Complex plant metabolomes guide fitness-relevant foraging decisions of 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 sugar perception in the western corn rootworm and benzoxazinoid production in maize, 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 even when both classes of chemical cues are almost completely absent. 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 lack 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

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

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

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

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

120 **Results**

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

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

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

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

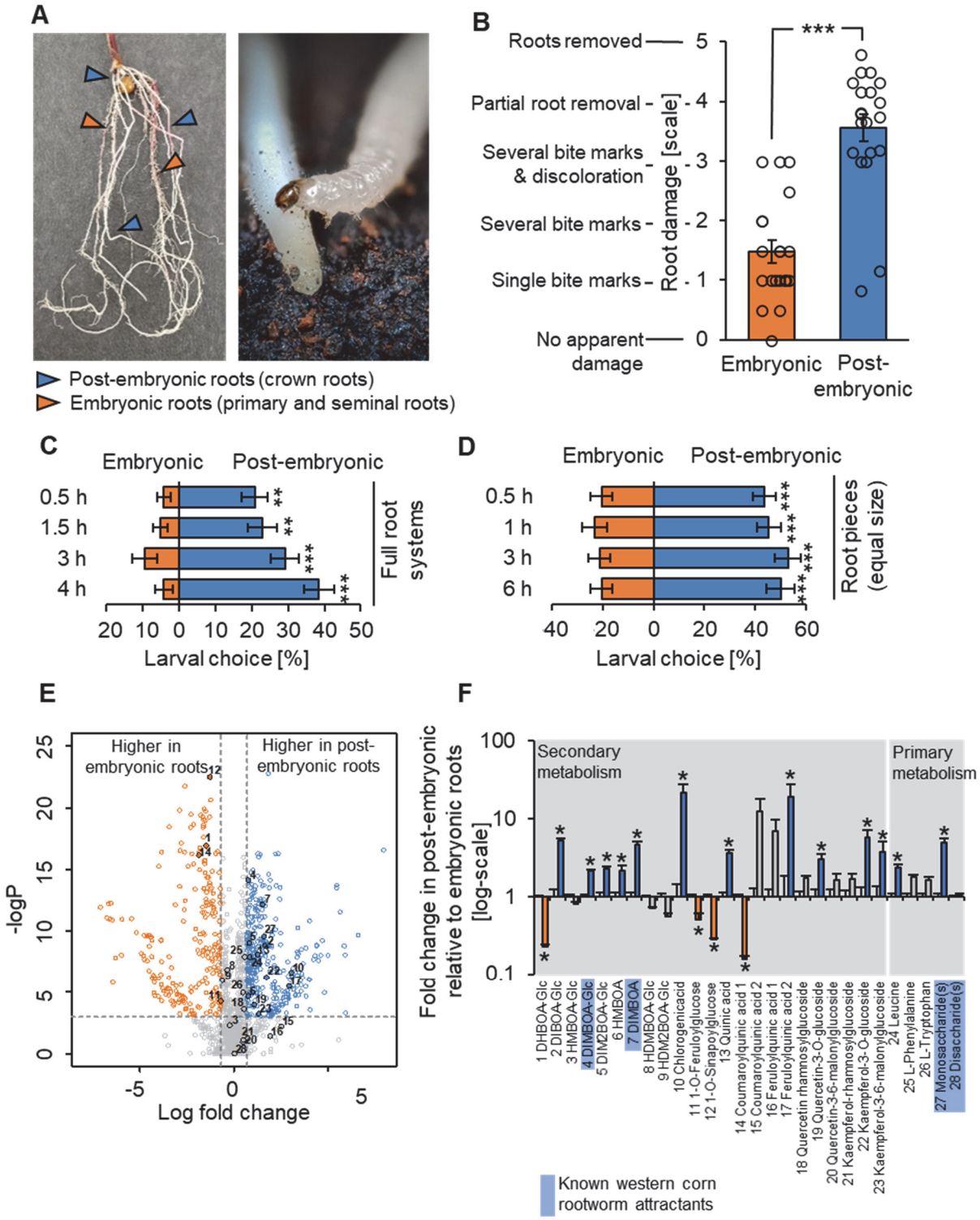


Fig. 1. *The western corn rootworm prefers to feed on root tissues that are rich in benzoxazinoids and soluble sugars.* (*A*) Left: young maize plants produce embryonic primary and seminal roots (orange arrows) and post-embryonic crown roots (blue arrows). Right: Western corn rootworm larvae are highly 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

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

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

192 *Independent manipulation of benzoxazinoids and sugars as foraging cues*

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

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

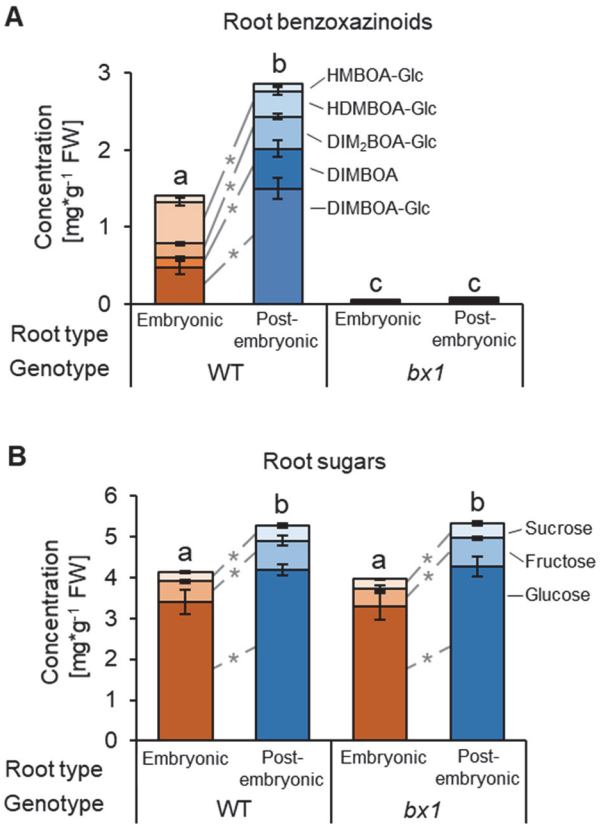
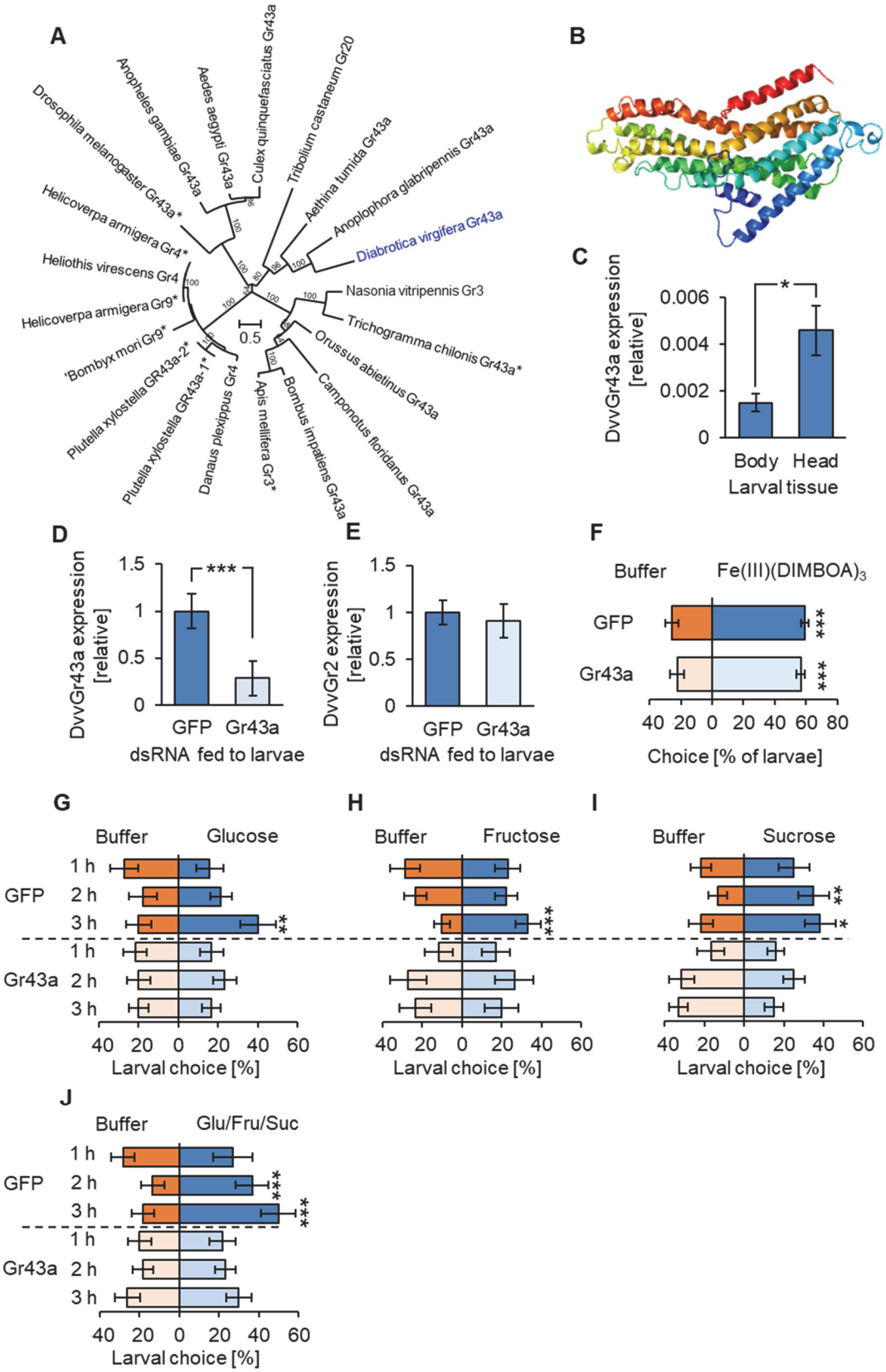


Fig. 2. *A mutation in the Bx1-gene suppresses root-type specific benzoxazinoid accumulation independently of root sugars*. (*A*) Concentrations of benzoxazinoids in embryonic and post-embryonic roots of wild type B73 and *bx1* mutant plants (n=11-18). (B) Concentrations of glucose, fructose and sucrose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

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



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

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

270 *Benzoxazinoids and sugars play distinct roles in root herbivore foraging*

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

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

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

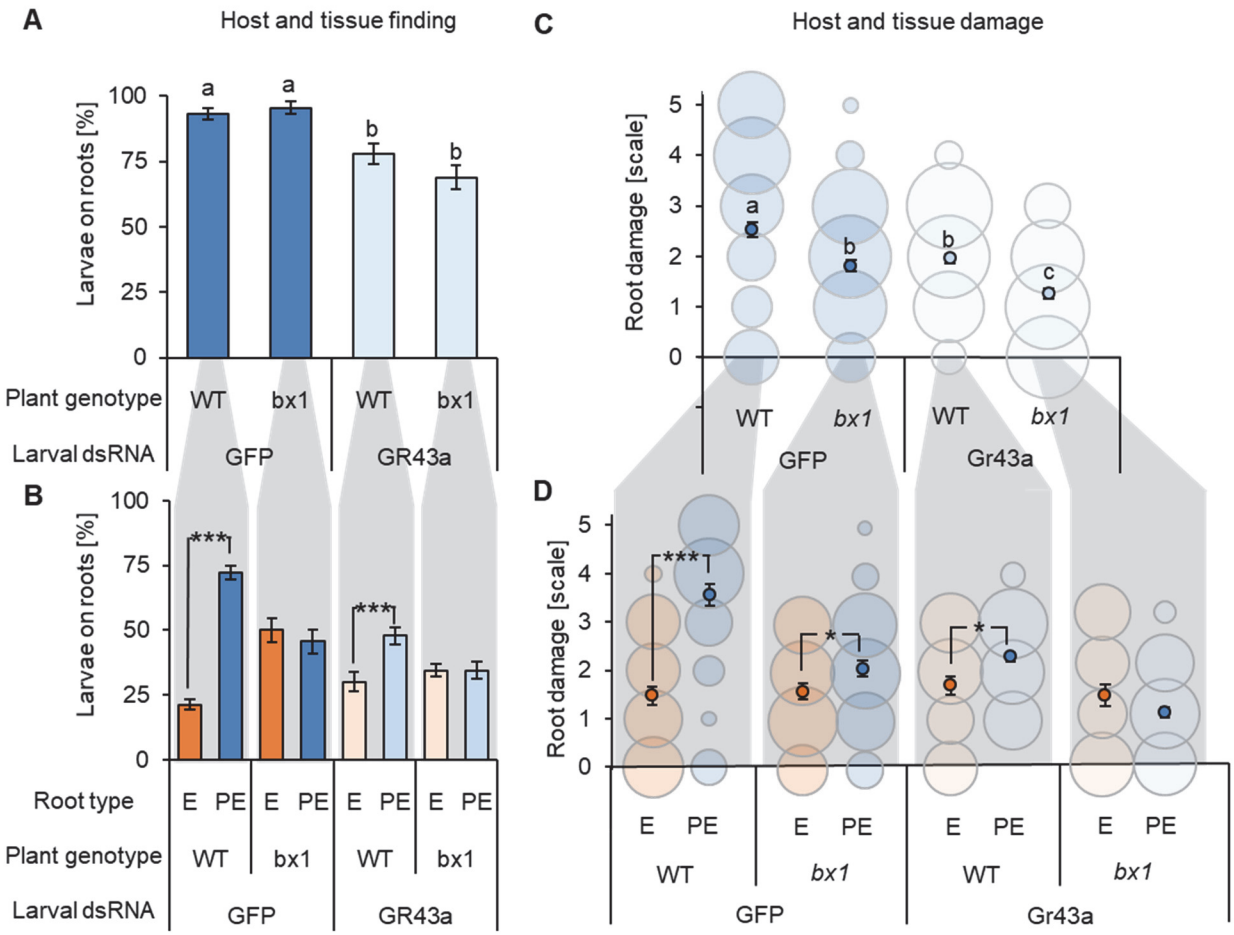

Fig. 4. *Benzoxazinoids and sugars play distinct roles in root herbivore foraging in vivo*. (*A*) Proportion of control (GFP) or *DvvGr43a*-silenced (Gr43a) larvae found on the roots of wild type B73 or *bx1* mutant roots 4 hours after their release. Letters indicate statistically significant differences 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

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

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

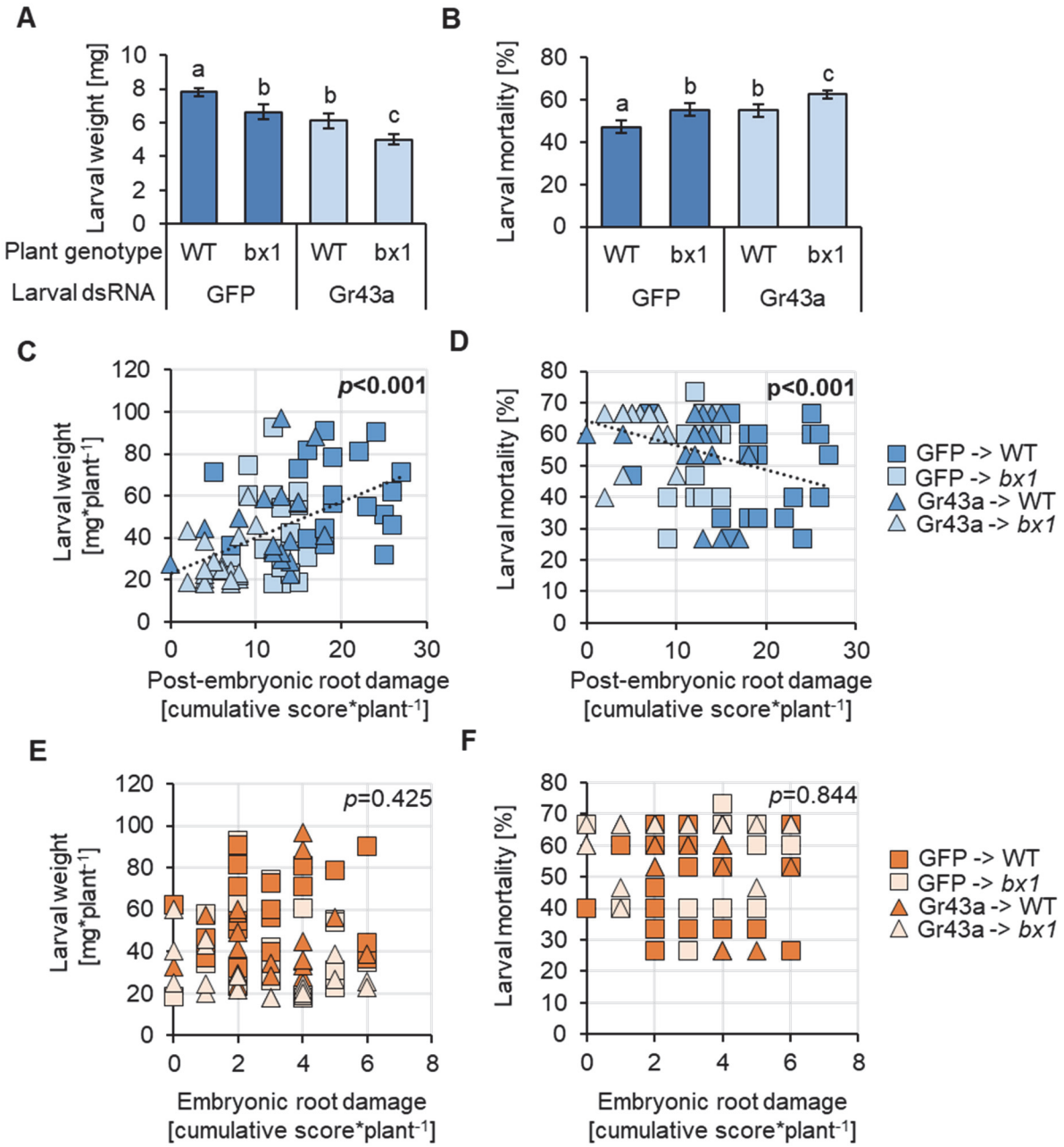

Fig. 5. *Using benzoxazinoids and sugars as foraging cues improves herbivore growth and survival.* (*A*) Larval weight of control (GFP) and DvvGr43a-silenced dsRNA fed western corn rootworm larvae feeding on wild type B73 or *bx1* mutant plants for 7 days. Note that in a no-choice situation, neither 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.

367 **Discussion**

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

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

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

403 Plants produce complex chemical mixtures, and herbivores likely combine many of these
404 chemicals to make appropriate foraging decisions (11, 12, 29). In line with this idea, recent *in vivo*
405 work shows that, while individual compound classes and insect gustatory receptors can be of major
406 importance for specific behavioral patterns, their disruption is not sufficient to fully suppress these
407 behaviors (22–24). In the case of the western corn rootworm, we find that even the suppression of
408 two major classes of foraging cues does not fully abolish its ability to find and accept a host plant,
409 at least under laboratory conditions. Several other plant-derived chemicals, including different
410 volatiles and fatty acids, are known to attract, arrest and stimulate feeding by the western corn
411 rootworm (61, 62, 64). These cues are likely to enable basic behavioral patterns in the absence of
412 sugars and benzoxazinoids. Whether the residual levels of benzoxazinoids that are still present in
413 *bx1* mutant roots contribute to foraging success remains to be determined. *bx1.igl* double mutants
414 that no longer produce benzoxazinoids are only available in a segregating genetic backgrounds,
415 thus making direct comparisons difficult (81). However, under no choice-conditions, western corn
416 rootworm larvae have been observed to attempt to feed on rice, a plant species that does not produce
417 benzoxazinoids (66), thus demonstrating that some degree of host acceptance also occurs in the
418 absence of these chemicals. We conclude that the flexible integration of complex blends of
419 chemical cues provides partial resistance to chemically mediated behavioral disruption. Recent
420 work highlights the importance of learning in herbivore foraging (12, 91), which likely increases
421 the capacity of herbivores to respond to dynamic chemical landscapes and to substitute between
422 different cues with redundant information content. How previous experiences influence the
423 foraging behavior of the western corn rootworm remains to be determined. All larvae used in our
424 experiments were reared on maize, reflecting their high degree of specialization on this host plant
425 (59). Western corn rootworm adults are more flexible with their diet (59, 92), and it would be
426 interesting to understand how their experience influences the responses of the next larval
427 generation to host plant cues.

428 The capacity of an herbivore to select a host plant and feed on specific tissues generally improves
429 its fitness (40, 42, 93). In line with these preference-performance relationships, diet experiments

430 often document strong associations between chemically-mediated herbivore feeding preferences
431 and herbivore performance (17). In many of these cases, herbivore feeding is directly elicited by
432 substances that improve performance (17). However, plant metabolomes are likely to complex to
433 be fully assessed by an herbivore, who thus need to rely on digestive feedbacks or subsets of
434 chemical cues to make decisions. To what extent the use of plant chemical cues enhances herbivore
435 performance by guiding herbivores to nutritious tissues is poorly understood, and interactions
436 between primary and secondary metabolite chemical cues have, to the best of our knowledge, not
437 been tested *in planta*. Our experimental approach rules out direct effects of benzoxazinoids and
438 sugar perception on western corn rootworm performance (66, 69) and thus allows us to quantify
439 their net impact as foraging cues. We find that the availability of sugars and benzoxazinoids during
440 foraging improves herbivore weight gain and survival individually by 8%, and in combination by
441 15%, benefits which are most likely driven by increased post-embryonic root feeding. Post-
442 embryonic roots of young maize plants are growing more vigorously than embryonic roots, receive
443 a higher share of photosynthates and are richer in carbohydrates, amino acids and total soluble
444 protein and increase western corn rootworm growth in no-choice experiments (69). In combination,
445 these findings demonstrate that the combined use of primary and secondary metabolites as foraging
446 cues enhance the performance of a specialist insect herbivore by guiding it to its most profitable
447 feeding niche.

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

462 in hemolymph sugar levels. A deeper molecular characterization could shed light on the precise
463 neurological and physiological roles of *DvvGr43a* in the western corn rootworm in the future.

464 In conclusion, the experiments presented here reveal how primary and secondary metabolites
465 interact to guide the foraging of a specialist herbivore. The distinct individual and combined roles
466 of these metabolites reveal finely tuned herbivore foraging strategies that are directly linked to
467 improved herbivore performance and survival. At the same time, foraging is remarkably robust to
468 perturbation, most likely due to the flexible use of redundant host plant cues. Our mechanistic
469 approach thus offers insights into the complex, multilayered interplay between plant metabolomes
470 and chemically guided herbivore foraging.

471 **Materials and Methods**

472 Plants and insects

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

486 Herbivore behavior

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

490 For western corn rootworm feeding preference experiments using whole plants, three-week-old
491 maize plants grown in 200 mL cylindrical plastic pots filled with sand and topped off with potting

492 soil were infested with fifteen second instar western corn rootworm larvae. Twenty plants per plant
493 genotype/type of larvae combination were evaluated (n=20). Seven days after infestation, plants
494 were gently excavated and inspected for root damage. Damage was recorded for each root
495 individually using the following scale. 0: no visual damage, 1: one-three bite marks, 2: more than
496 three bite marks, 3: several bite marks and brown discoloration, 4: root partially removed, and 5:
497 root fully removed.

498 Western corn rootworm feeding preference assays using petri dishes were conducted as described
499 (95, 96). Briefly, two-week-old maize plants grown as described above were gently excavated. The
500 root systems were washed and laid out onto a moist filter paper embedded in a petri dish (14 cm
501 diameter, Greiner Bio-one, Austria). A circular whole in the rim of the dishes accommodated the
502 plant stems, with the shoots remaining outside of the dishes. Five third instar western corn
503 rootworm larvae were released into each petri dish. Petri dishes were sealed and then covered with
504 aluminum foil. The number of western corn rootworm larvae feeding on each root type was
505 determined at different time points after their release.

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

513 To evaluate the attractiveness of CO_2 for western corn rootworm larvae, we conducted choice
514 experiments using belowground olfactometers (64). CO_2 levels were increased in one arm of the
515 olfactometer by delivering CO_2-enriched synthetic air (1% CO_2, Carbagas, Switzerland).
516 Olfactometer arms were closed on top using parafilm during CO_2 delivery. A manometer connected
517 to the synthetic air bottle allowed to fine-tune CO_2 delivery rates. CO_2 levels were measured using
518 a gas analyzer (Li7000, Li-Cor Inc., Lincoln, Nebraska, USA). Once desired CO_2 concentrations
519 were reached, CO_2 delivery was terminated, olfactometer arms were connected to olfactometer
520 central connectors and six second to third instar western corn rootworm larvae were release

521 immediately and their choice were evaluated within ten minutes of release. Six olfactometers per
522 larval type were assayed (n=6).

523 To evaluate the attractive effects of Fe(III)(DIMBOA)$_3$ for western corn rootworm larvae, we
524 release six second to third instar larvae in the middle of a moist sand-filled petri dish (9 cm
525 diameter, Greiner Bio-One GmbH, Frickenhausen, DE) where they encountered a filter paper disc
526 treated with 10 µl of Fe(III)(DIMBOA)$_3$ (1 µg/ml of water) or, on the opposite side, a filter paper
527 disc treated with water only. Ten independent petri dishes per larval type were evaluated (n=10).
528 Fe(III)(DIMBOA)$_3$ was prepared fresh by mixing $FeCl_3$ and DIMBOA at a 1:2 ratio as described
529 (96). Larval preferences were recorded 1 h after releasing the larvae.

530 To evaluate attractive effects of soluble sugars for western corn rootworm larvae, we followed the
531 procedure described by 97 with minor modifications. Briefly, we released six second to third instar
532 western corn rootworm larvae in the middle of a moist sand-filled petri dish (6 cm diameter, Greiner
533 Bio-One GmbH, Frickenhausen, DE) where they encountered a filter paper disc treated with 10 µl
534 of either glucose (30 mg/ml), fructose (30 mg/ml), sucrose (30 mg/ml), or a mixture of the three
535 (10 µl containing each sugar at a concentration of 30 mg/ml), or, on the opposite side, a filter paper
536 treated with water only. Fifteen independent petri dishes per treatment were assayed (n=15). Larval
537 preferences were recorded regularly for 3 h. In a second experiment, larvae were pre-fed on maize
538 roots or starved for 12 h before being released into petri dishes with filter paper discs treated with
539 a sugar mixture or water, as described above. Eight independent petri dishes with six larvae each
540 were assayed (n=8).

541 <u>Herbivore performance</u>

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

548 To evaluate the impact of *DvvGr43a*-silencing on western corn rootworm performance in a no-
549 choice setting, GFP and *DvvGr43a* fed first instar larvae were reared on 3-4-day old maize
550 seedlings, which only produce embryonic roots, in soil-filled plastic cups (n=40). Nine larvae were

551 used per cup. Fresh seedlings were provided to the larvae every three days for a total of six days,
552 after which the larvae were collected, counted, and weighted using a microbalance.

553 Root metabolite measurements

554 Untargeted root metabolomics was conducted as described (81). Embryonic and post-embryonic
555 roots of B73 plants were harvested separately, flash-frozen and ground in liquid nitrogen. Sixty to
556 eighty mg of plant tissues were then extracted in 800 µL of acidified H_2O/MeOH (50:50 v/v; 0.1%
557 formic acid). Resulting root extracts were analysed using an Acquity UHPLC system coupled to a
558 G2-XS QTOF mass spectrometer equipped with an electrospray source (Waters). Gradient elution
559 was performed on an Acquity BEH C18 column (2.1 × 50 mm i.d., 1.7 µm particle size) at 1-27.5%
560 B over 3.5 min, 27.5-100% B over 1 min, holding at 100% B for 1 min, and reconditioning at 1%
561 B for 1 min, where A =0.1% formic acid/water and B = 0.1% formic acid/acetonitrile. The flow
562 rate was 0.4 mL/min. The temperature of the column was maintained at 40°C, and the injection
563 volume was 1 µL. The QTOF MS was operated in negative mode. The data were acquired over an
564 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
565 energy ramp from 10 to 40 V. The capillary and cone voltages were set to 2 kV and 20 V,
566 respectively. The source temperature was maintained at 140°C, the desolvation gas was 400°C at
567 1000 L h-1 and cone gas flow was 50 L/hr. Accurate mass measurements (<2 ppm) were obtained
568 by infusing a solution of leucin encephalin at 200 ng/mL at a flow rate of 10 µL/min through the
569 Lock Spray probe (Waters). The chromatograms were processed using Progenesis QI (Nonlinear
570 Dynamics, Newcastle UK) with default settings for spectral alignment, peak picking, and
571 deconvolution, resulting in a total of 4956 mass features. Of these, 444 mass features eluting
572 between 0 and 0.3 min were discarded, since their lack of retention and excessive coelution made
573 reliable identification impossible.

574 Abundances for each remaining mass feature were normalised by ArcSin transformation, and mean
575 normalized abundances for post-embryonic and embryonic roots were compared by Student's t-
576 test. P-values were adjusted by Benjamini–Hochberg false discovery rate multiple testing
577 correction and a fold-change cut-off of 2.0 was applied to generate a list of compounds that
578 significantly differed between treatments. Volcano plots were used to visualize treatment
579 differences, and the list of differentially expressed features was tested against an in-house mass-
580 fragmentation database using Progenesis QI (Progenesis MetaScope v1.0.6253.26544, Nonlinear
581 Dynamics, Newcastle, UK). In addition, chromatograms were manually scanned for exact masses

582 of known plant metabolites, specifically amino acids, sugars, organic acids, and flavonols. Potential
583 matches were likewise tested against the mass fragmentation database, and putative identifications
584 were accepted based on exact mass, mass fragmentation patterns, and relative retention times.

585 Root benzoxazinoid quantification was conducted as described (98). For this, embryonic and post-
586 embryonic roots of B73 or *bx1* plants were harvested separately, flash-frozen and ground in liquid
587 nitrogen. Approximately 100mg of plant tissues were then extracted in 1 mL of acidified
588 H_2O/MeOH (50:50 v/v; 0.1% formic acid), and analyzed with an Acquity UHPLC system coupled
589 to a UV detector and a Waters QDa mass spectrometer equipped with an electrospray
590 source(Waters). Compounds were separated on an Acquity BEH C18 column (2.1×50 mm i.d., 1.7
591 µm particle size). Water (0.1% formic acid) and acetonitrile (0.1% formic acid) were employed as
592 mobile phases A and B. The elution profile was: 0-1 min, 10% B; 1-4 min, 10-30% B; 4-5 min,
593 30- 9740% B; 5-6 min, 40-100% B; 6-8.5 min, 100% B; followed by 1.5 min reconditioning at
594 10% B. The mobile phase flow rate was 0.4 mL/min. The column temperature was maintained at
595 40°C, and the injection volume was 3 µL. The MS was operated in negative mode and data were
596 acquired in single ion recording (SIR) mode, using $[M-H]^-$ and $[M+HCOO^-]^-$ adducts of
597 benzoxazinoid compounds. All other MS parameters were left at their default values as suggested
598 by the manufacturer. Benzoxazinoid peaks were identified from MS signals and quantified from
599 integrated UV signals at 265nm. External standards for HMBOA, DIMBOA, DIMBOA-Glc,
600 HDMBOA-Glc, and MBOA were used for absolute quantification. Where concentrations were too
601 low to result in reliable UV signals, a compound-specific MS-to-UV signal conversion factor was
602 estimated to calculate 'predicted' UV signals from the more sensitive MS signals before
603 quantification.

604 Root sugar levels were measured by an enzymatic/spectrophotometric method as described (99).
605 Embryonic and post-embryonic roots of B73 and *bx1* plants were harvested separately, flash-frozen
606 and ground in liquid nitrogen. Soluble sugars were extracted from 100 mg root tissue using 80%
607 (v/v) ethanol, followed by an incubation step (10 min at 78°C) with constant shaking at 800 rpm.
608 Pellets were re-extracted twice with 50% (v/v) ethanol (10 min at 78°C with constant shaking at
609 800 rpm). Supernatants from all extraction steps were pooled together, and sucrose, glucose and
610 fructose were quantified as described (100).

611 Identification of *DvvGr43a*

612 To identify the *Gr43a* receptor orthologues in the western corn rootworm, we used the *Gr43a*
613 receptor-encoding gene sequence of *Drosophila melanogaster* as query against publicly available
614 nucleotide sequences and against the western corn rootworm genome sequences (NCBI accession:
615 PXJM00000000.2) using the National Center for Biotechnology Information Basic Local
616 Alignment Search Tool (NCBI BLAST). The full gene sequence can be retrieved from the National
617 Center for Biotechnology Information (NCBI) data bank using the following accession number:
618 XM_028279548.1. Then, we reconstructed evolutionary relationships between the identified
619 DvvGr43a gene and several other putative Gr43a genes that have been functionally characterized
620 as glucose, fructose, and/or sucrose receptors (49, 50, 53, 54, 75–78). The evolutionary
621 relationships were inferred using the Maximum Likelihood method based on the Le Gascuel 2008
622 model in MEGA7 (101, 102). The tree with the highest log likelihood (-14287.71) is shown. The
623 percentage of trees in which the associated taxa clustered together is shown next to the branches.
624 Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and
625 BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting
626 the topology with superior log likelihood value. A discrete Gamma distribution was used to model
627 evolutionary rate differences among sites (5 categories (+G, parameter = 1.0811)). The tree is
628 drawn to scale, with branch lengths measured in the number of substitutions per site. There was a
629 total of 569 positions in the final dataset. Graphical representation and edition of the phylogenetic
630 tree were performed with the Interactive Tree of Life (version 3.5.1) (103). Protein tertiary
631 structures and topologies were predicted using Phyre2 (104). The protein sequences used to
632 reconstruct evolutionary relationships can be retrieved from the NCBI under the following
633 accession numbers: *Heliothis virescens* Gr4 (CAD31946.1), *Danaus plexippus* Gr4 (EHJ77681.1),
634 *Bombyx mori* Gr9(NP_001124345.1), *Bombus impatiens* Gr43a (XP_003486787.1), *Nasonia*
635 *vitripennis* Gr3 (NP_001164386.1), *Apis mellifera* Gr3 (XP_016768876.1), *Drosophila*
636 *melanogaster* Gr43a (NP_523650.2), *Tribolium castaneum* Gr20 (EFA05758.1), *Anopheles*
637 *gambiae* Gr43a (XP_318100.4), *Camponotus floridanus* Gr43a (EFN61344.1), *Aedes aegypti*
638 Gr43a (XP_001658898.3), *Culex quinquefasciatus* Gr43a (XP_001842305.1), *Orussus abietinus*
639 Gr43a (XP_012270798.1), *Aethina tumida* Gr43a (XP_019876958.1), *Anoplophora glabripennis*
640 Gr43a (XP_018574194.1), *Helicoverpa armigera* Gr4 (AGK90011.1), *Helicoverpa armigera* Gr9
641 (JX970522.1), *Trichogramma chilonis* Gr43a (QAY30709.1), and *Diabrotica virgifera* Gr43a
642 (XP_028135349.1). For *Plutella xylostella* GR43a-1 and *Plutella xylostella* GR43a-2, and *D.*

643 *melanogaster* gustatory receptors refer to: (53, 105), as these sequences have not been deposited
644 in a public repository.

645 Double stranded RNA production

646 Double stranded RNA targeting *DvvGr43a* was produced by *Escherichia coli* HT115. To this end,
647 electrocompetent *E. coli* HT115 cells were transformed with recombinant L4440 plasmids that
648 contained either a 240 bp long *DvvGr43a* gene fragment, or a 240 bp long green fluorescent protein
649 (GFP) gene fragment flanked by two T7 promotors in opposite directions. Gene fragments were
650 synthetized *de novo* (Eurofins, Germany). To induce the production of dsRNA, an overnight
651 bacterial culture was used to inoculate fresh Luria-Berthani broth (25 g/L, Luria/Miller, Carl Roth
652 GmbH, Karlsruhe, Germany). Once the bacterial culture reached an OD_{600} of 0.6-0.8, it was
653 supplemented with isopropyl β-D-1-thiogalactopyranoside, IPTG) (Sigma-aldrich, Switzerland) at
654 a final concentration of 2mM. Bacterial cultures were incubated at 37°C in an orbital shaker at 130
655 rpm for 16h. Bacteria were harvested by centrifugation and stored at -20°C for further use (106).

656 Gene silencing experiments

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

665 Gene expression measurements

666 Total RNA was isolated from approximately 10 mg of frozen, ground and homogenized western
667 corn rootworm larval tissue (3-7 larvae per biological replicate, n=8-11) using the GenElute
668 Universal Total RNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA). A NanoDrop
669 spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA) was used to
670 estimate RNA purity and quantity. DNase-treated RNA was used as template for reverse
671 transcription and first strand cDNA synthesis with PrimeScript Reverse Transcriptase (Takara Bio
672 Inc., Kusatsu, Japan). DNase treatment was carried out using the gDNA Eraser (Perfect Real Time)

673 following manufacturer's instructions (Takara Bio Inc.). For gene expression analysis, 2 µl of
674 undiluted cDNA (i.e. the equivalent of 100 ng total RNA) served as template in a 20 µl qRT-PCR
675 using the TB Green Premix Ex Taq II (Tli RNaseH Plus) kit (Takara Bio Inc.) and the Roche
676 LightCycler 96 system (Roche, Basel, Switzerland), according to manufacturer's instructions.
677 Transcript abundance of the following western corn rootworm genes were analysed: *DvvGr43a*
678 and *DvvGr2*. *Actin* was used as reference gene to normalize expression data across samples.
679 Relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (107).The following primers
680 were used: DvvGr43a-F GTCACATTCACCACGGGTCT, DvvGr43a-R
681 CGTTCGGTCTCTAACTTTGGC, DvvGr2-F GAACTAAGCGAGCTCCTCCA, DvvGr2-R
682 CAGAAGCACCATGCAATACG, DvvActin-F TCCAGGCTGTACTCTCCTTG, and DvvActin-
683 R CAAGTCCAAACGAAGGATTG.

684 Statistical analyses

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

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1011

1012 **Table S1.** Identified metabolites in maize post-embryonic and embryonic roots. Metabolites with retention times
1013 highlighted by asterisks (*) eluted together with many weakly-retained compounds that could not be separated by
1014 reversed-phase liquid chromatography (RP-UHPLC). Their identification is therefore tentative and based on accurate
1015 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	-

1016

1017 **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)

1018

1019

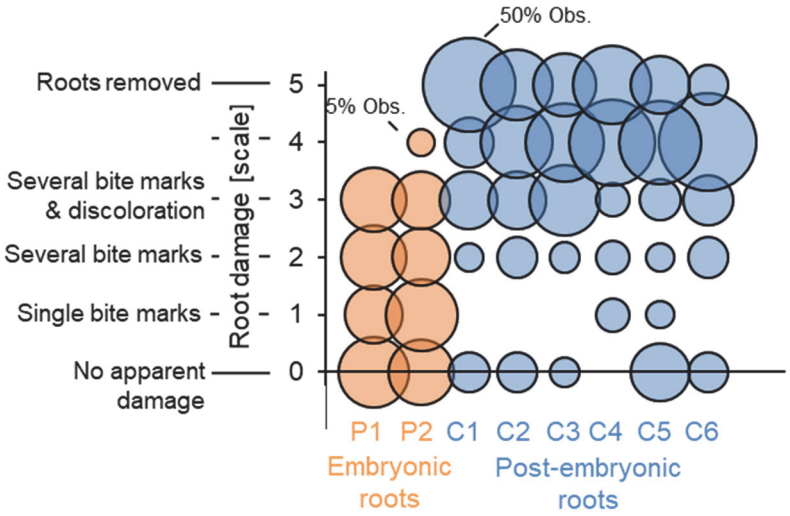


Fig. S1. *Frequency of different types of damage on individual embryonic and post embryonic roots*. Data is from experiment shown in Fig. 1*B* (n=20 plants). P1-P2 refer to individual embryonic roots, C1-C6 to individual post-embryonic roots per plant. Numbers were assigned randomly to embryonic and 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.

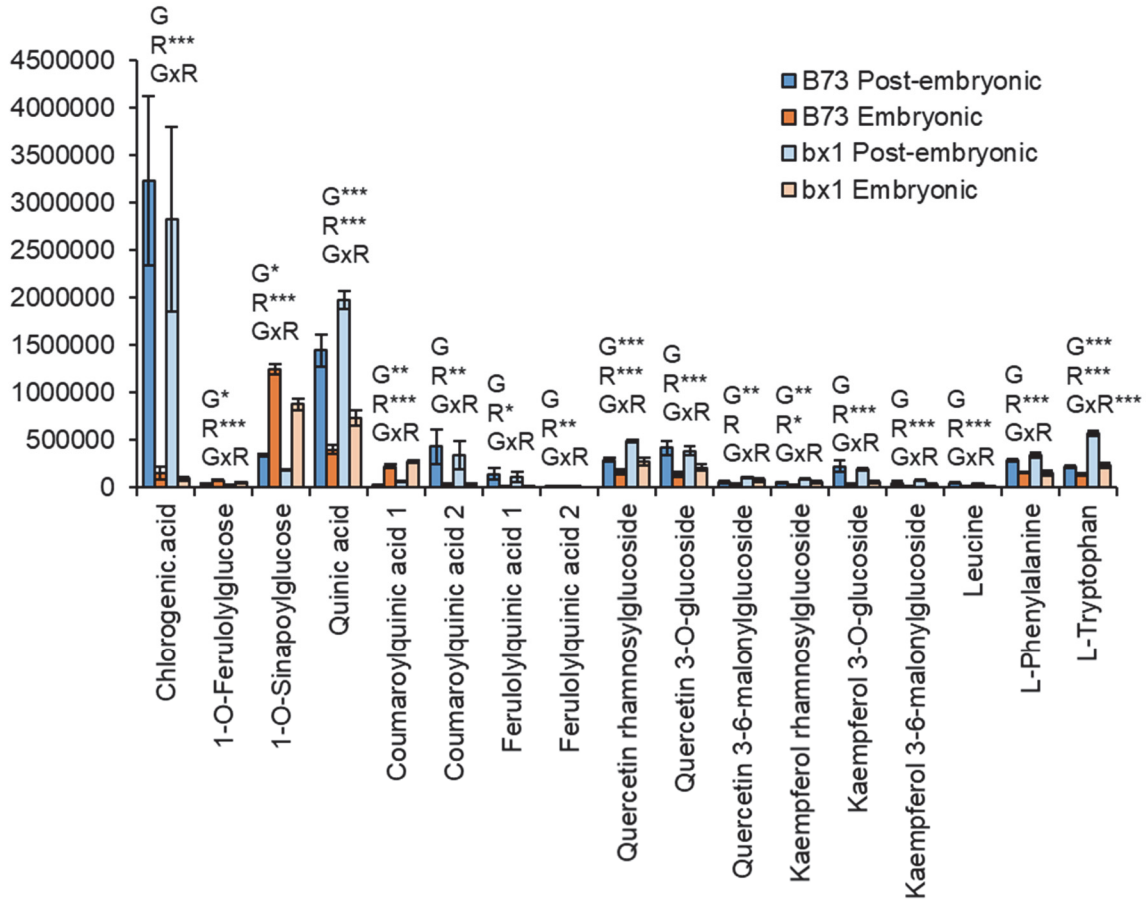


Fig. S2. *Metabolic differences between embryonic and post-embryonic roots are conserved in the bx1 mutant.* Relative abundances (signal intensities) of identified metabolic features in embryonic and post-embryonic roots of wild type B73 and *bx1* mutant plants (n=7-10). For sugars and benzoxazinoids, 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).

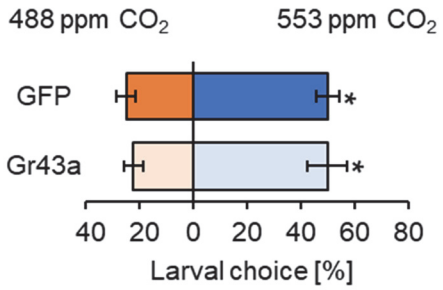
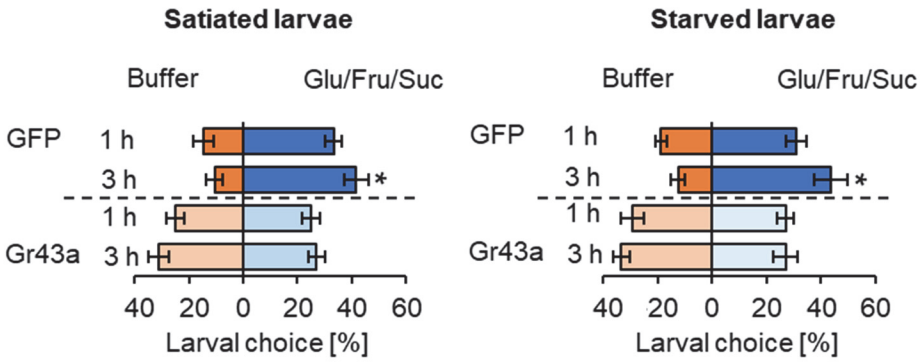


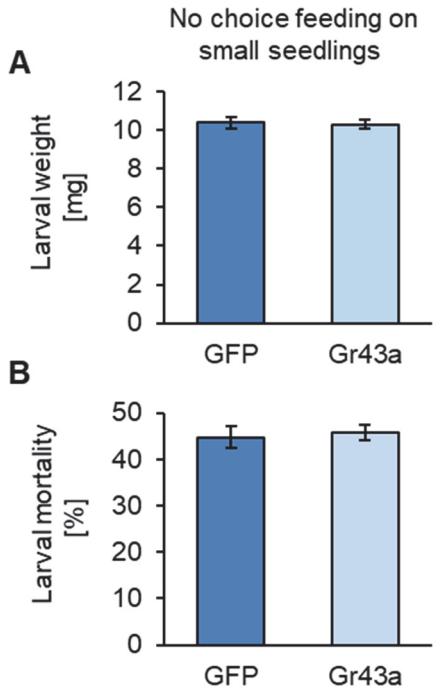
Fig. S3. *DvvGr43a does not influence the attraction of the western corn rootworm to CO_2.* Proportion of control (GFP) or *DvvGr43a*-silenced (Gr43a) larvae found on each arm of belowground olfactometers. Asterisks indicate significant differences between treatments (*$p<0.05$, FDR-corrected Least Square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



1037

1038 **Fig. S4.** *DvvGr43a mediates sugar preference independently of the feeding state of the western corn rootworm*.
1039 Preference of satiated (left) and starved (right) control or *DvvGr43a*-silenced larvae for buffer or a glucose, fructose,
1040 sucrose mixture on filter discs at different time points (*p<0.05, FDR-corrected Least Square Mean post hoc tests,
1041 eight petri plates with six larvae each were assayed, n=8). Error bars denote standard errors of means (SEM).

1042



1043

1044 **Fig. S5.** *DvvGr43a does not directly impair larval performance*. (*A*) Weight of western corn rootworm larvae fed on
1045 GFP and DvvGr43a dsRNA on young maize seedlings that produce embryonic roots only (no-choice setting) for 7
1046 days (n=40 cups with 9 larvae each). (*B*) Larval mortality within the same experiment.