Integration of plant metabolic complexity into fitness-relevant foraging by a specialist herbivore

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Abstract

Plants produce complex mixtures of primary and secondary metabolites. Herbivores use these metabolites as behavioral cues to increase their fitness. However, how herbivores integrate different metabolite classes into fitness-relevant foraging decisions *in planta* is poorly understood. We developed a molecular manipulative approach to modulate the availability of sugars and benzoxazinoid secondary metabolites as foraging cues for a specialist maize herbivore, the western corn rootworm. By disrupting benzoxazinoid biosynthesis in maize and sugar perception in the western corn rootworm, we show that sugars and benzoxazinoids act as distinct and dynamically integrated mediators of short-distance host finding and acceptance. While sugars improve the capacity of rootworm larvae to find a host plant and to distinguish post-embryonic from less nutritious embryonic roots, benzoxazinoids are specifically required for the latter. Host acceptance in the form of root damage is increased by benzoxazinoids and sugars in an additive manner. This pattern is driven by increasing damage to post-embryonic roots in the presence of benzoxazinoids and sugars. Benzoxazinoid- and sugar-mediated foraging directly improves western corn rootworm growth and survival. Interestingly, western corn rootworm larvae retain a substantial fraction of their capacity to feed and survive on maize plants in the absence of both classes of cues. This study unravels fine-grained differentiation and integration of primary and secondary metabolites into herbivore foraging and documents how the capacity to compensate for the absence of important chemical cues enables a specialist herbivore to survive within unpredictable metabolic landscapes.

Introduction

Herbivore foraging behavior contributes to the distribution and performance of herbivores and plants in natural and agricultural ecosystems (1–3). Insect herbivores often exhibit pronounced oviposition and feeding preferences for specific plant species, genotypes within species physiological states within genotypes (1, 4, 5). Most insect herbivores also show characteristic preferences for specific plant organs and tissues (6–8).

Herbivores establish preferences through different types of host-cues (9–12). Chemical cues, including plant primary and secondary metabolites, are particularly important for herbivores, as they provide specific information about the identity, physiological status and nutritional value of host plants and tissues (13–15). The overarching view in the field of chemical ecology is that primary metabolites are used as cues to identify nutritious hosts and tissues, while volatile and non-volatile secondary metabolites are used as indicators of toxicity and defense status, and as signature cues of specific host plant lineages and species (16–18). Specialized herbivores in particular are often attracted and stimulated by host-specific secondary metabolites (19). Over the past decades, herbivores have been found to respond to a multitude of plant primary and secondary metabolites in artificial diet experiments (13, 16, 17). An increasing number of studies now also document the importance of these metabolites *in planta* through molecular manipulative approaches (20–26).

Plants produce diverse sets of primary and secondary metabolites (27), and herbivores likely integrate many of these metabolites into their foraging behavior (16, 28, 29). Chemical cue integration is thought to allow herbivores to obtain more accurate information about the nutritional value and toxicity of complex host plant metabolomes (17) and thus to increase the robustness of their foraging decisions (12, 29). Many insect herbivores are known to be attracted to specific combinations of volatile chemicals, for instance (14, 29). Furthermore, some herbivores avoid combinations of secondary metabolites more strongly than individual compounds (30–32). A limited number of studies also indicate that herbivores may be able to integrate primary and secondary metabolites into their foraging strategies (17). Using artificial diet experiments, it was found that tannins reduce food intake by locusts at low protein:carbohydrate ratios, but not at high ratios, a behavior which mirrored the conditional impact of tannins on locust performance (33, 34). Modulation of prior exposure to secondary metabolites on subsequent food choice was observed for both insect herbivores (35) and mammals (36, 37). Conversely, sugars were found to mask the aversive taste of secondary metabolites (38). Despite these advances, we currently lack a detailed

70 understanding of how primary and secondary metabolites interact to determine herbivore behavior
71 under biologically realistic conditions (9). The paucity of manipulative experiments that test for
72 interactions between host-derived chemical foraging cues *in planta* limits our capacity to assess
73 the concerted impact of different metabolites on herbivore feeding preferences, and, more
74 generally, our understanding of the role of plant metabolic complexity in herbivore behavior and
75 plant-herbivore interactions.

76 An implicit assumption of herbivore foraging theory is that herbivore behavior improves herbivore
77 fitness (39). With some notable exceptions, herbivores generally prefer to oviposit and feed on
78 plants and tissues that increase their performance (40–42). Host plant chemicals likely play an
79 important role in these preference-performance relationships (12, 17). However, the contribution
80 of individual behavioral cues to herbivore fitness in the context of the full chemical complexity of
81 a given host plant has remained difficult to quantify (30, 43, 44). Targeted molecular manipulation
82 of the production and perception of chemical cues provides new opportunities in this context,
83 including the evaluation of the benefits of the integration of multiple chemical cues into herbivore
84 foraging (29), and the assessment of the importance of the flexible use of multiple foraging cues
85 with redundant information content (11, 12).

86 Insect herbivores use chemosensory receptors to detect volatile and non-volatile plant chemicals
87 (45, 46). Gustatory receptors (GRs) are required for the detection of a variety of chemicals (47–
88 49), including non-volatile plant chemicals such as alkaloids (24) and sugars (50). Knocking out
89 specific GRs reduces oviposition of swallowtail (*Papilio xuthus*) butterflies (24) and host
90 recognition by silkworm (*Bombyx mori*) larvae (23), thus demonstrating the functional importance
91 of individual GRs for herbivore behavior. Work in Drosophila (*Drosophila melanogaster*) revealed
92 that some GRs are broadly tuned and can mediate avoidance to many different compounds (51),
93 while others are narrowly tuned and confer responsiveness to specific compounds (52). Gr43a is a
94 highly conserved insect taste receptor that specifically responds to D-fructose in Drosophila (49),
95 the diamondback moth (*Plutella xylostella*) (53) and the cotton bollworm (*Helicoverpa armigera*)
96 (54). As Gr43a is responsible for the detection D-fructose in the hemolymph as a proxy for
97 carbohydrate supply, *Gr43a* silenced flies also become unresponsive to other dietary sugars (55).
98 Interestingly, different GRs can interact dynamically through competition, inhibition and activation
99 (47, 56, 57), resulting in substantial potential for GR-mediated integration of multiple chemical
100 cues into behavioral responses.

101 Here, we developed a manipulative approach to evaluate the importance of maize primary and
102 secondary metabolites for the foraging and foraging-dependent performance of the western corn
103 rootworm (*Diabrotica virgifera virgifera*). The western corn rootworm is an economically
104 damaging maize pest (58). Its larvae are highly specialized on maize roots (59). Over the last
105 decades, the chemical ecology of the western corn rootworm has been studied in detail (13, 58, 59).
106 Field and laboratory studies revealed tight associations between maize root chemistry and western
107 corn rootworm behavior. Western corn rootworm larvae respond behaviorally to a wide variety of
108 chemical cues, including CO_2 (60), sugars and fatty acids (61, 62), aromatic and terpene volatile
109 organic compounds (63, 64), conjugated phenolic acids (65) and benzoxazinoids (66). The
110 emerging picture is that these chemicals likely allow western corn rootworm larvae to locate plant
111 roots from a distance (67), to discriminate between plants of different quality (63–65, 68), and to
112 identify and feed on the most nutritious roots (66, 69). Several *in vitro* experiments also suggest
113 that the western corn rootworm can integrate multiple chemical cues for host finding and
114 acceptance (61), hinting at the substantial sensory capacity of this specialist root feeder. By
115 independently manipulating the availability of benzoxazinoids and sugars as foraging cues of the
116 western corn rootworm through plant genetics and insect RNA interference (RNAi), we
117 demonstrate that sugars and benzoxazinoids serve both specific and integrated roles as
118 determinants of the behavior and behaviorally driven performance of this specialist herbivore.

119 **Results**

120 *The western corn rootworm prefers to feed on root tissues that are rich in benzoxazinoids and*
121 *soluble sugars*

122 The root system of young maize plants consists of embryonic and post-embryonic roots (Fig. 1*A*).
123 Embryonic roots emerge directly from the embryo and comprise primary and seminal roots. Post-
124 embryonic roots emerge from the hypocotyl and stem and comprise crown roots, and, at later
125 developmental stages of the plant, internode-derived brace roots (70). To determine feeding
126 preferences of the western corn rootworm within the root system of young maize plants, we infested
127 soil-grown maize plants with western corn rootworm larvae for 7 days and then scored the damage
128 on the different root types. In line with earlier studies (66, 69), we found low amounts of damage
129 on embryonic roots and substantial damage on post-embryonic roots (Fig. 1*B*). While embryonic
130 roots showed scattered bite marks, post-embryonic roots were often partially or even fully removed
131 (Fig. S1). To test whether this feeding preference is reflected in the distribution of the larvae within
132 the root system, we carried out a series of behavioral experiments. First, we laid out intact maize

133 root systems on a filter paper and then recorded the position of second instar larvae at different
134 time points after their release. Five times more larvae were found on post-embryonic than
135 embryonic roots 30 min after the release of the larvae (Fig. 1*C*). This preference persisted over the
136 duration of the experiment. To test whether the preference of the larvae for post-embryonic roots
137 may be due to differences in abundance or the relative position of the two root types within the root
138 system (Fig. 1*A*), we offered post-embryonic and embryonic root pieces of equal size to the larvae.
139 Similar to what was observed for entire root systems, significantly more larvae were found on the
140 post-embryonic root pieces than the embryonic root pieces after 30 min (Fig. 1*D*). Thus, the
141 western corn rootworm preferentially stays and feeds on post-embryonic roots of young maize
142 plants.

143 To gain insights into the phytochemical differences that may drive the preference of the western
144 corn rootworm for post-embryonic roots, we performed untargeted metabolomics on methanolic
145 extracts of both root types. Using UHPLC-Q-TOF-MS and ESI-, 4512 mass features were detected,
146 358 and 250 of which were enriched in post-embryonic or embryonic roots based on more than
147 two-fold difference in normalized signal intensity at a statistical significance of $p<0.05$ (Fig. 1*E*).
148 Using exact masses and fragmentation patterns, 28 metabolites could be putatively identified
149 within the dataset. Identified metabolites included benzoxazinoids, phenolic acid derivatives,
150 amino acids and sugars (Fig. 1*F*, table S1). Seventeen of these metabolites showed root-type
151 specific accumulation patterns (Fig. 1*F*). Among the secondary metabolites, benzoxazinoids and
152 phenolic acids showed pronounced shifts in their profiles, with some metabolites accumulating in
153 higher amounts in post-embryonic roots and others accumulating in higher amounts in embryonic
154 roots (Fig. 1*F*). Among the detected primary metabolite features, amino acids and sugars were
155 more abundant in post-embryonic- than in embryonic roots, with the most pronounced shifts
156 observed for features matching leucin and monosaccharides (Fig. 1*F*). Thus, the preference of the
157 western corn rootworm for post-embryonic roots is associated with distinct primary and secondary
158 metabolite accumulation patterns in these tissues.

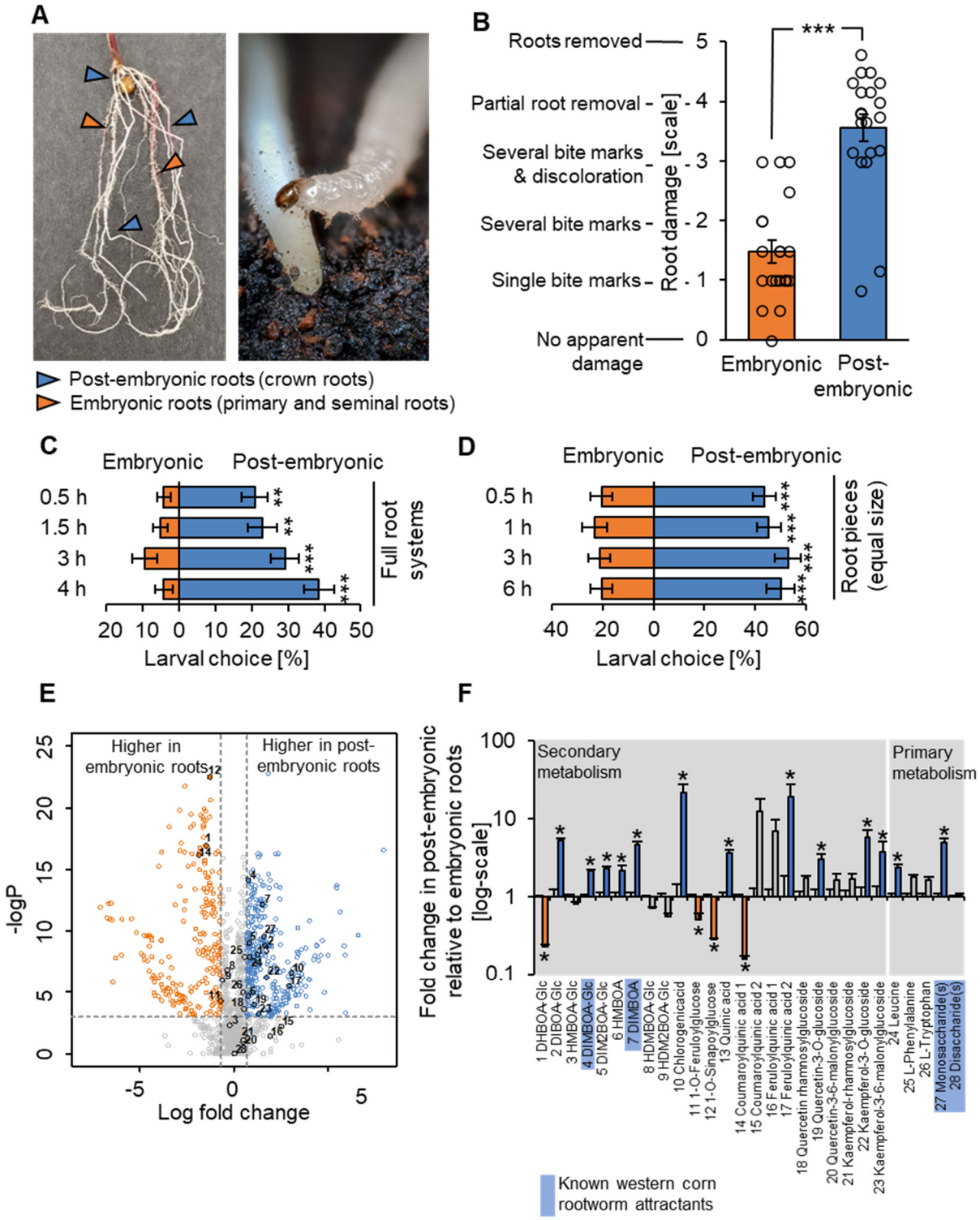


Fig. 1. *The western corn rootworm prefers to feed on root tissues that are rich in benzoxazinoids and soluble sugars.* (*A*) Left: young maize plants produce embryonic primary and seminal roots (orange arrows) and post-embryonic crown roots (blue arrows). Right: Western corn rootworm larvae are highly specialized maize root feeders that are very mobile in the second and third instar. A third instar larvae is shown. (*B*) Average damage score observed on post-embryonic and embryonic roots of soil-grown maize plants after 7 days of infestation by western corn rootworm larvae (***$p<0.001$, Wilcoxon Signed Rank test, n=20 plants with 15 larvae each, data from Fig. 4*D*). Dots represent damage scores on individual plants (averaged within root types). For frequency distributions of individual roots, refer to Fig. S1. (*C*) Preference of western corn rootworm larvae for post-embryonic and embryonic roots within the root system

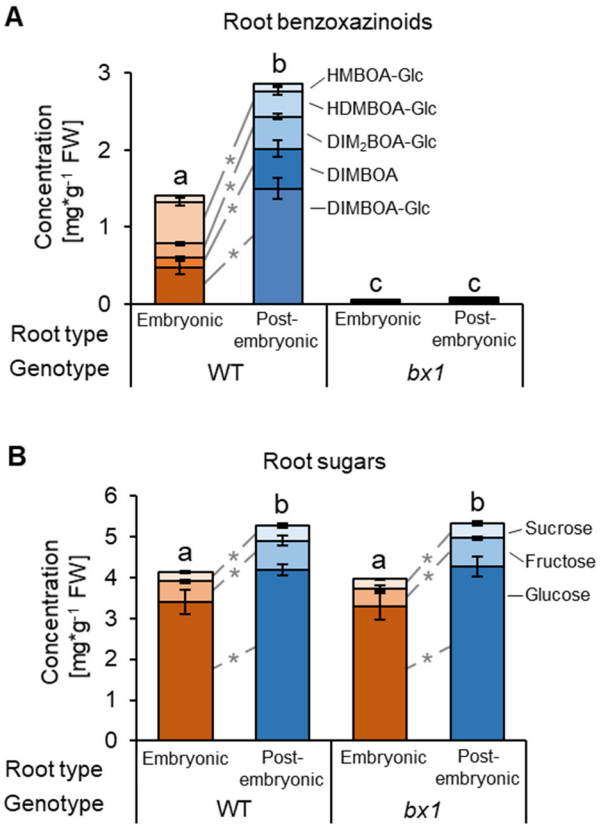
(**p<0.01, ***p<0.001, FDR-corrected Least Square Mean post hoc tests, n=16 dishes with 6 larvae each). (*D*) Preference of western corn rootworm larvae for root pieces of equal size of post-embryonic and embryonic roots (***p<0.001; FDR-corrected Least Square Mean post hoc tests, n=18 dishes with 5 larvae each). (*E*) Metabolomics profiles of methanolic extracts of post-embryonic and embryonic roots. Orange features are more abundant in embryonic roots, blue features are more abundant in post-embryonic roots (min. 2-fold difference, p<0.05, FDR-corrected Student's *T*-tests, n=9-10). Numbers denote features that were tentatively assigned to structures based on exact mass and fragment information (Table S2). (*F*) Relative abundance differences of tentatively identified metabolites between post-embryonic and embryonic roots (fold change of peak areas; *p<0.05, FDR-corrected Student's *T*-tests, n=9-10). Picture credits: Ricardo Machado, Lingfei Hu, Cyril Hertz. Error bars denote standard errors of means (SEM).

To narrow down the list of metabolites that may prompt the western corn rootworm to feed on post-embryonic roots, we performed a literature search on western corn rootworm attractants and feeding stimulants. Thirteen different compounds known to elicit behavioral responses in the western corn rootworm *in vitro* were found (Table S2). Cross-referencing this table with the list of chemicals that accumulated in post-embryonic roots at higher concentrations resulted in the identification of four candidate compounds that may mediate post-embryonic root preference: The monosaccharides glucose and fructose, which act as feeding stimulants (61) and the benzoxazinoids DIMBOA-Glc and DIMBOA, which form iron complexes in the rhizosphere that act as short-distance host preference and acceptance cues (66). Based on these results and earlier studies (66, 69), we hypothesized that benzoxazinoids and monosaccharides may interact to determine the preference of the western corn rootworm for post-embryonic roots. Given the observed overlap between post-embryonic root preference and overall plant attractiveness (66), we also hypothesized that the same compounds may mediate general host recognition and acceptance in this specialist herbivore.

Independent manipulation of benzoxazinoids and sugars as foraging cues

To test for the individual and combined roles of benzoxazinoids and sugars in mediating western corn rootworm behavior, we sought to independently manipulate their availability as foraging cues. The first dedicated step in benzoxazinoid biosynthesis is mediated by the indole-3-glycerol phosphate lyase *Bx1* (71). By consequence, *bx1* mutants are benzoxazinoid deficient (71, 72). To test if *bx1* mutant plants can be used to manipulate benzoxazinoids independently of root sugars, we performed a series of targeted analyses of wild type (WT) B73 and *bx1* roots. In accordance with our metabolomics screen and earlier studies (66, 69), post-embryonic roots of WT plants contained 4-fold higher DIMBOA levels and 2- to 3-fold higher DIMBOA-Glc and DIM_2BOA-Glc levels, but lower HDMBOA-Glc levels than embryonic roots (Fig. 2*A*). Benzoxazinoid levels in the *bx1* mutant were reduced by more than 97%, and residual benzoxazinoid concentrations were not significantly different between crown and primary roots (Fig. 2*A*). As expected from the

203 metabolomic analyses, post-embryonic roots of WT plants contained higher levels of the
204 monosaccharides glucose and fructose than embryonic roots (Fig 2*A*). Sucrose levels were also
205 higher in post-embryonic roots (Fig 2*A*). Sugar profiles were similar in WT and *bx1* roots (Fig.
206 2*A*), demonstrating that benzoxazinoid availability can be manipulated independently of sugar
207 availability. The differential abundance between and crown and primary roots of the other 17
208 identified metabolic features was conserved in the *bx1* mutant as well (Fig. S2). We only detected
209 a significant interaction between root type and genotype for tryptophan, which is also derived from
210 indole-3-glycerol phosphate (71). Tryptophan accumulated in higher amounts in post-embryonic
211 roots of both wild type and *bx1* mutant plants, and this difference was more pronounced in the *bx1*
212 mutant (Fig. S2). Thus, we conclude that apart from benzoxazinoids, the metabolic differences
213 between crown and primary roots are conserved in the *bx1* mutant, thus validating the use of this
214 genotype to assess the behavioral impact of benzoxazinoids on western corn rootworm behavior.



215 **Fig. 2.** *A mutation in the Bx1-gene suppresses root-type specific benzoxazinoid accumulation independently of root*
216 *sugars*. (*A*) Concentrations of benzoxazinoids in embryonic and post-embryonic roots of wild type B73 and *bx1* mutant
217 plants (n=11-18). (B) Concentrations of glucose, fructose and sucrose in embryonic and post-embryonic roots of wild
218 type B73 and *bx1* mutant plants (n=8). Letters indicate significant differences in total amounts between root types and
219 genotypes ($p<0.05$, Holm-Sidak post hoc tests). Asterisks indicate significant differences in the concentrations of
220 individual compounds between post-embryonic and embryonic roots ($p<0.05$, Holm-Sidak post hoc tests). Error bars
221 denote standard errors of means (SEM).

222 To manipulate the availability of sugars as foraging cues independently of benzoxazinoids, we
223 targeted sugar perception in the western corn rootworm. This approach was chosen because
224 manipulation of primary metabolism in plants results in pleiotropic effects (73, 74). Sugar
225 perception in insects is highly conserved and mediated by *Gr43a*-like genes (49, 50, 53, 54, 75–
226 78). BLAST search of publicly available nucleotide sequences revealed a single putative *Gr43a*
227 gene in the western corn rootworm, here named *DvvGr43a* (Fig. 3*A*). The full-length sequence of
228 this gene can be retrieved from NCBI [Accession: XM_028279548.1]. Similarity between
229 *DvvGr43a* and putative *Gr43a* genes from different insects, many of which have been functionally
230 characterized, was found to be between 42% and 64% at the protein level. No other closely related
231 genes were found in western corn rootworm genome. Structural prediction indicated that the
232 *DvvGr43a* gene encodes a 7-transmemrane domain protein, consistent with its putative role as a
233 gustatory receptor (Fig. 3*B*). Gene-expression profiling revealed stronger expression of *DvvGr43a*
234 in the head than the body of western corn rootworm larvae (Fig. 3C). To manipulate *DvvGr43a*,
235 we targeted its expression through RNA interference (RNAi) by feeding the larvae with a 240 bp
236 dsRNA targeting *DvvGr43a*. Compared to control larvae that were fed with dsRNA targeting green
237 fluorescent protein (GFP), this treatment resulted in a >70% reduction in *DvvGr43a* transcript
238 abundance (Fig. 3*D*). To assess silencing specificity, we measured the transcription of the putative
239 CO_2-receptor *DvvGr2* (67). Feeding dsRNA targeting *DvvGr43a* did not change the transcript
240 abundance of *DvvGr2* (Fig. 3*E*). Accordingly, the responsiveness of *DvvGr43a*-silenced larvae to
241 volatile CO_2 remained intact (Fig. S3). To evaluate whether *DvvGr43a* silencing changes the ability
242 of western corn rootworm to respond to benzoxazinoids, we conducted choice assays with the
243 behaviorally active benzoxazinoid iron complex Fe(III)(DIMBOA)$_3$ (66). After 1 hour, the
244 majority of western corn rootworm larvae were found on filter paper discs spiked with
245 Fe(III)(DIMBOA)$_3$, independently of *DvvGr43a* silencing. Next, we tested whether *DvvGr43a*
246 silencing changes the ability of the larvae to respond to sugars. Three hours after the start of the
247 experiment, control larvae preferred to locate on filter discs spiked with glucose, fructose, sucrose
248 or a mixture of the three compounds (Fig. 3*G-J*). These preferences were absent in larvae fed with
249 *DvvGr43a* dsRNA (Fig. 3 *G-J*), demonstrating that silencing *DvvGr43a* eliminates the capacity of
250 western corn rootworm larvae to respond behaviorally to three major plant sugars. *DvvGr43a*-
251 dependent sugar preference was similar in non-starved and starved western corn rootworm larvae
252 (Fig. S4). Thus, combining *bx1* plant mutants and *DvvGr43a*-silenced larvae can be used to
253 evaluate the individual and combined impact of benzoxazinoids and sugars on herbivore foraging.

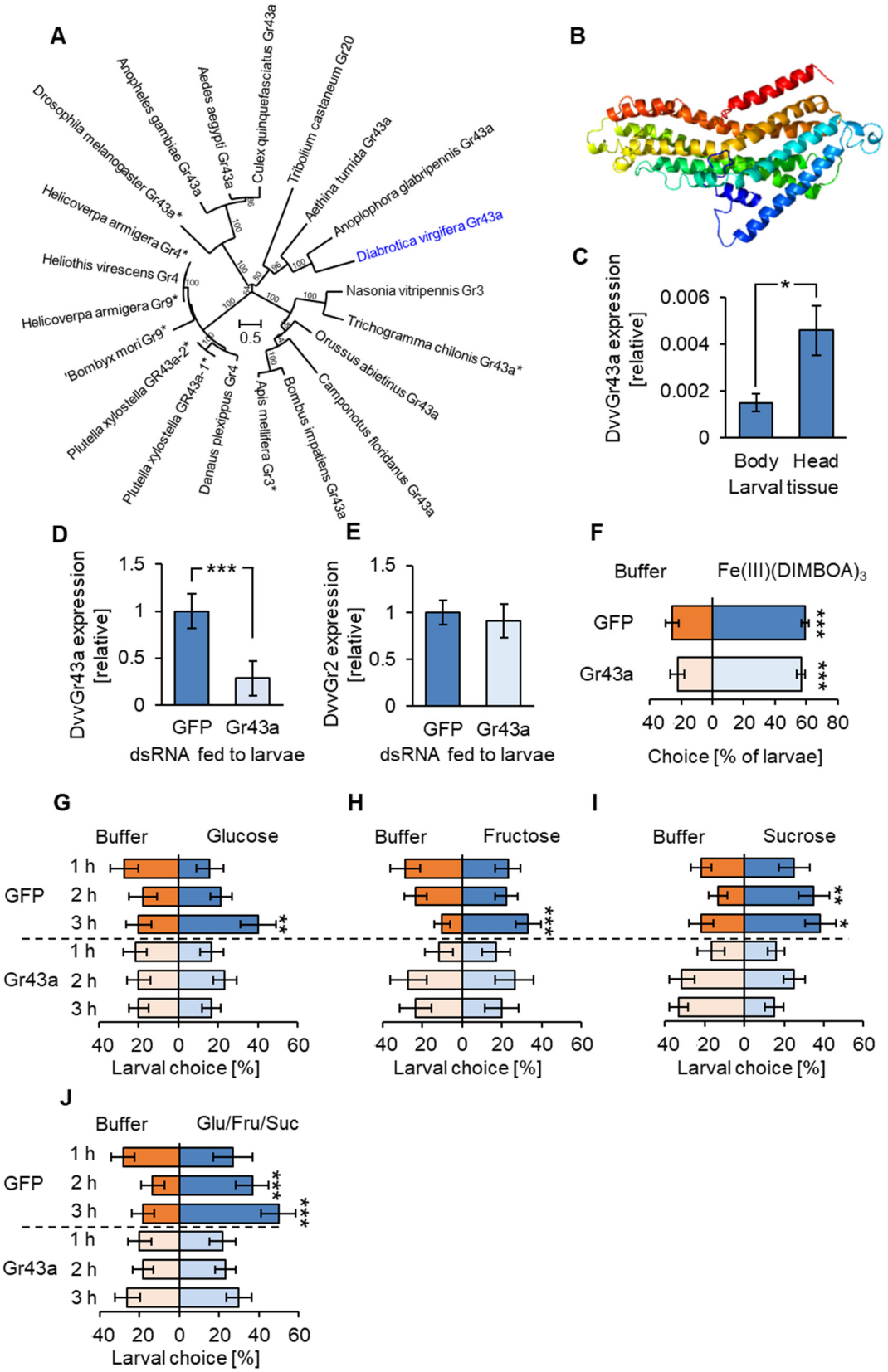


Fig. 3. *DvvGr43a mediates sugar preference of the western corn rootworm without influencing responsiveness to benzoxazinoids*. (*A*) Phylogenetic relationships between gustatory sugar receptors of different insects and *DvvGr43a*

of the western corn rootworm. The tree is based on protein sequences and drawn to scale, with branch lengths measured in the number of substitutions per site. Asterisks indicate functionally characterized receptors. (*B*) Protein tertiary structure of *DvvGr43a* as predicted with the Phyre2 algorithm. (*C*) *DvvGr43a* expression in the bodies (thorax and abdomen) or heads of second instar western corn rootworm larvae (*p<0.05, Student's *T*-test n=10). (*D*) *DvvGr43a* expression in western corn rootworm larvae fed with dsRNA targeting green fluorescent protein (control, GFP) or *DvvGr43a* (Gr43a, ***p<0.001, Student's *T*-test, n=11). (*E*) *DvvGr2* expression in western corn rootworm larvae fed with dsRNA targeting green fluorescent protein (control, GFP) or *DvvGr43a* (n=8). (*F*) Preference of GFP and *DvvGr43a* dsRNA fed larvae for buffer or Fe(III)(DIMBOA)$_3$ on filter discs after 1 h (***p<0.001, FDR-corrected Least Square Mean post hoc tests, n=10 dishes with 6 larvae each). (*G-J*) Preference of GFP or *DvvGr43a* dsRNA fed larvae for buffer or glucose, fructose, sucrose or a mixture of the three on filter discs at different time points (*p<0.05; **p<0.01; ***p<0.001, FDR-corrected Least Square Mean post hoc tests, n=15 dishes with 6 larvae each). Error bars denote standard errors of means (SEM).

Benzoxazinoids and sugars play distinct roles in root herbivore foraging

To understand the importance of benzoxazinoids and sugars in mediating host and tissue finding of the western corn rootworm in a neutral environment, we observed the behavior and feeding of control and *DvvGr43a*-silenced larvae on wild type and *bx1* mutant root systems laid out on moist filter paper. Ninety % of control larvae were found on the roots rather than the filter paper 4 hours after their release (Fig. 4*A*). This number dropped to 70% for *DvvGr43a* silenced larvae, independently of plant genotype. A different pattern was found when looking at the distribution of the larvae on the different root types within root systems (Fig. 4*B*). On wild type plants, significantly more control larvae were found on post-embryonic than embryonic roots. This difference was absent for control larvae feeding on *bx1* mutant plants. For *DvvGr43a* silenced larvae, more larvae were found on post-embryonic than embryonic roots, even though this preference was attenuated relative to control larvae. No preference was found for *DvvGr43a* silenced larvae feeding on *bx1* mutant roots (Fig. 4*B*). These results show that sugars contribute to root system finding and post-embryonic root preference but are not strictly required for either process. Benzoxazinoids on the other hand do not contribute to root system finding but are specifically required for the capacity of the western corn rootworm to distinguish post-embryonic from embryonic roots.

To evaluate whether benzoxazinoids and sugars affect host acceptance in the form of active feeding, we infested roots of soil-grown wild type and *bx1* mutant plants with control or *DvvGr43a*-silenced western corn rootworm larvae and inspected the roots for characteristic feeding marks and damage after 7 days. As feeding by the western corn rootworm cannot be observed directly in the soil, and root biomass is a poor proxy for root consumption due to confounding root regrowth effects and the disproportionate impact of severed roots (79), this approach was considered most suitable for the question at hand. The experiment revealed that root damage across different roots

293 was increased by the availability of benzoxazinoids and sugars as foraging cues in an additive
294 fashion (Fig. 4*C*). Frequent root removal was observed in wild type plants infested with control
295 larvae. Intermediate root damage in the form of frequent biting marks and brown discoloration was
296 observed for *bx1* mutant roots infested with control larvae and wild type roots infested with
297 *DvvGr43a*-silenced larvae. Infrequent biting marks and/or no visible damage was observed for *bx1*
298 mutant roots infested with *DvvGr43a*-silenced larvae. The same additive pattern was also visible
299 when looking at the distribution of damage patterns between the different root types (Fig. 4*D*).
300 Post-embryonic root damage was strongest and significantly more pronounced than embryonic root
301 damage in wild type plants infested with control larvae. Post-embryonic root damage and damage
302 differences with embryonic roots were intermediate for *bx1* mutants infested with control larvae
303 and wild type plants infested with *DvvGr43a*-silenced larvae. Post-embryonic root damage was
304 lower and no longer different from damage to embryonic roots for *bx1* mutants infested with
305 *DvvGr43a*-silenced larvae (Fig. 4*D*). Thus, both benzoxazinoids and sugars increase root damage
306 in an additive manner, and this pattern is driven by post-embryonic root damage. Nevertheless, bite
307 marks were still observed on 69% of *bx1* mutant roots infested with *DvvGr43a*-silenced larvae,
308 showing that the larvae attack maize roots even in the absence of these cues. Taken together, these
309 results reveal distinct and additive roles of benzoxazinoids and sugars in root and tissue location
310 and feeding preferences of the western corn rootworm.

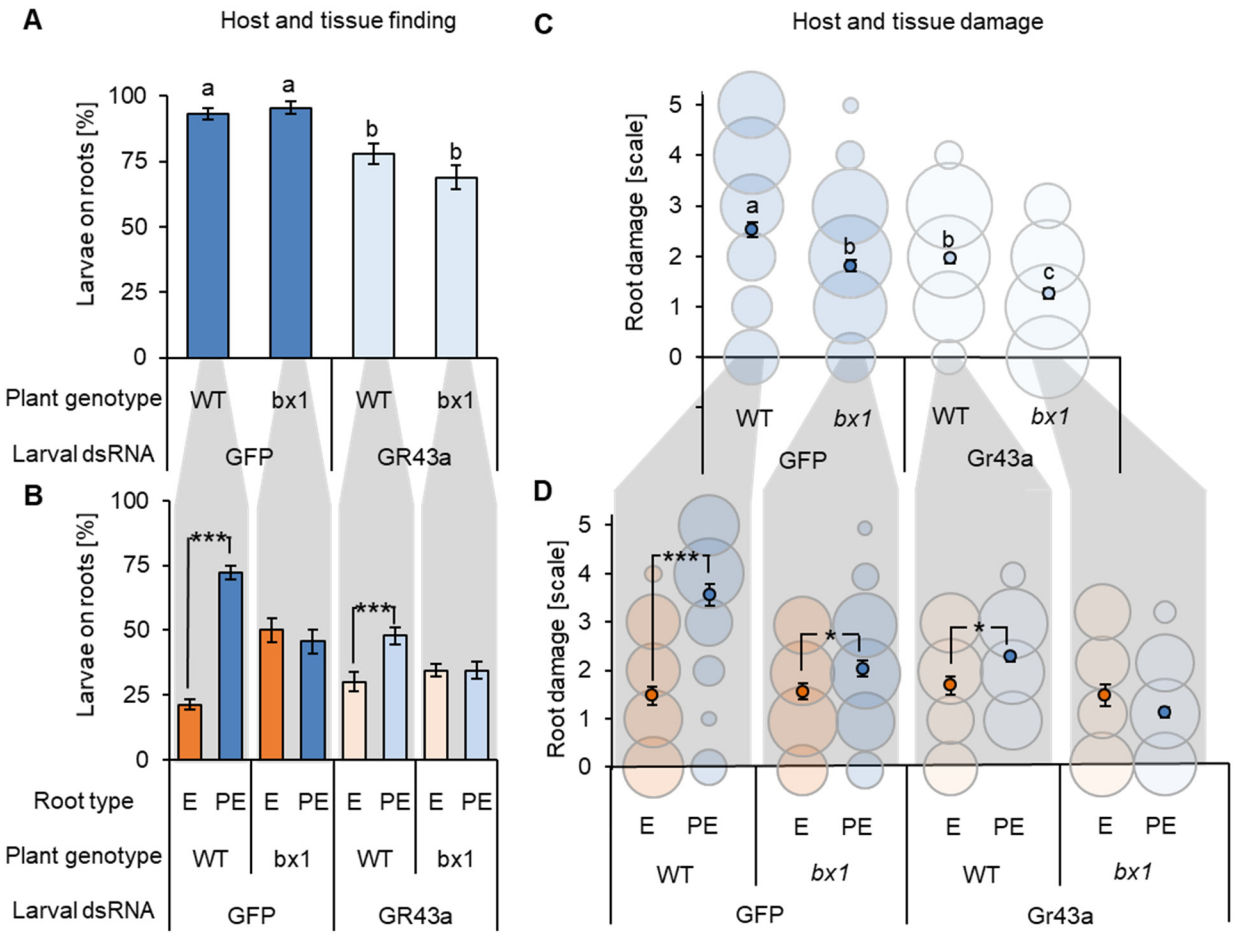


Fig. 4. *Benzoxazinoids and sugars play distinct roles in root herbivore foraging in vivo*. (*A*) Proportion of control (GFP) or *DvvGr43a*-silenced (Gr43a) larvae found on the roots of wild type B73 or *bx1* mutant roots 4 hours after their release. Letters indicate statistically significant differences between treatments (Holm-Sidak post-hoc tests, $p<0.05$, n=15 dishes with 6 larvae each). (*B*) Proportion of control (GFP) or *DvvGr43a*-silenced (Gr43a) larvae found on embryonic (E) and post-embryonic (PE) roots within the same experiment. Asterisks indicate significant differences between root types (***$p<0.001$, FDR-corrected Least Square Mean post hoc tests, n=15 dishes with 6 larvae each). (*C*) Average feeding damage by control (GFP) or *DvvGr43a*-silenced larvae per root of soil-grown wild type B73 or *bx1* mutant plants 7 days after infestation. Letters indicate statistically significant differences ($p<0.05$, Tukey's post hoc tests, n=20 plants with 15 larvae each). Bubble plots are shown for illustrative purposes. The sizes of the circles are proportional to the relative frequency (% within each treatment) of the different types of observed damage (each column sums up to 100%). For damage scale, refer to Fig. 1. (*D*) Average feeding damage on embryonic (E) or post-embryonic (PE) roots within the same experiment. Asterisks indicate significant differences in root damage between root types (*$p<0.05$, ***$p<0.001$ Wilcoxon Signed Rank tests, n=20 plants with 15 larvae each). Bubble plots are shown for illustrative purposes. The sizes of the circles are proportional to the relative frequency (% within each root type) of the different types of observed damage (each column sums up to 100%). Error bars denote standard errors of means (SEM).

Using benzoxazinoids and sugars as foraging cues improves herbivore growth and survival

Herbivore foraging theory predicts that herbivores use plant chemical cues to make fitness-relevant foraging decisions. To test whether the integration of primary and secondary metabolites into foraging improves herbivore performance and survival, we first evaluated whether the absence of

332 benzoxazinoids or the inability to detect sugars may have direct performance effects on the western
333 corn rootworm (as opposed to the effects mediated by changes in foraging behavior).
334 Benzoxazinoids improve western corn rootworm performance under iron-limiting conditions (66)
335 and upon attack by entomopathogenic nematodes (80, 81). When bioavailable iron is abundant and
336 natural enemies are absent, larval performance under no-choice conditions is not altered by
337 benzoxazinoids (66, 69). Thus, growing maize plants with bioavailable EDTA-Fe as iron source
338 allowed us to exclude direct effects of benzoxazinoids on larval performance. To test whether
339 silencing *DvvGr43a* has direct negative effects on western corn rootworm performance, we reared
340 control and *DvvGr43a*-silenced larvae on young maize seedlings that produce embryonic, but no
341 post-embryonic roots. Larval weight gain and mortality of control and *DvvGr43a*-silenced larvae
342 after 6 days was similar (Fig. S5), demonstrating that silencing *DvvGr43a* has no direct effects on
343 larval performance in a no-choice situation. Note that larval mortality in this experiment was >40%,
344 which is within the normal range for early instars of this species (82, 83).

345 Having established the validity of our experimental approach, we proceeded to evaluate the impact
346 of benzoxazinoid- and sugar-dependent foraging behavior on western corn rootworm performance.
347 Highest larval weight and lowest mortality was observed for control larvae feeding on wild type
348 plants (Fig. 5*A*-*B*). Intermediate weight gain and mortality was observed for control larvae feeding
349 on *bx1* mutant roots and for *DvvGr43a*-silenced larvae feeding on wild type roots. Lowest weight
350 gain and highest mortality was observed for *DvvGr43a*-silenced larvae feeding on *bx1* mutant
351 roots. Larval performance thus mirrored root damage patterns (Fig. 4*C*-*D*). This was confirmed by
352 correlation analysis which revealed a positive relationship between total larval weight and survival
353 on a given plant and the total damage observed on post-embryonic roots (Fig. 5*C*-*F*). In conclusion,
354 sugars and benzoxazinoids act together to improve western corn rootworm performance by acting
355 as foraging cues that guide the herbivore to feed on the most profitable host tissue.

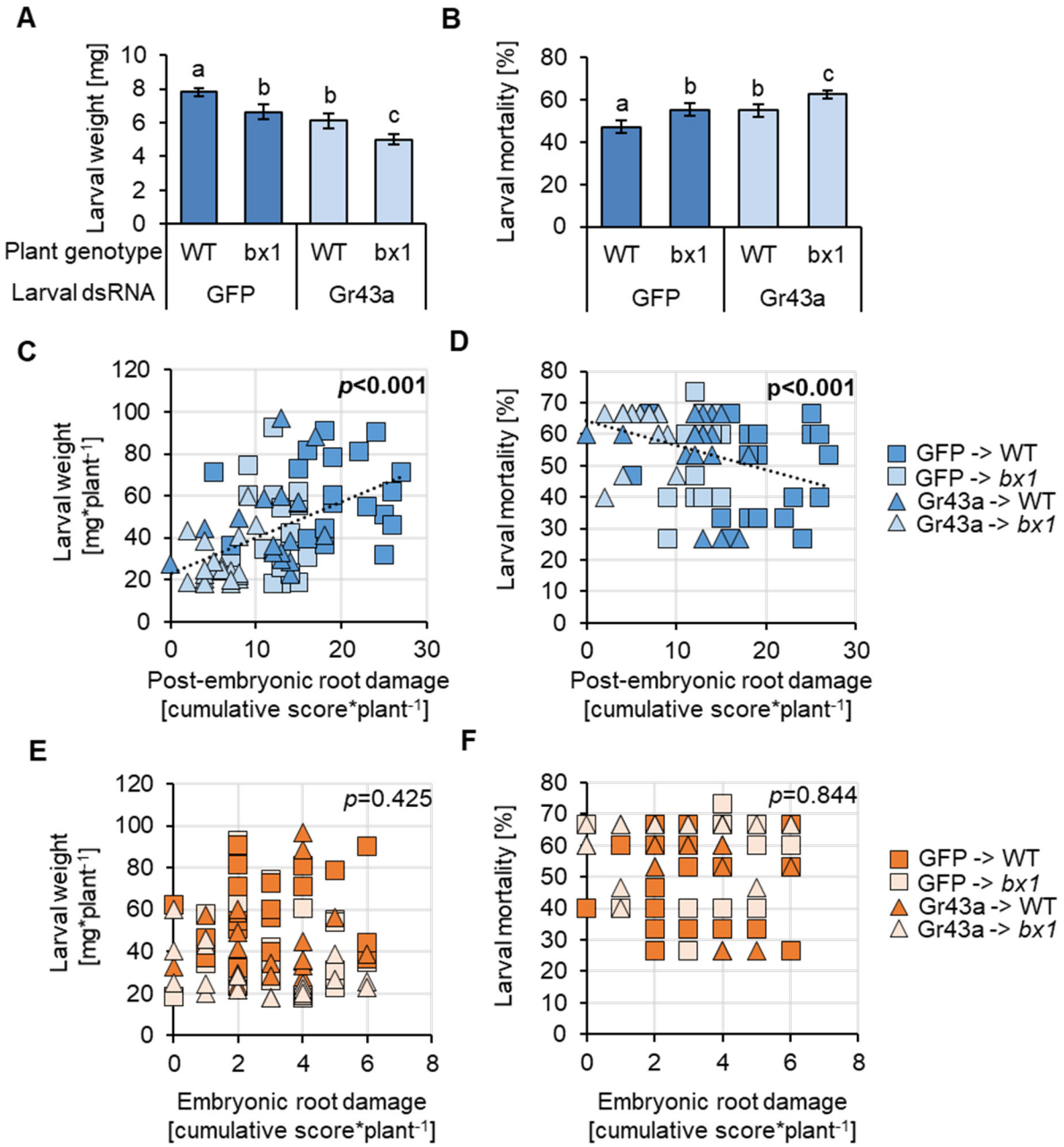


Fig. 5. *Using benzoxazinoids and sugars as foraging cues improves herbivore growth and survival.* (*A*) Larval weight of control (GFP) and DvvGr43a-silenced dsRNA fed western corn rootworm larvae feeding on wild type B73 or *bx1* mutant plants for 7 days. Note that in a no-choice situation, neither the *bx1* mutation nor DvvGr43a silencing reduce larval performance ((69) and Fig. S5). Letters indicate significant differences (p<0.05, Holm-Sidak post hoc tests, n=20 pots with 15 larvae each). (*B*) Larval mortality within the same experiment. Letters indicate significant differences (p<0.05, Holm-Sidak post hoc tests, n=20 pots with 15 larvae each). (*C-F*) Correlations between cumulative damage per plant and larval performance parameters for post-embryonic roots (*C-D*) and embryonic roots (*E-F*). Linear regressions are shown for significant correlations (p<0.05). *P*-values are shown for Spearman Rank Order correlations.

366 **Discussion**

367 Plant primary and secondary metabolites can act together to determine the behavior of generalist
368 herbivores on artificial diet (33, 34). The present work expands these findings by demonstrating
369 that primary and secondary metabolites can have distinct, overlapping, and additive roles as
370 determinants of the behavior, and, consequently, the performance of a specialist herbivore *in*
371 *planta*.

372 Herbivores are thought to use primary and secondary metabolites to make foraging decisions within
373 complex host metabolomes (16), but how they combine these two types of cues during different
374 stages of host finding and acceptance is not well understood. We find that the western corn
375 rootworm employs sugars, but not benzoxazinoids for host finding. Sugars increase the successful
376 localization of post-embryonic roots, but only in the presence of benzoxazinoids, which are
377 essential for the latter. In contrast to these distinct roles, sugars and benzoxazinoids have similar
378 roles in enhancing overall root damage and tissue-specific post-embryonic root damage, and act in
379 an additive manner in this context. Thus, western corn rootworm larvae employ primary and
380 secondary metabolites in varying combinations and hierarchies for different behavioral patterns.
381 This finding differs from current hypotheses and observations for polyphagous insects that suggest
382 sequential use of different foraging cues (84, 85). The explanation for this discrepancy is likely
383 found in the distribution patterns of plant primary and secondary metabolites in maize roots and
384 rhizosphere. Benzoxazinoids and sugars constantly accumulate in maize roots, with stable
385 concentration differences between post-embryonic and embryonic roots (66, 69). Both compound
386 classes are also exuded into the rhizosphere (86, 87). Current work using enzyme-inhibiting and
387 non-inhibiting extraction solvents (66, 69, 86) suggests that benzoxazinoids are likely exuded as
388 glucosides before being deglycosylated at the root surface, resulting in the release of aglucones and
389 glucose. Aglucones such as DIMBOA then form attractive complexes with free iron. (66). Thus,
390 both sugars and benzoxazinoids are present inside and outside of the roots, with outside
391 accumulation likely being more dynamic than inside accumulation. Taking into account that
392 benzoxazinoids are plant-specific, while sugars are more general cues (88, 89), one can envisage a
393 scenario where the western corn rootworm relies on sugars as general short-distance cues for the
394 presence of roots (with potentially high concentrations on the surface of maize roots due to
395 benzoxazinoid deglycosylation), followed by benzoxazinoids as specific within-plant tissue
396 selection cues. Benzoxazinoids may also be used as specific short-distance host recognition cues
397 in case roots of multiple plant species are intermingled. Following host and tissue finding, the

398 herbivore would then use both sugars and benzoxazinoids as stable host acceptance and feeding
399 cues. High resolution metabolite imaging (90) together with the manipulation of gustatory
400 receptors (23) could help to test these hypotheses and explore the connections between the
401 distribution dynamics of different plant metabolites and herbivore foraging.

402 Plants produce complex chemical mixtures, and herbivores likely combine many of these
403 chemicals to make appropriate foraging decisions (11, 12, 29). In line with this idea, recent *in vivo*
404 work shows that, while individual compound classes and insect gustatory receptors can be of major
405 importance for specific behavioral patterns, their disruption is not sufficient to fully suppress these
406 behaviors (22–24). In the case of the western corn rootworm, we find that even the suppression of
407 two major foraging cues does not fully abolish its ability to find and accept a host plant, at least
408 under laboratory conditions. Several other plant-derived chemicals, including different volatiles
409 and fatty acids, are known to attract, arrest and stimulate feeding by the western corn rootworm
410 (61, 62, 64). These cues are likely to enable basic behavioral patterns in the absence of
411 benzoxazinoids. We conclude that the flexible integration of complex blends of chemical cues
412 provides partial resistance to chemically mediated behavioral disruption. Recent work highlights
413 the importance of learning in herbivore foraging (12, 91), which likely increases the capacity of
414 herbivores to respond to dynamic chemical landscapes and to substitute between different cues
415 with redundant information content. How previous experiences influence the foraging behavior of
416 the western corn rootworm remains to be determined. All larvae used in our experiments were
417 reared on maize, reflecting their high degree of specialization on this host plant (59). Western corn
418 rootworm adults are more flexible with their diet (59, 92), and it would be interesting to understand
419 how their experience influences the responses of the next larval generation to host plant cues.

420 The capacity of an herbivore to select a host plant and feed on specific tissues generally improves
421 its fitness (40, 42, 93). In line with these preference-performance relationships, diet experiments
422 often document strong associations between chemically-mediated herbivore feeding preferences
423 and herbivore performance (17). In many of these cases, herbivore feeding is directly elicited by
424 substances that improve performance (17). However, plant metabolomes are likely to complex to
425 be fully assessed by an herbivore, who thus need to rely on digestive feedbacks or subsets of
426 chemical cues to make decisions. To what extent the use of plant chemical cues enhances herbivore
427 performance by guiding herbivores to nutritious tissues is poorly understood, and interactions
428 between primary and secondary metabolite chemical cues have, to the best of our knowledge, not

429 been tested *in planta*. Our experimental approach rules out direct effects of benzoxazinoids and
430 sugar perception on western corn rootworm performance (66, 69) and thus allows us to quantify
431 their net impact as foraging cues. We find that the availability of sugars and benzoxazinoids during
432 foraging improves herbivore weight gain and survival individually by 8%, and in combination by
433 15%, benefits which are most likely driven by increased post-embryonic root feeding. Post-
434 embryonic roots of young maize plants are growing more vigorously than embryonic roots, receive
435 a higher share of photosynthates and are richer in carbohydrates, amino acids and total soluble
436 protein and increase western corn rootworm growth in no-choice experiments (69). In combination,
437 these findings demonstrate that the combined use of primary and secondary metabolites as foraging
438 cues enhance the performance of a specialist insect herbivore by guiding it to its most profitable
439 feeding niche.

440 The gustatory receptor Gr43a is a highly conserved insect sugar receptor that is narrowly tuned to
441 D-fructose (49), but mediates feeding preferences for different sugars through dietary feedbacks
442 (55). Given the conserved function, predicted protein structure, tissue-specific expression pattern
443 and effect on sugar preferences, *DvvGr43a* is very likely to act as a sugar sensor in the western
444 corn rootworm as well. An interesting observation in this context is that *DvvGr43a*-dependent
445 larval feeding preferences for sugars and sugar mixtures were only established after 2-3 hours,
446 while feeding preferences for Fe(III)(DIMBOA)$_3$ were established more rapidly (66). In
447 Drosophila, Gr43a increases sugar intake under food limiting conditions and suppresses sugar
448 intake under satiation (49). We thus hypothesized that the delayed *DvvGr43a*-dependent sugar
449 feeding preference of western corn rootworm larvae for sugars may be the result of a starvation
450 response. However, we observed a similar lag time in sugar preference in pre-starved larvae. This
451 suggests that *DvvGr43a* likely functions as a sugar sensor rather than as a nutrient uptake regulator
452 in the western corn rootworm. The observed lag phase may be explained by the potential role of
453 *DvvGr43a* as an internal sugar sensor that triggers behavioral responses once sugars are taken up
454 and elicit changes in hemolymph sugar levels. A deeper molecular characterization could shed light
455 on the precise neurological and physiological roles of *DvvGr43a* in the western corn rootworm in
456 the future.

457 In conclusion, the experiments presented here reveal how primary and secondary metabolites
458 interact to guide the foraging of a specialist herbivore. The distinct individual and combined roles
459 of these metabolites reveal finely tuned herbivore foraging strategies that are directly linked to

460 improved herbivore performance and survival. At the same time, foraging is remarkably robust to
461 perturbation, most likely due to the flexible use of redundant host plant cues. Our mechanistic
462 approach thus offers a glimpse into the complex, multilayered interplay between plant
463 metabolomes and chemically guided herbivore foraging.

464 **Materials and Methods**

465 Plants and insects

466 Maize seeds (*Zea mays* L., inbred line B73) were provided by Delley Semences et Plantes SA
467 (Delley, CH). The near-isogenic benzoxazinoid deficient *bx1* mutant line in a B73 background was
468 obtained by backcrossing the original *bx1* mutant five times into B73 (94). Seedlings were grown
469 under greenhouse conditions (23 ± 2˚C, 60% relative humidity, 16:8 h L/D, and 250 mmol/m^2/s^1
470 additional light supplied by sodium lamps). Plantaaktiv® 16+6+26 Typ K fertilizer (Hauert HBG
471 Dünger AG, Grossaffoltern, Switzerland) was added twice a week after plant emergence following
472 the manufacturer's recommendations. When plants were used to feed insects, seedlings were
473 germinated in vermiculite (particle size: 2-4 mm; tabaksamen, Switzerland) and used within four
474 days after germination. Eggs of the non-diapausing *Diabrotica virgifera virgifera* strain were
475 originally supplied by USDA-ARS-NCARL, Brookings, SD. Insect colonies were subsequently
476 established and are maintained in the University of Bern and the University of Neuchatel. Upon
477 egg hatching, insects were maintained in organic soil (Selmaterra, Bigler Samen AG, Thun,
478 Switzerland) and fed freshly germinated maize seedlings.

479 Herbivore behavior

480 To evaluate the preference of western corn rootworm larvae for different plant genotypes and root
481 types, we use three different experimental setups: potted plants, root systems laid out in petri dishes
482 and detached roots in petri dishes.

483 For western corn rootworm feeding preference experiments using whole plants, three-week-old
484 maize plants grown in 200 mL cylindrical plastic pots filled with sand and topped off with potting
485 soil were infested with fifteen second instar western corn rootworm larvae. Twenty plants per plant
486 genotype/type of larvae combination were evaluated (n=20). Seven days after infestation, plants
487 were gently excavated and inspected for root damage. Damage was recorded for each root
488 individually using the following scale. 0: no visual damage, 1: one-three bite marks, 2: more than
489 three bite marks, 3: several bite marks and brown discoloration, 4: root partially removed, and 5:
490 root fully removed.

491 Western corn rootworm feeding preference assays using petri dishes were conducted as described
492 (95, 96). Briefly, two-week-old maize plants grown as described above were gently excavated. The
493 root systems were washed and laid out onto a moist filter paper embedded in a petri dish (14 cm
494 diameter, Greiner Bio-one, Austria). A circular whole in the rim of the dishes accommodated the
495 plant stems, with the shoots remaining outside of the dishes. Five third instar western corn
496 rootworm larvae were released into each petri dish. Petri dishes were sealed and then covered with
497 aluminum foil. The number of western corn rootworm larvae feeding on each root type was
498 determined at different time points after their release.

499 To evaluate western corn rootworm feeding preference using detached roots, pieces of equal sizes
500 of two post-embryonic roots and two embryonic roots from two-week-old maize plants were placed
501 next to each other onto a moist filter paper (diameter 90 mm, GE Healthcare, UK) embedded into
502 a petri plate (90 mm diameter, Greiner Bio-one, Austria). Five third instar western corn rootworm
503 larvae were released into each petri dish. Petri plates were sealed and then covered with aluminum
504 foil. The number of western corn rootworm larvae feeding on each root type was determined at
505 different time points.

506 To evaluate the attractiveness of CO_2 for western corn rootworm larvae, we conducted choice
507 experiments using belowground olfactometers (64). CO_2 levels were increased in one arm of the
508 olfactometer by delivering CO_2-enriched synthetic air (1% CO_2, Carbagas, Switzerland).
509 Olfactometer arms were closed on top using parafilm during CO_2 delivery. A manometer connected
510 to the synthetic air bottle allowed to fine-tune CO_2 delivery rates. CO_2 levels were measured using
511 a gas analyzer (Li7000, Li-Cor Inc., Lincoln, Nebraska, USA). Once desired CO_2 concentrations
512 were reached, CO_2 delivery was terminated, olfactometer arms were connected to olfactometer
513 central connectors and six second to third instar western corn rootworm larvae were release
514 immediately and their choice were evaluated within ten minutes of release. Six olfactometers per
515 larval type were assayed (n=6).

516 To evaluate the attractive effects of Fe(III)(DIMBOA)$_3$ for western corn rootworm larvae, we
517 release six second to third instar larvae in the middle of a moist sand-filled petri dish (9 cm
518 diameter, Greiner Bio-One GmbH, Frickenhausen, DE) where they encountered a filter paper disc
519 treated with 10 µl of Fe(III)(DIMBOA)$_3$ (1 µg/ml of water) or, on the opposite side, a filter paper
520 disc treated with water only. Ten independent petri dishes per larval type were evaluated (n=10).

521 Fe(III)(DIMBOA)$_3$ was prepared fresh by mixing $FeCl_3$ and DIMBOA at a 1:2 ratio as described
522 (96). Larval preferences were recorded 1 h after releasing the larvae.

523 To evaluate attractive effects of soluble sugars for western corn rootworm larvae, we followed the
524 procedure described by 97 with minor modifications. Briefly, we released six second to third instar
525 western corn rootworm larvae in the middle of a moist sand-filled petri dish (6 cm diameter, Greiner
526 Bio-One GmbH, Frickenhausen, DE) where they encountered a filter paper disc treated with 10 µl
527 of either glucose (30 mg/ml), fructose (30 mg/ml), sucrose (30 mg/ml), or a mixture of the three
528 (10 µl containing each sugar at a concentration of 30 mg/ml), or, on the opposite side, a filter paper
529 treated with water only. Fifteen independent petri dishes per treatment were assayed (n=15). Larval
530 preferences were recorded regularly for 3 h. In a second experiment, larvae were pre-fed on maize
531 roots or starved for 12 h before being released into petri dishes with filter paper discs treated with
532 a sugar mixture or water, as described above. Eight independent petri dishes with six larvae each
533 were assayed (n=8).

534 Herbivore performance

535 To evaluate the impact of benzoxazinoids and sugars on western corn rootworm performance,
536 three-week-old maize plants grown in 200 mL cylindric plastic pots (11 cm depth and 4 cm
537 diameter) filled with sand were infested with fifteen second instar western corn rootworm larvae
538 (n=20). Seven days after infestation, the larvae were collected, counted, and weighted using a
539 microbalance. Note that weight and survival was determined on the same plants as root damage
540 (see section "Herbivore behavior").

541 To evaluate the impact of *DvvGr43a*-silencing on western corn rootworm performance in a no-
542 choice setting, GFP and *DvvGr43a* fed first instar larvae were reared on 3-4-day old maize
543 seedlings, which only produce embryonic roots, in soil-filled plastic cups (n=40). Nine larvae were
544 used per cup. Fresh seedlings were provided to the larvae every three days for a total of six days,
545 after which the larvae were collected, counted, and weighted using a microbalance.

546 Root metabolite measurements

547 Untargeted root metabolomics was conducted as described (81). Embryonic and post-embryonic
548 roots of B73 plants were harvested separately, flash-frozen and ground in liquid nitrogen. Sixty to
549 eighty mg of plant tissues were then extracted in 800 µL of acidified H_2O/MeOH (50:50 v/v; 0.1%
550 formic acid). Resulting root extracts were analysed using an Acquity UHPLC system coupled to a

551 G2-XS QTOF mass spectrometer equipped with an electrospray source (Waters). Gradient elution
552 was performed on an Acquity BEH C18 column (2.1 × 50 mm i.d., 1.7 µm particle size) at 1-27.5%
553 B over 3.5 min, 27.5-100% B over 1 min, holding at 100% B for 1 min, and reconditioning at 1%
554 B for 1 min, where A =0.1% formic acid/water and B = 0.1% formic acid/acetonitrile. The flow
555 rate was 0.4 mL/min. The temperature of the column was maintained at 40°C, and the injection
556 volume was 1 µL. The QTOF MS was operated in negative mode. The data were acquired over an
557 m/z range of 100–1200 with scans of 0.15 s at collision energy of 4 V and 0.2 s with a collision
558 energy ramp from 10 to 40 V. The capillary and cone voltages were set to 2 kV and 20 V,
559 respectively. The source temperature was maintained at 140°C, the desolvation gas was 400°C at
560 1000 L h-1 and cone gas flow was 50 L/hr. Accurate mass measurements (<2 ppm) were obtained
561 by infusing a solution of leucin encephalin at 200 ng/mL at a flow rate of 10 µL/min through the
562 Lock Spray probe (Waters). The chromatograms were processed using Progenesis QI (Nonlinear
563 Dynamics, Newcastle UK) with default settings for spectral alignment, peak picking, and
564 deconvolution, resulting in a total of 4956 mass features. Of these, 444 mass features eluting
565 between 0 and 0.3 min were discarded, since their lack of retention and excessive coelution made
566 reliable identification impossible.

567 Abundances for each remaining mass feature were normalised by ArcSin transformation, and mean
568 normalized abundances for post-embryonic and embryonic roots were compared by Student's t-
569 test. P-values were adjusted by Benjamini–Hochberg false discovery rate multiple testing
570 correction and a fold-change cut-off of 2.0 was applied to generate a list of compounds that
571 significantly differed between treatments. Volcano plots were used to visualize treatment
572 differences, and the list of differentially expressed features was tested against an in-house mass-
573 fragmentation database using Progenesis QI (Progenesis MetaScope v1.0.6253.26544, Nonlinear
574 Dynamics, Newcastle, UK). In addition, chromatograms were manually scanned for exact masses
575 of known plant metabolites, specifically amino acids, sugars, organic acids, and flavonols. Potential
576 matches were likewise tested against the mass fragmentation database, and putative identifications
577 were accepted based on exact mass, mass fragmentation patterns, and relative retention times.

578 Root benzoxazinoid quantification was conducted as described (98). For this, embryonic and post-
579 embryonic roots of B73 or *bx1* plants were harvested separately, flash-frozen and ground in liquid
580 nitrogen. Approximately 100mg of plant tissues were then extracted in 1 mL of acidified
581 H_2O/MeOH (50:50 v/v; 0.1% formic acid), and analyzed with an Acquity UHPLC system coupled

582 to a UV detector and a Waters QDa mass spectrometer equipped with an electrospray
583 source(Waters). Compounds were separated on an Acquity BEH C18 column (2.1×50 mm i.d., 1.7
584 µm particle size). Water (0.1% formic acid) and acetonitrile (0.1% formic acid) were employed as
585 mobile phases A and B. The elution profile was: 0-1 min, 10% B; 1-4 min, 10-30% B; 4-5 min,
586 30- 9740% B; 5-6 min, 40-100% B; 6-8.5 min, 100% B; followed by 1.5 min reconditioning at
587 10% B. The mobile phase flow rate was 0.4 mL/min. The column temperature was maintained at
588 40°C, and the injection volume was 3 µL. The MS was operated in negative mode and data were
589 acquired in single ion recording (SIR) mode, using $[M-H]^-$ and $[M+HCOO^-]^-$ adducts of
590 benzoxazinoid compounds. All other MS parameters were left at their default values as suggested
591 by the manufacturer. Benzoxazinoid peaks were identified from MS signals and quantified from
592 integrated UV signals at 265nm. External standards for HMBOA, DIMBOA, DIMBOA-Glc,
593 HDMBOA-Glc, and MBOA were used for absolute quantification. Where concentrations were too
594 low to result in reliable UV signals, a compound-specific MS-to-UV signal conversion factor was
595 estimated to calculate 'predicted' UV signals from the more sensitive MS signals before
596 quantification.

597 Root sugar levels were measured by an enzymatic/spectrophotometric method as described (99).
598 Embryonic and post-embryonic roots of B73 and *bx1* plants were harvested separately, flash-frozen
599 and ground in liquid nitrogen. Soluble sugars were extracted from 100 mg root tissue using 80%
600 (v/v) ethanol, followed by an incubation step (10 min at 78°C) with constant shaking at 800 rpm.
601 Pellets were re-extracted twice with 50% (v/v) ethanol (10 min at 78°C with constant shaking at
602 800 rpm). Supernatants from all extraction steps were pooled together, and sucrose, glucose and
603 fructose were quantified as described (100).

604 Identification of *DvvGr43a*

605 To identify the *Gr43a* receptor orthologues in the western corn rootworm, we used the *Gr43a*
606 receptor-encoding gene sequence of *Drosophila melanogaster* as query against publicly available
607 nucleotide sequences and against the western corn rootworm genome sequences (NCBI accession:
608 PXJM00000000.2) using the National Center for Biotechnology Information Basic Local
609 Alignment Search Tool (NCBI BLAST). The full gene sequence can be retrieved from the National
610 Center for Biotechnology Information (NCBI) data bank using the following accession number:
611 XM_028279548.1. Then, we reconstructed evolutionary relationships between the identified
612 DvvGr43a gene and several other putative Gr43a genes that have been functionally characterized

613 as glucose, fructose, and/or sucrose receptors (49, 50, 53, 54, 75–78). The evolutionary
614 relationships were inferred using the Maximum Likelihood method based on the Le Gascuel 2008
615 model in MEGA7 (101, 102). The tree with the highest log likelihood (-14287.71) is shown. The
616 percentage of trees in which the associated taxa clustered together is shown next to the branches.
617 Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and
618 BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting
619 the topology with superior log likelihood value. A discrete Gamma distribution was used to model
620 evolutionary rate differences among sites (5 categories (+G, parameter = 1.0811)). The tree is
621 drawn to scale, with branch lengths measured in the number of substitutions per site. There was a
622 total of 569 positions in the final dataset. Graphical representation and edition of the phylogenetic
623 tree were performed with the Interactive Tree of Life (version 3.5.1) (103). Protein tertiary
624 structures and topologies were predicted using Phyre2 (104). The protein sequences used to
625 reconstruct evolutionary relationships can be retrieved from the NCBI under the following
626 accession numbers: *Heliothis virescens* Gr4 (CAD31946.1), *Danaus plexippus* Gr4 (EHJ77681.1),
627 *Bombyx mori* Gr9(NP_001124345.1), *Bombus impatiens* Gr43a (XP_003486787.1), *Nasonia*
628 *vitripennis* Gr3 (NP_001164386.1), *Apis mellifera* Gr3 (XP_016768876.1), *Drosophila*
629 *melanogaster* Gr43a (NP_523650.2), *Tribolium castaneum* Gr20 (EFA05758.1), *Anopheles*
630 *gambiae* Gr43a (XP_318100.4), *Camponotus floridanus* Gr43a (EFN61344.1), *Aedes aegypti*
631 Gr43a (XP_001658898.3), *Culex quinquefasciatus* Gr43a (XP_001842305.1), *Orussus abietinus*
632 Gr43a (XP_012270798.1), *Aethina tumida* Gr43a (XP_019876958.1), *Anoplophora glabripennis*
633 Gr43a (XP_018574194.1), *Helicoverpa armigera* Gr4 (AGK90011.1), *Helicoverpa armigera* Gr9
634 (JX970522.1), *Trichogramma chilonis* Gr43a (QAY30709.1), and *Diabrotica virgifera* Gr43a
635 (XP_028135349.1). For *Plutella xylostella* GR43a-1 and *Plutella xylostella* GR43a-2, and *D.*
636 *melanogaster* gustatory receptors refer to: (53, 105), as these sequences have not been deposited
637 in a public repository.

638 Double stranded RNA production

639 Double stranded RNA targeting *DvvGr43a* was produced by *Escherichia coli* HT115. To this end,
640 electrocompetent *E. coli* HT115 cells were transformed with recombinant L4440 plasmids that
641 contained either a 240 bp long *DvvGr43a* gene fragment, or a 240 bp long green fluorescent protein
642 (GFP) gene fragment flanked by two T7 promotors in opposite directions. Gene fragments were
643 synthetized *de novo* (Eurofins, Germany). To induce the production of dsRNA, an overnight

644 bacterial culture was used to inoculate fresh Luria-Berthani broth (25 g/L, Luria/Miller, Carl Roth
645 GmbH, Karlsruhe, Germany). Once the bacterial culture reached an OD_{600} of 0.6-0.8, it was
646 supplemented with isopropyl β-D-1-thiogalactopyranoside, IPTG) (Sigma-aldrich, Switzerland) at
647 a final concentration of 2mM. Bacterial cultures were incubated at 37°C in an orbital shaker at 130
648 rpm for 16h. Bacteria were harvested by centrifugation and stored at -20°C for further use (106).

649 Gene silencing experiments

650 To induce gene silencing in western corn rootworm, six to ten western corn rootworm larvae were
651 released in solo cups (30 mL, Frontier Scientific Services, Inc., Germany) containing approx. 2g
652 of autoclaved soil (Selmaterra, Bigler Samen AG, Thun, Switzerland) and 2-3 freshly germinated
653 maize seedling. Maize seedlings were coated with 1 ml of bacterial solution containing
654 approximately 200-500 ng of dsRNA targeting *DvvGr43a*. As controls, larvae were fed with
655 bacteria producing dsRNA targeting green fluorescent protein genes (GFP). Seedlings coated with
656 bacteria were provided every other day for three consecutive times. Two days after, larvae were
657 collected and used for experiments.

658 Gene expression measurements

659 Total RNA was isolated from approximately 10 mg of frozen, ground and homogenized western
660 corn rootworm larval tissue (3-7 larvae per biological replicate, n=8-11) using the GenElute
661 Universal Total RNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA). A NanoDrop
662 spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA) was used to
663 estimate RNA purity and quantity. DNase-treated RNA was used as template for reverse
664 transcription and first strand cDNA synthesis with PrimeScript Reverse Transcriptase (Takara Bio
665 Inc., Kusatsu, Japan). DNase treatment was carried out using the gDNA Eraser (Perfect Real Time)
666 following manufacturer's instructions (Takara Bio Inc.). For gene expression analysis, 2 µl of
667 undiluted cDNA (i.e. the equivalent of 100 ng total RNA) served as template in a 20 µl qRT-PCR
668 using the TB Green Premix Ex Taq II (Tli RNaseH Plus) kit (Takara Bio Inc.) and the Roche
669 LightCycler 96 system (Roche, Basel, Switzerland), according to manufacturer's instructions.
670 Transcript abundance of the following western corn rootworm genes were analysed: *DvvGr43a*
671 and *DvvGr2*. *Actin* was used as reference gene to normalize expression data across samples.
672 Relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (107).The following primers
673 were used: DvvGr43a-F GTCACATTCACCACGGGTCT, DvvGr43a-R
674 CGTTCGGTCTCTAACTTTGGC, DvvGr2-F GAACTAAGCGAGCTCCTCCA, DvvGr2-R

675 CAGAAGCACCATGCAATACG, DvvActin-F TCCAGGCTGTACTCTCCTTG, and DvvActin-
676 R CAAGTCCAAACGAAGGATTG.

677 Statistical analyses

678 Differences in root benzoxazinoid and root sugar levels as well as gene expression levels were
679 analyzed by One- and Two-Way Analyses of Variance (ANOVAs). Normality and equality of
680 variance were verified using Shapiro–Wilk and Levene's tests, respectively. Holm–Sidak post hoc
681 tests were used for multiple comparisons. Datasets from experiments that did not fulfill the
682 assumptions for ANOVA were natural log-, root square-, or rank-transformed before analysis.
683 Differences in damage scores between embryonic and post-embryonic roots were assessed using
684 Wilcoxon Signed Rank Tests. Differences in average root damage scores between treatments were
685 assessed using Kruskal-Wallis ANOVAs on ranks followed by Tukey's post hoc tests. The above
686 analyses were performed in Sigma Plot 12.0 (SystatSoftware Inc., San Jose, CA, USA) with the
687 support of the built-in Advisor Wizard function. Differences in larval preference were assessed
688 through Generalized Linear Models (GLM) with binomial distribution and corrected for
689 overdispersion with quasi-binomial function when necessary followed by analysis of variance
690 (ANOVA) and FDR-corrected Least Square Means post hoc tests. These analyses were followed
691 by residual analysis to verify the suitability of the error distribution and model fitting. These
692 Analyses were conducted using R 3.2.2 (43) using the packages "lme4", "car", "lsmeans" and
693 "RVAideMemoire" (108–112). Mass features were normalized using the function *arcsinh_x* in the
694 R package "bestNormalize" and compared between root types using functions *t.test* in base R and
695 *foldchange* in the package "gtools". P-values were FDR-corrected using the function *p.adjust*.

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1003

1004 **Table S1.** Identified metabolites in maize post-embryonic and embryonic roots. Metabolites with retention times
1005 highlighted by asterisks (*) eluted together with many weakly-retained compounds that could not be separated by
1006 reversed-phase liquid chromatography (RP-UHPLC). Their identification is therefore tentative and based on accurate
1007 masses only.

Nr	Compound class	Compound Name	Molecular formula	Retention time [min]	MS adducts	Progenesis QI fragmentation score	Characteristic fragments [m/z]
1	Benzoxazinoid	DHBOA-Glc	$C_{14}H_{17}NO_9$	1.30	M-H, M+FA-H, 2M-H	16.5	180.0303, 162.0200
2	Benzoxazinoid	DIBOA-Glc	$C_{14}H_{17}NO_9$	1.91	M-H, M+FA-H, 2M-H	2.6	180.0303, 162.0200, 134.0247
3	Benzoxazinoid	HMBOA-Glc	$C_{15}H_{19}NO_9$	2.11	M-H, M+FA-H, 2M-H	10.3	194.0459, 166.0511
4	Benzoxazinoid	DIMBOA-Glc	$C_{15}H_{19}NO_{10}$	2.18	M-H, M+FA-H, 2M-H	5.9	210.0406, 192.0302, 164.0356, 149.0119
5	Benzoxazinoid	DIM_2BOA-Glc	$C_{16}H_{21}NO_{11}$	2.22	M-H, M+FA-H, 2M-H	21.7	222.0408, 194.0458
6	Benzoxazinoid	HMBOA	$C_9H_9NO_4$	2.30	M-H	n/a	-
7	Benzoxazinoid	DIMBOA	$C_9H_9NO_5$	2.42	M-H	n/a	164.0353, 149.0118
8	Benzoxazinoid	HDMBOA-Glc	$C_{16}H_{21}NO_{10}$	2.74	M-H, M+FA-H, 2M-H	11.5	224.0564, 194.0460
9	Benzoxazinoid	HDM_2BOA-Glc	$C_{17}H_{23}NO_{11}$	2.74	M+FA-H	n/a	254.0789
10	Organic acid	Chlorogenic acid	$C_{16}H_{18}O_9$	1.77	M-H, 2M-H	24.3	191.0 562
11	Organic acid	Feruloylglucose	$C_{16}H_{20}O_9$	2.05	M-H	81.0	175.0 404
12	Organic acid	Sinapoylglucose	$C_{17}H_{22}O_{10}$	2.09	M-H	36.7	223.0615, 205.0507
13	Organic acid	Quinic acid	$C_{17}H_{12}O_6$	0.33*	M-H, 2M-H	70.1	-
14	Organic acid	Coumaroylquinic acid isomer	$C_{16}H_{18}O_8$	1.70	M-H	98.0	163.0409
15	Organic acid	Coumaroylquinic acid isomer	$C_{16}H_{18}O_8$	2.13	M-H	51.6	191.0561, 173.0455
16	Organic acid	Feruloylquinic acid isomer	$C_{17}H_{20}O_9$	2.35	M-H	46.1	191.0562
17	Organic acid	Feruloylquinic acid isomer	$C_{17}H_{20}O_9$	2.61	M-H	n/a	-
18	Flavonol	Quercetin rhamnosylglucoside	$C_{27}H_{30}O_{16}$	2.66	M-H	65.7	301.0353
19	Flavonol	Quercetin glucoside	$C_{21}H_{20}O_{12}$	2.75	M-H	26.0	301.0347, 271.0252
20	Flavonol	Quercetin malonylglucoside	$C_{24}H_{22}O_{15}$	2.91	M-H	75.1	505.0986, 301.0345

21	Flavonol	Kaempferol rhamnosylglucoside	$C_{27}H_{30}O_{15}$	2.94	M-H	98.9	285.0412
22	Flavonol	Kaempferol glucoside	$C_{21}H_{20}O_{11}$	3.04	M-H	88.6	285.0395, 255.0304
23	Flavonol	Kaempferol malonylglucoside	$C_{24}H_{22}O_{14}$	3.25	M-H	96.5	489.1037, 285.0400
24	Amino acid	Leucine	$C_6H_{13}NO_2$	0.80	M-H	n/a	-
25	Amino acid	Phenylalanine	$C_9H_{11}NO_2$	1.10	M-H	n/a	-
26	Amino acid	Tryptophan	$C_{11}H_{12}N_2O_2$	1.50	M-H, M+FA-H	n/a	-
27	Sugar	Monosaccharide	$C_6H_{12}O_6$	0.31*	M-H	n/a	-
28	Sugar	Disaccharide	$C_{12}H_{22}O_{11}$	0.34*	M-H, M+FA-H, 2M-H	3.2	-

1008

1009 **Table S2.** Root metabolites with behavioral activity towards western corn rootworm larvae.

Nr	Compound class	Compound Name	Behavioral effect	Reference.
1	Benzoxazinoid	MBOA	Host location (long-distance)	(113)
2	Benzoxazinoid	Fe-DIMBOA	Host location & acceptance (short-distance)	(66)
3	Benzoxazinoid	Fe-DIMBOA-Glc	Host location & acceptance (short-distance)	(66)
4	Sugar	Glucose	Feeding stimulation (within sugar blend)	(61)
5	Sugar	Fructose	Feeding stimulation (within sugar blend)	(61)
6	Sugar	Sucrose	Feeding stimulation (within sugar blend)	(61)
7	Fatty acid	Linoleic acid	Arrestment, feeding stimulation (within sugar blend)	(61)
8	Fatty acid	Oleic acid	Feeding stimulation (within sugar blend)	(61)
9	Fatty acid	Monoga-lactosyldiacylglycerol	Host recognition (tight turning behavior)	(62)
10	Aminobenzole	Methyl anthranilate	Host avoidance (repellent)	(63)
11	Terpene	(*E*)-β-caryophyllene	Host location and discrimination (long distance)	(43, 68)
12	Alkene	Ethylene	Host location and discrimination (long-distance	(64)
13	Inorganic compound	CO_2	Host location (long-distance)	(60)

1010

1011

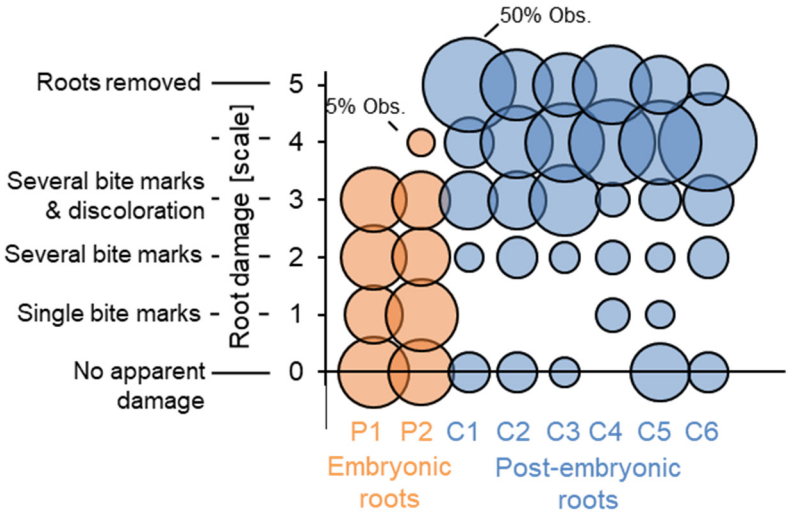


Fig. S1. *Frequency of different types of damage on individual embryonic and post embryonic roots*. Data is from experiment shown in Fig. 1*B* (n=20 plants). P1-P2 refer to individual embryonic roots, C1-C6 to individual post-embryonic roots per plant. Numbers were assigned randomly to embryonic and post embryonic roots within plants. The sizes of the circles are proportional to the relative frequency (% within each root) of the different types of observed damage.

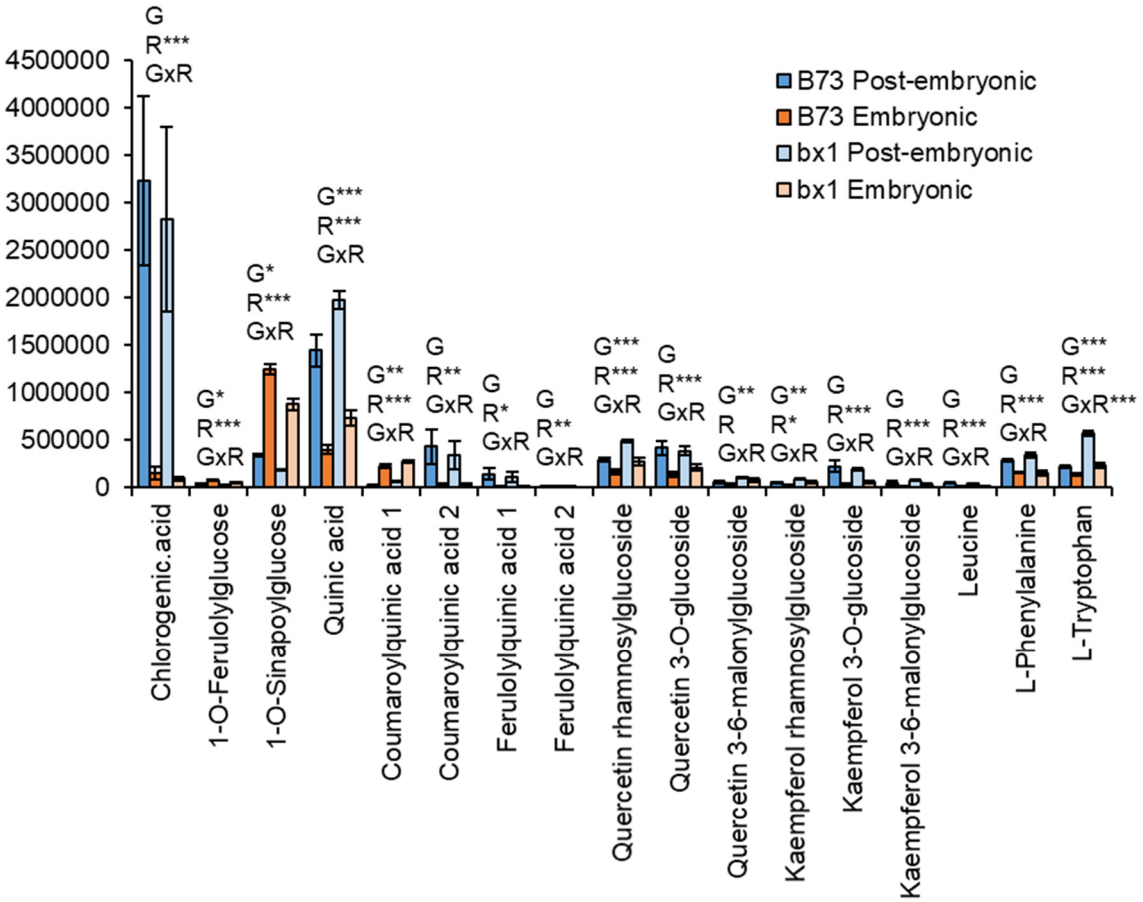


Fig. S2. *Metabolic differences between embryonic and post-embryonic roots are conserved in the bx1 mutant.* Relative abundances (signal intensities) of identified metabolic features in embryonic and post-embryonic roots of wild type B73 and *bx1* mutant plants (n=7-10). For sugars and benzoxazinoids, refer to Fig. 2. Results of Two-way ANOVAs for genotype effects (G), root type effects (R) and their interaction (GxR) are shown for each compound (***p<0.001, **p<0.01, *p<0.05). Error bars denote standard errors of means (SEM).

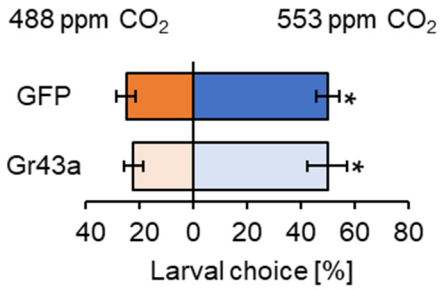


Fig. S3. *DvvGr43a does not influence the attraction of the western corn rootworm to CO_2.* Proportion of control (GFP) or *DvvGr43a*-silenced (Gr43a) larvae found on each arm of belowground olfactometers. Asterisks indicate significant differences between treatments (*$p<0.05$, FDR-corrected Least Square Mean post hoc tests, six two-arm olfactometers with 6 larvae each were evaluated, n=6).

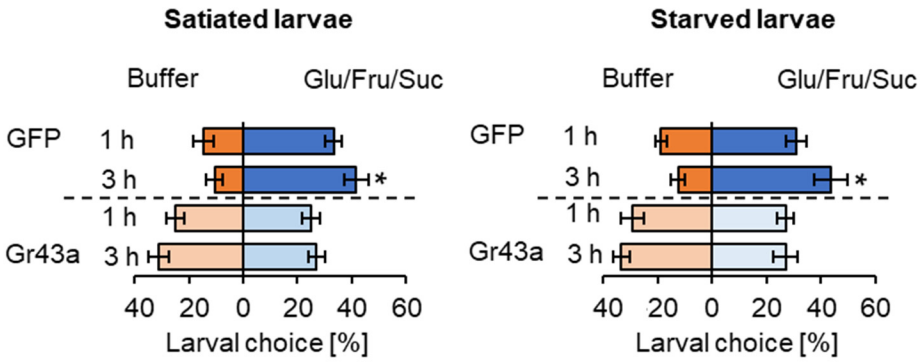
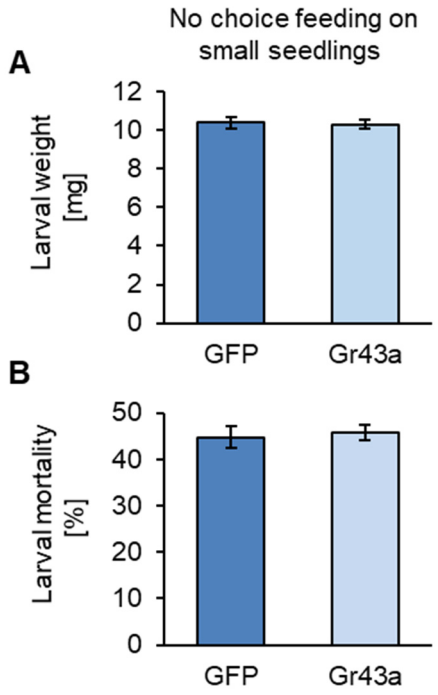


Fig. S4. *DvvGr43a mediates sugar preference independently of the feeding state of the western corn rootworm*. Preference of satiated (left) and starved (right) control or *DvvGr43a*-silenced larvae for buffer or a glucose, fructose, sucrose mixture on filter discs at different time points (*p<0.05, FDR-corrected Least Square Mean post hoc tests, eight petri plates with six larvae each were assayed, n=8). Error bars denote standard errors of means (SEM).

1034



1035

1036 **Fig. S5.** *DvvGr43a does not directly impair larval performance*. (*A*) Weight of western corn rootworm larvae fed on
1037 GFP and DvvGr43a dsRNA on young maize seedlings that produce embryonic roots only (no-choice setting) for 7
1038 days (n=40 cups with 9 larvae each). (*B*) Larval mortality within the same experiment.