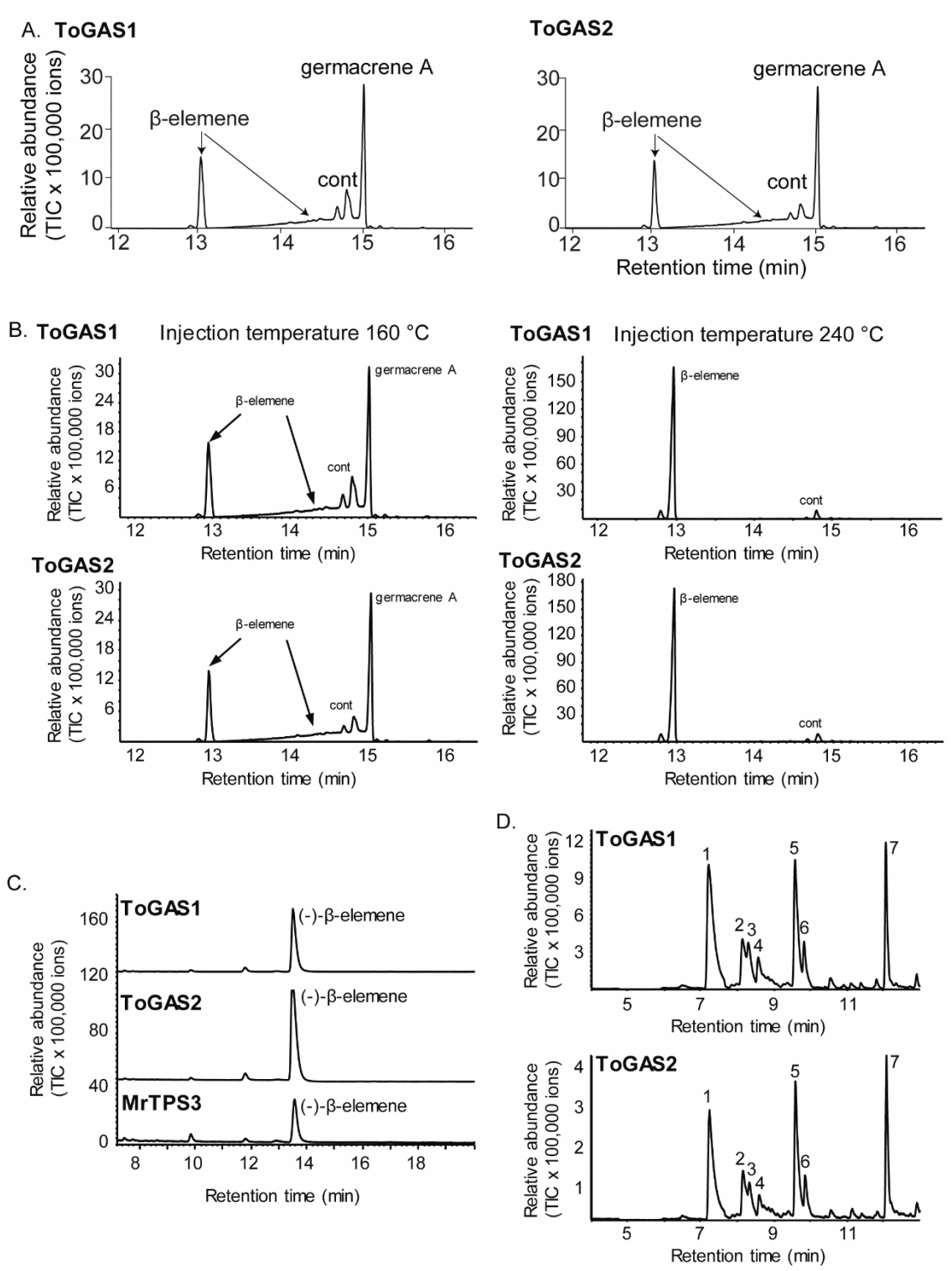


Fig. S1 Heterologous expression of germacrene A synthases (ToGAS1/2) in *Escherichia coli*. A. GC-MS analysis of enzyme products from recombinant ToGAS1/2 expressed in *Escherichia coli* and incubated with the substrate farnesyl diphosphate. Germacrene A is converted to β-elemene during hot GC injection. cont, contamination. B. GC-MS analysis of enzyme products from recombinant ToGAS1 and ToGAS2 incubated with the substrate FPP. Germacrene A produced by ToGAS1 and ToGAS2 is

converted to β-elemene during hot GC injection. cont, contamination. b. Chiral analysis of recombinant ToGAS1 and ToGAS2 enzyme products. Retention times and mass spectra of ToGAS enzyme products were compared to those of (-)-β-elemene obtained as a thermal rearrangement product of (+)-germacrene A synthesized by MrTPS3 from chamomile (Irmisch *et al.* 2012). c. GC-MS analysis of monoterpene products from recombinant ToGAS1 and ToGAS2 incubated with the substrate geranyl diphosphate. 1, myrcene; 2, limonene; 3, (*Z*)-β-ocimene; 4, (*E*)-β-ocimene; 5, terpinolene; 6, linalool; 7, α-terpineol. GC-MS = gas chromatograph coupled to a mass spectrometer. TIC = total ion count.

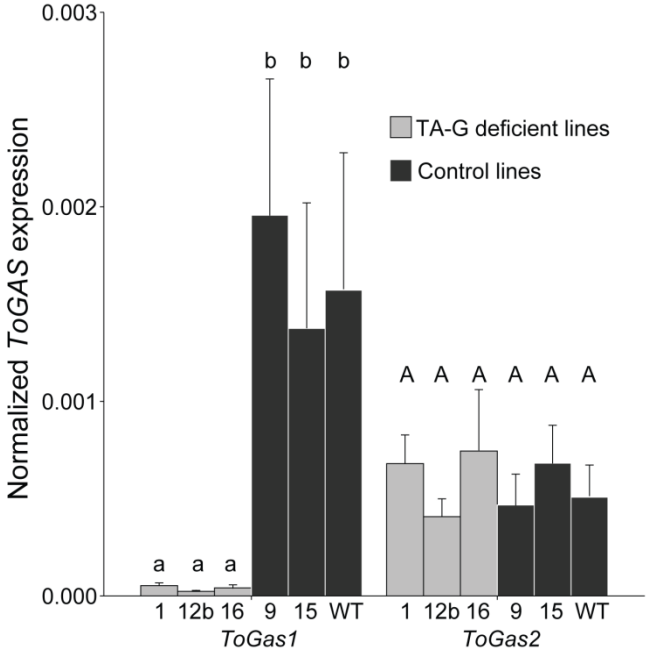


Fig. S2 Expression of *ToGAS1* and *ToGAS2* of the TA-G-deficient (RNAi -1, -12b, 16) and control (RNAi -9, -15, WT) lines normalized to the elongation factor *ToEF1α*. Different letters indicate significant differences in expression of *ToGAS1* (lower case) and *ToGAS2* (upper case) between the different lines in a generalized linear model. N = 4.

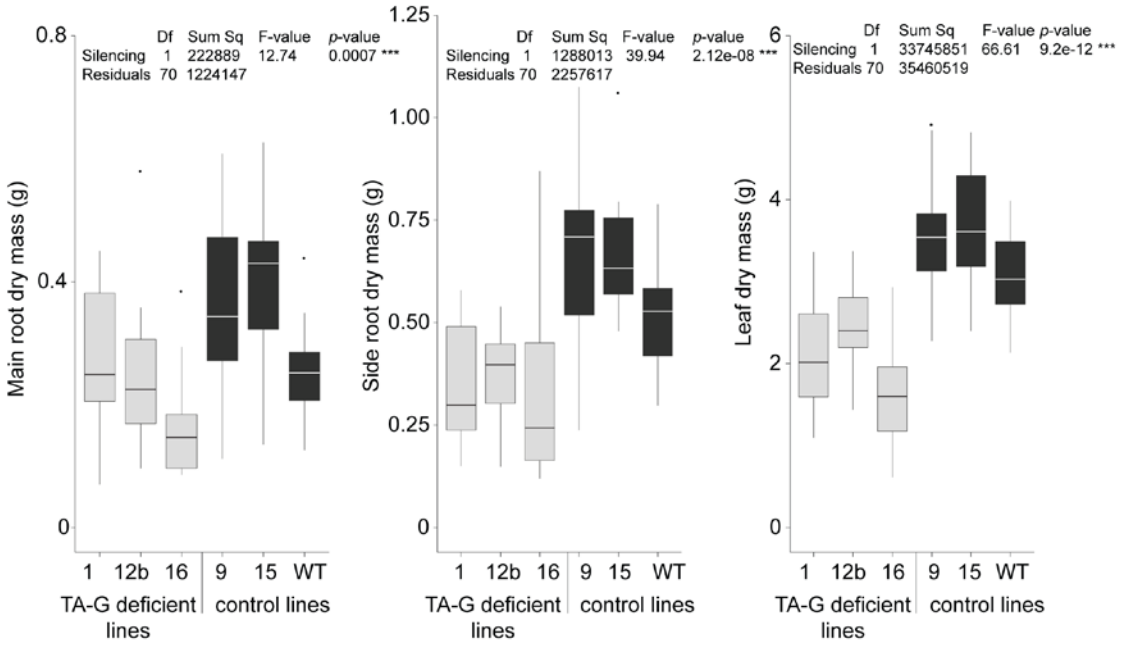


Fig. S3 Vegetative biomass of TA-G-deficient and control *T. officinale* lines at an age of 8 weeks as used for the no-choice experiment. Statistics show one-way ANOVA. Sum Sq = sum of squares. N = 12. TA-G = taraxinic acid β-D-glucopyranosyl ester.

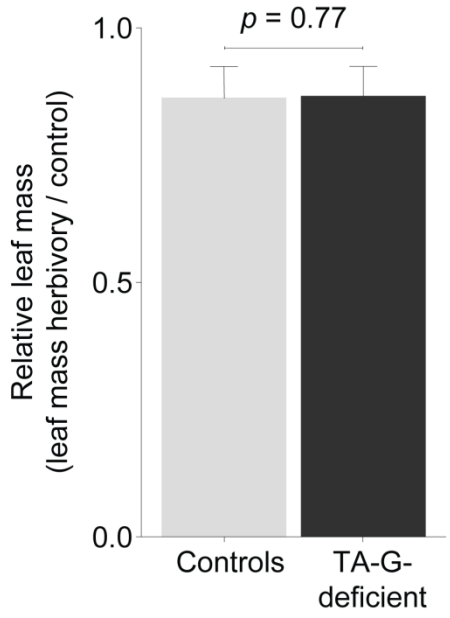


Fig. S4 Relative leaf mass of TA-G-deficient and control *T. officinale* lines upon *M. melolontha* attack. Larvae fed for 7 days on 8 week-old *T. officinale* seedlings. Relative leaf mass is the mass of each herbivore infested plant relative to the mean leaf mass of the control plants of its genotype. Statistics from Kruskal-Wallis rank sum test is shown. N = 12.

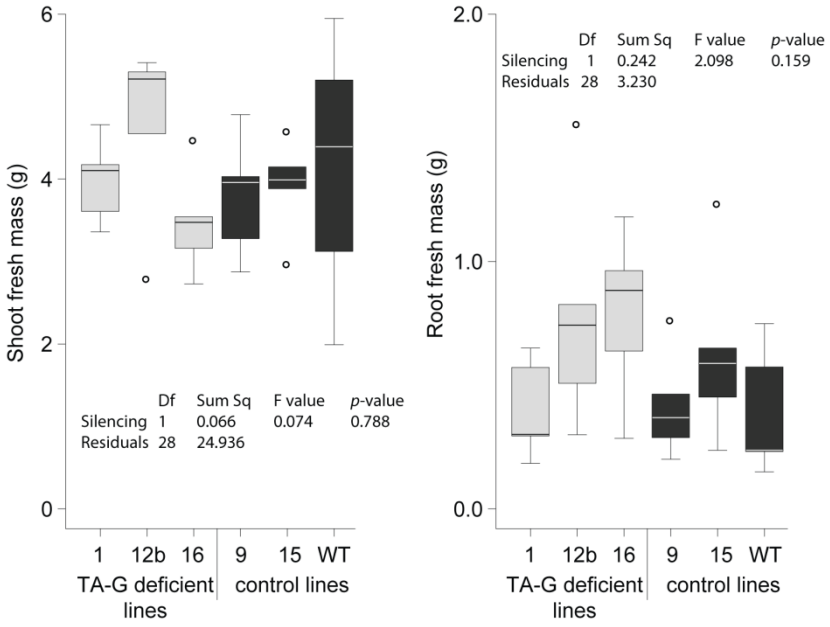


Fig. S5 Vegetative biomass of TA-G-deficient and control *T. officinale* lines at an age of 5 weeks as used for the choice experiment. Statistics show one-way ANOVA. Sum Sq = sum of squares. N = 5. TA-G = taraxinic acid β-D-glucopyranosyl ester.

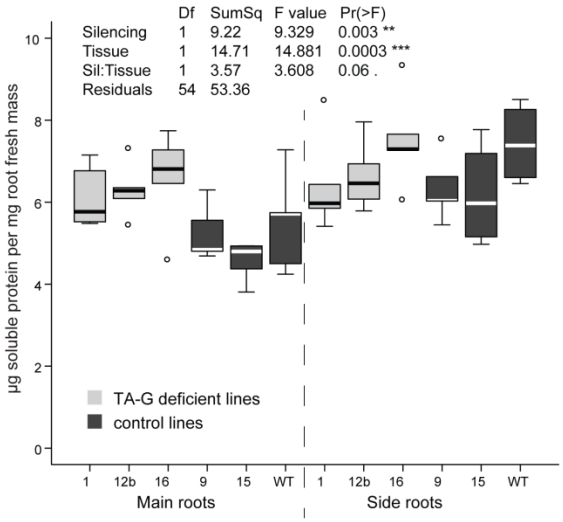


Fig. S6 Concentration of soluble proteins in roots of 8 week-old TA-G-deficient and control *T. officinale* lines. Statistics of two-way ANOVA is shown. Sum Sq = sum of squares; Pr(>F) = *p*-value N = 6. TA-G = taraxinic acid β-D-glucopyranosyl ester.

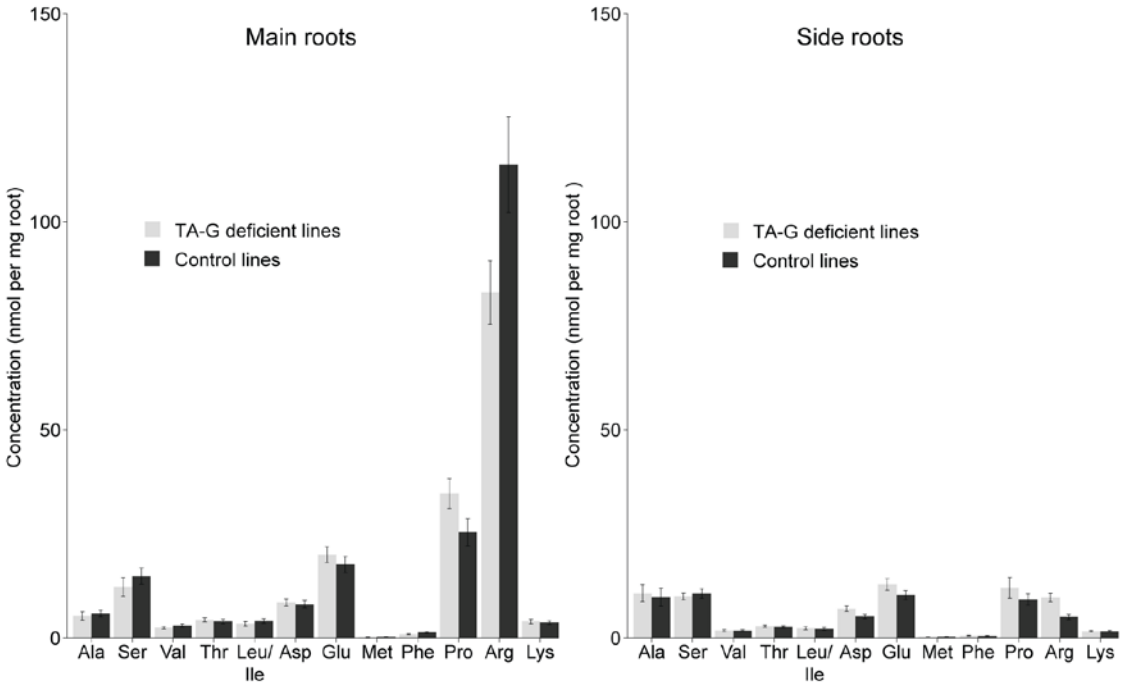


Fig. S7 Concentrations of free amino acids in roots of 8 week-old TA-G-deficient and control *T. officinale* lines. Data from the three TA-G-deficient (RNAi-1, -12b, -16) and control lines (wild type, RNAi-9, RNAi-15) were pooled. N = 6. TA-G = taraxinic acid β-D-glucopyranosyl ester.

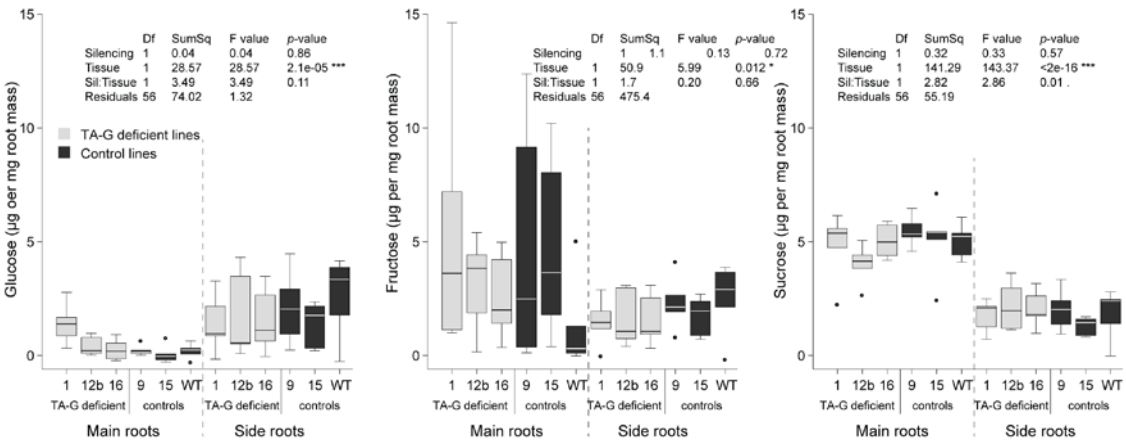


Fig. S8 Concentrations of soluble sugars in roots of 8 week-old TA-G-deficient and control *T. officinale* lines. Statistics of two-way ANOVA is shown. Sum Sq = sum of squares. N = 6. TA-G = taraxinic acid β-D-glucopyranosyl ester.

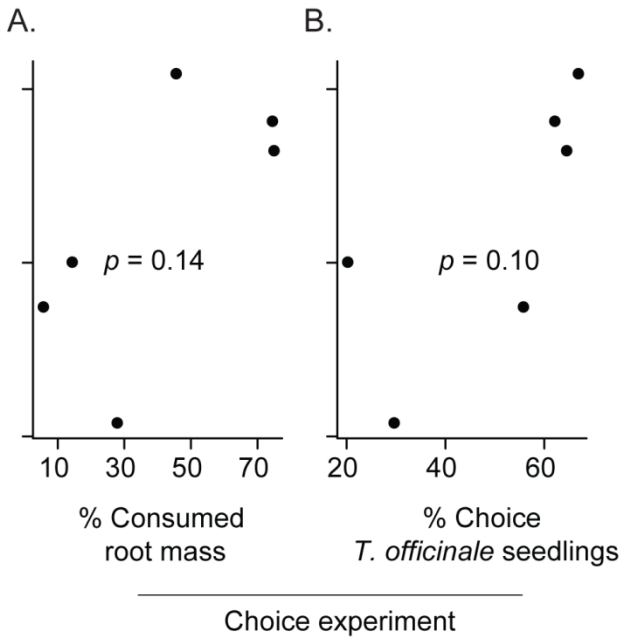


Fig. S9 Correlations of soluble protein concentration, main and side root dry mass of 8 week old transgenic lines with plant resistance and *M. melolontha* choice. *P*-values from Pearson product-moment correlations are shown.

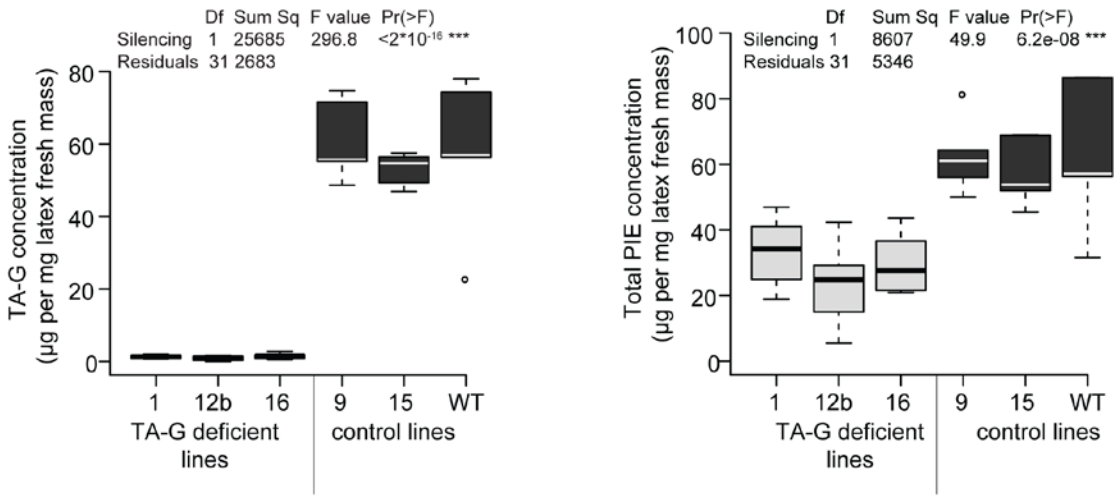


Fig. S10 TA-G and total PIE concentrations in the latex of 8 week-old TA-G-deficient and control *T. officinale* lines. TA-G = taraxinic acid β-D-glucopyranosyl ester; PIE = phenolic inositol ester. Statistics of one-way ANOVA is shown. Sum Sq = sum of squares; Pr(>F) = *p*-value. N = 6.

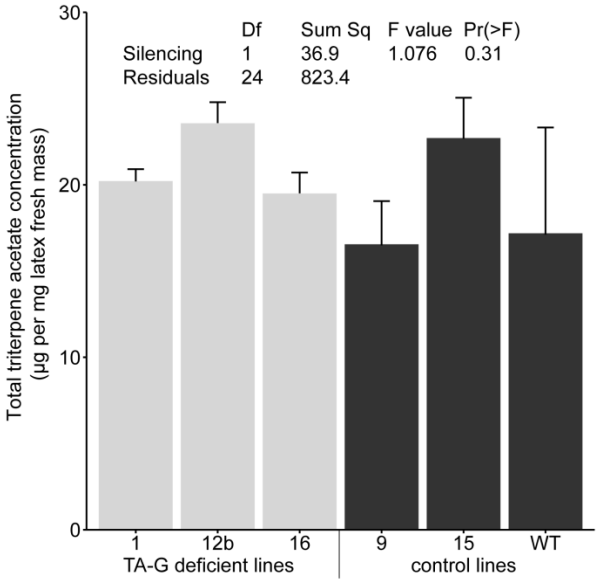


Fig. S11 Total triterpene acetate concentrations in latex of 8 week-old TA-G-deficient and control *T. officinale* lines. Statistics of one-way ANOVA is shown. Sum Sq = sum of squares; Pr(>F) = *p*-value. N = 6. TA-G = taraxinic acid β-D-glucopyranosyl ester.

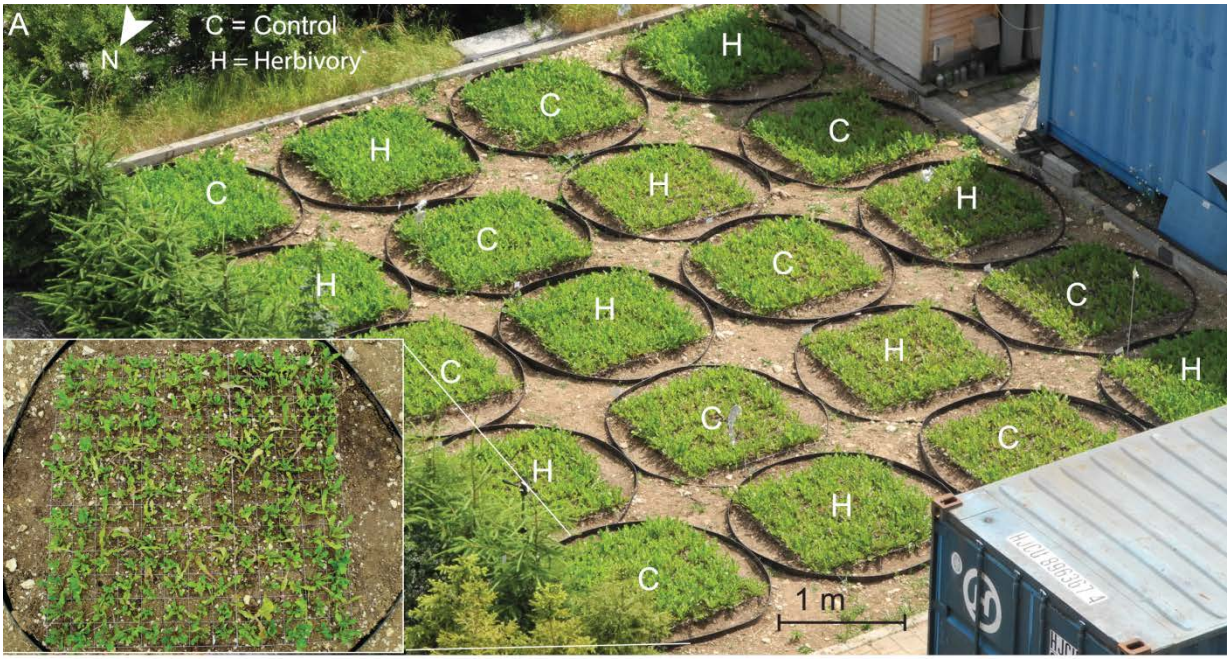


Fig. S12 Overview of common garden experiment.

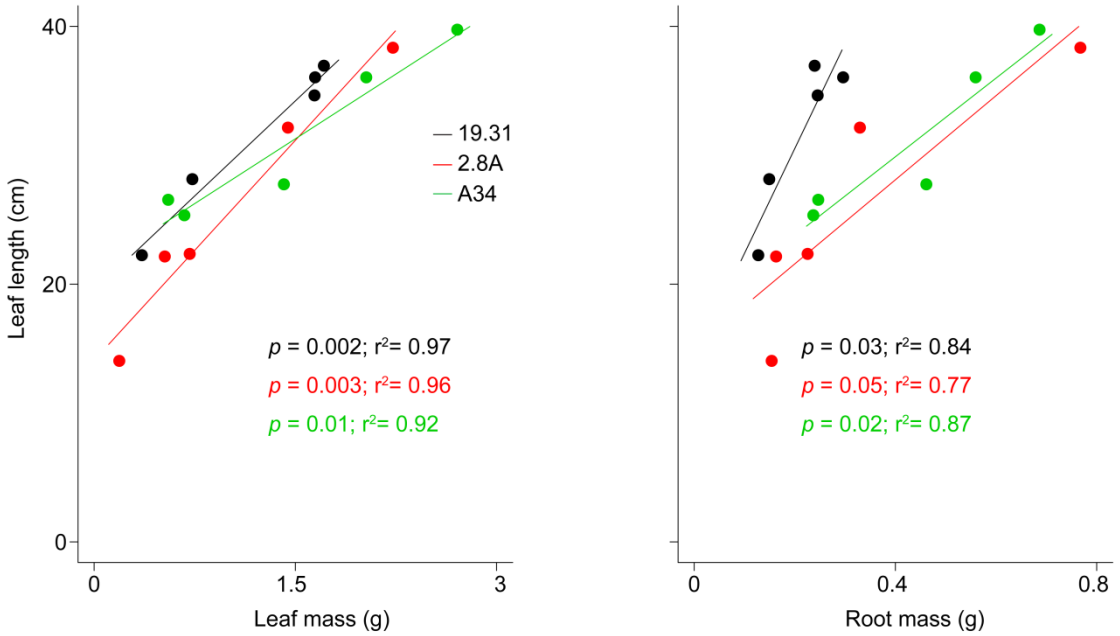


Fig. S13 Correlation between leaf length and leaf and root dry mass across three genotypes (19.31, 2.8A, A34) over a growth period of 5 weeks. 5 plants per genotype were harvested every week starting with 6 week-old plants cultivated in a growth chamber. Each data point represents the mean of each genotype and time point. Statistics from linear models are shown.

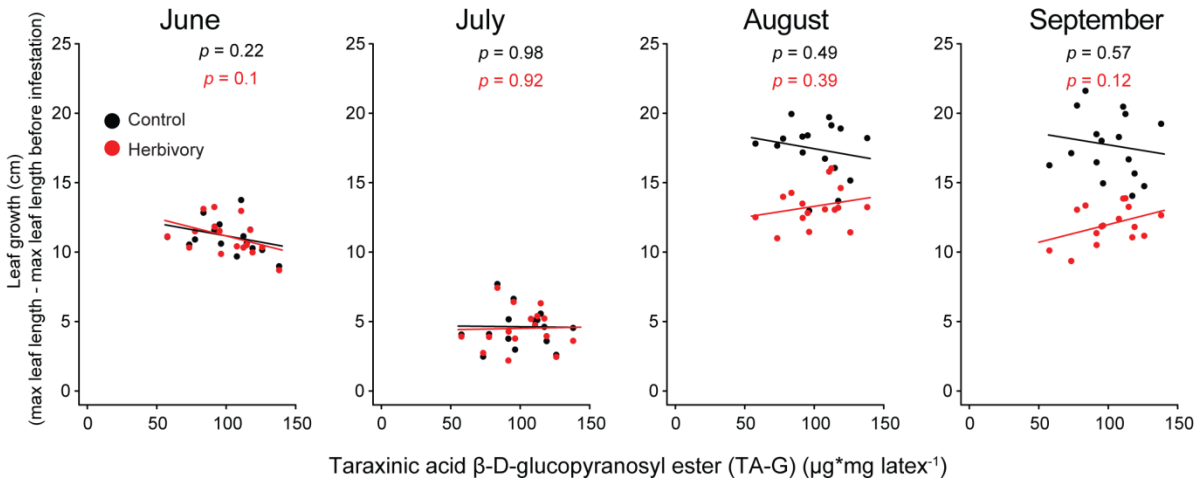


Fig. S14 Correlation between TA-G and leaf growth in a common garden experiment with and without *M. melolontha* infestation. TA-G concentration tended to be positively correlated to leaf growth (maximal leaf length of each month – maximal leaf length before infestation) under *M. melolontha* attack and negatively correlated to leaf growth in the control treatment towards the end of the growing season. Plants were infested in June. Each data point represents the mean of one genotype. *P*-values from linear models based on mean values of each genotype are shown.

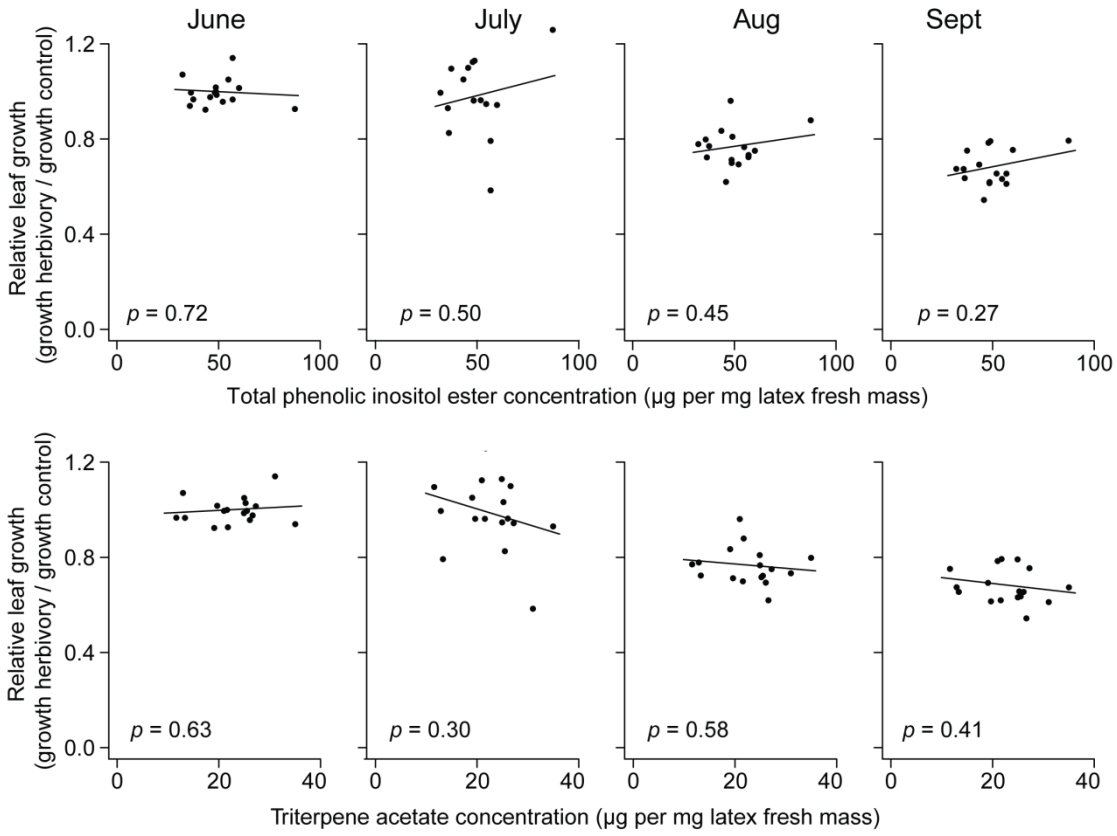


Fig. S15 Correlations of relative leaf growth with total PIE and total TritAc concentrations in a common garden. Relative leaf growth is the size increase of the longest leaf of the herbivore infested plants compared of the size of the longest leaf before infestation, expressed relative to the leaf growth of the control plants of each genotype. Each data point represents the mean relative leaf growth of one *T. officinale* genotype. Plants were infested in June. *P*-values from linear models based on mean values of each genotype are shown.

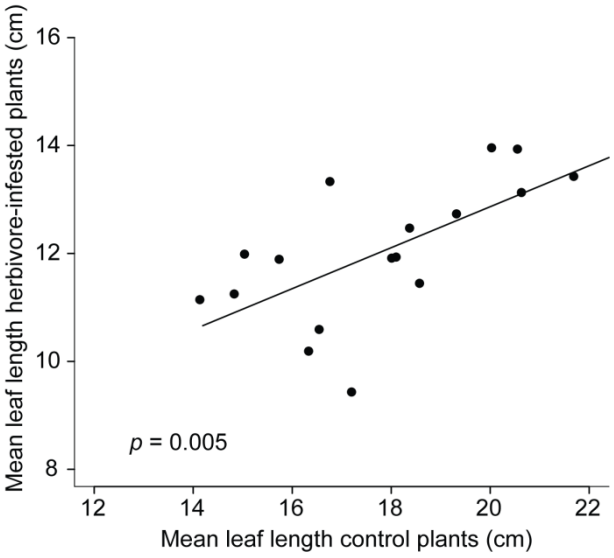
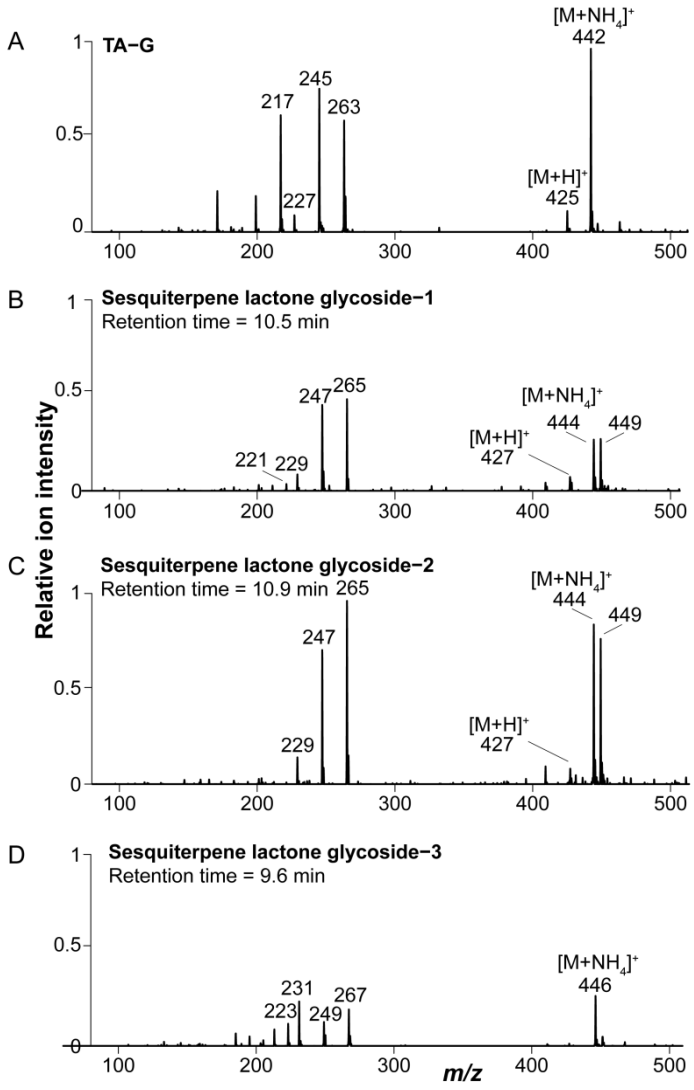


Fig. S16 Correlation between average leaf length of *M. melolontha*-infested plants and mean leaf length of non-infested plants in the common garden experiment in September. Herbivore-damage was proportional to plant size. The *P*-value of a Pearson product-moment correlation is shown. One data point represents the mean of one genotype.

Fig. S17 Picture of *M. melolontha* feeding on *T. officinale* roots.

Fig. S18



In-source fragmentation pattern of TA-G and putative sesquiterpene lactone glycosides. A. In-source fragmentation pattern of taraxinic acid β-D-glucopyranosyl ester (TA-G), obtained from a latex methanol extract of genotype A34. B-D. Putative sesquiterpene lactone glycosides. A latex methanol extract from genotype 17.20A was screened for fragmentation patterns resembling TA-G. All samples were analyzed on an Esquire 6000 ESI-Ion Trap mass spectrometer in positive ionization mode.

Table S1. Origin of 17 *T. officinale* genotypes.

Region	Country	Longitude	Latitude	Genotypes
Beroun	Czech Republic	14.0891	49.981	6.56
Hosingen	Luxembourg	6.0740	50.0149	3.48
Mühlheim am Main	Germany	8.8646	50.1172	4.26
Heteren	Netherlands	5.7509	51.9600	H72
Wageningen-Heteren	Netherlands	5.6627	51.9936	1.16A
Bockenem	Germany	10.1088	52.0121	8.13A
Ostbevern	Germany	7.8325	52.0328	2.2A; 2.8A
Gömnitz	Germany	10.7297	54.1148	10.8
Bentwisch	Germany	12.2326	54.123	11.4
Marum	Denmark	12.2784	56.0253	12.56; 12.57
Skaenninge	Sweden	15.1057	58.3629	15.47
Arboga	Sweden	15.8334	59.4139	16.14
Sikvik	Sweden	17.2982	60.6657	18.46
Haernoesand	Sweden	17.9268	62.6107	20.3B
Synthetic genotype	France x Netherlands			A34

Table S2. Accession numbers of protein sequences used for dendrogram analysis of Asteraceae terpene synthases.

Name	Species	Accession number
8-*epi*-cedrol synthase 1	*Artemisia annua*	AAF80333
8-*epi*-cedrol synthase 2	*Artemisia annua*	CAC08805
(*E*)-β-caryophyllene synthase	*Artemisia annua*	AAL79181
amorpha-4,11-diene synthase 1	*Artemisia annua*	CAB94691
amorpha-4,11-diene synthase 2	*Artemisia annua*	AAF61439
amorpha-4,11-diene synthase 3	*Artemisia annua*	AAF98444
(*E*)-β-farnesene synthase	*Artemisia annua*	AAX39387
germacrene A synthase	*Artemisia annua*	ABE03980
(-)-β-pinene synthase	*Artemisia annua*	AAK58723
(-)-(*3R*)-linalool synthase 1	*Artemisia annua*	AAF13357
(-)-(*3R*)-linalool synthase 2	*Artemsia annua*	AAF13356
germacrene A synthase 1	*Cichorium intybus*	AAM21658
germacrene A synthase 2	*Cichorium intybus*	AAM21659
germacrene A synthase 1	*Heliantus annuus*	ACA14463
germacrene A synthase 2	*Heliantus annuus*	ACA33925
germacrene A synthase 3	*Heliantus annuus*	ACZ50512
δ-cadinene synthase	*Heliantus annuus*	ACA33926
germacrene A synthase	*Ixeris dentata*	AAL92481
germacrene A synthase 1	*Lactuca sativa*	AAM11626
germacrene A synthase 2	*Lactuca sativa*	AAM11627
(*E*)-β-caryophyllene synthase	*Matricaria recutita*	AFM43734
α-isocomene synthase	*Matricaria recutita*	AFM43735
germacrene A synthase	*Matricaria recutita*	AFM43736
ocimene synthase	*Matricaria recutita*	AFM43737
germacrene D synthase	*Matricaria recutita*	AFM43738
(+)-germacrene D synthase	*Solidago canadensis*	AAR31144
(-)-germacrene D synthase	*Solidago canadensis*	AAR31145
(+)-germacrene A synthase	*Solidago canadensis*	CAC36896
kaurene synthase A	*Zea mays*	AAA73960

T**able S3. Density of *M. melolontha* per m^2 in common garden field experiment at the end of the flowering season in the second year.** Initial density of *M. melolontha* in the herbivory treatment was 23 *M. melolontha* larvae per m^2.

Treatment	*M. melolontha* density per m^2 ± standard error
Control	0 ± 0.0
Herbivory	0.86 ± 0.27

Text S1

To select 20 triploid dandelion genotypes that maximally vary in the latex chemical defenses, we screened 40 genotypes coming from a transect ranging from Czech Republic to Sweden in the greenhouse. 12 individuals of each genotype were cultivated in sand in a growth chamber for eight weeks in three temporally separated batches. During harvest, main root was cut 0.5 cm below the tiller, exuding latex collected into Eppendorf tubes and immediately frozen in liquid nitrogen. To measure coagulation rate, main root was cut once more and a 2 µl capillary was hold for 90 s into the exuding latex before recording the height of the latex inside the capillary. Leaf mass was dried for two days at 60 °C and weighed. Latex was extracted and analyzed on HPLC-DAD as described previously, and peak area of taraxinic acid β-D-glucopyranosyl ester and of phenolic inositol esters integrated at 275 nm. To select genotypes that maximally differed in the latex defenses, all genotypes that significantly differed in above ground biomass according to a Tukey post hoc test were excluded to reduce the effects of plant growth on plant resistance. Next, hierarchical clustering with Eucledian distance and Ward linkage was performed based on following latex chemical traits: concentration of taraxinic acid β-D-glucopyranosyl ester and major phenolic inositol esters, latex mass and coagulation rate. The dendrogram was then cut into 20 clusters and the most frequent genotype of each cluster was chosen. Data analysis was performed in R (RCoreTeam 2014) using the package agricolae (de Mendiburu 2014).

References

1. R Core Team. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2014.

2. Mendiburu Fd. agricolae: Statistical Procedures for Agricultural Research. R package version 1.2-0 ed2014.

Text S2

ToGAS1

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ToGAS2

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3.4 Manuscript IV

A below ground herbivore shapes root defensive chemistry in natural plant populations

Manuscript for Ecology Letters

A below ground herbivore shapes root defensive chemistry in natural plant populations

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Running title: A root herbivores shapes defensive chemistry

Key words: root herbivory, plant secondary metabolite, defense, evolution, selection, latex, fitness cost, sesquiterpene lactone, *Taraxacum officinale*, *Melolontha melolontha*

ABSTRACT

Plants display extensive intraspecific variation in secondary metabolites, but the selective forces shaping this diversity often remain unknown, especially below ground. Using *Taraxacum officinale* and its major native insect root herbivore *Melolontha melolontha*, we tested whether root herbivores drive the evolution of secondary metabolites. We found that historically high *M. melolontha* infestation levels are associated with high concentrations of major latex secondary metabolites across 27 central European *T. officinale* field populations. By cultivating offspring of these populations, we show that both heritable variation and phenotypic plasticity contribute to the observed differences. Using a combination of natural and transgenic *T. officinale* genotypes, we demonstrate that *M. melolontha* imposes divergent selection on the concentration of the latex-derived sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G), an effect that is likely mediated by the deterrent effects of TA-G. This study highlights the role of soil-dwelling insects for the evolution of plant defenses in nature.

INTRODUCTION

Heritable intraspecific variation is a common feature of many biological traits. Genetic variation results from heterogeneous selection pressures in relation to genetic architecture, population substructures and gene flow (Rainey & Travisano 1998; Newton *et al.* 1999; Lenormand 2002; Sinervo & Svensson 2002). In plants, local differences in both abiotic and biotic factors may drive trait evolution (Turner *et al.* 2010). Insect herbivores, the most abundant and diverse plant consumers, have long been suspected to play an important role in this context (Mauricio & Rausher 1997). Recent studies demonstrate that herbivore abundance can co-vary with the expression of plant defense metabolites (Züst *et al.* 2012), that the exclusion of phytophagous insects can lead to a relaxation of defensive syndromes within a few generations (Agrawal *et al.* 2012), and that defense genes are under differential selection across environments (Prasad *et al.* 2012; Kerwin *et al.* 2015), suggesting that the temporal and spatial variation in above ground herbivore communities can shape plant defensive chemistry.

In contrast to the ecological and evolutionary dynamics of above ground plant-herbivore interactions, below ground interactions have received little attention, despite the importance of roots for plant fitness and the high concentrations of secondary metabolites in below ground organs (Rasmann & Agrawal 2008; van Dam 2009; Rasmann *et al.* 2011). The rhizosphere differs from the phyllosphere in both biotic and abiotic conditions (Brown & Gange 1990). The selective forces shaping variation in secondary metabolites may therefore differ between the two environments. The evolution of root secondary metabolites may for instance be driven by herbivores (Rasmann & Agrawal 2008; van Dam 2009; Erb et al. 2013), pathogens (Sarwar et al. 1998) and symbionts (Mandal et al. 2010), as well as nutrient availability (Dixon & Paiva 1995), salt, drought and cold stress (Ramakrishna & Ravishankar 2011). Although root herbivores severely reduce plant performance in natural and agricultural ecosystems (Johnson et al. 2012), little is known about their impact on the evolution of plant defenses. By comparing one mainland and two island populations, Watts et al. (2011) showed that geographical isolation, including the escape from pocket gophers (Geomyidae), resulted in the evolutionary decline of root alkaloid concentrations of the host plant *Eschscholzia californica* (Papaveraceae), indicating the potential of root herbivores to shape the abundance and geographical distribution of plant secondary metabolites.

Among Europe's largest and most prevalent native root feeding insects are the larvae of the common cockchafer (*Melolontha melolontha*) (Coleoptera: Scarabaeidae). Due to its pest status and conspicuous appearance in the adult stage, *M. melolontha* has been monitored closely by federal authorities, local farmers and the public over the last decades (Keller 2004). *Melolontha melolontha* females lay their eggs close to their emergence site (Pener 2013), resulting in constant geographic distribution patterns. Although the larvae are highly polyphagous, they preferentially feed on the common dandelion (*Taraxacum officinale* agg., Flora Helvetica, 5th edition) during the final instar

(Hauss & Schütte 1976). *Taraxacum officinale* is a perennial that relies heavily on its strong tap root for resprouting and flowering in spring. The plant is native to and widely distributed in Europe (Scheenen *et al.* 2007), and has recently gained a cosmopolitan distribution due to human dispersal. *Taraxacum officinale* is described as a species complex with sexual, outcrossing diploids in central and southern Europe and a multitude of apomictic, clonal triploids across the globe (Verduijn *et al.* 2004). One of the factors that may explain the worldwide success of *T. officinale* is its capacity to produce latex in roots and other organs. Latex is the cytoplasm of the specialized laticifers cells, and typically contains high concentrations of toxic and sometimes sticky secondary metabolites (Agrawal & Konno 2009). The latex of plants is widely accepted to be defensive (reviewed in Agrawal & Konno 2009). The latex of *T. officinale* is dominated by three secondary metabolite classes: phenolic inositol esters (PIEs), triterpene acetates (TritAcs) and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G) (Huber *et al.* 2015). In a recent study, we showed that TA-G improves plant performance upon *M. melolontha* attack, opening up the possibility that this compound may be under natural selection by root herbivores (Huber *et al*., submitted manuscript).

In this study, we investigated whether *M. melolontha* shapes variation in latex secondary metabolites in *T. officinale*. We profiled the latex chemistry of natural *T. officinale* populations growing under different *M. melolontha* herbivore pressure, tested for inducibility, heritable variation and differential selection of the metabolites in the offspring of the field populations and investigated the deterrence of the candidate metabolite TA-G using transgenic knock-down lines. Through the above approaches, we provide evidence that *M. melolontha* can shape TA-G concentration in natural *T. officinale* populations, which unravels the potential of below ground herbivores to drive the evolution of chemical defenses in plants.

METHODS

Plant growth conditions

All greenhouse experiments were performed in a climate chamber operating under the following conditions: 16 h light 8 h dark; light supplied by sodium lamps (NH 360 FLX SUNLUX ACE Japan); light intensity at plant height 58 µmol m^2 s^{-1}; temperature: day 22 °C; night 20 °C; humidity: day 55%, night 65% (unless specified otherwise). Plants were grown in 1 l pots filled with potting soil.

Insects

Melolontha melolontha larvae were collected from meadows in Spessart, Germany. Experiments were conducted with third instar larvae. Insects were reared individually in 200 ml plastic beakers filled with potting soil and grated carrots in a phytotron operating under the following conditions: 12 h day 12 h night; temperature: day 13 °C, night 11 ° C; humidity: 70%; lighting: none.

Statistical analyses

All statistical analyses were performed in R version 3.1.1 (R Core Team 2014). Mixed linear effect models were analyzed with the nlme package (Pinheiro *et al.* 2014). Pairwise comparison were performed with the agricolae package (de Mendiburu 2014). Results were visualized using ggplot2 (Wickham 2009) and gridExtra (Auguie 2012). Maps were drawn with RgoogleMaps (Loecher 2014) and rworldmap (South 2011). Details on the individual statistics are provided in the experimental sections below.

T. officinale *field populations*

To investigate the influence of *M. melolontha* on the phenotypic variation of latex chemistry in natural *T. officinale* populations, we collected latex from a total of 324 *T. officinale* plants from 27 locations that differed in *M. melolontha* abundance over the last 20 years. We identified three regions with high *M. melolontha* abundance by contacting federal agricultural authorities (Fig. S1, Table S1). Within each region, we contacted local farmers who provided detailed information about the infestation histories of their fields over the last 20 years. Based on this information, plots were classified as historically *M. melolontha* infested ("local herbivory") or largely *M. melolontha* free ("local control"). As an additional control, we identified regions with low *M. melolontha* abundance at a distance of 50-100 km to the local herbivory and local control plots. These plots were identified based on data from Swiss and German authorities ("regional controls"). In each of the three areas (Germany, Swiss lowlands, Swiss Alps), we analyzed 3-4 local herbivory, local control and regional control populations. In the Swiss lowlands, no local control population could be established due to the lack of reliable records. Detailed information about the information sources for the individual plots and regions is listed in table S1. Plots were arranged in a pairwise manner so that each herbivore plot had a matching local

and regional control plot in the same area with similar environmental conditions, including altitude, exposition, inclination, land use and climate. At each location, we collected one fully ripened seed capsule from 12 *T. officinale* plants, with a minimal distance of 50 cm between plants. To characterize the present root herbivore community, we excavated a cube with 18 cm side length around each plant and recorded the identity and number of below ground herbivores in each. Furthermore, we collected latex from the main root of each plant into Eppendorf tubes and glass vials, which were immediately frozen in dry ice. Samples were stored at -80 °C until analysis. For extraction of TA-G and PIEs, 1 ml methanol containing 10 $\mu g*ml^{-1}$ loganin and 100 $\mu g*ml^{-1}$ salicin as internal standards for TA-G and PIEs, respectively, was added to the Eppendorf tubes. Tubes were vortexed for 5 min, centrifuged, and the supernatant was stored at -80 °C until analysis. Methanol samples were measured on a high pressure liquid chromatograph (HPLC 1100 series equipment, Agilent Technologies), coupled to a photodiode array detector (G1315A DAD, Agilent Technologies). For quantification, peak areas were integrated at 245 nm for TA-G and at 275 nm for PIEs, and quantified based on the internal standards. For extraction of the TritAcs, 1 ml hexane containing 0.1 $mg*ml^{-1}$ cholesteryl acetate as internal standard was added to the glass vials. Vials were shook for 5 min, centrifuged and supernatant stored at -80 °C until analysis. Hexane samples were analyzed on an Agilent series 6890 gas chromatograph coupled to a flame ionization detector (GC-FID). Individual triterpene acetates were quantified based on the internal standard. Methodological details for the analytical procedures are described in Huber *et al.* (2015).

Differences in root herbivore abundance between populations of different infestation histories were analyzed with Kruskal-Wallis rank sum tests using the mean values of each plot. The effect of infestation history on the concentrations of TA-G, total PIEs and total TritAcs was analyzed using mixed-effect models for each area separately, with metabolite concentrations as response variable, infestation history as fixed effect and the location as a hierarchical random effect. Two models using maximum likelihood estimations with and without infestation history were compared with one-way ANOVAs for each area and metabolite separately. Summary statistics of the models including infestation history are presented using the highly infested sites as the reference level.

Offspring analysis

In order to investigate heritable variation, inducibility and fitness effects of latex secondary metabolites, we grew offspring of the plants from the field survey with and without *M. melolontha* infestation. To balance the number of plots for the three different locations, the three plots with the most well-documented history of *M. melolontha* abundance for each infestation history and region were selected. Seeds were germinated in seedling trays in a growth chamber under standard conditions. After 4 weeks of growth in July 2013, the trays were placed outside for acclimatization (Jena, Germany: 50°54'34.8"N; 11°34'00.1"E). Each plant from the local herbivore plots was then potted together with either a plant from the matching regional control plot (“regional pair”) or a plant from the matching local control plot (“local pair”) inside a 2 l pot in potting soil, resulting in 216 combinations for the

regional pair and 144 combinations for the local pair. Two identical pots of each combination were set up, one of them was infested with one *M. melolontha* larva 7 weeks after germination, and the other was not manipulated ("control"). Plants were watered whenever necessary. At the end of November 2013, plants were moved to a 4-10 °C greenhouse to overwinter. Plants were moved outside again in early March 2014, and seeds of mature capitula were collected every day and weighed. Plants were harvested at the end of May when seed production had ceased. Latex from the main roots was collected in Eppendorf tubes and glass vials for secondary metabolite analysis and immediately frozen in liquid nitrogen. Latex in Eppendorf tubes and glass vials were extracted, analyzed and quantified as described above. The ploidy level of each accession was furthermore determined by flow-cytometry as described (Bubner *et al.* 2006).

To investigate the inducibility of latex secondary metabolites, TA-G, total PIE and total TritAc concentrations were compared between control and *M. melolontha*-infested plants with Kruskal-Wallis rank sum tests. We analyzed inducibility of TA-G (TA-G concentration herbivore-infested plants divided by TA-G concentration control plants) according to the infestation history using Kruskal-Wallis rank sum tests for the local and regional pair separately. To test for heritable variation in latex secondary metabolite concentrations, we analyzed constitutive TA-G, total PIE and total TritAc concentrations according to infestation history for each area separately with Kruskal-Wallis rank sum tests for both the local and the regional pairs. Triploid plants from the regional controls in the Swiss lowlands were excluded from the analysis to avoid confounding effects caused by their clonal life style. To measure selection on latex secondary metabolite concentrations in the presence and absence of *M. melolontha* herbivory, we calculated directional and disruptive/stabilizing selection gradients using regression coefficients for each metabolite class separately (Lande & Arnold 1983; Mitchell-Olds & Shaw 1987; Rausher 1992). To obtain the regression coefficients, relative fitness was calculated by dividing total seed mass by the maximal total seed mass of the population. Relative fitness was log-transformed to improve normal distribution. The directional selection gradient was calculated by linear regression of the relative fitness (log-transformed) to the concentration of latex secondary metabolites. An estimate for stabilizing/disruptive selection gradient was obtained by the second-order coefficient of a quadratic regression of the relative fitness (log-transformed) on the latex secondary metabolites. Only data from the regional pairs were used to avoid non-independence of data points. Individuals that contained other non-identified sesquiterpene lactones or that did not produce seeds were excluded from the analysis. Broad-sense heritability (H^2) of latex secondary metabolites was analyzed in the German triploid offspring of the field populations as these were the only triploid plants for which replicated individuals were present. The genotypic variance (variance of the mean latex secondary metabolite concentration across genotypes) was divided by the total phenotypic variance (variance of latex secondary metabolite concentration across all individuals) for the control and herbivore-infested plants separately.

M. melolontha *diet consumption*

In order to test whether TA-G is sufficient to affect *M. melolontha* herbivory, we measured food consumption by *M. melolontha* larvae on diet to which latex extracts from RNA interference-silenced, TA-G-deficient lines and latex extracts of control lines was added. 60 TA-G-deficient plants from three *ToGAS1*-silenced lines (RNAi-1, -12b, 16) and 60 non-silenced plants (RNAi-9, -15, WT) (Huber *et al*., submitted manuscript) were grown for 8 weeks under standard conditions. Latex from main roots was harvested into pre-weighed Eppendorf tubes, which were immediately frozen in liquid nitrogen. Latex was extracted with 1 ml methanol. Samples were pooled for each line, 2 ml water was added and the methanol was evaporated in a rotor-evaporator prior to freeze-drying of the samples. Freeze-dried samples were suspended in 2 µl water per mg latex fresh mass. For the assay, 500 mg diet consisting of cooked and mashed carrots, bean flower and agar (Huber *et al*., submitted manuscript) was supplemented with the latex water extracts from each line to reach a physiologically relevant concentration of 3 µg TA-G per mg diet for the non-silenced samples (Huber *et al*., submitted manuscript) (Fig. S2). *Melolontha melolontha* larvae were starved overnight before providing them a diet piece inside an empty 200 ml plastic beaker covered with a moist tissue paper. Leftover food was recovered after 24 h and weighed. Differences in food consumption between TA-G deficient latex and control latex were analyzed with a Kruskal-Wallis rank sum test.

RESULTS

The latex of *T. officinale* is dominated by three classes of secondary metabolites: Phenolic inositol esters, triterpene acetates and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G) (Huber *et al.* 2015). To explore the influence of *M. melolontha* abundance on the phenotypic variation in latex chemistry in nature, we profiled the major latex secondary metabolites across 27 *T. officinale* populations that evolved under different *M. melolontha* pressure over the last 20 years (Fig. 1A), as determined through public records, eye witness accounts and distribution maps (Fig. S1, Table S1). Populations were located in three areas, Germany, the Swiss lowland and the Swiss Alps, and categorized as (a) heavily infested by *M. melolontha* ("local herbivory"), (b) lightly infested few kilometers away ("local control"), and (c) lightly infested 50 to 100 kilometers away ("regional control"). In the Swiss lowlands, no local control plots could be identified because of a lack of available records. At each location, we recorded the identity and number of below ground herbivores, sampled root latex, and collected one fully ripened seed capitulum of each plant. As expected, *M. melolontha* was the major insect root herbivore in both number and size in the surveyed locations (Fig. 1B). The abundance of other root feeding insects did not differ significantly between individual sites and did not co-vary with *M. melolontha* abundance (Fig. 1B).

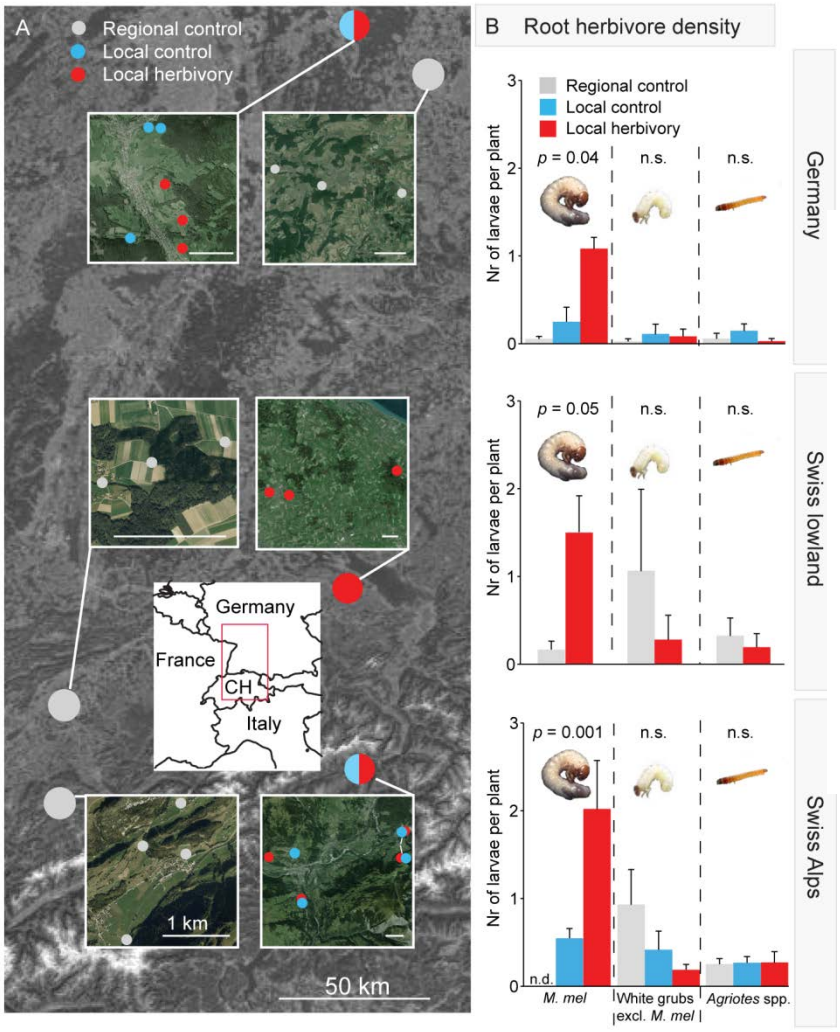

Fig. 1 Field locations *T. officinale* populations growing under different *M. melolontha* herbivory. A. Map of identified natural *T. officinale* populations evolving under high ("local herbivory") or low ("local control" and "regional control") *M. melolontha* infestation levels over the last 20 years. Circles inside insets denote individual sampling sites. CH = Switzerland. B. Mean number of root herbivore larvae per *T. officinale* plant found during a field survey in 2013. *M. mel* = *M. melolontha*. *P*-values of Kruskal-Wallis rank sum tests are shown

In the Swiss lowlands, no differences in the concentration of the three metabolite classes were observed, possibly due to the comparatively low *M. melolontha* densities in this region over the last 20 years. In contrast, in Germany and in the Swiss Alps, the concentration of TA-G was higher in the populations under strong *M. melolontha* pressure compared to local and regional control populations. Similarly, the concentrations of total PIEs and total TritAcs were higher in the heavily infested populations than in either the regional or the local control plots, although the patterns were weaker than for TA-G (Fig 2). These data indicate that *M. melolontha* can shape intraspecific phenotypic variation in the concentration of latex secondary metabolites, including TA-G, in natural *T. officinale* populations.

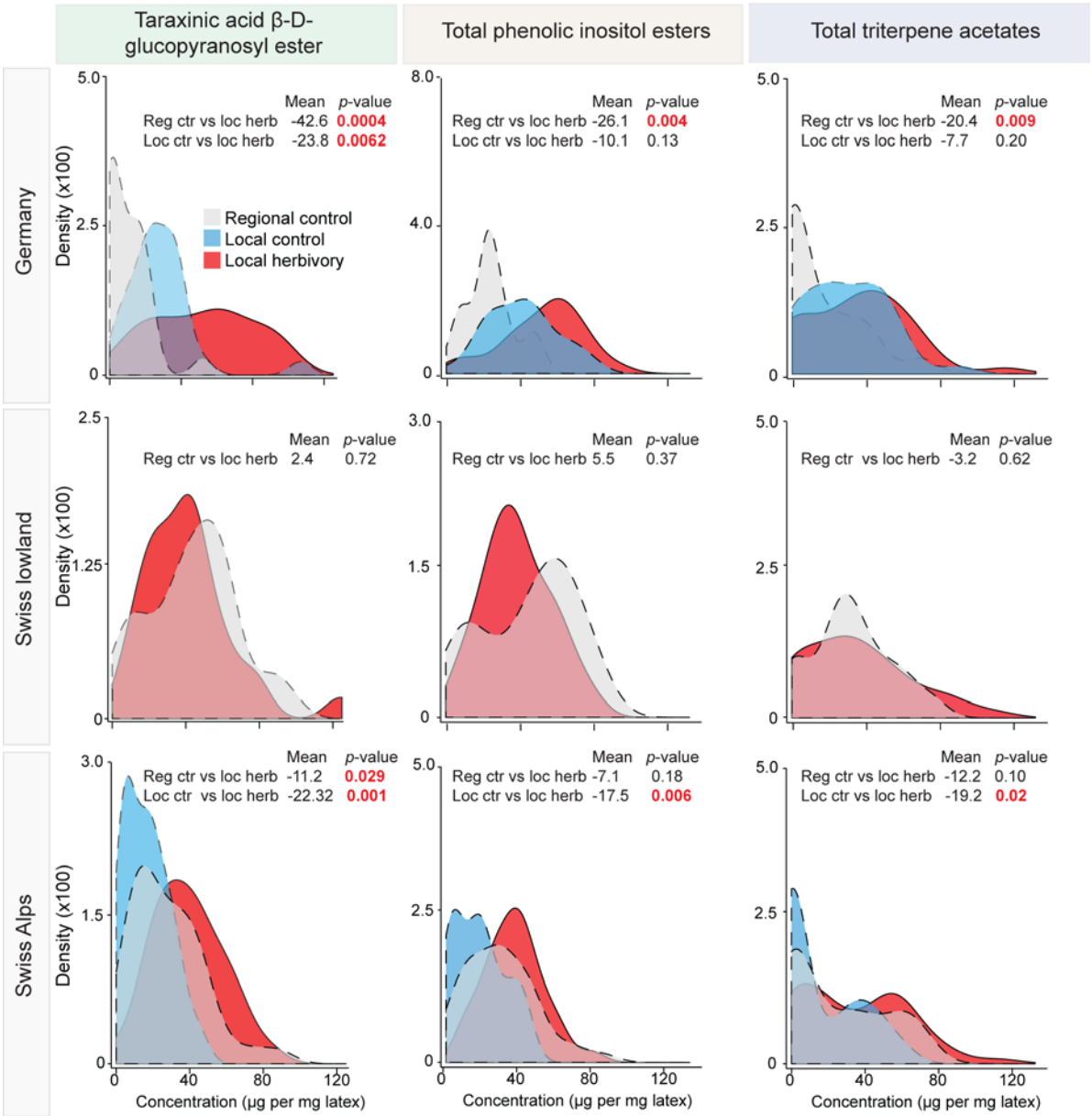

Fig. 2 Latex secondary metabolite concentrations in *T. officinale* field populations. High latex secondary metabolite concentrations were associated with present and historically high *M. melolontha* abundance in Germany and the Swiss Alps, but not in the Swiss lowlands. *P*-values of linear mixed effect models are shown.

The observed differences in latex secondary metabolite concentrations in natural *T. officinale* populations may result from phenotypic plasticity and/or heritable variation. To test these two hypotheses, we germinated seeds from the field-sampled plants ("offspring population"), determined ploidy levels to separate diploid and triploid *T. officinale* plants, infested half of the plants with *M. melolontha* larvae and measured seed set as well as latex secondary metabolite concentrations one year after infestation. Each offspring from the local herbivore plots was potted together with either a plant from the matching regional control plot ("regional pair") or a plant from the matching local control plot ("local pair"). The German populations consisted almost entirely of triploids. The Swiss lowland populations contained 40% diploids in the regional control populations and 100% diploids in the local herbivore populations ($p = 1.3 \times 10^{-7}$, proportion test). The Swiss Alps populations only contained diploids. On average, *M. melolontha* infestation increased TA-G concentration by 33% ($p < 0.001$, Kruskal-Wallis rank sum test), while total PIEs and total TritAcs concentrations were reduced by 10% (Fig. 3, PIEs: $p < 0.001$, TritAcs: $p = 0.002$, Kruskal-Wallis rank sum tests), suggesting that the inducibility of TA-G may have contributed to the field patterns. Inducibility of TA-G concentration did not differ between infestation histories (Fig. S3).

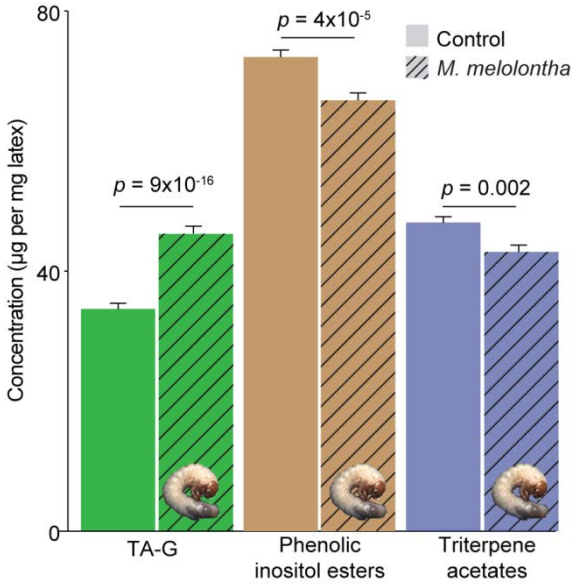

Fig. 3 Induction of latex secondary metabolites upon *M. melolontha* infestation. Taraxinic acid β-D-glucopyranosyl ester **(**TA-G) concentration was induced and total phenolic inositol ester and total triterpene acetate concentrations were suppressed by *M. melolontha* feeding in the offspring of the field populations. *P*-values from Kruskal-Wallis rank sum tests are shown.

We also observed heritable variation of latex secondary metabolite concentrations among *T. officinale* populations with different infestation histories. In the asexual, clonal German populations, constitutive TA-G concentrations were 60% higher than the regional controls (Fig. 4, $p = 0.02$, Kruskal-Wallis rank sum test). Constitutive total TritAc concentrations were 20-25% higher in the heavily infested populations in Germany compared to both local and regional controls (local controls: $p = 0.08$; regional controls: $p = 0.003$, Kruskal-Wallis rank sum tests). No difference in total PIE concentrations was observed between offspring populations from Germany. In the diploid, obligate outcrossing Swiss populations, the concentrations of the three latex metabolite classes did not differ between offspring populations, except that total PIE concentrations were 20% lower in the offspring from the heavily infested populations compared to the regional controls ($p = 0.02$, Kruskal-Wallis rank sum test). Taken

together, these data suggest heritable variation in TA-G and total TritAc concentrations among the genetically fixed triploid plants. Gene flow may have prevented the differentiation of heritable variation in the latex secondary metabolite concentrations among the diploid, obligate outcrossing populations, suggesting that the mode of reproduction is an important determinant of herbivore-dependent natural selection in *T. officinale*.

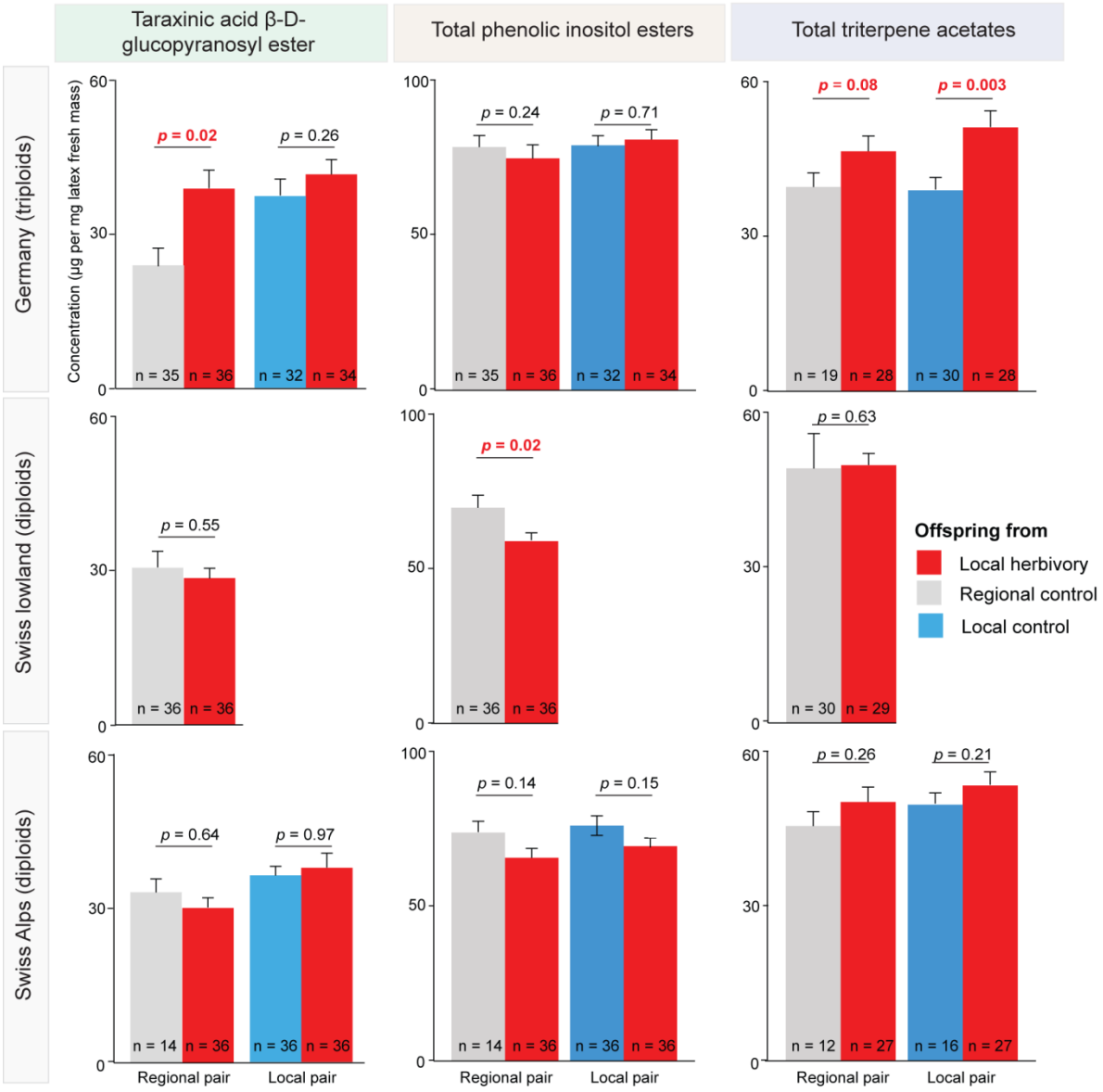

Fig. 4 Latex secondary metabolite concentrations in the offspring of the field populations grown in a common garden. Heritable variation in the constitutive concentration of taraxinic acid β-D-glucopyranosyl ester (TA-G) and total triterpene acetates were observed in the triploid, clonal offspring of the German populations but not in the diploid, sexual offspring of the Swiss populations. Two offspring from either regional control and local herbivory (regional pair) or local control and local herbivory (local pair) were grown together in one pot. *P*-values from Kruskal-Wallis rank sum tests are shown. Sample size is shown inside each bar.

The fixation of phenotypic variation among populations requires divergent selection and heritability. We correlated secondary metabolite concentrations with total seed mass and determined selection gradients in the offspring from the field-sampled plants. For all major latex secondary metabolites, the quadratic selection gradient did not different from zero (Table S2). For TA-G, the

directional selection gradient (β) was negative in the absence of *M. melolontha* (Fig. 5, β = -0.01 ± 0.005 (mean ± standard error), $p = 0.02$), and positive in its presence (β = 0.01 ± 0.005, $p = 0.01$). For total PIEs and TritAcs, no significant directional selection gradients were observed, although total PIE concentrations tended to be positively selected in the presence of *M. melolontha* (β = 0.008 ± 0.004, $p = 0.06$). Broad sense heritability of the three metabolite classes ranged between 0.49 and 0.78 in the absence and presence of *M. melolontha* (Fig. 5). Together, these experiments show that the concentrations of the three latex secondary metabolite classes are heritable, and that TA-G concentration but not total PIE or total TritAc concentrations are under *M. melolontha*-imposed divergent selection.

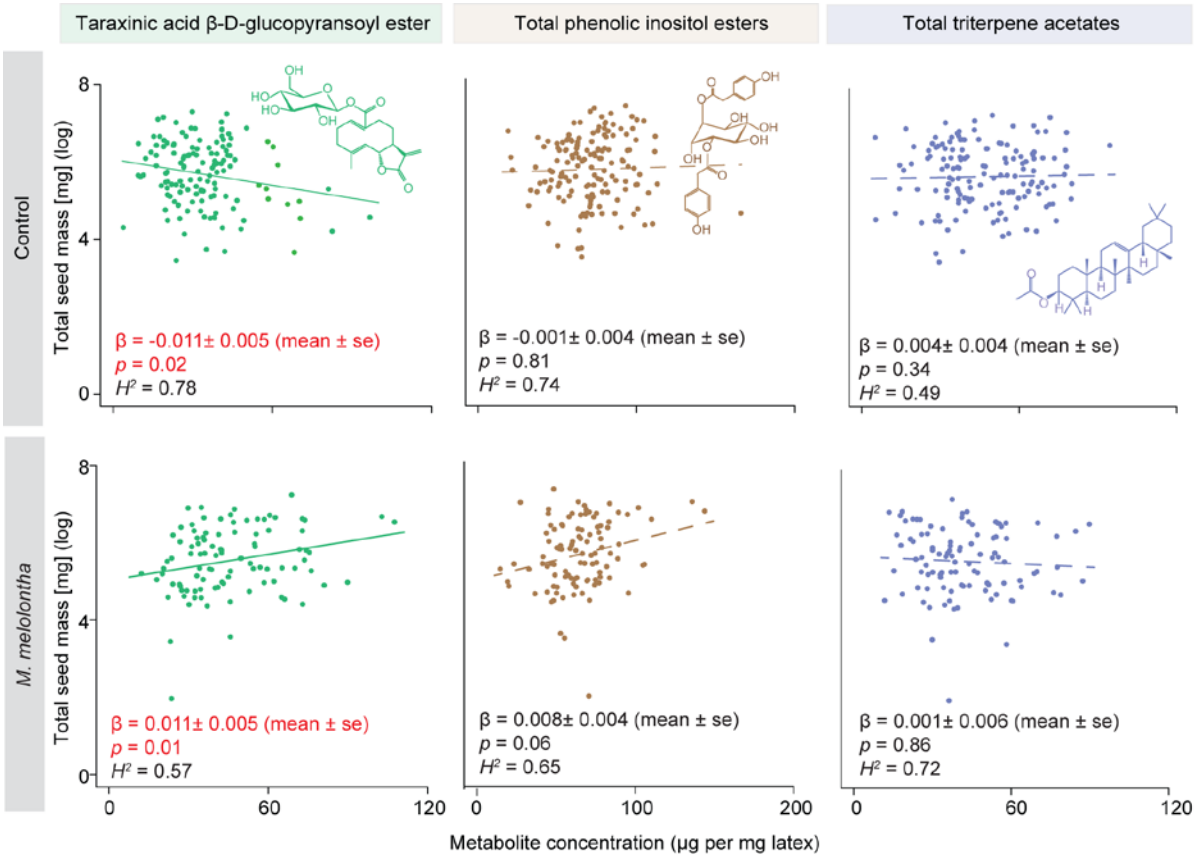

Fig. 5 Attack of *M. melolontha* leads to patterns of divergent selection in taraxinic acid β-D-glucopyranosyl ester (TA-G) concentrations. Correlations of TA-G, total PIE and total TritAc concentrations with total seed mass of different *T. officinale* genotypes in the absence (upper panel) and presence (lower panel) of *M. melolontha*. Broad sense heritability (H^2) and *p*-values of directional selection gradients (β) are shown. Representative chemical structures are depicted. se = standard error.

To verify that TA-G is sufficient to affect *M. melolontha* herbivory, we used transgenic *T. officinale* plants that were silenced in *ToGAS1* by RNA interference and that were almost completely devoid of TA-G ("TA-G-deficient lines") (Huber et al., submitted manuscript). We measured food consumption of *M. melolontha* in a non-choice experiment on diet to which latex extracts of TA-G-deficient and control lines was added in physiologically relevant concentrations. *Melolontha melolontha* larvae consumed more diet to which latex extracts of TA-G-deficient lines was added compared to diet with control latex extracts (Kruskal-Wallis rank sum test, $p = 0.01$). These results confirm the

hypothesis that TA-G decreases *M. melolontha* herbivory and may thereby improve plant fitness upon herbivore attack.

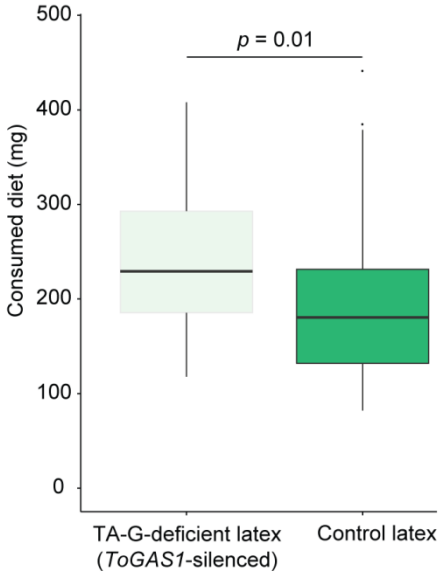

Fig. 6 TA-G deters *M. melolontha* feeding. Consumption of diet supplemented with latex extract of transgenic, TA-G-deficient lines and latex extracts of control lines by *M. melolontha* in a non-choice experiment. *P*-value of Wilcoxon-rank sum test is shown. N = 35-41.

DISCUSSION

In this study, we demonstrate that root herbivores can act as evolutionary drivers of plant defensive chemistry in nature. Four parallel lines of evidence support the hypothesis that *M. melolontha*, a major root herbivore of *T. officinale*, can shape variation in latex secondary metabolite concentrations in natural *T. officinale* populations, as discussed below.

Taraxacum officinale populations that were growing under high *M. melolontha* pressure over the last 20 years had higher concentrations of the three major secondary metabolites in their root latex than plants growing in areas with low *M. melolontha* abundance. The pattern was most pronounced for the sesquiterpene lactone TA-G in two out of three study areas. In the Swiss lowlands, latex secondary metabolite concentrations did not co-vary with *M. melolontha* abundance. The absence of this association may be due to lower past infestation levels than the other two regions or due to differences in ploidy levels and associated reproductive strategies – the heavily-infested populations consisted entirely of outcrossing diploids, while the low-infestation control populations consisted mostly of apomictic triploids. This screen reveals that *M. melolontha* can shape phenotypic variation of plant chemistry in nature, a pattern that has only rarely been observed either above (Prasad *et al.* 2012; Züst *et al.* 2012) or below ground (Watts *et al.* 2011).

We found that the phenotypic differences in the field were contributed by heritable variation. TA-G and total TritAc levels were higher in the offspring of the apomictic, triploid populations when grown in a common garden. In contrast, offspring populations of the diploid populations did not differ in the concentration of any of the three metabolite classes, except that the Swiss herbivore populations had 20% lower total PIE concentrations than regional controls. The lack of heritable variation between diploid populations may be due to outcrossing of the parental plants from heavily-infested populations with plants growing under low *M. melolontha* pressure. *Taraxacum officinale* is visited by a broad range of pollinators (Lázaro & Totland 2010); thus, pollen transport over several kilometers are likely to occur (Chifflet *et al.* 2011). Our observation also suggests that beneficial traits, such as protection against herbivores, can be selected faster in asexual than in sexual populations. This is in line with the general notion that differentiation between populations is established more rapidly when less gene flow occurs (Heywood 1991), and highlights the importance of gene flow for herbivore-dependent natural selection.

The establishment of heritable variation between populations requires differential selection and heritability. Selection gradient analyses of the three metabolite classes in the presence and absence of *M. melolontha* suggest that *M. melolontha* attack leads to divergent selection of TA-G. Negative correlations between the concentration of defensive metabolites and plant performance in the absence of herbivory are usually taken to indicate the potential fitness costs of compounds. However, the assumption that secondary metabolites are costly for plants is very controversial, with few examples of negative correlations between growth and defense (Paul-Victor *et al.* 2010; Züst *et al.* 2011) and many

other examples with no apparent correlation (Almeida-Cortez *et al.* 1999; Koricheva 2002). Fitness costs can include both directs costs, i.e. costs of production of the metabolites, and indirect costs, such as decreases in mutualistic interactions (Strauss *et al.* 2002). Although our experimental data cannot distinguish between these two types of costs, our data suggest that TA-G production is costly for *T. officinale* growing under low root herbivore pressure, but that the costs are outweighed at high levels of biotic stress. As we found high broad sense heritability of TA-G concentration, these data indicate that variation in *M. melolontha* abundance can lead to evolutionary changes of TA-G concentration over time.

Our study also presents direct evidence for TA-G as a resistance trait. *Melolontha melolontha* consumed more diet to which latex extracts of transgenic TA-G-deficient lines was added compared to diet with control latex extracts. Furthermore, the induction of TA-G upon *M. melolontha* infestation fits the role of TA-G as a defense. These results are in line with the general notion that sesquiterpene lactones are defensive (Picman 1986), and confirm our previous experiments showing that TA-G deters *M. melolontha* feeding and thereby protects root and improves plant fitness (Huber *et al*., submitted manuscript).

Taken together, the association of TA-G concentration with *M. melolontha* abundance in the field, the heritable differences in TA-G concentration among triploids, the patterns of divergent selection of TA-G concentration in the absence and presence of *M. melolontha*, the relatively high heritability estimates of TA-G concentration, and the deterrent effect of TA-G on *M. melolontha* feeding provide evidence that *M. melolontha* can shape variation in *T. officinale* root latex chemistry in nature. Further experiments involving reciprocal transplant experiments, population genetic data to track gene flow and to identify potential TA-G associated genes that are under selection may shed further light on the mechanistic underpinning of the observed patterns.

The tremendous diversity of plant secondary metabolites both within and between species has fascinated biologist for centuries. Recent studies suggest that the heterogeneous distribution of herbivores shapes plant defensive chemistry. While a study by Züst *et al.* (2012) and Prasad *et al.* (2012) showed that herbivores can select for dichotomous resistance traits, Agrawal *et al.* (2012) provided evidence that the concentration of secondary metabolites diverges in the absence and presence of the above ground herbivore community. Moreover, as study by Kerwin *et al.* (2015) showed that heterogeneity in the herbivore community maintains intraspecific variation in defense genes. While these examples provide evidence that above ground insects can drive the evolution of defensive metabolites, the potential of below ground herbivores to shape chemical defenses remained elusive (Watts *et al.* 2011). We show that variation in the abundance of a single root herbivore can contribute to the intraspecific variation in the concentration of a root secondary metabolite. These findings highlight the potential of root herbivores as evolutionary drivers of chemical defenses of their host plants. Thus,

we are now at the point of extending a central paradigm in plant-herbivore interactions to below ground environments.

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AUTHOR CONTRIBUTIONS

MH and ME conceived and designed the project and experiments. MH, ZB, JF, TB, ZA and ME performed experiments. MH analyzed data. JG and ME provided reagents/materials and analysis tools. MH and ME wrote the manuscript. All authors contributed to previous versions of the manuscript.

REFERENCES

1.

Agrawal, A.A., Hastings, A.P., Johnson, M.T.J., Maron, J.L. & Salminen, J.P. (2012). Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science*, 338, 113-116.

2.

Agrawal, A.A. & Konno, K. (2009). Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. *Annu Rev Ecol Evol S*, 40, 311-331.

3.

Almeida-Cortez, J.S., Shipley, B. & Arnason, J.T. (1999). Do plant species with high relative growth rates have poorer chemical defences? *Funct Ecol*, 13, 819-827.

4.

Auguie, B. (2012). gridExtra: functions in Grid graphics.

5.

Brown, V.K. & Gange, A.C. (1990). Insect herbivory below ground. *Advances in Ecological Research*, 20, 1-58.

6.

Bubner, B., Gase, K., Berger, B., Link, D. & Baldwin, I. (2006). Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue. *Plant Cell Rep*, 25, 668-675.

7.

Chifflet, R., Klein, E.K., Lavigne, C., Le Feon, V., Ricroch, A.E., Lecomte, J. *et al.* (2011). Spatial scale of insect-mediated pollen dispersal in oilseed rape in an open agricultural landscape. *Journal of Applied Ecology*, 48, 689-696.
8.

de Mendiburu, F. (2014). agricolae: Statistical Procedures for Agricultural Research.
9.

Dixon, R.A. & Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. *The Plant Cell*, 7, 1085-1097.
10.

Erb, M., Huber, M., Robert, C.A.M., Ferrieri, A.P., Machado, R.A.R. & Arce, C.C.M. (2013). The role of plant primary and secondary metabolites in root-herbivore behaviour, nutrition and physiology. In: *Behaviour and Physiology of Root Herbivores* (eds. Johnson, SN, Hiltpold, I & Turlings, TCJ). Elsevier Academic Press Inc San Diego, pp. 53-95.
11.

Hauss, R. & Schütte, F. (1976). Experiments on polyphagous habits of white grubs *Melolontha melolontha* on plants of grassland. *Anzeiger für Schädlingskunde, Pflanzenschutz, Umweltschutz*, 49, 129-132.
12.

Heywood, J.S. (1991). Spatial analysis of genetic variation in plant populations. *Annual Review of Ecology and Systematics*, 22, 335-355.
13.

Huber, M., Triebwasser-Freese, D., Reichelt, M., Heiling, S., Paetz, C., Chandran, J. *et al.* (2015). Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (*Taraxacum officinale* agg.). *Phytochemistry*, In press.
14.

Johnson, S.N., Clark, K.E., Hartley, S.E., Jones, T.H., McKenzie, S.W. & Koricheva, J. (2012). Aboveground-belowground herbivore interactions: a meta-analysis. *Ecology*, 93, 2208-2215.
15.

Keller, S. (2004). Engerlingsvorkommen und Bekämpfung in der Schweiz. *Nachrichtenblatt Deutscher Pflanzenschutzdienst*, 56, 88-90.
16.

Kerwin, R., Feusier, J., Corwin, J., Rubin, M., Lin, C., Muok, A. *et al.* (2015). Natural genetic variation in *Arabidopsis thaliana* defense metabolism genes modulates field fitness. *eLife*, 4.
17.

Koricheva, J. (2002). Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. *Ecology*, 83, 176-190.
18.

Lande, R. & Arnold, S.J. (1983). The measurement of selection on correlated characters. *Evolution*, 37, 1210-1226.
19.

Lázaro, A. & Totland, O. (2010). Population dependence in the interactions with neighbors for pollination: A field experiment with *Taraxacum officinale*. *American Journal of Botany*, 97, 760-769.
20.

Lenormand, T. (2002). Gene flow and the limits to natural selection. *Trends Ecol Evol*, 17, 183-189.

21.

Loecher, M. (2014). RgoogleMaps: Overlays on Google map tiles in R.
22.

Mandal, S.M., Chakraborty, D. & Dey, S. (2010). Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant Signaling & Behavior*, 5, 359-368.
23.

Mauricio, R. & Rausher, M.D. (1997). Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution*, 51, 1435-1444.
24.

Mitchell-Olds, T. & Shaw, R.G. (1987). Regression analysis of natural selection: statistical inference and biological interpretation. *Evolution*, 41, 1149-1161.
25.

Newton, A.C., Allnutt, T.R., Gillies, A.C.M., Lowe, A.J. & Ennos, R.A. (1999). Molecular phylogeography, intraspecific variation and the conservation of tree species. *Trends Ecol Evol*, 14, 140-145.
26.

Paul-Victor, C., Züst, T., Rees, M., Kliebenstein, D.J. & Turnbull, L.A. (2010). A new method for measuring relative growth rate can uncover the costs of defensive compounds in *Arabidopsis thaliana*. *New Phytol*, 187, 1102-1111.
27.

Pener, M.P. (2013). Hormonal effects on flight and migration. In: *Endocrinology II* (ed. Kerkut, GA). Elsevier, pp. 491-550.
28.

Picman, A.K. (1986). Biological activities of sesquiterpene lactones. *Biochem Syst Ecol*, 14, 255-281.
29.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, S. & team, R.c. (2014). nlme: Linear and Nonlinear Mixed Effects Models.
30.

Prasad, K.V.S.K., Song, B.-H., Olson-Manning, C., Anderson, J.T., Lee, C.-R., Schranz, M.E. *et al.* (2012). A gain-of-function polymorphism controlling complex traits and fitness in nature. *Science*, 337, 1081-1084.
31.

Rainey, P.B. & Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature*, 394, 69-72.
32.

Ramakrishna, A. & Ravishankar, G.A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, 6, 1720-1731.
33.

Rasmann, S. & Agrawal, A.A. (2008). In defense of roots: A research agenda for studying plant resistance to belowground herbivory. *Plant Physiol*, 146, 875-880.
34.

Rasmann, S., Erwin, A.C., Halitschke, R. & Agrawal, A.A. (2011). Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. *J Ecol*, 99, 16-25.

35.

Rausher, M.D. (1992). The measurement of selection on quantitative traits - biases due to environmental covariances between traits and fitness. *Evolution*, 46, 616-626.
36.

R Core Team (2014). A language and environment for statistical computing. R Foundation for Statistical Computing Vienna, Austria.
37.

Sarwar, M., Kirkegaard, J.A., Wong, P.T.W. & Desmarchelier, J.M. (1998). Biofumigation potential of brassicas - III. In vitro toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant Soil*, 201, 103-112.
38.

Scheenen, T.W.J., Vergeldt, F.J., Heemskerk, A.M. & Van As, H. (2007). Intact plant magnetic resonance imaging to study dynamics in long-distance sap flow and flow-conducting surface area. *Plant Physiol*, 144, 1157-1165.
39.

Sinervo, B. & Svensson, E. (2002). Correlational selection and the evolution of genomic architecture. *Heredity*, 89, 329-338.
40.

South, A. (2011). rworldmap: A New R package for Mapping Global Data. *The R Journal*, 3, 35-43.
41.

Strauss, S.Y., Rudgers, J.A., Lau, J.A. & Irwin, R.E. (2002). Direct and ecological costs of resistance to herbivory. *Trends Ecol Evol*, 17, 278-285.
42.

Turner, T.L., Bourne, E.C., Von Wettberg, E.J., Hu, T.T. & Nuzhdin, S.V. (2010). Population resequencing reveals local adaptation of *Arabidopsis lyrata* to serpentine soils. *Nature Genet.*, 42, 260-U242.
43.

van Dam, N.M. (2009). Belowground herbivory and plant defenses. *Annu Rev Ecol Evol S*, 40, 373-391.
44.

Verduijn, M.H., Van Dijk, P.J. & Van Damme, J.M.M. (2004). Distribution, phenology and demography of sympatric sexual and asexual dandelions (*Taraxacum officinale* s.l.): geographic parthenogenesis on a small scale. *Biol J Linn Soc*, 82, 205-218.
45.

Watts, S.M., Dodson, C.D. & Reichman, O.J. (2011). The roots of defense: Plant resistance and tolerance to belowground herbivory. *PLoS One*, 6.
46.

Wickham, H. (2009). *ggplot2: elegant graphics for data analysis*. Springer New York.
47.

Züst, T., Heichinger, C., Grossniklaus, U., Harrington, R., Kliebenstein, D.J. & Turnbull, L.A. (2012). Natural enemies drive geographic variation in plant defenses. *Science*, 338, 116-119.
48.

Züst, T., Joseph, B., Shimizu, K.K., Kliebenstein, D.J. & Turnbull, L.A. (2011). Using knockout mutants to reveal the growth costs of defensive traits. *P Roy Soc B-Biol Sci*, 278, 2598-2603.

3.4.1 Supplemental

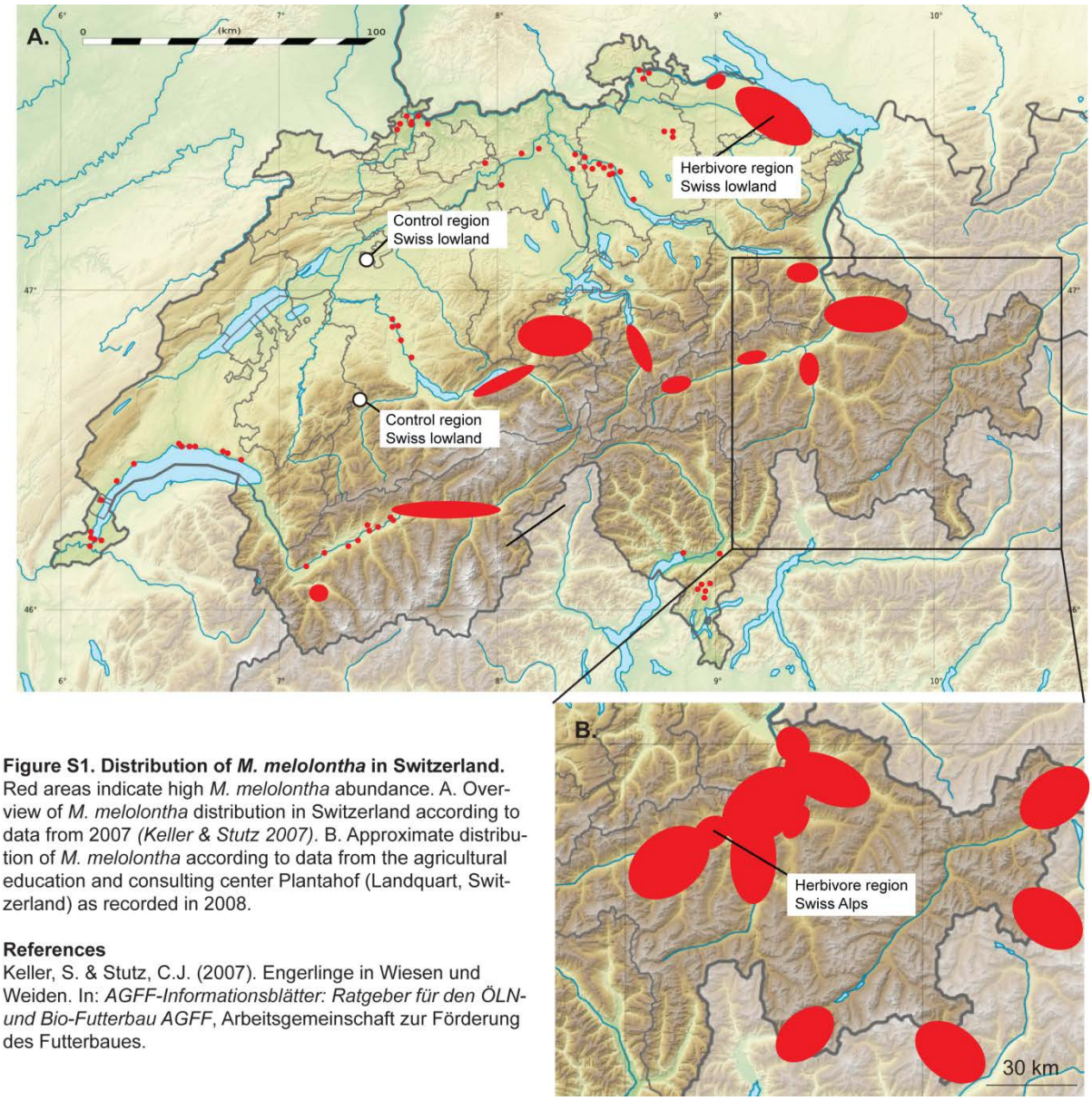

Figure S1. Distribution of *M. melolontha* in Switzerland. Red areas indicate high *M. melolontha* abundance. A. Overview of *M. melolontha* distribution in Switzerland according to data from 2007 *(Keller & Stutz 2007)*. B. Approximate distribution of *M. melolontha* according to data from the agricultural education and consulting center Plantahof (Landquart, Switzerland) as recorded in 2008.

References

Keller, S. & Stutz, C.J. (2007). Engerlinge in Wiesen und Weiden. In: *AGFF-Informationsblätter: Ratgeber für den ÖLN- und Bio-Futterbau AGFF*, Arbeitsgemeinschaft zur Förderung des Futterbaues.

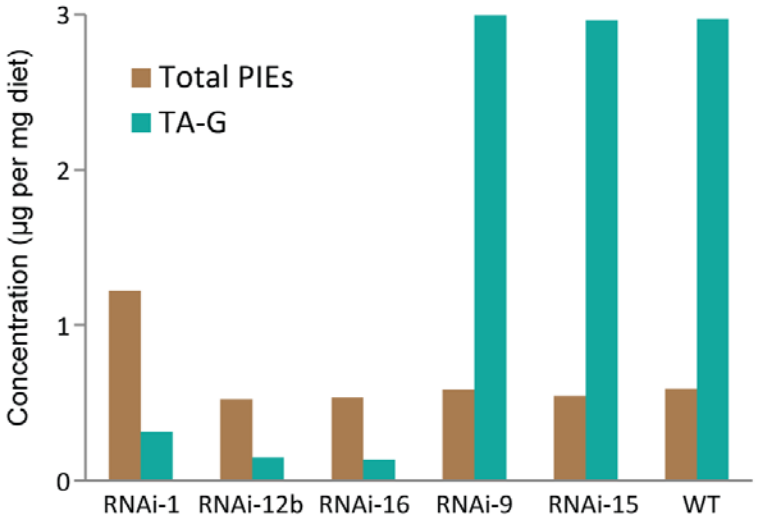

Figure S2. Concentrations of taraxinic acid β-D-glucopyranosyl ester (TA-G) and total phenolic inositol esters (PIEs) used for the feeding assay. Concentrations were measured in the latex water extract on an HPLC-UV before adding it to the diet, and concentrations per mg diet caluclated based on volume of the extract applied to the diet. N = 1.

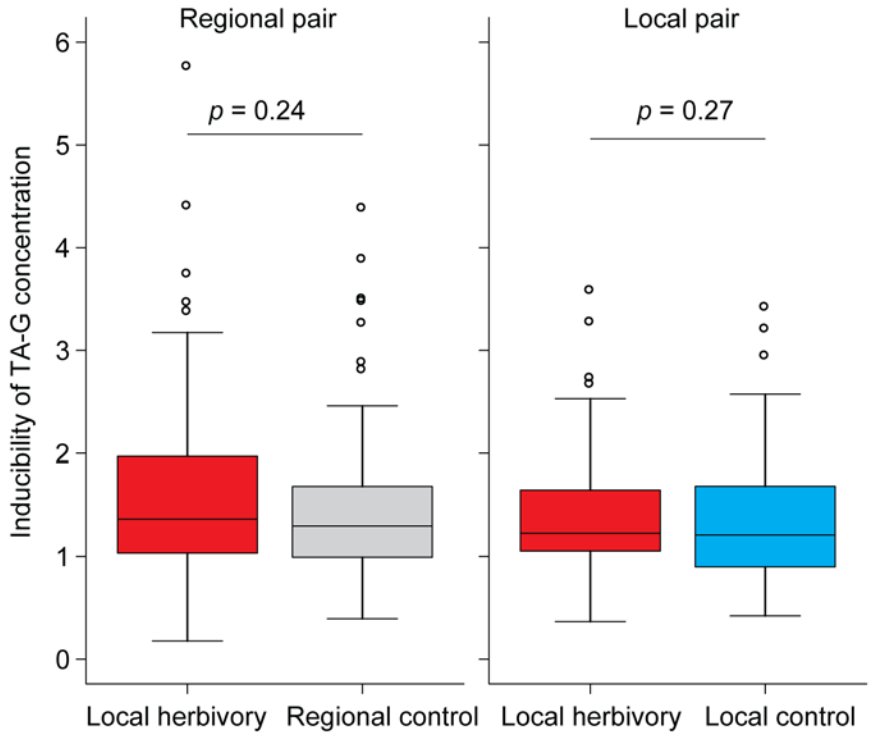

Figure S3. Inducibility of taraxinic acid β-D-glucopyranosyl ester (TA-G). Inducibility of TA-G (TA-G concentration herbivore-infested plant / TA-G concentration control plant) did not differ between infestation histories. *P*-values from Kruskal-Wallis rank sum tests are shown.

Table S1. Field sites of natural *T. officinale* populations. NA = no available information.

Nr	Area	Infestation history	Record	Information source	GPS-N	GPS-E	Altitude (m)	Exposition	Inclination	Characterization	Land use
1	Germany	Local herbivory	>20 years	Government bureau for viniculture in Kaiserstuhl; local farmer T. Hau and O. Fäth	49.91956	9.29467	372	North-west	slight slope	Extensively used grassland	Cutting and occasional sheep grazing
2	Germany	Local herbivory	>20 years	Government bureau for viniculture in Kaiserstuhl; local farmer T. Hau and O. Fäth	49.91402	9.29884	359	South-west	slope	Extensively used grassland	Hoarse grazing
3	Germany	Local herbivory	>20 years	Government bureau for viniculture in Kaiserstuhl; local farmer T. Hau and O. Fäth	49.90974	9.29882	335	South	slope	Intermediately rich pasture	NA
4	Germany	Local control	>20 years	Regional government bureau for viniculture in Kaiserstuhl; local farmer T. Hau and O. Fäth	49.92813	9.29368	369	West	slope	Extensively used grassland	Cutting and occasional grazing
5	Germany	Local control	>20 years	Regional government bureau for viniculture in Kaiserstuhl; local farmer T. Hau and O. Fäth	49.92825	9.2908	326	North	slope	Intermediately rich pasture	Cutting and occasional grazing
6	Germany	Local control	>20 years	Regional government bureau for viniculture in Kaiserstuhl; local farmer T. Hau and O. Fäth	49.9113	9.28641	359	South	slope	Intermediately rich pasture	Hoarse or sheep grazing
7	Germany	Regional control	>20 years	Regional government bureau for viniculture in Kaiserstuhl	49.72103	9.7307	283	South	slope	Extensively used grassland	Occasional grazing or cutting
8	Germany	Regional control	>20 years	Regional government bureau for viniculture in Kaiserstuhl	49.736657	9.603553	345	East	slope	Extensively used grassland	Occasional grazing or cutting
9	Germany	Regional control	>20 years	Regional government bureau for viniculture in Kaiserstuhl	49.72585	9.65052	323	South-east	slope	Extensively used grassland	Occasional grazing or cutting
10	Swiss lowlands	Local herbivory	>20 years	Regional educational center for agriculture, Arenenberg; local farmer W. Rutishauser	47.592226	9.271772	460	South-east	slope	Rich pasture	Cutting, occasional cow grazing
11	Swiss lowlands	Local herbivory	>20 years	Regional educational center for agriculture, Arenenberg; local inhabitant P. Reich	47.578017	9.178237	539	NA	flat	Rich pasture	Cutting
12	Swiss lowlands	Local herbivory	>20 years	Regional educational center for agriculture, Arenenberg; local inhabitant P. Reich	47.579609	9.161264	569	South	slope	Rich pasture	Cutting
13	Swiss lowlands	Regional control	>20 years	Swiss abundance map *M. melolontha*; local farmers K. Marti	47.068551	7.42436	539	South	slope	Rich pasture	Cow grazing & cutting
14	Swiss lowlands	Regional control	>20 years	Swiss abundance map *M. melolontha*; local farmers K. Marti	47.070144	7.430068	539	NA	flat	Rich pasture	Cutting
15	Swiss lowlands	Regional control	>20 years	Swiss abundance map *M. melolontha*; local farmers K. Marti	47.071755	7.438501	539	NA	flat	Rich pasture	Cutting
16	Swiss Alps	Local herbivory	>20 years	Regional educational center for agriculture, Plantahof; local farmer C. Guisep	46.78136	9.17917	944	South	slope	Rich pasture	Cutting and autumn cow grazing
17	Swiss Alps	Local herbivory	>20 years	Regional educational center for agriculture, Plantahof; local farmer P. Beeli	46.78884	9.28285	830	South-east	slight slope	Rich pasture	Cutting and autumn cow grazing
18	Swiss Alps	Local herbivory	>20 years	Regional educational center for agriculture, Plantahof; local farmer P. Beeli	46.78929	9.28346	829	South	slope	Nutrient-poor grassland	Cutting
19	Swiss Alps	Local herbivory	>20 years	Regional educational center for agriculture, Plantahof; local farmer M. Bühler	46.758603	9.205142	854	North-east	slope	Relatively nutrient poor meadow	Cutting
20	Swiss Alps	Local control	NA	Regional educational center for agriculture, Plantahof; local farmer C. Pieder	46.7832	9.19936	1061	South	slope	Rich pasture	Cutting and autumn cow grazing
21	Swiss Alps	Local control	>20 years	Regional educational center for agriculture, Plantahof; local farmer P. Beeli	46.78975	9.28363	842	South	slight slope	Rich pasture	Cutting
22	Swiss Alps	Local control	>20 years	Regional educational center for agriculture, Plantahof; local farmer P. Beeli	46.78947	9.28338	841	NA	flat	Rich pasture	Cutting
23	Swiss Alps	Local control	>20 years	Regional educational center for agriculture, Plantahof; local farmer M. Bühler	46.75834	9.20533	858	North-east	slope	Rich pasture	Cutting
24	Swiss Alps	Regional control	>20 years	Abundance map *M. melolontha*; local farmer U. Erb	46.63755	7.39325	1031	South	slope	Rich pasture	Cutting and autumn cow grazing
25	Swiss Alps	Regional control	>20 years	Abundance map *M. melolontha*; local farmer U. Erb	46.63068	7.39423	846	South-east	slope	Rich pasture	Cow grazing
26	Swiss Alps	Regional control	>20 years	Abundance map *M. melolontha*; local farmer U. Erb	46.631765	7.385793	935	East	slope	Intermediately rich pasture	Cutting and autumn cow grazing
27	Swiss Alps	Regional control	NA	Abundance map *M. melolontha*; local farmer U. Erb	46.61913	7.38262	828	NA	flat	Rich pasture	Cutting

4. DISCUSSION

In this thesis I investigated the composition, function and evolution of latex secondary metabolites in *T. officinale* in regard to below ground herbivory. Using natural variation, chemical manipulation and genetic modification, I found that the latex-derived sesquiterpene lactone, taraxinic acid β-D-glucopyranosyl ester (TA-G), deters *M. melolontha* feeding and thereby directly protects roots. Furthermore, *M. melolontha* imposes divergent selection on TA-G concentration, indicating that this root herbivore can tip the balance between herbivore-dependent fitness costs and benefits of this metabolite. The patterns of divergent selection were reflected in natural *T. officinale* populations that evolved under different *M. melolontha* densities for several decades, suggesting that *M. melolontha* can shape the defensive chemistry in *T. officinale* populations. Here, I would like to discuss the results in a broader context regarding the function and evolution of PSMs.

4.1 Storage of *T. officinale* secondary metabolites in latex: implications for physiology and function

In manuscript II, we described that the latex of *T. officinale* is dominated by three classes of secondary metabolites: Phenolic inositol esters (PIEs), triterpene acetates (TritAcs) and the sesquiterpene lactone TA-G. Each of the three metabolite classes accounts for 5-7% of the latex fresh mass. Such high concentrations are typical for latex secondary metabolites. For example, in the closely related *Lactuca sativa*, 14% of the latex fresh mass is accounted for by sesquiterpene lactones (Sessa *et al.* 2000). Similarly, the latex of *Chelidonium majus* (Papaveraceae) contains 20% isoquinoline alkaloids (Tome & Colombo 1995). As laticifers are - unlike other secretory ducts such as resin channels - metabolically active and fully functioning cells, the high concentration of secondary metabolites may impose substantial hurdles for the metabolism of these cells. To overcome toxicity of its own metabolites, the plants may compartmentalize the metabolites in specialized subcellular organelles, and/or store the metabolites as non-toxic protoxins.

Storage of reactive PSMs in subcellular compartments is a common theme in plant defense. Lipophilic compounds often accumulate in membranes, vesicles and dead cells (Wink 2010). For example, rubber molecules in the latex are typically enclosed by a monolayer membrane (Cornish, Wood & Windle 1999; Wood & Cornish 2000; Schmidt *et al.* 2010). Hydrophilic compounds, among them glucosinolates, cyanogenic glycosides, alkaloids, cardenolides and phenolic glycosides, are usually stored in the vacuole (Matile 1984; Mende & Wink 1987; Wink 1993). The vacuole is also a predominant storage compartment in laticifer cells: up to 500 mM morphine is found in the latex vacuoles of *Papaver somniferum* (Pham & Roberts 1991). No information regarding the storage of the secondary metabolites in the latex of *T. officinale* is available. The relative ease with which vacuoles

can be extracted from latex may allow rapid identification of the subcellular compartmentalization of latex secondary metabolites (Giordani & Marty 1983).

The storage of PSMs as non-reactive protoxins is another widespread strategy plants deploy to circumvent self-intoxication. Many PSMs, among them glucosinolates, benzoxazinoids, cardenolides, cardiac glycosides and salicinoids, are covalently bound to sugar moieties. The glycosylation of these compounds can have different, non-exclusive consequences (Gachon, Langlois-Meurinne & Saindrenan 2005). First, the sugar moiety may directly reduce the reactivity of the compound by stabilizing it through the protection of reactive groups (Jones & Vogt 2001). Cleavage of the sugar moiety results in the formation of unstable and reactive metabolites, a phenomenon observed for expample in glucosinolates (Bones & Rossiter 1996; Kliebenstein, Kroymann & Mitchell-Olds 2005; Halkier & Gershenzon 2006), benzoxazinoids (Sicker *et al.* 2000; Macias *et al.* 2004) and cyanogenic glycosides (Vetter 2000). Second, the activity of the metabolite can change upon glycosylation without alterations in its reactivity (Jones & Vogt 2001). For example, several phenolic glycosides from *Prunus avium* (Rosaceae) induced phytotoxin synthesis in *Pseudomonas syringae*, whereas the corresponding aglycone lacked the induction activity (Mo *et al.* 1995). Third, the sugar moiety increases solubility of the compounds and thus allows storage of high concentrations (Jones & Vogt 2001). Fourth, the sugar moiety can act as a biological flag controlling the transport and compartmentalization of the metabolite, for example by promoting the accumulation of the compounds in the vacuole (Wink 1998; Jones & Vogt 2001). For instance, cyanogenic glycosides (Lindberg Møller & Seigler 1998) and cardenolides (Christmann, Kreis & Reinhard 1993) are only transported in the glycosylated form.

In the latex of *T. officinale*, we found two classes of glycosylated compounds, PIEs and TA-G. While we did not obtain strong evidence for a defensive function of the PIEs, we found that TA-G deters *M. melolontha* feeding (manuscript III). Can we infer whether the toxicity of this compound may be affected by the glucose moiety based on the structure of TA-G? The vast majority of sesquiterpene lactones, among them TA-G, contain an exocyclic methylene group as part of an α-methylene-γ-lactone moiety (Herz 1977). This methylene group can react with nucleophilic targets, especially thiols, via Michael addition (Picman 1986; Schmidt 1999). TA-G additionally contains another α,β-unsaturated carbonyl group, an ester to which the glucose moiety is directly bound. While the reactivity of the exocyclic methylene group is likely not affected by deglycosylation due to its distance from the sugar, the second α,β-unsaturated carbonyl group may become more accessible to reaction upon deglycosylation. An alternative explanation for the glycosylation of TA-G is the potential effect of the glucose moiety on the transport and storage of TA-G within laticifers cells. However, further experiments are clearly needed to investigate whether deglycosylation takes place upon tissue rupture or feeding by insects, and whether the reactivity, toxicity or transport of TA-G differs from the aglycone both in the plant and in the insect. These experiments could provide insights into the mode of toxicity of the large class of glycosylated PSMs.

In manuscript III, we showed that TA-G reduces *M. melolontha* performance. The other latex secondary metabolites, PIEs and TritAcs, could act defensively as well and might enhance the effect of TA-G. Synergism of the three major classes of latex secondary metabolites could partially explain the tight co-regulation of these compounds across developmental stages in *T. officinale* (manuscript II). A possible scenario of synergistic interactions is as follows: The highly lipophilic triterpene acetates may disrupt the cell membrane in the midgut lumen and thereby allow TA-G and PIEs to enter the midgut cells. As an electrophile, TA-G may then conjugate to proteins or other thiols. The most abundant thiol in the cytosol of most cells is glutathione (Merrill *et al.* 1988; Woerdenbag *et al.* 1989; Jodynis-Liebert, Murias & Bloszyk 1999; Heilmann, Wasescha & Schmidt 2001). This tripeptide (L-y-glutamyl-L-cysteinylglycine) is found in nearly all organisms (Meister & Anderson 1983), and its conjugation to electrophiles serves as a major detoxification pathway for xenobiotics as well as for alleviating oxidative stress (Mittler 2002). Oxidative stress can be imposed by phenolic compounds under alkaline conditions (Egert *et al.* 2005), as phenolic rearrangement to reactive semiquinones is promoted at alkaline pH. Given the large quantities of TA-G in *T. officinale*, this substance might conjugate to and deplete glutathione levels in *T. officinale*-feeding herbivores making them more susceptible to PIEs that rearrange to reactive semiquinones in the alkaline gut environment of *M. melolontha*. Radioactive labelling to trace the fate of the metabolites upon ingestion, in combination with feeding assays with purified compounds and with transgenic plants devoid of one or several metabolite classes, would greatly facilitate the exploration of synergistic interactions between the various classes of latex secondary metabolites. Although synergism between metabolites has been proposed to account for much of the diversity and complexity of PSMs, experimental evidence for this phenomenon is scarce (Berenbaum & Neal 1985; Cipollini & Stiles 1992; Dyer *et al.* 2003; Steppuhn & Baldwin 2007).

4.2 Evolution of plant secondary metabolites

The capacity of plants to produce different chemical structures is unparalleled in nature. As PSMs mediate the interaction of plants with their abiotic and biotic environment, environmental stresses are hypothesized to drive PSM evolution. However, many PSMs often do not have one sole function and thus are likely under the selective force of different environmental pressures. For example, non-protein amino acid in the seeds of Fabaceae species can serve as both nitrogen storage and defense molecules (Rosenthal 1991). Similarly, PSMs may act against several biotic stresses in parallel: juglone, a compound present in the walnut family Juglandaceae, exhibits allelopathic effects against neighboring plants (Jose & Gillespie 1998; Willis 2000), is toxic to insects (Lindroth, Anson & Weisbrod 1990; Thiboldeaux, Lindroth & Tracy 1994) and has antimicrobial activity (Clark, Jurgens & Hufford 1990). It thus remains a major challenge to identify the major drivers of PSM evolution in nature. Putative selective forces include abiotic factors such as nutrient and water availability, temperature, radiation and the presence of toxic chemicals, as well as biotic factors including

herbivores, pollinators, beneficial and pathogenic microbes, neighboring plants, parasitoids and insect predators.

4.2.1 Insect herbivore-imposed selection

Among phytophagous animals, insects are the most abundant and species-rich plant consumers on earth (Gilbert *et al.* 1979; Bernays 1998). As the performance of many phytophagous insects decreases in the presence of PSMs, it has been hypothesized that herbivorous insects play a key role in the diversification of plant defense chemistry (Fraenkel 1959; Ehrlich & Raven 1964; Futuyma & Agrawal 2009). However, it has commonly been argued that the ambient selection pressure imposed by above ground feeding insects is not strong enough to drive the evolution of PSMs (Hairston, Smith & Slobodkin 1960; Jermy 1976; Jermy 1984; Schoonhoven, Jermy & van Loon 1998). Previous estimates suggested that insect herbivores consume about 10-20% of the plant primary production (Bray 1956; Coley, Bryant & Chapin 1985; Cyr & Pace 1993; Cebrian & Lartigue 2004). A recent review using data of broad taxonomic breath showed that phytophagous insects remove only 5% of the green leaf mass, with even lower estimates for non-woody plants (Turcotte *et al.* 2014). As slight biomass losses can sometimes lead to an overcompensation of sexual reproduction (Hendrix & Trapp 1981; Paige & Whitham 1987; Lennartsson, Tuomi & Nilsson 1997), it remains unclear to which extent vegetative biomass loss transfers to reproductive fitness costs. Thus, to evaluate the evolutionary consequences of herbivore feeding, measurements of plant reproductive success, ideally over several years, are of great value. Artificial removal of herbivores by application of insecticides demonstrated that the exclusion of the above ground herbivore community increases plant fitness (Simms & Rausher 1989), results in a rapid evolutionary divergence of plant genotype frequencies (Agrawal *et al.* 2012a) and changes species composition (Carson & Root 2000). Taken together, these data provide evidence that herbivores do impose selection pressure on plants, although the strength of selection may vary over space and time.

While ample evidence demonstrates that insect herbivores do impose selection pressure upon plants, it often remains unclear on which trait selection acts. As PSMs deter or decrease performance of herbivorous insects, plants should benefit accordingly, resulting in a positive selection of these metabolites under herbivory. Several studies indeed showed a positive correlation between concentration of PSMs and plant performance. For example, concentration of benzoxazinoids across different maize genotypes was negatively correlated with feeding damage (Niemeyer 2009). Nicotine deficient *Nicotiana attenuata* lines lost three fold more leaf area than wild type controls in the native habitat under ambient herbivore pressure (Steppuhn *et al.* 2004). Transgenic *Arabidopsis thaliana* plants deficient in rhizathalene A lost more root biomass than wild type controls upon attack by the opportunistic fungus gnat (*Bradysia* spp.) (Vaughan *et al.* 2013). Similarly, transgenic *A. thaliana* lines deficient in aliphatic glucosinolates were more strongly attacked by the Lepidoptera *Mamestra brassicae* compared to wild type (Beekwilder *et al.* 2008). Furthermore, iridoid glycoside concentrations across *Plantago lanceolata* (Plantaginaceae) genotypes were negatively correlated with

leaf consumption by *Spodoptera exigua* (Biere, Marak & van Damme 2004). In contrast, many studies failed to show a positive correlation between the variation of PSMs and plant performance upon herbivory. For example, the glucosinolate profile was not associated with plant performance upon herbivore attack in *Boechera stricta* (Brassicaceae) (Manzaneda, Prasad & Mitchell-Olds 2010). Similarly, total cardenolide concentration across different genotypes of the common milkweed (*Asclepias syriaca*) was not correlated to above ground biomass upon attack by the specialist root feeder *Tetraopes tetraophthalmus* (Rasmann *et al.* 2011). Furthermore, although PSMs may deter non-adapted insects and improve plant performance upon attack, PSMs may also reduce plant performance by attracting specialized herbivores that use these chemicals as oviposition (Mewis, Ulrich & Schnitzler 2002) and foraging cues (Gabrys & Tjallingii 2002; Miles, del Campo & Renwick 2005; Robert *et al.* 2012), as well as for sequestration (Malcolm & Brower 1989). For example, in a two year-long field study, Macel and Klinkhamer (2010) showed that pyrrolizidine alkaloid-containing genotypes of *Senecio jacobea* (Asteraceae) were more frequently attacked by specialized herbivores than genotypes lacking pyrrolizidine alkaloids. Moreover, the presence of anti-nutritional metabolites may force herbivores to consume more plant tissue to fulfill their nutritional requirements: Steppuhn and Baldwin (2007) showed that the presence of proteinase inhibitors, which reduce the nutritional quality of *Nicotiana attenuate* plants, increased plant tissue consumption by *Spodoptera exigua* larvae, but only in the absence of another toxic metabolite, nicotine. From these observations, three conclusions can be drawn:

1. **PSMs can reduce plant damage upon insect attack but not always.** The degree of metabolite-mediated fitness advantage greatly varies between different plant-herbivore interactions and environments. The variable effects of PSMs on plant resistance highlight how the heterogeneous composition of herbivore communities may shape different blends of defensive compounds.
2. **There is very little evidence demonstrating that natural variation in the concentration of a metabolite improves plant performance.** The clearest evidence for metabolite-mediated fitness advantages upon herbivory often involves transgenic knock-down mutants. While genetic modification is a powerful tool to study the function of a metabolite, the complete removal of a metabolite is less informative than to investigate how quantitative variation in metabolite concentrations affect plant performance upon herbivory.
3. **The net reproductive benefit of PSMs often remains unknown,** as plant performance is mostly measured under greenhouse conditions and on vegetative biomass. Further field studies are required to address the role of PSMs in nature.

Considering the above-mentioned limitations, it remains often unclear to which extent herbivorous insects drive the evolution of PSMs. One of the first experiments showing evidence for a PSM-mediated fitness advantage was conducted by Mauricio and Rausher (1997). By simultaneously applying insecticides and pesticides over one year, the authors showed that the elimination of natural enemies changed the patterns of selection for total glucosinolate concentration. Similarly, the patterns of selection for alkaloids in *Datura stramonium* (Solanceae) changed upon pesticide treatment (Shonle & Bergelson 2000). Furthermore, Agrawal *et al.* (2012a) demonstrated rapid changes in *Oenothera biennis* genotype frequencies upon removal of the insect community, which was accompanied by changes in the concentrations of several ellagitannins. Recently, Züst *et al.* (2012) showed that the frequency of *GS-ELONG* alleles, which determine the length of the glucosinolate side chain, co-varies with the abundance of two aphid species in nature. The geographic pattern was mimicked by a five generation-long selection experiment on a synthetic plant population exposed to different aphid treatments, suggesting that a heterogeneous distribution of the aphid species contributed to the observed patterns in the field. Prasad *et al.* (2012) showed that the *BCMA* locus, which determines the relative distribution of methionine-derived and branched-chain glucosinolates, was under differential selection. Together, these experiments provide evidence that ambient insect pressure can select for variation in PSMs. However, few studies have demonstrated a direct experimental link between the defensive function of a plant secondary metabolite, its associated fitness costs and benefits, and its herbivore-driven geographic variation. In our study (manuscripts III and IV), we attempted to fill in this gap of knowledge. Our results show that TA-G is defensive, under *M. melolontha*-imposed divergent selection, and that the concentration of TA-G co-varies with *M. melolontha* abundance in the field.

Based on current information on the evolution of PSMs above and below ground and our knowledge about the environmental conditions in the phyllo- and rhizosphere, can we infer whether herbivorous insects impose different selection pressure on roots compared to shoots? Roots are among the most common overwintering and storage organs in temperate ecosystems (Crawley 2009). The dependency of plants on roots for re-sprouting may result in a stronger selection of root defenses compared to leaf defenses. In addition, chemical defenses are likely of great importance in roots, since herbivore avoidance strategies such as leaf shedding or nutrient allocation may be deployed to a smaller extent in roots than in leaves due to the immediate requirement of roots for the survival of the plant. Roots are considered as an inferior food source compared to shoots, especially regarding nitrogen content (van Dam 2009). Although our literature survey did not support this assumption (manuscript I), chemical defenses that reduce the nutritional quality of the food source may be especially important in roots. As the soil is rich in microbes (Curtis, Sloan & Scannell 2002), PSMs that are active against both herbivores and pathogens may be under particularly strong positive selection in roots. The omnipresence of pathogens may also have resulted in the evolution towards higher constitutive and lower induced defenses. To evaluate the effects of root feeding insects on the evolution of PSMs, one should simultaneously consider the interaction of insect herbivores with other putative selective forces.

4.2.2 Other antagonistic interactions influencing PSM evolution

Pathogens are among the most devastating plant enemies (Harlan 1976; Dinoor & Eshed 1984). While a large body of data describes gene-for-gene evolution of pathogens with their host plants (Dangl & Jones 2001; Chisholm *et al.* 2006), little is known about the effects of pathogens on the evolution of PSMs. Application of PSMs to pathogens demonstrated that many compounds have anti-microbial activity (Sarwar *et al.* 1998; Chitwood 2002). Furthermore, the concentration of secondary metabolites *in planta* can be negatively correlated with pathogen performance, as was found for iridoid glycosides across *Plantago lanceolata* (Plantaginaceae) genotypes (Biere, Marak & van Damme 2004). Moreover, secondary metabolite concentrations can co-vary with environmental conditions and pathogen load: Talley, Coley and Kursar (2002) reported a positive correlation between the activity and diversity of antifungal compounds on the leaf surface of *Artemisia tridentata* (Asteraceae), the vapor pressure of the habitat, and fungal abundance. Similarly, in natural populations of *Capsicum chacoense* (Solanaceae), the ratio of pungent chili increased with moisture availability of the habitat (Haak *et al.* 2012). Unfortunately, manipulative field experiments that investigate changes of the selection gradients in the presence and absence of pathogens are scarce. Mauricio and Rausher (1997) showed that selection gradients for total glucosinolates differed in the presence and absence of biotic stresses, but the simultaneous application of both insecticides and fungicides does not allow disentangling the effects of insects and pathogens. Considering the strong putative fitness costs of pathogens for plants, the effect of pathogens for the evolution of PSMs clearly deserves more attention.

Although herbivores and pathogens dramatically differ in their effects on plants, they are closely entangled (Marquis & Alexander 1992), especially in the rhizosphere. First, pathogens often enter through wounds made by feeding insects (Friedli & Bacher 2001; Hatcher & Paul 2001; Kluth, Kruess & Tscharntke 2001). Second, although herbivores and pathogens tend to elicit different phytohormonal stress responses, substantial cross-talk between the phytohormonal pathways exists (McDowell & Dangl 2000; Nurnberger & Scheel 2001; Robert-Seilaniantz, Grant & Jones 2011). The simultaneous effect of herbivores and pathogens may thus impose a different selective regime on plant defenses than when herbivores and pathogens act alone. Third, despite the elicitation of different phytohormones, the early signaling cascades after herbivore and pathogen attack greatly overlap (Reymond *et al.* 2000; Hatcher *et al.* 2004; Taylor, Hatcher & Paul 2004). Thus, pathogens and herbivores may select for similar early signaling responses. Fourth, many plant secondary metabolites that are toxic to herbivores are also active against pathogens and vice versa (Mayer 2004). Fifth, pathogens can suppress plant immune responses, which will simultaneously affect herbivores (Chung *et al.* 2013). These observations suggest that defenses against herbivores and pathogens have likely evolved in a coordinated fashion.

Apart from pathogens and insect herbivores, mollusks, birds and mammals are among the most devastating plant enemies (Pollard 1192; Bryant & Kuropat 1980; McNaughton 1984; Bigger &

Marvier 1998). However, the very low number of studies on the interaction of PSMs with mollusks and birds does not allow assessing their influence on the evolution of PSMs. Mammalian herbivores are usually highly mobile and generalist feeders. Thus, it has been hypothesized that they may not show strong reciprocal co-evolution with plant defenses (Freeland & Janzen 1974; Palo & Robbins 1991). Nevertheless, mammalian herbivores do discriminate between food plants and selectively browse (Bryant *et al.* 1991), thereby imposing plant-specific selection pressure. Whereas multiple studies showed that mammals are often negatively affected or deterred by PSMs (Freeland & Janzen 1974; Bryant *et al.* 1991; Iason 2005), much less is known about the effect of mammals on the evolution of PSMs in nature (Iason 2005). In a 34 year-long rabbit enclosure experiment in English grasslands, Didiano *et al.* (2014) showed that the release from rabbit herbivory selected for genotypes with decreased tolerance to herbivory and reduced leaf number in three out of four grassland species. Unfortunately, data on PSMs are not available from this dataset. In the same experiment, exclusion of rabbits led to the evolutionary decline in the plant growth rate of another species, *Rumex acetosa* L. (Polygonaceae), but did not result in changes in tolerance and the concentrations of oxalate and tannic acid (Turley *et al.* 2013). Future experiments are clearly needed to investigate whether mammalian herbivores affect the evolution of PSMs.

Taken together, these data indicate that while insect herbivores can impose selection on PSMs, other abiotic and biotic factors may simultaneously drive the evolution of the very same metabolites. In this thesis, we showed that variation in *M. melolontha* abundance imposes divergent selection on TA-G concentration. Nevertheless, both mammals and pathogens may also impact the evolution of root latex secondary metabolites in *T. officinale*. Mice and voles can greatly reduce *T. officinale* abundance by feeding on the tap root over winter and thus can impose strong selection pressure on plant resistance traits. Pathogens may be especially important for the evolution of root secondary metabolites due to their high abundance in the soil and the frequent wounding of roots caused by lateral root branching. The disentanglement of the relative strength of the individual selective forces holds great promise to further elucidate the evolution of plant secondary chemistry.

4.3 Conclusions and outlook

Since the discovery of PSMs, substantial progress has been made to identify the function and evolution of plant chemicals. However, several obstacles have limited our understanding for PSM evolution especially in regard to herbivory to date:

1. **The major native herbivores for a given plant species are often unknown or have not been used in laboratory-based studies.** For many natural plant-herbivore interactions, the insects with the largest effects on plant fitness remain unknown. Likewise, many plant-herbivore interactions are based on non-native plant-herbivore systems, thereby limiting conclusions about PSM evolution.
2. **Many PSMs co-vary with other plant traits.** It is thus crucial to combine correlative and manipulative experiments to disentangle the effects of different plant traits.
3. **Plant resistance is often measured by herbivore performance.** Since herbivore performance does not necessarily reflect the fitness impact of herbivores on plants, it is important to measure plant resistance based on plant reproductive success.
4. **Plant resistance is measured predominantly in the laboratory.** As laboratory-based studies often overestimate the effect of herbivores on plant fitness, it is important to conduct realistic field experiments to evaluate the strength of this selective force.
5. **Historic records on herbivore abundance are scarce.** Together with the extensive temporal fluctuation of herbivores, the lack of reliable herbivore abundance data limits associative studies comparing metabolite concentrations or allelic variation with the nature of the herbivore community.
6. **Only one putative function of a metabolite is usually investigated.** The considerations of multiple functions of PSMs could greatly enhance our understanding for PSM evolution.

In this thesis, I deployed several approaches to overcome the above-mentioned limitations. I used a natural co-evolved root herbivore system and confirmed that *M. melolontha* is a major root herbivore of *T. officinale* by screening the root herbivore community of *T. officinale* in nature. The concentration of TA-G, but not the total concentrations of the other two major latex secondary metabolite classes, was positively correlated with plant resistance and negatively correlated with insect performance. Chemical complementation and genetic modifications of the candidate metabolite TA-G verified that TA-G is sufficient to affect both plant and insect performance. Two independent common garden experiments showed that TA-G can improve plant vegetative and reproductive fitness after herbivory in the field. I took advantage of historic records on *M. melolontha* abundance to show that *T. officinale* populations exposed to high *M. melolontha* herbivory during the last few decades exhibited higher TA-G concentration than local and regional control populations in nature. Together, these approaches indicate that *M. melolontha* can drive the evolution of TA-G in natural populations.

The following experiments are suggested to further elucidate the evolution of PSMs:

1. **Disentangle the effect of multiple selective forces.**
   A key question for the evolution of PSMs is the identification of the major selective forces. Thereto, the effect of a stepwise removal of the putative selective agents either by mechanical exclusion or pesticide application on PSM-mediated plant fitness could be monitored, ideally under natural conditions and over several years. These time- labor- and space-intensive experiments could give unique insights into the relative strength of putative selective forces in nature.
2. **Assess the synergistic and antagonistic effects of putative selective forces.**
   It has been proposed that different abiotic and biotic stresses exert selection pressure on different metabolites. Laboratory-based studies that systematically evaluate the relation between plant fitness, PSMs and allelic or gene expression variation in the presence and absence of a wide range of selective forces could greatly improve our understanding of the synergistic and antagonistic effects of different environmental stresses.
3. **Assess the effect of gene flow for PSM evolution.**
   Most studies so far have investigated PSM evolution in inbred or clonal plants. To understand the impact of herbivores in creating and maintaining intraspecific variation within and between populations, field experiments that track allelic frequencies of resistance loci in sexual and asexual conspecifics under different levels of biotic stress could be employed. These experiments would be best accompanied by modelling approaches that simulate changes in allele frequencies under different selection regimes and rates of gene flow.
4. **Evaluate putative fitness costs of PSMs.**
   Although costs are central to evolutionary theories explaining trait variation, we still know very little about the costs of PSMs. Evaluating the costs of PSMs could involve experiments that systematically manipulate the environmental conditions and measure the correlation between natural variation in PSMs and plant performance, or experiment that evaluate the relation of genetically modified quantities or qualities of PSMs and plant fitness.

Elucidating the evolution of PSMs holds great promise for understanding the general processes governing the emergence, maintenance and disappearance of plant traits. Although considerable effort has been made in this regard, most of the major questions remain unresolved. Nevertheless, the extensive intra- and interspecific variation in PSMs will continue to stimulate biologists to try to elucidate their function and evolution.

5. SUMMARY

The tremendous intra- and interspecific variation in PSMs has fascinated biologists for centuries. First regarded as waste products of primary metabolism, it is becoming increasingly clear that PSMs mediate the interaction of plants with their abiotic and biotic environment. Among biotic stresses, insect herbivores are the most abundant and species-rich plant consumers on earth. As many PSMs are deterrent or toxic to herbivorous insects, it has been hypothesized that these feeders drive the evolution of PSMs. While recent studies provided evidence that herbivores contribute to the maintenance of PSM variation above ground, the role of phytophagous insects for the evolution of PSMs below ground has received little attention. In this thesis, I aimed at improving our understanding of the function and evolution of PSMs in regard to below ground herbivory.

We first characterized the composition of secondary metabolites in the latex of *T. officinale*. We found that the latex is dominated by three classes of secondary metabolites: phenolic inositol esters, triterpene acetates and the sesquiterpene lactone taraxinic acid β-D-glucopyranosyl ester (TA-G). All of these metabolite classes are highly concentrated, each of them accounting for 5-7% of the latex fresh mass. We found that the composition and quantities of these metabolites vary across different organs, plant age and *T. officinale* genotypes, suggesting that variation in latex chemistry may be driven by biotic stresses.

We next elucidated the role of the three metabolite classes in defense against the larvae of the common cockchafer (*M. melolontha),* a major root herbivore of *T. officinale*. We found that TA-G was negatively correlated with *M. melolontha* larval growth, whereas no correlation was observed between the other two metabolite classes and *M. melolontha* mass gain. We thus studied the effect of TA-G on both *T. officinale* and *M. melolontha* performance by combining natural variation, phytochemical manipulation and genetic modification. We found that TA-G deters *M. melolontha* feeding and thereby directly protects the roots, resulting in improved plant performance upon *M. melolontha* attack under both greenhouse and field conditions. Furthermore, TA-G concentration was negatively correlated with plant fitness in the absence of *M. melolontha,* and positively correlated with plant fitness in its presence, indicating that *M. melolontha* imposes divergent selection on TA-G concentration.

To further elucidate the evolution of TA-G concentration, we investigated *T. officinale* field populations that evolved under different *M. melolontha* herbivory during the last decades. We found that *T. officinale* populations exposed to high levels of *M. melolontha* herbivory exhibited higher levels of TA-G than both geographically close and distant control populations under low *M. melolontha* pressure. By cultivating offspring of these field-measured plants, we found that these differences likely resulted from both induction of TA-G by *M. melolontha* feeding and from heritable variation in TA-G concentration. In the genetically fixed triploids, offspring from the high-herbivore populations had

higher TA-G concentration than geographically distant control populations. In contrast, no difference in TA-G concentration was found in the sexual outcrossing diploids, suggesting that gene flow may have limited the fixation of heritable variation between populations. This indicates that the mode of reproduction is an important determinant for the evolutionary response of *T. officinale* to herbivore pressure. Together, these results provide evidence that *M. melolontha* can drive the evolution of TA-G in *T. officinale* populations. Our study thus identifies root herbivores as important agents for the evolution of plant secondary chemistry, and highlights the role of biotic factors in shaping chemical variation in plants.

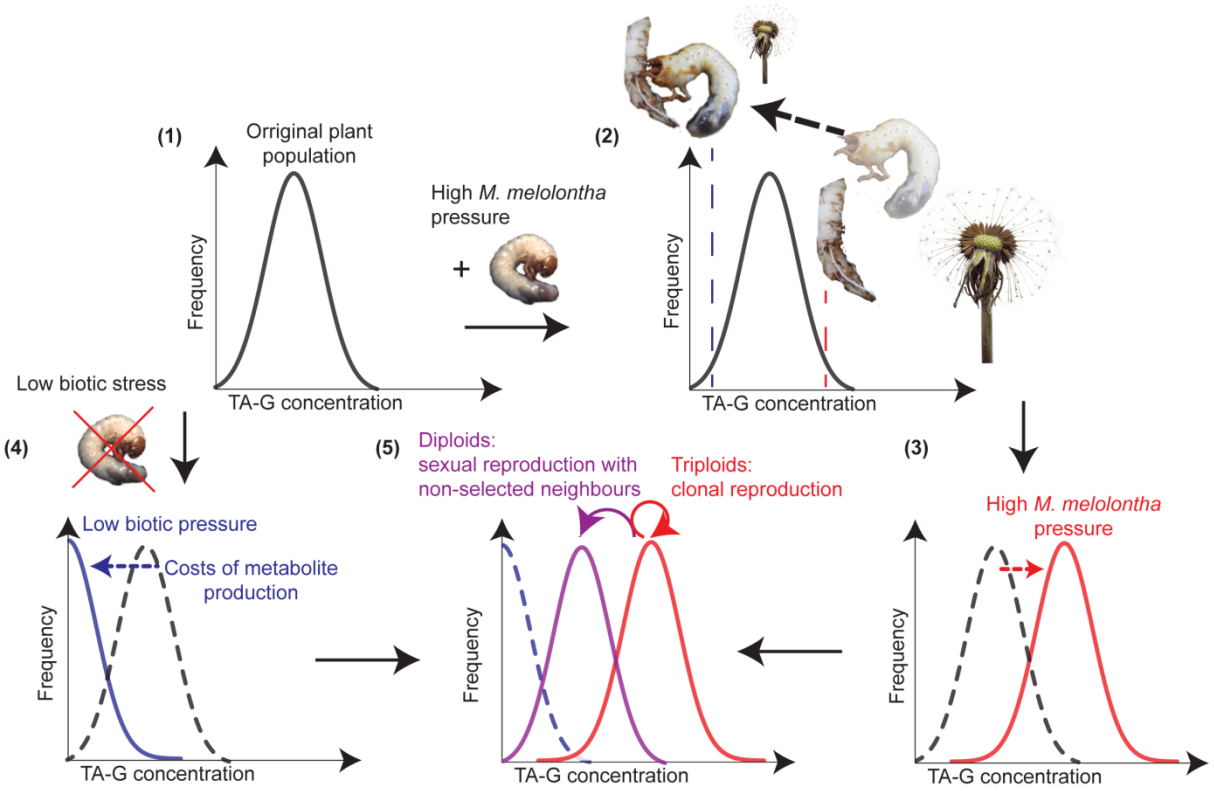

Figure 4 Current model for the evolution of taraxinic acid β-D glucopyranosyl ester (TA-G). At high *M. melolontha* densities, individuals with high TA-G concentration outperform their conspecifics as TA-G deters larval feeding (1-2), resulting in an increase of TA-G concentration in the population (3). Under low biotic stress, costs of TA-G production result in an evolutionary decline of TA-G concentration (4). The degree of gene flow between *T. officinale* plants under high and low *M. melolontha* pressure influences the extent of differentiation of TA-G concentration between populations (5).

6. ZUSAMMENFASSUNG

Die umfangreiche inter- und intraspezifische Diversität der Pflanzensekundärmetabolite (PSM) hat Biologen seit Jahrhunderten fasziniert. Zuerst als Abfallprodukte des Primärmetabolismus betrachtet, wurde mehr und mehr klar, dass PSM Interaktionen zwischen der Pflanze und ihrer abiotischen und biotischen Umwelt vermitteln. Zu den wichtigsten biotischen Faktoren gehören heterotrophe Insekten, die abundantesten und artenreichsten Frassfeinde der Pflanzen. Da viele PSM abstossend oder toxisch für phytophage Insekten sind, wurde hypothetisiert, dass diese Insekten die Evolution der PSM beeinflussen. Während kürzlich publizierte Studien darauf hinweisen, dass Herbivore zur Erhaltung der chemischen Variation in den oberirdischen Pflanzenorganen beitragen, wissen wir noch sehr wenig über die Rolle phytophager Insekten für die Evolution von PSM in der Wurzel. In dieser Thesis erstrebte ich unser Verständnis für die Funktion und Evolution von PSM bezüglich Wurzel-Herbivorie zu vertiefen.

Zuerst charakterisierten wir die Zusammensetzung der Sekundärmetabolite im Latex vom Löwenzahn (*Taraxacum officinale* agg.). Der Latex ist von drei Klassen von Sekundärmetaboliten dominiert: Phenolische Inositolester, Triterpenacetate und das Sesquiterpenlakton Taraxinsäure-glukopyranosyl-Ester (TA-G). Jede dieser drei Stoffklassen kommt für 5-7% der Latexfrischmasse auf. Wir fanden substanzielle Variation in der Zusammensetzung und den Konzentrationen dieser Sekundärmetabolite im Latex von verschiedenen Organen, Pflanzenalter und Löwenzahn-Genotypen, was darauf hinweisen könnte, dass biotischer Stress zur Evolution dieser Variabilität beigetragen hat.

Als nächstes untersuchten wir die Rolle der drei Sekundärmetabolit-Klassen für die Abwehr vom Löwenzahn gegen den Maikäferengerling (*Melolontha melolontha*), einer der Hauptschädlinge der Wurzel dieser Pflanze. Wir fanden eine negative Korrelation zwischen dem Wachstum der Maikäferlarven und der Konzentration von TA-G. Im Gegensatz dazu wurde keine Korrelation zwischen der Gewichtszunahme der Larven und den totalen Konzentrationen der anderen beiden Stoffklassen gefunden. Indem wir Experimente mit natürlichen Variationen zwischen Löwenzahngenotypen, phytochemischen Manipulationen und genetischen Modifizierungen kombinierten, untersuchten wir den Effekt von TA-G auf die Fitness der Maikäferlarven und des Löwenzahns. Wir zeigten, dass TA-G das Fressen der Larven reduziert und die Wurzel direkt vor Frass schützt, was die Fitness der Pflanze unter *M. melolontha*-Herbivorie im Gewächshaus und unter Feldbedingungen verbesserte. Des Weiteren fanden wir, dass in der Abwesenheit von Maikäferlarven TA-G negativ mit der Pflanzenfitness korreliert ist, während in der Präsenz von Maikäferengerlingen TA-G positiv mit der Pflanzenfitness assoziiert ist. Dies deutet darauf hin, dass Maikäferengerlinge divergente Selektion auf TA-G ausüben.

Um die Evolution von TA-G weiterführend zu untersuchen, analysierten wir Feldpopulationen vom Löwenzahn, welche seit Jahrzehnten unterschiedlichen Dichten von Maikäfern ausgesetzt sind. Löwenzahnpopulationen aus Gebieten mit viel Maikäfer-Herbivorie wiesen höhere TA-G Konzentrationen auf als Populationen aus Gebieten mit niedrigen Maikäferdichten. Indem wir die Nachkommen dieser Feldpopulationen unter standardisierten Bedingungen untersuchten, erhielten wir Hinweise, dass die im Feld observierten Unterschiede in der TA-G Konzentration durch Induktion und durch erbliche Variation hervorgerufen wurden. Die triploiden, asexuellen Nachkommen von den Maikäfer-infestierten Gebieten hatten höhere TA-G Konzentrationen als die geographisch weit entfernten Kontrollpopulationen. Die Nachkommen der diploiden, sexuellen Pflanzen zeigten keine Unterschiede in der TA-G Konzentration bezüglich der Infestationsgeschichte der Mutterpflanze, was darauf hindeutet, dass die Art der Reproduktion ein wichtiger Faktor für die evolutionäre Antwort auf Wurzelherbivorie ist. Zusammen weisen diese Daten darauf hin, dass Wurzelherbivore natürliche Selektion auf Wurzel-Sekundärmetabolite ausüben. Des Weiteren hebt diese Studie die Rolle von biotischen Faktoren für die Evolution der Pflanzennaturstoffe hervor.

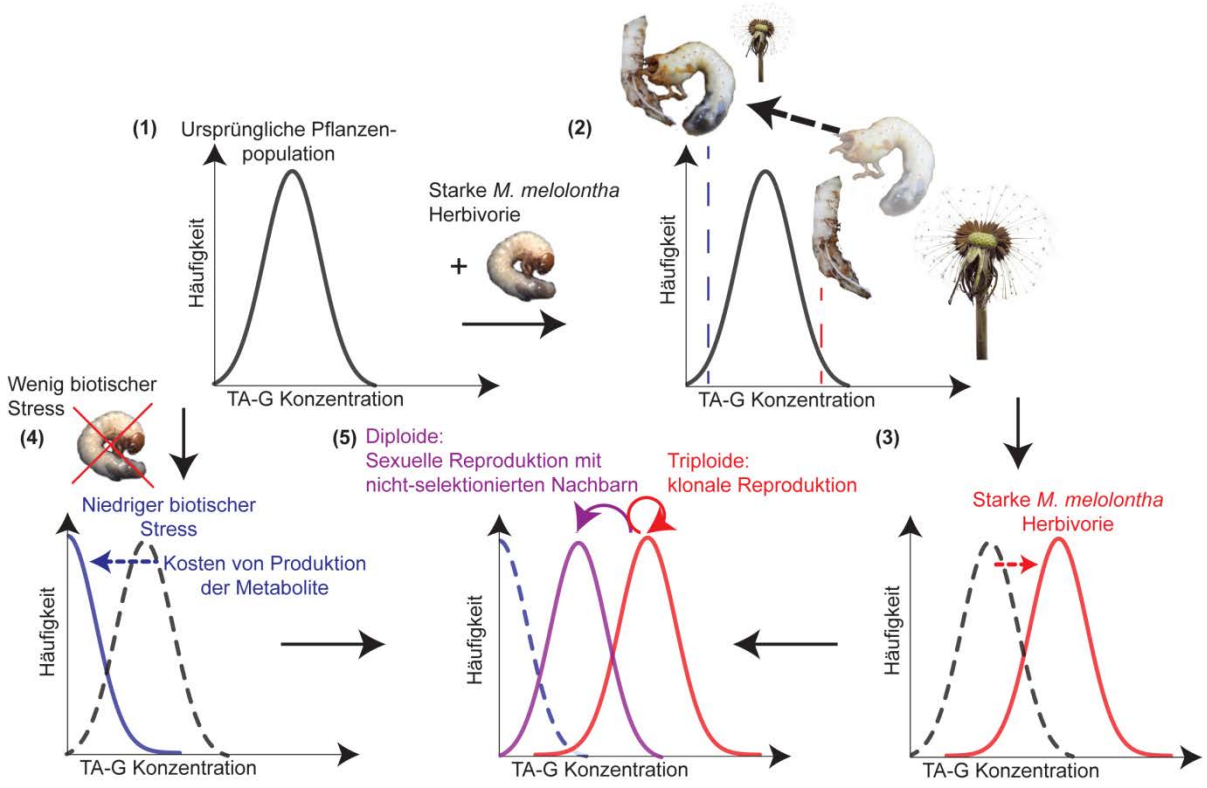

Figure 5 Derzeitiges Modell für die Evolution des Taraxinsäure-glukopyranosyl-Esters (TA-G). Unter hohem Maikäferfrass, Löwenzahn-Individuen mit hoher TA-G Konzentration haben höhere Fitness als Artgenossen mit niedriger TA-G Konzentration, da TA-G das Fressen der Larven reduziert (1-2). Die daraus resultierende höhere Reproduktion der Individuen mit hoher TA-G Konzentration führt zu einer Zunahme von TA-G in den Maikäfer-exponierten Populationen (3). Bei niedrigem biotischem Stress führen Kosten von TA-G Produktion zur evolutionären Abnahme von TA-G (4). Das Ausmass an Genfluss beeinflusst das Ausmass der Differenzierung zwischen Populationen unter unterschiedlicher Maikäfer-Herbivorie (5).

7. REFERENCES

Agrawal, A.A. (2005) Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. *Evolutionary Ecology Research,* **7,** 651-667.

Agrawal, A.A., Hastings, A.P., Johnson, M.T.J., Maron, J.L. & Salminen, J.P. (2012a) Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science,* **338,** 113-116.

Agrawal, A.A. & Konno, K. (2009) Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. *Annual Review of Ecology Evolution and Systematics,* **40,** 311-331.

Agrawal, A.A., Petschenka, G., Bingham, R.A., Weber, M.G. & Rasmann, S. (2012b) Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. *New Phytologist,* **194,** 28-45.

Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y., Martin, J., Smart, L., Glauser, G., Erb, M., Flors, V., Frey, M. & Ton, J. (2011) Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. *Plant Physiology,* **157,** 317-327.

Akhtar, N., Malik, A., Ali, S.N. & Kazmit, S.U. (1992) Proceragenin, an antibacterial cardenolide from *Calotropis procera*. *Phytochemistry,* **31,** 2821-2824.

Almeida-Cortez, J.S., Shipley, B. & Arnason, J.T. (1999) Do plant species with high relative growth rates have poorer chemical defences? *Functional Ecology,* **13,** 819-827.

Andersen, D.C. (1987) Belowground herbivory in natural communities - a review emphasizing fossorial animals. *Quarterly Review of Biology,* **62,** 261-286.

Auguie, B. (2012) gridExtra: functions in Grid graphics.

Baba, T. & Schneewind, O. (1998) Instruments of microbial warfare: bacteriocin synthesis, toxicity and immunity. *Trends Microbiol,* **6,** 66-71.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S. & Vivanco, J.M. (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology,* **57,** 233-266.

Baldwin, I. & Callahan, P. (1993) Autotoxicity and chemical defense: nicotine accumulation and carbon gain in solanaceous plants. *Oecologia,* **94,** 534-541.

Baldwin, I.T., Halitschke, R., Paschold, A., von Dahl, C.C. & Preston, C.A. (2006) Volatile signaling in plant-plant interactions: "talking trees" in the genomics era. *Science,* **311,** 812-815.

Beekwilder, J., van Leeuwen, W., van Dam, N.M., Bertossi, M., Grandi, V., Mizzi, L., Soloviev, M., Szabados, L., Molthoff, J.W., Schipper, B., Verbocht, H., de Vos, R.C.H., Morandini, P., Aarts, M.G.M. & Bovy, A. (2008) The impact of the absence of aliphatic glucosinolates on insect herbivory in *Arabidopsis*. *Plos One,* **3,** e2068.

Bennett, R.N. & Wallsgrove, R.M. (1994) Secondary metabolites in plant defense-mechanisms. *New Phytologist,* **127,** 617-633.

Berenbaum, M. & Neal, J. (1985) Synergism between myristicin and xanthotoxin, a naturally cooccurring plant toxicant. *Journal of Chemical Ecology,* **11,** 1349-1358.

Bergelson, J. (1994) The effects of genotype and the environment on costs of resistance in lettuce. *American Naturalist,* **143,** 349-359.

Bergelson, J. & Purrington, C.B. (1996) Surveying patterns in the cost of resistance in plants. *American Naturalist,* **148,** 536-558.

Bernays, E.A. (1998) Evolution of feeding behavior in insect herbivores - Success seen as different ways to eat without being eaten. *Bioscience,* **48,** 35-44.

Bernhardsson, C., Robinson, K.M., Abreu, I.N., Jansson, S., Albrectsen, B.R. & Ingvarsson, P.K. (2013) Geographic structure in metabolome and herbivore community co-occurs with genetic structure in plant defence genes. *Ecology Letters,* **16,** 791-798.

Bertin, C., Yang, X.H. & Weston, L.A. (2003) The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil,* **256,** 67-83.

Biere, A., Marak, H.B. & van Damme, J.M.M. (2004) Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? *Oecologia,* **140,** 430-441.

Bigger, D.S. & Marvier, M.A. (1998) How different would a world without herbivory be?: A search for generality in ecology. *Integrative Biology,* **1,** 60-67.

Blossey, B. & Hunt-Joshi, T.R. (2003) Belowground herbivory by insects: Influence on plants and aboveground herbivores. *Annual Review of Entomology,* **48,** 521-547.

Bones, A.M. & Rossiter, J.T. (1996) The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiologia Plantarum,* **97,** 194-208.

Bray, J.R. (1956) A study of mutual occurrence of plant-species. *Ecology,* **37,** 21-28.

Brown, V.K. & Gange, A.C. (1990) Insect herbivory below ground. *Advances in Ecological Research,* **20,** 1-58.

Bryant, J.P. & Kuropat, P.J. (1980) Selection of winter forage by subarctic browsing vertebrates: The role of plant chemistry. *Annual Review of Ecology and Systematics,* **11,** 261-285.

Bryant, J.P., Provenza, F.D., Pastor, J., Reichardt, P.B., Clausen, T.P. & Dutoit, J.T. (1991) Interactions between woody-plants and browsing mammals mediated by secondary metabolites. *Annual Review of Ecology and Systematics,* **22,** 431-446.

Carson, W.P. & Root, R.B. (2000) Herbivory and plant species coexistence: Community regulation by an outbreaking phytophagous insect. *Ecological Monographs,* **70,** 73-99.

Cate, P.C. (2004) Maikäferflüge in Österreich 1949-2000 - erste Auswertungen (Coleoptera, Scarabeidae, *Melolontha melolontha* (L.) und *M. hipoocastani* (F)). *Laimburg Journal,* **1,** 146-157.

Cebrian, J. & Lartigue, J. (2004) Patterns of herbivory and decomposition in aquatic and terrestrial ecosystems. *Ecological Monographs,* **74,** 237-259.

Chisholm, S.T., Coaker, G., Day, B. & Staskawicz, B.J. (2006) Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell*, **124,** 803-814.

Chitwood, D.J. (2002) Phytochemical based strategies for nematode control. *Annual Review of Phytopathology*, **40,** 221-249.

Chow, J.K., Akhtar, Y. & Isman, M.B. (2005) The effects of larval experience with a complex plant latex on subsequent feeding and oviposition by the cabbage looper moth: *Trichoplusia ni* (Lepidoptera : Noctuidae). *Chemoecology*, **15,** 129-133.

Christmann, J., Kreis, W. & Reinhard, E. (1993) Uptake, transport and storage of cardenolides in foxglove. Cardenolide sinks and occurrence of cardenolides in the sieve tubes of *Digitalis lanata*. *Botanica Acta*, **106,** 419-427.

Chung, S.H., Rosa, C., Scully, E.D., Peiffer, M., Tooker, J.F., Hoover, K., Luthe, D.S. & Felton, G.W. (2013) Herbivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences of the United States of America*, **110,** 15728-15733.

Cipollini, M.L. & Stiles, E.W. (1992) Antifungal activity of ripe ericaceous fruits - phenolic-acid interactions and palatability for dispersers. *Biochemical Systematics and Ecology*, **20,** 501-514.

Clark, A.M., Jurgens, T.M. & Hufford, C.D. (1990) Antimicrobial activity of juglone. *Phytotherapy Research*, **4,** 11-14.

Clark, K.E., Hartley, S.E. & Johnson, S.N. (2011) Does mother know best? The preference–performance hypothesis and parent–offspring conflict in aboveground–belowground herbivore life cycles. *Ecological Entomology*, **36,** 117-124.

Cole, R.A. (1987) Intensity of radicle fluorescence as related to the resistance of seedlings of lettuce to the lettuce root aphid and carrot to the carrot fly. *Annals of Applied Biology*, **111,** 629-639.

Coley, P.D., Bryant, J.P. & Chapin, F.S. (1985) Resource availability and plant antiherbivore defense. *Science*, **230,** 895-899.

Cornish, K., Wood, D.F. & Windle, J.J. (1999) Rubber particles from four different species, examined by transmission electron microscopy and electron-paramagnetic-resonance spin labeling, are found to consist of a homogeneous rubber core enclosed by a contiguous, monolayer biomembrane. *Planta*, **210,** 85-96.

Crawley, M. (2009) The structure of plant communities. *Plant Ecology* (ed. M. Crawley), pp. 475-531. Wiley.

Curtis, T.P., Sloan, W.T. & Scannell, J.W. (2002) Estimating prokaryotic diversity and its limits. *Proceedings of the National Academy of Sciences*, **99,** 10494-10499.

Cyr, H. & Pace, M.L. (1993) Magnitude and patterns of herbivory in aquatic and terrestrial ecosystems. *Nature*, **361,** 148-150.

Dangl, J.L. & Jones, J.D.G. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411,** 826-833.

Davies, J. (1990) What are antibiotics? Archaic functions for modern activities. *Mol Microbiol*, **4,** 1227-1232.

de Mendiburu, F. (2014) agricolae: Statistical Procedures for Agricultural Research.

Dell, B. & McComb, A.J. (1979) Plant resins—their formation, secretion and possible functions. *Advances in Botanical Research* (ed. H.W. Woolhouse), pp. 277-316. Academic Press.

Didiano, T.J., Turley, N.E., Everwand, G., Schaefer, H., Crawley, M.J. & Johnson, M.T.J. (2014) Experimental test of plant defence evolution in four species using long-term rabbit exclosures. *Journal of Ecology*, **102,** 584-594.

Dillon, P.M., Lowrie, S. & McKey, D. (1983) Disarming the "Evil Woman" - petiole constriction by a sphingid larva circumvents mechanical defenses of its host plant, *Cnidoscolus urens* (Euphorbiaceae). *Biotropica*, **15,** 112-116.

Dinoor, A. & Eshed, N. (1984) The role and importance of pathogens in natural plant communities. *Annual Review of Phytopathology*, **22,** 443-466.

Dixon, R.A. & Strack, D. (2003) Phytochemistry meets genome analysis, and beyond. *Phytochemistry*, **62,** 815-816.

Dobler, S., Dalla, S., Wagschal, V. & Agrawal, A.A. (2012) Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase. *Proceedings of the National Academy of Sciences of the United States of America*, **109,** 13040-13045.

Dussourd, D.E. & Denno, R.F. (1991) Deactivation of plant defense - correspondence between insect behavior and secretory canal architecture. *Ecology*, **72,** 1383-1396.

Dussourd, D.E. & Eisner, T. (1987) Vein-cutting behavior - insect counterploy to the latex defense of plants. *Science*, **237,** 898-901.

Dyer, L.A., Dodson, C.D., Stireman III, J.O., Tobler, M.A., Smilanich, A.M., Fincher, R.M. & Letourneau, D.K. (2003) Synergistic effects of three *Piper* amides on generalist and specialist herbivores. *Journal of Chemical Ecology*, **29,** 2499-2514.

Egert, M., Stingl, U., Bruun, L.D., Pommerenke, B., Brune, A. & Friedrich, M.W. (2005) Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* larvae (Coleoptera : Scarabaeidae). *Applied and Environmental Microbiology*, **71,** 4556-4566.

Ehrlich, P.R. & Raven, P.H. (1964) Butterflies and plants - a study in coevolution. *Evolution*, **18,** 586-608.

Enkerli, J., Widmer, F. & Keller, S. (2004) Long-term field persistence of *Beauveria brongniartii* strains applied as biocontrol agents against European cockchafer larvae in Switzerland. *Biological Control*, **29,** 115-123.

Fahn, A. (1988) Secretory-tissues in vascular plants. *New Phytologist*, **108,** 229-257.

Farrell, B.D., Dussourd, D.E. & Mitter, C. (1991) Escalation of plant defense - do latex and resin canals spur plant diversification? *American Naturalist*, **138,** 881-900.

Fraenkel, G.S. (1959) The raison d'être of secondary plant substances. *Science*, **129,** 1466-1470.

Freeland, W.J. & Janzen, D.H. (1974) Strategies in herbivory by mammals - role of plant secondary compounds. *American Naturalist*, **108,** 269-289.

Friedli, J. & Bacher, S. (2001) Mutualistic interaction between a weevil and a rust fungus, two parasites of the weed *Cirsium arvense*. *Oecologia*, **129,** 571-576.

Fröschle, M. (1996) Occurrence of the common cockchafer (*Melolonta melolontha* L.) in the State of Baden-Württemberg/Germany. *Bulletin OILB/SROP*, **19,** 1-4.

Futuyma, D.J. & Agrawal, A.A. (2009) Macroevolution and the biological diversity of plants and herbivores. *Proceedings of the National Academy of Sciences*, **106,** 18054-18061.

Gabrys, B. & Tjallingii, W.F. (2002) The role of sinigrin in host plant recognition by aphids during initial plant penetration. *Proceedings of the 11th International Symposium on Insect-Plant Relationships* (eds J. Nielsen, C. Kjær & L. Schoonhoven), pp. 89-93. Springer Netherlands.

Gachon, C.M., Langlois-Meurinne, M. & Saindrenan, P. (2005) Plant secondary metabolism glycosyltransferases: the emerging functional analysis. *Trends in Plant Science*, **10,** 542-549.

Gilbert, L.E., Horn, D.J., Stairs, G.R. & Mitchell, R.D. (1979) *Development of theory in the analysis of insect-plant interactions*. Ohio State Press.

Giordani, R. & Marty, F. (1983) *Isolement et purification des vacuoles des lacticifères articulés de lactuca sativa L.* Masson, Paris, FRANCE.

Gog, L., Berenbaum, M.R., DeLucia, E.H. & Zangerl, A.R. (2005) Autotoxic effects of essential oils on photosynthesis in parsley, parsnip, and rough lemon. *Chemoecology*, **15,** 115-119.

Gray, M.E., Sappington, T.W., Miller, N.J., Moeser, J. & Bohn, M.O. (2009) Adaptation and invasiveness of western corn rootworm: Intensifying research on a worsening pest. *Annual Review of Entomology*, **54,** 303-321.

Haak, D.C., McGinnis, L.A., Levey, D.J. & Tewksbury, J.J. (2012) Why are not all chilies hot? A trade-off limits pungency. *Proceedings of the Royal Society B-Biological Sciences*, **279,** 2012-2017.

Hagel, J.M., Yeung, E.C. & Facchini, P.J. (2008) Got milk? The secret life of laticifers. *Trends in Plant Science*, **13,** 631-639.

Hairston, N.G., Smith, F.E. & Slobodkin, L.B. (1960) Community structure, population control, and competition. *American Naturalist*, **94,** 421-425.

Halkier, B.A. & Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology*, pp. 303-333.

Harlan, J.R. (1976) Diseases as a factor in plant evolution. *Annual Review of Phytopathology*, **14,** 31-51.

Hartmann, T. (2007) From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochemistry*, **68,** 2831-2846.

Haslam, E. (1994) Secondary metabolism — evolution and function: Products or processes? *Chemoecology*, **5-6,** 89-95.

Hasler, T. (1986) Abundanz- und Dispersionsdynamik von *Melolontha melolontha (*L.*)* in Intensivobstanlagen. PhD, Eidgenössische Technische Hochschule Zürich.

Hatcher, P.E., Jason, M., Taylor, J.E., Tinney, G.W. & Paul, N.D. (2004) Phytohormones and plant-herbivore-pathogen interactions: Integrating the molecular with the ecological. *Ecology*, **85,** 59-69.

Hatcher, P.E. & Paul, N.D. (2001) Plant pathogen–herbivore interactions and their effects on weeds. *Biotic interactions in plant–pathogen associations* (eds M.J. Jeger & N.J. Spence), pp. 193-225. CAB International, Wallingford, UK.

Hauss, R. & Schütte, F. (1976) Experiments on polyphagous habits of white grubs *Melolontha melolontha* on plants of grassland. *Anzeiger für Schädlingskunde, Pflanzenschutz, Umweltschutz*, **49,** 129-132.

Hauss, R. & Schütte, F. (1978) Über die Eiablage des Maikäfers (*Melolontha melolontha* L.) in Abhängigkeit von den Wirtspflanzen des Engerlings. *Journal of Applied Entomology*, **86,** 167-174.

Heer, O. (1841) Ueber geographische Verbreitung und periodisches Auftreten der Maikäfer. *Verhandlungen der Schweizerischen Naturforschenden Gesellschaft*, **26**.

Heil, M. (2008) Indirect defence via tritrophic interactions. *New Phytologist*, **178,** 41-61.

Heilmann, J., Wasescha, M.R. & Schmidt, T.J. (2001) The influence of glutathione and cysteine levels on the cytotoxicity of helenanolide type sesquiterpene lactones against KB cells. *Bioorganic & Medicinal Chemistry*, **9,** 2189-2194.

Hendrix, S. & Trapp, E.J. (1981) Plant-herbivore interactions: Insect induced changes in host plant sex expression and fecundity. *Oecologia*, **49,** 119-122.

Herz, W. (1977) Sesquiterpene lactones in the Compositae. *The biology and chemistry of the compositae* (eds V.H. Heywood, J.B. Harborne & B.L. Turner). Academic Press INC., New York.

Huang, J., McAuslane, H.J. & Nuessly, G.S. (2003) Resistance in lettuce to *Diabrotica balteata* (Coleoptera : Chrysomelidae): The roles of latex and inducible defense. *Environmental Entomology*, **32,** 9-16.

Huber, M., Triebwasser-Freese, D., Reichelt, M., Heiling, S., Paetz, C., Chandran, J., Bartram, S., Schneider, B., Gershenson, J. & Erb, M. (2015) Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (*Taraxacum officinale* agg.). *Phytochemistry***,** In press.

Huiting, H.F., Moraal, L.G., Griepink, F.C. & Ester, A. (2006) Biology, control and luring of the cockchafer, *Melolontha melolontha*. Applied Plant Research, Wageningen.

Hunter, J.R. (1994) Reconsidering the functions of latex. *Trees-Structure and Function*, **9,** 1-5.

Iason, G. (2005) The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. *Proceedings of the Nutrition Society*, **64,** 123-131.

Irmisch, S., Krause, S.T., Kunert, G., Gershenzon, J., Degenhardt, J. & Köllner, T.G. (2012) The organ-specific expression of terpene synthase genes contributes to the terpene hydrocarbon composition of chamomile essential oils. *Bmc Plant Biology*, **12**.

Jacobsohn, M.K. & Jacobsohn, G.M. (1985) Production of a fungistat and the role of fungi during germination of *Digitalis purpurea* L. cv. Gloxiniaflora seeds. *Annals of Botany*, **56,** 543-552.

Jenke-Kodama, H., Müller, R. & Dittmann, E. (2008) Evolutionary mechanisms underlying secondary metabolite diversity. *Natural Compounds as Drugs Volume I* (eds F. Petersen & R. Amstutz), pp. 119-140. Birkhäuser Basel.

Jermy, T. (1976) Insect-host-plant relationship-co-evolution or sequential evolution? *Symposia Biologica Hungarica*, **16,** 109-113.

Jermy, T. (1984) Evolution of insect host plant relationships. *American Naturalist*, **124,** 609-630.

Jodynis-Liebert, J., Murias, M. & Bloszyk, E. (1999) Effect of several sesquiterpene lactones on lipid peroxidation and glutathione level. *Planta Medica*, **65,** 320-324.

Johnson, S.N., Barton, A.T., Clark, K.E., Gregory, P.J., McMenemy, L.S. & Hancock, R.D. (2011) Elevated atmospheric carbon dioxide impairs the performance of root-feeding vine weevils by modifying root growth and secondary metabolites. *Global Change Biology*, **17,** 688-695.

Johnson, S.N., Clark, K.E., Hartley, S.E., Jones, T.H., McKenzie, S.W. & Koricheva, J. (2012) Aboveground-belowground herbivore interactions: a meta-analysis. *Ecology*, **93,** 2208-2215.

Jones, C.G., Firn, R.D. & Malcolm, S.B. (1991) On the evolution of plant secondary chemical diversity. *Philosophical Transactions: Biological Sciences*, **333,** 273-280.

Jones, P. & Vogt, T. (2001) Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. *Planta,* **213,** 164-174.

Jose, S. & Gillespie, A.R. (1998) Allelopathy in black walnut (*Juglans nigra* L.) alley cropping. II. Effects of juglone on hydroponically grown corn (*Zea mays* L.) and soybean (*Glycine max* L. Merr.) growth and physiology. *Plant and Soil,* **203,** 199-205.

Karban, R., Baldwin, I.T., Baxter, K.J., Laue, G. & Felton, G.W. (2000) Communication between plants: induced resistance in wild tobacco plants following clipping of neighboring sagebrush. *Oecologia,* **125,** 66-71.

Keller, E., Keller, S., Buchi, R., Meier, W., Staub, A. & Wildbolz, T. (1986) Neuere Erkenntnisse über den Maikäfer. *Beiheft zu den Mitteilungen der Thurgauerischen Naturforschenden Gesellschaft,* **89,** 7-95.

Keller, S. (1984) Das andere Gesicht des Maikäfers. *Tagesanzeiger Magazin,* **19,** 27-33.

Keller, S. (2004) Engerlingsvorkommen und Bekämpfung in der Schweiz. *Nachrichtenblatt Deutscher Pflanzenschutzdienst,* **56,** 88-90.

Keller, S., Schweizer, C., Keller, E. & Brenner, H. (1997) Control of white grubs (*Melolontha melolontha* L) by treating adults with the fungus *Beauveria brongniartii*. *Biocontrol Science and Technology,* **7,** 105-116.

Kessler, A. & Baldwin, I.T. (2001) Defensive function of herbivore-induced plant volatile emissions in nature. *Science,* **291,** 2141-2144.

Kessler, D., Gase, K. & Baldwin, I.T. (2008) Field experiments with transformed plants reveal the sense of floral scents. *Science,* **321,** 1200-1202.

Kliebenstein, D.J., Kroymann, J. & Mitchell-Olds, T. (2005) The glucosinolate-myrosinase system in an ecological and evolutionary context. *Current Opinion in Plant Biology,* **8,** 264-271.

Kluth, S., Kruess, A. & Tscharntke, T. (2001) Interactions between the rust fungus *Puccinia punctiformis* and ectophagous and endophagous insects on creeping thistle. *Journal of Applied Ecology,* **38,** 548-556.

Kniep, H. (1904) *Über die Bedeutung des Milchsafts der Pflanzen*. München.

Kojima, M., Poulton, J.E., Thayer, S.S. & Conn, E.E. (1979) Tissue distributions of dhurrin and of enzymes involved in its metabolism in leaves of *Sorghum bicolor*. *Plant Physiology,* **63,** 1022-1028.

Konno, K., Hirayama, C., Nakamura, M., Tateishi, K., Tamura, Y., Hattori, M. & Kohno, K. (2004) Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. *Plant Journal,* **37,** 370-378.

Koricheva, J. (2002) Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. *Ecology,* **83,** 176-190.

Kossel, A. (1891) Über die chemische Zusammensetzung der Zelle. *Archiv für Physiologie,* 181-186.

Kushiro, T., Shibuya, M., Masuda, K. & Ebizuka, Y. (2000) A novel multifunctional triterpene synthase from *Arabidopsis thaliana*. *Tetrahedron Letters,* **41,** 7705-7710.

Lennartsson, T., Tuomi, J. & Nilsson, P. (1997) Evidence for an evolutionary history of overcompensation in the grassland biennial *Gentianella campestris* (Gentianaceae). *American Naturalist,* **149,** 1147-1155.

Levin, D.A. (1973) Role of trichomes in plant defense. *Quarterly Review of Biology,* **48,** 3-15.

Lewinsohn, T.M. (1991) The geographical distribution of plant latex. *Chemoecology,* **2,** 64-68.

Lindberg Møller, B. & Seigler, D.S. (1998) Biosynthesis of cyanogenic glycosides, cyanolipids, and related compounds. *Plant amino acids : biochemistry and biotechnology* (ed. B.K. Singh), pp. 563-609. Marcel Dekker Incorporated, New York.

Lindroth, R., Anson, B. & Weisbrod, A. (1990) Effects of protein and juglone on gypsy moths: Growth performance and detoxification enzyme activity. *Journal of Chemical Ecology,* **16,** 2533-2547.

Loecher, M. (2014) RgoogleMaps: Overlays on Google map tiles in R.

Lyman, J.C. & Ellstrand, N.C. (1984) Clonal diversity in *Taraxacum officinale* (Compositae), an apomict. *Heredity,* **53,** 1-10.

Macel, M. & Klinkhamer, P.G.L. (2010) Chemotype of *Senecio jacobaea* affects damage by pathogens and insect herbivores in the field. *Evolutionary Ecology,* **24,** 237-250.

Macias, F.A., Oliveros-Bastidas, A., Marin, D., Castellano, D., Simonet, A.M. & Molinillo, J.M.G. (2004) Degradation studies on benzoxazinoids. Soil degradation dynamics of 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) and its degradation products, phytotoxic allelochemicals from Gramineae. *Journal of Agricultural and Food Chemistry,* **52,** 6402-6413.

Mahlberg, P.G. (1993) Laticifers - an historical perspective. *Botanical Review,* **59,** 1-23.

Malcolm, S.B. & Brower, L.P. (1989) Evolutionary and ecological implications of cardenolide sequestration in the monarch butterfly. *Experientia,* **45,** 284-295.

Mandal, S.M., Chakraborty, D. & Dey, S. (2010) Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant Signaling & Behavior,* **5,** 359-368.

Manzaneda, A.J., Prasad, K.V. & Mitchell-Olds, T. (2010) Variation and fitness costs for tolerance to different types of herbivore damage in *Boechera stricta* genotypes with contrasting glucosinolate structures. *New Phytologist,* **188,** 464-477.

Marquis, R.J. & Alexander, H.M. (1992) Evolution of resistance and virulence in plant herbivore and plant pathogen interactions. *Trends in Ecology & Evolution,* **7,** 126-129.

Matile, P. (1984) The toxic compartment of plant cells. *Naturwissenschaften,* **71,** 18-24.

Mauricio, R. & Rausher, M.D. (1997) Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution,* **51,** 1435-1444.

Mayer, A.M. (2004) Resistance to herbivores and fungal pathogens: Variations on a common theme? A review comparing the effect of secondary metabolites, induced and constitutive, on herbivores and fungal pathogens. *Israel Journal of Plant Sciences,* **52,** 279-292.

McCormick, A.C., Irmisch, S., Reinecke, A., Boeckler, G.A., Veit, D., Reichelt, M., Hansson, B.S., Gershenzon, J., Köllner, T.G. & Unsicker, S.B. (2014) Herbivore-induced volatile emission in black poplar: regulation and role in attracting herbivore enemies. *Plant Cell and Environment,* **37,** 1909-1923.

McDowell, J.M. & Dangl, J.L. (2000) Signal transduction in the plant immune response. *Trends in Biochemical Sciences*, **25,** 79-82.

McNaughton, S.J. (1984) Grazing lawns - animals in herds, plant form, and coevolution. *American Naturalist*, **124,** 863-886.

Meister, A. & Anderson, M.E. (1983) Glutathione. *Annual Review of Biochemistry*, **52,** 711-760.

Mende, P. & Wink, M. (1987) Uptake of the quinolizidine alkaloid lupanine by protoplasts and isolated vacuoles of suspension-cultured *Lupinus polyphyllus* cells - diffusion or carrier-mediated transport. *Journal of Plant Physiology*, **129,** 229-242.

Menken, S.B.J., Smit, E. & DenNijs, H.C.M. (1995) Genetical population structure in plants: Gene flow between diploid sexual and triploid asexual dandelions (*Taraxacum* section *Ruderalia*). *Evolution*, **49,** 1108-1118.

Merrill, J.C., Kim, H.L., Safe, S., Murray, C.A. & Hayes, M.A. (1988) Role of glutathione in the toxicity of the sesquiterpene lactones hymenoxon and helenalin. *Journal of Toxicology and Environmental Health*, **23,** 159-169.

Metcalfe, C.R. (1967) Distribution of latex in the plant kingdom. *Economic Botany*, **21,** 115-127.

Mewis, I., Ulrich, C. & Schnitzler, W.H. (2002) The role of glucosinolates and their hydrolysis products in oviposition and host-plant finding by cabbage webworm, *Hellula undalis*. *Entomologia Experimentalis Et Applicata*, **105,** 129-139.

Miles, C.I., del Campo, M.L. & Renwick, J.A. (2005) Behavioral and chemosensory responses to a host recognition cue by larvae of *Pieris rapae*. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*, **191,** 147-155.

Mithöfer, A. & Boland, W. (2012) Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology*, **63,** 431-450.

Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, **7,** 405-410.

Mo, Y.Y., Geibel, M., Bonsall, R.F. & Gross, D.C. (1995) Analysis of sweet cherry (*Prunus avium* L.) leaves for plant signal molecules that activate the syrb gene required for synthesis of the phytotoxin, syringomycin, by *Pseudomonas syringae* pv *syringae*. *Plant Physiology*, **107,** 603-612.

Mogie, M. & Ford, H. (1988) Sexual and asexual *Taraxacum* species. *Biological Journal of the Linnean Society*, **35,** 155-168.

Moore, B.D., Andrew, R.L., Kulheim, C. & Foley, W.J. (2014) Explaining intraspecific diversity in plant secondary metabolites in an ecological context. *New Phytologist*, **201,** 733-750.

Morita, T., Sterk, A.A. & Den Nijs, J.C.M. (1990) The significance of agamospermous triploid pollen donors in the sexual relationships between diploids and triploids in *Taraxacum* Compositae. *Plant Species Biology*, **5,** 167-176.

Morris, W.F., Hufbauer, R.A., Agrawal, A.A., Bever, J.D., Borowicz, V.A., Gilbert, G.S., Maron, J.L., Mitchell, C.E., Parker, I.M., Power, A.G., Torchin, M.E. & Vázquez, D.P. (2007) Direct and interactive effects of enemies and mutualists on plant performance: A meta-analysis. *Ecology*, **88,** 1021-1029.

Niemeyer, H.M. (2009) Hydroxamic acids derived from 2-hydroxy-2h-1,4-benzoxazin-3(4h)-one: Key defense chemicals of cereals. *Journal of Agricultural and Food Chemistry*, **57,** 1677-1696.

Nurnberger, T. & Scheel, D. (2001) Signal transmission in the plant immune response. *Trends in Plant Science*, **6,** 372-379.

Nutt, K.A., O'Shea, M.G. & Allsopp, P.G. (2004) Feeding by sugarcane whitegrubs induces changes in the types and amounts of phenolics in the roots of sugarcane. *Environmental and Experimental Botany*, **51,** 155-165.

Paige, K.N. & Whitham, T.G. (1987) Overcompensation in response to mammalian herbivory - the advantage of being eaten. *American Naturalist*, **129,** 407-416.

Palo, R.T. & Robbins, C.T. (1991) *Plant defenses against mammalian herbivory*. Taylor & Francis.

Paul-Victor, C., Züst, T., Rees, M., Kliebenstein, D.J. & Turnbull, L.A. (2010) A new method for measuring relative growth rate can uncover the costs of defensive compounds in *Arabidopsis thaliana*. *New Phytologist*, **187,** 1102-1111.

Peach, K. (1950) *Biochemie und Physiologie der sekundären Pflanzenstoffe*. Springer, Berlin Göttingen Heidelberg.

Pener, M.P. (2013) Hormonal effects on flight and migration. *Endocrinology II* (ed. G.A. Kerkut), pp. 491-550. Elsevier.

Pham, T.T. & Roberts, M.F. (1991) Quantitative characterization of the contents of *Papaver somniferum* latex vacuoles. *Phytochemical Analysis*, **2,** 68-73.

Pickard, W.F. (2008) Laticifers and secretory ducts: two other tube systems in plants. *New Phytologist*, **177,** 877-887.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, S. & team, R.c. (2014) nlme: Linear and Nonlinear Mixed Effects Models.

Pollard, A.J. (1192) The importance of deterrence: responses of grazing animals to plant variation. *Plant resistance to herbivores and pathogens: ecology, evolution and genetics* (eds R.S. Fritz & E.L. Simms), pp. 216-239. University of Chicago Press, Chicago, USA.

Prasad, K.V.S.K., Song, B.-H., Olson-Manning, C., Anderson, J.T., Lee, C.-R., Schranz, M.E., Windsor, A.J., Clauss, M.J., Manzaneda, A.J., Naqvi, I., Reichelt, M., Gershenzon, J., Rupasinghe, S.G., Schuler, M.A. & Mitchell-Olds, T. (2012) A gain-of-function polymorphism controlling complex traits and fitness in nature. *Science*, **337,** 1081-1084.

Ramakrishna, A. & Ravishankar, G.A. (2011) Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, **6,** 1720-1731.

Rasmann, S. & Agrawal, A.A. (2008) In defense of roots: A research agenda for studying plant resistance to belowground herbivory. *Plant Physiology*, **146,** 875-880.

Rasmann, S., Erwin, A.C., Halitschke, R. & Agrawal, A.A. (2011) Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. *Journal of Ecology*, **99,** 16-25.

R Core Team (2014) A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reymond, P., Weber, H., Damond, M. & Farmer, E.E. (2000) Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. *Plant Cell*, **12,** 707-719.

Rezbanyai-Reser, L. (1986) Maikäferfänge mit Lichtfallen in der Schweiz 1983-1985 *(Melolontha melolontha* L. und *Melolontha hippocastani* F., Coleoptera). *Entomologische Berichte Luzern*, **15,** 39-46.

Richards, A.J. (1973) The origin of *Taraxacum* agamospecies. *Botanical Journal of the Linnean Society*, **66,** 189-211.

Richards, A.J. (1986) *Plant Breeding Systems*. George Allen & Unwin, London.

Robert-Seilaniantz, A., Grant, M. & Jones, J.D.G. (2011) Hormone crosstalk in plant disease and defense: More than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology, Vol 49* (eds N.K. VanAlfen, G. Bruening & J.E. Leach), pp. 317-343.

Robert, C.A.M., Veyrat, N., Glauser, G., Marti, G., Doyen, G.R., Villard, N., Gaillard, M.D.P., Köllner, T.G., Giron, D., Body, M., Babst, B.A., Ferrieri, R.A., Turlings, T.C.J. & Erb, M. (2012) A specialist root herbivore exploits defensive metabolites to locate nutritious tissues. *Ecology Letters*, **15,** 55-64.

Rosenthal, G.A. (1982) *Plant nonprotein amino acids and imino acids: Biological, biochemical and toxicological properties.* Adademic Press, New York, London.

Rosenthal, G.A. (1991) Nonprotein amino acids as protective allelochemicals. *Herbivores: Their interactions with secondary metabolites* (eds G.A. Rosenthal & M.R. Berenbaum). Academic Press, San Diego.

Sachs, J. (1873) *Lehrbuch der Botanik*. Leipzig.

Saito, T., Hirai, K. & Way, M.O. (2005) The rice water weevil, *Lissorhoptrus oryzophilus* Kuschel (Coleoptera : Curculionidae). *Applied Entomology and Zoology*, **40,** 31-39.

Sarwar, M., Kirkegaard, J.A., Wong, P.T.W. & Desmarchelier, J.M. (1998) Biofumigation potential of brassicas - III. In vitro toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant and Soil*, **201,** 103-112.

Schmelz, E.A., Grebenok, R.J., Ohnmeiss, T.E. & Bowers, W.S. (2002) Interactions between *Spinacia oleracea* and *Bradysia impatiens*: A role for phytoecdysteroids. *Archives of Insect Biochemistry and Physiology*, **51,** 204-221.

Schmidt, T., Lenders, M., Hillebrand, A., van Deenen, N., Munt, O., Reichelt, R., Eisenreich, W., Fischer, R., Prüfer, D. & Gronover, C.S. (2010) Characterization of rubber particles and rubber chain elongation in *Taraxacum koksaghyz*. *Bmc Biochemistry*, **11**.

Schmidt, T.J. (1999) Toxic activities of sesquiterpene lactones: Structural and biochemical aspects. *Current Organic Chemistry*, **3,** 577-608.

Schoonhoven, L.M., Jermy, T. & van Loon, J.J.A. (1998) Evolution: Who drives whom. *Insect-plant biology: From physiology to evolution*. Chapman & Hall.

Schutz, K., Carle, R. & Schieber, A. (2006) *Taraxacum* - A review on its phytochemical and pharmacological profile. *Journal of Ethnopharmacology*, **107,** 313-323.

Sessa, R.A., Bennett, M.H., Lewis, M.J., Mansfield, J.W. & Beale, M.H. (2000) Metabolite profiling of sesquiterpene lactones from *Lactuca* species - Major latex components are novel oxalate and sulfate conjugates of lactucin and its derivatives. *Journal of Biological Chemistry*, **275,** 26877-26884.

Sethi, A., McAuslane, H.J., Alborn, H.T., Nagata, R.T. & Nuessly, G.S. (2008) Romaine lettuce latex deters feeding of banded cucumber beetle: a vehicle for deployment of biochemical defenses. *Entomologia Experimentalis Et Applicata*, **128,** 410-420.

Shonle, I. & Bergelson, J. (2000) Evolutionary ecology of the tropane alkaloids of *Datura stramonium* L. (Solanaceae). *Evolution*, **54,** 778-788.

Sicker, D., Frey, M., Schulz, M. & Gierl, A. (2000) Role of natural benzoxazinones in the survival strategy of plants. *International Review of Cytology - a Survey of Cell Biology, Vol 198*, **198,** 319-346.

Simms, E.L. & Rausher, M.D. (1989) The evolution of resistance to herbivory in *Ipomoea purpurea*. 2. Natural selection by insects and costs of resistance. *Evolution*, **43,** 573-585.

Sirikantaramas, S., Yamazaki, M. & Saito, K. (2008) Mechanisms of resistance to self-produced toxic secondary metabolites in plants. *Phytochemistry Reviews*, **7,** 467-477.

South, A. (2011) rworldmap: A New R package for Mapping Global Data. *The R Journal*, **3,** 35-43.

Stahl, E. (1888) Pflanzen und Schnecken. *Jenaische Zeitschrift für Naturwissenschaften*, **22,** 557–684.

Steppuhn, A. & Baldwin, I.T. (2007) Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. *Ecology Letters*, **10,** 499-511.

Steppuhn, A., Gase, K., Krock, B., Halitschke, R. & Baldwin, I.T. (2004) Nicotine's defensive function in nature. *Plos Biology*, **2,** 1074-1080.

Stotz, H.U., Sawada, Y., Shimada, Y., Hirai, M.Y., Sasaki, E., Krischke, M., Brown, P.D., Saito, K. & Kamiya, Y. (2011) Role of camalexin, indole glucosinolates, and side chain modification of glucosinolate-derived isothiocyanates in defense of *Arabidopsis* against *Sclerotinia sclerotiorum*. *Plant Journal*, **67,** 81-93.

Strauss, S.Y., Rudgers, J.A., Lau, J.A. & Irwin, R.E. (2002) Direct and ecological costs of resistance to herbivory. *Trends in Ecology & Evolution*, **17,** 278-285.

Takanashi, K., Shitan, N., Sugiyama, A., Kamimoto, Y., Hamamoto, M., Iwaki, T., Takegawa, K. & Yazaki, K. (2008) Galactinol synthase gene of Coptis japonica is involved in berberine tolerance. *Biosci Biotechnol Biochem*, **72,** 398-405.

Talley, S.M., Coley, P.D. & Kursar, T.A. (2002) Antifungal leaf-surface metabolites correlate with fungal abundance in sagebrush populations. *Journal of Chemical Ecololgy*, **28,** 2141-2168.

Tas, I.C.Q. & Van Dijk, P.J. (1999) Crosses between sexual and apomictic dandelions (*Taraxacum*). I. The inheritance of apomixis. *Heredity*, **83,** 707-714.

Taylor, J.E., Hatcher, P.E. & Paul, N.D. (2004) Crosstalk between plant responses to pathogens and herbivores: a view from the outside in. *Journal of Experimental Botany*, **55,** 159-168.

Thiboldeaux, R., Lindroth, R. & Tracy, J. (1994) Differential toxicity of juglone (5-hydroxy-1,4-naphthoquinone) and related naphthoquinones to saturniid moths. *Journal of Chemical Ecology*, **20,** 1631-1641.

Tome, F. & Colombo, M.L. (1995) Distribution of alkaloids in *Chelidonium majus* and factors affecting their accumulation. *Phytochemistry*, **40,** 37-39.

Turcotte, M.M., Davies, T.J., Thomsen, C.J.M. & Johnson, M.T.J. (2014) Macroecological and macroevolutionary patterns of leaf herbivory across vascular plants. *Proceedings of the Royal Society B-Biological Sciences*, **281**.

Turley, N.E., Odell, W.C., Schaefer, H., Everwand, G., Crawley, M.J. & Johnson, M.T.J. (2013) Contemporary evolution of plant growth rate following experimental removal of herbivores. *American Naturalist*, **181,** S21-S34.

van Dam, N.M. (2009) Belowground herbivory and plant defenses. *Annual Review of Ecology Evolution and Systematics*, **40,** 373-391.

Vaughan, M.M., Wang, Q., Webster, F.X., Kiemle, D., Hong, Y.J., Tantillo, D.J., Coates, R.M., Wray, A.T., Askew, W., O'Donnell, C., Tokuhisa, J.G. & Tholl, D. (2013) Formation of the unusual semivolatile diterpene rhizathalene by the *Arabidopsis* class I terpene synthase TPS08 in the root stele is involved in defense against belowground herbivory. *Plant Cell*, **25,** 1108-1125.

Verduijn, M.H., Van Dijk, P.J. & Van Damme, J.M.M. (2004) The role of tetraploids in the sexual-asexual cycle in dandelions (*Taraxacum*). *Heredity*, **93,** 390-398.

Verhoeven, K.J.F., Van Dijk, P.J. & Biere, A. (2010) Changes in genomic methylation patterns during the formation of triploid asexual dandelion lineages. *Molecular Ecology*, **19,** 315-324.

Vetter, J. (2000) Plant cyanogenic glycosides. *Toxicon*, **38,** 11-36.

Vickers, C.E., Gershenzon, J., Lerdau, M.T. & Loreto, F. (2009) A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nature Chemical Biology*, **5,** 283-291.

War, A.R., Paulraj, M.G., Ahmad, T., Buhroo, A.A., Hussain, B., Ignacimuthu, S. & Sharma, H.C. (2012) Mechanisms of plant defense against insect herbivores. *Plant Signaling & Behavior*, **7,** 1306-1320.

Warmke, H.E. (1944) Self-fertilization in the Russian dandelion, *Taraxacum kok-saghyz*. *American Naturalist*, **78,** 285-288.

Watts, S.M., Dodson, C.D. & Reichman, O.J. (2011) The roots of defense: Plant resistance and tolerance to belowground herbivory. *Plos One*, **6**.

Wickham, H. (2009) *ggplot2: elegant graphics for data analysis*. Springer New York.

Willis, R.J. (2000) *Juglans* spp, juglone and allelopathy. *Allelopathy Journal*, **7,** 1-55.

Wink, M. (1993) The plant vacuole - a multifunctional compartment. *Journal of Experimental Botany*, **44,** 231-246.

Wink, M. (1998) Compartmentation of alkaloid biosynthesis, transport and storage. *Alkaloids: biochemistry, ecology and medicinal applications* (eds M.F. Roberts & M. Wink), pp. 239-258. Plenum Press, New York.

Wink, M. (2010) *Biochemistry of plant secondary metabolism*. Blackwell, London.

Woerdenbag, H.J., Lemstra, W., Malingre, T.M. & Konings, A.W. (1989) Enhanced cytostatic activity of the sesquiterpene lactone eupatoriopicrin by glutathione depletion. *Br J Cancer*, **59,** 68-75.

Wood, D.F. & Cornish, K. (2000) Microstructure of purified rubber particles. *International Journal of Plant Sciences*, **161,** 435-445.

Zalucki, M.P. & Brower, L.P. (1992) Survival of first instar larvae of *Danaus plexippus* (Lepidoptera: Danainae) in relation to cardiac glycoside and latex content of *Asclepias humistrata* (Asclepiadaceae). *Chemoecology*, **3,** 81-93.

Zalucki, M.P., Brower, L.P. & Alonso, A. (2001) Detrimental effects of latex and cardiac glycosides on survival and growth of first-instar monarch butterfly larvae *Danaus plexippus* feeding on the sandhill milkweed *Asclepias humistrata*. *Ecological Entomology*, **26,** 212-224.

Zalucki, M.P. & Malcolm, S.B. (1999) Plant latex and first-instar monarch larval growth and survival on three North American milkweed species. *Journal of Chemical Ecology*, **25,** 1827-1842.

Zhao, J., Davis, L.C. & Verpoorte, R. (2005) Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances*, **23,** 283-333.

Zhen, Y., Aardema, M.L., Medina, E.M., Schumer, M. & Andolfatto, P. (2012) Parallel molecular evolution in an herbivore community. *Science*, **337,** 1634-1637.

Zimmermann, G. (1992) Use of the fungus *Beauveria brongniartii* for the control of Euopean cockchafers *Melolontha spp* in Europe. *Use of Pathogens in Scarab Pest Management*, pp. 199-208. Jackson, T. A. Glare, T. R., Andover, UK.

Zulak, K.G. & Bohlmann, J. (2010) Terpenoid biosynthesis and specialized vascular cells of conifer defense. *Journal of Integrative Plant Biology*, **52,** 86-97.

Züst, T., Heichinger, C., Grossniklaus, U., Harrington, R., Kliebenstein, D.J. & Turnbull, L.A. (2012) Natural enemies drive geographic variation in plant defenses. *Science*, **338,** 116-119.

Züst, T., Joseph, B., Shimizu, K.K., Kliebenstein, D.J. & Turnbull, L.A. (2011) Using knockout mutants to reveal the growth costs of defensive traits. *Proceedings of the Royal Society B-Biological Sciences*, **278,** 2598-2603.

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9. EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, entsprechen der zur Zeit gültigen Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena, dass ich die vorliegende Promotionsarbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Personen, die zu dieser Arbeit beigetragen haben, sind am Beginn eines jeden Kapitels aufgeführt oder in der Danksagung erwähnt. Weder habe ich Hilfe eines Promotionsberaters beansprucht, noch haben Dritte geldwerte Leistungen für Arbeiten im Bezug zur vorliegenden Dissertation erhalten. Die vorgelegte Promotionsarbeit wurde zu keinem früheren Zeitpunkt als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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10. CURICULUM VITAE

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PUBLICATIONS

Huber, M., Bont, Z., Fricke J., Brillatz, T., Aziz, Z., Gershenzon, J., Erb, M. A below ground herbivore shapes root defensive chemistry in natural plant populations. In preparation.

Huber, M. Epping, J., Schulze Gronover, C., Fricke, J., Aziz, Z., Brillatz, T., Swyers, M., Köllner, T.G., Vogel, H., Hammerbacher, A., Triebwasser-Freese, D., Robert, C.A.M., Verhoeven, K., Preite, V., Gershenzon, J., Erb, M. A below ground herbivore increases plant fitness upon root herbivore attack. Submitted.

Huber, M., Triebwasser-Freese, D., Reichelt, M., Heiling, S., Paetz, C., Chandran, J., Bartram, S., Schneider, B., Gershenson, J. & Erb, M. (2015) Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (*Taraxacum officinale* agg.). *Phytochemistry***,** In press.

Erb, M., **Huber, M.**, Robert, C.A.M, Ferrieri, A.P., Marchado, RAR, Arce, CCM. (2013). The role of primary and secondary plant metabolites in root-herbivore behaviour, nutrition and physiology. *Advances in Insect Physiology*. (eds S.N. Johnson, I. Hiltpold & T.C.J. Turlings), pp. 53-95. Elsevier Academic Press Inc, San Diego.

Huber, M. and Linder, H. P. (2012). The evolutionary loss of aerenchyma limits both realized and fundamental niche in the Cape reeds (Restionaceae). *Journal of Ecology*. 100 (6): 1338-1348.

PRESENTATIONS

The dark matters: A below ground herbivore drives the evolution of root secondary metabolites in nature (2015). Talk (best talk award). International Max-Planck Research School Symposium, Dornburg (Germany).

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