Running head: Leaf attack triggers root herbivore avoidance

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Research area: Signaling and response

A physiological and behavioral mechanism for leaf-herbivore induced systemic root resistance

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One-sentence Summary: Leaf-herbivore attack changes free and conjugated phenolic acids in maize roots and thereby triggers an avoidance response in a specialist root herbivore.

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Abstract

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 leaf-infestation by *Spodoptera littoralis* caterpillars for the root-feeding larvae of the beetle *Diabrotica virgifera*, a major pest of maize. *Diabrotica virgifera* strongly avoided leaf-infested plants by recognizing systemic changes in soluble root components. The avoidance response occurred within 12 hours 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 which 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.

Introduction

Insect herbivores constantly compete for plants as 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 non-overlapping feeding niches, leaf and root herbivores determine each other's performance through shared host plants (Bezemer and Van Dam, 2005). While 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, but see 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 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., 2011b; Erb et al., 2009). In the opposite direction, leaf-feeding by *Spodoptera* caterpillars reduces *D. virgifera* growth and development in a sequence-specific manner in the laboratory and the field (Gill et al., 2011; Erb et al., 2011c). *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 non-volatile 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 trigger a short range avoidance response in *D. virgifera*. Through a combination of bioactivity guided fractionation of root extracts, 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

Diabrotica virgifera specifically recognizes and avoids leaf-infested plants

To test whether *D. virgifera* is able to distinguish between infested and non-infested plants in the soil, we offered maize seedlings that were infested in the leaves by *Spodoptera littoralis* or herbivore-free to the root feeding larvae of *D. virgifera* in a two-arm below ground 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 non-infested 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 non-infested 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* regurgitate, 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 regurgitate to the wounds elicited a behavioral response similar to 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 above ground atmosphere through the rhizosphere, we sealed off the soil and root system from the above ground atmosphere with an air-tight agarose/aluminum seal so that the only shoot-root contact was via the plant interior. *Diabrotica 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 non-soluble 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 non-structural 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 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-MS/MS. 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 to free phenolic acids, which were found in concentrations between 1 and 70 ng*g FW, soluble hydrolyzed phenolic acids were up to 100 times more abundant in the roots, with concentrations ranging from 0.1 to 8 µg*g FW (Fig. 2). Primary and crown roots differed in their phenylpropanoid patterns, with primary roots containing higher amounts of basic hydrolysable caffeic acid, free and acid hydrolysable ferulic acid and acid hydrolysable sinapic acid. Primary roots also had lower concentrations of basic hydrolysable *p*-coumaric acid, acid hydrolysable caffeic acid and basic hydrolysable ferulic acid than crown roots. Leaf-infestation by *S. littoralis* reduced the concentrations of all basic and acid hydrolysable 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 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 *Diabrotica 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 piperonylic acid (PA), which inhibits the conversion of cinnamic acid to *p*-coumaric acid through competitive inhibition of the cinnamate 4-hydroxylase C4H (Schalk, 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 (Fig. S1). Furthermore, we observed a slight reduction in free sinapic acid. However, contrary to what has been reported in other plant species (Schalk, 1998; Naseer et al., 2012) we did not observe a depletion of *p*-coumaric acid, caffeic acid or ferulic acid. Soluble acid hydrolysable 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, for instance through the production of *p*-coumaric acid from tyrosine (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 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 any more (Fig. 3A). A similar result was obtained with plants that were elicited by wounding and application of regurgitate (Fig. 3B). To understand this pattern in more detail, we complemented inhibited and non-inhibited control and induced plants with a 5.5 mM solution of *p*-coumaric acid. *P*-coumaric acid 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 *p*-coumaric acid 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 *p*-coumaric acid 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 (CAD) 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 (COMT), 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 to the near isogenic wild type line F2 (Guillaumie et al., 2007), the bm1 mutant is depleted in most soluble free phenolic acids, but over-accumulates soluble hydrolysable ferulic acid and sinapic acid, while the bm3 mutant is depleted in free phenolics without showing an over-accumulation of hydrolysable compounds (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 (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 regurgitate, *D. virgifera* chose the control side in the F2 and bm1 background, but did not show any preference in the bm3 mutant any more (Fig. 4B). The differential preference between real and simulated herbivory in the bm1 mutant was confirmed in a supplementary experiment that directly compared the two treatments (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.

Bioactivity-guided fractionation associates *D. virgifera* choice with differential accumulation of conjugated phenolic acids

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

215 *Discussion*

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

221 In the field, *D. virgifera* commonly co-occurs with many lepidopteran leaf-feeders, including
222 *Spodoptera spp*. (O'Day, 1998). Previous studies show that feeding by *S. frugiperda* on the leaves
223 reduces the survival of late-arriving *D. virgifera* larvae in the roots (Erb et al., 2011c; Gill et al., 2011),
224 especially in the upper layers of the rhizosphere (Erb et al., 2011d). As *D. virgifera*, which is highly
225 specialized on maize (Clark and Hibbard, 2004), can migrate up to 1 m in the soil to find new host plants
226 (Hibbard et al., 2003), it is conceivable that it may have developed the capacity to assess the quality of
227 different plant roots. *In vitro* assays have demonstrated that maize root extracts are strong arrestants of
228 *D. virgifera* larvae (Bernklau and Bjostad, 2005), and that monosacharides as well as free and galactose-
229 linked fatty acids (monogalactosyldiacylglycerols) stimulate their feeding (Bernklau et al., 2015;
230 Bernklau and Bjostad, 2008). Interestingly, it has also been demonstrated that contact with an inferior
231 host plants (e.g. *Glycine max*) changes the behavior of *D. virgifera* neonates from localized to wider-
232 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 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 non-infested plants even when the roots of the two types plants are tightly intertwined and presented together in the same volatile headspace and when above-ground cues were physically blocked by isolating the soil with agar and aluminium foil. Second, the preference was maintained in liquid extracts of the root metabolome, even after evaporation and re-solubilization. Fourth, the active metabolites could be separated from non-active compounds by fractionation using conventional reverse phase HPLC. Interestingly, the preference patterns were less strong when using root extracts compared to intact roots. It is therefore possible that short-range volatile and non-volatile 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 a UHPLC-TOF based metabolomics approach did not yield any clear candidate features (Marti et al., 2013). Using more targeted methods, we here provide several lines of evidence that phenolic acid conjugates can play a central role in mediating the preference of *D. virgifera* larvae for non-infested plants: First, our profiling assays demonstrate that the abundance of several hydrolysable phenolic acids in the roots decreases 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 hydrolysable 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 is derived from coumaric acid, can be conjugated to other phenylpropanoids, sugars, proteins, fatty acids and terpenoids (Cheynier et al., 2013; Quideau et al., 2011; Hoff et al., 1994; Koetter et al., 1994; Shimizu and Ohta, 1960), 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 signalling molecules (Brown, 2001) and enzymatic co-factors (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 mimick 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 phytopagous insects is a key process shaping plant-insect interactions. While 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 was 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 above-ground 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 below-ground 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 are avoided by *D. virgifera* larvae. 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.

Material and methods

Plants and insects

Maize plants (Zea mays) were grown as described previously (Erb et al., 2011c). Unless otherwise indicated, the hybrid Delprim (Delley DSP, Delley, Switzerland) was used for experiments. The brown midrib mutants bm1 and bm3 were bred at INRA Lusignan as described (Barriere *et al.*, 2004). Plants for experiments were 10-12 days old and had 2-3 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 1.5L PET 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 inner diameter) 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, were transferred carefully into the vessels. After 24 hours of acclimatization in a temperature and light-controlled environment (23°C, 16:8h L/D, 90µmol/m^2), 1.5 l PET bottles with their bottoms removed were attached to the glass pots upside down. Half of the plants were then infested with 20 second instar *S. littoralis* larvae over 48h, while the other half was left herbivore free. After this period, during which the leaves were damaged, but still had ample leaf biomass (>50%), 6 second instar *D. virgifera* larvae were introduced into the horizontal glass tubes (3 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 vs. treatment, see below) were laid out on a moist filter paper embedded in a large petri dish (12cm 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, 16:8h L/D, 90µmol/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, eleven different experiments were conducted. 1. Maize seedlings were infested with 20 second instar *S. littoralis* larvae for 48h. The herbivores were removed after this period, and roots from a control and an infested plants were offered to *D. virgifera* (n=19). 2. Plants were infested in the same manner, but offered to *D. virgifera* at different

time points ranging from 8 to 48 h (n=12). 3. Leaf-herbivory was simulated using three different treatments: i) scratching of about 1 cm^2 of leaf-tissue 6 times over 48h, until all leaves were damaged, ii) additional application of 10 µl of *S. littoralis* regurgitant to the scratched surfaces, as described in (Erb et al., 2009), iii) removal of leaf area by sequentially cutting off 50% of each leaf over 48h. 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). 4. Leaves were induced by scratching and application of *S. littoralis* regurgitate and offered to the larvae at different time points after induction (n=12-14). 5. 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 2cm layer of solidifying agar (2% Agarose in H_20, 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). 6. The potential effect of phenylpropanoids was investigated by C4H inhibition with piperonylic acid (PA). Plants were either infested with 20 *S. littoralis* larvae or left uninfested for 48 h. Half of the control and infested plants were treated with PA (Sigma Aldrich, Saint-Louis, MO, USA) by adding 10 mL of a 75 µM PA in 10 % EtOH solution to the soil every 24 h over the 48 h infestation period (three times in total). The other half of the plants were treated with buffer (10 % EtOH). 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). 7. The same setup as for experiment 6 was used, with the only difference that the plants were elicited by repeated wounding and regurgitate application over 48 h (n=12). 8. PA treated plants were complemented with 5.5mM *p*-coumaric acid (Sigma-Aldrich, St.-Louis, MO). In this experiment, we simultaneously tested the preference of *D. virgifera* as follows (n=23 for each experiment): (i) control versus leaf-elicited plants, both treated with PA, (ii) control versus leaf elicited plants, both treated with PA and complemented with *p*-coumaric acid (CA), (iii) control plants treated with buffer versus control plants complemented with CA, both treated with PA, (iv) 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 *p*-coumaric acid was achieved by watering the plants with 10 mL of a 5.5 mM CA in 1 % EtOH solution every 24 h over 48 h (two times in total). Non complemented plants were watered with 10 mL 1 % EtOH solution. 9. The choice of *D. virgifera* was evaluated for 3 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). 10. The preference of *D. virgifera* was tested in the three genotypes using artificial induction by wounding and regurgitate as described above (n=12). 11. 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 H_2O). 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 (5x5x5 mm) were cut and placed into new petri dishes (2 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 % r.h., no light). For 4 h, the position of the root herbivores in the dishes was recorded every 30 minutes. 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 semi preparative HPLC runs (see below). For each pair of fractions, we evaporated the solvents, resuspended the fractions in 10 µl AcN and diluted the dissolved fractions in 0.5 mL agarose (4%). This mixture was then diluted with 0.4 mL H_2O 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 crude root extract of non-infested 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 semi preparative 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 semi preparative reverse-phase HPLC (Marti et al., 2013). For the choice assay, extracts from roots of 400 control and 400 *S. littoralis* infested plants were lyophilized to yield 50 mg dry matter per treatment. The extracts were redissolved in 500 µl 50 % MeOH (v/v) and fractionated using a semi-preparative C18 column (C18, 250 × 10 mm i.d., 5 µm, XBridge™, Waters, UK) connected to a Varian 9012 Solvent Delivery System operating at a flow rate of 10 $ml*min^{-1}$. Injection volume was 250 µl. The solvent gradient started with 90 % H2O and 10 % can (both with 0.1 % v/v formic acid) for two minutes, followed by a ramp to 50:50 over 20 minutes and a ramp to 5:95 over 70 minutes. The 5:95 mix was held for 12

minutes, followed by a post equilibration at 90:10 for 10 minutes. Fractions were collected at 6 minute intervals between 2 and 62 minutes. After pooling the fractions from 2 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 that instead of 11 fractions, 5 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). Briefly, the fingerprints of each extract was obtained using a short UPLC BEH C18 Acquity column (50 × 1.0 mm i.d., 1.7 µm) (Waters, MA, USA). The mobile phase consisted of 0.1 % formic acid (FA) in water (phase A) and 0.1 % FA in acetonitrile (phase B). The linear gradient program was as follows: 98 % A over 0.2 minutes, to 100 % B over 4.9 minutes, held at 100 % B for a further 1.1 min, then returned to initial conditions (98 % A) in 0.1 min for 1.1 minutes of equilibration before next analysis. The flow rate was 0.3 ml/min; column temperature was kept at 40°C. Detection was performed by TOF-MS (LCT Premier, Waters, MA, USA) in W-mode in both electrospray (ESI) negative (NI) and positive (PI) ion modes in independent runs over a m/z range of 100-1000 Da. The MS was calibrated using sodium formiate, and leucine enkephalin was used as an internal reference. The injection volume was 1 µl and all samples were diluted at 0.5 mg/mL. The recorded profiles were normalized to 1000 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 hydrolysable 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). Briefly, all samples were ground in nitrogen to a fine powder using a mortar and a pestle. 600 µL 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 3 plants were pooled and separated into 3 aliquots: (i) 1mL aliquots were evaporated to dryness under a flow of nitrogen (Glas-Col, CAT No. 099A EV9624S, Terre Haute, USA) and resuspended in 50 µl 50% MeOH for the analysis of free phenolic acids. (ii) 50 µL aliquots were mixed with 50 µL of concentrated HCl (37%, Sigma Aldrich, Saint-Louis, MO, USA) and heated for 1 hour at 80° C for acid hydrolysis. 1 mL diethyl ether was added, and the organic phase was collected and

evaporated to dryness under a flow of nitrogen (Glas-Col, CAT No. 099A EV9624S, Terre Haute, USA) before resuspension in 50 µL 50% MeOH. (iii) 50 µL aliquots were mixed with 100 µL 2M NaOH (Sigma Aldrich, Saint-Louis, MO, USA) and left to stand for 3 h at ambient temperature for basic hydrolysis. The samples were then mixed with 50 µL concentrated HCl (37%, Sigma Aldrich, Saint-Louis, MO, USA) and 1 mL diethyl ether (Glas-Col, CAT No. 099A EV9624S, Terre Haute, USA), and the organic phase was recovered and evaporated under a nitrogen stream (Glas-Col, CAT No. 099A EV9624S, Terre Haute, USA) before resuspension in 50 µL 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 an Agilent 1260 Infinity HPLC system (Agilent Technologies, Boeblingen, Germany) coupled to an API 5000 tandem mass spectrometer (MS) (Applied Biosystems, Darmstadt, Germany). Briefly, the separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 0.5 µm; Agilent, Santa Clara, CA, USA) using formic acid (1%, Fisher Scientific, Geel, Belgium) in water and acetonitrile (Fisher Scientific UK, Loughborough, Leics, UK) as mobile phases A and B respectively. The employed elution gradient was as follow: 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 of 1.1 $mL.min^{-1}$. The column temperature was maintained at 25 ° C. The instruments parameters were optimized with infusion of pure standards (Sigma-Aldrich, St.-Louis, MO). The ion spray voltage was of -4500 eV. The turbo gas temperature was of 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 → product ion transitions as described in Table S1. Data acquisition and processing was performed on Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany). Dilution series of standard mixtures of each phenolic acid (purchased from Sigma-Aldrich, St.-Louis, MO) 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 (GLM with quasi-poisson distribution to take into account overdispersal, followed by analysis of variance (ANOVA)) (Robert et al., 2012) using R (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.

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Figure legends

Fig. 1: *The root herbivore* Diabrotica virgifera *specifically avoids leaf-infested plants by recognizing systemic changes in soluble root components.* A. Preference of *D. virgifera* larvae for roots of control vs. leaf-infested plants in a two-arm below ground choice system (n=15). B. Preference for roots of control vs. 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 defence 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 rhizo- and phyllosphere (n=12). G. Preference for root extracts of control and leaf-infested plants using agarose cubes (n=15). Preference is expressed as % choice corresponding to the proportions of independent replicates in which a given preference was observed (No choice: A-F: <10%; G: 21%). Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

Fig. 2: *Leaf-infestation alters soluble free and conjugated phenolic acids in the roots.* Average concentrations of different phenolic acids in control roots (grey 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 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. 3: *Manipulating the biosynthesis of phenolic acids through cinnamate 4-hydroxylase inhibition leads to the disappearance of the* D. virgifera *avoidance response towards 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 *p*-Coumaric acid (CA) complemented control and artificially induced plants. C4H was inhibited by application of the selective inhibitor piperonylic acid (n=23). Preference is expressed as % choice corresponding to the proportions of independent replicates in which a given preference was observed (No choice: <10%). Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

Fig. 4: *Genetic modification of the phenylpropanoid pathway leads to the disappearance of the* D. virgifera *avoidance response towards leaf-infested plants.* A. Preference of D. virgifera for roots of control and leaf-infested wild type (WT) plants, cinnamyl alcohol dehydrogenase (CAD) (bm1) mutant plants and Caffeic acid O-methyl transferase (COMT) (bm3) mutant plants (n=12-13). B. Preference of *D. virgifera* for roots of control and artificially induced WT, bm1 and bm3 plants (n=12). Preference is

expressed as % choice corresponding to the proportions of independent replicates in which a given preference was observed (No choice: <10%). Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

Fig. 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 hydrolysable phenolic acids. D. Concentrations of soluble, basic hydrolysable phenolic acids. Shaded areas correspond to the bioactive fraction.

Supplemental data

Fig. S1: Cinnamate 4-hydroxylase inhibition alters the accumulation of soluble free and conjugated phenolic acid in the roots.

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

Fig. S3: Genetic modification of the phenylpropanoid pathway does not alter root architecture of maize seedlings.

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

Fig. S5: Metabolomic fingerprints of active root fractions

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

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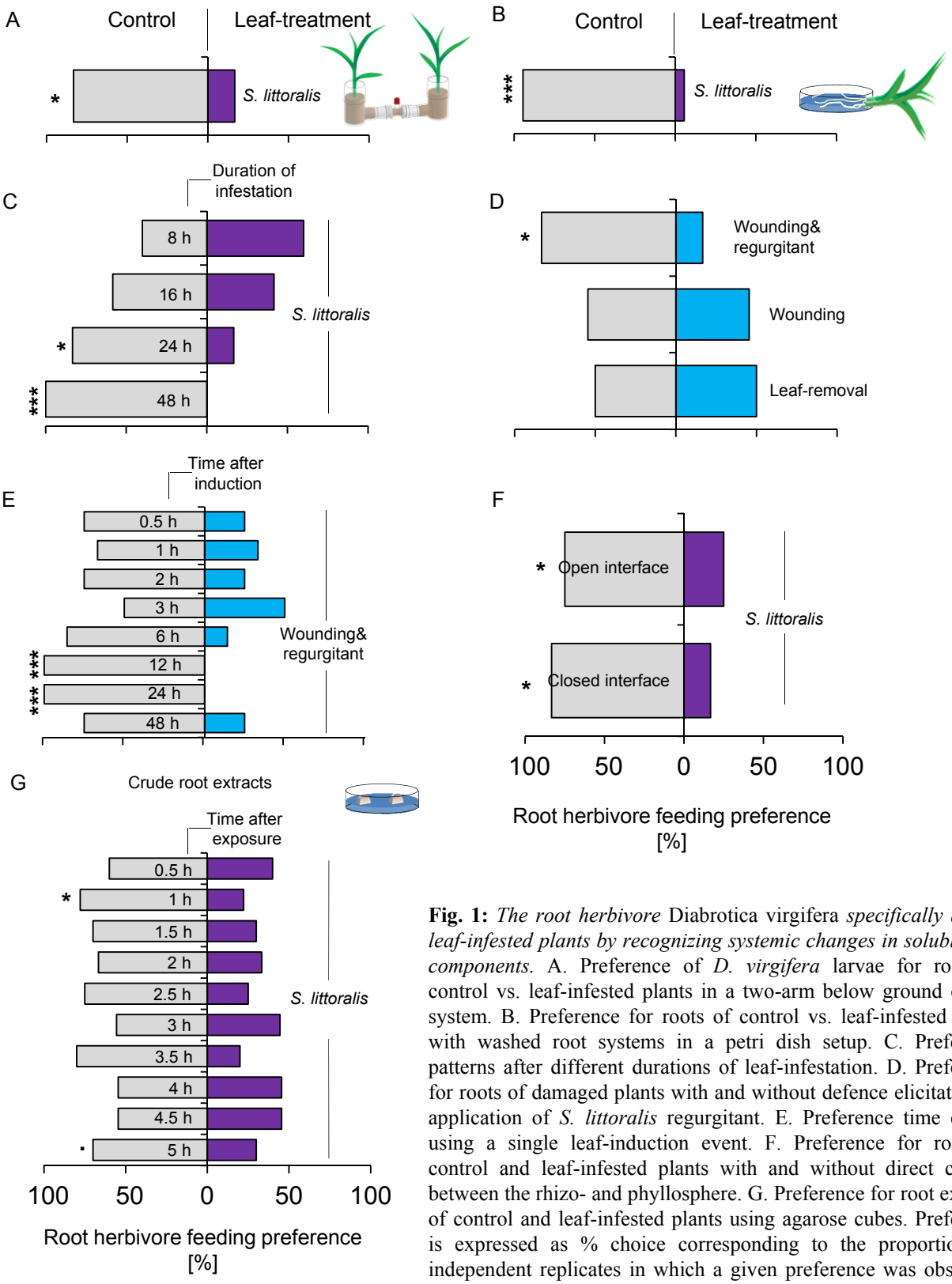

Fig. 1: *The root herbivore* Diabrotica virgifera *specifically avoids leaf-infested plants by recognizing systemic changes in soluble root components.* A. Preference of *D. virgifera* larvae for roots of control vs. leaf-infested plants in a two-arm below ground choice system. B. Preference for roots of control vs. leaf-infested plants with washed root systems in a petri dish setup. C. Preference patterns after different durations of leaf-infestation. D. Preference for roots of damaged plants with and without defence elicitation by application of *S. littoralis* regurgitant. E. Preference time course using a single leaf-induction event. F. Preference for roots of control and leaf-infested plants with and without direct contact between the rhizo- and phyllosphere. G. Preference for root extracts of control and leaf-infested plants using agarose cubes. Preference is expressed as % choice corresponding to the proportions of independent replicates in which a given preference was observed. Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

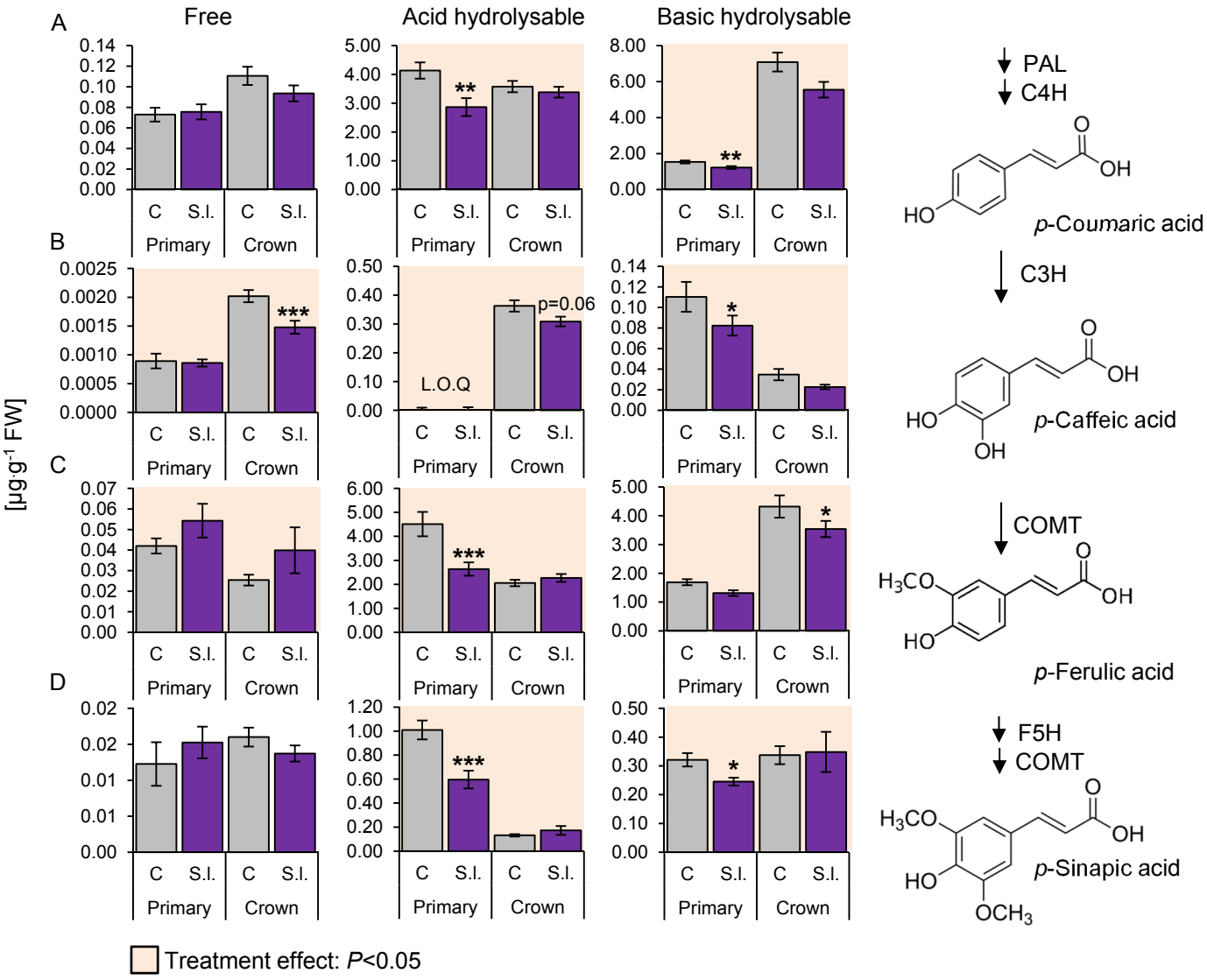

Fig. 2: *Leaf-infestation alters soluble free and conjugated phenolic acids in the roots.* Average concentrations of different phenolic acids in control roots (grey bars) and roots of leaf-infested plants (purple bars) are shown for crown and primary roots (± SE). Shading indicates a significant overall treatment effect determined by analysis of variance ($p<0.05$). Stars indicate significant pairwise differences between treatments within root types (Holm-Sidak post-hoc tests: *$p<0.05$, **$p<0.01$, ***$p<0.001$).

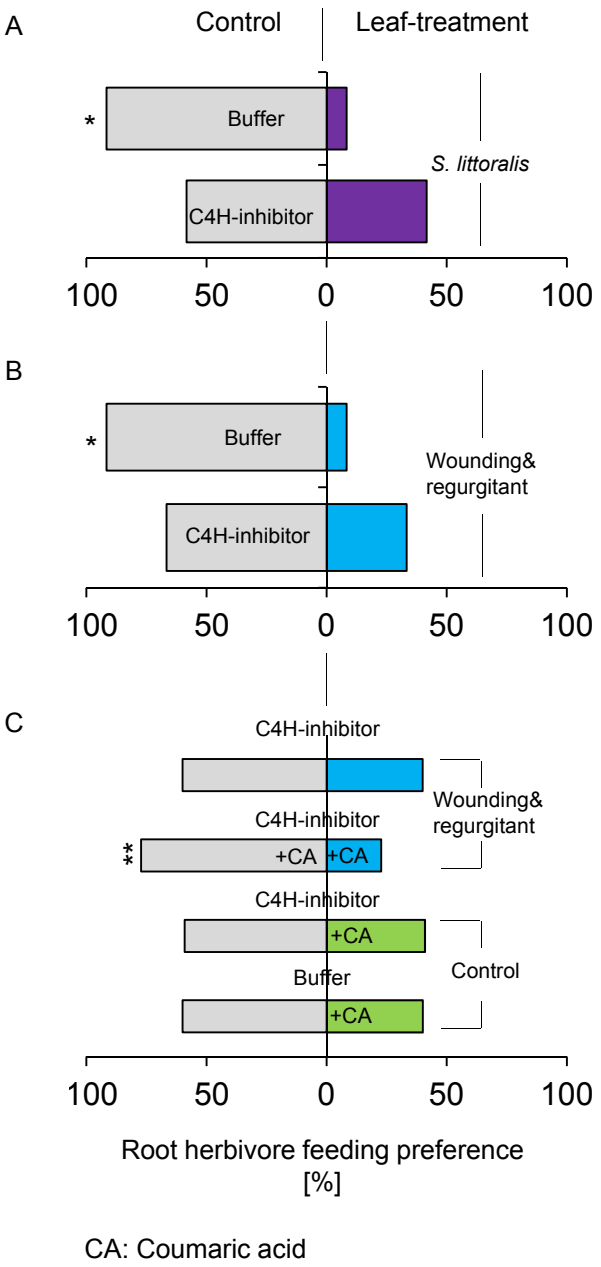

Fig. 3: *Manipulating the biosynthesis of phenolic acids through cinnamate 4-hydroxylase inhibition leads to the disappearance of the* D. virgifera *avoidance response towards leaf-infested plants.* A. Preference for roots of buffer treated and C4H-inhibited control and *S. littoralis* infested plants. B. Preference for roots of buffer treated and C4H-inhibited control and artificially induced plants. C. Preference for roots of buffer treated, C4H-inhibited and *p*-Coumaric acid (CA) complemented control and artificially induced plants. C4H was inhibited by application of the selective inhibitor piperonylic acid. Preference is expressed as % choice corresponding to the proportions of independent replicates in which a given preference was observed. Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

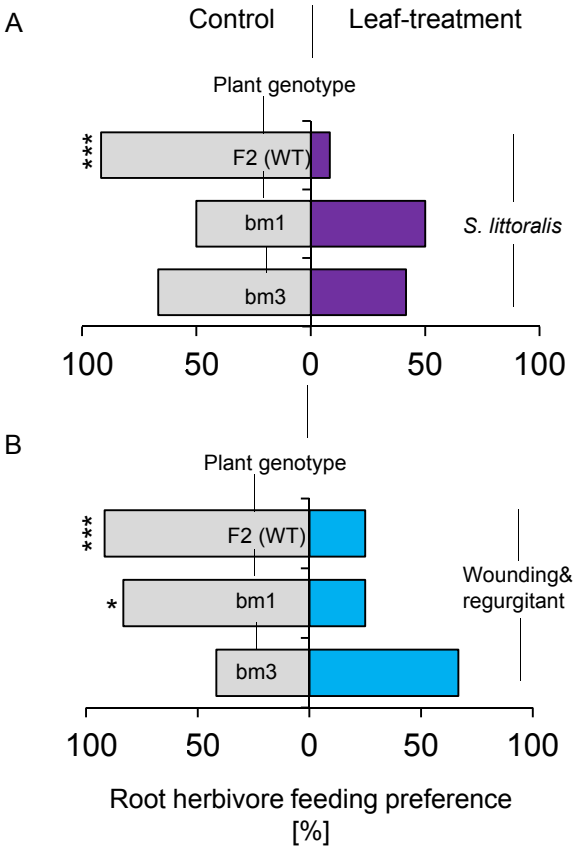

Fig. 4: *Genetic modification of the phenylpropanoid pathway leads to the disappearance of the* D. virgifera *avoidance response towards leaf-infested plants.* A. Preference of D. virgifera for roots of control and leaf-infested wild type (WT) plants, cinnamyl alcohol dehydrogenase (CAD) (bm1) mutant plants and Caffeic acid O-methyl transferase (COMT) (bm3) mutant plants. B. Preference of *D. virgifera* for roots of control and artificially induced WT, bm1 and bm3 plants. Preference is expressed as % choice corresponding to the proportions of independent replicates in which a given preference was observed. Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

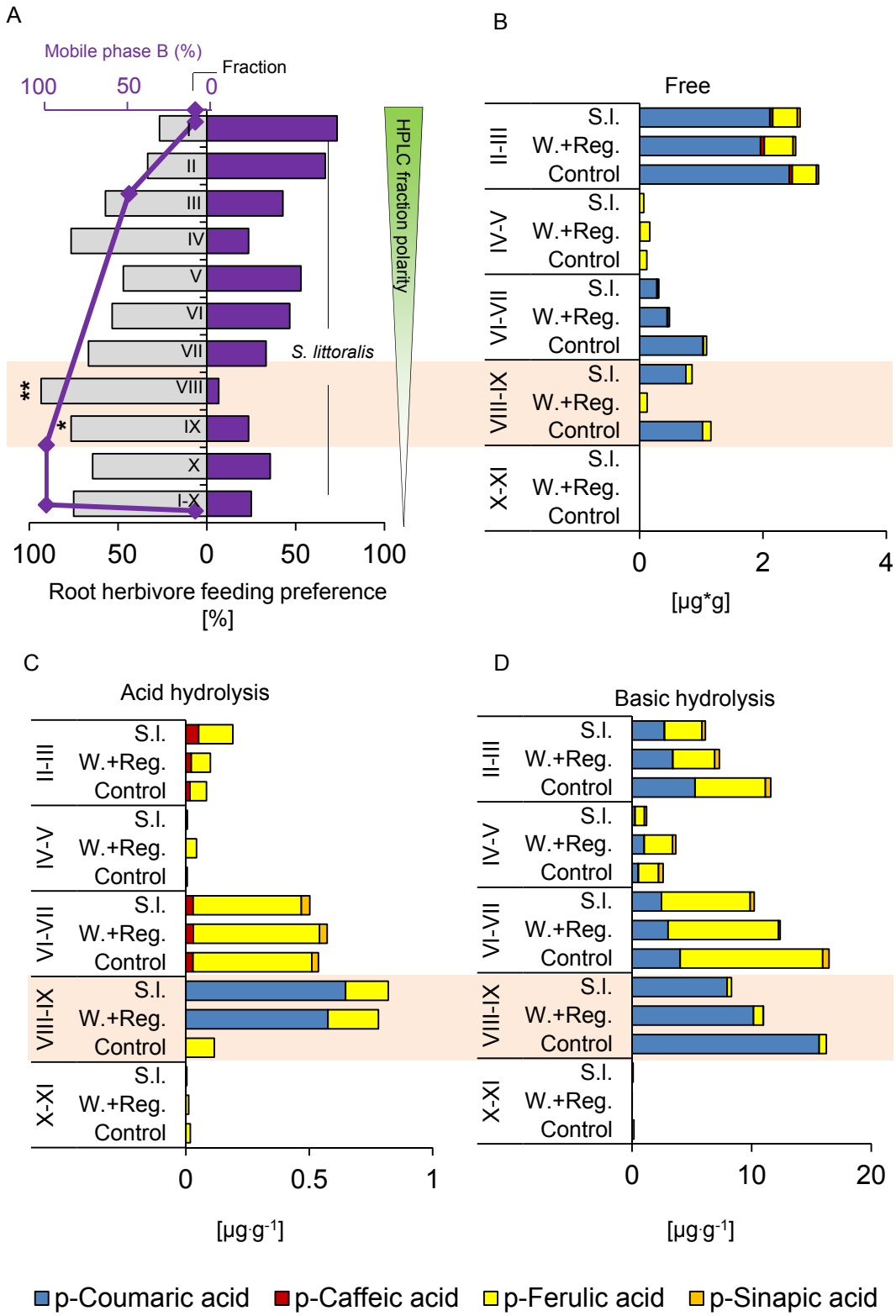

Fig. 5: *Bioactivity-guided fractionation links changes in conjugated phenolic acids with* D. virgifera *preference patterns.* A. Preference of *D. virgifera* for fractions of root extracts of control and leaf-infested plants. The polarity gradient of the fractionation setup is shown in purple. B. Concentrations of free phenolic acids in root extracts of control and leaf-induced plants across the polarity gradient. C. Concentrations of soluble, acid hydrolysable phenolic acids. D. Concentrations of soluble, basic hydrolysable phenolic acids. Shaded areas correspond to the bioactive fraction.

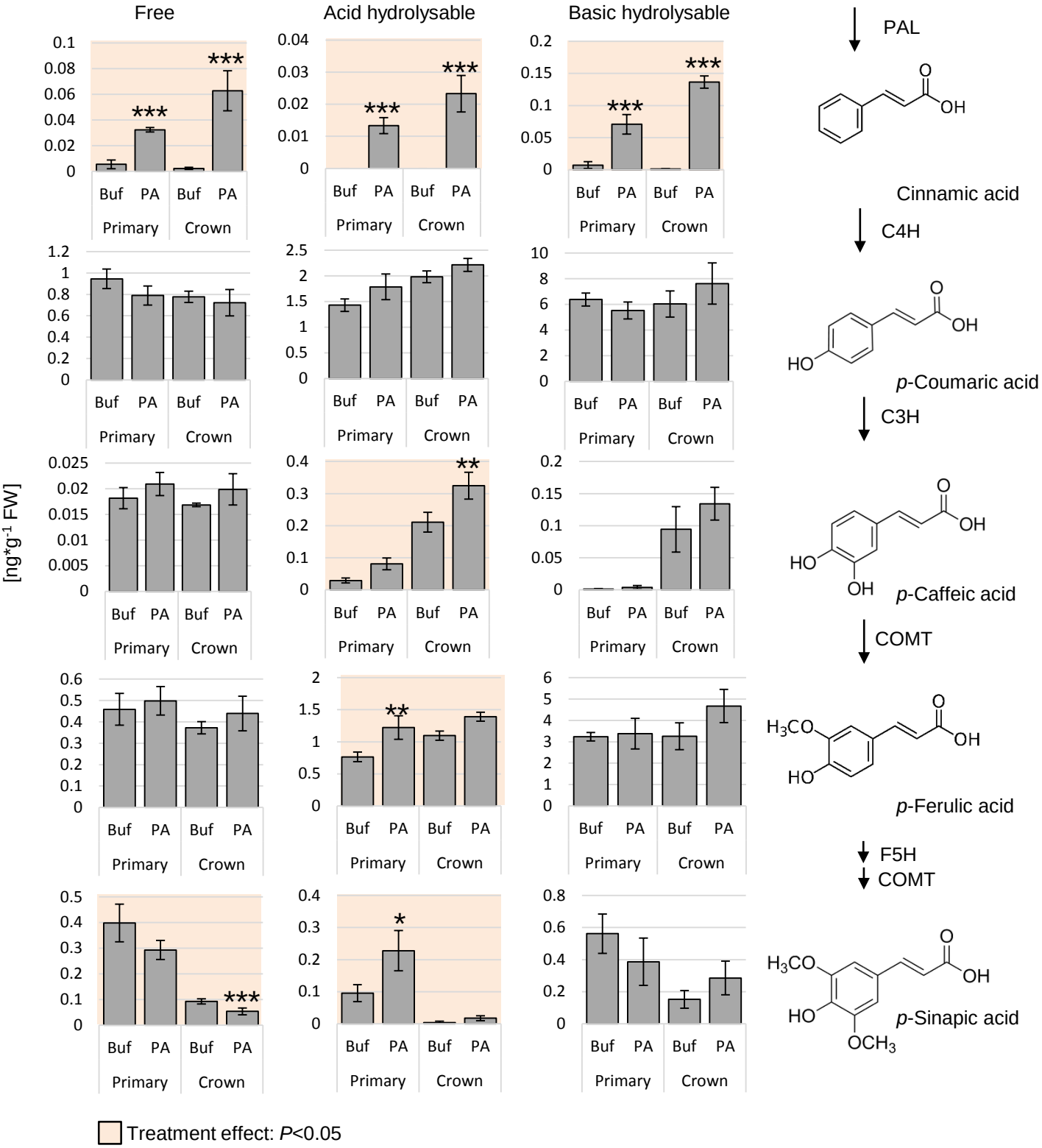

Fig. S1: *Cinnamate 4-hydroxylase inhibition alters the accumulation of soluble free and conjugated phenolic acid in the roots.* Average concentrations of different phenolic acids in buffer treated roots (Buf) and piperonylic acid treated, C4H inhibited roots (PA) are shown for crown and primary roots (± 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: *$p<0.05$, **$p<0.01$, ***$p<0.001$).

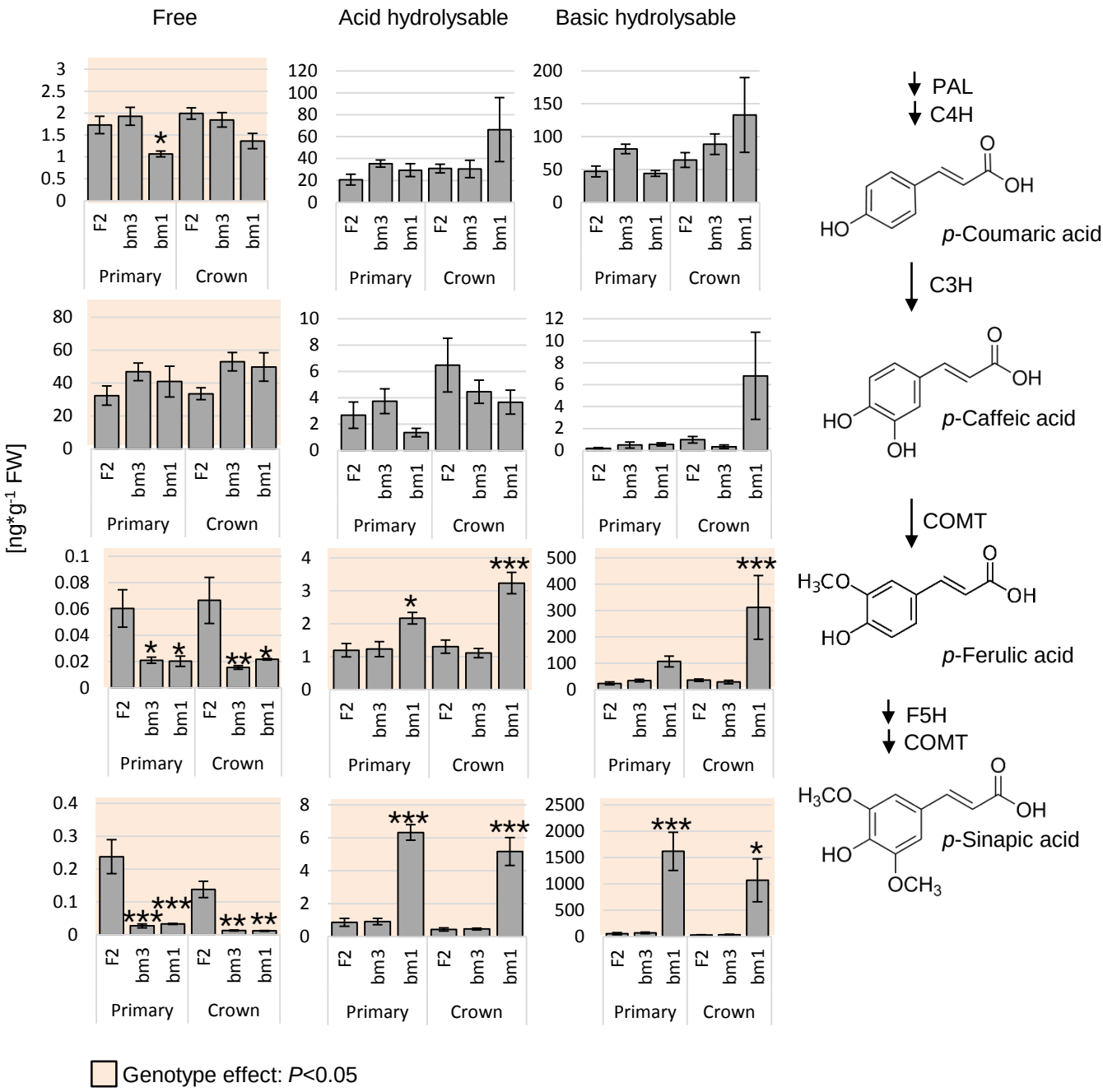

Fig. S2: *Genetic modification of the phenylpropanoid pathway alters the accumulation of soluble free and conjugated phenolic acid in the roots.* Average concentrations of different phenolic acids wild type (F2), bm1 and bm3 mutants are shown for crown and primary roots (± SE). Shading indicates a significant 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: *$p<0.05$, **$p<0.01$, ***$p<0.001$).

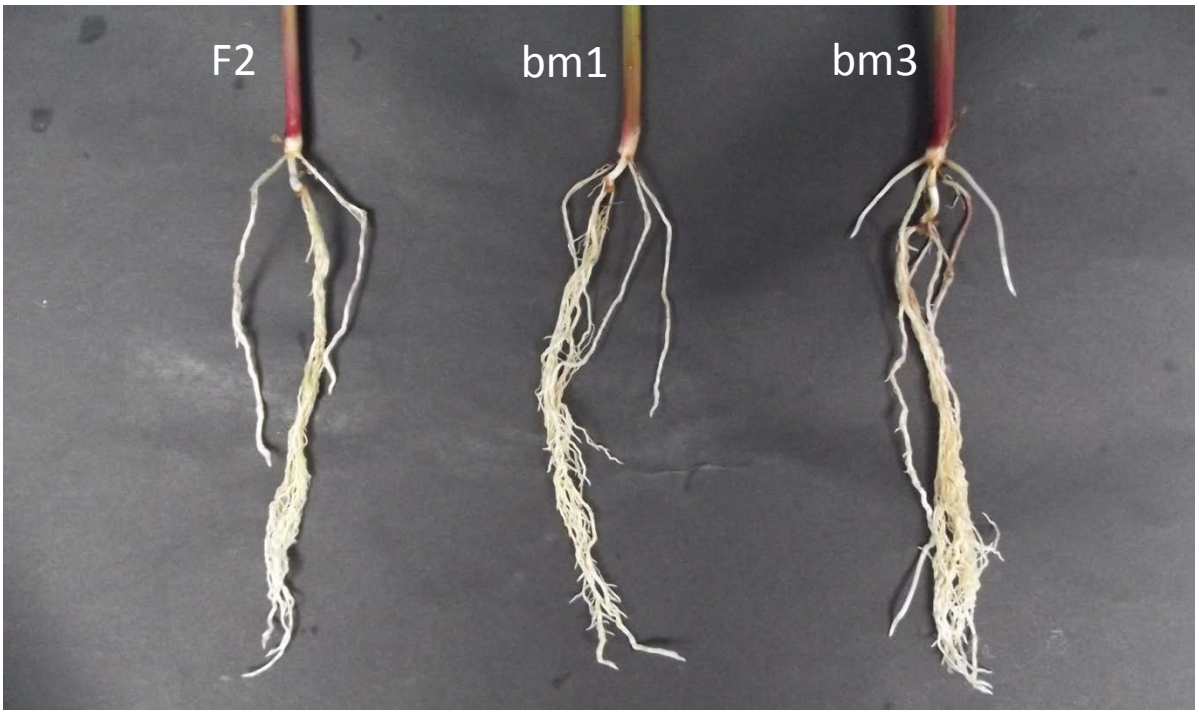

Fig. S3: *Genetic modification of the phenylpropanoid pathway does not alter root architecture of maize seedlings.* Pictures of wild type (F2), bm1 and bm3 roots of 12-day-old maize seedlings are shown.

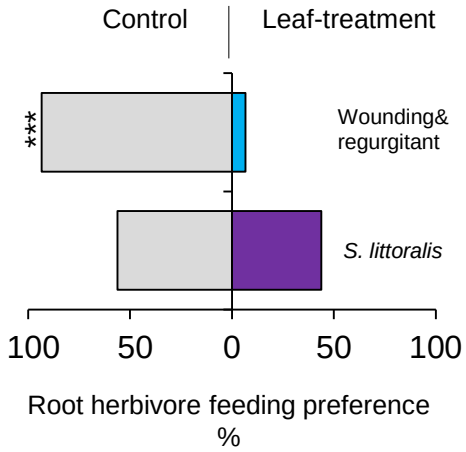

Fig. S4: *The bm1-dependent preference pattern of D. virgifera differs between S. littoralis infested and artificially elicited plants.* The preference for roots of leaf-induced bm1 mutant plants is shown. Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

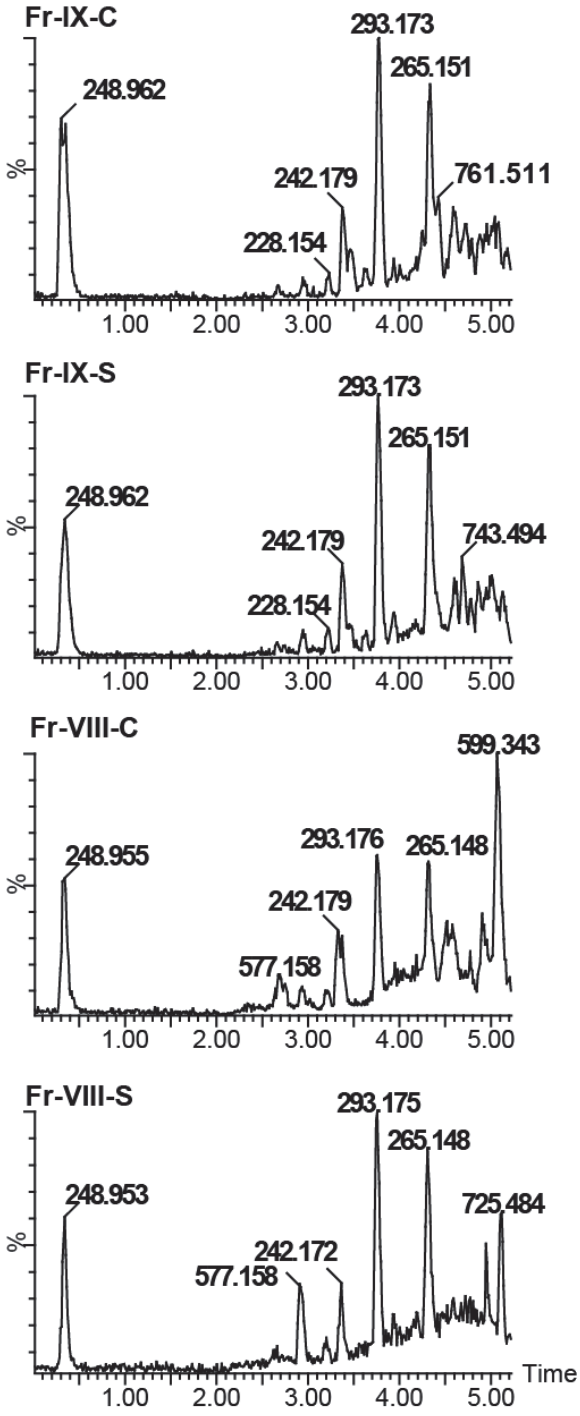

Fig. S5: *Metabolomic fingerprints of active root fractions.* LC-TOFMS chromatograms in ESI- of fraction VIII and IX from roots of control (C) and *S. littoralis* infested plants (S). Each chromatogram was blank-subtracted and normalized to 1000 counts. Peak extraction followed by univariate data analysis 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