The role of soluble sugars and starch in plant-herbivore interactions: signaling and ecological consequences

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GENERAL INTRODUCTION

Interesting, although underexplored, plant responses to herbivory are the local and systemic changes in primary metabolites [1-10]. In *N. attenuata*, for example, it has been documented that, upon *M. sexta* attack, the reallocation of carbon from shoots to roots is increased [10]. The newly reallocated carbon is thought to provide the metabolic energy necessary for shoot regrowth when insect pressure decreases and to reduce the nutritional value of leaf tissue for herbivores. The aim of this doctoral thesis was to investigate: i) whether *M. sexta* attack impacts soluble sugars and starch content in the leaves and the roots of *N. attenuata* plants ii) which potential plant signals and regulatory mechanisms are involved iii) whether the impact of *M. sexta* on soluble sugars and starch content affects *M. sexta* growth and/or *N. attenuata* regrowth capacity and iv) the evolution and conservation of the above traits.

Plant responses to insect herbivores

There is an enormous diversity of living organisms on earth. Some studies estimate the existence of three hundred thousand different species of plants and between three to six million species of insects, half of which are herbivorous [11, 12]. Although not all plant-insect interactions are antagonistic, insect herbivory typically has negative effects on plant fitness. Perhaps as consequence, plants and insects have been waging a long coevolutionary battle, with plants evolving strategies to fight off herbivore insects to minimize insect damage and its negative effects, and herbivores evolving strategies to counteract plant defenses to ensure their own survival [13].

Plants recognize insect attack by the perception of elicitors present in insect's oral secretions [14-18]. Upon herbivory perception, plants respond to the attack through the activation of an intricate signaling network that includes mitogen-activated proteinase kinase and calcium signaling, nitric oxide and reactive oxygen species production and the modulation of phytohormonal levels, resulting in a drastic reconfiguration of plant transcriptome, proteome and metabolome [19-21]. This reconfiguration eventually triggers the production of plant defense and tolerance [10, 19, 22]. Induced direct defenses include, for example, the production of toxic secondary metabolites and proteins that aim at deterring or directly

intoxicating insect attackers [23-25]. Indirect defenses include the emission of volatile organic compounds that attract natural enemies or the provision of cues, shelter and food to increase herbivore predation by natural enemies [26, 27]. While much research has focused on the identification and understanding plant traits that confer resistance to insect herbivory, plant tolerance traits have received less attention [10, 28-32]. Tolerance strategies include, for example, the induction of meristematic activity, photosynthesis and the reallocation of resources [28, 31]. The activation of such plant responses requires substantial amounts of energy and nutrients. Therefore, trade-offs between defense, tolerance and growth are expected under limiting conditions. Our knowledge on the signals that govern such trade-offs are still very limited [33].

Impact of herbivory on leaf and root carbohydrates

Increasing evidence suggests that insect attack alters carbon allocation patterns in plants. In some studies, it has been reported that actual and simulated herbivory trigger the remobilization of carbon from damaged and undamaged tissues to stems and roots [1-4, 6-10, 34]. While at the same time, other studies have reported that insect-attacked plants import more carbon into the leaves to support plant defenses [5, 35-39]. Although, these studies suggest that insect attack might reconfigure carbohydrate profiles in leaves and roots, only incipient evidence supports this notion. In *N. attenuata*, for example, soluble sugars −glucose, fructose and sucrose− have been shown to remain unaltered in the roots but decreased rapidly in the leaves upon simulated *M. sexta* attack [10]. While opposite patterns were observed in *Solanum lycopersicum* plants −glucose, fructose and sucrose were depleted in roots and remained unaltered in shoots [7]. It is worthy to mention that the carbohydrate measurements in these studies were carried out within hours of simulated insect attack, and it remains to be determined to what extent these carbohydrate profiles are maintained during a prolonged attack [40].

Regulation of plant carbohydrate pools

Numerous phytohormones have the potential to reprogram carbon allocation patterns and thereby reconfigure carbohydrate profiles in insect-attacked plants. The list of candidate signals includes gibberellins, auxins, cytokinins and jasmonates.

Gibberellins. Gibberellins are plant hormones involved in the regulation of plant growth and development, seed germination, stem elongation, leaf expansion, root growth as well as flower and seed development [41-46]. Gibberellins have a strong influence on photosynthesis and carbohydrate metabolism. For example, changes in photosynthesis-related genes and proteins have been observed in several plant species upon gibberellin homeostasis alterations [47-50]. These changes are often accompanied by an increase in phloem loading, sucrose synthesis, photosynthetic activity, soluble carbohydrates pools and starch degradation [51-57].

Auxins. Auxins are important phytohormones that regulate plant growth and development, plant responses to light, gravity, and to biotic and abiotic stressors [58-60]. Auxins modulate other hormone signaling pathways as salicylic acid, jasmonic acid, abscisic acid, and ethylene and can thereby alter plant defense responses [61-65]. Auxins influence cell wall polysaccharide composition [66-69], regulate sucrolytic enzyme activities [70], sugar transport [71] and sugar signaling [72, 73].

Cytokinins. Cytokinins play important roles in plant growth and development as well as in the regulation of cell proliferation and differentiation, plant senescence, shoot-to-root ratios balance, the transduction of nutritional signals and plant defenses [74-82]. Cytokinin signaling also modulate other plant hormonal signaling pathways including auxins, gibberellins, abscisic acid, jasmonates, ethylene, and salicylic acid [83-89]. Citokinnins influence sink/source relations and invertase activity [90-92], glucose transporters [91, 93], and photosynthesis [94, 95].

Jasmonates. Jasmonates are plant hormones that regulate plant responses to biotic and abiotic stress and influence plant growth and development [96]. Perhaps the best studied regulatory role of jasmonates is the induction of plant defenses upon insect attack [24, 97, 98]. Although they are thought to mainly regulate secondary metabolism, they might also be involved in primary metabolism homeostasis. Exogenous jasmonate application to the leaves reduce leaf starch concentration in poplar trees, stem sugars in tulip and leaf sugars in tobacco and cabbage [1, 99-102], suggesting that jasmonates might be a plant signal that reconfigures carbohydrate profiles upon insect attack. However, for *N. attenuata* plant genotypes with

reduced jasmonate biosynthetic capacity, simulated *M. sexta* herbivory was still capable of triggering the reallocation of photoassimilates from leaves to roots suggesting that jasmonates might not influence carbon allocation patterns or carbohydrate profiles [10].

Given that the above mentioned phytohormones are directly or indirectly involved in the regulation of plant primary metabolism, the manipulation of their signaling cascades through genetic transformation and/or pharmacological treatments might be a powerful tool to shed light on whether they influence carbon allocation patterns and/or carbohydrate pools in *M. sexta*-attacked plants.

The role of leaf carbohydrates in insect nutrition

Plants, as autotrophic organisms, utilize solar energy to fix carbon dioxide into carbohydrates as their primary source of energy that fuels their growth, development and reproduction. Insect herbivores, in turn, as heterotrophic organisms, rely on plant tissue consumption to obtain carbohydrates and other nutrients for similar purposes. The induced reallocation of carbon from shoots to roots observed in insect-attacked plants might alter carbohydrate pools in leaves and thereby affect herbivore consumers.

Many studies have addressed the question of how carbohydrate content in an insect's diet influences their growth and performance. The ratio between carbohydrates and protein seems to determine insect growth in a non-linear fashion, with sub-optimal ratios leading to a rapid reduction in growth rates [103-105]. Furthermore, protein and carbohydrate ratios influence the toxicity of secondary metabolites [106-108]. While these studies have unraveled fascinating aspects of insect nutrition, most of them have been carried out in chemically defined artificial environments. By contrast, plants as food sources in nature are inherently dynamic and variable. Insect attack alters nitrogen and carbon dynamics [1, 6, 35, 37], which often results in dramatic changes in primary and secondary metabolite pools [1, 7, 10, 100, 109]. In addition, primary and secondary metabolite pools vary diurnally in a developmental stage-dependent manner [110-113]. Combining the manipulation of carbohydrates *in vitro* and *in planta* would therefore be a promising approach to understand whether insect attack impacts plant carbohydrates and might, in turn, affect insect growth in a plant secondary chemistry context.

The role of root carbohydrates in tolerance to aboveground herbivory

Herbivory induced-reallocation of photoassimilates towards roots and stems observed in several plant species has been proposed to act as a putative tolerance mechanism by which root carbon pools are enriched, safeguarding plant resources necessary for future regrowth [10]. This hypothesis is supported by the fact that both the importance of root nonstructural carbohydrates and the ability of plants to reallocate resources from roots to shoots to support the regrowth of aboveground tissues upon defoliation are widely recognized [3, 114-123]. Any plant stressor that compromises the acquisition, storage, utilization and/or partitioning of plant carbohydrates might thereby affect the regrowth capacity of plants since the regrowth of new vegetative and reproductive tissues upon defoliation requires substantial quantities of both nutrients and carbohydrates [117, 124, 125].

The herbivore-induced reallocation of photoassimilates might consequently empower roots by providing them of more metabolic energy, which may result in an improved capacity of roots to regrow new shoots. Nevertheless, the newly acquired carbon might be used to support other root functions, such as sustaining growth, foraging and nutrient acquisition, maintaining symbiotic interactions with soil microbes, or the biosynthesis of defensive secondary metabolites [5, 8, 39, 126-133]. Strikingly, an enrichment of root carbohydrate pools upon insect attack has not been demonstrated so far. On the contrary, evidence suggests that root carbohydrates may be depleted, indicating that a reallocation of carbon to roots might not directly improve tolerance to herbivory *per se* [7]. Given these evident contradictions, an evaluation of root carbon dynamics and regrowth capacity followed insect attack in plant genotypes that differ in their carbon reallocation patterns would help to better understand the link between herbivory-induced reallocation of carbon and tolerance to foliar herbivory.

Evolution of tolerance traits to aboveground herbivory

Plants are constantly being challenged by herbivores. Perhaps as consequences, they have evolved a vast arsenal of divergent strategies to ensure the production of offspring. For example, among milkweeds (*Asclepias* spp.), tolerance to herbivory (regrowth) appears to have increased during the diversification of the genus, while resistance traits such as

cardenolides, latex and trichomes declined [134]. The prevalence of several divergent defensive strategies might be partially explained by the fact that their efficiency depends on the target organism [135-139]. For example, a single amino acid substitution in the α-subunit of the Na^+/K^+-ATPase gene is sufficient to confer cardenolide tolerance in leaf beetles [140], indicating that some plant defenses may lose their protective effect in nature rapidly. It is under these circumstances that other defensive strategies such as tolerance and regrowth may become particularly important [141]. The identification of the mechanisms that govern plant tolerance and their potential evolutive history are of crucial importance if we are to understand the mechanisms that drive the existence of the enormous diversity of living organisms on earth [11, 12, 142-146].

Several studies have stated the importance of non-structural root carbohydrates and the reallocation of resources from roots to shoots to support the regrowth of aboveground tissues upon defoliation [3, 114-123, 147], indicating that the bunkering of carbon in the roots might confer tolerance to herbivores and therefore an advantage in the face of herbivores that are well-adapted to chemical defenses [148]. Contrary to the degree of phylogenetic conservation of induced defenses [149-154], less studies have examined the evolution of tolerance in a phylogenetic framework [134]. Understanding the link between non-structural carbohydrate dynamics and tolerance to insect attack in several related plant species might help us to better understand the evolutive importance of plant defensive syndromes [155].

The interaction between *Manduca sexta* and solanaceous plants as a study system

Manduca sexta is a lepidopteran moth that belongs to the family Sphingidae. The larvae feed from various plants of the family Solanaceae, principally from members of the genera *Solanum*, *Nicotiana* and *Datura*. It has been used as a model organism to unravel the mechanisms of insect olfaction [156-158], host selection and oviposition [159], the effect of plant secondary chemical makeup on its growth and development [14, 98, 110, 160-163] and insect nutritional requirements [164-167].

The Solanaceae are a large plant family consisting of around 98 genera and 2700 species [168]. The family members exhibit highly diverse life styles from annual and perennial herbs to vines, lianas, epiphytes, shrubs, and trees. The family has a worldwide distribution, and the

greatest diversity in species is found in South and Central America. Apart from being widely cultivated as crops, some species are extensively used in research as it is the case of tomato (*Lycopersicum esculentum*), tobacco *(Nicotiana tabacum)* and coyote tobacco (*Nicotiana attenuata*).

Perhaps one of the best studied plants to understand plant defensive strategies at the molecular and ecological level is *N. attenuata*. *N. attenuata* is a wild annual tobacco native to the Southwestern USA that germinates after fires from long-lived seed banks [169-171]. The short generation time, the availability of a vast amount of transgenic genotypes, and a well-established genetic transformation protocol make *N. attenuata* a promising model to study plant defensive strategies and plant-herbivore interactions. .

Experimental approach

Impact of *M. sexta* attack on soluble sugars and starch content. To evaluate the impact of *M. sexta* attack on plant carbohydrates, I adapted and optimized two already established protocols to quantify soluble sugars (glucose, fructose and sucrose) and starch in plant tissue [172, 173], and evaluated carbohydrate dynamics in *M. sexta* attacked plants under different developmental stages and times of day.

Potential plant signals and regulatory mechanisms of *M. sexta*-induced reconfiguration of plant carbohydrates. To investigate the potential signals that reconfigure plant carbohydrates profiles in *M. sexta*-attacked plants, I quantified soluble sugars and starch in plant genotypes impaired in jasmonate production (irAOC) and perception (irCOI1) upon *M. sexta* attack. To understand the potential mechanisms by which jasmonates deplete soluble sugars, I measured chlorophyll content and the activities of carbohydrate metabolizing enzymes in jasmonate perception and/or biosynthesis-impaired *N. attenuata* plants.

Ecological consequences of carbohydrate depletion in *M. sexta*-attacked plants. To understand the potential ecological consequences of plant carbohydrates depletion, I exploited genetic manipulation, natural genetic variability, micrografting and *in vitro* complementation techniques to manipulate carbohydrate pools and evaluated plant's capacity

to regrow (as a proxy for tolerance) and to suppress *M. sexta* growth (as a proxy for resistance).

The evolution and conservation of *M. sexta*-induced changes in root carbohydrates and tolerance. To investigate the evolutionary history and phylogenetic conservation of herbivory-induced tolerance, I evaluated the regrowth capacity and root carbohydrate dynamics upon *M. sexta* attack in eight solanaceous species covering four genera −*Petunia*, *Datura*, *Nicotiana* and *Solanum*− and estimated the phylogenetic signal of these two traits using different evolutionary models.

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Manuscript I.

Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling

2013

Ricardo A.R. Machado, Abigail P. Ferrieri, Christelle A.M. Robert, Gaétan Glauser, Mario Kallenbach, Ian T. Baldwin, Matthias Erb

In this study, we found that *M. sexta* attack depletes non-structural root carbohydrates and reduces the regrowth capacity of *N. attenuata* plants. By using micrografting techniques and combining genetic and natural variation, we identified leaf-derived jasmonates as the major regulators of the observed carbohydrate and regrowth phenotypes. We concluded that *M. sexta*-induced jasmonates constrain tolerance to herbivory, which may result in divergent tolerance strategies in nature.

I designed and carried out all the experiments, analyzed data and wrote the manuscript. AF carried out experiments and analyzed data. CR, GG and MK supported the analytical measurements. IT designed experiments and contributed to writing the manuscript. ME conceived the study, designed and carried out experiments, analyzed data and wrote the manuscript. All coauthors were involved in the discussion of results. All co-authors read and approved the final version of the manuscript.

Manuscript II

Jasmonate-dependent depletion of soluble sugars compromises plant resistance to *Manduca sexta*

2015

Ricardo A.R. Machado, Carla C.M. Arce, Abigail P. Ferrieri, Ian T. Baldwin, Matthias Erb

New Phytologist (in press).

In this study, we demonstrated that constitutive and *M. sexta*-induced jasmonates reduce glucose and fructose concentrations in the leaves of *Nicotiana attenuata*. We identified the inhibition of sucrolytic enzymes as the potential mechanism by which jasmonates reduce leaf carbohydrates. We manipulated sugar levels *in vivo* through genetic engineering and *in vitro* through complementation with synthetic sugars and found that JA-dependent sugar depletion benefits *M. sexta* growth, and thereby constrains JA-mediated plant resistance.

I designed and carried out all the experiments, analyzed data and wrote the manuscript. CA and AF carried out experiments and analyzed data. IT conceived the study, designed experiments and contributed to writing the manuscript. ME conceived the study, designed experiments, analyzed data and wrote the manuscript. All coauthors were involved in the discussion of results. All co-authors read and approved the final version of the manuscript.

Manuscript III

Rapid evolution and strong correlation between herbivory-induced root carbohydrate responses and defoliation tolerance among eight solanaceous species

Ricardo A.R. Machado, Wenwu Zhou, Abigail P. Ferrieri, Carla C.M. Arce, Ian T. Baldwin, Shuqing Xu, and Matthias Erb

In this study, we found that, *M. sexta*-induced changes in root carbohydrates pools are strongly correlated with regrowth capacity among eight solanaceous species: plants species that suffered from carbohydrate depletion upon simulated *M. sexta* herbivory regrew less, while species that maintained root carbohydrate homeostasis tolerated herbivore attack. Moreover, we did not find a phylogenetic signal for the evolution of these two traits, indicating that induced tolerance responses can evolve rapidly.

I designed and carried out all the experiments, analyzed the data and wrote the manuscript. WZ, AF and CA carried out experiments and contributed to writing the manuscript. IT analyzed data and contributed to writing the manuscript. ME conceived the study, designed experiments, analyzed data and wrote the manuscript. SX designed experiments, analyzed data and wrote the manuscript. All co-authors read and approved the final version of the manuscript.

Manuscript I

Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling

2013

Ricardo A.R. Machado, Abigail P. Ferrieri, Christelle A.M. Robert, Gaétan Glauser, Mario Kallenbach, Ian T. Baldwin, Matthias Erb

Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling

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Summary

• Herbivore attack leads to resource conflicts between plant defensive strategies. Photoassimilates are required for defensive compounds and carbon storage below ground and may therefore be depleted or enriched in the roots of herbivore-defoliated plants. The potential role of belowground tissues as mediators of induced tolerance–defense trade-offs is unknown.

• We evaluated signaling and carbohydrate dynamics in the roots of *Nicotiana attenuata* following *Manduca sexta* attack. Experimental and natural genetic variability was exploited to link the observed metabolite patterns to plant tolerance and resistance.

• Leaf-herbivore attack decreased sugar and starch concentrations in the roots and reduced regrowth from the rootstock and flower production in the glasshouse and the field. Leaf-derived jasmonates were identified as major regulators of this root-mediated resource-based trade-off: lower jasmonate levels were associated with decreased defense, increased carbohydrate levels and improved regrowth from the rootstock. Application and transport inhibition experiments, in combination with silencing of the sucrose non-fermenting (SNF) -related kinase GAL83, indicated that auxins may act as additional signals that regulate regrowth patterns.

• In conclusion, our study shows that the ability to mobilize defenses has a hidden resource-based cost below ground that constrains defoliation tolerance. Jasmonate- and auxin-dependent mechanisms may lead to divergent defensive plant strategies against herbivores in nature.

Introduction

Plants have evolved mechanisms that allow them to maximize protection against herbivorous insects whilst minimizing deviations from optimal growth and fitness (Meldau *et al.*, 2012). Defensive metabolites and proteins, for instance, have been found to accumulate on herbivore attack (Green & Ryan, 1972; Baldwin *et al.*, 1998; Zhu-Salzman *et al.*, 2008; Glauser *et al.*, 2011). However, herbivores can adapt to toxic and deterrent compounds (Zangerl & Berenbaum, 1990; Berenbaum & Zangerl, 1994; Lindigkeit *et al.*, 1997; Mao *et al.*, 2006). Possibly as a consequence, plants have evolved alternative strategies that may reduce herbivore-imposed fitness costs even in the face of resistant attackers. The induction of meristematic activity, photosynthesis and reallocation of resources, for instance, may increase plant tolerance and help attacked individuals to grow and produce offspring even under heavy herbivore pressure (reviewed by Strauss & Agrawal, 1999; Tiffin, 2000; Schwachtje & Baldwin, 2008). Recent evidence suggests that many plants employ mixed tolerance–resistance strategies (Leimu &

Koricheva, 2006; Núñez-Farfán *et al.*, 2007; Carmona & Fornoni, 2013), allowing them to cope with the spatially and temporally diverse herbivore communities which they face in nature.

A growing body of evidence suggests that plants respond to actual and simulated herbivory by increasing the remobilization of resources from damaged and undamaged tissues to stems and roots, a process termed 'herbivory-induced resource sequestration' (Dyer *et al.*, 1991; Briske *et al.*, 1996; Holland *et al.*, 1996; Babst *et al.*, 2005, 2008; Bazot *et al.*, 2005; Schwachtje *et al.*, 2006; Kaplan *et al.*, 2008; Gómez *et al.*, 2010, 2012). The molecular basis of this phenomenon has been studied in *Nicotiana attenuata*. Schwachtje *et al.* (2006) found that the β-subunit of the sucrose non-fermenting-related kinase (SnRK1), GAL83, is down-regulated in source leaves within hours following simulated attack by *Manduca sexta*. GAL83-silenced plants constitutively allocated 10% more photoassimilates to the roots and had a prolonged flowering period on water deprivation (Schwachtje *et al.*, 2006). Although these results speak in favor of the hypothesis that induced resource sequestration may be part of a plant's

tolerance response, other studies report that attacked plants import more resources into the leaves to support plant defenses (Arnold & Schultz, 2002; Arnold *et al.*, 2004; Appel *et al.*, 2012; Ferrieri *et al.*, 2012, 2013). Indeed, resource reallocation does not necessarily result in increased carbohydrate pools (Schwachtje *et al.*, 2006), as the translocated assimilates may be used for other processes, such as exudation into the rhizosphere (Holland *et al.*, 1996; Frost & Hunter, 2008), respiration (Clayton *et al.*, 2010) and the synthesis of defensive metabolites (Shoji *et al.*, 2000). It is noteworthy in this context that no increase in root primary metabolite pools following leaf attack has been demonstrated so far in any of the above plant systems (see, for example, Schwachtje *et al.*, 2006). On the contrary, a recent study in tomato has shown that *M. sexta* attack triggers carbon (C) reallocation to the roots and, at the same time, leads to a depletion of carbohydrates (Gómez *et al.*, 2012). Clearly, the connection between resource reallocation and tolerance deserves more attention if we are to understand the role of primary metabolism in plant defensive strategies.

Jasmonates (JA) are important regulators of plant resistance (reviewed by Liechti & Farmer, 2002) and have been implicated in systemic signaling between leaves and roots (Zhang & Baldwin, 1997). On herbivore attack, JA levels increase in local and systemic tissues and trigger the biosynthesis of many defensive metabolites (Howe & Jander, 2008), including, for instance, nicotine in *N. attenuata* (Steppuhn *et al.*, 2004). JA deficiency results in a strong decrease in induced defenses and renders plants susceptible to a wide range of herbivores (Paschold *et al.*, 2007; Kallenbach *et al.*, 2012). At the same time, JA reduce plant growth and defoliation tolerance, suggesting that they mediate herbivore-induced trade-offs between resistance and tolerance (Zavala *et al.*, 2006). Two explanations have been proposed for this phenomenon. First, JA may antagonize gibberellin (GA), cytokinin and auxin signaling, which may reduce cell elongation and growth directly (Chen *et al.*, 2011; Yang *et al.*, 2012). Second, JA-dependent defenses may deplete plant resources and thereby limit plant growth. The role of JA in tolerance–resistance trade-offs is complicated by the finding that the exogenous application of methyl jasmonate (MeJA) induces C reallocation to the roots of poplar and *Arabidopsis thaliana* (Babst *et al.*, 2005, 2008; Ferrieri *et al.*, 2013) and nitrogen in tomato (Gómez *et al.*, 2010), whereas changes in C reallocation were found to be independent of JA in *N. attenuata* (Schwachtje *et al.*, 2006). As JA mediate systemic signals that reprogram both primary and secondary metabolism in the leaves and the roots, detailed mechanistic studies will be crucial to evaluate the role of JA signaling in defense and tolerance.

We aimed to obtain an understanding of the role of plant roots in tolerance–defense trade-offs in *N. attenuata*. This plant responds to herbivory by the synthesis of alkaloids in the roots which are then transported to the leaves for defense (Baldwin *et al.*, 1997). At the same time, root allocation of photoassimilates increases (Schwachtje *et al.*, 2006), making the species a suitable choice to investigate the role of roots in tolerance and resistance. In the current study, we specifically focused on whether the enrichment or depletion of photoassimilates in the

roots following leaf-herbivore attack alters the regrowth capacity of *N. attenuata* from the roots. To this end, we used genetically engineered *N. attenuata* lines and a set of diverse field-collected genotypes which vary in their defensive strategies. To understand the metabolic processes that govern tolerance–defense trade-offs, we measured phytohormone, defensive metabolite and major carbohydrate levels in the leaves and roots of *N. attenuata*. Our results provide a comprehensive picture of the impact of leaf induction on the metabolism and regrowth capacity of plants as a function of herbivore-induced phytohormone signaling across experimental and natural genetic variation, and suggest that roots play an important and possibly underestimated role in tolerance–defense trade-offs.

Materials and Methods

Plant material and planting conditions

The following plant material was used in the present study: wild-type *N. attenuata* Torr. Ex. Watson plants of the 31st inbred generation derived from seeds collected at the Desert Inn Ranch in Utah, UT, USA in 1988; an anti-sense line silenced in the expression of the sucrose non-fermenting 1 (SNF1)-related kinase GAL83 (asGAL83; Schwachtje *et al.*, 2006); a JA-deficient inverted repeat allene oxide cyclase line (irAOC; Kallenbach *et al.*, 2012); a transgenic control line transformed with an empty vector construct (EV, line A-03-9-1); a set of 120 ecotypes grown from seeds that were collected over 20 yr from different locations in the Great Basin Desert (UT, USA). Before planting, all seeds were surface sterilized and germinated on Gamborg's B5 medium, as described by Krügel *et al.* (2002). For glasshouse experiments, the seedlings were transferred to Teku pots (Pöppelmann GmbH & Co. KG, Lohne, Germany) 10 d after germination and, 10–12 d later, the seedlings were planted into 1-l pots filled with washed sand. Plants were grown as described by Krügel *et al.* (2002). For the field experiment, seeds of the transformed *N. attenuata* lines were imported under APHIS notification number 07-341-101n and experiments were conducted under notification number 06-242-02r. Plants were grown as described by Schuman *et al.* (2012).

Regrowth capacity from the roots following *M. sexta* attack

To understand how *Manduca sexta* (Linnaeus) attack affects the regrowth capacity of *N. attenuata* from the roots, we placed six neonate larvae on wild-type plants and let them feed freely for 6 d ($n = 30$). Non-infested plants ($n = 30$) were used as controls. To specifically investigate the role of the roots in supplying resources for leaf growth, all stems and leaves were removed (hereafter referred to as 'shoot removal'), together with the larvae, 6 d after infestation, leaving only the root system and the lowest 0.5 cm of the stalk for regrowth (hereafter referred to as 'remaining shoot'). Complete shoot removal by browsing mammals can occur under natural conditions (Baldwin, 1998). The regrowth and fitness of the regrowing plants were monitored by

determining the average rosette diameter, branch length and number of flowers. All these parameters have been found to be positively correlated with total seed production (Glawe *et al.*, 2003). To determine whether the presence of the main stem affects the regrowth capacity following *M. sexta* attack, we induced plants by simulated herbivory (W + OS) and either removed the complete shoot or only the leaves. Regrowth was then measured as described above (*n* = 5). Herbivory was simulated by wounding (W) three leaves with a pattern wheel to produce three rows on each side of the midvein and treating the wounds immediately with 10 µl of a 1 : 5-diluted *M. sexta* oral secretions (OS) solution. This treatment mimics the defense induction triggered by *M. sexta* without removing extensive amounts of leaf-tissue (Qu *et al.*, 2004). Every 48 h, three leaves per plant were treated over a total of 6 d, resulting in nine treated leaves per plant.

Regrowth capacity from the roots in the field

To evaluate the consequences of leaf induction for the regrowth capacity of *N. attenuata* under natural conditions, we conducted a field experiment in the Lytle Ranch Preserve (UT, USA). EV and JA-deficient irAOC plants were planted on 14 May 2012 in quadruplets. Once the plants reached the rosette stage, one EV and one irAOC plant per quadruplet were subjected to simulated herbivory (W + OS) as described above (*n* = 14). Control plants were left untreated. One day after the last treatment, the shoots were removed as described above. Nine days after shoot removal, the rosette diameters of the regrowing shoots were measured. Plants that died during the regrowing phase were removed from the analysis (EV W + OS, 2; EV control, 2; irAOC W + OS, 5; irAOC control, 4).

Resource mobilization for regrowth

To investigate whether *N. attenuata* roots can mobilize resources to regrow shoots in the absence of residual photosynthetic activity, we induced plants as described above and removed their shoots 6 d after the first treatment. Half of the plants were harvested immediately after shoot removal, and their root and remaining shoot biomass was determined (*n* = 15). The other half was left to regrow in total darkness for 9 d, after which their root, remaining shoot and regrown leaf biomasses were determined (*n* = 15). One plant was excluded from the analysis as a result of pathogen contamination of the rootstock.

Effect of induction on regrowth capacity under water stress

Under natural conditions, soil desiccation is thought to function as an abiotic signal that is used by *N. attenuata* to mobilize its remaining root resources for a final reproductive effort at the end of the growing season (Schwachtje *et al.*, 2006). However, water stress also induces C reallocation to the roots (Geiger & Servaites, 1991). To determine whether soil moisture characteristics affect the regrowth capacity of *N. attenuata* from the roots following leaf induction, we treated EV and JA-deficient irAOC

plants with wounding and *M. sexta* oral secretions (W + OS) or wounding and water (W + W) as described above, and gradually reduced the water supply during the 6 d of induction treatments. For this, pots of control plants were weighed daily, and the weight loss caused by evaporation and transpiration was compensated by watering. Water-stressed plants only received a fraction of the water they had lost: over the 6 d of water stress, the amount of resupplied water was reduced from 90% to 50%. After shoot removal, all the plants were again watered normally (*n* = 12).

Regrowth capacity of JA-deficient and allocation-altered transgenic plants

An additional experiment was conducted to evaluate the contribution of JA signaling and the SNF1-related kinase GAL83 to the regrowth capacity of *N. attenuata* in detail. asGAL83 plants have been shown to constitutively allocate more carbohydrates to the roots (Schwachtje *et al.*, 2006). Thus, we hypothesized that these plants would have an increased capacity to regrow from the rootstock. Plants transformed with an empty vector (EV) were used as controls (*n* = 12). Plants were induced by wounding and applications of *M. sexta* oral secretions or water as described above. Control plants were left intact. In addition, irAOC plants were complemented with MeJA to restore JA signaling. For this, 75 µg of MeJA in lanolin paste were applied to one leaf every other day for 6 d. Following the different treatments, the shoots of all plants were removed and the regrowth capacity was monitored as described above.

The role of leaf- and root-derived JA as regulators of regrowth

Root- and leaf-derived JA are likely to have different functions in plant stress responses, growth and development (reviewed by Wasternack & Hause, 2013). To determine the role of leaf- and root-derived JA on the regrowth responses of *N. attenuata*, we monitored the regrowth capacity after leaf herbivory of different JA-deficient chimeric plants. The chimeric plants were created by micrografting according to the procedures described in Fragoso *et al.* (2011). The following micrografting combinations were evaluated: EV/EV, plants with intact JA signaling (*n* = 10); EV/irAOC, plants silenced in root JA production (*n* = 8); irAOC/irAOC, plants silenced in both root and leaf JA production (*n* = 10). The different grafting combinations were treated and monitored for regrowth as described above.

Herbivory-induced reconfiguration of primary and secondary metabolism in leaves and roots

To determine whether leaf herbivory reconfigures leaf and root primary and secondary metabolism, we treated leaves of EV, asGAL83 and irAOC plants as described above, harvested the shoots and roots 6 d after the first treatment and 6 d after the start of regrowth, and measured nicotine, soluble sugars and starch in the different tissues (*n* = 3). To determine the

concentration of nicotine, previously described procedures were followed (Keinänen *et al.*, 2001). Soluble sugars were extracted from plant tissue using 80% (v/v) ethanol, followed by an incubation step (10 min at 78°C) with constant shaking at 800 rpm. Pellets were re-extracted twice with 50% (v/v) ethanol (10 min at 78°C with constant shaking at 800 rpm). Supernatants from all extraction steps were pooled together, and sucrose, glucose and fructose were quantified as described by Velterop & Vos (2001). The remaining pellets were used for an enzymatic determination of starch content as described previously (Smith & Zeeman, 2006).

Phytohormone measurements

To find possible systemic signals that trigger the herbivory-induced reduction in regrowth from the rootstock, we evaluated the induction of phytohormones in response to *M. sexta* attack. For this, three rosette leaves were wounded and immediately treated with 30 µl of a 1:5 (v/v) milliQ water-diluted *M. sexta* oral secretions solution (W + OS). Wounded and water-treated plants (W + W), as well as intact plants, were used as controls. Roots and leaves were harvested 30 min, 1 and 3 h after treatment. The extraction and quantification of phytohormones were carried out as described by Glauser *et al.* (2012) with some modifications (see Supporting Information Note S1 for details).

JA signaling and regrowth in natural populations

To examine potential trade-offs between JA signaling and the regrowth capacity in natural populations of *N. attenuata*, we analyzed the regrowth capacity and herbivory-induced JA production of 120 different individuals originating from different natural accessions. To quantify the herbivore-induced jasmonic acid production, the S1 leaf was wounded by rolling a fabric pattern wheel three times on one side of the midvein. The wounds were immediately treated with 10 µl of a 1:5 (v/v) milliQ water-diluted *M. sexta* oral secretions solution. The S1 leaf is the youngest fully developed leaf and highly responsive to insect attack (Zavala & Baldwin, 2004). After 60 min, the tissue of the treated leaf side was collected and immediately frozen in liquid nitrogen. JA was quantified as described previously (Stitz *et al.*, 2011). To phenotype the regrowth capacity, the shoots of all plants were removed at the end of the flowering period and the regrowth capacity was measured as described above. This experiment was embedded in a large sampling campaign that measured a number of phenotypic traits in different *N. attenuata* accessions. Additional results from this experiment will be published elsewhere.

The role of GAs as regulators of regrowth

To determine the possible role of GAs in the observed regrowth effects, we induced plants as described above and monitored the internode elongation of regrowing branches. Internode elongation has been used as a downstream marker to determine JA-induced alterations in GA signaling (Yang *et al.*, 2012). The average internode length was determined by measuring the length of the longest branch and counting its number of internodes. In addition, we determined the number of branches and total length of the branches (cumulative branching; $n = 10$).

The role of auxins as regulators of regrowth

To determine the importance of auxin (indole-3-acetic acid, IAA) on the regulation of root responses to insect attack, we applied either 0.7 mg IAA or 0.3 mg *trans*-cinnamic acid (TCA) dissolved in lanolin paste to the petioles of *N. attenuata* immediately after W + OS elicitation. TCA has been shown to alter auxin polar transport (An *et al.*, 1999). W + OS elicitation was carried out as described above. The procedure was carried out every other day for 6 d. Non-elicited plants and plants treated with lanolin paste without IAA or TCA were used as controls ($n = 10$). The regrowth capacity after shoot removal of asGAL83, irAOC and EV plants after the mentioned treatments was monitored as described previously. IAA and TCA concentrations were chosen following previous studies (Baldwin *et al.*, 1997; An *et al.*, 1999).

Chlorophyll content of induced shoots

To determine whether the decrease in soluble sugar contents following leaf herbivory could be explained by a reduction in chlorophyll, we determined chlorophyll concentrations in induced leaves of EV and irAOC plants. Treatments were performed as described above ($n = 17$). The chlorophyll content was quantified using a portable chlorophyll meter (SPAD 502; Konica Minolta, Tokyo, Japan).

Herbivore resistance of regrowing leaves

To evaluate whether regrowing leaves of previously infested plants were more resistant to subsequent attack, we measured *M. sexta* larval performance on regrowing leaves of previously treated plants. EV and irAOC plants were treated as described above ($n = 17$), and one *M. sexta* neonate per plant was placed on the regrowing leaves 6 d after shoot removal and left to feed freely for 10 d. Larval mass was determined using a microbalance (Sartorius TE214S; Data Weighing Systems Inc., Elk Grove, IL, USA).

Statistical analyses

All statistical tests were carried out with Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA) using analysis of variance (ANOVA). Levene's and Shapiro–Wilk tests were applied to determine error variance and normality. Fitness parameters to determine the effect of *M. sexta* attack on regrowth capacity were tested in a one-way ANOVA and Dunn's *post-hoc* tests. Fitness parameters of regrowing EV, irAOC and asGAL83 plants, as well as metabolite reconfiguration before and after shoot removal, were tested individually for each genotype in a one-way ANOVA and Holm–Sidak *post-hoc* tests. A non-

parametric Kruskal–Wallis one-way ANOVA on ranks was carried out for variables that did not conform to normality. Two-way ANOVA and Holm–Sidak *post-hoc* tests, with treatment and genotype as factors, were used to determine the effect of simulated *M. sexta* attack on regrowth capacity in the field, fitness changes of regrowing grafted plants, chlorophyll contents after herbivore attack, larval performance on regrowing leaves, the contribution of stems to regrowth capacity and the role of GAs in regrowth. Two-way ANOVA and Holm–Sidak *post-hoc* tests were carried out to assess herbivory-induced phytohormone levels, with time and treatment as factors, for each time point individually, as well as to evaluate the role of auxin in regrowth capacity, with treatment and OS elicitation as factors. Fitness parameters to determine the effect of simulated *M. sexta* attack (W + OS) and soil moisture characteristics on regrowth capacity were assessed in a three-way ANOVA and Holm–Sidak *post-hoc* tests, with treatment, water condition and genotype as factors. Correlations between JA signaling and regrowth capacity were tested using Pearson product moment tests.

Results

M. sexta attack constrains *N. attenuata* regrowth and fitness in the glasshouse and the field

Leaf-herbivore-induced C reallocation to the roots has been suggested as a potential tolerance mechanism in *N. attenuata* (Schwachtje *et al.*, 2006). However, we observed that *M. sexta* attack reduces regrowth from the roots. Shoots regrowing from rootstocks of *M. sexta*-attacked plants had smaller rosettes, shorter branches and produced fewer flowers than regrowing shoots of control plants (Fig. 1a–e). Similar effects were observed for wounded plants treated with *M. sexta* oral secretions (W + OS) in the field (Fig. 1f–h). In contrast with EV plants, no differences were observed between the W + OS and control treatment in JA-deficient irAOC plants (Fig. 1g), suggesting that JA-dependent induced defenses might constrain regrowth.

Caterpillars typically only remove the leaves and rarely feed from stems. To confirm the patterns of the somewhat artificial shoot removal treatment (which aimed to specifically evaluate the contribution of rootstocks to regrowth) in a more realistic set-up, we defoliated plants without removing the stem. Plants with intact stems produced significantly more flowers at the end of the flowering period than plants that were regrowing from the rootstocks (Fig. S1). However, W + OS induction decreased flower production of regrowing plants independent of the type of shoot removal (Fig. S1), suggesting that our shoot removal treatment yields biologically meaningful data.

Roots supply resources for leaf regrowth

To understand whether resources can be mobilized from the roots to support shoot regrowth, we performed an experiment in complete darkness. This enabled us to determine shoot biomass accumulation in the absence of any photosynthetic activity. We found that *N. attenuata* plants were able to regrow from the
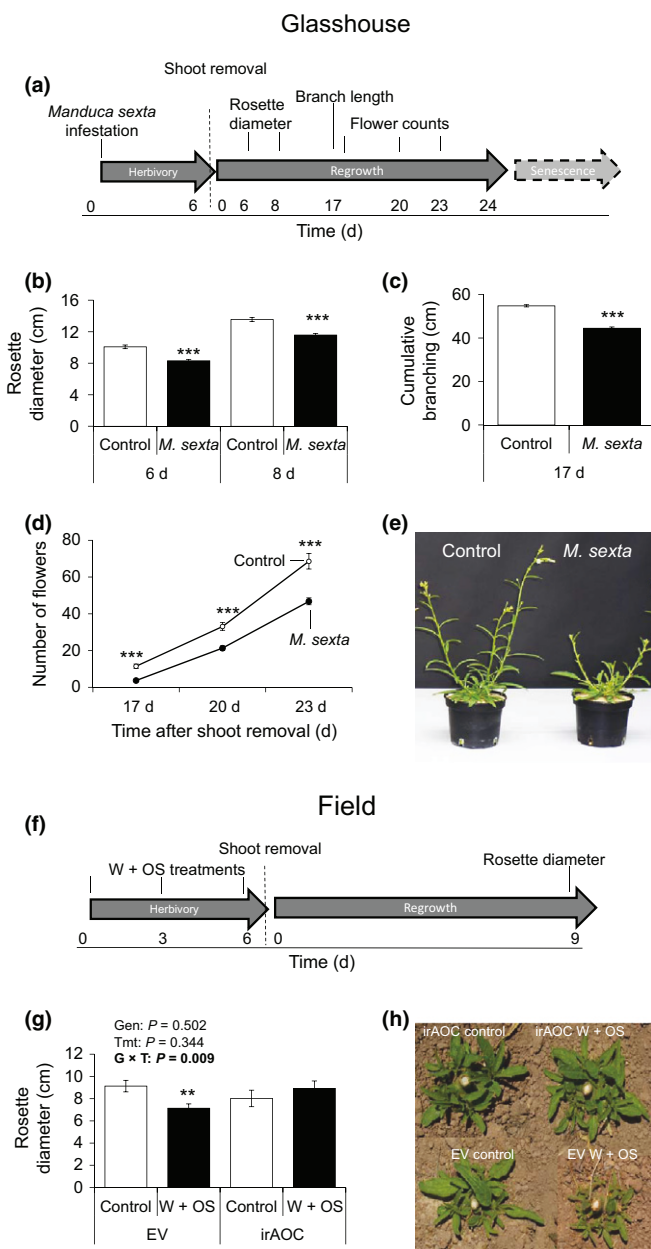

Fig. 1 Simulated and actual *Manduca sexta* herbivory reduces regrowth from the rootstock in the glasshouse and the field. (a) Timing of the glasshouse experiment, (b) average ($\pm$ SE) rosette diameter, (c) cumulative branching, (d) number of flowers, (e) representative pictures of regrowing plants, (f) timing of the field experiment, (g) rosette diameter of empty vector (EV) and jasmonate-deficient irAOC plants, (h) representative pictures of regrowing plants in the field. (b,c) asterisks indicate significant differences among treatments within time points (***, $P < 0.001$). Results of two-way ANOVAs are shown for (g). Gen, genotype; Tmt, treatment; G $\times$ T, genotype by treatment; OS, oral secretions; W, wounding. Asterisks indicate significant differences among treatments within genotypes (**, $P < 0.01$).

rootstock in complete darkness. Both the remaining shoot and the regrowing leaves gained a significant amount of biomass (Fig. S2b,c). At the same time, root biomass was reduced, implying resource remobilization from belowground tissues (Fig. S2a). The aboveground plant parts accumulated *c.* 30 mg of dry

matter, whereas the roots lost 100 mg. W + OS pretreatment reduced the biomass of the regrowing leaves by > 50%, whereas the reduction in root biomass was not altered significantly (Fig. S2a,c). Six days of W + OS treatment did not reduce root biomass compared with untreated controls (Fig. S2a), suggesting that W + OS treatment reduced root quality or conversion efficiency to support leaf regrowth.

Water stress improves plant regrowth capacity in a herbivore-independent manner

Water shortage is an important determinant of a plant's capacity to regrow as it changes C reallocation patterns (Geiger & Servaites, 1991) and co-occurs with herbivore attack of *N. attenuata* in nature (Schwachtje *et al.*, 2006). We found that both EV and irAOC plants that were subjected to water stress for 6 d regrew significantly better from the rootstock. However, water stress did not affect the herbivory-induced reduction of regrowth (Fig. 2). Although W + OS treatment of EV plants reduced rosette diameter, branch length and number of flowers of regrowing plants, wounding of the leaves with a pattern wheel and application of water to the wounds (W + W) did not affect either branch length or flower production (Fig. 2), demonstrating that herbivore-associated molecular patterns are important to trigger the plant response. Again, irAOC plants did not display any significant changes in regrowth following either W + W or W + OS treatment.

Herbivore-induced constraints in regrowth are GAL83 and JA dependent

An earlier study demonstrated that the herbivore-induced down-regulation of GAL83 increases C allocation to the roots and improves flower production in herbivore-attacked *N. attenuata* plants in a JA-independent manner (Schwachtje *et al.*, 2006). We therefore tested whether silencing this gene changes the regrowth patterns from the rootstock after simulated herbivory. Indeed, in contrast with EV plants, asGAL83 plants did not suffer from any fitness consequences on W + OS treatment (Fig. 3). To further understand the role of JA in the system, we also included irAOC plants and MeJA applications. Contrary to W + W and W + OS, MeJA treatment reduced regrowth in irAOC plants (Fig. 3).

Leaf- and root-derived JA constrain herbivore-induced regrowth

JA have been shown to regulate plant stress responses, growth and development (reviewed by Wasternack & Hause, 2013) in a tissue-specific manner (Nalam *et al.*, 2012). By employing a grafting protocol to create chimeric plants that were either silenced in root (EV/irAOC) or root and leaf (irAOC/irAOC) JA production, we investigated the contributions of leaf- and root-derived JA to regrowth. Similar to our previous observations on non-grafted plants, W + OS-treated EV/EV plants had smaller rosettes, smaller branches and fewer flowers than control EV/EV

Three-way ANOVAs:

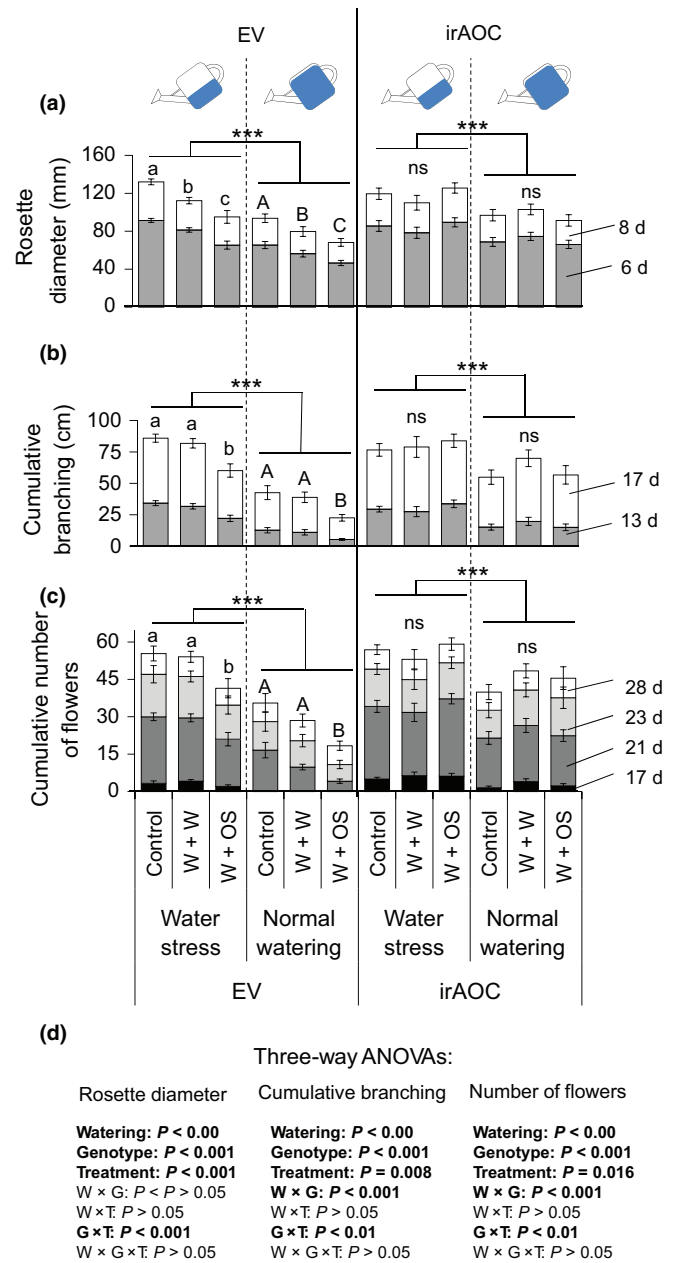|	Rosette diameter	Cumulative branching	Number of flowers
Watering:	**P < 0.00**	**P < 0.00**	**P < 0.00**
Genotype:	**P < 0.001**	**P < 0.001**	**P < 0.001**
Treatment:	**P < 0.001**	P = 0.008	P = 0.016
W × G:	P < 0.05	**W × G: P < 0.001**	**W × G: P < 0.001**
W × T:	P > 0.05	W × T: P > 0.05	W × T: P > 0.05
G × T: P < 0.001		**G × T: P < 0.01**	**G × T: P < 0.01**
W × G × T: P > 0.05		W × G × T: P > 0.05	W × G × T: P > 0.05

Fig. 2 The suppression of regrowth occurs in a herbivory-specific manner independent of the soil moisture conditions. Control, untreated plants; W + W, wounded and water-treated plants; W + OS, wounded and *Manduca sexta* oral secretion-treated plants. EV, empty vector; irAOC, jasmonate-deficient plants. Water stress, water supply was gradually reduced over 6 d. Normal watering, *c.* 50 ml of water per plant every day during the treatment period. (a) Average (± SE) rosette diameter, (b) cumulative branching, (c) number of flowers and (d) detailed statistical analysis. W, watering regime; G, genotype; T, treatment. Different letters indicate significant differences among treatments within watering regimes and genotypes (*P* < 0.05). Asterisks indicate differences among watering regimes within genotypes (***, *P* < 0.001). Statistical data (three-way ANOVAs) are given for the last measured time point of each fitness parameter. n.s, not significant.

plants (Fig. 4). No induction effect was observed in irAOC/irAOC plants. W + OS-treated EV/irAOC plants showed a reduction in shoot regrowth from the rootstock relative to non-treated
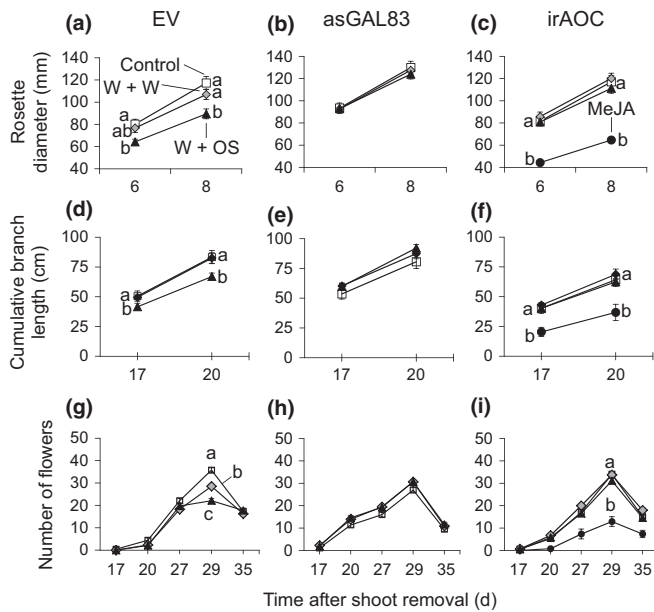
Fig. 3 The herbivore-induced regrowth reduction is jasmonate and GAL83 dependent. Control, untreated plants (open squares); W + W, wounded and water-treated plants (gray diamonds); W + OS, wounded and *Manduca sexta* oral secretion-treated plants (closed triangles); MeJA, methyl jasmonate-treated plants (closed circles). EV, empty vector plants; asGAL83, constitutively translocate more photoassimilates to roots; irAOC, jasmonate-deficient plants. Average ($\pm$ SE) rosette diameter of EV plants (a), asGAL83 plants (b) and irAOC plants (c), (d–f) average ($\pm$ SE) cumulative branching, (g–i) average ($\pm$ SE) number of flowers. Different letters indicate significant differences among treatments within time points and genotypes ($P < 0.05$).

Fig. 4 Leaf- and root-derived jasmonates regulate the regrowth of induced plants. Control, untreated plants (squares); W + OS, wounded and *Manduca sexta* oral secretion-treated plants (triangles); EV/EV, rootstock and scion from EV plants; irAOC/irAOC, rootstock and scion from irAOC plants; EV/irAOC, rootstock from irAOC and scion from EV plants. EV, empty vector plants; irAOC, jasmonate-deficient plants. (a–c) Average ($\pm$ SE) rosette diameter, (d–f) average ($\pm$ SE) cumulative branching, (g–i) average ($\pm$ SE) number of flowers. Asterisks indicate significant differences among treatments within time points and genotypes (*, $P < 0.05$; **, $P < 0.01$).

EV/irAOC plants (Fig. 4). Interestingly, the relative difference between control and W + OS-treated plants was intermediate between EV/EV and irAOC/irAOC plants (Fig. S3), suggesting that both root and leaf JA contribute to the reduction in regrowth capacity.

Simulated herbivory reconfigures the primary and secondary metabolism of shoots and roots in a Gal83- and JA-dependent manner

The induction of defense on herbivory attack might deplete plant resources that could otherwise be used to regrow. To test this hypothesis, we profiled soluble sugars and starch in herbivore-attacked plants. Six days after W + OS induction, concentrations of starch and soluble sugars were strongly reduced in EV leaves and roots (Fig. 5a), suggesting resource depletion. Nicotine, however, was induced in roots after OS elicitation (W + OS) and in leaves after both W + W and W + OS treatments (Fig. 5b). asGAL83 plants showed reduced levels of starch and elevated levels of nicotine in wounding and water (W + W)-treated leaves, but no effect of the application of oral secretions was observed (Fig. 5a,c). Accordingly, sugar contents in the leaves and roots of asGAL83 plants remained unaltered (Fig. 5b). irAOC plants did not display any changes in sugars and nicotine, and starch levels were only reduced in wounded leaves (Fig. 5a–c). MeJA application to irAOC plants restored EV patterns (Fig. 5a–c). Taken
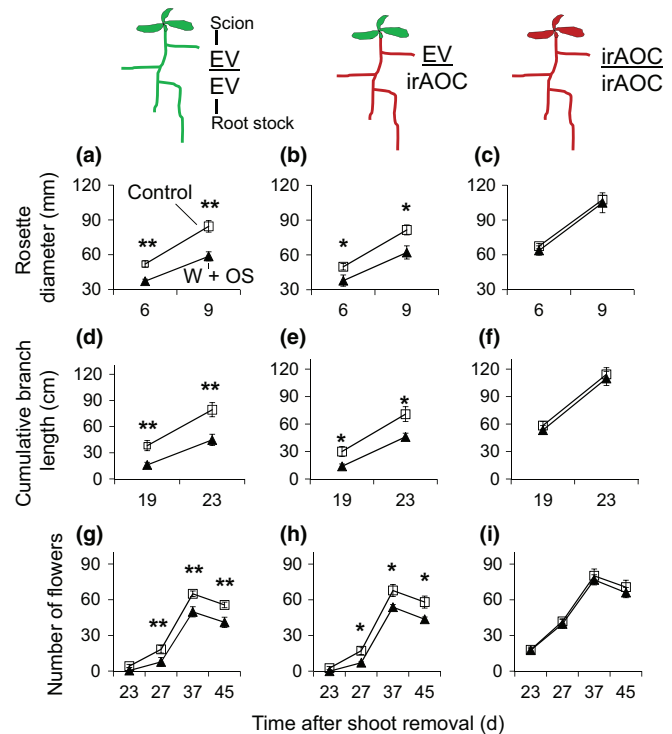
together, these results show that the induction of nicotine and the depletion of carbohydrates in the roots are positively correlated, and that root C depletion is correlated with a reduction in regrowth from the rootstock. The regrowing leaves of EV and asGAL83 plants did not differ in starch and sugar concentrations (Fig. 5d,e). Nicotine levels were increased more strongly in EV than in asGAL83 plants (Fig. 5f). Regrowing leaves in irAOC plants had higher levels of starch on W + W and W + OS pre-treatment, whereas nicotine levels were reduced (Fig. 5d–f). This pattern was restored to wild-type levels on MeJA application (Fig. 5d–f). This suggests that regrowing leaves are better defended, but potentially also more costly to produce, for EV plants.

Foliar herbivory induces JA and IAA in the roots and leaves

Systemic signals may be required to trigger the herbivory-induced reduction in regrowth from the rootstock. We therefore evaluated the induction of phytohormones in response to *M. sexta* attack. As demonstrated previously, JA and (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) were up-regulated within 1 h after wounding of EV plants (Fig. 6a–f). The application of oral secretions (W + OS) amplified this response. The JA burst was also observed in
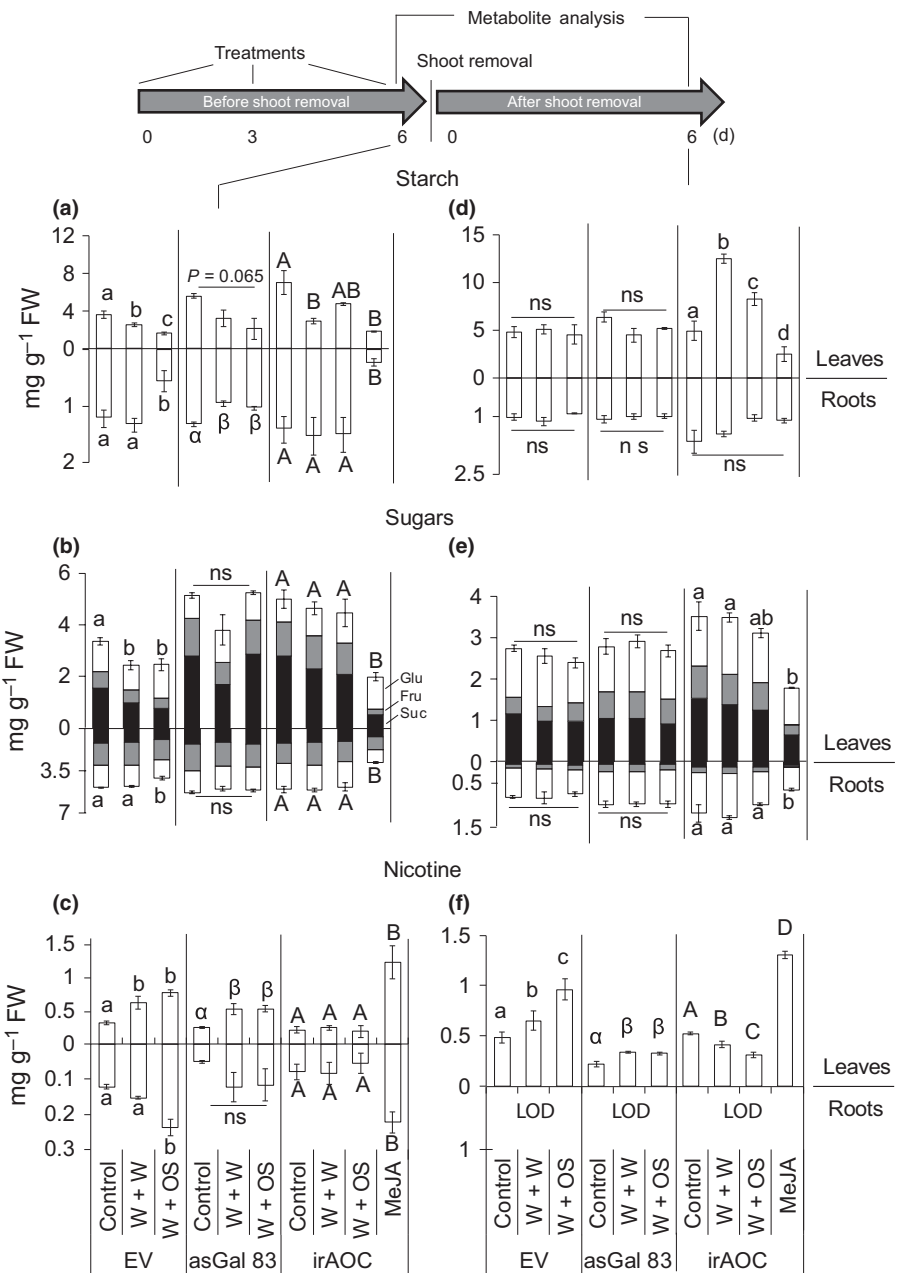
Fig. 5 Jasmonates and GAL83 regulate herbivore-induced changes in primary and secondary metabolism of leaves and roots before and after regrowth. Control, untreated plants; W + W, wounded and water-treated plants; W + OS, wounded and *Manduca sexta* oral secretion-treated plants; MeJA, methyl jasmonate-treated plants. EV, empty vector plants; asGal83, constitutively translocate more photoassimilates to roots; irAOC, jasmonate-deficient plants. (a, d) Average (± SE) starch content, (b, e) glucose (Glu, white bars), fructose (Fru, grey bars) and sucrose (Suc, black bars) content, (c, f) nicotine. Different letters indicate significant differences among treatments within genotypes (*P* < 0.05). LOD, limit of detection; n.s, not significant.

asGAL83 plants, even though induced JA-Ile levels were slightly lower. JA were not induced in irAOC leaves. Neither JA nor JA-Ile were up-regulated in the roots of EV and irAOC plants. However, JA-Ile was up-regulated in the roots of asGAL83, 3 h after W + OS treatment. Within 1 h after leaf elicitation, IAA was highly up-regulated in the leaves and roots of all genotypes (Fig. 6g–i). IAA levels were maintained above controls in W + OS-treated asGAL83 and irAOC plants over 3 h, whereas they dropped to control levels in EV plants 1 h after elicitation. ABA levels remained unaffected in the roots and were slightly induced in the leaves (Fig. S4a–c). Salicylic acid (SA) was highly induced in the leaves 3 h after treatment in all genotypes. SA levels in the roots remained unaffected (Fig. S4d–f), apart from a slight increase in asGAL83 plants 1 h after W + OS treatment.

Taken together, these results show that the JA burst is conserved in asGAL83 plants and that auxin may act as a systemic leaf-to-root signal in *N. attenuata*.

JA signaling and regrowth capacity are negatively correlated in natural accessions

To confirm the role of JA in determining the regrowth responses among different natural *N. attenuata* populations, we measured both parameters in 120 field-collected individuals from different populations. We found a significant negative correlation between W + OS-induced JA production and flower production of regrowing shoots (Pearson's correlation *r* = −0.217, *P* = 0.0182; Fig. 7). The relatively low correlation coefficient suggests the
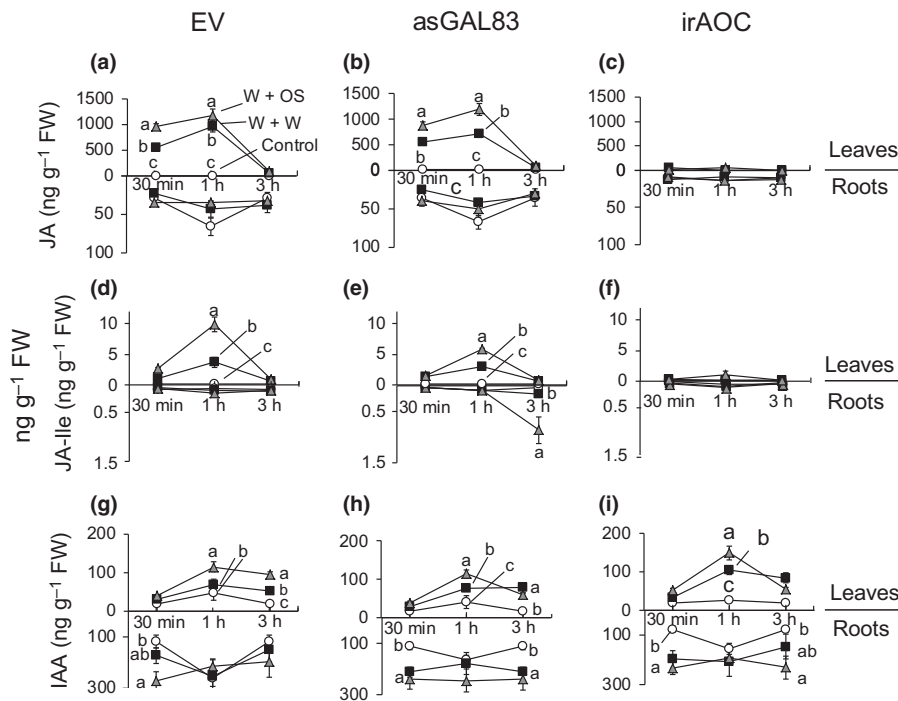
Fig. 6 Herbivore-induced phytohormones are regulated locally and systemically in a jasmonate- and GAL83-dependent manner. EV, empty vector plants; asGAL83, constitutively translocate more photoassimilates to roots; irAOC, jasmonate-deficient plants. Control, untreated plants (open circles); W + W, wounded and water-treated plants (closed squares); W + OS, wounded and *Manduca sexta* oral secretion-treated plants (gray triangles); JA, jasmonic acid; JA-Ile, (+)-7-*iso*-jasmonoyl-L-isoleucine; IAA, indole acetic acid. (a–c) Average ($\pm$ SE) JA, (d–f) JA-Ile, (g–i) IAA. Different letters indicate significant differences among treatments within genotypes and time points ($P < 0.05$).

presence of JA-independent regulatory elements that determine regrowth patterns.

Little evidence for the involvement of GAs in regrowth responses

Plants seem to prioritize defense over growth via an antagonistic crosstalk between JA and GA signaling (Yang *et al.*, 2012). Monitoring of the changes in branch architecture as a GA signaling downstream marker, however, provided little evidence for the involvement of GAs in root responses to insect attack. W + OS-treated EV plants had shorter branches and fewer internodes than, but similar internode lengths to, control plants 18 and 20 d after shoot removal (Fig. S5a,d,g,j). W + OS elicitation did not affect significantly the branch architecture of asGAL83 plants, apart from a slight reduction in the average internode length 20 d after shoot removal (Fig. S5b,e,h,k). Simulated herbivory did not affect the branch architecture of irAOC plants (Fig. S5c,f,i,l).

IAA application restores wild-type patterns in asGAL83, but not in irAOC, plants

Nicotiana attenuata responds to *M. sexta* herbivory by inducing auxin levels in both leaves and roots. To explore the possible role of auxin homeostasis in tolerance responses, we applied IAA or TCA to alter auxin transport and monitored plant regrowth following elicitation. The application of the auxin transport inhibitor *trans*-cinnamic acid to EV plants attenuated the fitness cost of simulated *M. sexta* herbivory (Fig. 8a,d,g). IAA application, however, did not change the regrowth pattern of EV plants. However, IAA application to asGAL83 plants resulted in an increase in regrowth from the rootstock in controls, but not in
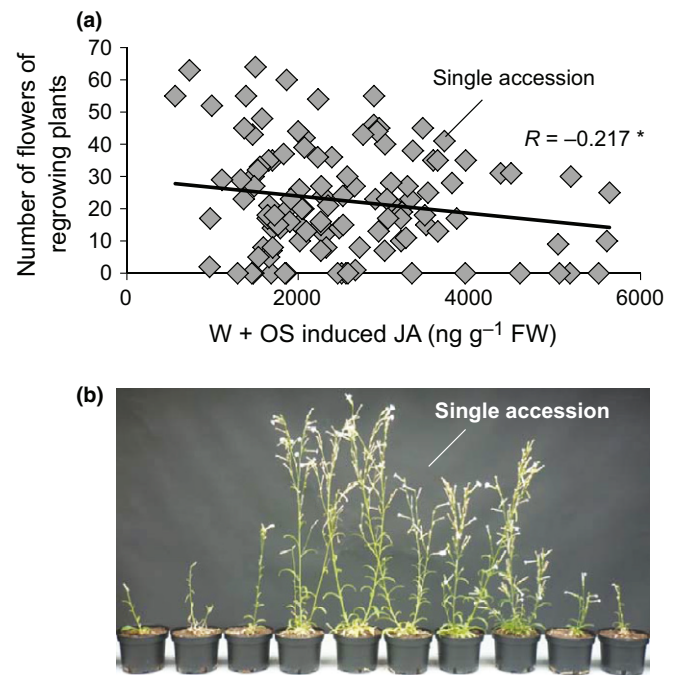

Fig. 7 The jasmonate burst and regrowth capacity are negatively correlated across *Nicotiana attenuata* ecotypes. (a) *y*-axis, number of flowers at the end of the flowering period; *x*-axis, jasmonic acid (JA) produced 1 h after elicitation with *Manduca sexta* oral secretions (W + OS). Each point corresponds to one individual from an independent accession. (b) Number of flowers produced by regrowing shoots. *Significant statistical difference, $P < 0.05$.

W + OS-treated plants. As a consequence, IAA-supplemented asGAL83 plants behaved like EV plants in terms of their herbivore-induced regrowth patterns (Fig. 8b,e,h). TCA application
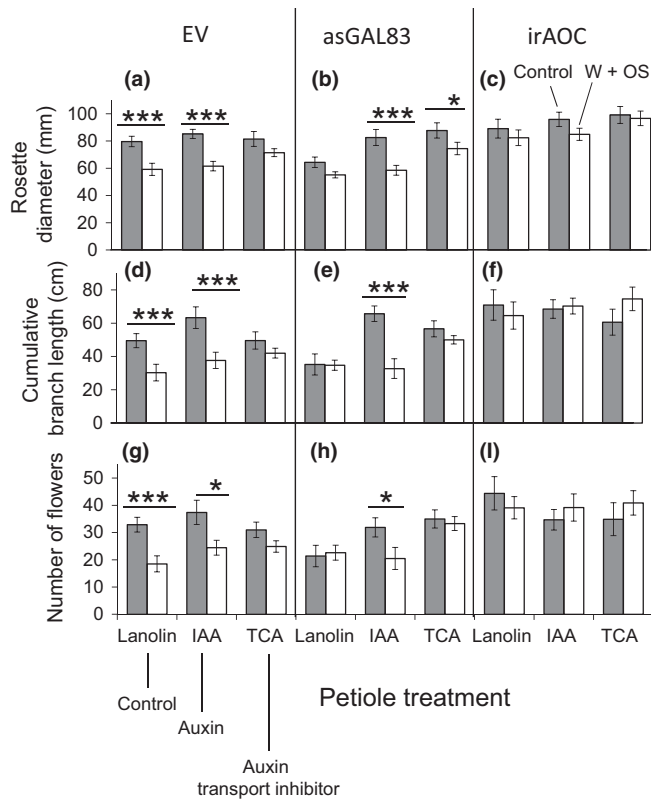
Fig. 8 Auxin regulates the regrowth response in a jasmonate (JA)-independent manner. EV, empty vector plants; asGal83, constitutively translocate more photoassimilates to roots; irAOC, jasmonate-deficient plants. Control, plants without OS induction (closed bars); W + OS, wounded and *Manduca sexta* oral secretion-treated plants (open bars); IAA, indole-3-acetic acid in lanolin paste; TCA, *trans*-cinnamic acid in lanolin paste; Lanolin, lanolin-treated plants. (a–c) Average (± SE) rosette diameter, (d–f) average (± SE) cumulative branching, (g–i) average (± SE) number of flowers at the end of the flowering period. Asterisks indicate significant differences between control and W + OS treatments within genotypes and auxin manipulation type (*, $P < 0.05$; ***, $P < 0.001$).

also increased the growth of asGAL83 plants. irAOC plants were not influenced by either IAA or TCA application (Fig. 8c,e,i). Taken together, these experiments suggest a role for IAA in determining the herbivory-induced regrowth patterns in EV and irGAL83 plants.

Attacked leaves have lower chlorophyll contents

The reduction in non-structural carbohydrates in herbivore-attacked plants might be explained by an increase in energy demand or a decrease in photosynthetic energy supply. We therefore measured chlorophyll content as an indication of photosynthetic capability. Overall, chlorophyll contents did not differ between EV and irAOC plants (Fig. S6). Wounding reduced the chlorophyll contents of the treated leaves in both genotypes. The application of oral secretions further reduced chlorophyll levels in EV, but not in irAOC, plants. These results indicate that, in addition to the energy required for the biosynthesis of defensive compounds, reduced photoassimilation may contribute to the herbivory-induced JA-dependent depletion of carbohydrates.

JA signaling determines the induced resistance of regrowing leaves

Our previous results suggested that regrowing shoots of previously attacked plants might be better defended (Fig. 5f) and therefore more costly to produce. To further evaluate the defensive status of regrowing leaves, we measured *M. sexta* performance on regrowing pretreated plants. Overall, caterpillars performed better on regrowing shoots of irAOC than EV plants. Plants growing from EV rootstocks that had previously been subjected to simulated herbivory supported less *M. sexta* growth than untreated plants (Fig. S7). Surprisingly, *M. sexta* performed better on regrowing shoots of irAOC plants that had been wounded before. No effect of W + OS treatment was observed in irAOC plants, whereas MeJA treatment reduced *M. sexta* performance (Fig. S7).

Discussion

In this study, we provide evidence for a role of roots in resource-based trade-offs between resistance and tolerance to herbivory. We found that simulated *M. sexta* herbivory reduces starch and sugar contents and induces nicotine production in the roots and leaves of *N. attenuata*. The reduction of non-structural carbohydrates in roots was correlated with a reduction in regrowth from the rootstock in both the glasshouse and the field. In many plants, an increase in C transport from both damaged and undamaged tissues to the roots has been observed (Dyer *et al.*, 1991; Briske *et al.*, 1996; Holland *et al.*, 1996; Babst *et al.*, 2005, 2008; Bazot *et al.*, 2005; Schwachtje *et al.*, 2006; Kaplan *et al.*, 2008; Gómez *et al.*, 2010, 2012; Ferrieri *et al.*, 2012). Although it has been proposed that the herbivory-induced resource sequestration acts as a putative tolerance mechanism by increasing root reserves for future regrowth (Schwachtje *et al.*, 2006), the lack of evidence for an actual accumulation of carbohydrate resources in the roots has called this view into question (Steinbrenner *et al.*, 2011; Gómez *et al.*, 2012). In our experiments, we expected that induced resource sequestration would increase the capacity of *N. attenuata* to regrow after shoot removal. However, in none of the investigated plant genotypes and environmental conditions was such an effect observed. Although Schwachtje *et al.* (2006) demonstrated that silencing GAL83 increases C allocation to the roots and prolongs flowering in *N. attenuata*, that study did not provide any direct evidence for herbivore-induced plant tolerance via increased C storage. Early flower production in wild-type plants was reduced on elicitation (Schwachtje *et al.*, 2006). Our results confirm this finding and show that the down-regulation of GAL83 improves regrowth on herbivory. It is noteworthy that C reallocation documented by Schwachtje *et al.* (2006) was independent of JA, whereas the C depletion and regrowth patterns in this study were influenced by JA. Based on this evidence, we propose that *M. sexta*-induced resource sequestration in *N. attenuata* does not increase root C storage and defoliation tolerance *per se*, but may support the synthesis of plant defensive metabolites below ground (Shoji *et al.*, 2000) and/or buffer against a breakdown of

root primary functions in the face of carbohydrate depletion. A good mechanistic understanding of resource allocation processes and C fluxes will be necessary to test these hypotheses in a more comprehensive manner in the future. In this context, it will also be important to compare regrowth patterns across different plant species. *Nicotiana attenuata* synthesizes large amounts of nicotine in the roots which are then transported to the leaves. Plants that do not directly engage their roots in defensive processes may show different regrowth patterns.

Our study demonstrates that JA play a central, but not exclusive, role in root-mediated growth–defense trade-offs: Contrary to wild-type plants, JA-deficient irAOC plants did not suffer from herbivory-induced reduction of root carbohydrates and were not impaired in their regrowth capacity in the glasshouse and the field. Furthermore, the induction of defenses was abolished in irAOC plants, and the regrowing leaves were more susceptible to *M. sexta* attack. MeJA application restored wild-type patterns in irAOC plants. The central role of JA as determinants of the defensive make-up in nature is illustrated by the fact that the JA burst is negatively correlated with defoliation tolerance across field-collected *N. attenuata* genotypes. A reduction in the herbivore-induced suppression of leaf growth has also been documented in JA-deficient asLOX3 plants (Zavala & Baldwin, 2006), and it has been proposed that a JA-dependent reduction in leaf photosynthesis may be responsible for this effect (Nabity et al., 2009, 2013). In accordance with this hypothesis, we found that simulated herbivory reduces leaf chlorophyll concentrations in a JA-dependent manner. It is therefore likely that JA signaling depletes the plant's C pools by inducing the production of defensive metabolites on the one hand and reducing photoassimilation on the other. Even though our micrografting approach suggested that the aboveground JA burst is sufficient to trigger a reduction in the plant's regrowth capacity from the rootstock, we found that plants silenced in root JA production displayed an intermediate regrowth phenotype. It is therefore likely that the *de novo* synthesis of JA in the roots (Wang et al., 2008; Bonaventure et al., 2011) also contributes to the regulation of plant tolerance, for example by contributing to induced nicotine biosynthesis.

Resource-based trade-offs between growth and defense have long been discussed (McKey, 1974; van der Meijden et al., 1988; de Jong & van der Meijden, 2000; Schwachtje & Baldwin, 2008; Anten & Pierik, 2010; Orians et al., 2011) and hormonal crosstalk has been proposed as a possible mechanism (Chen et al., 2011; Yang et al., 2012). A recent study in rice and *A. thaliana* proposed that JA may reduce plant growth by interfering with the GA-mediated promotion of internode elongation (Yang et al., 2012). We found little morphological evidence of JA/GA crosstalk in regrowing shoots, and propose that the depletion of storage carbohydrates, rather than a hormone-dependent reduction in cellular activity, may restrain *N. attenuata* regrowth. Nevertheless, the relatively weak correlation between JA production and regrowth among natural accessions suggests that regulatory elements other than JA may influence the plant's root storage regime and regrowth capacity. One prominent candidate in this context is IAA, which has been proposed as a negative regulator of nicotine biosynthesis and JA accumulation in *Nicotiana* sp.

(Baldwin et al., 1997; Shi et al., 2006; Onkokesung et al., 2010). In contrast with our expectations, we found that IAA rapidly accumulated in the leaves and roots of herbivore-attacked *N. attenuata* plants, and that the root auxin response was prolonged in both asGAL83 and irAOC lines. The fact that the inhibition of auxin transport reduced the herbivore-induced reduction in regrowth in EV plants, whereas IAA application restored wild-type patterns in asGAL83 plants, strongly suggests that auxin homeostasis is an important determinant of plant tolerance against herbivory.

Our understanding of how plants coordinate and fine tune JA and IAA signaling is still limited. Glucose signaling has been proposed to coordinate plant growth by interfering with auxins (Moore et al., 2003; Sairanen et al., 2012), and SNF-related serine/threonine-protein kinases (SnRK kinases) can regulate carbohydrate partitioning (reviewed by Halford & Paul, 2003; Rolland et al., 2006) and mediate the binding of the 26S proteasome to SCF ubiquitin ligases (Farras et al., 2001), suggesting that the SnRK1 kinases promote auxin signaling via the activation of auxin-responsive gene transcription. Furthermore, the *A. thaliana* auxin-responsive genes GH3.3, GH3.5 and GH3.6 have been found to conjugate jasmonic acid to amino acids, leading either to JA degradation or affecting the synthesis of JA-Ile (Gutierrez et al., 2012). Therefore, it is conceivable that a tight interplay between root/shoot auxin ratios and GAL83-mediated resource partitioning coordinates growth and root defenses in *N. attenuata* in addition to JA. Further experiments involving a tight spatio-temporal control of auxin and carbohydrate fluxes will be necessary to disentangle the exact mechanisms in detail.

In conclusion, we have shown that herbivore attack results in the depletion of non-structural carbohydrates in the roots which is likely to constrain the plant's capacity to regrow and, at the same time, enable the deployment of effective defenses. Both JA and IAA regulate trade-offs between induced defenses and tolerance. The underlying regulatory network is likely to provide a robust mechanistic basis for the divergent intraspecific strategies that plants display to survive in a hostile environment.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Fitness of regrowing plants on defoliation and complete shoot removal.

Fig. S2 Changes in root and shoot biomass following simulated leaf-herbivore attack and regrowth in the dark.

Fig. S3 Relative changes in regrowth in grafted plants.

Fig. S4 Phytohormone induction in response to foliar herbivory.

Fig. S5 Elongation patterns of regrowing shoots.

Fig. S6 Chlorophyll content of induced shoots.

Fig. S7 Herbivore resistance of regrowth leaves.

Notes S1 Methodological details for phytohormone analysis.

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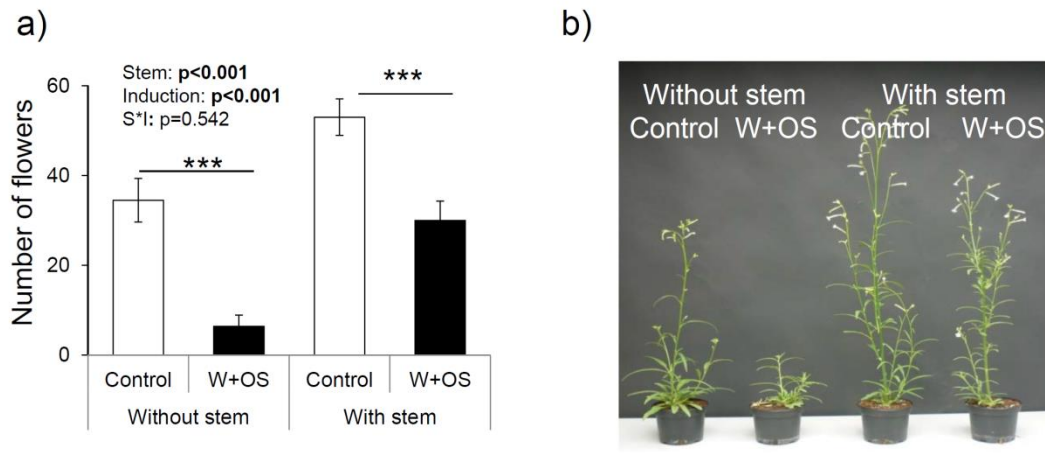# Supporting information

Figure S1

Fig. S1. Fitness of regrowing plants upon defoliation and complete shoot removal. Control: untreated plants; W+OS: wounded and *M. sexta* oral secretion-treated plants. (a) Number of flowers of regrowing plants. (b) Representative picture of regrowing plants attacked and non-attack plants with and without stems. Asterisks indicate significant differences among treatments within genotypes (***, p <0.01).
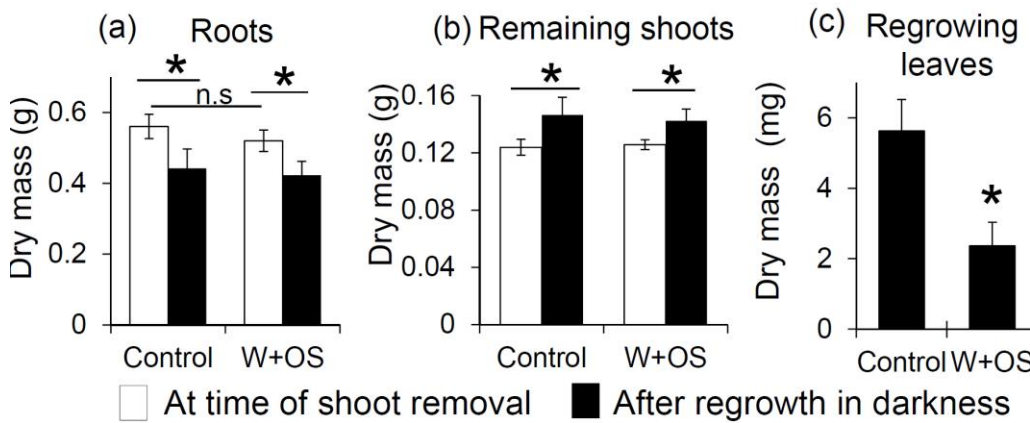
Figure S2.

Fig. S2. Changes in root and shoot biomass following simulated leaf-herbivore attack and regrowth in the dark. Control: untreated plants, W+OS: wounded and *M. sexta* oral secretion-treated plants. Average (± SE) dry biomass of roots (a), remaining shoots (b) and regrowth leaves (c) before shoot removal in light and after shoot removal in darkness. Asterisks indicate significant differences among treatments (*, p <0.05).

Figure S3

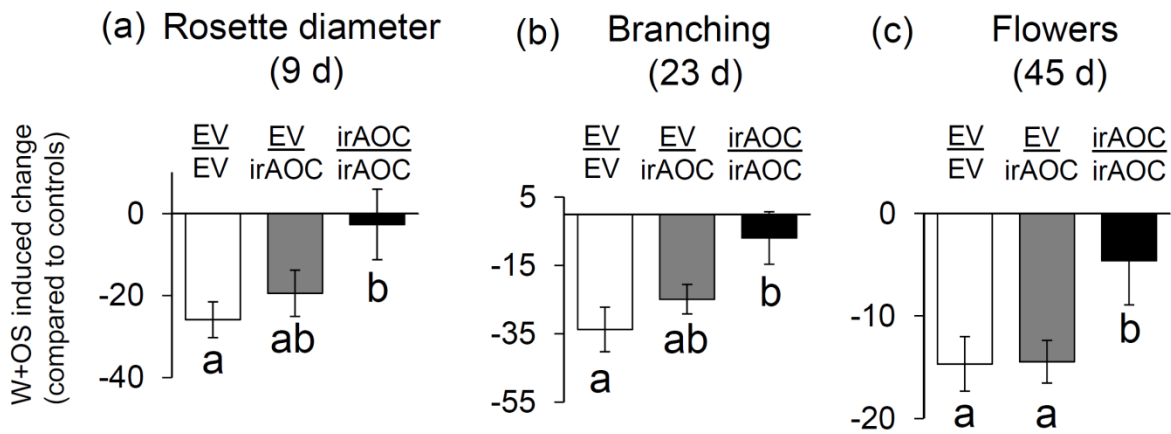(a) Rosette diameter (9 d)   (b) Branching (23 d)   (c) Flowers (45 d)

Fig. S3. Relative changes of regrowth in grafted plants. Average (± SE) rosette diameter (a), cumulative branching (b), number of flowers (c). Letters indicate significant differences among chimeric plants (p <0.05).
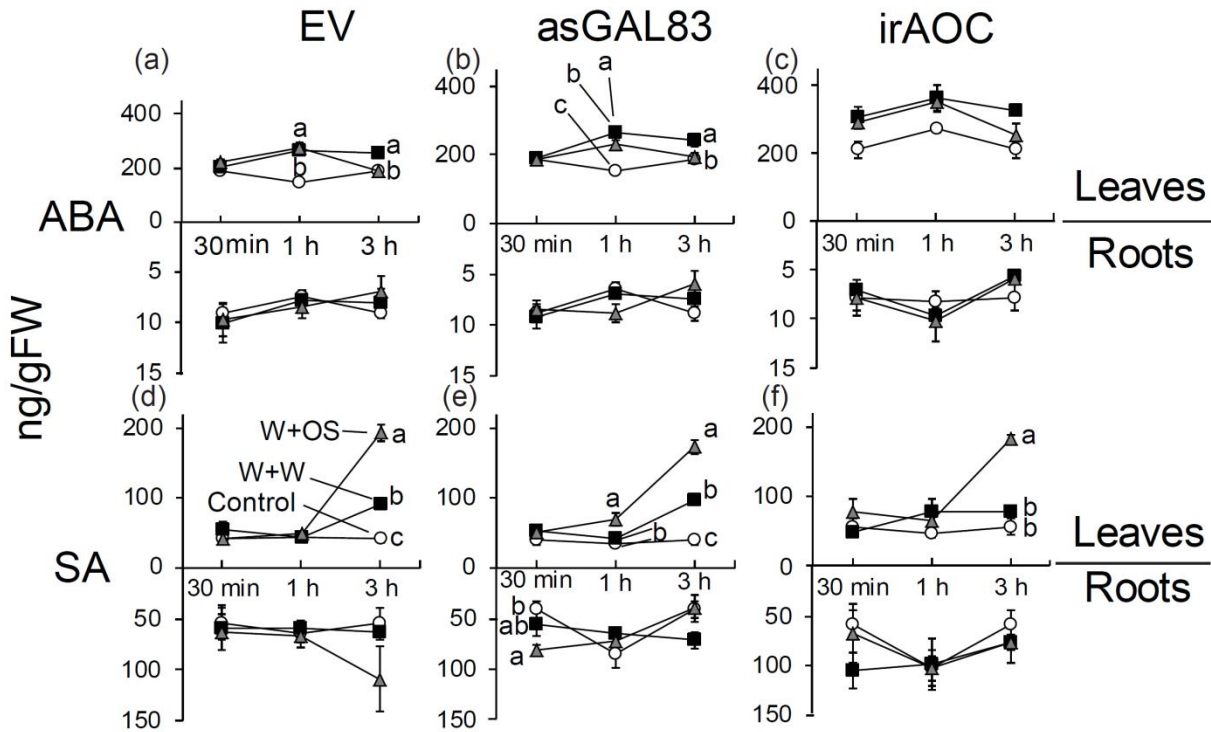
Figure S4

Fig. S4. Phytohormone induction in response to foliar herbivory. Control: untreated plants; W+W: wounded and water-treated plants; W+OS: wounded and M. sexta oral secretions-treated plants; ABA: abscisic acid; SA: salicylic acid. Different letters indicate significant differences among treatments within genotypes and time points (p <0.05).
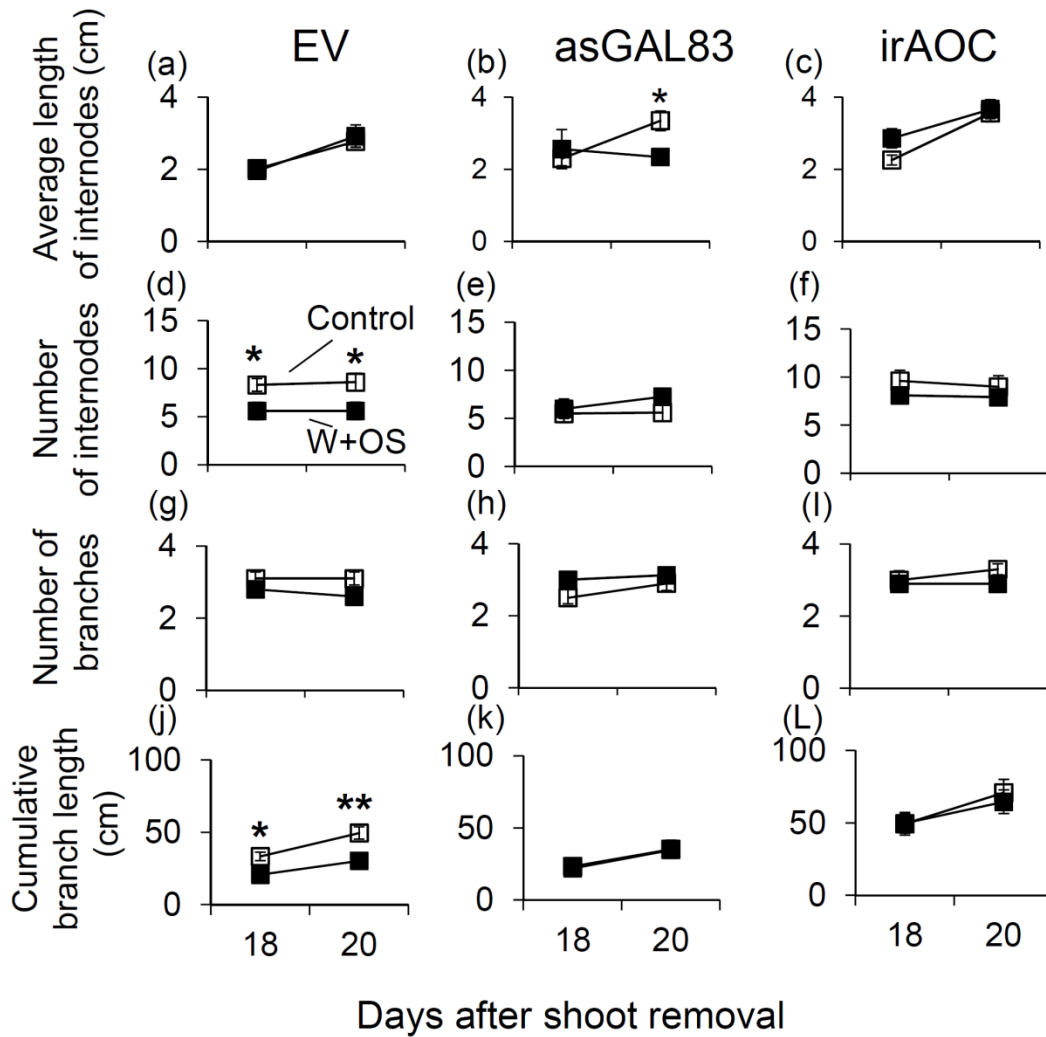
35

Figure S5

Fig. S5. Elongation patterns of regrowing shoots. EV: empty vector plants. AsGal83: Constitutively translocate more photoassimilates to roots. IrAOC: jasmonate-deficient plants. W+OS: wounded and *M. sexta* oral secretion-treated plants. Asterisks indicate significant differences among treatments within genotypes (*, $p < 0.05$; **, $p < 0.01$).
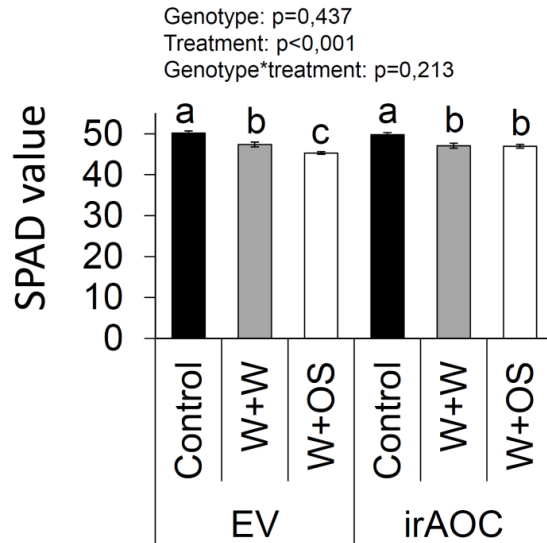
Figure S6

Genotype: p=0,437
Treatment: p<0,001
Genotype*treatment: p=0,213

Fig. S6. Chlorophyll content of induced shoots. Control: untreated plants; W+W: wounded and water-treated plants; W+OS: wounded and *M. sexta* oral secretion-treated plants. EV: empty vector. IrAOC: jasmonate-deficient plants. Different letters indicate significant differences among treatments within genotypes (p <0.05). SPAD value: Index value displayed by Konica Minolta Chlorophyll meters positively correlated with chlorophyll density.

Figure S7

Larval performance

Genotype: p<0.001
Treatment : p<0.001
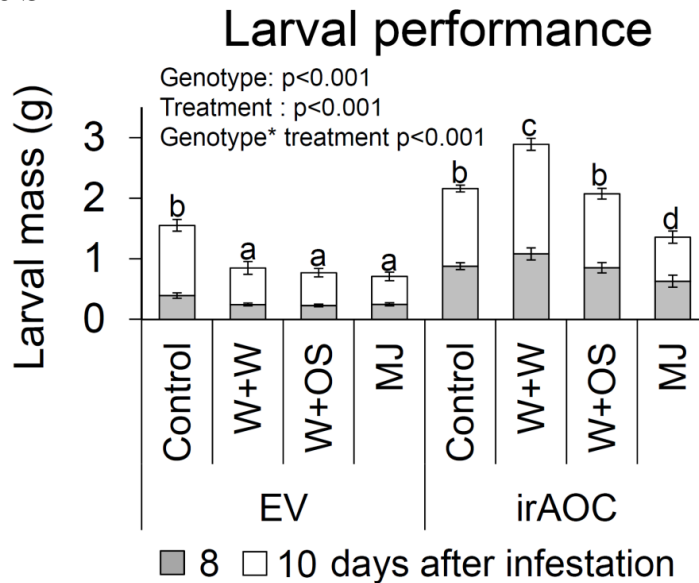Genotype* treatment p<0.001

Fig. S7. Herbivore resistance of regrowth leaves. EV: empty vector plants. IrAOC: JA-impaired plants. Control: untreated plants, W+W: wounded and water-treated plants, W+OS: wounded and *M. sexta* oral secretions-treated plants, MJ: Methyl jasmonate-treated plants. Grey bars: 8 d old larvae, grey plus open bars: 10 d old larvae. Different letters indicate significant differences among treatments within genotypes (p <0.05).

Supporting Information Note S1

Methodological details for phytohormone analysis, including IAA

One hundred mg of plant tissue per sample were extracted with 1 mL ethyl acetate: formic acid (99.5:0.5) containing the following phytohormone standards: 40ng of 9,10-D_2-9,10-dihydrojasmonic acid (JA), 40 ng of D_4-salicylic acid (SA), 40 ng of D_6-Abscissic acid (ABA) (Santa Cruz Biotechnology, USA), 8 ng of jasmonic acid-[$^{13}C_6$] isoleucine(JA-Ile) and 20 ng of D_5-indole-3-acetic-acid (IAA). JA-[$^{13}C_6$]-Ile conjugate was synthesized as described by Kramell *et al.* (1988) using [$^{13}C_6$]-Ile (Sigma). All samples were then vortexed for 10 min and centrifuged at 14.000 rpm for 20 min at 4 °C. Supernatants were evaporated to dryness in a SpeedVac at room temperature (Eppendorf 5301, Germany). Remaining pellets were resuspended in 50 µL methanol:water (70:30) and dissolved using an ultrasonic bath (Branson 1210, USA) for 5 min. JA, SA, JA-Ile and ABA were analyzed using liquid chromatography (Agilent 1260 infinity, HPLC system (Agilent Technologies)) coupled to a mass spectrometer (API 5000, Applied Biosystems) using a procedure adapted from Vadassery *et al.* (2012). Briefly, separation was performed on a Zorbax Eclipse XDB-C18 column (50 x 4,6 mm, 1,8 mm; Agilent). Mobile phases A and B were 0.05% acetic acid in water and acetonitrile, respectively. Compared to Vadassery *et al.* (2012), acetic acid was chosen as mobile phase additive instead of formic acid since it was shown to provide higher sensitivity for all hormones measured. The following gradient was employed: 0 to 0.50 min, 5% B; 0.50 to 9.50 min, 5% to 58% B; 9.50 to 9.52 min, 58% to 100% B; 9.52 to 11.00 min, 100% B; and 11.10 to 14.00 min, 5% B he mobile phase flow rate was of 1.1 mL.min^{-1} and the column temperature was maintained at 25°C. The API 5000 tandem mass spectrometer (Applied Biosystems, Carlsbad, CA, US) equipped with a Turbospray ion source was operated in the negative ionization mode. The ion spray voltage was set at -4500 eV and the turbo gas temperature at 700 °C. Nebulizing gas, curtain gas and collision gas were set at 60, 25 and 7 psi respectively. Multiple reaction monitoring was used to monitor analyte parent ion → product ion: mass-to charge ratio [m/z] 136.9 → 93.0 (collision energy [CE], 222; declustering potential [DP], 235 V) for salicylic acid; m/z 140.9 → 97.0 (CE, 222 V; DP, 2 35 V) for D_4-SA; m/z 209.1 → 59.0 (CE, 224 V; DP, 235 V) for JA; m/z 213.1 → 56.0 (CE, 224 V; DP, 235 V) for 9,10-D_2-9,10-JA; m/z 263.0 → 153.2 (CE, 222 V; DP, 235 V) for ABA; m/z 269.0 → 159.2 (CE, 222 V; DP, 235 V) for D_6-ABA; m/z 322.2 → 130.1 (CE, 230 V; DP, 250 V) for JA-Ile; and m/z 328.2 → 136.1 (CE, 230 V; DP, 250 V) for JA-[$^{13}C_6$]Ile. Both Q1 and Q3 quadrupoles were maintained at unit resolution. IAA was analyzed using the same system and solvents except that the elution profile was as follows: 0 to 0.50 min, 5% B; 0.50 to 6.00 min, 5% to 37.4% B; 6.00 to 6.02 min, 37.4% to 100% B; 6.02 to 7,50 min, 100% B; 7.50 to 7.60 min, 100 to 5% B; and 7.60 min to 10.50 min, 5 % B. The mass spectrometer was operated in the positive ionization mode, with an ion spray voltage of + 5 500 eV and a turbo gas temperature of 700 °C. Nebulizing gas, curtain gas and collision gas were set at 60, 30 and 4 psi respectively. IAA was measured by monitoring the transition m/z 176,00 → 130,00 (CE, 19 V; DP, 31 V) for IAA; m/z 181,00 → 135,00 (CE, 19 V; DP, 31 V) for D_5-IAA. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Data acquisition and processing was performed using Analyst 1.5 software (Applied Biosystems, Carlsbad, CA, US). Phytohormones were quantified using the signal of their corresponding internal standard.

Manuscript II

Jasmonate-dependent depletion of soluble sugars compromises plant resistance to *Manduca sexta*

2015

Ricardo A.R. Machado, Carla C.M. Arce, Abigail P. Ferrieri, Ian T. Baldwin, Matthias Erb

Jasmonate-dependent depletion of soluble sugars compromises plant resistance to *Manduca sexta*

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Summary

- Jasmonates regulate plant secondary metabolism and herbivore resistance. How they influence primary metabolites and how this may affect herbivore growth and performance are not well understood.
- We profiled sugars and starch of jasmonate biosynthesis-deficient and jasmonate-insensitive *Nicotiana attenuata* plants and manipulated leaf carbohydrates through genetic engineering and *in vitro* complementation to assess how jasmonate-dependent sugar accumulation affects the growth of *Manduca sexta* caterpillars.
- We found that jasmonates reduce the constitutive and herbivore-induced concentration of glucose and fructose in the leaves across different developmental stages. Diurnal, jasmonate-dependent inhibition of invertase activity was identified as a likely mechanism for this phenomenon. Contrary to our expectation, both *in planta* and *in vitro* approaches showed that the lower sugar concentrations led to increased *M. sexta* growth. As a consequence, jasmonate-dependent depletion of sugars rendered *N. attenuata* plants more susceptible to *M. sexta* attack.
- In conclusion, jasmonates are important regulators of leaf carbohydrate accumulation and this determines herbivore growth. Jasmonate-dependent resistance is reduced rather than enhanced through the suppression of glucose and fructose concentrations, which may contribute to the evolution of divergent resistance strategies of plants in nature.

Introduction

Jasmonates regulate plant responses to biotic and abiotic stress and influence plant growth and development. They are part of the regulatory networks of plant–symbiont (Pozo & Azcón-Aguilar, 2007; Stein *et al.*, 2008; Jacobs *et al.*, 2011), plant–pathogen (Landgraf *et al.*, 2012) and plant–herbivore interactions (reviewed by Wu & Baldwin, 2010), and are involved in the regulation of seed germination (Corbineau *et al.*, 1988), root growth and development (Staswick *et al.*, 1992), leaf movement (Nakamura *et al.*, 2006) and flower development (Li *et al.*, 2004). Perhaps the best known function of jasmonates is their stimulatory effect on plant secondary chemistry. Plants impaired in jasmonate production or perception generally display reduced levels of constitutive and induced secondary metabolites (Chen *et al.*, 2006; Paschold *et al.*, 2007; Shoji *et al.*, 2008; Zhang *et al.*, 2011).

Although our understanding of several aspects of jasmonate signaling is increasing, knowledge about its possible role as a regulator of primary metabolism in plants is unclear. Recently, leaf glucose and fructose concentrations were found to be constitutively higher and less depleted in response to simulated *Manduca*

sexta herbivory in jasmonate biosynthesis-deficient *Nicotiana attenuata* plants, an effect that can be mimicked by the exogenous application of jasmonic acid (JA; Machado *et al.*, 2013). Moreover, exogenous jasmonate application to the leaves reduced leaf starch concentration in poplar trees, stem sugars in tulip, leaf sugars in tobacco, and leaf sugars and amino acids in cabbage (Babst *et al.*, 2005; Skrzypek *et al.*, 2005; van Dam & Oomen, 2008; Hanik *et al.*, 2010; Tytgat *et al.*, 2013), suggesting that jasmonates might act as negative regulators of plant primary metabolism. By contrast, starch concentrations in jasmonate signaling-impaired tobacco plants were significantly lower (Wang *et al.*, 2014) and jasmonate application to the leaves induced amino acids in tobacco leaves (Hanik *et al.*, 2010), suggesting that jasmonates can also promote starch and amino acid accumulation. A detailed analysis of primary metabolites in jasmonate signaling-impaired plants is therefore required to clarify the potential role of endogenous jasmonates in the regulation of plant primary metabolism. Constitutive and induced jasmonate levels change over plant development (Abdala *et al.*, 2002; Diezel *et al.*, 2011), a phenomenon that correlates with a reduction in the magnitude of induction of jasmonate-dependent secondary metabolites and defensive proteins (van Dam *et al.*, 2001; Kaur *et al.*, 2010;

Onkokesung *et al.*, 2012). It is therefore possible that the impact of jasmonates on leaf carbohydrates is dependent on a plant's developmental stage.

Sugars are the dominant soluble leaf carbohydrates of plants. They are produced through the incorporation of carbon dioxide (CO_2) into ribulose-1,5-bisphosphate (RuBP) by the action of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), followed by the spontaneous formation of two molecules of 3-phosphoglyceric acid (3PGA). RuBisCO activase (RCA) activates RuBisCO by removing inhibitory sugar phosphates from the active site. 3PGA is subsequently converted to glucose, fructose, sucrose and starch via several enzymatic steps. Sucrose and starch can be stored, transported and/or metabolized further (Braun *et al.*, 2014). Soluble sugars and starch accumulate during the day and are catabolized during the night to meet the energy demand of the plant. Therefore, their concentrations rise and fall in a diurnal manner. Diurnal patterns therefore need to be taken into account when studying the impact of jasmonates on leaf carbohydrates.

Phytophagous insects feed on plants to acquire nutrients to fuel growth, development and reproduction, and are therefore affected directly by the metabolic make up of their food source. Both primary and secondary metabolites influence insect performance (Roeder & Behmer, 2014). Secondary metabolites are directly toxic or reduce the digestibility of the plant material in a quantitative manner (Bennett & Wallsgrove, 1994). The influence of primary metabolites on herbivores is more context dependent (Behmer, 2008). The ratio between carbohydrates and protein, for instance, determines insect growth in a nonlinear fashion, with suboptimal ratios leading to a rapid reduction in growth rates (Thompson & Redak, 2000; Simpson & Raubenheimer, 2009; Roeder & Behmer, 2014). Furthermore, protein and carbohydrate ratios influence the toxicity of plant secondary metabolites (Raubenheimer & Simpson, 1990; Raubenheimer, 1992; Simpson & Raubenheimer, 2001). Most studies on insect nutrition have been carried out in chemically defined artificial environments. However, plants as food sources in nature are inherently variable. Herbivore attack, for instance, alters nitrogen and carbon dynamics (Arnold & Schultz, 2002; Babst *et al.*, 2005; Gómez *et al.*, 2010; Appel *et al.*, 2012), which often results in dramatic changes in primary and secondary metabolite pools (Babst *et al.*, 2005; Skrzypek *et al.*, 2005; Schwachtje *et al.*, 2006; Steinbrenner *et al.*, 2011; Gómez *et al.*, 2012; Machado *et al.*, 2013) that might affect the nutritional quality of foliar tissue and could potentially affect herbivore nutrition. If we are to understand the importance of carbohydrates for insect nutrition, combining *in vitro* assays with experiments *in planta* would therefore be a promising approach.

One approach to manipulate plant chemistry is to target defensive signals. Jasmonates, for instance, have been silenced in a number of plant species, and the susceptibility of the jasmonate signaling-impaired plants to herbivores has subsequently been attributed to deficiencies in secondary metabolite production and accumulation (Steppuhn *et al.*, 2004; Paschold *et al.*, 2007; Steppuhn & Baldwin, 2007; Heiling *et al.*, 2010). Given that jasmonates also regulate primary metabolites in plants (Machado *et al.*,

2013; Wang *et al.*, 2014) and that the primary metabolites can be equally important for insect performance (Fernstrom, 1987; Cohen *et al.*, 1988; Waldbauer & Friedman, 1991; Thompson & Redak, 2000; Simpson & Raubenheimer, 2009; Roeder & Behmer, 2014), the question arises as to whether they could be responsible for the observed susceptibility of jasmonate-deficient plants. We investigated this potentially overlooked aspect of plant–herbivore interactions by studying the role of jasmonates in the regulation of carbohydrate accumulation in *N. attenuata* leaves, including potential underlying mechanisms, and the contribution of jasmonate-dependent carbohydrate depletion to herbivore resistance. To answer the first question, we measured sugar concentrations and invertase activity in *N. attenuata* genotypes that are impaired to different degrees in their jasmonate biosynthesis, signaling and/or perception. To answer the second question, we evaluated *M. sexta* growth when feeding on plants, artificial and semi-artificial diets with different sugar concentrations. Our results reveal that soluble sugar concentrations reduce rather than enhance jasmonate-dependent plant resistance.

Materials and Methods

Plant material

Transgenic inverted repeat (ir) and empty vector (EV) control (A-03-9-1) *Nicotiana attenuata* Torr. Ex. Watson plants were used in this study. The characteristics of these previously characterized different genotypes are summarized in Table 1. In addition, we produced a hemizygous cross between inverted repeat allene oxide cyclase (irAOC; line A-07-457-1) and inverted repeat ribulose-1,5-bisphosphate carboxylase/oxygenase activase (irRCA; line A-03-462-7-1) lines by removing anthers from flowers of irRCA plants before pollen maturation and pollinating the stigmas with pollen from irAOC plants.

Planting conditions

Before planting, all seeds were surface sterilized and germinated on Gamborg's B5 medium (Krügel *et al.*, 2002). Ten-day old seedlings were transferred to Teku pots for another 10 d (Pöppelmann GmbH & Co. KG, Lohne, Germany) before planting them into 1-l pots filled with washed sand or standard substrate. Plants were grown at 45–55% relative humidity and 24–26°C during days and 23–25°C during nights under 16 h of light (06:00–22:00 h). Plants were watered twice every day.

Soluble sugar, starch and protein concentrations in jasmonate signaling-impaired lines across different developmental stages

To investigate the possible role of jasmonates in the regulation of primary metabolism in *N. attenuata*, we measured glucose, fructose, sucrose, starch and soluble protein concentrations in the rosette leaves of jasmonate biosynthesis-deficient irAOC and jasmonate perception-impaired inverted repeat coronatine insensitive 1 (irCOI1) plants. As endogenous jasmonate levels change

Table 1 Characteristics of the inverted repeat (ir) *Nicotiana attenuata* transgenic lines used in the present study

Genotype	Gene silenced	Impaired function	Phenotype	Reference
irSIPK	Salicylic acid-induced protein kinase	Early jasmonate signaling	Reduced levels of jasmonates	Meldau *et al.* (2009)
irWIPK	Wound-induced protein kinase			
irGLA1	Glycerolipase A1	Jasmonate biosynthesis		Bonaventure *et al.* (2011)
irAOS	Allene oxide synthase			Kallenbach *et al.* (2012)
irAOC	Allene oxide cyclase			
irOPR3	12-oxo-phytodienoic acid reductase			
irJAR4/6	JA-Ile synthetase		Reduced levels of JA-Ile	Wang *et al.* (2008)
irCOI1	Coronatine-insensitive 1	JA-Ile perception	Reduced JA-Ile perception	Paschold *et al.* (2007)
irRCA	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase	Photosynthesis	Reduced photosynthetic activity	Mitra & Baldwin (2008)
irAOC × irRCA	Allene oxide cyclase and ribulose-1,5-bisphosphate carboxylase/oxygenase activase	Jasmonate biosynthesis and sugar metabolism	Reduced sugar concentrations compared with irAOC plants	Present study

JA-Ile, jasmonoyl-L-isoleucine.

over plant development (Abdala *et al.*, 2002), we measured sugar concentrations at four different developmental stages: early rosette (32 d after germination; DAG), rosette (38 DAG), elongation (44 DAG) and early flowering (50 DAG). Sugar and starch concentrations were quantified as described by Machado *et al.* (2013). Briefly, soluble sugars were extracted from plant tissue using 80% (v/v) ethanol, followed by an incubation step (20 min at 80°C). Pellets were re-extracted twice with 50% (v/v) ethanol (20 min at 80°C). Supernatants from all extraction steps were pooled together, and sucrose, glucose and fructose were quantified enzymatically as described by Velterop & Vos (2001). The remaining pellets were used for an enzymatic determination of starch (Smith & Zeeman, 2006). In addition, total soluble protein was quantified (Bradford, 1976). As protein solubility is affected by pH, total soluble protein levels may be underestimated by this method. Five independent replicates of each genotype and developmental stage were analyzed. Plant leaves were harvested at 13:00 h and flash frozen in liquid nitrogen for analysis.

Constitutive jasmonate and soluble sugar concentrations in jasmonate-deficient plants

To assess the importance of jasmonates for sugar accumulation, we evaluated eight different genetically engineered lines that differ in their capacity to produce jasmonates because they are deficient in either jasmonate biosynthesis or in the upstream signaling network. We measured glucose, fructose and sucrose concentrations, as well as constitutive JA and jasmonoyl-L-isoleucine (JA-Ile), in the leaves of rosette stage plants of all genotypes. Phytohormone measurements were carried out as described by Machado *et al.* (2013). Plants were harvested at 10:00 h ($n = 5$). Sugars were then correlated with phytohormone levels.

Diurnal changes in invertase activity and soluble sugar concentrations in jasmonate-deficient irAOC and EV plants

Invertases cleave sucrose into glucose and fructose following a diurnal pattern (Sturm & Tang, 1999; Nägele *et al.*, 2010).

Higher invertase activity might therefore lead to higher glucose and fructose pools. To investigate whether the higher glucose and fructose concentrations observed in jasmonate biosynthesis-deficient irAOC plants can be attributed to higher invertase activity, we measured the activity of soluble and insoluble invertases and correlated the ratio of sucrose (precursor) to glucose and fructose (products) with the measured enzyme activities. Invertase activities and sugar concentrations were measured from leaf extracts of rosette stage EV and irAOC plants at five times of the day: 07:00, 10:00, 13:00, 17:00 and 21:00 h. Five independent replicates (plants) of each genotype were harvested per time point. Enzyme activities (Ferrieri *et al.*, 2013) and sugar concentrations were measured as described by Machado *et al.* (2013).

Effect of soluble sugars on caterpillar growth

Low secondary metabolite levels are generally assumed to be responsible for the increased larval growth of herbivores on jasmonate signaling-impaired plants (Halitschke & Baldwin, 2003; Rayapuram & Baldwin, 2006; Paschold *et al.*, 2007). To determine whether the higher soluble sugar concentrations in jasmonate-deficient plants contribute to the increased *M. sexta* larval growth, we manipulated sugar concentrations *in planta* and *in vitro* and measured caterpillar growth in five different experiments as follows.

Caterpillar growth on sugar-restored, jasmonate biosynthesis-deficient plants To decrease soluble sugars in irAOC plants, we produced a hemizygous irAOC × irRCA line by crossing an irAOC line with an irRCA line. Silencing RCA slightly impairs photosynthetic activity in *N. attenuata* (Mitra & Baldwin, 2008), and we therefore hypothesized that a reduction in the photosynthetic capacity should reduce sugar concentrations and, consequently, the hemizygous jasmonate-deficient plants should have restored wild-type (WT) sugar concentrations. To test the validity of this assumption, sugar concentrations were measured in the leaves of EV, irRCA, irAOC and irAOC × irRCA plants at 05:00 h (end of the dark period) and 13:00 h (middle of the light period). As *M. sexta* herbivory has been shown to reduce sugar

concentrations in *N. attenuata* (Machado *et al.*, 2013), we also measured sugar concentrations after simulated (wounding and *M. sexta* oral secretion treatments, W + OS) and actual (three neonates per plant for 6 d) *M. sexta* herbivory. Intact plants served as controls (*n* = 5). *Manduca sexta* herbivory (W + OS) was simulated by rolling a fabric pattern wheel three times on each side of the midvein of fully developed rosette leaves. The wounds were immediately treated with 20 µl of a 1 : 5 (v/v) milliQ water-diluted *M. sexta* oral secretion solution. The treatments were repeated three times every other day. Following the validation of this *in vivo* approach, we determined caterpillar growth on the different genotypes. Two *M. sexta* neonates were placed on rosette stage plants and allowed to feed freely (*n* = 25). Seven and 9 d later, their mass was determined using a microbalance (Sartorius TE214S; Data Weighing Systems Inc., Elk Grove, IL, USA). *Manduca sexta* eggs were derived from an in-house colony and reared as described by Grosse-Wilde *et al.* (2011).

Caterpillar growth on plants with reduced photosynthetically active radiation (PAR) As an alternative means of reducing soluble sugars in irAOC plants, we reduced the amount of PAR by covering rosette leaves with a green filter (Roscolux #4430; Rosco Laboratories Inc., Stamford, CT, USA). We hypothesized that reducing PAR supply should reduce sugar concentrations in the leaves. Plants covered with a clear filter (Roscolux #000; Rosco Laboratories Inc.) were used as controls. Sugar concentrations were quantified 3 d after the start of the PAR reduction treatment as described by Machado *et al.* (2013). Plants for the sugar measurements were harvested at 09:00 h. Head space temperature and humidity, red to far-red ratios, starch (Machado *et al.*, 2013), soluble proteins (Bradford, 1976), average internode length and number of flowers were also quantified to assess whether the filters elicited shade avoidance responses and other secondary effects. To evaluate caterpillar growth, two *M. sexta* neonates were placed on rosette stage plants (*n* = 30) and allowed to feed freely. Seven and 9 d later, larval mass was determined as described earlier.

Caterpillar growth on semi-artificial diets *Manduca sexta*-induced jasmonate signaling depletes soluble sugars and induces secondary defensive metabolites in the leaves of *N. attenuata* plants; in contrast with EV plants, sugars are not depleted and secondary defensive metabolites are not induced in response to *M. sexta* simulated herbivory in jasmonate biosynthesis-deficient irAOC plants (Machado *et al.*, 2013). To understand whether the better *M. sexta* growth on jasmonate-deficient plants is a result of their increased sugar concentration and/or their decreased levels of secondary metabolites, we performed an experiment with semi-artificial diets in which sugars were complemented to match those of WT and control plants. The diets were prepared as described later, but the wheat germ was replaced with 25 g of dried *N. attenuata* leaves. To generate the necessary plant material, we treated irAOC and EV plants with *M. sexta* oral secretions (W + OS induction) as described by Machado *et al.* (2013). These treatments induce plant defenses and deplete sugars in the leaves of *N. attenuata* in a JA-dependent manner

(Machado *et al.*, 2013). After the treatments, we collected and dried the leaves (24 h at 50°C). Plants were harvested at 13:00 h. Diets prepared with untreated plant material served as controls. Sugar concentrations in the semi-artificial diets were determined, and subsets of diet cubes were complemented with pure sugars to match WT and control levels. *Manduca sexta* growth on the different diets was then measured over 12 d. Forty-four neonates per diet type (four larvae per plate; 11 plates per diet type) were fed *ad libitum* and the diet cubes were replaced every other day. In addition, caterpillar survivorship was recorded. As sugar complementation of plants may induce secondary responses (Rolland *et al.*, 2006), the above approach allowed us to test the direct contribution of soluble sugars to herbivore growth in a plant matrix.

Caterpillar growth on artificial diets enriched in glucose and fructose To evaluate the individual effect of glucose and fructose on *M. sexta* growth, we prepared artificial diets with different concentrations of glucose and/or fructose (see later, Fig. 7, for treatment combinations) and measured *M. sexta* growth. The diets were prepared essentially as described by Pohlon & Baldwin (2001) without sucrose, plant material and antibiotics. Briefly, 17 g of agar were dissolved in 500 ml of water at 50°C and mixed with 55 g wheat germ, 12 g yeast extract, 9 g Wesson salt mixture, 3.5 g ascorbic acid, 2.5 g cholesterol, 1.5 g sorbic acid, 5 ml raw linseed oil, 1.5 ml formalin and 9 ml vitamin mixture (100 mg nicotinic acid, 500 mg riboflavin, 233.5 mg thiamine, 233.5 mg pyridoxine, 233.5 mg folic acid and 20 mg l^{-1} biotin in water). The produced food was aliquoted into small plastic boxes and kept at 8°C until use. Glucose and fructose were dissolved in water and added to the diet cubes. Diets were freshly prepared and replaced every other day. Forty caterpillars (four larvae per plate; 10 plates per diet type) were fed *ad libitum* in a climate chamber (45–55% relative humidity, 24–26°C during days and 23–25°C during nights under 16 h of light). Larval mass was determined as described earlier, 7 and 9 d after the beginning of the experiment (*n* = 40).

Interaction between protein and soluble sugars The performance of insect herbivores depends, among other factors, on protein : carbohydrate ratios (Raubenheimer *et al.*, 2005; Simpson & Raubenheimer, 2009; Roeder & Behmer, 2014). To investigate whether the observed negative effect of increased dietary soluble sugars on *M. sexta* growth changes with the amount of available protein, we prepared artificial diets with variations in sugar and protein concentration and measured *M. sexta* larval mass and the amount of ingested diet, and calculated the efficiency of conversion of ingested food (Waldbauer, 1968). Diets were prepared according to Pohlon & Baldwin (2001). Sucrose was replaced by increasing concentrations of glucose and fructose (1, 6 and 12 mg g^{-1} of diet) to mimic the actual differences in soluble sugar profiles between jasmonate biosynthesis-deficient irAOC and WT plants. To increase the protein concentration of the diets, casein was added at concentrations of 50 or 150 mg g^{-1} FW. Soluble protein concentrations in plants estimated by the Bradford method can reach 14.47 mg g^{-1} in the leaves of some plant species (Ruiz & Romero, 1999). Thirty neonates (three

larvae per plate; 10 plates per diet type) were fed *ad libitum* and the above parameters were determined 9 d after the beginning of the experiment ($n = 30$).

Statistics

Unless otherwise stated, statistical tests were carried out with Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA) using analysis of variance. Levene's and Shapiro–Wilk tests were applied to determine error variance and normality. Holm–Sidak and Dunn's *post hoc* tests were used for pairwise or multiple comparisons. Datasets from experiments that did not fulfil the assumptions for ANOVA were natural log-, root square- or rank-transformed before analysis. The effect of semi-artificial diets on caterpillar survivorship was analyzed in R (R Development Core Team, 2012) using generalized linear models (GLMs), under a quasibinomial distribution with *F*-test. Residual analysis was carried out to verify the suitability of error distribution and model fitting. Details on specific tests carried out in each experiment are provided in Supporting Information Notes S1.

Results

Jasmonate signaling negatively affects leaf glucose and fructose concentrations

Leaf soluble protein levels of jasmonate perception-impaired irCOI1 and jasmonate biosynthesis-deficient irAOC plants were similar to those observed in jasmonate-competent EV plants across different developmental stages (Fig. 1a–d). Starch did not differ between genotypes at early rosette (Fig. 1e), rosette (Fig. 1f) and elongated (Fig. 1g) stages. Early flowering irCOI1, but not irAOC, plants contained higher leaf starch concentrations than EV plants (Fig. 1h). Glucose and fructose concentrations were higher in jasmonate signaling-impaired plants compared with EV controls at early rosette (Fig. 1i), elongated (Fig. 1k) and early flowering (Fig. 1l) stages. At the rosette stage, only fructose concentrations in irAOC plants were elevated compared with EV controls (Fig. 1j). Sucrose concentrations did not differ between genotypes at any of the evaluated developmental stages (Fig. 1i–l).

JA concentrations are negatively correlated with leaf soluble sugars across different jasmonate-deficient genotypes

Across eight jasmonate-deficient transgenic lines (Fig. 2a), we found significant variations in soluble sugar, JA and JA-Ile concentrations (Fig. 2b,c). A significant negative correlation between soluble sugar and constitutive JA concentrations ($P < 0.001$) was observed (Fig. 2b). By contrast, no significant correlation between soluble sugar and JA-Ile concentrations ($P = 0.213$) was found (Fig. 2c). Together, the above experiments demonstrate that jasmonates negatively affect soluble sugar concentrations in *N. attenuata* leaves.

Jasmonate-dependent sugar suppression is correlated with decreased invertase activity

Over the course of the day, we found that leaves of jasmonate biosynthesis-deficient irAOC plants accumulated less sucrose from 13:00 to 17:00 h, but more glucose and fructose from 10:00 to 21:00 h, compared with EV controls (Fig. 3a,b). Moreover, soluble invertase activity was increased in jasmonate biosynthesis-deficient irAOC plants compared with EV plants from 13:00 to 21:00 h (Fig. 3c), an effect which correlated negatively with the ratios of sucrose to fructose and glucose across the different samples and time points (Fig. 3d). By contrast, insoluble invertase activity was not altered by jasmonates and not correlated with soluble sugar ratios (Fig. 3e,f).

Jasmonate-dependent sugar suppression improves herbivore weight gain

To understand whether higher leaf sugar concentrations in jasmonate biosynthesis-deficient irAOC plants improve *M. sexta* growth, we reduced sugar concentrations in irAOC plants by silencing RCA activity. A reduction in RCA activity did not affect soluble sugars in EV plants, but decreased glucose and fructose concentrations in irAOC plants (Fig. 4, S1). The partial restoration of WT sugar concentrations in the irAOC × irRCA crosses was even more pronounced in herbivory-induced plants (Fig. 4). Sucrose and soluble protein concentrations remained largely unchanged (Figs 4, S2), apart from a slight increase in constitutive sucrose concentrations in irAOC × irRCA plants. From these results, we deduced that silencing RCA partially restored WT sugar concentrations in jasmonate biosynthesis-deficient irAOC plants, and these lines could be used to test the influence of soluble sugars on *M. sexta* growth *in planta*. Our initial expectation was that increased glucose and fructose concentrations would increase *M. sexta* growth. In contrast with this hypothesis, we found that the reduced sugar concentrations in irAOC × irRCA plants increased *M. sexta* growth even beyond the highly increased mass gain in the irAOC plants (Fig. 4j). This result suggests that jasmonate-dependent sugar depletion reduces rather than enhances plant resistance.

Manduca sexta gains more weight on PAR-limited, sugar-deprived plants

As a second approach to manipulate plant sugar concentrations, we reduced PAR supply by 43% (Fig. 5a). As expected, reducing PAR significantly reduced sugar concentrations in both EV and irAOC plants (Fig. 5b). Although the green filters slightly changed the red : far-red ratios (Fig. S3a), we found little phenotypic evidence for the activation of shade avoidance responses in PAR-reduced plants (Fig. S4a,b). We also found no changes in head space temperature and humidity (Fig. S3b,c). Starch and soluble protein concentrations also remained unaltered (Fig. S3c,d). Overall, *M. sexta* larvae gained more weight on irAOC plants than on EV plants (Fig. 5c). PAR reduction significantly increased caterpillar weight gain independent of the
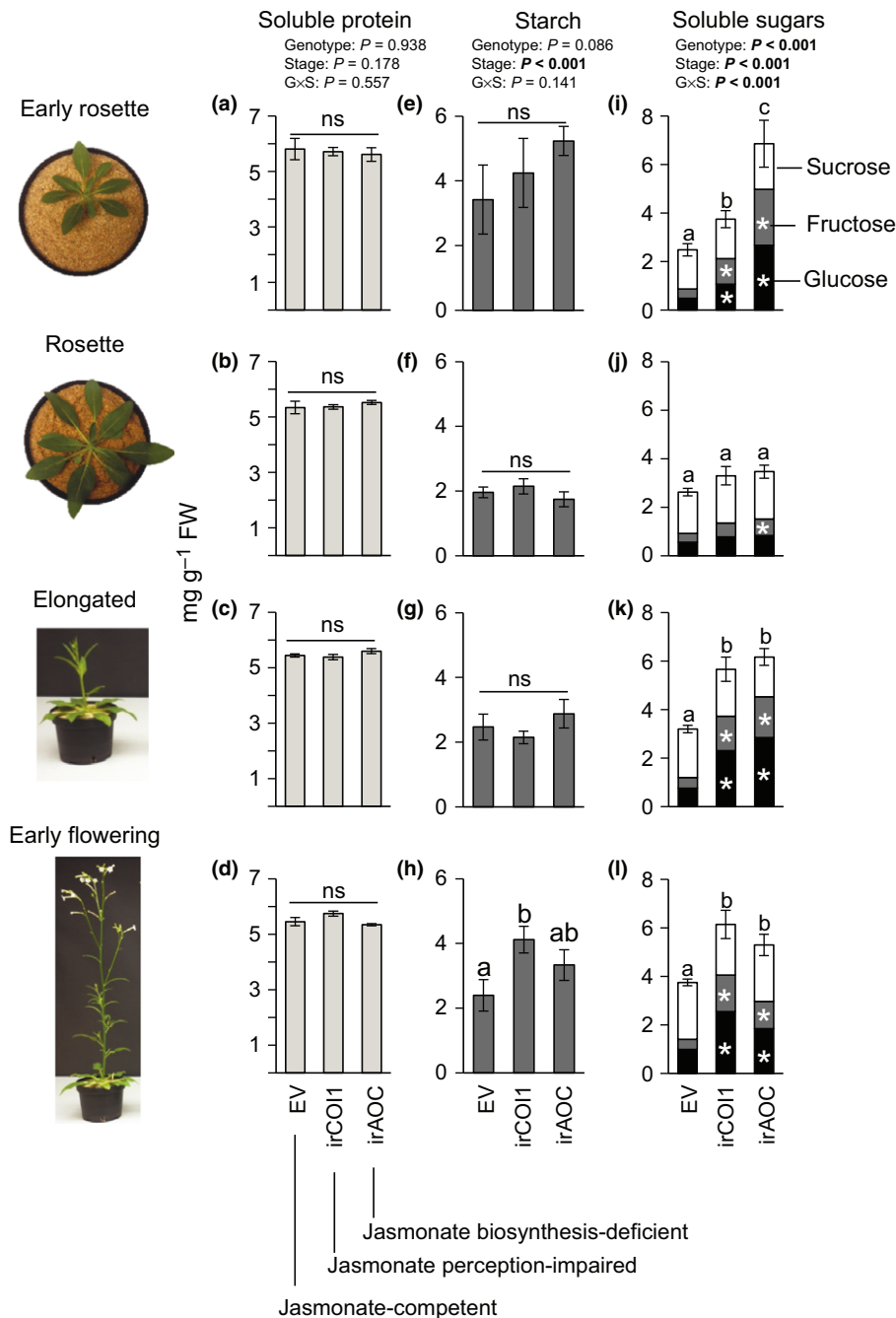
Fig. 1 Jasmonate signaling-impaired *Nicotiana attenuata* plants accumulate higher sugar concentrations than jasmonate-competent empty vector (EV) plants across different developmental stages. Average (± SE) (a–d) soluble protein, (e–h) starch and (i–l) soluble sugar concentrations of rosette leaves at (a, e, i) early rosette, (b, f, j) rosette, (c, g, k) elongated and (d, h, l) early flowering stages. Different letters indicate significant differences ($P < 0.05$, Holm–Sidak *post hoc* tests) in total sugar (glucose, fructose and sucrose) concentration among genotypes within developmental stages. Asterisks indicate significant differences of each individual metabolite (glucose, fructose or sucrose) concentration among genotypes (irAOC or irCOI1) compared with EV within developmental stages (Holm–Sidak *post hoc* tests: *, $P < 0.05$; ns, not significant) ($n = 5$).

plant's capacity to produce jasmonates (Fig. 5c). As irAOC plants contain higher sugar levels, but also lower secondary metabolites, compared with EV plants, both factors potentially contribute to the observed caterpillar growth rates.

Manduca sexta grows better on low secondary metabolite and low sugar-containing semi-artificial diets

In a third approach, we complemented semi-artificial diets with soluble sugars. This resulted in seven different diets with different sugar concentrations and added plant materials (Fig. 6a). Sugar complementation effectively increased control EV sugar concentrations to match those of control irAOC plants, and herbivory-suppressed EV sugar concentrations to match those of control EV and herbivory-induced irAOC concentrations (Fig. 6a). *Manduca sexta* weight gain did not differ between larvae that fed on uninduced EV and irAOC diets (Fig. 6b, bars A, C). When sugar concentrations in uninduced EV diets were complemented to uninduced irAOC concentrations, *M. sexta* growth was reduced (Fig. 6b, bar B). When feeding on W + OS-induced plant material, *M. sexta* growth was lower on EV than on irAOC diet (Fig. 6b, bars D, G). When sugar concentrations of W + OS-induced EV plant material were complemented to uninduced EV controls or to uninduced irAOC concentrations, *M. sexta* growth

Fig. 2 Constitutive jasmonic acid and soluble sugar concentrations are negatively correlated. (a) Schematic representation of the jasmonate signaling cascade. (b, c) Correlation between average ($\pm$ SE) soluble sugars (glucose, fructose and sucrose; Glu + Fru + Suc) and (b) average ($\pm$ SE) constitutive jasmonic acid levels (JA) or (c) constitutive jasmonoyl-L-isoleucine (JA-Ile) levels. GLA1, glycerolipase A1; SIPK, salicylic acid-induced protein kinase; FACs, fatty acid–amino acid conjugates; WIPK, wound-induced protein kinase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR3, 12-oxophytodienoic acid (OPDA) reductase; OPC-8:0, 3-oxo-2-(2′-pentenyl)-cyclopentane-1-octanoic acid; JAR 4/6, JA-amino acid synthetase; COI1, coronatine insensitive 1; JAZ, Jasmonate ZIM-domain protein; TF, transcription factors. Metabolite analysis was carried out in five independent replicates of each *Nicotiana attenuata* genotype.
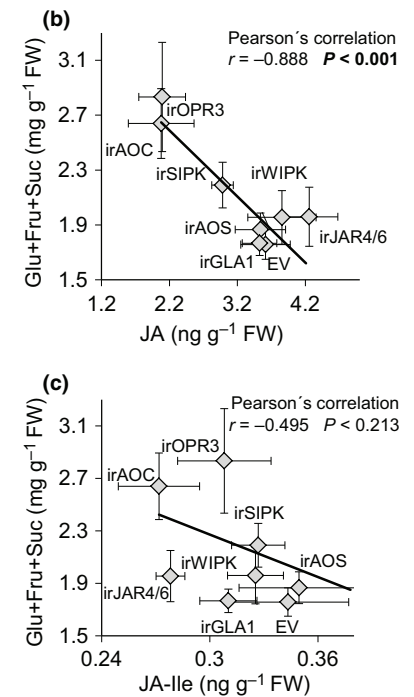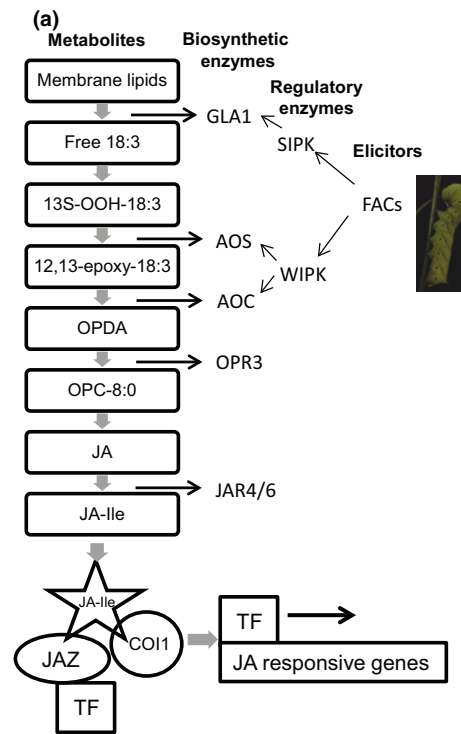
was further reduced (Fig. 6b, bars E, F). A similar pattern was observed for caterpillar survival (Fig. 6c). Taken together, these results show that the W + OS-triggered induction of defense reduces *M. sexta* growth and survival in a jasmonate-dependent manner, but that the jasmonate-dependent W + OS-induced sugar depletion increases *M. sexta* growth and thereby compromises induced resistance.

Manduca sexta grows better on low sugar-containing artificial diets

To disentangle the individual contribution of glucose and fructose to *M. sexta* growth suppression, we complemented artificial diets with different combinations of the two sugars at physiologically relevant concentrations. *Manduca sexta* growth strongly decreased with increasing amounts of glucose and fructose in a dose-dependent manner, independent of the combination of sugars (Fig. 7). On an individual basis, increased fructose decreased *M. sexta* growth to a greater extent than did increased glucose (Fig. 7).

Excess protein reverses the negative effect of soluble sugars on *M. sexta* weight gain

At a protein supply of 50 mg g^{-1} FW, *M. sexta* grew less well on higher soluble sugar diets in a dose-dependent manner (Fig. 8a). However, when excess protein at a concentration of 150 mg g^{-1} FW was offered, the opposite effect was observed: *M. sexta* now gained more weight on sugar-rich diets (Fig. 8a). We also found that the amount of ingested diet decreased with increasing sugar concentrations in a protein-independent manner (Fig. 8b). The

efficiency of conversion of ingested food did not change under normal protein supply, but tended to increase with sugar concentrations under excess protein (Fig. 8c). These results demonstrate that the negative effect of soluble sugars on *M. sexta* growth depends on the protein content of the food source. Under natural conditions, the amount of available protein in the leaves would result in a negative effect of sugars on *M. sexta* growth, as protein concentrations in plant leaves are below 50 mg g^{-1} FW.

Discussion

Our experiments demonstrate that jasmonates inhibit soluble invertases and reduce glucose and fructose concentrations in *N. attenuata* leaves, and that this reduction directly compromises plant resistance by increasing *M. sexta* growth and survival.

Across different developmental stages, times of day, and transgenic events, jasmonate signaling negatively influenced the concentrations of glucose and fructose in *N. attenuata* leaves, whereas only minor changes in starch and sucrose were found. This suggests that jasmonates specifically influence soluble monosaccharide concentrations. The differences between WT and jasmonate signaling-impaired plants were weaker at the late rosette stage than in younger and older plants. It remains to be determined to what extent this variation is a result of variation in jasmonate biosynthesis or downstream signaling. Constitutive and induced jasmonate levels are reduced in flowering *N. attenuata* plants, an effect that is correlated with the lower expression of jasmonate-dependent defenses (van Dam *et al.*, 2001; Kaur *et al.*, 2010; Diezel *et al.*, 2011; Onkokesung *et al.*, 2012). The fact that jasmonate-dependent sugar regulation did not follow this pattern
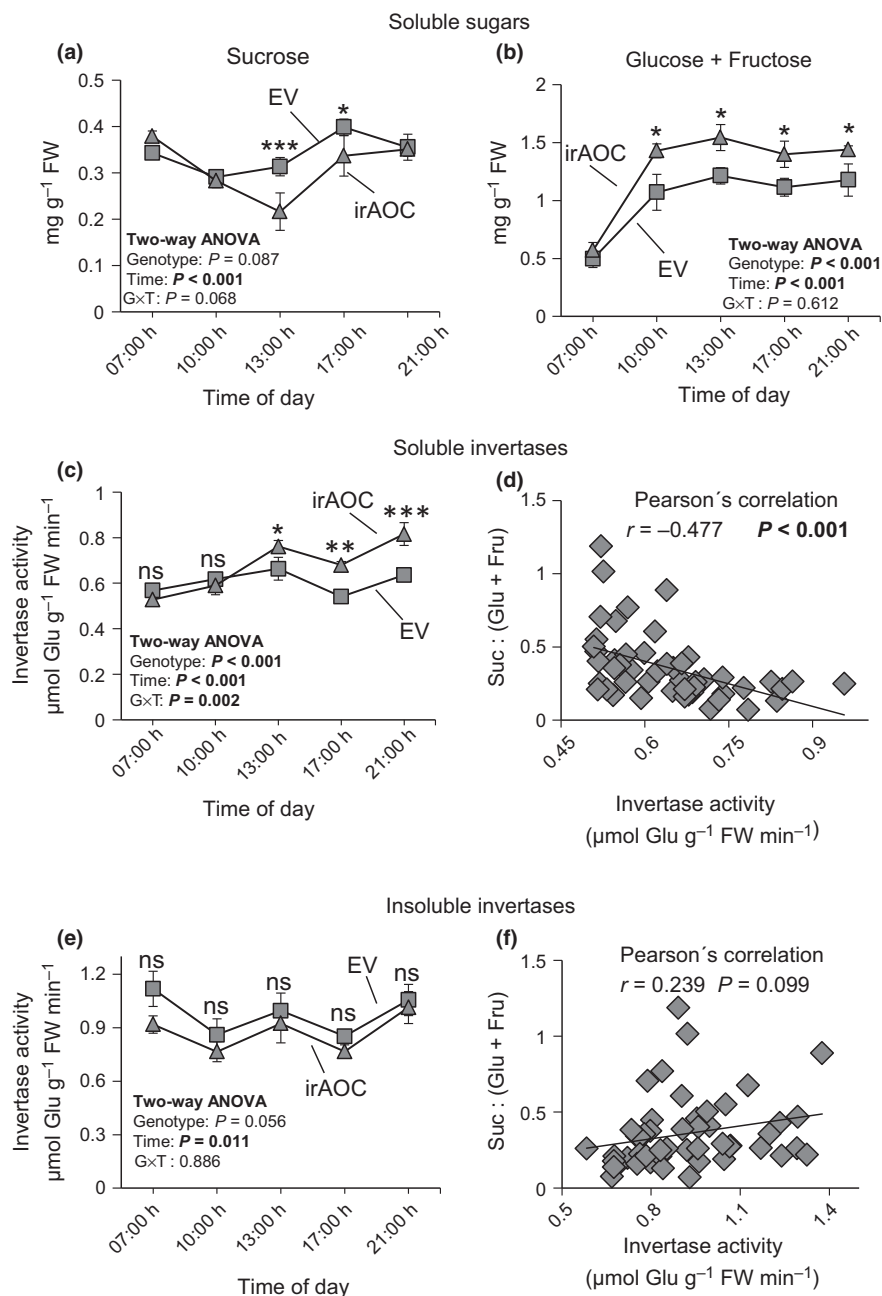
Soluble sugars

(a) Sucrose

(b) Glucose + Fructose

Soluble invertases

(c)

(d) Pearson´s correlation
$r = -0.477$ $P < 0.001$

Insoluble invertases

(e)

(f) Pearson´s correlation
$r = 0.239$ $P = 0.099$

Fig. 3 Diurnal jasmonate-dependent suppression of soluble sugars is associated with decreased invertase activity. Average ($\pm$ SE) (a) sucrose and (b) glucose and fructose concentrations. Average ($\pm$ SE) (c) soluble and (e) insoluble invertase activity. (d, f) Correlation between the ratio of sucrose (Suc) to glucose (Glu) and fructose (Fru) and (d) soluble invertase activity or (f) insoluble invertase activity. Asterisks indicate significant differences within each time point (Holm–Sidak *post hoc* tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant). Metabolite and enzyme activity measurements were carried out in five independent replicates (individual *Nicotiana attenuata* plants) for each genotype and time point. EV, empty vector; irAOC, allene oxide cyclase-silenced plants.

suggests a role of downstream signaling, rather than a direct effect of jasmonate biosynthesis, on the observed developmental patterns.

We found that the total soluble protein remained constant in the leaves of *N. attenuata* plants across different developmental stages, times of day, transgenic events and plant treatments. It is important to note that we measured protein concentration by the Bradford method. As this method requires acidic conditions, and as the solubility of RuBisCO, one of the most abundant proteins, is decreased under low pH, we may have underestimated the total protein concentrations. However, using ^{15}N labeling and LC-MS[E], it was demonstrated that investment into RuBisCO biosynthesis is not changed in jasmonate-deficient inverted repeat

lipoxygenase (irLOX3) *N. attenuata* plants (Ullmann-Zeunert *et al.*, 2013). However, herbivore attack decreased RuBisCO levels in WT and, albeit to a lesser extent, irLOX3 plants (Ullmann-Zeunert *et al.*, 2013). A detailed analysis of the most abundant soluble proteins in jasmonate signaling-impaired plants using similar approaches might help us to understand whether and how jasmonate signaling regulates soluble protein levels in plants in more detail.

Over the course of the day, we observed that deficiencies in jasmonate signaling increased glucose and fructose concentrations, as well as invertase activity, in the leaves of rosette stage *N. attenuata* plants during the light phase. We also noted a slight suppression in sucrose concentrations around midday. Based on
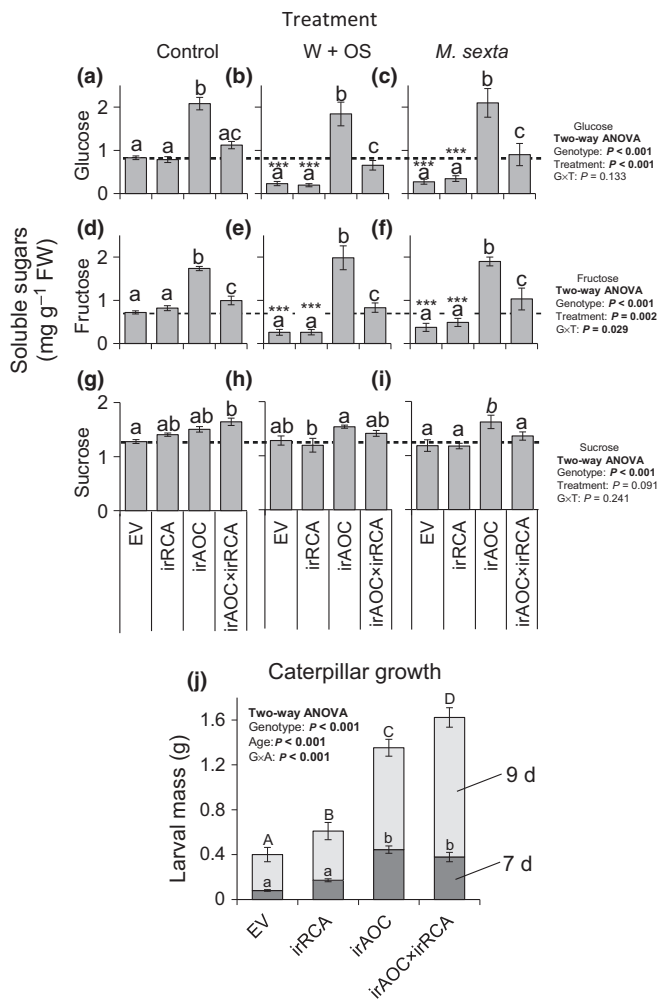
Fig. 4 Reducing soluble sugar concentrations through ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RCA) inhibition increases *Manduca sexta* growth. Average ($\pm$ SE) (a, b, c) glucose, (d, e, f) fructose and (g, h, i) sucrose concentrations of (a, d, g) control, (b, e, h) wounding and *M. sexta* oral secretion (W + OS)-treated and (c, f, i) *M. sexta*-attacked plants harvested at 13:00 h. (j) Average ($\pm$ SE) mass of *M. sexta* larvae reared on different *Nicotiana attenuata* genotypes (n = 35–50). irRCA, ribulose-1,5-bisphosphate carboxylase/oxygenase activase-silenced plants; irAOC, allene oxide cyclase-silenced plants; irRCA $\times$ irRCA, hemizygous crosses between AOC- and RCA-silenced plants; EV, empty vector-transformed plants. Different letters indicate significant differences (Holm–Sidak *post hoc* tests: $P < 0.05$) among genotypes within each treatment and metabolite for (a–i) and within time point for (j). Asterisks indicate significant differences among each treatment and control plants within each genotype and metabolite (Holm–Sidak *post hoc* tests: ***, $P < 0.001$). Dashed lines indicate the levels of each metabolite for intact EV plants. Metabolite measurements were carried out in five independent replicates of each genotype and treatment. (j) The lower portion represents the weight that caterpillars reached after 7 d, and the total height of the bars represents the weight that caterpillars reached after 9 d. Therefore, the upper portion represents only the weight that caterpillars gained between day 7 and 9.

Fig. 5 Reduction in photosynthetically active radiation (PAR) in *Nicotiana attenuata* plants decreases soluble sugars and increases *Manduca sexta* growth in a jasmonate-independent manner. (a) PAR under PAR-reduced and normal glasshouse PAR conditions. (b) Average ($\pm$ SE) sugar concentrations of *N. attenuata* plants under PAR-reduced and normal conditions. (c) Average ($\pm$ SE) mass of 7- and 9-d-old *M. sexta* larvae feeding on plants grown under PAR-reduced and normal conditions (n = 36–48). Asterisks in (a) indicate a significant effect of filter treatments on PAR (Holm–Sidak *post hoc* tests: ***, $P < 0.001$). Asterisks in (b) indicate significant differences in sugar concentration between plants under PAR-reduced and normal PAR conditions within each genotype (Holm–Sidak *post hoc* tests: *, $P < 0.05$). Different letters indicate significant differences (Holm–Sidak *post hoc* tests: $P < 0.05$) among treatments within caterpillar age. Metabolite measurements were carried out in five independent replicates of each genotype and treatment. EV, empty vector; irAOC, allene oxide cyclase-silenced plants.

the positive correlation between invertase activities and precursor to product ratios of the different sugars, we propose that jasmonates might regulate sugar concentrations through the suppression of invertase activity. Invertases are well known to control the
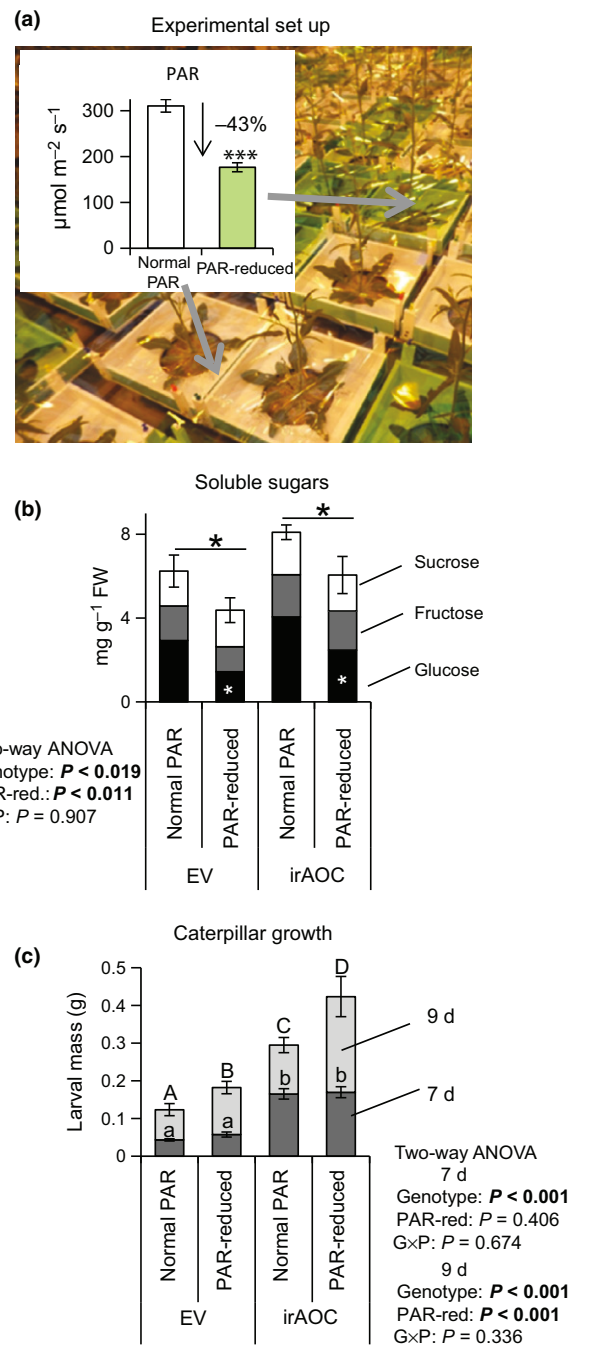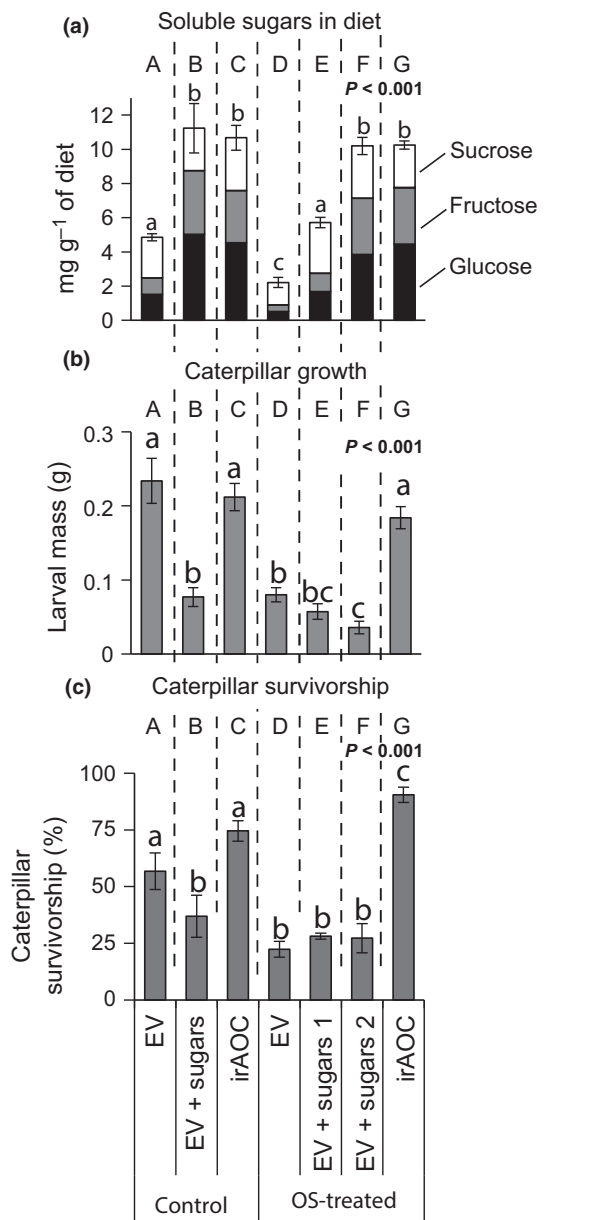
Fig. 6 Soluble sugar complementation of a semi-artificial diet reduces *Manduca sexta* growth and survival. (a) Average (± SE) soluble sugars in diets. (b) Average (± SE) mass of *M. sexta* larvae reared on semi-artificial diets that differ in their primary and secondary chemical profiles (*n* = 10–38). (c) Average (± SE) proportion of living caterpillars at the end vs the beginning of the experiment. Different letters in (a) and (b) indicate significant differences in total sugar and caterpillar growth (Dunn's *post hoc* tests: *P* < 0.05). Different letters in (c) indicate significant differences in caterpillar survival (*F*-test: *P* < 0.05). Uppercase letters (A–G) serve as a guide for the comparisons. EV, empty vector; irAOC, allene oxide cyclase-silenced plants.

Fig. 7 *Manduca sexta* grow less well on artificial diets containing higher glucose and fructose concentrations. Average (± SE) mass of 7- and 9-d-old *M. sexta* larvae reared on artificial diets containing variable amounts of glucose and fructose (*n* = 32–39). Different letters indicate significant differences within a time point (Holm–Sidak *post hoc* tests: *P* < 0.05).

correlation between the ratio of hexoses to sucrose and acid-soluble invertase activity in different potato cultivars. Silencing of a soluble acid invertase resulted in lower ratios of hexoses to sucrose. It is noteworthy to mention that other studies in poplar and thale cress have documented that exogenous jasmonate application increases invertase activity (Arnold & Schultz, 2002; Bogatek *et al.*, 2002; Arnold *et al.*, 2004; Ferrieri *et al.*, 2013; Horibe *et al.*, 2013). The fact that constitutive jasmonate deficiency led to higher invertase activity in *N. attenuata* leaves suggests that the outcome of induced jasmonates on invertase activity might be determined by their endogenous concentrations.

Although alteration in invertase activity can change the ratio of glucose and fructose to sucrose, the increase in glucose and fructose is not always proportional to the decrease in sucrose concentration (Tang *et al.*, 1999; Bhaskar *et al.*, 2010), indicating that total sugar pools might also be regulated by other factors, including, for example, changes in photosynthetic efficiency, carbon assimilation, glycolytic activity, sucrose synthase activity, sucrose transporter activities and mechanisms of phloem loading/unloading. Indeed, reducing the activity of RCA, which modulates the activity of RuBisCO, the enzyme that carries out the first major step of CO_2 fixation in plants (Raines, 2003), in jasmonate biosynthesis-deficient irAOC plants reduced glucose and fructose concentrations by 54% and 57%, respectively, suggesting that jasmonate deficiency might affect sugar accumulation via the regulation of this enzyme. Although we have no evidence for changes in glycolytic enzyme activity, sucrose synthase activity, sucrose transporter activities and mechanisms of phloem loading/unloading, future studies might investigate their contribution to the higher accumulation of glucose and fructose in jasmonate signaling-impaired plants.

Given the importance of jasmonates for plant–herbivore interactions, we were interested in understanding whether and how the jasmonate-dependent reduction of soluble sugars influences the resistance of *N. attenuata* to *M. sexta* caterpillars. Through four orthogonal lines of evidence, we show that *M. sexta* growth is reduced, rather than enhanced, through increased dietary

ratio of glucose and fructose to sucrose (Zrenner *et al.*, 1996; Ohyama & Hirai, 1999; Tang *et al.*, 1999; Jin *et al.*, 2009; Bhaskar *et al.*, 2010). Silencing of a vacuolar invertase gene in potato, *Solanum tuberosum*, was found to decrease vacuolar invertase activity, increase sucrose and reduce glucose and fructose (Bhaskar *et al.*, 2010). Similarly, Zrenner *et al.* (1996) found a positive
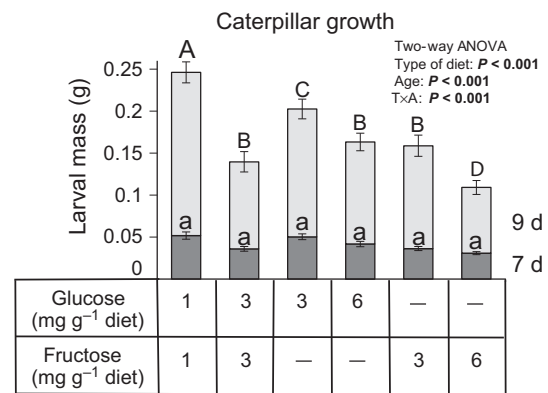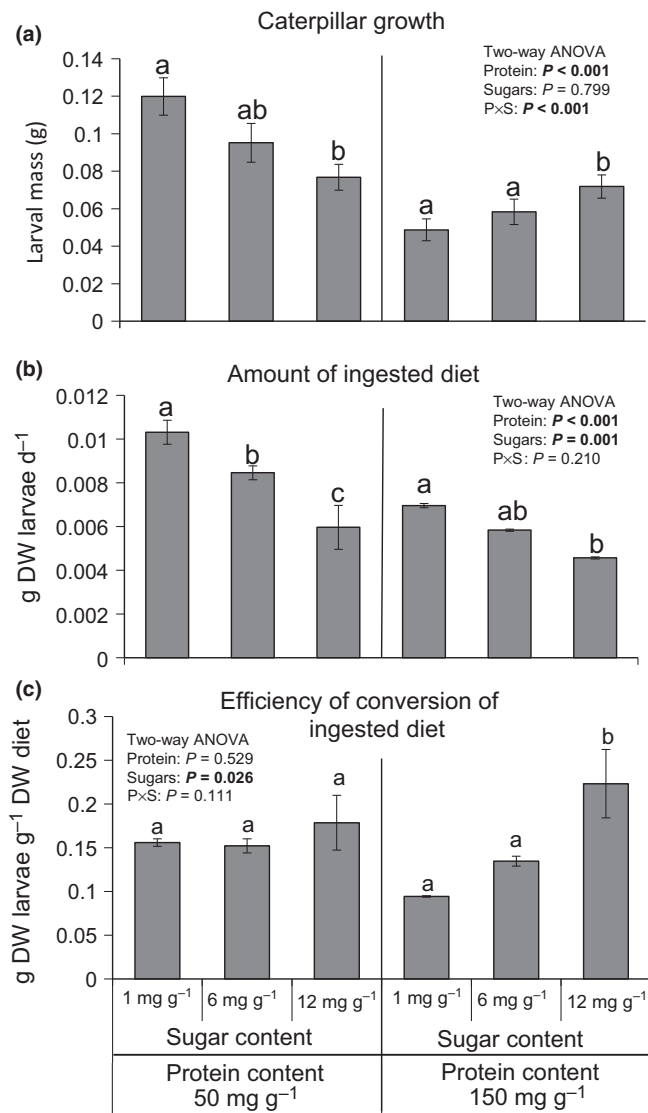
49

(a)

Caterpillar growth

Two-way ANOVA
Protein: **P < 0.001**
Sugars: P = 0.799
P×S: **P < 0.001**

(b)

Amount of ingested diet

Two-way ANOVA
Protein: **P < 0.001**
Sugars: **P = 0.001**
P×S: P = 0.210

(c)

Efficiency of conversion of ingested diet

Two-way ANOVA
Protein: P = 0.529
Sugars: **P = 0.026**
P×S: P = 0.111

Fig. 8 Increasing protein supply beyond natural concentrations inverts the effect of sugars on *Manduca sexta* growth. (a) Average (± SE) mass of *M. sexta* larvae reared on artificial diets containing different amounts of protein and soluble sugars (n = 23–30). (b) Average (± SE) amount of ingested diets by *M. sexta* larvae. (c) Average (± SE) efficiency of conversion of ingested food. Different letters indicate significant differences (Holm–Sidak *post hoc* tests: P < 0.05) within type of diet.

carbohydrates, an effect that is associated with a reduction in the amount of ingested food. First, reducing sugar concentrations through RCA silencing in jasmonate-deficient plants significantly improved *M. sexta* growth. irAOC plants are almost completely deficient in many defensive secondary metabolites (Machado *et al.*, 2013; Fragoso *et al.*, 2014). Therefore, this result points to a secondary metabolite-independent growth effect of soluble sugars on *M. sexta*. The better growth of *M. sexta* on nonhemizygous irRCA plants has been attributed to the reduced levels of secondary metabolites, an effect driven by the RCA-mediated redirection of the bioactive oxylipin JA-Ile to the inactive methyl jasmonate (Mitra & Baldwin, 2008, 2014). However, it is possible that this effect is also the result of reduced sugar

concentrations in this line at certain times of the day (see Supporting Information, Fig. S1). Second, reducing sugars by limiting PAR supply also improved *M. sexta* growth independent of the plant's capacity to produce jasmonates. Although PAR reduction may have additional effects on plant physiology, we did not find any evidence for either the shade avoidance response or changes in starch and protein production in plants growing under PAR-reduced conditions. Furthermore, the fact that we observed similar PAR effects on caterpillar growth in EV and irAOC plants allows us to exclude secondary effects on plant secondary metabolites as key determinants of insect growth (Paschold *et al.*, 2007). Third, supplementing minimal artificial diets containing dried plant material with sugars reduced *M. sexta* growth and survival. Artificial diets containing plant material have been used in previous studies to assess the contribution of jasmonate-induced changes in plant metabolism to *M. sexta* growth (Pohlon & Baldwin, 2001). *Manduca sexta* grows less well on artificial diets containing jasmonate-treated *N. attenuata* plant material than on diets containing nontreated plant material, an effect that is positively correlated with the jasmonate-dependent induction of protease inhibitors (PIs) and nicotine (Pohlon & Baldwin, 2001). The fact that caterpillars grew less well on induced EV than irAOC plants confirms that this assay can be used to reproduce natural resistance patterns, whilst allowing for the complementation of semi-artificial diet with soluble sugars without the confounding effect of sugar signaling on plant physiology. Fourth, complementing artificial diets with physiologically relevant concentrations of individual soluble sugars confirmed their negative, dose-dependent effect on *M. sexta* growth at physiologically relevant protein concentrations.

Carbohydrate-rich artificial diets have been shown to reduce insect performance (Raubenheimer & Simpson, 1997; Lee *et al.*, 2003; Raubenheimer *et al.*, 2005; Babic *et al.*, 2008; Merkx-Jacques *et al.*, 2008) and survival (Raubenheimer *et al.*, 2005) in earlier studies, effects that are associated with a greater propensity to store the excess of ingested carbohydrate as body fat (Chippindale *et al.*, 1996; Simpson *et al.*, 2004; Warbrick-Smith *et al.*, 2006). Although we did not determine lipid concentrations in the caterpillars, the fact that the semi-artificial diet experiment showed that larval survival and weight gain were positively correlated indicates that the heavier caterpillars are not necessarily fatter, but also fitter. Direct measurements of body fat would be necessary to confirm this hypothesis. The post-ingestion mechanisms that insects use to cope with an excess of dietary carbohydrates include the down-regulation of carbohydrate-catabolizing enzymes (Kotkar *et al.*, 2009; Clissold *et al.*, 2010), the increase in respiration rates (Zanotto *et al.*, 1997), the up-regulation of glucose-oxidizing enzymes (Merkx-Jacques & Bede, 2005) and the increase in carbohydrate egestion (Telang *et al.*, 2003; Babic *et al.*, 2008). In addition to the storage of excess dietary carbohydrates as body fat, the earlier mentioned mechanisms might result in metabolic costs for caterpillars that potentially reduce their optimal growth and development.

Insect guts host an enormous and phylogenetically diverse group of microorganisms (Engel & Moran, 2013). Although their beneficial roles are increasingly being recognized (Salem

et al., 2013, 2014), they are also potentially deleterious (Basset *et al.*, 2000; Nehme *et al.*, 2007; Buchon *et al.*, 2013). Alteration in the gut microbial homeostasis is known to influence insect behavior (Sharon *et al.*, 2010) and probably also affects insect performance. We hypothesize that increasing dietary carbohydrates could negatively impact *M. sexta* growth in two ways. The ingestion of high levels of carbohydrates might increase the size of microbial communities to levels that first outcompete *M. sexta* for limiting nutrients, such as nitrogen, and, second, alter the microbial community homeostasis so as to increase the prevalence of pathogenic microorganisms. Consistent with the first hypothesis, we found that the negative effects of ingesting an excess of dietary carbohydrates on *M. sexta* growth were reversed by increasing the amount of dietary protein. It remains to be investigated to what extent this process might be driven by changes in the *M. sexta* gut microbial community.

We found that the efficiency of conversion of ingested food tended to increase with increasing dietary protein concentration and, as a consequence, *M. sexta* might have been able to cope better with excess dietary carbohydrates. Protein quality and quantity are subject to considerable variation (Bloem & Duffey, 1990; Felton, 1996), and proteins can interact with plant secondary chemistry to affect digestibility (Zucker, 1983). Trypsin proteinase inhibitors (TPIs) could also modulate the effect of dietary protein content on tissue digestibility. It is worth mentioning that locusts have been shown to perform better on low-nitrogen plants (Cease *et al.*, 2012), indicating species-specific responses to this nutritional parameter. An experimental approach to manipulate protein levels *in planta* is to target RuBisCO, one of the most abundant leaf proteins (Felton, 1996; Taiz & Zeiger, 1998) and one of the main dietary proteins for herbivores (Felton, 1996, 2005). Mitra & Baldwin (2008) reduced the transcript levels of RuBisCO in *N. attenuata* by an *Agrobacterium*-mediated transformation, resulting in a decrease in this protein of up to 1.5-fold. Silencing the expression of this gene, together with reducing PAR and RCA transcripts, and/or sugar supplementation to semi-artificial diets, may allow for a better understanding of the contribution of protein, carbohydrates and their ratios to insect performance in a plant secondary chemistry context. It is important to note that the negative effect of sugars on caterpillar growth was only inverted when protein levels were increased significantly beyond those typically found in leaves. We therefore expect the magnitude, but not the direction, of the jasmonate-dependent sugar depletion effect to change with more modest changes in protein levels.

The results of our study are of potential significance for the evolution of plant defense syndromes. Natural *N. attenuata* populations exhibit high phenotypic plasticity in herbivory-induced jasmonate production (Machado *et al.*, 2013), which is positively correlated with secondary metabolite biosynthesis (Gaquerel *et al.*, 2009) and might therefore be correlated with herbivore resistance (Royo *et al.*, 1999; Halitschke & Baldwin, 2003; Li *et al.*, 2004; Kallenbach *et al.*, 2012). We found that jasmonate deficiency leads to higher sugar concentrations in leaves which may, in turn, reduce *M. sexta* performance. Therefore, this jasmonate-dependent sugar depletion might lead to

trade-offs that contribute to natural variation in jasmonate signaling in nature and may favor jasmonate-independent resistance mechanisms.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Silencing RuBisCO activase (RCA) reduces sugar concentrations in the leaves at the end of the dark phase.

Fig. S2 Soluble protein concentrations remain unaltered in response to simulated and actual *Manduca sexta* herbivory in empty vector (EV), inverted repeat allene oxide cyclase (irAOC), inverted repeat RuBisCO activase (irRCA) and irAOC × irRCA plants.

Fig. S3 Photosynthetically active radiation (PAR) reduction does not alter temperature and humidity in the plant headspace, but slightly reduces red : far-red ratios.

Fig. S4 No evidence of shade avoidance responses in photosynthetically active radiation (PAR)-reduced plants.

Notes S1 Detailed statistical tests.

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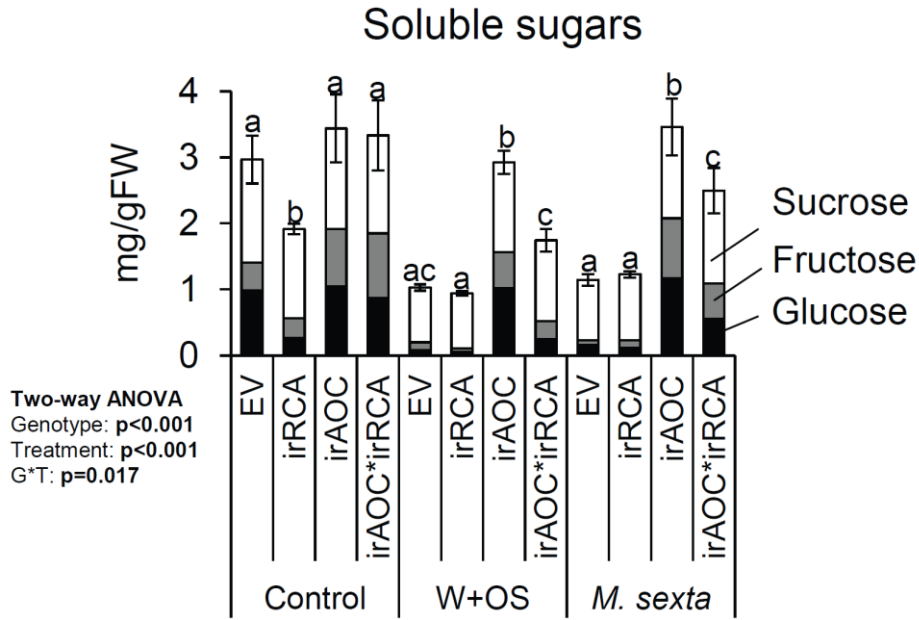
Supporting information

Figure S1

Figure S1. *IrAOC plants have higher, irRCA*irAOC intermediate and EV and irRCA plants lower sugar concentrations in the leaves.* Average (±SE) glucose, fructose and sucrose levels of control, W+OS-treated and *M. sexta*-attacked plants harvested at 5 am. IrRCA: RCA-silenced plants; irAOC: allene oxide cyclase-silenced plants; irRCA*irRCA: Hemizygous crosses between allene oxide cyclase and RCA-silenced plants; EV: empty vector tranformed-plants. Different letters indicate significant differences (p<0.05) among genotypes within each treatment.
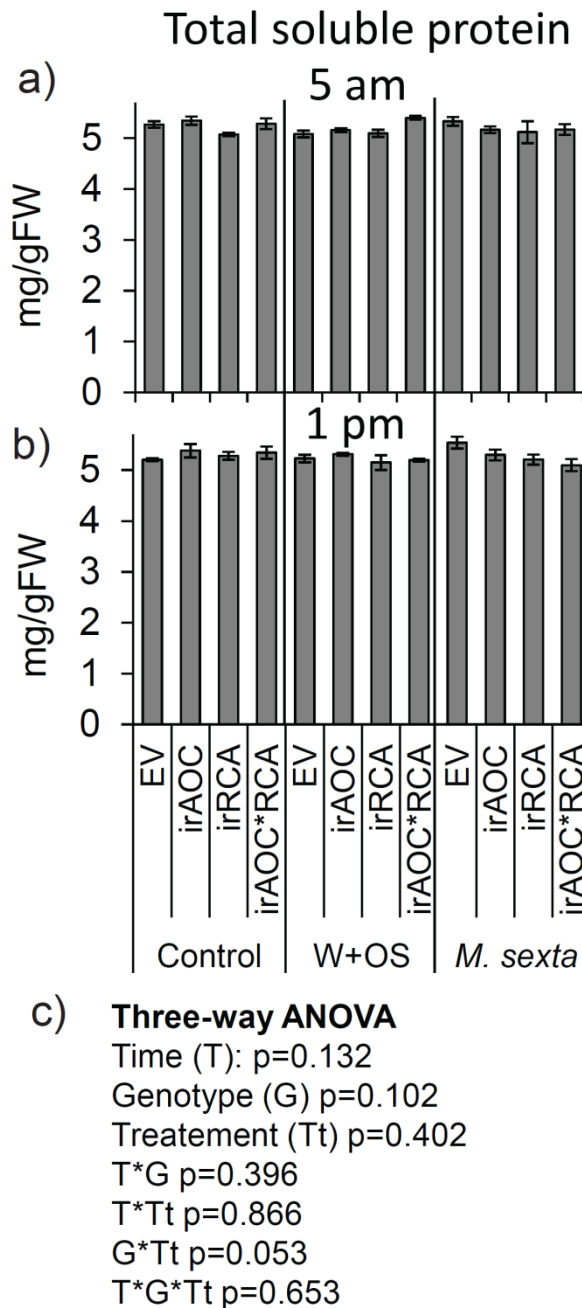
Figure S2

Figure S2. *Soluble protein levels remain unaltered in response to simulated and actual M. sexta herbivory in EV, irAOC, irRCA and irAOC*irRCA plants.* Average (±SE) soluble protein content of leaves harvested at 5 am (a) and 1 pm (b). Results of a three-way ANOVA are shown (c). Note that the Bradford assay used here cannot determine RuBisCO levels accurately, which have been shown to decrease upon herbivore attack.
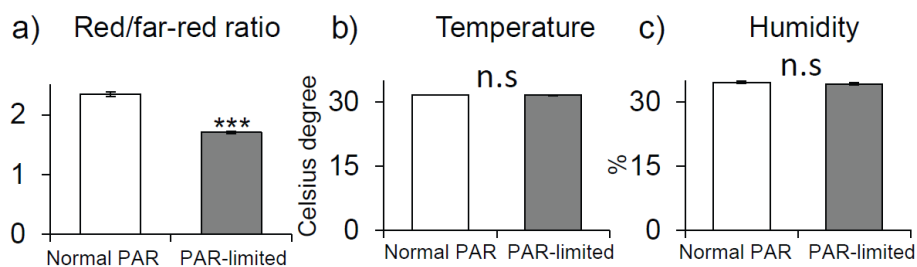
Figure S3

Figure S3. *PAR-reduction treatments do not alter temperature and humidity in the plant headspace, but slightly reduce red/far-red ratios.* Average (±SE) red/far-red ratio (a), temperature (b) and humidity in the plant headspace (c). Asterisks indicate significant differences (***, p<0.001). n.s: not significant.

Figure S4

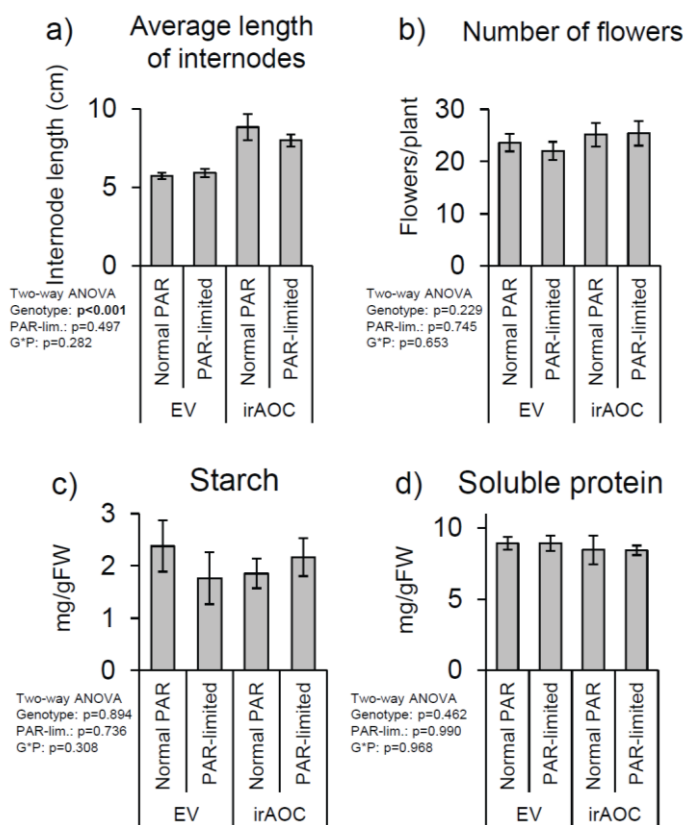

Figure S4. *No evidence of shade avoidance responses in PAR-reduced plants.* Average (±SE) length of internodes (a), number of flowers (b), starch content (c) and soluble protein (d).

Note S1. Detailed Statistical tests

Unless otherwise stated, all statistical tests were carried out with Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA) using analysis of variance (ANOVA). Levene's and Shapiro–Wilk tests were applied to determine error variance and normality. Two-way ANOVA and Holm–Sidak *post-hoc* tests were carried out to test the effect of jasmonate-deficiency on leaf sugar (each sugar type was tested individually), starch and protein levels with genotype and developmental stage as factors. Correlations between jasmonic acid (JA) and jasmonoyl-L-Isoleucine (JA-Ile) constitutive levels and soluble sugar content were tested using Pearson product moment tests. To assess the effect of jasmonate-deficiency on sugar levels and invertase activity, two-way ANOVA and Holm–Sidak *post-hoc* tests, with time of harvesting and genotype as factors, were carried out. Correlations between soluble sugars and invertase activity were tested using Pearson product moment tests. To test the effect of simulated and actual *M. sexta* herbivory on sugar and protein levels in EV, irRCA, irAOC and irAOC*RCA plants, two-way ANOVA and Holm–Sidak *post-hoc* tests, with genotype and treatment as factors were carried out for each time point individually. Caterpillar mass on EV, irRCA, irAOC and irAOC*irRCA plants was assessed by two-way ANOVA and Holm–Sidak *post-hoc* tests, with genotype and caterpillar age as factors. To test the effect of PAR-reduction on sugar, starch and soluble protein levels and average length of internodes and number of flowers, two-way ANOVA and Holm–Sidak *post-hoc* tests, with genotype and type of filter as factors, were carried out. Caterpillar mass on PAR-reduced plants was tested by two-way ANOVA and Holm–Sidak *post-hoc* tests, with genotype and type of filter as factors within each caterpillar age individually. The effect of glucose and fructose on caterpillar mass was tested by a two-way ANOVA and Holm–Sidak *post-hoc* tests, with type of diet and caterpillar age as factors. Sugar levels of and caterpillar mass on semi-artificial diets were assessed by one-way ANOVA and Dunn's *post-hoc* tests. In the caterpillar experiments, due to practical restrictions, we had multiple larvae per box and multiple boxes per type of diet (replicates). We assessed the effect of the diet type on caterpillar mass using either each caterpillar as an independent replicate or the mean values within each box as an independent replicate; similar statistical results were obtained. The effect of semi-artificial diets on caterpillar survivorship was analyzed in R (R Development Core Team, 2012) using Generalized Linear Models (GLM), under Binomial distribution with Chi-square tests. Residual analysis was carried out to verify the suitability of error distribution and model fitting. When the data were over dispersed, a quasi-binomial distribution with F-test was carried out. Caterpillar mass, amount of ingested food and efficiency of conversion of ingested food on artificial diets with variable concentrations of protein/and or sugars were tested by two-way ANOVA and Holm–Sidak *post-hoc* tests, with sugar and protein content as factors. Datasets from experiments that did not fulfill the assumptions for ANOVA were natural log-, root square- or rank-transformed prior to analysis.

Manuscript III

Rapid evolution and strong correlation between herbivory-induced root carbohydrate responses and defoliation tolerance among eight solanaceous species

Ricardo A.R. Machado, Wenwu Zhou, Abigail P. Ferrieri, Carla C.M. Arce, Ian T. Baldwin, Shuqing Xu, and Matthias Erb

Rapid evolution and strong correlation between herbivory-induced root carbohydrate responses and defoliation tolerance among eight solanaceous species

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Running title: Plant tolerance strategies evolve rapidly.

Key words: *Manduca sexta*, Solanaceae, herbivory-induced tolerance, phylogenetic signal, root carbohydrates, regrowth suppression

Abstract

Root carbohydrate pools are commonly assumed to determine plant defoliation tolerance. However, the connection between herbivory-induced changes in root soluble carbohydrates and regrowth remains unclear, as different plant species show divergent patterns. We attempted to link these two traits in a phylogenetic context by using eight solanaceous plant species from the genera *Petunia*, *Datura*, *Nicotiana* and *Solanum*. Changes in soluble root carbohydrate pools were strongly correlated with the plant's regrowth capacity: species that reduced root carbohydrate pools in response to simulated *Manduca sexta* herbivory regrew less, while species that maintained root carbohydrate levels tolerated herbivore attack. No species accumulated more soluble carbohydrates or increased their regrowth capacity in response to *M. sexta* attack. Furthermore, we found no phylogenetic signal in these responses. Even within the four closely related *Nicotiana* species, both negative and neutral carbohydrate and regrowth patterns were observed. These results suggest that root-mediated tolerance strategies are highly divergent and subject to rapid evolution within the Solanaceae. As induced tolerance and resistance in the Solanaceae are regulated by jasmonic acid signaling and subject to similar resource constraints, trade-offs between the two strategies may shape plant defenses of this family.

Introduction

Insect herbivores threaten the performance and fitness of plants. To minimize the impact of herbivory, plants have evolved several mitigating strategies, including resistance and tolerance. Resistance traits include the production of secondary metabolites and proteins that deter or intoxicate herbivores, or act as cues which increase herbivore predation by natural enemies (Baldwin 1998; Green& Ryan 1972; Heil 2015; Schuman *et al.* 2012; Zhu-Salzman *et al.* 2008). In turn, herbivores have evolved strategies to counteract plant defenses, for instance by detoxifying or excreting secondary metabolites, avoiding toxic tissues or minimizing predation risk (Berenbaum& Zangerl 1994; Kaplan& Thaler 2010; Lindigkeit *et al.* 1997; Mao *et al.* 2006; Zangerl& Berenbaum 1990). Resistance traits may therefore only be of limited value for plants facing specialized herbivores (Lefort *et al.* 2015; Machado *et al.*

2013; Mao *et al.* 2006; Robert *et al.* 2012). It is under these circumstances that tolerance traits may become particularly important (Agrawal& Fishbein 2008; Robert *et al.* 2014). Plant tolerance to herbivory is defined as the capacity of plants to reduce the fitness costs imposed by herbivore damage and is typically expressed as fitness as a function of damage (Strauss& Agrawal 1999). Induced tolerance traits include the induction of dormant meristems, increase growth rates, increased photosynthesis, phenological delays and bunkering of carbon (Schwachtje& Baldwin 2008; Strauss& Agrawal 1999). Together, these strategies may allow plants to sustain growth and produce offspring even under attack by specialized herbivores (Strauss& Agrawal 1999; Tiffin 2000). While much research has focused on identifying and understanding plant traits that confer induced resistance to insect herbivory, induced tolerance traits have received less attention (Fornoni *et al.* 2003; Juenger& Bergelson 2000; Schwachtje *et al.* 2006; Stahl 1888; Stowe *et al.* 2000; Strauss& Agrawal 1999; Tiffin 2000).

The importance of non-structural root carbohydrates and the reallocation of resources from roots to shoots to support the regrowth of aboveground tissues in response to defoliation are widely recognized (Alberda 1966; Bokhari 1977; Briske *et al.* 1996; Cook 1966; Danckwerts& Gordon 1989; Detling *et al.* 1979; Donart& Cook 1970; Lee *et al.* 2009; Machado *et al.* 2013; Myers& Kitajima 2007; Ryle& Powell 1975; White 1973). Accordingly, the herbivore-induced allocation of photoassimilates towards roots and stems observed in several plant species has been proposed to be an induced tolerance mechanism that enables plants to bunker carbohydrates and use them for future regrowth (Babst *et al.* 2005; Babst *et al.* 2008; Briske *et al.* 1996; Dyer *et al.* 1991; Gómez *et al.* 2012; Holland *et al.* 1996; Kaplan *et al.* 2008; Schwachtje *et al.* 2006). However, an increased allocation of photoassimilates to roots and/or stems does not necessarily increase plant tolerance to aboveground herbivores through the enrichment of root carbohydrates. The newly allocated carbon may, for instance, be used for other purposes including the synthesis of plant defenses, root respiration and/or exudation (Barber& Martin 1976; Clayton *et al.* 2010; Erb *et al.* 2009; Ferrieri *et al.* 2013; Ferrieri *et al.* 2005; Frost& Hunter 2008; Holland *et al.* 1996; Keith *et al.* 1986; Machado *et al.* 2013; Shoji *et al.* 2000). Furthermore, the newly allocated carbon may be sequestered and may not be available for subsequent reallocation to the shoots (Marshall& Sagar 1965).

Interestingly, despite the large number of tracer studies demonstrating a higher net carbon flux to the roots in response to herbivore attack, there is little evidence for an increase in non-structural carbohydrates in the roots (Castrillón-Arbeláez *et al.* 2012; Gómez *et al.* 2012; Machado *et al.* 2013; Schwachtje *et al.* 2006; Steinbrenner *et al.* 2011). On the contrary, a number of studies have shown that roots are depleted in sugars and starch following leaf-herbivore attack (Gómez *et al.* 2012; Machado *et al.* 2013). Therefore, the link between the herbivory-induced carbon allocation to roots and tolerance deserves more attention.

Two recent studies have investigated the link between herbivory-induced changes in root carbohydrate pools and tolerance to aboveground herbivory. Machado *et al.* (2013) found that aboveground herbivory depletes root carbohydrate pools and constrain the regrowth capacity of *Manduca sexta*-attacked *Nicotiana attenuata* plants. Both effects were absent in jasmonate-deficient transgenic plants impaired in the production of plant resistance metabolites, indicating that the plants face a trade-off between resistance and tolerance, or that jasmonate signaling regulate both resistance and tolerance traits. In contrast, Korpita *et al.* (2014) found that *Solanum lycopersicum* plants regrew better when attacked by *M. sexta* larvae, despite the herbivore-induced reduction in root carbohydrates in this species (Gómez *et al.* 2012). The contrasting results of these two studies suggest that the connection between herbivore-induced changes in root carbohydrates and tolerance can be explored within the Solanaceae using a comparative evolutionary framework.

In contrast to the numerous studies of phylogenetic conservation of induced defenses (Agrawal 2007; Campbell& Kessler 2013; Gonzales-Vigil *et al.* 2012; Haak *et al.* 2014; Kempel *et al.* 2011; Thaler& Karban 1997), few studies have examined induced tolerance responses in a phylogenetic context (Agrawal& Fishbein 2008). Constitutive tolerance and resistance strategies were predicted to be mutually exclusive (van der Meijden *et al.* 1988) and subject to resource-based trade-offs (Fineblum& Rausher 1995; Herms& Mattson 1992). Among different milkweed species (*Asclepias* spp.), tolerance to herbivory appears to have increased, while resistance traits such as cardenolides, latex and trichomes declined during the diversification of the genus (Agrawal 2007; Agrawal& Fishbein 2006, 2008), lending some

support to this concept. However, a recent meta-analysis suggests that resistance and tolerance evolve independently (Leimu& Koricheva 2006), and that most plants follow mixed strategies (Núñez-Farfán *et al.* 2007). The role of inducibility in the context of resistance tolerance trade-offs remains unexplored.

Based on the above considerations, we studied the dynamics of root carbohydrate responses and regrowth capacity in eight solanaceous plant species following *M. sexta* attack. *Manduca sexta* is specialized on the Solanaceae and is among the most damaging insect herbivores in this plant family (Kessler *et al.* 2004). We specifically focused on the following two questions: 1) Do changes in root carbohydrate pools correlate with herbivore tolerance across species? 2) Are the two traits phylogenetically conserved? Our results suggest that there is a strong correlation between changes in root carbohydrates and regrowth capacity across different species, and that root carbohydrate mediated tolerance and susceptibility strongly diverge between closely related species and are therefore likely to evolve quickly.

Materials and methods

Plant material

The following plant species were used in this study: *Datura wrightii, Petunia axillaris axillaris, Solanum lycopersicum* LA2696*, Solanum peruvianum* LA2744*, Nicotiana attenuata, Nicotiana miersii, Nicotiana pauciflora* and *Nicotiana obtusifolia* (Goldberg *et al.* 2010). Together, these species sample most branches of the phylogenetic tree of the Solanaceae. The seeds of *Datura wrightii* and *Nicotiana obtusifolia* were collected in Utah (USA) and inbred for one generation. *Nicotiana miersii* and *Nicotiana pauciflora* seeds were obtained from the United States *Nicotiana* germplasm collection and inbred for one generation. Seeds of *Solanum lycopersicum* LA2696 were obtained from the tomato genetics resource center (TRGC) at Davis University in California (USA) and inbred for one generation. The seeds of *S. peruvianum* LA2744 were initially obtained from the TRGC and propagated by bulk pollination. *Petunia axillaris axillaris* seeds were derived from a wild accession by self-fertilization. *Nicotiana attenuata* seeds were originally collected in Utah (USA) and inbred for 31 generations.

Germination and planting conditions

N. attenuata, N. miersii, N. pauciflora, N. obtusifolia and *P. axillaris* seeds were germinated on Gamborg's B5 medium as described by Krügel *et al.* (2002). *N. attenuata* seeds were smoke-treated to trigger germination. Approximately 9-10 days later, all seedlings were transferred to Teku pots (Pöppelmann GmbH & Co. KG, Lohne, Germany) for 12 days before transferring them into 1 L pots filled with sand. *Datura wrightii*, *S. lycopersicum* LA2696 and *S. peruvianum* LA2744 seeds were germinated directly in Teku pots. Between 16 and 20 days later, the seedlings were transferred into 1 L pots filled with sand. To synchronize the first day of flowering of all plant species, *D. wrightii* seeds were germinated first, followed by *S. lycopersicum* LA2696, *S. peruvianum* LA2744, *N. attenuata, N. pauciflora* and *N. obtusifolia* 5 days later and *N. miersii* and *P. axillaris* another 5 days later (Fig. S1a). All plants were grown at 45-55% relative humidity and 23-25 °C during days and 19-23 °C during nights under 16 h of light (6am-10pm). Plants were watered twice every day by a flood irrigation system.

Manduca sexta-induced changes in regrowth

Upon *M. sexta* attack, *N. attenuata* plants regrowth less while *S. lycopersicum* plants regrow better (Korpita *et al.* 2014; Machado *et al.* 2013). We determined whether the *M. sexta*-induced changes in regrowth differ between different species grown under similar conditions. Simulated *M. sexta* attack and the regrowth capacity evaluation were carried out as described by (Machado *et al.* 2013)(Fig. S1b,c). Briefly, a pattern wheel was rolled over the leaf 3-4 times on each side of the midvein and the resulting wounds (W) were immediately treated with 20-30 µL of a 1:5 (v/v) milliQ water-diluted *M. sexta* oral secretion (OS) solution. Three to four leaves per plant were treated every time and the treatments were repeated every other day three times to obtain a total of 9-12 treated leaves per plant over 6 days of treatments (Fig. S1b). The number of treated leaves and the amount of applied OS to the wounds were proportionally increased in plant species that produce greater number or size of leaves to ensure that all plants were treated in a standardized manner. Intact plants served as controls (N=19). To specifically evaluate the contribution of belowground tissues to the

regrowth capacity of aboveground tissues, all plant species were defoliated 24 h after the last treatment, leaving only the roots and the lowest 0.5 cm of the main stem. Since *S. lycopersicum* and *S. peruvianum* do not recover from full shoot defoliation, plants were defoliated, and the stem base (10 cm above the shoot-root junction) with two axillary meristems was left to regrow. Regrowth was monitored for all species until senescence (Fig. S1c). A subset of plants was used for the quantification of root carbohydrates (see below).

Manduca sexta-induced changes in root carbohydrates

Non-structural root carbohydrates are depleted upon *M. sexta* herbivory in *N. attenuata* and *S. lycopersicum* (Gómez *et al.* 2012; Machado *et al.* 2013) and correlate to the magnitude of regrowth upon defoliation in *N. attenuata* (Machado *et al.* 2013). To determine whether other solanaceous species respond in a similar manner, we measured root carbohydrates upon simulated *M. sexta* herbivory across eight species. Root carbohydrate pools (glucose, fructose, sucrose and starch) were determined by an enzymatic/spectrophotometric method (N=5) adapted and optimized by Machado *et al.* (2013) from Smithand Zeeman (2006) and Velteropand Vos (2001). The sum of glucose, fructose, sucrose and starch was used as a proxy of total non-structural carbohydrates (Bokhari 1977; Cook 1966; Smith 1973; White 1973).

Phylogenetic analysis

To gain insight into the evolutionary history and phylogenetic conservation of herbivory-induced tolerance, we evaluated the regrowth capacity and root carbohydrate response upon simulated *M. sexta* herbivory in eight species covering four genera (*Petunia*, *Datura*, *Nicotiana* and *Solanum*) of Solanaceae (Goldberg *et al.* 2010). The phylogenetic relationship among the studied species was determined based on two DNA regions: one nuclear – internal transcribed spacer region of nuclear 5.8S ribosomal DNA (ITS1 and ITS2) and one chloroplastic – tRNA-Leu gene, inter gene spacer, and tRNA-Phe gene (trnLF), which have been broadly used in phylogenetic studies (Chase *et al.* 2003; Goldberg *et al.* 2010). The DNA sequences were obtained from the Genbank (www.ncbi.nlm.nih.gov/genbank) and the accession numbers are listed in Supplementary

Table 1. The DNA sequences of ITS and trnLF were aligned using MUSCLE (v. 3.8.31) and manually curated (Edgar 2004). The phylogenetic trees based on both nuclear and chloroplastic DNA sequences were constructed using PhyML (v. 20140206) with a molecular evolution model estimated by jModeltest2 (v.2.1.5) (Darriba *et al.* 2012; Guindon *et al.* 2010). The phylogenetic signal of *M. sexta*-induced regrowth suppression and root carbohydrate depletion was analyzed using three approaches following Haak *et al.* (2014): K statistics (Blomberg *et al.* 2003), Pagel's lambda (λ) (Pagel 1999), and three fit of trait evolution models: Brownian motion (BM), Ornstein-Uhlenbeck (OU), and white noise (WN). K statistics provides a Brownian-motion-based estimate of phylogenetic signal and indicates if the evolution of a trait is equivalent ($K=1$), above ($K > 1$) or below ($K < 1$) the expected trait similarity among related taxa. Pagel's lambda (λ) calculates trees that have complete or no phylogenetic structure by multiplying the off diagonal elements of the variance/covariance matrix and indicates weak (0) or strong (1) phylogenetic signal. Brownian motion (BM) assumes single random walk rates, with the correlation structures among trait values being proportional to the extent of shared ancestry. Ornstein-Uhlenbeck (OU) incorporates an additional parameter to characterize the central tendency in addition to the rate of the random walk. White noise (WN) assumes no covariance structure among species. The different models were compared using akaike information criterion (AIC) values, weights (ω), and likelihood ratio testing (LRT). In addition, phylogenetic generalized least squares (PGLS) models were constructed to estimate the relationship between regrowth and root carbohydrates. Phylogenetic signal analyses were performed based on the phylogenetic tree constructed from both ITS and trnLF DNA sequences, respectively. Since both analyses showed highly similar results, we only present the results based on the phylogenetic tree derived from ITS sequences. The *M. sexta* induced regrowth suppression and root carbohydrate reduction were normalized to the control samples in each species. All phylogenetic analysis were performed in R (v.3.1.1) using the packages picante (K statistics), geiger (λ and fit of trait evolution models) and caper (PGLS analysis) (Harmon *et al.* 2008; Kembel *et al.* 2010; Orme *et al.* 2012; R Development Core Team 2012).

Statistics

Unless otherwise stated, statistical tests were carried out with Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA) using analysis of variance (ANOVA). Levene's and Shapiro–Wilk tests were applied to determine error variance and normality. Holm–Sidak *post hoc* tests were used for multiple comparisons. The effect of simulated herbivory on all vegetative and reproductive parameters measured in regrowing plants was tested by two-way repeated measures ANOVA with time and treatment as factors. The effect of simulated herbivory on soluble sugars, starch and total non-structural carbohydrates was evaluated by two-way ANOVA with plant species and treatment as factors. Datasets from experiments that did not fulfill the assumptions for ANOVA were natural log-, root square- or rank-transformed before analysis.

Results

The regrowth response in response to simulated *M. sexta* attack is species-specific

Across the eight tested solanaceous plant species, we observed neutral to negative regrowth patterns in plants under simulated *M. sexta* attack compared to unelicited controls (Fig. 1). While simulated *M. sexta* attack did not affect either the vegetative growth or reproductive output of *P. axillaris*, *S. lycopersicum, N. miersii* and *N. obtusifolia*, it reduced the leaf number, branch length and number of flowers in *S. peruvianum*, *N. pauciflora* and *N. attenuata*. In *D. wrightii*, simulated *M. sexta* attack did not affect the growth of vegetative tissues, but strongly reduced the number of flowers (Fig. 1).
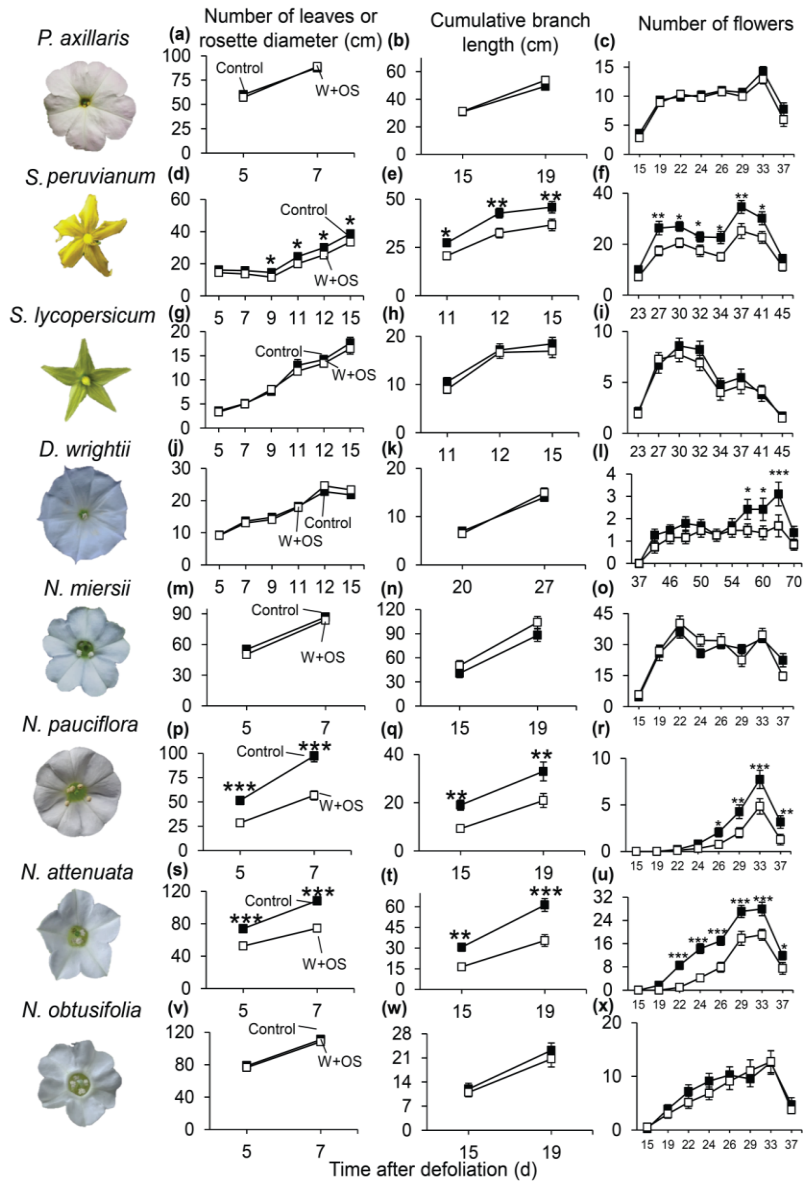
Figure 1. *Impact of M. sexta attack on regrowth capacity of eight solanaceous plant species.* Average (±SE) rosette diameter (a, m, p, s, v), number of leaves (d, g j), cumulative branch length (b, e, h, k, n, q, t, w) and number of flowers (c, f, i, l, o, r, u, x) of regrowing *Petunia axillaris* (a-c), *Solanum peruvianum* (d-f), *Solanum lycopersicum* (g-i), *Datura wrightii* (j-l), *Nicotiana miersii* (m-o), *Nicotiana pauciflora* (p-r), *Nicotiana attenuata* (s-u) and *Nicotiana obtusifolia* (v-x) plants. Asterisks indicate significant differences (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$) among W+OS-treated and control plants within each plant species and time point (N=19). Control (closed squares): intact plants; W+OS (open squares): plants treated with wounding and *M. sexta* oral secretions (simulated *M. sexta* attack).

The root carbohydrate response upon simulated *M. sexta* attack is species specific

Similar to the measured regrowth responses, we found neutral to negative effects of simulated *M. sexta* herbivory on root carbohydrate pools (Fig. 2). Total non-structural carbohydrate pool—calculated as the total amount of glucose, fructose, sucrose and starch—in *P. axillaris*, *S. lycopersicum, N. miersii* and *N. obtusifolia* plants remained unaffected by leaf induction (Fig. 2). By contrast, a significant reduction in total root carbohydrates was observed *S. peruvianum*, *D. wrightii*, *N. pauciflora* and *N. attenuata*. Glucose and fructose contributed most strongly to these changes and correlated with the overall response (Fig. 2). One exception was *N. obtusifolia*, in which simulated *M. sexta* herbivory reduced root glucose and fructose levels, but not total non-structural carbohydrates, due to the slight, non-significant increase in starch. The only positive response was observed for starch levels in *S. lycopersicum*, which increased in the roots of leaf-induced plants. Total soluble carbohydrates were not changed in the roots of *S. lycopersicum.*

***Manduca sexta* attack-induced changes in root carbohydrates and regrowth are strongly correlated, but not phylogenetically conserved**

The solanaceous species used in this study form three clades: i) all *Nicotiana* spp; ii) *Solanum* spp. and *D. wrightii* and iii) *P. axillaris* (Fig. 3a) (Goldberg *et al.* 2010). Using the reconstructed phylogeny, we analyzed the phylogenetic conservation of herbivory-induced regrowth and carbohydrate suppression and found no phylogenetic signal for both traits. This result was supported by three independent analyses. First, the *K* values for both traits were low and non-significant (Table 1), indicating the similarity of measured trait among related taxa were below the expectation under Brownian evolution models. Second, Pagel's λ were very close to 0 (Table 1), suggesting weak phylogenetic signal. Third, we found that the white noise (WN) model, which assumes no phylogenetic signal, is the best-fit model for all tested traits (Table 2). In contrast to their phylogenetic conservatism, carbohydrate depletion and growth suppression were tightly correlated across species (β=1.02, *P*=0.003, Table S2); depletion of root carbohydrates was always accompanied by a suppression of regrowth, while species that did not suffer from carbohydrate depletion also suffered no apparent tolerance

penalty (Fig. 3b). Among the individual metabolites, glucose was the strongest predictor of regrowth (β=0.64, P=0.039). Across species, visual two-dimensional component analysis revealed no clear first order regression, but a clear grouping effect that separated susceptible from tolerant species according to the magnitude of their root carbohydrate depletion and flower production reduction.
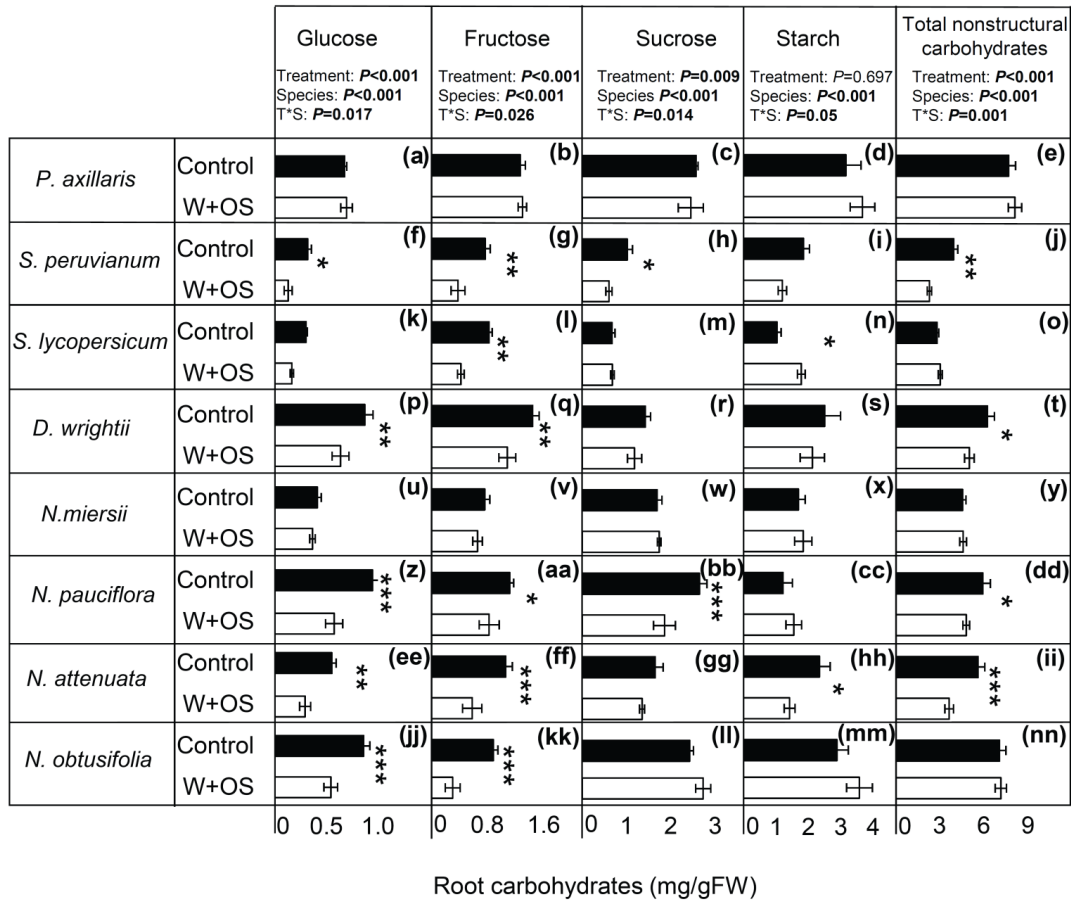

Figure 2. *Impact of M. sexta attack on root carbohydrate pools of eight solanaceous plant species.* Average (±SE) glucose (a, f, k, p, u, z, ee jj), fructose (b, g, l, q, v, aa, ff, kk), sucrose (c, h, m, r, w, b, gg, ll), starch (d, i, n, s, x, cc, hh, mm) and total nonstructural carbohydrates (e, j, o, t, y, dd, ii, nn) of *Petunia axillaris* (a-d), *Solanum peruvianum* (f-j), *Solanum lycopersicum* (k-o), *Datura wrightii* (p-t), *Nicotiana miersii* (u-y), *Nicotiana pauciflora* (z-dd), *Nicotiana attenuata* (ee-ii) and *Nicotiana obtusifolia* (jj-nn) plants. Asterisks indicate significant differences (*: P<0.05; **: P<0.01; ***: P<0.001) among W+OS-treated and control plants within each plant species and metabolite (N=5). Control: intact plants; W+OS: plants treated with wounding and *M. sexta* oral secretions (simulated *M. sexta* attack).
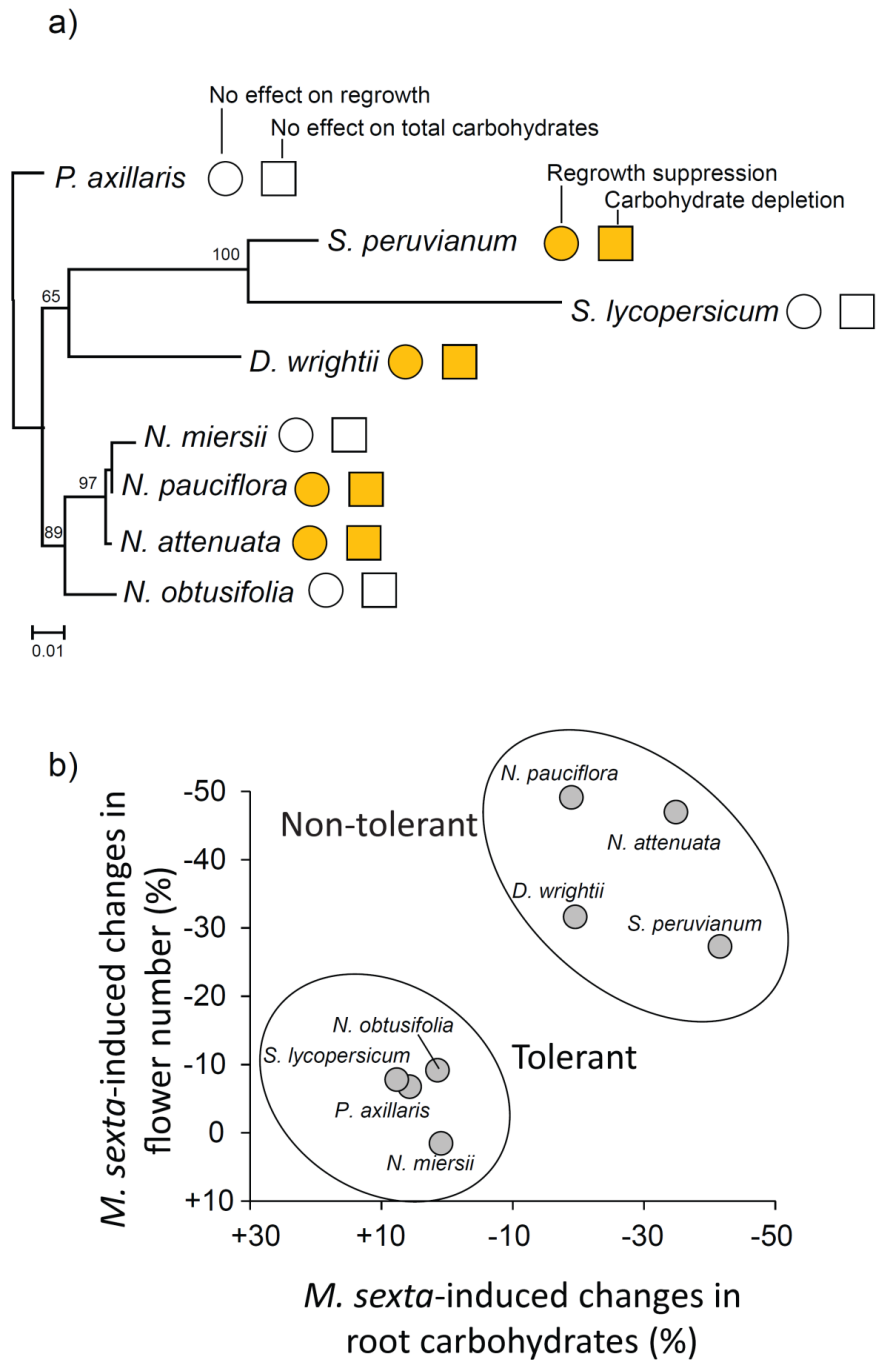
Figure 3. *Total nonstructural root carbohydrates and regrowth capacity upon M. sexta attack are positively correlated, but show no phylogenetic signal.* Intraspecific variation of the impact of *M. sexta* attack on root carbohydrates and regrowth capacity (a). Correlation between the impact of *M. sexta* attack on total flower production of regrowing plants and concentration of total nonstructural root carbohydrates at the beginning of regrowth (b).

Table 1. Linear mixed-effects models of tolerance and root carbohydrates

Trait	K statistics		λ-estimation	
	K	λ	SE	p value
Flower Number	0.087351	$2.05*10^{-06}$	0.1814	0.6655
Glucose	0.492937	$1.79*10^{-209}$	0.1896	0.2215
Fructose	0.352583	$2.82*10^{-140}$	0.1953	0.2200
Sucrose	0.124222	$7.05*10^{-145}$	$9.63*10^{-216}$	0.4740
Starch	0.101341	$3.62*10^{-22}$	$9.53*10^{-9}$	0.5640
Total monosaccharides	0.248507	$7.12*10^{-218}$	0.1094	0.2040

Table 2. Results of trait evolution model-fitting models

Model	White noise			Brownian motion			Ornstein-Uhlenbeck		
	AICc	dAICc	ω	AICc	dAICc	ω	AICc	dAICc	ω
Flower Number	2.02	0.00	**1.00**	15.19	13.17	0.00	19.24	17.23	0.00
Glucose	2.50	0.00	**0.88**	6.76	4.27	0.10	11.47	8.98	0.01
Fructose	2.97	0.00	**0.94**	8.92	5.95	0.05	13.47	10.50	0.01
Sucrose	3.80	0.00	**0.99**	13.16	9.35	0.01	17.34	13.53	0.00
Starch	14.42	0.00	**0.99**	25.09	10.67	0.00	29.28	14.86	0.00
Total monosaccharides	2.61	0.00	**0.93**	8.20	5.59	0.06	12.67	10.06	0.01

Discussion

In this study, we show that induced root carbohydrate depletion and regrowth capacity are two closely related, but highly divergent traits. Our findings are consistent with the hypothesis that root carbohydrate pools are important determinants of plant tolerance to defoliation and indicate that herbivore tolerance through the maintenance of root reserves can evolve rapidly, possibly via changes in the plant's regulatory network.

The eight plant species evaluated in this study can be divided into two groups. The first group consists of plant species whose regrowth capacity is reduced by simulated leaf herbivory and includes *S. peruvianum*, *D. wrightii*, *N. pauciflora* and *N. attenuata*. The second group includes plant species that maintain their capacity to regrow after herbivore

attack and includes *P. axillaris*, *S. lycopersicum*, *N. miersii* and *N. obtusifolia*. Concomitantly, the two groups also differ markedly in their capacity to maintain root carbohydrate homeostasis upon *M. sexta* attack, with the non-tolerant species suffering from carbohydrate depletion. From a mechanistic point of view, the strong correlation of these two traits across species may be due to several non-exclusive reasons. First, it is likely that the changes in root carbohydrates directly determine a plant's capacity to produce new photosynthetic tissues at the end of the defoliation process. Plants with lower levels of non-structural carbohydrates in the roots often regrow smaller shoots (Alberda 1966; Bokhari 1977; Lee *et al.* 2009; Machado *et al.* 2013; Smith& Silva 1969). Second, both traits may share a common regulatory basis. Variation in both regrowth and root carbohydrate depletion is tightly associated with jasmonate signaling in *N. attenuata*. In both transgenic and naturally occurring jasmonate-deficient *N. attenuata* lines, neither carbohydrate depletion nor a reduction in regrowth are observed, a result in stark contrast to those of jasmonate-competent plants (Machado *et al.* 2013). Another study recently demonstrated that *M. sexta* attack induces a much weaker jasmonate response in *N. miersii* and *N. obtusifolia* than in *N. pauciflora* and *N. attenuata* (Xu *et al.* 2015), which is consistent with the carbohydrate and tolerance profiles found in this study. The fact that *S. lycopersicum* plants showed high *M. sexta*-induced jasmonate accumulation (Paschold *et al.* 2008) but no root carbohydrate depletion and regrowth suppression suggests that in addition to jasmonate-mediated processes, other mechanisms are also likely involved. Interestingly, in *S. lycopersicum*, the starch content was increased by simulated *M. sexta* attack. This is likely the result of increased starch synthesis rather than an increase in root carbohydrate allocation, as total non-structural carbohydrate pools remained unchanged. The production of defensive compounds is tightly linked to primary metabolism since amino acids, sugars, and organic acids act as precursors, carbon skeletons, and as substrates to produce the required energy for their biosynthesis (Arnold& Schultz 2002; Bolton 2009; Broeckling *et al.* 2005; Schwachtje& Baldwin 2008). Plant species that synthesize their most abundant secondary metabolites in the roots might consequently suffer from greater carbon depletion in the roots and stronger reduction of regrowth capacity. The synthesis of alkaloids

in *S. lycopersicum* occurs mainly in the leaves, in contrast with *N. attenuata* that occurs in the roots, and might therefore explain the contrasting carbohydrate profiles of these two species (Baldwin 1999; Itkin *et al.* 2013; Itkin *et al.* 2011). The fact that this pattern is not observed in other *Nicotiana* or *Solanum* species might be due to additional factors as, for example, plant life history. Perennial plants might favor the storage of root carbohydrates over the utilization of root resources for the production of defenses (Chapin *et al.* 1990).

Using three different phylogenetic analyses, our results consistently indicated no intrinsic phylogenetic constraint on the evolution of herbivory induced changes in root carbohydrates and regrowth in different solanaceous species (Tables 1 and 2). Although the relatively low sample size and unevenly distributed number of species within each genus might have resulted in an under-estimation of phylogenetic conservatism, the high divergence within the genus *Nicotiana*, which included four closely related *Nicotiana* species, supports the lack of phylogenetic signal. The pattern found here is similar to the pattern of *M. sexta*-induced defense traits observed in *Solanum*, which also showed no phylogenetic conservatism (Haak *et al.* 2014). This indicates that these two traits: herbivore induced defense and regrowth capacity after herbivore attack can evolve rapidly, likely in response to local environmental conditions. The induction of toxic secondary metabolites and anti-digestive proteins are well-known plant responses against insect attackers. However, these strategies might not be sufficient in the face of well-adapted organisms (Berenbaum& Zangerl 1994; Lindigkeit *et al.* 1997; Mao *et al.* 2006; Zangerl& Berenbaum 1990). A single amino acid substitution in the α-subunit of the Na^+/K^+-ATPase gene is sufficient to confer cardenolide tolerance in leaf beetles (Labeyrie& Dobler 2004), indicating how rapidly plant defenses may lose their protective effect in nature. Perhaps as a consequence, plants have evolved divergent strategies to minimize the insect imposed fitness costs, such as induced regrowth, a phenomenon reflected in the low conservatism of *M. sexta*-induced regrowth suppression in the plant family Solanaceae.

More than 20 years ago, resource-based growth defense trade-offs were proposed to be a major force that shape the evolution of plant resistance and tolerance strategies (Fineblum&

Rausher 1995; Herms& Mattson 1992). Recent evidence suggests however that most plants use mixed strategies (Carmona& Fornoni 2013) and that tolerance and resistance may evolve independently (Leimu& Koricheva 2006). Our work highlights that induced resistance and tolerance can be negatively linked through jasmonate regulation (Machado *et al.* 2013) where root carbohydrate pools may supply resources for defensive metabolites and regrowth. We therefore propose that inducibility may be the missing link between traditional tolerance resistance predictions and contemporary observations of their variability among plant species.

Acknowledgements

Supporting information

a) Planting conditions

b) Plant treatments

c) Regrowth capacity evaluation

Figure S1. *Experimental set up.* Planting conditions (a), plant treatments (b) and timing of regrowing plant phenotyping (c).
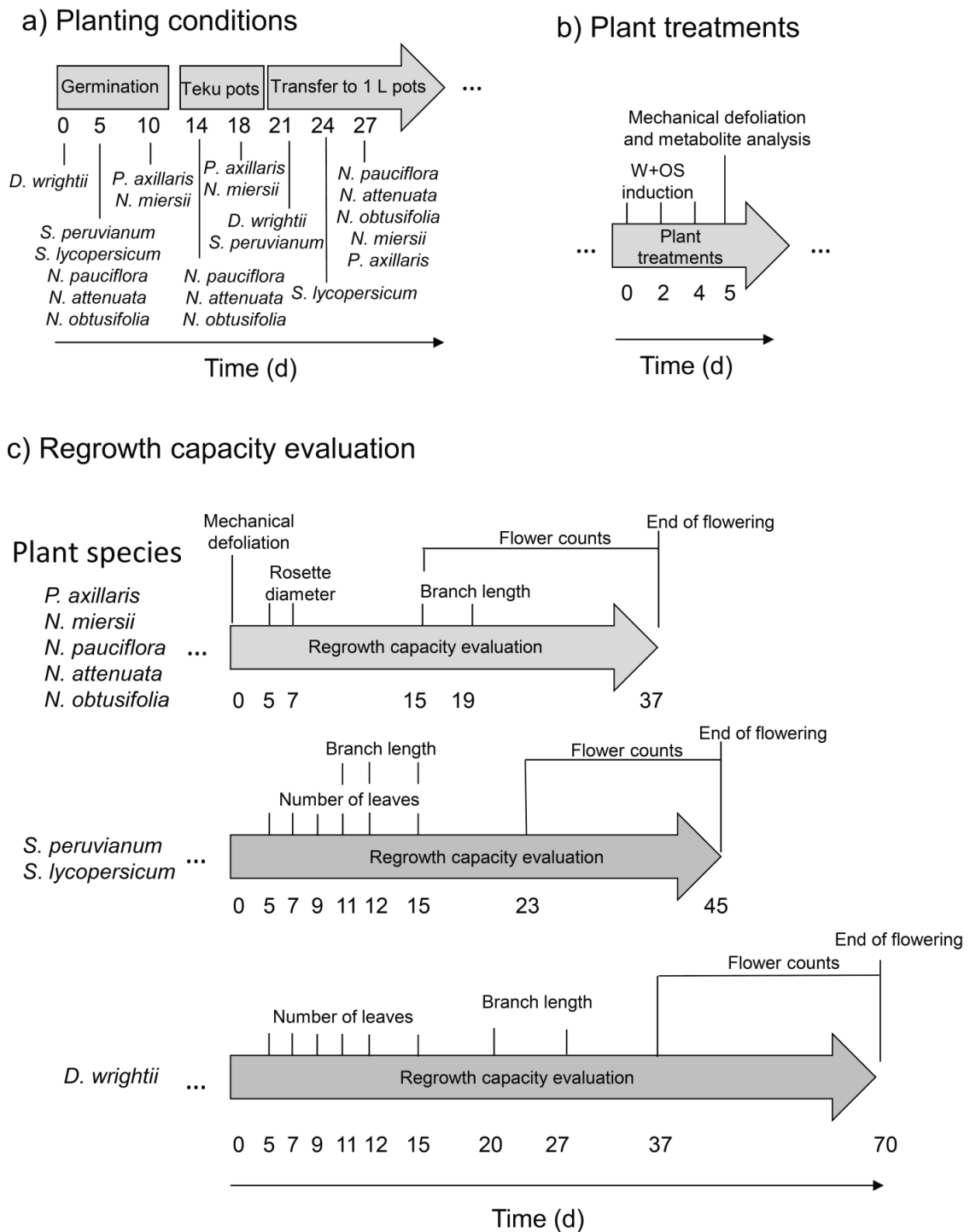
Table S1. Gene bank accession numbers of tRNA-Leu gene, inter gene spacer, and tRNA-Phe gene (trnLF) and internal transcribed spacer (ITS) regions of nuclear 5.8S ribosomal DNA (ITS1 and 2).

	TrnLF	**ITS1 and 2**
N. attenuata	AJ577401	AJ492427.1
N. miersii	AJ577423	AJ492429.1
N. obtusifolia	AJ577438	GQ120451.1
N. pauciflora	AJ577407	AJ492428.1
P. axillaris	AY098702	AJ300213.1
S. lycopersicum	AY098703	KC213749.1
S. peruvianum	AB515411.1	AJ300210.1
D. wrightii	JX467585.1	JX467602.1

Table S2. Correlation between number of flowers of regrowing plants and root carbohydrates across plant species.

Trait	**β**	**R-squared**	$F_{1,6}$	***p***
Glucose	0.644	0.537	6.955	**0.039**
Sucrose	0.617	0.466	5.244	0.062
Fructose	0.242	0.072	0.469	0.519
Starch	0.233	0.239	1.887	0.219
Total monosaccharides	1.019	0.801	24.140	**0.003**

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GENERAL DISCUSSION

The results of my thesis provide new insights into the regulatory role of constitutive and herbivory-induced jasmonates in leaf and root soluble sugars and starch in *N. attenuata* plants. They demonstrate that the jasmonate-dependent depletion of plant soluble sugars and starch reduce both plant resistance and tolerance to *M. sexta* herbivory and suggest that the above traits are highly divergent and subject to rapid evolution in the Solanaceae. Here, I discuss the potential mechanisms by which jasmonates regulate sugars as well as the ecological consequences of sugar depletion in the context of plant resistance and tolerance to herbivory. Furthermore, I propose a number of explanations for the prevalence of the observed phenotype in nature and suggest a number of possible future experiments.

M. sexta attack negatively impacts carbohydrate pools in the leaves and the roots of *N. attenuata* plants: Potential Regulatory Mechanisms

I found that *Manduca sexta* attack dramatically decreased carbohydrates, including glucose, fructose, sucrose and starch, in *Nicotiana attenuata* leaves and roots. These effects were absent in jasmonate-deficient transgenic plants, and could be restored by methyl jasmonate (MeJA) treatments, showing that jasmonates are negative regulators of soluble carbohydrates in *N. attenuata*. Jasmonates promote the synthesis of defensive secondary metabolites, reduce photosynthetic capacity, affect sugar metabolizing enzymes, and antagonize other phytohormonal networks [1]. All these processes can potentially explain the jasmonate-mediated reduction in leaf and root carbohydrates observed in *M. sexta*-attacked *N. attenuata* plants, as discussed in detail below.

Plant defensive secondary metabolites. Upon herbivore attack, jasmonate levels increase in local and systemic tissues and trigger the biosynthesis of many defensive metabolites, including, for instance, glucosinolates [2, 3], benzoxazinoids [4-6], terpenoids [7], alkaloids [8, 9], phenolics [10] and tannins [11, 12] in several plant species. The production of defensive compounds is tightly linked to primary metabolism since amino acids, sugars, and organic acids act as precursors, carbon skeletons, and as substrates to produce the required energy for their biosynthesis [10, 12-17]. Upon insect attack or methyl jasmonate treatments, for example, an increased import of carbon with a concomitant increased in condensed

tannins, cinnamic acid and phenolic glycosides have been observed in Poplar and Cress Thale plants [12, 18-20]. Similarly, *M. sexta* attack induces the accumulation of diterpene glycosides (DTGs) in *N. attenuata* in a jasmonate-dependent manner. DTGs consist of an acyclic C20 17-hydroxygeranyllinalool skeleton conjugated to sugar groups [21]. The observed *M. sexta*-induced, jasmonate-dependent depletion of soluble sugars and starch may therefore be explained by an increased demand of carbon and energy to support the biosynthesis of secondary defensive metabolites. Other primary compounds such as amino acids serve as precursors for the biosynthesis of secondary metabolites [22], including the jasmonate-dependent nicotine [23]. Amino acids are also synthesized from glucose, and therefore, a greater demand of amino acids for the synthesis of secondary metabolites may still lead to carbohydrate depletion. Consistent with this hypothesis, jasmonate treatments were found to deplete carbohydrates in the leaves of tobacco plants and at the same time induce amino acids pools and secondary defensive metabolites [24]. No clear connection between secondary metabolite biosynthesis and protein turnover was found in *N. attenuata* [25], indicating that the biosynthesis of secondary metabolites might rely directly on plant carbohydrates. Spatio-temporally resolved measurements of carbohydrate and amino acid fluxes in plants impaired in the production of one or several defensive metabolites might help to understand whether the production of *M. sexta*-induced defensive secondary metabolites leads to the depletion of plant carbohydrates or whether additional jasmonate-mediated antagonistic processes might play are role in this context.

Photosynthetic capacity and chlorophyll content. Sugars are produced through the incorporation of carbon dioxide into ribulose-1,5-bisphosphate by the action of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBiSCO), followed by the spontaneous formation of two molecules of 3-phosphoglyceric acid. 3-phosphoglyceric acid is subsequently converted to glucose, fructose, sucrose and starch via several enzymatic steps [26]. Any interference with the photosynthetic machinery might therefore directly reduce the production of sugars. Transcript levels of photosynthetically related genes as well as protein abundance are diminished after foliar herbivory in several plant species [27-32], an effect that is also reflected at the physiological level. For example, the removal of 5% of leaf area by caterpillar feeding reduced photosynthesis by 20% in the remaining foliage in wild parsnip [33], and the

decline in photosynthesis in the remaining leaf tissue of an oak sapling was proportional to the actual removal of leaf tissue [34]. On the other hand, *M. sexta*-induced jasmonates have been shown to specifically decrease photosynthetically activity in *N. attenuata* plants [35]. It is therefore possible that insect attack reduce carbohydrate pools by reducing plant's photosynthetic capacity via jasmonate signaling. Consistent with this notion, I observed that *M. sexta*-induced jasmonates reduce chlorophyll content in the leaves of *N. attenuata* and reducing the activity of RuBiSCO activase (RCA), which modulates the activity of RuBisCO, the enzyme that carries out the first major step of CO_2 fixation in plants, in jasmonate-deficient plants reduced glucose and fructose concentrations.

Invertase activity. Invertases are sucrolytic enzymes that cleave sucrose into glucose and fructose, and are essential for the regulation of carbohydrate metabolism and source-sink relationships [36]. Invertase activity is affected by several abiotic and biotic stressors such as mechanical damage and herbivore and pathogen attack [37, 38]. Both gene expression and enzyme activity of invertases have been found to increase upon wounding in several plant species including pea, tomato, carrot and sugar beet [39-42]. I found that the jasmonate-dependent inhibition of invertase activity positively correlates with soluble sugars content in the leaves of *N. attenuata*, indicating that jasmonates might regulate sugar pools by inhibiting sucrolytic enzymes. Consistent with this hypothesis, Ferrieri *et al.*, (2015) found that *N. attenuata* transgenic plants silenced in the expression of a jasmonate-dependent, *M. sexta*-induced cell-wall invertase inhibitor showed higher invertase activity and a greater depletion of glucose, fructose and starch upon simulated *M. sexta* attack, providing a clear link between invertase activity, jasmonates and soluble sugar pools in *M. sexta*-attacked *N. attenuata* plants [43].

Hormonal signaling crosstalk. Hormonal signaling crosstalk is thought to shape and orchestrate diverse plant responses to biotic and abiotic stressors [44-46]. Direct evidence for the involvement of jasmonates (JAs), cytokinins (CKs) and auxins [47-49], and only indirect evidence for the involvement of gibberellins [50] in *N. attenuata* responses to *M. sexta* attack have been demonstrated so far. Cross-talk between *M. sexta*-induced jasmonates and other hormonal pathways might explain the depletion of soluble sugars in *N. attenuata*-attacked plants. Gibberellins, cytokinins and auxins modulate sugar metabolism in plants, and

jasmonates have been shown to antagonize their signaling pathways [49, 51, 52]. For example, jasmonic acid promotes the degradation of JASMONATE ZIM-DOMAIN (JAZ) proteins and, in turn, frees the transcriptional regulation activity of MYC2, the major transcription factor of jasmonate-mediated gene expression, and MYC2 directly binds the promoter regions of *PLT1* and *PLT2,* two well-known auxin-inducible genes, repressing their expression [51]. Similarly, jasmonates delay the gibberellin-mediated DELLA protein degradation, thereby interferes with gibberellin signaling [52]. Jasmonate biosynthesis and signaling repress gene expression of RRA5, a CK-signaling gene marker [49]. Although, it is plausible, the exact molecular mechanisms by which jasmonate-mediated crosstalk with other phytohormonal pathways might deplete soluble sugars upon *M. sexta* attack still remains to be investigated.

Increased sink strength. Another potential explanation for the depletion of soluble sugars in *N. attenuata* plants is the diversion of carbohydrates into other plant structural components such as cellulose, hemicellulose or pectin. Upon simulated *M. sexta* herbivory, ethylene and jasmonate signaling act synergistically to repress cell wall remodeling-associated genes such as pectin methylesterase and expansin [27, 53], suggesting that herbivory might directly affect the composition of cell walls. Direct measurement of cellulose, hemicellulose and pectin levels upon herbivory are required to further test this hypothesis, since the existing evidence is still controversial. For example, cellulose and hemicellulose levels decrease upon methyl jasmonate treatments [54] but are also induced in other plants [55-57]. On the other hand, *M. sexta* attack increases stem thickness in tomato [58], which might potentially be explained by an increased cellulose deposition, thereby depleting soluble sugars.

Combinations of the above effects. The dramatic depletion of soluble sugars observed in *M. sexta*-attacked *N. attenuata* plants may also be the result of a combination of the above factors. A reduction in carbon fixation together with an increase in assimilate demand for the production of defenses, for instance, may result in an additive negative effect on soluble sugar pools. Disentangling the relative contribution of the different factors will be a challenging task, but may ultimately provide a more detailed picture of the physiological context and potential constraints that lead to sugar depletion under herbivore attack.

***M. sexta* attack negatively impacts carbohydrate pools in the leaves and the roots of *N. attenuata* plants: Ecological Consequences**

I found that the *M. sexta*-induced, jasmonate-dependent depletion of soluble sugars in leaves and roots brings negative consequences for plant fitness expressed as a lower capacity to resist *M. sexta* attack and a decreased capacity to regrow after *M. sexta* attack. Here, I discuss these two aspects in detail.

Resistance against M. sexta attack. Combining carbohydrate manipulation *in vitro* and *in planta*, I showed that *M. sexta* growth is increased, rather than reduced, through decreased dietary carbohydrates. As a consequence, the *M. sexta*-induced, jasmonate-dependent depletion of leaf sugars renders *N. attenuata* plants less resistant to *M. sexta* attack. Although counterintuitive, it has been widely reported that insect performance [59-63] and survival [62, 64] are compromised with increasing concentration of dietary carbohydrates. Ingesting an excess of dietary carbohydrates is avoided via behavioral regulation of nutrient intake through both food selection and amounts of food eaten [65-69]. Apart from behavioral mechanisms, insects use additional post-ingestion mechanisms to cope with an excess of dietary carbohydrates such as the down regulation of carbohydrate-catabolizing enzymes [70, 71], the increase of respiration rates [72], the up-regulation of glucose-oxidizing enzymes [73], the increase of carbohydrate egestion [63, 74] and greater rates of carbohydrate deposition as body fat [68, 75, 76]. I found that the effect of excessive dietary carbohydrates in *M. sexta* growth is tightly linked to the amount of food consumed and to changes in the efficiency of conversion of ingested food: the amount of ingested food decreased with increasing sugar concentrations in a protein independent-manner and greater protein availability increases efficiency of conversion of ingested food, reversing the detrimental effect of excessive dietary carbohydrates in *M. sexta* growth.

Tolerance to M. sexta attack. I found that *M. sexta* attack constrains *N. attenuata* regrowth capacity in a jasmonate-dependent manner: plants genotypes impaired in jasmonate biosynthesis do not suffer any fitness consequences upon *M. sexta* attack. By using plant genotypes that constitutively translocate more carbon to the roots but are intact in jasmonate signaling, I show that the depletion of non-structural root carbohydrates are a likely

mechanism by which jasmonates constrains regrowth capacity in *M. sexta*-attacked *N. attenuata* plants. Upon defoliation, the regrowth of new photosynthetic tissue requires substantial amounts of energy, nutrients, and carbon and nitrogen skeletons [77, 78]. The contribution of roots in providing such resources has been widely recognized [47, 79-89]. Plants with lower levels of root carbohydrates often regrow smaller shoots [47, 83, 88-90], indicating that the depletion of sugars and starch in the roots might directly constrain plant's capacity to regrow new vegetative tissue and thereby constrains tolerance to aboveground herbivory. However, the direct link between jasmonate induction, root carbon depletion and regrowth capacity in herbivore-attacked plants still needs further experiments. Roots are often considered as a sink tissue that requires the constant supply of photoassimilates that are used as energy source to fuel root primary functions [91]. However, when plants are severely defoliated, storage organs like roots should become source tissue and provide the necessary resources to regrow new photosynthetic tissues [92]. While our mechanistic understanding on how leaf sources provide carbohydrates to sink tissues, less is known on how roots carry out such functions in defoliated plants [93-96] and several fundamental questions remain still unclear in this respect [97]. For example, what are the pathways of the starch metabolism in root amyloplasts? How are the resulting sugars transported to aboveground "sink tissues" to support their growth and development? Is it a xylem- or a phloem-mediated process, in other words, are sugar transporters involved? [98-101]. The understanding of these fundamental questions will greatly improve our knowledge on the link between herbivory-induced depletion of root carbohydrates and tolerance to aboveground herbivory.

Evolutive consequences of *M. sexta*-induced, jasmonate-dependent whole plant soluble sugars and starch depletion

The results of my thesis show that the *M. sexta*-induced, jasmonate-dependent depletion of plant carbohydrates significantly constrains herbivore tolerance and resistance in *N. attenuata*, The question that arises here is why *N. attenuata*, with its high risk of herbivore attack [102], shows this phenotype and has not evolved strategies to minimize sugar depletion, as other species have done?. Here, I propose several hypothetical scenarios that could lead to benefits of the observed phenotype in nature. Furthermore, I argue that *M. sexta*

by itself, may, in fact, exert selective pressure against induced jasmonate signaling in *N. attenuata*.

Potential benefits of sugar depletion in *M. sexta*-attacked *N. attenuata* plants

Under natural conditions, *N. attenuata* is attacked by herbivores from more than 20 different taxa that vary greatly in their feedings guild, from mammals to microorganisms [102]. Plant responses to the attack of an initial herbivore may result in resistance to the subsequent attack of the same species, but might also affect the performance of subsequent attackers, which again may lead to important effects on plant fitness and thereby influence the evolution of plant defense traits [103].

Reduction in nutrient availability to limit subsequent attack. The colonization and survival of pathogens upon infection is tightly linked to the availability of resources. Bacteria and fungi, for instance, co-opt plant sugar efflux carriers and invertases during infection, creating sink strength that results in greater sugar supply that benefits pathogen growth and survival [37, 104-110]. Caterpillar feeding causes wounds to plant tissue creating potential entry sites for plant pathogens [111, 112]. The observed depletion of soluble sugars in *M. sexta*-attacked plants might therefore limit the proliferation of plant pathogens through wounded tissues, thereby providing an indirect benefit for *N. attenuata*. Under natural conditions, plant pathogens such as *Pseudomonas* spp., *Alternaria* spp., and *Fusarium* spp. have been observed invading *N. attenuata* plants, with large negative consequences [113, 114]. A potential constrain of depleting sugars as a mean of restricting subsequent pathogen attack is that it might also impact other potentially beneficial microbial communities, as for example, soil bacteria and mycorrhiza that are commonly found interacting with *N. attenuata* [115-120] and can provide resistance against root pathogens [121]. Since most of the soil bacteria are recruited before germination, it remains to be determined whether the *M. sexta*-induced sugar depletion might affect microbial communities.

Other potential group of organisms that might be affected by a decrease in plant sugar content upon *M. sexta* attack is that of piercing-sucking insects. Under natural conditions, *Tupiocuris notatus* mirid bugs and *Empoasca* leafhoppers are frequently found attacking *N. attenuata* plants [103, 122, 123]. Piercing-sucking insects heavily relay in the acquisition of nutrients

such as sugars and amino acids feeding from both phloem and mesophyll [124], therefore, any change in the quality of their food source might directly affect their performance [125]. *Empoasca* leafhoppers feeding preference in *N. attenuata* is driven by jasmonate-mediated processes, independently of nicotine, proteinase inhibitor and diterpene glycoside accumulation or volatile emissions [122]. A plausible scenario is that jasmonate-dependent changes in sugar accumulation might mediate the observed *Empoasca* feeding preference, and that the *M. sexta*-induced reduction in soluble sugars might consequently affect the performance of these insects. Contrary to the effects of *T. notatus* feeding on *M. sexta* performance, the negative effects of *M. sexta* feeding on *Empoasca* leafhoppers or *T. notatus* have not been documented [103]. Hence, whether *M. sexta*-induced sugar depletion affects *T. notatus* or *Empoasca* performance, and whether this represents a fitness benefit for *N. attenuata*, remains open. A tightly controlled experiment, taking into consideration the sequence of arrival, together with the manipulation of leaf soluble sugars, and the evaluation of insect performance and plant fitness might help to understand whether a decrease in soluble sugars might alleviate the consequences of sequential insect attack [103, 126]

Diversion of sugars to other less digestive compounds and defenses. Cellulose-based leaf toughness has been suggested as a resistance factor that determines insect performance [127-132], especially in lepidopteran insect, since cell wall digestibility is very low or absent in this order [133-136]. An increased deposition of soluble sugars in cell wall polysaccharides such as cellulose, hemicellulose or pectin, might therefore increase both leaf toughness and reduce tissue digestibility and might, as consequence, bring potential benefits for *N. attenuata* to compensate the large detrimental effects of sugar depletion on resistance against *M. sexta* attack [137, 138]. Tracer studies and direct measurements of cell wall composition upon insect attack followed by the assessment of insect performance on plant genotypes altered in the expression of herbivory-induced cell wall-remodeling enzymes might help to determine whether the diversion of soluble sugars into cell wall constituents might be a factor that confer resistance against *M. sexta* or another group of insects and help to compensate for the negative consequences of soluble sugar depletion.

Soluble sugars might also be diverted to toxic secondary metabolite biosynthesis. In *N. attenuata*, the genetic manipulation of secondary metabolite biosynthetic enzymes has clearly

demonstrated their defensive role against insect attack [21, 139-141]. It is possible that the benefit of secondary metabolite production outweighs the negative effects of sugar depletion, for instance, during an attack by generalist herbivores [139, 140]. The asymmetry of the fitness trade-off may subsequently have favored the maintenance of the sugar depletion phenotype in *N. attenuata*. Testing the above mentioned hypothesis will help us to understand why, at least some *N. attenuata* accessions, have not evolved strategies to minimize sugar depletion, as other solanaceous plants have done, as discussed below.

Does *M. sexta* exert selective pressure against jasmonate signaling in nature?

Jasmonates regulate the production of secondary metabolites that confer resistance to insect attack [142] but also compromise herbivory tolerance [47, 143], suggesting that jasmonates mediate trade-offs between resistance and tolerance. Highly specialized insects have evolved strategies to circumvent plant resistance strategies [144-148]. For example, *M. sexta* is able to efficiently excrete and metabolize nicotine [149-153], one of the most potent jasmonate-dependent defensive secondary metabolites in *N. attenuata* [154]. *M. sexta* defoliation rates are often similar in jasmonate-deficient *N. attenuata* plant genotypes compared to jasmonate-competent ones [155, 156] and silencing defensive secondary metabolites as proteinase inhibitors (PIs), have been shown to provide little fitness benefits in nature [157]. One can imagine a hypothetical scenario in which, under the attack of a well-adapted herbivore like *M. sexta*, both jasmonate-competent and jasmonate-deficient genotypes might suffer similar defoliation regimes, but those deficient in jasmonate signaling will regrow better and produce more offspring, enriching seed banks of jasmonate-deficient genotypes. Consistent with this hypothesis, populations of *N. attenuata* have been shown to harbor significant genetic diversity among individuals within a population, and among populations [158], that is reflected at the jasmonate signaling level: there are plant genotypes whose *M. sexta*-induced jasmonate burst is almost absent while, in others, it is severalfold greater [47, 122, 159]. It is then plausible that *M. sexta* might be a driving force that selects for jasmonate deficient plants in nature, while less specialized herbivores may have the opposite effect.

***M. sexta*-induced root carbohydrate responses and defoliation tolerance are highly correlated and subject to rapid evolution in the Solanaceae**

I found that changes in non-structural root carbohydrate are strongly correlated with plant regrowth capacity across several solanaceous species: species that suffered from carbohydrate depletion upon simulated *M. sexta* herbivory regrew less, while species that maintained root carbohydrate levels tolerated herbivore attack. Additionally, I found no phylogenetic signal on both herbivory-induced root carbohydrate depletion and regrowth suppression among the studied plant species, indicating that root-mediated tolerance strategies are highly divergent and subject to rapid evolution within the Solanaceae. This finding is consistent with the idea that Solanaceous plants differ strongly in their defensive strategies, and that sugar depletion may be under strong local selection.

Plants employ two general defensive strategies against herbivores: resistance and tolerance. Resistance traits aim at reducing the amount of herbivore damage and include, for example, the production of secondary metabolites and proteins that deter or intoxicate herbivores and the provision of cues, shelter and food to increase herbivore predation by natural enemies [160-164]. Plant tolerance to herbivory is defined as the capacity of plants to reduce the fitness costs imposed by herbivore damage and is classically expressed as plant fitness as a function of damage [77]. Induced tolerance traits include the induction of dormant meristems, increase growth rates, increased photosynthesis, phenotypical delays and bunkering of carbon [77]. In turn, herbivores have evolved strategies to counteract plant defenses, for instance by detoxifying or excreting secondary metabolites, avoiding toxic tissues or minimizing predation risk [144-148]. *M. sexta*, for example, efficiently excrete and metabolize nicotine [149-153], one of the most potent jasmonate-dependent defensive secondary metabolites in *N. attenuata* [154]. Resistance traits may therefore only be of limited value for plants facing specialized herbivores [47, 146, 165, 166], indicating how rapidly plant defenses may lose their protective effect in nature. On the face of well-adapted insect, the evolution of divergent strategies such as tolerance might be therefore of particular importance to minimize the insect imposed fitness costs [167, 168]. Consistently, I found a low conservatism of *M. sexta*-induced tolerance in the Solanaceae, indicating that plants rapidly evolve divergent defensive strategies as a part of an evolutive arm-race that alleviate the herbivore plant defense-

counteracting strategies. The evolution of induced resistance and tolerance in the Solanaceae is clearly worthy of further study.

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SUMMARY

Insect attack leads to a strong reconfiguration of a plant's metabolome, which influences the performance of the attacker. While the causes and consequences of induced changes in plant secondary metabolites are well understood, less is known about primary metabolites. To contribute to filling this gap of knowledge, I studied the role of sugars and starch in the interaction between *Nicotiana attenuata* and *Manduca sexta*.

I found that *M. sexta* attack dramatically decreases soluble carbohydrates, including glucose, fructose, sucrose, and starch, in *N. attenuata* leaves and roots. I did not observe this effect in jasmonate-deficient transgenic plants, suggesting that jasmonates are a plant signal that reprograms carbohydrate metabolism in plants. Based on the above observation, I evaluated the ecological consequences of the observed jasmonate-dependent sugar and starch depletion in the context of plant resistance and tolerance by asking: Does the impact of *M. sexta* attack on leaf carbohydrates affect plant nutritional value and in turn plant resistance? And does it reduce the capacity of roots to supply energy for regrowth and herbivore tolerance? Finally, I evaluated the degree of phylogenetic conservatism of the impact of *M. sexta* attack on both root carbohydrate reconfiguration and tolerance in the Solanaceae.

My results suggest that i) lower sugar concentrations in the leaves lead to an increased *M. sexta* growth, suggesting that leaf carbohydrate depletion constrains rather than enhances plant resistance against herbivores; ii) the depletion of root carbohydrates significantly constrains the plant's regrowth capacity and fitness, indicating that root carbohydrate depletion constrains tolerance to herbivory; and iii) changes in soluble root carbohydrate pools are strongly correlated with regrowth capacity across several solanaceous plant species and iv) that both traits do not show a phylogenetic signal, indicating that root carbohydrates are important determinants of tolerance to aboveground herbivory and that these two traits are subject of rapid evolution in the Solanaceae.

The results of my thesis highlight the importance of herbivory-induced changes in plant primary metabolism as determinants of plant resistance and tolerance. Moreover, they demonstrate the potential trade-off between jasmonate-dependent primary and secondary metabolites that may help to explain natural variation in jasmonate signaling and induced defenses.

Zusammenfassung

Insektenbefall führt zu einer starken Rekonfiguration des pflanzlichen Metabolismus, was wiederum die Performance des Angreifers beeinflusst. Während man die Ursachen und Folgen der induzierten Veränderungen der sekundären Pflanzenstoffe gut versteht ist nur wenig über Veränderungen der Primärstoffwechselprodukte bekannt. Um zur Schließung dieser Wissenslücke beizutragen habe ich die Rolle von Zucker und Stärke in der Interaktion zwischen *Nicotiana attenuata* und *Manduca sexta* studiert.

Ich fand, dass ein Angriff von *M. sexta* die Konzentration von löslichen Kohlenhydraten, einschließlich Glucose, Fructose, Saccharose und Stärke, in den Wurzeln und Blättern von *N. attenuata* drastisch reduziert. Dieser Effekt wurde in Jasmonat-defizienten transgenen Pflanzen nicht beobachtet, was darauf hindeutet dass Jasmonate als Signale an der induzierten Kohlenhydrat-Reduktion beteiligt sind. Basierend auf diesen Beobachtungen habe ich die ökologischen Konsequenzen der Jasmonat-abhängigen Zucker und Stärke-Reduktion im Kontext der Pflanzenresistenz und Toleranz untersucht. Dabei habe ich mich auf folgende Fragen konzentriert: Verändert die induzierte Reduktion von Kohlenhydraten den Nährwert und damit die Resistenz der Pflanze gegenüber *M. sexta*? Reduziert diese Reaktion die Fähigkeit der Wurzeln, Energie für das Wiederaustreiben der Blätter nach dem Befall bereitzustellen? Schlussendlich habe ich auch die Phylogenetische Konservation des Einflusses von *M. sexta* auf die Kohlenhydrat-Rekonfiguration und Herbivoren-Toleranz in den Nachtschattengewächsen untersucht.

Meine Ergebnisse zeigen i) dass niedrige Zuckerkonzentration in den Blättern zu einem erhöhten *M. sexta* Wachstum führt, was darauf hindeutet, dass die Kohlenhydrat-Reduktion die induzierte Resistenz gegen Herbivoren einschränkt; ii) dass die Reduktion der Kohlenhydrate in der Wurzel die Kapazität des Wiederaustreibens reduziert und so die Herbivoren-Toleranz der Pflanze schwächt; und iii) dass die Änderungen des Wurzel-Kohlenhydrat-Pools und des Wiederaustreibens innerhalb der Nachtschattengewächse stark korrelieren, aber iv) dass diese Eigenschaften kein phylogenetisches Signal zeigen, was weiter darauf hinweist dass diese beiden Eigenschaften eng verwandt sind, aber sich innerhalb der Nachtschattengewächse sehr rasch entwickeln.

Die Ergebnisse meiner Arbeit unterstreichen die Bedeutung der Primärstoffwechselprodukte in der dynamischen Interaktion zwischen Pflanzen und Herbivoren und zeigen dass die Herbivoren-induzierten Veränderungen der Kohlenhydrate die Pflanzenresistenz und Toleranz maßgeblich mitbestimmen. Darüber hinaus zeigen sie dass die Jasmonat-abhängigen Veränderungen des Primär- und Sekundärstoffwechsels zu einem Konflikt für die Pflanze führen, welcher die oft beobachtete Variation der induzierten Jasmonat-Antwort in verschiedenen Spezies und Ökotypen erklären könnte.

Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena, 14th April 2015   Ricardo A.R. Machado

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufenhatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstesPromotionsverfahren überhaupt.

Jena, 14th April 2015   Ricardo A.R. Machado

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Education

2011-present	PhD student at Max Planck Institute for Chemical Ecology. Root-Herbivore Interactions Group. Jena (Türingen, Germany).
2009 – 2011	Friedrich Schiller Jena Universität. Jena (Türingen, Germany). Master degree in Microbiology. **Average grade (1.2).**
2003-2008	Universidad Nacional de Colombia. Medellin (Antioquia, Colombia). Diploma in Agronomic Engineering. **Top graduating student.**

Honors, scholarships, prizes and awards

3/2015	**Student Travel Award** to participate at the 31st conference of the international Society of Chemical Ecology.
10/2011	**Top grade (1.00) for Master Thesis.** Friedrich Schiller Jena Universität. Jena (Türingen, Germany).
12/2008	**Top Graduating Student**. Recognition given to the student who has excelled academically with the highest average among graduating students of the diploma in Agronomic Engineering.
7/2009	**Colfuturo Scholarship**. Support given to outstanding candidates based on academic achievement to pursue a postgraduate programme in a recognized University around the world.
8/2009	**DAAD Fellowship**. Financial support given to pursue a postgraduate program in a recognized University in Germany.

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Place, date   Ricardo A.R. Machado

Publications

1. **Machado RAR,** Zhou W, Ferrieri AP, Arce C, Baldwin IT, Xu S, Erb M. Rapid evolution and strong correlation between herbivory-induced root carbohydrate responses and defoliation tolerance across eight solanaceous species. *(Submitted to Molecular Ecology)*
2. Ferrieri AP, Arce C, **Machado RAR**, Meza-Canales ID, Lima E, Baldwin IT, Erb M. 2015. The invertase inhibitor NaCWII reduces growth and increases secondary metabolite biosynthesis in herbivore attacked *Nicotiana attenuata*. *New Phytologist* (*in press*).
3. Jimenez-Aleman GH, **Machado RAR,** Görls H, Baldwin IT, Boland W. 2015. Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones. Organic & Biomolecular Chemistry. (*in press*).
4. **Machado RAR**, Arce C, Ferrieri A, Baldwin IT, Erb M. 2015. Jasmonate-dependent depletion of soluble sugars compromises plant resistance to *Manduca sexta*. *New Phytologist* (*in press*).
5. Robert C, Ferrieri, R, Schirmer S, Babst B, Alexoff D, Schueller M, **Machado RAR**, Arce C, Hibbard B, Gershenzon J, Turlings T, Erb M. 2014. Induced carbon reallocation and compensatory growth as root herbivore tolerance mechanisms. *Plant, Cell & Environment* 37:2613-2622.
6. Erb M, Huber M, Robert C, Ferrieri A, **Machado RAR**, Arce C. 2013. The role of plant primary and secondary metabolites in root herbivore behavior, nutrition and physiology. In P. Evans, V. Wigglesworth (Eds.), *Advances in Insect Physiology* 53-95. Burlington: Elsevier.
7. **Machado RAR**, Ferrieri A, Robert C, Glauser G, Kallenbach M, Baldwin IT, Erb M. 2013. Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling. New Phytologist 200:1234-1246.
8. **Machado RAR**. 2010. Derechos humanos en materia de desplazamiento forzado por el conflicto socio-politico en Colombia. *El Ágora USB* 10(1):183-196
9. **Machado RAR**. 2008. "Evaluación de diferentes métodos de muestreo para estimar la infestación de la broca del café *Hypothenemus hampei* (Ferrari) (Col: Curculionidae) y reconocimiento de parasitoides establecidos en campo". In: XXXV Congreso de la Sociedad Colombiana de Entomología SOCOLEN.

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Conference presentations

1. **Machado RAR.** 2015. Jasmonate-dependent depletion of plant carbohydrates constrains resistance and tolerance against herbivores. Talk presented at 14th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE.
2. **Machado RAR.** 2014. Jasmonate-dependent depletion of soluble sugars constrains herbivore resistance and reveals the importance of carbohydrates for insect nutrition in planta. Talk presented at: Universidade Federal de Viçosa, Viçosa, BR
3. **Machado RAR.** 2014. Efecto de los metabolitos primarios y secundarios de las plantas en el desarrollo de *Manduca sexta* L. (Lepidoptera: Sphingidae). Talk presented at: Congreso de la Sociedad Colombiana de Entomología, Cali, CO
4. **Machado RAR.** 2014. Auxin as an herbivory-induced signal in *Nicotiana attenuata*. Talk presented at: 13th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE
5. **Machado RAR.** 2013. Auxins and jasmonates coordinate regrowth responses from rootstocks in *Nicotiana attenuata*. Talk presented at: Society for Experimental Biology Annual Main Meeting 2013, Society for Experimental Biology, Valencia, ES
6. **Machado RAR.** 2008. Evaluación de diferentes métodos de muestreo para estimar la infestación de la broca del café *Hypothenemus hampei* (Ferrari) (Col: Curculionidae) y reconocimiento de parasitoides establecidos en campo. Talked presented at: XXXV Congreso de la Sociedad Colombiana de Entomología SOCOLEN. Cali (Colombia)..
7. **Machado RAR.** 2008. Tenencia y uso del suelo en Colombia. Talked presented at: I Academic Journey: "Agricultural Politics". Medellin (Colombia).
8. **Machado RAR.** 2008. Tenencia de la tierra en Colombia: Repercusiones en la Seguridad Alimentaria de la Población Rural Desplazada. Talked presented at: Academic Forum "Socialismo, Neolibreralismo y derechos humanos". Medellin (Colombia).
9. **Machado RAR.** 2008. Derecho a la propiedad de la Población Rural Desplazada por el Conflicto Socio-Político en Colombia. In: "Desplazamiento Forzado en Colombia". University Lecture. Universidad Nacional de Colombia, Medellin (Colombia).

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Poster presentation

1. Ferrieri A., Gulati J., Gaquerel E., **Machado R.A.R.,** Erb M., Santhanam R., Weinhold A., Wang M., Wilde J., Groten K., Oh Y., Fragoso V., Kim S.G., Xu S., Baldwin I.T. (2014). Roots: where it all starts. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE
2. Ferrieri A., **Machado R.A.R.,** Xu S., Baldwin I.T., Erb M. (2014). Ecophysiology of the *Nicotiana attenuata* root system. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE
3. **Machado R.A.R.,** Robert C., Arce C., Ferrieri A., Xu S., Baldwin I.T., Erb M. (2014). Auxin as an herbivory-induced signal in *Nicotiana attenuata*. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE
4. Ferrieri A., **Machado R.A.R.,** Baldwin I.T., Erb M. (2013). Root foraging in *Nicotiana attenuata*. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE
5. **Machado R.A.R.** (2013). Auxins and jasmonates coordinate regrowth responses from rootstocks in *Nicotiana attenuata*. Poster presented at 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, DE
6. **Machado R.A.R.** (2013). Roots mediate resource-based trade-offs between shoot regrowth and herbivore defense via jasmonate and auxin signaling. Poster presented at Max-Planck-Day, Max-Planck-Society, Jena, DE
7. **Machado R.A.R.** (2012). Leaf-herbivory depletes root carbon reserves and constrains *Nicotiana attenuata* regrowth. Poster presented at SAB Meeting 2012, MPI for Chemical Ecology, Jena, DE
8. **Machado R.A.R.,** Baldwin I.T., Erb M. (2012). The cost of vengeance: Herbivore-attack improves resistance and reduces fitness of regrowing shoots. Poster presented at 28th ISCE Meeting, International Society of Chemical Ecology, Vilnius, LT

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