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LETTER

A specialist root herbivore exploits defensive metabolites to locate nutritious tissues

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Abstract

The most valuable organs of plants are often particularly rich in essential elements, but also very well defended. This creates a dilemma for herbivores that need to maximise energy intake while minimising intoxication. We investigated how the specialist root herbivore *Diabrotica virgifera* solves this conundrum when feeding on wild and cultivated maize plants. We found that crown roots of maize seedlings were vital for plant development and, in accordance, were rich in nutritious primary metabolites and contained higher amounts of the insecticidal 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and the phenolic compound chlorogenic acid. The generalist herbivores *Diabrotica balteata* and *Spodoptera littoralis* were deterred from feeding on crown roots, whereas the specialist *D. virgifera* preferred and grew best on these tissues. Using a 1,4-benzoxazin-3-one-deficient maize mutant, we found that *D. virgifera* is resistant to DIMBOA and other 1,4-benzoxazin-3-ones and that it even hijacks these compounds to optimally forage for nutritious roots.

Keywords

Diabrotica virgifera, DIMBOA, optimal defence, optimal foraging, plant-insect interactions, root herbivore, *Zea mays*.

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INTRODUCTION

Plants possess a wide arsenal of toxic secondary metabolites to defend themselves against herbivores (Sicker *et al.* 2000; Steppuhn *et al.* 2004; Gershenzon & Dudareva 2007). The concentrations of these compounds vary considerably between (Kaplan *et al.* 2008) and within tissues (Shroff *et al.* 2008), as well as over the course of plant development (Cambier *et al.* 2000). A proposed evolutionary explanation for this variability is that, because of metabolic costs, the concentrations of secondary defence metabolites in a particular tissue at a given developmental stage should reflect its relative fitness value (Rhoades & Cates 1976). As a part of the 'optimal defense' hypothesis, this concept has found considerable support in the literature (Stamp 2003). However, as plant organs with a high reproductive value such as young leaves or developing flowers receive a substantial amount of nutrients and photo-assimilates (Pommel *et al.* 2006; Li *et al.* 2009), these are also the tissues that are potentially valuable food sources for herbivores (Awmack & Leather 2002). To optimise their intake of energy per unit of time (Macarthur & Pianka 1966), herbivores should therefore attempt to feed on the most valuable, and, consequently, best defended plant tissues. This creates an intriguing dilemma for the herbivores, which is solved if they can overcome the plant's defences, thus allowing them to feed on the most nutritious tissues and maximise their fitness. Indeed, specialist herbivores have been shown to be able to develop on highly defended plant organs (Kimmerer & Potter 1987; Ishimoto & Chrispeels 1996). The capacity to cope with plant defences is thought to have favoured adaptive radiation of herbivores (Wheat *et al.* 2007), and is therefore widely recognised as a major driver of plant-insect co-evolution.

Although the above interactions have received considerable attention above ground, little is known about how strategies of defence and foraging shape the interplay between plants and herbivores below ground (Yanai & Eissenstat 2002; Johnson & Gregory 2006; van Dam 2009). This prompted us to carry out a series of experiments on the interaction between wild and cultivated maize (*Zea mays* L. spp) and its most important root pest, *Diabrotica virgifera virgifera* (L.). By combining behavioural and performance experiments with analytical and molecular methods, we show how *D. virgifera* has successfully solved the optimal foraging dilemma. Our experiments also reveal how a counter adaption of a below ground herbivore to a chemical plant defence determines its distribution and abundance in the soil.

MATERIAL AND METHODS

Plants and insects

The maize hybrid Delprim (*Zea mays* L. ssp. *mays*) was obtained from Delley DSP (Delley, Switzerland). The *bx1* mutant (428G) and its wild

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type parental line (H88) were ordered from the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu). Plants were grown as described (Erb *et al.* 2011b) and used when they were 12–14 days old and had three fully developed leaves, unless specified otherwise. Teosinte (*Z. mays* L. ssp. *mexicana*) seeds were collected from two wild populations near Texcoco (Mexico) in 1998. As the teosinte plants grew more slowly than cultivated maize, they were left in the phytotron for 20 days until they had three fully developed leaves. Maize plants for the $^{11}CO_2$ labelling experiments (see below) were germinated in petri dishes and transplanted into cylindrical glass cells (20 cm height, 10 cm diameter) containing a growth medium consisting of 1.6 g Hoagland modified basal salt mixture (PhytoTechnology Laboratories TM, Shawnee Mission, KS, USA) and 0.55 g of 2-(N-Morpholino) ethanesulfonic acid (MES) hydrate (Sigma Life Science, St. Louis, MO, USA) in 1 L of distilled water. After adjusting the pH to 5.8 with a few droplets of sodium hydroxide, 2.5 g of Gelzan TM CM (Sigma Life Science) was added. *Diabrotica virgifera virgifera* (LeConte) and *Spodoptera littoralis* (Boisduval) were reared as described before (Erb *et al.* 2011a). *Diabrotica balteata* (LeConte) was reared under identical conditions as *D. virgifera*.

Relative value of root types for the plant

The root systems of maize consists of primary and secondary roots that grow directly from the embryo (also called embryonic roots), as well as crown roots that originate from the stem (also called adventitious or post-embryonic roots; Hochholdinger & Tuberosa 2009). To determine the relative value of these different root types for the development of maize seedlings, two separate experiments were carried out. In a first assay, either crown roots or primary and secondary roots were excised from individual maize seedlings ($n = 11$). To remove the different roots without otherwise damaging the plant, the topsoil was washed off under a gentle stream of warm water, until the different roots were visible and could be accessed with surgical scissors. After excision, the missing substrate in the pots was replaced with fresh moist soil. Plants were checked every 5 days over a period of 30 days, and regrowing roots were cut over the first 20 days to simulate an ongoing herbivore attack by *D. virgifera*. Complete pruning of crown roots by *D. virgifera* occurs regularly in the field, resulting in plants losing their stability (Oleson *et al.* 2005). Senescence symptoms for each emerging leaf were recorded using a scale from 0 (no senescence) to 4 (leaf yellow and curling). Three weeks after the beginning of the experiment, the leaves of the plants were harvested, and their biomass was determined. A second experiment was carried out using the same procedure as above, except that the plants were transplanted into 2 L pots before treatment ($n = 6$), which enabled them to grow more vigorously and possibly enabled additional compensatory growth. For this experiment, crown, primary and secondary roots were excised separately, and untreated controls were included. Leaf-growth was determined by measuring the height of the plant every 2 days.

Determination of free amino acids, total protein, starch, sugars and resource allocation

To measure concentrations of free amino acids in primary and crown roots, 12-day-old maize seedlings were harvested, and their roots were gently washed. Crown and primary roots were separated and immediately frozen in liquid nitrogen ($n = 6$). Amino acids were determined following a previously described method (Knill *et al.* 2008). Total soluble protein was determined on crown and primary roots ($n = 5$–6) using an adapted Bradford assay (Jongsma *et al.* 1994). To determine free sugars in the different roots, a 50 mg aliquot of freshly ground material was lyophilized over 48 h and analysed using a method based on a previously published procedure (Rovio *et al.* 2007). Starch concentrations ($n = 8$) were determined as published previously (Smith & Zeeman 2006). To measure the allocation of photo assimilates to the different roots, plants were grown in an agar-based growth medium for 20 days (see above). Single plants were then pulsed with 30 mCi of $^{11}CO_2$ (< 1 p.p.m CO_2) for 30 s using the methodology described previously (Babst *et al.* 2005) ($n = 8$). Two and a half hours after exposure, roots were excised, and the accumulation of radioactivity in individual roots was measured with a beta/gamma-counter. As the plants already had three generations of crown roots at this stage, these were separated according to their growth stage. For visualisation purposes, an individual plant was treated as described above, the roots were extracted from the gel and the full plant was visualised using beta-imaging.

Extraction and analysis of 1,4-benzoxazin-3-ones

To determine the concentrations of 1,4-benzoxazin-3-one derivatives (BXDs, see Fig. S1 in Supporting Information) in different root types, five different experiments were carried out. In the first two experiments, basal BXDs were determined in crown and primary roots of maize plants ($n = 8$; $n = 9$). In the third experiment, we determined BXDs in teosinte ($n = 11$). Maize plants were included as a positive control. In the fourth experiment, maize plants were infested with six second instar *D. virgifera* larvae over 24 h ($n = 8$). Finally, BXD concentrations in crown, primary and secondary roots of maize were determined in control plants and plants treated with 200 μM jasmonic acid (JA) for 24 h ($n = 8$) following a previously described protocol (Erb *et al.* 2009). Roots of all experiments were extracted in 1 mL of acidified H_2O/MeOH (50:50 v/v; 0.5% formic acid) as described (Erb *et al.* 2009) and analysed using UPLC-QTOF-MS (Glauser *et al.* 2011). To measure BXD concentrations in root exudates, we developed a method based on liquid extraction surface analysis using the Advion TriVersaNanomatechip-based infusion nanoESI system (Advion bioscience, NY, USA, see Appendix S1). With this system, exudation of benzoxazinoids from crown and primary roots was quantified ($n = 6$).

Extraction and analysis of phenolic compounds and analysis of gene expression

Phenolic compounds were analysed using 100 mg samples of the roots from the JA experiment ($n = 8$, see above). The extraction and analysis of phenolics were similar to that of BXDs, except for the following modifications: the extraction solvent was MeOH/H_2O (80:20, v/v); The injection volume was 2.5 μL and gradient analysis was performed at 400 μL min^{-1} under the following conditions: A = water + formic acid 0.05%, B = acetonitrile + formic acid 0.05%; 2–30% B in 2.5 min, 30–100% in 3 min, 100% B for 2 min, re-equilibration at 2% B for 1 min. The expression of defence marker genes in different roots after JA induction was measured using previously established methods and primers (Erb *et al.* 2010) on the same material as above ($n = 8$).

Herbivore feeding preference

The preference of *D. virgifera* for different root types was determined by infesting 12-day-old seedlings with six second instar *D. virgifera* larvae (n = 11). In the field, egg densities have been estimated to be over 200 per plant, and infestation with 10 or more larvae per plant is not uncommon (Hibbard *et al.* 2004). Timing of attack varies with climatic conditions, but *D. virgifera* larvae with an extended diapause can hatch as early as April, shortly after maize plants start to develop in the field. Thus, the chosen experimental conditions reflect a possible field situation. After 4 days of feeding, the root systems were washed and the different root types were rated for damage using a scale from 0 (no visible damage) to 3 (pruned or completely tunnelled roots). In the second approach, maize or teosinte seedlings with 2–3 fully developed leaves were removed from pots and their roots were gently washed under a stream of warm water. The root systems were then laid onto a moist filter paper embedded in a large petri dish (12 cm diameter). The petri dish had a cavity on the side, into which the stem was laid, leaving the leaves of the plant freely outside of the dish. Six second instar larvae were introduced into the dish, which was sealed with its lid and laid out on a table supplied with plant lights. To guarantee moisture saturated air around the exposed roots, water-drenched paper tissue was wrapped around the petri dishes, followed by a layer of aluminium foil to shade the roots from light. Every 3 h for 24 h, the foil and paper were removed and the position of the larvae was recorded. Four independent experiments were carried out using this procedure: First, the preference of *D. virgifera* for the different root types was determined by exposing larvae to the root systems of maize (n = 8) or teosinte (n = 18). In the third experiment, the generalist root herbivore *D. balteata* and the leaf-herbivore *Spodoptera littoralis* were observed on independent root systems of maize and compared with *D. virgifera* (n = 15). *S. littoralis* larvae will accept belowground tissues as food source if leaves are unavailable. Finally, the behaviour of *D. virgifera* on wild type (H88) and BXD mutant plants (*bx1*) was compared (n = 16). The *bx1* mutant produces only trace amounts of BXDs (Frey *et al.* 1997a). An additional choice assay between H88 and *bx1* plants was realised using the same setup, but by combining the root systems of the two genotypes in the same dish before adding *D. virgifera* larvae and observing their behaviour (n = 25).

Diabrotica virgifera performance

To measure the performance of *D. virgifera* on different root types, root systems of maize seedlings were gently washed and either the primary or crown roots were re-potted in 50 mL falcon tubes filled with moist soil (n = 12). A single second instar *D. virgifera* larva was weighed and introduced into the falcon tube, which was then sealed at the top using plastic film, leaving just a small opening for the roots. The falcon tube together with the rest of the root system was then buried in a bigger pot filled with soil, thereby guaranteeing that all roots of the plants were able to grow in an adequate environment. After 6 days, the larvae were recovered from the falcon tubes and re-weighed. To evaluate growth of *D. virgifera* under more controlled conditions, a second experiment was carried out. Roots of maize plants were washed, and either the primary or a crown root was gently slid into a 200 µL micro-capillary (n = 6). Twenty microlitres of water were added to the capillary to guarantee adequate water supply. A single, pre-weighed second instar *D. virgifera* larva was then introduced into the capillary, which was sealed at the top and bottom using aluminium foil. The capillary together with the rest of the root system were put on a moist paper tissue and covered with another piece of wetted paper. After 24 h, the larvae were removed from the capillaries and re-weighed.

Statistical procedures

Average ratings of leaf-senescence, final biomass of the plants in the root-removal experiments and herbivore performance in the capillary assays were compared using one-way analysis of variance (ANOVA). Where data did not conform to normality, $\log_{10}$ + 1 transformation was carried out. Mann–Whitney Rank Sum Tests were used for data that could not be normalised by transformation. Differences in free amino acids, soluble sugar and BXD concentrations in different roots of the same plants were tested using paired t-tests (plant as subject, type of root as treatment). JA induction of defence markers, BXDs and phenolic compounds was tested using Two-way ANOVAs (root type and treatment as factors, roots sampled on independent plants).

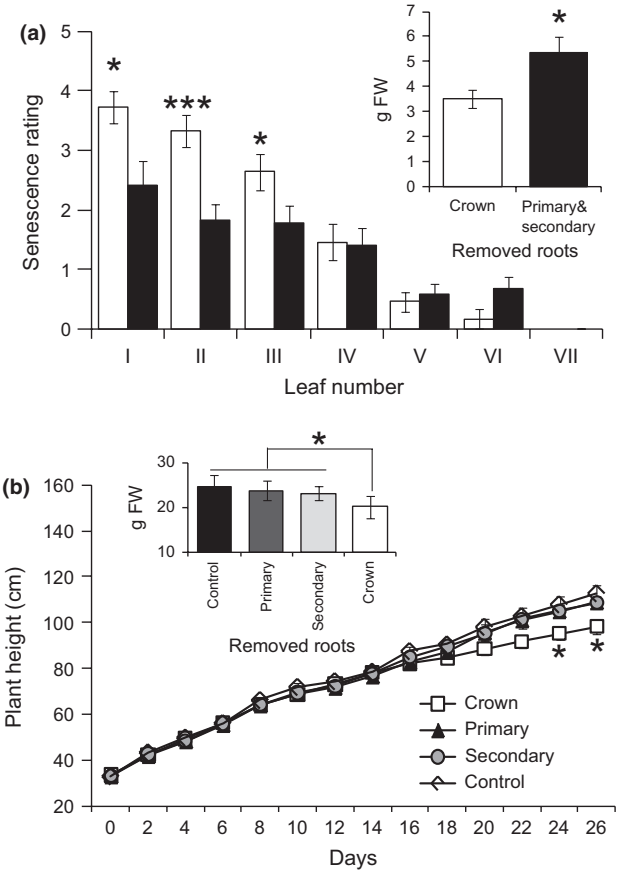

Figure 1 Crown roots are more valuable for young maize plants than primary and secondary roots. (a) Average (±SE) leaf senescence (main) and leaf biomass (inset) of maize plants 20 days after the removal of crown (white bars) or primary and secondary roots (black bars). Senescence rating goes from 0 (no visible symptoms) to 5 (leaf yellow and wilting). Leaf numbers correspond to the number of fully developed leaves on maize seedlings from I (oldest leaf) to VII (youngest). (b) Average (±SE) plant height (main) and leaf biomass (inset) of maize plants at different times after removal of different root parts in a separate experiment. FW, fresh weight. Stars indicate significant differences between treatments (*P < 0.05, **P < 0.01, ***P < 0.001).

The amount of radioactivity in the different roots, the damage ratings for *D. virgifera* and *D. virgifera* performance on crown and primary roots were tested using Two-way ANOVAs (plant and root type as factors). Holm-Sidak *post hoc* tests were used for pairwise comparisons following ANOVAs. Preference of *D. virgifera* for mutant or wild type roots was tested using a chi-squared test. Herbivore preference for the different root types in the petri-dish assays was determined using average counts of larva for each root type over 24 h using a log-linear model as described (Erb *et al.* 2010).

RESULTS

Crown roots are more valuable and nutritious than primary and secondary roots

Thirty days after selective excision of different roots, the leaves of maize plants without crown roots showed stronger symptoms of senescence (Fig. 1a) and had a lower leaf biomass than plants without embryonic roots (Fig. 1a inset). These results were confirmed in a second experiment showing that plants without crown roots grew significantly less tall (Fig. 1b) and accumulated less biomass than control plants or plants without primary or secondary roots (Fig. 1b inset). Crown roots also contained significantly higher amounts of most measured free amino acids (Fig. 2a), total soluble protein (Fig. 2a inset), sucrose (Fig. 2b) and starch (Fig. 2b inset) than primary roots. Moreover, crown roots were the primary sink for newly fixed CO_2: Two hours after the administration of $^{11}CO_2$ to source leaves of 20-day-old plants, the highest activity could be detected in the emerging and elongating crown roots (Fig. 2c and d).

Crown roots have higher concentrations of BXDs and phenolics

To test whether embryonic and post-embryonic roots contain different amounts of defensive metabolites, we analysed crown and primary roots for BXDs, which are key resistance factors of grasses (Niemeyer 2009). Crown roots had higher total concentrations of BXDs than primary roots (Fig. 3a inset). Especially the concentration of the highly toxic aglucone DIMBOA was fivefold higher in crown roots (Fig. 3a). HDMBOA-Glc and HDM2BOA-Glc, were present in slightly lower concentrations, resulting in a root-specific BXD profile. No difference was found between primary and secondary roots (see Fig. S2a). Teosinte plants contained four times less BXDs than the commercial maize hybrid (Fig. 3b, inset). The BXD distribution pattern, however, was similar with crown roots containing pronouncedly higher amounts of DIMBOA and lower amounts of HDMBOA-Glc and HMBOA-Glc (Fig. 3b). Although *D. virgifera* infestation did not significantly change BXD concentrations in maize (see Fig. S2a), JA treatment induced DIMBOA and HDM2BOA-Glc and reduced HDMBOA-Glc in crown roots, but not in embryonic

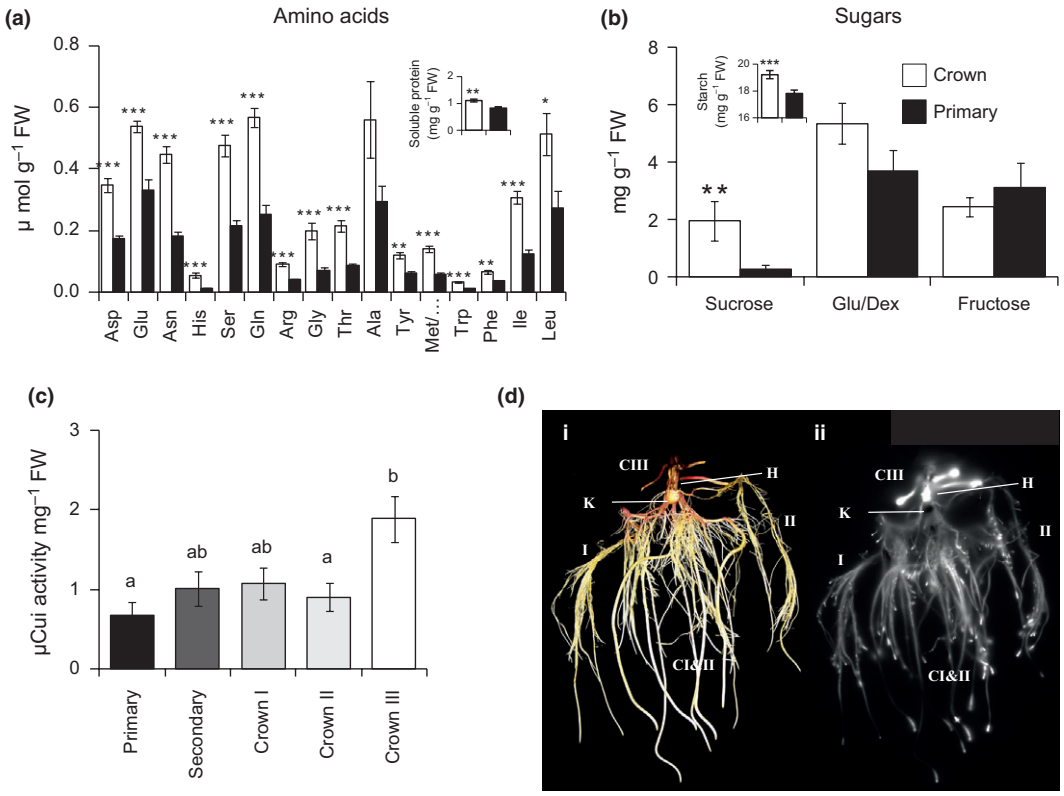

Figure 2 Crown roots are major sinks for photoassimilates and nutrients. (a) Average free amino acid concentrations (±SE) in crown (white bars) and primary roots (black bars) of maize seedlings. "Met/…." stands for "Met/Val". Inset: Total soluble protein (±SE) in crown and primary roots. (b) Average concentrations (±SE) of sucrose, glucose/dextrose and fructose in the different root types. Inset: Starch concentrations (±SE) in crown and primary roots. Stars indicate significant differences between root types (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (c) Average accumulation of ^{11}C in different root types 90 min after $^{11}CO_2$ administration to leaves of 20 day old maize plants. Crown root generations are shown separately from I (oldest) to III (youngest, emerging roots). Different letters indicate significant differences between root types ($P < 0.05$). (d) Photograph (i) and beta-image (ii) of roots of a $^{11}CO_2$ exposed maize plant. Brighter white indicates higher accumulation of ^{11}C assimilates. H, hypocotyl; K, Kernel; I, primary root; II, secondary root; C, crown roots from I (oldest) to III (youngest). FW, fresh weight.

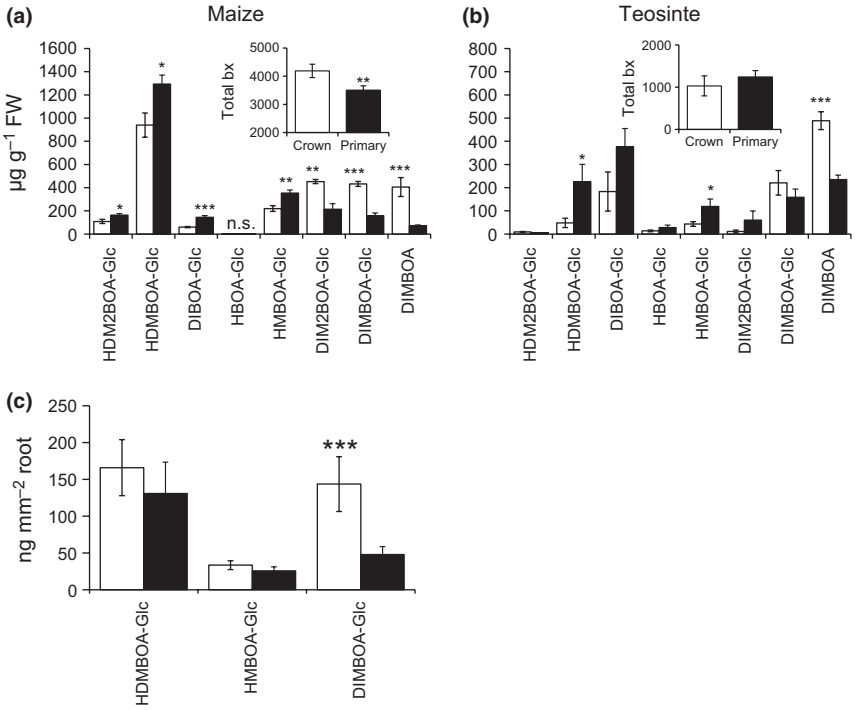

Figure 3 Crown roots have a specific profile of defensive metabolites. Average concentrations (±SE) of 1-4-benzoxazin-3-ones in crown (white bars) and primary roots (black bars) in maize (a) and teosinte (b) as well as on the root surface (c). Insets: Average total 1-4-benzoxazin-3-ones in crown and primary roots (separate experiment for maize). FW, fresh weight. Stars indicate significant differences between root types (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

roots (see Fig. S2b). Crown roots also contained 10-times more of the phenolic compound cholorogenic acid than primary and secondary roots (see Fig. S2a). Chlorogenic acid concentrations in crown roots were constitutively higher and did not change significantly following JA treatment. The gene *Zm-bx1*, which encodes for the first dedicated enzymatic step of BXD production and *Zm-Pal*, which is involved in the biosynthesis of phenolics, were most strongly expressed in crown roots (Fig. S3b). Leaf-defence markers coding for proteinase inhibitor (PI) homologues (Erb *et al.* 2009) showed a strong induction after JA treatment. For all tested PIs, crown roots were more inducible by JA than primary and secondary roots, while basal levels were slightly higher in the latter (Fig. S3b). We found BXDs to be the dominant compounds in maize root exudates (see Fig. S4). In accordance with the endogenous pattern, crown roots of maize plants also exuded significantly more DIMBOA-Glc than primary roots (Fig. 3b).

In contrast to generalist herbivores, the specialist *Diabrotica virgifera* preferentially feeds on crown roots

When we infested young maize plants with root-feeding larvae of the maize specialist *D. virgifera*, we observed that second instar larvae preferably fed on crown roots: After 4 days of infestation, crown roots of infested plants showed obvious signs of damage and were often chewed off, while primary and secondary roots were largely intact (Fig. 4a and b). *In vivo* observations confirmed the pronounced preference of the larvae for crown roots over other root tissues (Fig. 4c) over the full observation period of 24 h (Fig. 4c inset). This preference pattern was similar on teosinte plants (Fig. 4d). In contrast to maize, we rarely observed *D. virgifera* larvae feeding on secondary roots, which were much smaller and sometimes entirely absent from the teosinte seedlings. When comparing the behaviour of the specialist with generalist herbivores, *D. virgifera* again showed a pronounced preference for crown roots. When embryonic roots (primary and secondary) were compared with post-embryonic roots (crown roots), the preference of *D. virgifera* for the latter was highly significant (Fig. 5a inset). The generalists *S. littoralis* and *D. balteata*, on the other hand, showed a tendency to settle on primary roots and strongly preferred embryonic over post-embryonic roots (Fig. 5a). *S. littoralis*, which normally feeds on leaves, had a lower feeding activity compared with the two root-feeders (Fig. 5b).

Diabrotica virgifera uses BXDs as foraging cues

To test whether *D. virgifera* grows differently on different root types and whether its development and preference are affected by BXDs, we confined larvae to feed on either crown or primary roots of wild-type (H88), or of *bx1* mutant maize plants, which only produce trace amounts of BXDs (Frey *et al.* 1997b). *D. virgifera* larvae gained over 50% more weight on crown than on primary roots on both wild-type and mutant plants (Fig. 6a). In an additional experiment, we confirmed that *D. virgifera* also grows better on crown than on primary roots when feeding on the BXD producing maize hybrid Delprim over 24 h (Fig. 6b). From preliminary observations, it appeared that *D. virgifera* showed no preference for crown roots in the mutant plants. We therefore hypothesised that the specialist uses BXDs to distinguish between crown and primary roots. Indeed, while the choice pattern in the WT line H88 was similar to our previous observations with the hybrid Delprim, *D. virgifera* no longer distinguished primary from crown roots when feeding on mutant plants (Fig. 6c).

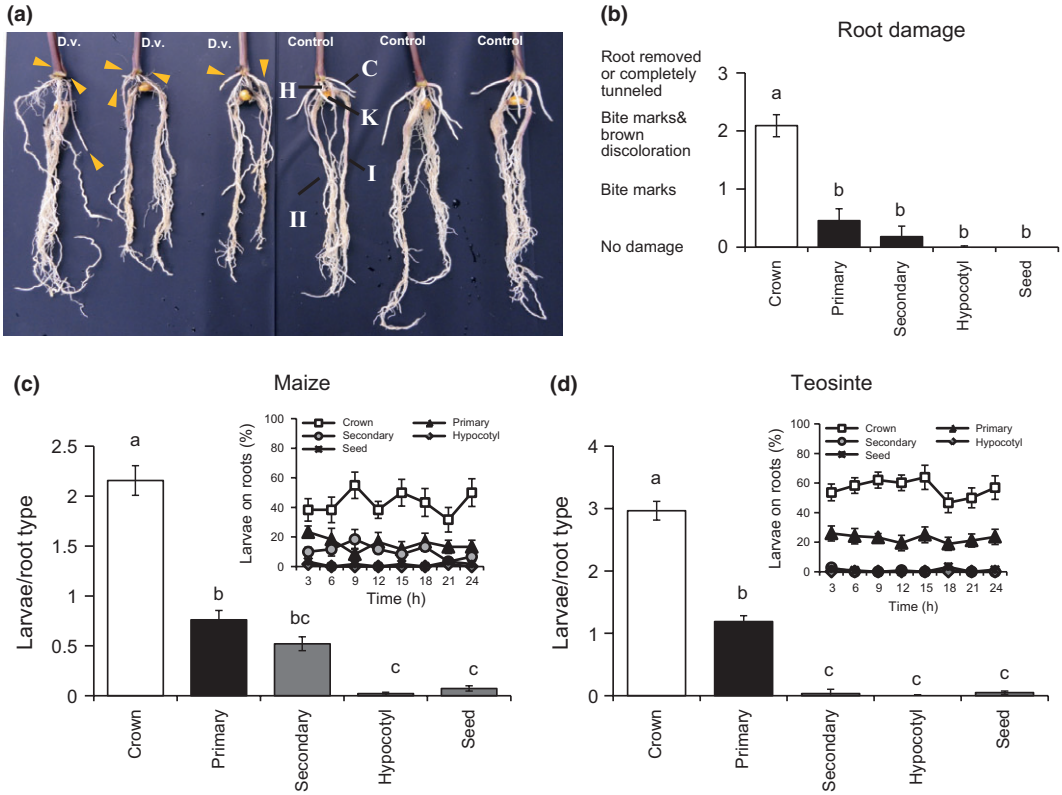

Figure 4 The specialist *Diabrotica virgifera* feeds preferentially on crown roots. (a) Representative photographs of 10 day old maize seedlings 4 days after *D. virgifera* attack (left, D.v.) compared with unattacked plants (right, controls). Orange triangles point to pruned or attacked crown roots. H, hypocotyl; K, Kernel; I, primary root; II, secondary root; C, crown roots. (b) Average values (±SE) of visual damage rating of roots after *D. virgifera* attack from 0 (no visible damage) to 3 (root removed or completely tunnelled). (c) and (d) Average number of larvae on each root type (mean over 24 h) of maize and teosinte. Insets: Percentage (±SE) of larvae observed on different root types over an observation period of 24 h. Different letters indicate significant differences between root types ($P < 0.05$).

DISCUSSION

The root removal experiments demonstrate that crown roots are more important for maize development than the primary and secondary roots (Fig. 1). These results are in accordance with the typical development of many gramineous root systems, including teosinte: While in the first day after germination, primary and secondary roots are responsible for nutrient and water uptake, the plants start to grow multiple layers of shoot-borne crown roots soon after, which take over these functions and become essential for overall plant performance (Hochholdinger & Tuberosa 2009). Thus, analogous to what has been found aboveground (Ohnmeiss & Baldwin 2000; Rostás & Eggert 2008), newly developing belowground tissues have the highest relative value for the plant. The fact that crown roots are metabolically more active than primary and secondary roots becomes evident from our $^{11}CO_2$ allocation data (Fig. 2c and d) as well as sugar and amino acid measurements (Fig. 2a and b). These differences are likely to be the result of an increased investment into the growth of these newly forming roots.

For herbivores, newly emerging crown roots would seem an ideal food source, as they are rich in carbohydrates and amino acids as well as soluble proteins (Fig. 2). However, our results suggest that differences in defensive chemistry may reduce the quality of post-embryonic roots for non-adapted consumers: Crown roots contained and exuded higher amounts of basal and JA-inducible BXDs (Fig. 3 and S2b), which are considered a particularly important resistance factor of grasses (Macias *et al.* 2009; Niemeyer 2009). Especially the 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), which was increased fivefold in crown roots compared with embryonic tissues (Fig. 3) has been shown to be toxic against a wide variety of insect herbivores (Rostas 2007; Niemeyer 2009; Glauser *et al.* 2011). Interestingly, *D. virgifera* infestation, contrary to JA treatment, did not induce DIMBOA in crown roots. It remains to be elucidated if *D. virgifera* has the ability to suppress the production of BXDs, or if the induction by the herbivore, contrary to a systemic JA treatment, is predominantly local (Hiltpold *et al.* 2011), making it hard to detect when analysing entire roots. Untreated crown roots also contained 10 times more chlorogenic acid than primary roots (see Fig. S3a), a phenolic compound that has been implicated in maize resistance (Nuessly *et al.* 2007) and expressed proteinase inhibitor genes, which have an important role in induced resistance (Cordero *et al.* 1994; Ton *et al.* 2007), at higher induced levels (see Fig. S3b). The higher levels of defensive compounds may explain why the generalist *D. balteata* and the leaf-feeder *S. littoralis* avoided crown roots and preferred to feed on embryonic roots instead (Fig. 5a insets). From the experiment with teosinte (Fig. 3b), it becomes clear that at least the higher levels of DIMBOA in crown roots are not an artefact of plant breeding, but a conserved preferential allocation pattern that may help the plant to defend its most valuable belowground tissues against environmental threats. It is interesting to note that cultivated maize contained over four times more BXDs than the wild teosinte plants (Fig. 3), and it

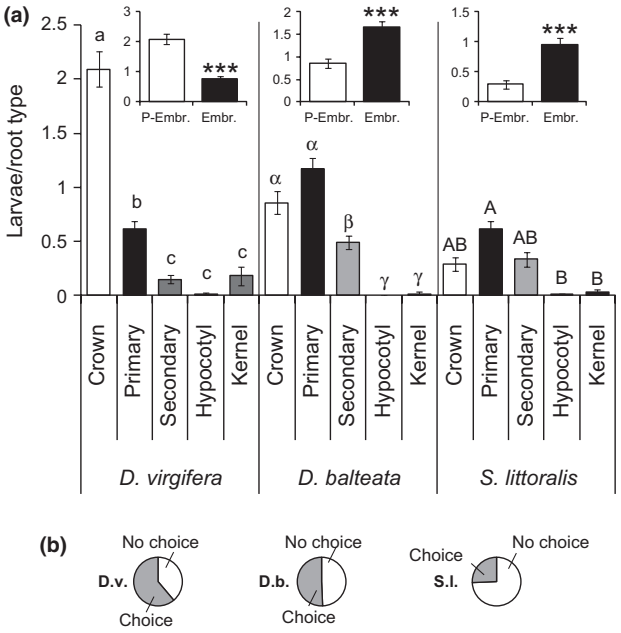

Figure 5 Unlike the specialist *Diabrotica virgifera,* generalist herbivores prefer embryonic roots. (a) Average number (±SE) of larvae of *D. virgifera*, *Diabrotica balteata* and *Spodoptera littoralis* observed on different root types over an observation period of 24 h. Different letters indicate significant differences between root types within species ($P < 0.05$). Insets: Average number of larvae on post-embryonic (white bars: crown roots) and embryonic roots (black bars: Primary and secondary roots). Stars indicate a significant preference (***$P < 0.001$). (b) Average proportion of larvae feeding on the roots (grey) vs. inactive individuals (white).

remains to be determined if this is due to positive selection for insect resistance during cultivation, or if BXDs have other, yet unknown functions in *Z. mays* spp. that were targeted by plant breeders.

Interestingly, the alleged BXD defence strategy of maize and teosinte does not seem to work against the specialist *D. virgifera*: The larvae of the root feeder strongly preferred crown roots over all other parts of the root system (Fig. 4). That this behaviour may be adaptive for *D. virgifera* is evident from the fact that the herbivore grows pronouncedly better on crown roots (Fig. 6a). In artificial diet assays, it was found that protein quality is an important determinant of *D. virgifera* fitness (Pleau *et al.* 2002), and it is likely that the insect is able to take advantage of the higher concentrations of free amino acids, soluble proteins and photo-assimilates, including sucrose and starch (Fig. 2). The performance results are in accordance with an earlier study reporting that *D. virgifera* develops better on growing root systems than older plants in the generative phase (Hibbard *et al.* 2008). The fact that *D. virgifera* gains more weight on crown roots, despite the fact that they contain more active 1-4-benzoxazin-3-ones begs the question if the herbivore is resistant to this type of defence. Earlier studies found positive correlations between DIMBOA contents and resistance against *D. virgifera* in some, but not all cases in the field (Assabgui *et al.* 1995; Davis *et al.* 2000). However, none of these studies took into account the differential distribution of 1-4-benzoxazin-3-ones among root types, and the employed analytical techniques did not permit to separate the full profile of 1-4-benzoxazin-3-ones. In our assays, it was evident that *D. virgifera* was not negatively affected by the presence of 1-4-benzoxazin-3-ones in the roots (Fig. 6a). *D. virgifera* is known for its capacity to rapidly evolve resistance or tolerance to pest control methods, including novel compounds like the *bt* toxin (Meihls *et al.* 2008), and our results show that it possesses effective tolerance or detoxification mechanisms against the naturally occurring 1-4-benzoxazin-3-ones and possibly also the other defensive traits of maize roots, thereby enabling it to fully exploit the higher nutrient content of crown roots. Additional research will be necessary to elucidate the mechanism that enables *D. virgifera* to tolerate BXDs. As yet, nothing is known about BXD detoxification in chrysomelids.

Diabrotica virgifera does not only tolerate 1-4-benzoxazin-3-ones, but even uses them to identify the most nutritional roots: When given a choice, the herbivore settled on 1-4-benzoxazin-3-one containing roots rather than those of the *bx1* mutants (Fig. 6b), and in the absence of BXDs, it did no longer distinguish between crown and primary roots (Fig. 6c). MBOA, a breakdown product of DIMBOA and HDMBOA, has previously been reported to attract *D. virgifera* larvae *in vitro* (Bjostad & Hibbard 1992): In choice assays, *D. virgifera* larvae preferred volatile compounds coming from MBOA-treated glass wool within a CO_2 background. Another study, however, reported that the application of pure DIMBOA and MBOA to maize roots deterred *D. virgifera* (Xie *et al.* 1992). The experiments in that case were conducted with BXD producing roots that were dipped into ethanol solutions containing 250–1000 p.p.m of additional DIMBOA or MBOA, concentrations which are well above the amounts that are typically exuded by maize roots (Fig. 3). Although the contradictory results of these two studies indicate that *D. virgifera* is highly sensitive to 1-4-benzoxazin-3-ones, they also illustrate how challenging it is to develop biologically meaningful *in vitro* assays to test the role of BXDs in *D. virgifera* feeding behaviour. Our experiments involving a BXD deficient mutant circumvent potential problems associated with purification and dose for *in vitro* tests and show that *D. virgifera* is not repelled by the higher DIMBOA concentrations in crown roots, but uses BXD patterns to select optimal roots. That *D. virgifera* also showed a preference for crown roots of teosinte (Fig. 4d), despite the fact that the total amount of BXDs was similar between crown and primary roots (Fig. 3b inset), suggests that *D. virgifera* may either use single BXDs like DIMBOA or ratios between compounds (e.g. DIMBOA/HDMBOA-Glc) to select roots. In general, it should be noted that knocking out BXD production may also affect the root metabolism and other defensive processes. Using a different set of mutants, it was recently shown that BXDs have a positive effect on callose deposition following plant treatment with fungal elicitors (Ahmad *et al.* 2011). Wound-induced JA-accumulation in the leaves on the other hand is not affected by the *bx1* mutation (Huffaker *et al.* 2011). Future research should aim at determining whether such indirect effects, possibly also in combination with changes in nutritional patterns, may affect the feeding behaviour of *D. virgifera*. Generating maize lines that have an altered capacity to express *bx1* downstream genes will make it possible to further disentangle the role of individual BXDs in below ground plant-insect interactions. Overall, our study shows that *D. virgifera* hijacks a plant defence that is most probably targeted at deterring attackers from nutritious tissues. Thereby, it effectively turns the tables on maize to maximise its own fitness.

Evolving the capacity to cope with plant defences is thought to drive radiation of insect species, and, consequently, the co-evolution among plants and insects (Wheat *et al.* 2007). The capacity of *D. virgifera* to tolerate BXDs may therefore have facilitated its ongoing spread and success through maize growing regions around the world, contrary to other *Diabrotica* species, which have remained much less

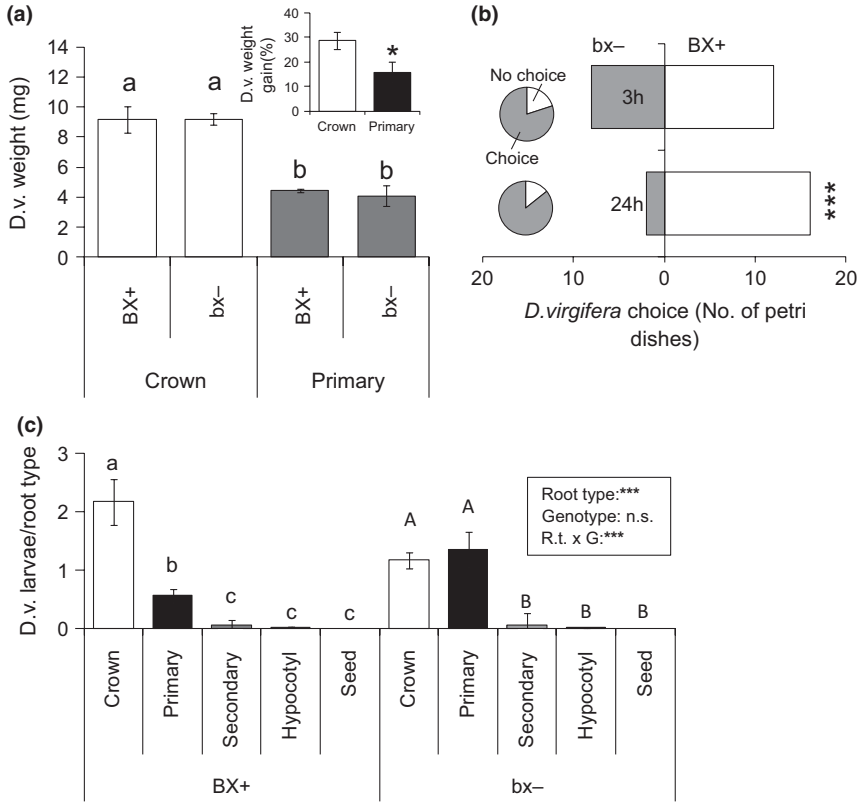

Figure 6 *Diabrotica virgifera* requires 1-4-benzoxazin-3-ones to locate optimal roots.(a) *D. virgifera* weight (±SE) 7 days after feeding on crown or primary roots (no-choice assay) of plants producing 1-4-benzoxazin-3-ones (BX+) or *bx1* mutants without these compounds (bx−). Different letters indicate significant differences between root types and genotypes. Inset: Average relative growth of *D. virgifera* feeding on crown or primary roots of the 1-4-benzoxazin-3-one producing hybrid Delprim over 24 h ($P < 0.05$). (b) Choice of *D. virgifera* for BX+ (white bars) or bx− roots (grey and black bars) at 3 h (top) and 24 h (bottom). Numbers of petri dishes with a choice of the larvae for either genotype are shown. Pie charts show the proportion of dishes with equal distribution of larvae (no choice situation, white). Stars indicate a significant preference (***$P < 0.001$). (c) Average number of *D. virgifera* larvae on each root type (mean over 24 h) in a no-choice situation. Significance levels (***$P < 0.001$) of a Two-way ANOVA are shown for the factors 'root type', 'genotype' and the interaction term (root type*genotype). Different letters indicate significant differences between root types (Tukey's HSD: $P < 0.05$) within the BX+ (small letters) and BX− (big letters) plant genotypes.

important. The current study shows that plant defences may not only determine the large scale abundance of insect herbivores (Johnson & Gregory 2006), but also their distribution within the plant system: Small scale adaptive behaviour enabled the generalists *D. balteata* and *S. littoralis* to escape toxic compounds, whereas it facilitated the aggregation of *D. virgifera* on the most nutritious tissues. The distribution of herbivores within a root system is evidently an important determinant of the fitness of both plant (Fig. 1) and insect (Fig. 6). We therefore conclude that understanding plant-insect interactions at this scale is important to determine the factors that shape plant and herbivore abundance in natural and agricultural systems.

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AUTHORSHIP

C.A.M. designed and carried out behavioural experiments, quantitative PCR, secondary metabolite analysis and $^{11}CO_2$ labelling and analysed data. N.Ve., G.D. and N.Vi. carried out behavioural experiments and analysed data. G.G. and G.M. developed analytical procedures and carried out secondary metabolite analyses. M.D.P.G. carried out quantitative PCR experiments. T.G.K. measured amino acids and analysed data. D.G. and M.B. measured sugars and analysed data. B.A.B. and R.A.F. carried out experiments with $^{11}CO_2$ and analysed data. T.C.J.T. contributed to writing the manuscript. M.E. designed the study, performed experiments, analysed data and wrote the first draft of the manuscript. All authors contributed to writing the final version of this article.

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SUPPORTING INFORMATION

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

Appendix S1 Method for surface extraction and quantification of 1,4-benzoxazin-3-ones using the NanoMate technology.

Figure S1 Structures and full names of 1,4-benzoxazin-3-ones in maize roots.

Figure S2 Induction of 1,4-benzoxazin-3-ones by *Diabrotica virgifera* and jasmonic acid.

Figure S3 Basal and induced phenolic compounds and proteinase inhibitors in maize roots.

Figure S4 UPLC-TOF-MS profile of maize root exudates.

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