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Functional validation of the carbon dioxide receptor in labial palps of *Helicoverpa armigera* moths

Chao Ning, Ke Yang, Meng Xu, Ling-Qiao Huang, Chen-Zhu Wang*

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

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ABSTRACT

Adult moths possess an organ in their labial palps, the labial-palp pit organ, which is specialized for sensing carbon dioxide (CO_2). They use CO_2 as a cue to detect healthy plants and find food or lay eggs on them. The molecular bases of the CO_2 receptor in *Drosophila melanogaster* and *Aedes aegypti* have been reported, but the molecular mechanisms of the CO_2 receptor in Lepidoptera remains elusive. In this study, we first re-examined three putative *Helicoverpa armigera* CO_2 gustatory receptor genes (*HarmGr1*, *HarmGr2*, and *HarmGr3*), and then analyzed expression patterns of them. RT-PCR results verified they were predominantly expressed in the labial palps of *H. armigera*. Thus, we used *in situ* hybridization to localize the expression of three genes in the labial palps. We found that all three genes were co-expressed in the same cells of the labial palps. Next, we employed the *Xenopus laevis* oocyte expression system and the two-electrode voltage-clamp recording to study the function of the three genes. Results showed that only oocytes co-expressing HarmGr1 and HarmGr3 or co-expressing HarmGr1, HarmGr2 and HarmGr3 gave robust responses to $NaHCO_3$. Finally, we confirmed that the sensory cells in labial palps of both females and males show dose dependent responses to CO_2 stimuli by using single sensillum recording. Our work uncovers that HarmGr1 and HarmGr3 are indispensable and sufficient for CO_2 sensing in labial palps of *H. armigera*.

1. Introduction

Almost all organisms produce carbon dioxide (CO_2) when they generate the energy required for their life processes by oxidizing the biomass. As a ubiquitous sensory cue, CO_2 not only helps hematophagous insects detect and orientate to host vertebrates, but also plays important roles in foraging and oviposition of phytophagous insects (Guerenstein and Hildebrand, 2008).

Insects possess specialized receptor cells that can detect low concentrations of CO_2 (Guerenstein and Hildebrand, 2008). These cells are located in the maxillary palps in mosquitos and labial palps in Lepidoptera adults (Bogner et al., 1986; Grant et al., 1995; Kellogg, 1970; Kent et al., 1986). Basiconic sensilla on the maxillary palps of female *Aedes aegypti* respond to concentrations of CO_2 as low as 150–300 ppm and can detect increments as small as 50 ppm (Grant et al., 1995). In the labial palps of adult Lepidoptera, there is an organ called labial-palp pit organ (LPO), which is specialized for sensing CO_2 (Bogner et al., 1986; Kent et al., 1986). Before foraging and ovipositing, moths probe the surface of a plant with their LPOs to measure the gradient of CO_2 levels generated by host plants (Stange et al., 1995; Thom et al., 2004).

The molecular mechanisms underlying chemoreception of CO_2 stimuli at the level of receptor-cell dendrites were first unraveled from *Drosophila melanogaster* (Jones et al., 2007b; Kwon et al., 2007). Two gustatory receptor (Gr) genes, *DmelGr21a* and *DmelGr63a* are co-expressed in all CO_2 receptor cells of *D. melanogaster*. Such configuration is necessary and sufficient for the CO_2 receptor cells to respond to CO_2, and it is thought that the two Grs form a heterodimeric receptor (Jones et al., 2007b; Kwon et al., 2007). Three putative CO_2 receptor genes have been identified in the maxillary palps of three mosquito species, *Anopheles gambiae*, *Aedes aegypti*, and *Culex pipiens quinquefasciatus* (Kent et al., 2008; Robertson and Kent, 2009), and their function has been studied in *Aedes aegypti* (Erdelyan et al., 2012). *AaGr1*, *AaGr2* and *AaGr3* are co-expressed in the same receptor cells of the maxillary palps in *A. aegypti*. RNA interference (RNAi)-mediated gene knockdown of either *AaGr1* or *AaGr3* resulted in a loss of CO_2 sensitivity in both

* Corresponding author. State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing, China.
E-mail address: czwang@ioz.ac.cn (C.-Z. Wang).

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male and female mosquitoes, but RNAi-mediated knockdown of *AaGr2* expression had no impact on CO_2 reception, suggesting that *AaGr1* and *AaGr3*, like the *Drosophila* orthologues, function as a heterodimer (Erdelyan et al., 2012; Jones et al., 2007a; Kwon et al., 2007; McMeniman et al., 2014). Three putative CO_2 receptor genes have also been identified in five Lepidoptera species, *Bombyx mori, Helicoverpa armigera*, *Danaus plexippus, Heliconius melpomene*, and *Popilio xuthus* (Anderson et al., 2009; Briscoe et al., 2013; Robertson and Kent, 2009; Xu and Anderson, 2015), but their functional identity has not been completely validated. Xu and Anderson (2015) reported that three putative CO_2 receptor genes, *HarmGR1*, *HarmGR2* and *HarmGR3*, were specifically expressed in the labial palps of *Helicoverpa armigera*. When the three Grs were expressed individually in insect Sf9 cells, only HarmGR3 was significantly activated by $NaHCO_3$ (Xu and Anderson, 2015). Therefore, whether the co-expression of multiple Grs is required for CO_2 detection in *H. armigera* and other lepidopteran species is still unknown.

In the present study, we first re-examine the coding sequences of three putative CO_2 Grs in *H. armigera*, and then analyze their expression patterns in tissues of adults and larvae and used *in situ* hybridization to localize the cells expressing the three genes in labial palps. We further used *Xenopus* oocyte expression system and two-electrode voltage-clamp recording to study the functions of three Grs in different combinations. Finally, we verified electrophysiological responses of the CO_2-sensitive cells in labial palps of *H. armigera*.

2. Materials and methods

2.1. Animals rearing

H. armigera were reared at 27 ± 1 °C with a photoperiod of 16 h: 8 h, D: L in the laboratory of Institute of Zoology, Chinese Academy of Sciences, Beijing. Eggs were hatched on a pad of cotton gauze, and larvae were individually transferred into glass tubes (i.d. 2.5 cm, length 8 cm) with an artificial diet mainly containing wheat germ, yeast and tomato paste as previously reported (Jiang et al., 2015). Pupae were sexed and males and females were put into separate cages for eclosion. 10% honey was used as the diet for adults. One-to-three-day-old virgin adults and fifth instar larvae were used in the experiments.

Xenopus laevis frogs were kindly provided by Prof. Qinghua Tao's laboratory in School of Life Sciences, Tsinghua University in Beijing, and reared with pork liver as food in our laboratory at 20 ± 1 °C. *Xenopus laevis* were anesthetized by submersion in 1 g/L 3-aminobenzoic acid ethyl ester, and the oocytes were surgically collected before experiments.

2.2. Chemicals

$NaHCO_3$ (>99.5%) (Sigma, USA), ethephon (>98%) (Guangtaiweiye Company, Beijing, China), 2,3-butanedione (>97%) (Sigma, USA), D-(−)-fructose (>97%) (Sigma, USA), D-(+)-glucose (>99.5%) (Sigma, USA), (Z)-11-hexadecenal (Z11-16:Ald, >92%) (Shin-Etsu Chemical, Tokyo Japan), (Z)-9-hexadecenal (Z9-16:Ald, >90%) (Shin-Etsu Chemical, Tokyo Japan), citral (>96%) (Sigma, USA), geraniol (>96%) (Sigma, USA) were used in experiments. Before experiments, stock solutions (0.5 M) of $NaHCO_3$, ethephon, 2,3-butanedione, D-(−)-fructose, and D-(+)-glucose were diluted in ND96 Buffer (96 mM NaCl/2 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/5mMHEPES pH 7.5). The stock solutions (200 mM) of citral, geraniol, Z11-16:Ald, and Z9-16:Ald were first prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C, and then dissolved in ND96 Buffer before use. ND96 Buffer and ND96 Buffer containing 0.1% DMSO were used as negative controls respectively.

2.3. Re-examination of putative carbon dioxide receptor genes

To understand the molecular mechanism of CO_2 detection in *H. armigera*, we first used the transcriptome data of *H. armigera* from the NCBI sequence read archive (SRA) (http://www.ncbi.nlm.nih.gov/sra/?term=SRP041166) provided by Liu et al. (2014) and from our laboratory to recheck the coding sequences of three putative CO_2 receptor genes, *HarmGR1* (KF768713.1), *HarmGR2* (KF768714.1) and *HarmGR3* (KF768715.1), which were previously identified by Xu and Anderson (2015) from the transcriptome of *H. armigera* in Australia. The SRA Toolkit was used to convert the data to fastq format. The data of twelve tissues including ours were trimmed using Trimmomatic-0.30, merged, assembled using Trinityrnaseu-r2013-02-25, removed redundancy using Tgicl, and finally capped using Cap3 to get the unigene. The annotation of unigenes was performed using Blastx, and the database is the non-redundant protein sequences (nr) and UniProtKB/Swiss-Prot (swissprot). The hit sequences were compared with the coding sequences of the CO_2 Grs of *D. malanogaster* and *A. aegypti* using Genwise-2-4-1. The coding sequences and amino acid sequences of the three Grs of *H. armigera* were aligned using DNAMAN version 8.

2.4. RT-PCR and qRT-PCR of putative carbon dioxide receptor genes

We used RT-PCR to study the expression pattern of three putative CO_2 receptor genes in different tissues or organs of *H. armigera* adults and larvae. Labial palps, female antennae, male antennae, female proboscis, male proboscis, female foreleg tarsi, and female wings of the adults, and maxillae, foreguts, midguts, hindguts, and fat body of the fifth instar larvae were separately collected from up to 150 individuals, and then stored at −80 °C. Frozen tissues were transferred to a liquid nitrogen cooled mortar and ground with a pestle. The homogenate was covered with 1 mL of TriZol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA extractions were performed following the manufacturer's instructions. Total RNA of each tissue or organ was dissolved in H_2O, and RNA integrity was verified by gel electrophoresis. RNA quantity was determined by absorbance at 260 nm, 280 nm, and 230 nm on a Nanodrop ND-2000 spectrophotometer (Nano-Drop products, Wilmington, DE, USA). cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI, USA) from the total RNA.

RT-PCR was performed using gene-specific primers designed with Primer Premier 5.0 based on the full length mRNA sequences of three putative CO_2 receptor genes from our *H. armigera* transcriptome. The primer sequences are listed in Table 1. *Actin* gene of *H. armigera* was used as the control to monitor the quality of each cDNA samples. The PCR program was as follows: 98 °C for 2 min; 94 °C for 3 min; 26 cycles at 94 °C for 30s, 53 °C for 30s, 72 °C for 90s, and 72 °C for 10 min for Actin, and 98 °C for 2 min; 94 °C for 3 min; 35 cycles at 94 °C for 30s, 53 °C for 30 s, 72 °C for 90s, and 72 °C for 8 min for *HarmGr1*, *HarmGr2* and *HarmGr3*. The RT-PCR reactions were repeated three times, and the products were analyzed by using 1.2% agarose gels (Jiang et al., 2015).

qRT-PCR was further performed to quantify the relative expression levels of *HarmGr1*, *HarmGr2* and *HarmGr3* in labial palps of *H. armigera* adults. Total RNA was isolated from labial palps of 50 pairs of female and male adults using RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany), in which genomic DNA was removed by gDNA Eliminator. cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI, USA) from the total RNA. Real-time PCR were carried out using Mx3005P qPCR System (Agilent Technologies, CA, USA). The primer sequences are listed in Table 1. All reactions were performed in triplicate in a total volume of 20 μL containing 10 μL SYBR Premix Ex TaqII(TaKaRa, Otsu, Japan) and 0.4 mM of each primer under the following conditions: 95 °C

Table 1
The primer sequences used in RT-PCR, qRT-PCR, and PCR for cloning the full-length coding sequences of three putative CO_2 receptor genes of *Helicoverpa armigera*.

Gene	Name	Primer sequences (5′-3′)
Gr1	RT-PCR/F	TGTTTCAGCGGATTGATGAGAAGG
	RT-PCR/R	GAGACCCCATGAGAACACACACAG
	qRT-PCR/F	TGCAATGCATCTGGCACCGC
	qRT-PCR/R	TGTGCCACTTTGAGCGCCGG
	EcoRI/F	GAATTC**GCCACC**ATGAACAAAGAAGAACATGGGTT
	XhoI/R	CTCGAGTCATTCCTCATCAACTTCATCAGTAA
Gr2	RT-PCR/F	TCATCTCAAGATGGCGACACGTGCGAG
	RT-PCR/R	GTTGATCATTGTAATAATGTGGTA
	qRT-PCR/F	TTGGGGCGTGGCTCATCAAG
	qRT-PCR/R	TTTCGCCAGTCACGCGGTAG
	EcoRI/F	GAATTC**GCCACC**ATGACGATCCCGGATCATCTGTTTG
	XhoI/R	CTCGAGTCACGTTGCAACAATTTGTGGGTCC
Gr3	RT-PCR/F	TATCTATGAGAACATCAAACCCGTC
	RT-PCR/R	CTCAAAGTCTCGCAGAGTAGGTACCA
	qRT-PCR/F	ACATGCTTGGAGGATACTGG
	qRT-PCR/R	AGCCATAGAGCGCGGTATTC
	EcoRI/F	GAATTC**GCCACC**ATGTCGTTTCATACCAGTAACAGTTTAT
	XhoI/R	CTCGAGTTAATTCTTCTTTTTCTTCTTGGCGAGCG
actin	RT-PCR/F	ACCAACTGGGACGACATGGAG
	RT-PCR/R	CGTCAGGATCTTCATGAGGTAGTC
	qRT-PCR/F	GGCATGGGGCAGAAGGACTC
	qRT-PCR/R	ATGATGCCGTGCTCGATGGG

F: forward strand; R: reverse strand, the underlined indicate restriction recognition sites, the bold indicate Kozak sequence.

for 30 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s, and 72 °C for 30 s, 1 cycle 95 °C for 15s, 60 °C for 1 min, 95 °C for 15 s. Expression levels of *HarmGr1, HarmGr2 and HarmGr3* were calculated using the $2^{-\Delta\Delta Ct}$ method, with an actin gene transcript as an internal control for sample normalization. The experiment was repeated three times using three independent RNA samples.

2.5. In situ hybridization

Two-color double *in situ* hybridizations were performed following protocols reported previously (Jiang et al., 2014; Krieger et al., 2002). The following sense (s) and antisense (as) primers were used to synthesize the gene-specific probes from the open reading frames (ORFs): Gr1, s5′- AAGATGGAGCCTTGTTGGATG -3′ and as5′- TCAGCAGACGCACTTGGAA-3'; Gr2, s5′- TCCACGACCAGTTCTACAGAG -3′ and as5′- GAGCAATCAACGAAGATGAAGAG -3'; Gr3, s5′- GCTATCCTCGTGTTCCTATCTCT -3′ and as5′- TGTGCGTCAGTGTTCATCCA -3'. The length of *HarmGr1*, *HarmGr2*, and *HarmGr3* was 954, 868, and 812 nucleotides, respectively. Both digoxin (Dig)-labeled and biotin (Bio)-labeled probes were synthesized with DIG RNA labeling Kit version 12 (SP6/T7) (Roche, Mannheim, Germany), with Dig-NTP or Bio-NTP (Roche, Mannheim, Germany) labeling mixture, respectively. Antisense and sense probes were generated from linearized recombinant pGEM-T vector using the T7/SP6 RNA transcription system (Roche, Basel, Switzerland) following recommended protocols. RNA probes were subsequently fragmented to an average length of about 300 bp by incubation in carbonate buffer.

Labial palps were dissected from 3-day-old female moths, embedded in JUNG tissue freezing medium (Leica, Nussloch, Germany) and frozen at −80 °C until use. Sections (12 μm) of palps were then mounted on SuperFrost Plus slides (Boster, Wuhan, China). After series of fixing and washing procedures, 100 μL hybridization solution (Boster, Wuhan, China) containing both Dig and Bio probes was placed onto the tissue sections. After adding a coverslip, slides were incubated in a humid box at 55 °C overnight. After hybridization, slides were washed twice for 30 min in 0.1 × saline-sodium citrate (SSC) at 60 °C, treated with 1% blocking reagent (Roche) in TBST for 30 min at room temperature, and then incubated for 60 min with anti-digoxigen (Roche, Mannheim, Germany) and Strepavidin-HRP (PerkinElmer, Boston, USA). Visualization of hybridization signals was performed by incubating the sections first for 30 min with HNPP/Fast Red (Roche, Mannheim, Germany), followed by three 5 min washes in TBS, with 0.05% Tween-20 (Tianma, Beijing, China) at room temperature with shaking. The sections were incubated with Biotinyl Tyramide Working Solution for 8 min at room temperature followed by the TSA kit protocols (PerkinElmer, USA). After three additional washings for 5 min in TBS with 0.05% Tween-20 at room temperature with shaking, sections were finally mounted in Antifade Mounting Medium (Beyotime, Beijing, China). All the sections were analyzed under a Zeiss LSM710 Meta laser scanning microscope (Zeiss, Oberkochen, Germany). Photoshop (Adobe systems, San Jose, CA) was used to arrange figures and the images were only altered to adjust brightness and contrast.

2.6. Functional characterization of HarmGr1, HarmGr2, HarmGr3

Full-length coding sequences of *HarmGr1, HarmGr2,* and *HarmGr3* were expressed in *Xenopus laevis* oocytes individually or in all possible combinations, and thus total seven groups of oocytes expressing different *Grs* were obtained: (1) *HarmGr1,* (2) *HarmGr2,* (3) *HarmGr3*, (4) *HarmGr1* + *HarmGr2*, (5) *HarmGr1* + *HarmGr3*, (6) *HarmGr2* + *HarmGr3*, (7) *HarmGr1* + *HarmGr2* + *HarmGr3*. Oocytes were challenged with concentrations series (from 0.001 to 0.3 M) of sodium bicarbonate ($NaHCO_3$) and other chemicals including ethephon, 2,3-butanedione, D-(−)-fructose, and D-(+)-glucose at the concentration of 0.1 M, citral, geraniol, Z11-16:Ald, and Z9-16:Ald at the concentration of 0.1 mM. Two-electrode voltage-clamp recording was used to detect the whole cell current (Jiang et al., 2014).

Total RNA was isolated from the labial palps collected from 100 adult moths, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. M-MLV Reverse Transcriptase (Promoga, Wisconsin, WI, USA) was used to synthesis cDNA. To obtain the full-length coding sequences of the three putative gustatory receptor genes, PCR was carried out using gene-specific primers with Kozak consensus sequence and Restriction Enzyme cutting site based on the mRNA sequences of *HarmGr1*, *HarmGr2* and *HarmGr3*. The primer sequences are listed in Table 1. The PCR program included initial denaturation 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s; and 72 °C for 8 min. The coding sequences of three *HarmGrs* were cloned into pGEM-T easy vector (Promega, Madison, WI, USA), then subcloned into pCS2+ vector. The pCS2+ vectors were linearized by using NotI (Takara shuzo, Shiga, Japan), cRNAs were synthesized from the linearized pCS2+ vectors with mMESSAGE mMACHINE SP6 (Ambion, Austin, TX, USA). Each cRNA of three *HarmGrs* were dissolved in RNAase-free water and stored at −80 °C.

Mature healthy oocytes were treated with 2 mg/mL of collagenase type I (Sigma-Aldrich) in Ca^{2+}-free saline solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5) for 1–2 h at room temperature. Each oocyte was microinjected with 23.6 nL (50 ng) of each *HarmGr* cRNA or their mixtures with the ratio of 1:1 or 1:1:1. Oocytes injected with RNAase-free water were used as a negative control. Injected oocytes were incubated for 3–4 days at 18 °C in a bath solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 100 μg/mL gentamycin and 550 μg/mL sodium pyruvate.

Whole-cell currents were recorded with a two-electrode voltage clamp. Intracellular glass electrodes were filled with 3 M KCl and presented resistances of 0.2–2.0 MΩ. Signals were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT, USA) at a

holding potential of −80 mV, low-pass filtered at 50 Hz and digitized at 1 kHz. Sodium bicarbonate ($NaHCO_3$) was diluted, and the pH was adjusted to 7.5 in Ringer's solution before being introduced to the oocyte recording chamber using a perfusion system. Data acquisition and analysis were carried out with Digidata 1322A and pCLAMP software (Axon Instruments Inc., Foster City, CA, USA). Dose-response data were analyzed using GraphPad Prism 6.

2.7. Single sensillum recording

The insect was placed inside a 1 mL disposable Eppendorf pipette tip with the narrow end cut to allow the head and the antenna to protrude. The head and labial palps were immobilized with dental wax under a stereomicroscope. The reference electrode was inserted into the second segment of the labial palp, and a sharpened tungsten recording electrode was inserted into the base of the sensilla housed in LPO. The recorded signals were then amplified through a IDAC interface amplifier (IDAC-4, Syntech, Germany). The software Autospike (Syntech, Germany) was used to store and analyze data.

A continuous stream of purified and humidified air was directed on the LPO (12.5 mL/s) from the outlet of a steel tube (i.d. 6 mm, length 15 cm), positioned 2 cm from the LPO. Test odors were injected into the air stream using a stimulus flow controller (CS-55, Syntech, Germany), which generated 200 ms air pulses through the odor cartridge at a flow rate of 10 mL/s, and a compensating air flow was provided to keep a constant current. CO_2 of different concentrations was given by several customized 20 mL syringes. 100% CO_2 was bought from Qianxi Gas (Beijing). Other concentrations were achieved via mixing 100% CO_2 with the air.

2.8. Data analysis

Data were analyzed using unpaired Student's *t*-test for comparing two sets of data and one-way analysis of variance (ANOVA) with Tukey LSD tests for comparing multiple sets of data. The level of significance was set as $P < 0.05$. The dose-response curve was analyzed for EC_{50} using the logistic regression. Statistical analysis was done using SPSS 21.0 software.

3. Results

3.1. Re-examination of the coding sequences of three putative CO_2 Grs in H. armigera

We used the established transcriptome data to re-check the coding sequences of three putative CO_2 receptor genes, *HarmGR1* (KF768713.1), *HarmGR2* (KF768714.1) and *HarmGR3* (KF768715.1) previously identified by Xu and Anderson (2015). The coding sequences of the three genes we re-examined had some differences comparing with the previously identified, therefore we resubmitted the coding sequences of *HarmGr1*, *HarmGr2* and *HarmGr3* to GenBank, with the accession numbers as KU664806, KU664807 and KU664808, respectively. The coding sequence of *HarmGr1* (KU664806) has 1404 nucleotides, which is 105 nucleotides shorter than that of *HarmGR1* (KF768713.1) with 88.27% similarity, and the coding sequence of *HarmGr2* (KU664807) and *HarmGr3* (KU664808) has 1302 and 1434 nucleotides, which are the same in length while sharing 96.15% and 96.16% similarity with that of *HarmGR2* (KF768714.1) and *HarmGR3* (KF768715.1), respectively (Supplementary material). It is necessary to clarify that Jiang et al. (2015) also named a different gustatory receptor gene of *H. armigera* as *HarmGR1* (GenBank accession number JX982535) almost in the same time with Xu and Anderson (2015). In this paper, we adopt the name system of Xu and Anderson (2015) in sake of convenience, and the Gr named as *HarmGR1* (JX982535) stays for future rectifying.

3.2. Expression patterns of three putative CO_2 receptor genes in H. armigera

All three putative CO_2 receptor genes are predominantly expressed in the labial palps of adults. However, the expression patterns of the three genes in other tissues are quite different (Fig. 1). HarmGr1 is specifically expressed in the labial palps; HarmGr3 appears mainly in the labial palps, female proboscis, and also in female antennae, male proboscis, larval maxilla and larval hindgut; HarmGr2 is expressed in almost all the tissues although very weakly in wings and larval fat body. qRT-PCR analysis showed the transcript levels of *HarmGr1, HarmGr2 and HarmGr3* in the labial palps of *H. armigera* did not differ sharply, and their ratio was about 1:1.1:1.3 respectively (Fig. 2).

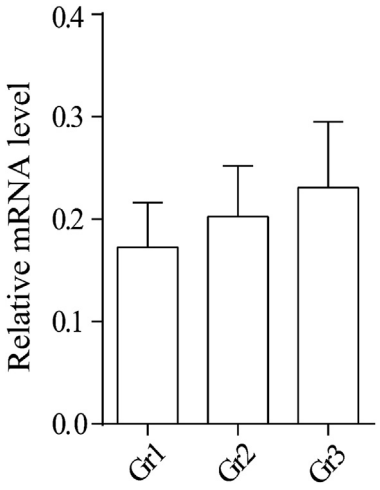

Fig. 2. Relative transcript levels of *HarmGr1*, *HarmGr2* and *HarmGr3* in labial palps of *Helicoverpa armigera* by qRT-PCR analysis. Error bars indicate SEMs from the analysis of three replications.

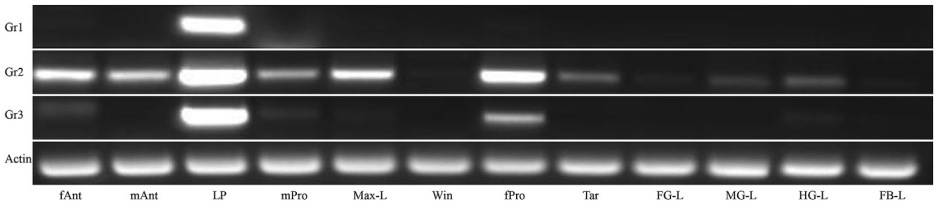

Fig. 1. RT-PCR analysis of *HarmGr1*, *HarmGr2* and *HarmGr3* in tissues and organs of adults and larvae of *Helicoverpa armigera*. fAnt, female antennae; mAnt, male antennae; LP, adult labial palps; mPro, male proboscis; Max-L, larval maxilla; Win, wings; fPro, female proboscis; Tar, adult tarsi; FG-L, larval foregut; MG-L, larval midgut; HG-L, larval hindgut; FB-L, fat body. The actin gene was used as a control.

3.3. Localization of the putative CO_2 gustatory receptors in labial palps

To identify the cells expressing the 3 putative CO_2 receptor genes in labial palps, double *in situ* hybridization experiments were performed. Employing Dig-labeled HarmGr1 and HarmGr2, Bio-labeled HarmGr2 and HarmGr3, we assembled three pairs of 2-color *in situ* hybridization, namely HarmGr1-Dig/HarmGr2-Bio, HarmGr1-Dig/HarmGr3-Bio and HarmGr2-Dig/HarmGr3-Bio. We found that HarmGr1 and HarmGr2, HarmGr1 and HarmGr3, and HarmGr2 and HarmGr3 were co-expressed in the same cell (Fig. 3). Taken together, we confirm that all the 3 Grs are co-expressed in the same cells of labial palps.

3.4. Functional analysis of HarmGr1, HarmGr2, HarmGr3 and their combinations

To investigate the function of the three genes and their mutual interactions, we examined electrophysiological responses of *Xenopus* oocytes expressing them individually and in all possible combinations, *HarmGr1, HarmGr2, HarmGr3, HarmGr1+HarmGr2, HarmGr1+HarmGr3, HarmGr2+HarmGr3, HarmGr1+HarmGr2+HarmGr3*. We found that only the oocytes expressing *HarmGr1+HarmGr3* and *HarmGr1+HarmGr2+HarmGr3* showed responses to $NaHCO_3$ (Fig. 4). However, there was no significant difference between responses of oocytes expressing *HarmGr1+HarmGr3* and those expressing

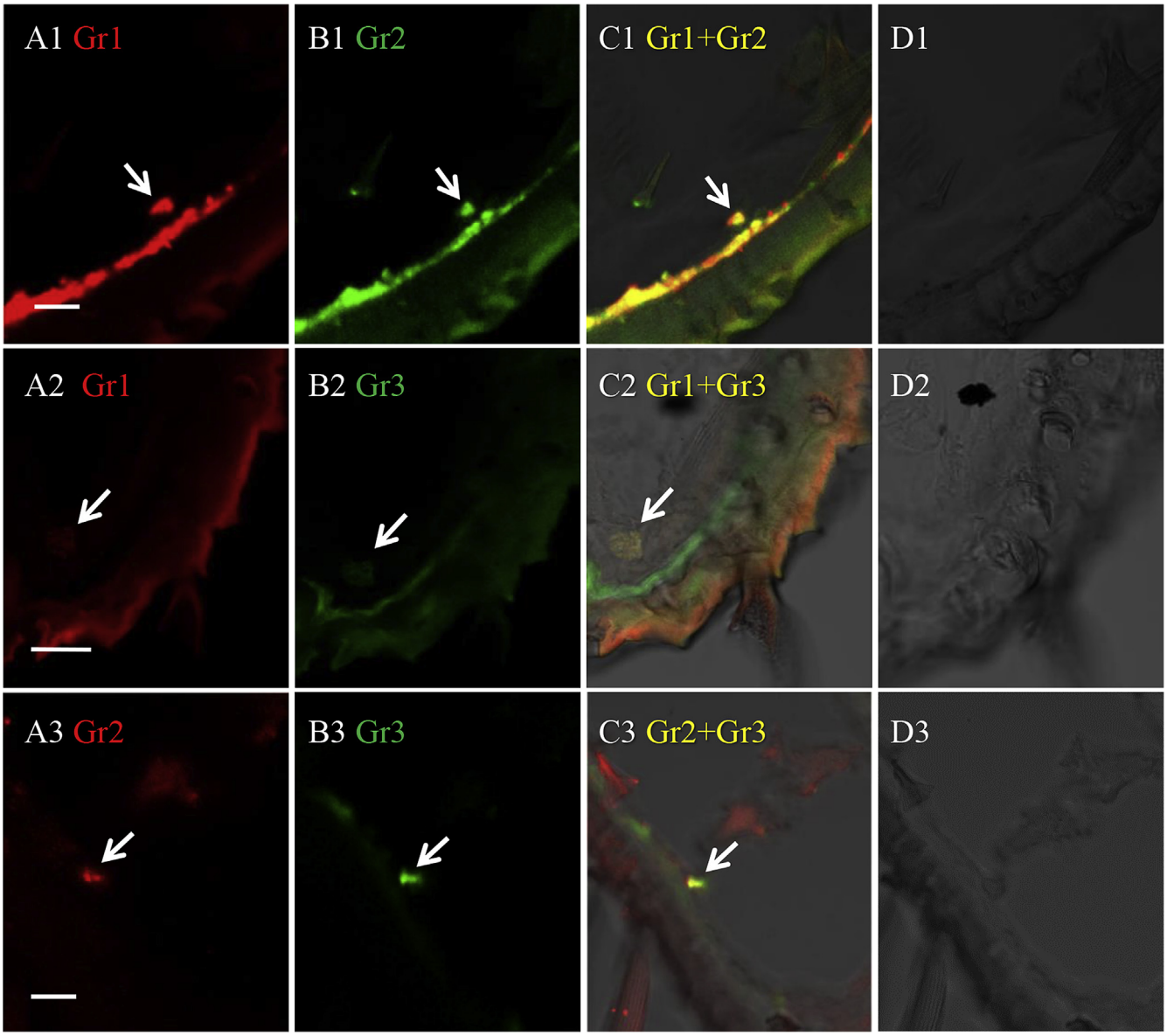

Fig. 3. Co-expression of HarmGr1, HarmGr2 and HarmGr3 in cells of labial palps of female *Helicoverpa armigera*. Double-FISH with female labial palps using combinations of differentially labeled Gr probes and visualization of cells bearing distinct Gr transcripts by red (DIG) (A1-3) and green (biotin) (B1-3) fluorescence, respectively. Co-labelling of cells by both Gr probes appears as yellow/orange color in the overlay of the red and green fluorescence channels (C1-3). The bright-field images are presented as references (D1-3). Arrows indicate the cell location. Scale bars: 20 μm. (A1, B1, C1, D1) The HarmGr1 and HarmGr2 probes label the same cell. (A2, B2, C2, D2) The HarmGr1 and HarmGr3 probes label the same cell. (A3, B3, C3, D3) The HarmGr2 and HarmGr3 probes label the same cell.

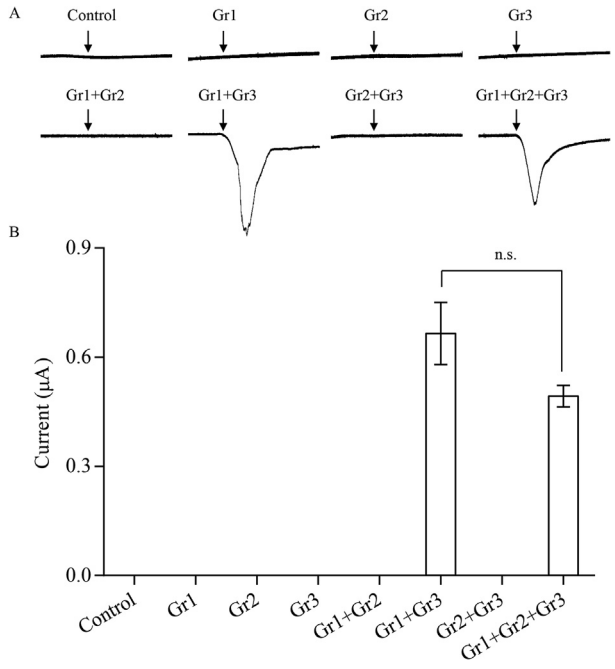

Fig. 4. Responses of *Xenopus* oocytes injected with the cRNA of *HarmGr1*, *HarmGr2*, *HarmGr3*, and their combinations to $NaHCO_3$. Only the oocytes co-expressing HarmGr1 and HarmGr3 and the oocytes co-expressing HarmGr1, HarmGr2 and HarmGr3 produce responses to $NaHCO_3$, while all the other combinations did not. The experiments were repeated 6 times for each treatment using different oocytes. $NaHCO_3$ (0.1 M) was applied for 5 s at the times indicated by arrows. (A) the Representative current traces of oocytes expressing Gr1+Gr3 and Gr1+Gr2+Gr3; (B) Responses (mean ± SEM) of oocytes expressing different Grs. n.s.: no significance (*t*-test, n = 6, P = 0.05).

HarmGr1+HarmGr2+HarmGr3 (t = 1.905, P = 0.086, n = 6). The dose response curve of oocytes expressing *HarmGr1+HarmGr3* showed that the $NaHCO_3$ induced current increased significantly from the concentration of 0.01 to 0.3 M with an EC_{50} of 0.032 M (Fig. 5A, B).

To probe the selectivity of the pair HarmGr1/HarmGr3 we tested responses of oocytes expressing both genes to other chemicals, including two pheromone components (Z9-16:Ald, Z11-16:Ald), two plant volatile compounds (citral and geraniol) at 0.1 mM, and fructose, glucose, ethephon and 2,3-butanedione at 0.1 M. The cells only responded to 0.1 M $NaHCO_3$ with a strong signal (Fig. 5C), indicating that they are very finely tuned to CO_2.

3.5. Electrophysiological responses of the CO_2-sensitive cells in labial palps to CO_2

Electrophysiological recording from labial palps of both female and male adults of *H. armigera* showed the presence of CO_2-sensitive cells (Fig. 6). Females produced stronger action potentials than in males, with an apparent threshold at 1%. However, the firing frequency did not change much from 1% to 10% of CO_2.

4. Discussion

Carbon dioxide plays an important behavioral role in foraging and oviposition of lepidopteran insects. Adults use CO_2-specifically sensitive neurons in their labial palps for detecting CO_2 (Kent et al., 1986). Xu and Anderson (2015) identified three putative CO_2 receptor genes, *HarmGR1*, *HarmGR2*, and *HarmGR3* from *H. armigera*

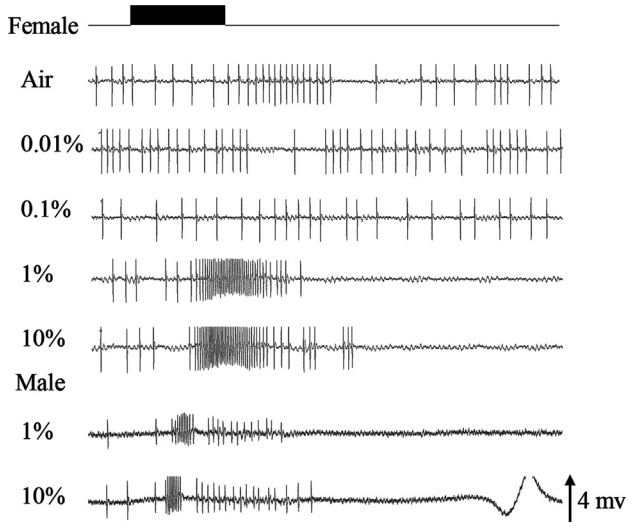

Fig. 6. Electrophysiological responses of a palp-pit receptor cell in female and male adults of *Helicoverpa armigera* to ambient air and to different concentrations of CO_2 (0.01%, 0.1%, 1% and 10%). Bars: stimulus duration (0.2 s).

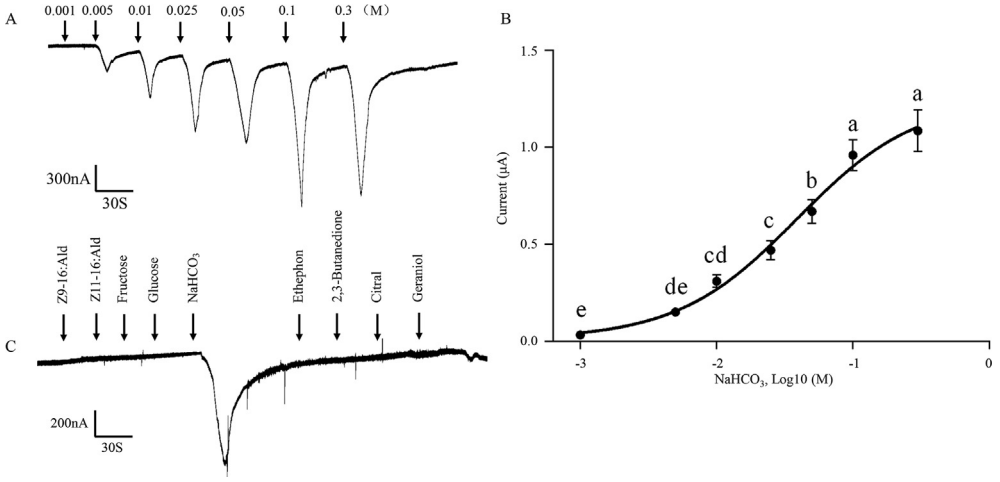

Fig. 5. Dose–response curve and specificity of *Xenopus* oocytes injected with the cRNAs of HarmGr1 and HarmGr3 to $NaHCO_3$. (A and B) Dose–response curves. (C) Oocyte responses to different stimuli. Chemicals were applied for 5 s at the times indicated by arrows in (A and C). Data in (B) are presented as mean ± SEM. Different letters above the bars indicate the significant differences (ANOVA, P = 0.05).

and found that only HarmGR3 is significantly activated by $NaHCO_3$ when expressed in insect Sf9 cells. Here we further uncover that all three genes are co-expressed in the same cells of labial palps, and prove that HarmGr1 and HarmGr3 are indispensable and sufficient for CO_2 sensing.

By measuring the electrophysiological responses to HCO_3^- of *Xenopus* oocytes expressing different combinations of three putative CO_2 receptor genes, we provide evidence that HarmGr1 and HarmGr3 are components of the CO_2 receptor in *H. armigera.* Like the CO_2 receptors in *D. melanogaster* and *Aedes aegypti* (Erdelyan et al., 2012; Jones et al., 2007a; Kwon et al., 2007), the CO_2 receptor in *H. armigera* appears to act as a heterodimer of HarmGr1 and HarmGr3, because neither of the two genes is functional in detecting HCO_3^- when expressed individually. Based on significant sequence similarities, HarmGr1 and HarmGr3 are the likely orthologues of Gr63a and Gr21a of *Drosophila* and AaGr1 and AaGr3 of *A. aegypti*, respectively although none of the other *H. armigera* Gr protein sequences showed more than 64% identity to the CO_2 receptor proteins in *D. melanogaster* and *A. aegypti* (Table 2).

Up to now, the full-length protein sequences of three putative CO_2 receptor genes have been identified in five lepidopteran species, *B. mori, H. armigera*, *D. plexippus, H. melpomene,* and *Papilio xuthus* (Briscoe et al., 2013; Engsontia et al., 2014; Liu et al., 2014; Robertson and Kent, 2009; Xu and Anderson, 2015; Zhan et al., 2011). Using neighbor-joining analysis, we analyzed their phylogeny together with the homologues from *D. melanogaster*, *A. aegypti*, and *Tribolium castaneum* (Fig. 7). The three Grs make three monophyletic clades distinct from one another, with the sequences of five lepidopteran species clustered together. This suggests that the five lepidopteran species might use the orthologues of the three Gr identified in *H. armigera,* possibly with the same modalities for detecting CO_2. In the genome of honeybees and ants, however, such orthologues cannot be found, suggesting that their CO_2–sensitive receptors evolved from different precursors (Galizia and Lledo, 2013; Robertson and Kent, 2009).

The fine structure and primary sensory projections of sensilla located in the labial-palp pit organ of *H. armigera* has been reported (Zhao et al., 2013). Our SSR results show that CO_2 perception in adult *H. armigera* is mediated by CO_2-sensitive sensory neurons housed in the sensilla of labial palps, and *in situ* hybridization results indicate that HarmGr1 and HarmGr3, together with HarmGr2 are co-expressed within the same cells of labial palps. However, HarmGr2 has no apparent role in CO_2 detection, and therefore its function remains unclear, although all three genes are expressed at comparable levels in the labial palps of *H. armigera* (Fig. 2). Given that the CO_2 receptor functions as a heterodimer, equal quantities of each subunit might confer optimal CO_2 reception. However, Kwon et al. (2007) found that ectopic over-expression of either *DmGr21a* or *DmGr63a* resulted in increased sensitivity to CO_2, which implies that equal expression of the two genes is not essential for enhanced reception (Kwon et al., 2007). Larvae of *H. armigera* also preferred sources of high CO_2 alone (Rasch and Rembold, 1994), and it is suggested that some sensilla on the maxillary palps of *H. armigera* larvae are the most likely organs for CO_2 detection (Keil, 1996). Our RT-PCR results showed only a weak expression of HarmGr3 in larval maxilla. On the other hand, we analyzed the transcriptome of the larval antennae and maxilla of *H. armigera* as a mixture (unpublished data) and revealed the presence of the three Gr genes, but at much lower levels than in the adult head. All together, it seems that the molecular mechanisms of sensing CO_2 in larvae still need deeper investigation.

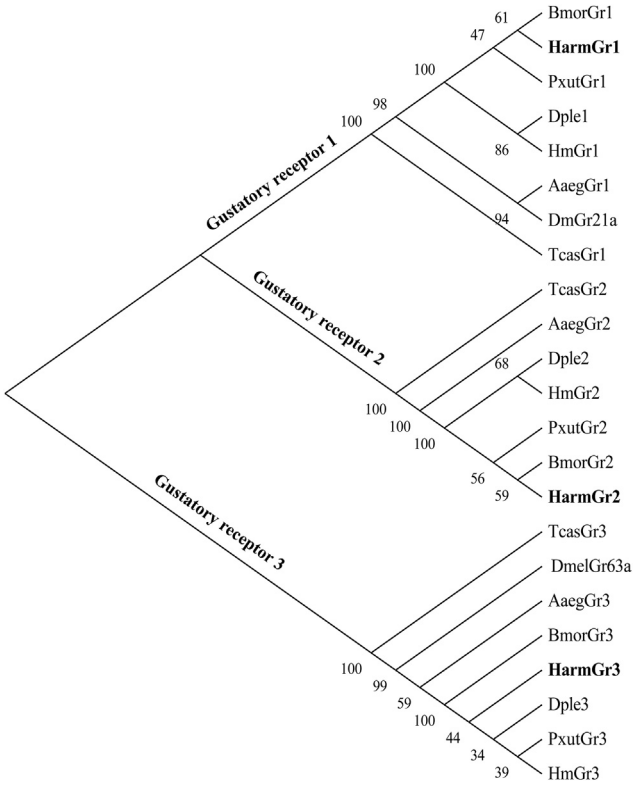

Fig. 7. Phylogenetic relationships of three putative CO_2 gustatory receptors in 5 lepidopteran species, *Helicoverpa armigera* (Harm), *Bombyx mori* (Bmor), *Danaus plexippus* (Dple), *Heliconius melpomene* (Hmel), and *Papilio xuthus* (Pxut), and three reference insect species, *Drosophila melanogaster* (Dmel), *Aeges aegypti* (Aaeg), and *Tribolium castaneum* (Tcas).

Olfactory reception in insects requires in most cases the interaction of one unique ligand-specific odorant receptor (Or) and one highly conserved co-receptor (Orco) (Vosshall and Hansson, 2011). Two proteins form a heteromeric complex to function as an olfactory sensor in insects (Benton et al., 2006; DeGennaro et al., 2013; Larsson et al., 2004; Neuhaus et al., 2005). Similarly, two Grs seem also to be coupled for CO_2 reception in insects, as reported for *D. melanogaster*, *A. aegypti*, and *H. armigera*. It is therefore possible that one Gr can selectively bind CO_2 functioning as an odor-tuned receptor, transport of the ligand or assisting in other ways the

Table 2
Percent identities of the amino acid sequences of three putative CO_2 receptor genes in *Helicoverpa armigera* (Harm), *Drosophila melanogaster* (Dmel) and *Aeges aegypti* (Aaeg) calculated with the Needleman-Wunsch global alignment algorithm in EMBOSS.

	HarmGr2	HarmGr3	AaegGr1	AaegGr2	AaegGr3	DmelGr21a	DmelGr63a
HarmGr1	34.4	26.5	57.4	35.3	23.6	55.7	25
HarmGr2		24.8	37.5	63	28	35.3	25.9
HarmGr3			26.5	23.4	54.6	24.5	50.2
AaegGr1				36.4	27	64	24.7
AaegGr2					27	36	25
AaegGr3						25.7	52.1
DmelGr21a							22.9

signal transduction process. When HarmGr1 and HarmGr3 were expressed individually in insect Sf9 cells, only HarmGr3 was significantly activated by $NaHCO_3$ (Xu and Anderson, 2015), therefore it is reasonable to suggest that HarmGr3 binds CO_2 and HarmGr1 acts as an Orco-like co-receptor.

It has yet to be established whether the CO_2 receptor binds CO_2 gas or as bicarbonate (HCO_3^-). Our results from *Xenopus* oocytes stimulated with $NaHCO_3$ suggest that the CO_2 receptor can sense CO_2 through the formation of HCO_3^-. However, we could not exclude that CO_2 as a gas could interact with the CO_2 receptor.

In this study, we clarify that HarmGr1 and HarmGr3 are two indispensable and sufficient elements for sensing CO_2 in labial palps of adult *H. armigera*, a serious agricultural pest worldwide. The results will help to better understand the behavioral roles that CO_2 plays in interactions between insects and their host plants. By identifying the proteins involved in CO_2 reception and further uncovering their structure, it is possible to design effective inhibitors or attractants to manipulate the oviposition and foraging behaviors of moth species and eventually develop new methods for crop pest control.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.04.002.

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