Plant-derived CO₂ 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 CO₂, 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 CO₂ concentrations. To address this gap of knowledge, we manipulated CO₂ perception of the larvae of the western corn rootworm (*Diabrotica virgifera virgifera*; WCR) through RNA interference and studied how CO₂ 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 CO₂ 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 CO₂ scrubbing, we demonstrate that WCR larvae use CO₂ as a long-range host plant finding cue, but employ other volatiles for short-range host location. We furthermore show that the larvae use CO₂ as a fitness-relevant long-distance indicator of plant nutritional status: Maize plants that are well-fertilized emit more CO₂ 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 CO₂ through *DvvGr2*. This study unravels how CO₂ 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 (CO₂) in plant-herbivore interactions. CO₂ 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 CO₂ due to their ability to fix carbon in the dark reactions of photosynthesis, they also release CO₂ 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). CO₂ 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 CO₂ during times of high nectar availability; as hawkmoth pollinators are attracted to CO₂, 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 CO₂ release and attract *Bactrocera tryoni* fruit flies. As CO₂ at corresponding concentrations is attractive to the flies, it has been suggested that they may use plant-derived CO₂ to locate suitable oviposition sites (*Stange, 1998*).

Root feeding insects are also highly attracted to CO₂ *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 CO₂ 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 CO₂ 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 CO₂ as a host-location cue for root feeders has been questioned due to a number of reasons: i) CO₂ 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 CO₂ 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 CO₂ background (*Agus et al.,*

2010; Eilers *et al.*, 2012; Erb *et al.*, 2013; Hansen, 1977; Hibbard and Bjostad, 1988; Hiltbold 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 CO₂ 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 CO₂ 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 CO₂ 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 (Hiltbold *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 CO₂ 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 CO₂ levels can outweigh the attractive effects of other maize volatiles, it was suggested that CO₂ may be the only relevant volatile attractant for WCR larvae (Bernklau and Bjostad, 1998b). However, under conditions where CO₂ 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 CO₂ receptors from transcriptomic data (Rodrigues *et al.*, 2016) makes this species a suitable model system to investigate the role of CO₂ in plant-herbivore interactions. Ongoing efforts to use CO₂ 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 CO₂ for WCR foraging in the soil, we manipulated the insect's capacity to perceive CO₂. We reduced the expression levels of three putative WCR CO₂ receptor-encoding genes through RNA interference (RNAi), resulting in the identification of *DvGr2* as an essential gene for CO₂ perception. Using *DvGr2*-silenced larvae in combination with CO₂-removal, we then assessed the importance of CO₂ perception for WCR behaviour and foraging in olfactometers and soil arenas. Our experiments reveal how root-derived CO₂

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 CO₂.

Results

The western corn rootworm (WCR) genome encodes three putative CO₂ receptors

To identify genes encoding putative CO₂ receptors in WCR, we used known CO₂ 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, *DvGr1*, *DvGr2*, and *DvGr3*, 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 CO₂ receptors of WCR and the protein sequences of other coleopteran insects such as *Tribolium castaneum* (Fig. 1A). Expression levels of *DvGr1* and *DvGr2* 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 *DvGr3* (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 (CO₂) receptors. (A) Phylogenetic relationships between putative CO₂ 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*, Dv: *Diabrotica virgifera virgifera* (Western corn rootworm, WCR). Mean (±SEM) relative gene expression levels of group 1 (*DvGr1*) (B), group 2 (*DvGr2*) (C), and group 3 (*DvGr3*) (D) CO₂ 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 comparisons test; n.s.: not significant). (B-E) Predicted protein tertiary structure (left) and transmembrane protein topology (right) of: (B) *DvGr1*; (C) *DvGr2*; and (D) *DvGr3* according to the Phyre2 algorithm.

***DvvGr2* expression is specifically required for behavioural responses of WCR larvae to CO₂**

To determine the importance of *DvvGr1*, *DvvGr2*, and *DvvGr3* for the responsiveness of WCR larvae to CO₂, we knocked down the expression of each gene individually through double-stranded RNA (dsRNA)-mediated RNA interference (RNAi) and conducted initial behavioural experiments with carbonated water as a CO₂ source (Fig. 2). Oral administration of dsRNA targeting either *DvvGr1*, *DvvGr2*, or *DvvGr3* reduced the expression levels of these genes by 80%, 83% and 66% compared to WCR larvae fed with dsRNA of the green fluorescent protein (GFP) gene (herein referred as wild type, WT) (Fig. 2A). All RNAi constructs were confirmed to be gene specific (Fig 2B). Olfactometer arms containing carbonated water released approximately 100 ppm more CO₂ than their counterparts filled with distilled water (Fig. 2B, Fig. S1A) and were more attractive to WT larvae than arms containing distilled water (Fig 2C). Silencing *DvvGr1* or *DvvGr3* expression did not alter this behaviour. In contrast, *DvvGr2*-silenced larvae did not show any preference any more (Fig 2C). To further explore the role of *DvvGr2* in WCR behaviour, we conducted a series of additional experiments. First, we assessed the impact of silencing *DvvGr2* on the capacity of WCR larvae to respond to other volatile and non-volatile host cues. *DvvGr2*-silenced larvae responded similarly to the repellent volatile methyl anthranilate (*Bernklau et al., 2016b; Bernklau et al., 2018a*) as WT larvae (Fig. 2D). Responsiveness to non-volatile attractants, Fe(III)(DIMBOA)₃ and a blend of glucose, fructose and sucrose (*Bernklau et al., 2018b; Hu et al., 2018*) was also unaltered in *DvvGr2*-silenced larvae (Figs 2E, F), demonstrating that knocking down *DvvGr2* expression does not alter the capacity of WCR larvae to respond to other important chemical cues. Second, we assessed the contribution of *DvvGr2* to CO₂ responsiveness using synthetic CO₂ at different concentrations (Fig. 3, Fig. S1B). WT larvae showed characteristic dose-dependent behavioural responses to CO₂. While they did not respond to 22 ppm CO₂ above ambient CO₂ levels, they were attracted to CO₂ concentrations between 59-258 ppm above ambient, and repelled by CO₂-enriched air at 950 ppm above ambient CO₂ levels and above (Fig. 3). In contrast, *DvvGr2*-silenced larvae did not respond to CO₂ enrichment at any of the tested concentrations (Fig. 3). These experiments show that WCR larvae are attracted to CO₂ enriched environments within the physiological range of the maize rhizosphere (Fig. S1) and that *DvvGr2*-silencing fully and specifically suppresses CO₂ responsiveness in WCR larvae.

Figure 2 The carbon dioxide group 2 receptor (*DvGr2*) is specifically required for behavioural responses of WCR to CO₂. (A) Mean (±SEM) relative gene expression levels of group 1 (*DvGr1*), group 2 (*DvGr2*), and group 3 (*DvGr3*) CO₂ receptors after WCR larvae were fed with dsRNA-expressing bacteria targeting green fluorescent protein (GFP, herein referred as WT), *DvGr1*, *DvGr2*, or *DvGr3* genes (n=11-13). Different letters indicate significant differences of gene expression levels ($p < 0.05$ by one-way ANOVA with Holm's multiple comparisons test). (B) Mean (±SEM) CO₂ levels in each arm of the two-arm belowground olfactometers used for behavioural experiments (n=4-8). Asterisk indicates significant differences in CO₂ 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 CO₂ 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 CO₂ in each arm.

Figure 3 *DvGr2* is required for dose-dependent behavioural responses of WCR to CO₂. (A) Olfactometer setup used for behavioural experiments. (A) Mean (±SEM) CO₂ 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. ΔCO₂ values are given for each level. Asterisks indicate significant differences between CO₂ 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 CO₂ in each arm.

***DvGr2* expression does not impair larval motility or short-range host location**

To assess the impact of *DvGr2* on larval motility, we followed the trajectories of individual larvae in Petri plates that were outfitted with a CO₂ point releaser (Fig. 4A-C). WT larvae made frequent turns, but consistently oriented themselves towards the CO₂ release point. Once they reached the CO₂ release point, they stopped moving (Fig. 4A). *DvGr2*-silenced larvae exhibited similar turning behaviour as WT larvae, but did not move towards the CO₂ release point (Fig. 4B). WT larvae spend more time on CO₂ release point than *DvGr2*-silenced larvae (Fig. 4C). During the movement phase, the mean speed of WT larvae and *DvGr2*-silenced larvae was similar (Fig. 4C), but the distance covered by *DvGr2*-silenced larvae was higher, as they did not stop at the CO₂ release point. In a second experiment, we followed the trajectories of individual larvae in Petri plates with maize roots (Fig. 4D, E). Speed and distance covered were similar between WT and *DvGr2*-silenced larvae (Fig. 4F). Surprisingly, both WT and *DvGr2*-silenced larvae oriented themselves towards the maize roots and reached the maize roots after a similar amount of time (Fig. 4F). This result shows that *DvGr2* expression is required for the location and detection of CO₂, but does not influence WCR motility nor its ability to locate maize roots over short distances (i.e.: <9 cm).

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187 **Figure 4** Silencing the carbon dioxide group 2 receptor (*DvGr2*) impairs WCR responses to CO₂ without changing motility
188 or search behavior. (A-B) Trajectories of individual WT (A) and *DvGr2*-silenced (B) WCR larvae in Petri plates with a CO₂
189 source. The blue circles represent larval release points. The red circles represent CO₂ sources consisting of a fine needle that
190 releases CO₂ at 60 ppm above ambient. (C) Mean (±SEM) speed and distance covered during the movement phase, and time
191 spent at the CO₂ source during the first 3 min of the experiment. (D-E) Trajectories followed by WT (D) and by *DvGr2*-
192 silenced (E) WCR larvae on Petri plates containing maize seedling roots. The blue circles represent larval release points. The
193 yellow circles represent maize seedling roots. (F) Mean (±SEM) speed and distance covered during the movement phase, and
194 time necessary to reach the maize root during the first 3 min of the experiment. (F). For both experiments, six Petri plates
195 with one larva each were assayed (n=6). Asterisks indicate statistically significant differences between mobility parameters
196 of WT and *DvGr2*-silenced larvae (***: $p < 0.001$ by one-way ANOVA followed by Holm's multiple-comparisons test).

***DvGr2*-dependent CO₂ responsiveness mediates long-range host location**

To further explore the role of CO₂ and *DvGr2* in volatile-mediated host location, we performed a series of olfactometer experiments with maize plants grown in sand on one side and sand only on the other side. We varied the distance between the volatile sources and the release points of the larvae between 9 and 18 cm (Fig. 5). We also manipulated CO₂ emission of maize roots in a subset of olfactometers by adding a layer of CO₂-absorbing soda lime into the olfactometer arms. CO₂ measurements revealed that the presence of a host root system increased CO₂ concentrations by approx. 100 ppm above ambient CO₂ levels in the corresponding olfactometer arm (Fig. 5, Fig. S1C). The soda lime reduced ambient CO₂ concentrations in the olfactometer arms by approx. 100 ppm, and equalized CO₂ concentrations between arms with and without a host plant (Fig. 5), thus validating the CO₂ scrubbing approach. Larvae did not have direct access to the plant, the plant growth medium or the soda lime, and received no visual cues, and thus had to rely on host plant volatiles for orientation. When released at distance of 9 cm from the volatile sources, both WT and *DvGr2*-silenced larvae showed a clear preference for the olfactometer arms leading to host plants (Fig. 5A). This preference was still intact in olfactometers outfitted with a soda lime filters, confirming that volatiles other than CO₂ are sufficient for volatile-mediated host location at a short distance. At a distance of 18 cm from the volatile sources, WT larvae showed a similarly strong preference for arms leading to host plants (Fig. 5B). By contrast, *DvGr2*-silenced larvae did not exhibit any preference (Fig. 5B). In the presence of a soda lime filter, neither WT nor *DvGr2*-silenced larvae were attracted to arms with a host plant (Fig. 5B). Taken together, these experiments demonstrate that WCR larvae require plant-derived CO₂ to locate host plants over longer distances in a *DvGr2*-dependent manner.

Figure 5 Plant-derived CO₂ mediates *DvGr2*-dependent long-distance host location. (A, B) Choice (mean±SEM) of WCR larvae in two-arm olfactometers. Control olfactometers allowed for plant-derived CO₂ to diffuse into the choice chamber, while CO₂ scrubber olfactometers were outfitted with soda lime to suppress CO₂ diffusion. ΔCO₂ represents the difference in CO₂ in the arms with plants compared to arms without plants (***p<0.001). For detailed data on CO₂ 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 *DvGr2*-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.

Root-derived CO₂ enhances volatile-mediated host location over longer distances

WCR larvae can move up to 1 m in the soil. Second and third instar larvae in particular are known to move between maize plants across rows in maize fields (*Hibbard et al., 2003*). To test whether *DvGr2*-mediated CO₂ responsiveness mediates host location over longer distances in a soil context, we planted maize plants in soil-filled plastic trays, released WCR larvae at distances of 16, 32, 48 or 64 cm from the maize plants and evaluated larval positions after 8 h (Fig. 6). This time point was chosen based on preliminary observations showing that larvae take approx. 8 h to cross the soil arenas. The CO₂ emitted by maize roots formed a gradient in the soil, starting at about 506 ppm in the rhizosphere (Zone 1), and 430 ppm at distances of 16-32 cm from the plant (Zone 2) (Fig. 6B, Fig. S1F). At distances of more than 32 cm from the plant, the CO₂ levels were around 400 ppm and statistically indistinguishable from soil without plants or ambient air (Fig. S1F). To confirm that larval motility is not altered by *DvGr2*-silencing in a soil context, we first released WT and *DvGr2*-silenced larvae into the middle of a set of arenas without a host plant and evaluated larval positions after 8 h. We found that the larvae dispersed equally across the arenas, without any difference between WT and *DvGr2*-silenced larvae (Fig. 6A). Eight hours after releasing the larvae into arenas that included host plants on one side, 53% of WT larvae that were released at 64 cm from the plant were retrieved from the rhizosphere, i.e.: in Zone 1 (Fig. 6C). In contrast, only 33% of the *DvGr2*-silenced larvae that were released at the same distance were recovered from the maize rhizosphere (Fig. 6C). Significantly more *DvGr2*-silenced larvae were recovered further away from the plants, in zones 3 and 4 (Fig. 6C). The number of WT and *DvGr2*-silenced WCR larvae found close to the host plant increased with decreasing release distance, as did the difference between WT and *DvGr2*-silenced larvae (Fig. 6C-F). At a release distance of 16 cm, only slightly more WT than *DvGr2*-silenced larvae were found close to the plant roots (Fig. 6F). To further confirm the role of *DvGr2* in mediating host plant location over long distances in the soil, we performed a time-course experiment where we released WT and *DvGr2*-silenced larvae in Zone 5 (64 cm away from the host plant) and then recorded how rapidly they reached Zone 1 containing host plants (Fig. S2). The capacity of the larvae to directly feed on the host roots was impeded using a volatile-permeable root barrier (Fig. S2A). Within 10 h, 36% of the released WT larvae were found in Zone 1, and within 32 h, this number had increased to 90% (Fig. S2B). By contrast, only 24% of the released *DvGr2*-silenced larvae were found in Zone 1 after 10 h, and after 32 h, this value had only increased to 56% (Fig. S2B). Thus, the capacity to detect CO₂ gradients contributes to successful host location by WCR larvae in a distance specific manner in the soil.

Figure 6 Root-derived CO₂ enhances WCR host location over long distances in the soil. (A) Mean (±SEM) proportion of WT (dark green) or *DvGr2*-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) CO₂ levels detected in the soil gas phase of each tray zone (n=3-4). Different letters indicate significant differences in CO₂ levels ($p < 0.05$ by one-way ANOVA with Holm's multiple-comparisons test). (C-F) Mean (±SEM) proportion of WT (dark green) or *DvGr2*-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 *DvGr2*-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 CO₂ levels, refer to Fig. S1E.

Root-derived CO₂ is used for host quality assessment by WCR larvae

Plant nutritional status determines plant growth and defense, and can thus modulate plant-herbivore interactions (Wetzel *et al.*, 2016). To test for a possible connection between plant nutritional status, host suitability and CO₂-dependent herbivore attraction, we varied the nutrient supply of maize plants and then carried out CO₂ measurements, and behavioural and insect performance experiments (Fig. 7). Plants that were well fertilized released higher levels of CO₂ into the soil than did plants that received a medium (50 % of optimally fertilized plants) or low (10% of optimally fertilized plants) dose of fertilizer (Fig 7A, B). As observed before, soil CO₂ levels decreased with increasing distance from the plants and were lowest in the middle of the experimental trays (Fig. 7A, B). In choice experiments with maize plants planted approx. 50 cm apart, which corresponds to row spacing used for high planting densities in maize cultivation, WT larvae showed a significant preference for well-fertilized over medium- or low-fertilized plants (Fig 7C, D). *DvvGr2*-silenced larvae did not distinguish between plants subjected to the different fertilizer regimes (Fig. 7C, D). In no-choice experiments, WCR larvae gained most weight on well-fertilized maize plants than on plants treated with medium or low doses of fertilizer (Fig. 7E). Hence, intact CO₂ perception is required for WCR larvae to express host quality related preferences for maize plants growing at agriculturally relevant distances.

Figure 7 Root-derived CO₂ 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) CO₂ levels detected in the soil gas phase of each tray zone (n=10). Different letters indicate statistically significant differences in CO₂ levels ($p < 0.05$ by one-way ANOVA with Holm's multiple comparisons 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 comparisons tests). For P -values of Generalized Linear Models (GLMs), refer to Table S1. For detailed data on CO₂ levels, refer to Fig. S1D.

Discussion

Volatile chemicals have a strong impact on the behaviour and performance of organisms across the tree of life (Arce et al., 2017; Bentley and Day, 1989; Gang and Hallem, 2016; Hansson and Stensmyr, 2011; Johnson and Gregory, 2006; Miller and Strickler, 1984; Veyrat et al., 2016; Webster and Cardé, 2017; Ye et al., 2018). Many organisms, including bacteria, fungi, nematodes, plants, insects, fish and mammals respond to CO₂, for instance (Bahn and Mühlischlegel, 2006; Cummins et al., 2014; Eilers et al., 2012; Hallem and Sternberg, 2008; Hester et al., 2012; Huckstepp and Dale, 2011; Jones et al., 2007; Perry and Abdallah, 2012; Raguso et al., 2005; Stange and Stowe, 1999). However, the role of CO₂ in plant-herbivore interactions is poorly understood and has not been studied *in vivo* through molecular manipulation approaches.

In this study, we conducted gene sequence similarity analyses, phylogenetic relationship reconstructions, RNA interference and behavioural experiments with different setups to explore the biological relevance of root-derived CO₂ for plant-herbivore interactions. We found that the WCR genome contains at least three putative CO₂ receptor-encoding genes: *DvvGr1*, *DvvGr2*, and *DvvGr3*, which is consistent with previous transcriptomic-based studies (Rodrigues et al., 2016). Protein tertiary structure and topology prediction models show that the identified genes code for proteins that contain seven transmembrane domains, which is consistent with the protein topology of gustatory and odor receptors (Dahanukar et al., 2005; Hallem et al., 2006). Larval behaviour and gene silencing based-functional characterization of the three identified WCR putative CO₂ receptor genes revealed that the intact expression of *DvvGr2*, is essential for the attractive effects of CO₂ to WCR larvae. Knocking down *DvvGr2* rendered larvae fully unresponsive to synthetic and plant-derived CO₂. In *Aedes aegypti*, *Helicoverpa armigera* and *Drosophila melanogaster*, both carbon dioxide receptors *Gr1* and *Gr3* are required for CO₂ detection (Erdelyan et al., 2012; Jones et al., 2007; Kwon et al., 2007; McMeniman et al., 2014; Ning et al., 2016; Suh et al., 2004). In *Culex quinquefasciatus*, both *Gr2* and *Gr3* carbon dioxide receptors are required, while *Gr1* act as a modulator (Xu et al., 2020). In *Aedes aegypti*, the involvement of *Gr2* in carbon dioxide responsiveness is still under debate (Erdelyan et al., 2012; Kumar et al., 2019). Taken together, the molecular elements required for carbon dioxide perception may be species-specific. Our results support this notion, as *DvvGr2*, but not *DvvGr1* and *DvvGr3*, are crucial for CO₂ responsiveness. The role of *DvvGr1* and *DvvGr3* for WCR remains to be determined, but their presence and expression may hint at additional complexity in developmental and/or tissue-specific patterns of CO₂ responsiveness in this species.

Despite the inability of *DvvGr2*-silenced WCR larvae to respond to differences in CO₂ levels, the larvae were still able to orient towards maize roots at short distances of 8-10 cm. Olfactometer experiments in combination with a CO₂ scrubber that removes plant-derived CO₂ demonstrates that other volatile cues can be used by WCR larvae to locate maize plants at short distances. Earlier studies found that (*E*)- β -caryophyllene, which is emitted from the roots of certain maize genotypes when they are attacked by root herbivores, attracts second and third instar WCR larvae and allows them to aggregate on maize plants and thereby enhance their fitness (Robert et al., 2012b), while

neonate larvae are not attracted to this volatile (*Hiltpold and Hibbard, 2016*). Ethylene has also been shown to attract WCR larvae (*Robert et al., 2012a*), and MBOA or its breakdown products have also been proposed as volatile attractants (*Bjostad and Hibbard, 1992*). Methyl anthranilate on the other hand has been shown to repel WCR larvae (*Bernklau et al., 2016b; Bernklau et al., 2018a*). Many other leaf- and root feeding herbivores are known to respond to plant volatiles other than CO₂ (*Bruce et al., 2005*). Given the low reliability of CO₂ as a host-specific cue, it is probably not surprising that WCR, as a highly specialized maize feeder, can use other volatile cues to locate host plants. Integrating other volatile cues likely allows WCR larvae to locate maize plants even in the absence of reliable CO₂ gradients in the soil, thus increasing the robustness of its foraging behaviour at short distances. An intriguing result in this context is the fact that WCR larvae show the same efficiency in locating maize roots at short distances in the absence of a CO₂ gradient, suggesting that this volatile may not play a role as a cue at close range.

Although intact CO₂ perception was not required for host location at short distances, it had a strong impact on the capacity of WCR larvae to reach the maize rhizosphere at long distances. The advantages of CO₂ are that it is abundantly produced through respiration by most organisms, that it is relatively stable (*Jones and Coaker, 1977; Li et al., 2016*), and that it diffuses rapidly in air, water and soil (*Hashimoto and Suzuki, 2002; Ma et al., 2013*). CO₂ may thus be a suitable long-range cue to locate organisms with high respiratory rates, such as mammals and heterotroph plant parts, including roots (*Johnson and Nielsen, 2012*). Aboveground insects can be attracted to CO₂ traps located as far away as 10 m, and it is estimated that this distance could even be as long as 60 m under optimal environmental circumstances (*Guerenstein and Hildebrand, 2008; Zollner et al., 2004*). For belowground insects, this distance is hypothesized to be within the lower cm-range as CO₂ diffusion is substantially decreased within the soil matrix compared to the air (*Bernklau et al., 2005; Doane et al., 1975; Doane and Klingler, 1978; Klingler, 1966*). Other volatiles that are less abundant and diffuse even less well through the soil such as (E)- β -caryophyllene are unlikely to be detectable at distances of more than 10 cm (*Chiriboga et al., 2017; Hiltpold and Turlings, 2008*). In support of this, we did not succeed in the past to detect maize volatiles in the soil at distance of more than 10 cm, even using a highly sensitive combination of solid-phase microextraction (SPME) and gas chromatography/mass spectrometry (GC/MS). These volatiles are thus likely useful host location cues at short, but not long distances in the soil. The finding that WCR integrates CO₂ perception with other environmental cues and that attraction to CO₂ is context dependent is in line with patterns reported for other insects such as mosquitoes, whose response to stimuli such as color, temperature and human body odors is enhanced by CO₂ (*McMeniman et al., 2014; van Breugel et al., 2015*), and pollinating hawkmoths, that use CO₂ as a redundant volatile distance stimulus in a sex-specific manner (*Goyret et al., 2008*).

A recent study shows that a CO₂ receptor in *Drosophila* flies is also involved in the detection and behavioral responses to other volatiles (*MacWilliam et al., 2018*). We observed that *DvGr2*-silenced larvae were repelled by methyl anthranilate, a potent maize root repellent, to a similar extent than WT larvae, suggesting that their

sensitivity to other plant volatiles is unchanged (*Bernklau et al., 2016b*). In *Drosophila* flies, the CO₂ receptor *Gr63a* is required for spermidine attractiveness over short time spans, i.e.: less than 1 min, but not over longer time spans (hours), when other receptors likely become more important (*MacWilliam et al., 2018*). In the present experiments, WCR behaviour was evaluated after one or more hours. The CO₂ scrubber experiment provides further evidence that the foraging patterns observed in this study are not due to different sensitivity of *DvGr2*-silenced larvae to other root volatiles.

Apart from acting as a long-distance host location cue, CO₂ also links plant fertilization to herbivore behaviour by guiding WCR to well fertilized plants. As WCR larvae are resistant to root defenses of maize (*Robert et al., 2012b*), it is likely to benefit from increased fertilization, independently of the plant's defensive status. As the plant nutritional status and host quality for WCR larvae is associated with higher CO₂ release from the roots, following the highest concentrations of CO₂ in the soil may be adaptive for the herbivore, as it may increase its chance not only to find a maize plant *per se*, but also to identify a plant that has the resources to grow vigorously and that is a better host. WCR larvae are specialized maize pests that have evolved with intense maize cultivation in the corn belt of the US (*Gray et al., 2009*) and are resistant to maize defense metabolites (*Robert et al., 2012b*). Following the strongest CO₂ gradient in an equally spaced maize monoculture may indeed be a useful strategy for this root feeder to locate suitable food sources. An association between CO₂ emission and food-source profitability was also suggested for *Datura* flowers, that emit the highest level of CO₂ in times when nectar is most abundant (*Guerenstein et al., 2004; Thom et al., 2004*). These findings support the hypothesis that CO₂ is used as a marker of metabolic activity that allows for an assessment of the vigour and profitability of a wide variety of hosts.

In summary, this work demonstrates how an herbivore uses its capacity to perceive CO₂ to locate host plants and to distinguish plants of different quality. Volatiles other than CO₂ are also integrated into host finding behaviour in the soil, but their effects are more important at shorter distances. Thus, evidence is now accumulating that CO₂ acts as a fitness-relevant host location cue in different insects, likely because of its unique role as a highly conserved long-range marker of metabolic activity within complex sensory landscapes.

Materials and Methods

Plants and planting conditions

Maize seeds (*Zea mays* L., var. Akku) were provided by Delley Semences et Plantes SA (Delley, CHE). Seedlings were grown under greenhouse conditions (23 ± 2°C, 60% relative humidity, 16:8 h L/D, and 250 mmol/m²/s¹ additional light supplied by sodium lamps). Plantaaktiv® 16+6+26 Typ K fertilizer (Hauert HBG Dünger AG, Grossaffoltern, Switzerland) was added twice a week after plant emergence following the manufacturer's recommendations. The composition of the fertilizer is: total nitrogen (N) 16%, nitrate 11%, ammonium 5%, phosphate (P₂O₅) 6%, potassium oxide (K₂O) 26%, magnesium oxide (MgO) 3.3%, boron (B) 0.02%, copper (Cu, EDTA-chelated) 0.04%, iron (Fe, EDTA-chelated) 0.1%, manganese (Mn, EDTA-chelated) 0.05%, molybdenum

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

Insects and insect rearing

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

Identification of CO₂ receptor genes

To identify CO₂ receptor orthologues in WCR, we used CO₂ receptor-encoding gene sequences of *Tribolium castaneum* and several sequences from other insects as queries against publicly available WCR genome sequences (NCBI accession: PXJM000000000.2) using the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST)(*Robertson and Kent, 2009; Wang et al., 2013; Xu and Anderson, 2015*). The full gene sequences can be retrieved from the National Center for Biotechnology Information (NCBI) data bank using the following accession numbers: XM_028276483.1 (*DvGr1*), XM_028280521.1 (*DvGr2*), and XM_028272033.1 (*DvGr3*). These gene sequences were translated to obtain protein sequences. The obtained protein sequences and the protein sequences of CO₂ receptors from different insects were used to infer evolutionary relationships using the Neighbor-Joining method in MEGA 7 (*Kumar et al., 2016; Robertson and Kent, 2009; Rodrigues et al., 2016; Saitou and Nei, 1987*). The optimal tree with the sum of branch length = 4.44068889 is provided in Fig 1a. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (*Felsenstein, 1985*). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (*Zuckerkandl and Pauling, 1965*) and are in the units of the number of amino acid substitutions per site. A total of 242 amino acid positions were included in the final dataset. Graphical representation and edition of the phylogenetic tree were performed with the Interactive Tree of Life (version 3.5.1)(*Letunic and Bork, 2016*). Protein tertiary structures and topologies were predicted using Phyre2 (*Kelley et al., 2015*).

Production of double stranded RNA

Escherichia coli HT115 were transformed with recombinant L4440 plasmids that contained a 211-240 bp long gene fragment targeting one of the three CO₂ receptors. Cloned nucleotide sequences were synthesized *de novo* (Eurofins, Germany). To induce the production of dsRNA, an overnight bacterial culture was used to inoculate fresh Luria-Berthani broth (25 g/L, Luria/Miller, Carl Roth GmbH, Karlsruhe, Germany). Once the bacterial

culture reached an OD₆₀₀ of 0.6-0.8, it was supplemented with isopropyl β-D-1-thiogalactopyranoside, IPTG) (Sigma-aldrich, Switzerland) at a final concentration of 2mM. Bacterial cultures were incubated at 37°C in an orbital shaker at 130 rpms for 16 additional hours. Bacteria were harvested by centrifugation and stored at -20°C for further use (*Kim et al., 2015*).

Gene silencing experiments

To induce gene silencing in WCR, six to ten second instar WCR larvae were released in solo cups (30 mL, Frontier Scientific Services, Inc., Germany) containing approx. 2g of autoclaved soil (Selmaterre, Bigler Samen AG, Thun, Switzerland) and 2-3 freshly germinated maize seedlings. Maize seedlings were coated with 1 ml of bacterial solution containing approximately 200-500 ng of dsRNA, targeting the different CO₂ receptor genes. As controls, larvae were fed with bacteria producing dsRNA targeting green fluorescent protein genes (GFP), which are absent in the WCR genome (*Rodrigues et al., 2016*). Fresh bacteria and seedlings were added to solo cups every other day for three consecutive times. Two days after the last dsRNA/bacteria application, larvae were collected and used for experiments.

Gene expression measurements

Total RNA was isolated from approximately 10 mg of frozen, ground and homogenized WCR larval tissue (3-7 larvae per biological replicate, n=8) using the GenElute Universal Total RNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA). A NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA) was used to estimate RNA purity and quantity. DNase-treated RNA was used as template for reverse transcription and first strand cDNA synthesis with PrimeScript Reverse Transcriptase (Takara Bio Inc., Kusatsu, Japan). DNase treatment was carried out using the gDNA Eraser (Perfect Real Time) following manufacturer's instructions (Takara Bio Inc.). For gene expression analysis, 2 µl of undiluted cDNA (i.e. the equivalent of 100 ng total RNA) served as template in a 20 µl qRT-PCR using the TB Green Premix Ex Taq II (Tli RNaseH Plus) kit (Takara Bio Inc.) and the Roche LightCycler 96 system (Roche, Basel, Switzerland), according to manufacturer's instructions. Transcript abundances of the following WCR genes were analysed: *DvGr1*, *DvGr2*, and *DvGr3* (*Rodrigues et al., 2016*). *Actin* was used as reference gene to normalize expression data across samples. Relative gene expression levels were calculated by the 2^{-ΔΔCt} method (*Livak and Schmittgen, 2001*). The following primers were used: *DvGr1*-F CGTTAATTTAGCTGCTGTGG, *DvGr1*-R GTTTTCTGTTGCTAGAGTTGC, *DvGr2*-F GAACTAAGCGAGCTCCTCCA, *DvGr2*-R CAGAAGCACCATGCAATACG, *DvGr3*-F GCAACGCTTTCAGCTTTACC, *DvGr3*-R GTGCATCGTCATTCATCCAG, *DvActin*-F TCCAGGCTGTACTCTCCTTG, and *DvActin*-R CAAGTCCAAACGAAGGATTG.

Belowground olfactometer experiments with *DvGr1*-, *DvGr2*-, *DvGr3*-silenced WCR larvae

To determine whether silencing putative CO₂ receptor genes impairs the ability of WCR larvae to behaviourally respond to CO₂, we silenced *DvGr1*, *DvGr2*, and *DvGr3* as described above and conducted behavioural experiments using belowground olfactometers. Larvae that were fed bacteria expressing dsRNA targeting green fluorescent protein (GFP) were used as controls (herein referred as wild type larvae; WT). We conducted choice experiments using two-arm belowground olfactometers with carbonated water as a CO₂ source (*Bernklau and Bjostad, 1998a; Huang et al., 2017; Jewett and Bjostad, 1996*). For this, a plastic cup containing 50 ml of carbonated water was placed in one arm of the olfactometer and a plastic cup containing 50 ml of distilled water was placed in the opposite arm (Fig. 2B). Five larvae were released in the middle of the olfactometer (indicated by a red arrow) and their positions were recorded 1h after their release. Seven olfactometers per larval type and experiment were assessed. The experiment was repeated twice. CO₂ levels on each arm of the olfactometer were measured by gas chromatography coupled to a flame ionization detector (GC-FID) as described (*Zhang et al., 2019*).

Insect behavioural experiments

To determine larval responses to plant volatile organic compounds other than CO₂, we evaluated insect behaviour to methyl anthranilate. For this, we evaluated insect preferences for seedling roots or for seedling roots placed next to a filter paper disc treated with methyl anthranilate in choice experiments following a similar experimental procedure as described (*Bernklau et al., 2018a*). To this end, five 2nd-3rd instar WCR larvae were released in the middle of a sand-filled petri plate (9 cm diameter, Greiner Bio-One GmbH, Frickenhausen, DE) where they encountered seedling roots in one side or seedlings roots and a filter paper disc treated with 10 µl of methyl anthranilate solution (10 mg/ml of water) in the other side. Ten Petri plates per larval type with five larvae each were evaluated (n=10). Methyl anthranilate was purchased from Sigma (CAS: 134-20-3; Sigma Aldrich Chemie, CHE). Larval preferences were recorded 1h after releasing the larvae.

To investigate larval responses to non-volatile root chemicals, we tested two well established attractants, Fe(III)(DIMBOA)₃ and a sugar blend (*Bernklau et al., 2018b; Hu et al., 2018*). To evaluate the attractive effects of Fe(III)(DIMBOA)₃, we released six 2nd-3rd instar WCR larvae in the middle of a moist sand-filled Petri plate (6 cm diameter, Greiner Bio-One GmbH, Frickenhausen, DE) where they had a choice between a filter paper disc 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 water only. Twenty Petri plates with six larvae each were evaluated (n=20). Fe(III)(DIMBOA)₃ was prepared fresh by mixing FeCl₃ and DIMBOA at a 1:2 ratio as described. Larval preferences were recorded 1h after releasing the larvae. To evaluate attractive effects of soluble sugars for WCR larvae, we followed the procedure described by (*Bernklau et al., 2018b*). Briefly, we released six 2nd-3rd instar WCR larvae in the middle of a moist sand-filled petri plate (6 cm diameter, Greiner Bio-One GmbH, Frickenhausen, DE) where they had a choice between a filter

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

To evaluate the attractiveness of CO₂ for WCR larvae, we conducted choice experiments using two-arm belowground olfactometers. CO₂ levels were increased in one arm of the olfactometer by delivering CO₂-enriched synthetic air (1% CO₂, Carbagas, Switzerland). Olfactometer arms were closed on top using parafilm during CO₂ delivery. A manometer connected to the synthetic air bottle allowed to fine-tune CO₂ delivery rates. CO₂ levels were measured using a gas analyzer (Li7000, Li-Cor Inc., Lincoln, Nebraska, USA). CO₂ levels were increased at different levels from 22 to 1832 ppm above ambient CO₂ levels. Once the desired CO₂ concentrations were reached, CO₂ delivery was terminated, olfactometer arms were connected to olfactometer central connectors and six 2nd-3rd instar WCR larvae were release immediately, and their choice were evaluated within ten minutes of release. Three olfactometers per larval type were assayed (n=3).

To assess the impact of *DvGr2* on larval motility, we followed the trajectories of individual larvae in Petri plate that were outfitted with a CO₂ point releaser. For this, Petri plates (9 cm diameter, Greiner Bio-one, Austria) were pierced in their centers to allow the insertion of a needle that released CO₂ at 60 ppm above ambient level. Larvae were released at the rim of the Petri plates (4.5 cm apart from CO₂ source). Petri plates were overlaid with moist filter paper (9cm diameter, GE Healthcare, UK). Insect behavior was observed for three minutes. Six Petri plates with one larva each were assayed (n=6). In a second experiment, we followed the trajectories of individual larvae in Petri plate with maize roots. For this, two root pieces (3-4 cm long) of four-day-old maize seedlings were placed at the rim of a Petri plate overlaid with moist filter paper. Then, on the opposite rim (9cm apart), we released one 2nd-3rd instar WCR larva and followed their trajectories during three minutes. Six Petri plates with one larva each were assayed (n=6). Crawled distances were measured using ImageJ 1.53a.

Host location experiments

To determine the importance of plant-derived CO₂ for distance-dependent host-location by WCR larvae, we used two experimental setups: two-arm belowground olfactometers and soil-filled trays (Fig. 5, Fig. 6). For behavioural experiments using belowground olfactometers, we used two types of olfactometers: one with short arms that allowed for the release of larvae at 9 cm (Fig. 5A) from plant volatile sources, and one with long arms that allow to release the larvae at 18 cm (Fig. 5B) from plant volatiles sources. To specifically investigate the importance of plant derived CO₂, we contrasted insect behavioural responses to intact plant odours and to plant odours without CO₂. CO₂ was experimentally removed using soda lime (Carl Roth, Karlsruhe, Germany). For this, a layer of 5g of soda lime granules (2-4mm) were placed between Teflon connectors and sand 30 min before the experiments. Olfactometers without soda lime served as controls. Olfactometer arms were filled with sand 48h before the experiment and one three-week-old maize plant was transplanted to one arm of the olfactometer. Four

olfactometers with six larvae each were assayed (n=4). CO₂ levels were measured using a gas analyzer (Li7000, Li-Cor Inc., Lincoln, Nebraska, USA). For behavioural experiments using soil-filled trays, second instar WCR larvae were released at 16, 32, 48, or 64 cm from four or five 2-to 3-week-old maize plants. Larval release points are indicated by colour arrows (Fig. 6B). Plants were planted in plastic trays (80cmx15cmx4.5cm)(Migros do it + garden, Switzerland) filled with soil. Eight h after releasing the larvae, their positions were recorded. Six trays per larval type and distance with twenty larvae each were assayed (n=6). CO₂ levels in the soil gas phase of each tray zone were measured by GC-FID as described (*Zhang et al., 2019*).

Host finding behavioural experiments with nutrient-deficient plants

Optimally fertilized plants emit higher levels of respiratory CO₂ than sub-optimally fertilized plants (*Zhu and Lynch, 2004*). To test whether WCR larvae orient towards and prefer optimally fertilized maize plants and to evaluate the importance of CO₂ detection through *DvvGr2* in this context, we silenced this gene and conducted choice experiments to contrast larval preference for plants that were differentially fertilized. To this end, we fertilized maize plants with three doses of fertilizer: 0.1% (optimally fertilized), 0.05% (medium fertilized), or 0.01% (low fertilized). Plantaaktiv® 16+6+26 Typ K fertilizer (Hauert HBG Dünger AG, Grossaffoltern, Switzerland) was dissolved to the abovementioned concentrations and applied following manufacture's indications. Fertilizer macro- and micronutrient composition are described above (Plants and planting conditions). For the choice experiments, plants were grown under the different fertilizer regimes. 24 hr before the choice experiment, five 15-day-old, optimally fertilized plants were transferred and re-planted in one corner of an 80 cm long plastic tray (Migros do it + garden, Switzerland) filled with soil (Figs 5a, b). At the opposite side, plants grown either under low or medium fertilizer regimes were equally transferred and re-planted (Figs 7A, B). Then, twenty 2th instar WCR larvae were released in the middle of the tray (Zone 3, Fig 6A, B, red arrows). Six independent trays per larval type and fertilizer regime pair were evaluated (n=6). Eight hours after releasing the larvae, larval positions were recorded.

Effect of plant nutritional status on larval growth

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

Statistical analyses

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

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Author contributions

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 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, 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.; Writing – review & editing, R.A.R.M., M.E., B.C.J.S., and C.C.M.A.

Declaration of interests

The authors declare no competing interests

Data availability

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

Supplementary Figures

Figure S1 Carbon dioxide measurements for the different experiments. Mean ($\pm$ SEM) CO₂ levels measured in behavioural experiments of Figure 2 (A), Figure 3 (B), Figure 5 (C), Figure 7 (D, E), Figure 6 (F). Different letters indicate significant differences in CO₂ levels ($p < 0.05$ by one-way ANOVA with Holm's multiple-comparisons test). Asterisks indicate significant differences in CO₂ levels (***: $p < 0.001$ by ANOVA with Holm's multiple-comparisons test). Gr2: *DvGr2*-silenced larvae. For details on CO₂ sampling points, refer to the main figures.

Figure S2 Root-derived CO₂ is required for host location by WCR larvae at long distances. (A) Schematic representation (photomontage) of experimental set up used. (B) Mean ($\pm$ 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 comparisons test). For P -values of the different factors and their interactions, refer to Table S1.

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