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The role of abscisic acid and water stress in root herbivore-induced leaf resistance

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Summary

• Herbivore-induced systemic resistance occurs in many plants and is commonly assumed to be adaptive. The mechanisms triggered by leaf-herbivores that lead to systemic resistance are largely understood, but it remains unknown how and why root herbivory also increases resistance in leaves.

• To resolve this, we investigated the mechanism by which the root herbivore *Diabrotica virgifera* induces resistance against lepidopteran herbivores in the leaves of *Zea mays*.

• *Diabrotica virgifera* infested plants suffered less aboveground herbivory in the field and showed reduced growth of *Spodoptera littoralis* caterpillars in the laboratory. Root herbivory did not lead to a jasmonate-dependent response in the leaves, but specifically triggered water loss and abscisic acid (ABA) accumulation. The induction of ABA by itself was partly responsible for the induction of leaf defenses, but not for the resistance against *S. littoralis*. Root-herbivore induced hydraulic changes in the leaves, however, were crucial for the increase in insect resistance.

• We conclude that the induced leaf resistance after root feeding is the result of hydraulic changes, which reduce the quality of the leaves for chewing herbivores. This finding calls into question whether root-herbivore induced leaf-resistance is an evolved response.

Key words
aboveground–belowground interactions, abscisic acid, *Diabrotica virgifera,* induced resistance, *Spodoptera littoralis,* water stress, *Zea mays.*

Introduction

Many plants increase their resistance systemically upon attack by pathogens and insects. Along with constitutive defenses and tolerance mechanisms, induced resistance can have important consequences for the associated organisms and thus may strongly affect ecosystem dynamics (Johnson *et al.*, 2003; Kaplan *et al.*, 2007, 2008). Most of the mechanisms leading to systemic resistance have been at least partly unraveled. After pathogen attack for example, noninfested leaves become more resistant against other pathogens, a phenomenon termed systemic acquired resistance (SAR). This is dependent on the phytohormone salicylic acid (SA) which accumulates both locally and distally upon pathogen infection (Metraux *et al.*, 1990). The search for the systemically translocated signal responsible for SAR has led to a list of candidates including SA (Malamy *et al.*, 1990), its methylated form methyl salicylate (MeSA) (Park *et al.*, 2007) and jasmonic acid (JA) (Truman *et al.*, 2007). The importance of each of these ubiquitous plant hormones has been questioned (Delaney *et al.*, 1994; Attaran *et al.*, 2009). Recently, azealic acid (AzA) has also been implicated in SAR (Jung *et al.*, 2009).

Upon mechanical damage or leaf-attack by herbivores, plants also activate their defenses in uninfested leaves (Orians, 2005), an effect that is referred to as wound-induced resistance (WIR). The expression of WIR is predominantly regulated by bioactive jasmonates (Howe & Jander, 2008) that accumulate both locally and systemically in response to wounding (Glauser *et al.*, 2008). Although there is increasing evidence for JA as the long-distance signal mediating WIR (Stratmann, 2003), some recent studies suggests that other signals may be involved (Heil & Ton, 2008; Koo *et al.*, 2009).

Compared with these well-described effects, virtually nothing is known about what causes an increase in leaf

defense and resistance upon root attack by herbivorous insects and vice versa (Bezemer *et al.*, 2003; Wäckers & Bezemer, 2003; van Dam *et al.*, 2005; Erb *et al.*, 2009a,c). Root herbivore-induced shoot resistance (RISR) seems to be a common and abundant phenomenon with important consequences for multitrophic interactions and ecosystem dynamics (van der Putten *et al.*, 2001; Bardgett & Wardle, 2003; Soler *et al.*, 2005, 2007; Kaplan *et al.*, 2008). It has been proposed that RISR could be a WIR-like phenomenon extending from the roots to the leaves or a priming effect similar to ISR (Erb *et al.*, 2008). Early work on the impact of root herbivores on shoot resistance also led to the hypothesis that changes in plant water balance may lead to altered performance of aboveground herbivores (Masters *et al.*, 1993).

While systemic induced resistance in the leaves is commonly thought to be adaptive for the plant (Heidel & Dong, 2006; Walling, 2009), as the same attacker is likely to feed on different leaves over time, the situation is much less clear for RISR. Why would plants increase their leaf-resistance after root attack? Wäckers & Bezemer (2003) proposed three explanations for increased shoot defenses upon root herbivory: (1) plant adaptation to an increased likelihood of aboveground herbivory after root attack; (2) root herbivore manipulation to mobilize defenses against competing aboveground herbivores; or (3) increased shoot defenses as a consequence of a plant physiological constraint. To date, none of these hypotheses have been explicitly tested. The lack of knowledge about the physiological basis of RISR, in particular, has hampered efforts to elucidate its adaptive value (Wäckers & Bezemer, 2003) and possible ecological importance (Wardle *et al.*, 2004).

In maize, RISR has been shown to be effective against both herbivores and pathogens (Erb *et al.*, 2009a). An induction of ABA and a reduction of leaf-water contents have been observed in this system (Erb *et al.*, 2009a), leading to the hypothesis that hydraulic changes and/or ABA-signaling might mediate the increase in resistance. However, as for other cases of RISR, the causal factors linking the resistance phenotype to the physiological changes have remained unclear. By altering root-water supply and ABA-biosynthesis, the current study aims at unraveling the relative contribution of ABA and water loss for RISR in maize. Combined with results from behavioral assays and field experiments, the molecular and chemical data presented here show that root-herbivore induced leaf-resistance is mediated by changes in the plant's water balance and therefore may not be an evolved plant defense response.

Materials and Methods

Field experiment

To determine the influence of root infestation on leaf-herbivore resistance in the field, 12 plots (plot dimensions: 9.3 × 3.7 m, 56 plants, two rows) of maize (*Zea mays* var. Delprim) were sown at the Bradford Research and Extension Center of the University of Missouri, Columbia, USA, at the end of May 2008. The plots were interspersed with different commercial varieties that were arranged in a randomized complete block design (C Zwahlen *et al.*, unpublished). Two weeks after planting, eight plots were infested with *Diabrotica virgifera* by applying 11 800 eggs over a distance of 3.6 m to one row per plot. Taking into account a viability of 75%, this equaled *c.* 400 viable eggs per plant, with 22 infested plants per plot. Four plots were left root-herbivore free. The root herbivore density used is within the natural range of infestation (Pierce & Gray, 2006). At the beginning of July, 1 month after application of the eggs when the *D. virgifera* larvae had reached their second instar and maize plants had six or seven fully developed leaves (growth stage V8), the plants were sampled for aboveground herbivore damage. All the normally developed, *D. virgifera*-infested plants and corresponding controls were examined. The number of damaged leaves was noted, as well as the number of the longitudinal- and shotgun-shaped holes. A leaf was considered damaged when clear surface removal by herbivores was visible. Small white traces caused by flea beetles and thrips were not taken into account. Herbivores encountered were photographed or conserved in alcohol for later identification. For statistical analysis, data from all plants within one plot were pooled and treated as one independent replicate.

Laboratory experiments – plants and insects

To further investigate the mechanism underlying root herbivore-induced changes in leaf-resistance, additional experiments were carried out in the laboratory. Maize plants were grown in bottom-pierced, aluminum-wrapped plastic pots (diameter, 4 cm; depth, 11 cm) in a phytotron (23 ± 1°C, 60% RH, 16 light : 8 h dark, and 50 000 lumen m^{-2}). Before planting, the seeds were rinsed with water to remove any storage residuals and, unless mentioned otherwise, sown in sand (lower 8 cm) topped with commercial potting soil (upper 3 cm, Ricoter Aussaaterde, Aarberg, Switzerland). Plants used for experiments had two fully expanded primary leaves and were 9–10 d old. Plants were watered with 10 ml of tap water every day until the beginning of the experiments. All experiments were carried out under light benches in a climatized laboratory (25 ± 2°C, 40 ± 10% RH, 16 light : 8 h dark, and 8000 lumen m^{-2}). *Spodoptera littoralis* eggs were provided by Syngenta (Stein, Switzerland) and larvae were reared on artificial diet as described by Turlings *et al.* (2004). *Diabrotica virgifera* eggs and larvae were obtained from CABI (Delémont, Switzerland) and from the USDA-ARS-NCARL (Brookings, SD, USA) and kept on freshly germinated maize seedlings until use.

Leaf-herbivore performance experiments

To determine the dynamics of *D. virgifera*-induced changes in leaf-herbivore resistance, we measured the growth, survival and leaf-consumption of *S. littoralis* caterpillars in three independent experiments. For the experiments, maize plants were either left uninfested (controls) or were infested with six L2 *D. virgifera* larvae by placing them on the soil with a fine brush (n = 15). The root herbivores were then left to feed on the roots for 48 h, after which individual 2nd instar *S. littoralis* larvae were placed on the second true leaf of the 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 and put into the cages, and the cages were then gently slid over one half of the maize leaves, exposing *c.* 0.5 cm^2 of tissue to each larva. The caterpillars were reweighed with a microbalance after 6, 12 and 24 h of feeding, and the cages were moved to a different position on the leaves after 6 h and 12 h of feeding to ensure ample food supply. After 24 h, the caterpillars were directly placed on the plants to feed freely for the rest of the experiment. To stop the larvae from escaping, PET-tubes (30 cm height, cone-shaped with a top-diameter of 8 cm) were put over the plants and attached to the pots with Parafilm. They were covered by a fine nylon mesh (0.3 mm diameter) on top.

The experiment was repeated a second time without weighing the larvae (n = 15). Only the survival of the larvae was recorded daily in order to obtain a sufficient number of total replicates for the analysis of survival curves. In an additional independent experiment, we analysed the first 6 h of *S. littoralis* feeding in more detail by recording both larval growth and leaf-consumption (n = 30). The procedure was as described above, but the caterpillars were weighed, left on the plants for 6 h, reweighed and removed. The leaves were then scanned, and the consumed leaf-area was determined using Adobe Photoshop.

Alteration of root water supply

Root herbivory by *D. virgifera* is known to influence the water status of plants both in the field and the laboratory (Godfrey *et al.*, 1993; Riedell & Reese, 1999; Erb *et al.*, 2009a). To investigate the contribution of water supply on root-herbivore induced leaf-resistance, we subjected maize seedlings to different water regimes and measured leaf-water contents and growth of *S. littoralis* larvae. For this experiment, maize seedlings were either left root-herbivore free or were infested with *D. virgifera* as described earlier (n = 24). Infested and uninfested plants were then divided into three watering regimes. One-third of the plants received no water over the 48 h of root-herbivore infestation. This resulted in a gradual drying of the soil. No phenotypical changes in the leaves were observed, indicating only mild water limitation. One-third of the plants received normal watering (10 ml d^{-1}) and one-third was supplied with water *ad libitum* by placing the pots in a tray with a shallow layer of water at the bottom. The water was taken up to saturation through the bottom holes in the pot, resulting in constantly elevated soil humidity. All the plants also grew normally in this case. After 48 h, *S. littoralis* growth was measured for the six treatment combinations over 6 h of feeding as described earlier. Leaves were then harvested and weighed immediately to determine their FW. The DW was determined after drying them for 48 h at 80°, and relative water contents (RWC) were determined using the formula RWC = 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. Roots were washed, harvested and their DW was determined as described above.

Influence of root-feeding location

Because *D. virgifera* larvae were often observed to feed on the hypocotyl and just below on the primary roots of maize seedlings, we tested the effect of this behavior on root herbivore growth and leaf-resistance. To be able to confine *D. virgifera* to different parts of the belowground tissues, we used fine nylon screens (mesh size 0.3 mm). Roots of maize plants penetrated the nets easily, as the fine root tips could grow through and could then stretch and expand the mesh as they thickened. However, belowground herbivores, at least at the L2 larval stage used here, were unable to move through the screen. Three experiments were performed using this method. In the first experiment, a small PVC tube (2 cm diameter, 4 cm height) was covered at the bottom with the nylon mesh. The tube was then placed in a planting tube filled up to 7 cm with potting soil. After having added another 2 cm of potting soil to the small PVC-tube itself, the maize seeds were planted into the tube and covered again with soil. In this way, the plants developed their top root system within the PCV-tube, while the rest of the root system grew through the nylon mesh into the normal planting pot. In a second setup, we aimed at controlling for possible size- and root density effects that may have arisen from the different size of the compartments. To do so, a much bigger PVC-tube (diameter 3.8 cm, height 10 cm) was covered with a nylon mesh at the bottom, filled with soil, and slid into the planting pots to a depth of 9 cm. This created a bottom root-compartment of 2 cm (equally filled with soil), into which the roots grew down. For both setups, individual *D. virgifera* larvae were weighed and added to the different root compartments by either putting them on the top of the soil of the PCV tubes (allowing them to feed only on the upper root part) or by carefully introducing them to

the bottom of the root system through the holes in the plastic pot that were closed with aluminum foil afterwards (giving the larvae access only to the lower compartments; $n = 24$). After 7 d, the pots were emptied and the larvae retrieved and weighed again. For the third experiment, the small PCV-tube system was used again. The maize plants were infested with 62nd instar *D. virgifera* larvae released either in the top or the bottom compartment and left to feed for 48 h. Control plants were left uninfested. All plants received 10 ml of water per day ($n = 24$). The growth of *S. littoralis* larvae as well as the RWC were then determined as described earlier.

Leaf-hormones and defense marker genes

To measure the effect of root herbivory on leaf-hormones and defense-marker genes, we carried out three independent experiments. In a first experiment, we infested normally watered maize plants with six L2 *D. virgifera* larvae over a period of 48 h. Control plants were left root-herbivore free. Plants were harvested and immediately frozen in liquid nitrogen and ground to a fine powder. Leaves of six plants were pooled to obtain enough plant material for both hormone- and gene-expression analysis. In total, nine independent pools of six plants were analysed ($n = 6 \times 9$). For the hormone analysis, an aliquot of 150 mg per sample was transferred to FastPrep tubes (MP Biomedicals, Heidelberg, Germany) and mixed with 1 ml ethyl acetate containing 200 ng of D_6-ABA, D_2-JA, D_4-SA and $^{13}C_6$-JA-Ile as internal standards. The mixture was homogenized and centrifuged before transferring the supernatant to a 2 ml 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% methanol. Ten microliters of each sample were then injected into an HPLC-MS equipped with a ProntoSIL C18 Column (MAC-MOD Analytical Inc., Chadds Ford, PA, USA). The 1200L LC/MS system (Varian, Palo Alto, CA, USA) was operated at a flow rate of 0.1 ml min^{-1}. 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. The collision energy was 15 V for SA, 12 V for JA, 9 V for ABA and 19 V for JA-Ile.

For gene expression analysis, total RNA was extracted from the same leaf-pools ($n = 6 \times 9$) using Qiagen 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 manufacturer's 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 carried out using gene-specific primers (Erb *et al.*, 2009a). The q-PCR mix consisted of 5 µl Quantace Sensimix (Biolabo SA, Chatel-St-Denis, Switzerland) containing Sybr Green I, 3.4 µl H_2O, 100 nmol of each primer (2×0.3 µl H_2O) and 1 µl of cDNA sample. The Q-PCR was carried out using 45 cycles with the following temperature curve: 10 s at 95°C, 20 s at 60°C and 15 s 72°C. The final melt curve was obtained by ramping from 68°C to 98°C in 1°C steps every 5 s. To determine primer efficiencies and optimal quantification thresholds, a dilution series of a cDNA mix consisting of 4 ul 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 cycle threshold (Ct) values (using the automated threshold determination feature of the Rotor-Gene 6000 software (Biolabo SA, Chatel-St-Denis, Switzerland)) were corrected for the housekeeping gene *GapC* (Frey *et al.*, 2000) and normalized to control levels to obtain average fold changes of treated plants.

In two additional independent experiments, plants were subjected to different water regimes (drench or drought treatment, as described earlier) and either infested with six *D. virgifera* larvae or left uninfested. After 48 h of infestation, individual plants belonging to one of the four treatments were harvested and used for hormonal analysis ($n = 12$) or gene expression measurements (independent experiment; $n = 9$) as described above.

Total nitrogen and free amino acids

Because earlier studies have indicated that root-herbivore attack may alter leaf nitrogen concentrations (Gange & Brown, 1989), we measured total carbon and nitrogen contents and free amino acid concentrations of *D. virgifera* infested and uninfested plants. To determine carbon : nitrogen ratios, we used the dried plant material from the short-term *S. littoralis* performance experiment (6 h of infestation; $n = 30$) described above. The dried shoots were ground to a fine powder using a ball mill, and total carbon as well as total nitrogen were determined from 2 to 3 mg per sample using an elemental analyser. Free amino acid concentrations were measured in an independent experiment. For this, plants were subjected to two watering regimes (drench or drought treatment, as described earlier) and either infested with *D. virgifera* or left uninfested ($n = 9$). Leaves were then harvested, immediately frozen in liquid nitrogen and freeze-dried. The analysis was then

carried out following the procedure described in Knill *et al.* (2008).

Genetic and chemical inhibition of ABA biosynthesis

To test whether the observed increase in defense marker gene expression and resistance against *S. littoralis* in the leaves after root herbivore attack is dependent on ABA, we used two approaches. First, transgenic maize lines expressing *Zm-nced(vp14)* (the main regulatory gene in ABA biosynthesis) in antisense direction were compared with wild-type plants. The antisense lines have been characterized before and are known to have reduced ABA contents and inducibility without showing the strong phenotypic changes of *vp14* mutants (Voisin *et al.*, 2006). Because in the previous experiments *Zm-nced(vp14)* was only induced when water supply was limiting (see the Results section), the experiments were carried out under drought conditions (n = 8, as described earlier). For the gene expression experiment, two independently transformed lines were planted and infested with six L2 *D. virgifera* larvae for 48 h. The leaves were then harvested and immediately frozen in liquid nitrogen. Genotyping of the transgenic lines was carried out using the procedure described previously (Voisin *et al.*, 2006). Gene expression analysis was carried out as described above. For statistics, the two transformed lines were pooled (resulting in four treatment groups: controls of wild-type plants, controls of antisense plants, *D. virgifera* infested wild-type plants and *D. virgifera* infested antisense plants). In an independent experiment, wild type and antisense plants were treated as described earlier (n = 24), but were used to measure *S. littoralis* growth 6 h and 12 h after infestation using clip-cages. Leaves were harvested and genotyped after the performance experiment.

In a second approach, we treated maize seedlings with 10 mM of the ABA inhibitor sodium tungstate (Fonseca *et al.*, 2005) (n = 24). This concentration had first been determined to cause no major phenotypical changes in maize leaves under well-watered conditions and in preliminary experiments, concentrations of up to 100 mM sodium tungstate did not have any impact on *D. virgifera* performance or mortality over a feeding period of 48 h (M. Erb, unpublished). Because the inhibited plants were much more susceptible to water stress-induced wilting, plants were well watered (10 ml d^{-1}) for this experiment. *S. littoralis* growth was then measured over 6 h of feeding, and leaves were then harvested to determine their RWC.

Statistical procedures

Differences in survival of *S. littoralis* were tested using Kaplan–Meier's survival analysis of log-ranks. An ANOVA was carried out on the rest of the experiments. For pairwise comparisons, the Student's t-test was used. For experiments involving one or two classes of factors, one-way and two-way ANOVAs followed by Holm–Sidak *post hoc* tests were applied. Normality and equality of variance was verified using Kolmogorov–Smirnov and Levene's tests, respectively. Data that did not pass these tests were transformed ($\log_{10}$ + 1 or square-root). Where transformation did not resolve normality or equality of variance, nonparametric tests (ANOVA on ranks, Mann–Whitney rank sum test) were used.

Results

Root herbivory by *D. virgifera* increases leaf-resistance in the field and the laboratory

Maize plants in the field showed typical traces of first and second instar *Ostrinia nubilalis* feeding as well as damage caused by *Spodoptera frugiperda* and other lepidopteran larvae. 'Shotgun-like' holes were also found frequently, which can be caused by several herbivores including *O. nubilalis* and *Sphenophorus maidis*. *D. virgifera* infestation of the roots caused a reduction of leaf surface damage by almost 50% (Student's t-test: P = 0.033; Fig. 1a). This difference was also reflected in a significant reduction of the number of longitudinal feeding traces on leaves (Student's t-test: P = 0.021; Fig. 1b). Natural infestation by *Diabrotica virgifera* does not normally occur in the area where the experiments were conducted (no *D. virgifera* adults were found to emerge from the control plots at a later stage of the experiment), and occurrence of other *Diabrotica* species was rare (C. Zwahlen, unpublished).

In the laboratory, similar effects of *D. virgifera* on leaf-herbivore performance could be observed: Fig. 1(c) shows the average cumulative growth of the larvae (n = 15). Root infestation affected caterpillar growth significantly (ANOVA: P = 0.0196), and pairwise comparisons showed significantly lower larval weights at time-points 6 h, 12 h and 24 h (Holm–Sidak *post hoc* test: $P < 0.05$). This trend persisted over the whole observation period (Fig. 1c). Over two experimental runs, 25% of the larvae reached the pupal stage, of which 73% had been feeding on plants without the root herbivore (n = 30; Fig. 1d). The relatively low number of pupating larvae may have been the result of the high susceptibility of *S. littoralis* to maize defenses. Furthermore, the frequent handling during the weighing process may have weakened the larvae. The survival curves obtained 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). An independent experiment confirmed that caterpillar growth after 6 h of feeding was reduced on plants with *D. virgifera*-infested roots (Student's t-test: P = 0.037; see the Supporting Information, Fig. S1a), an effect that was also reflected in a reduction in leaf surface damage (Student's t-test: P = 0.046; Fig. S1b).

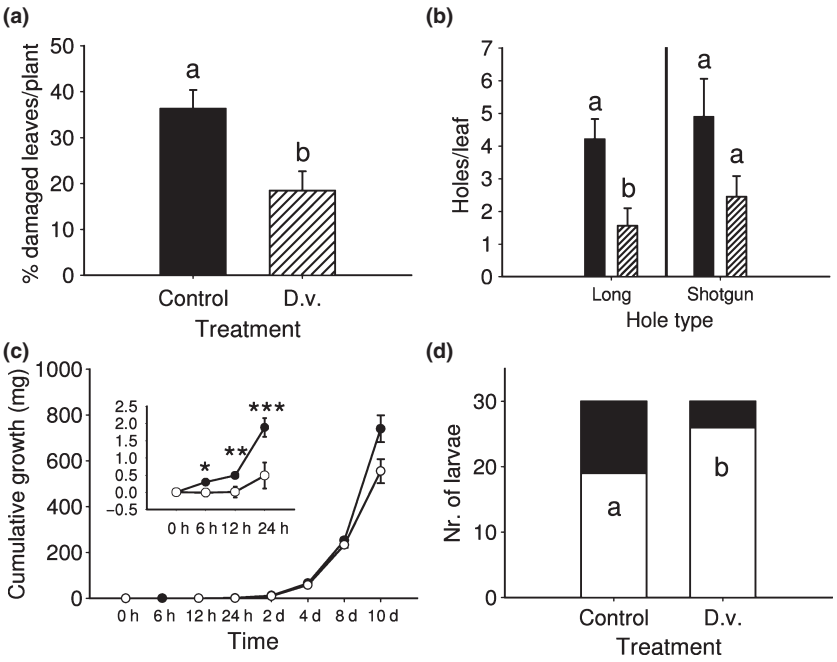

Fig. 1 Root herbivore induced resistance in the field and the laboratory. (a) Average percentage of damaged leaves per plant (+ SE) in uninfested plots (closed bars) and plots infested with *Diabrotica virgifera* (hatched bars). (b) Average number (+ SE) of longitudinal (left) and 'shotgun' holes (right) per plant. (c) Average cumulative growth (± SE) of *Spodoptera littoralis* caterpillars over 10 d of feeding on plants infested with *D. virgifera* in the roots (open circles) or uninfested control plants (closed circles) in the laboratory. Asterisks denote significant differences (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). (d) Total numbers of *S. littoralis* caterpillars reaching the pupal stage (closed) or dying (open) on infested vs uninfested plants. Different letters indicate significant differences between treatments ($P < 0.05$).

Changes in leaf water contents are required for the increase in resistance

To investigate whether the hydraulic changes imposed by the root herbivore influence the systemic resistance, we subjected maize seedlings to different water regimes and measured leaf-water contents and growth of *S. littoralis* larvae on plants with and without *D. virgifera* infestation. *Spodoptera littoralis* growth was most strongly reduced on *D. virgifera*-infested plants with low water supply (Holm–Sidak *post hoc* test: $P < 0.001$, Fig. 2a). A negative trend was still visible for normally watered plants (Holm–Sidak *post hoc* test: $P = 0.070$), while no effect was observed under the high water regime. *Diabrotica virgifera* reduced leaf-water contents under medium and low water supply ($P < 0.001$), while it had no significant impact on water contents under high water supply (Fig. 2b). The reduction of RWC by *c.* 3% resulted in visible wilting symptoms, indicating that the *D. virgifera*-infested plants were indeed water stressed under low water supply. Analysis of root dry weights showed that *D. virgifera* significantly reduced root biomass of maize seedlings (ANOVA: $P = 0.005$), but the imposed water regime had no effect on root biomass and the extent of root removal by the larvae (ANOVA: $P = 0.890$; Fig. S2).

We also tested if the exact location where *D. virgifera* feeds is important for its development and induced leaf resistance. *Diabrotica virgifera* larvae confined to the top 2 cm of the rhizosphere grew significantly more over a period of 7 d than larvae excluded from this part of the rhizosphere (Student's *t*-test: $P = 0.046$, Fig. S3a). Equally, when confined to the lowest 2 cm or the upper part of the roots, larvae feeding on the upper part grew significantly larger (Student's *t*-test: $P < 0.001$, Fig. S3b). *Diabrotica virgifera* only affected *S. littoralis* growth when they were feeding on the top 2 cm of the root system (Holm–Sidak *post hoc* test: $P = 0.003$, Fig. 2c). Similarly , shoot water contents were significantly reduced when *D. virgifera* fed on the upper root system and hypocotyl (Dunn's *post hoc* test: $P < 0.05$, Fig. 2d), while only a trend remained when the larvae fed on the lower parts.

Water supply determines induction of ABA, defense markers and free amino acids in the leaves

Under normal watering conditions, *D. virgifera* attack by six L2 larvae over a period of 4 d results in an increase in leaf ABA concentrations and expression of defense marker genes (Erb *et al.*, 2009a). Here we confirm these results and show that the effect occurs already after 48 h of infestation (Fig. S4). Of the measured phytohormones (JA, JA-Ile, SA and ABA), only ABA increased in concentration in the leaves after root herbivore attack (Fig. S4a–d; Mann-Whitney rank sum test ABA: $P > 0.05$). *Diabrotica virgifera* furthermore induced several defense markers (Student's *t*-test: $P > 0.05$) including two pathogenesis related genes, *Zm-pr1* and *Zm-pr5*, (Morris *et al.*, 1998), three proteinase inhibitors, *Zm-cysII*, *Zm-serpin*, *Zm-cyst* (Ton et al., 2007), and the regulatory gene for hydroxamic acid biosynthesis *Zm-bx1* (Frey *et al.*, 1997) (Fig. S4e; Erb *et al.*, 2009a). The hormonal measurements show that JA, JA-Ile and SA concentrations were neither affected by the root herbivore, nor by the plant water status (two-way ANOVAs; Fig. 3a–

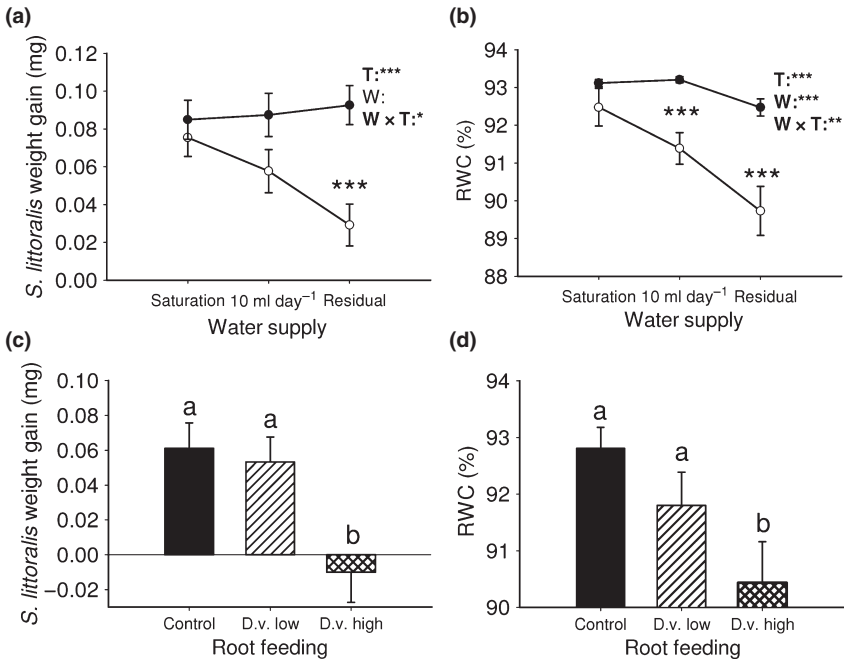

Fig. 2 Influence of water supply and belowground feeding site on root herbivore induced shoot resistance. (a) Average weight gain (+ SE) of *Spodoptera littoralis* larvae after 6 h of feeding on *Diabrotica virgifera* infested (open circles) and control plants (closed circles) under different water regimes. Saturation = soil drench (48 h); 10 ml d^{-1} = 10 ml H_2O d^{-1} (48 h); Residual = no watering (48 h). Asterisks denote significant differences (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). (b) Average relative water content (+ SE) of maize shoots infested in the roots with *D. virgifera* (closed circles) and control plants (open circles) under different water regimes. (c) Average weight gain (+ SE) of *S. littoralis* larvae after 6 h of feeding on control plants (closed bars), plants infested with *D. virgifera* confined to the upper 2 cm of the soil (cross-hatched bar) or the lower part of the roots (hatched bar). All plants received 10 ml^{-1} of water d^{-1}. (d) Average shoot water contents (+ SE) of plants infested with *D. virgifera* on upper and lower parts of the roots. All plants received 10 ml^{-1} of water d^{-1}. Different letters indicate significant differences between treatments ($P < 0.05$).

c). By contrast, ABA was affected by both water status (ANOVA: $P = 0.036$) and *D. virgifera* feeding (ANOVA: $P = 0.012$) and there was a strong interaction between the two stresses (ANOVA: $P = 0.032$): ABA was most strongly induced by *D. virgifera* when the plants were not watered over the 48 h of infestation. Average concentrations increased to 160 ng g^{-1} FW under this condition, which is *c.* 40 times the concentration of control plants. Interestingly, this effect was almost completely absent under excess water supply (Fig. 3d). The ABA concentrations were only weakly elevated in the unwatered controls, indicating that the watering regime by itself did not heavily stress the plants. The systemic induction of defense markers by *D. virgifera* was affected by the plant's water supply: *Zm-pr10*, *Zm-serpin* and *Zm-bx1* were more strongly induced under water limiting conditions (ANOVA $P < 0.05$). *Zm-cysII* was more responsive when the plants were well watered, while the induction of *Zm-pr1*, *Zm-pr5* and *Zm-cyst* was not influenced by the plant's water status (Fig. 3e). The most pronounced reaction was measured for the gene that regulates ABA biosynthesis in maize: *Zm-nced(vp14)*. In accordance with ABA content measurements (Fig. 3d), *Zm-nced(vp14)* was induced by *D. virgifera* much more strongly when the plants were water stressed (Fig. 3e).

The carbon : nitrogen ratio analyses showed no difference between the treatments (*t*-test: $P > 0.05$; Fig. 4a). Free amino acid (AS) patterns were unchanged under high water supply. conversely, several measured AS increased in concentration when the plants were infested by *D. virgifera* under low water supply (two-way ANOVAs; interaction herbivory × water: $P < 0.05$; Fig. 4b).

ABA affects the induction of defense markers, but not induced resistance

The transcriptional profiling confirmed that *Zm-nced(vp14)* was suppressed in the antisense lines, whereas it was induced after root attack in the wild-type plants (Fig. 5a). The marker genes *Zm-cysII*, *Zm-cyst* and *Zm-bx1* were not induced by *D. virgifera* in the antisense plants (two-way ANOVAs: genotype × treatment interaction, $P < 0.05$). Other genes, including *Zm-pr10* and *Zm-serpin* were induced similarly in the transgenic and control plants (Fig. 5a). Root removal by *D. virgifera* on antisense plants was the same as for wild-type plants (Fig. S5) and induced shoot resistance against *S. littoralis* (reduced growth) was similar for both plant types after 6 h and 12 h of feeding (Figs 5b–c). These results imply that the induction of *Zm-nced(vp14)* upon root

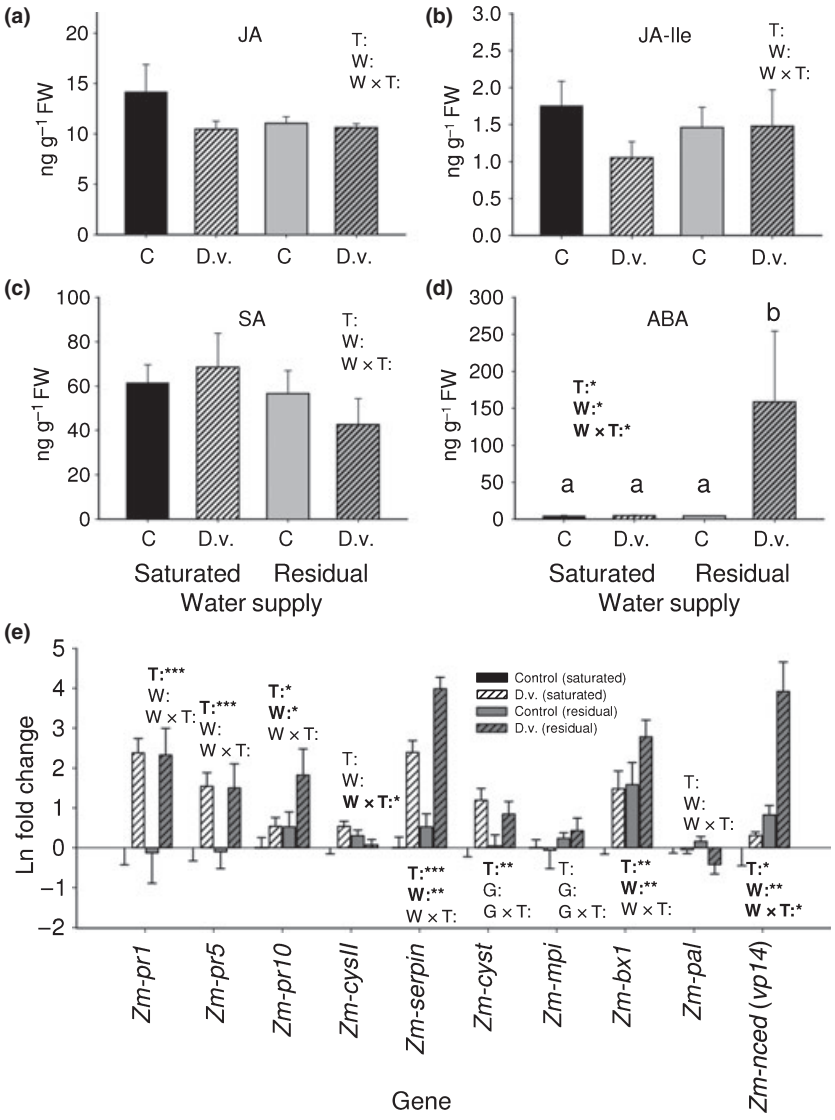

Fig. 3 Influence of root herbivory and water stress on shoot phytohormone levels and defense gene expression. Average shoot concentrations (+ SE) of jasmonic acid (JA) (a), JA-Ile (b), salicylic acid (SA) (c) and ABA (d) upon root stress are shown. Hatched bars indicate *Diabrotica virgifera* infested roots. The left bars (open and closed) show concentrations for well-watered plants, while the right bars (tinted) indicate plants with low water supply. Different letters indicate significant differences between the treatments ($P < 0.05$). Significance levels are also shown for two-way ANOVAS (T = herbivore infestation; W = water treatment; T × W = interaction). Asterisks denote significant ANOVA effects (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). (e) Expression levels (Ln fold change + SE relative to well-watered controls) of defense marker genes upon stress treatments. Different letters indicate significant differences between the treatments ($P < 0.05$).

herbivore attack under water limiting conditions was not responsible for the observed increase in resistance.

This was also confirmed by the experiment involving chemical inhibition of ABA biosynthesis. 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 losing their capacity to remain upright. This observation was reflected in a two-way ANOVA of relative water contents showing significant effects of *D. virgifera* and sodium tungstate as well as an interaction (ANOVA: $P = 0.034$). As shown in Fig. S6(a), *D. virgifera* infested plants suffered much more from water stress when treated with the ABA inhibitor. *Diabrotica virgifera* feeding again reduced growth of *S. littoralis* (ANOVA: $P = 0.010$), the effect being even more pronounced in ABA-inhibited plants (Holm–Sidak *post hoc* test: $P = 0.004$) than in untreated plants, where only a trend was visible in this assay (Fig. S6b).

Discussion

Our results reveal different mechanisms that lead to systemic changes in aboveground tissues upon belowground herbivory. First, *D. virgifera* larvae induce defenses aboveground independently of the plant's water status. This is illustrated in Fig. 3(e), which shows that several defense marker genes including the serine protease *Zm-serpin* and the pathogenesis-related genes *Zm-pr1* and *Zm-pr5* are induced under high as well as low water supply. Second, water supply can be an important factor influencing the induction of leaf defense by *D. virgifera*. This involves the upregulation of ABA (Fig. 3d) and increased expression of a number of marker genes including the regulatory gene for ABA biosynthesis, *Zm-nced(vp14)* (Tan *et al.*, 1997) and *Zm-bx1* (Fig. 3e), which codes for a gene implicated in the biosynthesis of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a well-known antifeedant of

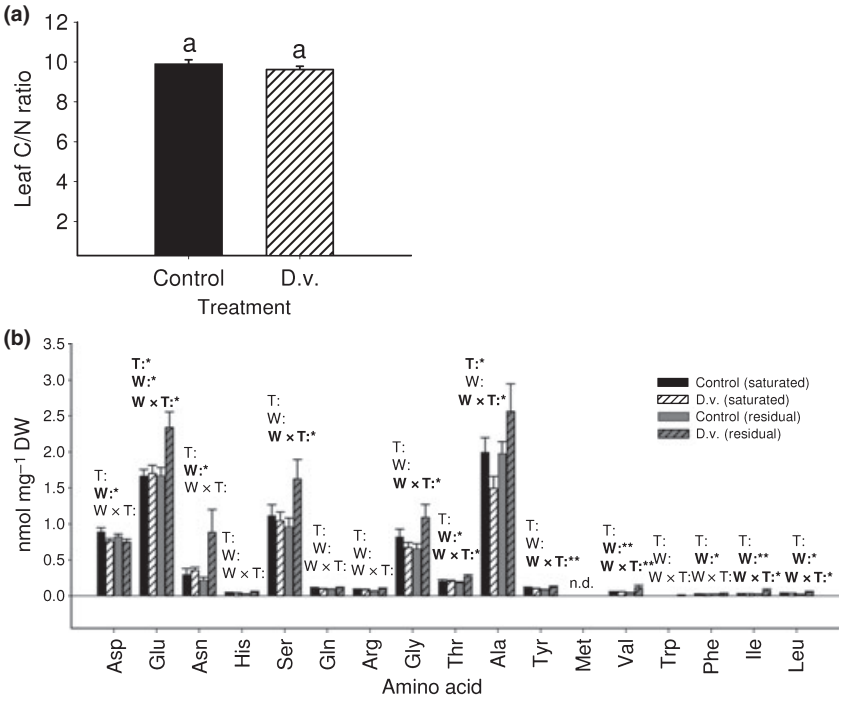

Fig. 4 Influence of root herbivory on C : N ratios and free amino acids. (a) Average carbon (C) : nitrogen (N) ratios (+ SE) of maize shoots infested in the roots with *Diabrotica virgifera* (hatched bar) and control plants (closed bar) under normal water supply. Different letters denote significant differences between treatments ($P < 0.05$). (b) Average concentration of 17 free amino acids (+ SE) in root-stressed plants. Hatched bars indicate *D. virgifera* infested roots. The left bars (open and closed) show concentrations for well-watered plants, while the right bars (tinted) indicate plants with low water supply. Significance levels are shown for two-way ANOVAs (T = herbivore infestation; W = water treatment; T × W = interaction). Asterisks denote significant ANOVA effects (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

maize (Frey *et al.*, 1997). Changes in free amino acids were also dependent on the plant's water status (Fig. 4b). Third, several, but not all of the *D. virgifera*-induced marker genes seem to be dependent on water-stress induced ABA. The induction of *Zm-bx1*, for example, is absent in *Zm-nced (vp14)* antisense plants (Fig. 5a). This fits well with earlier findings showing that DIMBOA is induced by *D. virgifera* and application of exogenous ABA (Erb *et al.*, 2009a,b). Interestingly, the expression of two putative cystatin protease genes, *Zm-cyst* and *Zm-cysII*, was reduced in the antisense plants (Fig. 5a), but not specifically induced under water stress (Fig. 3e). This suggests that they are positively regulated by ABA, but suppressed by an additional signal that is specifically present under water stress conditions. A number of genes including *Zm-pr1*, *Zm-pr5* and *Zm-pal* seem to follow this same pattern (Fig. 5a). Overall, our experiments demonstrate the important, but not exclusive role of water stress and ABA-signaling for *D. virgifera* induced changes in leaf defense.

The increase in resistance against *S. littoralis* was closely related to the changes in relative leaf water contents after root herbivory, as is evident from Fig. 2, where it is shown that the weight gain of the larvae is considerably reduced when *D. virgifera* has a strong negative impact on the water supply of the maize plant. As the induction of ABA and ABA-dependent defenses is most pronounced under water-limiting conditions (Fig. 3d–e), ABA was expected to be responsible for the increased resistance. Evidence for its role comes for example from research on *Arabidopsis thaliana*, for which it has been found that ABA-deficient mutants are highly susceptible to *S. littoralis* (Bodenhausen & Reymond, 2007). Yet, our results strongly suggest that ABA is not required for root herbivore induced shoot resistance in maize. This is most evident from the fact that the induction of resistance by *D. virgifera* also occurred after genetic or chemical inhibition of ABA signaling (Figs 5b–c and S6). We therefore postulate that ABA-independent hydraulic changes are the causal factor in *D. virgifera* induced shoot resistance in maize. The upset water balance causes reduced leaf-turgor, which may directly impair feeding by *S. littoralis* larvae: The larvae normally display so called 'windowpane-feeding', where the epidermis of only one side of the leaf is ingested together with the inner parenchyma tissue. This enables the herbivore to gain access to the easily digestible inner cell layers, while avoiding the tough cuticle and epidermal layers of the other leaf-side. Our experiments show that under heavy leaf-water stress caused by *D. virgifera*, this feeding strategy is no longer possible and *S. littoralis* larvae have to ingest both epidermal layers and cuticles. This effect is independent of ABA signaling, as it can be observed in both wild-type and ABA-impaired plants (M. Erb, pers. obs.). Apart from such mechanical effects, the experiments also demonstrate that certain defense markers like *Zm-pr10* are induced by *D. virgifera* imposed water stress in an ABA-independent manner (Figs 3e and 5a). Some defenses are thus specifically responsive to ABA-independent hydraulic changes. *Spodoptera littoralis* may be particularly sensitive to

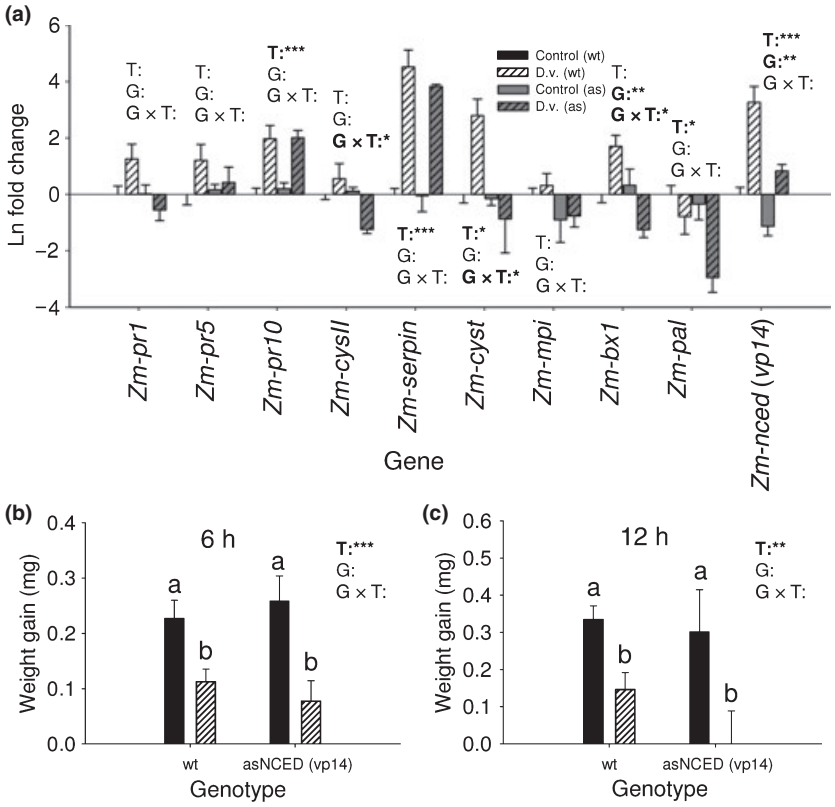

Fig. 5 The role of the ABA-biosynthesis gene *Zm-nced(vp14)* on root-herbivore induced shoot defenses. Wild-type (*wt*) and antisense lines (asNCED(vp14)) were tested under low water supply. (a) Ln fold change (+ SE) of defense marker genes for the different treatments and genotypes. Significance levels are shown for two-way ANOVAS (T = herbivore infestation; G = genotype; T × G = interaction). Asterisks denote significant ANOVA effects (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). Average weight gain (+ SE) of *Spodoptera littoralis* larvae after 6 h (b) and 12 h (c) of feeding on *Diabrotica virgifera* infested (hatched bars) and control plants (closed bars) is shown. Different letters indicate significant differences between treatments ($P < 0.05$).

these effectors, and further research could aim at characterizing them in more detail.

The finding that hydraulic changes are responsible for the increase in leaf resistance is of potential importance for a variety of induced resistance phenomena. Numerous root herbivores change the water balance of aboveground plant parts (Gange & Brown, 1989; Murray & Clements, 1998; Blossey & Hunt-Joshi, 2003; Staley *et al.*, 2008), and the involvement of water stress in changes in aboveground resistance has been proposed in early models of aboveground–belowground interactions (Masters *et al.*, 1993). Depending on the feeding strategy of the leaf herbivore, such changes can either increase or decrease plant resistance. Phloem feeding aphids, for example, may benefit from the increased AS concentrations in leaves under water stress (Fig. 4b), whereas chewing herbivores are negatively affected by the increased defenses (Huberty & Denno, 2004).

Our experiments also suggest that studies conducted in the laboratory or the glasshouse may underestimate the systemic effects of insect infestation, as these effects may depend on slight changes in abiotic factors such as water supply. In nature, plants are continuously exposed to various mild stress events, and our data clearly suggest that these fluctuations should be taken into account when looking at induced resistance phenomena. Highly sensitive methods that capture the plant's water status beyond relative water contents may contribute to unraveling the importance of hydraulic conductivity in induced resistance in more detail.

The adaptive value of root herbivore-induced shoot resistance has remained unresolved (Wäckers & Bezemer, 2003). The current study favors the hypothesis that RISR may be the result of a plant physiological constraint. The later larval stages of *D. virgifera* larvae often attack the upper root system (Strnad & Bergman, 1987; Hibbard *et al.*, 2008), which we found to be the site where the larvae develop much better (Fig. S3). For the plant, this feeding behavior poses a significant threat to its water supply (Fig. 2b), especially at early developmental stages of the seedling, when the root system relies on few connective elements. The increase in ABA biosynthesis following belowground attack seems to be a tolerance response of the plant to reduce the negative effects of water loss. Under conditions where the metabolic and physiological changes are not sufficient, water concentrations in the shoot decrease nevertheless (Fig. 2b), sometimes even to a point where acute wilting occurs. It is under these circumstances that the aboveground herbivore *S. littoralis* is most negatively affected (Fig. 2a,c). 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. Interestingly, the root herbivore *D. virgifera* seems to benefit from feeding on the most

vulnerable part of the root system (Fig. S3a,b). Whether this is only because of 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. It is known that plants under water stress increase their investment in root growth (Reid & Renquist, 1997), and it is possible that *D. virgifera* directly profits from this. Another exciting option that deserves further attention is a possible manipulation by the root herbivore to increase phloem transport of leaf assimilates for its own benefit, a phenomenon known for parasitic root-feeding nematodes (Caillaud *et al.*, 2008). It seems unlikely that *D. virgifera* manipulates the plant's water balance to fend off aboveground competitors, as this effect depends on environmental conditions and may not be very efficient against nonlepidopteran leaf-feeders. Therefore, the results suggest that the increase in leaf resistance is neither intentionally initiated by *D. virgifera* nor by its host plant, but rather the indirect result of their intimate interaction and the physiological struggle of the plant to optimize its chances of surviving the attack.

Conclusions

Root attack by *D. virgifera* has a profound impact on the shoot physiology of maize plants, thereby causing enhanced resistance against aboveground herbivores. The most important effect leading to this change in resistance is the water stress imposed by the root herbivore. Herbivore-induced hydraulic changes and the subsequent tolerance response of the plant should be considered as an additional factor contributing to a systemic increase in plant resistance.

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Supporting Information

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

Fig. S1 Influence of root herbivory on short-term growth and consumption of *Spodoptera littoralis*.

Fig S2 Influence of water stress and root herbivory on root biomass.

Fig. S3 Growth of *Diabrotica virgifera* confined to different parts of the root system.

Fig. S4 Influence of root herbivory on leaf phytohormones and defense gene expression.

Fig. S5 Influence of plant genotype on root biomass removal by *Diabrotica virgifera*.

Fig. S6 Impact chemical ABA inhibition on root herbivore induced resistance and water contents.