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Combinatorial Olfactory Signaling in Short-Distance Determines Host Plant Recognition in Locust

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Abstract: Selecting palatable plants matters for insect herbivores' survival, especially for food-restricted oligophagous and monophagous species. However, the definite selection strategy to distinguish host plants from nonhost plants, as well as the underlying sensory basis, remains controversial. Here, we investigated the olfactory recognition of host plants in oligophagous migratory locusts. By establishing one novel behavioral paradigm that allowed the free-moving locusts to make olfactory choices in short-distance, we demonstrated that palps were required to differentiate host plants apart from nonhost counterparts sensitively. Specifically, the characteristic odors between the host plant and nonhost plant defined the behavioral differentiation of food sources, and this process required intact palps. Further, single nonhost odor suppressed the behavioral potency to host plant extraction, while single host odor attenuated the behavioral repulsion to nonhost plant. We also identified the palps odorant receptors (ORs) repertoire that modulated the short-range recognition of key volatiles from host plants and nonhost and demonstrated that combinatorial olfactory signaling controls food choice. Our results support a "pull–push" model in which olfactory signaling on locust palps acts as a key tuning modulator in host plant recognition, expanding the knowledge of insect chemosensation.

Keywords: host plant selection; locust; olfaction; palps; odorant receptor; oligophagous

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1. Introduction

One critical driver for insect herbivores to search for and select appropriate plants to eat is to acquire sufficient nutrients for survival. More nutritious compounds and less hazardous substances mostly define the host plants that fuel the processes of development and reproduction. Plants containing all the nutrients required by insects are selectively eaten, whereas nonhost plants are actively avoided [1–3]. Insects are capable of determining their food spectrum when given a choice because they are equipped with sensitive sensory organs and efficient metabolic processes to distinguish and adapt to host and nonhost plants [3,4]. However, a critical question is, therefore, how does an insect herbivore localize the nutritious host plants among the external complexity, especially before they eat? Specialist lepidopteran insects localize a food source based on attractive host odor components [5–7]; for example, checkerspot butterflies detect iridoid glycosides [8] and cabbage butterflies detect glucosinolates [9]. However, studies with bark beetles show that avoidance of nonhost volatiles is important in their host location process [10]. In addition, whether the foraging strategy differs between poly and oligophagous insects in choosing host plants remains largely enigmatic [11–13]. One ideal candidate would be the various plant-produced chemical cues that provide the relevant traits to facilitate herbivores to locate and assess the quality of the potential foods [14–16].

The molecular mechanisms of insect olfaction have been extensively elucidated [17]. Insects detect external signals using several families of receptor proteins, including odorant

receptors (ORs) and ionotropic receptors [18]. Previous studies have found that insect ORs are involved in the recognition of plant volatiles. For example, AlinOR59 is involved in chemoreception of floral scent in *Adelphocoris lineolatus* [19]. In addition, larval antenna-specific OR, LstiOR5, of the meadow moth, *Loxostege sticticalis*, is narrowly tuned to a foraging-induced volatile of the host plant's leaves [20]. However, these studies did not demonstrate the possibility of the combinational recognition of plant volatiles by multiple ORs. Considering the extremely diverse odorant composition derived from the host/nonhost plants, it is reasonable to speculate on the involvement of multiple ORs in food selection as a molecular strategy for combinatorial coding to odorants [21].

The migratory locust, *Locusta migratoria*, is a notorious oligophagous herbivore insect that feeds on a small number of gramineous species, such as corn and wheat, as well as *Cyperus* spp. Once the plant is touched, the locust vibrates the palps frequently, in close proximity to potential food, to rapidly collect chemical cues called palpation [22]. Many reports have focused on the gustatory roles of palpation in judging food palatability, for example, the active physiological responses to taste and nutrients [23–26]. Previously, other authors and we reported that locust palps react actively to various odors in a broad response intensity, both physiologically and behaviorally [27–29]. In addition, we identified complex encoding patterns between olfactory receptors and volatile ligands, as well as established spatial compartmentalization between olfactory sensilla and volatile ligands on the palps [30]. Fewer ORs may be expressed on the palps than on the antennae, which provides a favorable system for studying the combinational function of multiple ORs. Thus, in this study, we examined the olfactory roles of palpation in distinguishing host plants from nonhost plants in a novel free-moving olfactory assay. We observed that, within a short distance, olfactory coding on the palps was associated with opposite behavioral effects between host and nonhost plants. We also identified the single key odors that elicited the opposite behavioral performances and presented a "pull–push" model for the locust to understand the host-plant selection mechanism in an oligophagous insect.

2. Materials and Methods

2.1. Locusts

The locusts (*Locusta migratoria*) used in the experiments were maintained at the China Agricultural University under relative humidity of 30–50% and temperature of 28–32 °C. Locusts were fed with fresh wheat plants every day. Fifth-instar nymphs were used for RNA interference (RNAi) and all behavioral tests. Three kinds of locusts treated with amputation were used for behavioral tests, namely, antenna-intact locusts (Ant+, Palp−), antenna-amputated locusts (Ant−, Palp+), and both antenna- and palp-amputated locusts (Ant−, Palp−). Unless otherwise specified, the "palp+ locust" in the text refers to antenna-amputated locusts.

2.2. Plants

Corn (*Zea mays*) and wheat (*Triticum aestivum*) were selected as host plants, while soybean (*Glycine max*) and cotton (*Gossypium hirsutum*) were selected as nonhost plants. Soil culture was used for all plants.

2.3. Chemicals Preparation

Three forms of plant volatiles were used, comprising plant grinds, plant juice, and odorant solution. The "odorant solution" refers to a solution containing a single odorant. "Plant juice" is the supernatant solution of homogenized plant tissues after concentration. "Plant grinds" refers to homogenized plant tissues without concentration. Each type was prepared as follows. To prepare plant grinds, fresh leaves were ground to powder in liquid nitrogen in a sterile mortar. Two grams of young plant leaves were ground per trial. For plant juice, the plant leaf powder was transferred to a 2 mL centrifuge tube for 15 min centrifugation at the highest speed. The supernatant was mixed thoroughly with the solvent dichloromethane (1:10, v/v) to obtain the plant juice. For the odorant solution,

all single volatiles used for behaviors were purchased with the highest purity. Working solutions were prepared with paraffin oil. Information for all chemical compounds is listed in Table S1.

2.4. Volatile Collection of Host and Nonhost Plants

The volatiles of host and nonhost plants were collected by static solid phase microextraction (SPME; Supelco) for a short period (30 min). In brief, a fiber (PDMS/DVB 65 μm) was introduced into a 1L Erlenmeyer flask (20 cm high × 13 cm internal diameter), and the plant was placed in the bottom of the flask to avoid direct contact with the extraction fiber. The plants used for behavioral experiments and volatiles collection were the same, both with 2 g leaves. The SPME volatiles collected from an empty Erlenmeyer flask for 30 min served as a control. The fiber with adsorbed odors was subjected to chemical analysis.

2.5. Chemical Analysis

Odor compositions from a short distance were analyzed for host and nonhost plants by gas chromatography–mass spectrometry. Characteristic volatiles were categorized into three groups: host-specific, nonhost-specific, and commonly shared (see Table S1, Figure S1A–C). We also expanded the panel of test compounds used in this study, comprising two host-specific volatiles ((*E*)-2-pentenal and 2-methyl-2-pentenal), two nonhost volatiles (heptanal and 2-octanone), and two common chemicals ((*E*)-2-hexenal and pentanal) [31–39]. The procedure details are as follows. A gas chromatograph (GC) (SHIMADZU 2010 plus) was used to quantify volatiles in the SPME samples. The GC was equipped with a non-polar DB-1MS column (30 m × 0.25 mm inner diameter × 0.25 μm Rice Film Thickness, Agilent Technologies). The GC furnace temperature on the DB-1MS column was maintained at 40 °C for 5 min, then increased to 240 °C at the rate of 5 °C/min. The injector temperature was maintained at 250 °C, and the nitrogen flow rate was constant at 1.0 mL/min. The fibers were inserted into the inlet operating in the forkless injection mode and held for 15 min. By comparison of the retention time with synthetic reference materials on the same column, the volatile compounds were identified.

2.6. Duration of Excitatory Response Assay

We defined the duration of palpation-like behavior as a new quantitative parameter to better characterize olfactory discrimination of volatiles under a free-moving state. When a locust approaches the odor source, the palps open and close alternately and frequently in close proximity to the stimulus, which is similar to one of the feeding steps, termed palpation. The locust also moves around the stimulus to perform repeated detection actions. We considered the state in which a locust exhibits one of such behavioral steps as an "excitatory response", and we defined the total duration of locusts exhibiting the series of behaviors as the duration of excitatory response (DER). The DER was quantified as a preference index (PI_DER), which was calculated according to the following formula: PI_DER = (Odor − CK)/(Odor + CK), where Odor and CK are the DERs of locusts at the odor zone and the control zone, respectively. The DER was manually recorded.

For the DER assay configuration, 3- to 5-day-old fifth-instar nymphs were starved for 10 h and then individually introduced to the center of an acrylic tube (length, 30 cm; diameter, 3 cm) with a modified syringe. Filter paper (0.5 cm × 2.0 cm) was placed at two opposite ends of the tube. One end was loaded with 10 μL of an odorant solution, plant juice, or plant grinds, and the other with 10 μL of the corresponding solvent (dichloromethane or paraffin oil). All assays were tested at 15% (*v*/*v*) dilutions of pure chemicals. Hemispherical wire mesh was set as a physical barrier between the insect and odor sources. The wire meshes were wrapped in perforated tinfoil to block visual discrimination and allow airflow. Air was pumped into the tube to carry the volatiles of the odor source through both ends of the device into the middle discharge point. Back-illumination from LED arrays provided a uniform and stable light source to facilitate better video capture. The video camera (Logitech C920e) was fixed in parallel with the longitudinal axis of the acrylic tube to

record the locusts' behavior. The video was used for trajectory analysis using EthoVision XT software (v.8, Noldus Information Technology) to measure two behavioral parameters: the total moving distance and the duration in the whole arena or each half. The visit of each zone was defined as the state that locusts passed the central release area and stayed in each zone. The preference index of distance was equal to (*distance_O* − *distance_C*) divided by (*distance_O* + *distance_C*), where *distance_O* and *distance_C* are the total distance of the locust traveled in the odor zone or the CK zone during the test period, respectively. The preference index of duration was equal to (*duration_O* − *duration_C*) divided by (*duration_O* + *duration_C*), where the *duration_O* and *duration_C* are the total length of time that locusts stayed in the odor zone or the CK zone during the test period, respectively. Replication numbers for each DER assay were listed in each figure legend.

2.7. Palp Opening Response Assay

Palp opening response (POR) experiments were conducted as described previously [29]. In brief, 3- to 5-day-old fifth-instar antenna-amputated nymphs were starved for 10 h and then placed individually inside a 1.5 mL Eppendorf tube to allow the head and palps to move freely. An aliquot (10 µL) of chemicals (5%, *v*/*v*) was added to the filter papers (0.5 cm × 2 cm) for quick stimulation. The stimulants were moved rapidly toward the locust mouthparts but without contacting the palps. The locust number was counted if one or more palps extended over the labrum sulcus during stimulation. The opened palp was defined as POR-Y, and the closed palp was defined as POR-N. The POR index was defined as (POR-Y)/[(POR-Y) + (POR-N)]. The filter paper was changed every 10 insects. Each experimental group comprised 30 locusts, and each experimental group was replicated three times with an interval of 10 min to recover palp activity. All experiments were conducted at 28–30 °C under white light.

2.8. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Candidate OR genes were identified by a BLAST-based search for homologous sequences. The tissue-specific expression of the candidate OR genes (accession numbers and gene names are listed in Supplementary Table S2) was analyzed with RT-PCR. The olfactory organs of locusts were collected and transferred to Eppendorf tubes chilled on liquid nitrogen. Subsequently, they were homogenized with ceramic beads for 240 s at 60 Hz in a Tissue Lyser. Total RNA was isolated using TRIzol Reagent (Invitrogen, Waltham, MA, USA) following the manufacturer's protocol. The extracted total RNA was dissolved in RNase-free water. The concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA). Synthesis of cDNA for PCR was performed with 1 µg total RNA using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA). Each PCR was performed using the following program: 95 °C for 3 min, then 30 cycles at 95 °C for 15 s, 55~60 °C (the T_m varied depending on the primer) for 20 s, and extension at 72 °C for 30 s. Sequences of gene-specific primers and the T_m are listed in Supplementary Table S2. To assess the integrity of the cDNA preparation, primers for the *L. migratoria Actin* gene were used. The PCR products were electrophoresed on 1.2% agarose gels and visualized with Gel-stain dye (Transgene, Shenzhen, China). The PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The specificity of the bands was determined by sequencing the purified PCR products by RuiBiotech (Beijing, China).

2.9. RNA Interference

RNA interference was used to suppress LmigOR expression levels in locusts specifically. T7 promoter sequence-conjugated PCR primers for double-stranded RNA (dsRNA) synthesis were between 300 and 500 bp, as described in Table S3. The PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) and used as templates. The dsRNA was synthesized using the T7 RiboMAX™ Express RNAi System (Promega). Green fluorescent protein (GFP)-derived dsRNA (dsRNA-GFP) was used as

a control in both behavioral and RT-PCR assays. The concentration of dsRNA was determined with a NanoDrop ND-2000 spectrophotometer. The dsRNA was then diluted to 1000 ng/μL with ddH_2O and stored at −20 °C until use. Each locust was injected with 5 μg dsRNA. The dsRNA was injected into each locust's dorsal vessel through the abdomen's intersegmental membrane (first day of fifth-instar nymphs) using an IM-9B microinjector (Narishige, Tokyo, Japan) equipped with a glass capillary. All RNAi-treated locusts were used for the behavioral experiment between the third and fourth days. Silencing efficiency was checked after the behavioral experiments as follows: Intact palps were isolated and immediately frozen in liquid nitrogen or stored at −80 °C. Total RNAs were then extracted using TRIzol Reagent. The silencing efficiency of RNAi was checked by RT–PCR using the above-mentioned primers.

2.10. Statistical Analysis

Data were analyzed and plotted using GraphPad Prism9 (GraphPad Software, San Diego, CA, USA). One-way ANOVA with Fisher's LSD multiple comparisons tests, two-way ANOVA with Bonferroni post-tests and unpaired Student's *t*-test were used for statistical analysis. Data are presented as the mean ± SEM.

3. Results

3.1. Establishment of Olfactory Feeding Behavior Responses of Palps

In this study, we established the duration of palpation-like behavior as a new quantitative parameter (duration of excitatory response, DER) to characterize the olfactory discrimination of the locust to volatiles under a free-moving state (Figure 1A). We observed that locusts were active in visiting both sides with wheat grinds and the solvent (Figure 1B). We analyzed additional parameters of the recorded trajectory response to wheat volatiles. Walking speed, movement distance, and duration between the wheat extraction and solvent were not significantly different, whereas locusts visited the plant extraction side longer than the control side, regardless of whether it was wheat or cotton (Figure S2A–D). We examined the locust actions near the stimulus in greater detail. After approaching the host odors, the locusts vibrated their palps and moved around the stimulus more frequently than the control solvent, which mimicked palpation under a natural situation (see Video S1).

Locusts responded to the negative control (solvent in both arms) with a fairly low PI_DER index; however, wheat volatiles elicited a high index when paired with the solvent, which was not related to the extraction method (juice and grinds) (Figure 1C). Later in the assay, ground wheat seedlings (wheat grinds) were used as the host-plant stimulus. First, an antenna was not required in short-range palpation because antennae-amputated locusts still showed a high PI-DER to the stimulus. However, intact palps were indispensable to inducing active searching toward the stimulus (Figure 1D). Second, using DER, volatiles between host plants (wheat and corn) and nonhost plants (cotton and soybean) could definitely be discriminated in the pairing with the solvent (Figure 1E). Third, locusts were not capable of distinguishing volatiles between two host plants or between two nonhost plants (Figure 1F), but they sensitively preferred host wheat odors over nonhost cotton odors (Figure 1G). Furthermore, the palps of locusts responded to volatiles in a dose-dependent fashion using DER (Figure S3). Overall, the DER paradigm was not only feasible to quantify the olfactory preference for host plants but also the aversion to nonhost plants at short range. Thus, we established a novel behavioral paradigm to determine the olfactory roles of the palps in food choice.

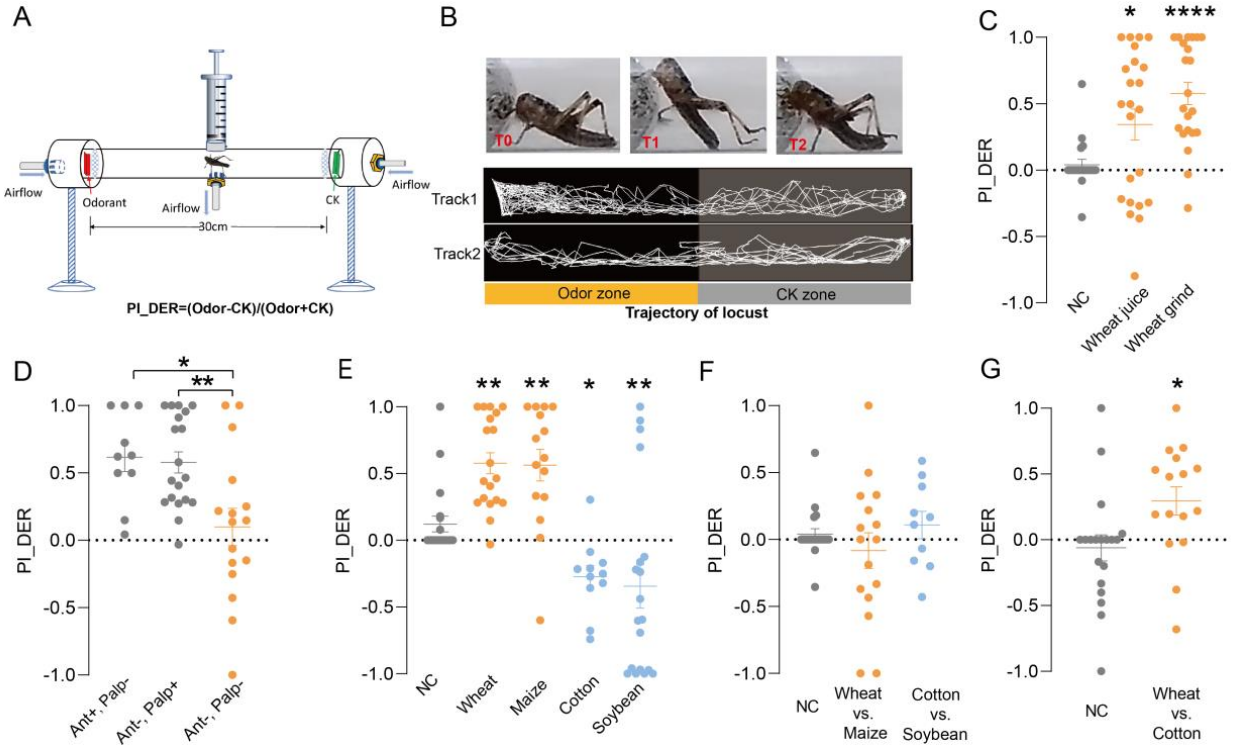

Figure 1. Locusts discriminate host plant volatiles through palp sensation. (**A**): Schematic diagram of the duration of excitatory response (DER) behavior assay. (**B**): Examples of locust behavior in the testing arena. Top, palpation-like behavior in close proximity to wheat grinds. T0–T2, temporal order; bottom, trajectories of two locusts (track 1 and 2) between wheat grinds (odor zone) and solvent (CK zone). (**C**): PI_DER of Ant−/palp+ locusts to wheat juice and wheat grinds. N = 14–23. (**D**): PI_DER of antenna-intact locusts (Ant+, Palp−), palp-intact locusts (Ant−, Palp+) and both antenna- and palp-amputated locusts (Ant−, Palp−) to wheat grinds. N = 10–19. (**E**): PI_DER of locusts to four plant grinds. N = 11–19. (**F**): PI_DER of locusts to plant grinds between wheat and corn or between soybean and cotton. N = 10–16. (**G**): PI_DER of locusts to plant grinds between wheat and cotton. N = 16–18. One-way ANOVA with Fisher's LSD multiple comparisons tests in (**C**–**F**) and unpaired Student's t-test in (**G**). Error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

3.2. Volatiles from Host and Nonhost Plants Contribute to Food Selection in Locust

Volatiles derived from plants are probably the critical cues that guide host recognition by insect herbivores. However, the olfactory strategy in the insect–plant interaction remains unresolved. First, we observed that locusts preferred two host-specific volatiles and two common volatiles over the control solvent (Figure 2A,B, Video S2), whereas they refused two non-host-specific volatiles (Figure 2C, Video S3). Butyric acid has been reported to be a sensitive volatile stimulus to trigger the palp opening response in a previous study [29]. Therefore, we used butyric acid as a positive control. Similarly, butyric acid elicited an obvious DER response (Figure 2D). These results supported the contention that plant-specific volatiles confer reverse effects on locust palpation. Based on the release ratio of several volatiles in corn and cotton plants, we reproduced the mixed volatiles (Figure S1D,E). As expected, mix B, which mimicked the chemical composition in cotton, repelled locusts significantly; however, mix A, which mimicked the chemical composition in corn, did not attract locusts (Figure S1F). These results suggested that either inhibitory heptanol outperforms the feeding attraction of hexanal or more hedonic chemicals in corn are needed to mimic the feeding preference.

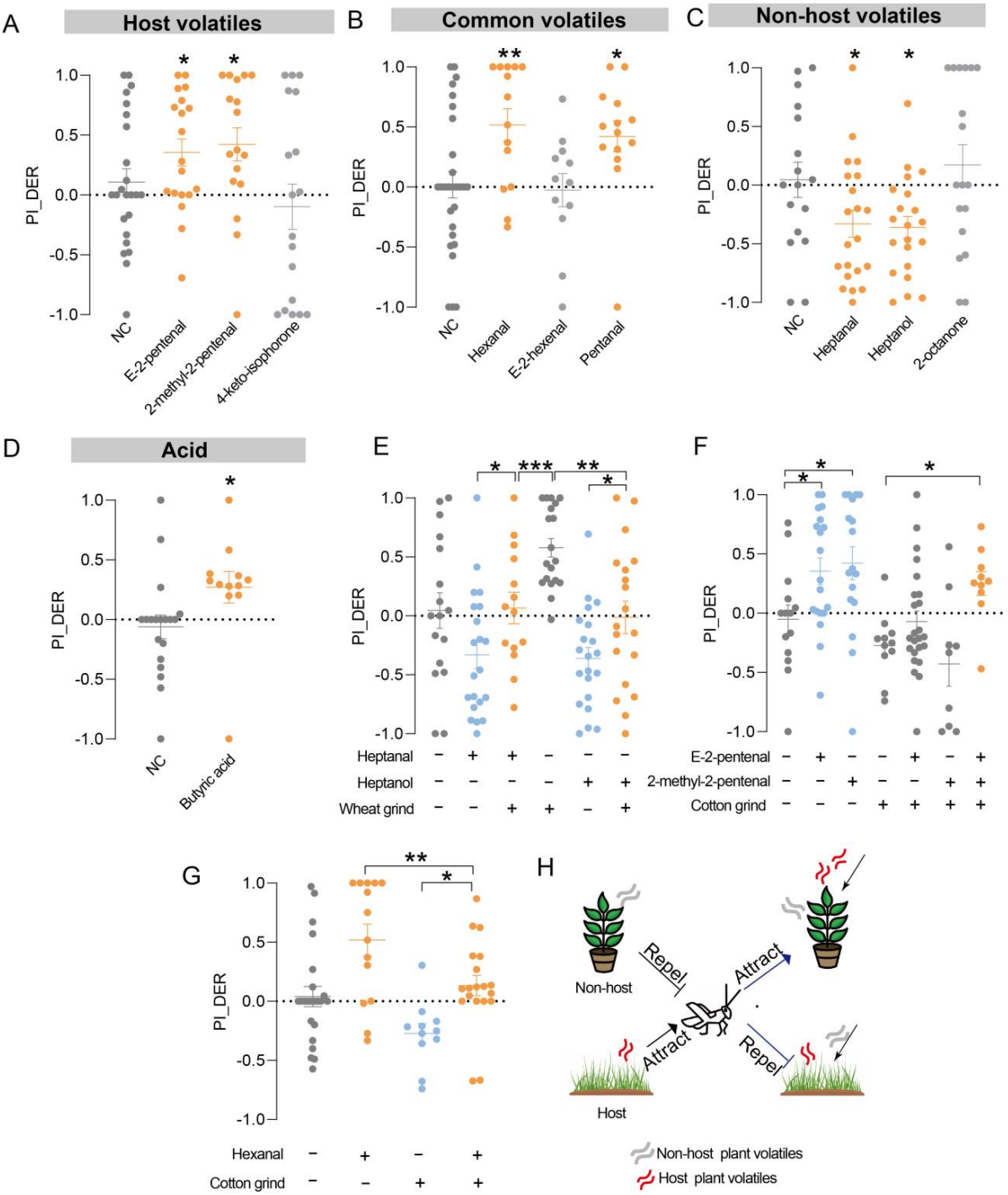

Figure 2. Olfactory discrimination among single odorants at close range. (**A**–**D**): PI_DER of Ant−/Palp+ locusts to host-plant-specific volatiles (**A**), common volatiles (**B**), non-host-specific volatiles (**C**), and butyric acid (**D**) as a positive control. N = 12–25. (**E**): PI_DER of Ant−/Palp+ locusts to wheat grinds mixed with single nonhost volatiles. N = 14–21. (**F**): PI_DER of Ant−/Palp+ locusts to cotton grinds mixed with single host volatiles. N = 9–24. (**G**): PI_DER of Ant−/Palp+ locusts to cotton grinds mixed with the common volatile hexanal. N = 11–21. (**H**): Working model of locust palps action in distinguishing host plants from nonhosts by detecting volatiles. Red undulating lines represent host-specific volatiles. One-way ANOVA with Fisher's LSD multiple comparisons tests in (**A**–**C**,**E**–**G**), and unpaired Student's t-test in (**D**). Error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

We further demonstrated the sufficiency of plant odors in manipulating locust foraging behavior. The addition of nonhost odors into the wheat grinds reduced the total preference of wheat extraction (Figure 2E), whereas supplementation of two host odors into the cotton grinds significantly increased the palpation-like behavior (Figure 2F). In addition, the attractive common volatile hexanal was sufficient to confer the hedonic valence of cotton extraction (Figure 2G). Together, these findings revealed that both host and nonhost plant volatiles contribute to the food selection of locusts in a reverse manner (Figure 2H).

3.3. Odorant Receptors in Palps Involved in Detection of Odors from Host and Nonhost Plants

We systematically profiled the OR genes expressed on palps in previous transcriptomic analyses [27] and identified six novel ORs (Figure 3A), among which LmigOR12 was specifically expressed in the palps. We coupled RNAi with the POR, which was previously used to deorphanize the locust ORs by comparing the olfactory opening frequency of the palps (Figure 3B) [29]. Knockdown of the palp-specific OR12 (dsOR12) led to broad attenuation of the olfactory response among the tested odors, indicating that OR12 might be generally required to tune volatiles on the palps (Figure 3C). Similarly, we knocked down the other five ORs and observed strongly differentiated tuning activities between each other or a combinatorial coding pattern (Figure 3D,E). The dsOR12/dsOR17 injections reduced the POR for most of the tested volatiles, suggesting that LmigOR12 and LmigOR17 were responsible for the detection of host and nonhost plant odors (Figure 3C,D). In addition, LmigOR15 was weakly involved in the detection of (*E*)-2-pentenal, 2-methyl-2-pentenal, and three common volatiles. In contrast, suppression of LmigOR19 led to narrowly reduced responses to (*E*)-2-pentenal and the common volatile pentanal. LmigOR21 was involved in the detection of common volatiles, the host volatile (*E*)-2-pentenal, and the nonhost volatile heptanol (Figure 3D). LmigOR22 was involved in the detection of (*E*)-2-pentenal and 2-methyl-2-pentenal, two common volatiles (*E*)-2-hexenal and pentanal, and the nonhost volatile heptanal (Figure 3E). Notably, OR12, OR21, and OR22 were all needed to modulate the nonhost heptanal. Together, these results supported the conclusion that locust palps employ a combinatorial coding strategy to detect plant volatiles; that is, one receptor can recognize multiple plant odors, and one plant odor can be detected by multiple ORs (Figure 3F).

3.4. Combinatorial Olfactory Signaling Controls the Locust's Food Choice

To directly measure the connection between ORs and cognate ligands in a free-moving locust, we aimed to link gene suppression with the DER assay. First, ddH_2O- or dsGFP-injected locusts did not differ significantly from the wild-type groups (Figure 4A), thus providing a solid control for dsORs injection. Next, dsORs-injected locusts were challenged between wheat extraction and the solvent in the DER assay. At least five LmigORs were associated with reduced foraging behavior after RNAi, and the knockdown of dsOR12 and dsOR15 led to the worst behavioral attenuation to the food cue mixture (Figure 4B). We further separated the volatile mixture into single odors and observed that suppression of single ORs was sufficient to reverse the foraging tendency in response to all three odor types. For instance, down-regulation of either OR12, OR21, or OR22 changed the positive valence of host-specific and common odors, as well as the negative valence of nonhost heptanal (Figure 4C–E). However, OR15 was only required for the positive valence of host-specific and common odors, instead of the negative valence of nonhost odors (Figure 4F). Thus, the critical volatiles-induced free-moving preference is also regulated in a combinatorial olfactory coding mode. Taken together, we conclude that locust palps express olfactory receptors that mediate short-range attraction and repellence to specific plant odors to mediate host-plant selection (Figure 4G).

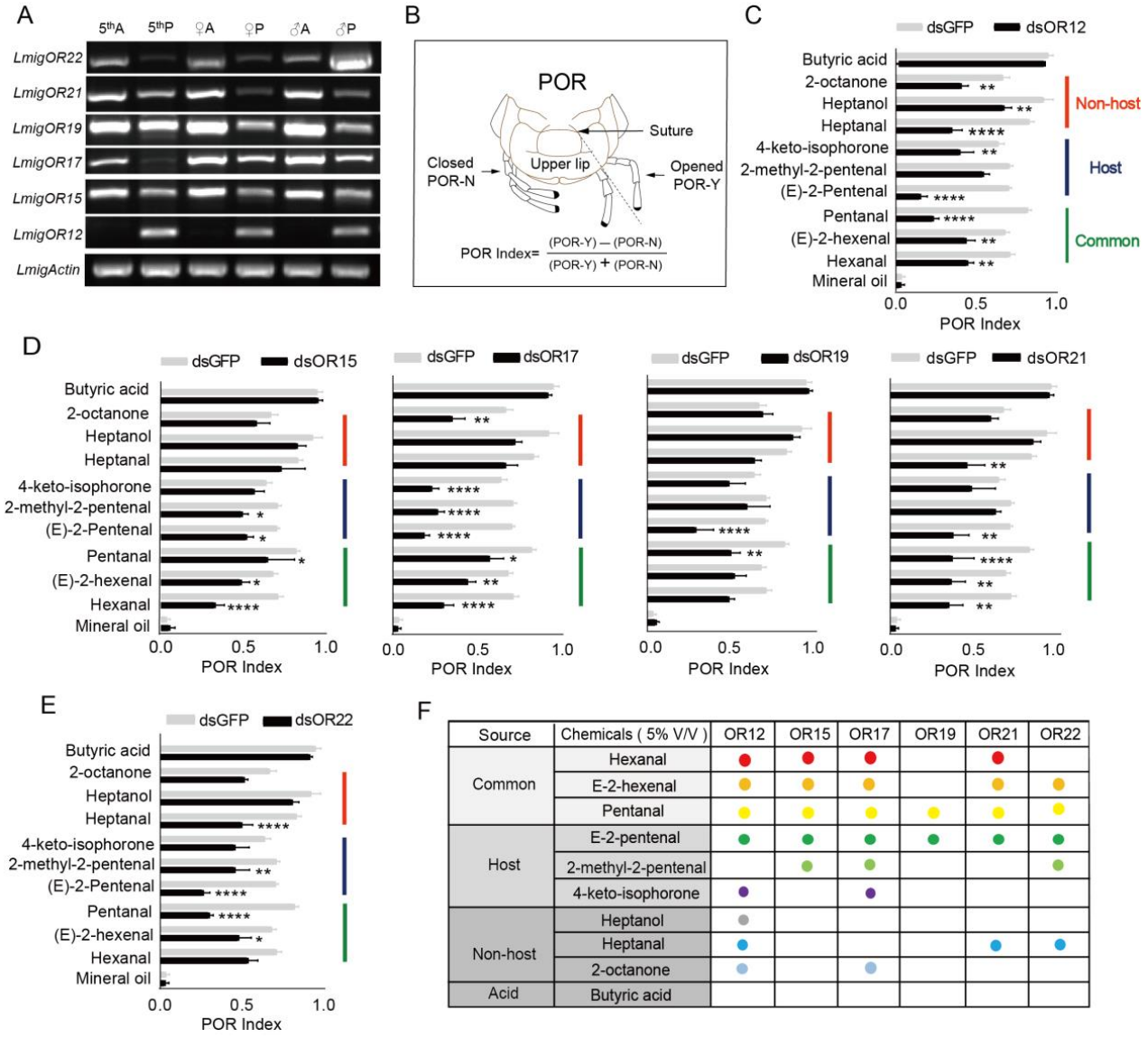

Figure 3. Multiple odorant receptors (ORs) are required for the odorant-induced palp opening response. (**A**): Spatial and temporal expression patterns of locust palp ORs. LmigActin was the positive control. 5thA: antennae of fifth-instar nymphs, 5thP: mouthpart palps of fifth-instar nymphs, ♀A: antennae of female adults, ♀P: mouthpart palps of female adults, ♂A: antennae of male adults; ♂P: mouthpart palps of male adults. (**B**): Diagrammatic illustration of the palp opening response (POR) assay. (**C**–**E**): POR index of Ant−/Palp+ locusts toward odorants when injected with dsRNA of OR12 (**C**), OR15/17/19/21 (**D**), and OR22 (**E**). dsGFP was injected as the control. Two-way ANOVA with Bonferroni post-tests. $N = 3$ (30 locusts per replicate) for each assay. Error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (**F**): Summary of POR results in response to plant odors. Circles of the same color represent the ORs that recognize the same plant odors.

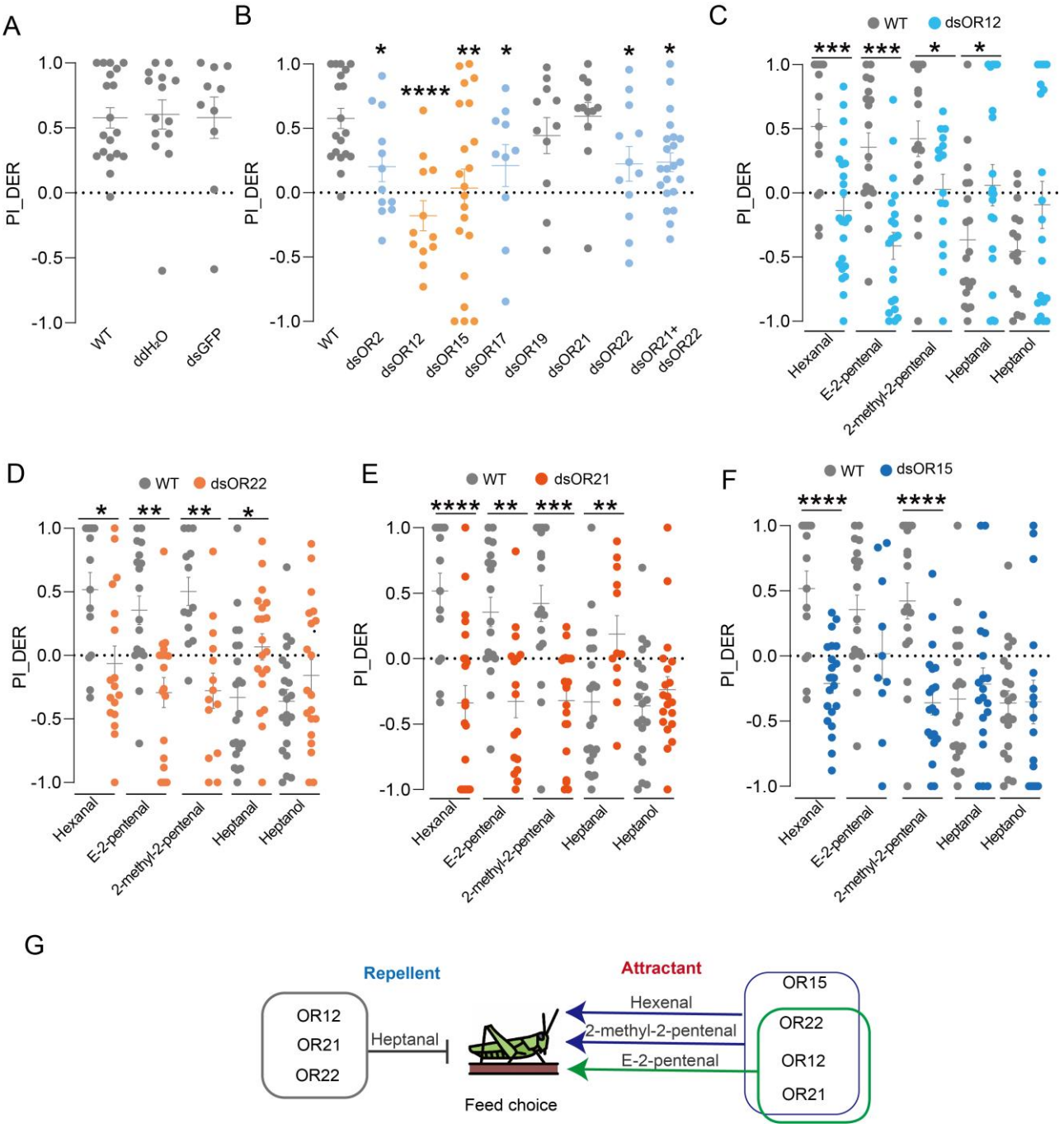

Figure 4. Multiple odorant receptors (ORs) are required for plant odors discrimination. (**A**): PI_DER to wheat grinds in wild-type locusts (WT) or locusts injected with water (ddH_2O) or dsRNA of GFP (dsGFP). N = 10–18. (**B**): PI_DER to wheat grinds in locusts injected with dsRNA against multiple ORs. N = 10–22. (**C**–**F**): PI_DER to single odorants in locusts injected with dsRNA against OR12 (**C**), OR22 (**D**), OR15 (**E**), and OR21 (**F**). Chemical dose: 15% (v/v). N = 10–23. (**G**): Working model of combinatorial olfactory signaling of host-plant recognition by a locust based on the present results. One-way ANOVA with Fisher's LSD multiple comparisons tests. Error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion

How insect herbivores select the correct plants to eat is an unresolved and crucial question in insect–plant interactions. Several hypotheses and theories have been proposed

to account for this common ecological communication process in different insect–plant combinations [1,14,40–42]. However, how the selection strategy was shaped and diverged among insect groups remains enigmatic. This study revealed that short-range olfactory coding to characteristic volatiles in both host and nonhost plants facilitated critical decision-making with regard to food sources in the oligophagous locust.

An olfactometer is widely used to evaluate the behavioral effects of plants on insect feeding preference, for instance, Y-tube or T-tube types [43,44]. The insects are observed for their response to loaded volatiles in an open arena, and several parameters are measured, such as the preference to approach each loaded volatile and the time or distance they stay in corresponding spaces. One of the drawbacks of these traditional paradigms is the uncertainty of where and when the olfactory process occurs, which is of particular importance to pinpoint the insect–plant interaction strategy. The DER is designed to visualize the olfactory contribution of locust palps to food cues at short range. Locusts move freely in the tunnel offering two choices containing volatiles, and then vibrate the palps intensely in close proximity to an attractive stimulus, which is reminiscent of palpation. Using DER in this study, we observed that oligophagous locusts sensitively discriminated hedonic wheat extraction from repulsive cotton extraction, and this selection depended on short-range olfactory pathways on the palps. The DER assay allowed us to release the restricted insects and observe their olfactory choice toward stimulus delivery at a short range. This assay could also be modified for testing other insect herbivores.

It has long been considered that volatile cues emitted by plants contribute largely to influencing insect behavior during host plant searching and selection [13,15,21,45]. Herbivorous insects are exposed to plant volatiles in a complex environment, but not all plant volatiles may carry information that is useful for host-plant decisions. With consideration of polyphagous insects, in particular, there can be few or even no common volatiles that are indicative of the plant spectrum of interest in a broad range of host plants. Nevertheless, oligophagous insects are thought to choose food plants relatively more easily because limited hosts are associated with specific attractive infochemicals that are indicative of plant localization. It has been reported that insects appear to choose host plants via recognition of specific blends of certain ubiquitous odors. The signal value of this blend seems to be interpreted by the blend composition as a whole instead of the summed effects of individual components [42]. Although greater focus has been paid to the identification and behavioral tests of host-plant-derived volatiles [46,47], whether nonhost plants release inhibitory odors to repel oligophagous insects remains unclear. The present results supported the conclusion that characteristic individual volatiles from host and nonhost plants have opposite behavioral effects, and this "double-check" would greatly enhance the accuracy of final decision-making. This delicate "pull–push" strategy in locusts complements the host-plant selection theory, especially in understanding how specialist insects select their food source.

During the foraging behavior of insects, they will encounter, detect, and behaviorally respond to plant volatiles from long range to short range. As the distance changes, the content and concentration of the compound changes over long to short distances. Generally, insects initially trace the common chemicals that are emitted by host and nonhost plants over long distances. While approaching a host, they need to accurately judge the plant species based on the characteristic volatiles released by host and nonhost plants. This study showed that locusts were attracted by two host-specific volatiles, (*E*)-2-pentenal and 2-methyl-2-pentenal but were repulsed by the non-host-specific heptanol and heptanal volatiles at short-range in the DER assay. A previous study [28] reported that the host-specific volatiles (*E*)-2-pentenal and 2-methyl-2-pentenal had significant effects on the biting response in locusts, but the non-host-plant volatiles heptanol and heptanal showed no effect on biting behavior. In addition, the common volatile hexanal resulted in locust biting. These observations are consistent with the present findings. In addition, it suggests that olfaction and taste together determine the locust feeding selection in the short range,

and olfaction occurs before taste. The chemical cues are used to decide whether to eat, and then the quality of the food is further evaluated through taste.

Gustatory and tactile factors have been shown to guide the short-range evaluation of food plants, whereas volatiles are believed to act in long-range attraction via antennal olfactory signaling [25,28]. For orthopteran locusts, the domes of the maxillary and labial palps among the mouthparts are sparsely affiliated with olfactory basiconica sensilla and canonical ORco-based olfactory signaling was demonstrated to tune common green-leaf volatiles of host plants [30,48]. In this study, suppression of olfactory signaling, putatively in the palps, disrupts host-plant selection in the short range. This demonstrated the direct roles of olfactory coding in the close differentiation of plants. An interesting question is how are the opposite behavioral effects for host and nonhost plant volatiles encoded in the olfactory receptor neurons (ORNs) and then relayed and presented in the olfactory center in the brain? First, the cellular expression modes between ORs involved in sensing wheat and cotton odors should be determined in the palps. The compartmentalization of ORNs for opposite plant odors would be expected. In addition, whether this spatial separation of ORNs is associated with a physiological reaction to volatiles with opposite valence needs to be addressed. Second, how do the plant odors of wheat and cotton trigger opposite behavioral consequences? One possibility is that different ORNs that receive host and nonhost odors project into spatially separated areas in the brain. An early report supported the hypothesis that odor-induced vomiting is combinatorially triggered by palp ORNs that project to the lobus glomerulatus (LG) in the locust brain [30]. It would be of particular interest to check the projection patterns in the LG between olfactory inputs from single odors of wheat and cotton. Genetically labeling of specific ORs identified in this study could clarify this question in the future.

5. Conclusions

In summary, we designed one novel behavioral paradigm that allowed the free-moving locusts to make olfactory choices in short-distance and provided an alternative way to measure the olfactory roles of palps in short range. Our results suggested that palps were required to differentiate host plants apart from nonhost counterparts sensitively and elucidated the delicate "push–pull" model that plants employ to influence the feeding choice of locusts. We also revealed that this model relies on the combinatorial coding logic of locust odorant receptors (ORs) in the determination of host- and nonhost plant odorants in short distances.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13051030/s1, Figure S1: Quantification of plant volatiles by GC-MS. Figure S2: Characterization of novel DER behavioral assay; Figure S3: Dose-dependent behavioral curve of three plant volatiles; Figure S4: RNAi efficiency determined with RT-PCR. Table S1: Details of odors used in the behavioral assays; Table S2: Primers used for RT-PCR and the Tm; Table S3: Sequence of primers used in RNAi experiments. Video S1: 15% 2-methyl-2-pentenal attract locust; Video S2: 15% hexenal attract locust; Video S3: 15% heptanol repel locust.

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