Induced Immunity Against Belowground Insect Herbivores-Activation of Defenses in the Absence of a Jasmonate Burst

Matthias Erb · Gaetan Glauser · Christelle A. M. Robert

Abstract Roots respond dynamically to belowground herbivore attack. Yet, little is known about the mechanisms and ecological consequences of these responses. Do roots behave the same way as leaves, or do the paradigms derived from aboveground research need to be rewritten? This is the central question that we tackle in this article. To this end, we review the current literature on induced root defenses and present a number of experiments on the interaction between the root herbivore *Diabrotica virgifera* and its natural host, maize. Currently, the literature provides no clear evidence that plants can recognize root herbivores specifically. In maize, mild mechanical damage is sufficient to trigger a root volatile response comparable to *D. virgifera* induction. Interestingly, the jasmonate (JA) burst, a highly conserved signaling event following leaf attack, is consistently attenuated in the roots across plant species, from wild tobacco to Arabidopsis. In accordance, we found only a weak JA response in *D. virgifera* attacked maize roots. Despite this reduction in JA-signaling, roots of many plants start producing a distinct suite of secondary metabolites upon attack and reconfigure their primary metabolism. We, therefore, postulate the existence of additional, unknown signals that govern induced root responses in the absence of a jasmonate burst. Surprisingly, despite the high phenotypic plasticity of plant roots, evidence for herbivore-induced resistance below ground is virtually absent from the literature. We propose that other defensive mechanisms, including resource reallocation and compensatory growth, may be more important to improve plant immunity below ground.

Keywords *Diabrotica virgifera · Zea mays ·* Jasmonic acid · Induced resistance · Root herbivory · Plant defenses · Plant tolerance · Plant immunity

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The Root Immune System

Plants possess an inducible immune system that helps them to cope with pathogens, nematodes, and arthropod herbivores. The functional components of this system above ground are well understood, and the current paradigm distinguishes at least four different steps from recognition to response: First, arthropod herbivores are perceived by plants via elicitors, also called herbivore and damage associated molecular patterns (HAMPs and DAMPs) (Felton and Tumlinson 2008), and wounding events. Second, a regulatory cascade is triggered, with jasmonates (JA) as central signaling components (Koo and Howe 2009). Third, both primary and secondary metabolisms are reprogrammed (Berenbaum and Zangerl 2008; Schwachtje and Baldwin 2008), resulting in plant phenotypical changes. Fourth and as a final result, plants become immune against the attacker via increased resistance and/or tolerance (Nunez-Farfan et al. 2007).

An overwhelming portion of the evidence supporting these central paradigms comes from studies above ground (van Dam 2009). However, to what extent are they valid for belowground plant parts? This question is particularly important, given the fact that arthropod herbivores of at least 25 insect families feed below ground, including many

important agricultural pests (Hunter 2001). Root herbivores can have a strong impact on plant fitness (Blossey and Hunt-Joshi 2003), and it can be expected that, just as the leaves do, plant roots should possess an inducible immune system to defend themselves. Especially the combination of low probability of attack and high potential fitness cost should have favored the evolution of inducibility below ground (Zangerl and Rutledge 1996). Yet, despite an increasing number of studies that document dynamic root responses following insect attack (Puthoff and Smigocki 2006; van Dam and Raaijmakers 2006; Hiltpold et al. 2011), few attempts have been made to understand the mechanistic basis and adaptive value of induced root responses.

Roots, since their appearance in the first land plants 400 million years ago (Raven and Edwards 2001), are highly specialized structures that differ both morphologically as well as physiologically from their aboveground counterparts: Instead of chloroplasts for example, which play a central role not only in photosynthesis but also in defensive processes (Howe and Browse 2001), roots possess leucoplasts (Itoh and Fujiwara 2010), which are not pigmented and can serve as storage organelles. Also, instead of extracuticular barriers like waxes and trichomes (Valkama et al. 2005), roots interact with their environment directly via the apoplastic space that is separated from the vascular system by casparian strips (Waisel et al. 2002). From a resource perspective, roots have direct access to the major nutrients and water, but need to import assimilated carbon from the leaves. This high degree of differentiation makes it likely that the root immune system may have a mechanistic underpinning different from leaves (van Dam 2009).

In this paper, we combine three approaches to test the central paradigms of plant immunity below ground. First, we review the available literature on root immunity by using a comparative approach with aboveground studies. Second, we use the current understanding of general root physiology to predict specific differences in induction mechanisms. Third, we present a series of experiments on the reaction of maize plants to infestation by larvae of the specialist root feeder *Diabrotica virgifera virgifera*. Based on these three approaches, we present a series of general patterns and testable hypotheses about the mechanisms and consequences of herbivore-induced defenses below ground.

Do Roots Perceive Herbivores?

To be able to respond appropriately, plants have to recognize that they are under attack. Aboveground attackers are recognized by a series of associated molecular patterns. First, the wounds inflicted by chewing mouth-parts trigger the release and oxidation of otherwise contained and compartmentalized molecules, which then act as elicitors of

defensive reactions (Ryan 2000; Huffaker et al. 2011). This process is also called damaged self-recognition (Heil 2009; Koo and Howe 2009), and the respective compounds are referred to as damage associated molecular patterns (DAMPS) (Boller and Felix 2009). Second, specific compounds in the saliva of insects, so called herbivore-associated molecular patterns (HAMPs) are recognized by the plant and boost the wound-induced immune response (Felton and Tumlinson 2008). A number of HAMPs have been structurally characterized for chewing herbivores (Alborn et al. 1997; Schmelz et al. 2007), and resistance genes that encode for receptor-like proteins have been discovered to mediate immunity against hemipteran phloem-feeders (Dogimont et al. 2010), which, taken together, leave little doubt that plants possess powerful and specific recognition systems to detect leaf-feeding herbivores (Erb et al. 2012). However, to what extent can plants recognize root attackers?

Several studies document that root herbivore attack induces pronounced defensive reactions below ground (van Dam 2009), which demonstrates that roots can perceive this type of biotic stress in some form. For phytopathogenic nematodes, the existence of resistance (R) genes hints at specific perception (Milligan et al. 1998). However, for insect herbivores, it is much less clear if roots have evolved the capacity to specifically recognize them, or if the measured responses are non-specific consequences of tissue rupturing. In maize, feeding by the root herbivore *Diabrotica virgifera virgifera* induces the emission of the sesquiterpene *(E)*-β-caryophyllene, which can be used by entomopathogenic nematodes to locate their host. Contrary to herbivore attack, "stabbing" the roots with a cork-borer did not result in the attraction of entomopatogenic nematodes (Rasmann et al. 2005). Similarly, mechanical wounding was not enough to elicit attraction of entomopathogenic nematodes to citrus trees (Ali et al. 2010). Additionally, different root herbivores have been found to elicit different quantities of the *(E)*-β-caryophyllene (Rasmann and Turlings 2008), and a number of other studies report on differential responses of roots to wounding and herbivory (Blossey and Hunt-Joshi 2003). Until now, it is unclear if the observed differences are due to herbivore-specific recognition or to different degrees of wounding (Blossey and Hunt-Joshi 2003; Rasmann and Turlings 2008). In a microarray study, we found that wounding roots with a knife is enough to regulate 80% of the maize transcripts that are responsive to *D. virgifera* (Erb 2009), suggesting that cues associated with mechanical damage dominate recognition in herbivore-induced root responses. Testing this hypothesis will require further targeted experiments. Comparing wound-induced patterns with and without the application of insect oral secretions to the roots or using herbivores with ablated salivary glands (Musser et al. 2002) would help to answer the question whether plants use HAMPS to specifically recognize

herbivores below ground. Another important aspect in this context is a role for microorganisms, which may invade the wound-sites and trigger defensive reactions. Experiments under sterile conditions may help to evaluate their contribution as eliciting factors below ground.

Overall, from an evolutionary perspective, there may be less selection pressure for roots to use specific molecular patterns to recognize herbivores than for leaves: The above-ground parts are constantly damaged by wind, wind-transported particles, rain, and heavier animals, which may favor specific recognition mechanisms as a way of reducing costs of "false alarm". Roots, however, may experience fewer abiotic mechanical damage events, and, therefore, wounding itself may be enough to reliably indicate the presence of an herbivore. From a physiological perspective, the capacity of plants to detect HAMPs below ground will depend on the presence of specific extra- or intracellular receptors in the roots. Plant roots can sense mechanical resistance (Hofmann 2009), nutrients (Schachtman and Shin 2007), and water (Kobayashi et al. 2007) as well as beneficial and pathogenic microorganisms (Mathesius and Noorden 2011), and, therefore, are likely to have a considerable array of environmental sensors and PAMP receptors at their disposition. Environmental sensing below ground is further facilitated by the enormous contact surface between roots and the surrounding matrix: At a macro scale, fine roots maximize contact surface, and at a micro-scale, the apoplastic space multiplies this surface many fold (Waisel et al. 2002). Thus, given sufficient selection pressure by herbivores, plant roots could very quickly have evolved the capacity to recognize HAMPS. The recognition of DAMPS on the other hand is a general mechanism for stress perception, as it involves compounds that are liberated from the plant following attack (Heil 2009). From a physiological perspective, the "damaged self" below ground is likely to be strikingly different from what is known in the leaves: Because roots have a distinct chemical composition, including different membrane lipids, sugars, amino acids, and secondary metabolites, wounding by herbivores could also result in differential DAMP release. It will be interesting to assess whether roots have evolved to recognize their own "damaged self" or whether the same DAMPs trigger wound-reactions above and below ground.

To understand in detail whether mechanical wounding is enough to trigger root defenses in maize, we performed an experiment with 4-day-old maize seedlings, which show a similar volatile response to herbivore attack as older seedlings (personal observations). The seedlings were either pierced 4 times with a fine needle or infested with 12 *D. virgifera* larvae. We then determined the release of (E)-β-caryophyllene, the major herbivore-induced volatile in the roots, every 2 hours over a period of 18 hours *in vivo* using solid phase microextraction (SPME) as described (Robert et

al. 2012). As shown in Fig. 1, wounding the roots was sufficient to trigger a burst of (E)-β-caryophyllene, which was only matched in intensity after 10 hours of continuous *D. virgifera* feeding. From this experiment, it can be concluded that herbivore-derived cues are not strictly required to trigger (E)-β-caryophyllene emission in maize roots, at least at this young seedling stage. Relatively mild mechanical damage is, in fact, enough to prompt a quick and robust response that is similar to herbivore feeding, suggesting low specificity of recognition. Further experiments will be necessary to exclude a potentiating effect of HAMPS from *D. virgifera* on induced defenses in maize roots.

Induced Root Signaling and the Elusive Role of Jasmonates

Following recognition by DAMPs and HAMPs in the leaves, plants start deploying intricate signaling cascades. Quick membrane depolarization by Ca^{2+} influx is followed by the generation of reactive oxygen species (ROS), the induction of mitogen activated protein kinases (MAPKs) and WRKY transcription factors, and, eventually, a pronounced increase in phytohormones (Wu and Baldwin 2009). The burst in fatty acid derived oxidative products, so called oxilipins, with jasmonic acid isoleucine (JA-Ile) as the main active jasmonate, is widely accepted to be the master regulator of induced responses against chewing herbivores (Howe and Jander 2008). However, a number of other phytohormones also mediate defensive responses, either by acting as modulators of the JA pathway, as is the case for ethylene (ET) (Onkokesung et al. 2010) and abscisic acid (ABA) (Bodenhausen and Reymond 2007; Erb et al. 2011b), or as independent inducers of distinct immune responses, as
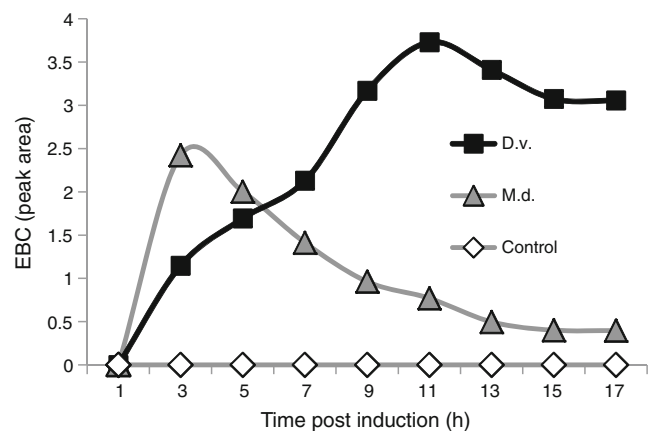

Fig. 1 (E)-β-caryophyllene is induced by mechanical damage and *Diabrotica virgifera* feeding. Quantities of (E)-β-caryophyllene (peak area/20 min) emitted from maize seedlings over time are shown. Seedlings were either untreated (Control, white diamonds), mechanically damaged with a needle (M.d., grey triangles), or infested with *D. virgifera* larvae (D.v., black squares). Amounts represent cumulative quantities from 3 equally treated seedlings

shown for salicylic acid (SA), a key player in resistance against hemipteran feeders (Gao et al. 2008). Hormones like cytokinins (CKs) and auxins that have formerly been associated with growth regulation are increasingly recognized as defense regulators as well (Erb et al. 2012). Does herbivory trigger similar signaling events below ground?

Several recent studies have measured the jasmonate response below ground following herbivore attack or mechanical injury. In *Medicago truncatula*, mechanical wounding during root harvest led to a modest increase in JA levels. Interestingly, the systemic JA response in the leaves upon root stress was stronger than the local response in the roots (Tretner et al. 2008). Similar effects were reported for *Arabidopsis thaliana* (McConn et al. 1997; Hasegawa et al. 2011). Maize roots were found to respond to herbivory by increasing their JA levels 2-fold (Erb et al. 2009a), which is modest compared to the pronounced JA response in the leaves following her-

bivory (Schmelz et al. 2003). Finally, in *Nicotiana attenuata*, a species that shows a pronounced wound-induced jasmonate burst above ground, wounding of the roots led to a modest 2-fold increase in JA levels (Bonaventure et al. 2011). Taken together, this is strong evidence that the JA-burst in plant roots generally is attenuated compared to the leaves (Fig. 2). Nevertheless, several studies point to a role for jasmonates in root defense: Exogenous application of JA or its methyl-ester MeJA increased the belowground production of ectosteroids in spinach (Schmelz et al. 1998), volatiles in maize (Erb et al. 2011a), glucosinolates in *Brassica spp.* (Pierre et al. 2012), nicotine in *Nicotiana spp.* (Baldwin 1989), a subset of sugar beet root maggot induced genes in *Beta vulgaris* (Puthoff and Smigocki 2007) and defense related transcripts in *Arabidopsis thaliana* (Hasegawa et al. 2011).

We propose three hypotheses that may reconcile the seemingly contrasting results that i) root defenses are JA-

Fig. 2 Induction of phytohormones in the roots. A–D: Average quantities (ng/mg FW) of phytohormones produced by maize roots at different time points after onset of *Diabrotica virgifera* attack. (A) Jasmonic acid (JA), (B) jasmonic acid isoleucine conjugate (JA-Ile), (C) salicylic acid (SA), and (D) abscisic acid (ABA) are shown for control plants (Control, white diamonds) and *D. virgifera* infested plants (D.v., black squares). Values correspond to average values from 6 equally treated plants. E: Average fold change of JA levels in the roots and leaves of different plants following local induction. Asterisk indicates datasets that measured JA in roots and leaves following root induction only. For references, see text
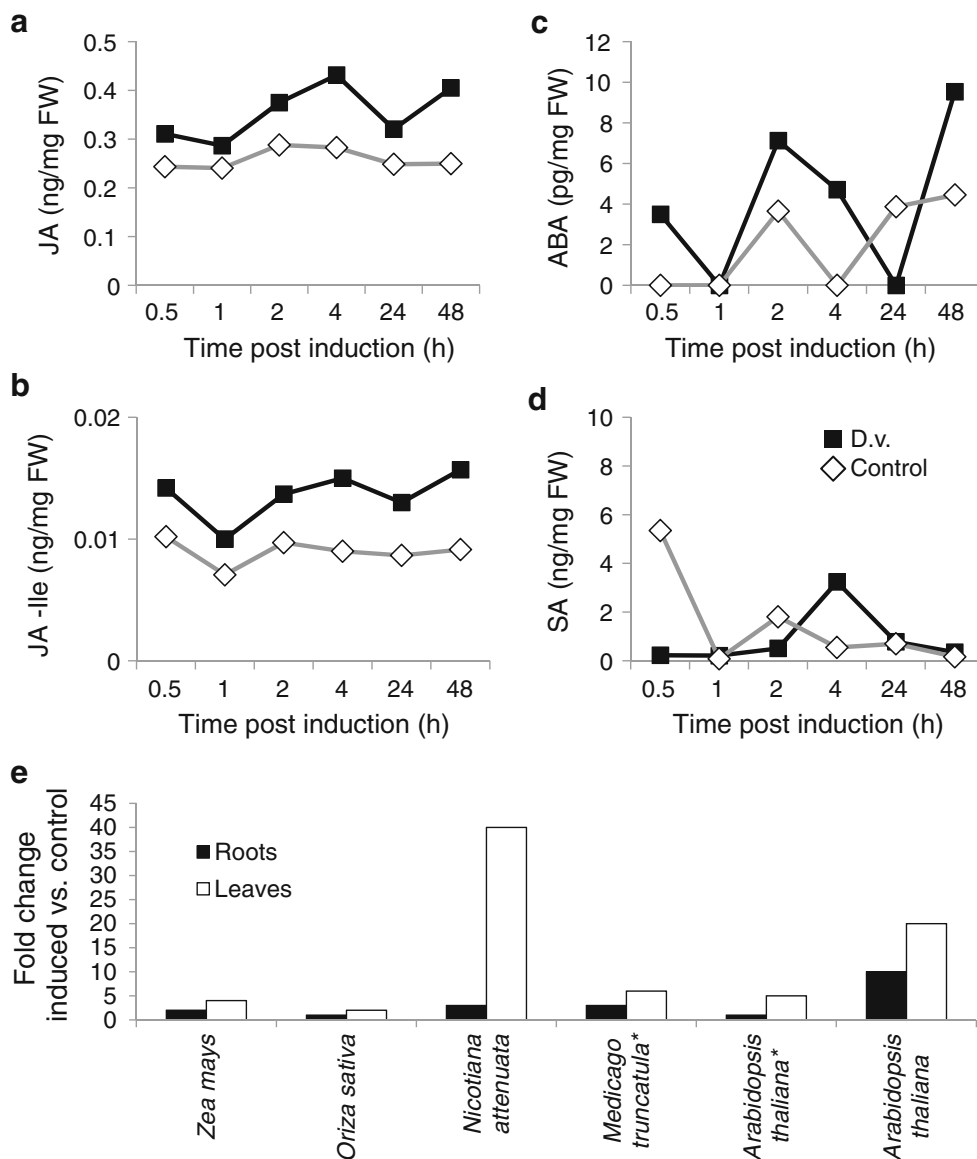
inducible, but that ii) roots produce little JA in response to herbivore attack. First, the sensitivity of roots to jasmonates may be higher, making it possible that even small changes in jasmonates are sufficient to trigger defensive reactions. Second, JA-derivatives other than JA-Ile may be the triggering signals in the roots. Third, the activation of JA-responsive elements may occur indirectly via other signals. Until now, little is known about a possible involvement of other phytohormones in induced root immunity. In our studies, we did not find any induction of ABA or SA in *D. virgifera* attacked maize roots (Erb et al. 2009a), despite the fact that ABA seems to be a herbivore-induced root-shoot signal (Erb et al. 2009b). Furthermore, the application of SA to broccoli roots could not mimic the induction of glucosinolates by *Delia radicum* (Pierre et al. 2012). The potential of SA and ET to modulate root-herbivore induced responses has been discussed by Puthoff and Smigocki (2007), who found several root maggot induced genes in *Beta vulgaris* to be responsive to these hormones. The involvement of growth hormones like auxins and CKs in induced-responses below ground has not been investigated. However, given the fact that i) belowground structures exhibit extensive regrowth and tolerance patterns following herbivory (Rubio and Lynch 2007; Poveda et al. 2010), and that ii) auxin and CKs regulate root growth patterns and branching (Aloni et al. 2006), we hypothesize that these hormones may play an important role in induced responses below ground.

From a physiological perspective, roots can employ the same signaling molecules as leaves. Major phytohormones, for example, can be detected in roots (Erb et al. 2009a). However, while the molecular vocabulary of roots and shoots may be the same, they speak a very different language: Auxin for example inhibits lateral branching above ground, but promotes root elongation below ground (Benjamins and Scheres 2008). Abscisic acid is important for stomatal regulation in leaves and regulates root growth (Cutler et al. 2010). Therefore, it can be expected that the signaling processes involved in induced root immunity will also differ from the leaves. Furthermore, the capacity of roots to synthesize specific signals following herbivory may be different from the leaves: The JA precursor linolenic acid, for example, is much less abundant in the roots than in the leaves. Instead, roots contain higher concentrations of linoleic acid (Li et al. 2003), the precursor of dihydro-JA, one of many other jasmonates with biological activity (Blechert et al. 1995; Erb and Glauser 2010). It is tempting to speculate that other jasmonates than JA-Ile may be important for defense signaling in the roots.

We measured the changes in several phytohormones in the roots of 12-day-old maize plants following *D. virgifera* attack as described (Hiltpold et al. 2011) using a previously described protocol (Erb et al. 2011b). The results show that both JA and JA-Ile increased locally by only about 50% 30

minutes after *D. virgifera* attack. The levels then remained stable over the two days of infestation (Fig. 2). On the other hand, SA and ABA did not show any clear induction pattern. The phytohormone responses are in strong contrast to the volatile production, measured in the same samples: (E)-β-caryophyllene emissions start about 4 hours after onset of feeding, and then increase exponentially (Hiltpold et al. 2011). The hormonal response of maize roots measured here adds to the growing evidence that roots do not show a characteristic JA-burst upon elicitation (Fig. 2). Furthermore, while volatile production and JA-elicitation are strongly correlated in the leaves (Schmelz et al. 2003), there is an obvious disconnect between maize root JA induction and (E)-β-caryophyllene, indicating that signals other than JA may be able to trigger the activation of sesquiterpene biosynthesis. Investigation of the regulatory mechanisms of root induction should therefore be a priority of future research on belowground defenses.

Induced Chemical and Phenotypical Changes in Roots

In the leaves, herbivore-induced defense signals trigger a variety of phenotypical changes. Typically, the reprogramming involves i) Induction of volatile organic compounds (Hare 2011), ii) induction of non-volatile secondary metabolites (Glauser et al. 2011), iii) induction of defensive proteins (Zhu-Salzman et al. 2008), iv) redirection of assimilate fluxes (Schwachtje and Baldwin 2008), and v) long term morphological changes (Strauss and Agrawal 1999; Traw and Dawson 2002). Does root herbivory result in similar phenotypical changes?

Recent studies show that attack by root-feeding insects triggers distinct phenotypical changes below ground that rival their aboveground counterparts in both diversity and magnitude. Several recent reviews have discussed induced phytochemical responses in detail (Kaplan et al. 2008b; Rasmann and Agrawal 2008; van Dam 2009), and a number of remarkable trends are emerging. First, roots produce different inducible secondary metabolites from the leaves. An illustrative example is the release of volatile compounds of herbivore attacked maize plants: Leaves of herbivore-attacked plants produce over 30 different compounds in response to real or simulated herbivory, including green leaf volatiles (GLVs), aromatic compounds, homo, mono, and sesquiterpenes. Two sesquiterpenes, (E)-β-farnesene and (E)-α-bergamotene, are dominating the induced leaf-blend (Erb et al. 2010). Attacked maize roots on the other hand produce only a few sesquiterpenes and traces of the aromatic compound indole (Hiltpold et al. 2011). The sesquiterpene (E)-β-caryophyllene dominates the root blend, while (E)-β-farnesene and (E)-α-bergamotene cannot be detected at all. Tissue specific patterns also have been observed for

herbivore induced volatiles of Swingle (*Citrus paradisi* x *Poncirus trifoliata*) (Ali et al. 2011). Furthermore, non-volatile secondary metabolites also are produced differentially in the roots. Alkaloids are a prominent example in this context: Although they are induced by leaf-herbivory and accumulate above ground, their biosynthesis often occurs exclusively in the roots (Ziegler and Facchini 2008). Additionally, Brassica plants can produce specific root glucosinolates against nematode herbivores (van Dam et al. 2009). Conversely, insecticidal phenolics such as maysin are produced only in the leaves and silks of maize plants (Nuessly et al. 2007), but not in the roots (Robert et al. 2012). Roots are thus unique bioreactors, and the cocktails of secondary compounds that they produce following herbivore attack are highly distinct.

A second emerging trend is that even compounds that are produced by both leaves and roots may differ in their inducibility between the two tissues. The insecticidal benzoxazinoid HDMBOA-Glc for example is highly inducible in maize leaves (Glauser et al. 2011), but constitutively produced below ground (Robert et al. 2012). The furanocoumarin xanthotoxin, on the other hand, is produced in constitutively high levels in the leaves of wild parsnip, but inducible in the roots (Zangerl and Rutledge 1996). These findings underscore the fact that the root secondary metabolism is specifically regulated.

Most inducible secondary metabolites are activated in a quantitative manner by JA signaling (Howe and Jander 2008), but root attack leads to an attenuated JA burst compared to the leaves (Fig. 2). We therefore reason that, if jasmonates are the predominant signals regulating root responses, the wound- and herbivore-induced production of defensive compounds in the roots should generally be reduced compared to the leaves. Surprisingly however, a recent meta-analysis found that root secondary metabolites are, overall, as inducible as their leaf-counterparts (Kaplan et al. 2008b). Because this study excluded mechanical damage treatments, but included pathogens and nematodes, we recompiled data from the literature focusing on secondary metabolites that i) occur in the leaves and roots, and ii) have been measured in both leaves and roots after local induction by herbivores or mechanical damage. Apart from data from 9 published studies (Zangerl and Rutledge 1996; Schmelz et al. 1998; Bezemer et al. 2003, 2004; Soler et al. 2005; van Dam and Raaijmakers 2006; Rasmann et al. 2009; Glauser et al. 2011; Robert et al. 2012), we included unpublished data on hydroxycinnamoyl tyramines from maize (G. Marti, unpublished). Two principal trends become visible (Fig. 3): First, for many defensive metabolites, leaves are indeed more inducible than roots (5 out of 7). Second, some specific secondary metabolites, namely phytoecdysteroids in spinach (Schmelz et al. 1998) and xanthotoxins in wild parsnip (Zangerl and Rutledge 1996) are more inducible in
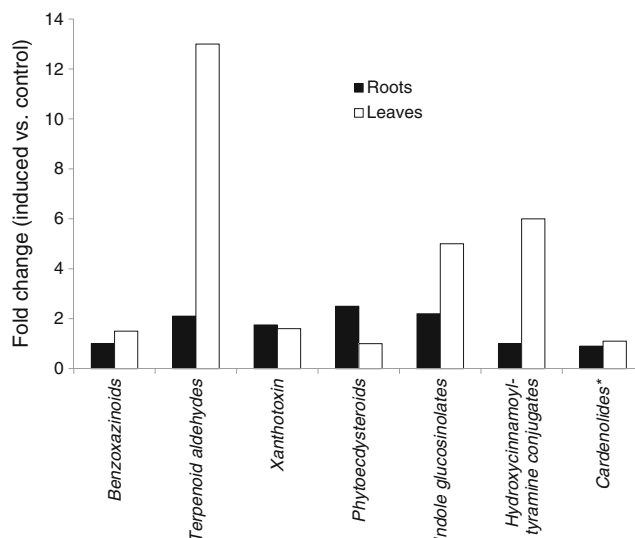

Fig. 3 Average fold change of secondary metabolites in the leaves and roots of locally induced plants. Induction was achieved by either mechanical damage or insect herbivory. *Induction of cardenolides was not significant. Data from Zangerl and Rutledge 1996; Schmelz et al. 1998; Bezemer et al. 2003, 2004; Soler et al. 2005; van Dam and Raaijmakers 2006; Rasmann et al. 2009; Glauser et al. 2011; Robert et al. 2012; G. Marti, unpublished

the roots. It would be informative to measure the root JA-burst in these two plant species to see whether their higher inducibility below ground is correlated with a high JA responsiveness, or whether, just as for (E)-β-caryophyllene in maize, the existence of alternative signals has to be envisaged. It should be emphasized that some secondary compounds may be less inducible in the roots because they are already present in high constitutive amounts. This is for example the case for glucosinolates (van Dam et al. 2009) and benzoxazinoids (Robert et al. 2012).

To get insight into the herbivore-induced changes of the root secondary metabolism of maize, we performed a metabolomics screen on *D. virgifera* induced roots of 12-day-old maize seedlings. The employed methodology was the same as published previously (Erb et al. 2011a; Glauser et al. 2011) and covered both volatile and non-volatile secondary metabolites. A similar experiment in maize leaves found a pronounced induction of several non-volatile secondary metabolites (Glauser et al. 2011). *Diabrotica virgifera* infestation led to clear changes in the root volatile profile, leading to a separation of controls and induced plants along the first principal component (PC) axis (Fig. 4). The only significant change was the induction of (E)-β-caryophyllene, as reported by previous studies (Rasmann et al. 2005). Surprisingly, however, we could not find any clear induction of non-volatile secondary metabolites in either positive or negative detection mode with UHPLC-QTOF-MS (Fig. 4). This is in contrast to the leaves (Glauser et al. 2011) and shows that the root secondary metabolome of maize is pronouncedly less responsive to herbivory
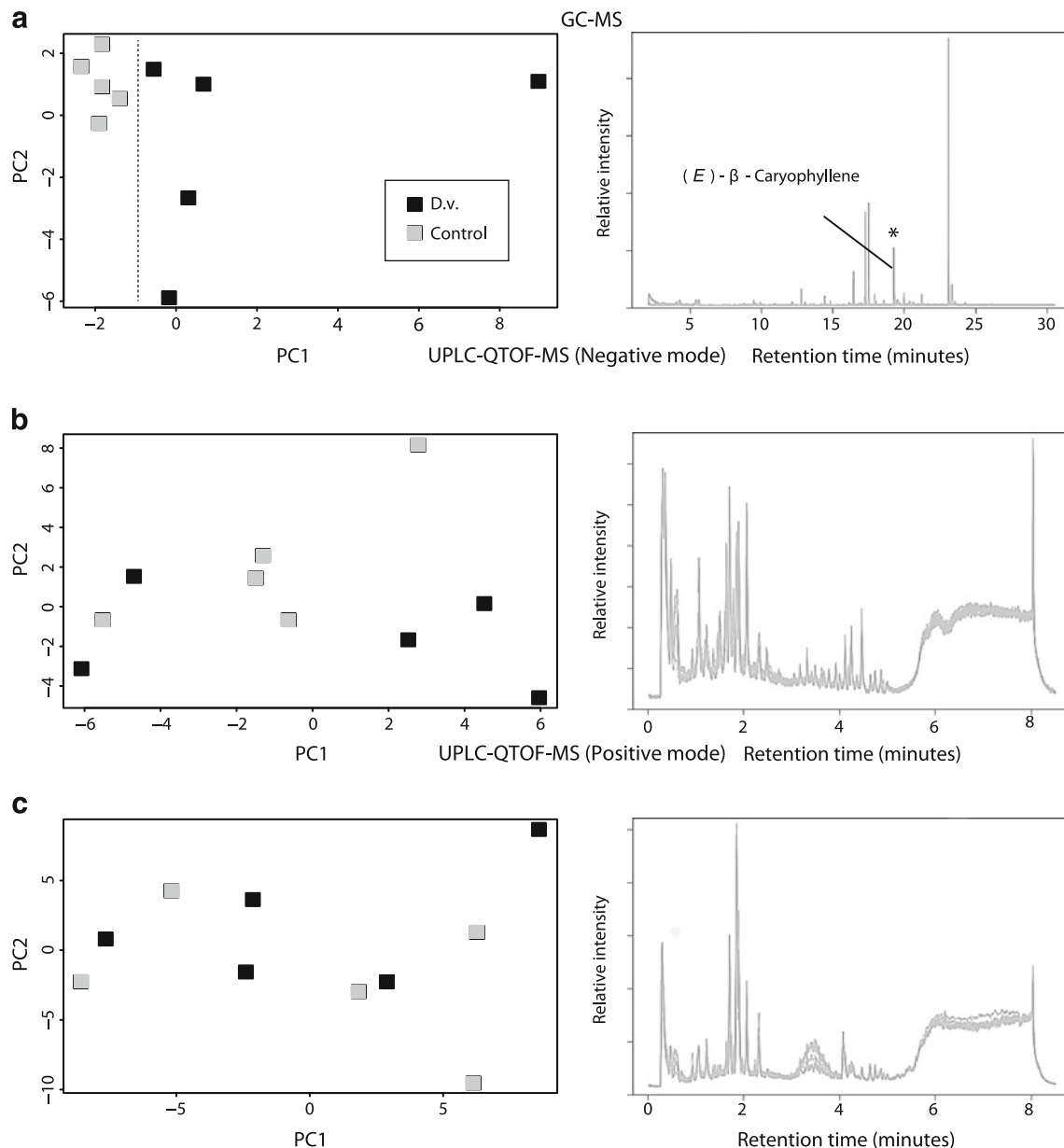
Fig. 4 Metabolomics screen of *Diabrotica virgifera* attacked maize roots. Results of principal component analyses (PCAs, left) and total ion chromatograms (TICs; right) for volatiles detected by GC-MS (**A**) and non-volatile metabolites detected with UHPLC-QTOF-MS in negative (**B**) and positive (**C**) ionization modes are shown. PCAs:

Individual control samples (grey squares) and *D. virgifera* induced samples (*black bars*) are depicted. Compounds that were different between treatments $P > 0.01$, fold change > 1.5) are denoted with an asterisk in the TICs

than the leaf metabolome. The hypothesis that *D. virgifera*, as a specialist, may suppress root responses in maize is unlikely, as we found similar results for plants that were attacked by the generalist *D. balteata* (data not shown). This finding underscores the trend that roots are generally less inducible than leaves, but that they nevertheless produce some specific secondary metabolites following herbivore attack.

Compared to low-molecular weight secondary metabolites, less is known about the induction of defensive proteins in roots following herbivore attack. Maize lines with a leaf

insect-inducible cysteine protease (Mir1-CP) were found to be resistant against *D. virgifera*, indicating that Mir1-CP may accumulate in attacked roots as well (Torrence et al. 2011). Chitinase activity was increased in *Diaprepes abbreviatus* infested citrus rootstocks (Mayer et al. 1995). We furthermore found that *D. virgifera* feeding induces the expression of several proteinase inhibitor genes (C.A.M. Robert, unpublished data), indicating that proteinase inhibitor activity may increase in infested roots. So far, both Mir1-CP and PI induction have been observed in both

above- and belowground organs (Lopez et al. 2007; Erb et al. 2009a). Untargeted proteomic approaches will be necessary to find out whether roots express specific protein-based defenses against insects. As the cost for nitrogen acquisition may be lower below than above ground, while the inverse may be true for carbon, it is well possible that evolution may have favored nitrogen- over carbon-consuming defenses below ground (Erb et al. 2009c). Apart from nitrogen containing secondary metabolites like alkaloids, defensive proteins may thus be more important in the roots than in the leaves.

Can roots reallocate resources to other tissues in the case of an attack? A study on root-herbivore tolerant *Centaurea maculosa* plants suggests that this may indeed be the case: When attacked by the root boring *Agapeta zoegana*, *C. maculosa* allocated more nitrogen to the shoots, especially under low nitrogen supply (Newingham et al. 2007), a behavior which may help the plant sustain high photosynthetic activity for compensatory growth. Further indirect evidence for changes in resource allocation comes from a study on potato that showed an increase in non-attacked tuber weight following infestation by root herbivores (Poveda et al. 2010). The JA-application to one half of a tomato root system also leads to an increase in carbon allocation to the non-treated half (Henkes et al. 2008). Resource reallocation following leaf-attack has been attributed to changes in sink-source relationships: Herbivore-attacked tobacco plants increase invertase activity in the roots (Kaplan et al. 2008a), which may increase root sink strength for carbon. When the roots are under attack, this process could simply be reversed: A reduction of invertase activity would likely lead to increased allocation to the aboveground parts of the plant. How nitrogen reallocation may be achieved remains to be determined (Lalonde et al. 2004), but it is possible that an increase in photosynthetic activity following root herbivore attack leads to an increased demand in, and, consequently, transport of nitrogen to the leaves (Godfrey et al. 1993).

Tightly linked to resource reallocation are root growth responses and morphological changes following herbivore attack. Upon root damage, certain plants start growing new roots to replace the lost tissue. *Medicago sativa* plants attacked by the root feeding larvae of *Sitona hispidula* for example react with a pronounced regrowth response that can even result in overcompensation (Quinn and Hall 1992). A particularly interesting morphological response was found in white clover plants attacked by *S. lepidus*: Five days after infestation, the tissue density of the different roots had almost tripled (Care et al. 2000), which might be the result of additional lignification, which again could lead to tougher and more resistant roots (Johnson et al. 2010). Care and co-authors also note that clover genotypes with long fine roots suffered less from root herbivory than genotypes, with short,

thick roots (Care et al. 2000). It is tempting to speculate that upon root herbivore attack, plants may change the structure of their root system to spread the risk of attack from a few main roots to many fine roots. New techniques for root visualization may help to unravel root morphological responses to belowground attack (Clark et al. 2011b). In this context, it is important to note that different belowground tissues can vary in their physiological properties and defensive status, and that resolving root defenses spatially is important to understand their effect on root herbivores (van Dam and Vrieling 1994; Robert et al. 2012).

Do Plants Use Alternative Strategies to Survive a Root Attack?

Evidence is accumulating that many leaf-responses improve plant immunity: Induced resistance, for instance, improved the fitness of wild radish and tobacco under high herbivore pressure (Agrawal 1998; Baldwin 1998). Application of extrafloral nectar as a means of attracting natural enemies increased growth rates and flower numbers of wild lima bean (Kost and Heil 2008). Activation of a carbon reallocation response by silencing a SNF1-related kinase delayed senescence and prolonged flowering in wild tobacco (Schwachtje et al. 2006). The testing for benefits of induced responses for the plant is crucial to understanding their adaptive value, and to distinguishing actual defenses from artifacts (Karban and Myers 1989). Do induced root responses improve plant immunity?

Despite an increasing number of studies on the topic, evidence for induced root resistance (i.e., the reduction of herbivore damage due to induced direct defenses) remains remarkably scarce. During our literature search, we found only one example where infestation by a belowground herbivore triggered an increase in root resistance against the same species: Vine weevils (*Otiorhynchus sulcatus*) feeding on raspberry plants that had previously been infested with conspecifics grew 19% less than larvae feeding on control plants (Clark et al. 2011a). In contrast, larvae of the onion root fly *Delia antiqua* were found to survive best on slightly damaged onion bulbs (Hausmann and Miller 1989), and *D. radicum* larvae tended to grow better on previously infested turnip plants (Pierre et al. 2012). In addition, our own experiments show that *D. virgifera* larvae perform better on roots of previously infested maize plants than on uninduced controls (Robert et al. in press). More experiments are needed to determine the prevalence of induced resistance in roots, but given the current literature, it seems that induced root resistance may not be as common as induced leaf resistance. Interestingly, many studies document that roots do have the potential to increase their resistance, given the right stimulus: The application of JA increases resistance of

broccoli roots against *D. radicum* larvae (Pierre et al. 2012) and resistance of grapevine against the root feeding grape phylloxera *Daktulosphaira vitifoliae* (Omer et al. 2000). Furthermore, leaf herbivory induces systemic resistance in roots (Soler et al. 2007; Erb et al. 2011c; Torrence et al. 2011). This suggests that, while roots have the capacity to increase their direct defenses and resistance, root herbivory simply may not trigger the appropriate signaling events. A possible evolutionary explanation for the absence of induced root resistance may be that induced resistance *per se* provides little benefit to the plant. For example, many root herbivores are specialists and, therefore, potentially resistant to inducible defenses (Robert et al. 2012). Furthermore, because movement of root herbivores is restricted in the soil and because they often tunnel into larger roots, it may be more difficult for a plant to deter attackers by inducible defenses, a putative strategy above ground that may enable plants to gain a competitive advantage, as the neighboring plants will suffer from the emigrating herbivores (van Dam et al. 2000). Clearly, further research is required to understand whether induced responses to belowground herbivores can contribute to improved plant fitness, or whether other defensive strategies generally are more effective, and, consequently, favored by evolution.

One proposed alternative strategy to induced direct resistance is the release of herbivore-induced root volatiles, which may protect plants against belowground feeders by attracting natural enemies (see Hiltpold and Turlings, this issue). Maize plants attacked by *D. virgifera,* for example, release a sesquiterpene signal that is used by entomopathogenic nematodes to locate and kill the herbivore (Rasmann et al. 2005). Comparable effects have been found in citrus (Ali et al. 2010) and rapeseed (Ferry et al. 2007) The application of nematodes to *Asclepias syriaca* plants infested with larvae of the root-boring beetle *Tetraopes tetraophthalmus* prevented the loss of biomass to root herbivory (Rasmann et al. 2011), thus hinting at the possibility that attracting nematodes may be beneficial to the plant. However, just as in the aboveground case (Heil 2008), clear evidence that herbivore-induced root volatiles are indeed an indirect defense against herbivores is lacking. On the contrary, phytopathogenic nematodes (Ali et al. 2010) as well as herbivores themselves (Robert et al. in press) may use induced root signals to find host plants (see Rasmann et al., this issue). Until now, it remains unclear whether the defensive responses of roots are directly targeted at herbivores, or are deployed to avoid secondary infections by opportunistic pathogens (Kurtz et al. 2010), which are a constant threat below ground.

Compared to induced direct and indirect defenses, there is little doubt that induced tolerance responses to root herbivory in the form of rapid regrowth improve plant immunity. *Medicago sativa* plants that regrow root nodules to equal or higher numbers after denodulation by larvae of *S. hispidulus,* for example, do not suffer from a reduction of biomass (Quinn and Hall 1992), while *Trifolium repens* plants lose a significant amount of their nodules and, consequently, suffer negative consequences from *Sitona lepidus* attack (Murray et al. 2002). A locally adapted potato variety from the Columbian Andes even increases tuber production and aboveground biomass when attacked by the Guatemalan potato moth (*Tecia solanivora*) in low densities (Poveda et al. 2010), a reaction that implicitly increases plant fitness compared to potato varieties that fail to show this type of response. Finally, the capacity of maize plants to regrow roots following *D. virgifera* infestation can significantly reduce yield loss (Prischmann et al. 2007). It remains to be demonstrated whether other putative root-herbivore tolerance strategies such as changes in resource allocation (Newingham et al. 2007; Orians et al. 2011) or increased photosynthesis (Godfrey et al. 1993) can be linked to improved plant immunity. Overall, it appears that tolerance strategies may be more commonplace than induced resistance below ground.

Conclusions

Root defenses are special. The current literature provides ample evidence for this statement and indicates that roots: i) perceive herbivores differently; ii) use different signals to react to herbivore attack; iii) synthesize specific defensive compounds; and iv) may use distinct survival strategies to improve their immunity in the face of a herbivore threat. However, many questions remain open, and mechanistic aspects have not been tackled with the same rigor as above ground, thus making many of the above conclusions tentative. Understanding the mechanisms of induced root immunity will not only broaden our horizon on plant defensive strategies, but may eventually lead to novel, paradigm-shifting insights into the secret life of roots and belowground herbivores.

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