Plant-derived CO_2 mediates long-distance host location and quality assessment by a root herbivore

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Summary

Insect herbivores can use volatile and visual cues to locate and select suitable host plants from a distance. The importance of CO_2, arguable the most conserved volatile marker of metabolic activity, is not well understood in this context, even though many herbivores are known to respond to minute differences in CO_2 concentrations. To address this gap of knowledge, we manipulated CO_2 perception of the larvae of the western corn rootworm (*Diabrotica virgifera virgifera*; WCR) through RNA interference and studied how CO_2 perception impacts their interaction with their host plant, maize (*Zea mays*). We show that the expression of a putative Group 2 carbon dioxide receptor, *DvvGr2*, is specifically required for dose-dependent larval responses to CO_2 in the ppm range. Silencing *DvvGr2* has no effect on the ability of WCR larvae to locate host plants at short distance (<9 cm), but impairs host location at greater distances. Using soil arenas and olfactometer experiments in combination with *DvvGr2* silencing and CO_2 scrubbing, we demonstrate that WCR larvae use CO_2 as a long-range host plant finding cue, but employ other volatiles for short-range host location. We furthermore show that the larvae use CO_2 as a fitness-relevant long-distance indicator of plant nutritional status: Maize plants that are well-fertilized emit more CO_2 from their roots and are better hosts for WCR than plants that are nutrient-deficient, and the capacity of WCR larvae to distinguish between these plants depends exclusively on their capacity to perceive CO_2 through *DvvGr2*. This study unravels how CO_2 can mediate plant-herbivore interactions by serving as a distance-dependent host location and quality assessment cue.

Keywords: Plant-herbivore interactions, foraging, volatile perception, behaviour, host location.

Introduction

Insect herbivores can use both visual and olfactory cues to locate suitable host plants from a distance. Volatile cues in particular can convey information about the identity and physiological status of a host plant and are integrated by herbivores to locate host plants for oviposition and feeding (***Visser and Avé, 1978***). Over the years, many attractive and repellent plant volatiles were identified (***Bruce et al., 2005; Späthe et al., 2013; Webster and Cardé, 2017***), and the importance of individual compounds and volatile blends was documented using synthetic chemicals (***Bruce and Pickett, 2011; Carrasco et al., 2015; Fraenkel, 1959; Natale et al., 2003; Visser and Avé, 1978***). More recently, molecular manipulative approaches were used to manipulate plant volatile production and herbivore perception *in vivo (**Fandino et al., 2019; Halitschke et al., 2008; Robert et al., 2013**)*, thus confirming the important role of plant volatiles in plant-herbivore interactions.

While the role of plant volatiles such as green-leaf volatiles, aromatic compounds and terpenes is well understood, much less is known about the role of plant-derived carbon dioxide (CO_2) in plant-herbivore interactions. CO_2 is a ubiquitous primary product of oxidative metabolism that is produced through the citric acid cycle (***Krebs and Johnson, 1937***), and is thus arguably the most conserved volatile marker of biological activity on the planet. Although plants are net sinks of CO_2 due to their ability to fix carbon in the dark reactions of photosynthesis, they also release CO_2 through respiration from metabolically active heterotrophic tissues such as flowers and roots, and autotrophic tissues such as leaves that operate below the light compensation point (e.g. at night). CO_2 may thus be integrated into herbivore foraging activities as a marker of plant metabolic activity. *Datura wrightii* flowers for instance emit the highest levels of CO_2 during times of high nectar availability; as hawkmoth pollinators are attracted to CO_2, they may thus use this cue to locate rewarding flowers (***Goyret et al., 2008; Guerenstein et al., 2004; Guerenstein and Hildebrand, 2008; Stange, 1996; Stange, 1999; Stange and Stowe, 1999; Thom et al., 2004***). Similarly, lesions in apples result in high CO_2 release and attract *Bactrocera tryoni* fruit flies. As CO_2 at corresponding concentrations is attractive to the flies, it has been suggested that they may use plant-derived CO_2 to locate suitable oviposition sites (**Stange, 1998**).

Root feeding insects are also highly attracted to CO_2 *in vitro* (***Bernklau and Bjostad, 1998a, 1998b; Eilers et al., 2012; Hibbard and Bjostad, 1988; Jones and Coaker, 1978; Klingler, 1966; Nicolas and Sillans, 1989; Rogers et al., 2013; Strnad et al., 1986; Strnad and Dunn, 1990***). Given that CO_2 is produced and released by plant roots and diffuses relatively well through the soil, a likely explanation for this phenomenon is that root herbivores use CO_2 as a host location cue (***Bernklau and Bjostad, 1998a, 1998b; Doane et al., 1975; Erb et al., 2013; Johnson and Gregory, 2006; Johnson and Nielsen, 2012***), However, the reliability of CO_2 as a host-location cue for root feeders has been questioned due to a number of reasons: i) CO_2 can be emitted by many other sources apart from host plant roots, including decaying organic matter, microorganisms, and non-host plants, ii) there is a strong diurnal fluctuation in plant CO_2 emissions that does not necessarily match with insect foraging habits and iii) other plant-released chemicals can be used by root herbivores for host location within a CO_2 background (***Agus et al.,***

2010; Eilers et al., 2012; Erb et al., 2013; Hansen, 1977; Hibbard and Bjostad, 1988; Hiltpold and Turlings, 2012; Johnson and Nielsen, 2012; Reinecke et al., 2008; Weissteiner et al., 2012). A model that may reconcile these different views is that CO_2 may be used as an initial cue at long-distances, while other, more host specific volatiles may be used at shorter distances (***Erb et al., 2013; Johnson et al., 2006; Johnson and Nielsen, 2012***). So far, this model has not been experimentally validated, and the precise role of plant-derived CO_2 as a host location cue by herbivores in general, and root herbivores in particular, remains unclear (***Eilers et al., 2016***). To the best of our knowledge, no studies so far have investigated the role of plant-derived CO_2 in plant-herbivore interactions *in vivo* using molecular manipulative approaches.

The larvae of *Diabrotica virgifera virgifera* (western corn rootworm, WCR) feed exclusively on maize roots and cause major yield losses in the US and Eastern Europe (***Ciosi et al., 2008; Gray et al., 2009; Meinke et al., 2009; Toepfer et al., 2015***). The larvae rely on a number of volatile and non-volatile chemicals to identify and locate host plants, distinguish between suitable and less-suitable maize plants and forage within the maize root system (***Hiltpold et al., 2013; Johnson and Gregory, 2006; Johnson and Nielsen, 2012; Robert et al., 2012c; Schumann et al., 2018***). Non-volatile primary metabolites such as sugars and fatty acids as well as secondary metabolites such as benzoxazinoids and phenolic acid conjugates modulate larval behaviour (***Bernklau et al., 2011; Bernklau et al., 2015; Bernklau et al., 2016a; Bernklau et al., 2018b; Erb et al., 2015b; Hu et al., 2018; Huang et al., 2017; Robert et al., 2012c***). Volatiles including (*E*)-β-caryophyllene, ethylene and CO_2 attract the larvae (***Bernklau and Bjostad, 1998a, 1998b; Robert et al., 2012b; Robert et al., 2012a***), while methyl anthranilate repels them (***Bernklau et al., 2016b***). Based on the finding that high CO_2 levels can outweigh the attractive effects of other maize volatiles, it was suggested that CO_2 may be the only relevant volatile attractant for WCR larvae (***Bernklau and Bjostad, 1998b***). However, under conditions where CO_2 levels are similar, WCR larvae reliably choose between host plants of different suitability using other volatile cues (***Erb et al., 2011; Erb et al., 2015a; Huang et al., 2017; Lu et al., 2016; Robert et al., 2012b; Robert et al., 2012a***). The demonstrated ability of WCR larvae to respond to different volatile cues and the recent identification of putative CO_2 receptors from transcriptomic data (***Rodrigues et al., 2016***) makes this species a suitable model system to investigate the role of CO_2 in plant-herbivore interactions. Ongoing efforts to use CO_2 as a bait to control WCR in the field (***Bernklau et al., 2004; Schumann et al., 2014b; Schumann et al., 2014a***) provide further motivation to assess the importance of this volatile for WCR foraging.

To understand the importance of CO_2 for WCR foraging in the soil, we manipulated the insect's capacity to perceive CO_2. We reduced the expression levels of three putative WCR CO_2 receptor-encoding genes through RNA interference (RNAi), resulting in the identification of *DvvGr2* as an essential gene for CO_2 perception. Using *DvvGr2*-silenced larvae in combination with CO_2-removal, we then assessed the importance of CO_2 perception for WCR behaviour and foraging in olfactometers and soil arenas. Our experiments reveal how root-derived CO_2

modulates the interaction between maize and its economically most damaging root pest and expand the current repertoire of adaptive explanations for the attraction of insect herbivores to CO_2.

Results

The western corn rootworm (WCR) genome encodes three putative CO_2 receptors

To identify genes encoding putative CO_2 receptors in WCR, we used known CO_2 receptor-encoding gene sequences as queries against the WCR genome (available from the National Center for Biotechnology Information (NCBI)). Three putative carbon dioxide receptor candidates, *DvvGr1, DvvGr2,* and *DvvGr3*, were identified, matching three candidate genes that were found in previous transcriptome analyses (Rodrigues *et al.*, 2016). Phylogenetic reconstruction based on *in silico* predicted protein sequences revealed orthologous relationships for the three WCR candidate receptors and the receptors of several other insects (Fig. 1A). Consistent with their taxonomy, we observed close homology between the protein sequences of the CO_2 receptors of WCR and the protein sequences of other coleopteran insects such as *Tribolium castaneum* (Fig. 1A). Expression levels of *DvvGr1* and *DvvGr2* were found to be significantly higher in the head than in the rest of the body (torax and abdomen) of 2nd instar WCR larvae (Figs. 1B, C). A similar trend was observed for *DvvGr3* (Fig. 1D). Protein tertiary structure and topology models indicated that all three genes encode for 7-transmembrane domain proteins, which is consistent with their roles as receptors (Fig. 1B-D).

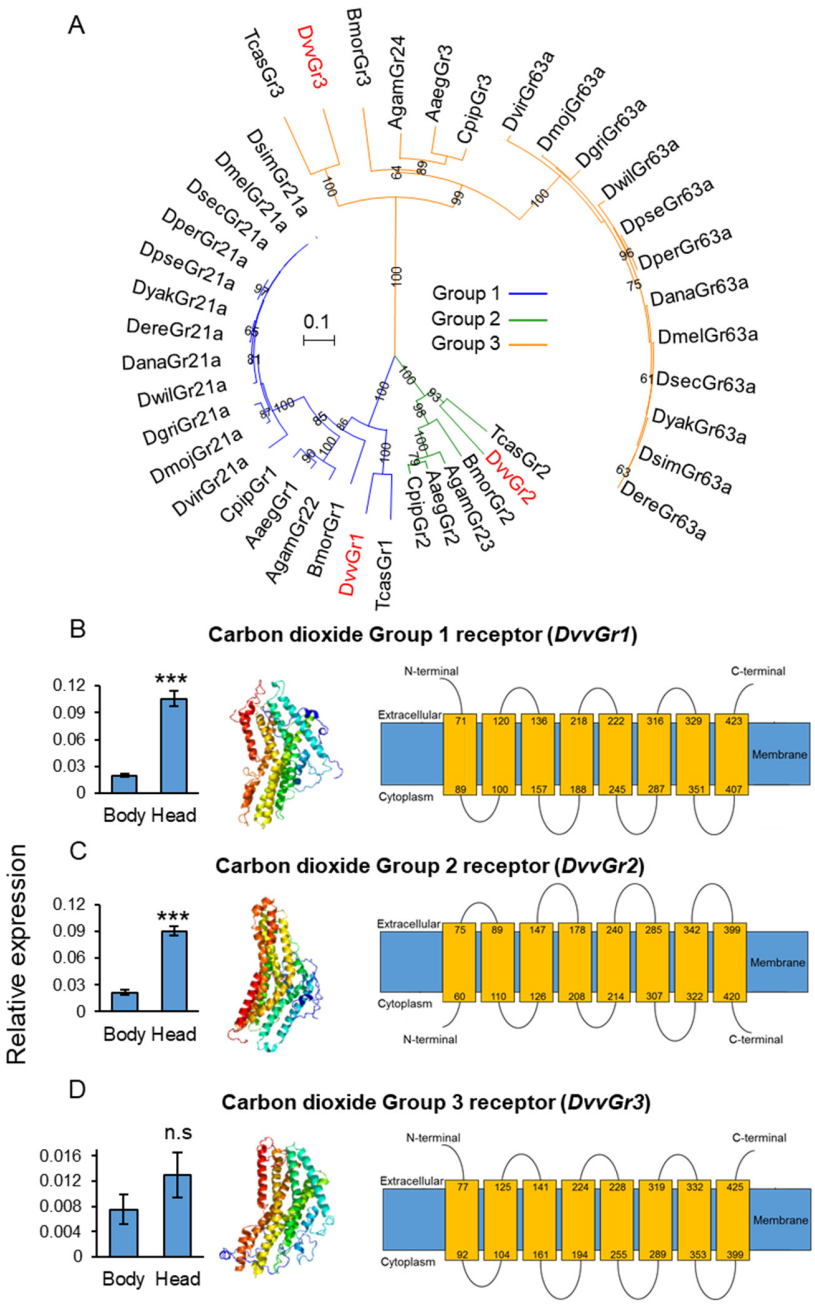

Figure 1 *The WCR genome contains three putative carbon dioxide (CO_2) receptors.* (A) Phylogenetic relationships between putative CO_2 receptors based on protein sequences of different insects. Dmel: *Drosophila melanogaster*, Dsim: *Drosophila simulans*, Dsec: *Drosophila sechellia*, Dyak: *Drosophila yakuba*, Dere: *Drosophila erecta*, Dana: *Drosophila ananassae*, Dper: *Drosophila persimilis*, Dpse: *Drosophila pseudoobscura*, Dwil: Drosophila willistoni, Dgri: *Drosophila grimshawi*, Dmoj: *Drosophila mojavensis*, Dvir: *Drosophila virilis*, Agam: *Aedes. gambiae*, Aaeg: *Aedes aegypti*, Cqui: *Culex quinquefasciatus*, Bmor: *Bombix mori*, Tcas *Tribolium castaneum,* Dvv*: Diabrotica virgifera virgifera* (Western corn rootworm, WCR). Mean (±SEM) relative gene expression levels of group 1 (*DvvGr1*) (B), group 2 (*DvvGr2*)(C), and group 3 (*DvvGr3*)(D) CO_2 receptors in the bodies (thorax and abdomen) or heads of 2nd instar WCR larvae (n=10). Asterisks indicate statistically significant differences between tissue types within genes (***: $p<0.001$ by one-way ANOVA with Holm's multiple comparisions test; n.s.: not significant). (B-E) Predicted protein tertiary structure (left) and transmembrane protein topology (right) of: (B) *DvvGr1*; (C) *DvvGr2*; and (D) *DvvGr3* according to the Phyre2 algorithm.

122 ***DvvGr2* expression is specifically required for behavioural responses of WCR larvae to CO_2**

123 To determine the importance of *DvvGr1, DvvGr2,* and *DvvGr3* for the responsiveness of WCR larvae to CO_2, we
124 knocked down the expression of each gene individually through double-stranded RNA (dsRNA)-mediated RNA
125 interference (RNAi) and conducted initial behavioural experiments with carbonated water as a CO_2 source (Fig.
126 2). Oral administration of dsRNA targeting either *DvvGr1*, *DvvGr2*, or *DvvGr3* reduced the expression levels of
127 these genes by 80%, 83% and 66% compared to WCR larvae fed with dsRNA of the green fluorescent protein
128 (GFP) gene (herein referred as wild type, WT) (Fig. 2A). All RNAi constructs were confirmed to be gene specific
129 (Fig 2B). Olfactometer arms containing carbonated water released approximately 100 ppm more CO_2 than their
130 counterparts filled with distilled water (Fig. 2B, Fig. S1A) and were more attractive to WT larvae than arms
131 containing distilled water (Fig 2C). Silencing *DvvGr1* or *DvvGr3* expression did not alter this behaviour. In
132 contrast, *DvvGr2*-silenced larvae did not show any preference any more (Fig 2C). To further explore the role of
133 *DvvGr2* in WCR behaviour, we conducted a series of additional experiments. First, we assessed the impact of
134 silencing *DvvGr2* on the capacity of WCR larvae to respond to other volatile and non-volatile host cues. *DvvGr2*-
135 silenced larvae responded similarly to the repellent volatile methyl anthranilate (***Bernklau et al., 2016b; Bernklau***
136 ***et al., 2018a***) as WT larvae (Fig. 2D). Responsiveness to non-volatile attractants, Fe(III)$(DIMBOA)_3$ and a blend
137 of glucose, fructose and sucrose (***Bernklau et al., 2018b; Hu et al., 2018***) was also unaltered in *DvvGr2*-silenced
138 larvae (Figs 2E, F), demonstrating that knocking down *DvvGr2* expression does not alter the capacity of WCR
139 larvae to respond to other important chemical cues. Second, we assessed the contribution of *DvvGr2* to CO_2
140 responsiveness using synthetic CO_2 at different concentrations (Fig. 3, Fig. S1B). WT larvae showed characteristic
141 dose-dependent behavioural responses to CO_2. While they did not respond to 22 ppm CO_2 above ambient CO_2
142 levels, they were attracted to CO_2 concentrations between 59-258 ppm above ambient, and repelled by CO_2-
143 enriched air at 950 ppm above ambient CO_2 levels and above (Fig. 3). In contrast, *DvvGr2*-silenced larvae did not
144 respond to CO_2 enrichment at any of the tested concentrations (Fig. 3). These experiments show that WCR larvae
145 are attracted to CO_2 enriched environments within the physiological range of the maize rhizosphere (Fig. S1) and
146 that *DvvGr2*-silencing fully and specifically suppresses CO_2 responsiveness in WCR larvae.

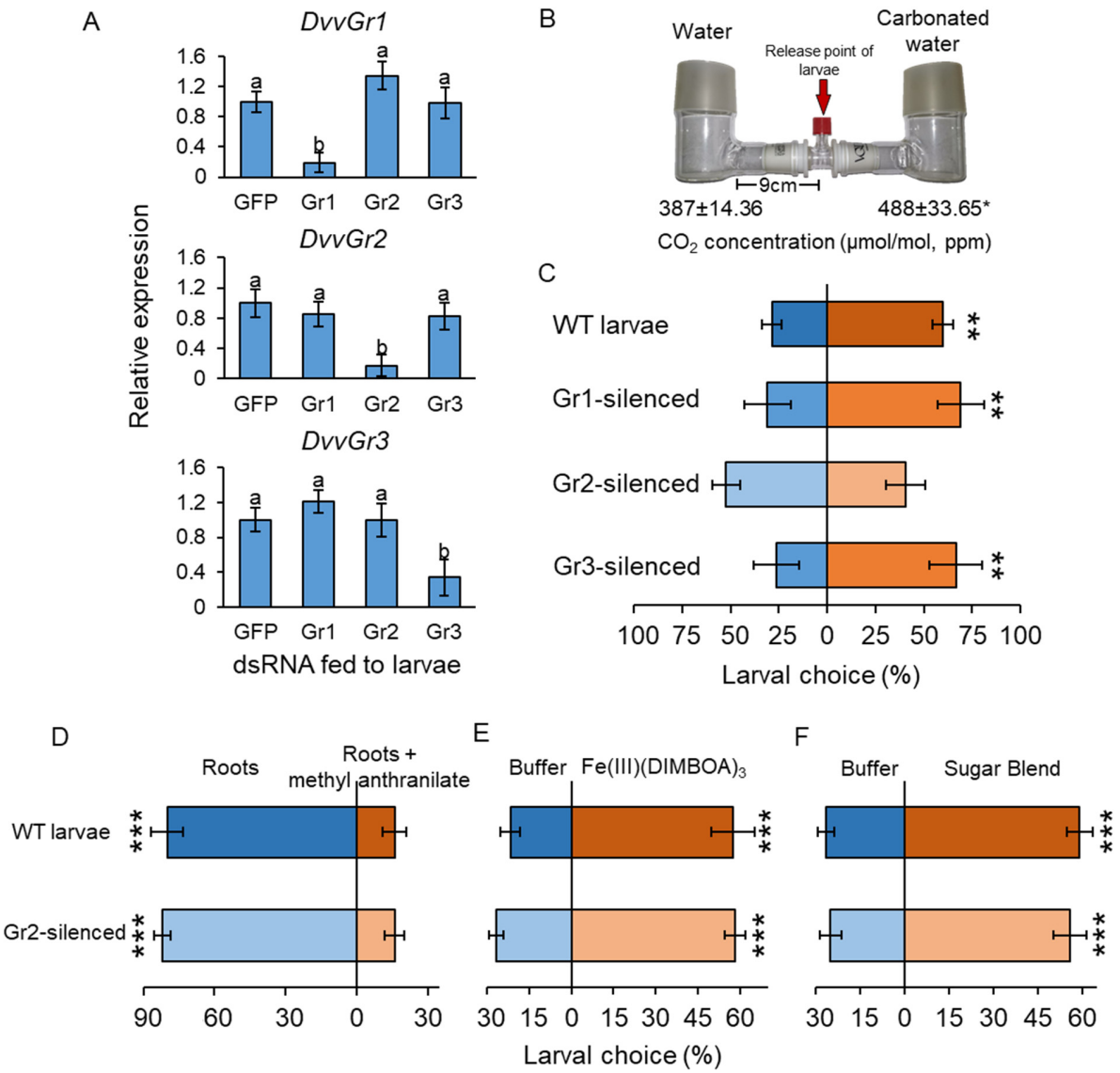

Figure 2 *The carbon dioxide group 2 receptor (DvvGr2) is specifically required for behavioural responses of WCR to* CO_2. (A) Mean (±SEM) relative gene expression levels of group 1 (*DvvGr1*), group 2 (*DvvGr2*), and group 3 (*DvvGr3*) CO_2. receptors after WCR larvae were fed with dsRNA-expressing bacteria targeting green fluorescent protein (GFP, herein referred as WT), *DvvGr1*, *DvvGr2*, or *DvvGr3* genes (n=11-13). Different letters indicate significant differences of gene expression levels ($p<0.05$ by one-way ANOVA with Holm's multiple comparisions test). (B) Mean (±SEM) CO_2 levels in each arm of the two-arm belowground olfactometers used for behavioural experiments (n=4-8). Asterisk indicates significant differences in CO_2 levels (*: $p<0.05$ by one-way ANOVA with Holm's multiple-comparisons test). Mean (±SEM) proportion of WCR larvae observed in the olfactometer arms with higher CO_2 levels (carbonated water side) or in control arms (distilled water side). Seven olfactometers with six larvae each were assayed (n=7). Mean (±SEM) proportion of WCR larvae observed on roots or on roots placed next to filter paper discs impregnated with methyl anthranilate (D), on filter paper discs impregnated with buffer or with $Fe(III)(DIMBOA)_3$ (E), and on filter paper discs impregnated with buffer or with a blend of glucose, fructose, and sucrose (F). For panel D, ten choice arenas with five larvae each were assayed (n=10). For panels E and F, twenty choice arenas with six larvae each were assayed (n=20). Asterisks indicate statistically significant differences in larval choices between treatments (***: $p<0.001$ by two-way ANOVA followed by FDR-corrected post hoc tests). For *P*-values of Generalized Linear Models (GLMs), refer to Table S1. Refer to Fig. S1A for quantities of CO_2 in each arm.

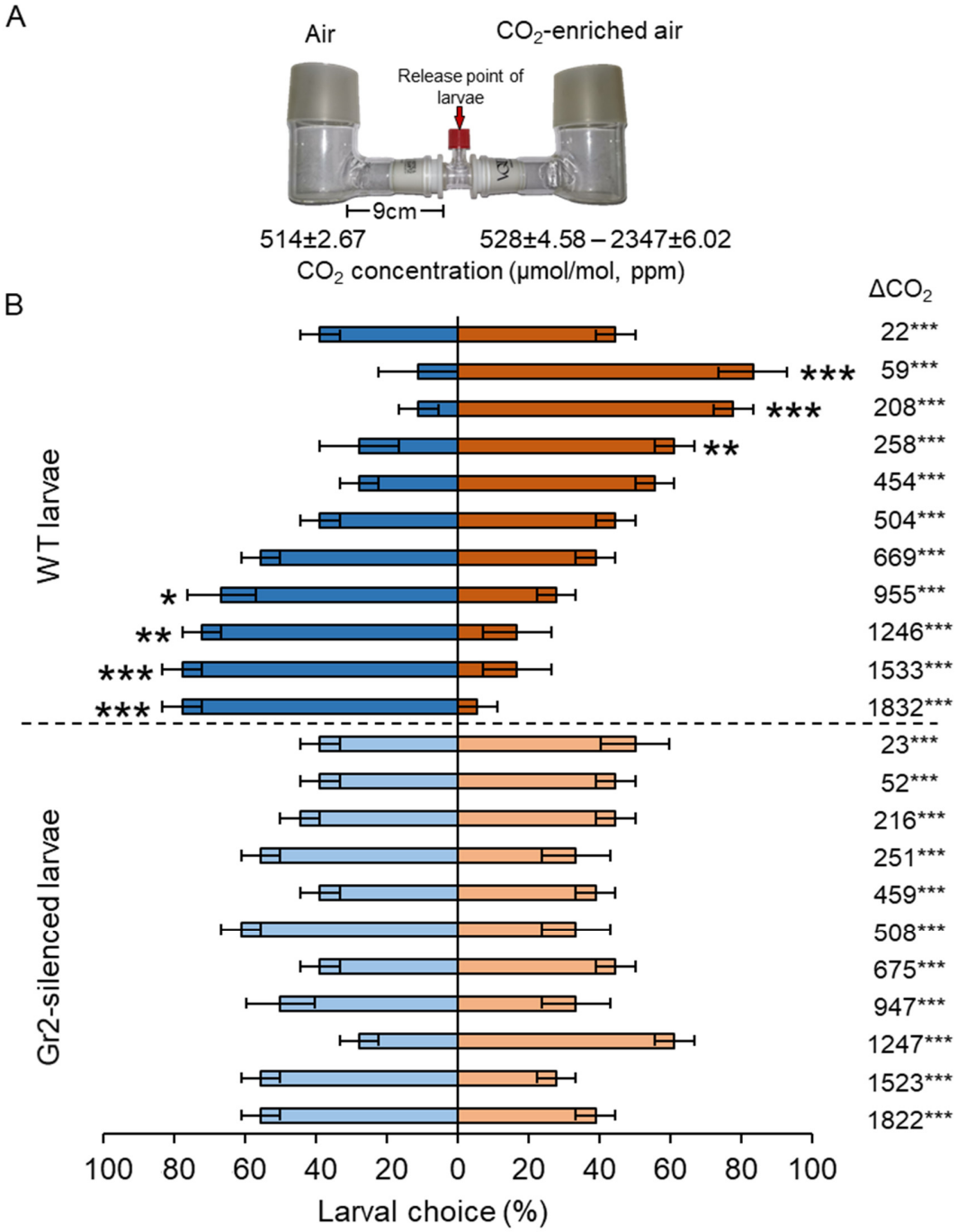

Figure 3 *DvvGr2 is required for dose-dependent behavioural responses of WCR to CO_2.* (A) Olfactometer setup used for behavioural experiments. (A) Mean (±SEM) CO_2 levels in each arm of the olfactometers (n=3). (B) Mean (±SEM) proportion of WCR larvae observed in each arm of the olfactometers. Three olfactometers with six larvae each were assayed (n=3). Asterisks indicate statistically significant differences between larval choices (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ by three-way ANOVA followed by FDR-corrected post hoc tests). For *P*-values of Generalized Linear Models (GLMs), refer to Table S1. ΔCO_2 values are given for each level. Asterisks indicate significant differences between CO_2 levels in the two olfactometer arms (***: p<0.001 by three-way ANOVA followed by FDR-corrected post hoc tests). Refer to Fig. S1B for quantities of CO_2 in each arm.

172 ***DvvGr2* expression does not impair larval motility or short-range host location**

173 To assess the impact of *DvvGr2* on larval motility, we followed the trajectories of individual larvae in Petri plates
174 that were outfitted with a CO_2 point releaser (Fig. 4A-C). WT larvae made frequent turns, but consistently oriented
175 themselves towards the CO_2 release point. Once they reached the CO_2 release point, they stopped moving (Fig.
176 4A). *DvvGr2*-silenced larvae exhibited similar turning behaviour as WT larvae, but did not move towards the CO_2
177 release point (Fig. 4B). WT larvae spend more time on CO_2 release point than *DvvGr2*-silenced larvae (Fig. 4C).
178 During the movement phase, the mean speed of WT larvae and *DvvGr2*-silenced larvae was similar (Fig. 4C), but
179 the distance covered by *DvvGr2*-silenced larvae was higher, as they did not stop at the CO_2 release point. In a
180 second experiment, we followed the trajectories of individual larvae in Petri plates with maize roots (Fig. 4D, E).
181 Speed and distance covered were similar between WT and *DvvGr2*-silenced larvae (Fig. 4F). Surprisingly, both
182 WT and *DvvGr2*-silenced larvae oriented themselves towards the maize roots and reached the maize roots after a
183 similar amount of time (Fig. 4F). This result shows that *DvvGr2* expression is required for the location and
184 detection of CO_2, but does not influence WCR motility nor its ability to locate maize roots over short distances
185 (i.e.: <9 cm).

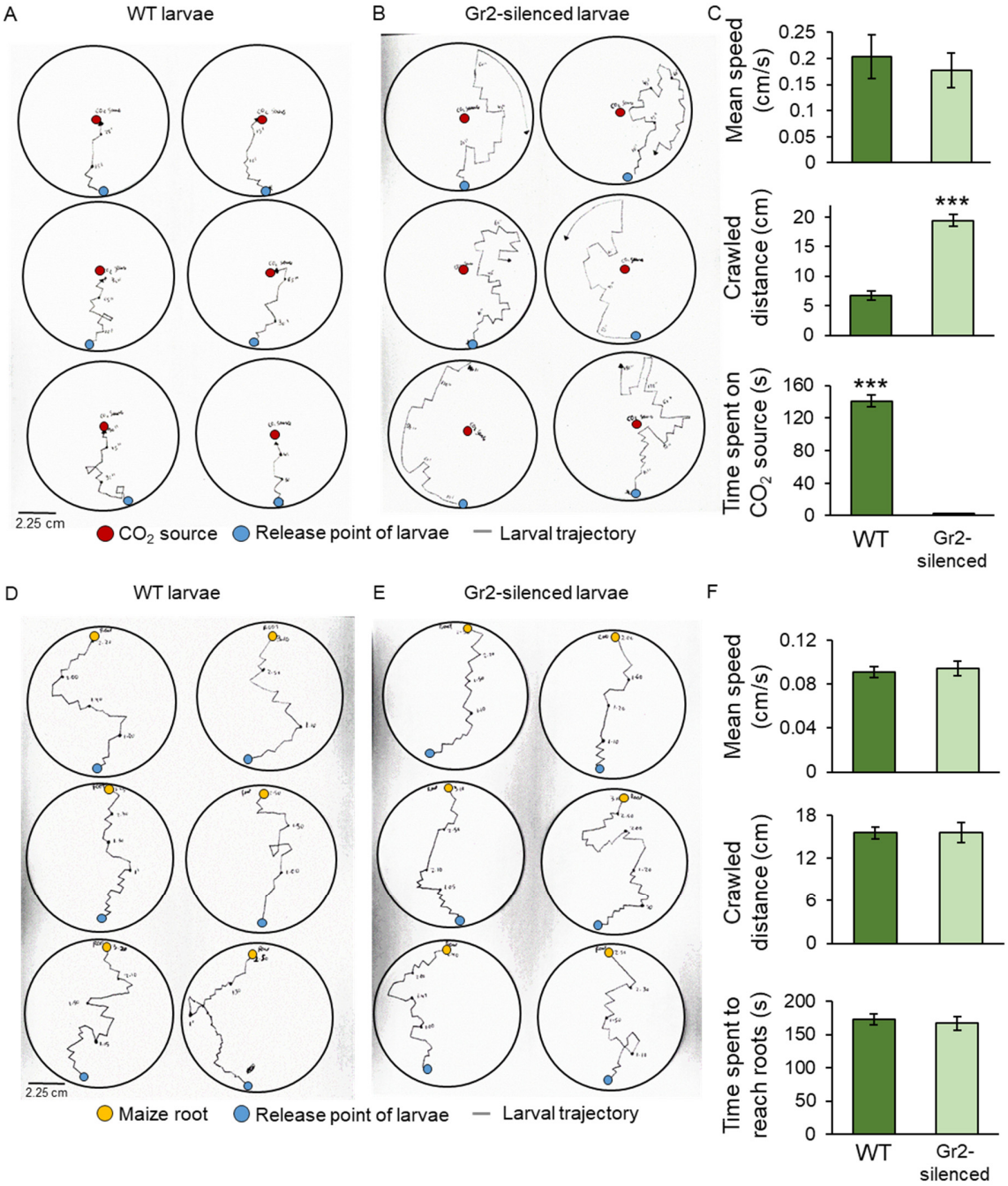

Figure 4 *Silencing the carbon dioxide group 2 receptor (DvvGr2) impairs WCR responses to* CO_2 *without changing motility or search behavior.* (A-B) Trajectories of individual WT (A) and *DvvGr2*-silenced (B) WCR larvae in Petri plates with a CO_2 source. The blue circles represent larval release points. The red circles represent CO_2 sources consisting of a fine needle that releases CO_2 at 60 ppm above ambient. (C) Mean (±SEM) speed and distance covered during the movement phase, and time spent at the CO_2 source during the first 3 min of the experiment. (D-E) Trajectories followed by WT (D) and by *DvvGr2*-silenced (E) WCR larvae on Petri plates containing maize seedling roots. The blue circles represent larval release points. The yellow circles represent maize seedling roots. (F) Mean (±SEM) speed and distance covered during the movement phase, and time necessary to reach the maize root during the first 3 min of the experiment. (F). For both experiments, six Petri plates with one larva each were assayed (n=6). Asterisks indicate statistically significant differences between mobility parameters of WT and *DvvGr2*-silenced larvae (***: $p<0.001$ by one-way ANOVA followed by Holm's multiple-comparisons test).

197 ***DvvGr2*-dependent CO_2 responsiveness mediates long-range host location**

198 To further explore the role of CO_2 and *DvvGr2* in volatile-mediated host location, we performed a series of
199 olfactometer experiments with maize plants grown in sand on one side and sand only on the other side. We varied
200 the distance between the volatile sources and the release points of the larvae between 9 and 18 cm (Fig. 5). We
201 also manipulated CO_2 emission of maize roots in a subset of olfactometers by adding a layer of CO_2-absorbing
202 soda lime into the olfactometer arms. CO_2 measurements revealed that the presence of a host root system increased
203 CO_2 concentrations by approx. 100 ppm above ambient CO_2 levels in the corresponding olfactometer arm (Fig. 5,
204 Fig. S1C). The soda lime reduced ambient CO_2 concentrations in the olfactometer arms by approx. 100 ppm, and
205 equalized CO_2 concentrations between arms with and without a host plant (Fig. 5), thus validating the CO_2
206 scrubbing approach. Larvae did not have direct access to the plant, the plant growth medium or the soda lime, and
207 received no visual cues, and thus had to rely on host plant volatiles for orientation. When released at distance of 9
208 cm from the volatile sources, both WT and *DvvGr2*-silenced larvae showed a clear preference for the olfactometer
209 arms leading to host plants (Fig. 5A). This preference was still intact in olfactometers outfitted with a soda lime
210 filters, confirming that volatiles other than CO_2 are sufficient for volatile-mediated host location at a short distance.
211 At a distance of 18 cm from the volatile sources, WT larvae showed a similarly strong preference for arms leading
212 to host plants (Fig. 5B). By contrast, *DvvGr2*-silenced larvae did not exhibit any preference (Fig. 5B). In the
213 presence of a soda lime filter, neither WT nor *DvvGr2*-silenced larvae were attracted to arms with a host plant
214 (Fig. 5B). Taken together, these experiments demonstrate that WCR larvae require plant-derived CO_2 to locate
215 host plants over longer distances in a *DvvGr2*-dependent manner.

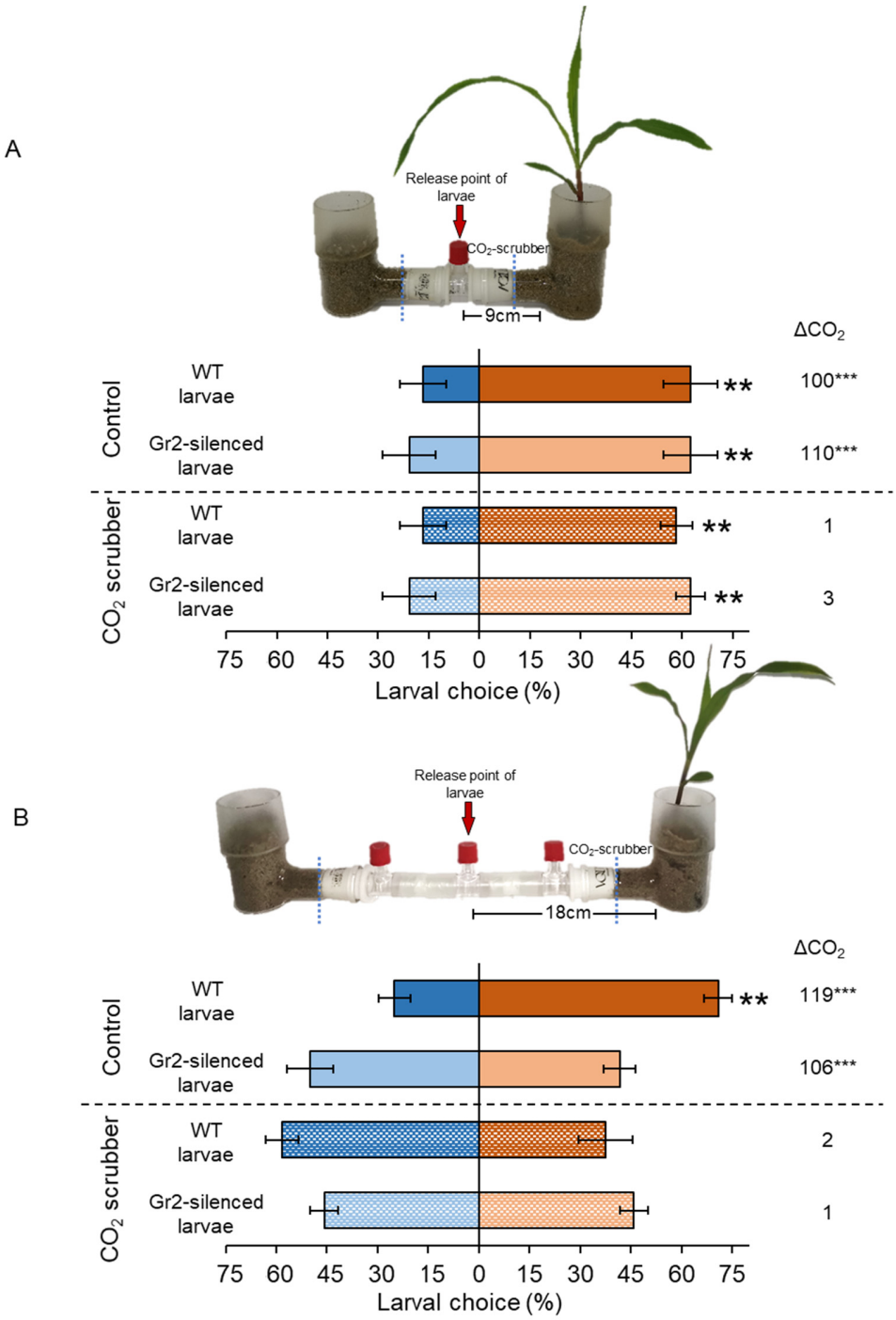

Figure 5 *Plant-derived CO_2 mediates DvvGr2-dependent long-distance host location.* (A, B) Choice (mean±SEM) of WCR larvae in two-arm olfactometers. Control olfactometers allowed for plant-derived CO_2 to diffuse into the choice chamber, while CO_2 scrubber olfactometers were outfitted with soda lime to suppress CO_2 diffusion. ΔCO_2 represents the difference in CO_2 in the arms with plants compared to arms without plants (***p<0.001). For detailed data on CO_2 levels, refer to Fig. S1C. Olfactometer arms were either 9 cm (A) or 18 cm (B) in length. Four olfactometers with six larvae each were assayed using WT or *DvvGr2*-silenced larvae (n=4). Asterisks indicate statistically significant differences between treatments (**: $p<0.01$ by four-way ANOVA followed by FDR-corrected post hoc tests). For *P*-values of Generalized Linear Models (GLMs), refer to Table S1.

225 **Root-derived CO_2 enhances volatile-mediated host location over longer distances**

226 WCR larvae can move up to 1 m in the soil. Second and third instar larvae in particular are known to move between
227 maize plants across rows in maize fields (***Hibbard et al., 2003***). To test whether *DvvGr2*-mediated CO_2
228 responsiveness mediates host location over longer distances in a soil context, we planted maize plants in soil-filled
229 plastic trays, released WCR larvae at distances of 16, 32, 48 or 64 cm from the maize plants and evaluated larval
230 positions after 8 h (Fig. 6). This time point was chosen based on preliminary observations showing that larvae take
231 approx. 8 h to cross the soil arenas. The CO_2 emitted by maize roots formed a gradient in the soil, starting at about
232 506 ppm in the rhizosphere (Zone 1), and 430 ppm at distances of 16-32 cm from the plant (Zone 2) (Fig. 6B, Fig.
233 S1F). At distances of more than 32 cm from the plant, the CO_2 levels were around 400 ppm and statistically
234 indistinguishable from soil without plants or ambient air (Fig. S1F). To confirm that larval motility is not altered
235 by *DvvGr2*-silencing in a soil context, we first released WT and *DvvGr2*-silenced larvae into the middle of a set
236 of arenas without a host plant and evaluated larval positions after 8 h. We found that the larvae dispersed equally
237 across the arenas, without any difference between WT and *DvvGr2*-silenced larvae (Fig. 6A). Eight hours after
238 releasing the larvae into arenas that included host plants on one side, 53% of WT larvae that were released at 64
239 cm from the plant were retrieved from the rhizosphere, i.e.: in Zone 1 (Fig. 6C). In contrast, only 33% of the
240 *DvvGr2*-silenced larvae that were released at the same distance were recovered from the maize rhizosphere (Fig.
241 6C). Significantly more *DvvGr2*-silenced larvae were recovered further away from the plants, in zones 3 and 4
242 (Fig 6C). The number of WT and *DvvGr2*-silenced WCR larvae found close to the host plant increased with
243 decreasing release distance, as did the difference between WT and *DvvGr2*-silenced larvae (Fig. 6C-F). At a
244 release distance of 16 cm, only slightly more WT than *DvvGr2*-silenced larvae were found close to the plant roots
245 (Fig 6F). To further confirm the role of *DvvGr2* in mediating host plant location over long distances in the soil,
246 we performed a time-course experiment where we released WT and *DvvGr2*-silenced larvae in Zone 5 (64 cm
247 away from the host plant) and then recorded how rapidly they reached Zone 1 containing host plants (Fig. S2).
248 The capacity of the larvae to directly feed on the host roots was impeded using a volatile-permeable root barrier
249 (Fig. S2A). Within 10 h, 36% of the released WT larvae were found in Zone 1, and within 32 h, this number had
250 increased to 90% (Fig. S2B). By contrast, only 24% of the released *DvvGr2*-silenced larvae were found in Zone 1
251 after 10 h, and after 32 h, this value had only increased to 56% (Fig. S2B). Thus, the capacity to detect CO_2
252 gradients contributes to successful host location by WCR larvae in a distance specific manner in the soil.

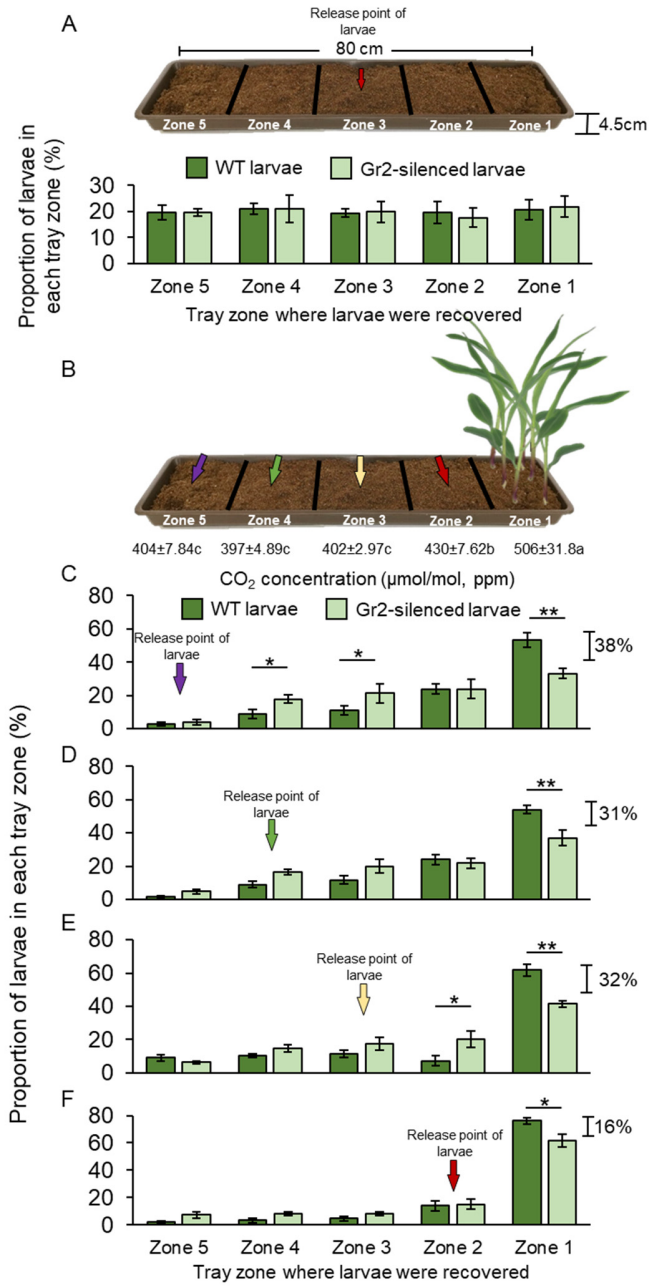

Figure 6 *Root-derived CO_2 enhances WCR host location over long distances in the soil.* (A) Mean (±SEM) proportion of WT (dark green) or *DvvGr2*-silenced (light green) WCR larvae observed in the different tray zones eight hours after releasing the larvae in the centre of soil-filled trays without plants. Three trays per larval type with twenty larvae each were assayed (n=3). (B) Schematic representation (photomontage) of experimental setup used for behavioural experiments depicting mean (±SEM) CO_2 levels detected in the soil gas phase of each tray zone (n=3-4). Different letters indicate significant differences in CO_2 levels ($p<0.05$ by one-way ANOVA with Holm's multiple-comparisons test). (C-F) Mean (±SEM) proportion of WT (dark green) or *DvvGr2*-silenced (light green) WCR larvae observed in the different tray zones eight hours after releasing the larvae at distances of 64 cm (C), 48 cm (D), 32 cm (E), or 16 cm (F) from the plants. Six trays per larval type and distance combination with twenty larvae each were assayed (n=6). Asterisks indicate statistically significant differences in the proportion of WT and *DvvGr2*-silenced larvae found in each tray zone (*: $p<0.05$; **: $p<0.01$ by three-way ANOVA followed by FDR-corrected post hoc tests). For *P*-values of Generalized Linear Models (GLMs), refer to Table S1. For detailed data on CO_2 levels, refer to Fig. S1E.

266 **Root-derived CO_2 is used for host quality assessment by WCR larvae**

267 Plant nutritional status determines plant growth and defense, and can thus modulate plant-herbivore interactions
268 (***Wetzel et al., 2016***). To test for a possible connection between plant nutritional status, host suitability and CO_2-
269 dependent herbivore attraction, we varied the nutrient supply of maize plants and then carried out CO_2
270 measurements, and behavioural and insect performance experiments (Fig. 7). Plants that were well fertilized
271 released higher levels of CO_2 into the soil than did plants that received a medium (50 % of optimally fertilized
272 plants) or low (10% of optimally fertilized plants) dose of fertilizer (Fig 7A, B). As observed before, soil CO_2
273 levels decreased with increasing distance from the plants and were lowest in the middle of the experimental trays
274 (Fig. 7A, B). In choice experiments with maize plants planted approx. 50 cm apart, which corresponds to row
275 spacing used for high planting densities in maize cultivation, WT larvae showed a significant preference for well-
276 fertilized over medium- or low-fertilized plants (Fig 7C, D). *DvvGr2*-silenced larvae did not distinguish between
277 plants subjected to the different fertilizer regimes (Fig. 7C, D). In no-choice experiments, WCR larvae gained
278 most weight on well-fertilized maize plants than on plants treated with medium or low doses of fertilizer (Fig. 7E).
279 Hence, intact CO_2 perception is required for WCR larvae to express host quality related preferences for maize
280 plants growing at agriculturally relevant distances.

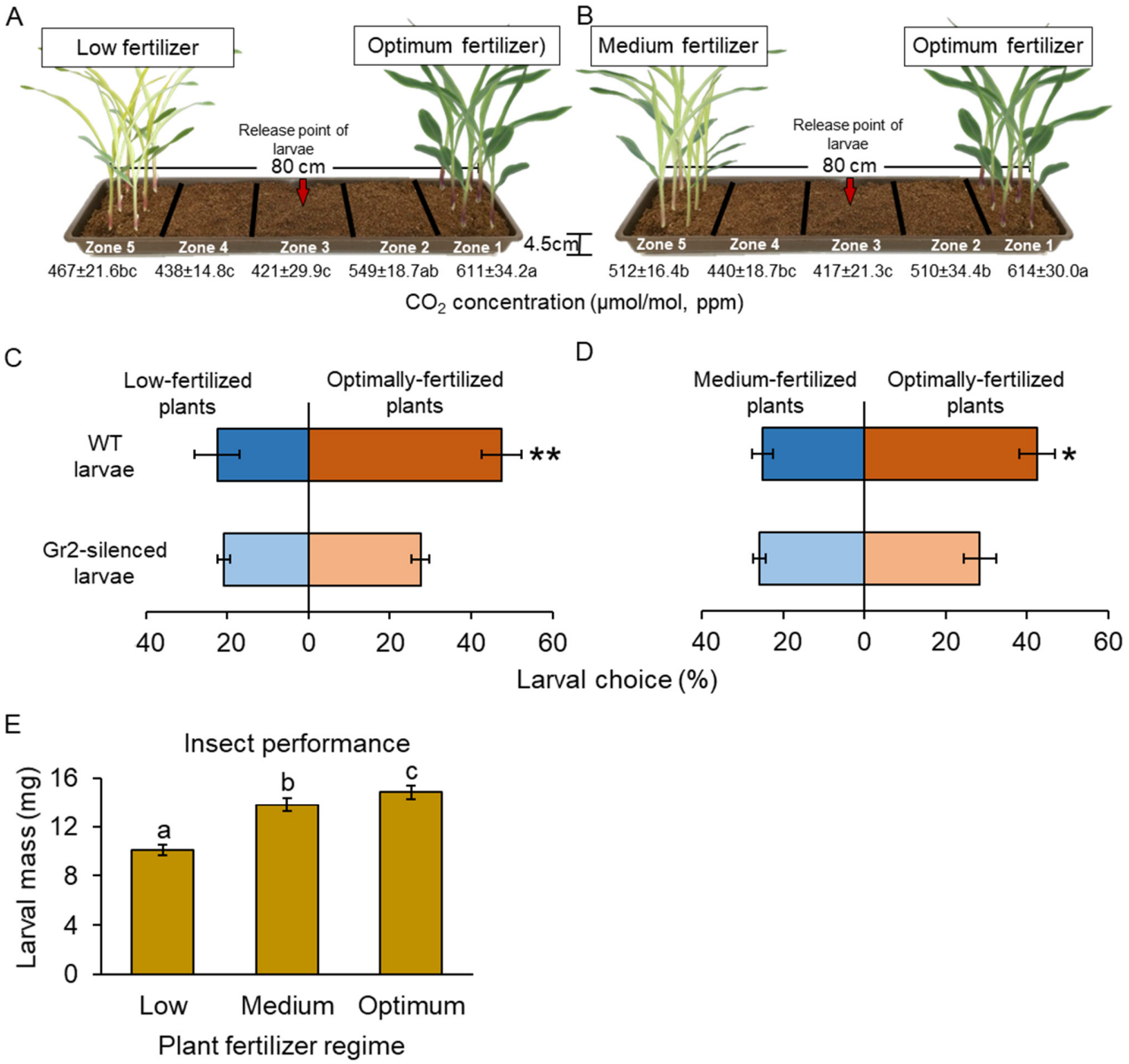

Figure 7 *Root-derived* CO_2 *is used for host quality assessment by WCR larvae in the soil.* Schematic representation (photomontage) of experimental set up used to evaluate insect preference for plants fertilized with low or optimum fertilizer doses (A), and for plants fertilized with medium or optimum fertilizer doses (B) depicting mean (±SEM) CO_2 levels detected in the soil gas phase of each tray zone (n=10). Different letters indicate statistically significant differences in CO_2 levels (p<0.05 by one-way ANOVA with Holm's multiple comparisions test). (C, D) Mean (±SEM) proportion of WCR larvae recovered from the rhizosphere (either Zone 1, or Zone 5) in choice assays contrasting larval preference for plants fertilized with low or optimum fertilizer doses (C), and for plants fertilized with medium or optimum fertilizer doses (D) eight hours after releasing the larvae. Six trays with twenty larvae per tray were assayed (n=6). Different letters indicate statistically significant differences in larval preferences (*: p<0.05; **: (*: p<0.01 by two-way ANOVA followed by FDR-corrected post hoc tests). (E) Mean (±SEM) weight of WCR larvae after 7d feeding on plants fertilized with low, medium or optimum fertilizer doses. Twenty solo cups with 4 to 7 larvae each were assayed (n=20). Different letters indicate statistically significant differences in larval mass (p<0.05 by one-way ANOVA followed by Holm's multiple comparisions tests). For P-values of Generalized Linear Models (GLMs), refer to Table S1. For detailed data on CO_2 levels, refer to Fig. S1D.

295 **Discussion**

296 Volatile chemicals have a strong impact on the behaviour and performance of organisms across the tree of life
297 (***Arce et al., 2017; Bentley and Day, 1989; Gang and Hallem, 2016; Hansson and Stensmyr, 2011; Johnson***
298 ***and Gregory, 2006; Miller and Strickler, 1984; Veyrat et al., 2016; Webster and Cardé, 2017; Ye et al., 2018***).
299 Many organisms, including bacteria, fungi, nematodes, plants, insects, fish and mammals respond to CO_2, for
300 instance (***Bahn and Mühlschlegel, 2006; Cummins et al., 2014; Eilers et al., 2012; Hallem and Sternberg, 2008;***
301 ***Hester et al., 2012; Huckstepp and Dale, 2011; Jones et al., 2007; Perry and Abdallah, 2012; Raguso et al.,***
302 ***2005; Stange and Stowe, 1999***). However, the role of CO_2 in plant-herbivore interactions is poorly understood
303 and has not been studied *in vivo* through molecular manipulation approaches.

304 In this study, we conducted gene sequence similarity analyses, phylogenetic relationship reconstructions, RNA
305 interference and behavioural experiments with different setups to explore the biological relevance of root-derived
306 CO_2 for plant-herbivore interactions. We found that the WCR genome contains at least three putative CO_2 receptor-
307 encoding genes: *DvvGr1*, *DvvGr2*, and *DvvGr3*, which is consistent with previous transcriptomic-based studies
308 (***Rodrigues et al., 2016***). Protein tertiary structure and topology prediction models show that the identified genes
309 code for proteins that contain seven transmembrane domains, which is consistent with the protein topology of
310 gustatory and odor receptors (***Dahanukar et al., 2005; Hallem et al., 2006***). Larval behaviour and gene silencing
311 based-functional characterization of the three identified WCR putative CO_2 receptor genes revealed that the intact
312 expression of *DvvGr2*, is essential for the attractive effects of CO_2 to WCR larvae. Knocking down *DvvGr2*
313 rendered larvae fully unresponsive to synthetic and plant-derived CO_2. In *Aedes aegypti, Helicoverpa armigera*
314 and *Drosophila melanoganster*, both carbon dioxide receptors *Gr1* and *Gr3* are required for CO_2 detection
315 (***Erdelyan et al., 2012; Jones et al., 2007; Kwon et al., 2007; McMeniman et al., 2014; Ning et al., 2016; Suh et***
316 ***al., 2004***). In *Culex quinquefasciatus*, both *Gr2* and *Gr3* carbon dioxide receptors are required, while *Gr1* act as a
317 modulator (***Xu et al., 2020***). In *Aedes aegypti,* the involvement of *Gr2* in carbon dioxide responsiveness is still
318 under debate (***Erdelyan et al., 2012; Kumar et al., 2019***). Taken together, the molecular elements required for
319 carbon dioxide perception may be species-specific. Our results support this notion, as *DvvGr2,* but not *DvvGr1*
320 and *DvvGr3*, are crucial for CO_2 responsiveness. The role of *DvvGr1* and *DvvGr3* for WCR remains to be
321 determined, but their presence and expression may hint at additional complexity in developmental and/or tissue-
322 specific patterns of CO_2 responsiveness in this species.

323 Despite the inability of *DvvGr2*-silenced WCR larvae to respond to differences in CO_2 levels, the larvae were still
324 able to orient towards maize roots at short distances of 8-10 cm. Olfactometer experiments in combination with a
325 CO_2 scrubber that removes plant-derived CO_2 demonstrates that other volatile cues can be used by WCR larvae to
326 locate maize plants at short distances. Earlier studies found that (*E*)-β-caryophyllene, which is emitted from the
327 roots of certain maize genotypes when they are attacked by root herbivores, attracts second and third instar WCR
328 larvae and allows them to aggregate on maize plants and thereby enhance their fitness (***Robert et al., 2012b***), while

329 neonate larvae are not attracted to this volatile (***Hiltpold and Hibbard, 2016***). Ethylene has also been shown to
330 attract WCR larvae (***Robert et al., 2012a***), and MBOA or its breakdown products have also been proposed as
331 volatile attractants (***Bjostad and Hibbard, 1992***). Methyl anthranilate on the other hand has been shown to repel
332 WCR larvae (***Bernklau et al., 2016b; Bernklau et al., 2018a***). Many other leaf- and root feeding herbivores are
333 known to respond to plant volatiles other than CO_2 (***Bruce et al., 2005***). Given the low reliability of CO_2 as a host-
334 specific cue, it is probably not surprising that WCR, as a highly specialized maize feeder, can use other volatile
335 cues to locate host plants. Integrating other volatile cues likely allows WCR larvae to locate maize plants even in
336 the absence of reliable CO_2 gradients in the soil, thus increasing the robustness of its foraging behaviour at short
337 distances. An intriguing result in this context is the fact that WCR larvae show the same efficiency in locating
338 maize roots at short distances in the absence of a CO_2 gradient, suggesting that this volatile may not play a role as
339 a cue at close range.

340 Although intact CO_2 perception was not required for host location at short distances, it had a strong impact on the
341 capacity of WCR larvae to reach the maize rhizosphere at long distances. The advantages of CO_2 are that it is
342 abundantly produced through respiration by most organisms, that it is relatively stable (***Jones and Coaker, 1977;***
343 ***Li et al., 2016***), and that it diffuses rapidly in air, water and soil (***Hashimoto and Suzuki, 2002; Ma et al., 2013***).
344 CO_2 may thus be a suitable long-range cue to locate organisms with high respiratory rates, such as mammals and
345 heterotroph plant parts, including roots (***Johnson and Nielsen, 2012***). Aboveground insects can be attracted to
346 CO_2 traps located as far away as 10 m, and it is estimated that this distance could even be as long as 60 m under
347 optimal environmental circumstances (***Guerenstein and Hildebrand, 2008; Zollner et al., 2004***). For belowground
348 insects, this distance is hypothesized to be within the lower cm-range as CO_2 diffusion is substantially decreased
349 within the soil matrix compared to the air (***Bernklau et al., 2005; Doane et al., 1975; Doane and Klingler, 1978;***
350 ***Klingler, 1966***). Other volatiles that are less abundant and diffuse even less well through the soil such as (*E*)-β-
351 caryophyllene are unlikely to be detectable at distances of more than 10 cm (***Chiriboga et al., 2017; Hiltpold and***
352 ***Turlings, 2008***). In support of this, we did not succeed in the past to detect maize volatiles in the soil at distance
353 of more than 10 cm, even using a highly sensitive combination of solid-phase microextraction (SPME) and gas
354 chromatography/mass spectrometry (GC/MS). These volatiles are thus likely useful host location cues at short,
355 but not long distances in the soil. The finding that WCR integrates CO_2 perception with other environmental cues
356 and that attraction to CO_2 is context dependent is in line with patterns reported for other insects such as mosquitoes,
357 whose response to stimuli such as color, temperature and human body odors is enhanced by CO_2 (***McMeniman et***
358 ***al., 2014; van Breugel et al., 2015***), and pollinating hawkmoths, that use CO_2 as a redundant volatile distance
359 stimulus in a sex-specific manner (***Goyret et al., 2008***).

360 A recent study shows that a CO_2 receptor in *Drosophila* flies is also involved in the detection and behavioral
361 responses to other volatiles (***MacWilliam et al., 2018***). We observed that *DvvGr2*-silenced larvae were repelled
362 by methyl anthranilate, a potent maize root repellent, to a similar extent than WT larvae, suggesting that their

363 sensitivity to other plant volatiles is unchanged (***Bernklau et al., 2016b***). In *Drosophila* flies, the CO_2 receptor
364 *Gr63a* is required for spermidine attractiveness over short time spans, i.e.: less than 1 min, but not over longer
365 time spans (hours), when other receptors likely become more important (***MacWilliam et al., 2018***). In the present
366 experiments, WCR behaviour was evaluated after one or more hours. The CO_2 scrubber experiment provides
367 further evidence that the foraging patterns observed in this study are not due to different sensitivity of *DvvGr2*-
368 silenced larvae to other root volatiles.

369 Apart from acting as a long-distance host location cue, CO_2 also links plant fertilization to herbivore behaviour by
370 guiding WCR to well fertilized plants. As WCR larvae are resistant to root defenses of maize (***Robert et al., 2012b***),
371 it is likely to benefit from increased fertilization, independently of the plant's defensive status. As the plant
372 nutritional status and host quality for WCR larvae is associated with higher CO_2 release from the roots, following
373 the highest concentrations of CO_2 in the soil may be adaptive for the herbivore, as it may increase its chance not
374 only to find a maize plant *per se*, but also to identify a plant that has the resources to grow vigorously and that is
375 a better host. WCR larvae are specialized maize pests that have evolved with intense maize cultivation in the corn
376 belt of the US (***Gray et al., 2009***) and are resistant to maize defense metabolites (***Robert et al., 2012b***). Following
377 the strongest CO_2 gradient in an equally spaced maize monoculture may indeed be a useful strategy for this root
378 feeder to locate suitable food sources. An association between CO_2 emission and food-source profitability was
379 also suggested for *Datura* flowers, that emit the highest level of CO_2 in times when nectar is most abundant
380 (***Guerenstein et al., 2004; Thom et al., 2004***). These findings support the hypothesis that CO_2 is used as a marker
381 of metabolic activity that allows for an assessment of the vigour and profitability of a wide variety of hosts.

382 In summary, this work demonstrates how an herbivore uses its capacity to perceive CO_2 to locate host plants and
383 to distinguish plants of different quality. Volatiles other than CO_2 are also integrated into host finding behaviour
384 in the soil, but their effects are more important at shorter distances. Thus, evidence is now accumulating that CO_2
385 acts as a fitness-relevant host location cue in different insects, likely because of its unique role as a highly
386 conserved long-range marker of metabolic activity within complex sensory landscapes.

387 **Materials and Methods**

388 **Plants and planting conditions**

389 Maize seeds (*Zea mays* L., var. Akku) were provided by Delley Semences et Plantes SA (Delley, CHE). Seedlings
390 were grown under greenhouse conditions (23 ± 2˚C, 60% relative humidity, 16:8 h L/D, and 250 mmol/m^2/s^1
391 additional light supplied by sodium lamps). Plantaaktiv® 16+6+26 Typ K fertilizer (Hauert HBG Dünger AG,
392 Grossaffoltern, Switzerland) was added twice a week after plant emergence following the manufacturer's
393 recommendations. The composition of the fertilizer is: total nitrogen (N) 16%, nitrate 11%, nmmonium 5%,
394 phosphate (P_2O_5) 6%, potassium oxide (K_2O) 26%, magnesium oxide (MgO) 3.3%, borum (B) 0.02%, cooper (Cu,
395 EDTA-chelated) 0.04%, iron (Fe, EDTA-chelated) 0.1%, manganese (Mn, EDTA-chelated) 0.05%, molybdenum

396 (Mo) 0.01%, and zink (Zn, EDTA-chelated) 0.01%. When plants were used as insect food, seedlings were
397 germinated in vermiculite (particle size: 2-4 mm; tabaksamen, Switzerland) and used within 4 days after
398 germination.

399 **Insects and insect rearing**

400 *Diabrotica virgifera virgifera* (Western corn rootworm, WCR) insects used in this study were derived from a non-
401 diapausing colony reared at the University of Neuchâtel. The eggs used to establish the colony were originally
402 supplied by USDA-ARS-NCARL, Brookings, SD. Upon hatching, insects were maintained in organic soil
403 (Selmaterra, Bigler Samen AG, Thun, Switzerland) and fed freshly germinated maize seedlings (var. Akku).

404 **Identification of CO_2 receptor genes**

405 To identify CO_2 receptor orthologues in WCR, we used CO_2 receptor-encoding gene sequences of *Tribolium*
406 *castaneum* and several sequences from other insects as queries against publicly available WCR genome sequences
407 (NCBI accession: PXJM00000000.2) using the National Center for Biotechnology Information Basic Local
408 Alignment Search Tool (NCBI BLAST)(***Robertson and Kent, 2009; Wang et al., 2013; Xu and Anderson, 2015***).
409 The full gene sequences can be retrieved from the National Center for Biotechnology Information (NCBI) data
410 bank using the following accession numbers: XM_028276483.1 (*DvvGr1*), XM_028280521.1 (*DvvGr2*), and
411 XM_028272033.1 (*DvvGr3*). These gene sequences were translated to obtain protein sequences. The obtained
412 protein sequences and the protein sequences of CO_2 receptors from different insects were used to infer evolutionary
413 relationships using the Neighbor-Joining method in MEGA 7 (***Kumar et al., 2016; Robertson and Kent, 2009;***
414 ***Rodrigues et al., 2016; Saitou and Nei, 1987***). The optimal tree with the sum of branch length = 4.44068889 is
415 provided in Fig 1a. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap
416 test (100 replicates) are shown next to the branches (***Felsenstein, 1985***). The tree is drawn to scale, with branch
417 lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary
418 distances were computed using the Poisson correction method (***Zuckerkandl and Pauling, 1965***) and are in the
419 units of the number of amino acid substitutions per site. A total of 242 amino acid positions were included in the
420 final dataset. Graphical representation and edition of the phylogenetic tree were performed with the Interactive
421 Tree of Life (version 3.5.1)(***Letunic and Bork, 2016***). Protein tertiary structures and topologies were predicted
422 using Phyre2 (***Kelley et al., 2015***).

423 **Production of double stranded RNA**

424 *Escherichia coli* HT115 were transformed with recombinant L4440 plasmids that contained a 211-240 bp long
425 gene fragment targeting one of the three CO_2 receptors. Cloned nucleotide sequences were synthetized *de novo*
426 (Eurofins, Germany). To induce the production of dsRNA, an overnight bacterial culture was used to inoculate
427 fresh Luria-Berthani broth (25 g/L, Luria/Miller, Carl Roth GmbH, Karlsruhe, Germany). Once the bacterial

428 culture reached an OD_{600} of 0.6-0.8, it was supplemented with isopropyl β-D-1-thiogalactopyranoside, IPTG)
429 (Sigma-aldrich, Switzerland) at a final concentration of 2mM. Bacterial cultures were incubated at 37°C in an
430 orbital shaker at 130 rpms for 16 additional hours. Bacteria were harvested by centrifugation and stored at -20°C
431 for further use (***Kim et al., 2015***).

432 **Gene silencing experiments**

433 To induce gene silencing in WCR, six to ten second instar WCR larvae were released in solo cups (30 mL, Frontier
434 Scientific Services, Inc., Germany) containing approx. 2g of autoclaved soil (Selmaterra, Bigler Samen AG, Thun,
435 Switzerland) and 2-3 freshly germinated maize seedlings. Maize seedlings were coated with 1 ml of bacterial
436 solution containing approximately 200-500 ng of dsRNA, targeting the different CO_2 receptor genes. As controls,
437 larvae were fed with bacteria producing dsRNA targeting green fluorescent protein genes (GFP), which are absent
438 in the WCR genome (***Rodrigues et al., 2016***). Fresh bacteria and seedlings were added to solo cups every other
439 day for three consecutive times. Two days after the last dsRNA/bacteria application, larvae were collected and
440 used for experiments.

441 **Gene expression measurements**

442 Total RNA was isolated from approximately 10 mg of frozen, ground and homogenized WCR larval tissue (3-7
443 larvae per biological replicate, n=8) using the GenElute Universal Total RNA Purification Kit (Sigma-Aldrich, St.
444 Louis, MO, USA). A NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA)
445 was used to estimate RNA purity and quantity. DNase-treated RNA was used as template for reverse transcription
446 and first strand cDNA synthesis with PrimeScript Reverse Transcriptase (Takara Bio Inc., Kusatsu, Japan). DNase
447 treatment was carried out using the gDNA Eraser (Perfect Real Time) following manufacturer's instructions
448 (Takara Bio Inc.). For gene expression analysis, 2 µl of undiluted cDNA (i.e. the equivalent of 100 ng total RNA)
449 served as template in a 20 µl qRT-PCR using the TB Green Premix Ex Taq II (Tli RNaseH Plus) kit (Takara Bio
450 Inc.) and the Roche LightCycler 96 system (Roche, Basel, Switzerland), according to manufacturer's instructions.
451 Transcript abundances of the following WCR genes were analysed: *DvvGr1, DvvGr2, and DvvGr3* (***Rodrigues et***
452 ***al., 2016***). *Actin* was used as reference gene to normalize expression data across samples. Relative gene expression
453 levels were calculated by the $2^{-\Delta\Delta Ct}$ method (***Livak and Schmittgen, 2001***). The following primers were used:
454 DvvGr1-F CGTTAATTTAGCTGCTGTGG, DvvGr1-R GTTTTCTGTTGCTAGAGTTGC, DvvGr2-F
455 GAACTAAGCGAGCTCCTCCA, DvvGr2-R CAGAAGCACCATGCAATACG, DvvGr3-F
456 GCAACGCTTTCAGCTTTACC, DvvGr3-R GTGCATCGTCATTCATCCAG, DvvActin-F
457 TCCAGGCTGTACTCTCCTTG, and DvvActin-R CAAGTCCAAACGAAGGATTG.

458 **Belowground olfactometer experiments with *DvvGr1*-, *DvvGr2*-, *DvvGr3*-silenced WCR larvae**

459 To determine whether silencing putative CO_2 receptor genes impairs the ability of WCR larvae to behaviourally
460 respond to CO_2, we silenced *DvvGr1*, *DvvGr2*, and *DvvGr3* as described above and conducted behavioural
461 experiments using belowground olfactometers. Larvae that were fed bacteria expressing dsRNA targeting green
462 fluorescent protein (GFP) were used as controls (herein referred as wild type larvae; WT). We conducted choice
463 experiments using two-arm belowground olfactometers with carbonated water as a CO_2 source (***Bernklau and***
464 ***Bjostad, 1998a; Huang et al., 2017; Jewett and Bjostad, 1996***). For this, a plastic cup containing 50 ml of
465 carbonated water was placed in one arm of the olfactometer and a plastic cup containing 50 ml of distilled water
466 was placed in the opposite arm (Fig. 2B). Five larvae were released in the middle of the olfactometer (indicated
467 by a red arrow) and their positions were recorded 1h after their release. Seven olfactometers per larval type and
468 experiment were assessed. The experiment was repeated twice. CO_2 levels on each arm of the olfactometer were
469 measured by gas chromatography coupled to a flame ionization detector (GC-FID) as described (***Zhang et al.,***
470 ***2019***).

471 **Insect behavioural experiments**

472 To determine larval responses to plant volatile organic compounds other than CO_2, we evaluated insect behaviour
473 to methyl anthranilate. For this, we evaluated insect preferences for seedling roots or for seedling roots placed next
474 to a filter paper disc treated with methyl anthranilate in choice experiments following a similar experimental
475 procedure as described (***Bernklau et al., 2018a***). To this end, five 2nd-3rd instar WCR larvae were released in the
476 middle of a sand-filled petri plate (9 cm diameter, Greiner Bio-One GmbH, Frickenhausen, DE) where they
477 encountered seedling roots in one side or seedlings roots and a filter paper disc treated with 10 µl of methyl
478 anthranilate solution (10 mg/ml of water) in the other side. Ten Petri plates per larval type with five larvae each
479 were evaluated (n=10). Methyl anthranilate was purchased from Sigma (CAS: 134-20-3; Sigma Aldrich Chemie,
480 CHE). Larval preferences were recorded 1h after releasing the larvae.

481 To investigate larval responses to non-volatile root chemicals, we tested two well established attractants,
482 $Fe(III)(DIMBOA)_3$ and a sugar blend (***Bernklau et al., 2018b; Hu et al., 2018***). To evaluate the attractive effects
483 of $Fe(III)(DIMBOA)_3$, we released six 2nd-3rd instar WCR larvae in the middle of a moist sand-filled Petri plate (6
484 cm diameter, Greiner Bio-One GmbH, Frickenhausen, DE) where they had a choice between a filter paper disc
485 treated with 10 µl of $Fe(III)(DIMBOA)_3$ (1 µg/ml of water) or, on the opposite side, a filter paper disc treated with
486 water only. Twenty Petri plates with six larvae each were evaluated (n=20). $Fe(III)(DIMBOA)_3$ was prepared fresh
487 by mixing $FeCl_3$ and DIMBOA at a 1:2 ratio as described. Larval preferences were recorded 1h after releasing the
488 larvae. To evaluate attractive effects of soluble sugars for WCR larvae, we followed the procedure described by
489 (***Bernklau et al., 2018b***). Briefly, we released six 2nd-3rd instar WCR larvae in the middle of a moist sand-filled
490 petri plate (6 cm diameter, Greiner Bio-One GmbH, Frickenhausen, DE) where they had a choice between a filter

491 paper disc treated with 10 µl of a mixture of glucose, fructose, and sucrose (30 mg/ml of each sugar), or, on the
492 opposite side, a filter paper treated with water only. Twenty Petri plates with six larvae each were assayed (n=20).
493 Larval preferences were recorded 3h after release.

494 To evaluate the attractiveness of CO_2 for WCR larvae, we conducted choice experiments using two-arm
495 belowground olfactometers. CO_2 levels were increased in one arm of the olfactometer by delivering CO_2-enriched
496 synthetic air (1% CO_2, Carbagas, Switzerland). Olfactometer arms were closed on top using parafilm during CO_2
497 delivery. A manometer connected to the synthetic air bottle allowed to fine-tune CO_2 delivery rates. CO_2 levels
498 were measured using a gas analyzer (Li7000, Li-Cor Inc., Lincoln, Nebraska, USA). CO_2 levels were increased at
499 different levels from 22 to 1832 ppm above ambient CO_2 levels. Once the desired CO_2 concentrations were
500 reached, CO_2 delivery was terminated, olfactometer arms were connected to olfactometer central connectors and
501 six 2nd-3rd instar WCR larvae were release immediately, and their choice were evaluated within ten minutes of
502 release. Three olfactometers per larval type were assayed (n=3).

503 To assess the impact of *DvvGr2* on larval motility, we followed the trajectories of individual larvae in Petri plate
504 that were outfitted with a CO_2 point releaser. For this, Petri plates (9 cm diameter, Greiner Bio-one, Austria) were
505 pierced in their centers to allow the insertion of a needle that released CO_2 at 60 ppm above ambient level. Larvae
506 were released at the rim of the Petri plates (4.5 cm apart from CO_2 source). Petri plates were overlaid with moist
507 filter paper (9cm diameter, GE Healthcare, UK). Insect behavior was observed for three minutes. Six Petri plates
508 with one larva each were assayed (n=6). In a second experiment, we followed the trajectories of individual larvae
509 in Petri plate with maize roots. For this, two root pieces (3-4 cm long) of four-day-old maize seedlings were placed
510 at the rim of a Petri plate overlaid with moist filter paper. Then, on the opposite rim (9cm apart), we released one
511 2nd-3rd instar WCR larva and followed their trajectories during three minutes. Six Petri plates with one larva each
512 were assayed (n=6). Crawled distances were measured using ImageJ 1.53a.

513 **Host location experiments**

514 To determine the importance of plant-derived CO_2 for distance-dependent host-location by WCR larvae, we used
515 two experimental setups: two-arm belowground olfactometers and soil-filled trays (Fig. 5, Fig. 6). For behavioural
516 experiments using belowground olfactometers, we used two types of olfactometers: one with short arms that
517 allowed for the release of larvae at 9 cm (Fig. 5A) from plant volatile sources, and one with long arms that allow
518 to release the larvae at 18 cm (Fig. 5B) from plant volatiles sources. To specifically investigate the importance of
519 plant derived CO_2, we contrasted insect behavioural responses to intact plant odours and to plant odours without
520 CO_2. CO_2 was experimentally removed using soda lime (Carl Roth, Karlsruhe, Germany). For this, a layer of 5g
521 of soda lime granules (2-4mm) were placed between Teflon connectors and sand 30 min before the experiments.
522 Olfactometers without soda lime served as controls. Olfactometer arms were filled with sand 48h before the
523 experiment and one three-week-old maize plant was transplanted to one arm of the olfactometer. Four

524 olfactometers with six larvae each were assayed (n=4). CO_2 levels were measured using a gas analyzer (Li7000,
525 Li-Cor Inc., Lincoln, Nebraska, USA). For behavioural experiments using soil-filled trays, second instar WCR
526 larvae were released at 16, 32, 48, or 64 cm from four or five 2-to 3-week-old maize plants. Larval release points
527 are indicated by colour arrows (Fig. 6B). Plants were planted in plastic trays (80cmx15cmx4.5cm)(Migros do it +
528 garden, Switzerland) filled with soil. Eight h after releasing the larvae, their positions were recorded. Six trays per
529 larval type and distance with twenty larvae each were assayed (n=6). CO_2 levels in the soil gas phase of each tray
530 zone were measured by GC-FID as described (***Zhang et al., 2019***).

531 **Host finding behavioural experiments with nutrient-deficient plants**

532 Optimally fertilized plants emit higher levels of respiratory CO_2 than sub-optimally fertilized plants (***Zhu and***
533 ***Lynch, 2004***). To test whether WCR larvae orients towards and prefer optimally fertilized maize plants and to
534 evaluate the importance of CO_2 detection through *DvvGr2* in this context, we silenced this gene and conducted
535 choice experiments to contrast larval preference for plants that were differentially fertilized. To this end, we
536 fertilized maize plants with three doses of fertilizer: 0.1% (optimally fertilized), 0.05% (medium fertilized), or
537 0.01% (low fertilized). Plantaaktiv® 16+6+26 Typ K fertilizer (Hauert HBG Dünger AG, Grossaffoltern,
538 Switzerland) was dissolved to the abovementioned concentrations and applied following manufacture's
539 indications. Fertilizer macro- and micronutrient composition are described above (Plants and planting conditions).
540 For the choice experiments, plants were grown under the different fertilizer regimes. 24 hr before the choice
541 experiment, five 15-day-old, optimally fertilized plants were transferred and re-planted in one corner of an 80 cm
542 long plastic tray (Migros do it + garden, Switzerland) filled with soil (Figs 5a, b). At the opposite side, plants
543 grown either under low or medium fertilizer regimes were equally transferred and re-planted (Figs 7A, B). Then,
544 twenty 2th instar WCR larvae were released in the middle of the tray (Zone 3, Fig 6A, B, red arrows). Six
545 independent trays per larval type and fertilizer regime pair were evaluated (n=6). Eight hours after releasing the
546 larvae, larval positions were recorded.

547 **Effect of plant nutritional status on larval growth**

548 To test whether larval preference for optimally fertilized plants is reflected in their growth, we measured larval
549 weights of larvae feeding on roots of plants that were grown under the different fertilizer regimes. Fertilizer
550 regimens are described above. For this, seven 1th instar larvae were released into solo cups (30 mL, Frontier
551 Scientific Services, Inc., DE) containing approx. 2g of organic soil (Selmaterra, Bigler Samen AG, Thun, CHE).
552 Fresh roots of 15-day old plants were provided everyday *ad libitum*. Roots were washed thoroughly to remove
553 fertilizer traces from the root surface. Twenty solo cups per treatment were included (n=20). Eight days after the
554 beginning of the experiment, larvae were weighed using a micro balance. Four to seven larvae were recovered per
555 experimental unit at the end of the experiment.

556 **Statistical analyses**

557 Differences in gene expression levels, carbon dioxide concentrations, and larval performance were analysed by
558 analysis of variance (ANOVA) using Sigma Plot 12.0 (SystatSoftware Inc., San Jose, CA, USA). Normality and
559 equality of variance were verified using Shapiro–Wilk and Levene's tests, respectively. Holm–Sidak post hoc tests
560 were used for multiple comparisons. Datasets from experiments that did not fulfill the assumptions for ANOVA
561 were natural log-, root square-, or rank-transformed before analysis. The differences in larval preference were
562 assessed using Generalized Linear Models (GLM) under binomial distribution and corrected for overdispersion
563 with quasi-binomial function when necessary followed by analysis of variance (ANOVA) and FDR-corrected post
564 hoc tests. All analyses were followed by residual analysis to verify the suitability of the error distribution and
565 model fitting. All The above analyses were conducted using R 3.2.2 (43) using the packages "lme4", "car",
566 "lsmeans" and "RVAideMemoire" (***Bates et al., 2014; Fox and Weisberg, 2011; Herve, 2015; Lenth, 2016;***
567 ***Team, 2014***).

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574 **Author contributions**

575 Conceptualization, M.E. and R.A.R.M.; Methodology, R.A.R.M. and M.E.; Formal analysis, R.A.R.M., M.E., and
576 C.C.M.A.; Investigation, R.A.R.M., C.C.M.A., V.T., B.C.J.S., and G.J.; Visualization, R.A.R.M.; Supervision,
577 R.A.R.M. and M.E.; Funding Acquisition, M.E., and R.A.R.M Writing – original draft, R.A.R.M. and M.E.;
578 Writing – review & editing, R.A.R.M., M.E., B.C.J.S., and C.C.M.A.

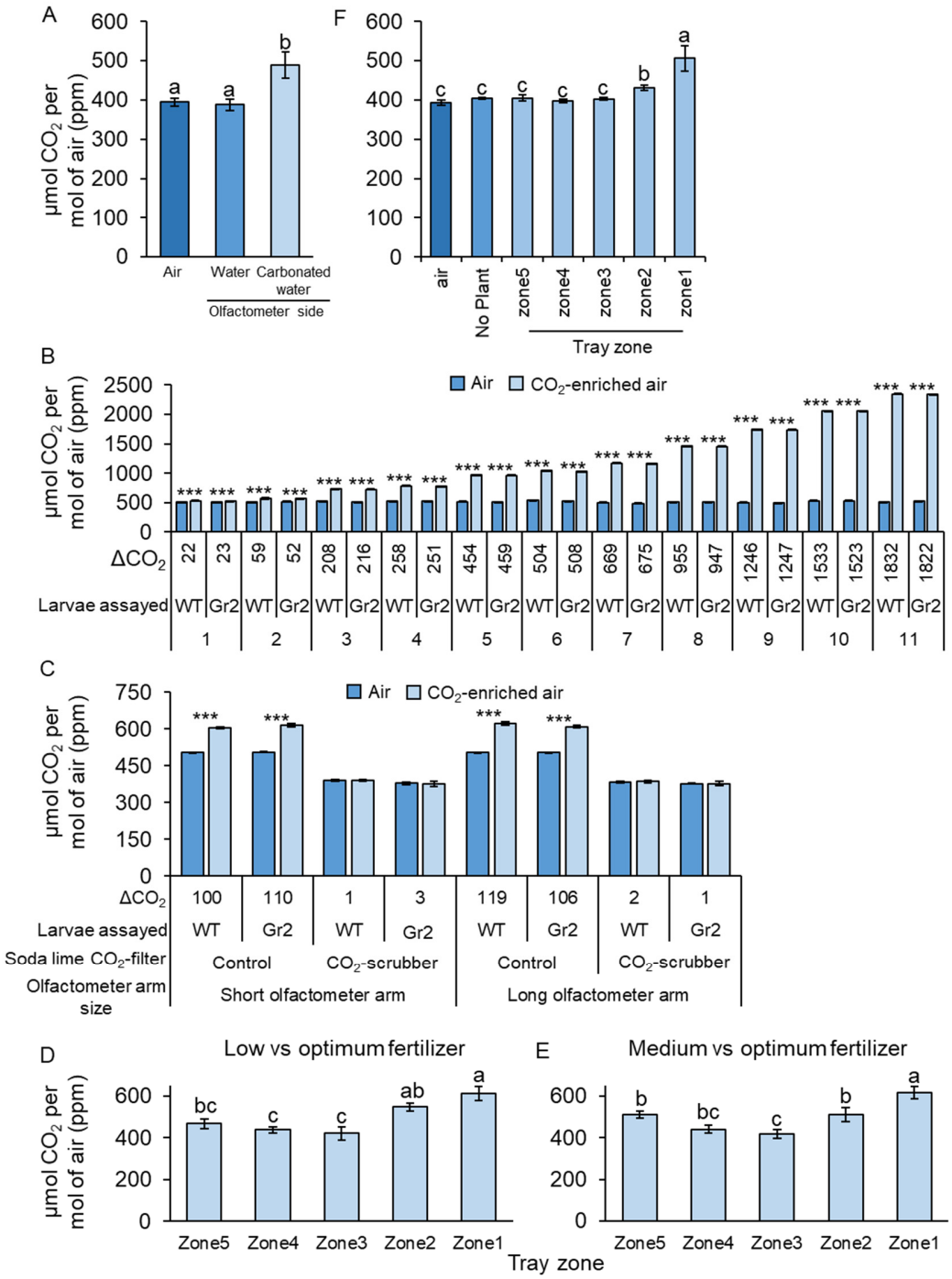
579 **Declaration of interests**

580 The authors declare no competing interests

581 **Data availability**

582 All data sets are available from Dryad (to be added at a later stage).

583 **Supplementary Figures**



584

585 **Figure S1** *Carbon dioxide measurements for the different experiments.* Mean (±SEM) CO_2 levels measured in behavioural
586 experiments of Figure 2 (A), Figure 3 (B), Figure 5 (C), Figure 7 (D, E), Figure 6 (F). Different letters indicate significant
587 differences in CO_2 levels (p<0.05 by one-way ANOVA with Holm's multiple-comparisons test). Asterisks indicate significant
588 differences in CO_2 levels (***: p<0.001 by ANOVA with Holm's multiple-comparisons test). Gr2: *DvvGr2*-silenced larvae.
589 For details on CO_2 sampling points, refer to the main figures.

590

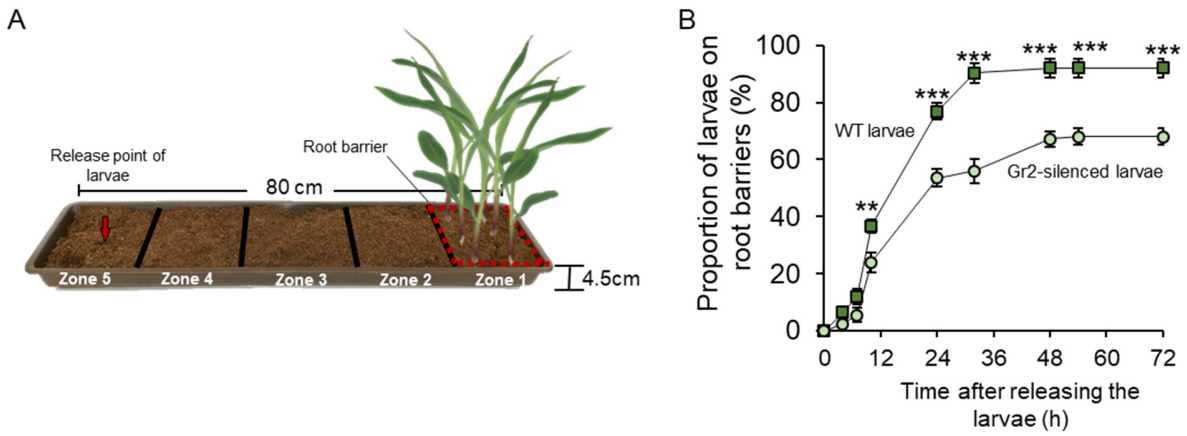


Figure S2 *Root-derived CO_2 is required for host location by WCR larvae at long distances.* (A) Schematic representation (photomontage) of experimental set up used. (B) Mean (±SEM) proportion of WCR larvae retrieved at close vicinity of root systems (i.e.: on root barriers). Twenty-five larvae were released at 64cm from the plants and five arenas were assayed (n=5). Soil surrounding root barriers (indicated by dashed red lines) were carefully inspected and the observed larvae were collected and counted regularly. Asterisks indicate statistically significant differences in the proportion of larvae retrieved at each time point (**: $p<0.01$; ***: $p<0.001$ by two-way repeated measures ANOVA with Holm's multiple comparisions test). For *P*-values of the different factors and their interactions, refer to Table S1.

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