A Physiological and Behavioral Mechanism for Leaf Herbivore-Induced Systemic Root Resistance[1][OPEN]

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Indirect plant-mediated interactions between herbivores are important drivers of community composition in terrestrial ecosystems. Among the most striking examples are the strong indirect interactions between spatially separated leaf- and root-feeding insects sharing a host plant. Although leaf feeders generally reduce the performance of root herbivores, little is known about the underlying systemic changes in root physiology and the associated behavioral responses of the root feeders. We investigated the consequences of maize (*Zea mays*) leaf infestation by *Spodoptera littoralis* caterpillars for the root-feeding larvae of the beetle *Diabrotica virgifera virgifera*, a major pest of maize. *D. virgifera* strongly avoided leaf-infested plants by recognizing systemic changes in soluble root components. The avoidance response occurred within 12 h and was induced by real and mimicked herbivory, but not wounding alone. Roots of leaf-infested plants showed altered patterns in soluble free and soluble conjugated phenolic acids. Biochemical inhibition and genetic manipulation of phenolic acid biosynthesis led to a complete disappearance of the avoidance response of *D. virgifera*. Furthermore, bioactivity-guided fractionation revealed a direct link between the avoidance response of *D. virgifera* and changes in soluble conjugated phenolic acids in the roots of leaf-attacked plants. Our study provides a physiological mechanism for a behavioral pattern that explains the negative effect of leaf attack on a root-feeding insect. Furthermore, it opens up the possibility to control *D. virgifera* in the field by genetically mimicking leaf herbivore-induced changes in root phenylpropanoid patterns.

Insect herbivores constantly compete for plants as a primary terrestrial source of organic carbon and nitrogen (Denno et al., 1995). Consequently, resource competition is thought to be a major determinant of the distribution and abundance of insects in natural and agricultural systems (Begon et al., 2006). Recent evidence suggests, however, that in many cases, insect herbivore competition may not follow the traditional theoretical assumptions of direct interference and/or resource exploitation, but may be determined by indirect plant-mediated effects (Kaplan and Denno, 2007; Poelman et al., 2008). Among the most striking examples of indirect plant-mediated interactions is the interplay between root- and leaf-feeding insects (Blossey and Hunt-Joshi, 2003). Despite their nonoverlapping feeding niches, leaf and root herbivores determine each other's performance through shared host plants (Bezemer and van Dam, 2005). Although root feeders can have positive or negative effects on leaf feeders (van Dam and Heil, 2011), the effect of leaf herbivores on root consumers is predominantly negative (Johnson et al., 2012; Huang et al., 2014).

Despite the increasing number of examples demonstrating negative effects of leaf attack on root herbivores (Tindall and Stout, 2001; Blossey and Hunt-Joshi, 2003; Soler et al., 2007; Gill et al., 2011), the mechanisms underlying this form of systemic induced resistance remain poorly understood (Erb et al., 2008; Rasmann and Agrawal, 2008). *Pieris brassicae*, for instance, was found to increase glucosinolate levels in the roots, which correlated with a reduced survival of the root feeder *Delia radicum* (Soler et al., 2007). Understanding why root feeders perform worse on leaf-infested plants would allow for more detailed investigations regarding the adaptive and evolutionary context of the phenomenon, and may allow for its exploitation in agriculture (for instance, by triggering root resistance through targeted leaf treatments).

A promising system to study the mechanisms and agroecological consequences of plant-mediated interactions between herbivores is maize (*Zea mays*) and its associated pests. In the field, maize is attacked by a suite of herbivores, including leaf feeders, stem borers, and root feeders. The highly specialized root-feeding larvae of the western corn rootworm *Diabrotica virgifera virgifera* cause significant plant damage and yield loss in the United States and Eastern Europe. Earlier studies

demonstrated that *D. virgifera* attack increases leaf resistance against *Spodoptera* spp. by triggering drought stress responses (Erb et al., 2009, 2011b). In the opposite direction, leaf feeding by *Spodoptera* spp. caterpillars reduces *D. virgifera* growth and development in a sequence-specific manner in the laboratory and the field (Erb et al., 2011c; Gill et al., 2011). *D. virgifera* was subsequently demonstrated to avoid leaf-infested plants by detecting and responding to a reduction in root ethylene emissions (Robert et al., 2012). However, it remains unclear whether nonvolatile chemical changes in the roots of leaf-infested maize plants affect *D. virgifera* foraging and performance. In this study, we explored the hypothesis that leaf infestation by *Spodoptera* spp. caterpillars triggers a short-range avoidance response in *D. virgifera*. Through a combination of bioactivity-guided fractionation of root extracts and biochemical and molecular manipulation, we show that systemic changes in soluble phenylpropanoid derivatives trigger a strong avoidance response in *D. virgifera*. We furthermore demonstrate that this avoidance response is mediated by systemic internal signals and is triggered specifically by herbivory, suggesting that *D. virgifera* actively and specifically recognizes and avoids leaf-infested plants.

RESULTS

D. virgifera Specifically Recognizes and Avoids Leaf-Infested Plants

To test whether *D. virgifera* is able to distinguish between infested and noninfested plants in the soil, we offered maize seedlings that were infested in the leaves by *Spodoptera littoralis* or were herbivore free to the root-feeding larvae of *D. virgifera* in a two-arm belowground system (Robert et al., 2012; Fig. 1). After 48 h of foraging activity, significantly more larvae were recovered on control plants than infested plants (Fig. 1A). As root volatiles may mediate *D. virgifera* foraging behavior (Robert et al., 2012), we conducted an additional experiment in which root systems of leaf-infested and noninfested plants were intertwined and offered to *D. virgifera* together in a single petri dish, so that larvae could not distinguish between the root systems of individual plants by using volatiles as long-distance cues. Again, *D. virgifera* showed a pronounced preference to feed on noninfested plants (Fig. 1B), indicating that changes in root volatiles are not necessary to trigger the avoidance response. A time course revealed that the avoidance response started 24 h after the beginning of leaf attack by *S. littoralis* and was most pronounced after 48 h (Fig. 1C). To test whether *D. virgifera* responds specifically to herbivore-induced changes in the plants, we wounded leaves and treated a subset of them with *S. littoralis* regurgitant, which induces a plant response similar to real herbivory (Erb et al., 2009). Wounding and leaf removal did not trigger an avoidance response (Fig. 1D). By contrast, adding regurgitant to the wounds elicited a behavioral response similar to a real *S. littoralis* attack, demonstrating that *D. virgifera* specifically recognizes leaf-infested plants. The response to a single, artificial elicitation event started 12 h after treatment and subsided between 24 and 48 h (Fig. 1E), suggesting a slow and transient change in root chemistry upon a single leaf elicitation. To understand whether internal leaf-to-root signals are responsible for the elicited behavior, or whether signals pass externally from the aboveground atmosphere through the rhizosphere, we sealed off the soil and root system from the aboveground atmosphere with an air-tight agarose/aluminum seal so that the only shoot-root contact was via the plant interior. *D. virgifera* responded by avoiding *S. littoralis*-infested plants irrespective of direct contact between the phyllosphere and the rhizosphere (Fig. 1F), demonstrating that a systemic change in the roots mediated by internal signaling is responsible for the reduction in attractiveness of the roots. To evaluate whether the systemic changes are due to water-soluble or nonsoluble substances, we obtained liquid fractions from the roots and mixed them with agarose to test the feeding preference of *D. virgifera* in an agarose cube choice assay. *D. virgifera* larvae preferred to feed on control fractions over leaf-induced fractions (Fig. 1G), showing that nonstructural chemical changes in the roots are sufficient to explain the observed behavior.

Leaf Infestation Changes Root Phenylpropanoid Accumulation

As phenolic compounds have been associated with changes in root herbivore performance in other plant

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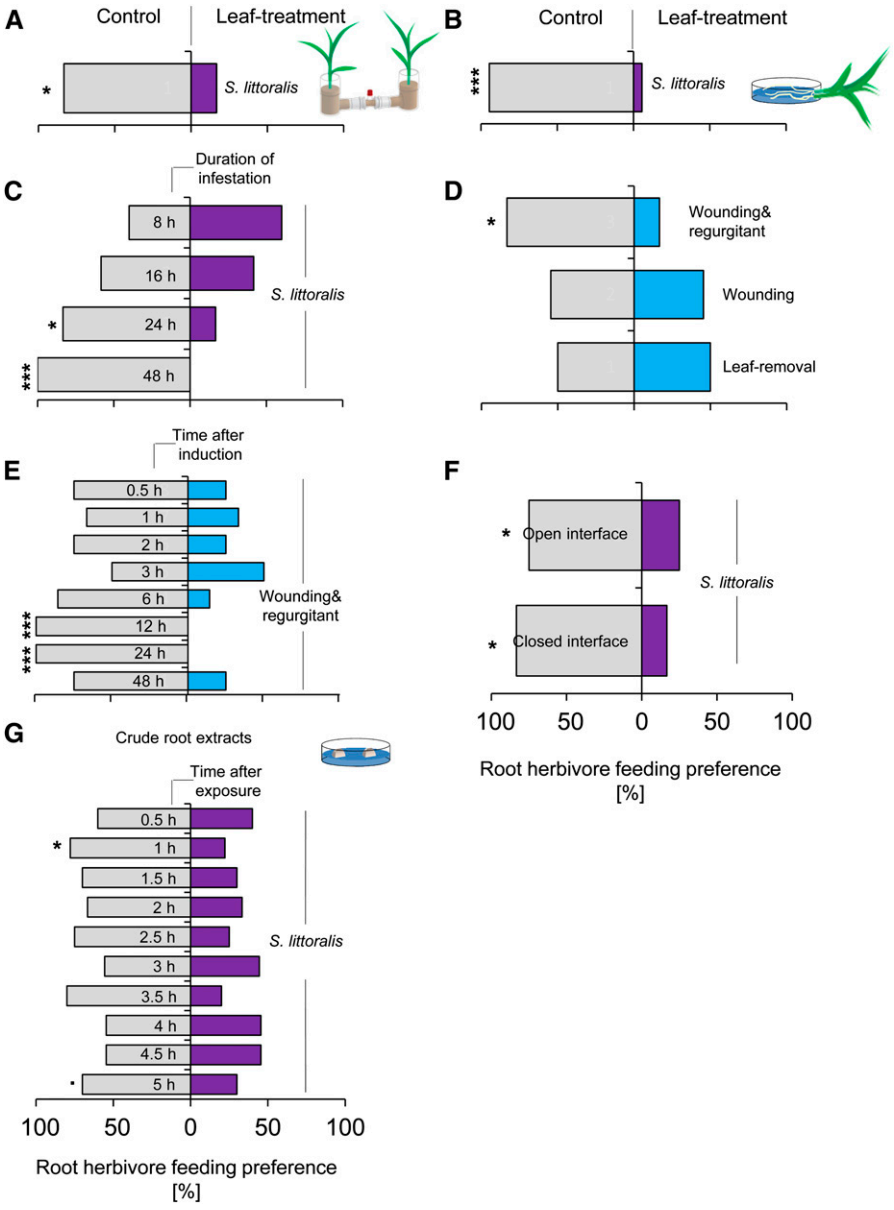

Figure 1. The root herbivore *D. virgifera* specifically avoids leaf-infested plants by recognizing systemic changes in soluble root components. A, Preference of *D. virgifera* larvae for roots of control versus leaf-infested plants in a two-arm belowground choice system (n = 15). B, Preference for roots of control versus leaf-infested plants with washed root systems in a petri dish setup (n = 19). C, Preference patterns after different durations of leaf infestation (n = 12). D, Preference for roots of damaged plants with and without defense elicitation by application of *S. littoralis* regurgitant (n = 12). E, Preference time course using a single leaf-induction event (n = 12–14). F, Preference for roots of control and leaf-infested plants with and without direct contact between the rhizosphere and phyllosphere (n = 12). G, Preference for root extracts of control and leaf-infested plants using agarose cubes (n = 15). Preference is expressed as percent choice corresponding to the proportions of independent replicates in which a given preference was observed (no choice: A–F, <10%; G, 21%). Asterisks indicate significant differences between treatments (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

species (Johnson et al., 2011), we hypothesized that changes in the phenylpropanoid pathway may be responsible for the change in *D. virgifera* behavior. To evaluate whether leaf infestation changes root phenolic acids, we analyzed crown and primary roots of leaf-infested plants by HPLC-tandem mass spectrometry. Based on the results of our choice experiments, we focused on soluble rather than cell wall bound phenolic acids. As soluble phenylpropanoids can be conjugated to proteins and other metabolites and may thereby escape detection (Nicoletti et al., 2013), we subjected soluble extracts to acid and basic hydrolysis to release ester- and ether-bound soluble phenolic acids. Both hydrolysis protocols resulted in the release of significant quantities of phenolic acids. Compared with free phenolic acids, which were found in concentrations between 1 and 70 ng g^{-1} fresh weight, soluble hydrolyzed phenolic acids were up to 100 times more abundant in the roots, with concentrations ranging from 0.1 to 8 μg g^{-1} fresh weight (Fig. 2). Primary and crown roots differed in their phenylpropanoid patterns, with primary roots containing higher amounts of basic hydrolyzable caffeic acid, free and acid-hydrolyzable ferulic acid, and acid-hydrolyzable sinapic acid. Primary roots also had lower concentrations of basic hydrolyzable *p*-coumaric acid (CA), acid-hydrolyzable caffeic acid, and basic hydrolyzable ferulic acid than crown roots. Leaf infestation by *S. littoralis* reduced the concentrations of all basic and acid-hydrolyzable phenolic acids as well as free caffeic acid in the roots (Fig. 2). By contrast, we observed a small but consistent average increase in soluble ferulic acid in the roots of leaf-infested

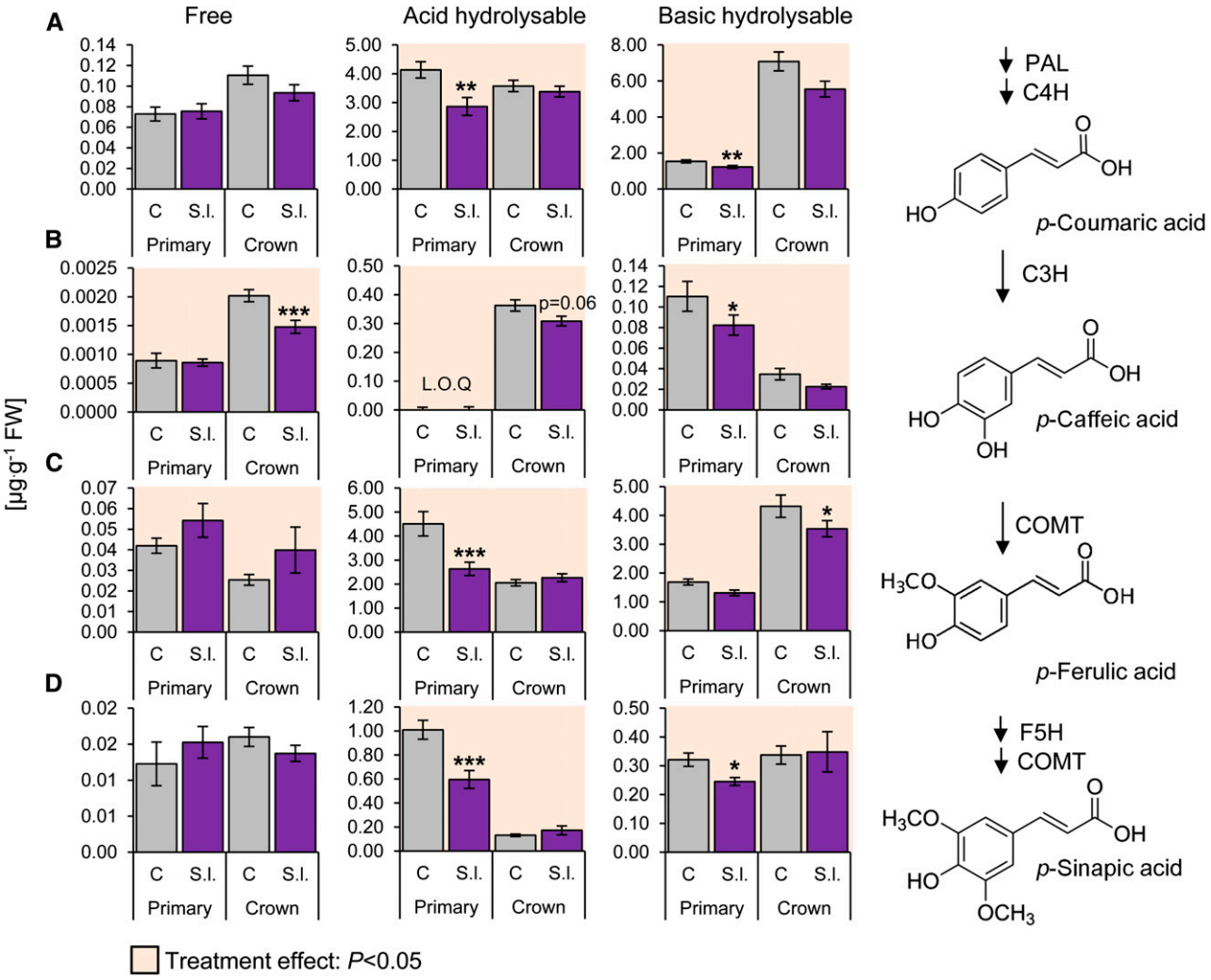

Figure 2. Leaf infestation alters soluble free and conjugated phenolic acids in the roots. Average concentrations of different phenolic acids in control roots (gray bars) and roots of leaf-infested plants (purple bars) are shown for crown and primary roots (±SE). Shading indicates a significant overall treatment effect determined by ANOVA ($P < 0.05$). Asterisks indicate significant pairwise differences between treatments within root types (Holm-Sidak post hoc tests: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$). COMT, Caffeic acid *O*-methyl transferase; PAL, phenylalanine lyase; C, control; S.l., *S. littoralis*.

plants. Pairwise comparisons revealed that different phenolic acids were reduced in primary and crown roots, even though the overall trends stayed the same, and no significant interactions between root type and leaf treatment were detected by two-way ANOVA ($P > 0.05$).

Manipulating the Phenylpropanoid Pathway Disrupts *D. virgifera* Host Choice

To test whether the leaf herbivore-induced changes in root phenolic acids are responsible for the reduced attractiveness of maize roots to *D. virgifera*, we performed a series of manipulative experiments (Fig. 3). First, we treated maize roots with PA, which inhibits the conversion of cinnamic acid to CA through competitive inhibition of C4H (Schalk et al., 1998). To confirm the efficacy of the treatment, we measured cinnamic acid accumulation in the roots following PA application. As expected, we observed a strong accumulation of soluble free and conjugated cinnamic acid (Supplemental Fig. S1). Furthermore, we observed a slight reduction in free sinapic acid. However, contrary to what has been reported in other plant species (Schalk et al., 1998; Naseer et al., 2012), we did not observe a depletion of CA, caffeic acid, or ferulic acid. Soluble acid, hydrolyzable caffeic acid, ferulic acid, and sinapic acid even increased in concentration in C4H-inhibited plants, suggesting that they are formed and induced by PA through a C4H-independent pathway, such as through the production of CA from Tyr (Rösler et al., 1997). As the PA treatment significantly changed the synthesis of free and conjugated phenolic acids, we concluded that this treatment is nevertheless suitable to

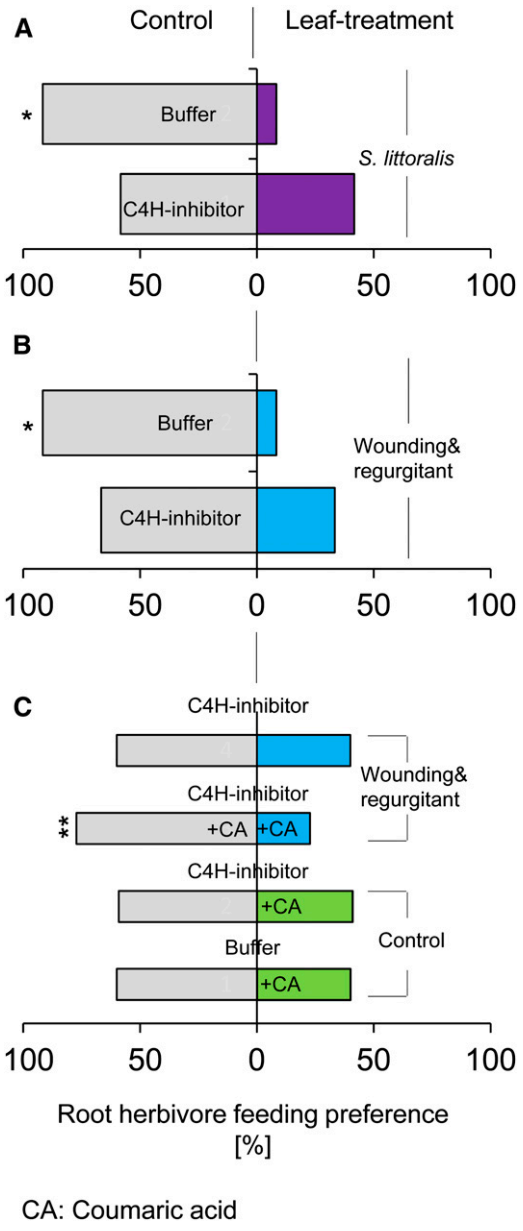

Figure 3. Manipulating the biosynthesis of phenolic acids through cinnamate 4-hydroxylase (C4H) inhibition leads to the disappearance of the *D. virgifera* avoidance response toward leaf-infested plants. A, Preference for roots of buffer-treated and C4H-inhibited control and *S. littoralis*-infested plants ($n = 12$). B, Preference for roots of buffer-treated and C4H-inhibited control and artificially induced plants ($n = 12$). C, Preference for roots of buffer-treated, C4H-inhibited, and CA-complemented control and artificially induced plants. C4H was inhibited by application of the selective inhibitor piperonylic acid (PA; $n = 23$). Preference is expressed as percent choice corresponding to the proportions of independent replicates in which a given preference was observed (no choice, <10%). Asterisks indicate significant differences between treatments (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

gain first insights into the potential involvement of this metabolite class in leaf herbivore-induced root resistance. When *D. virgifera* larvae were offered a choice between buffer-treated control and *S. littoralis*-infested plants, they showed the usual preference for control plants. However, when C4H was inhibited, no choice was observed (Fig. 3A). A similar result was obtained with plants that were elicited by wounding and application of regurgitant (Fig. 3B). To understand this pattern in more detail, we complemented inhibited and noninhibited control and induced plants with a 5.5-mM solution of CA. CA complementation in the absence of induction did not elicit a preference response in *D. virgifera* (Fig. 3C). However, complementing a C4H-inhibited, leaf-induced plant restored the preference pattern of the larvae, suggesting that C4H-dependent CA is necessary for the repellent effect of the roots, and that induction by leaf herbivory is specifically required to elicit this response.

In maize, several mutants have been characterized that are defective in their capacity to produce CA-derived phenolic acids and lignin (Halpin et al., 1998). We used two brown-midrib mutants, *bm1* and *bm3*, to further understand the importance of phenolic acid derivatives for *D. virgifera* host choice (Fig. 4). *bm1* is defective in cinnamyl alcohol dehydrogenase activity required to convert phenolic aldehydes into their alcoholic forms (Halpin et al., 1998). The *bm3* mutant has a defective caffeic acid *O*-methyl transferase, which is necessary for the production of sinapic acid-type phenolics and lignin (Vignols et al., 1995). Both mutations exert feedback effects on phenylpropanoid biosynthesis (Guillaumie et al., 2007). Our own analyses showed that, compared with the near-isogenic wild-type line F2 (Guillaumie et al., 2007), the *bm1* mutant is depleted in most soluble free phenolic acids, but overaccumulates soluble hydrolyzable ferulic acid and sinapic acid, whereas the *bm3* mutant is depleted in free phenolics without showing an overaccumulation of hydrolyzable compounds (Supplemental Fig. S2). Furthermore, both mutants accumulated slightly higher levels of caffeic acid. No phenotypic differences in root system architecture were observed between wild-type and mutant lines (Supplemental Fig. S3). Lignin levels at the seedling stage are low, as most lignin deposition occurs after the end of internode elongation (Müse et al., 1997; Riboulet et al., 2009). When given a choice between *S. littoralis*-infested and control F2 wild-type plants, *D. virgifera* exhibited a strong preference for the controls. In both *bm1* and *bm3* mutants, however, *D. virgifera* was no longer able to distinguish leaf-infested from control plants (Fig. 4A). When leaves were elicited by wounding and regurgitant, *D. virgifera* chose the control side in the F2 and *bm1* background, but no longer showed any preference in the *bm3* mutant (Fig. 4B). The differential preference between real and simulated herbivory in the *bm1* mutant was confirmed in a supplemental experiment that directly compared the two treatments (Supplemental Fig. S4). These data confirm that an intact phenylpropanoid pathway is required for the negative effect of leaf herbivory on root attractiveness. Furthermore, they illustrate that *bm1* is required for *D. virgifera* to recognize *S. littoralis*-infested, but not artificially elicited, plants.

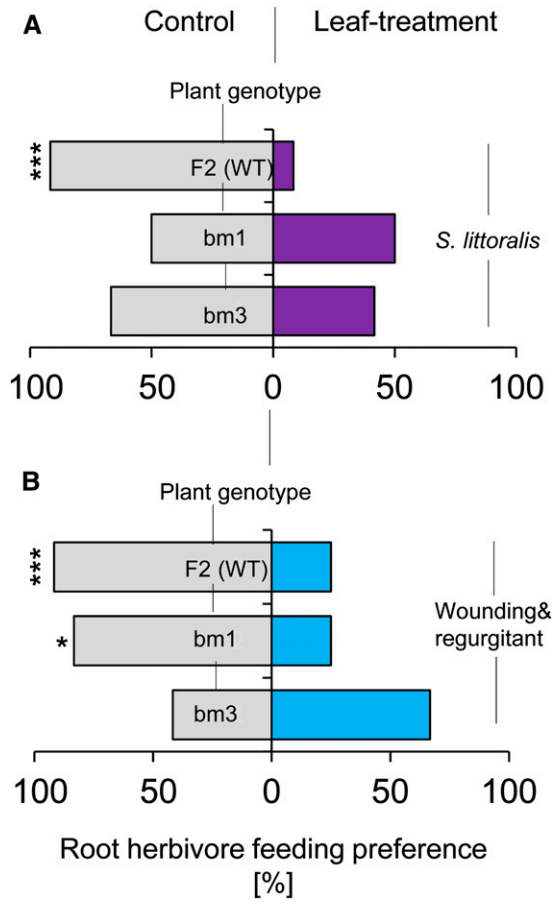

Figure 4. Genetic modification of the phenylpropanoid pathway leads to the disappearance of the *D. virgifera* avoidance response toward leaf-infested plants. A, Preference of *D. virgifera* for roots of control and leaf-infested wild-type (WT) plants, cinnamyl alcohol dehydrogenase (*bm1*) mutant plants, and caffeic acid *O*-methyl transferase (*bm3*) mutant plants ($n = 12–13$). B, Preference of *D. virgifera* for roots of control and artificially induced wild-type, *bm1*, and *bm3* plants ($n = 12$). Preference is expressed as percent choice corresponding to the proportions of independent replicates in which a given preference was observed (no choice, <10%). Asterisks indicate significant differences between treatments (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

Bioactivity-Guided Fractionation Associates *D. virgifera* Choice with Differential Accumulation of Conjugated Phenolic Acids

To further confirm the role of phenolic acids in leaf herbivore-induced root resistance, we collected soluble root fractions from control and *S. littoralis*-infested plants, redissolved them in 50% MeOH, and fractionated them further by reverse-phase semipreparative HPLC. Each fraction was then tested for activity by mixing it with agarose and offering it to *D. virgifera* in a choice assay (Fig. 5). Two nonpolar fractions (VIII and IX) were identified to exhibit activity and elicit a significant preference for control over *S. littoralis*-infested extracts (Fig. 5A). As conventional metabolomics fingerprinting by ultra-HPLC time-of-flight (TOF)-mass spectrometry (MS) did not reveal any differentially accumulating peaks in the active fractions (Supplemental Fig. S5), we conducted a second fractionation run and analyzed the active fraction for free and hydrolyzable phenolic acids by HPLC-tandem MS. This approach enabled us to separate conjugated phenolic acids in intact form and assess their abundance in each fraction individually through hydrolysis. Free phenolic acids were mostly contained in the polar fractions (Fig. 5B), whereas conjugated phenolics occurred across the entire polarity gradient (Fig. 5, C and D). In the bioactive fraction VIII-IX, hydrolysis revealed an herbivore-induced increase in acid hydrolyzable and a decrease in basic hydrolyzable CA (Fig. 5, C and D). Decreasing concentrations of free and hydrolyzable phenolic acids were observed in several inactive fractions. These data support the hypothesis that leaf herbivory changes the pattern of phenolic acid conjugates in the roots, and that these changes are associated with a decreased attractiveness of the roots for *D. virgifera*.

DISCUSSION

Although leaf herbivory often reduces the fitness of root feeders (Blossey and Hunt-Joshi, 2003; Johnson et al., 2012), the physiological and behavior mechanisms behind this phenomenon are poorly understood. Our results link systemic changes in conjugated phenolic acids to a strong avoidance response of a root feeder and thereby provide a physiological and behavioral explanation for the reduced abundance of *D. virgifera* larvae on the roots of leaf-attacked plants.

In the field, *D. virgifera* commonly co-occurs with many lepidopteran leaf feeders, including *Spodoptera* spp. (O'Day, 1998). Previous studies show that feeding by *Spodoptera frugiperda* on the leaves reduces the survival of late-arriving *D. virgifera* larvae in the roots (Erb et al., 2011c; Gill et al., 2011), especially in the upper layers of the rhizosphere (Erb et al., 2011d). As *D. virgifera*, which is highly specialized in maize (Clark and Hibbard, 2004), can migrate up to 1 m in the soil to find new host plants (Hibbard et al., 2003), it is conceivable that it may have developed the capacity to assess the quality of different plant roots. In vitro assays have demonstrated that maize root extracts are strong arrestants of *D. virgifera* larvae (Bernklau and Bjostad, 2005), and that monosaccharides as well as free and Gal-linked fatty acids (monogalactosyldiacylglycerols) stimulate their feeding (Bernklau and Bjostad, 2008; Bernklau et al., 2015). Interestingly, it has also been demonstrated that contact with an inferior host plants (e.g. soybean [*Glycine max*]) changes the behavior of *D. virgifera* neonates from localized to wider-ranging search behavior (Strnad and Dunn, 1990). Our experiments show that, if given a choice, *D. virgifera* larvae can also assess qualitative differences within genotypes and avoid inferior leaf-infested plants.

Theoretically, *D. virgifera* may use different cues to avoid leaf-infested plants. Possibilities include direct cues from the phyllosphere-like leaf volatiles, larvae, or

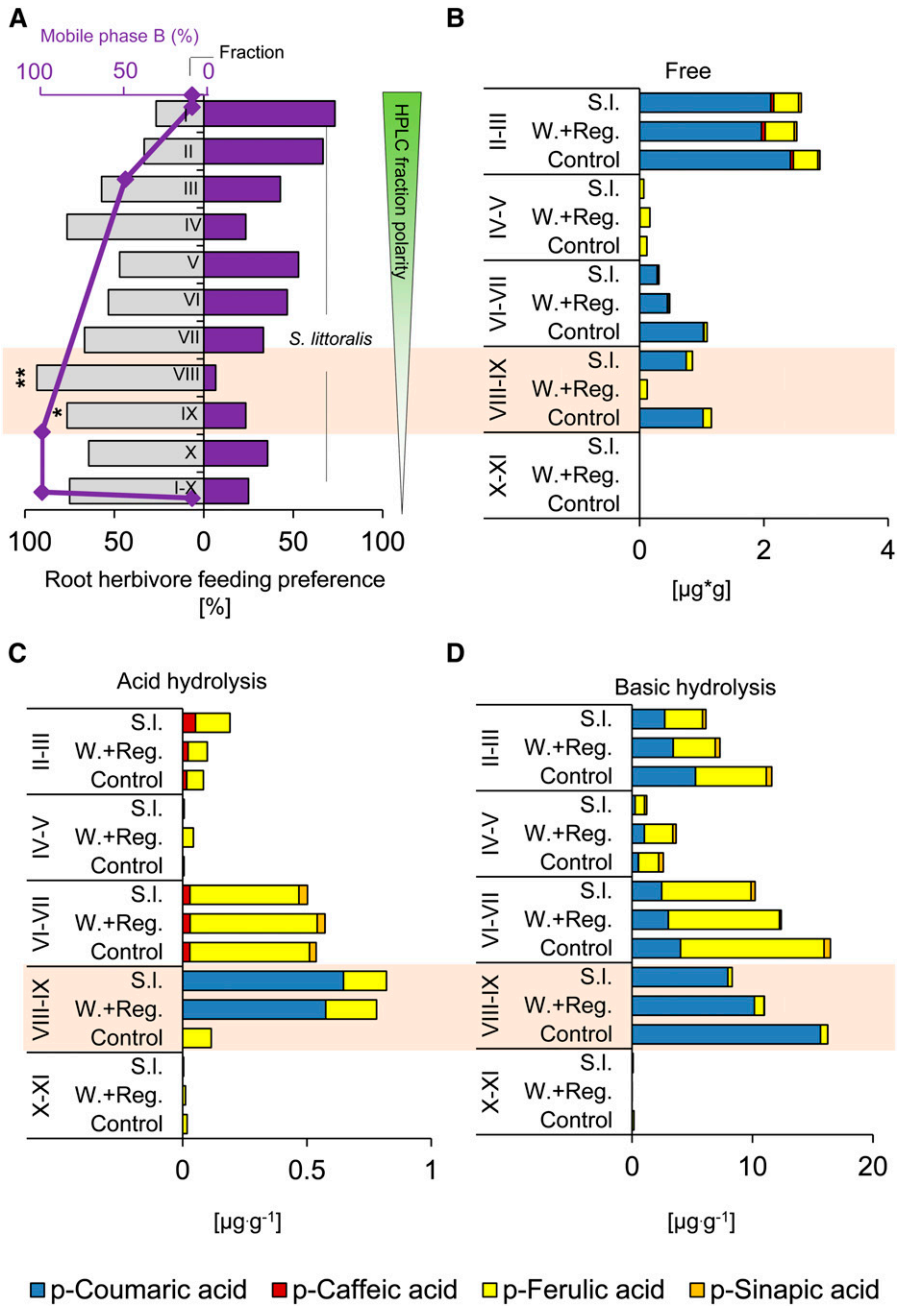

Figure 5. Bioactivity-guided fractionation links changes in conjugated phenolic acids with *D. virgifera* preference patterns. A, Preference of *D. virgifera* for fractions of root extracts of control and leaf-infested plants ($n = 15$). The polarity gradient of the fractionation setup is shown in purple. B, Concentrations of free phenolic acids in root extracts of control and leaf-induced plants across the polarity gradient. Note that analyzed fractions cover the range of two fractions of experiment A. C, Concentrations of soluble, acid hydrolyzable phenolic acids. D, Concentrations of soluble, basic hydrolyzable phenolic acids. Shaded areas correspond to the bioactive fraction. S.l., *S. littoralis*; W., wounding; Reg., regurgitant.

their frass; changes in root exudates or root volatile patterns; modification of the root-associated bacterial community; structural changes on the root surface; and changes in the root metabolite profile. In an earlier study, we found that *D. virgifera* can use changes in volatile organic compounds to avoid leaf-infested plants (Robert et al., 2012). The experiments presented here show that, in addition, changes in soluble root chemicals are sufficient to dramatically reduce the attraction of *D. virgifera*. The following findings supports this conclusion. First, *D. virgifera* distinguished infested from noninfested plants even when the roots of the two types of plants are tightly intertwined and presented together in the same volatile headspace, and when aboveground cues were physically blocked by isolating the soil with agar and aluminum foil. Second, the preference was maintained in liquid extracts of the root metabolome, even after evaporation and resolubilization. Third, the active metabolites could be separated from nonactive compounds by fractionation using conventional reverse-phase HPLC. Interestingly, the preference patterns were less strong when using root extracts compared with intact roots. It is therefore possible that short-range volatile and nonvolatile cues act in a synergistic manner.

Despite the evidence pointing to stable, soluble root chemicals as causal factors in the interaction, our earlier attempts to identify root metabolites that respond to leaf infestation through an ultra-HPLC-TOF-based metabolomics approach did not yield any clear candidate features (Marti et al., 2013). Here, using more targeted methods, we provide several lines of evidence that phenolic acid conjugates can play a central role in mediating the preference of *D. virgifera* larvae for noninfested plants. First, our profiling assays demonstrate that the abundance of several hydrolyzable phenolic acids in the roots decrease in leaf-infested plants. Second, chemical and molecular interference with phenolic acid biosynthesis led to the disappearance of the differential preference exhibited by *D. virgifera*. Third, the bioactive fraction of root extracts contained significant amounts of hydrolyzable phenolic acids, of which the abundance strongly changed with leaf infestation. However, despite the presented evidence, several open questions regarding the biosynthesis, regulation, and identity of the foraging cues remain. Phenolic acids, a majority of which are derived from coumaric acid, can be conjugated to other phenylpropanoids, sugars, proteins, fatty acids, and terpenoids (Shimizu and Ohta, 1960; Hoff et al., 1994; Koetter et al., 1994; Quideau et al., 2011; Cheynier et al., 2013), resulting in a large number of possible soluble and insoluble structures, many of which are biologically active (Cheynier et al., 2013). Our fractionation/hydrolysis approach reveals that conjugated phenolic acids are both highly abundant and diverse. Orthogonal approaches, including, for instance, hyphenated NMR, will be necessary to identify the actual metabolites that are recognized by *D. virgifera*. Phenylpropanoid derivatives are known to serve as signaling molecules (Brown et al., 2001) and enzymatic cofactors (Šukalović et al., 2005). Furthermore, despite the bioactivity of our HPLC fractions pointing at a direct effect, the possibility that changes in *D. virgifera* preference are not due to changes in phenolic acid content, but rather due to other metabolites that are regulated by phenolic acids, cannot be fully excluded at this point (Maag et al., 2015). Another interesting observation concerns the fact that the *bm1* mutation made it impossible for *D. virgifera* to distinguish control from *S. littoralis*-infested plants, but still allowed it to distinguish control from artificially elicited seedlings. This finding suggests that the application of oral secretions to wounded leaves does not fully mimic the systemic changes in the roots that are elicited by real herbivory. Further experiments will be necessary to determine whether the intensity and speed of wounding or labile elicitors in the oral secretions of *S. littoralis* are responsible for this remarkable degree of specificity.

Host plant selection in phytophagous insects is a key process shaping plant-insect interactions. Although much is known about how leaf feeders find and choose their food source (Bernays and Chapman, 1994), it remains poorly understood how root herbivores accomplish this task. So far, it is not known if root herbivores might escape plant-mediated competition with aboveground feeders by specifically recognizing systemic changes in the roots of leaf-infested plants. Our experiments show that root-feeding *D. virgifera* larvae actively engage in host selection, and that leaf herbivory specifically influences their host choice by altering phenylpropanoid patterns in the roots. This implies that aboveground herbivores may have a strong effect on the distribution and abundance of soil-dwelling organisms via systemic changes in root metabolites and could thereby shape entire belowground food webs.

From an applied point of view, our findings open up two potential strategies to improve the management of one of the world's most damaging maize pests. First, by altering root phenylpropanoid biosynthesis, *D. virgifera* may be tricked into feeding on inferior (i.e. leaf-infested) host plants, which may reduce its performance and overall damage in the field. Second, it may eventually be possible to mimic leaf infestation at a genetic level and thereby produce maize plants that *D. virgifera* larvae will avoid. The currently available *bm* mutants may be a good starting point to assess whether changes in phenylpropanoid and lignification patterns can be used to alter the behavior of *D. virgifera* and reduce its damage under field conditions. Root-specific silencing of the corresponding genes could be a next step to harness the positive effect of these alterations without compromising the resistance of the plants to leaf pests and pathogens.

MATERIALS AND METHODS

Plants and Insects

Maize (*Zea mays*) plants were grown as described previously (Erb et al., 2011c). Unless otherwise indicated, the hybrid Delprim (Delley DSP, Delley) was used for experiments. The *bm1* and *bm3* mutants were bred at INRA Lusignan as described (Barrière et al., 2004). Plants for experiments were 10 to 12 d old and had two to three fully expanded leaves. The herbivores *Diabrotica virgifera virgifera* and *Spodoptera littoralis* were reared following previously established protocols (Robert et al., 2012). Third instar *D. virgifera* and second-instar *S. littoralis* larvae were used in all experiments. All plants were covered with 1.5-L polyethylene bottles as described (Erb et al., 2011a) to prevent leaf herbivore escape.

Root Herbivore Choice Patterns in the Soil

To assess the choice of *D. virgifera* when exposed to leaf-infested and herbivore-free plants, we used several different behavioral setups. First, we developed a system composed of two L-shaped glass pots to assess *D. virgifera* choice in the soil. The pots were 5 cm in diameter and 10 cm deep. At the bottom of the pots, an open glass tube (4 cm long, 1.5-cm i.d.) extended the rhizosphere system horizontally. The lowest 2 cm of the pots (including the glass tubes) was filled with soil, before individual plants, together with the soil and sand medium from their cultivation system, and were transferred carefully into the vessels. After 24 h of acclimatization in a temperature- and light-controlled environment (23°C, 16/8 h of light/dark, 90 μmol m^{-2}), 1.5-L polyethylene bottles with their bottoms removed were attached to the glass pots upside down. One-half of the plants were then infested with 20-s instar *S. littoralis* larvae over 48 h, whereas the other one-half was left herbivore free. After this period, during which the leaves were damaged but still had ample leaf biomass (>50%), 6-s instar *D. virgifera* larvae were introduced into the horizontal glass tubes (three on each side). The openings of the glass tubes of a control and a leaf-infested plant were then connected and sealed using plastic film ($n = 15$). In this way, the root herbivores had access to the differentially treated plants via a 10-cm glass tube filled with soil. *D. virgifera* larvae were left to move freely

between the two plants for 48 h, after which the system was disassembled, and the position of the root herbivores was recorded.

Root Herbivore Choice Patterns with Superposed Root Systems

To assess whether *D. virgifera* can use tactile cues to distinguish leaf-infested from uninfested plants, we developed a petri dish assay. First, maize seedlings were treated in their normal growth environment (see below). Plants were then removed from their pots, and the roots were gently washed under a stream of warm water. The root systems of two plants (control versus treatment; see below) were laid out on moist filter paper embedded in a large petri dish (12-cm diameter). Roots were mixed to create a random pattern of roots from the two plants. The petri dish had a cavity on the side, into which the stems were laid, leaving the leaves of the plant free in the air. Six-second instar *D. virgifera* larvae were then introduced into the dish, which was sealed with its lid and laid out on an experimental bench supplied with plant growth lights (23°C, 16/8 h of light/dark, 90 μmol m^{-2}). To guarantee moisture-saturated air around the exposed roots, water-drenched paper tissue was wrapped around the petri dish, followed by a layer of aluminum foil to shade the roots from light. The position of the larvae was recorded 30 min, 1 h, 2 h, 3 h, and 4 h after introduction into the choice arena. Using this setup, 11 different experiments were conducted. First, maize seedlings were infested with 20-s instar *S. littoralis* larvae for 48 h. The herbivores were removed after this period, and roots from a control plant and an infested plant were offered to *D. virgifera* (n = 19). Second, plants were infested in the same manner, but offered to *D. virgifera* at different time points ranging from 8 to 48 h (n = 12). Third, leaf herbivory was simulated using three different treatments: (1) scratching of approximately 1 cm^2 of leaf tissue six times over 48 h, until all leaves were damaged; (2) additional application of 10 μL of *S. littoralis* regurgitant to the scratched surfaces, as described in Erb et al. (2009); and (3) removal of leaf area by sequentially cutting off 50% of each leaf over 48 h. All treatments started at the lowest leaf and ended with the youngest, freshly developed leaf, which corresponds to the order of attack by *S. littoralis* (Köhler et al., 2014). Plants of each treatment were paired with untreated control plants, and the superposed roots were offered to *D. virgifera* larvae (n = 12). Fourth, leaves were induced by scratching and application of *S. littoralis* regurgitant and offered to the larvae at different time points after induction (n = 12–14). Fifth, plants were induced by *S. littoralis*, but the root system and soil were sealed off from the aboveground environment of the plant by pouring a 2-cm layer of solidifying agar (2% [w/w] agarose in water, 45°C) onto the soil in the pots, resulting in an air-tight seal around the stem. Furthermore, the stem was sealed off by two layers of tightly wrapped aluminum foil, ensuring that the stem was the only physical connection between leaves and roots. After removing the agar seal and aluminum from the plants, roots were washed and exposed to *D. virgifera* as described above. A control set of plants without seal was included in the assay (n = 12). Sixth, the potential effect of phenylpropanoids was investigated by C4H inhibition with PA. Plants were either infested with 20 *S. littoralis* larvae or left uninfested for 48 h. One-half of the control and infested plants were treated with PA (Sigma-Aldrich) by adding 10 mL of 75 μM PA in 10% ethanol solution to the soil every 24 h over the 48-h infestation period (three times in total). The other one-half of the plants were treated with buffer (10% ethanol). After this time, the preference of six *D. virgifera* larvae for control or leaf-infested plants within PA- or buffer-treated plants was evaluated (n = 12). Seventh, the same setup as for experiment 6 was used, with the only difference being that the plants were elicited by repeated wounding and regurgitant application over 48 h (n = 12). Eighth, PA-treated plants were complemented with 5.5 mM CA (Sigma-Aldrich). In this experiment, we simultaneously tested the preference of *D. virgifera* as follows (n = 23 for each experiment): (1) control versus leaf-elicited plants, both treated with PA; (2) control versus leaf-elicited plants, both treated with PA and complemented with CA; (3) control plants treated with buffer versus control plants complemented with CA, both treated with PA; and (4) control plants treated with buffer versus control plants complemented with CA. Maize leaves were induced as described above. Control plants remained undamaged. PA treatment was performed as described above. Complementation with CA was achieved by watering the plants with 10 mL of 5.5 mM CA in 1% ethanol solution every 24 h over 48 h (two times in total). Noncomplemented plants were watered with 10 mL of 1% ethanol solution. Ninth, the choice of *D. virgifera* was evaluated for three different maize genotypes: the near-isogenic line F2 and the mutants *bm1* and *bm3* (Guillaumie et al., 2007). Plants were infested with *S. littoralis* as described (n = 12–13). Tenth, the preference of *D. virgifera* was tested in the three genotypes using artificial induction by wounding and regurgitant as described above (n = 12). Finally, in the last choice experiment, the choice of *D. virgifera* on *S. littoralis*-infested and artificially induced *bm1* mutants was compared directly using conditions and treatments as described above (n = 15).

Root Herbivore Choice Patterns with Plant Extracts

To test whether *D. virgifera* can detect leaf herbivore-induced, systemic metabolic changes in root extracts, we conducted a separate experiment using root extracts in agarose. For this, roots of control plants and plants infested with 20 *S. littoralis* larvae were removed from the pots, washed gently, and flash frozen in liquid nitrogen. After grinding the roots to a fine powder with mortar and pestle in liquid nitrogen, the root material was centrifuged (2 min at 17,500*g*), and the supernatant was recovered and stored at −80°. In this way, we recovered about 50% of the root fresh mass in liquid form. During our assays, we found that filling root material into a 5-mL syringe tube and pressing it with the plunger was equally effective and much faster to extract root liquid, and we used this technique for large-scale isolation (see below). For behavioral assays, agarose solutions were prepared (2% agarose in water). Just before solidification of the solutions (45°C), we added different root extracts and stirred the mix. The solutions were then poured into petri dishes and left to solidify. From the different gels, cubes (5 × 5 × 5 mm) were cut and placed into new petri dishes (two per dish with different treatments). Six-second instar *D. virgifera* larvae were then introduced to each dish, and the dishes were placed in a humidity-controlled phytotron (23°C, 95% relative humidity, no light). For 4 h, the position of the root herbivores in the dishes was recorded every 30 min. Using this procedure, we offered cubes containing extracts from control and infested plants (diluted 1:1 in water) to the root herbivores (n = 15). In a second experiment, we tested 10 fractions of control and induced root extracts from 400 plants for each treatment obtained from semipreparative HPLC runs (see below). For each pair of fractions, we evaporated the solvents, resuspended the fractions in 10 μL acetonitrile, and diluted the dissolved fractions in 0.5 mL of agarose (4%). This mixture was then diluted with 0.4 mL of water to reach concentrations that were equivalent to root concentrations and the 50% dilution of crude root extracts in agarose. To provide a metabolite background for the choice experiments, 125 μL of crude root extract of noninfested plants was added to each test fraction. *S. littoralis* choice was then assessed for each pair of fractions (n = 15).

Fractionation of Root Extracts by Semipreparative HPLC

To facilitate the identification of the metabolites that are used by *D. virgifera* larvae to distinguish between leaf-damaged and control plants, we carried out two fractionation runs using semipreparative reverse-phase HPLC (Marti et al., 2013). For the choice assay, extracts from the roots of 400 control and 400 *S. littoralis*-infested plants were lyophilized to yield 50 mg of dry matter per treatment. The extracts were redissolved in 500 μL of 50% MeOH (v/v) and fractionated using a semipreparative C18 column (C18, 250- × 10-mm i.d., 5 μm, XBridge; Waters) connected to a Varian 9012 Solvent Delivery System operating at a flow rate of 10 mL min^{-1}. The injection volume was 250 μL. The solvent gradient started with 90% water and 10% can (both with 0.1% [v/v] formic acid) for 2 min, followed by a ramp to 50:50 over 20 min and a ramp to 5:95 over 70 min. The 5:95 mix was held for 12 min, followed by a post-equilibration at 90:10 for 10 min. Fractions were collected at 6-min intervals between 2 and 62 min. After pooling the fractions from two different runs per treatment, they were lyophilized and evaporated to dryness under nitrogen flow before being redissolved for biological experiments. Dry weights for the different fractions were between 0.1 and 0.9 mg. For phenolic acid analysis, pools of 18 control, 18 *S. littoralis*–infested, and 18 artificially induced plants were fractionated using the same setup, with the only difference being that, instead of 11 fractions, five different fractions were collected, with fraction VII-IX corresponding to the bioactive window as determined by biological experiments.

Metabolomic Fingerprinting of Active Root Fractions

Metabolic fingerprinting of the active fractions was carried out as described previously (Marti et al., 2013). In brief, the fingerprints of each extract were obtained using a short UPLC BEH C18 Acquity column (50- × 1.0-mm i.d., 1.7 μm; Waters). The mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The linear gradient program was as follows: 98% A over 0.2 min, to 100% B over 4.9 min, held at 100% B for a further 1.1 min, then returned to initial conditions (98% A) in 0.1 min for 1.1 min of equilibration before the next analysis. The flow rate was 0.3 mL min^{-1};

column temperature was kept at 40°C. Detection was performed by TOF-MS (LCT Premier; Waters) in W-mode in both electrospray negative- and positive-ion modes in independent runs over a mass-to-charge ratio range of 100 to 1,000 D. The MS was calibrated using sodium formate, and Leu-enkephalin was used as an internal reference. The injection volume was 1 μL, and all samples were diluted at 0.5 mg mL^{-1}. The recorded profiles were normalized to 1,000 counts, and peaks were extracted using MZmine v 2.12 (Pluskal et al., 2010) followed by univariate data analysis with Microsoft Excel.

Root Phenolic Acid Profiling of Leaf-Infested Plants

Soluble free and hydrolyzable root phenolic acids were profiled in three different experiments. To evaluate changes in root phenolic acids upon leaf infestation, maize seedlings were infested with 20 *S. littoralis* larvae. Control plants remained uninfested. After 48 h, maize primary and crown roots were collected separately, washed in a stream of tap water, immediately frozen in liquid nitrogen, and stored at −80°C until use ($n = 12$). The extraction procedure was adapted from de Ascensao and Dubery (2003). In brief, all samples were ground in nitrogen to a fine powder using a mortar and a pestle. Six hundred microliters of 100% MeOH was added to 100 mg of root powder, vortexed, and centrifuged at 17,500 rpm for 20 min at 4°C. The supernatant was collected and used for the next extraction steps. For each biological replicate, extracts of three plants were pooled and separated into three aliquots. First, 1-mL aliquots were evaporated to dryness under a flow of nitrogen (Glas-Col, catalog number 099A EV9624S) and resuspended in 50 μL of 50% MeOH for the analysis of free phenolic acids. Second, 50-μL aliquots were mixed with 50 μL of concentrated HCl (37%; Sigma-Aldrich) and heated for 1 h at 80°C for acid hydrolysis. One milliliter of diethyl ether was added, and the organic phase was collected and evaporated to dryness under a flow of nitrogen (Glas-Col, catalog number 099A EV9624S) before resuspension in 50 μL of 50% MeOH. Third, 50-μL aliquots were mixed with 100 μL of 2 M NaOH (Sigma-Aldrich) and left to stand for 3 h at ambient temperature for basic hydrolysis. The samples were then mixed with 50 μL of concentrated HCl (37%; Sigma-Aldrich) and 1 mL of diethyl ether (Glas-Col, catalog number 099A EV9624S), and the organic phase was recovered and evaporated under a nitrogen stream (Glas-Col, catalog no. 099A EV9624S) before resuspension in 50 μL of 50% MeOH. The three different types of extracts were then analyzed by HPLC as described below.

HPLC-MS Analysis of Phenolic Acids

Chromatography was conducted on a 1260 Infinity HPLC system (Agilent Technologies) coupled to an API 5000 tandem mass spectrometer (Applied Biosystems). In brief, the separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 0.5 μm; Agilent) using formic acid (1%; Fisher Scientific) in water and acetonitrile (Fisher Scientific) as mobile phases A and B, respectively. The elution gradient used was as follows: 0 to 0.5 min, 10% B; 0.5 to 4 min, 10% to 90% B; 4 to 4.02 min, 90% to 100%; 4.02 to 4.5 min, 100% B; 4.5 to 4.51 min, 10%; and 4.51 to 7 min, 10% B. The flow rate of the mobile phase was 1.1 mL min^{-1}. The column temperature was maintained at 25°C. The instrument parameters were optimized with infusion of pure standards (Sigma-Aldrich). The ion spray voltage was −4,500 eV. The turbo gas temperature was 700°C. Nebulizing gas was set at 60°C, curtain gas at 25°C, heating gas at 60°C, and collision gas at 7°C. Multiple-reaction monitoring was used to measure the parent ion to product ion transitions as described in Supplemental Table S1. Data acquisition and processing were performed on Analyst 1.5 software (Applied Biosystems). Dilution series of standard mixtures of each phenolic acid (purchased from Sigma-Aldrich) were used for quantification. Peak areas of cis and trans isomers were summed up for quantification.

Data Treatment and Statistical Analysis

To test the preference of *D. virgifera* in the two-arm belowground system, we used the statistical procedure outlined previously (generalized linear model with quasi-poisson distribution to take into account overdispersal, followed by ANOVA; Robert et al., 2012) using the R Project for Statistical Computing (version 3.2.1.). To assess larval choice in petri dish assays, a choice differential was calculated from each replicate by subtracting the average number of larvae on the control side from the average number of larvae on the treatment side. The differentials were then compared against the null hypothesis (equal preference for both sides, resulting in a differential of 0) using ANOVA in R. Differences in phenolic acid profiles were evaluated by ANOVAs followed by Holm-Sidak post hoc tests in Sigma Plot 12.5.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. C4H inhibition alters the accumulation of soluble free and conjugated phenolic acid in the roots.

Supplemental Figure S2. Genetic modification of the phenylpropanoid pathway alters the accumulation of soluble free and conjugated phenolic acid in the roots.

Supplemental Figure S3. Genetic modification of the phenylpropanoid pathway does not alter root architecture of maize seedlings.

Supplemental Figure S4. The *bm1*-dependent preference pattern of *D. virgifera* differs between *S. littoralis*-infested and artificially elicited plants.

Supplemental Figure S5. Metabolomic fingerprints of active root fractions.

Supplemental Table S1. Multiple reaction monitoring parameters for phenolic acid analysis.

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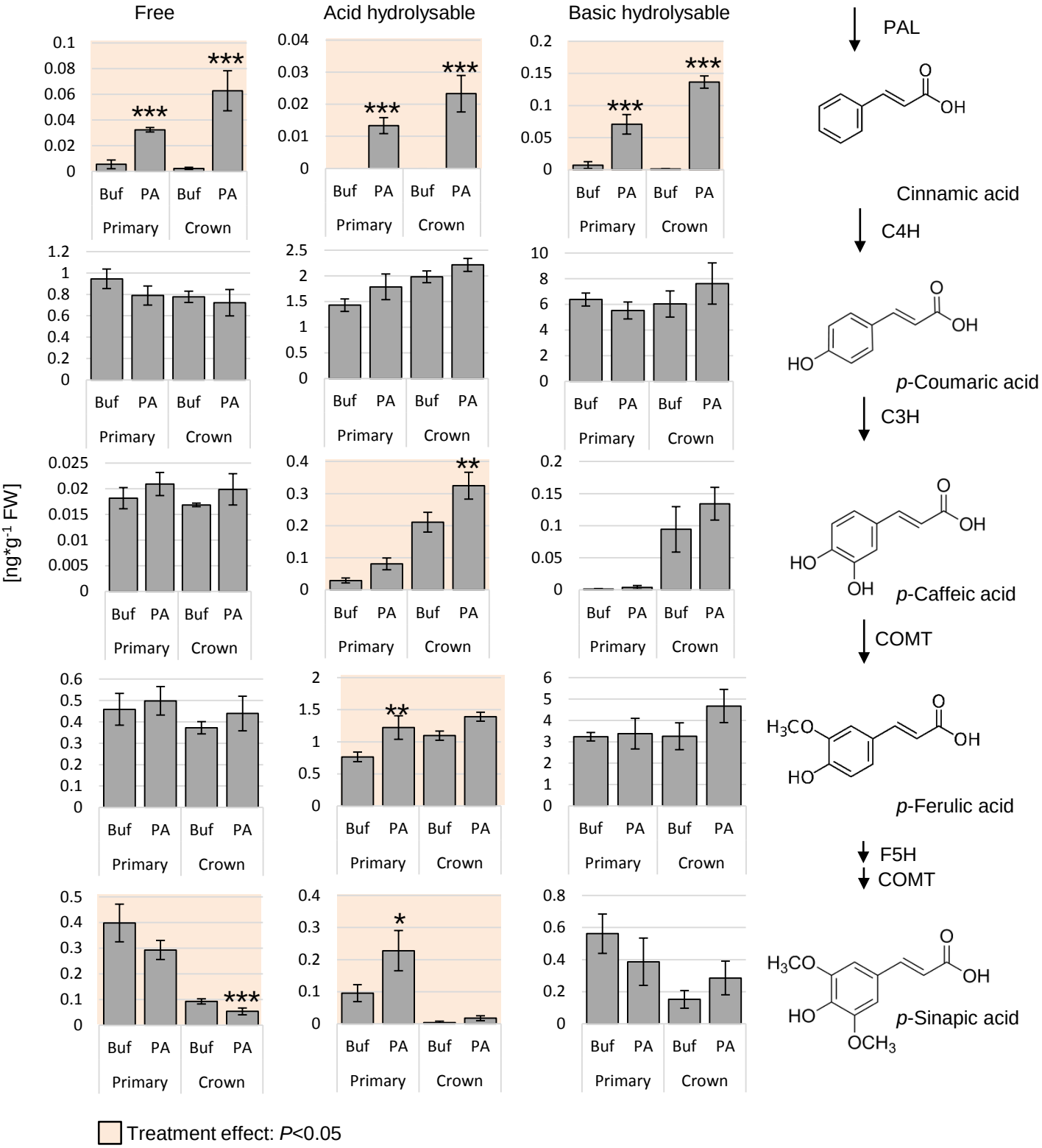

Fig. S1: *Cinnamate 4-hydroxylase inhibition alters the accumulation of soluble free and conjugated phenolic acid in the roots.* Average concentrations of different phenolic acids in buffer treated roots (Buf) and piperonylic acid treated, C4H inhibited roots (PA) are shown for crown and primary roots (± SE). Shading indicates a significant overall treatment effect determined by analysis of variance ($p<0.05$). Stars indicate significant pairwise differences between treatments within root types (Holm-Sidak post-hoc tests: *$p<0.05$, **$p<0.01$, ***$p<0.001$).

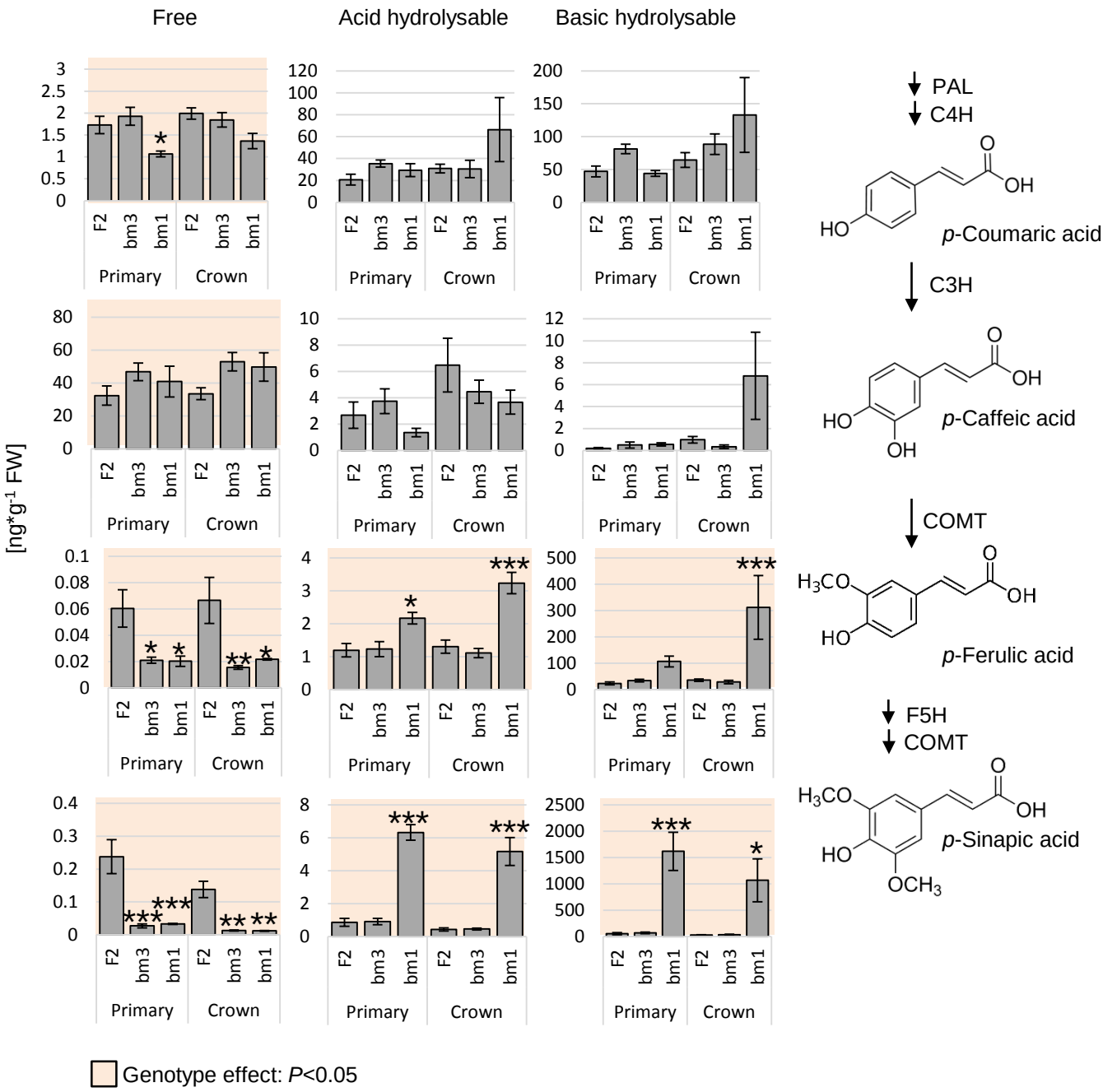

Fig. S2: *Genetic modification of the phenylpropanoid pathway alters the accumulation of soluble free and conjugated phenolic acid in the roots.* Average concentrations of different phenolic acids wild type (F2), bm1 and bm3 mutants are shown for crown and primary roots (± SE). Shading indicates a significant overall treatment effect determined by analysis of variance ($p<0.05$). Stars indicate significant pairwise differences between treatments within root types (Holm-Sidak post-hoc tests: *$p<0.05$, **$p<0.01$, ***$p<0.001$).

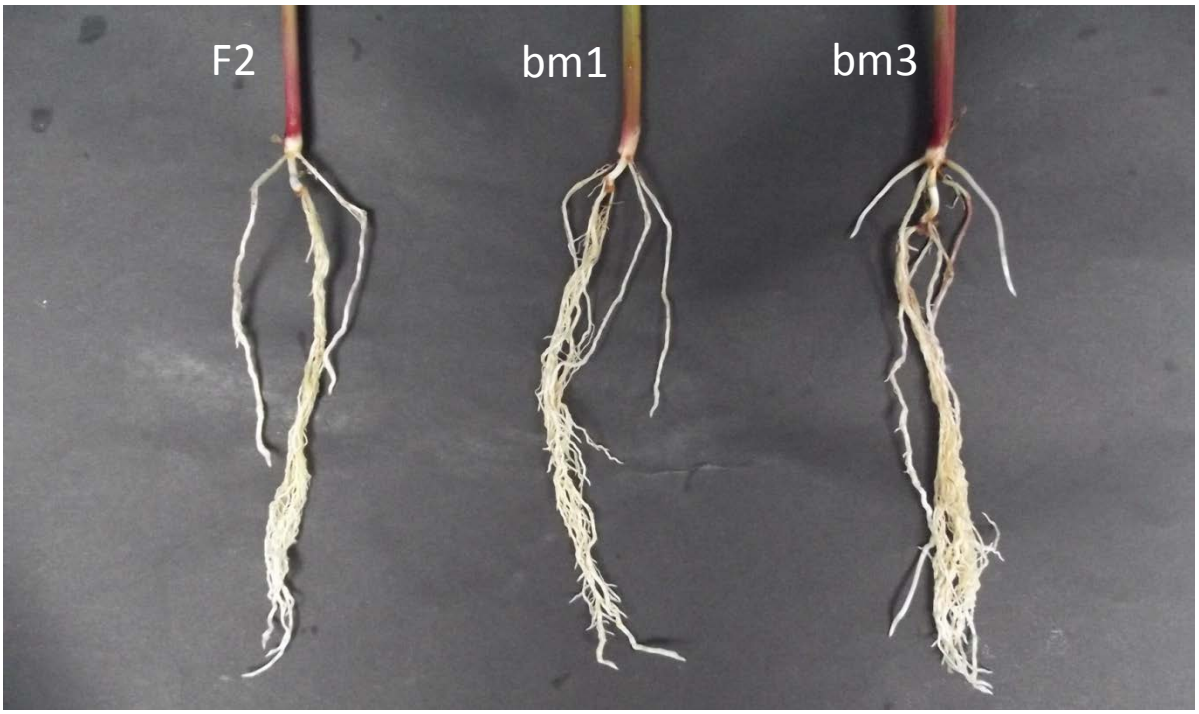

Fig. S3: *Genetic modification of the phenylpropanoid pathway does not alter root architecture of maize seedlings.* Pictures of wild type (F2), bm1 and bm3 roots of 12-day-old maize seedlings are shown.

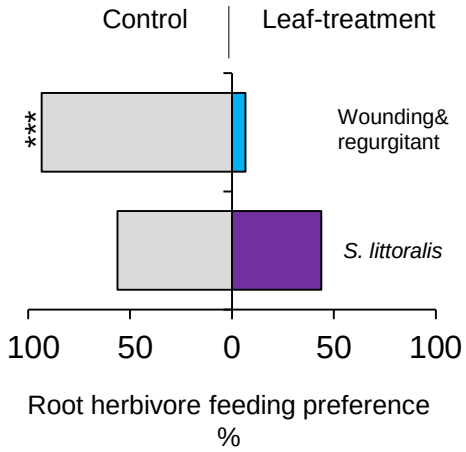

Fig. S4: *The bm1-dependent preference pattern of D. virgifera differs between S. littoralis infested and artificially elicited plants.* The preference for roots of leaf-induced bm1 mutant plants is shown. Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

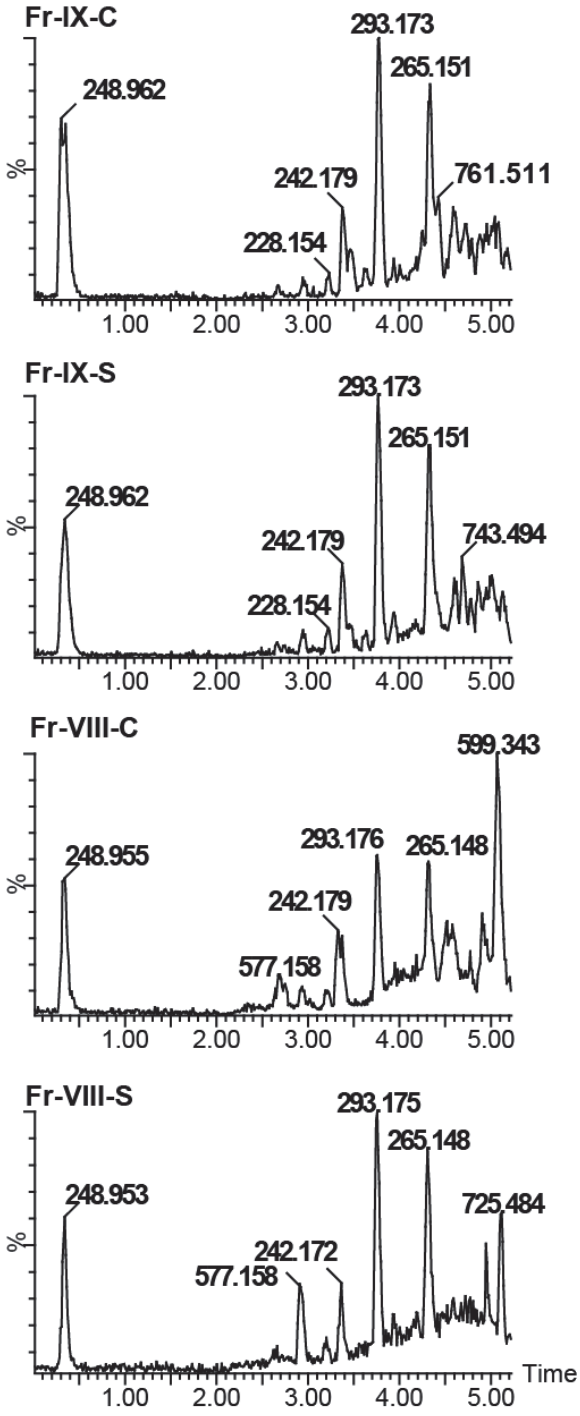

Fig. S5: *Metabolomic fingerprints of active root fractions.* LC-TOFMS chromatograms in ESI- of fraction VIII and IX from roots of control (C) and *S. littoralis* infested plants (S). Each chromatogram was blank-subtracted and normalized to 1000 counts. Peak extraction followed by univariate data analysis did not reveal any clear differences in ESI- or ESI+ modes.

Table S1. *Multiple reaction monitoring parameters for phenolic acid analysis*. Q1: Parent ion → Q3: product ion: mass to charge ratio [m/z]. ID: compound name; DP: Declustering potential; CE: Collision energy.

Q1 mass (Dalton)	**Q3 mass (Dalton)**	**ID**	**DP (Volts)**	**CE (Volts)**
147	102.8	Cinnamic acid	-65	-16
163	118.9	Coumaric acid	-60	-20
179	134.9	Caffeic acid	-55	-22
193.1	133.9	Ferulic acid	-75	-22
223	149	Sinapic acid	-65	-26