# 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 larval responses to $CO_2$. Silencing *DvvGr2* has no major effect on the ability of WCR larvae to locate host plants at short distance (<10 cm), but increasingly impairs host location at greater distances, suggesting that WCR larvae integrate $CO_2$ with other volatile cues for host finding in a distance-specific manner. We further 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) root herbivores have been found to use other plant-released chemicals 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., 2015a; 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., 2015b; 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, we then assessed the importance of $CO_2$ perception for WCR behaviour and foraging. 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 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 publicly available WCR genome sequences available from the National Center for Biotechnology Information (NCBI). Three putative carbon dioxide receptor candidates, *DvvGr1, DvvGr2,* and *DvvGr3*, were identified, matching the three candidates that were identified earlier using transcriptome data (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. A similar trend was observed for *DvvGr3* (Fig 1b). Protein tertiary structure and topology models indicated that, consistent with their roles as receptors, all three genes encode for 7-transmembrane domain proteins (Fig 1c-e).

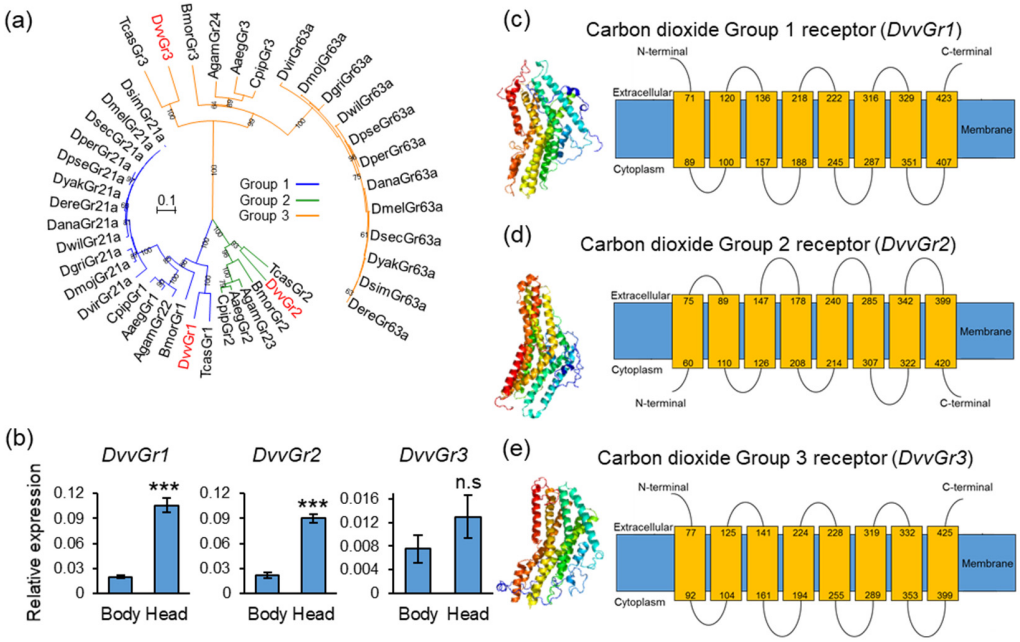


**Figure 1** *The WCR genome contains at least three putative carbon dioxide ($CO_2$) receptor-encoding genes.* (a) Phylogenetic relationships between putative $CO_2$ receptors based on protein sequences of different insects. Dmel: *Drosophila melanogaster*, Dsim: *Drosophila simulans*, Dsec: *Drosophila 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).* (b) Mean (±SEM) relative gene expression levels of group 1 (*DvvGr1*), group 2 (*DvvGr2*), or group 3 (*DvvGr3*) $CO_2$ 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). (c-e) Predicted protein tertiary structure (left) and transmembrane protein topology (right) of: (c) *DvvGr1*; (d) *DvvGr2*; and (e) *DvvGr3*. Models were predicted using Phyre2.

### *DvvGr2* expression is required for behavioural responses of WCR larvae to $CO_2$ gradients

To determine the importance of *DvvGr1, DvvGr2,* and *DvvGr3* for the responsiveness of WCR larvae to $CO_2$, we silenced the expression of each gene individually through double-stranded RNA (dsRNA)-mediated RNA interference (RNAi) and conducted behavioural experiments with carbonated water as a $CO_2$ source (Fig 2a). Olfactometer arms containing carbonated water released approximately 100 ppm more $CO_2$ than their counterparts filled with distilled water (Fig 2b). 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 2c). All RNAi constructs were confirmed to be gene specific (Fig 2c). In belowground olfactometer experiments, WT larvae preferred olfactometer arms with carbonated water (Fig 2d). Silencing *DvvGr1* or *DvvGr3* expression did not significantly alter this behaviour. In contrast, silencing *DvvGr2* disrupted the attractive effects of $CO_2$, as *DvvGr2*-silenced larvae showed no preference for carbonated over distilled water (Fig 2d). Hence, *DvvGr2* expression is necessary for WCR larvae to respond to differences in environmental $CO_2$.

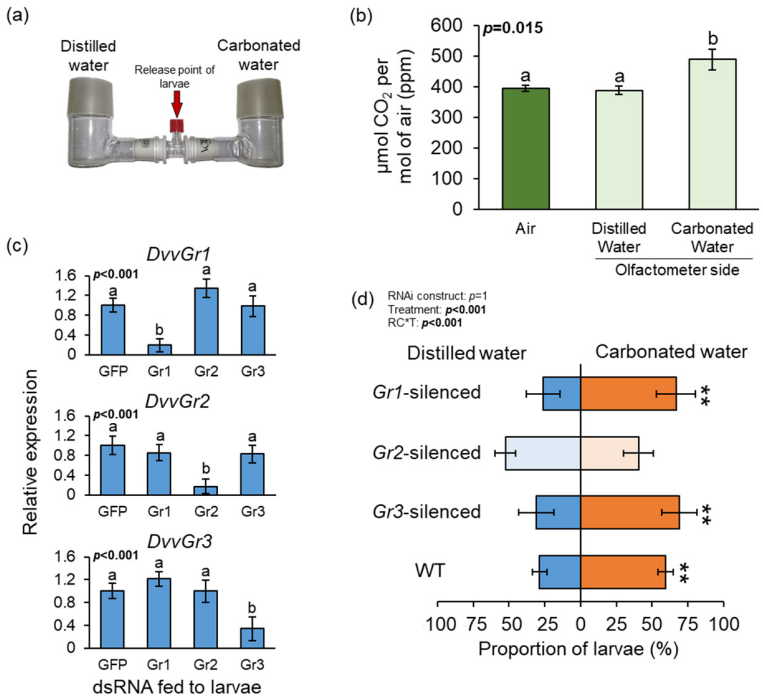


**Figure 2** *The carbon dioxide group 2 receptor (DvvGr2) is required for behavioral responses of WCR to $CO_2$.* (a) Schematic representation of the two-arm belowground olfactometer used for behavioral experiments. (b) Mean (±SEM) environmental $CO_2$ levels (air) and $CO_2$ levels in each arm of the two-arm belowground olfactometers (n=4-8). Different letters indicate significant differences in $CO_2$ levels ($p<0.05$ by one-way ANOVA with Holm's multiple-comparisons test). (c) Mean (±SEM) relative gene expression levels of group 1 (*DvvGr1*), group 2 (*DvvGr2*), or 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). (d) 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 olfactometer pairs with six larvae each were assayed (n=7). Asterisks indicate statistically significant differences between treatments (*: $p<0.05$; **: $p<0.01$ by two-way ANOVA followed by FDR-corrected post hoc tests). *P*-values of Generalized Linear Models (GLMs) are given for different factors and their interactions.

**Root-derived $CO_2$ is not required for volatile-mediated host location at short distances**

To test whether root-derived $CO_2$ mediates host location by WCR larvae at short distances, we conducted behavioural experiments using sand-grown maize plants in belowground olfactometers (Fig 3a). This experimental setup allowed us to release WCR larvae at a distance of 8-10 cm from the maize root system and to assess their behaviour in response to root volatiles while excluding visual and tactile cues. Olfactometer arms containing sand-grown plants released approximately 80 ppm more $CO_2$ than olfactometer arms containing sand only (Fig 3b). Both WT and *DvvGr2*-silenced larvae showed a clear preference for olfactometer arms containing a plant (Fig 3c,d). Over the duration of the experiment, the preference of WT larvae was slightly more pronounced than that of *DvvGr2*-silenced larvae (Fig 3d). Thus, the overall capacity to detect differences in $CO_2$ has only a minor effect on host location by WCR larvae at distances shorter than 10 cm.

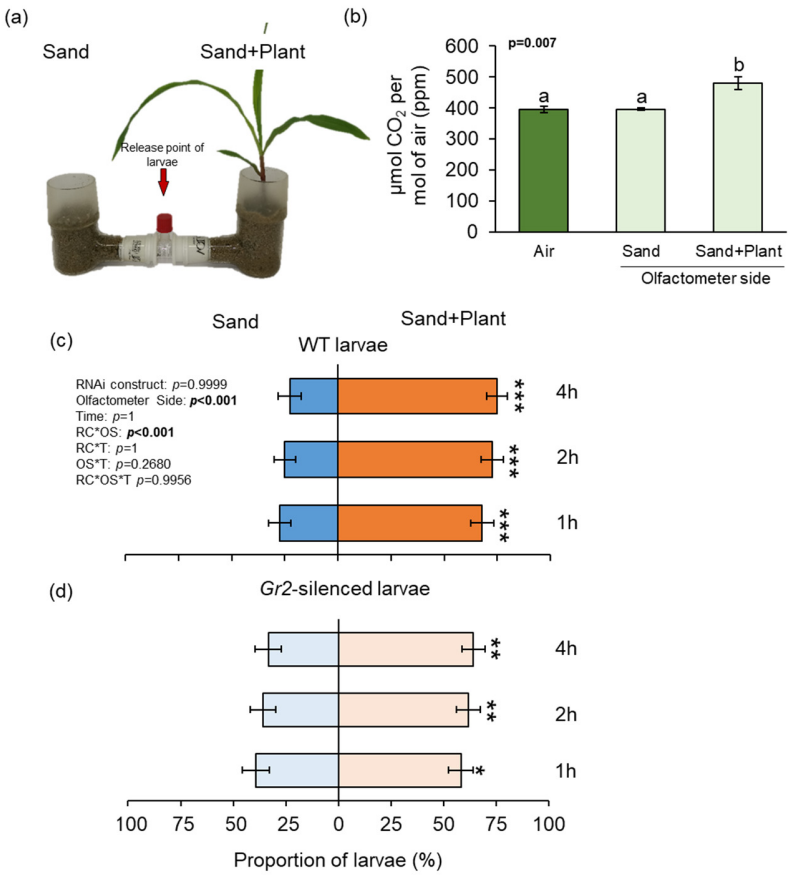


**Figure 3** *Root-derived CO2 is not required for volatile-mediated host location at short distances.* (a) Schematic representation of the two-arm belowground olfactometers used for behavioral experiments. (b) Mean (±SEM) $CO_2$ levels in the air and in each arm of the two-arm belowground olfactometers (n=5-8). Different letters indicate significant differences in $CO_2$ levels ($p<0.05$ by one-way ANOVA with Holm's multiple comparisions test). Mean (±SEM) proportion of: (c) WT, or (d) Gr2-silenced WCR larvae observed in arms with higher $CO_2$ levels (plants growing in sand side) and in control arms (sand only side) 1, 2 or 4h after release. Fourteen independent olfactometer pairs with five larvae each were assayed (n=14). Asterisks indicate statistically significant differences between treatments within time point and RNAi construct (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ by three-way ANOVA followed by FDR-corrected post hoc tests). *P*-values of Generalized Linear Models (GLMs) are given for different factors and their interactions.

**Root-derived $CO_2$ 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 *DvvGr2*-mediated $CO_2$ responsiveness is required for volatile-mediated host location over distances greater than 10 cm, 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 4a). This time point was chosen based on preliminary observations showing that larvae take approx. 8 h to cross the soil arenas. The $CO_2$ emitted by maize roots formed a gradient in the soil, starting at about 500 ppm in the rhizosphere (Zone 1), and 430 ppm at distances of 16-32 cm from the plant (Zone 2) (Fig 4b). At distances of more than 32 cm from the plant, the $CO_2$ levels were around 400 ppm and statistically indistinguishable from soil without plants or ambient air (Fig 4b). Eight hours after their release, 53% of WT larvae that were released at 64 cm from the plant were retrieved from the rhizosphere, i.e.: in Zone 1 (Fig 4c). In contrast, only 33% of the *DvvGr2*-silenced larvae that were released at the same distance were recovered from the maize rhizosphere (Fig 4c). Significantly more *DvvGr2*-silenced larvae were recovered further away from the plants, in zones 3 and 4 (Fig 4c). The number of WT and *DvvGr2*-silenced WCR larvae found close to the host plant increased with decreasing release distance, as did the difference between WT and *DvvGr2*-silenced larvae (Fig 4c-f). At a release distance of 16 cm, only slightly more WT than *DvvGr2*-silenced larvae were found close to the plant roots (Fig 4f). To test whether the observed behaviour can be explained by reduced locomotory responses of *DvvGr2*-silenced larvae, we released either WT or *DvvGr2*-silenced larvae in soil-filled plastic trays without plants and evaluated larval dispersal patterns (Fig S1). We observed that after 8 h, all larvae had dispersed evenly in the soil-filled trays. No differences in dispersal patterns between WT and *DvvGr2*-silenced larvae were observed (Fig S1). To determine whether *DvvGr2*-silencing impairs larval responses to other plant volatiles, we evaluated insect behaviour to methyl anthranilate, a known repellent of the western corn rootworm (***Bernklau et al., 2016b; Bernklau et al., 2018a***). Both WT and *DvvGr2*-silenced larvae showed similar aversive behaviour to methyl anthranilate (Fig S2). Thus, the capacity to detect $CO_2$ gradients contributes to host location by WCR larvae in a distance specific manner and is more important at long than at short distances.

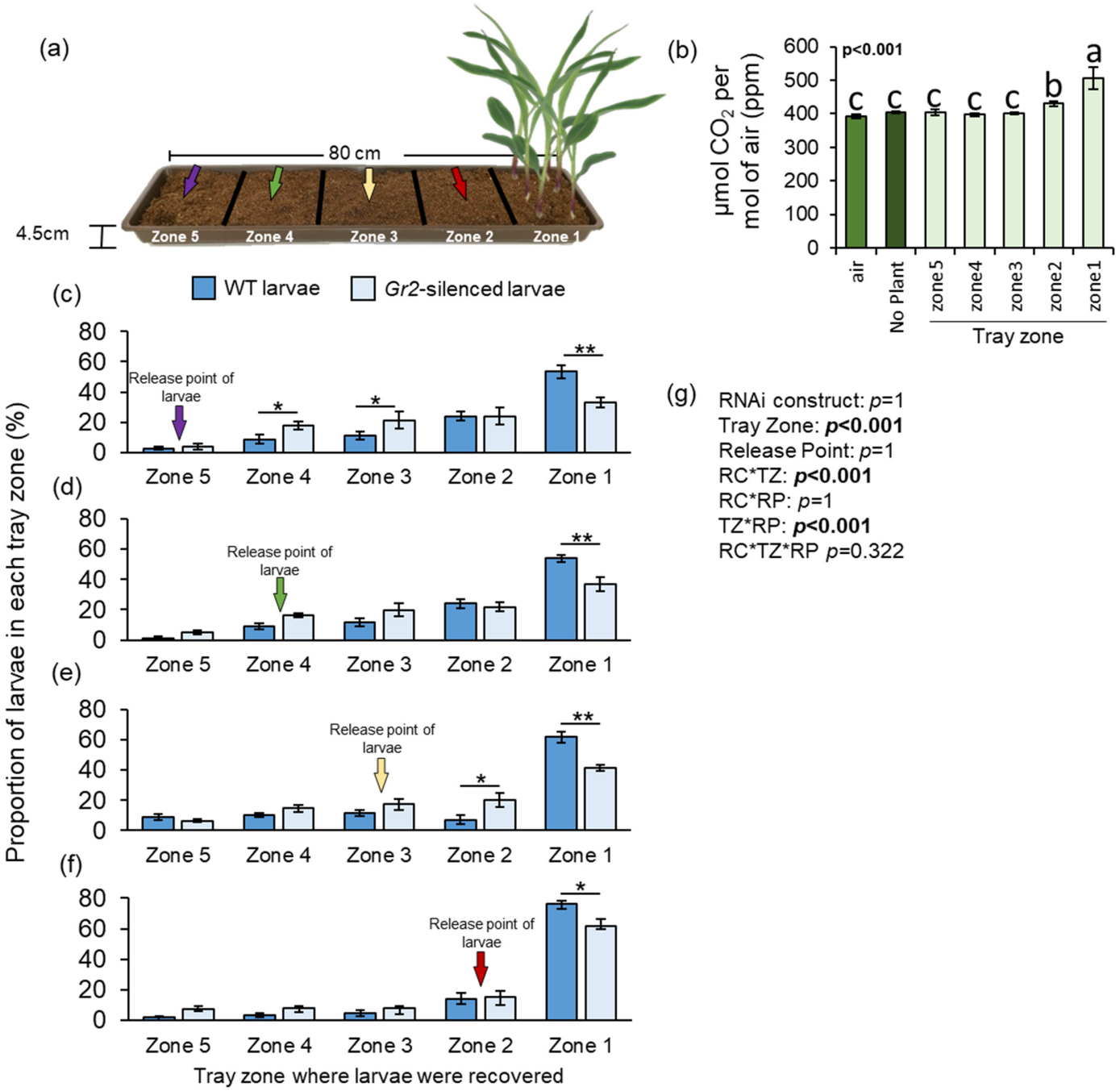


**Figure 4** *Root-derived $CO_2$ enhances WCR host location over long distances.* (a) Schematic representation (photomontage) of experimental setup used for behavioral experiments. (b) Mean (±SEM) $CO_2$ levels in the air, in the soil gas phase of trays without plants (no plant), or in the gas phase of soil-filled trays with plants (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 or *DvvGr2*-silenced 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. Twenty WT or twenty *DvvGr2*-silenced larvae were released in each tray. Six identical trays per larval type and distance 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). (g) *P*-values of Generalized Linear Models (GLMs) are given for different factors and their interactions.

**Root-derived $CO_2$ 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 $CO_2$-dependent herbivore attraction, we varied the nutrient supply of maize plants and then carried out $CO_2$ measurements, and behavioural and insect performance experiments (Fig 5). Plants that were well fertilized released higher levels of $CO_2$ 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 5c,d). As observed before, soil $CO_2$ levels decreased with increasing distance from the plants and were lowest in the middle of the experimental trays (Fig 5c,d). 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 5e,f). *DvvGr2*-silenced larvae were again less successful in reaching the rhizosphere of the plants, and did not distinguish between well-, medium- or low-fertilized plants (Fig 5g,h). 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 5i). Hence, intact $CO_2$ perception is required for WCR larvae to express host quality related preferences for maize plants growing at agriculturally relevant distances.

**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 $CO_2$, for instance (***Bahn and Mühlschlegel, 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 $CO_2$ 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 to explore the biological relevance of root-derived $CO_2$ for plant-herbivore interactions. We found that the WCR genome contains at least three putative $CO_2$ 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 $CO_2$ receptor genes revealed that the intact expression of only one, *DvvGr2*, is crucial for the attractive effects of $CO_2$ to WCR larvae. In contrast, silencing either *DvvGr1* or *DvvGr3* did not disrupt $CO_2$ attractiveness in WCR. In *Aedes aegypti, Helicoverpa armigera* and *Drosophila melanoganster*, both carbon dioxide receptors *Gr1* and *Gr3* are required for $CO_2$ 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***).

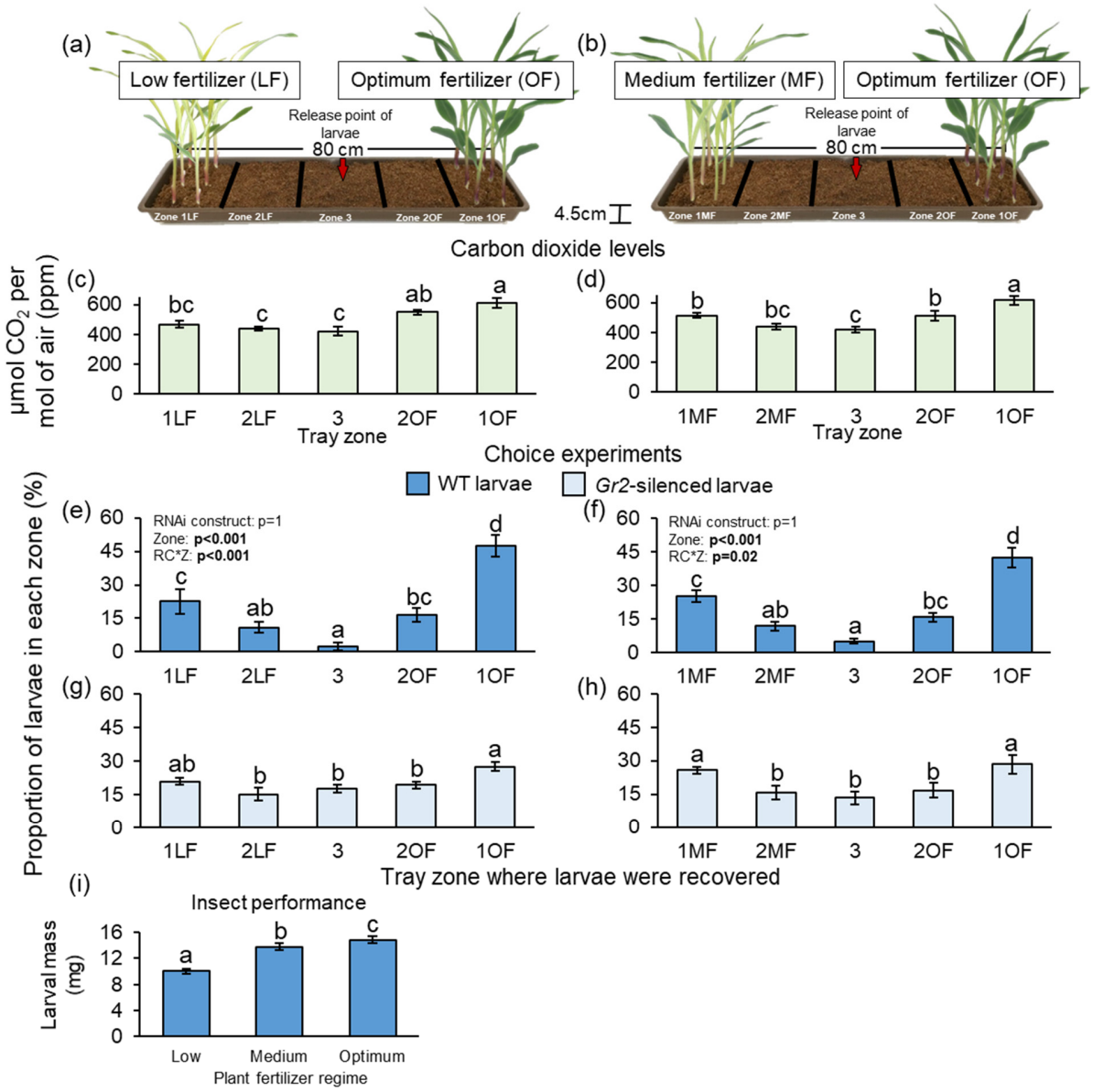


**Figure 5** *Root-derived CO2 is used for host quality assessment by WCR larvae.* Schematic representation (photomontage) of experimental set up used to evaluate insect preference for plants fertilized with: (a) low or optimum fertilizer doses, (b) medium or optimum fertilizer doses. (c, d) Mean (±SEM) $CO_2$ levels contained in the soil gas phase at different tray zones (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). Mean (±SEM) proportion of: WT (e, f), or *DvvGr2*-silenced (g, h) WCR larvae in the different tray zones in choice experiments contrasting larval preferences for plants fertilized with low or optimum fertilizer doses (e, g), or for plants fertilized with medium or optimum fertilizer doses (f, h). Six trays with twenty WT or twenty *DvvGr2*-silenced larvae per tray were assayed (n=6). Different letters indicate statistically significant differences in the proportion of larvae found in each tray zone ($p<0.05$ by two-way ANOVA followed by FDR-corrected post hoc tests). *P*-values of Generalized Linear Models (GLMs) are given for different factors and their interactions. (i) 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). LF: Low fertilizer; MF: medium fertilizer; OF: optimum fertilizer dose.

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 $CO_2$ 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 $CO_2$ responsiveness in this species.

Despite the inability of *DvvGr2*-silenced WCR larvae to respond to differences in $CO_2$ levels, the larvae were still able to orient towards volatiles from maize roots at short distances of 8-10 cm. This result implies 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 $CO_2$ (***Bruce et al., 2005***). Given the low reliability of $CO_2$ as a host-specific cue, it is probably not surprising that WCR, as a highly specialized maize feeder, can use not only $CO_2$ but also 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 $CO_2$ gradients in the soil, thus increasing the robustness of its foraging behaviour at short distances.

Although intact $CO_2$ 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 $CO_2$ 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***). $CO_2$ 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 $CO_2$ traps located as far away as 10 m, and it is estimated that this distance could even be as long as 60 m under 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 $CO_2$ 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 $CO_2$ perception with other environmental cues and that attraction to $CO_2$ 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 $CO_2$ (***McMeniman et al., 2014; van Breugel et al., 2015***), and pollinating hawkmoths, that use $CO_2$ as a redundant volatile distance stimulus in a sex-specific manner (***Goyret et al., 2008***).

A recent study shows that a $CO_2$ receptor in *Drosophila* flies is also involved in the detection and behavioral responses to other volatiles (***MacWilliam et al., 2018***). We observed that *DvvGr2*-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 $CO_2$ 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. Taken together, these aspects indicate that the foraging patterns observed in this study are not due to different sensitivity of *DvvGr2*-silenced larvae to other root volatiles.

Apart from acting as a long-distance host location cue, $CO_2$ 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 $CO_2$ release from the roots, following the highest concentrations of $CO_2$ 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 $CO_2$ 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 $CO_2$ emission and food-source profitability was also suggested for *Datura* flowers, that emit the highest level of $CO_2$ in times when nectar is most abundant (***Guerenstein et al., 2004; Thom et al., 2004***). These findings support the hypothesis that $CO_2$ 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 $CO_2$ to locate host plants and to distinguish plants of different quality. Volatiles other than $CO_2$ are also integrated into host finding behaviour in the soil, but their effects are more important at shorter distances. Thus, $CO_2$ is emerging as a volatile that interacts with other environmental cues to determine the behaviour of pollinators and herbivores, likely reflecting its role as a highly conserved marker of metabolic activity within complex sensory landscapes.

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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., V.T., C.C.M.A., and B.C.J.S.; Resources, M.E. and C.C.M.A.; Visualization, R.A.R.M.; Supervision, R.A.R.M. and M.E.; Funding Acquisition, M.E. 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).

**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/$m^2$/$s^1$ 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 ($P_2O_5$) 6%, Potassium Oxide ($K_2O$) 26%, Magnesium Oxide (MgO) 3.3%, Borum (B) 0.02%, Cooper (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 2-4 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 (Selmaterra, Bigler Samen AG, Thun, Switzerland) and fed freshly germinated maize seedlings (var. Akku).

**Identification of $CO_2$ receptor genes**

To identify $CO_2$ receptor orthologues in WCR, we used $CO_2$ receptor-encoding gene sequences of *Tribolium castaneum* and several sequences from other insects as queries against publicly available WCR genome sequences (NCBI accession: PXJM00000000.2) 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 number: XM_028276483.1 (*DvvGr1*), XM_028280521.1 (*DvvGr2*), and XM_028272033.1 (*DvvGr3*). These gene sequences were translated to obtain protein sequences. The obtained protein sequences and the protein sequences of $CO_2$ 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***).

**Creation of double stranded RNA-producing *E. coli***

*Escherichia coli* HT115 were transformed with recombinant L4440 plasmids that contained a 211-240 bp long gene fragment targeting one of each of the three $CO_2$ receptors. Cloned nucleotide sequences were synthetized *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 $OD_{600}$ 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 (Selmaterra, Bigler Samen AG, Thun, Switzerland) and 2-3 freshly germinated maize seedling. Maize seedlings were coated with 1 ml of bacterial solution containing approximately 200-500 ng of dsRNA, targeting the different $CO_2$ receptor genes. As controls, larvae were fed with bacteria producing dsRNA targeting green fluorescent protein genes (GFP), which are absent in the western corn rootworm 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 analyzed: *DvvGr1, DvvGr2, and DvvGr3* (***Rodrigues et al., 2016***). *Actin* was used as reference gene to normalize expression data across samples. Relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (***Livak and Schmittgen, 2001***). The following primers were used: DvvGr1-F CGTTAATTTAGCTGCTGTGG, DvvGr1-R GTTTTCTGTTGCTAGAGTTGC, DvvGr2-F GAACTAAGCGAGCTCCTCCA, DvvGr2-R CAGAAGCACCATGCAATACG, DvvGr3-F GCAACGCTTTCAGCTTTACC, DvvGr3-R GTGCATCGTCATTCATCCAG, DvvActin-F TCCAGGCTGTACTCTCCTTG, and DvvActin-R CAAGTCCAAACGAAGGATTG.

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

To determine whether silencing putative $CO_2$ receptor genes impairs the ability of WCR larvae to behaviorally respond to $CO_2$, we silenced *DvvGr1*, *DvvGr2*, and *DvvGr3* as described above and conducted behavioral 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 $CO_2$ 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 (Figure 2A). Five larvae that were fed dsRNA-expressing bacteria targeting *gfp*, *DvvGr1*, *DvvGr2*, or *DvvGr3* genes were released in the middle of the olfactometer (indicated by a red arrow) and their positions were recorded 1 h after their release. Seven independent olfactometer pairs were assessed per larval type and experiment, and the entire experiment was repeated twice. $CO_2$ 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***).

**Host location behavioural experiments with *DvvGr2*-silenced larvae**

To determine the importance of $CO_2$ perception for distance-dependent host-location, we used two experimental setups: belowground olfactometers to test for short distance effects, and soil filled trays to test for long distance effects (Figs 3a, 4a). Based on our results showing that *DvvGr2* is crucially required for WCR larvae to respond to $CO_2$, we contrasted the behaviour of *DvvGr2*-silenced and WT larvae in the different experimental setups. For this, either five control (fed with dsRNA-producing bacteria targeting *gfp* genes) or five *DvvGr2*-silenced WCR larvae (fed with dsRNA-producing bacteria targeting *DvvGr2* genes) were released in the middle of a two-arm belowground olfactometer and allowed to choose between a pot with a sand grown plant and a pot with only sand. Larval positions were recorded 1, 2 and 4 hr after their release (Fig 3a). Fourteen independent olfactometers per larval type were assayed. For soil-filled tray experiments, twenty second instar

WT WCR larvae or twenty second instar *DvvGr2*-silenced WCR larvae were released at 16, 32, 48, or 64 cm from 4 or 5 2-3-week-old maize plants. Larval release points are indicated by colour arrows. Plants were planted in plastic trays (80cmx15cmx4.5cm)(Migros do it + garden, Switzerland) filled with soil (Fig 4a). Six independent trays per type of larvae were assayed. 8 hr after releasing the larvae, their positions were recorded. $CO_2$ levels on each arm of the olfactometer, and inside each tray zone were measured by GC-FID as described (***Zhang et al., 2018***).

**Larval dispersal patterns and responses to methyl anthranilate**

To evaluate the effect of silencing *DvvGr2* genes on larval dispersion, we released twenty WT or twenty *DvvGr2*-silenced larvae in the middle of 80 cm, soil-filled plastic trays without plants. Eight h after release, larvae in each tray zone were recovered and counted. To determine larval responses to plant volatile organic compounds other than $CO_2$, 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 $2^{nd}$-$3^{rd}$ instar WT or five $2^{nd}$-$3^{rd}$ instar *DvvGr2*-silenced larvae were released in the middle of a sand-filled petri dish (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). Ten independent peteri plates per larval type 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.

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

Optimally fertilized plants emit higher levels of respiratory $CO_2$ than sub-optimally fertilized plants (***Zhu and Lynch, 2004***). To test whether WCR larvae orients towards and prefer optimally fertilized maize plants and to evaluate the importance of $CO_2$ 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 5a, b). Then, twenty $2^{th}$ instar WT or twenty $2^{th}$ instar *DvvGr2*-silenced 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. 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 $1^{th}$ 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 thoroughly washed to remove fertilizer traces from the root surface. Twenty solo cups per treatment were included. 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***).

## Supplementary Figures

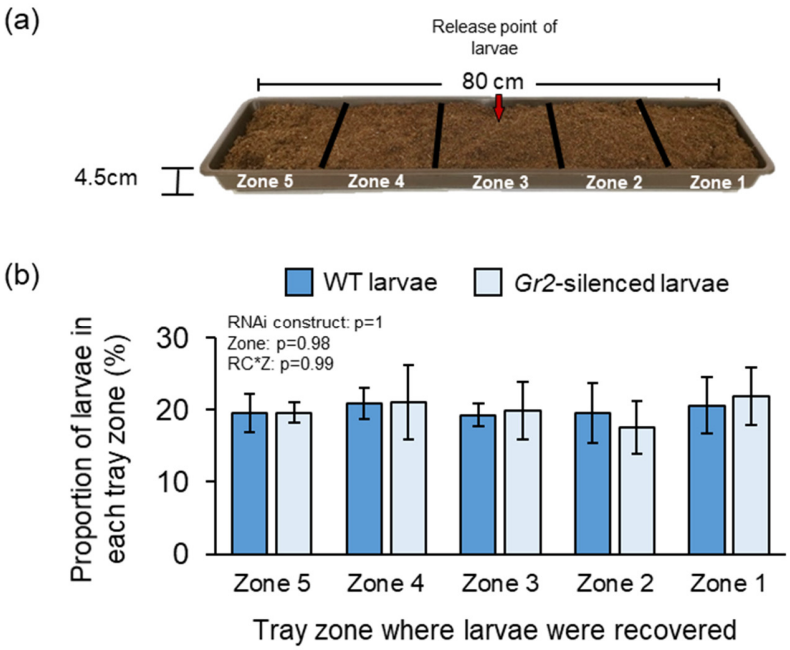


**Figure S1** *DvvGr₂ CO2 receptor does not regulate WCR locomotion.* (a) Schematic representation of experimental setup used for behavioural experiments. (b) Mean (±SEM) proportion of WT or *DvvGr2*-silenced WCR larvae observed in the different tray zones eight hours after releasing the larvae in the middle of the tray. Twenty WT or twenty *DvvGr2*-silenced larvae were released. Three identical trays per larval type were assayed (n=3). No statistical differences in the proportion of WT and *DvvGr2*-silenced larvae found in each tray zone were observed ($p<0.05$ by two-way ANOVA followed by FDR-corrected post hoc tests). *P*-values of Generalized Linear Models (GLMs) are given for different factors and their interactions.

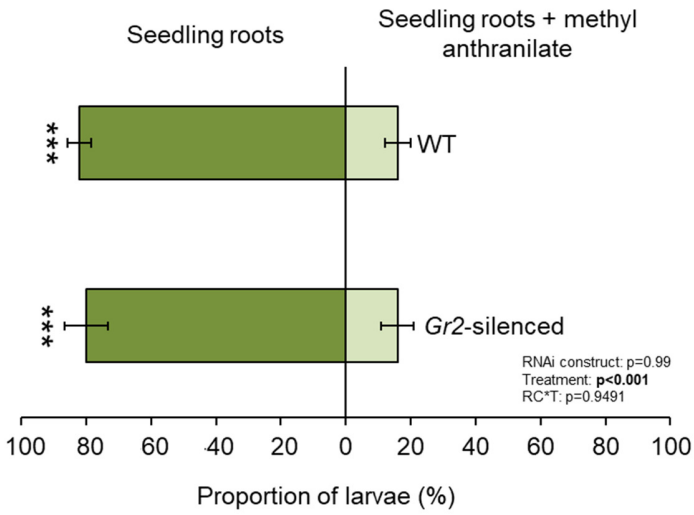


**Figure S2** *DvvGr2-silenced larvae respond to the plant volatile methyl anthranilate in a similar manner as WT larvae.* Mean (±SEM) proportion of WCR larvae observed in each side of choice arenas. Ten choice arenas with five larvae each were assayed (n=7). Asterisks indicate statistically significant differences between treatments (***: $p<0.001$ by two-way ANOVA followed by FDR-corrected post hoc tests). *P*-values of Generalized Linear Models (GLMs) are given for different factors and their interactions.

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