MODIFICATION OF PLANT RESISTANCE AND METABOLISM BY ABOVE- AND BELOWGROUND HERBIVORES

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MATTHIAS ERB

Institute of Biology
Faculty of Sciences

Accepted by recommendation of the thesis committee:

Prof. Ted C.J. Turlings (thesis director)
Prof. Jörg Degenhardt
Prof. Jonathan Gershenzon
Prof. Felix Kessler
Dr. Jurriaan Ton

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Secrétariat-Décanat de la faculté
- Rue Emile-Argand 11
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Modification of plant resistance and metabolism by above- and belowground herbivores

Matthias ERB

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel,
sur le rapport des membres du jury

MM. T. Turlings (directeur de thèse),
F. Kessler, J. Gershenzon (Jena D),
J. Degenhardt (Halle D) et J. Ton (Harpenden, UK)

autorise l'impression de la présente thèse.

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Le doyen :
F. Kessler

UNIVERSITE DE NEUCHATEL
FACULTE DES SCIENCES
Secrétariat - décanat de la faculté
Rue Emile-Argand 11 - CP 158
CH-2009 Neuchâtel

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Summary and introduction

SUMMARY

Plants are often attacked by above- and belowground herbivores. As a result, they have evolved defense mechanisms to protect both their roots and shoot. However, physiological processes in roots and shoots are tightly connected, and attack of one of these plant parts can dramatically alter primary and secondary metabolism of the other (chapter 1). It is therefore important to understand how the plant reacts aboveground upon belowground insect attack and *vice versa*. This thesis investigates how shoots of maize plants respond to root attack by lavae of the beetle *Diabrotica virgifera* and *vice versa*, how roots react to shoot attack by *Spodoptera littoralis* caterpillars. This is one of the first studies highlighting the physiology and potential evolutionary significance of plant-mediated above-belowground interactions.

The results obtained show that root infestation by *D. virgifera* broadly increases defenses in maize leaves, mostly after prolonged infestation (chapters 2-4). Many of these processes were found to be inducible by absisic acid (ABA), a well-known stress-hormone that increased in concentration aboveground after prolonged belowground attack by *D. virgifera*. The increase of ABA coincided with a decrease of leaf-water content, and our experiments suggest that the observed ABA-dependent defense reaction is the consequence of a physiological stress induced by the root herbivore. The changes in shoot physiology boosted the plant's resistance against the necrotrophic pathogen *Setosphaeria turcica* and the herbivore *S. littoralis* in the laboratory, as well as against lepidopteran pests in the field, demonstrating that *D. virgifera* has an ecologically important impact on aboveground interactions. While the observed ABA response can explain the reduction of *S. turcica* growth in the leaves (chapter 2), *S. littoralis* was negatively affected by the reduction of leaf-water contents (chapter 3).

Shoot herbivory by *S. littoralis* profoundly altered root gene expression, even early after infestation (chapter 4). The reaction in the roots was entirely different from the changes in shoot transcriptional profiles, suggesting that the root-shoot signal(s) are dissimilar to the known systemic shoot defense signals. *S. littoralis* had a strong impact on root protein biosynthesis, a novel finding that demands further attention. The strong effect of *S. littoralis* infestation on root physiology was reflected in a dramatically increased resistance of attacked maize plants against *D. virgifera*.

In conclusion, both root- and shoot herbivores change the physiology of plants not only locally, but also in the unattacked parts. These changes increase the resistance of the plant against herbivores and pathogens. Root-herbivore induced shoot resistance seems to be caused by

physiological constraints rather than plant adaptive behaviour, while shoot-herbivore induced root resistance is likely to be the result of the plant’s integrated, systemic defensive system.

INTRODUCTION

The two worlds of plants and insects

If you look out of the window (unless you're living in the desert, the city centre of London, the North Pole or some other hostile environment), you will immediately realize how successful plants are in colonizing our planet (Kreft and Jetz, 2007). They grow out of cracks in roads, appear magically from the bottom of sweetwater lakes, they colonize bare land left behind by retreating glaciers and erupting volcanoes and they cover whole continents with a green, impenetrable thicket. Plants probably owe their success to a very simple concept: Independence. They have found a way of using abundantly occurring molecules in the atmosphere and the earth crust to sustain their growth and development (Taiz and Ziegler, 2006). To be able to do that, they had to develop two distinct structural elements: Shoots and leaves to access the world above the surface, the phyllosphere, and roots to penetrate deeply into the soil, the rhizosphere (Waisel et al., 2002). It is the beautiful functional orchestration of these organs that assures the availability of almost all essential elements to create plant life in all its nuances.

However, success attracts free-riders, and plants are no exeption to this. After all, eating a birchermüesli in the morning, a little mixed salad for lunch and a healthy fruit during afternoon-break is nothing else but a direct profit from the plant's extraordinary ability to assimilate inorganic matter. However, over evolutionary time, plant consumption by humans probably is a negligible factor. Indeed, a famous evolutionary biologist once said that "to a first approximation, all multicellular species on Earth are insects" (May, 1986). These six-legged creatures are old (Grimaldi and Engel, 2005), they form the most species-rich family of higher organisms on the planet (May, 1986), and- most importantly- most of them are herbivores (Strong et al., 1984). Insects, apart from microorganims, are the most important antagonists of plants. They attack them both above- and belowground, at all life-stages, in any environment, at any time of the day and any season of the year (Schoonhoven et al., 2005). Very few plants are lucky enough not to be under pressure from these herbivores.

No wonder that plants have come up with strategies to withstand extermination by insects (Karban and Baldwin, 1997; Howe and Jander, 2008). Their defensive reactions are stunningly diverse, ranging from the production of powerful toxins to the salvation of resources for later regrowth (Ort, 2008). Plants also use the advantage of having access to the soil and the air to coordinate their response to insect attack. Several toxic compounds are produced and stored in the roots, where they are safe from leaf-eating beasts, and are transported up in times of need (Smith, 1961; Baldwin et al., 1994; Lopez et al., 2007). Similarly, plants start reallocating more sugars into the roots as soon as they are attacked in the leaves, most probably to be able to regrow their aboveground parts once the raid is over (Babst et al., 2005; Schwachtje et al., 2006). However, they also behave in seemlingly strange ways sometimes, for example by increasing their leaf-defenses upon insect attack in the roots (Bezemer et al., 2003; Hol et al., 2004; Soler et al., 2005; van Dam et al., 2005). The opposite effect, i.e. and increase in root defenses upon shoot herbivory has also been observed (Soler et al., 2007). The reason why plants increase their defenses in tissues that are not attacked has remained unclear to ecologists to this day (Erb et al., 2008). What is obvious from the above examples however is that if we want to understand the defensive strategies of plants (for example to increase the resistance of crops to insects), we need to take a step back and look at them in their full functional and structural splendour.

Another important and fascinating aspect of the spatial duality of plant reactions to insects is that it makes them mediators between two very different environments: the rhizosphere, and the phyllosphere (van der Putten et al., 2001; Wardle et al., 2004). While many living creatures spend their life aboveground, crawling around on the surface or happily buzzing through the air, others live in the soil, hidden in the dark (Borror et al., 1989; Rasmann and Agrawal, 2008). Most of these organisms complete their full life cycle either above- or belowground. A notable exeption again are certain insects that develop as larvae in the soil and spend their adulthood aboveground (Vidal et al., 2005), but even here, the separation in space is only relieved by a development in time. Plants, as they are in permanent contact with the phyllosphere and the rhizosphere, willingly or not, serve as ambassadors on both sides (Bezemer et al., 2003). Sugar hoarding in the roots after aboveground insect attack in tobacco for example increases the fecundity of soil nematodes (Kaplan et al., 2008), while the increase in cabbage root toxins after shoot attack reduces the growth of the cabbage root maggot (Soler et al., 2007). Conversely, root attack by the same maggot also increases toxins in the shoot and therefore gives aboveground herbivores a hard time (van Dam et al., 2005). Such plant-mediated effects do not even stop there: If plant-feeding insects perform worse, higher trophic levels will also be negatively affected (Soler et al., 2007). That means that herbivores, by sharing the same plant, can enter into indirect competition with each other (Denno et al., 1995; Kaplan and Denno, 2007), and that whole food-web dynamics can change because of a seemingly unimportant event "in the other world" of a plant's spatial existence (Rasmann and Turlings, 2007).

Maize- a model system for above-belowground interactions

To study plant-mediated above-belowground interactions and their importance for ecology and agriculture, several general approaches can be taken, each with its distinct advantages and disadvantages. Ecologists have started to work with multiple species (Bezemer and van der Putten, 2007) in order to increase the possibility of finding generally important factors and broadly applicable concepts. More mechanistic work on the other hand can only be done on a handful of well characterized model systems (van Poecke and Dicke, 2004), bringing with it the challenge of verifying the findings in a broader ecological context. Agricultural scientists, however, prefer to work directly with crop species, mainly because of the immediate applicability of the resulting gain of knowledge. While there is a certain danger that ecologically important effects are overlooked with artificially selected agricultural plants, actual discoveries in crops often translate into natural settings as well (Gouinguené et al., 2001).

Research on above-belowground interactions has until now mainly been carried out on weakly characterized wild and agricultural species including *Senecio jacobea*, *Gossypium herbaceum*, *Spinacia oleracea* and *Brassica spp.* (chapter 1, Table 1), which possibly explains why so little is known about the physiology behind insect-mediated root-shoot interactions. The two prime natural model species for plant-insect interactions, *Arabidopsis thaliana* and *Nicotiana attenuate,* are of little use to alleviate this situation, because no ecologically or agriculturally relevant root herbivores are known to feed on them. One of the few model plants that is both relatively well characterized on the physiological and molecular level and known to be attacked by agriculturally important insect pests both above- and belowground is maize (*Zea mays*). Maize, since the beginning of its cultivation in Mexico 6000 years ago (Piperno and Flannery, 2001), has become one of the most important crops worldwide (Oerke and Dehne, 1997). Its genome is almost fully sequenced, and whole genome-microarrays are available alongside with transposon-mutant libraries and transformation systems. Furthermore, maize is attacked by a number of pest insects, with *Spodoptera* and *Diabrotica* species among the most harmful (Levine and Oloumisadeghi, 1991; Buntin et al., 2001). The larvae of the beetle *Diabrotica virgifera* for example feed on the root system of maize plants and thereby pose a major threat to the structural and functional integrity of the plant (Vidal et al., 2004). While *D. virgifera* is mostly specialized on grasses (Oyediran et al., 2004), *Spodoptera littoralis* is a generalist noctuid moth that develops on the leaves of a broad variety of plants (Brown and Dewhurst, 1975), thereby causing major yield losses. A considerable research effort over the last years has led to fascinating insights into the ecology and physiology of the interaction between maize, *D. virgifera* and *S. littoralis*, (Turlings et al., 1990; Friedrich et al., 1996; Frey et al., 1997; Schmelz et al., 2003; Rasmann et al., 2005; Rasmann and Turlings, 2007; Ton et al., 2007; Kollner et al., 2008), making the plant a suitable model to study the physiology of above-belowground interactions.

Thesis outline

I hope to have convinced you by now how important it is to study both roots and shoots of plants and their involvement with the environment *simultaneously* rather than separately, and that maize is an excellent model system for this purpose, because this is essentially what this thesis is about. I investigated several novel aspects of above-belowground interactions in maize, and you are cordially invited to browse through the manuscripts resulting from this adventure. Chapter 1 gives a general introduction of the subject from a plant physiological perspective (Erb et al., 2008). In chapter 2, I present results on the effects of root herbivore attack on shoot physiology, insect and pathogen performance. It is one of the first studies addressing above-belowground interactions on a plant physiological level. Chapter 3 follows these results and provides a possible answer to the question why plants become more resistant in the shoots upon root attack. Chapter 4 is a molecular study taking a holistic perspective of the plant's transcriptional reaction in roots and shoots after insect attack, a unique dataset harbouring many clues for future research. Chapter 5 finally is a supplementary project on the effects of a piercing-sucking insect on plant defenses.

Chapter 1: Interactions between arthropod-induced aboveground and belowground defenses in plants

Matthias Erb, Jurriaan Ton, Jörg Degenhardt and Ted C.J. Turlings

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INTRODUCTION

Plants can be attacked by arthropods both above- and belowground. The ensuing systemic defense response of the plant can affect even the most distant tissues. Both primary and secondary metabolic profiles of shoots can be altered upon root herbivory and *vice versa* (Gange and Brown, 1989; Bezemer et al., 2003; Hol et al., 2004; Schwachtje et al., 2006), making plants powerful mediators of interactions between otherwise loosely connected food webs (van der Putten et al., 2001; Bardgett and Wardle, 2003). While the ecological relevance of such processes has been recognized and the role of primary and secondary metabolites acknowledged (for reviews, see Blossey and Hunt-Joshi, 2003; van Dam et al., 2003; Bezemer and van Dam, 2005), it remains to be explored exactly how plants coordinate their root and shoot responses against herbivores.

We propose that results from current research into the mechanisms governing plant-stress responses might provide several starting points to explore the physiological basis of plant-mediated above- and belowground interactions. Priming (Ryals et al., 1996; van Wees et al., 1999; Ton et al., 2005; Conrath et al., 2006) and plant volatile signaling (Engelberth et al., 2004; Heil and Kost, 2006; Ton et al., 2007) may be particularly relevant, and we attempt to place these novel insights in the context of interactions between above- and belowground plant defense responses.

Because of the scope of this special issue, we limit our review to arthropod-induced plant defense responses. We do not discuss induced changes in primary metabolites, which can be of substantial importance (Mattson, 1980; Gange and Brown, 1989; Babst et al., 2005; Schwachtje et al., 2006). We also acknowledge the importance of putting the current findings in an appropriate ecological context and the necessity to include micro-organisms as important players in both rhizosphere and phyllosphere interactions. Several excellent reviews cover these and other intricacies of above-belowground interactions (van der Putten et al., 2001; Blossey and Hunt-Joshi, 2003; van Dam et al., 2003; Bonkowski, 2004; Wardle et al., 2004).

PLANT DEFENSE RESPONSES UPON ABOVE- AND BELOWGROUND HERBIVORY

Changes of defenses in non-attacked tissues

Various studies on interactions between above- and belowground plant responses have found an increase in basal levels of shoot defenses (defined here as the level of shoot defenses in absence of aboveground herbivores) following root herbivory, artificial damage and plant defense hormone application (Table I). Root treatments have been shown to increase shoot concentrations of terpenoids in *Gossypium herbaceum* and *Zea mays* (Bezemer et al., 2003; Bezemer et al., 2004; Rasmann et al., 2005), phenolics in *Brassica nigra* (van Dam et al., 2005), pyrrolizidine alkaloids in *Senecio jacobea* (Hol et al., 2004), certain glucosinolates in *Brassica spp.* (Birch et al., 1992; van Dam et al., 2004; Soler et al., 2005; van Dam and Raaijmakers, 2006; Soler et al., 2007), phytoectosteroids in *Spinacia oleracea* (Schmelz et al., 1998), proteinase inhibitors in *Nicotiana attenuata* (van Dam et al., 2001) and extrafloral nectar in *G. herbaceum* (Wäckers and Bezemer, 2003). Within this wide array of defensive metabolites, negative effects of root herbivory on basal levels of shoot defenses are also possible in some plant genotypes (Hol et al., 2004) and under certain experimental conditions (van Dam et al., 2005). Current results are as yet inconclusive about whether the generally observed increase of shoot defensive compounds is a result of active defense signaling and *de novo* synthesis in the shoot or if the metabolites are translocated from the roots to the shoot. We discuss both possibilities below.

In the reverse direction, effects of shoot herbivores on basal levels of root defenses have been observed (Table I). Shoot herbivory or treatment with jasmonic acid can increase root concentrations of nicotine and proteinase inhibitors in *N. attenuata* (Baldwin et al., 1994; van Dam et al., 2001) as well as glucosinolates in *Brassica campestris* and *B. nigra* (Ludwig-Müller et al., 1997; Soler et al., 2007). In contrast, reduced concentrations of other defense-related compounds can also be observed, such as in the case of pyrrolizidine alkaloids in the roots of *S. jacobea* after herbivory on shoots (Hol et al., 2004). Other studies found no clear effects of shoot treatments on basal levels of root defensive compounds including terpenoids in *G. herbaceum* and *Z. mays* (Bezemer et al., 2003; Bezemer et al., 2004; Rasmann and Turlings, 2007) phytoectosteroids in *S. oleracea* (Schmelz et al., 1998), pyrrolizidine alkaloids in *Cynoglossum officinale* (van Dam and Vrieling, 1994) and glucosinolates in *Brassica oleracea* and *B. nigra* (van Dam et al., 2004). Various patterns can be found, even for different genotypes of the same species (van Dam and Vrieling, 1994), making it difficult to draw general conclusions on how shoot treatments affect basal levels of root defenses.

Above-belowground changes of induced defenses

The above examples deal with single challenges of plant tissue that affect non-attacked parts of the plant. However, recent studies show that effects of herbivory on distant tissues do not always result in changes of defense substances, but rather in how these tissues respond when they themselves are subsequently attacked (Table I). This is the principle of priming for defense, a cost-effective way of "getting ready for battle" that results in faster and stronger defense responses upon attack (Conrath et al., 2006; van Hulten et al., 2006). While several studies indicate that root-herbivory results in enhanced resistance against aboveground attackers (Bezemer et al., 2003; Hol et al., 2004; Soler et al., 2005; van Dam et al., 2005), the importance of priming has not been thoroughly investigated in this context. Van Dam et al. (2005) found that *Delia radicum* attack of the roots resulted in lower initial glucosinolate levels in the shoot of *B. nigra*. Upon leaf damage by *Pieris rapae*, however, aboveground glucosinolate levels increased more strongly in these plants, suggesting that *B. nigra* leaves were primed for defense. In contrast, Soler et al. (2005) found no clear effect of belowground herbivory on glucosinolate levels in *B. nigra* leaves attacked by *Pieris brassicae*, implying that above-belowground responses may depend on the herbivore combination. Because priming often merely involves a faster defense reaction upon attack, its occurrence can easily be missed if measurements are taken only at one time point. Intensity and timing of direct defenses might be most easily observed by measuring the expression of defense marker genes and hormone levels (Engelberth et al., 2004; Ton et al., 2007) rather than a small sub-sample of defense-related secondary metabolites present in a plant. It has also been found that root herbivory can reduce herbivore-induced defense responses in the shoot, specifically the production of volatile terpenoids as shown for *B. nigra* (Soler et al., 2007) and *Z. mays* (Rasmann and Turlings, 2007, own research). A suppression of inducible plant defenses could be of benefit if the plant has to "set priorities" in cases of resource limitations and differential effects on fitness.

Table I: Summary of the literature on effects of root treatments (herbivory, mechanical damage or defense hormone application) on shoot defenses and *vice versa*. MD= Mechanical Damage, MJ= Methyl jasmonate, JA= Jasmonic acid, SA= Salicilic acid, ST= Shoot treatment, RT= Root treatment, AB= Aboveground, BG= Belowground, n.a.=Not applicable. For a complementary table, see also Rasmann and Agrawal (2008).

Effects of root treatments on shoot defenses

Plant	Root treatment	Induced root defense	Altered basal shoot defense		Shoot treatment	Altered ST induced shoot defense		Influences on herbivore AG	Reference
Gossypium herbaceum	*Agriotes lineatus*	Terpenoids	+	Increase of terpenoids	*Spodpotera exigua*	0	None	Reduced growth	Bezemer et al., 2003
Gossypium herbaceum	*Agriotes lineatus*, MD	Terpenoid aldehydes	+	Higher terpenoid aldehyde levels	*Spodoptera exigua*	0	None	n.a.	Bezemer et al., 2004
Brassica oleracea, Brassica napus	*Delia floralis*	Glucosinolates, indole-based compounds	+/-	Higher glucosinolate contents, lower indole- based compounds	n.a		n.a.	n.a.	Birch et al., 1992
Senecio jacobea	MD	Pyrrolizidine alkaloids	+	Partially increased pyrrolizidine alkaloids (genotype)	*Mamestra brassicae*	0	None	Partially reduced survival	Hol et al., 2004
Brassica campestris	*Delia radicum*	Unknown volatiles	+	Induced volatiles	n.a.		n.a.	n.a.	Neveu et al., 2002
Zea mays	*Diabrotica virgifera*	(E)-β-caryophyllene	+	Increased (E)-β-caryophyllene (foilage)	n.a.		n.a	n.a.	Rasmann et al., 2005
Zea mays	*Diabrotica virgifera*	(E)-β-caryophyllene	0	None (headspace)	*Spodoptera littoralis*	-	Reduced volatiles (Trend)	n.a.	Rasmann & Turlings, 2007
Spinacia oleracea	MD, MJ	20-hydroxyecdysone	+	Small induction of 20E	*Spodoptera exigua*, MD, MJ	0	None	n.a	Schmelz et al., 1998
Spinacia oleracea	*Otiorhynchus sulcatus*	20-hydroxyecdysone	0	None	n.a.		n.a.	n.a.	Schmelz et al., 1999
Brassica nigra	*Delia radicum*	n.a.	+	Higher sinigrin levels	*Pieris brassicae*	0/+	None/ Trend for increased sinigrin levels (young leaves)	Reduced growth	Soler et al., 2005
Brassica nigra	*Delia radicum*	n.a.	+	More volatile sulfides (headspace)	*Pieris brassicae*	-/+	Altered volatile profile	n.a.	Soler et al., 2007
Nicotiana attenuata	MJ	Proteinase inhibitors	+	Higher proteinase inhibitor levels	n.a.		n.a.	n.a.	van Dam et al., 2001
Brassica oleracea, Brassica nigra	JA/ SA	Glucosinolates (JA)	+	Induced glucosinolates (JA)	JA, SA	+	More total glucosinolates (JA/JA)	n.a.	van Dam et al., 2004
Brassica nigra	*Delia radicum*	n.a.	-/+	Less total glucosinolates, more phenolics	*Pieris Rapae*	+	More total phenolics	Reduced growth and survival	van Dam et al., 2005
Brassica oleracea, Brassica nigra	*Delia radicum*	Indole glucosinolates	0/+	None/ Higher glucosinolate levels (plant species)	n.a.		n.a.	n.a.	van Dam & Raaijmakers, 2006
Gossypium herbaceum	*Agriotes lineatus*, MD	n.a.	+	Induced extrafloral nectar	n.a.		n.a.	n.a.	Wäckers & Bezemer, 2003

Effects of shoot treatments on root defenses

Plant	Shoot treatment	Induced shoot defense	Altered basal root defense		Root treatment	Altered RH induced root defense		Influences on herbivore BG	Reference
Niccotiana attenuata	MD	Nicotine	+	Nicotine	n.a.		n.a.	n.a.	Baldwin et al., 1994
Gossypium herbaceum	*Spodpotera exigua*	Terpenoids	0	None	*Agriotes lineatus*	-	Non-signifcant reduction of terpenoids	None	Bezemer et al., 2003
Gossypium herbaceum	*Spodoptera exigua*	Terpenoid aldehydes	0	None	*Agriotes lineatus*, MD	-	Reduced terpenoid aldehydelevels	n.a.	Bezemer et al., 2004
Senecio jacobea	*Mamestra brassicae*	None (Pyrrolizidine alkaloids)	-	Reduced pyrrolizidine alkaloids	MD	0/-	Partially reduced pyrrolizidine alkaloids (genotype)	n.a.	Hol et al., 2004
Brassica campestris	JA, SA	Glucosinolates	+	Higher level of glucosinolates	n.a.		n.a.	n.a.	Ludwig-Müller et al., 1997
Zea mays	*Spodoptera littoralis*	(E)-β-caryophyllene	0	None	*Diabrotica virgifera*	-	Reduced (E)-β-caryophyllene	n.a.	Rasmann & Turlings, 2007
Spinacia oleracea	*Spodoptera exigua*, MD, MJ	None (20E)	0	None	n.a.		n.a.	n.a.	Schmelz et al., 1998
Brassica bigra	*Pieris brassicae*	n.a.	+	Higher indole glucosinolate levels	*Delia radicum*		n.a.	Reduced survival & size	Soler et al., 2007
Nicotiana attenuata	MJ, MD	Proteinase inhibitors	+	Higher level of trypsin proteinase inhibitors	n.a.		n.a.	n.a.	van Dam et al., 2001
Brassica oleracea, Brassica nigra	JA, SA	Glucosinolates	0	None	JA, SA	0	None discussed	n.a.	van Dam et al., 2004
Cynoglossum officinale	MD	Pyrrolizidine alkaloids	+/-	Higher/lower level of pyrrolizidine alkaloids (genotype)	n.a.		n.a.	n.a.	van Dam & Vrieling, 1994

The effects of shoot herbivory on belowground-herbivore induced root defenses have received little attention. Bezemer et al. (2003; 2004) found that shoot attack leads to a reduction of root treatment induced terpenoids and terpenpoid aldehydes in *G. herbaceum*. A similar phenomenon was observed for terpenoid volatiles in *Z. mays* (Rasmann and Turlings, 2007). We are not aware of any study that reports an increase of belowground-herbivore induced root defenses upon shoot herbivory, and it has been speculated that when attacked by both above- and belowground herbivores simultaneously, plants preferentially allocate their defenses to the shoot (Bezemer et al., 2004; Rasmann and Turlings, 2007). This hypothesis awaits further testing. Another exciting possibility is that herbivores themselves manipulate plant defenses in their favor, which could also result in changes in distant tissues. This could simply be suppression of defense responses (Musser et al., 2002) or the activation of defenses that are ineffective against the herbivore itself, but might affect other attackers. Such "decoy strategies" could be of major ecological significance and should be kept in mind when investigating above- belowground interactions.

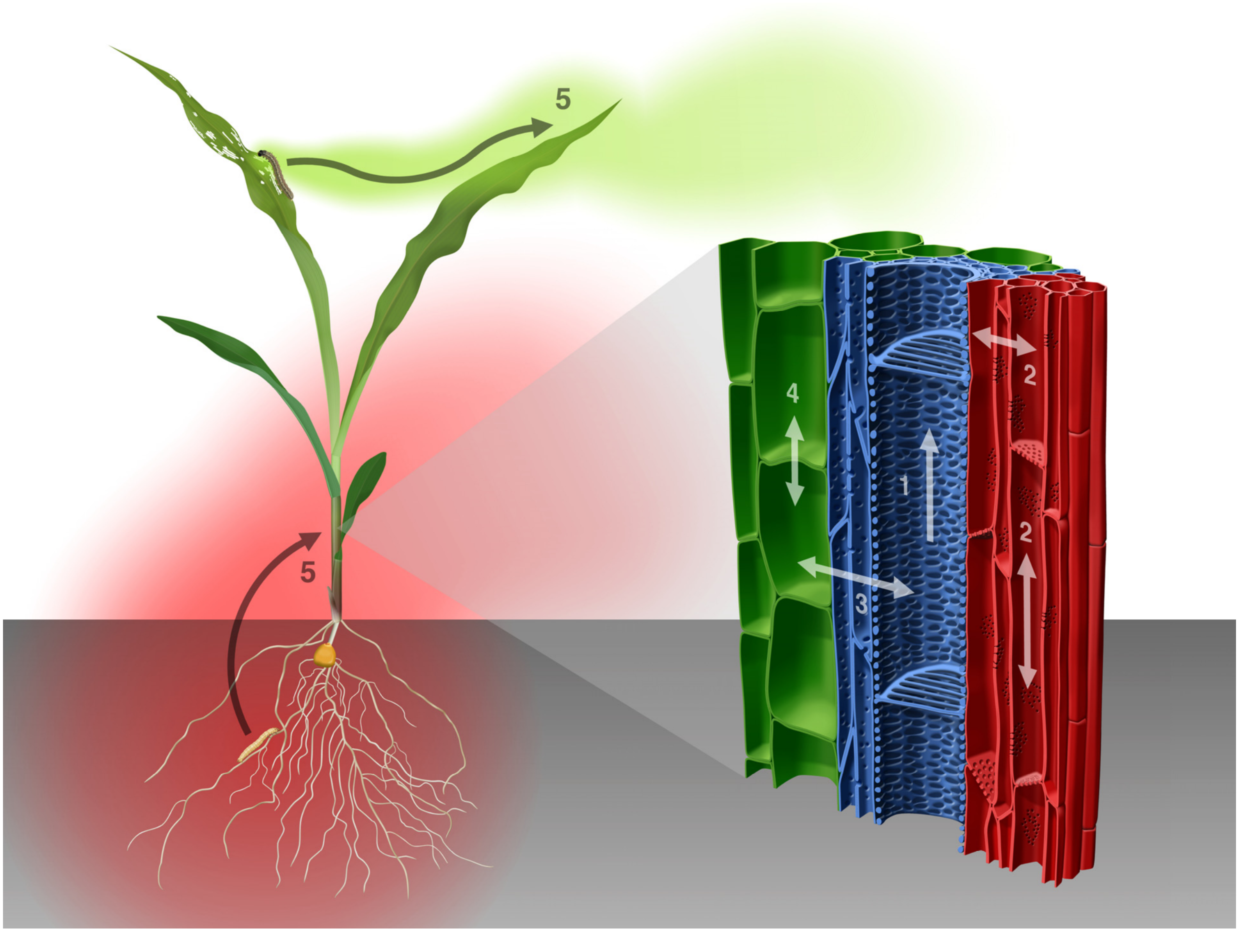

Figure 1: Model of the signaling processes behind plant-mediated above belowground interactions. Herbivores attack roots and shoot of a plant resulting in the production of various stress-related signals. As depicted in the enlarged section of a monocotyledonous vascular bundle (right), above-belowground signaling will most probably involve root to shoot transport via xylem vessels (1), bidirectional translocation via the phloem (2), exchange between the vascular tissue and the surrounding cells (3) and non-vascular cell-to-cell signaling (4). External communication with volatile compounds that can reach distant parts of the plant is also possible (5), as illustrated for a maize seedling (left). Possible mediators of the interactions are typical stress signals such as plant hormones and volatiles as well as bioactive non-hormonal metabolites

THE PHYSIOLOGICAL BASIS OF ROOT-SHOOT INTERACTIONS

The findings discussed in the previous section strongly suggest that signals are exchanged between roots and shoot upon herbivore attack. Root-shoot communication likely follows either the internal vascular network of the plant (i.e. phloem and xylem bundles, Orians, 2005; Atkins and Smith, 2007) or the external route via volatile signaling. These possible routes and preferential flows are depicted in Figure 1. It remains largely unclear which signals and/or compounds are mediating the interactions between root and shoot. The extremely variable effects of root herbivores on shoot responses and *vice versa* make it unlikely that one specific signal or process is involved. We discuss three classes of compounds that could be of major importance in this context: plant hormones, volatile organic compounds and non-hormonal secondary metabolites.

Plant Hormones

Plant hormones are crucial components of the regulatory network underlying plant growth, development and defense reactions. Several hormones have been implicated in root-shoot communication and might therefore mediate above- belowground interactions in response to herbivory.

Auxin is readily translocated from the shoot to the roots (Reed et al., 1998), where it promotes root cell proliferation and elongation (Hager et al., 1971). Belowground attack can result in compensatory root growth (Steinger and Müller-Schärer, 1992), thereby likely affecting the auxin-cytokinin balance (Woodward and Bartel, 2005), which is of major importance in regulating above- and belowground metabolic states. Application of synthetic auxin (1-naphtaleneaic acid) to spinach roots has been found to enhance levels of root phytoecdysteroids (Schmelz et al., 1999) and causes root/shoot dry mass ratios to shift. This shift indicates higher resource allocation to the roots (Schmelz et al., 1999) and implicates auxin's role as a possible regulator of above-belowground feedback. Indeed, transcriptional upregulation of the auxin-marker gene *Zm-SAUR2* in the roots of maize upon belowground feeding by *Diabrotica virgifera* was found (own results, unpublished), indicative of increased auxin shoot-root translocation or biosynthesis in the roots.

Abiscisic acid (ABA) represents a classical example of a xylem-translocated root-shoot hormone (Davies and Zhang, 1991; Jackson, 1997; but see Christmann et al., 2005; Christmann et al., 2007). While ABA is traditionally associated with responses to drought stress (Davies and Zhang, 1991), it is becoming evident that it may also have an important role in herbivore defense

(Anderson et al., 2004). Schmelz et al. (1999) found that application of ABA to the roots of spinach decreased the concentration of the defensive phytoecdysteroid 20E in the shoot. ABA-deficiency has been shown to enhance the performance of both *Spodoptera exigua* on *Solanum lycopersicum* and *Spodoptera littoralis* on *A. thaliana* (Thaler and Bostock, 2004; Bodenhausen and Reymond, 2007). Furthermore, root herbivory can elicit drought-like responses in plants (Gange and Brown, 1989, own observations), which may represent an additional link between ABA and above-belowground interactions. This is expected to be especially important when herbivores severely damage root systems, as is the case for various chewing insects. Hence, further research into the role of ABA in plant mediated-interactions between root and shoot herbivores is certainly warranted.

Jasmonic acid (JA) is often considered to be the central hormone governing systemic plant responses to herbivory aboveground (Farmer and Ryan, 1992; Howe et al., 1996; McConn et al., 1997) and probably has a similar role belowground (McConn et al., 1997; Schmelz et al., 1999; Puthoff and Smigocki, 2007). Compounds of the jasmonic acid family are suggested to be responsible for long distance wound signaling (Stratmann, 2003; Wasternack et al., 2006), a fact supported by the ability of methyl jasmonate (MJ) to move readily along both xylem and phloem pathways (Thorpe et al., 2007) as well as through the air (Farmer and Ryan, 1990). The potential of JA as an above-belowground regulator is indicated by the fact that when applied to the leaves of *Nicotiana sylvestris,* it seems to be transported to the roots, where it induces nicotine synthesis (Zhang and Baldwin, 1997). Furthermore, application of JA (or MJ) to roots induces shoot defenses (Baldwin, 1996; van Dam et al., 2001; van Dam et al., 2004), providing additional evidence for its key role in root-shoot interactions.

Salicylic acid (SA) is usually implicated in defense responses to pathogens, but can also be involved in plant responses upon herbivore attack (Zarate et al., 2007). It is not clear, however, in what respect SA functions as a systemic signal. It is unlikely that SA is the translocated signal inducing resistance in plant-pathogen interactions (Ryals et al., 1996), and van Dam et al. (2004) found no systemic effects of SA applied to either roots or shoots on glucosinolate levels in two *Brassica* species. However, the methylated form of SA (MeSA) is a mobile signal that is required for systemic resistance induction in tobacco plants (Park et al., 2007). MeSA and may also function as an airborne signal (Shulaev et al., 1997). Root systems damaged by herbivores can be assumed to have an increased risk of colonization by microorganisms, be it from the oral secretions of the attacker itself or from the rhizosphere. Hence, SA-related defenses induced in response to herbivory could be adaptive and also modulate aboveground defenses, for example via SA/JA crosstalk (Niki et al., 1998).

Finally, ethylene and its precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) have a well known function in positive root-shoot signaling (Bradford and Yang, 1980; Jackson, 1997). Research focusing on plant hormonal cross-talk has shown the importance of ethylene in modulating responses to biotic stress aboveground (Xu et al., 1994; Odonnell et al., 1996; van Loon et al., 2006), which includes activity upon attack by arthropod herbivores (Kendall and Bjostad, 1990; von Dahl and Baldwin, 2007). Puthoff and Smigocki (2007) found an upregulation of genes responsive to root herbivory in *Beta vulgaris* upon ethylene treatment, a first indication that ethylene is also involved in root defenses. Because of its volatility, ethylene can either diffuse through the vascular tissue directly into the shoot (Jackson and Campbell, 1975) or travel externally, diffusing from the rhizosphere (Jackson and Campbell, 1975) to the phyllosphere. Since it is likely that ethylene is involved in volatile defense signaling within and between plants (Ruther and Kleier, 2005), it is imperative to study this compound as a possible root-shoot signal in plant-arthropod interactions.

Volatile organic compounds as root-shoot signals

Apart from ethylene, a wide range of other volatile organic compounds (VOCs) are synthesized and released after herbivore attack above- and belowground (see for example Rasmann et al., 2005; D'Alessandro et al., 2006). Plant volatiles, in particular induced volatiles, have long been implicated in plant-plant communication. The benefit of such communication for the emitting plant is questionable, unless the information is passed on to a closely related plant. Moreover, volatile signals can be exploited by herbivores (Carroll et al., 2006) and even parasitic plants (Runyon et al., 2006). A more adaptive functioning of volatiles is in overcoming the plant’s vascular constraints and communicating between parts of the same plant (Frost et al., 2007; Heil and Silva Bueno, 2007). There is increasing evidence that green leaf volatiles (GLVs) play an important role in this context (Arimura et al., 2001; Engelberth et al., 2004; Ruther and Furstenau, 2005). Some GLVs belong to the family of reactive electrophile species (RES), which have recently been implicated as stress and defense signals (Farmer and Davoine, 2007). Several RES are very short-lived and therefore could be ideal short-range signals. We have found evidence that GLVs, despite their name, are also released from crushed roots of *Z. mays* (unpublished). In the only study that looked for belowground GLVs, Steeghs et al. (2004) did not detect any emission from artificially damaged *A. thaliana* roots, possibly because the ecotype they used (Col-0) carries a mutation severely affecting HPL activity and C_6 volatile synthesis

(Duan et al., 2005). GLVs, if indeed produced by the roots, and other volatiles are likely to diffuse into the phyllosphere and change the physiological state of plants aboveground (Fig 1). Research on the biochemistry of GLVs and other VOCs is progressing rapidly (Matsui, 2006; Schnee et al., 2006; D'Auria et al., 2007), revealing new experimental approaches to test for their effects.

Translocation of non-hormonal secondary metabolites

Secondary metabolites with defensive properties are by no means bound to either the roots or the shoot of a plant, and their translocation could account for many of the observed effects of cross-resistance and interactions between above- and belowground plant defenses. Nicotine for example is the prime example of a secondary metabolite that it synthesized in the roots of *Niccotiana spp*. and then translocated to the shoots to unleash its anti-herbivore properties (Shoji et al., 2000 and references therein). Van Dam and Vrieling (1994) report a negative relationship between changes in wound-induced pyrrolizidine alkaloid contents in the roots and the shoots of *Cynoglossum officinale*, which can be seen as an indication for within-plant transport of this class of compounds. Rasmann et al. (2005) found increased levels of (E)-β-caryophyllene in maize shoots upon root feeding by *Diabrotica virgifera virgifera*. Köllner et al. (in preparation) found no indication of higher transcriptional activity of the corresponding terpene synthase in the shoot upon *D. virgifera* feeding on the roots, indicating that it is the compound itself that is translocated from the roots to the shoot. A recent study on terpenoid synthesis in carrots found (E)-β-caryophyllene to be independently synthesized in the roots and the shoot (Hampel et al., 2005). These indicative results underpin the possibility that it is not necessarily only the activation of aboveground defenses that leads to higher concentrations of secondary compounds in the shoot upon root herbivory, but also simple translocation, be it active transport or passive diffusion.

CONCLUSIONS

Plant-mediated interactions between above- and belowground arthropod herbivores can have profound effects on natural and agricultural food-webs. Although only few studies have specifically looked at defense responses of plants that have been subjected to both root- and shoot herbivory, it is clear that there is considerable complexity, which depends on a variety of biotic and abiotic factors. Even with our limited knowledge, we can conclude that it is unlikely that all effects are the result of the same physiological processes. Research into the mechanisms as well as the ecological significance of root-shoot feedback effects is sorely needed, and current progress in plant biochemistry and targeted molecular manipulation is likely to reveal which genes and pathways are involved. Recent discoveries focusing on priming for defense and the role of volatiles as external cues involved in plant defense responses show great promise for a better understanding of within-plant signaling. Applying this knowledge for a comprehensive insight into the ecological relevance of cross-effects between above and belowground interactions requires close collaboration between plant physiologists and ecologists.

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Chapter 2: Signal signature of aboveground induced resistance upon belowground herbivory in maize

Matthias Erb, Victor Flors, Danielle Karlen, Elvira de Lange, Chantal Planchamp, Marco D’Allessandro, Ted C.J. Turlings and Jurriaan Ton

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SUMMARY

Plants activate local and systemic defence mechanisms upon exposure to stress. This innate immune response is partially regulated by plant hormones and involves accumulation of defensive metabolites. While local defence reactions to herbivores are well studied, less is known about the impact of root herbivory on shoot defence. Here, we examined the effects of belowground infestation by the western corn rootworm *Diabrotica virgifera virgifera* on aboveground resistance in maize. Belowground feeding by *D. virgifera* induced aboveground resistance against the generalist herbivore *Spodoptera littoralis* and the necrotrophic pathogen *Setosphaeria turcica.* Furthermore, *D. virgifera* increased shoot levels of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and primed induction of chlorogenic acid upon subsequent infestation by *S. littoralis.* To gain insight into signaling network behind this below- and aboveground defence interaction, we compiled a comprehensive set of 32 defence-related genes whose transcriptional profile can mark activities of different hormone response pathways. Belowground attack by *D. virgifera* triggered an abscisic acid (ABA)-inducible transcription pattern in the shoot. Quantification of defence hormones showed a local increase in production of oxylipins after root and shoot infestation by *D. virgifera* and *S. littoralis*, respectively. On the other hand, ABA accumulated locally and systemically upon belowground attack by *D. virgifera*. Furthermore, *D. virgifera* reduced aboveground water content, whereas removal of similar amounts of root biomass had no effect. Our study shows that root herbivory by *D. virgifera* specifically alters the plant's aboveground defence status against biotic and abiotic stress and suggests that ABA plays a role in the signaling network mediating this interaction.

INTRODUCTION

To cope with environmental stress, plants possess an inducible immune system that provides protection against many potentially harmful organisms (Agrawal, 1998; Tollrian and Harvell, 1998). Inducible defenses, such as the production of defensive metabolites and proteins, are controlled by signaling pathways that are specifically activated upon perception of stress-derived signals. While jasmonic acid (JA) and ethlylene (ET) play predominant roles in the regulation of defensive responses to herbivory (Odonnell et al., 1996; Farmer et al., 2003), pathogen resistance involves a broad range of regulatory mechanisms, which are controlled by different hormone-dependent defence pathways including salicylic acid (SA) (Delaney et al., 1994; Loake and Grant, 2007), MeJA (Glazebrook, 2005) , ET (van Loon et al., 2006) and ABA (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2007; Flors et al., 2008). ABA also plays a key role in the tolerance response to abiotic stress and has been reported to act as a systemically transported signal from the roots to shoots (Jackson, 1997).

While plant stress responses can be relatively specific (De Vos et al., 2005), there is increasing evidence that stress-induced signaling pathways can interact with each other. This signaling cross-talk is thought to integrate multiple stress signals into one appropriate and specific defence response (Pozo et al., 2004). Examples of signaling cross-talk are the antagonistic interaction between the JA- and SA-dependent pathways (Pieterse and Van Loon, 2004; Beckers and Spoel, 2006), the synergistic function of ET on JA- and SA-inducible defenses (Lorenzo et al., 2003), and the cross-effects between ABA, JA-, and ET-dependent stress responses (Anderson et al., 2004; Mauch-Mani and Mauch, 2005). Depending on the type of interaction, pathway cross-talk can have positive and negative outcomes on plant resistance (Stout et al., 1998).

Striking examples of interacting stress responses come from plant-mediated interactions between above- and belowground herbivores (van der Putten et al., 2001; Bardgett and Wardle, 2003; Kaplan et al., 2008). Recent evidence suggests that root herbivory modulates shoot defenses, thereby altering shoot herbivore performance and even the behaviour of organisms at higher trophic levels (Wäckers and Bezemer, 2003; van Dam et al., 2005; Rasmann and Turlings, 2007; Soler et al., 2007; Soler et al., 2007). Because of their potential to influence entire food webs and ecosystems, interactions between below- and aboveground plant defenses are highly relevant from an ecological point of view. It remains, however, unclear if these interactions are adaptive, and if they are, to whom (Wäckers and Bezemer, 2003)? Answering this question has been hampered by the fact that the physiological basis of below- and aboveground interactions is poorly understood (Erb et al., 2008).

In this study, we characterized the defence response of maize to belowground attack by larvae of the beetle *Diabrotica virgifera virgifera*. We show that infestation by this specialist root herbivore induces aboveground resistance against chewing herbivores and pathogens and boosts systemic production of defensive metabolites. Furthermore, we provide evidence that belowground attack by *D. virgifera* triggers a local and systemic increase in ABA accumulation, as well as ABA-inducible gene transcription in the leaves The fact that root herbivory also caused desiccation of the leaves suggests that this ABA response is related to an osmotic stress reaction of the plant, which influences the plant's interaction with aboveground attackers.

RESULTS

Root herbivory by D. virgifera *induces aboveground resistance against the leaf herbivore* S. littoralis *and the necrotrohpic fungus* S. turcica.

To investigate the impact of root herbivory on aboveground resistance, we quantified levels of resistance to the generalist herbivore *Spodoptera littoralis* and the necrotrophic fungus *Setosphaeria turcica* in leaves of *D. virgifera*-infested maize seedlings. Compared to un-infested control plants, *D. virgifera*-infested plants allowed significantly less growth of *S. littoralis* larvae over a 11-hour time-interval (Figure 1A, see also Figure S1). Similarly, *S. turtica* caused significantly smaller lesions and developed shorter hypha on *D. virgifera*-infested plants at 3 days after inoculation (Figure 1B). Hence, belowground infestation by *D. virgifera* induces aboveground resistance against both *S. littoralis* and *S. turcica*.

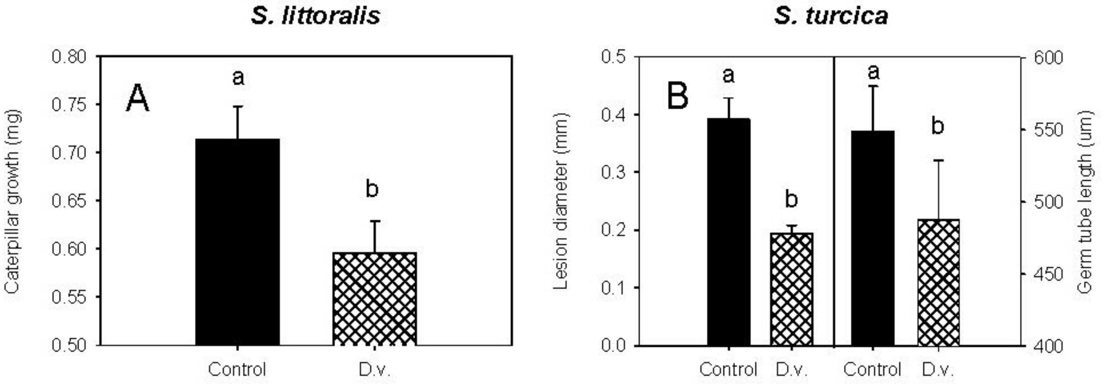

Figure 1: Root herbivore-induced resistance in maize leaves against *S. littoralis and S. turcica*. Leaf challenge with *S. littoralis* caterpillars and *S. turcica* spores was performed at 4 days after application of *D. virgifera* larvae to the roots. A: Average growth (+SE) of *S. littoralis* caterpillars over a feeding period of 11 hours on control plants, and *D. virgifera*-infested plants *(D.v.)*. B: Average lesion diameters (+SE, left) and average hyphal lengths of germination tubes (+SE, right) in leaves of *S. turcica*-infested plants at 3 days after inoculation. Different letters indicate significant differences between treatments ($p<0.05$).

Root herbivory induces shoot DIMBOA and primes for chlorogenic acid induction.

To examine the impact of belowground infestation on aboveground defence compounds, we profiled secondary metabolite composition in the leaves after root feeding by *D. virigfera* and subsequent leaf infestation by *S. littoralis*. HPLC-DAD quantification of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) showed that root attack by *D. virgifera* directly increases DIMBOA levels in the leaves (Figure 2A). DIMBOA was induced to even higher levels by shoot infestation of *S. littoralis*,. This level of induction was not influenced by belowground *D. virgifera* infestation. DIMBOA glucoside (DIMBOA-glc) levels were not significantly affected by the different herbivore treatments (Figure 2B). UPLC-MS analysis of phenolic compounds revealed that ferrulic acid levels remain unaltered in response to all herbivore treatments (Figure 2E), whereas caffeic acid production was significantly suppressed upon infestation by *D. virgifera* or *S. littoralis* (Figure 2D). Chlorogenic acid was significantly induced by *S. littoralis* feeding, but not by *D. virgifera*. Interestingly, however, *D. virigifera* infested plants showed augmented production of chlorogenic acid following *S. littoralis* attack (Figure 2C), suggesting that belowground herbivory primes chlorogenic acid production aboveground.

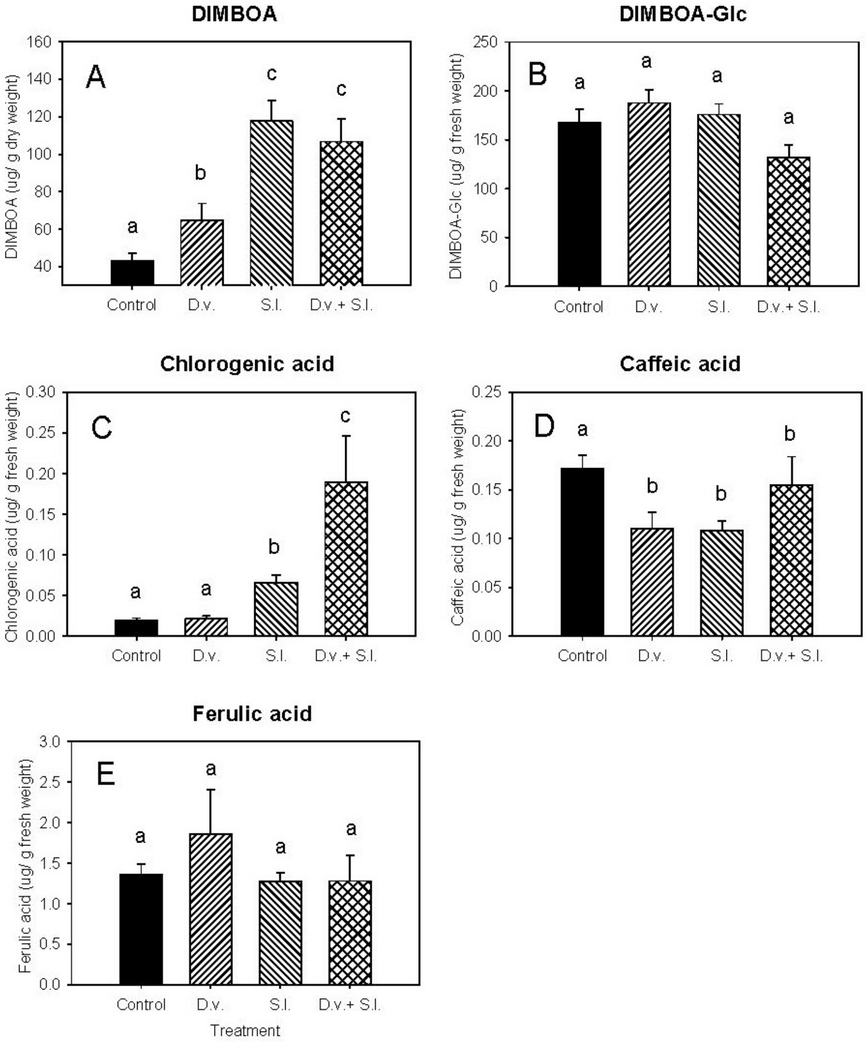

Figure 2: Average concentrations of DIMBOA (**A**), DIMBOA-glucoside (**B**), chlorogenic acid (**C**), caffeic acid (**D**) and ferulic acid (**E**) in leaves of herbivore-infested plants. Leaves were collected after 4 days of belowground infestation by *D. virgifera (D.v.)*, 2 days of aboveground infestation by *S. littoralis* (S.l.), or simultaneous infestation by *D. virgifera* (4 days) and *S. littoralis* (2 days; *D.v+ S.l.)*. Values presented are concentrations in µg/g fresh or dry weight (+SE). Different letters indicate significant differences between the treatments ($p<0.05$).

A transcriptional marker system to differentiate between hormone-dependent defence responses to (a)biotic stress.

To further examine the impact of belowground *D. virgifera* on aboveground defence, we developed a transcriptional marker system to distinguish between different stress and defence pathways. To this end, we designed primers against 32 stress- and hormone-inducible genes for reverse-transcriptase quantitative PCR (RT-qPCR) analysis (Table S1). To test if this set of marker genes can differentiate between different (a)biotic stress responses, we analysed shoots of plants after exposure to various stress treatments, such as aboveground attack by *S. littoralis* and

S. turcica, as well as mechanical leaf damage and belowground salt stress. Hierarchical clustering (HC) and principal component (PC) analysis showed that the transcription profiles can be used reliably to distinguish different stress reactions in the plant (Figures 3 and S3). To investigate if these transcriptional stress responses involve regulation by hormones, we treated maize seedlings with JA, ABA, 1-aminocyclopropane-1-carboxylate (ACC, the direct precursor of ethylene) (Adams and Yang, 1979) and benzothiadiazole (BTH, a functional homologue of SA) (Friedrich et al., 1996), after which the resulting transcription profiles were compared to stress-induced profiles (Figures 3 and S3). The effectiveness of these hormone treatments was confirmed by quantifying levels of hormone-induced resistance against *S. littoralis*, *S. turcica* and salt stress (Figure S2). HC analysis of the combined samples revealed that the JA-induced gene profile is related to those elicited by *S. littoralis* feeding or *S. littoralis* regurgitant (Figure 3). Both HC and PC analysis indicated similarity between *S. turcica*-infected profiles and ACC- and BTH-induced profiles (Figures 3 and S3), suggesting that the plant's defence response to *S. turcica* involves regulation by ET and SA. Although HC did not reveal a close relationship

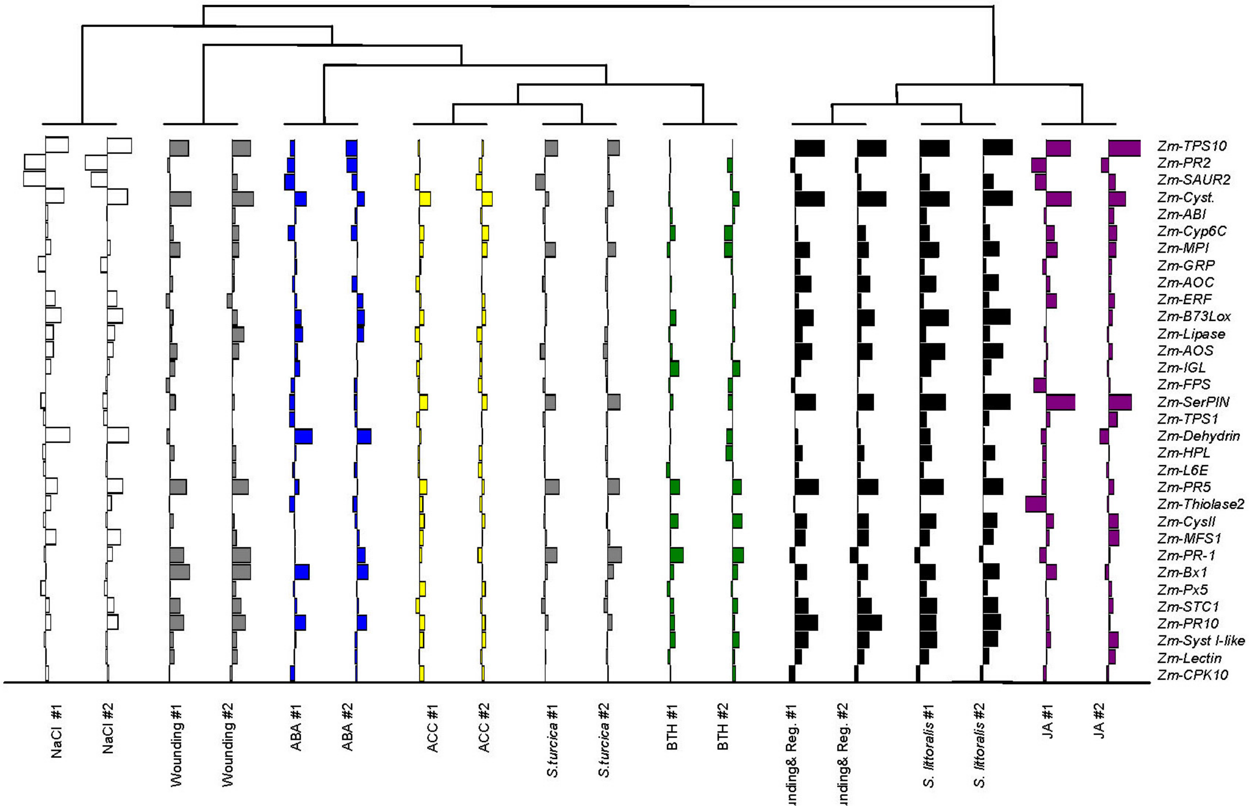

Figure 3: Hierarchical cluster analysis of gene induction profiles in maize leaves after treatment with (a)biotic stress or defence hormones. Leaves were collected at 1 day after soil-drenching with salt (NaCl; 150 mM), abscisic acid (ABA; 300 µM), jasmonic acid (JA; 300 µM), 1-aminocyclopropane-1-carboxylate (ACC; 2 mM), or at 2 days after spraying the leaves with benzothiadiazole (BTH; 5 mM). Leaves from wounded plants with or without 50% *S. littoralis* regurgitant were collected at 1 day after treatment (Wounding& Reg. and Wounding, respectively). Leaves from herbivore- and pathogen-treated plants were collected at 2 days after application of *S. littoralis* caterpillars and 3 days after inoculation with *S. turcica* spores. Bar width of induced (to the right, scale from 0 to 6) or repressed (to the left, scale from 0 to -6) genes is proportional to the ln-transformed fold-induction values of each gene relative to the control treatment. Average linkage clustering (black trees) shows relative similarities between the transcription profiles upon the different treatments.

between salt-stressed and ABA-treated plants (Figure 3), the transcription profiles of both treatments clustered relatively closely in the PC diagram (Figure S3), which suggests partial involvement of ABA in the plant's response to salt stress. Together, these results benchmark our transcriptional marker system as a suitable method to quantify activities of SA-, JA-, ET-, and ABA-dependent signaling activities in (a)biotic stress reactions.

Root herbivory alters hormone-dependent gene expression in the leaves

Using our transcriptional marker system, we quantified basal and *S. littoralis*-inducible gene profiles in leaves of *D. virgifera*-infested plants. Leaf material from 3 independent experiments was collected at 4 days after application of *D. virgifera* larvae to the roots and at 2 days after application of *S. littoralis* caterpillars to the leaves. HC analysis of the different transcription profiles revealed clearly distinctive patterns of gene expression in response to the different herbivore treatments (Figure 4A and Table 1). Whereas *D. virgifera*-induced profiles clearly clustered apart from *S. littoralis*-induced profiles, the transcription profiles of double-infested plants showed an intermediate clustering (Figure 4A). In response to *D. virgifera* infestation, the defence-related genes *Zm-Bx1* and *Zm-Cyst* showed statistically significant levels of induction. Furthermore, *D. virgifera* elicited a remarkably pronounced systemic induction of the ABA-dependent *Zm-Dehydrin* gene. This suggests involvement of ABA in the aboveground response to *D. virgifera*. Infestation of the leaves by *S. littoralis* triggered statistically significant inductions of 15 genes (*Zm-AOS, Zm-B73LOX, Zm-Syst-1-like, Zm-Cyst, Zm-CystII, Zm-SerPIN, Zm-MPI, Zm-Bx1, Zm-IGL, Zm-STC1, Zm-TPS10, Zm-PR10, Zm-PR1, Zm-PR5 and Zm-MFS1*; Table 1).

Table 1: Fold-induction (±SE) of 32 genes in maize leaves of herbivore-infested plants. Leaf material was collected after 4 days of belowground infestation by *D. virgifera*, 2 days of aboveground infestation by *S. littoralis*, or after simultaneous infestation by *D. virgifera* (4 days) and *S. littoralis* (2 days). Asterisks indicate statistically significant differences in gene expression compared to controls (Student's T-test; *p<0.05, **p<0.01, ***p<0.001). Different letters indicate significant differences between treatments (One-Way-ANOVA followed by a Holm-Sidak test for pairwise multiple comparisons (p<0.05).

Gene Name	*S. littoralis*	*S. littoralis* and *D. virgifera*	*D. virgifera*	*F* value	*P* value
Zm-CPK10	1.34±0.34	0.95±0.01*	0.74±0.4	1.61	0.275
Zm-AOC	1.09±0.05	0.65±0.15	0.93±0.49	0.55	0.602
Zm-ABI	0.81±0.23	0.27±0.13	0.39±0.07*	1.91	0.228
Zm-Px5	0.95±0.26	0.44±0.3	0.5±0.28	1.23	0.356
Zm-CysII	24.39±4.11***(a)	12.92±8.67*(a)	0.83±0.29(b)	14.97	0.005
Zm-L6E	1.06±0.33	0.52±0.11	0.42±0.08	2.43	0.168
Zm-SerPIN	458.07±158.08***(a)	34.85±19**(b)	1.13±0.05(c)	65.25	0.000
Zm-Lipase	2.05±0.36	4.2±1*	1.92±0.38	3.51	0.098
Zm-GRP	1.87±0.28(a)	0.69±0.16(b)	0.7±0.17(ab)	7.09	0.026
Zm-Cyst	1958.63±1413.87*(a)	264.4±37.37***(a)	3.74±0.72*(a)	6.36	0.033
Zm-Lectin	1152.89±694.52	596.77±500.23	186.63±160.75	0.36	0.714
Zm-Bx1	44.1±16.63**	20.34±16.74	1.98±0.21**	3.91	0.082
Zm-Thiolase2	1.01±0.15	3.57±1.19	1.62±0.61	2.56	0.157
Zm-HPL	3.78±1.35	1.64±0.74	1.23±0.33	2.36	0.175
Zm-FPS	1.12±0.52	4.52±2.92	1.86±1.29	0.69	0.537
Zm-STC1	25.24±6*	20.63±8.82	5.78±2.63	3.17	0.115
Zm-B73Lox	292.32±78.76***(a)	131.34±68.51**(a)	5.31±3.37(b)	17.07	0.003
Zm-MPI	8.97±3.21**	14.06±9.18	1.58±0.78	2.19	0.193
Zm-SAUR2	0.86±0.34	0.58±0.27	0.43±0.28	0.70	0.531
Zm-Syst I-like	42.44±7.19**(a)	10.83±6.18(a)	1.08±0.62(b)	13.94	0.006
Zm-MFS1	3.95±0.88**(ab)	9.91±3.15**(a)	2.13±0.31(b)	7.19	0.026
Zm-PR-1	15.86±8.48*	268.26±181.07*	135.18±128.94	0.76	0.507
Zm-Dehydrin	1.46±0.76(a)	3212.91±1790.86**(b)	1535.45±1440.72*(b)	14.03	0.005
Zm-IGL	20.98±6.97**(a)	10.29±4.26*(ab)	1.44±0.55(b)	8.68	0.017
Zm-ERF	2.01±0.96	14.54±4.76**	2.76±2.14	4.69	0.059
Zm-Cyp6C	1.05±0.34	0.99±0.26	0.76±0.17	0.26	0.782
Zm-PR2	1.06±0.57	0.78±0.73	0.39±0.28	0.63	0.565
Zm-PR10	78.32±10.94***	50.1±10.16**	22.16±17.93	4.11	0.075
Zm-TPS10	11852.28±2609.75***(a)	11211.28±5264.86***(a)	2.96±2.02(b)	60.03	0.000
Zm-PR5	112.1±69.03**	31.84±15.19*	6.73±4.94	3.64	0.092
Zm-TPS1	1.62±0.35	0.51±0.17	0.55±0.19	3.17	0.115
Zm-AOS	13.01±2.02**(a)	13.36±9.23(a)	1.25±0.53(b)	7.33	0.025

To assess the role of plant hormones in the aboveground response to *D. virgifera*, we compared herbivore- and hormone-induced gene profiles by multivariate statistical analysis. Feeding by *S. littoralis* triggered transcriptional profiles that resembled JA-induced profiles, while the transcriptional patterns of *D. virgifera* infested plants clustered relatively closely to the profiles of ABA-treated plants (Figure 4B). This leads to the conclusion that belowground infestation by *D. virgifera* predominantly promotes ABA-inducible gene expression in the leaves.

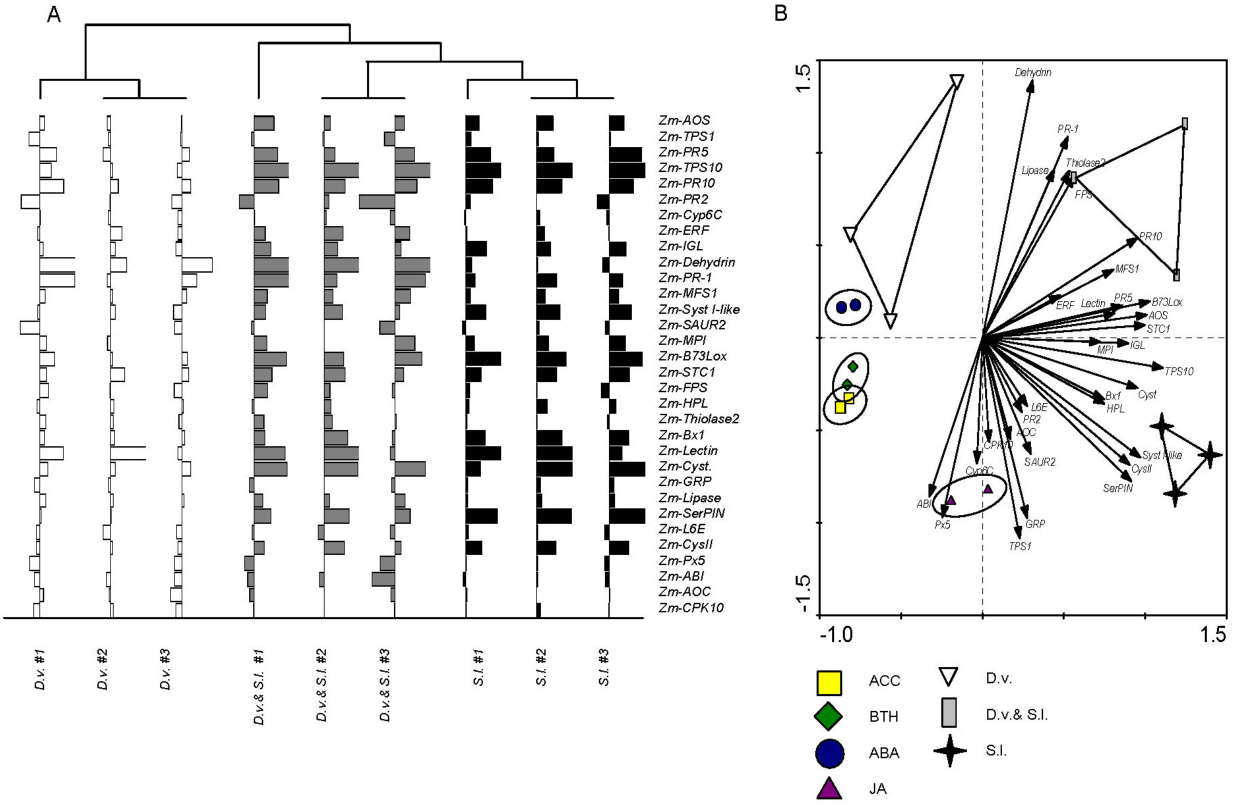

Figure 4: Gene expression profiles in maize leaves after 4 days of belowground infestation by *D. virgifera (D.v.)*, 2 days of aboveground infestation by *S. littoralis (S.l)*, or after simultaneous infestation by *D. virgifera* (4 days) and *S. littoralis* (2 days; *D.v.+ S.l.).* **A:** Hierarchical cluster analysis of gene induction profiles upon treatments. For details, see legend to Figure 1A. **B:** Principal component analysis of the combined ln+1-transformed gene expression values.

Impact of above- and belowground herbivory on defence hormones in roots and shoots.

Our observation that belowground infestation by *D. virgifera* elicits ABA-dependent gene expression in the leaves prompted us to quantify levels of different defence-related hormones (ABA, JA, its precursor 12-oxo-phytodienoic acid (OPDA) and SA), in leaves and roots of herbivore-infested plants. Aboveground attack by *S. littoralis* caterpillars induced a strong local induction of JA and its 12-oxo-phytodienoic acid (OPDA) and a relatively modest induction of ABA (Figure 5). This aboveground infestation had no systemic effects on hormone levels in the roots (Figure 5). Belowground attack by *D. virgifera* caused a local increase in JA, OPDA and ABA that was statistically significant (Figure 5). Interestingly, *D. virgifera* infestation also increased ABA levels in the leaves, whereas JA, OPDA and SA remained unaltered. The systemic induction of ABA production by *D. virgifera* plants was even more pronounced when plants were subsequently infested by *S. littoralis* (Figure 5C). Thus, OPDA, JA and ABA are enhanced locally by *D. virgifera* and *S. littoralis* attack, but the only hormone responding systemically to belowground *D. virgifera* attack is ABA. This *D. virgifera*-induced ABA is even further boosted by subsequent *S. littoralis* attack.

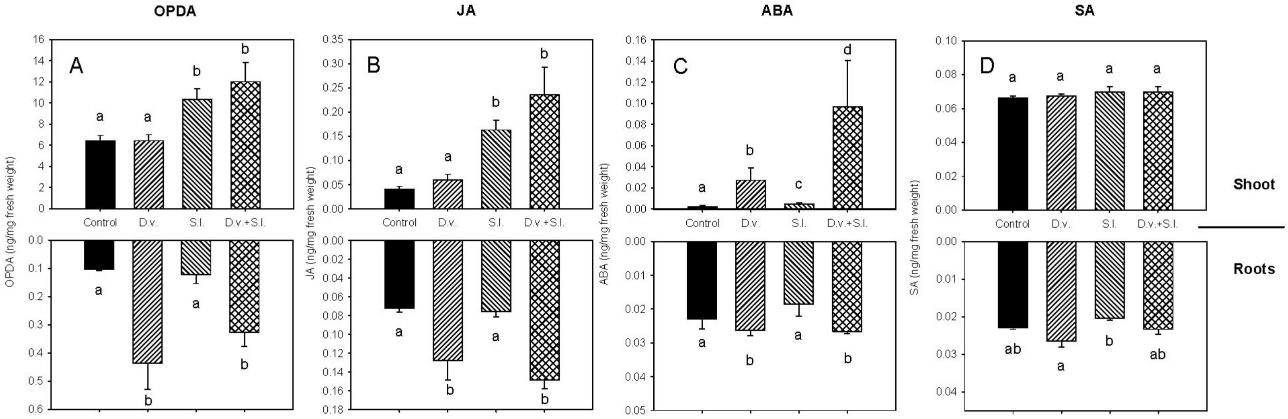

Figure 5: Average concentrations (+SE) of 12-oxo-phytodienoic acid (OPDA; **A**), jasmonic acid (JA; **B**), abscisic acid (ABA; **C**) and salicylic acid (SA; **D**) and in maize leaves and roots of herbivore-infested plants. Leaves were collected after 4 days of belowground infestation by *D. virgifera (D.v.)*, 2 days of aboveground infestation by *S. littoralis* (S.l.), or simultaneous infestation by *D. virgifera* (4 days) and *S. littoralis* (2 days; *D.v+ S.l.)*. Values presented are concentrations in ng/mg fresh weight (+SE). Different letters indicate significant differences between the treatments (p<0.05).

Root treatment with ABA induces resistance to S. turcica *but not against* S. littoralis

To investigate if exogenous ABA application to the roots can mimic *D. virgifera*-induced resistance in the leaves, plants were soil-drenched with ABA and subsequently tested for induced

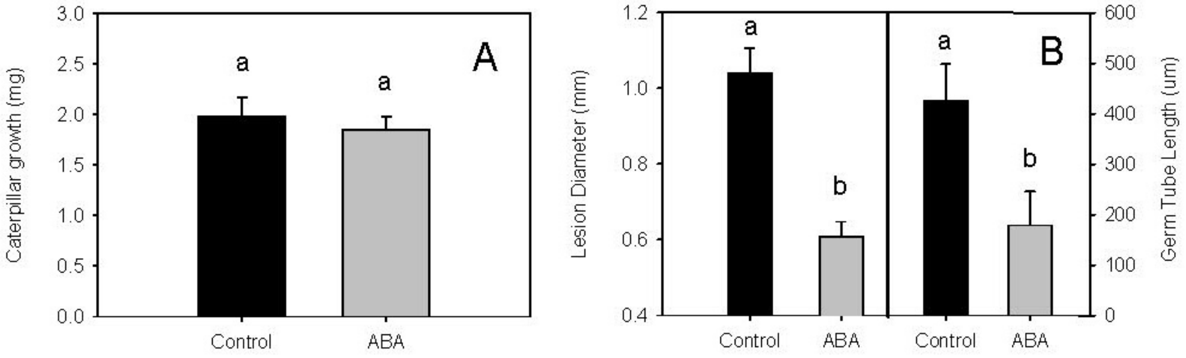

Figure 6: ABA-induced shoot resistance of maize plants. ABA (300 µM) was applied to the soil and resistance against *S. littoralis* and *S. turcica* was measured 24 hours later. **A:** Average growth (+SE) of *S. littoralis* caterpillars over a feeding period of 11 hours. **B:** Average lesion diameters (+SE, left) and hyphal lengths of germination tubes (+SE, right) in leaves of *S. turcica*-infested plants at 3 days after inoculation. Different letters indicate significant differences between treatments (p<0.05).

resistance against *S. littoralis* and *S. turtica*. ABA-treated plants allowed similar levels of *S. littoralis* growth on their leaves as control plants (Figure 6A), indicating that *D. virgifera*-induced ABA production is not solely responsible for the induced resistance against *S. littoralis*. On the other hand, ABA-treated plants developed reduced levels of disease at 3 days after inoculation with *S. turtica* spores, which correlated with a statistically significant reduction in hyphal lengths (Figure 6B). Hence, *D. virgifera*-induced stimulation of ABA in the leaves is likely to contribute to induced resistance against *S. turcica*

D. virgifera *induces osmotic stress in the leaves*

To test if the systemic induction of ABA during *D. virgifera* infestation is related to osmotic stress, we quantified water contents in leaves after *D. virgifera* infestation. As an extra control treatment, artificial root damage was imposed by removing comparable amounts of root-biomass as *D. infestation* larvae over a period of 4 days (Figure 7A). As is shown in Figure 7B, *D. virgifera* reduced leaf-water content by 2% in comparison to control plants. Conversely, artificial root damage did not alter shoot water content (Figure 7B). Hence, *D. virgifera* disturbs the plant's aboveground osmotic balance, an effect that cannot be mimicked artificially by removing similar amounts of root biomass.

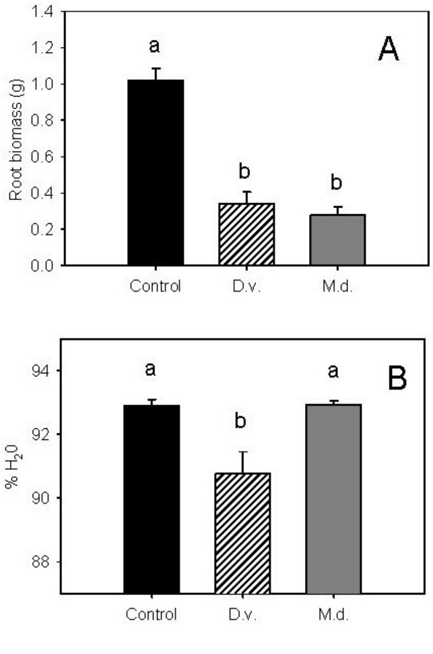

Figure 7: Root biomass (g fresh weight ± SE) (**A**) and relative shoot water contents (± SE) (**B**) after 4 days of infestation by *D. virgifera (D.v.)* or after 4 days of daily mechanical damage application (M.d.). Water content is expressed as the percentage water per unit fresh weight (w/w). Different letters indicate significant differences between the treatments ($p<0.05$).

DISCUSSION

Belowground infestation by *D. virgifera* larvae triggers aboveground resistance against *S. littoralis* and *S. turcica* (Figure 1). While a variety of negative and positive interactions have been reported between root and shoot herbivores (Erb et al., 2008), our current study appears to be the first to show that a root herbivore can induce aboveground resistance against both herbivores and pathogens. In theory, induction of aboveground resistance by root herbivory could be mediated by translocation of defensive compounds. However, our gene expression profiling clearly demonstrates induction of defence-related genes in the leaves, indicating regulation by long-distance defence signals. For instance, *D. virgifera* systemically enhanced expression of the *Zm-Bx1* gene (Table 1), which encodes an enzyme that catalyzes the first step in the biosynthesis of DIMBOA (Frey et al., 2000). Hence, the observed increase of DIMBOA in leaves of *D. virgifera*-infested plants (Figure 2A) likely results from the systemic up-regulation of DIMBOA biosynthesis. Since DIMBOA has been reported to suppress mycelial growth of *S. turcica* and to act as a feeding-deterrent on *S. littoralis* caterpillars (Rostas, 2007), it is possible that this metabolite contributes to the observed systemic resistance response.

In addition to direct induction of defenses, we also provide evidence that belowground attack by *D. virgifera* can prime aboveground defenses. Although *D. virgifera* directly induced systemic ABA and *Zm-Dehydrin* transcript accumulation, *D. virgifera*-infested plants displayed even higher levels of ABA and *Zm-Dehydrin* induction after subsequent *S. littoralis* attack (Figure 4; Table 1). Thus, *D. virgifera* infestation not only activates shoot ABA responses directly, but it also primes for augmented ABA responses to subsequent attack by *S. littoralis* caterpillars. Furthermore, induction of chlorogenic acid by *S. littoralis* was strongly potentiated when plants were concomitantly infested by *D. virigifera* (Figure 2C). Since chlorogenic acid has been associated with resistance to *Spodoptera frugiperda* and *Helicoverpa zea* (Nuessly et al., 2007 and references therein), priming of this defence compound may also have contributed to *D. virigifera*-induced resistance in the leaves. Together with the direct effects on defensive mechanisms, these results demonstrate that belowground herbivory has a profound impact on the defensive capacity of the aboveground plant tissues.

Belowground attack by *D. virgifera* and aboveground attack by *S. littoralis* stimulated OPDA and JA production locally (Figures 5A and B). Interestingly, however, JA and OPDA were not significantly induced in the roots after root attack by *S. littoralis*, nor were they induced in the leaves after root attack by *D. virgifera* (Figures 5A and B). Of all defence hormones tested, ABA was the only hormone that accumulated systemically to belowground attack by *D. virgifera*

(Figure 5), which is supported by the induction of an ABA-related transcription profile in the leaves (Figure 4). ABA is known to be synthesized in plant roots upon drought stress and increased salt concentrations (Jackson, 1997). Moreover, ABA can be transported from the roots to the shoot (Wilkinson and Davies, 2002), where it mediates closure of stomata and induction of defensive gene expression (Dodd, 2003; Boudsocq and Lauriere, 2005). Finally, we observed that root infestation by *D. virgifera* caused a statistically significant reduction in leaf water content (Figure 7). This suggests that the ABA induction is the result of an osmotic stress reaction of the host plant to tolerate *D. virgifera*-induced drought stress. In support of this, a reduction in water uptake and stomatal conductance has been reported to occur under greenhouse and field conditions in *D. virgifera* infested plants (Riedell and Reese, 1999). Interestingly, however, artificial removal of similar amounts of root biomass did not cause any reduction in leaf water content (Figure 7). This indicates that *D. virgifera* employs a highly efficient strategy to influence the plant's water potential, which cannot be explained by reduction in root biomass only. Whether this manipulation is based on a specific mode of feeding, or by additional mechanisms, requires further investigation. It seems, nevertheless, tempting to speculate that the root herbivore manipulates the water balance in the host plant to increase photo-assimilate transport into the roots.

Our finding that soil-drench treatment with ABA induced resistance against *S. turcica* (Figure 6B) suggests that *D. virgifera*-induced shoot ABA is sufficient to induce resistance against this fungus. Interestingly, ABA is emerging as a novel regulatory signal in pathogen resistance (Ton and Mauch-Mani, 2004; Mauch-Mani and Mauch, 2005; Ton et al., 2005). Furthermore, the chemical agent beta-aminobutyric acid (BABA) has been shown to induce resistance against nectrotrophic fungi in an ABA-dependent manner, which is based on a priming of cell wall defenses (Ton and Mauch-Mani, 2004; Ton et al., 2005). Recent evidence also suggests involvement of ABA in the plant's response to herbivory ((Reymond et al., 2000)Bodenhausen and Reymond 2007). It has been suggested that leaf herbivores actively attempt to suppress drought-related responses in the plant, possibly for their own benefit (Van Dam et al., 2003). This hypothesis is supported by our finding that *S. littoralis* evoked a relatively mild induction of ABA in the leaves (Figure 5). On the other hand, when plants were simultaneously subjected to belowground herbivory, *S. littoralis* caterpillars triggered a strongly augmented ABA accumulation. The accompanying changes in shoot physiology may have contributed to the induced resistance against *S. littoralis*. However, from our ABA soil-drench experiments, it appears that ABA alone cannot be responsible for the root herbivore-induced resistance against *S. littoralis* (Figure 6). Consequently, the exact contribution of ABA to the aboveground resistance against *S. littoralis* remains to be evaluated. We, therefore, conclude that *D. virgifera*-induced resistance against *S. littoralis* is either based on an ABA-independent mechanism that is related to leaf water-loss (Huberty and Denno, 2004), or that the induced resistance requires an another, yet unknown, signal in addition to ABA (Bodenhausen and Reymond 2007).

In conclusion, our study shows that root attack by *D. virgifera* profoundly alters the plant's aboveground physiology, resulting in direct induction of defence-related genes and defence compounds, priming of defence mechanisms, and a change in the plant's water potential. Although ABA is a strong candidate to act as a systemic signal in this interaction, we conclude that the aboveground resistance likely involves additional layers of regulation. Together, our results provide a physiological basis for future research on the ecological implications of plant-mediated interactions between below- and aboveground defence responses.

EXPERIMENTAL PROCEDURES

Plants, insects and fungi

Plants (*Zea mays*, variety Delprim) were grown in plastic pots (diameter, 4cm; depth, 11cm) under controlled conditions in a climate chamber (CLF plant climatics, Percival; 30°C; photoperiod: 16 hours). Plants for hormone-induced transcription profiling and induced resistance assays were grown in commercial potting soil (Ricoter Aussaaterde, Aarberg, Switzerland) . For experiments involving *D. virgifera*, all seedlings were grown in a sand-vermiculite mixture (3/1) to facilitate harvesting of roots. Plants for transcription profiling had 2 fully expanded primary leaves and were between 10 and 12 days old. *S. littoralis* eggs were provided by Syngenta Switzerland and reared on artificial diet as described before (Turlings et al., 2004). Second instar larvae of *D. virgifera* were obtained from CABI Delémont (Switzerland) and kept on maize seedlings until use. Spores of *S. turticia* were isolated as described by (Rostas et al., 2006).

Chemical and biological treatments

JA, ABA, ACC, and salt (NaCl) were applied by soil-drenching the plants to a final soil concentration of 500 µM, 300 µM, 2 mM, or 150 mM, respectively. Control plants were treated with an equal volume (15 mL) of water. BTH was applied by spraying a 5 mM solution (25% active ingredient formulation) onto the leaves. Root infestation by *D. virgifera* was achieved by placing 6 second instar larvae onto the soil surface around the stem of the maize plants. Infestation by *S. littoralis* caterpillars was performed by applying about 20 second instar larvae in the whorls of the leaves. Wounding was performed by scratching the underside of 2 leaves at 2 different locations over an area of about 1 cm^2 on both sides of the central vein with a razor blade, after which 10 µL water or 50% (v/v) *S. littoralis* regurgitant was distributed over the wounded leaf areas. Regurgitant was collected from fourth- and fifth-instar *S. littoralis* larvae that had been feeding on maize leaves for at least 2 days, and stored at -76°C until use (Turlings et al, 1993). Infection by *S. turcica* was performed by spreading 100 µl spore suspension (6×10^4 spores/ml; 0.01% Silwet) over second and third leaves, as described by Rostas et al. (2006). Control plants were mock-inoculated in the same manner with 0.01% Silwet solution. Plant material for transcriptional profiling was harvested at 1 day after treatment with JA, ABA, ACC, NaCl, *S. littoralis* caterpillars, wounding, or wounding and *S. littoralis* regurgitant, at 2 days after treatment with BTH and *S. littoralis* caterpillars (above belowground experiment), at 3 days after inoculation with *S. turcica* and at 4 days after *D. virgifera* infestation. Mechanical damage of the roots was achieved by inserting a knife blade (1 cm) into the soil at a distance of approx

0.7 cm from the stem. This was repeated over a period of four days (damaging a different side around the stem every 24h), and resulted in the gradual removal of around 70% of root biomass.

Gene expression analysis

RNA extraction, cDNA synthesis and RT-qPCR analysis, was performed as described by Ton *et al.* (2007). For each replicate sample, RNA was extracted from 2 - 3 plants, which were pooled for the synthesis of cDNA. For transcriptional profiling of *D. virgifera*-infested plants, 12 plants per treatment were harvested in experimental blocks of 2 plants. To ensure selection of sufficiently infested plants, plants were analyzed for *D. virgifera*-induced emission E-*β*-Caryophyllene by SPME GC-MS analysis, as described by Rasmann and Turlings (2007). RNA was only extracted from 3 blocks showing the highest *E-β-Caryophyllene* values (data not shown), resulting in 6 plants per treatment that were pooled block-wise for cDNA synthesis (n=3x2). Primers were designed based on publicly available sequences of stress-inducible maize genes or on ESTs identified in a differential hybridization screen for *S. littoralis*-inducible genes (Ton et al., 2007). Primers sequences, genebank accession numbers and putative functions of genes are listed in Table S1. Specificity of primers was tested by conventional PCR (40 cycles) of cDNA followed by 1.5% agarose gel electrophoresis or by qPCR followed by melt point analysis.

Induced resistance assays

Resistance against *S. littoralis* was quantified by determining average weight gain of 10 second instar larvae per plant over a period of 11 hours of infestation, as described previously (Ton et al., 2007). Induced resistance assays upon hormone treatments were based on 5–6 plants per treatment. For *D. virgifera*-induced resistance assays, sample sizes were increased to 20-22 plants per treatment to compensate for the relatively high variation in herbivory levels. Plants from which less than 60% of the applied caterpillars could be recovered were excluded from the analysis. Resistance against *S. turcica* was assessed based on lesion diameters and lengths of germination hyphae from *S. turcica* spores at 3-4 days after inoculation with 5 x 10^4 spores/ml. Lesion diameters were measured using a calibrated loupe. Lengths of *S. turcica* germination tubes were examined under a light microscope (Olympus BX50W1) and quantified using AnalySIS-D software (Soft Imaging System GmbH, Germany). Analysis of *S. turcica* germination tubes was performed in randomly selected leaves (hormone assays: n=6 plants, 75 germination tubes; root herbivore assays: n=10 plants, 164 germination tubes). Tolerance against osmotic stress was quantified as the number of surviving/wilting plants upon repeated soil-drench treatment to a final concentration in the soil of 150 mM NaCl (every 4 days over a period of 20 days; n=8). Herbivores, fungi and the first salt treatment were applied at 24 hours (for ABA, JA, and ACC) or 48 hours (for BTH) after hormone treatments and 4 days after application of *D. virgifera* larvae.

Quantification of hormones and phenolic compounds

To determine changes in ABA, JA, OPDA and SA levels upon herbivory, maize plants were subjected to herbivore infestation as described above (n=9). Shoots and roots were harvested, frozen in liquid nitrogen and pulverized to fine powder (0.5 g per plant). Before extraction, a mixture of internal standards containing 100ng [2H6]-ABA, 100ng dihydrojasmonic acid, 100ng prostaglandin B1 (Pinfield-Wells et al., 2005) , 100ng d6-SA and 100ng parabene were added. The frozen tissue was immediately homogenized in 2.5 ml of ultra-pure water and centrifuged (5000 g, 40 min), after which the supernatant was recovered, acidified and partitioned against diethyl-ether as described in (Flors et al., 2008). After evaporation to dryness, the solid residue was re-suspended in 1 ml of a water/methanol (90:10) solution and filtered through a 0.22 lm cellulose acetate filter. A 20 µL aliquot of this solution was then directly injected into an ultra-performance Waters Acquity liquid chromatography (UPLC™) system (Waters, Milford, MA, USA). The UPLC was interfaced to a triple quadrupole tandem mass spectrometer (TQD, Waters Micromass, Manchester, UK) using an orthogonal Z-spray electrospray interface. LC separation was performed using an Acquity UPLC BEH C18 analytical column (Waters, 2.1 × 50 mm, 1.7 µm) at a flow rate of 300 µL/min. Standard curves for all hormones were obtained by injecting a mixture pure compounds at different concentrations (10ng, 25ng, 50ng, 70ng, 100ng, 150ng). Quantifications were carried out with Mass Lynx (v 1.4, Mycromass) software using the internal standards as reference for extraction recovery and the standard curves as quantifiers.

Quantification of hydroxamic acids

DIMBOA and DIMBOA-Glc were quantified in plant material from the same plants as used for phytohormone measurements. Approximately 10 mg of lyophilized plant material was resuspended in 1 mL extraction buffer (98% methanol 2% acetic acid) and sonicated for 10 min. After 10 min. of centrifugation at 12.000 g, 800 uL supernatant was collected for HPLC injection (10 µl). Samples were analyzed on a Shimadzu prominence HPLC with diode array detector (detection at 254 nm), using a thermal hypersil C-18 column. (150 mm × 4.6 mm, 5-µm) at a flow rate of 1 ml/min. Elution was carried out for 2 min under isocratic conditions of 100% solvent A (H_2O), 9 min. linear gradient to 50% solvent A and 50% solvent B (methanol/isopropanol(95/5) + 0.025% acetic acid) and 5 min. isocratic conditions at 50% solvent A and 50% solvent B.

Quantification of root biomass and leaf water contents

Roots and shoots were harvested and weighed at the end of the bioassays to determine root biomass (FW) and shoot fresh weight (FW). Subsequently, shoots were carefully put in paper cooking-bags and dried at 80° over 4 days. Shoot dry weight (DW) was then determined using the same balance as before. Relative leaf-water contents were calculated assuming equal turgid weights using the formula $\%H_20=(FW-DW)/FWx100)$.

Statistical analysis of transcription profiles

Gene expression levels were calculated relative to the expression of 2 constitutively expressed reference genes: *Zm-GAPC* and *Zm-Actin1*. Fold-inductions were calculated relative to gene expression levels in control or mock treatments. Hierarchical cluster (HC) analysis was based on ln-transformed fold-induction values, using MultiExperiment Viewer software (Saeed et al., 2003). Metric selection for HC analysis was based on Eucledian distance using average linkage clustering. Principal component (PC) analysis of gene distribution was adjusted to the methods described for analysis of microarray data (Held et al., 2004). To determine the appropriate model for description of gene distribution, a Detrended Correspondence Analysis (DCA) was performed. The given dimensionless value for the length of gradient of the first ordination axis was < 3 (< 1,047 for the hormone treatments, < 1,228 for the above-belowground treatments), indicating that the values should be fitted by a linear distribution model. Therefore, PC analysis for comparison of gene expression values was based on a linear model. PCA was performed on ln+1 transformed fold-induction ratios, using the Canoco 4.5 package (Ter Braak and Smilauer, 2002). For all gene profiling experiments involving *D. virgifera*, normality of the data was verified using the Kolmogorov-Smirnov test, while the Leveen test for homogeneity of variance was carried out to ensure equal variances. Ln-transformed fold-induction values were tested against controls using a Student's T-tests. One-Way ANOVA followed by pairwise multiple comparisons (Holm-Sidak) was used to identify genes with differential responsiveness to more than 2 treatments.

Statistical analysis of bioassays and UPLC/ HPLC results

Multiple comparisons were analysed by one-way ANOVA followed by pairwise multiple comparisons (Holm-Sidak test). Comparisons between two treatments were analyzed by a Student's t-test. Normality of the data was verified using the Kolmogorov-Smirnov test and equality of variances was tested using a Leveen test ($p<0.05$). In case of non-normality and/or unequal variances, data were transformed were possible or analyzed by a Mann-Whitney rank sum test or an ANOVA based on ranks, followed by a Dunn's test for multiple comparisons (unequal sample sizes) or a Student-Newman-Keuls test (equal sample sizes), respectively. Effects of hormone treatments on salt-stress tolerance were analyzed using standard Kaplan-Meier survival analysis on log-ranks.

ACKNOWLEDGEMENTS

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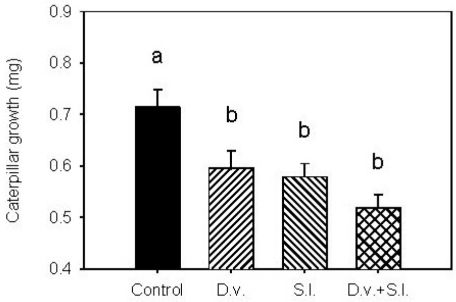

Figure S1: Induced resistance against *S. littoralis* in the leaves upon attack by Different herbivores. Shown are average weight gain values (+SE) of *S. littoralis* caterpillars over a feeding period of 11 hours on control plants, *D. virgifera*-infested plants *(D.v.)*, *S. littoralis* pre-infested plants (*S.l.*) and double (pre-) infested plants (*D.v.+S.l.)*. Different letters indicate significant differences between treatments ($p<0.05$).

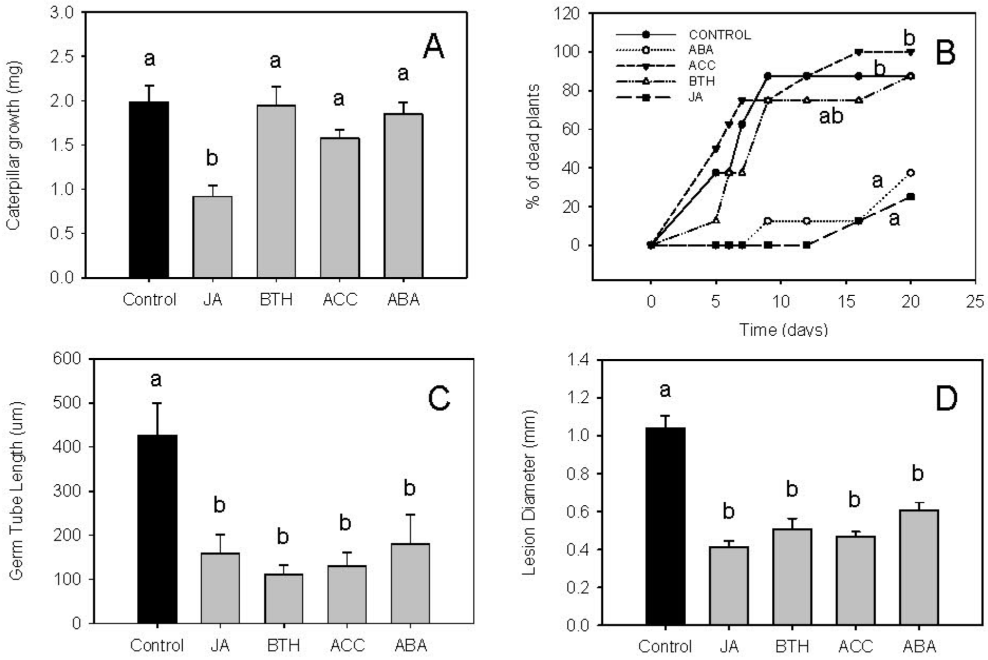

Figure S2: Hormone-induced resistance in the maize leaves against *S. littoralis*, salt stress and *S. turtica*. Stress treatments were applied at 1 day after soil-drench treatment with ABA (300 µM), JA (500 µM), ACC (2 mM) and at 2 days after spraying the leaves with BTH (5 mM). Different letters indicate statistically significant differences between treatments ($p<0.05$). **A:** Average growth (+SE) of *S. littoralis* caterpillars over a feeding period of 11 hours. **B:** Average Lesion Diameters (+SE) in leaves of *S. turcica*-infested plants at 3 days after inoculation. **C:** Average hyphal lengths of germination tubes from *S. turcica* spores (+SE) at 3 days after inoculation. **D:** Percentage of wilted plants at different days after growth under elevated salt concentrations.

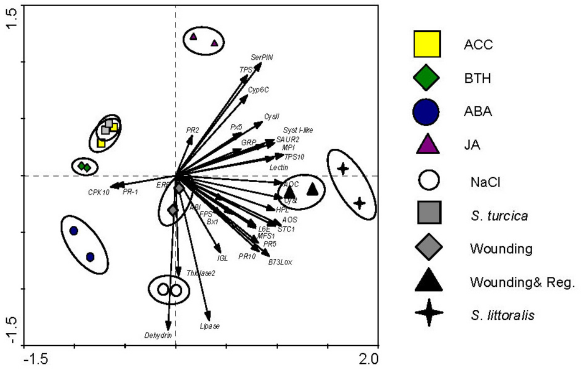

Figure S3: Principal component analysis of the gene expression profiles in maize leaves after treatment with (a)biotic stress or defence hormones. Leaves were collected at 1 day after soil-drenching with salt (NaCl; 150mM), abscisic acid (ABA; 300µM), jasmonic acid (JA; 300µM), 1- aminocyclopropane-1-carboxylate (ACC; 2mM), or at 2 days after spraying the leaves with benzothiadiazole (BTH; 5mM). Leaves from wounded plants with or without 50% *S. littoralis* regurgitant were collected at 1 day after treatment (Wounding& Reg. and Wounding, respectively). Leaves from herbivore- and pathogen-treated plants were collected at 2 days after application of *S. littoralis* caterpillars and 3 days after inoculation with *S. turcica* spores. Principal component analysis was performed on the ln+1-transformed gene induction values.

Table S1 Gene names, GenBank accession numbers, putative gene functions, literature references and corresponding primer sequences of genes that were used in this study for qPCR analysis.

Gene name	**Reference**	**Genbank no.**	**Putative function**	**Left primer**	**Right primer**
Zm-ABI	NCBI direct submission	X12564	Homology to glycin-rich protein	gcgagatcctcgactccaag	gggcttggttaacggtgatg
Zm-Actin1	NCBI direct submission	MZEACT1G	Actin	ccatgaggccacgtacaact	ggtaaaaccccactgagga
Zm-AOC	NCBI direct submission	AY488136	Allene oxide cyclase	ccccttcaccaacaaggtgt	accgagatgtggccgtagtc
Zm-AOS	NCBI direct submission	AY488135	Allene oxide synthase	acctgttcacgggcacctac	cgaggagcgaggagaagttg
Zm-B73Lox	(Ton et al., 2007)	AF465643	B73 lipoxygenase	gcgacaccatgaccatcaac	gctcggtgaagttccagctc
Zm-Bx1	NCBI direct submission	AY254103	DIMBOA biosynthesis gene	cccgagcacgtaaagcagat	cttcatgcccctggcatact
Zm-CPK10	(Murillo et al., 2001)	AJ007366	Calcium-dependent protein kinase	gagcagggcatattcgagga	cggatgccgtagaacttcgt
Zm-Cyp6C	(Persans et al., 2001)	T15323	Cytochrome P450 monooxygenase	gagagcaaggagcagcagaa	ttgcctatggagcaggttg
Zm-CysII	(Ton et al., 2007)	D38130	Cystatin II proteinase inhibitor	tgccctgctcatactgcttg	gcgagttcctggaggtgaag
Zm-Cyst.	(Ton et al., 2007)	CK371502	Cystatin proteinase inhibitor	caaggagcacaacaggcaga	ggacatgagctggcgatttt
Zm-Dehydrin	NCBI direct submission	X15290	Dehydrin	accagtacggcaacccagtc	gccggtcttgtgctcctc
Zm-ERF	NCBI, direct submission	AY672654	Homology to ERF1 transcription factor	aaggtggaggcacagactca	taagggatgccgaggaagtt

Zm-FPS	NCBI direct submission	AF330036	Homology to famesyl Pi phosphatase	cgtgctgatgagagccaaaa	ctgggcttcaatgtctgcaa
Zm-GAPC	(Farag et al., 2005)	X07156	Glyceraldehyde phosphate dehydrogenase	gcatcaggaaccctgaggaa	catgggtgcatctttgcttg
Zm-GRP	(Ton et al., 2007)	-	Glycine-rich protein	ggcgacgataaatttgaatgc	tcaaaagccagacacatgcac
Zm-HPL	NCBI direct submission	AY540745	Hydroperoxide lyase	acttcggcttcaccatcctg	gtagtagcccggccagatga
Zm-IGL	(Frey et al., 2000)	AF271383	Indole-3-glycerol phosphate lyase	gcctcatagttcccgacctc	gaatcctcgtgaagctcgtg
Zm-L6E	(Ton et al., 2007)	AY103559	L6E ribosomal protein	tcaagtctggcctgctcctt	acttggcgacatcaacacca
Zm-Lectin	(Ton et al., 2007)	CF032590	Lectin	tcgtcgtccttggagagctt	catctgccaagtccccttct
Zm-Lipase	(Ton et al., 2007)	AI820221	Lipase/esterase	ccaagagcctcatcatcgtg	cgtggtagtggtccgtgttg
Zm-MFS1	(Simmons et al., 2003)	CA452753	Multiflux efflux synthase	cactgtgggctgtgagcagt	gcaggccgaaatgtcttgat
Zm-MPI	(Cordero et al., 1994)	AY549620	Maize proteinase inhibitor	atgagctccacggagtgc	tcagccgatgtggggtgtc
Zm-PR-1	(Morris et al., 1998)	U82200	Pathogenesis-related gene 1	ctgggtgtccgagaagcagt	cgggttgtagctgcagatgat
Zm-PR10	NCBI direct submission	AY953127	Pathogenesis-related gene 10	gtcatgccgttcagcttcat	tgttcttgcactcgacttg
Zm-PR2	(Alleman et al., 2006)	DQ417752	Pathogenesis-related gene 2	gtgactcgacggagctgttc	gccgtctcaagcttctcctt
Zm-PR5	(Morris et al., 1998)	U82201	Pathogenesis-related gene 5	tgcatgcatgggctagtgat	cgcacacaaatccagctacg
Zm-Px5	NCBI direct submission	BG837605	Peroxidase	ggattgatcctgcgctgag	gactcgaagaggcccaggtt
Zm-SAUR2	(Knauss et al., 2003)	X79211	Auxin biosynthesis gene	gtgccttagcacccctgtct	ggctcctctcctgagcaaac
Zm-SerPIN	(Ton et al., 2007)	BM382058	Serine proteinase inhibitor	gacggaggaggaaggaggag	acctgatgcactgcttgcac
Zm-STC1	(Shen et al., 2000)	AF296122	Sesquiterpenecyclase	agggatctgctgagccttca	atctcgagcgcacgctttat
Zm-Cyst I-like	(Ton et al., 2007)	CK827737	Cystatin-like proteinase inhibitor	agggcttgttcggttaggtg	tgcagaataaggagccatgc
Zm-Thiolase2	NCBI direct submission	AF113522	Thiolase	ttcgcccaagtttcaaggag	gccgcatctgcatatcctct
Zm-TPS1	(Schnee et al., 2006)	AF529266	Sesquiterpene cyclase	tgctggcaccatgttctctc	tcgtcccacatctcaaccaa
Zm-TPS10	(Schnee et al., 2006)	AY928079	Sesquiterpene synthase	tgtgtccacggtccaatgtt	gtccgctgtccttgcaaaat

Chapter 3: Root herbivory by the western corn rootworm induces shoot resistance in maize by reducing leaf-water contents

Matthias Erb, Jörg Degenhardt, Jurriaan Ton, Claudia Zwahlen, Bruce Hibbard and Ted C.J. Turlings

SUMMARY

Root herbivory can change shoot physiology and increase resistance of plants against aboveground attackers. It is however not known if such root-shoot interactions have any adaptive value or if they are a mere consequence of physiological constraints. We therefore investigated if, how and why maize seedlings infested with the western corn rootworm *Diabrotica virgifera* become more resistant against lepidopteran herbivores in the leaves. *D. virgifera* infested plants suffered less herbivory in the field and showed reduced growth of *Spodoptera littoralis* caterpillars in the laboratory. Root herbivory furthermore reduced leaf-water contents and triggered ABA accumulation. By chemically and genetically altering ABA-biosynthesis, we show that the induction of ABA by itself is not responsible for the increased resistance phenomenon. Instead, *S. littoralis* is sensitive to changes in the plant's water balance caused by root herbivory. These changes are most pronounced under water-limited conditions and when *D. virgifera* is allowed to feed on the upper root system, which in turn is the feeding side that was found to be optimal for the development of the root herbivore. We conclude that *D. virgifera* effectively upsets the plant's water balance, and that the resulting physiological changes increase the plant's resistance against *S. littoralis.*

INTRODUCTION

Because plants are in intimate contact with both the soil and the phyllosphere, they are powerful mediators between above- and belowground food-webs (van der Putten et al., 2001; Bardgett and Wardle, 2003; Kaplan et al., 2008). Changes in shoot defenses and physiology upon root attack for example can dramatically alter shoot herbivore performance and even the behaviour of organisms at higher trophic levels (Wäckers and Bezemer, 2003; van Dam et al., 2005; Rasmann and Turlings, 2007; Soler et al., 2007; Soler et al., 2007; Kaplan et al., 2008). However, despite the increasing number of studies documenting such effects, it remains unclear if they are adaptive, and if so, for whom (Wäckers and Bezemer, 2003)? It has for example been proposed that root herbivores might try to protect the shoots of their host plant for their own benefit (Wäckers and Bezemer, 2003), as intact foliage might promote shoot-root assimilate flow and root regrowth. Alternatively, plants could increase defenses in the shoot upon root herbivory if belowground herbivory is correlated with an increased probability of future aboveground attack. *Diabrotica spp* for example feed on the roots as larvae, while the emerging adult beetles feed on the aboveground parts (Vidal et al., 2004). Plants under pressure from these herbivores might benefit from anticipating aboveground attack after root infestation. Finally, the removal of root biomass and the induction of root defenses could by themselves lead to non-adaptive physiological changes in the leaves that influence aboveground resistance. Answering the question why shoot resistance is altered upon root herbivory has been particulary hampered by the fact that the physiological basis of below- and aboveground interactions is poorly understood (Erb et al., 2008).

We have previously shown that root attack by the larvae of the beetle *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) increases the resistance of maize shoots against both the pathogen *Setosphaeria turcica* and the herbivore *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Erb et al., submitted). Our experiments furthermore demonstrated enhanced abscisic acid (ABA) levels and ABA inducible defense gene expression in the leaves of these plants. As ABA soil-drench enhanced resistance against *S. turcica* but not against *S. littoralis*, we concluded that another unknown process might account for the reduced growth of the shoot herbivore upon root attack (Erb et al., submitted). Here we confirm that feeding by *D. virgifera* on maize roots indeed has an effect on aboveground resistance to herbivores in a natural setting as well as in a laboratory experiment with *S. littoralis* larvae. By using a combination of behavioural, molecular and physiological assays, we address the question if the loss of shoot water or the induction of ABA-dependent defenses upon *D. virgifera* attack are responsible for the increased resistance against *S. littoralis*. By measuring fitness parameters of all organisms involved, we aim to gain insight into possible benefits and adaptive value of root-herbivore induced shoot resistance.

RESULTS

Root herbivory by D. virgifera *reduces aboveground damage by lepidopteran pests in the field*

In order to get insight into how root infestation by *D. virgifera* influences aboveground herbivores in maize-agroecoystems, we carried out a manipulative field experiment in central Missouri (US) comparing leaf-herbivore damage in plots that were left root-herbivore free with plots that had been artificially infested with *D. virgifera.* The dominant aboveground herbivore species encountered in knee-high maize (growth stage V8) in this part of Missouri are the European corn borer (*Ostrinia nubilalis*) and the fall armyworm (*Spodoptera frugiperda;* (O'Day, 1998)). Indeed, *O. nubilalis* was regularly encountered in the plots, and we were also able to confirm the presence of *S. frugiperda* larvae. Flea beetles *(Chaetocnerma pulicaria)* as well as several species of grasshoppers were encountered occasionally. Maize leaves showed typical traces of first and second instar *O. nubilalis* feeding as well as damage caused by *S. frugiperda*. Shotgun-like holes were also found frequently, which can be caused by several herbivores including *O. nubilalis* and *C. pulicaria* (O'Day, 1998). The high diversity of non-penetrating small feeding traces on the leaves furthermore pointed to the presence of other but only marginally important insects. On average, 27% of all maize leaves were severely damaged by herbivores (not taking into account small feeding marks), with an overall average of 2.9 longitudinal and 3.7 shotgun-like wholes per plant. In addition, 22 minor feeding marks per maize shoot were counted on average.

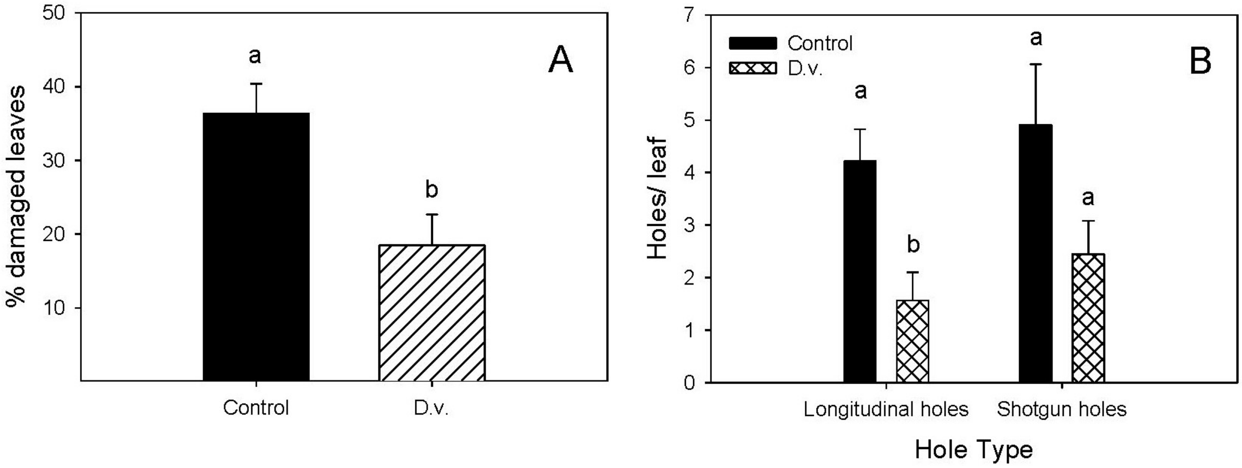

Figure 1: Root herbivore induced resistance in the field. **A:** Average percentage of damaged leaves per plant (+SE) in uninfested plots (closed bars) and plots infested with D. virgifera (crossed bars). **B:** Average number (+SE) of longitudinal (left) and shotgun holes (right) per plant. **C:** Average number of non penetrating feeding traces per plant. Different letters denote significant differences between treatments ($p<0.05$).

The percentage of damaged leaves per plant was reduced by almost 50% (Student's T-test: $p=0.033$; Figure 1A) in the presence of *D. virgifera* in the roots. This dramatic difference was also reflected in a significant reduction of longitudinal feeding traces on leaves (Student's T-test: p=0.021; Figure 1B). Shotgun-like holes also showed a trend to be less in root-infested plots (Student's T-test: p=0.115; Figure 1B), while the number of minor feeding marks was not affected by root herbivore infestation (data not shown). *D. virgifera* infestation thus negatively affected herbivore feeding aboveground, a phenomenon that was most pronounced for damage typically caused by lepidopteran larvae.

Root herbivory by D. virgifera *reduces growth and survival of* S. littoralis *in the laboratory*

To determine the precise dynamics of *D. virgifera*-induced changes in shoot resistance against herbivores, we followed the growth and survival of *S. littoralis* caterpillars on the leaves of

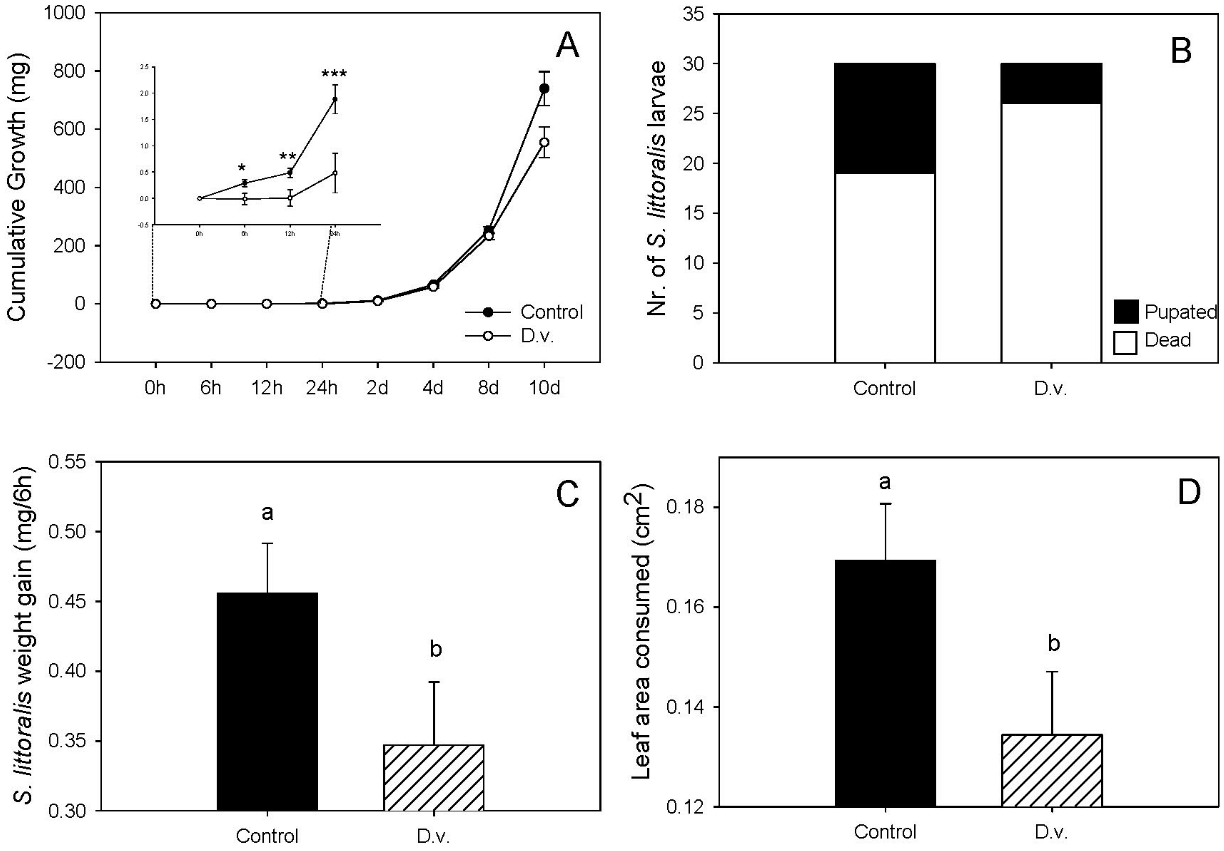

Figure 2: Induced shoot restistance upon root stress. **A:** Average cumulative growth (±SE) of *S. littoralis* caterpillars over 10 days of feeding on plants infested with *D. virgifera* in the roots (white circles) or uninfested control plants (black circles). Stars denote significant differences (*p<0.05, **p<0.01, ***p<0.001). **B:** Total numbers of S. littoralis caterpillars reaching the pupal stage (black) or dying while trying (white) on infested vs. uninfested plants. **C:** Average weight gain (+SE) of *S. littoralis* larvae after 6 hours of feeding on *D. virgifera* infested (crossed lines) and control plants (black). Different letters denote significant differences between treatments (p<0.05). **D:** Average leaf are consumed (+SE) of *S. littoralis* larvae after 6 hours of feeding on *D. virgifera* infested (crossed lines) and control plants (black). Different letters denote significant differences between treatments (p<0.05).

plants with and plants without the root herbivore over a time period of ten days until pupation in the laboratory. Figure 2A shows the average cumulative growth of the larvae (n=15). Root infestation affected caterpillar growth significantly (ANOVA: p=0.0196), and pair-wise comparisons showed significantly lower larval weights at time-points 6h, 12h and 24h (Holm-Sidak Post-Hoc Test: p<0.05). This trend persisted over the whole observation period (Figure 2A). Because the final number of surviving larvae was too low to carry out a meaningful survival analysis, the experiment was repeated. For this second run, only survival of the larvae was recorded. In total, 25% of the larvae reached the pupal stage, of which 73% had been feeding on plants without the root herbivore (Figure 2B). The obtained survival curves showed a significant difference between the treatments, with caterpillars on *D. virgifera* infested plants having a reduced chance of reaching the pupal stage (Log-Rank Test: p=0.036). In an independent experiment, we analyzed the first 6 hours of *S. littoralis* feeding in more detail by recording both larval growth and leaf-consumption (n=24). Again, caterpillar growth aboveground was reduced on plants infested with *D. virgifera* in the roots (Holm-Sidak Post-Hoc Test: p=0.0327; Figure 2C), an effect that was also reflected in a reduction in leaf consumption (Holm-Sidak Post-Hoc Test: p=0.0096; Figure 2D).

Root herbivory does not alter leaf C/N ratios, but reduces leaf-water content

Because root herbivory has previously been shown to shift nitrogen allocation and shoot water contents (Blossey and Hunt-Joshi, 2003), we tested the leaves of the same maize seedlings used for the 6h performance experiment for C/N ratios and relative water contents. *D. virgifera* infestation did not change C/N ratios significantly compared to controls (Figure 3A), but did reduce leaf water content (Holm-Sidak Post-Hoc Test: p=0.0013, Figure 3B).

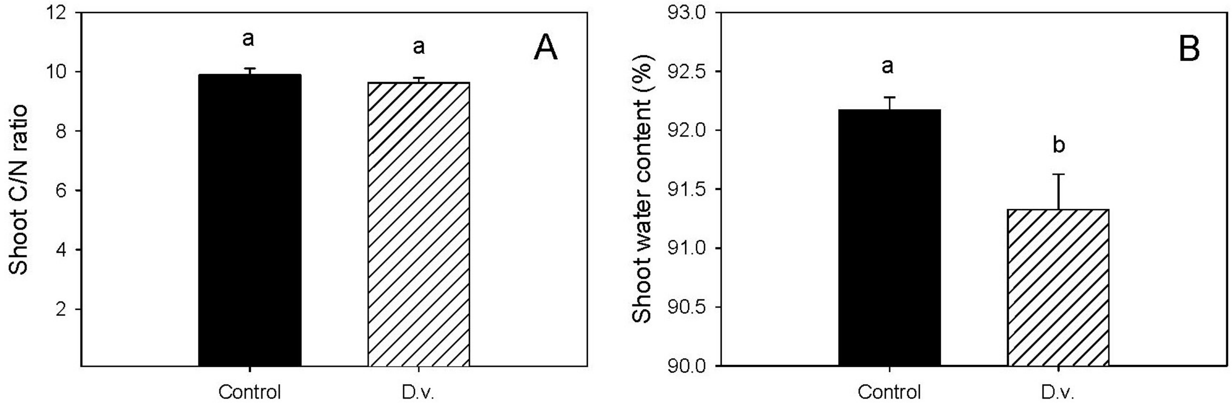

Figure 3: Root stress induced changes in shoot physiology. **A:** Average C/N ratios (+SE) of maize shoots infested in the roots with *D. virgifera* (crossed lines) and control plants (black). Different letters denote significant differences between treatments (p<0.05). **B:** Average relative water content (+SE) of maize shoots infested in the roots with *D. virgifera* (crossed lines) and control plants (black). Different letters denote significant differences between treatments (p<0.05).

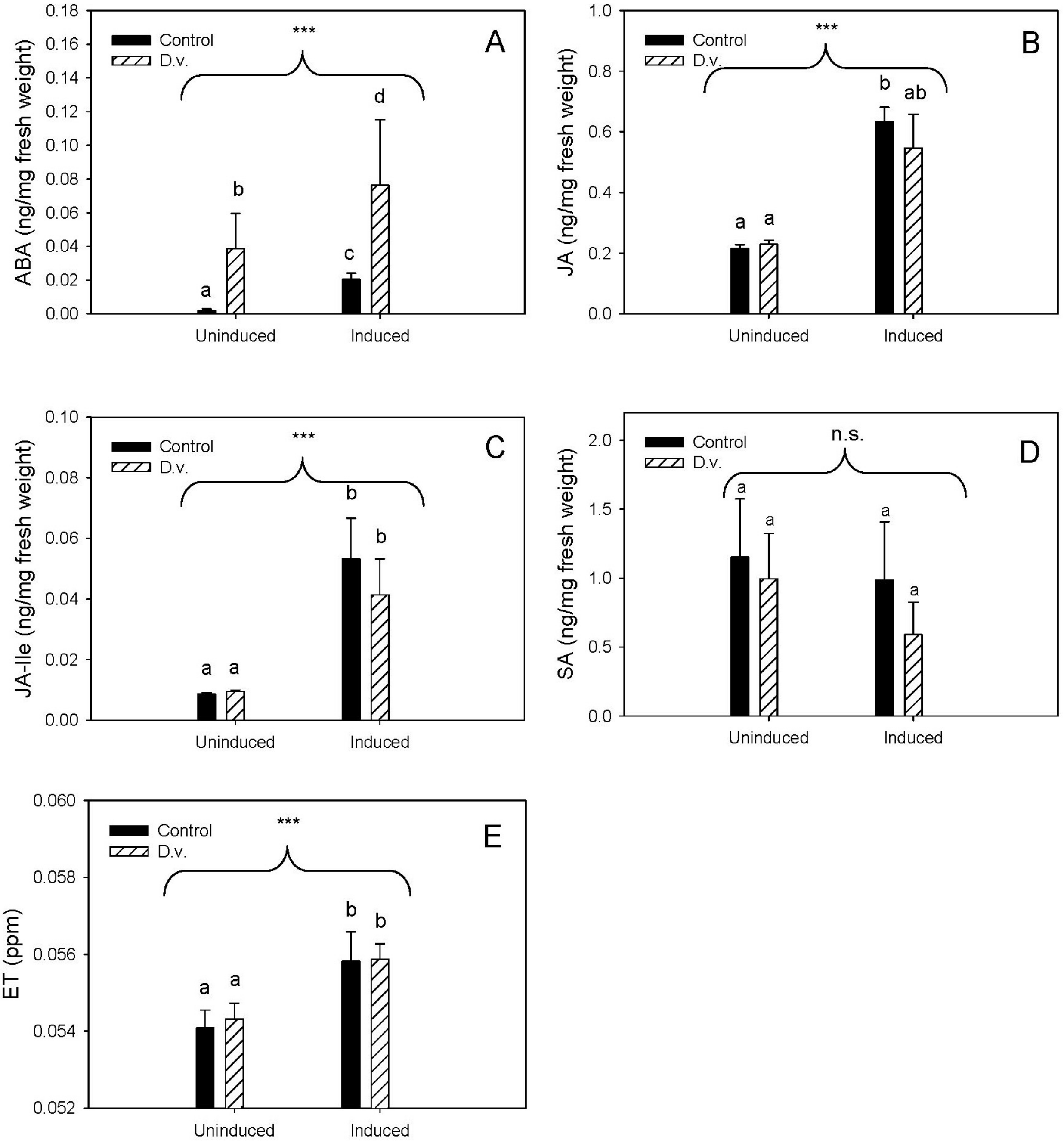

Figure 4: Influence of root stress on shoot phytohormone levels. Average shoot concentrations (+SE) of ABA (**A**), JA (**B**), JA-Ile (**C**) and SA (**D**) upon root stress (left groups) and upon root stress and shoot induction with *S. littoralis* regurgitate (right groups). Black bars denote unstressed roots and crossed lines for *D. virgifera* infested roots. Different letters indicate significant differences between the treatments within its respective group ($p<0.05$). Stars show the level of significance for differences between shoot induced and uninduced plants (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Root herbivory increases shoot ABA levels, but do not alter basal or induced JA, SA or ET

To test the effect of root herbivory on basal and induced concentrations of plant defense hormones in the shoot, we analyzed *D. virgifera* infested plants using HPLC-MS and GC-FID. ABA, SA, ET and JA as well as JA-Ile, the isoleucin conjugate of jasmonic acid, were tested. We also included shoot-induced plants (scratched with a razor blade and treated with *S. littoralis* regurgitant) harvested at a priming-sensitive time point (60 minutes after induction) to detect possible priming effects, which have been shown to be visible on the metabolite level for JA in maize (Engelberth et al., 2004). *D. virgifera* attack in the roots enhanced ABA levels in the shoots (Student-Newman Keuls Post-Hoc test: $p<0.05$), while JA, JA-Ile SA and ET concentrations remained unaltered (Figure 4). Additional shoot induction with *S. littoralis* regurgitate resulted in a higher ABA response in plants with root attack as compared to plants with undamaged roots (Student-Newman Keuls Post-Hoc test: $p<0.05$). Independent of the root treatment, shoot induction with *S. littoralis* regurgitant increased shoot ABA, JA, JA-Ille and ET (Holm-Sidak Post-Hoc test: $p<0.0001$) in all plants, while SA levels remained unaffected (Figure 4). Root herbivory and mechanical damage thus synergistically induce ABA, but do not prime for any of the measured phytohormones.

Inhibiting ABA biosynthesis leads to strong wilting and increased shoot resistance after D. virgifera *attack*

To get further insight into the role of ABA in the interaction between root attack and shoot physiology, we treated maize seedlings with 10mM of the ABA inhibitor sodium tungstate (n=24). This concentration had previously been determined to cause no significant phenotypical changes in maize shoots. Furthermore, concentrations of up to 100mM sodium tungstate did not have any impact on *D. virgifera* mortality over a feeding period of 48 hours (unpublished). Interestingly, while control plants showed no or minor wilting symptoms upon inhibitor treatment, plants infested with *D. virgifera* exhibited a strong wilting phenotype, with all leaves curling and loosing their capacity to remain upright. This observation was reflected in the Two-Way-ANOVA showing significant effects of *D. virgifera* and sodium tungstate as well as an interaction (ANOVA: $p=0.034$). As seen in Figure 5B, *D. virgifera* infested plants suffered much more from water stress when treated with the ABA inhibitor. *D. virgifera* feeding again reduced growth of *S. littoralis* (ANOVA: $p=0.010$), the effect being more pronounced in ABA inhibited plants (Holm-Sidak Post-Hoc Test: $p=0.004$) than in untreated plants (Figure 5A).

Sense and antisense expression of the ABA biosynthesis gene NCED (vp14) *does not influence* D. virgifera *induced shoot resistance*

Because of the possible pleiotrophic effects of sodium tungstate, we also used a genetic approach to modify ABA biosynthesis. Transgenic maize lines expressing *NCED (vp14)* in either sense or antisense direction were infested with *D. virgifera*, and the induction of resistance against *S. littoralis* was measured. The lines used have been charcterized before and are known to have altered ABA contents and inducibility without showing the strong phenotypic changes of *vp14* mutants (Voisin et al., 2006). Our results show that antisense expression of *NCED* did not alter root herbivore induced shoot resistance after 6h and 12h of *S. littoralis* feeding (Figure 6). Sense expression of *NCED* on the other hand reduced the effect of induced resistance after 6h, but not after 12h of feeding (Figures 6C and 6D).

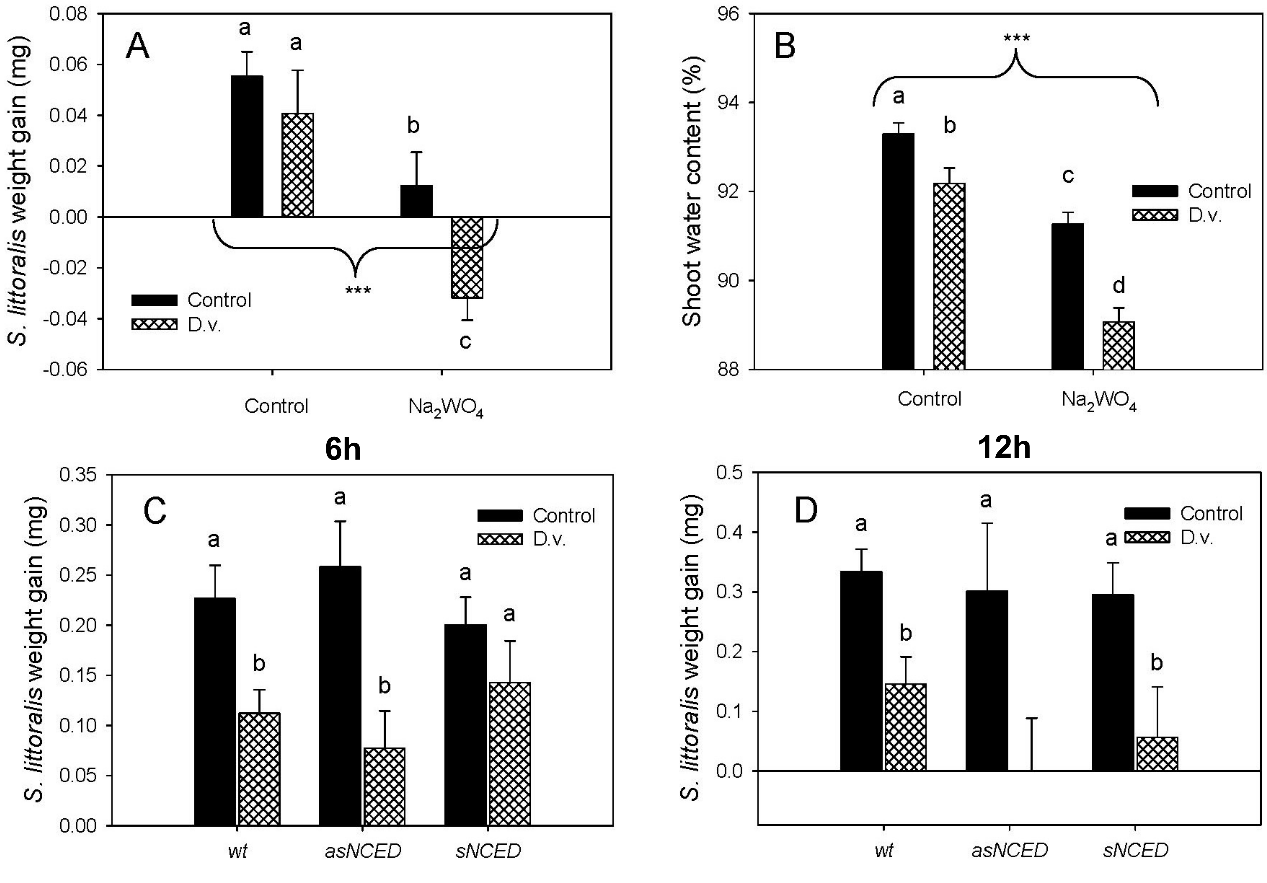

Figure 5: Impact of chemical and genetic modification of ABA biosynthesis on root herbivore induced shoot resistance. **A**: Average weight gain (+SE) of S. littoralis larvae after 6 hours of feeding on *D. virgifera* infested (crossed bars) and control plants (black bars) with and without ABA inhibitor soil-drench. **B:** Average relative water content (+SE) of maize shoots infested in the roots with *D. virgifera* (crossed bars) and control plants (black bars) with and without ABA inhibitor soil-drench. (**A**) Average weight gain (+SE) of *S. littoralis* after 6 hours of feeding on *D. virgifera* infested (crossed bars) and control plants (crossed bars) altered in *NCED (vp14)* expression. *wt*= wild type plants; *asNCED*= plants expressing *NCED* in antisense direction; *sNCED*= plants expressing *NCED* in sense direction. (**B**) Average weight gain (+SE) of *S. littoralis* after 12 hours of feeding. Different letters indicate significant differences between the treatments ($p<0.05$).

Root herbivore induced shoot resistance is influenced by the plant's water status

To elucidate the role of water supply in the observed resistance phenomenon, we subjected maize seedlings to different water regimes. Plants were either permanently supplied with water (lowest 3 cm of root system bottom-drenched), supplied with 10ml of water per day or left without water over a period of two days. Growth of *S. littoralis* larvae was then measured over a 6 hour feeding period on *D. virgifera* infested vs. control plants (n=24). After the experiment, roots and shoots of the experimental plants were harvested to determine dry and fresh weight as well as relative water contents. Root herbivory by *D. virgifera* significantly influenced the growth of *S. littoralis* (ANOVA: $p<0.001$), while the different water regimes alone had no clear effect. *S. littoralis* growth was most strongly reduced on *D. virgifera*-infested plants with low water supply, as reflected by a significant interaction effect between *D. virgifera* and water status (ANOVA: $p=0.038$, Figure 6A). Pairwise comparisons yielded a non-significant effect of *D. virgifera* under soil drench regime, a trend for reduced *S. littoralis* growth at a medium water supply (Holm-Sidak Post-Hoc Test: $p=0.070$) and a clear negative effect under drought conditions (Holm-Sidak Post-Hoc Test: $p<0.001$). Relative water content of the shoots was influenced by both *D. virgifera* and water regime (Holm-Sidak Post-Hoc Tests: $p<0.001$), and the interaction was again significant with *D. virgifera* infested plants loosing more water under drought conditions (Holm-Sidak Post-Hoc Test: $p=0.005$; Figure 6B). Pair-wise comparisons yielded a significant effect of *D. virgifera* under medium water supply and drought conditions (Holm-Sidak Post-Hoc Test: $p<0.001$). Analysis of root dry weight showed that the imposed water regime had no significant effect on biomass removal by *D. virgifera* (data not shown).

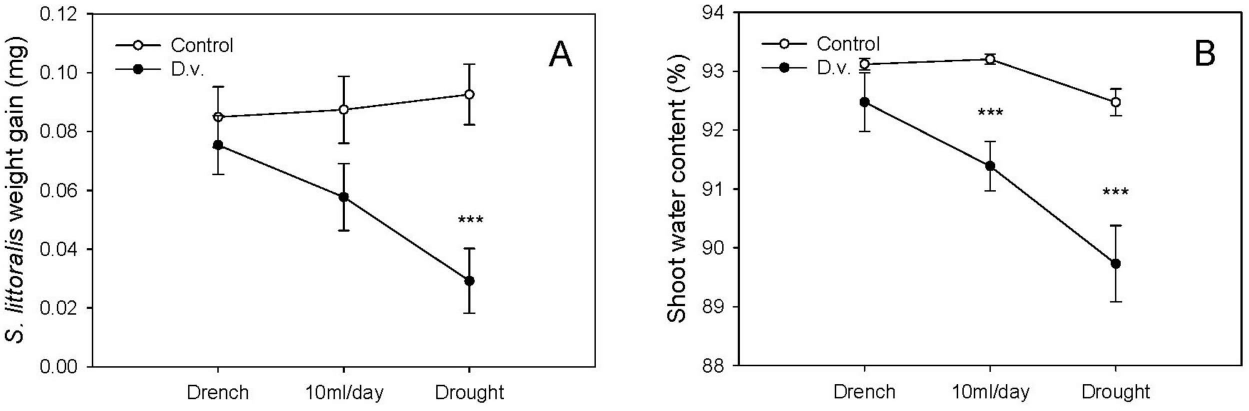

Figure 6: Influence of different water regimes on root herbivore induced shoot resistance. **A:** Average weight gain (+SE) of *S. littoralis* larvae after 6 hours of feeding on *D. virgifera* infested (black points) and control plants (white points) under different water regimes. **B:** Average relative water content (+SE) of maize shoots infested in the roots with *D. virgifera* (black points) and control plants (white points) under different water regimes. Stars denote a significant influence of *D. virgifera* (*p<0.05, **p<0.01, ***p<0.001).

Water limitation accentuates D. virgifera *induced changes in aboveground defense gene expression*

Our earlier results showed that *D. virgifera* infestation in the roots influences aboveground defense gene expression. Namely the serine proteinase inhibitor *SerPIN* and the hydroxamic acid biosynthesis gene *Bx1* were induced after root herbivore feeding (Erb et al., submitted). To confirm these results, we assessed changes in expression of several marker genes including three pathogenesis related genes (*PR1, PR5 and PR10*), four proteinase inhibitors (*SerPIN, MPI, Cyst, CysII*) and two genes involved in secondary metabolite synthesis (*Bx1, PAL*). We then subjected plants to different water regimes to assess wether water status has an influence on *D. virgifera*

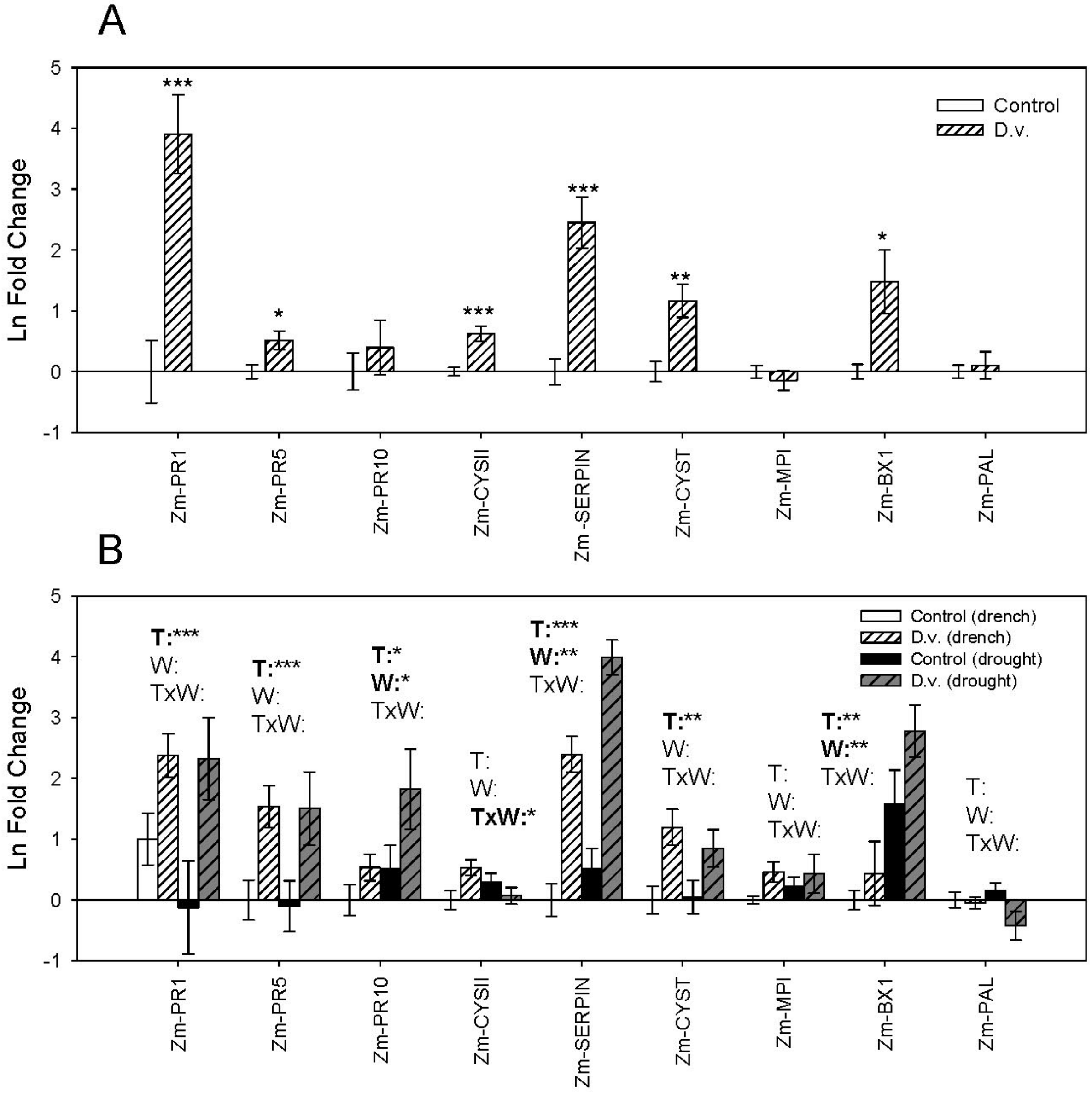

Figure 7: Influence of water status on *D. virgifera*-induced shoot defense gene expression. (**A**) Average ln-fold change (+SE) of defense marker genes upon root herbivore attack relative to unattacked control plants under normal water supply (10ml/day). Stars denote significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001). (**B**) Average ln-fold change (+SE) of root herbivore infested plants relative to unattacked control plants under different water regimes. Stars denote significant effects of herbivore treatment (T), water regime (W) and interactions (TxW; *p<0.05, **p<0.01, ***p<0.001).

induced shoot defense gene expression. Our results confirm that *D. virgifera* induces a variety of aboveground defenses (Figure 7A) including *PR1, PR5, CysII, SerPIN, Cyst* and *Bx1*. Furthermore, the induction of *SerPIN, PR10* and *Bx1* was more pronounced under water-limiting conditions (Figure 7B). *CysII* on the other hand was more responsive when the plants were well watered (Figure 7B).

D. virgifera *performs better and exclusively induces resistance against* S. littoralis *when feeding on the upper roots*

Because *D. virgifera* was often observed to feed on the mesophyl and the upper roots of maize seedlings, we tested if this behaviour was advantageous for the root herbivore by measuring weight gain of individuals feeding on upper or lower parts of the roots. *D. virgifera* larvae confined to the top 2 cm of the root system grew significantly more over a period of 7 days than larvae excluded from this part of the rhizosphere (Student's T-Test: p=0.046, Figure 8A). To

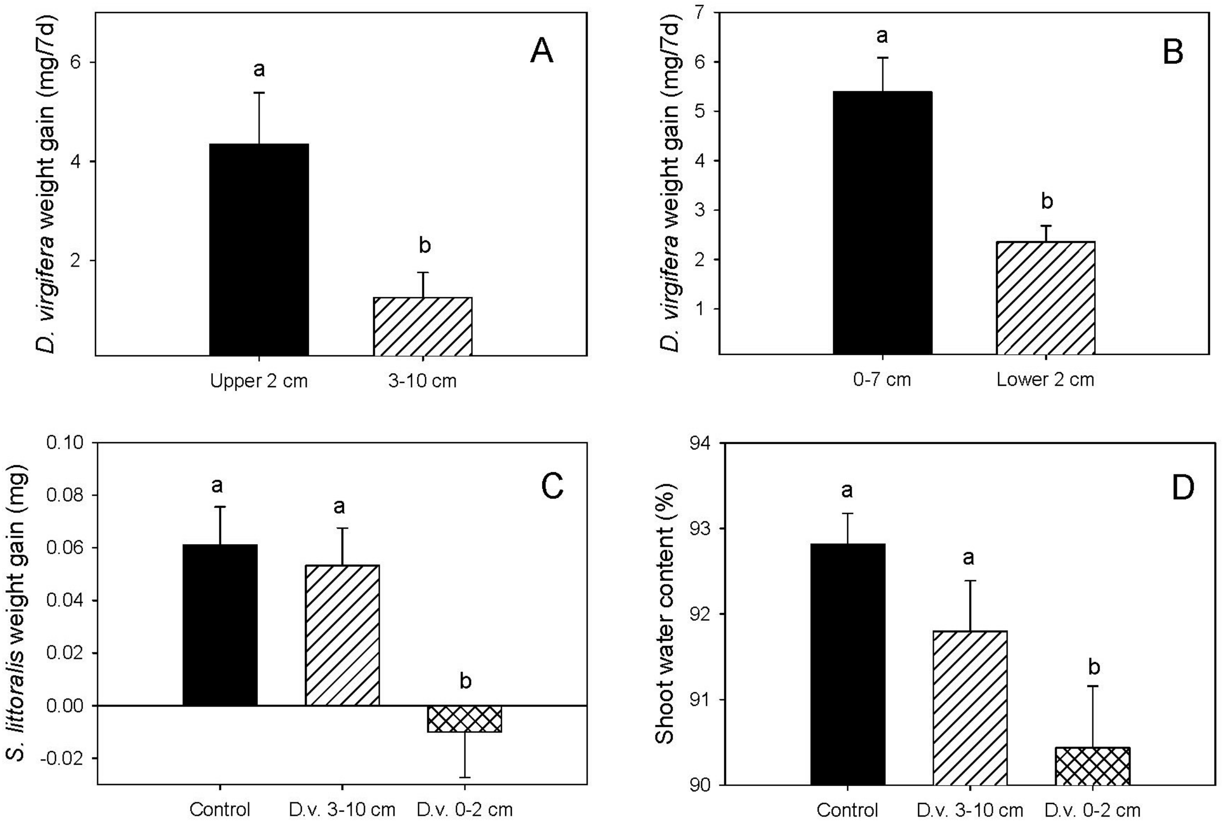

Figure 8 Growth of *D. virgifera* confined to different parts of the root system and influence on *S. littoralis* weight gain and shoot water contents. Average weight gain (+SE) is given for (**A**) *D. virgifera* larvae feeding on the top 2 cm vs. the rest of the root system, (**B**) *D. virgifera* larvae feeding on the bottom 2 cm vs. the rest of the roots and (**C**) S. littoralis feeding on plants infested with *D. virgifera* confined to the upper or the lower parts of the roots. (**D**) Average shoot water contents (+SE) of plants infested with *D. virgifera* on upper and lower parts of the roots. Different letters indicate significant differences between treatments (p<0.05).

exclude size-effects of the different compartments, we performed a second experiment confining the larvae to the lowest 2cm of the root system. Again, larvae feeding on the upper part grew significantly better (Student's T-Test: $p<0.001$, Figure 8B). To test if the behaviour of *D. virgifera* influenced the resistance induced in the shoot, we measured *S. littoralis* growth on plants infested by *D. virgifera* in either the lower or the top part of the roots. *D. virgifera* only affected *S. littoralis* growth when they were feeding on the top 2 cm of the root system (Holm-Sidak Post-Hoc Test: $p=0.003$, Figure 8C), whereas feeding on the lower parts had no measurable impact on *S. littoralis* performance. Similarly, shoot water contents were significantly reduced when *D. virgifera* fed on the upper root system and mesophyll (Dunn's Post-Hoc Test: $p<0.05$, Figure 8D), while only a trend remained when the larvae fed on the lower parts.

DISCUSSION

Adaptiveness of root herbivore induced shoot resistance

Three central hypotheses about why shoot defenses may be affected by root herbivory have been postulated by (Wäckers and Bezemer, 2003): Plant adaptation, root herbivore adaptation and plant physiological constraints. The current study supports the latter possibility to be the principal cause of the enhanced resistance in maize shoots after attack by a root herbivore. *D. virgifera* larvae often attack the upper root system (own observations), which was found to be the site were they develop much better (Figure 8). For the plant, this feeding behaviour poses a significant threat to its shoot water supply (Figure 3), especially at early developmental stages. The increase in ABA biosynthesis following root attack seems to be a tolerance response of the plant, as it reduces water loss (Figure 5B). However, under conditions where the resulting metabolic and physiological modifications are not sufficient, water concentrations in the shoot decrease nevertheless (Figure 6B), under certain conditions even to a point where a clear wilting phenotype is observed. It is under these circumstances that the aboveground herbivore *S. littoralis* is most negatively affected in its development (Figure 6A). This phenomenon is unlikely to be adaptive for the plant, as a loss of leaf-turgor to increase shoot resistance is a very unlikely defense strategy for an organism that heavily depends on an effective water supply for growth and survival. This is also reflected in the observed ABA-mediated drought-tolerance response, indicating that the plant tries to maintain leaf-tugor. Interestingly, the root herbivore *D. virgifera* seems to benefit from feeding on the most vulnerable part of the root system (Figure 8A and B). If this is only due to better access to leaf-assimilates or if changes in the plant's water balance are advantageous for *D. virgifera per se* remains to be determined. Indeed, it is well known that plants under water stress increase their investment in root growth (Reid and Renquist, 1997), and it is possible that *D. virgifera* directly profits from this. Another exciting possibility that deserves further attention is a possible manipulation of the root herbivore to increase phloem-transport of leaf-assimilates for its own benefit comparable to what is known from parasitic root-feeding nematodes (Caillaud et al., 2008). However, independently of the causal agent responsible for the observed changes in shoot physiology, it is likely that the increase in shoot resistance is a secondary effect resulting from the battle between *D. virgifera* and its host plant. Root herbivore-induced shoot resistance in this system by itself is therefore expected to be a non-adaptive phenomenon,

Physiological explanations for the increased shoot resistance upon shoot water loss

The fact that *S. littoralis* growth was strongly reduced on *D. virgifera*-infested plants that had suffered a reduction of leaf- water contents suggests that the related changes in plant physiology were unfavourable for the shoot herbivore. Traditionally, changes in C/N ratios have been considered to be the driving factor in changing shoot herbivore performance under water-limiting conditions (Huberty and Denno, 2004). Surprisingly, *D. virgifera* attack did not alter leaf C/N ratios in our system (Figure 3B), implying that other factors led to the increased resistance. We have shown that *D. virgifera* attack profoundly changes levels of secondary metabolites and defense gene expression in the shoot (Erb et al., submitted; Figure 7A) and it is likely that these changes contributed significantly to the observed enhanced resistance. Moreover, concentrations of secondary metabolites implicated in plant defense are known to increase in maize shoots under water stress (Richardson and Bacon, 1993; Hura et al., 2008), possibly because they also have a function in abiotic stress tolerance.

Our transcriptional data shows that the induction of several defense-related genes by *D. virgifera* is more pronounced when the plant suffers from water stress (Figure 7B), adding further evidence to the observation that the combination of these two stressors has the most profound impact on shoot resistance. That shoot herbivores may actively try to avoid drought-like responses of their host has been suggested by (Reymond et al., 2000), and it is tempting to speculate about the possibility that *D. virgifera* infestation in the roots triggers changes in shoot physiology that offset the herbivore's effort to suppress effective plant defenses. The fact that neither chemical nor genetic inhibition of ABA biosynthesis reduced *D. virgifera* induced shoot resistance (Figures 5) strongly suggests that ABA is not strictly required for this phenomenon. On the contrary, ABA inhibition even slightly increased the expressed resistance, possibly via a negative feedback on the plant's water status. The opposite effect (i.e. increased drought tolerance and therefore reduced water loss) could explain why the resistance was initially weaker on NCED overexpressor plants (Figure 5C). Further analysis will aim at clarifying these phenomena.

General relevance of the phenomenon for natural systems

The field experiment demonstrates that *D. virgifera* can have a strong impact on aboveground herbivores under semi-natural conditions. That damage by lepidopteran herbivores was reduced in *D. virgifera* infested plots (Figure 1) corresponds well to the feeding-deterrent effect observed in the laboratory (Figure 2). Our findings support observations by (Alleman et al., 2006) reporting a reduction of corn borer (*Ostrinia nubilalis*) numbers in *D. virgifera* infested field plots. That *D. virgifera* can alter the plant's water uptake and stomatal conductance has been documented in both field and greenhouse studies (Godfrey et al., 1993; Riedell and Reese, 1999). It has also been found that the impact of root herbivory in maize is strongest when water supply is limited (Dunn and Frommelt, 1998), a finding that is well in tune with our results. It appears that the impact of *D. virgifera* on the water balance of maize is a widespread phenomenon and could therefore have important consequences for the distribution and abundance of aboveground herbivores in agroecosystems. Effects of root herbivores on the water balance of plants have also been documented for other plant-herbivore systems (Gange and Brown, 1989; Murray and Clements, 1998; Staley et al., 2008), indicating that such effects are of general relevance in above-belowground interactions. It should be emphasized that drought-related processes can have both positive and negative effects on shoot herbivores (Huberty and Denno, 2004), and different feeding-guilds may show different responses. Furthermore, non-chewing and slow-feeding root herbivores can be expected to have a less detrimental effect on plant physiology than a voracious pest like *D. virgifera*, further contributing to the diversity of possible outcomes of indirect interactions between root and shoot feeders.

CONCLUSIONS

Root attack by *D. virgifera* has a profound impact on the shoot physiology of maize and thereby alters the plant's resistance against aboveground herbivores. Our study demonstrates that his phenomenon is likely to be a physiological constraint rather than an adaptation by either the plant or the root herbivore. This study thus provides experimental evidence for the ongoing debate about the ecological and evolutionary significance of plant-mediated interactions between above- and belowground herbivores.

MATERIAL AND METHODS

Field experiment

Maize plants were sown in a field in Columbia (Missouri) in spring 2008. Different maize varieties were randomly arranged in 96 patches of 4 rows with 20 plants/ row each. The experimental set-up included 12 patches with the variety Delprim as well as different *bt*- and non-*bt* varieties (Zwahlen et al., unpublished). Eight patches of Delprim within the field were randomly selected and infested with *D. virgifera* larvae by applying 20’000 eggs to the soil of one row, while 4 patches were left root-herbivore free. In the beginning of July, one month after application of the eggs when the *D. virgifera* larvae had reached their second instar and maize plants had 6-7 fully developed leaves (growth stage V8), the maize plants of the infested rows were sampled for aboveground herbivore damage. In the 4 control plots, two rows were sampled to compensate for the lower plot number. From each row, all the normally developed plants (apart from the two on the row margins) were examined, resulting in a total of 196 recordings. The number of damaged leaves was recorded alongside with the number of the longitudinal holes and window-frass (mostly caused by lepidopteran larvae) and shotgun-shaped holes (caused by both lepidopteran and coleopteran larvae). A leaf was considered damaged when clear surface removal by herbivores was visible. Almost all leaves showed little white traces caused by minor insect pests like flea beetles and thrips, and these were counted separately. Encountered herbivores were photographed or conserved in alcohol for later identification. For statistical analysis, results from all plants from one plot were pooled and treated as one independent replicate.

Laboratory plants and insects

For the laboratory experiments, maize plants (variety Delprim [ecological and physiological experiments] and Ames 188 [*NCED* experiment]) were grown in plastic pots (diameter, 4cm; depth, 11cm) wrapped in aluminium foil in a phytotron (CLF plant climatics, Percival; 30°C; photoperiod: 16 hours of light and 8 hours of night). The seeds were rinsed with water to remove any storage residuals and sown in sand (lower 8 cm) topped with commercial potting soil (upper 3 cm, Ricoter Aussaaterde, Aarberg, Switzerland). Plants used in *D. virgifera* performance experiments were sown in commercial potting soil to ensure equal mobility in the rhizoshpere. Plants used for experiments had 2 fully expanded primary leaves and were 9-10 days old. *S. littoralis* eggs were provided by Syngenta Switzerland and larvae were reared on artificial diet as described by (Turlings et al., 2004). Second instar larvae of *D. virgifera* were obtained from CABI Delémont (Switzerland) and from the USDA-ARS-NCARL Brookings (United States) and kept on freshly germinated maize seedlings until use.

Genotying of NCED plants

To distinguish transgenic from wildtype plants, maize seedlings were rescued after the experiment and treated with BASTA F1 one week later. Plants showing herbicide resistance were classified as transgenics, plants that showed necrosis after 48 hours were classified as wildtype. Plants that could not be regrown because of extensive herbivore damage were harvested and genotyped using PCR as described in (Voisin et al., 2006).

Root treatments

Maize seedlings were infested with *D. virgifera* by releasing six 2nd instar larvae into the soil at a depth of approximately 6 cm 48 or 96 hours prior to the experiments. Water supply of the plant's root system was manipulated by either watering the plants with a fixed volume per day (10ml), leaving them without water for 48h (dry) or putting the plants into a 3 cm deep tray filled with water, leading to a drench of the soil from the bottom of the pierced pots (drench). To inhibit ABA biosynthesis, plant seedlings were subjected to two soil drench treatments of 5 mM of sodium tungstate (dissolved in 10 ml distilled water) over 48 hours. Root access of *D. virgifera* was restricted by using a fine nylon screen to separate different compartments of the pot. This screen was easily penetrated by maize roots, but not by *D. virgifera* larvae (own observation).

Shoot resistance measurements

To quantify shoot resistance of maize seedlings upon root stress, individual 2nd instar *S. littoralis* larvae were placed on the second true leaf of plants using clip-cages. Clip cages consisted of two black lids held together with a rubber band. Fine metal screens on both sides ensured air supply to the cages. The *S. littoralis* larvae were weighed, put into the cages and the cages were then gently slid over one half of the maize leaves, exposing around $0.5cm^2$ of tissue to each larva. For the short-term performance experiments (n=30, two independent replicate experiments), clip-cages were removed after 6 hours and the caterpillars were re-weighed. The damaged leaves were then scanned into Photoshop and the consumed leaf-area was determined using the same software. For the NCED experiments, one series of caterpillars was left to feed on the plant for 6h in clip-cages, while a second series was left for 12 hours on the opposite side of the leaf. For the long-term performance experiment, the caterpillars were weighed 6, 12 and 24 hours after putting them on the plant, and the cages were moved to a different position of the leaves every time to ensure ample food supply. After 24 hours, the caterpillars were put on the plant to feed freely for the rest of the experiment. To stop the larvae from escaping, the plants were placed in PET-tubes with a circular top opening covered by a fine nylon mesh.

Root herbivore growth measurements

Individual *D. virgifera* larvae were weighed and added to the different root compartments by either putting them on the top of the soil or by carefully releasing them to the bottom of the root system through a whole in the plastic pot that was closed with aluminium foil afterwards. After 7 days, the pots were emptied and the larvae retrieved and weighed again.

Determination of C/N ratios and relative water content

Shoots of the plants from the short term performance experiment (n=30) were cut and weighed immediately to determine their fresh weight (FW). Dry weight (DW) was determined after drying them for 48 hours at 80°, and relative water contents (%WC) were determined from FW and DW using the formula %WC=100-(FW-DW/FW*100). Constant turgid weight was used in the calculations, as the measured leaves were of equal growth stage and quality in the different treatments. The dried shoots were then ground to a fine power using a ball mill, and total carbon (C) as well as total nitrogen (N) were determined from 2-3 mg/ sample using an elemental analyzer.

Quantification of phytohormones

Maize seedlings were subjected to root stress as described above. Half of the plants (n=6x9) were then mechanically damaged on their second leaf using a razor blade, with witch their underside was scratched on a surface of 1cm2 on both sides. The scratched leaf-area was then treated with 10ul of undiluted herbivore regurgitate from 3rd instar *S. littoralis* larvae that had previously fed on maize plants. Sixty minutes after elicitation, the second leaf of scratched and unscratched plants was harvested and immediately frozen in liquid nitrogen. Leaves of 6 plants were pooled and ground to a fine powder in liquid nitrogen. An aliquot of 150mg per sample was then transferred to FastPrep tubes and mixed with 1ml ethylacetate containing 200ng of D_6-ABA, D_2-JA, D_4-SA and $^{13}C_6$-JA-Ile as internal standards. The mixture was then homogenized and centrifuged before transferring the supernatant to a 2ml Eppendorf tube. After repeating the extraction procedure and combining the supernatants, the solvent was evaporated in a vacuum concentrator and the pellet redissolved in 70% MeOH. 10ul of each sample were then injected into a HPLC-MS equipped with a ProntoSIL C18 Column. The 1200L LC/MS system (Varian, Palo Alto, CA, USA) was operated a flow rate of 0.1 mL/min. A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in acetonitrile) was used in gradient mode for separation. The compounds were detected in the ESI negative mode. Molecular ions (M–H) with *m/z* 137, 209, 263 and 322 for SA, JA, ABA and JA-Ile and 141, 213, 269 and 328 for the respective internal standards were fragmented and daughter ions 93, 59 153 and 130 (compounds) and 97, 59, 159 and 136 (internal standards) were recorded for quantification. Collision energy was 15V for SA, 12V for JA, 9V for ABA and 19V for JA-Ile. For ethylene measurements, maize seedlings (n=6) were treated as described above. Sixty minutes after elicitation, residue regurgitate was washed off with water and the leaves were carefully put in 20ml gastight vials. The wounded leaf-parts had been sealed with small pieces of aluminium foil before. After incubation at room temperature overnight, 1ml headspace samples were withdrawn from the vials with a 2.5ml gastight syringe and directly injected into a gas chromatograph equipped with a flame ionization detector (GC-FID). The GC-FID was operated in split-mode (2:1) with a liner temperature of 60° C, a column temperature of 50° C and a detector temperature of 300°C. For separation, a GS-Alumina column was used at a constant flow-rate of 4.8ml/min. Ethylene was identified by comparison of the retention time with that of the pure compound. Absolute quantification was based on a standard-curve obtained by injecting different concentrations of pure ethylene.

Quantification of gene expression

Maize seedlings were subjected to root herbivory and different water regimes as described above. Leaves of 6 plants were pooled and ground to a fine powder in liquid nitrogen. Total RNA was extracted using Quiagen RNA-Easy extraction kits following the manufacturer's instructions. The quality of the RNA was assessed by photometry and gel electrophoresis. To remove contaminant genomic DNA, all samples were treated with Ambion DNAse following the standard protocol. cDNA was then synthesized using Invitrogen Super-Script III reverse transcriptase according to the manufacturer's instructions. Quantitative reverse transcriptase real time polymerase chain reactions (q-PCR) were then carried out using gene-specific primers (Erb et al., submitted). The q-PCR mix consisted of 5ul Quantace Sensimix containing Sybr Green I, 3.4ul H_20, 100nmol of each primer (2x0.3ul H_20) and 1ul of cDNA sample. Q-PCR was carried out using 45 cycles with the following temperature curve: 10s 95°C, 20s 60°, 15s 72°. The final melt curve was obtained by ramping from 68 to 98°C in 1°C steps every 5s. To determine primer efficiencies and optimal quantification thresholds, a dilution series of a cDNA mix consisting of 4ul solution from every sample was created. Six 10-fold dilution steps were carried out and the standard curve was included into every q-PCR run. The final obtained Ct values (using the automated threshold determination feature of the Rotor-Gene 6000 software) were corrected for the housekeeping gene GapC (Frey et al., 2000) and normalized to control levels to obtain average fold changes of treated plants.

Statistical procedures

Differences in survival of *S. littoralis* were tested using Kaplan-Meier's Survival Analysis of Log-Ranks. As *S. littoralis* growth over time did not conform to normality and equality of variance, it was assessed using a Two-Way Repeated Measures ANOVA on Ranks followed by Holm-Sidak Post-Hoc Tests. Two-Way ANOVAs (with a block-factor for two independent replicate experiments) and Holm-Sidak Post-Hoc Tests were used to test *S. littoralis* growth and leaf consumption over 6 hours on *D. virgifera* infested plants. The same procedure was also applied for leaf C/N ratios. Leaf water contents were tested using Two-Way ANOVAs on Ranks followed by Holm-Sidak Post-Hoc Tests. Hormonal concentrations were tested using Two-Way ANOVAs (followed by Holm-Sidak Post-Hoc Tests) or Two-Way ANOVAs on ranks (followed by Student-Newman-Keuls Tests) with root herbivory and shoot induction as factors. The relative influence of root water supply and *D. virgifera* infestation as well as the influence of ABA-inhibition was assessed using Two-Way ANOVAs followed by Holm-Sidak Post-Hoc Tests. Changes in gene expression were assessed using Student's T-Tests (*D. virgifera* vs. control plants) and Two-Way ANOVAS (*D. virgifera* vs. control plants, drought vs. soil drench) on ln Fold-change values relative to control expression. Weight gain of *D. virgifera* and *S. littoralis* as well as shoot water contents in the confined feeding experiments was tested using Student's T-Tests and One-Way ANOVAs followed by Holm-Sidak Post-Hoc Tests respectively.

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Chapter 4: Transcriptional changes in roots and shoots of herbivore-attacked maize plants

Matthias Erb*, Claudia Lenk*, Christelle Robert, Ted C.J. Turlings and Jörg Degenhardt

*These authors contributed equally to the work

SUMMARY

Plants can be attacked by insect herbivores at both roots and shoots, and the ensuing defense responses involve not only the attacked tissues, but can be systemic, affecting non-attacked parts as well. As yet, knowledge about how plants react upon root herbivory is very limited, as is our knowledge about physiological changes in the shoots upon root herbivore attack and *vice versa*. Using whole-transcriptome microarrays, we therefore investigated how roots and shoots of *Zea mays* respond to aboveground attack by the larvae of the noctuid moth *Spodoptera littoralis* and belowground infestation by the larvae of the beetle *Diabrotica virgifera*. The results show that the systemic transcriptional changes are profoundly different from local induction by either herbivore, suggesting specific root-shoot and shoot-root signals. A significant proportion of transcripts is similarly activated locally both upon root- and shoot attack, illustrating that above- and belowground defenses share many common elements. Roots displayed specific reactions after both root and shoot herbivory, with *D. virgifera* inducing genes involved in various transport processes and *S. littoralis* changing transcripts with functions in protein metabolism. The ecological implications of the dramatic changes in root metabolism upon shoot herbivory are indicated by a 70% reduction in *D. virgifera* growth when plants are attacked aboveground by *S. littoralis*.

INTRODUCTION

Plants under attack by leaf-feeding insects undergo extensive transcriptional reprogramming. Starting with the recognition of wound- and insect derived elicitors (Alborn et al., 1997; Schmelz et al., 2006) and the activation of intra- and inter-cell signaling cascades (Kandoth et al., 2007; Maffei et al., 2007), the expression of a significant proportion of the genome is changed, enabling the plant to specifically adjust its phenotype to the occurring threat (De Vos et al., 2005). The synthesis of defensive proteins and metabolites for example is a well characterized, ubiquitous phenomenon that helps plants to reduce herbivore damage and the loss of vital tissue (Howe and Jander, 2008). Whole genome microarray analysis has greatly facilitated the discovery of novel regulatory patterns and processes involved in plant defense and tolerance to herbivory, at least for a handful of model systems including *Arabidopsis thaliana* (De Vos et al., 2005; Kempema et al., 2007; Ehlting et al., 2008) and *Oryza sativa* (Yuan et al., 2008).

Studies on transcriptional changes upon insect encounter have been limited to the leaves, but it is evident that a plant's herbivore-induced responses also involve the root system. Important insect toxins for example are synthesized in the roots of plants, from where they are transported into the shoots upon insect attack. These include gossypol (Smith, 1961), an important defensive secondary metabolite in *Gossypium herbaceum* (Mao et al., 2007), pyrrolizidine alkaloid N-oxides in *Senecio vulgaris* (Hartmann et al., 1988), as well as nicotine (Baldwin et al., 1994), the primary insecticidal alkaloid of *Nicotiana attenuata* (Steppuhn et al., 2004). The powerful *Zea mays* cysteine protease MIR1 is also thought to be synthesized belowground and then transported into the leaves upon attack (Lopez et al., 2007). Conversely, recent research suggests that plants move assimilates away from the leaves into the roots for future regrowth as soon as they experience insect herbivory (Babst et al., 2005; Schwachtje et al., 2006). Despite the fact that roots seem to harbor many central functions in leaf-defenses, their systemic transcriptional reprogramming has not yet been investigated.

Roots are not only involved in defense against aboveground herbivores, but have to resist direct threats as well. Herbivorous insects are abundant soil inhabitants (Blossey and Hunt-Joshi, 2003), and they can have a significant impact on plant survival in nature and on yield of agricultural crops (Rasmann and Agrawal, 2008). *Diabrotica virgifera* and *Lissorhoptrus oryzophilus* for example are economically important pests of *Zea mays* (Vidal et al., 2005) and *Oryza sativa* (Saito et al., 2005), with *D. virgifera* alone causing losses of more than a billion US$ per year in the United States. While a considerable research effort is being undertaken to develop novel pest control strategies against these herbivores, very little is known about the physiology of inducible defenses in the roots upon belowground attack (Erb et al., 2008; Rasmann and Agrawal, 2008).

The systemic changes in plant metabolism upon attack can also have dramatic indirect consequences for food-web dynamics. Plants, because they reach into both the rhizosphere and the phyllosphere, are powerful mediators between above- and belowground biotic systems (van der Putten et al., 2001; Wardle et al., 2004), and several recent studies highlight the plant-medaited effects of leaf-herbivory on root feeders and *vice versa*. Increased sugar translocation into the roots upon shoot attack by herbivorous insects in *Nicotiana tabacum* for example is likely to be responsible for the increased fecundity of *Meloidogyne incognita*, a phytopathogenic nematode (Kaplan et al., 2008). Conversely, increased levels of indole glucosinulates upon leaf attack by *Pieris brassicae* in *Brassica nigra* have been speculated to contribute to the reduced performance of the root-herbivore *Delia radicum* and its associated parasitoid *Trybliographa rapae* (Soler et al., 2007). We have recently demonstrated that root herbivory by *D. virgifera* in *Z. mays* on increases shoot resistance against both the pathogen *Setosphaeria turcica* and the herbivore *Spodoptera littoralis* (Erb et al., submitted), adding further evidence to the general trend that chewing root herbivores decrease the performance of aboveground attackers. From these examples, it becomes clear that understanding the systemic physiological changes occurring after insect attack is of considerable interest not only for plant physiologists, but also for ecologists (Erb et al., 2008).

As a start to filling the gap in our knowledge of plant transcriptional activity involved in plant-insect interactions, we conducted micro-array experiments that yielded a comprehensive dataset covering the local and systemic transcriptional changes of maize plants to insect attack both in the roots and the shoot. This whole-transcriptome microarray study presented here is one of the first to cover i) root-herbivore induced defenses in plants ii) changes in root physiology upon shoot herbivory and *vice versa* and iii) plant insect interactions in *Z. mays* in particular.

RESULTS

Timing and systemic effects of belowground herbivory

While the dynamics of aboveground responses of maize upon *Spodoptera spp.* attack or contact with its regurgitant are well studied (Schmelz et al., 2003; Ton et al., 2007), nothing is known about the temporal dynamics of root induction of herbivore infested plants. We therefore measured the effects of *D. virgifera* attack on phytohormone levels, emission of the belowground volatile organic compound E-β-caryophyllene and expression of the corresponding terpene synthase, TPS-23, at different time-points. Using a root compartmentalization system also enabled us to distinguish between local and systemic induction. E-β-Caryophyllene, the dominant volatile organic compound emitted by maize roots upon *D. virgifera* attack (Rasmann et al., 2005) was strongly induced after 4 hours of infestation at the site of *D. virgifera* feeding. A systemic effect upstream in the root system was also visible, albeit later (after 24 hours) and at a lower scale (Figure 1). The corresponding synthase gene *Zm-TPS23* (Köllner et al 2008) was induced locally after 1 hour of infestation already and was also upregulated systemically after 24 hours in the roots (Figure 1). Phytohormone analysis of the same plant material showed that local induction of both jasmonic acid (JA) and its isoleucin conjugate (JA-Ile) occurred rapidly after 30 minutes of root infestation (Figure 2) and persisted over the course of the experiment. JA and JA-Ile concentrations also increased systemically after 2-4 hours. No significant increase in salicylic acid (SA) was detected, while ABA levels showed an idiosyncratic behaviour, with locally higher levels after 2, 4 and 48 hours, but lower concentrations after 24 hours (Figure 2).

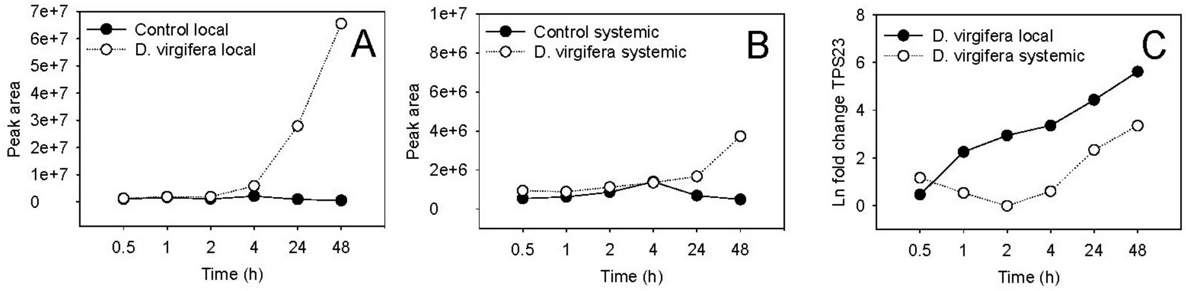

Figure 1: Temporal dynamics of local and systemic defense elicitation by *D. virgifera*. (**A**) (E)-β-Caryophyllene accumulation at the feeding site of *D. virgifera* attacked roots. (**B**) (E)-β-Caryophyllene accumulation in the roots above the *D. virgifera* attacked site. (**C**) Local and systemic expression of the (E)-β-Caryophyllene-synthase (*Zm-TPS-23)* after *D. virgifera* attack relative to non-attacked control-plants. Means are given for 6 pooled plants.

General structure of transcriptional changes after root- and shoot herbivory

Based on the temporal dynamics of defense elicitation by *D. virgifera* and the available literature on aboveground plant responses to herbivore attack (Schmelz et al., 2003; Ton et al., 2007), we chose a time point of 18 hours after initial feeding for microarray analysis of roots and shoots of plants infested with *S. littoralis* and *D. virgifera*. Genes showing more than 2-fold changes upon herbivore attack at a significance level of p<0.01 were selected for further analysis. This resulted in a total number of 1065 differentially expressed genes in the different herbivore treatments. Because of the occurence of false positives and negatives, which has been estimated to be around 15% in this type of experiment (Claudia Lenk, unpublished), we decided to perform a general meta-analysis of the data rather than a detailed discussion of single genes. Aboveground attack by *S. littoralis* induced 209 genes in the leaves, while 3 were suppressed (Figure 3). In the roots, 168 genes showed higher expression upon shoot attack, while 201 were suppressed. *D. virgifera* attack belowground induced 467 genes in the roots, while 99 were suppressed. Transcript levels

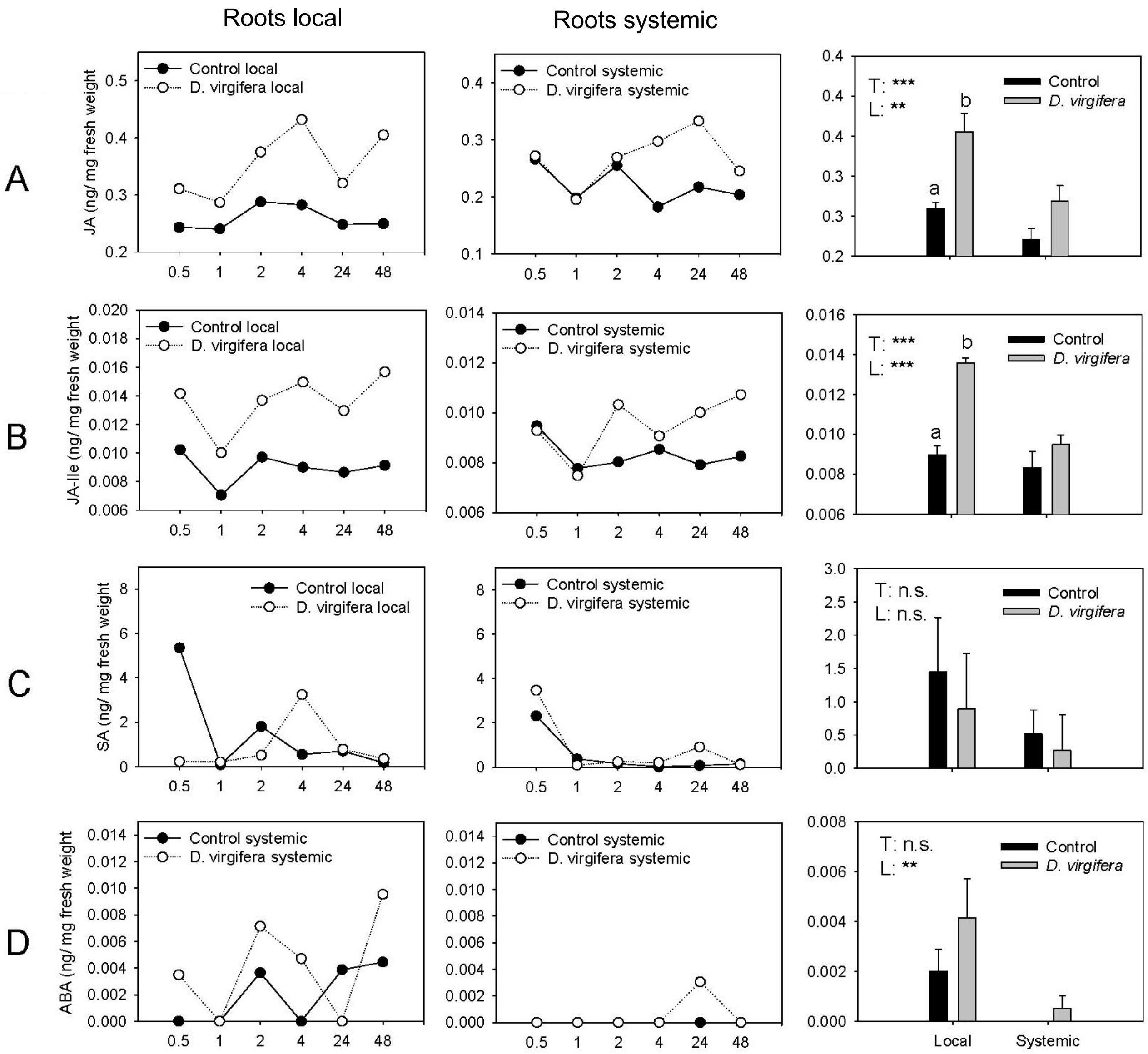

Figure 2: Temporal dynamics of local and systemic phytohormone accumulation after *D. virgifera* attack. Means are given for (**A**) JA, (**B**) JA-Ile, (**C**) SA and (**D**) ABA. Bargraphs represent means (+SE) of each of the different time points. Significance values (*p<0.05, **p<0.01, ***p<0.001) are given for two-way ANOVAs with T= treatment and L= location). Different letters indicate significant differences between treatments according to post-hoc tests.

of 17 genes were enhanced in the leaves upon root infestation, while 21 were reduced (Figure 3). To gain further insight into the functional distribution of obtained gene expression patterns, we translated the differentially expressed maize genes into *Arabidopsis thaliana* homologues (Calderon-Vazquez et al., 2008) using PLEXdb (Wise et al., 2006). Sequences that had no correspondence in the database were compared manually using the NCBI BLASTx function. Double alignments were removed manually to obtain a non-redundant list of *Arabidopsis* homologues. In total 61% of the differentially regulated maize transcripts corresponded to known *Arabidopsis* sequences, a similar percentage as the non-annotated genes in the original transcript annotation list. Although wrong annotations possibly leading to erroneous interpretations of single genes cannot be excluded, this procedure was considered robust enough to permit a general functional classification with the MapMan software (Thimm et al., 2004). The analysis shows that the majority of functionally annotated genes in shoots of *S. littoralis* attacked are assumed to be involved in stress responses, secondary metabolism, hormone metabolism, amino acid and protein metabolism (Figure 4A). A comparable pattern was observed belowground upon *D. virgifera* attack (Figure 4D), with the exception of a strikingly bigger proportion of upregulated genes involved in transport processes. Interestingly, while there was no clear expression pattern in shoots of root-infested plants (Figure 4B), roots of shoot-infested plants changed markedly in their expression of genes involved in protein synthesis and degradation (Figure 4C).

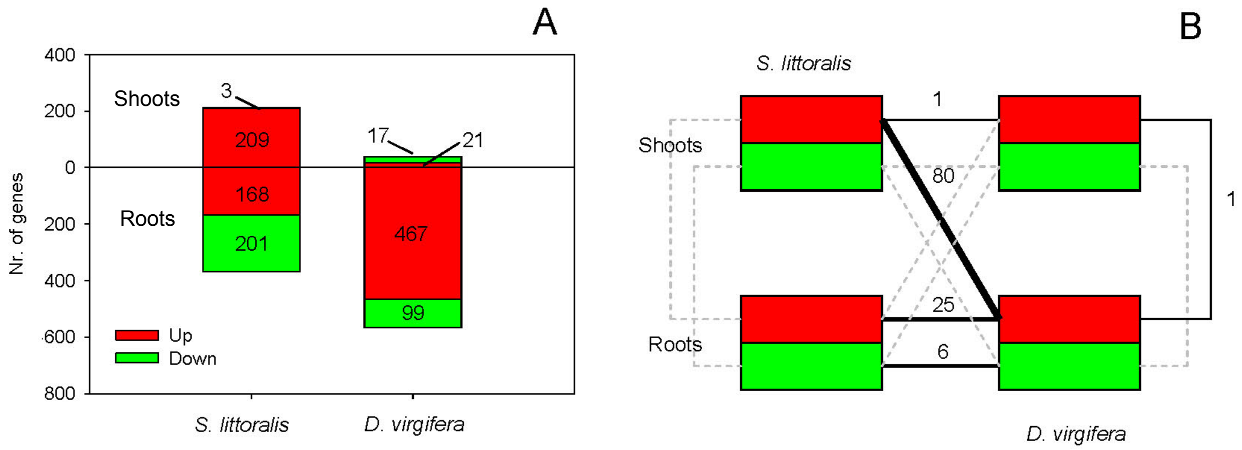

Figure 3: Overview of transcriptional changes in herbivore-infested plants. (**A**) Absolute number of significantly induced (red, dark) and suppressed (green, light) transcripts upon attack by *S. littoralis* and *D. virgifera* in the shoots (upper half) and the roots (lower half). (**B)** Number of genes that show the same behavior (up- or downregulation) upon different treatments.

Overlap between wound-inducible and D. virgifera inducible transcripts

To test if the transcriptional changes caused by *D. virgifera* attack are a general wound response or a specific reaction of the plant, we also analyzed roots that were damaged mechanically with a knife over a period of 18 hours. Similar to *D. virgifera* attack, mechanical root damage resulted mostly in changes in gene expression belowground and only few changes aboveground. Analysis with FiRe (Garcion and Metraux, 2006) revealed that more than 50% of the genes induced by *D. virgifera* were also wound-responsive (Figure 5B). However, the aboveground changes were different for the two root-stress treatments, with only one gene showing the same response (Figure 5B). Furthermore, only 5% of the genes that were downregulated by *D. virgifera* in the roots showed the same behavior upon mechanical wounding (Figure 5B). Functional analysis

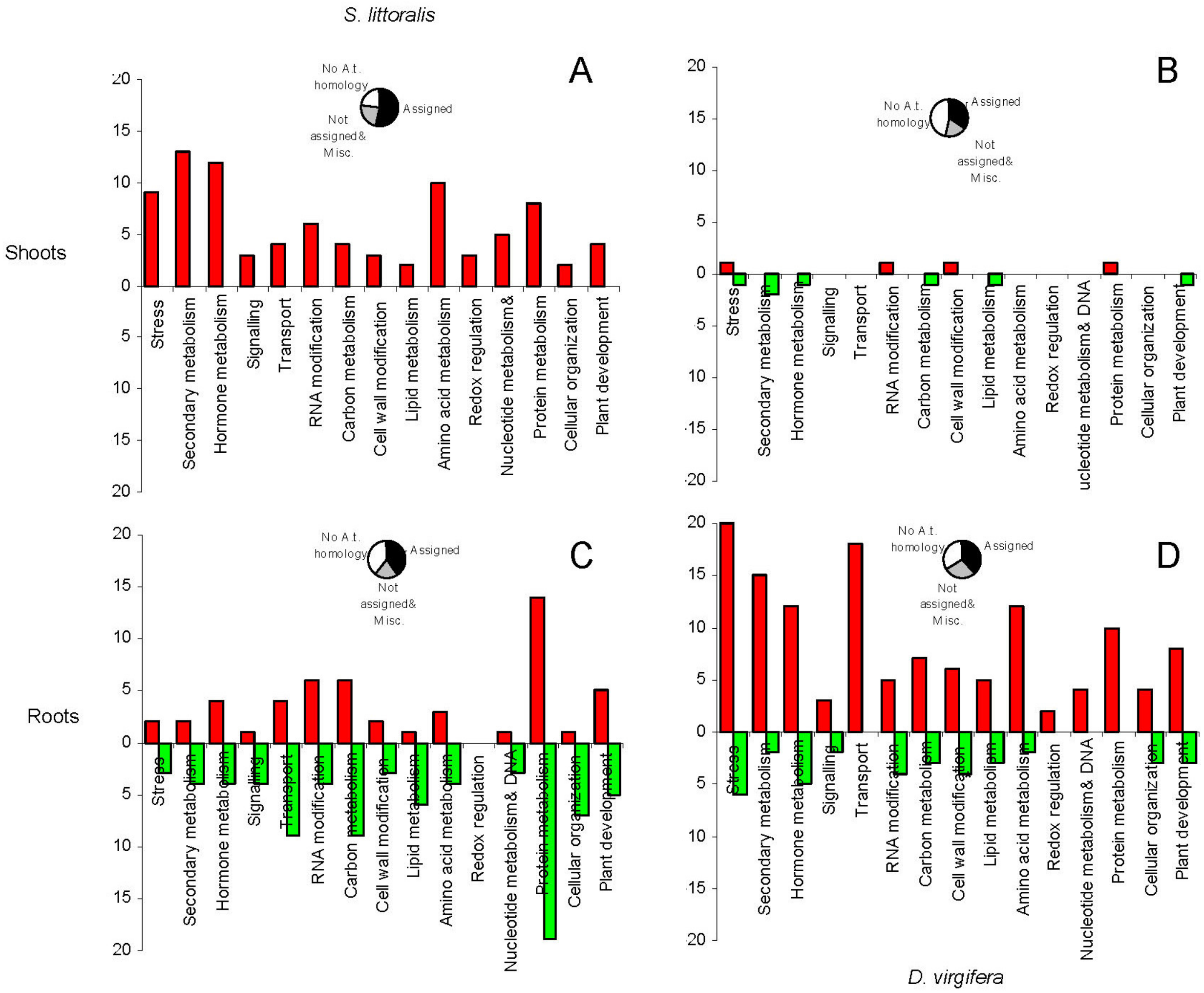

Figure 4: Functional classification of transcriptional changes in herbivore-infested plants. Y-Axes indicate absolute number of genes. (**A**) Shoots of *S. littoralis* infested plants. (**B**) Shoots of *D. virgifera* infested plants. (**C**) Roots of *S. littoralis* infested plants. (**D**) Roots of *D. virgifera* infested plants. Red (dark) bars, up= induced genes. Green (light) bars, down= suppressed genes.

revealed a strong correlation in abundance of the functional groups (Figures 4D and 6), with the exception of genes involved in protein metabolism, which were more strongly induced upon mechanical wounding (23 transcripts) than *D. virgifera* attack (10 transcripts).

Overlap in changes in gene expression upon root versus shoot herbivory

Analysis with FiRe showed that root- and shoot changes upon herbivore attack were remarkably specific, with no overlapping elements between root- and shoots in *S. littoralis* infested plants

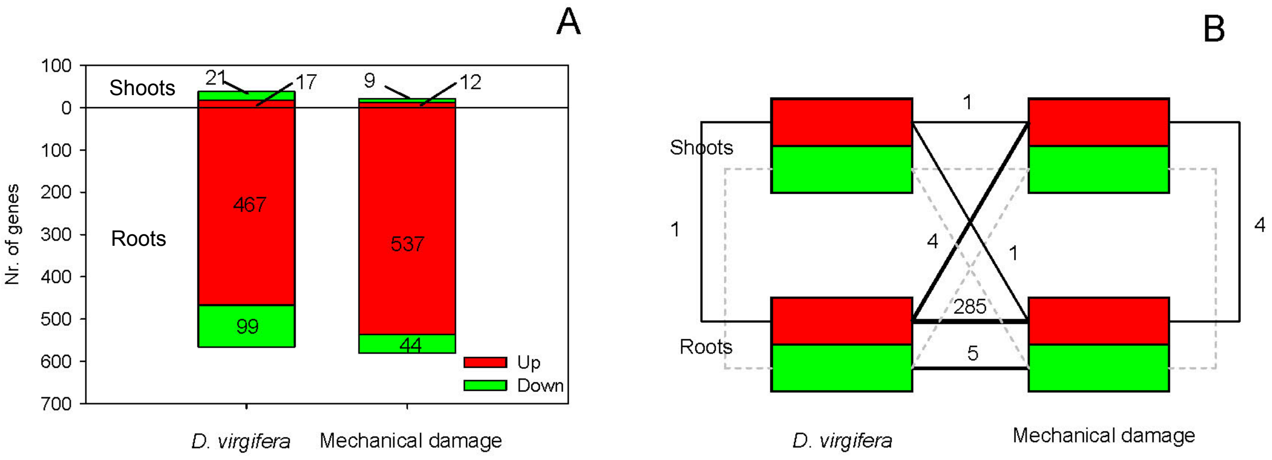

Figure 5: Overview of transcriptional changes in root stressed plants. (**A**) Absolute number of significantly induced (red) and suppressed (green) transcripts upon attack by *D. virgifera* and mechanical root damage in the shoots (upper half) and the roots (lower half). (**B)** Number of genes that show the same behavior (up- or downregulation) upon different treatments.

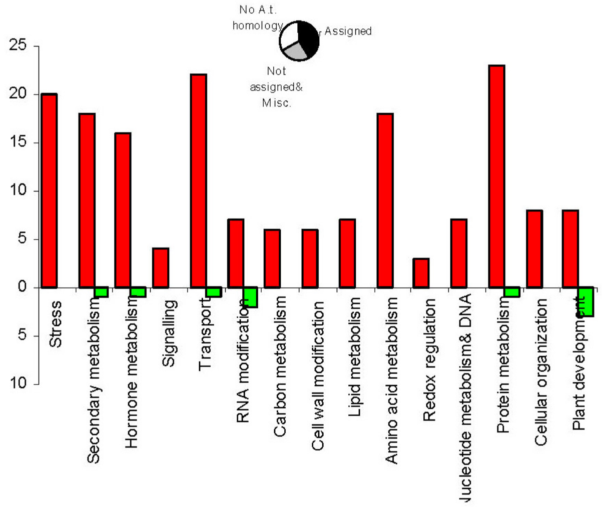

Figure 6: Functional classification of transcriptional changes in roots of mechanically damaged plants. Y-Axes indicates absolute number of genes. . Red (dark) bars= induced genes. Green (light) bars= suppressed genes.

(Figure 1C) and only one gene being induced in both roots and shoots after attack by *D. virgifera* (a putative class III chitinase). Interestingly, 38% of the transcripts that were upregulated in the shoot after *S. littoralis* attack were also upregulated in the roots after *D. virgifera* attack (Figure 1B). Closer analysis of these 80 locally induced, herbivore-responsive genes showed a significant enrichment of transcripts associated with JA-biosynthesis and general defense activation. Four O-Methyltransferases and two transcripts annotated as Glutathione-S-transferases were also induced both in roots and shoots upon local attack. Functional classification with MapMan confirmed this general pattern by showing induction of homologues involved in stress reaction, secondary metabolism, hormone metabolism, amino acid and protein metabolism. 73% of the transcripts responsive to herbivory were also induced upon mechanical root damage, indicating that a majority among them is generally wound-inducible.

We furthermore found 25 transcripts that were induced in the roots both upon local attack by *D. virgifera* and aboveground attack by *S. littoralis*. This included two putative proline oxidases as well as two peroxidases (Passardi et al., 2005). Further analysis showed that four putative proteinase inhibitors were induced in the roots upon both shoot and root attack as well. 61% of the root-responsive genes were inducible by belowground mechanical wounding.

Involvement of defense signals in root and shoot defenses

Both shoot herbivory by *S. littoralis* and root infestation by *D. virgifera* changed local transcript levels of genes involved in defense signaling. The most striking induction was measured for genes involved in the octadecanoid pathway and JA-signaling. Transcripts involved in ethylene (ET) signaling showed a positive response locally after infestation by *D. virgifera* and *S. littoralis* as well. Interestingly, while *D. virgifera* did not induce any JA, ET, SA or ABA transcripts in the shoot, *S. littoralis* feeding in the leaves induced a putative SAMT in the roots, while it suppressed a JMT. Apart from genes involved in hormonal signaling, we detected an increase in the expression of several transcription factors and other regulatory elements. Comparison with the transcriptional profile evoked belowground by mechanical root damage showed a weak overlap of 25%, indicating that a majority of the signaling related genes were specifically induced by the insect herbivores.

Induction of defensive proteins by root and shoot herbivores

Attack by the two herbivores induced several transcripts coding for defensive proteins. Both *S. littoralis* and *D. virgifera* induced a series of proteins possibly exerting inhibitor activity towards various proteases. Both herbivores also induced a local accumulation of transcripts coding for chitinase-like genes (Collinge et al., 1993) and beta-glucanases. Furthermore, both herbivores induced a variety of PR-proteins (van Loon et al., 2006) as well as other transcripts implicated in protein-mediated defenses. Overall, *D. virgifera* in the roots induced 41 defensive proteins in the roots, while 2 were suppressed. Aboveground feeding by *S. littoralis* induced 18 defensive proteins in the shoot. Closer analysis also showed that 68% of the defensive protein coding genes induced by the herbivores were responsive to mechanical root damage.

Activation of secondary metabolite synthesis upon herbivore attack

Root and shoot herbivory increased transcript levels of various genes involved in secondary metabolite synthesis. *S. littoralis* and *D. virgifera* induced several genes involved in the synthesis of phenylpropanoids, alkaloids, hydroxamic acids and isoprenoids. Comparisons with the mechanical root damage treatment showed that 57% of the genes involved in secondary metabolite synthesis were also wound-inducible belowground. The remaining 43% of the genes were herbivore-specific in their reaction.

Changes in root transporter activity after D. virgifera attack

The striking number of upregulated transcripts involved in transport processes upon root attack by *D. virgivera* in the roots (Figure 4) prompted us to perform a closer analysis of these genes. Analysis with MapMan and functional annotation with the help of publicly available databases revealed five genes that are implicated in carbohydrate transport including an STP1 homologue possibly involved in root-elongation (Beemster and Baskin, 2000), 3 genes implicated in amino acid transport, 5 drug-resistance associated transcripts as well as transporters involved in translocation of oligopeptides, sulfates, potassium and water. While 48% of the transporter genes reacted in an herbivore-specific manner, 52% were generally wound-inducible in the roots.

Changes in root protein metabolism upon shoot attack by S. littoralis

As the functional analysis of *S. littoralis*-induced changes in the roots revealed that a considerable number of genes were involved in protein metabolism (Figure 4C), we performed a detailed analysis of the differentially regulated transcripts, which showed that they belong to several functional subclasses: Protein biosynthesis (8 transcripts) including regulation of ribosomal proteins and translation initiation factors, posttranslational modification (4 transcripts), protein targeting factors (4 transcripts) and protein degradation (16 transcripts), including multiple proteases. None of these genes responded to *D. virgifera* infestation, whereas only 8% of the transcripts were changed by mechanical root damage, suggesting a high degree of specificity in their responsiveness to leaf herbivory.

Influence of S. littoralis infestation on root-herbivore performance

To test if the dramatic changes in root transcriptional activity upon shoot herbivory influenced the development of root herbivores, we measured the growth of *D. virgifera* larvae over 7 days on uninfested and *S. littoralis* infested plants. Plants that had been infested with 4 L2 *S. littoralis* larvae retained at least part of their foliage over the whole course of the experiment, but plant under high shoot herbivore pressure (12 L2 *S. littoralis*) were almost entirely defoliated after 5 days of infestation. Overall, 46% of the root herbivores were retrieved from the pots after 7 days. While 60% of the *D. virgifera* larvae were retrieved from control plants, 48% were found in plants under low aboveground herbivore pressure and 30% in plants under high infestation. Irrespective of the severity of aboveground infestation, *D. virgifera* larva grew significantly less on infested than on uninfested plants. This effect was more pronounced for larvae that had been feeding on the upper part of the root system, were aboveground herbivory reduced their growth by almost 70% (Figure 7). This was reflected by a significant interaction between treatment and location (two-way ANOVA treatment*location, p<0.05).

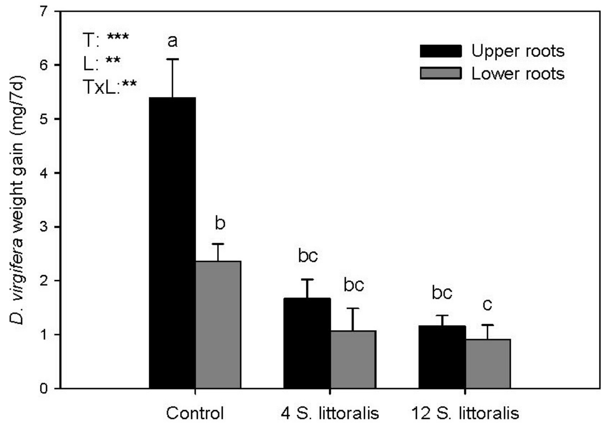

Figure 7: Effect of aboveground *S. littoralis* attack on *D. virgifera* growth belowground. Average weight gain (+SE) of *D. virgifera* over 7 days of feeding on unattacked plants, plants infested with 4 L2 *S. littoralis* and 12 L2 *S. littoralis* larvae is shown. Black bars indicate weight gain of *D. virgifera* larvae feeding on the upper roots, gray bars indicate weight gain of larvae feeding on the lower roots. Significance values (*p<0.05, **p<0.01, ***p<0.001) are given for two-way ANOVAs with T= treatment and L= location).

DISCUSSION

The transcriptional data show that both *S. littoralis* and *D. virgifera* attack resulted in an upregulation of genes involved in plant defense reactions. Both herbivores triggered the expression of transcripts involved in the biosynthesis of jasmonic acid (JA) and related metabolites as well as ethylene (ET) in the attacked tissue. It is well known that *Spodoptera spp.* induce JA and ET aboveground in maize (Schmelz et al., 2003; Schmelz et al., 2003), and we provide first evidence that root herbivory triggers the same major signaling cascades in the roots. This is confirmed by our root phytohormone profiling showing that *D. virgifera* indeed induces JA and JA-Ile locally (Figure 2). Interestingly, *S. littoralis* feeding also induced a putative jasmonic acid carboxyl transferase in the leaves that might be responsible for the synthesis of MeJA (Seo et al., 2001) and a putative salicylic acid (SA) carboxyl methyltransferase implicated in the production of methyl-salicylate was induced locally by both herbivores as well (Zubieta et al., 2003). Overall, 75% of the signaling-related genes were induced upon herbivory, but not upon mechanical damage, indicating that they are involved in insect-specific reactions. Other well-known defensive processes observed to be activated in both roots and shoots of attacked maize seedlings include the synthesis of a variety of defensive proteins and secondary metabolites. While 68% of the defensive proteins were also wound-inducible in the roots, 57% of the genes involved in secondary metabolite synthesis showed an insect-specific reaction. This suggests that many of these defenses are of general importance for the wound-response of the plant, but others react specifically to herbivorous insects. Remarkably, leaf-attack by *S. littoralis* increased the transcription of 209 genes, while only 3 were downregulated. This is in contrast with other plant-insect systems, where much more significant downregulation of genes involved in photosynthesis was observed upon insect attack (Ehlting et al., 2008 and references therein). It seems that upon insect attack, maize does not reduce its investment in growth and development aboveground, but instead accelerates its metabolism.

It is known from aboveground studies that plant defenses are not only induced at the site of attack, but systemically throughout the leaves (Turlings and Tumlinson, 1992; Orians, 2005), and we demonstrate here that root defenses are also induced systemically within the root-system (Figure 1 and 2). JA and JA-Ile concentrations increased upstream of the wounding site after a few hours already, while the response of TPS23 and (E)-β-caryophyllene was detectable after 24 hours. Interestingly, while (E)-β-caryophyllene production increased exponentially over 48 hours, JA did not follow this pattern. Both jasmonates and MeSA have been implicated in aboveground long-distance signaling (Heil and Ton, 2008), and jasmonic acid itself has even been proposed as a mobile shoot-root signal (Baldwin et al., 1994). In this context, it is striking that there is almost no overlap between root and shoot transcriptional responses of maize plants upon local herbivore attack (Figure 3). This suggests that the systemic root-to-shoot and shoot-to-root signals are not identical to the systemic within-root and within-shoot signals, but have an entirely different structure and/or mode of action.

The local responses of roots and shoots showed considerable overlap. Almost 40% of the genes induced by *S. littoralis* in the roots were also induced by *D. virgifera* in the roots (Figure 3). This overlap is comparable to the similarity of transcriptional responses in *Arabidopsis* upon attack by different chewing herbivores aboveground (Ehlting et al., 2008). Our analyses show that many of the common locally induced genes belong to well-known plant-defense processes including, as discussed above, JA and ET biosynthesis as well as the pathogenesis related proteins, proteinase inhibitors, chitinases, O-methyltransferases, gluthatione-S-transferases and many other defense-related transcripts. The fact that 73% of these genes were also responsive to mechanical damage in the roots suggests that these are general wound-inducible genes, possibly with similar roles in above- and belowground defenses.

Apart from this strong overlap we also found some remarkable differences in the response of maize seedlings to root and shoot herbivores (Figure 5). The induction of defensive proteins, for example, was much more pronounced in the roots after attack by *D. virgifera* than in the shoots after *S. littoralis* infestation. There was a strong overrepresentation of proteinase inhibitor coding transcripts as well as PR-proteins belowground. Root insect attack, by wounding the plant tissue, can increase the possibility for abundantly present soil microorganisms and pathogens to colonize the plant, and it is therefore not surprising that we found many pathogen-response associated transcripts to respond in the roots after local attack. This notion is further strengthened by the fact that mechanical wounding was sufficient to induce many of these transcripts in the roots, suggesting that the difference is not necessarily caused by an insect specific reaction of maize roots, but possibly by secondary effectors present in the environment.

Functionally, the most dramatic difference between locally induced root- and shoot-herbivore transcripts was found for genes implicated in transport processes (Figure 5). Several explanations might account for this result. First, root herbivory can lead to increased root growth, as the plant tries to compensate for the loss of belowground tissue. The sugar transporter STP1 for example, a homologue of which was induced by *D. virgifera* in maize roots, has been implicated in root elongation in *Arabidopsis* (Beemster and Baskin, 2000). This suggests that the plant indeed started to increase its energetic investment in growth belowground. A second explanation is that the plants might start to move resources away from the attacked site, as it has been shown to happen aboveground (Babst et al., 2005; Schwachtje et al., 2006). However, for sugars, this would most probably be accomplished by reducing the sink-strength of roots, and the increase in expression levels of sugar transporters would very likely lead to the opposite effect. In this context, it is tempting to speculate about a possible manipulation of the plant by the specialist *D. virgifera* to obtain more assimilates from the shoots of maize plants. We have shown earlier that the root herbivore strongly upsets the plant's water balance (Erb et al., submitted), and it is possible that *D. virgifera* obtains assimilates by inducing osmotic stress in the roots. This hypothesis is further strengthened by the fact that that several of the induced transporters were unresponsive to mechanical root damage. The activation of amino acid transporters on the other hand might be a strategy of the plant to allocate nitrogen away from the attacked roots into the shoot (Newingham et al., 2007). Three amino acid transporter homologues were induced by *D. virgifera*, possibly contributing to increased xylem loading and translocation into the shoot. Further research is clearly required to assess the impact of root herbivory on carbon and nitrogen partitioning in maize.

In contrast with our earlier results (Erb et al., submitted), we found no strong impact of *D. virgifera* attack or mechanical wounding on shoot transcriptional activity (Figure 3). As mentioned before (chapters 2 and 3), most changes in shoot metabolism upon root herbivory seem to have been related to drought-like conditions and physiological stress, and this experiment confirms this by showing that during an early stage of root infection, there is no large scale reorganization of shoot metabolism (yet). Interestingly however, the root transcriptome showed dramatic changes after shoot attack by *S. littoralis* (Figure 3). The total number of genes showing differential regulation was larger in the roots than the shoots of maize seedlings attacked in the leaves, demonstrating just how strongly the belowground plant parts are implicated in the aboveground response to herbivory. Closer analysis revealed 25 transcripts that are activated in the roots both upon above- and belowground attack including 4 proteinase inhibitors, an O-methyltransferase (Held et al., 1993), a pathogen related protein and several genes with unknown function. These genes deserve further attention, as they represent root-specific transcripts that might function in tolerance and defense against both root and shoot herbivores. The most strongly affected functional group in the roots upon shoot herbivory by *S. littoralis* consisted of genes involved in protein metabolism (Figure 4), many of which were active in protein degradation processes. Most of these genes were exclusively induced or repressed by aboveground herbivory. The ubiquitin/26S-proteasome pathway is increasingly recognized as an important regulatory element of stress reactions in plants (Dreher and Callis, 2007), including ethylene-mediated interactions (McClellan and Chang, 2008), drought (Cho et al., 2008) and oxidative stress (Kurepa et al., 2008). We show here that *S. littoralis* differentially regulates several homologues of genes involved in ubiquitin-dependent degradation in the roots. All these genes were specifically root responsive after *S. littoralis* infestation, suggesting a specific function of root metabolism to act in response to shoot attack. The function and relevance of this type of response upon insect attack is entirely unresolved, and it remains to be investigated if changes in belowground protein metabolism serve to increase the availability of nitrogen for the synthesis of aboveground-defense compounds and compensatory shoot-growth, or if they play an important role in the plant's defense signaling network

The differential regulation of root gene expression upon shoot herbivory can be expected to have a profound impact on the rhizosphere via changes in plant exudate composition, but also on resistance to root-feeding insects. In contrast to (Kaplan et al., 2008) who found that root-feeding nematodes profited from shoot herbivory, we demonstrate here that aboveground attack by *S. littoralis* reduces the growth of *D. virgifera* in the roots (Figure 7). That the effect was observed even when the leaves were attacked only by 4 larvae that did not defoliate the plant until shortly before the end of the experiment implies that the reduced growth was not just due to a lack of assimilates from the shoots, but caused by the induced changes in root metabolism upon shoot herbivory. The dramatic reduction of *D. virgifera* weight gain by almost 70% furthermore suggests a strong insecticidal effect, and the genetic patterns identified in this study should help to elucidate the mechanisms behind this phenomenon.

CONCLUSIONS

Taken together, our results show that roots are specifically involved in the plant's response against both above- and belowground herbivores. The role of belowground plant tissues in plant defenses has until today received little attention, and this study provides a first basis for more detailed investigations.

MATERIAL AND METHODS

Plants and Insects

Maize plants (*Zea mays*, variety Delprim) were grown in plastic pots (diameter, 4cm; depth, 11cm) wrapped in aluminium foil in a phytotron (CLF plant climatics, Percival; 30°C; photoperiod: 16 hours of light and 8 hours of night). To distinguish between local and systemic root responses, plants were sown in a multilayer environment: The lowest 1 cm of the pot was filled with standard potting soil (Ricoter Aussahterde, Aarberg, Switzerland). A fine nylon mesh (0.25mm, stretchable) penetrable by roots, but not by *D. virgifera* larvae) was then placed on the layer, and a second plastic tube (diameter: 3.8 cm: depth: 11cm) was pushed into the outer plastic pot and filled with sand up to 3 cm. The seeds were placed on this sand layer and covered with 3 cm of standard potting soil again. Plants for microarray analysis were sown in the same type of pots filled with sand and covered with 3 cm of standard potting soil. All plants were fertilized twice with standard mineral fertilizer (Mio-Plant Gemüse- und Kräuterdünger, MIGROS Deutschland GmbH, Lörrach) after 4 and 8 days of growth. Plants used for experiments were 9-10 days old, had two fully expanded primary leaves and their roots had reached to bottom of the pots. *S. littoralis* eggs were provided by Syngenta Switzerland and larvae were reared on artificial diet as described before (Turlings et al., 2004). Second instar larvae of *D. virgifera* were obtained from CABI Delémont (Switzerland) and kept on freshly germinated maize seedlings until use.

Herbivore and mechanical treatments

For the restricted feeding experiment (n=6x6), maize seedlings were infested with eight 2nd instar *D. virgifera* larvae by releasing them into the lower compartment of the pots via 4 previously drilled holes that had been sealed with aluminium foil. After applying the larvae, the wholes were covered again with foil to prevent the herbivores from escaping. The roots were harvested at different time points after infestation (0.5h, 1h, 2h, 4h, 24h and 48h, n=6). The roots from the harvested plants were harvested, carefully rinsed with water, excised from the shoots and immediately frozen in liquid nitrogen. For each plant, roots were separated into local tissue (root parts that had been in direct contact with *D. virgifera*) and systemic tissue (roots above the nylon mesh that had not been directly fed upon by herbivores).

For the microarray experiment (n=4), roots of maize seedlings were infested with *D. virgifera* by releasing 12 early 2nd instar larvae into the soil at a depth of approximately 6 cm. Shoots of maize plants were infested with *S. littoralis* by placing 24 early 2nd instar larvae into the whorl of the seedlings. Larvae were kept from escaping by placing a PET tube cages (40 cm height) with circular top openings (diameter: 10 cm) over each plant. One other set of four plants received the same shoot treatment, but no *S. littoralis* larvae, and a third group was left unharmed (control plants). Both above- and belowground herbivores were left to feed on the plants for 18 hours. To test the effect of mechnical damage to the roots, a knife-blade (1cm wide) was inserted to a depth of 8 cm into the soil at a distance of 1 cm from the stem and removed again, this was repeated 4 times over 18 hours on different sides, resulting in a gradual cut and detachment of around 50 percent of the root system in a square around the stem. The plant material was harvested as described above.

The root-herbivore performance experiment (n=25) was carried out by releasing one L2 *D. virgifera* larva in the lower compartment of the two-layer pots (see above) and one in the upper part by directly placing them on the soil. Plants had been infested 48 hours before with 3 (low shoot herbivory) or 12 (high shoot herbivory) L2 *S. littoralis* larvae in the shoots. Aboveground herbivores had been released directly into the whorl of plants and were kept on the plants using PET-cages (see above).

Quantification of root volatiles and phytohormones

Root material from the restricted feeding experiment as described in the previous section was used to measure induction of (E)-β-caryophyllene and phytohormones over time. To determine the relative amounts of induced volatiles, 0.3 g of ground root material was transferred to a glass vial sealed with a teflon-coated septum. Analysis was then carried out using SMPE-GC-MS as described by (Rasmann and Turlings, 2007). For phytohormone analysis, an aliquot of 150mg per sample was transferred to FastPrep tubes and mixed with 1ml ethylacetate containing 200ng of D_6-ABA, D_2-JA, D_4-SA and $^{13}C_6$-JA-Ile as internal standards. The mixture was then homogenized and centrifuged before transferring the supernatant to a 2ml Eppendorf tube. After repeating the extraction procedure and combining the supernatants, the solvent was evaporated in a vacuum concentrator and the pellet redissolved in 70% MeOH. 10ul of each sample was then injected into a HPLC-MS equipped with a ProntoSIL C18 Column. The 1200L LC/MS system (Varian, Palo Alto, CA, USA) was operated a flow rate of 0.1 mL/min. A mobile phase composed of solvent A (0.05% formic acid in water) and solvent B (0.05% formic acid in acetonitrile) was used in gradient mode for separation. The compounds were detected in the ESI negative mode. Molecular ions (M–H) with *m/z* 137, 209, 263 and 322 for SA, JA, ABA and JA-Ile and 141, 213, 269 and 328 for the respective internal standards were fragmented and daughter ions 93, 59 153 and 130 (compounds) and 97, 59, 159 and 136 (internal standards) were recorded for quantification. Collision energy was 15V for SA, 12V for JA, 9V for ABA and 19V for JA-Ile.

Quantification of Zm-TPS23 expression

Total RNA was extracted using Quiagen RNA-Easy extraction kits following the manufacturer's instructions. The quality of the RNA was assessed by photometry and gel electrophoresis. To remove contaminant genomic DNA, all samples were treated with Ambion DNAse following the standard protocol. cDNA was then synthesized using Invitrogen Super-Script III reverse transcriptase according to the manufacturer's instructions. Quantitative reverse transcriptase real time polymerase chain reactions (q-PCR) were then carried out using TPS-23 specific primers (L: tctggatgatgggagtcttctttg; R: gcgttgccttcctctgtgg). The q-PCR mix consisted of 5ul Quantace Sensimix containing Sybr Green I, 3.4ul H_20, 100nmol of each primer (2x0.3ul H_20) and 1ul of cDNA sample. Q-PCR was carried out using 45 cycles with the following temperature curve: 10s 95°C, 20s 60°, 15s 72°. The final melt curve was obtained by ramping from 68 to 98°C in 1°C steps every 5s. To determine primer efficiencies and optimal quantification thresholds, a dilution series of a cDNA mix consisting of 4ul solution from every sample was created. Six 10-fold dilution steps were carried out and the standard curve was included into every q-PCR run. The final obtained Ct values (using the automated threshold determination feature of the Rotor-Gene 6000 software) were corrected for the housekeeping gene GapC and normalized to average control levels to obtain fold changes of treated plants.

Microarray analysis

To profile the transcriptional changes in leaves and roots of herbivore-attacked plants, we used the maize long oligonucleotide 46k array (version 1) provided by the University of Arizona. Frozen plant material was ground in liquid nitrogen into a fine powder and total RNA was extracted using the RNeasy Plant Mini Kit (Quiagen, Hilden). According to the manufactures advice a DNA digestion using Promega DNAse (Promega, WI, USA) was performed on-column. Using the Amino Allyl MessageAmp™II aRNA Ampilifaction Kit (Ambion, TX, UA), amplified RNA was produced and labeled with Cy3/Cy5 Mono-Reactive Dye Pack (GE Healthcare, Freiburg) according to the instruction of the manufactures. The labeled RNA probes were purified (RNeasy MinElute Cleanup, Quiagen, Hilden), mixed and hybridized with the long oligonucleotide microarrays according to the protocols provided by the University of Arizona (http://ag.arizona.edu/microarray/methods.html). To eliminate dye-specific bias, reverse labeling experiments were performed. Therefore, for each treatment, the RNA was labeled with Cy3 and Cy5 in the reverse experiment. Including the reverse labeling experiments, a total of 4 biological replicates and 2 technical replicates were included. After hybridization, the microarray slides were washed and scanned immediately using the DNA microarray scanner (system: G2565AA/BA from Agilent, CA, USA) and images were processed by Feature Extraction software (Agilent,CA, USA).

Quantification of D. virgifera weight gain

To assess the weight gain of *D. virgifera* larvae on *S. littoralis* infested and uninfested plants, the root herbivores were weighed individually before and after 7 days feeding on maize plants, using a microbalance. Absolute weight gain was then calculated. Larvae that could not be retrieved or had died during the experiment were excluded from further analysis.

Statistical analyses

The induction of phytohormones in the roots after *D. virgifera* infestation was tested using two-way ANOVAs with treatment as one factor and location (local vs. systemic) as second variable. An interaction term (TxL) was also included. Normality of the data was verified using the Kolmogorov-Smirnov test and equality of variances was tested using a Leveen test ($p<0.05$). Pairwise comparisons were carried out using Holm-Sidak Post-Hoc Tests. For the microarry data, the R-based open source software Bioconductor (http://www.bioconductor.org) was used to analyze the resulting datafiles. Local background subtraction and Lowess normalization was applied for each microarray slide. To calculate the p-values and $\log_2$ – ratios between arrays the linear models and empirical Bayes methods from the limma package of Bioconductor were used. Genes that showed an average change of more than 2-fold at a p-value of <0.01 were analyzed. Oligos with a different identifier, but with the same matching sequence were pooled. Genes that shared the same annotation, but aligned with different matching sequences were left separate.

ACKNOWLEDGEMENTS

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Chapter 5: A robust partnership: A tritrophic system in maize resists disturbance by a phloem feeder

Matthias Erb, Nicolas Foresti and Ted C.J. Turlings

SUMMARY

The attraction of parasitoids to volatiles of host-infested plants is a well studied phenomenon. However, less is known about how non-host herbivores may affect these tritrophic interactions. As phloem feeders in particular have been shown to interfere with plant defense responses, we investigated how attack by the cicadellid *Euscelidius variegatus* influences volatile releases and parasitoid attraction of healthy maize seedlings and seedlings infested by the larvae of the moth *Spodoptera littoralis*. The results show that the parasitoid *Cotesia marginiventris* strongly prefers volatiles of plants infested with its host *S. littoralis* over plants infested with *E. variegatus*. While terpenoid emission was induced to the same extent by both herbivores, green leaf volatiles and indole were emitted in greater quantities by plants infested with *S. littoralis* caterpillars, possibly allowing the wasps to distinguish host and non-host infested plants. Furthermore, the presence of *E. variegatus* on *S. littoralis* infested plants did not affect the attraction of *C. marginiventris*. In accordance with this, induction of defense marker genes and volatiles upon *S. littoralis* attack was similar for maize plants that were previously infested with *E. variegatus*. These findings imply that induced volatiles convey specific information about the type of herbivore that is attacking a plant, even in a complex environment with multiple herbivores on one plant.

INTRODUCTION

Parasitoids can use herbivore induced plant volatiles (HIPVs) as host-searching cues (Turlings et al., 1990). As HIPVs can be used as a reliable indicator for the presence of suitable oviposition targets (Vet and Dicke, 1992), enhancing the attraction of parasitoids of agricultural pests represents a potential novel biocontrol strategy (Degenhardt et al., 2003; Turlings and Ton, 2006). In nature however, plants are often attacked by non-hosts or by multiple herbivores simultaneously, possibly reducing the reliability and detectability of HIPV signals. While specialist parasitoids can distinguish between plants attacked by hosts and plants attacked by non-hosts using HIPV cues (De Moraes et al., 1998), the impact of non-hosts feeding *on the same plant* as the host might be particularly problematic. Non-hosts can for example change plant resistance (Poelman et al., 2008) and therefore influence feeding behaviour and subsequent HIPV induction of hosts. They can also change the induction of plant defenses on the physiological level via positive or negative cross-talk (Walling, 2000) or induce volatile bouquets that either mask (Schroeder and Hilker, 2008), distort (Shiojiri et al., 2001; Soler et al., 2007) or synergize (de Boer et al., 2008) host-finding cues. Little is known about such plant-mediated interactions between hosts and non-hosts and their effects on tritrophic systems.

Chewing herbivores predominantly activate jasmonic acid (JA) and ethylene (ET) dependent defenses (Odonnell et al., 1996; Farmer et al., 2003), whereas many piercing sucking insects appear to induce defense-pathways commonly associated with pathogens (Walling, 2000). Silverleaf whitefly for example induces salicylic acid-dependent defenses and suppresses JA dependent plant reactions in *Arabidopsis* (Zarate et al., 2007), possibly via classical JA/SA cross-talk (Pieterse and Van Loon, 2004; Beckers and Spoel, 2006). Their evident potential to manipulate plant defenses makes piercing sucking insects important factors to consider in multitrophic systems requiring functional plant defenses. Yet, so far, the impact of such herbivores on chewing HIPVs and parasitoid attraction has remained elusive.

Among the piercing sucking insects, leaf and plant hoppers are particularly important agricultural pests. *Nilparavata lugens* for example is a phloem-feeder of rice causing considerable yield losses (Watanabe and Kitagawa, 2000). In maize, leafhoppers can transmit *Spiroplasma kunkelii* (Alivizatos and Markham, 1986) and thereby indirectly reduce crop quality. While the volatile response of rice to *N. lugens* infestation has been characterized (Lou et al., 2005), nothing is known about the response of maize plants to leafhopper infestation. The importance of these abundant insects for top-down control of lepidopteran pests by parasitoids in maize-agroecosystems is equally unclear.

This prompted us to conduct experiments on the impact of the leafhopper *Euscelidius variegatus* (Hemiptera : Cicadellidae; Kirschbaum 1858) on volatile emission of maize and the effects on the tritrophic interaction involving maize, the lepidopteran pest *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the generalist lepidopteran parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae). *Euscelidius variegatus* is naturally occurring in many maize agroecosystems and can transmit *S. kunkelii* (Alivizatos, 1987), making it a logical candidate for the current study. We compared the HIPVs profiles and expression of defense marker genes of *E. variegates*- and *S. littoralis*-infested plants, as well as those of plants infested by both herbivores. In addition, the attractiveness of plants with these different infestation types was tested for female *C. marginiventris* wasps.

RESULTS

C. marginiventris strongly prefers odours from S. littoralis *infested plants*

To test whether the parasitoid *C. marginiventris* is able to distinguish between volatile blends from plants infested with its host *S. littoralis* and volatiles emitted by plants infested with the non-host *E. variegatus*, we performed an olfactometer assay giving the wasps a choice between uninfested, *S. littoralis*-, and *E. variegatus*-infested plants. Previous volatile collections had shown that *E. variegatus* induced plant volatile emission was strongest after 48 hours of

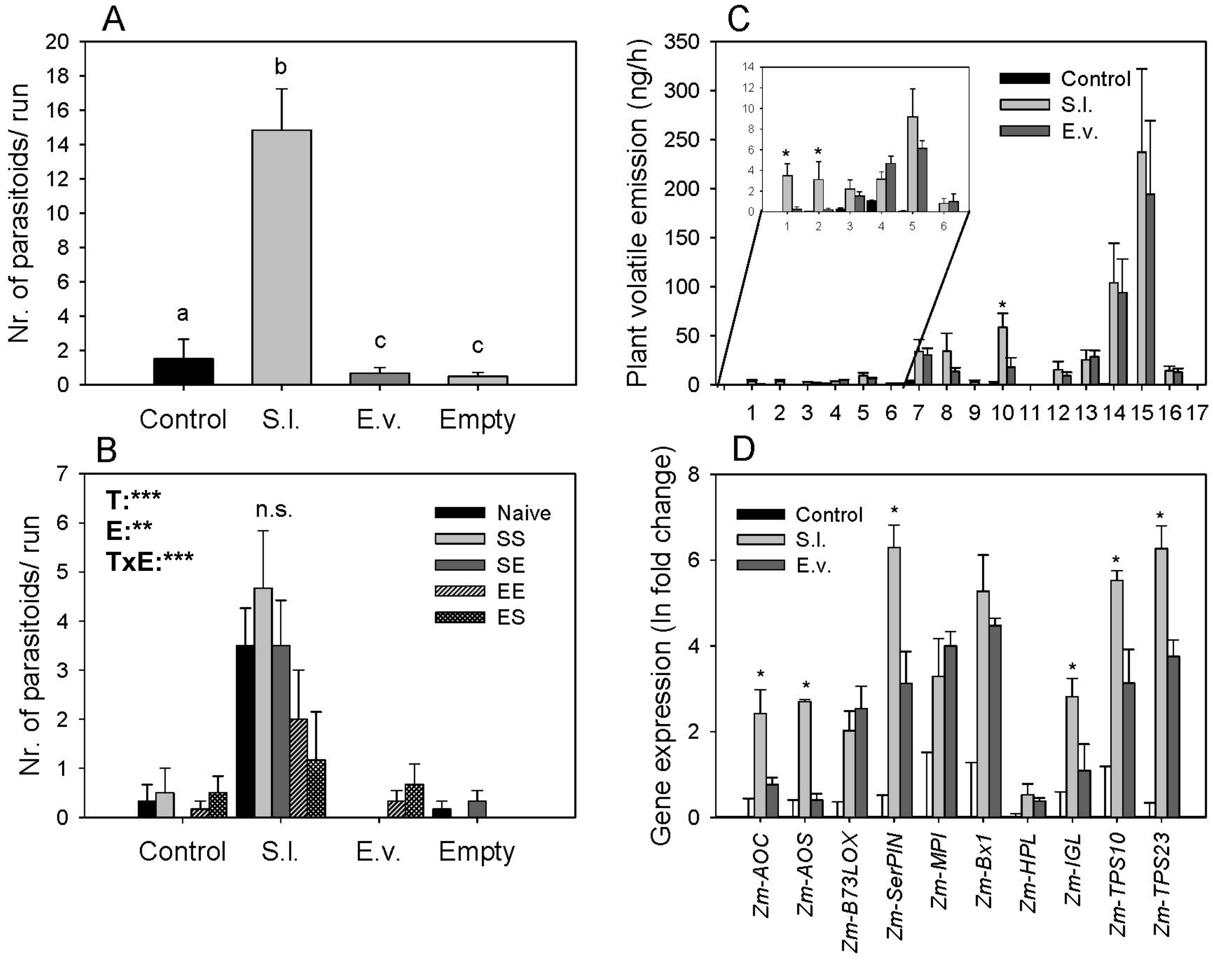

Figure 1: Influence of *E. variegatus* and *S. littoralis* infestationon on parasitoid attraction, volatile emission and defense gene expression. **A**: Average choice of *C. marginiventris* (+SE) in a six-arm olfactometer. Control= Uninfested plant; S.l.= *S. littoralis* infested plant; E.v.= *E. variegatus* infested plant; Empty= Empty arms. Different letters indicate significant differences between treatments ($p<0.05$). **B**: Average choice of *C. marginiventris* parasitoids with different previous host- or non-host experience. Naive= No experience; SS= Host-presence with *S. littoralis* induced plant odours; SE= Host presence with *E. variegatus* induced plant odours. EE= Non-host presence with *E. variegatus* induced plant odours; ES= Non-host presence with *S. littoralis* induced odours. Stars denote significant effects of treatment (T), experience (E) and the interaction (TxE) (*$p<0.05$, **$p<0.01$, ***$p<0.001$). **C**: Average volatile emission (+SE) of herbivore infested maize seedlings. 1=(Z)-3-hexenal; 2=(E)-2-hexenal; 3= (Z)-3-hexen-1-ol; 4= β-myrcene; 5=(Z)-3-hexenyl acetate; 6=(Z)--β-ocimeneN; 7=Linalool; 8= (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT); 9=Phenethyl acetate; 10=Indole; 11= Methyl antranilate; 12=Gerany lactetae; 13=E- β-caryophyllene; 14=(E)-α-bergamotene; 15=E-β-farnesene; 16= β-sesquiphellandreneN. Compounds denoted with N are only tentatively identified. Stars denote significant differences between *S. littoralis* and *E. variegatus* induced plants ($p<0.05$). **D**: Average change in gene expression (+SE) of herbivore infested maize seedlings relative to uninfested control plants. Stars in graphs **C** and **D** denote significant differences between *S. littoralis* and *E. variegatus* induced plants ($p<0.05$).

infestation by adult cicadellids (N. Foresti, unpublished). We therefore chose this time point for the behavioral experiments. Based on preliminary experiments (N. Foresti, unpublished), we chose to test plants infested with 40 *E. variegatus* adults against seedlings infested with 3 L2 *S. littoralis* larvae, which resulted in comparable overall induction. Irrespective of their previous experience, *C. marginiventris* exhibited a strong preference for odors from host-infested plants (Figure 1A and 1B). *E. variegatus*-infested plants were even less attractive than control plants. Interestingly, previous contact with *E. variegatus* adults over a period of 2 minutes drastically reduced the overall responsiveness of the wasps and the choice for the odor of host-infested plants by almost 50% (Figure 1B).

E. variegatus *induces all major classes of maize HIPVs*

The volatile profiles analyzed from the olfactometer experiment described above show that *E. variegatus*-infested plants emitted similar amounts of terpenoids as *S. littoralis*-infested seedlings (Figure 1C), indicating that the overall systemic induction was indeed comparable. Unlike *S. littoralis*, *E. variegatus* did not induce the green leaf volatiles (GLVs) (Z)-3-hexanal and (E)-2 hexenal. Emission of the other two GLVs, (Z)-3-hexen-1-ol and (E)-2-Hexenylacetate was not significantly different between the two herbivore treatments. Of the other compounds, only indole was emitted in lesser amounts by *E. variegatus*-infested plants compared to *S. littoralis*-infested plants (Figure 1C).

C. marginiventris *is not repelled* by E. variegatus-*infested plants*

Because *C. marginiventris* seemed to prefer control plants over *E. variegatus*-infested plants in the first olfactometer assay, we tested if the *E. variegatus* infestation had a repellent effect on the parasitoid. When offered an uninfested plant and a plant hopper infested plant only, *C. marginiventris* showed a preference for odors from *E. variegatus*-infested plants (Figure 2A), irrespective of previous experience (Figure 2B). The analyzed volatile profiles confirmed that *E. variegatus* induces the same 18 compounds commonly found in *S. littoralis*-infested plants, apart from the two GLVs (Z)-3-hexanal and (E)-2-hexenal (Figure 2C).

The attraction of C. marginiventris *to* S. littoralis *infested plants is not affected by* E. variegatus

To assess whether *E. variegatus* influenced the plants' response to *S. littoralis* and the subsequent attraction of *C. marginiventris*, we simultaneously infested plants with 40 *E. variegatus* adults (48 hours prior to experiment) and 9 L2 *S. littoralis* larvae (12 hours prior to experiment) and compared their reaction to plants infested with *S. littoralis* only. To exclude possible effects of differenial *S. littoralis* feeding activity, we weighed the larvae before and after the experiment. The larvae gained similar amounts of weight irrespective of the presence of *E. variegatus* (Figure 4). Overall, *C. marginiventris* was equally attracted to double infested plants compared to plants infested with *S. littoralis* only (Figure 3A). Parasitoids with previous positive experience, while perceiving the odor of double infested plants, shifted their preference in favor of this odor (Figure 3B). The volatile profiles induced by the two treatments were qualitatively and quantitatively similar (Figure 3C).

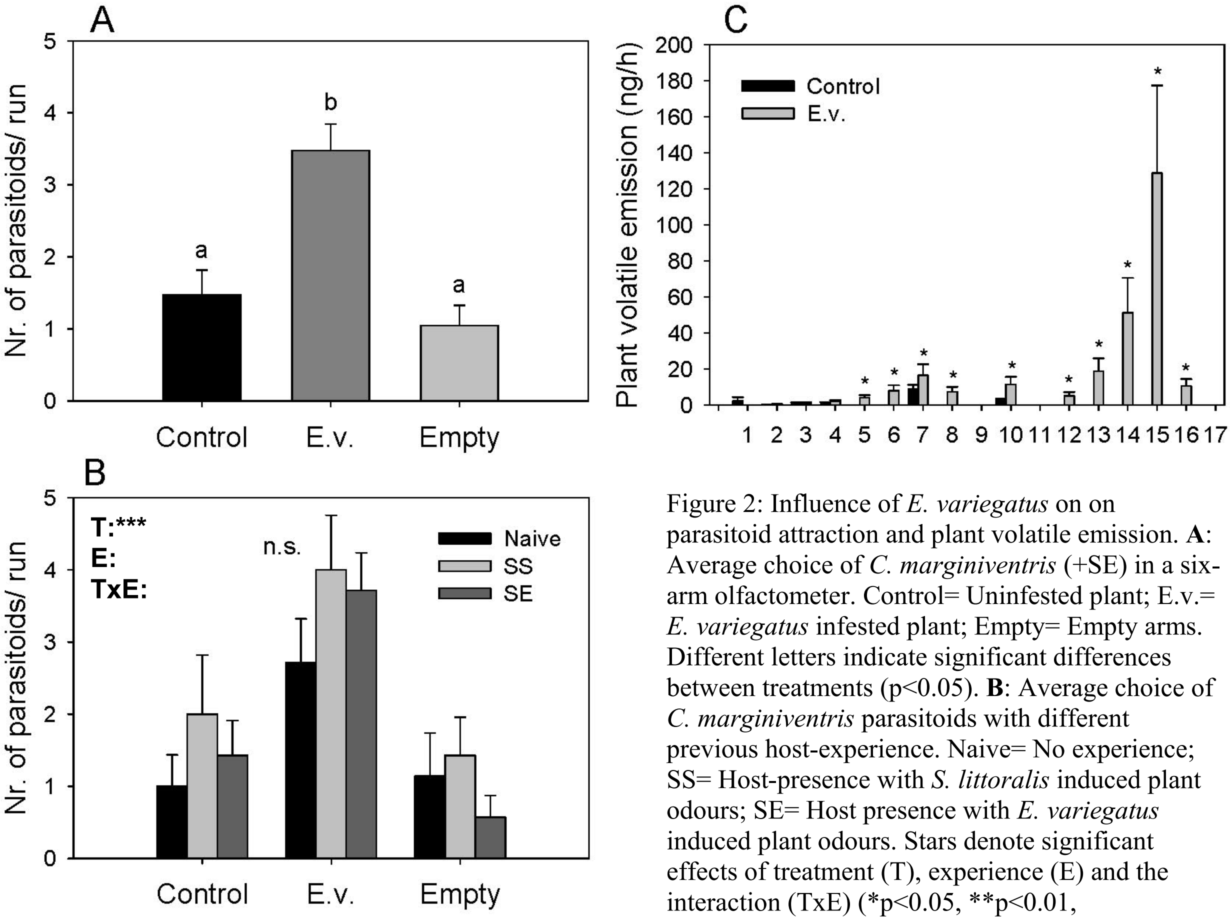

Figure 2: Influence of *E. variegatus* on on parasitoid attraction and plant volatile emission. **A**: Average choice of *C. marginiventris* (+SE) in a six-arm olfactometer. Control= Uninfested plant; E.v.= *E. variegatus* infested plant; Empty= Empty arms. Different letters indicate significant differences between treatments ($p<0.05$). **B**: Average choice of *C. marginiventris* parasitoids with different previous host-experience. Naive= No experience; SS= Host-presence with *S. littoralis* induced plant odours; SE= Host presence with *E. variegatus* induced plant odours. Stars denote significant effects of treatment (T), experience (E) and the interaction (TxE) (*$p<0.05$, **$p<0.01$, ***$p<0.001$). **C**: Average volatile emission (+SE) of *E. variegatus* infested maize seedlings. For compound descriptions, see legend in Figure 1. Stars denote significant differences between *E. variegatus* induced and uninfested plants ($p<0.05$).

E. variegatus induces genes involved in JA-biosynthesis, direct defenses and volatile production

To gain insight into the molecular basis of the observed volatile responses, we profiled gene expression patterns of plants infested by *E. variegatus*, *S. littoralis* and both herbivores simultaneously. We used *Zm-B73LOX* (Ton *et al.*, 2007), *Zm-AOC* and *Zm-AOS* (Gao et al., 2008) as markers for the induction of the octadecanoid pathway, *Zm-SerPIN* (Ton *et al.*, 2007), *Zm-MPI* (Cordero *et al.,* 2005) and *Zm-Bx1* (Frey et al., 1997) as markers for the induction of direct defenses and *Zm-HPL* (Farag et al., 2005), *Zm-TPS10* (Schnee et al., 2006), *Zm-TPS23* (Kollner et al., 2008) and *Zm-IGL* (Frey et al., 2000) as markers for volatile induction. Octadecanoid markers were induced by both *E. variegatus* and *S. littoralis*, with *Zm-AOS*

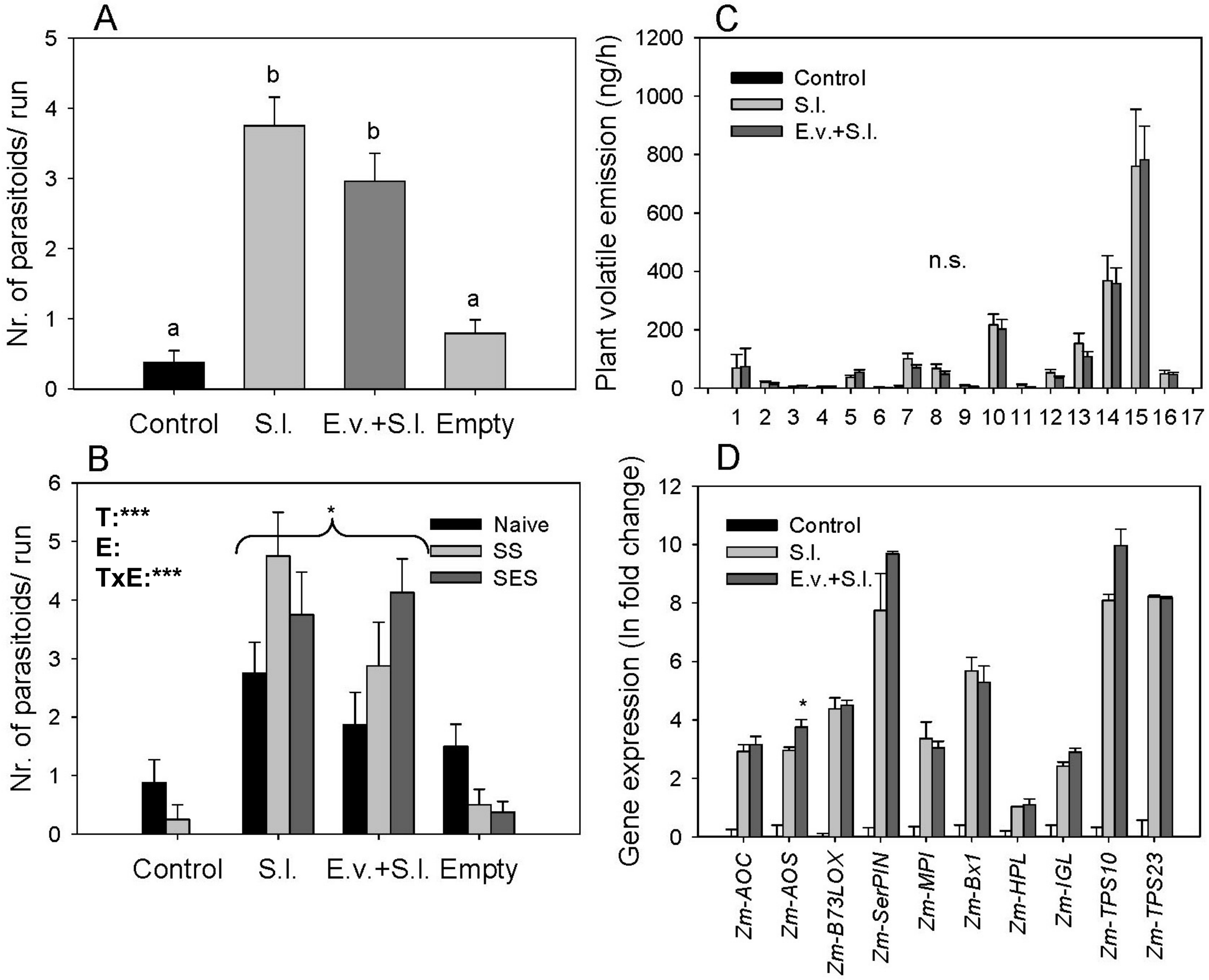

Figure 3: Influence of *E. variegatus* on *S. littoralis* induced parasitoid attraction, volatile emission and defense gene expression. **A**: Average choice of *C. marginiventris* (+SE) in a six-arm olfactometer. Control= Uninfested plant; S.l.= *S. littoralis* infested plant; E.v.+S.l.= *E. variegatus* and *S. littoralis* infested plant; Empty= Empty arms. Different letters indicate significant differences between treatments ($p<0.05$). **B**: Average choice of *C. marginiventris* parasitoids with different previous host-experience. Naive= No experience; SS= Host-presence with *S. littoralis* induced plant odours; SES= Host presence with *E. variegatus* and *S. littoralis* induced plant odours. The star denotes a significant interaction between treatment and experience ($p<0.05$). Stars denote significant effects of treatment (T), experience (E) and the interaction (TxE) (*$p<0.05$, **$p<0.01$, ***$p<0.001$). **C**: Average volatile emission (+SE) of herbivore infested maize seedlings. For compound descriptions, see legend in Figure 1. **D**: Average change in gene expression (+SE) of herbivore infested maize seedlings relative to uninfested control plants. Stars denote significant differences between *S. littoralis* and *E. variegatus* and *S. littoralis* induced plants ($p<0.05$).

showing a reaction to *S. littoralis* only and *Zm-AOC* being more strongly induced by the caterpillar (Figure 1D). The same was true for *Zm-SerPIN, Zm-MPI* and *Zm-BX1*, with *Zm-SerPIN* showing a stronger reaction to *S. littoralis*. *E. variegatus* also induced *Zm-IGL*, *Zm-TPS10* and *Zm-TPS23,* with *S. littoralis* again evoking the stronger response. *Zm-HPL* was only significantly induced by *S. littoralis*, but there was no significant difference compared to the transcriptional activity upon *E. variegatus* attack (Figure 1D). Plants attacked simultaneously by both *E. variegatus* and *S. littoralis* showed similar levels of expression for all genes under investigation (Figure 3D), with the exception of *Zm-AOS*, which showed a more pronounced response upon double attack compared to *S. littoralis* only.

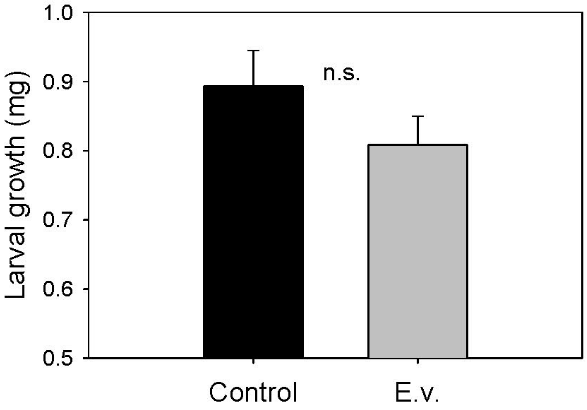

Figure 4: Average *S. littoralis* growth (+SE) over 18 hours on uninfested (Control) and *E. variegatus* infested (E.v.) plants.

DISCUSSION

Differences in volatile profiles and defenses induced by S. littoralis and E. variegatus

Surprisingly, *E. variegatus* induced volatile profiles that resembled the ones induced by *S. littoralis* in many aspects. Both herbivores induced a variety of mono- homo- and sesquiterpenes, the shikimik acid pathway derived indole and volatile metabolites from the oxlipin cascade, (Z)-3-hexen-1-ol and (E)-2-hexenylacetate (Figure 1C and 2C). This suggests that the plant's response to the two herbivores is not fundamentally different, at least not for the measured HIPVs. Sesquiterpene emissions in maize are strongly correlated with induction of JA, resulting from activation of the octadecanoid pathway (Schmelz et al., 2003). Similarly, *Zm-IGL*, the gene responsible for indole formation, is inducible by methyl jasmonate (Frey et al., 2004). GLVs (green leaf volatiles) finally are derived from linoleic acid, which is by itself a precursor for JA production. The notion that both herbivores induce the octadecanoid pathway is further strengthened by the transcriptional data showing induction of genes involved in JA biosynthesis (*Zm-B73LOX* and *Zm-AOC,* Figure 1D). Taken together, we suggest that *E. variegatus* does induce JA-dependent volatile production rather than suppress this type of defense, as has been shown for other piercing-sucking insects (Zarate et al., 2007). The fact that 40 *E. variegatus* adults were needed to achieve emission of a similar quantity of volatiles as 3 L2 *S. littoralis* larvae implies that the induction of defenses is much weaker on a per individual basis. The transcriptional profiling supports this by showing that *E. variegatus* induction of some defense-related genes (*Zm-SerPIN*, *Zm-AOS*) was weaker than that of *S. littoralis*, even when many more leafhoppers were present on the plant (Figure 1D). This effect can be attributed to the lower amount of cell damage inflicted by the stylets of *E. variegatus* compared to the brute-force chewing mouthparts of *S. littoralis*. This is supported by the fact that the upstream GLVs (Z)-3-hexanal and (E)-2-hexenal were not detected in *E. variegatus*-infested plants. These two volatiles are predominantly released locally upon caterpillar feeding and mechanical damage. That LOX- HPL-, ADH- and CHAT-like enzymes (D'Auria et al., 2007) nevertheless exhibited increased activity upon *E. variegatus* attack is is evident from the release of (Z)-3-hexen-1-ol and (E)-2-hexenyl acetate upon hopper attack. Apart from direct damage, volatile release in maize has been shown to be triggered by herbivore-derived elicitors like volicitin (Alborn et al., 1997). It is not known if *E. variegatus* saliva also contains these or other volatile elicitors, but it is tempting to speculate that the stronger volatile burst observed after *S. littoralis* attack is partially due to elicitor activity that is reduced or absent in *E. variegatus*-attacked plants.

The fact that *E. variegatus* did not reduce *S. littoralis* induced volatile emissions (Figure 3C) and did not reduce the induced resistance the caterpillars encountered in the leaves is further evidence for the absence of negative cross-talk between defense pathways in our study system. Obviously, the response to caterpillar attack was not significantly changed by the presence of *E. variegatus*. This is confirmed by our transcriptional data, showing similar induction of most defense-related genes upon *S. littoralis* attack irrespective of the presence of *E. variegatus* (Figure 3D). Only *Zm-AOS* was even more induced in the double treatment than by caterpillar attack. *S. littoralis* showed a weak trend for reduced growth on *E. variegatus*-infested plants (Figure 4), which was probably the result of a slight activation of effective plant defenses by the leafhopper, as indicated by our gene expression profiles (Figure 1C). However, this growth reduction was insignificant, and the volatiles induced by both species simultaneously showed no clear synergistic effect (Figure 3C), possibly because the volatile burst upon infestation with 9 *S. littoralis* larvae was approximately 4 times stronger than the average induction by *E. variegatus* (Figures 2C and 3C), making the hopper's contribution to overall HIPV emissions negligible in this experiment.

Specific attraction of C. marginiventris

C. marginiventris showed a clear preference for *S. littoralis*-induced blend of volatiles (Figure 1A), suggesting that the parasitoid can readily distinguish between the two odors. While indole has been shown to have no positive effect on the attractiveness of maize plants (D'Alessandro et al., 2006), we have shown previously that naïve *C. marginiventris* individuals are strongly attracted to freshly damaged plants, and experienced wasps prefer a mix between fresh and older damage (Hoballah and Turlings, 2005). Attraction thus seems to depend strongly on volatiles emitted by freshly wounded leaves. These types of volatiles were clearly more prominent in *S. littoralis*-infested plants, which exhibited physical tissue damage and consequently released more (Z)-3-hexanal and (E)-2-hexenal (Figure 1C). We thus provide further evidence for the notion that the key attractants for parasitoids of lepidopteran pests are likely to be found in the blend directly released from wounded sites. As the attractiveness of these compounds is not reduced by positive experience with alternative volatile blends (Figure 1B), this seems to be ea strong innate preference of *C. marginiventris*. The results also indicate that this strategy could be adaptive, as it enables the parasitoid to distinguish between plants attacked by chewing herbivores (potential hosts) and insects with other feeding modes (non-hosts). The observation that the choice fidelity of *C. marginiventris* slightly decreased after having been in contact with *E. variegatus* adults for 2 minutes (Figure 1B) is noteworthy in this context, as this could indicate that the parasitoids employ a strategy to reduce their responses to no-rewarding odors.

However, the fact that *C. marginiventris* was attracted to *E. variegatus* infested plants when no hosts were present (Figure 2A) demonstrates that there is no repellent effect of *E. variegatus* induced volatiles. Several reasons could be responsible for the behavior of the parasitoid. First, it is possible that the key attractants responsible for the attractiveness of the *S. littoralis* induced blend were also emitted by *E. variegatus* infested plants, albeit in much lower quantities, prompting the insect to respond to them only in the absence of stronger cues. Second, it is possible that *C. marginiventris* simply chooses to follow “the most promising trail” present in an environment by using secondary cues in the absence of primary attractants. This behavioral plasticity could be especially important for generalist parasitoids, as they have to be able to exploit a broad range of host-induced cues.

The attractiveness of *S. littoralis*-infested plants was not significantly reduced when *E. variegatus* was present on the same plants (Figure 3A), indicating a robust host-finding behavior of the parasitoid. This contrasts with other studies documenting hard-to-explain effects of multiple herbivory on tritrophic systems. Rodriguez-Sanoa et al. (2005) found that *C. marginiventris* was attracted to tomato plants infested with both the aphid *Macrosiphum euphorbiae* and the caterpillar *S. exigua*, while plants infested with *S. exigua* only were not attractive at all. De Boer et al. (2008) again showed a positive effect of *Spodoptera exigua* on the attraction of predatory spider mites to *Tetranychus urticae* induced plants. This illustrates that the behaviour of natural enemies depends specifically on the plant-herbivore combination under investigation. Unfortunately, none of the studies discussed here took into account the learning capacity of parasitoids and predators, which might be an important factor increasing their foraging success (Turlings and Wackers, 2004).

Interestingly, in our system, the parasitoid was able to discriminate double- from single-infested plants after oviposition-experience in presence of the respective blends (Figure 3B). Rasmann and Turlings (2007) found something similar when they tested the attraction of *C. marginiventris* to maize plants that were simultaneously attacked by *S. littoralis* and a belowground herbivore. In both cases the learned behavior cannot be explained by the measurable volatile profiles, as they did not differ between the two treatments (Figure 3C). This suggests that minor compounds influenced by the presence of the additional herbivore can be learned and affect the wasps responses. As concluded in earlier studies (D'Alessandro and Turlings, 2005; Rasmann and Turlings, 2007), future research will have to focus on these “hidden signals” in order to unravel the functional complexity of herbivore-induced volatiles in detail.

CONLCUSIONS

Our data show that plant-mediated signaling in the tritrophic system comprising maize, its lepidopteran pest *S. littoralis*, and the associated parasitoid *C. marginiventris* was not disrupted by a non-host phloem feeder. This demonstrates that the interaction is robust and that the attraction of natural enemies of herbivores to plant signals also functions when plants are attacked by multiple antagonists. Furthermore, our investigations provide the basis for further research on cicadellid-induced volatiles and their possible function as host-location cues for their specialized parasitoids.

MATERIAL AND METHODS

Insects and Insect Treatments

The cicadellid *E. variegatus* was reared on 3-5 week old barley plants in plastic Bugdorm cages (Megaview, Taiwan) under constant conditions (25°C, 16:8 h L/D). The caterpillar *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the solitary endoparasitoid *C. marginiventris* (Cresson) (Hymenoptera: Braconidae) were reared as previously described (Turlings et al., 2004). Adult parasitoids were kept in plastic cages at a male/female ratio of approximately 1:2 and were provided with moist cotton wool and honey as food source. Cages were kept in incubators incubators (25°C; 16:8 h L/D) and transferred to the laboratory 30 min before the experiments. Two to four day old naive and experienced females were tested. For details on the training setup see (D'Alessandro et al., 2006). Naïve wasps did not have any previous oviposition experience and had never before been in contact with plant odours. To experience wasps they were either brought into contact with their host (20 L2 *S. littoralis* larvae) until they had oviposited 3-5 times, or with the non-host (10 adult *E. variegatus*) during 2 minutes, while they were exposed to the odor from either *E. variegatus* or *S. littoralis* infested plants. This resulted in 4 different experience groups. The different groups of wasps were kept separately in small plastic boxes and released into the olfactometer 1–3 hr after their experience.

Plants and odor sources

Maize (*Z. mays*, var. Delprim) was sown in plastic pots (10 cm high, 4 cm diam) with commercial potting soil (Ricoter Aussaaterde, Aarberg, Switzerland) and placed in a climate chamber (23°C, 60% r.h., 16:8 h L/D, 50’000 lm/m2). Plants used for the experiments were 10–12 d old and had 2-3 fully developed leaves. The evening before the experiments, plants were transferred to glass vessels (Turlings et al., 2004) and infested with 3 second instar *S. littoralis* (released in the whorl of the youngest leaf) or 30 adult *E. variegatus* (released freely into the vessel). For the experiment involving double-infestation, maize seedlings were inoculated with 30 adult *E. variegatus* 48 hours before the olfactometer assay. Nine L2 *S. littoralis* larvae were added 24 hours later. After infestation, the vessels with plants were attached to the air supply of The olfactometer and kept under laboratory conditions (25°C, 50% r.h., 16:8 h L/D, humidified airflow 0.3l/min, 8000 lm/m2). Olfactometer experiments were done the following day, between 10 A.M. and 4 P.M. The groups of *S. littoralis* larvae assigned to each plant were weighed before and after the experiment with a microbalance.

Olfactometer bioassays

All odor sources were tested for attractiveness to parasitoids in a six-arm olfactometer as described in (Turlings et al., 2004). Cleaned and humidified air entered each odor source vessel at 1.1 l/min (adjusted by a manifold with four flowmeters; Analytical Research System, Gainesville, FL, USA) via Teflon tubing and carried the volatiles through to the olfactometer compartment. Half of the air (0.6 l/min/olfactometer arm) was pulled out via a volatile collection trap that was attached to the system above the odor source vessels (see "Collection and analyses of HIPVs"). Incoming and outgoing air were balanced by a Tygon tube connected to a vacuum pump via another flow meter and a pressure gauge. Empty arms were connected to empty vessels and carried clean, humidified air only. The position of the odour sources was randomly altered between experimental runs to avoid position-bias. Wasps were released in groups of six into the central part of the olfactometer, alternating between groups of naive and experienced wasps, and after 30 min the wasps that had entered an arm of the olfactometer were counted and removed. Wasps that did not enter an arm after this time were removed from the central part of the olfactometer and considered as "no choice." Experiments were replicated on 6-8 different days.

Collection and analysis of HIPVs

HIPVs of each odor source were collected during the olfactometer bioassay on a Super-Q trap (25 mg, 80–100 mesh; Alltech Associates, Deerfield, IL, USA, described by Heath and Manukian, 1992). Each trap was attached horizontally to the elbow of an odor source vessel and connected via Tygon tubing to a flowmeter (Analytical Research System) and a vacuum pump. Air carrying the volatiles was pulled through each trap at a rate of 0.6 l/min during each behavioral bioassay. Afterwards, the traps were extracted with 150 µl dichloromethane (Suprasolv; Merck, Dietikon, Switzerland), and 200 ng of n-octane and n-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µl dichloromethane were added to the samples as internal standards. All extracts were stored at -76°C until analyses. Traps were washed with 3 ml dichloromethane before they were reused for a next collection. HIPVs of the experiments were identified with a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectrometer that operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33–280 amu). A 2-µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness; Alltech Associates). Helium at constant flow (0.9 ml/min) was used as carrier gas. After injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min followed by a postrun of 5 min at 250°C. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 02 library, by comparison of their spectra and retention times with those of authentic standards, and by comparison of retention times with those in previous analyses (D'Alessandro and Turlings, 2005). Compounds that were not identified by comparing retention times and spectra with those of pure standards are indicated in Fig. 2 with superscript N, and their identity should be considered tentative. Precise quantification of the identified volatiles was carried out using an Agilent 6850 gas chromatograph with a flame ionization detector. A 3-µl aliquot of each sample was injected in pulsed splitless mode onto the same type of column as above at a constant pressure of 18.55 psi. The column temperature ramping was as described above.

Analysis of gene expression

The leaves from plants used in the olfactometer experiments were harvested and flash-frozen in liquid nitrogen. Based on the volatile profiles, 3 representative samples were chosen and ground to a fine power under liquid nitrogen. Total RNA was then extracted using Quiagen RNA-Easy extraction kits following the manufacturer's instructions. The quality of the RNA was assessed by photometry and gel electrophoresis. To remove contaminant genomic DNA, all samples were treated with Ambion DNAse following the standard protocol. cDNA was then synthesized using Invitrogen Super-Script III reverse transcriptase according to the manufacturer's instructions. Quantitative reverse transcriptase real time polymerase chain reactions (q-PCR) were then carried out using the following gene-specific primers: *Zm-AOS* L:acctgttcacgggcacctac; R:cgaggagcgaggagaagttg. *Zm-AOC* L: ccccttcaccaacaaggtgt; R: accgagatgtggccgtagtc. *Zm-B73LOX* L: gcgacaccatgaccatcaac; R: gctcggtgaagttccagctc. *Zm-SerPIN* L: gacggaggaggaaggaggag; R: acctgatgcactgcttgcac. *Zm-MPI* L: atgagctccacggagtgc; R: acctgatgcactgcttgcac. *Zm-BX1* L: cccgagcacgtaaagcagat; R: cttcatgcccctggcatact. *Zm-HPL* L: acttcggcttcaccatcctg; R: gtagtagcccggccagatga; Zm-IGL L: gcctcatagttcccgacctc; R: gaatcctcgtgaagctcgtg. *Zm-TPS10* L: tgtgtccacggtccaatgtt; R: gtccgctgtccttgcaaaat. *Zm-TPS23* L: tctggatgatgggagtcttctttg; R: gcgttgccttcctctgtgg. The q-PCR mix consisted of 5ul Quantace Sensimix containing Sybr Green I, 3.4ul H20, 100nmol of each primer (2x0.3ul H20) and 1ul of cDNA sample. Q-PCR was carried out using 45 cycles with the following temperature curve: 10s 95°C, 20s 60°, 15s 72°. The final melt curve was obtained by ramping from 68 to 98°C in 1°C steps every 5s. To determine primer efficiencies and optimal quantification thresholds, a dilution series of a cDNA mix consisting of 4ul solution from every sample was created. Six 10-fold dilution steps were carried out and the standard curve was included into every q-PCR run. The final obtained Ct values (using the automated threshold determination feature of the Rotor-Gene

6000 software) were corrected for the housekeeping gene GapC 1 and normalized to control levels to obtain average fold changes of treated plants.

Statistical analysis

The functional relationship between parasitoids' behavioral responses and the different odor sources offered in the six-arm olfactometer was examined with a log-linear model (a generalized linear model, GLM). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of wasps within the olfactometer (Turlings et al., 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (R: A language and Environment for Statistical Computing, Version 1.9.1, Vienna, Austria, 2006, ISBN 3-900051-07-0 http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals. The amounts of volatiles and gene expression data were analyzed by using ANOVAs followed by Holm-Sidak post-hoc tests. Datasets that were not normally distributed were transformed prior to analysis. Were transformation did not resolve non-normality or unequal variances, ANOVA's on ranks followed by Dunn's or Student-Newman-Keul's post-hoc tests were used. Comparisons involving two treatments were made using Student's T-tests.

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Discussion, outlook and conclusions

Above-belowground interactions in the context of community ecology

Plant-mediated interactions between above- and belowground herbivores can be integrated into several theoretical and empirical frameworks of different biological disciplines. Community ecology for instance has a long standing tradition in examining interspecific interactions between phytophagous insects (Denno et al., 1995). In this context, herbivores sharing the same host plant are viewed as resource competitors. While the importance of resource-based competition for insect community structure has been challenged on several occasions (Lawton and Strong, 1981; Jermy, 1985), extensive meta-analysis showed that 76% of the examined pair wise interactions indeed exhibited interspecific competition, i.e. a negative influence of one herbivore on the other (Denno et al., 1995). Interestingly, in a novel synthesis, Kaplan and Denno (Kaplan and Denno, 2007) suggest that a majority of the competitive interactions are plant quality mediated rather than being the result of direct interference or the removal of plant biomass. Changes in plant physiology are thus increasingly recognized as important factors shaping indirect competition between insect herbivores. Importantly, this also includes temporally or spatially separated insects that do not experience direct contact and will not directly compete for plant tissue (Kaitaniemi et al., 1998; Bezemer et al., 2003; Poelman et al., 2008). Interactions between root and shoot feeding herbivores fall into this category, and our results provide compelling evidence for the importance of plant quality traits for indirect interactions between herbivorous insects: *D. virgifera*, by attacking the roots, changes the quality of maize leaves, both in terms of water household and secondary metabolism (chapter 2 and 3), while in turn, leaf-attack by *S. littoralis* profoundly influences the quality of roots in turn (chapter 4). Both herbivores thereby affect each other negatively. The influence of the leaf-hopper *E. variegatus* on *S. littoralis* on the other hand (chapter 5) is an example of a neutral spatially and temporally confounded interaction, adding to the emerging view that it is not necessarily the direct contact and overlapping resource use *per se* that enforces competition.

While traditional theory about interspecific competition predicts a reciprocal struggle for resources, there is a clear trend for asymmetric competition between insect herbivores sharing the same host-plant (Kaplan and Denno, 2007). In other words, the effect of one competitor on the other often is significantly stronger than *vice versa*. While the inferior competitor has often been shown to still have a minor influence on the superior species (Kaplan and Denno, 2007), it is the latter that is the major driver of the interaction and a potentially important factor shaping the herbivore community surrounding the host plant. The results presented in chapters 2, 3, and 4 enable us to gain first insights into the symmetry of the host-mediated competiton between *D. virgifera* and *S. littoralis*. After 7 days of growth on plants attacked by four *S. littoralis*, *D.*

virgifera larvae feeding on the upper root system had gained 70% less weight than larvae feeding on control plants. Conversely, *S. littoralis* larvae feeding over the same period on plants that had been attacked by six *D. virgifera* larvae lost less than 10% of their weight (Figure 1). From these results, it seems that the shoot-root effect on plant quality is stronger than the root-shoot effect after herbivore attack, thus making *S. littoralis* the superior competitor on maize. This is also confirmed by several experiments (Chaper 3 and 4), clearly demonstrating that i) the early impact of shoot herbivory on root metabolism is much more dramatic than *vice versa*, and ii) that the increased resistance in the shoot only appears after prolonged root infestation and is most pronounced under severe stress. In accordance with this, the impact of shoot herbivory on root volatile release seems to be stronger than the effect *vice versa* in the same system (Rasmann and Turlings, 2007). Taken together, our study provides the ideal basis for further research on plant-mediated, indirect competition between herbivorous insects in maize. It could for example be investigated in detail to what extent belowground herbivory drives aboveground assemblage of phytophagous insects (chapter 3), or if, as we suggest here, the opposite effect is dominant. Answering this question will require laboratory and field experiments were the performance of both herbivores is determined simultaneously and in a density-dependent manner. Furthermore, the temporal effects will have to be assessed and correlated with field observations to determine if it is an advantage to arrive first on the host plant, or if the outcome of the competition is independent from this factor (Viswanathan et al., 2007).

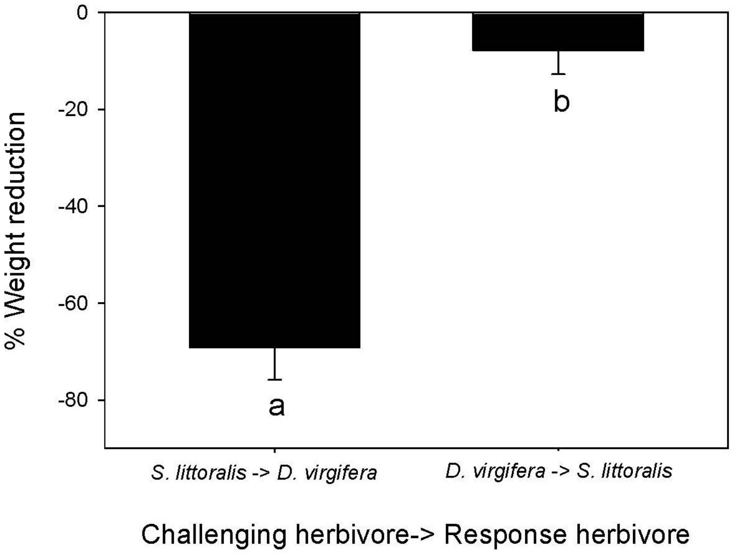

Figure 1: Average relative changes (+SE) in weight gain over 7 days of *D. virgifera* feeding on *S. littoralis* infested plants (left) and *S. littoralis* feeding on *D. virgifera* infested plants (right). Different letters indicate statistically significant differences ($p<0.05$). For experimental details, see Chapters 3 and 4.

Physiology and adaptive value of above-belowground interactions

Apart from community ecology, plant physiologists have established an alternative theoretical framework to explain plant-mediated interactions between herbivores, the central hypothesis being that plants integrate and optimize their defense response after attack (Beckers and Spoel, 2006) while herbivores in turn try to highjack these fine-tuning mechanisms for their own benefit (Walling, 2000). Depending on the attacker combination, this can lead to either a positive outcome, where one herbivore benefits from the suppression of plant defenses by the other (Rodriguez-Saona et al., 2005; Zarate et al., 2007), or to negative results, where the plant's anti-herbivore response negatively affects a second attacker (Voelckel and Baldwin, 2004). The main difference to the competition-framework lies in the fact that plants are not seen as mere "resource" with a certain "quality", but as active players with a functional, specific immune system (Jones and Dangl, 2006). This view has important consequences for the assessment of plant-mediated interactions between herbivores, as it leads to the question if a plant shows an adaptive reaction, for example by anticipating future attack upon an initial herbivore encounter (Voelckel and Baldwin, 2004; Frost et al., 2008), or by prioritizing resource allocation to protect its most vital tissues (Zangerl, 1986).

Initially, we were predicting a decision-making problem by the plant caused by resource trade-offs between root- and leaf defenses. This hypothesis was inspired by the fact that root and shoot responses of maize involve the synthesis of the same classes of defensive secondary metabolites including terpenoids (Turlings and Tumlinson, 1992; Rasmann et al., 2005) and nitrogen-containing, aromatic compounds (Frey et al., 1997; Schmälzlin, 2003). The results from chapters 2 and 4 however clearly show that there are no trade-off effects present that have an impact on plant resistance, as attack in the roots increases shoot resistance and *vice versa*. While the plant's shoot reaction upon root herbivory likely to be adaptive for the plant (chapter 3), the increased resistance *per se* is unlikely to be an optimal strategy of the plant defense system, as it is mainly determined by the severity of the stress from which the plant suffers (chapter 3). While it is possible that *D. virgifera* actively increases shoot stress for it's own benefit (chapters 2 and 4), it is equally unlikely that the observed resistance in the shoots is a manipulation by the root herbivore to fight possible aboveground competitors, as i) this could be achieved much more efficiently than via indirect changes in the plants water status and ii) would probably require to be more effective to have a positive effect on root-herbivore fitness. Our current model of root-herbivore induced shoot resistance based on plant physiological data (Figure 2) thus suggests that root-herbivore induced shoot resistance is a non-adaptive phenomenon resulting from indirectly related physiological responses of the host plant to *D. virgifera* attack. The consequences nevertheless remain important for community ecology and agroecology, but the potential to exploit this phenomenon in a future crop protection strategy, unlike other root-induced shoot resistance phenomena like pseudomonas-induced ISR (Kloepper et al., 2004), will most probably remain limited, simply because there is a clear trade-off with important plant-fitness parameters.

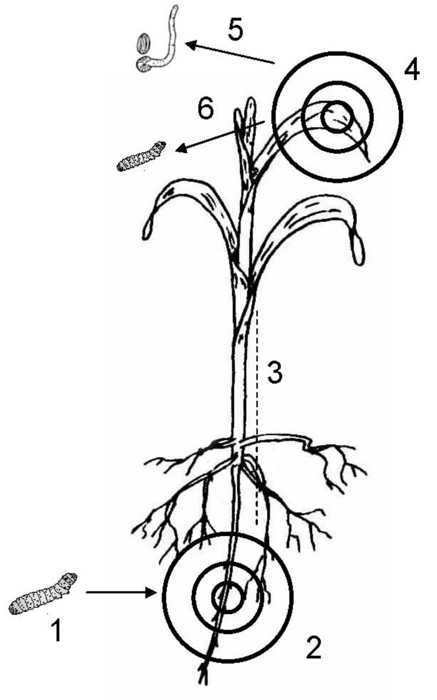

Figure 2: General model of root herbivore induced shoot resistance. **1**. *D. virgifera* attacks the root system, often at its most vulnerable point (Chapters 2 and 3). **2**. The roots respond with extensive transcriptional reprogramming involving the activation of OPDA, JA and ABA (Chapter2) and of multiple transport-related genes (Chapter4). **3**. While there is little change in shoots early after attack (Chapter4), but after 48 hours, the plant starts suffering from reduced water supply from the root system and in response increases shoot ABA (Chapter2). **4**. Shoot physiology is adjusted to the increasing root stress (Chapter2 and 3). **5**. The increase in ABA-dependent defenses and secondary metabolites renders the plant more resistant to the pathogen *S. turcica*. **6**. The loss of leaf-water and induction of ABA-independent defenses increases shoot resistance against *S. littoralis*.

The precise role of both plant and aboveground herbivore in shoot-herbivore induced root resistance on the other hand remains to be determined. To gain insight into a possible adaptive behaviour of the players involved, a better understanding of the physiology underlying the resistance phenomenon will be required. It is possible that the root-herbivore suffers from a lack of assimilates from the shoots upon attack, although the opposite effect has been reported (Babst et al., 2005; Schwachtje et al., 2006; Kaplan et al., 2008). Alternatively, aboveground herbivores might increase their competitive power (Figure 1) by specifically inducing root defenses. A third possibility is that the root-herbivore suffers from the fact that shoot defenses rely on compounds that are synthesized in the roots (discussed below). The mechanisms behind *S. littoralis*-induced root resistance clearly deserves further attention, as its strong negative effect on an economically important root pest might lead to the development of novel, plant-based pest control strategies. Our transcriptional data (chapter 4) represent an ideal starting point for such an undertaking.

The role of roots in plant responses against herbivores

Insect-induced changes in the aboveground parts of plants have received a tremendous amount of attention over the last years (Howe and Jander, 2008). Yet, little is known about the role of roots in insect defenses against above- and belowground herbivores (Erb et al., 2008; Rasmann and Agrawal, 2008). This is somewhat surprising, given the fact that i) several important agricultural pests feed on plant roots (Saito et al., 2005; Vidal et al., 2005), and ii) many important secondary metabolites required for leaf-defenses appear to be synthesized belowground (Kaplan et al., 2008, Chapter 4). The current state of plant physiological theory is that root defenses often resemble their aboveground counterparts in terms of chemical composition (Rasmann and Agrawal, 2008), but that they are not necessarily similar. The bouquet of herbivore-induced volatiles in maize for instance is distinctly different in the roots compared to the shoots (Rasmann et al., 2005), a phenomenon that can also be observed in *Arabidopsis* (Steeghs et al., 2004). Our results largely confirm these trends by showing that while the root phytohormonal response upon *D. virgifera* attack is comparable to elicitation in the leaves by *S. littoralis* (chapter 2) and almost 40% of the induced transcripts aboveground are also reactive belowground, there are also pronounced differences in local root and shoot responses at the transcriptional level (chapter 4). These can be attributed to at least three possible factors: i) differences in the feeding behaviour and elicitor composition between *D. virgifera* and *S. littoralis*; ii) direct, physiological differences in the plant's above- and belowground response and iii) indirect effects caused by the surrounding microbial community. Future molecular studies will have to disentangle these effects, for example by applying the same herbivores to roots and shoots (an achievable task with *S. littoralis* (Carroll et al., 2008)) and by performing experiments under sterile conditions. This will eventually lead to the identification of root-specific, herbivore responsive genes that can be used to increase the performance of marker-guided research on root-defenses.

That the magnitude of inducibility can vary between roots and shoots of plants has been observed in several plant systems (Zangerl and Rutledge, 1996, S. Rasmann, unpublished), begging the question if the preceding signaling processes are comparable belowground to what is known from aboveground plant-insect interactions. Most published evidence indeed points to an equally important role of the octadecanoid pathway in the local defense response of roots compared to shoots (McConn et al., 1997; Schmelz et al., 2002; Puthoff and Smigocki, 2007; Gao et al., 2008). However, clear evidence for the induction and signaling of JA-related compounds however has remained scarce. The octadecanoid pathway is required for root development (Creelman and Mullet, 1997; Vellosillo et al., 2007; Gao et al., 2008; Hummel et al., 2009), and mutants therefore are likely to have an altered root-phenotype (Gao et al., 2008), possibly confounding conclusions about insect resistance. In this context, it is remarkable that the only study involving mutant plants deficient in JA production to measure root insect resistance does not contain any information on root morphology (McConn and Browse, 1996; McConn et al., 1997) and belowground induction of JA. Our results demonstrate for the first time that root herbivory indeed increases OPDA and JA levels in roots (chapter 2), both locally and systemically within the roots and over a prolonged period of time (chapter 4). Furthermore, *D. virgifera* induces several genes involved in the biosynthesis of JA belowground (chapter 4), adding to our general conclusion that an activation of the octadecanoid pathway is part of the local root defense-response. Remarkably, however, we did not observe a strong burst after infestation and no clear correlation with belowground (E)-β-caryophyllene emissions, as has been reported to occur aboveground in maize (Schmelz et al., 2003). Instead, JA and JA-Ile levels increased slightly after attack and remained at the same elevated level over a period of 48 hours, while (E)-β-caryophyllene emissions increased exponentially (chapter 4). This points to subtle differences in the role of the octadecanoid pathway above- and belowground, and further research should aim at unravelling its precise role for belowground plant-insect interactions. Mutants deficient in the expression of OPR7, a possible key gene involved in insect-dependent JA-induction belowground (chapter 4), are currently being characterized (M. Kolomiets, personal communication), and might help to achieve this.

The fact that several inducible plant secondary metabolites and proteins are synthesized in the roots upon shoot herbivory does not only show that these tissues are important for aboveground defenses, but also implies that shoot-root signals are deployed upon insect attack. Baldwin *et al.* (Baldwin et al., 1997) reported an increase in root JA after 3h after leaf damage in *Nicotiana sylvestris* and showed that radiolabelled $2C^{14}$JA can be translocated from the shoots to the roots (Zhang and Baldwin, 1997). While systemin has been proposed as a long-distance signal in tomato, the current view is that JA is the actual long-distance signal, at least aboveground, in tomato (Schilmiller and Howe, 2005). The observation that several leaf-inducible genes are also induced in the roots upon mechanical wounding and MeJA treatment in hybrid poplar finally led Major and Constable (Major and Constabel, 2007) to the conclusion that JA or a JA derived signal might be moving from the shoots to the roots. The results presented here, however, do not support this hypothesis. First, we did not deteced an increase of JA in the roots upon shoot attack by *S. littoralis* at a timepoint where shoot levels were clearly elevated (chapter 2). Although a timecourse would be desirable to confirm this result, other experiments suggest that in maize, the systemic induction of both volatiles and JA is exclusively directed from the wounding site to the tissues above (T. Köllner and I. Hiltpold, unpublished). Secondly, our transcriptional profiles show no overlap between the roots and the shoots of *S. littoralis* attacked plants. This strongly suggests that the signal traveling from the shoot into the roots is dissimilar from the local and systemic signals within the shoot. Further research could aim at elucidating the shoot-root signal in maize, an effort certainly warranted, given the possible importance of roots in induced defenses against insects and the current lack of knowledge thereof. A first step in this direction could be the identification of overrepresented binding-motivs in the roots of shoot-attacked plants using the transcriptional information from the microarray experiment (chapter 4) and the publicly available maize genome sequence.

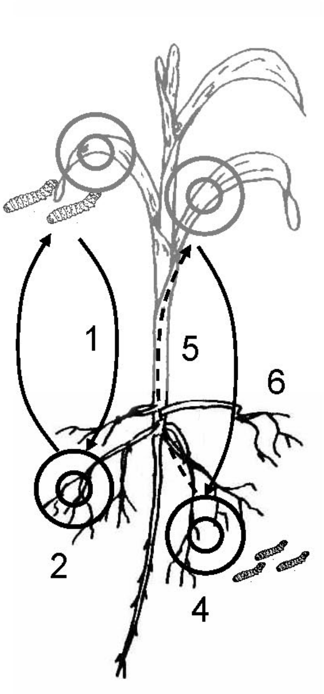

Figure 3: Model of the central role for roots in plant-insect interactions. **1.** Unknown signals move from the shoot to the roots after attack by *S. littoralis* (Chapter2 and 4). **2.** The roots undergo substantial transcriptional reprogramming (Chapter4). **3.** Changes in root physiology most likely have a feed-back effect on the leaves (e.g. supply of nitrogen or defensive compounds). **4.** Root herbivory by *D. virgifera* changes the metabolic state of roots (Chapters 2 and 4). **5.** Initial (weak) signaling occurs between attacked roots and shoots, and water transport decreases (Chapters 2,3 and 4). **6.** The changes in root physiology possibly alter assimilate distribution and flows from the shoot (Chapter4).

In general, the insect-induced molecular reprogramming of roots appears to be of central importance for plant-insect interactions including i) root herbivore induced changes in shoot physiology, ii) root herbivore induced root defenses iii) shoot herbivore induced root resistance and iv) shoot herbivore induced defense and tolerance responses (Figure 3), and this thesis provides a basis to explore the role of roots in all these processes, a challenge that has the potential to considerably improve our understanding on plant-insect interactions.

CONCLUSIONS

This thesis demonstrates how novel techniques and theoretical frameworks from ecology, behaviour and plant physiology can be combined to unravel the mechanism and adaptive value of otherwise hard to explain, counterintuitive phenomena. While ecology provides the necessary knowledge about the relevance of above-belowground interactions in nature and can help to develop hypotheses about their evolutionary significance, modern plant physiology contributes the necessary tools to explain cause and effect *in planta*. Thereby, it allows validation of ecological theory from a functional perspective. By using this multi-disciplinary approach, we provide a concise explanation for root herbivore induced shoot resistance in maize and open up several new lines of research, including the role of roots in shoot herbivore defense and shoot herbivore induced root resistance. It is my hope that future projects on above-belowground interactions in maize will benefit from the insight gained here.

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