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Signal signature of aboveground-induced resistance upon belowground herbivory in maize

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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 the accumulation of defensive metabolites. Although 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 herbivory by *D. v. virgifera* induced aboveground resistance against the generalist herbivore *Spodoptera littoralis*, and the necrotrophic pathogen *Setosphaeria turcica*. Furthermore, *D. v. virgifera* increased shoot levels of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), and primed the induction of chlorogenic acid upon subsequent infestation by *S. littoralis*. To gain insight into the signalling network behind this below- and aboveground defence interaction, we compiled a set of 32 defence-related genes, which can be used as transcriptional marker systems to detect activities of different hormone-response pathways. Belowground attack by *D. v. virgifera* triggered an ABA-inducible transcription pattern in the shoot. The quantification of defence hormones showed a local increase in the production of oxylipins after root and shoot infestation by *D. v. virgifera* and *S. littoralis*, respectively. On the other hand, ABA accumulated locally and systemically upon belowground attack by *D. v. virgifera*. Furthermore, *D. v. virgifera* reduced the aboveground water content, whereas the removal of similar quantities of root biomass had no effect. Our study shows that root herbivory by *D. v. virgifera* specifically alters the aboveground defence status of a maize, and suggests that ABA plays a role in the signalling network mediating this interaction.

Keywords
above- and belowground defence interactions, induced resistance, defence hormones, *Zea mays*, *Diabrotica virgifera*, priming

INTRODUCTION

Plants possess an inducible immune system that provides protection against many potentially harmful organisms (Agrawal, 1998; Tollrian and Harvell, 1998). Inducible defences, such as the production of defensive metabolites and proteins, are controlled by signalling pathways that are specifically activated upon perception of stress-derived signals. Whereas 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), methyl jasmonate (MeJA; Glazebrook, 2005), ethylene (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 signalling pathways can interact with each other. This signalling crosstalk is thought to integrate multiple stress signals into one appropriate and specific defence

response (Pozo *et al.*, 2004). Examples of signalling crosstalk 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 defences (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 crosstalk 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 defences, 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.*, 2007a,b). Because of their potential to influence entire food webs and ecosystems, interactions between below- and aboveground plant defences are highly relevant from an ecological point of view. It remains, however, unclear if these interactions are adaptive, and if they are, for which organisms (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 the systemic production of defensive metabolites. Furthermore, we provide evidence that belowground attack by *D. v. 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. v. virgifera* induces aboveground resistance against the leaf herbivore *Spodoptera littoralis* and the necrotrohpic fungus *Setosphaeria turcica*

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

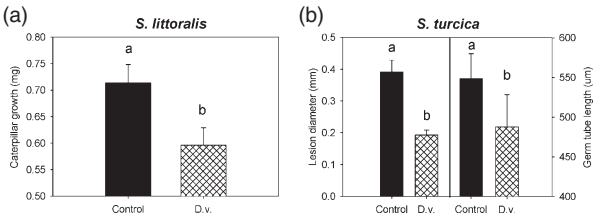

Figure 1. Root herbivore-induced resistance in maize leaves against *Spodoptera littoralis and Setosphaeria turcica*. Leaf challenge with *S. littoralis* caterpillars and *S. turcica* spores was performed 4 days after the application of *Diabrotica virgifera virgifera* larvae to the roots.
(a) Average growth (+SE) of *S. littoralis* caterpillars over a feeding period of 11 h on control plants and on *D. v. virgifera*-infested plants (*D.v.*).
(b) Average diameters of lesions (+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 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), and primes chlorogenic acid induction

To examine the impact of belowground infestation on aboveground defence compounds, we profiled secondary metabolite composition in leaves after root herbivory by *D. v. virigfera*, and subsequent leaf infestation by *S. littoralis*. The HPLC-DAD quantification of DIMBOA showed that root attack by *D. v. 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. v. 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. v. virgifera* or *S. littoralis* (Figure 2d). Chlorogenic acid was significantly induced by *S. littoralis* herbivory, but not by *D. v. virgifera*. Interestingly, however, *D. v. virigifera*-infested plants showed augmented production of chlorogenic acid following *S. littoralis* attack (Figure 2c), suggesting that belowground herbivory primes chlorogenic acid production aboveground.

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

To further examine the impact of belowground *D. v. virgifera* on aboveground defence, we developed a transcrip-

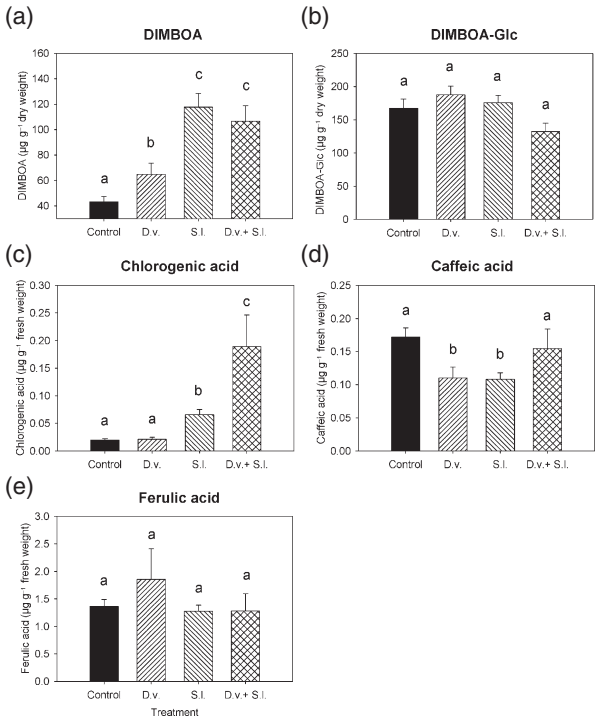

Figure 2. Average concentrations of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (a), DIMBOA-glucoside (b), chlorogenic acid (c), caffeic acid (d) and ferulic acid (e) in leaves of herbivore-infested plants. Leaves were collected after 4 days of belowground infestation by *Diabrotica virgifera virgifera* (*D.v.*), 2 days of aboveground infestation by *Spodoptera littoralis* (*S.l.*), or simultaneous infestation by *D. v. virgifera* (4 days) and *S. littoralis* (2 days; *D.v + S.l.)*. The values presented are concentrations in µg g^{-1} fresh or dry weight (+SE). Different letters indicate significant differences between treatments ($P < 0.05$).

tional 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 the 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 to reliably 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 with stress-induced profiles (Figures 3 and S3). The effectiveness of these hormone treatments was confirmed by quantifying levels of 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* herbivory or regurgitant (Figure 3). Both the HC and PC analyses indicated a similarity between the *S. turcica*-infected profiles, and the ACC- and BTH-induced profiles (Figures 3 and S3), suggesting that the defence response of the plant to *S. turcica* involves regulation by ET and SA. Although HC did not reveal a close relationship 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 the partial involvement of ABA in the response of the plant 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 signalling activities in (a)biotic stress reactions.

Root herbivory alters hormone-dependent gene expression in leaves

Using our transcriptional marker system, we quantified basal and *S. littoralis*-inducible gene profiles in the leaves of *D. v. virgifera*-infested plants. Leaf material from three independent experiments was collected at 4 days after the application of *D. v. virgifera* larvae to the roots, and at 2 days after the 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; Table 1). Whereas the *D. v. virgifera*-induced profiles clearly clustered away from *S. littoralis*-induced profiles, the transcription profiles of double-infested plants showed an intermediate clustering (Figure 4a). In response to *D. v. 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 the involvement of ABA in the aboveground response to *D. v. virgifera*. Infestation of the leaves by *S. littoralis* triggered the statistically significant induction 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).

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

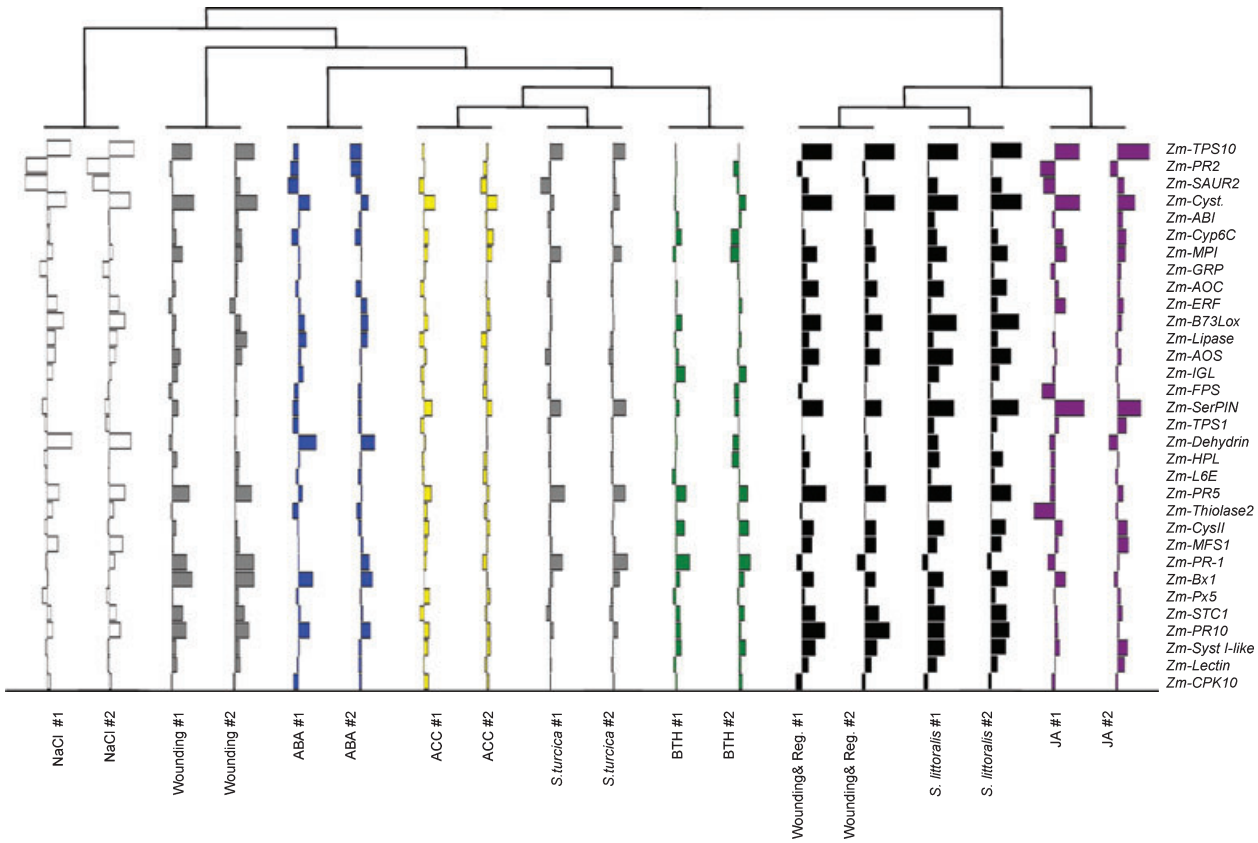

Figure 3. Hierarchical cluster analysis of gene induction profiles in maize leaves after treatment with (a)biotic stress or defence hormones. Leaves were collected at 1 day after soil-drenching with salt (NaCl, 150 mM), abscisic acid (ABA, 300 μM), jasmonic acid (JA, 300 μM) or 1-aminocyclopropane-1-carboxylate (ACC, 2 mM), or at 2 days after spraying the leaves with benzothiadiazole (BTH, 5 mM). Leaves from wounded plants with or without 50% *Spodoptera 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 the application of *S. littoralis* caterpillars, and 3 days after inoculation with *Setosphaeria turcica* spores. The bar widths of induced (to the right, scale from 0 to 6) or repressed (to the left, scale from 0 to −6) genes are 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 different treatments.

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

Our observation that belowground infestation by *D. v. virgifera* elicits ABA-dependent gene expression in leaves prompted us to quantify the 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. The aboveground attack by *S. littoralis* caterpillars induced a strong local induction of JA and 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. v. virgifera* caused a local increase in JA, OPDA and ABA that was statistically significant (Figure 5). Interestingly, *D. v. virgifera* infestation also increased ABA levels in the leaves, whereas JA, OPDA and SA remained unaltered. The systemic induction of ABA by *D. v. 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. v. virgifera* and *S. littoralis* attack, but the only hormone responding systemically to belowground *D. v. virgifera* attack is ABA. This *D. v. virgifera*-induced ABA response is boosted even further by subsequent attack by *S. littoralis*.

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

To investigate if the exogenous application of ABA to the roots can mimic *D. v. virgifera*-induced resistance in the leaves, plants were soil-drenched with ABA, and subsequently tested for induced resistance against *S. littoralis* and *S. turcica*. ABA-treated plants allowed similar levels of *S. littoralis* growth on their leaves as control plants (Figure 6a), indicating that *D. v. 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. turcica* spores, which correlated with a statistically significant reduction in hyphal

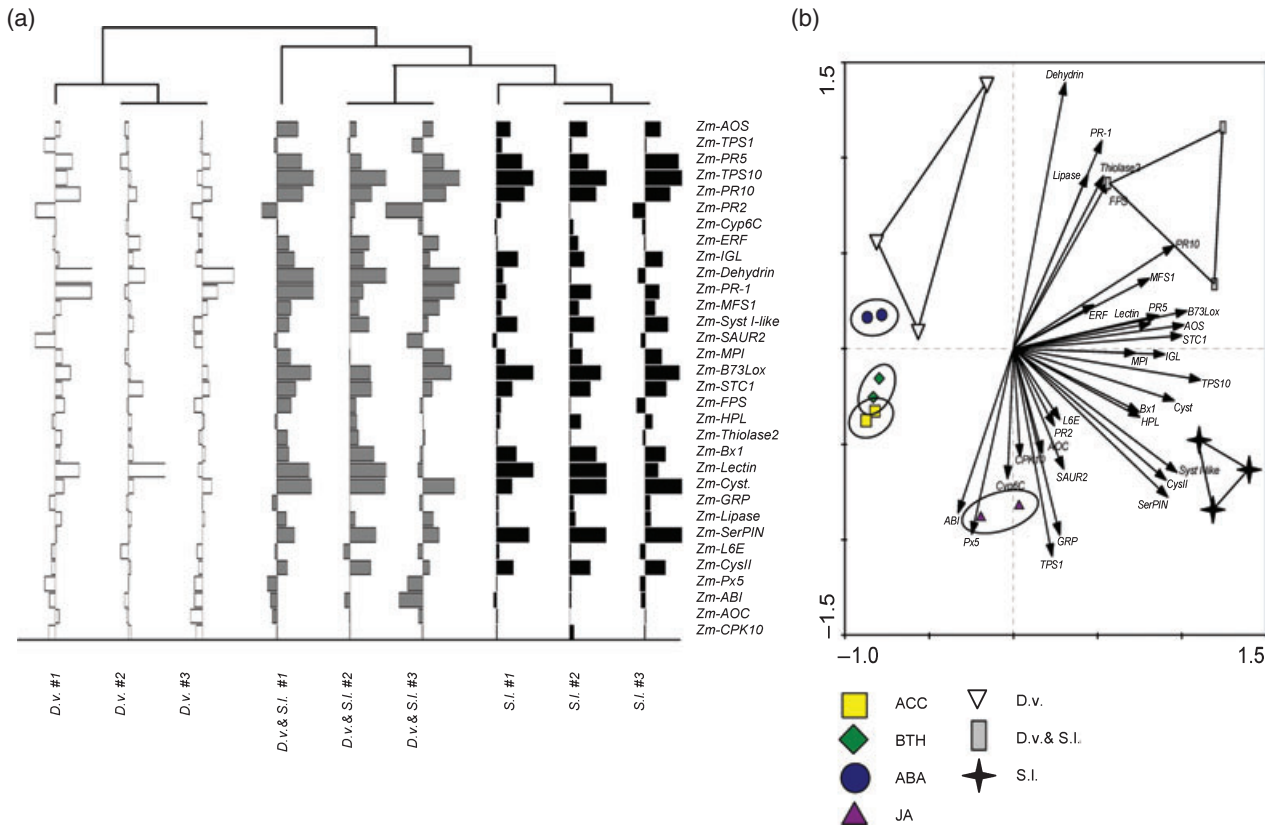

Figure 4. Gene expression profiles in maize leaves after 4 days of belowground infestation by *Diabrotica virgifera virgifera* (*D.v.*), 2 days of aboveground infestation by *Spodoptera littoralis* (*S.l.*), or after simultaneous infestation with *D. v. virgifera* (4 days) and *S. littoralis* (2 days;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

lengths (Figure 6b). Hence, the *D. v. virgifera*-induced stimulation of ABA in the leaves is likely to contribute to the induced resistance against *S. turcica*.

D. v. virgifera induces osmotic stress in leaves

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

DISCUSSION

The belowground infestation by *D. v. virgifera* larvae triggers aboveground resistance against *S. littoralis* and *S. turcica* (Figure 1). Although a variety of negative and positive interactions have been reported between root and shoot herbivores (Erb *et al.*, 2008), our study shows 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 the translocation of defensive compounds. However, our gene expression profiling clearly demonstrates the induction of defence-related genes in the leaves, indicating regulation by long-distance defence signals. For instance, *D. v. virgifera* systemically enhanced the expression of the *Zm-Bx1* gene (Table 1), which encodes an enzyme that catalyses the first step in the biosynthesis of DIMBOA (Frey *et al.*, 2000). Hence, the observed increase of DIMBOA in leaves of *D. v. virgifera*-infested plants (Figure 2a) is likely to result in the systemic upregulation of DIMBOA biosynthesis. As DIMBOA has been reported to suppress the 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.

Table 1 Fold induction (±SE) of 32 genes in maize leaves of herbivore-infested plants

Gene name	*S. littoralis*	*S. littoralis* and *D. v. virgifera*	*D. v. 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

Leaf material was collected after 4 days of belowground infestation by *Diabrotica virgifera virgifera*, 2 days of aboveground infestation by *Spodoptera littoralis* or after simultaneous infestation by *D. v. virgifera* (4 days) and *S. littoralis* (2 days). Asterisks indicate statistically significant differences in gene expression compared with 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 pair-wise multiple comparisons ($P < 0.05$).

In addition to the direct induction of defences, we also provide evidence that belowground attack by *D. v. virgifera* can prime aboveground defences. Although *D. v. virgifera* directly induced systemic ABA and *Zm-Dehydrin* transcript accumulation, *D. v. virgifera*-infested plants displayed even higher levels of ABA and *Zm-Dehydrin* induction after subsequent *S. littoralis* attack (Figure 4; Table 1). Thus, *D. v. virgifera* infestation not only activates shoot ABA responses directly, but also primes for augmented ABA responses after subsequent attack by *S. littoralis* caterpillars. Furthermore, the induction of chlorogenic acid by *S. littoralis* was strongly potentiated when plants were concomitantly infested by *D. v. virigifera* (Figure 2c). As chlorogenic acid has been associated with resistance to *Spodoptera frugiperda* and *Helicoverpa zea* (Nuessly *et al.*, 2007, and references therein), the priming of this defence compound may also have contributed to *D. v. 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. v. virgifera* and aboveground attack by *S. littoralis* stimulated OPDA and JA production locally (Figure 5a,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. v. virgifera* (Figure 5a,b). Of all the defence hormones tested, ABA was the only hormone that accumulated systemically with belowground attack by *D. v. 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; Jia *et al.*, 2002). Moreover, ABA can be transported from the roots to the shoot (Wilkinson and Davies, 2002), where it mediates

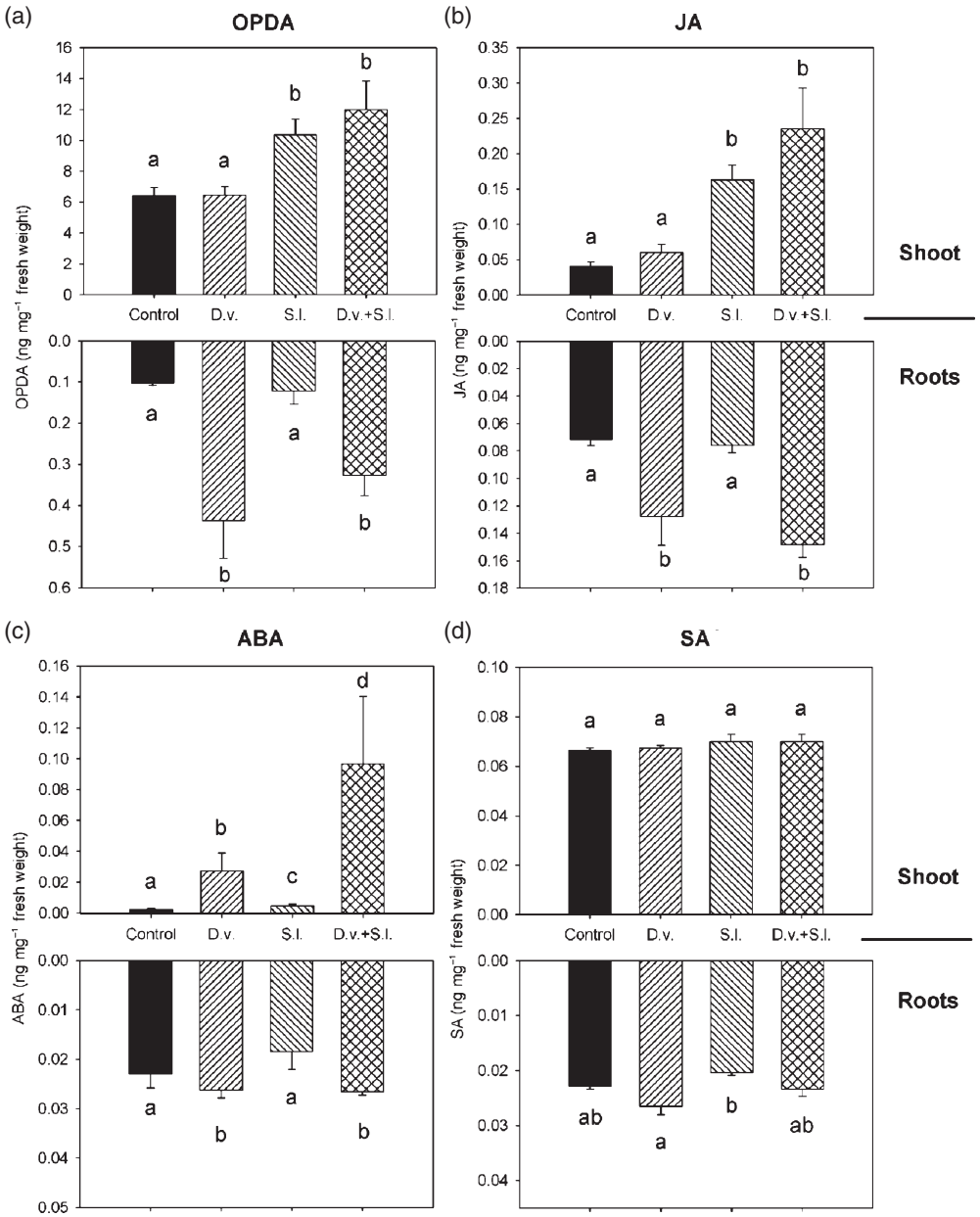

Figure 5. Average concentrations (+SE) of 12-oxo-phytodienoic acid (OPDA; a), jasmonic acid (JA; b), abscisic acid (ABA; c) and salicylic acid (SA; d), and in maize leaves and roots of herbivore-infested plants. Leaves were collected after 4 days of belowground infestation by *Diabrotica virgifera virgifera* (*D.v.*), 2 days of aboveground infestation by *Spodoptera littoralis* (*S.l.*), or simultaneous infestation by *D. v. virgifera* (4 days) and *S. littoralis* (2 days; *D.v. + S.l.)*. The values presented are concentrations in ng mg^{-1} fresh weight (+SE). Different letters indicate significant differences between the treatments ($P < 0.05$).

the closure of stomata and the induction of defensive gene expression (Dodd, 2003; Boudsocq and Lauriere, 2005). Finally, we observed that root infestation by *D. v. virgifera* caused a statistically significant reduction in leaf water content (Figure 7). This suggests that the induction of ABA is the result of an osmotic stress reaction of the host plant to tolerate *D. v. virgifera*-induced drought stress. In support of this, a reduction in water uptake and stomatal conductance has been reported to occur under glasshouse and field conditions in *D. v. virgifera*-infested plants (Godfrey *et al.*, 1993; Riedell and Reese, 1999). Interestingly, however, the artificial removal of similar quantities of root biomass did not cause any reduction in leaf water content (Figure 7). This indicates that *D. v. virgifera* employs a highly efficient strategy to influence the water potential of the plant, which cannot be explained by the reduction in root biomass only. Whether this manipulation is based on a specific mode of feeding, or by additional mechanisms, requires further

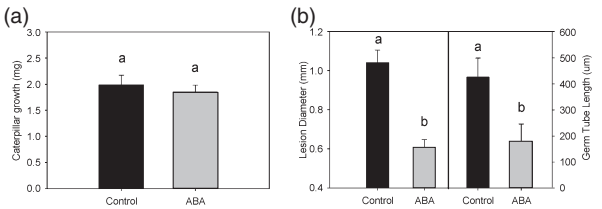

Figure 6. The ABA-induced shoot resistance of maize plants. ABA (300 μM) was applied to the soil, and resistance against *Spodoptera littoralis* and *Setosphaeria turcica* was measured 24 h later.
(a) Average growth (+SE) of *S. littoralis* caterpillars over a feeding period of 11 h.
(b) Average lesion 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 ($P < 0.05$).

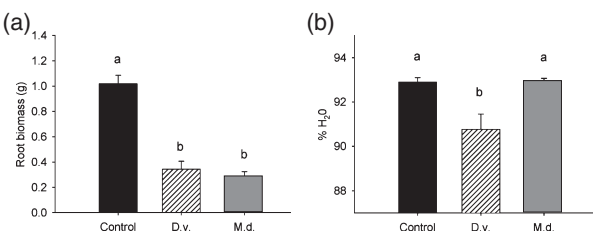

Figure 7. Root biomass (g fresh weight ± SE) (a) and relative shoot water content (±SE) (b) after 4 days of infestation by *Diabrotica virgifera virgifera* (*D.v.*), or after 4 days of daily application of mechanical damage (M.d.). Water content is expressed as the percentage of water per unit of fresh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

investigation. It seems tempting, nevertheless, to speculate that the root herbivore manipulates the water balance in the host plant to increase photoassimilate transport into the roots.

Our finding that soil-drench treatment with ABA induced resistance against *S. turcica* (Figure 6b), suggests that *D. v. 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 β-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 defences (Ton and Mauch-Mani, 2004; Ton *et al.*, 2005). Recent evidence also suggests the involvement of ABA in the response of plants 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* is still to be evaluated. We conclude, therefore, that *D. v. 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 another, yet unknown, signal, in addition to ABA (Bodenhausen and Reymond, 2007).

In conclusion, our study shows that root attack by *D. v. virgifera* profoundly alters the plant's aboveground physiology, resulting in the direct induction of defence-related genes and defence compounds, the priming of defence mechanisms and a change in the water potential of the plant. Although ABA is a strong candidate to act as a systemic signal in this interaction, we conclude that the aboveground resistance is likely to involve 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, 4 cm; depth, 11 cm) under controlled conditions in a climate chamber (30°C and 16-h photoperiod; CLF plant climatics, Percival Scientific, Inc., http://www.percival-scientific.com). The plants for hormone-induced transcription profiling and induced resistance assays were grown in commercial potting soil (Ricoter Aussaaterde, http://www.ricoter.ch). For experiments involving *D. v. virgifera*, all seedlings were grown in a sand/vermiculite mixture (3:1) to facilitate the harvesting of roots. Plants for transcription profiling had two fully expanded primary leaves, and were 10–12-days old. *S. littoralis* eggs were provided by Syngenta (http://www.syngenta.com) and reared on an artificial diet, as described previously (Turlings *et al.*, 2004). Second-instar larvae of *D. v. virgifera* were obtained from CABI Delémont (http://www.cabi.org), and were maintained on maize seedlings until use. Spores of *S. turcica* were isolated as described by Rostas *et al.* (2006).

Chemical and biological treatments

Solutions containing JA, ABA, ACC and salt (NaCl) were applied as a soil-drench 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. v. virgifera* was achieved by placing 6 sec-instar larvae onto the soil surface around the stem of the maize plants. Infestation by *S. littoralis* caterpillars was performed by applying about 20 sec-instar larvae in the whorls of the leaves. Wounding was

performed by scratching the underside of two leaves at two 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 was stored at −76°C until use (Turlings *et al.*, 1993). Infection by *S. turcica* was performed by spreading 100 μL of spore suspension (6×10^4 spores ml^{-1}; 0.01% Silwet) over the 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. The 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- and belowground experiment), at 3 days after inoculation with *S. turcica* and at 4 days after *D. v. virgifera* infestation. Mechanical damage of the roots was achieved by inserting a knife blade into the soil (at a depth of 10 cm), at a distance of approximately 0.7 cm from the stem. This was repeated over a period of 4 days (damaging a different side around the stem every 24 h), and resulted in the gradual removal of around 70% of the 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 – two or three plants, which were pooled for the synthesis of cDNA. For transcriptional profiling of *D. v. virgifera*-infested plants, 12 plants per treatment were harvested in experimental blocks of two plants. To ensure the selection of sufficiently infested plants, plants were analysed for *D. v. virgifera*-induced emission (*E*)-β-caryophyllene by solid-phase micro-extraction (SPME) GC-MS analysis, as described by Rasmann and Turlings (2007). RNA was only extracted from three blocks showing the highest (*E*)-β-caryophyllene values (data not shown), resulting in six plants per treatment that were pooled block-wise for cDNA synthesis ($n = 3 \times 2$). Primers were designed based on publicly available sequences of stress-inducible maize genes, or on expressed sequence tags (ESTs) identified in a differential hybridization screen for *S. littoralis*-inducible genes (Ton *et al.*, 2007). The primer sequences, GenBank accession numbers and putative functions of genes are listed in Table S1. The specificity of primers was tested by conventional PCR (40 cycles) of cDNA followed by 1.5% agarose gel electrophoresis, or by qPCR followed by melting point analysis.

Induced resistance assays

Resistance against *S. littoralis* was quantified by determining the average weight gain of 10 sec-instar larvae per plant over a period of 11 h of infestation, as described previously (Ton *et al.*, 2007). Induced resistance assays upon hormone treatments were based on five or six plants per treatment. For *D. v. 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 <60% of the applied caterpillars could be recovered were excluded from the analysis. Resistance against *S. turcica* was assessed based on the diameters of lesions and lengths of germination hyphae from *S. turcica* spores at 3–4 days after inoculation with 5×10^4 spores ml^{-1}. The diameters of lesions were measured using a calibrated loupe. The lengths of *S. turcica* hyphae were examined under a light microscope (BX50W1; Olympus, http://www.olympus-global.com), and then quantified using AnalySIS-D software (Soft Imaging System GmbH, http://www.soft-imaging.ne). Analysis of *S. turcica* hyphae was performed in randomly selected leaves (hormone assays, $n = 6$ plants, 75 hyphae; root herbivore assays, $n = 10$ plants, 164 hyphae). The 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 h (for ABA, JA, and ACC) or 48 h (for BTH) after hormone treatments and at 4 days after application of *D. v. 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 then pulverized to a fine powder (0.5 g per plant). Before extraction, a mixture of internal standards containing 100 ng [$^2H_6$]ABA, 100 ng dihydrojasmonic acid, 100 ng prostaglandin B1 (Pinfield-Wells *et al.*, 2005), 100 ng d6-SA and 100 ng parabene were added. The frozen tissue was immediately homogenized in 2.5 mL of ultrapure water and then 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 resuspended in 1 mL of a water/methanol (90:10) solution, and then filtered through a 0.22 mm cellulose acetate filter. A 20-μL aliquot of this solution was then directly injected into an ultraperformance Waters Acquity liquid chromatography (UPLC™) system (Waters, http://www.waters.com). The UPLC was interfaced to a triple quadrupole tandem mass spectrometer (TQD; Waters) using an orthogonal Z-spray electrospray interface. LC separation was performed using an Acquity UPLC BEH C18 analytical column (2.1×50 mm, 1.7 μm; Waters) at a flow rate of 300 μL min^{-1}. Standard curves for all hormones were obtained by injecting a mixture of pure compounds at different concentrations (10, 25, 50, 70, 100 and 150 ng). Quantifications were carried out with MassLynx v1.4 (Waters) using the internal standards as a reference for extraction recovery, and the standard curves as quantifiers.

Quantification of hydroxamic acids

Both DIMBOA and DIMBOA-Glc were quantified in plant material from the same plants as were used for the phytohormone measurements. Approximately 10 mg of lyophilized plant material was resuspended in 1 mL of extraction buffer (98% methanol, 2% acetic acid) and then sonicated for 10 min. After 10 min of centrifugation at 12 000 ***g***, 800 μL of supernatant was collected for HPLC injection (10 μL). Samples were analysed on a Shimadzu prominence HPLC with a diode array detector (detection at 254 nm), using a thermal hypersil C-18 column (150×4.6 mm, 5 μm), at a flow rate of 1 mL min^{-1}. Elution was carried out for 2 min under isocratic conditions of 100% solvent A (H_2O), 9 min with a linear gradient to 50% solvent A and 50% solvent B [methanol/isopropanol (95:5) + 0.025% acetic acid], and then for 5 min under isocratic conditions with 50% solvent A and 50% solvent B.

Quantification of root biomass and leaf water content

Roots and shoots were harvested and weighed at the end of the bioassays to determine root biomass (fresh weight, FW) and shoot FW. Subsequently, shoots were carefully put in paper cooking bags, and then dried at 80°C over 4 days. The shoot dry weight (DW) was then determined using the same balance as before. The relative leaf water contents were calculated assuming equal turgid weights using the formula $\%H_2O = (FW - DW)/(FW \times 100)$.

Statistical analysis of transcription profiles

The gene expression levels were calculated relative to the expression of two constitutively expressed reference genes: *Zm-GAPC* and *Zm-Actin1*. The -fold inductions were calculated relative to gene expression levels in control or mock treatments. The HC analysis was based on ln-transformed -fold induction values, using MultiExperiment Viewer (Saeed *et al.*, 2003). Metric selection for HC analysis was based on Eucledian distance using average linkage clustering. The PC analysis of gene distribution was adjusted to the methods described for the analysis of microarray data (Held *et al.*, 2004). To determine the appropriate model for the description of gene distribution, a detrended correspondence analysis (DCA) was performed. The dimensionless value given for the length of the gradient of the first ordination axis was <3 (i.e. <1.047 for the hormone treatments; <1.228 for the above- and belowground treatments), indicating that the values should be fitted by a linear distribution model. Therefore, the PC analysis for the comparison of gene expression values was based on a linear model. PCA was performed on ln + 1-transformed -fold induction ratios, using Canoco 4.5 package (Ter Braak and Smilauer, 2002). For all gene profiling experiments involving *D. v. virgifera*, the normality of the data was verified using the Kolmogorov–Smirnov test, whereas the Leveen test for homogeneity of variance was carried out to ensure equal variances. The ln-transformed -fold induction values were tested against controls using a Student's *t*-test. A one-way ANOVA, followed by pair-wise multiple comparisons (Holm–Sidak test), was used to identify genes with differential responsiveness to more than two treatments.

Statistical analysis of bioassays and UPLC/HPLC results

Multiple comparisons were analysed by one-way ANOVA followed by pair-wise multiple comparisons (Holm–Sidak test). Comparisons between two treatments were analysed by a Student's *t*-test. The normality of the data was verified using the Kolmogorov–Smirnov test, and the equality of variances was tested using a Leveen test ($P < 0.05$). In the case of non-normality and/or unequal variances, data were transformed where possible, or were analysed 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's–Newman–Keuls test (equal sample sizes), respectively. Effects of hormone treatments on salt-stress tolerance were analysed using standard Kaplan–Meier survival analysis on log ranks.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Induced resistance against *Spodoptera littoralis* in leaves upon attack by different herbivores.

Figure S2. Hormone-induced resistance against *S. littoralis*, salt stress and *S. turcica* in maize leaves.

Figure S3. Principal component analysis of the gene expression profiles in maize leaves after treatment with (a)biotic stress or defence hormones.

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

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