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

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1 **Summary**

2 • Herbivore induced systemic resistance occurs in many plants and is commonly

3 assumed to be adaptive. The mechanisms triggered by leaf-herbivores that lead to

4 systemic resistance are largely understood, but it remains unknown how and why

5 root herbivory increases resistance in leaves.

6 • To resolve this, we investigated the mechanism by which the root herbivore

7 *Diabrotica virgifera* induces resistance against lepidopteran herbivores in the

8 leaves of *Zea mays*.

9 • *D. virgifera* infested plants suffered less aboveground herbivory in the field and

10 showed reduced growth of *Spodoptera littoralis* caterpillars in the laboratory.

11 Root herbivory did not lead to a jasmonate-dependent response in the leaves, but

12 specifically triggered water loss and abscisic acid (ABA) accumulation. The

13 induction of ABA by itself was partially responsible for the induction of leaf

14 defenses, but not for the resistance against *S. littoralis*. Root-herbivore induced

15 hydraulic changes in the leaves on the other hand were crucial for the increase in

16 insect resistance.

17 • We conclude that the induced leaf resistance after root feeding is the result of

18 hydraulic changes, which reduce the quality of the leaves for chewing herbivores.

19 This finding calls into question whether root-herbivore induced leaf-resistance is

20 an evolved response.

21

22 Key-words: Above-belowground interactions, *Zea mays, Diabrotica virgifera,*

23 *Spodoptera littoralis*, induced resistance, abscisic acid, water stress

24

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 partially unravelled. After pathogen attack for example, non-infested leaves become more resistant against other pathogens, a phenomenon termed systemic acquired resistance (SAR). SAR 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 lead 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 been implicated in SAR (Jung *et al.*, 2009).

Upon mechanical damage or leaf-attack by herbivores, plants activate their defenses in uninfested leaves as well (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).

1   Compared to these well described effects, virtually nothing is known about what

2   causes an increase in leaf defense and resistance upon root attack by herbivorous insects

3   and *vice versa* (Bezemer *et al.*, 2003; Wäckers & Bezemer, 2003; van Dam *et al.*, 2005;

4   Erb *et al.*, 2009a; Erb *et al.*, 2009b). Root herbivore induced shoot resistance (RISR)

5   seems to be a common and abundant phenomenon with important consequences for

6   multitrophic interactions and ecosystem dynamics (van der Putten *et al.*, 2001; Bardgett

7   & Wardle, 2003; Soler *et al.*, 2005; Soler *et al.*, 2007; Kaplan *et al.*, 2008). It has been

8   proposed that RISR could be a WIR-like phenomenon extending from the roots to the

9   leaves or a priming effect similar to ISR (Erb *et al.*, 2008). Early work on the impact of

10   root herbivores on shoot resistance also led to the hypothesis that changes in plant water

11   balance may lead to altered performance of aboveground herbivores (Masters *et al.*,

12   1993).

13   While systemic induced resistance in the leaves is commonly thought to be

14   adaptive for the plant (Heidel & Dong, 2006; Walling, 2009), as the same attacker is

15   likely to feed on different leaves over time, the situation is much less clear for RISR.

16   Why would plants increase their leaf- resistance after root attack? Wäckers et al. (2003)

17   proposed three explanations for increased shoot defenses upon root herbivory: 1) plant

18   adaptation to an increased likelihood of aboveground herbivory after root attack, 2) root

19   herbivore manipulation to mobilize defenses against competing aboveground herbivores,

20   or 3) increased shoot defenses as a consequence of a plant physiological constraint. So

21   far, none of these hypotheses have been explicitly tested. Particularly the lack of

22   knowledge about the physiological basis of RISR has hampered efforts to elucidate its

1   adaptive value (Wäckers & Bezemer, 2003) and possible ecological importance (Wardle

2   *et al.*, 2004).

3   In maize, RISR has been shown to be effective against both herbivores and

4   pathogens (Erb *et al.*, 2009a). An induction of abscisic acid (ABA) and a reduction of

5   leaf-water contents has been observed in this system (Erb *et al.*, 2009a), leading to the

6   hypothesis that hydraulic changes and/or ABA-signalling might mediate the increase in

7   resistance. However, as for other cases of RISR, the causal factors linking the resistance

8   phenotype to the physiological changes have remained unclear. By altering root-water

9   supply and ABA-biosynthesis, the current study aims at unravelling the relative

10   contribution of ABA and water loss for RISR in maize. Combined with results from

11   behavioural assays and field experiments, the molecular and chemical data presented here

12   show that root-herbivore induced leaf-resistance is mediated by changes in the plant's

13   water balance and therefore may not be an evolved plant defense response.

14

15

Methods

Field experiment

To determine the influence of root infestation on leaf-herbivore resistance in the field, twelve plots (plot dimensions: 9.3x3.7m, 56 plants, two rows) of maize (*Zea mays*; variety 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 (Zwahlen *et al.*, unpublished). Two weeks after planting, eight plots were infested with *D. virgifera* by applying 11'800 eggs over a distance of 3.6m to one row per plot. Taking into account a viability of 75%, this equaled about 400 viable eggs/ 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 and Gray, 2006). At the beginning of July, one month after application of the eggs when the *D. virgifera* larvae had reached their second instar and maize plants had 6-7 fully developed leaves (growth stage V8), the 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. Encountered herbivores 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.

1   *Laboratory experiments- plants and insects*

2   To further investigate the mechanism underlying root herbivore induced changes

3   in leaf-resistance, additional experiments were carried out in the laboratory. Maize plants

4   were grown in bottom-pierced, aluminium-wrapped plastic pots (diameter, 4cm; depth,

5   11cm) in a phytotron (23±1°C, 60% r.h., 16:8 hr L/D, and 50,000 lm/m^2). Before

6   planting, the seeds were rinsed with water to remove any storage residuals and, unless

7   mentioned otherwise, sown in sand (lower 8 cm) topped with commercial potting soil

8   (upper 3 cm, Ricoter Aussaaterde, Aarberg, Switzerland). Plants used for experiments

9   had two fully expanded primary leaves and were 9-10 days old. Plants were watered with

10   10ml of tap water every day until the beginning of the experiments. All experiments were

11   carried out under light benches in a climatized laboratory (25±2°C, 40±10% r.h., 16:8 hr

12   L/D, and 8000 lm/m^2). *S. littoralis* eggs were provided by Syngenta (Stein, Switzerland)

13   and larvae were reared on artificial diet as described (Turlings *et al.*, 2004). *D. virgifera*

14   eggs and larvae were obtained from CABI Delémont (Switzerland) and from the USDA-

15   ARS-NCARL Brookings (US) and kept on freshly germinated maize seedlings until use.

16

17   *Leaf-herbivore performance experiments*

18   To determine the dynamics of *D. virgifera*-induced changes in leaf-herbivore

19   resistance, we measured the growth, survival and leaf-consumption of *S. littoralis*

20   caterpillars in three independent experiments. For the experiments, maize plants were

21   either left uninfested (controls) or were infested with 6 L2 *D. virgifera* larvae by placing

22   them on the soil with a fine brush (n=15). The root herbivores were then left to feed on

23   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 about $0.5cm^2$ 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 and 12 h

of feeding to ensure ample food supply. After 24 h, the caterpillars were directly placed

on the plant to feed freely for the rest of the experiment. To stop the larvae from

escaping, PET-tubes (30cm height, conal shape, top-diameter: 8cm) were put over the

plants and attached to the pots with parafilm. They were covered by a fine nylon mesh

(0.3mm 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 analyzed 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 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

1   leaf-resistance, we subjected maize seedlings to different water regimes and measured

2   leaf-water contents and growth of *S. littoralis* larvae. For this experiment, maize

3   seedlings were either left root-herbivore free or were infested with *D. virgifera* as

4   described above (n=24). Infested and uninfested plants were then divided into three

5   watering regimes: One third of the plants received no water over the 48h of root-

6   herbivore infestation. This resulted in a gradual drying of the soil. No phenotypical

7   changes in the leaves were observed, indicating only mild water limitation. Another third

8   of the plants received normal watering (10ml/day), and one third was supplied with water

9   *ad libitum* by placing the pots in a tray with a shallow layer of water at the bottom. The

10   water was taken up to saturation through the bottom holes in the pot, resulting in

11   constantly elevated soil humidity. All the plants grew normally in this case as well. After

12   48 h, *S. littoralis* growth was measured for the 6 treatment combinations over 6 h of

13   feeding as described above. Leaves were then harvested and weighed immediately to

14   determine their fresh weight (FW). Dry weight (DW) was determined after drying them

15   for 48 h at 80°, and relative water contents (RWC) were determined using the formula

16   RWC=100-(FW-DW/FW*100). Constant turgid weight was used in the calculations, as

17   the measured leaves were of equal growth stage and quality in the different treatments.

18   Roots were washed, harvested and their DW was determined as described.

19

20   ***Influence of root-feeding location***

21   Because *D. virgifera* larvae were often observed to feed on the hypocotyl and just

22   below on the primary roots of maize seedlings, we tested the effect of this behaviour on

23   root herbivore growth and leaf-resistance. To be able to confine *D. virgifera* to different

1   parts of the belowground tissues, we used fine nylon screens (mesh size: 0.3mm). Roots

2   of maize plants penetrated the nets easily, as the fine root tips could grow through and

3   could then stretch and expand the mesh as they thickened. The belowground herbivores

4   on the other hand, at least at the L2 larval stage used here, were not able to move through

5   the screen. Three experiments were performed using this method: In the first experiment,

6   a small PVC tube (2cm diameter, 4cm height) was covered at the bottom with the nylon

7   mesh. The tube was then placed in a planting tube filled up to 7 cm with potting soil.

8   After having added another 2cm of potting soil to the small PVC-tube itself, the maize

9   seeds were planted into the tube and covered again with soil. Like this, the plants

10   developed their top root system within the PCV-tube, while the rest of the root system

11   grew through the nylon mesh into the normal planting pot. In a second setup, we aimed at

12   controlling for possible size- and root density effects that may have arisen from the

13   different size of the compartments. To do so, a much bigger PVC-tube (diameter 3.8cm,

14   height 10cm) was covered with a nylon mesh at the bottom, filled with soil, and slid into

15   the planting pots to a depth of 9 cm. This created a bottom root-compartment of 2cm

16   (equally filled with soil), into which the roots grew down. For both setups, individual *D.*

17   *virgifera* larvae were weighed and added to the different root compartments by either

18   putting them on the top of the soil of the PCV tubes (allowing them to feed only on the

19   upper root part) or by carefully introducing them to the bottom of the root system through

20   the holes in the plastic pot that were closed with aluminium foil afterwards (giving the

21   larvae access only to the lower compartments; n=24). After 7 days, the pots were emptied

22   and the larvae retrieved and weighed again. For the third experiment, the small PCV-tube

23   system was used again. The maize plants were infested with 6 2nd instar *D. virgifera*

1 larvae released either in the top or the bottom compartment and left to feed for 48 h.

2 Control plants were left uninfested. All plants received 10ml of water per day (n=24).

3 The growth of *S. littoralis* larvae as well as the RWC were then determined as described

4 above.

5

6 ***Leaf-hormones and defense marker genes***

7   To measure the effect of root herbivory on leaf-hormones and defense-marker

8 genes, we carried out 3 independent experiments. In a first experiment, we infested

9 normally watered maize plants with 6 L2 *D. virgifera* larvae over a period of 48h.

10 Control plants were left root-herbivore free. Plants were harvested and immediately

11 frozen in liquid nitrogen and ground to a fine powder. Leaves of 6 plants were pooled to

12 obtain enough plant material for both hormone- and gene expression analysis. In total,

13 nine independent pools of 6 plants were analyzed (n=6x9). For the hormone analysis, an

14 aliquot of 150 mg per sample was transferred to FastPrep tubes and mixed with 1 ml

15 ethylacetate containing 200ng of D_6-ABA, D_2-JA, D_4-SA and $^{13}C_6$-JA-Ile as internal

16 standards. The mixture was homogenized and centrifuged before transferring the

17 supernatant to a 2 ml Eppendorf tube. After repeating the extraction procedure and

18 combining the supernatants, the solvent was evaporated in a vacuum concentrator and the

19 pellet redissolved in 70% MeOH. Ten µl of each sample were then injected into an

20 HPLC-MS equipped with a ProntoSIL C18 Column. The 1200L LC/MS system (Varian,

21 Palo Alto, CA, USA) was operated at a flow rate of 0.1 ml/min. A mobile phase

22 composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in

23 acetonitrile) was used in gradient mode for separation. The compounds were detected in

1   the ESI negative mode. Molecular ions (M–H) with *m/z* 137, 209, 263 and 322 for SA,

2   JA, ABA and JA-Ile and 141, 213, 269 and 328 for the respective internal standards were

3   fragmented and daughter ions 93, 59 153 and 130 (compounds) and 97, 59, 159 and 136

4   (internal standards) were recorded for quantification. Collision energy was 15V for SA,

5   12V for JA, 9V for ABA and 19V for JA-Ile.

6   For gene expression analysis, total RNA was extracted from the same leaf-pools

7   (n=6x9) using Quiagen RNA-Easy extraction kits following the manufacturer's

8   instructions. The quality of the RNA was assessed by photometry and gel electrophoresis.

9   To remove contaminant genomic DNA, all samples were treated with Ambion DNAse

10   following the manufacturer's protocol. cDNA was then synthesized using Invitrogen

11   Super-Script III reverse transcriptase according to the manufacturer's instructions.

12   Quantitative reverse transcriptase real time polymerase chain reactions (q-PCR) were

13   carried out using gene-specific primers (Erb *et al.*, 2009a). The q-PCR mix consisted of

14   5ul Quantace Sensimix containing Sybr Green I, 3.4ul H_2O, 100nmol of each primer

15   (2x0.3ul H_2O) and 1ul of cDNA sample. Q-PCR was carried out using 45 cycles with the

16   following temperature curve: 10s 95°C, 20s 60°, 15s 72°. The final melt curve was

17   obtained by ramping from 68 to 98°C in 1°C steps every 5s. To determine primer

18   efficiencies and optimal quantification thresholds, a dilution series of a cDNA mix

19   consisting of 4ul solution from every sample was created. Six 10-fold dilution steps were

20   carried out and the standard curve was included into every q-PCR run. The final obtained

21   Ct values (using the automated threshold determination feature of the Rotor-Gene 6000

22   software) were corrected for the housekeeping gene GapC (Frey *et al.*, 2000) and

23   normalized to control levels to obtain average fold changes of treated plants.

1   In two additional independent experiments, plants were subjected to different

2   water regimes (drench or drought treatment, as described above) and either infested with

3   6 *D. virgifera* larvae or left uninfested. After 48 h of infestation, individual plants

4   belonging to one of the 4 treatments were harvested and used for hormonal analysis

5   (n=12) or gene expression measurements (independent experiment; n=9) as described

6   above.

7

8   *Total nitrogen and free amino acids*

9   Because earlier studies have indicated that root-herbivore attack may alter leaf-

10   nitrogen concentrations (Gange & Brown, 1989), we measured total carbon and nitrogen

11   contents and free amino acid concentrations of *D. virgifera* infested and uninfested

12   plants. To determine C/N ratios, we used the dried plant material from the short-term *S.*

13   *littoralis* performance experiment (6h of infestation; n=30) described above. The dried

14   shoots were ground to a fine powder using a ball mill, and total carbon (C) as well as

15   total nitrogen (N) were determined from 2-3 mg/ sample using an elemental analyzer.

16   Free amino acid concentrations were measured in an independent experiment. For this,

17   plants were subjected to two watering regimes (drench or drought treatment, as described

18   above) and either infested with *D. virgifera* or left uninfested (n=9). Leaves were then

19   harvested, immediately frozen in liquid nitrogen and freeze-dried. The analysis was then

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

21

22   *Genetic and chemical inhibition of ABA biosynthesis*

1   To test whether the observed increase in defense marker gene expression and

2   resistance against *S. littoralis* in the leaves after root herbivore attack is dependent on

3   ABA, we used two approaches: First, transgenic maize lines expressing *Zm-nced(vp14)*

4   (the main regulatory gene in ABA biosynthesis) in antisense direction were compared

5   with wild type plants. The antisense lines have been characterized before and are known

6   to have reduced ABA contents and inducibility without showing the strong phenotypic

7   changes of *vp14* mutants (Voisin *et al.*, 2006). Because in the previous experiments, *Zm-*

8   *nced(vp14)* was only induced when water supply was limiting (see results), the

9   experiments were carried out under drought conditions (n=8; as described above). For the

10   gene expression experiment, two independently transformed lines were planted and

11   infested with 6 L2 *D. virgifera* larvae for 48 h. The leaves were then harvested and

12   immediately frozen in liquid nitrogen. Genotyping of the transgenic lines was carried out

13   using the procedure described previously (Voisin *et al.*, 2006). Gene expression analysis

14   was carried out as described above. For statistics, the two transformed lines were pooled

15   (resulting in 4 treatment groups: Controls of wild type plants, controls of antisense plants,

16   *D. virgifera* infested wildtype plants and *D. virgifera* infested antisense plants). In an

17   independent experiment, wild type and antisense plants were treated as described above

18   (n=24), but were used to measure *S. littoralis* growth 6h and 12h after infestation using

19   clip-cages as described. Leaves were harvested and genotyped after the performance

20   experiment.

21   In a second approach, we treated maize seedlings with 10mM of the ABA

22   inhibitor sodium tungstate (Fonseca *et al.*, 2005) (n=24). This concentration had first

23   been determined to cause no major phenotypical changes in maize leaves under well-

1   watered conditions and in preliminary experiments, concentrations of up to 100mM

2   sodium tungstate did not have any impact on *D. virgifera* performance or mortality over a

3   feeding period of 48 h (M. Erb, unpublished). Because the inhibited plants were much

4   more susceptible to water-stress induced wilting, plants were well watered (10ml/day) for

5   this experiment. *S. littoralis* growth was then measured over 6h of feeding as described,

6   and leaves were then harvested to determine their RWC.

7

8   ***Statistical procedures***

9   Differences in survival of *S. littoralis* were tested using Kaplan-Meier's Survival

10   Analysis of Log-Ranks. Analysis of variance (ANOVA) was carried out on the rest of the

11   experiments. For pairwise comparisons, the Student's t-Test was used. For experiments

12   involving one or two classes of factors, one-way and two-way ANOVAs followed by

13   Holm-Sidak Post-Hoc tests were applied. Normality and equality of variance was verified

14   using Kolmogorov-Smirnov and Levene's tests, respectively. Data that did not pass these

15   tests were transformed ($\log_{10}+1$ or square-root). Where transformation did not resolve

16   normality or equality of variance, non-parametric tests (ANOVA on ranks, Mann-

17   Whitney rank sum test) were used.

18

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 *D. 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. 1c shows the average cumulative growth of the larvae (n=15). Root infestation affected caterpillar growth significantly (ANOVA: p=0.0196), and pair-wise comparisons showed significantly lower larval weights at time-points 6h, 12h and 24h (Holm-Sidak Post-Hoc Test: p<0.05). This trend persisted over the whole observation period (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 obtained survival curves

showed a significant difference between the treatments, with caterpillars on *D. virgifera* infested plants having a reduced chance of reaching the pupal stage (Log-Rank Test: p=0.036). An independent experiment confirmed that caterpillar growth after 6h of feeding was reduced on plants with *D. virgifera*-infested roots (Student's T-test: p=0.037; Fig. S1a), an effect that was also reflected in a reduction in leaf surface damage (Student's t-test: p=0.046; Fig. S1b).

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. *S. 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. *D. 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 around 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).

1   We also tested if the exact location where *D. virgifera* feeds is important for its

2   development and induced leaf resistance. *D. virgifera* larvae confined to the top 2 cm of

3   the rhizosphere grew significantly more over a period of 7 days than larvae excluded

4   from this part of the rhizosphere (Student's T-Test: p=0.046, Fig. S3a). Equally, when

5   confined to the lowest 2 cm or the upper part of the roots, larvae feeding on the upper

6   part grew significantly larger (Student's T-Test: p<0.001, Fig. S3b). *D. virgifera* only

7   affected *S. littoralis* growth when they were feeding on the top 2 cm of the root system

8   (Holm-Sidak Post-Hoc Test: p=0.003, Fig. 2c). Similarly, shoot water contents were

9   significantly reduced when *D. virgifera* fed on the upper root system and hypocotyl

10   (Dunn's Post-Hoc Test: p<0.05, Fig. 2d), while only a trend remained when the larvae

11   fed on the lower parts.

12

13   ***Water supply determines induction of absisic acid, defense markers and free amino***

14   ***acids in the leaves***

15   Under normal watering conditions, *D. virgifera* attack by 6 L2 larvae over a

16   period of 4 days results in an increase of leaf-ABA levels and expression of defense

17   marker genes (Erb *et al.*, 2009a). Here we confirm these results and show that the effect

18   occurs already after 48 h of infestation (Fig. S4). Of the measured phytohormones (JA,

19   JA-Ile, SA and ABA), only ABA increased in concentration in the leaves after root

20   herbivore attack (Fig. S4 a-d; Mann-Whitney rank sum test ABA: p>0.05). *D. virgifera*

21   furthermore induced several defense markers (Student's T-test: p>0.05) including two

22   pathogenesis related genes, *Zm-pr1* and *Zm-pr5*, (Morris *et al.*, 1998), three proteinase

23   inhibitors, *Zm-cysII, Zm-serpin, Zm-cyst (Ton et al., 2007)*, and the regulatory gene for

1   hydroxamic acid biosynthesis *Zm-bx1* (Frey *et al.*, 1997) (Fig. S4e; Erb *et al.*, 2009a).

2   The hormonal measurements show that JA, JA-Ile and SA concentrations were neither

3   affected by the root herbivore, nor by the plant water status (Two-way ANOVAs; Fig. 3

4   a-c). ABA on the other hand was affected by both water status (ANOVA: p=0.036) and

5   *D. virgifera* feeding (ANOVA: p=0.012) and there was a strong interaction between the

6   two stresses (ANOVA: p=0.032): ABA was most strongly induced by *D. virgifera* when

7   the plants were not watered over the 48 h of infestation. Average concentrations

8   increased to 160 ng/g fresh weight (FW) under this condition, which is about 40 times the

9   concentration of control plants. Interestingly, this effect was almost completely absent

10 under excess water supply (Fig. 3d). ABA levels were only weakly elevated in the

11 unwatered controls, indicating that the watering regime by itself did not heavily stress the

12 plants. The systemic induction of defense markers by *D. virgifera* was affected by the

13 plant's water supply: *Zm-pr10*, *Zm-serpin* and *Zm-bx1* were more strongly induced under

14 water limiting conditions (ANOVA p<0.05). *Zm-cysII* was more responsive when the

15 plants were well watered, while the induction of *Zm-pr1, Zm-pr5* and *Zm-cyst* was not

16 influenced by the plant's water status (Fig. 3e). The most pronounced reaction was

17 measured for the gene that regulates ABA biosynthesis in maize: *Zm-nced(vp14)*. In

18 accordance with ABA content measurements (Fig. 3d), *Zm-nced(vp14)* was induced by

19 *D. virgifera* much more strongly when the plants were water stressed (Fig. 3e).

20   The C/N analyses showed no difference between the treatments (T-test: p>0.05;

21 Fig. 4a). Free amino acid (AS) patterns were unchanged under high water supply. On the

22 other hand, several of the measured AS increased in concentration when the plants were

1   infested by *D. virgifera* under low water supply (Two-way ANOVAs; interaction

2   herbivory*water: p<0.05; Fig. 4b).

3

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

5   The transcriptional profiling confirmed that *Zm-nced(vp14)* was suppressed in the

6   antisense lines, whereas it was induced after root attack in the wildtype plants (Fig. 5a).

7   The marker genes *Zm-cysII*, *Zm-cyst* and *Zm-bx1* were not induced by *D. virgifera* in the

8   antisense plants (Two-way ANOVAs: Genotype*Treatment interaction, p<0.05). Other

9   genes, including *Zm-pr10* and *Zm-serpin* were induced similarly in the transgenic and

10   control plants (Fig. 5a). Root removal by *D. virgifera* on antisense plants was the same as

11   for wild type plants (Fig. S5) and induced shoot resistance against *S. littoralis* (reduced

12   growth) was similar for both plant types after 6h and 12h of feeding (Figs. 5 b-c). These

13   results imply that the induction of *Zm-nced(vp14)* upon root herbivore attack under water

14   limiting conditions was not responsible for the observed increase in resistance.

15   This was also confirmed by the experiment involving chemical inhibition of ABA

16   biosynthesis. Interestingly, while control plants showed no or minor wilting symptoms

17   upon inhibitor treatment, plants infested with *D. virgifera* exhibited a strong wilting

18   phenotype, with all leaves curling and losing their capacity to remain upright. This

19   observation was reflected in a Two-Way-ANOVA of relative water contents showing

20   significant effects of *D. virgifera* and sodium tungstate as well as an interaction

21   (ANOVA: p=0.034). As shown in Fig. S6a, *D. virgifera* infested plants suffered much

22   more from water stress when treated with the ABA inhibitor. *D. virgifera* feeding again

23   reduced growth of *S. littoralis* (ANOVA: p=0.010), the effect being even more

1   pronounced in ABA inhibited plants (Holm-Sidak Post-Hoc Test: p=0.004) than in

2   untreated plants, where only a trend was visible in this assay (Fig. S6b).

3

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. 3e, 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 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; Erb *et al.*, 2009c). 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

22

1   important, but not exclusive role of water stress and ABA-signalling for *D. virgifera*

2   induced changes in leaf-defense.

3   The increase in resistance against *S. littoralis* was closely related to the changes in

4   relative leaf water contents after root herbivory, as is evident from Fig. 2, were it is

5   shown that the weight gain of the larvae is considerably reduced when *D. virgifera* has a

6   strong negative impact on the water supply of the maize plant. As the induction of ABA

7   and ABA-dependent defenses is most pronounced under water limiting conditions (Fig.

8   3d-e), ABA was expected to be responsible for the increased resistance. Evidence for its

9   role comes for example from research on *A. thaliana*, for which it has been found that

10   ABA-deficient mutants are highly susceptible to *S. littoralis* (Bodenhausen & Reymond,

11   2007). Yet, our results strongly suggest that ABA is not required for root herbivore

12   induced shoot resistance in maize. This is most evident from the fact that the induction of

13   resistance by *D. virgifera* also occurred after genetic or chemical inhibition of ABA-

14   signalling (Fig. 5b-c and S6). We therefore postulate that ABA-independent hydraulic

15   changes are the causal factor in *D. virgifera* induced shoot resistance in maize. The upset

16   water balance causes reduced leaf-turgor, which may directly impair feeding by *S.*

17   *littoralis* larvae: The larvae normally display so called "windowpane-feeding", where the

18   epidermis of only one side of the leaf is ingested together with the inner parenchyma

19   tissue. This enables the herbivore to gain access to the easily digestible inner cell layers,

20   while avoiding the tough cuticle and epidermal layers of the other leaf-side. Our

21   experiments show that under heavy leaf-water stress caused by *D. virgifera*, this feeding

22   strategy is no longer possible and *S. littoralis* larvae have to ingest both epidermal layers

23   and cuticles. This effect is independent of ABA signalling, as it can be observed in both

23

1   wildtype and ABA-impaired plants (M. Erb, personal observations). Apart from such

2   mechanical effects, the experiments also demonstrate that certain defense markers like

3   *Zm-pr10* are induced by *D. virgifera* imposed water stress in an ABA-independent

4   manner (Fig. 3e and 5a). Some defenses are thus specifically responsive to ABA-

5   independent hydraulic changes. *S. littoralis* may be particularly sensitive to these

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

7   The finding that hydraulic changes are responsible for the increase in leaf

8   resistance is of potential importance for a variety of induced resistance phenomena.

9   Numerous root herbivores change the water balance of aboveground plant parts (Gange

10   & Brown, 1989; Murray & Clements, 1998; Blossey & Hunt-Joshi, 2003; Staley *et al.*,

11   2008), and the involvement of water stress in changes in aboveground resistance has been

12   proposed in early models of above-belowground interactions (Masters *et al.,* 1993).

13   Depending on the feeding strategy of the leaf herbivore, such changes can either increase

14   or decrease plant resistance. Phloem feeding aphids for example may benefit from the

15   increased AS concentrations in leaves under water stress (Fig. 4b), whereas chewing

16   herbivores are negatively affected by the increased defenses (Huberty & Denno, 2004).

17   Our experiments also suggest that studies conducted in the laboratory or the

18   greenhouse may underestimate the systemic effects of insect infestation, as such effects

19   may depend on slight changes in abiotic factors like water supply. In nature, plants are

20   continuously exposed to various mild stress events, and our data clearly suggest that these

21   fluctuations should be taken into account when looking at induced resistance phenomena.

22   Highly sensitive methods that capture the plant's water status beyond relative water

1 contents may contribute to unravel the importance of hydraulic conductivity in induced

2 resistance in more detail.

3   The adaptive value of root herbivore induced shoot resistance has remained

4 unresolved (Wäckers & Bezemer, 2003). The current study favours the hypothesis that

5 RISR may be the result of a plant physiological constraint. The later larval stages of *D.*

6 *virgifera* larvae often attack the upper root system *(*Strnad & Bergman*, 1987;* Hibbard *et*

7 *al.*, 2008), which we found to be the site where the larvae develop much better (Fig. S3).

8 For the plant, this feeding behaviour poses a significant threat to its water supply (Fig.

9 2b), especially at early developmental stages of the seedling, when the root system relies

10 on few connective elements. The increase in ABA biosynthesis following belowground

11 attack seems to be a tolerance response of the plant to reduce the negative effects of water

12 loss. Under conditions where the metabolic and physiological changes are not sufficient,

13 water concentrations in the shoot decrease nevertheless (Fig. 2b), sometimes even to a

14 point where acute wilting occurs. It is under these circumstances that the aboveground

15 herbivore *S. littoralis* is most negatively affected (Fig. 2a and 2c). This phenomenon is

16 unlikely to be adaptive for the plant, as a loss of leaf-turgor to increase shoot resistance is

17 a very unlikely defense strategy for an organism that heavily depends on an effective

18 water supply for growth and survival. Interestingly, the root herbivore *D. virgifera* seems

19 to benefit from feeding on the most vulnerable part of the root system (Fig. S3a and b).

20 Whether this is only due to better access to leaf-assimilates or if changes in the plant's

21 water balance are advantageous for *D. virgifera per se* remains to be determined. It is

22 known that plants under water stress increase their investment in root growth (Reid &

23 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, which is 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 non-lepidopteran 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.

Acknowledgements

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10

11

Figure legends

Figure 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 *D. virgifera* (hatched bars). **(b):** Average number (+SE) of longitudinal (left) and shotgun holes (right) per plant. **(c):** Average cumulative growth (±SE) of *S. littoralis* caterpillars over 10 days of feeding on plants infested with *D. virgifera* in the roots (white circles) or uninfested control plants (black circles) in the laboratory. Stars denote significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$). **(d):** Total numbers of *S. littoralis* caterpillars reaching the pupal stage (black) or dying (white) on infested vs. uninfested plants. Different letters indicate significant differences between treatments ($p<0.05$).

Figure 2: Influence of water supply and belowground feeding site on root herbivore induced shoot resistance. **(a):** Average weight gain (+SE) of *S. littoralis* larvae after 6 h of feeding on *D. virgifera* infested (white points) and control plants (black points) under different water regimes. Saturation= Soil drench (48h); 10 ml/day= 10 ml H_2O per day (48h); Residual= No watering (48h). Stars 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* (black points) and control plants (white points) under different water regimes. **(c):** Average weight gain (+SE) of *S. littoralis* larvae after 6 h of feeding on control plants (filled bars), plants infested with *D. virgifera* confined to the upper 2cm of the soil (hatched bar) or the lower part of the roots (crossed bar). All plants received 10/ml of water per day. **(d)**: Average shoot water contents (+SE) of plants

1   infested with *D. virgifera* on upper and lower parts of the roots. All plants received 10/ml

2   of water per day. Different letters indicate significant differences between treatments

3   (p<0.05).

4

5   *Figure 3:* Influence of root herbivory and water stress on shoot phytohormone levels and

6   defense gene expression. Average shoot concentrations (+SE) of JA (**a**), JA-Ile (**b**), SA

7   (**c**) and ABA (**d**) upon root stress are shown. Hatched bars stand for *D. virgifera* infested

8   roots. The left bars (white and black) show concentrations for well-watered plants, while

9   the right bars (grey) stand for plants with low water supply. Different letters indicate

10   significant differences between the treatments (p<0.05). Significance levels are also

11   shown for two-way ANOVAS (T=Herbivore infestation; W= Water treatment; TxW=

12   Interaction). Stars denote significant ANOVA effects (*p<0.05, **p<0.01, ***p<0.001).

13   (**e**): Expression levels (Ln fold change +SE relative to well-watered controls) of defense

14   marker genes upon stress treatments. Different letters indicate significant differences

15   between the treatments (p<0.05).

16

17   *Figure 4:* Influence of root herbivory on C/N ratios and free amino acids. (**a**): Average

18   C/N ratios (+SE) of maize shoots infested in the roots with *D. virgifera* (hatched bar) and

19   control plants (black bar) under normal water supply. Different letters denote significant

20   differences between treatments (p<0.05). (**b**): Average concentration of 17 free amino

21   acids (+SE) in root stressed plants. Hatched bars stand for *D. virgifera* infested roots.

22   The left bars (white and black) show concentrations for well-watered plants, while the

23   right bars (grey) stand for plants with low water supply. Significance levels are shown for

1   two-way ANOVAS (T=Herbivore infestation; W= Water treatment; TxW= Interaction).

2   Stars denote significant ANOVA effects (*p<0.05, **p<0.01, ***p<0.001).

3

4   *Figure 5:* The role of the ABA-biosynthesis gene *Zm-nced(vp14)* on root-herbivore

5   induced shoot defenses. Wildtype (*wt*) and antisense lines (asNCED(vp14)) were tested

6   under low water supply. **(a)**: Ln fold change (+SE) of defense marker genes for the

7   different treatments and genotypes. Significance levels are shown for two-way ANOVAS

8   (T=Herbivore infestation; G= Genotype; TxG= Interaction). Stars denote significant

9   ANOVA effects (*p<0.05, **p<0.01, ***p<0.001). Average weight gain (+SE) of *S.*

10   *littoralis* larvae after 6 h (**b**) and 12 h (**c**) of feeding on *D. virgifera* infested (hatched

11   bars) and control plants (black bars) is shown. Different letters indicate significant

12   differences between treatments (p<0.05).

13

14

Supplementary figure legends

Figure S1: Influence of root herbivory on short-term growth and consumtion of *S. littoralis*. Average growth (**a**) and leaf consumption (**b**) of *S. littoralis* caterpillars (+SE) over a 6 hour feeding period on control (black bars) and root herbivore infested plants (hatched bars). Different letters denote significant differences between treatments (p<0.05).

Figure S2: Influence of water stress and root herbivory on root biomass. Average root dry weight (+SE) of control plants (black circles) and root herbivore infested plants (white circles) on plants grown under different water regimes. Significance levels are shown for a two-way ANOVA (T=Herbivore infestation; W= Water treatment; TxW= Interaction). Stars denote significant ANOVA effects (*p<0.05, **p<0.01, ***p<0.001).

*Figure S3:* Growth of *D. virgifera* confined to different parts of the root system. Average weight gain (+SE) is given for (**a**) *D. virgifera* larvae feeding on the top 2 cm vs. the rest of the root system, (**b**) *D. virgifera* larvae feeding on the bottom 2 cm vs. the rest of the roots above. Different letters denote significant differences between treatments (p<0.05).

Figure S4: Influence of root herbivory on leaf phytohormones and defense gene expression. Average shoot concentrations (+SE) of JA (**a**), JA-Ile (**b**), SA (**c**) and ABA (**d**) upon root stress are shown. Hatched bars stand for *D. virgifera* infested roots. Different letters denote significant differences between treatments (p<0.05). **(e):** Ln fold

1 change (+SE) of defense marker genes in the leaves of root herbivore infested plants

2 (hatched bars). Stars denote significant differences (*p<0.05, **p<0.01, ***p<0.001).

3

4 *Figure S5:* Influence of plant genotype on root biomass removal by *D. virgifera*. Average

5 root fresh weight (+SE) of wildtype plants (black bars) and *asNCED(vp14)* plants

6 (hatched bars) infested with *D. virgifera*.

7

8 *Figure S6:* Impact chemical ABA inhibition on root herbivore induced resistance and

9 water contents. **(a):** Average relative water content (+SE) of maize shoots infested in the

10 roots with *D. virgifera* (crossed bars) and control plants (black bars) with and without

11 ABA inhibition under normal water supply. Different letters indicate significant

12 differences between the treatments (p<0.05). **(b)**: Average weight gain (+SE) of *S.*

13 *littoralis* larvae after 6 h of feeding on *D. virgifera* infested (crossed bars) and control

14 plants (black bars) in untreated plants (Control) and plants treated with an ABA inhibitor

15 (Na_2WO_4). Significance levels are shown for two-way ANOVAs (T=Herbivore

16 infestation; I= Inhibitor treatment; TxI= Interaction). Stars denote significant ANOVA
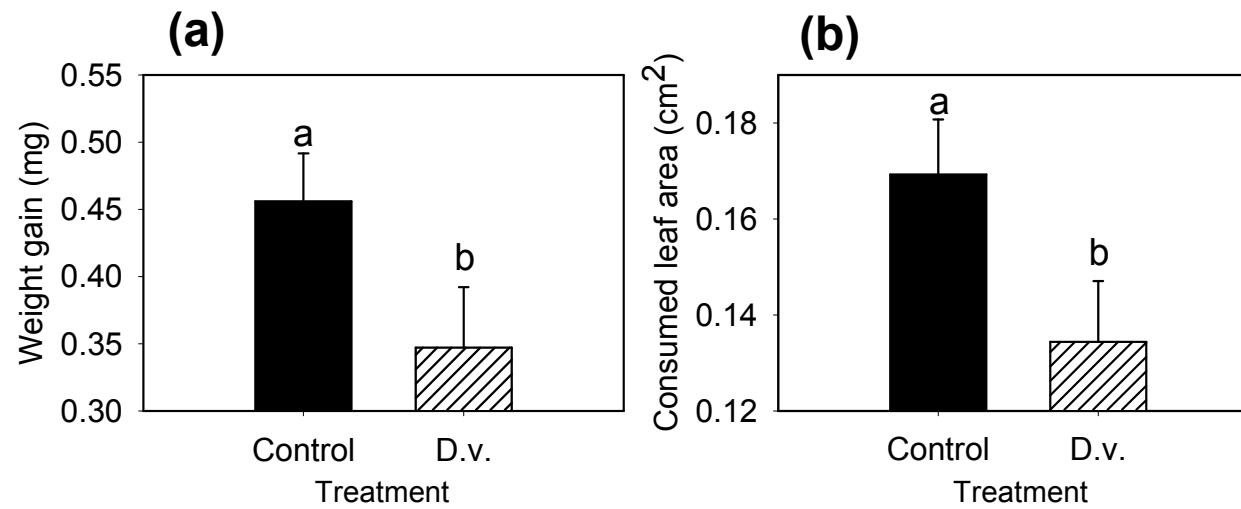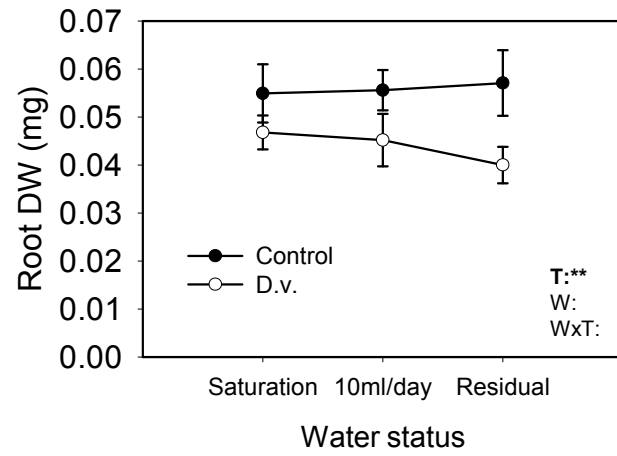
17 effects (*p<0.05, **p<0.01, ***p<0.001).

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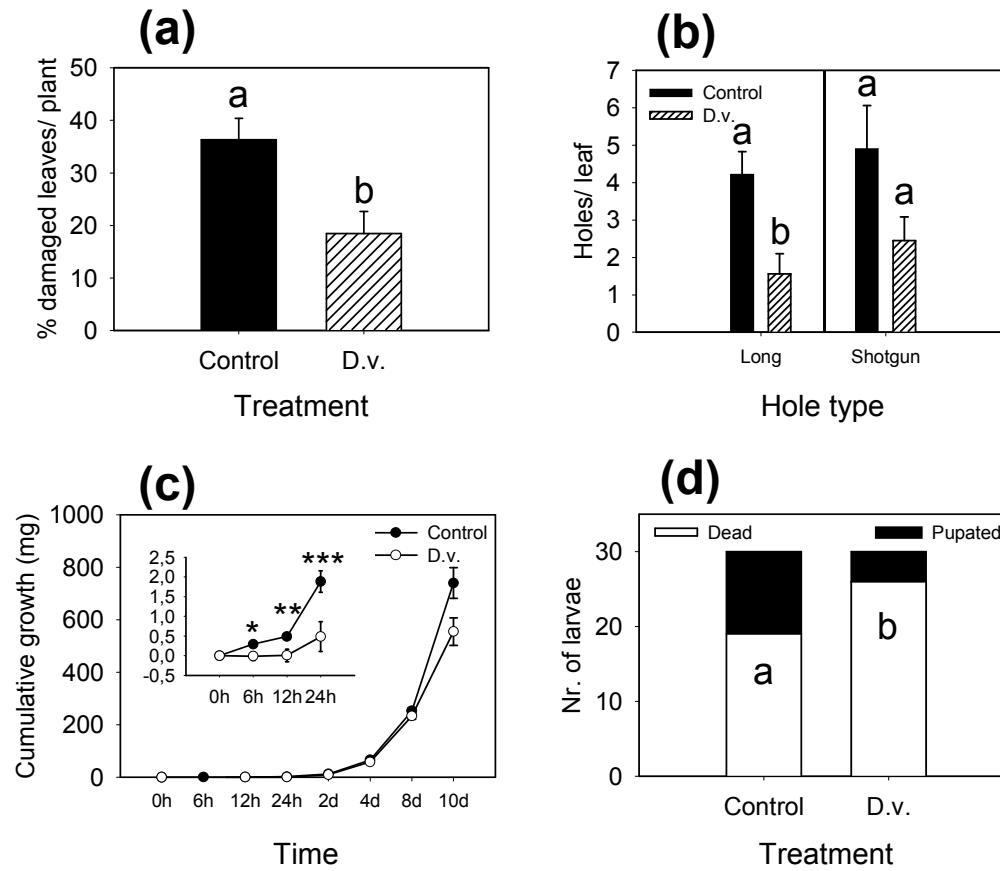
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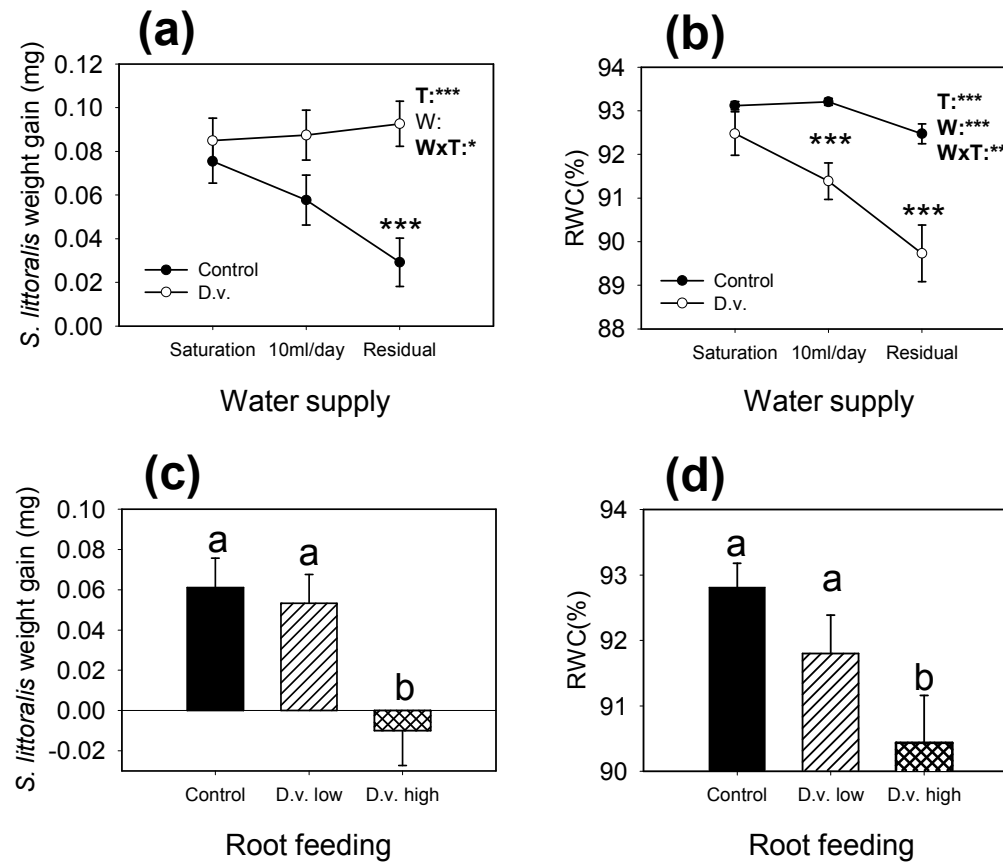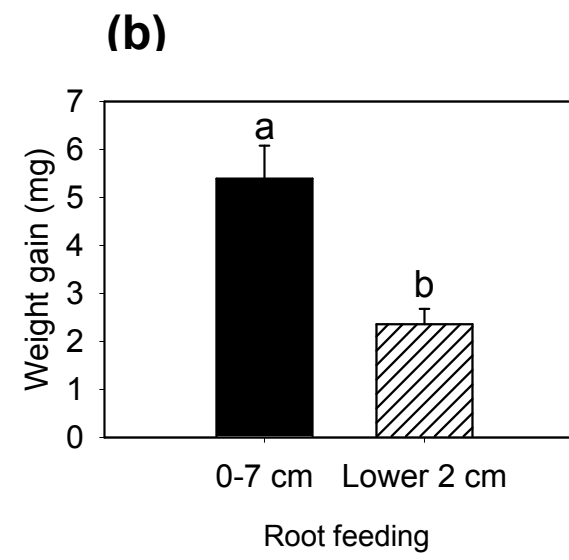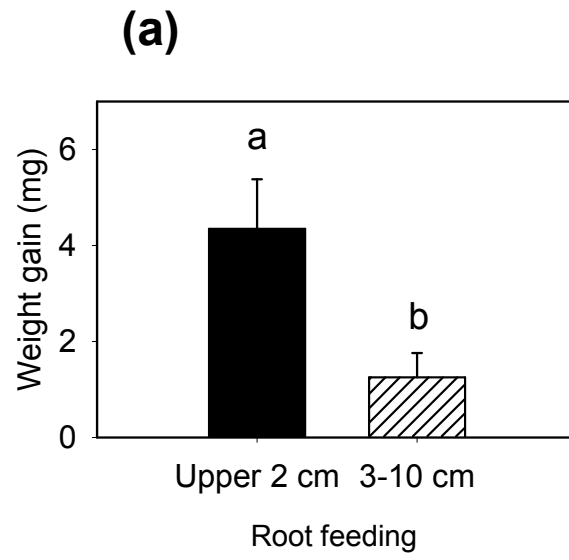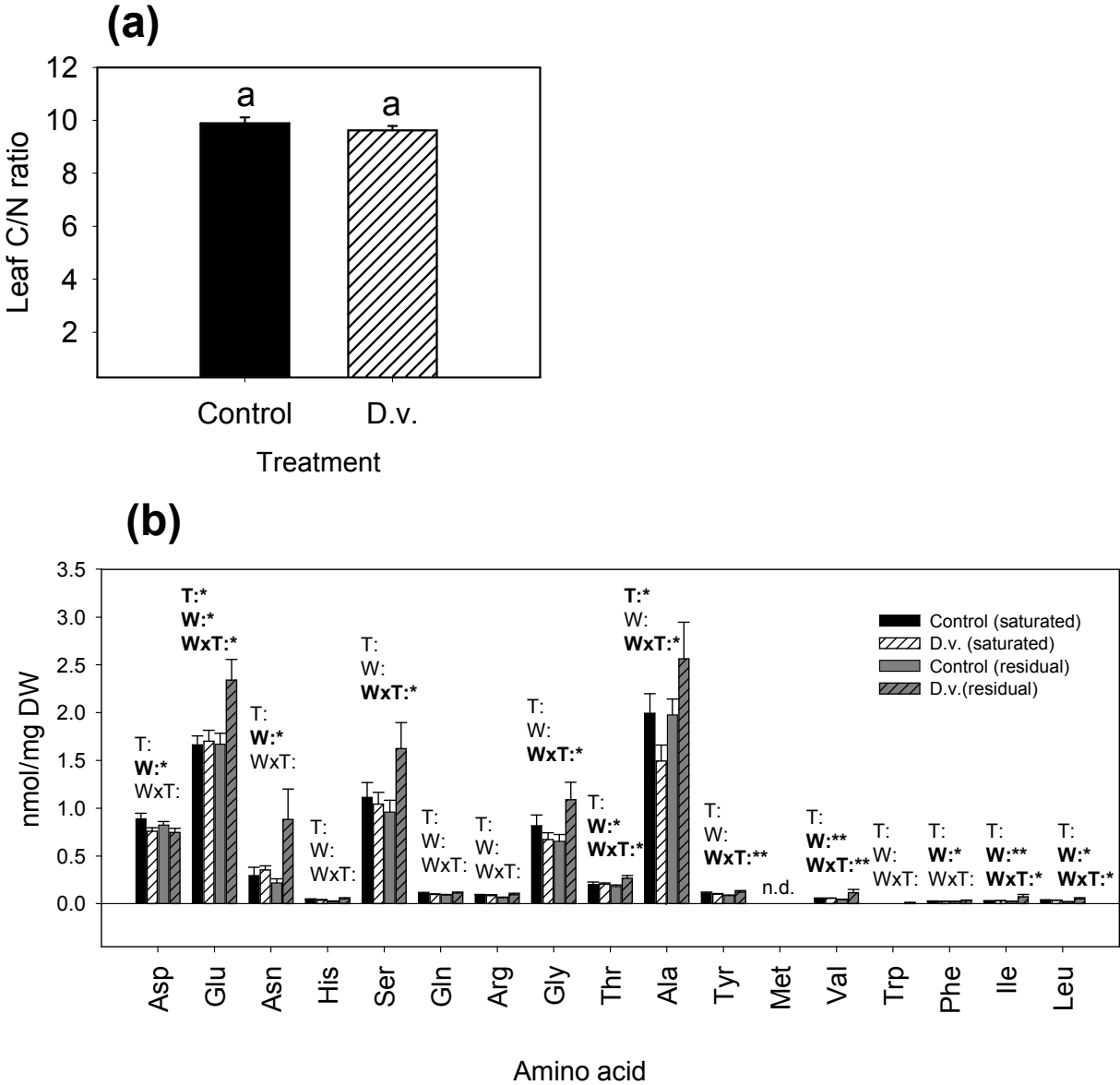
Figure 3

Figure 4

Figure S1

(a)

(b)
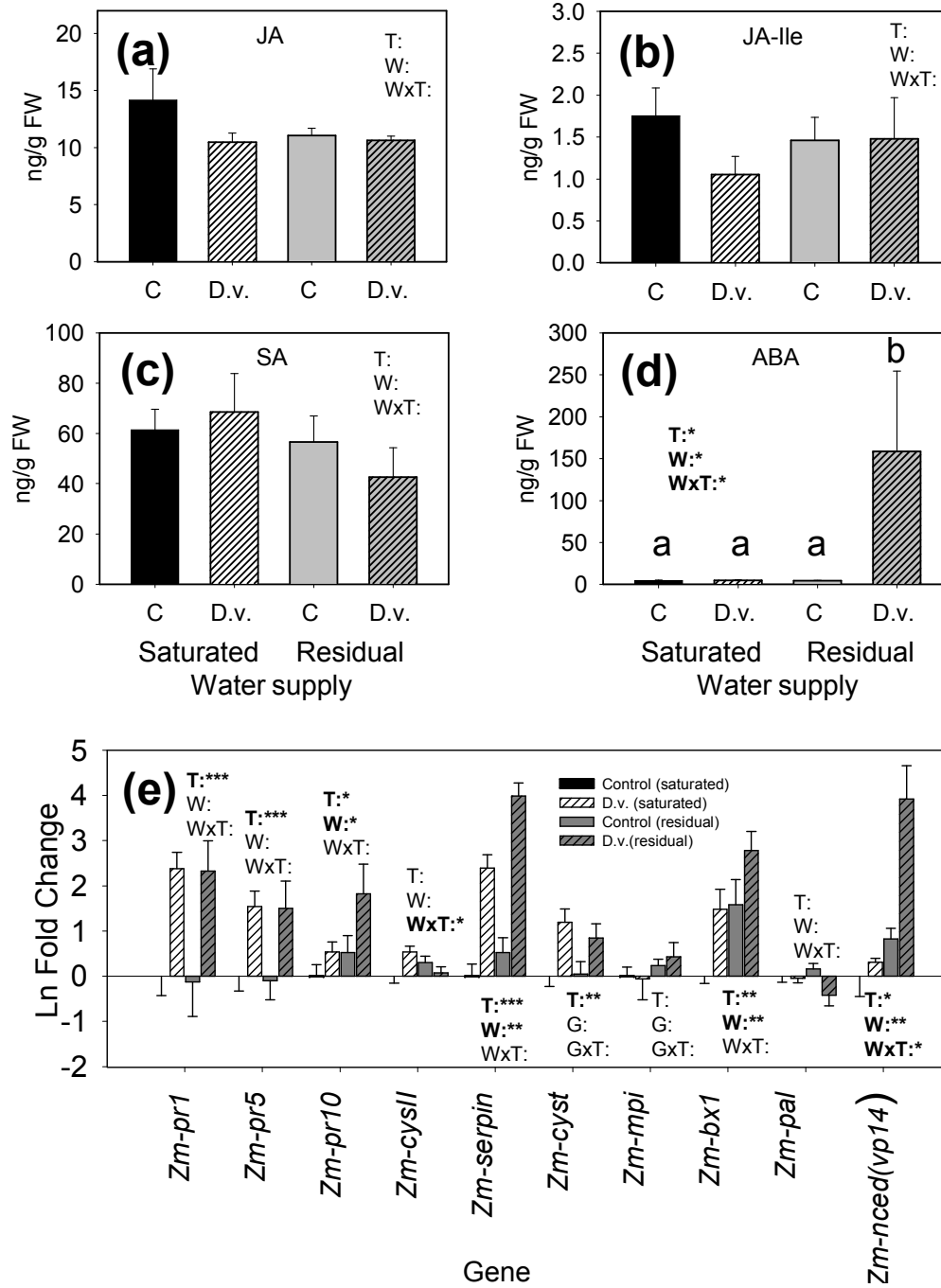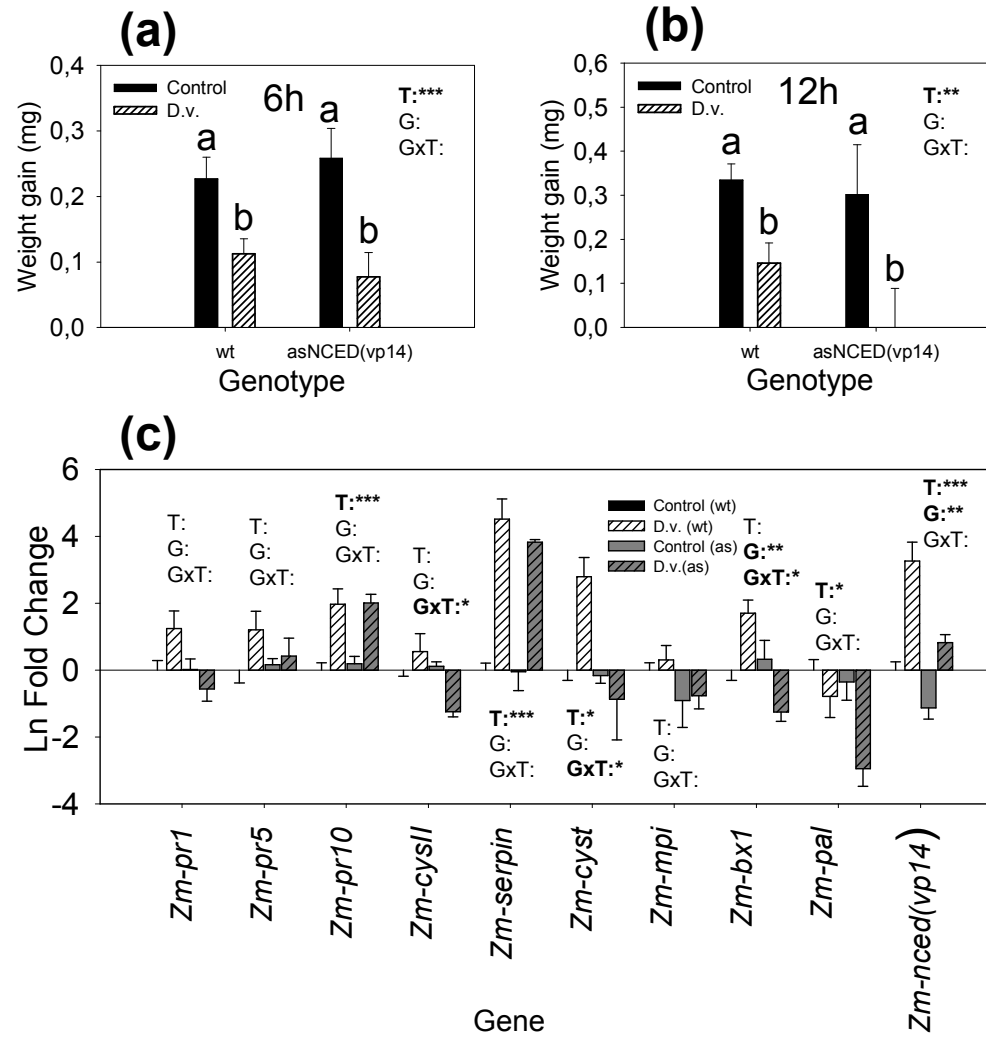
Figure S2

Figure S3

(a)

(b)

Figure S4

Figure S5

Figure S6

Figure S7