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Plant Volatiles Activating Specific Olfactory Receptor Neurons of the Cabbage Moth *Mamestra brassicae* L. (Lepidoptera, Noctuidae)

S. Ulland[1], E. Ian[1], M. Stranden[1], A.-K. Borg-Karlson[2] and H. Mustaparta[1]

[1]Neuroscience Unit, Department of Biology, Norwegian University of Science and Technology, NO-7489 Trondheim, Norway and [2]Ecological Chemistry Group, Department of Chemistry, Royal Institute of Technology, SE-100 44 Stockholm, Sweden

Correspondence to be sent to: Hanna Mustaparta, Neuroscience Unit, Department of Biology, Norwegian University of Science and Technology, NO-7489 Trondheim, Norway. e-mail: Hanna.Mustaparta@bio.ntnu.no

Abstract

Herbivore insects are suitable model organisms for studying how plant odor information is encoded in olfactory receptor neurons (RNs). By the use of gas chromatography linked to electrophysiological recordings from single RNs, screening for sensitivity to naturally produced plant odorants is possible in order to determine the molecular receptive ranges of the neurons. Using this method, we have in this study of the cabbage moth, *Mamestra brassicae*, classified 21 types of olfactory RNs according to their responses to odorants present in the host plants of Brassicae, in the related species of *Arabidopsis*, as well as in essential oils of nonhost plants like ylang-ylang. Most of the RNs were tuned to one or a few structurally similar compounds, showing minimal overlap of their molecular receptive ranges. Whereas some RNs displayed a novel tuning, others were tuned to the same compounds as neurons in other insect species. We also found colocation in the same sensillum of 3 RN types with the same response characteristics and tuning as 3 colocated types described in heliothine moths living on different host plants. The presence of similar RN types across different insect species implies conservation or reappearance of the RN types, independent of the evolution of host plant ranges.

Key words: GC-SCR, *Mamestra brassicae*, molecular receptive range, receptor neuron

Introduction

In the search by an insect for a proper mate or a suitable host plant, the olfactory system meets the challenge of recognizing relevant information about volatiles released by conspecifics or sympatric species as well as by plants. Whereas much knowledge has been acquired concerning pheromone detection in many insect species, much less data about relevant plant odorants have been reported. In recent years, the use of gas chromatography (GC) linked to electrophysiological recordings from single receptor neurons (RNs) (GC linked to single-cell recordings [GC-SCR]) has resulted in reliable information about relevant plant odorants in some species of moths, weevils, and other beetles (e.g., Blight et al. 1995; Larsson et al. 2001; Barata et al. 2002; Stranden et al. 2003; Bichão, Borg-Karlson, Wibe, et al. 2005; Røstelien et al. 2005). Plants may emit more than 1000 volatile compounds (Dudareva et al. 2004), and the insects must be able to discriminate among these volatiles in order to choose a suitable host. The important objective in this context is to identify the relevant odorants utilized by the insects in their orientation toward a host. A fruitful approach in resolving the problem seems to be identification of the odorants among the numerous volatiles that are detected by single RNs and then to test the behavioral significance of these odorants.

Another aspect of the results from studies using GC-SCR are data on the specificity of olfactory RNs for relevant odorants. Such knowledge is of interest for olfaction in general. Because each olfactory RN typically expresses only one type of receptor proteins as shown in insects and vertebrates (Clyne et al. 1999; Störtkuhl and Kettler 2001; Wetzel et al. 2001; Krieger et al. 2002; Keller and Vosshall 2003; Hallem and Carlson 2004; Mombaerts 2004), we can assume that the specificity of a RN reflects the specificity of the particular receptor protein type expressed in the neuron. By screening via the GC all volatiles present in the blends of various plants on single neurons, the molecular receptive ranges (MRRs) of olfactory RNs have been identified in several herbivorous species. According to this, the neurons have appeared as distinct functional types like in heliothine moths (Røstelien et al. 2005). In general, each type seems to be specialized for one odorant, called the primary odorant, and

responds weaker to a few structurally similar odorants. Thus, the MRR of each type is narrow, and in heliothine moths, a minimal overlap is found between the neuron types. Comparison of the functional types of olfactory RNs within closely and distantly related species may shed light upon the evolution of the specificity of olfactory receptor proteins. This study allowed comparison of the olfactory RNs with another polyphagous species (*Mamestra brassicae*) preferring different hosts than the heliothines that live on plants of sunflower, cotton, corn, etc.

Mamestra brassicae is a polyphagous insect species that survives on many species of plants. However, these insects often choose host plants of the genus *Brassica* (McKinlay 1992; CAB International 2005). The female moths, having a large number of antennal sensilla for plant odorants, use odor cues to locate the host plants where she may lay clusters of 50–300 eggs under the leaves. Feeding by the caterpillars causes severe damage on the plants in monocultures, and the species is an economically important pest in agriculture. Research aiming at identifying behaviorally modifying odorants and understanding the olfactory mechanisms involved in host plant location in this species may contribute to integrated pest management. Previous studies of olfaction in this species concern pheromone detection (Renou and Lucas 1994), whereas studies have just started on how plant odor information is encoded in single RNs.

In the present study of olfaction in *M. brassicae*, we have used GC-SCR to identify relevant plant odorants detected by single RNs. Volatiles collected from the host plants *Brassica napus* L. and *Brassica oleracea* (Italica), as well as ecotypes of the related species *Arabidopsis thaliana*, served as test materials. In addition, we tested volatiles of other plants used in studies of heliothine moths. We present 53 olfactory RNs classified into 21 RN types. The RNs showed a narrow tuning and minimal overlap of their MRRs.

Materials and methods

Insect material

Mamestra brassicae pupae were supplied by The Norwegian Crop Research Institute, Ås, Norway. Prior to pupation, the larvae were fed on a white bean diet. The sexed pupae were stored in separate containers placed in climate chambers (22 °C, 14:10 light:dark photoperiod, onset of dark cycle at 10 AM). After eclosion, the adult insects were kept in cylindrical containers with access to water containing sucrose (5%). The age of adult insects used in the experiments ranged from 2 to 14 days (showing no differences in responses depending on age). Both sexes were used in the experiments.

Chemicals and headspace samples

Volatiles were collected from several plant species by the use of a headspace technique (Byrne et al. 1975; Pham-Delegue et al. 1989; Røstelien et al. 2000). The plants were placed in a closed oven bag (Look) through which purified air was suctioned (less than 40 ml\min) and led into glass tubes (2.5 × 120 mm) packed with the adsorbents (Tenax TA and Porapak Q, 1:1). The air was purified in a filter of activated charcoal before the intake to the bag, and aeration was carried out for 24 or 48 h. The trapped volatiles were eluted by filling the glass tube with the solvent (hexane and ethyl acetate, ratio 1:1) and leading it drop by drop into different vials that were stored in a freezer. The most concentrated samples, consisting of the first 2 or 3 drops, were usually used as test samples on the RNs. In addition to these plant materials, essential oils and synthetic compounds were also included as test materials. The essential oil of ylang-ylang (*Cananga odorata*), previously tested by our group, was particularly selected because it contains numerous compounds with identified retention times. The synthetic compounds were tested alone or in mixtures (standards) at concentrations ranging from 10^{-7} to 10^{-4} (v/v). The dilution of the compounds constituting the standards was 10^{-4}. Dilutions of all test materials were made in hexane. In general, the purity of the synthetic compounds was high (95–98%). Using GC-SCR, the impurities would not influence the results. Table 1 gives an overview of the materials used.

Direct stimulation via cartridges

Direct stimulation via glass cartridges ($v \sim 1$ ml) was used for screening the RN sensitivity to the various samples of headspace and other mixtures before the tests were performed via the GC. Five microliters of each test sample were applied to a filter paper placed inside a cartridge letting the solvent evaporate before use. The RN was exposed for the test sample by puffing 4 ml of air during 0.5 s through the cartridge and over the insect antenna. Between stimulations, the antenna was exposed to a continuous flow (500 ml/min) of purified air.

GC linked to single-cell recordings

The insects were mounted in a Plexiglas holder, and the head and antennae were stabilized with tape and wax as described by Røstelien et al. (2000). Electrophysiological recordings from single RNs were made by the use of electrolytically sharpened tungsten microelectrodes. The recording electrode was intended to be placed into the base of an olfactory sensillum, verified by an acceptable signal to noise ratio. However, the sensillum from which the recording was actually made could not be determined because of the high density of the sensilla hairs. Both the female and the male antennae possess large numbers of *sensilla trichodea* of varying length (Figure 1) (Renou and Lucas 1994), but other types are also present. When obtaining spike activity, we first screened via cartridges the neurons for sensitivity to the various test samples. Those eliciting responses were then tested via the GC by injecting 0.5–1 µl of the solution into the GC column. The column was equipped with a splitter at the end,

leading half of the effluent to the flame ionization detector (FID) and the other half into a constant airflow (500 ml/min) blowing over the insect antenna. This made it possible, together with the simultaneous single-cell recording, to determine which compounds in the mixture elicited the responses. The electrophysiological recording and gas chromatogram were recorded in the software GC-EAD (Syntech, Kirchzarten, the Netherlands). Separation in the polar column (DBwax [25 m, inner diameter {i.d.} 0.25 mm, film thickness 0.25 μm, J&W Scientific, Palo Alto, CA]) was performed with 2 different programs. The first and most frequently used program started at the initial temperature of 80 °C with an increase rate of 6 °C/min to 180 °C and a further increase rate of 15 °C/min to 220 °C, which was held until the sample was eluted. The second program was used to achieve better separation of the compounds in some of the samples: performed from the initial temperature of 50 °C isothermal for 2 min followed by a 3 °C/min increase to 180 °C and a final increase of 15 °C/min to 220 °C. The FID temperature was set to 230 °C for all programs. The GC was equipped with an on-column injector.

Identification of the compounds activating the RNs, GC linked to mass spectrometry

The active compounds were identified by GC linked to mass spectrometry (GC–MS) using a Finnigan SSQ7000 MS instrument connected to a Varian 3400 GC. Ion source was at 150 °C, 70 eV, and scan range 30–400 m/z. A DBwax column (30 m, i.d. 0.25 mm, and film thickness 0.25 μm; J&W Scientific) was used for separation of the compounds. Temperature program was 40 °C for 1 min followed by 4 °C/min to 220 °C for 12 min and injector temperature 200 °C. Helium was the carrier gas at 10 psi. The compounds were identified by means of mass spectra and GC retention times of commercially available standards.

Spike analysis and cell classification

Electrophysiological recordings from RNs were stored and analyzed in the software program Spike 2 (Cambridge Electronic Design Limited, Cambridge, UK). Separation of the cell types in the recordings was based on differences in spike amplitudes and waveforms. The RNs were classified according to which odorant elicited the strongest response (primary odorant) as well as those having weaker effects (secondary odorants).

Results

The results presented in this paper are based on recordings from more than 120 RNs on the antennae of *M. brassicae* females and males. Fifty-three of these RNs responded to stimulation with plant odorants. No differences were noticed between RNs obtained in the 2 sexes. Most RNs ($n = 51$) were investigated using GC-SCR. The recordings lasted from 30 min up to 3 days, allowing stimulation of each olfactory RN 1–52 times via the GC. This included tests with the numerous plant volatiles present in the headspace samples of plants, essential oils, and synthetic standards. Thus, the long-lasting cell contacts enabled repeated tests, including retesting the same samples to verify which compounds the RNs responded to. All responses were recorded as increased firing rate that followed the concentration profile of the GC peak. The 53 RNs were classified into 21 distinct types (Table 2) according to the compounds eliciting the responses, MRR. In general, there was little overlap between the MRRs of the 21 cell types. Only 3 pairs of the RN types showed overlap of single compounds; *racemic* linalool (RNs 8 and 10), (Z3)-hexenyl acetate (RNs 11 and 13), and *p*-methylanisole (RNs 15 and 16). Below, the RNs are presented according to the group of compounds to which they responded and named by the primary odorant or the odorant eliciting the strongest response.

Terpenoides

RN type 1: E,E-α-farnesene

Screening by cartridges with the various samples resulted in responses to the essential oil of ylang-ylang and standard 8 by 4 RNs, classified as type one. Figure 2A shows a gas chromatogram of standard 8, containing a mixture of synthetic farnesene isomers (standard 8) and the simultaneously recorded responses of one of the RNs. One marked strong response was elicited by *E*,*E*-α-farnesene, whereas the 2 structurally similar compounds *E*-β-farnesene and *Z*,*E*-α-farnesene elicited only weak responses. Spike analysis of the responses to the 3 compounds confirmed that they originated from the same RN. Three of the 4 RNs were recorded together with other RN types displaying different spike amplitudes and waveforms, probably appearing in the same sensillum. These neurons, classified as RN type 2, 3, and 4 are presented below. In one recording, all 4 RN types appeared in the same sensillum.

RN type 2: E,E-farnesol

One RN, recorded together with RN type 1, responded to a terpenoid eluted later than *E*,*E*-α-farnesene when stimulated with ylang-ylang oil. GC–MS analyses indicated the active compound to be *E*,*E*-farnesol. However, verification by retesting with a synthetic sample of *E*,*E*-farnesol needs to be carried out. This RN was classified as type 2. Spike analysis of the recording showed that the responses to *E*,*E*-α-farnesene and *E*,*E*-farnesol originated from 2 neurons with different spike amplitudes and waveforms, that is, RN types 1 and 2.

RN type 3: E-β-ocimene

Two RNs were classified as type 3. Both were recorded together with RN type 1 and in one recording together with

Table 1 Constituents of the test materials used to stimulate the RNs

Synthetic standards	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
	Camphor[a]	1-Heptanol[f]	*Racemic* Citronellol	Benzylalcohol[j]	2,3-Butandiol[k]
	Isoborneol[b]	(2*E*)-Hexenal[g]	Dihydromyrcene	Benzyl cyanide[j]	Butyl caprylate[k]
	racemic Linalool[c]	1-Hexanol[h]	Dihydromyrcenol	(+)-3-Carene[j]	iso-Butyl hexanoate[k]
	Methyl benzoate[d]	(3*Z*)-Hexen-1-ol[i]	Geraniol	Citral [geranial and neral 1:1][j]	Butyl pentanoate[k]
	Methyl salicylate[e]	(2*E*)-Hexen-1-ol[h]	Myrcene	Indole[j]	Ethyl 2-methylpropanoate[k]
	(*Z*)- and (*E*)-β-Ocimene (70%)/limonene (25%)[c]	(2*Z*)-Hexen-1-ol[h]	Myrcenol		Hexyl butyrate[k]
		(3*Z*)-Hexenyl acetate[g]	(*Z*)- and (*E*)-β-Ocimene		Hexyl formate[k]
	(+)-*trans*-Verbenol[b]	1-Octanol[i]	3,7-Dimethyl-1-octanol		*racemic* 2-Methyl-1-butanol[k]
		3-Octanone[b]			Methyl methylbutyrate[k]
					Propyl butyrate[k]
					iso-Propyl butyrate[k]
					Pentyl hexanoate[k]
	Standard 6	Standard 7	Standard 8	Standard 9	Standard 10
	Butyl propionate[k] Ethyl butyrate[k]	Butyl butyrate[k] Butyl hexanoate[k]	*E*-β-Farnesene[k] Farnesenes mix of isomers[k]	(−)-Verbenone[i]	Allyl isothiocyanate[k]
	Ethyl 2,4-decadienoates, mix.[k]	Ethyl 2-methylbutyrate[k]	Farnesol[b]		3-Butenyl isothiocyanate[k]
	Hexyl hexanoate[k]	(2*E*)-Hexenal[k]	Farnesol, mix of isomers[b]	(−)-*trans*-Verbenol[i]	Butyl thiocyanate[k]
	Hexyl 2-methylbutyrate[k]	(2*E*)-Hexenyl acetate[k]			3-Methylbutyl thiocyanate[k]
	Hexyl propanoate[k]	(3*Z*)-Hexenyl hexanoate[k]			Phenethyl isothiocyanate[k]
	3-Methyl-1-butanol[k] 2-Methylbutyl butyrate[k] 2-Methylbutyl hexanoate[k] 2-Methylbutyl propanoate[k]	3-Methylbutyl hexanoate[k] 2-Methylhexyl butanoate[k] *sec*-propyl butanoate[k]		(+)-*cis*-Verbenol[i]	Phenethyl thiocyanate[k] Phenyl isothiocyanate[k]
	Pentyl butyrate[k]				

Table 1 Continued

Synthetic compounds, headspace samples, and essential oils	Terpenoids	Aromatics	Aliphatics	Headspace samples	Essential oils
	Camphor[a]	Ethyl benzoate[d]	3-Octanone[b]	*Arabidopsis thaliana* (Cape Verdi)[m]	Basil (*Ocimum basilicum* L.)[o]
	racemic Citronellol			*A. thaliana* (Columbia)[m]	Bergamot (*Citrus bergamia*)[o]
	Dihydrolinalool[b]	Eugenol[h]	*racemic* 1-Octen-3-ol[h]	*A. thaliana* (Landsberger) [m]	Clove bud (*Syzygium aromaticum* L.)[o]
	Geraniol			*A. thaliana* (Wassilewskija)[m]	Clove (*S. aromaticum* L.)[o]
	(*S*)-(+)-Linalool[b]	*m*-Methylanisole[h]	(*S*)-(+)-1-Octen-3-ol[l]	*Brassica napus* L.[m]	Cinnamon (*Cinnamomum zeylanicum* L.)[o]
	racemic Linalool[c]			*Brassica oleracea* (Italica)[m]	Fennel (*Foeniculum vulgare* Mill.)[o]
	racemic Tetrahydrolinalool[h]	*o*-Methylanisole[h]	Methyl jasmonate[d]	Cotton induced *Gossypium herbaceum L* (infected by larvae of *Spodoptera littoralis* B.)[n]	Lemon (*Citrus medica* L.)[o]
	Linalyl acetate[h]	*p*-Methylanisole[h]			Lilac (*Syringa vulgaris* L.)[b]
					Peppermint (*Mentha x piperata*)[o]
					Ylang-ylang (C*ananga odorata* Hook)[g]

[a]Kebo.
[b]A.-K. Borg-Karlson (KTH), Stockholm, Sweden.
[c]Fluka, Buchs, Switzerland.
[d]Lancaster, Lancashire, UK.
[e]Merck, Darmstadt, Germany.
[f]Jansen-Chemica, Geel, Belgium.
[g]Dragoco RGB, Surrey, UK.
[h]Aldrich, Oslo, Norway.
[i]Sigma, Oslo, Norway.
[j]J. Anderson; (KTH), Stockholm, Sweden.
[k]I. Liblikas (KTH), Stockholm, Sweden.
[l]J. Fäldt (KTH), Stockholm, Sweden.
[m]S. Ulland (NTNU), Trondheim, Norway.
[n]S. Gouinguené and T. Turlings (UniNE), Neuchatel, Switzerland.
[o]NMD (Norsk Medisinal Depot), Oslo, Norway.

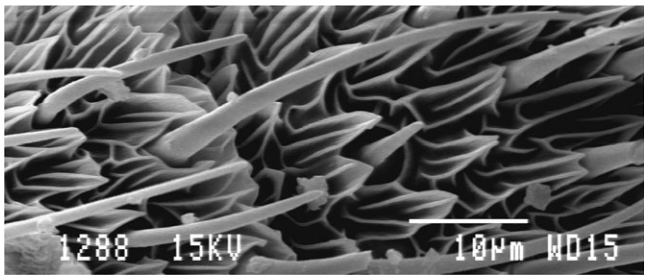


Figure 1 Scanning electron microscope (SEM) picture of a part of the antenna of a female *Mamestra brassicae*. The sensilla hairs containing the olfactory RNs are densely distributed over the antenna.

type 2. They responded strongest to *E*-β-ocimene and weaker to the structurally similar compound β-myrcene. Both compounds were present in the headspace sample of induced cotton (infected by larvae of *Spodoptera littoralis* B.). Recordings of RN type 3 were obtained twice.

RN type 4: 4,8,12-trimethyl-1,3,7,11-tridecatetraene

Two experiments showed a RN that responded when stimulated with the cartridge containing the headspace sample of induced cotton. These RNs classified as type 4 appeared together with RN types 1, 2, and 3 and responded during elution of the terpenoid *E*,*E*-4,8,12-trimethyl-1,3,7,11-tridecatetraene (*E,E*-TMTT) when stimulated with induced cotton via the GC. Responses to other compounds were not obtained. Figure 2B shows the responses of RN type 1 and RN type 4 to the GC-eluted compounds, *E*,*E*-α-farnesene and *E*,*E*-TMTT, respectively. Analysis of spike waveforms and amplitudes showed that the responses to *E*,*E*-TMTT and *E*,*E*-α-farnesene originated from 2 different RNs. In spite of the structural similarity of these compounds, they did not show a reciprocal secondary effect on the other RN type.

RN type 5: citronellol

A RN type was activated during screening with cartridges containing the essential oils of clove bud, clove, and cinnamon. Injection of these essential oils in the GC showed that a compound with the retention time of citronellol activated the RN (Figure 3A). Injection of synthetic citronellol verified the response to this compound. Altogether, 3 RNs responded to citronellol and were classified as RN type 5. The responses were demonstrated 8 times when stimulating via the GC with essential oils and synthetic compounds. Separation of citronellol in a chiral column was not made. No responses were obtained to the other plant compounds present in the material tested via the GC. Unfortunately, headspace samples were not tested on this RN type.

RN type 6: 1,8-cineol

One RN responded to the essential oils of peppermint, ylang-ylang, and cinnamon during direct screening with cartridges. Injection of peppermint essential oil in the GC followed by GC–MS analysis showed that the RN was activated by 1,8-cineol. Further tests by injection of ylang-ylang and cinnamon essential oils confirmed the response to 1,8-cineol. Dose-dependent responses to this compound was shown by a long-lasting firing to the high concentration in the peppermint oil and a weak response decaying with the GC peak to the low concentration present in the essential oils of cinnamon and ylang-ylang.

RN type 7: γ-terpinene

One RN was excited during screening with cartridges containing the essential oil of peppermint and cinnamon. Injection of peppermint essential oil in the GC column elicited 4 responses, the strongest to γ-terpinene and weaker responses to β-myrcene, α-phellandrene, and β-phellandrene. In addition, this RN responded weakly to α-phellandrene when stimulated via the GC with cinnamon essential oil and standard 1. The compounds were identified by their mass spectra.

RN type 8: (+)-trans-verbenol

One RN responded during screening with a cartridge containing the standard 1. Stimulation via the GC showed that the response was to (+)-*trans*-verbenol. In addition, weak responses were obtained to *racemic* linalool, methyl benzoate, and an unknown compound (trace amount) present in the standard 1. (–)-*trans*-Verbenol was not tested because it was not available during the recording. This RN was colocated with a RN tuned to linalool (type 10). Separation of activity originating from the 2 RN types was based on analysis of spike waveform and amplitude.

RN type 9: fenchone

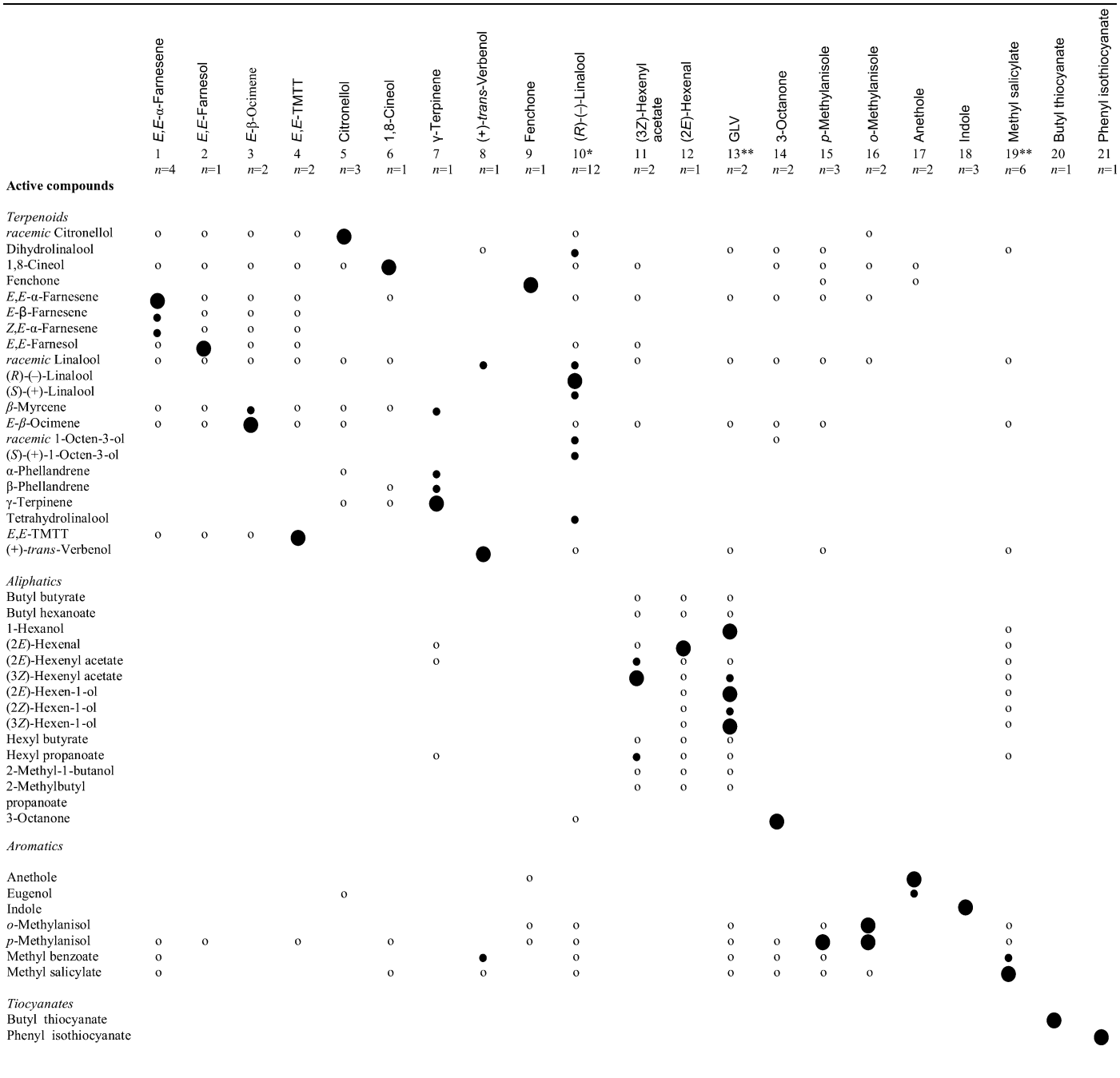
The RN classified as type 9 responded to stimulation with a cartridge containing the essential oil of fennel. Screening with the other essential oils, headspace samples, and synthetic standards did not activate this RN type. Injecting fennel oil in the GC showed that the RN responded to a compound present in high amount (Figure 3B). This compound was identified by GC–MS as fenchone. The RN type appeared in 2 recordings, both showing weak responses to the high concentration of fenchone in the fennel oil, which suggests that fenchone might not be the primary odorant for this RN type. Unfortunately, chiral separation of fenchone was not performed.

Aliphatics

RN type 11: (3Z)-hexenyl acetate

Responses were obtained when stimulating a RN type via cartridges with the essential oil of ylang-ylang and standard 2. Injection of ylang-ylang oil and standard 2 in the GC resulted in responses at the retention time of (3*Z*)-hexenyl

Table 2 Overview of the olfactory RN types identified in *Mamestra brassicae* and their responses to compounds tested via the GC



The compounds tested were either synthetic or in headspace samples and essential oils (identified by GC–MS). The number of neurones recorded within each RN type *(n)* is given. The relative response strengths of the compounds are indicated by the size of the dots (large dot for strongest response). Zero indicates no response. Empty spaces are used when the compound was not tested. The indicated response strengths can only be compared within each RN type. GLV, green leaf volatiles.

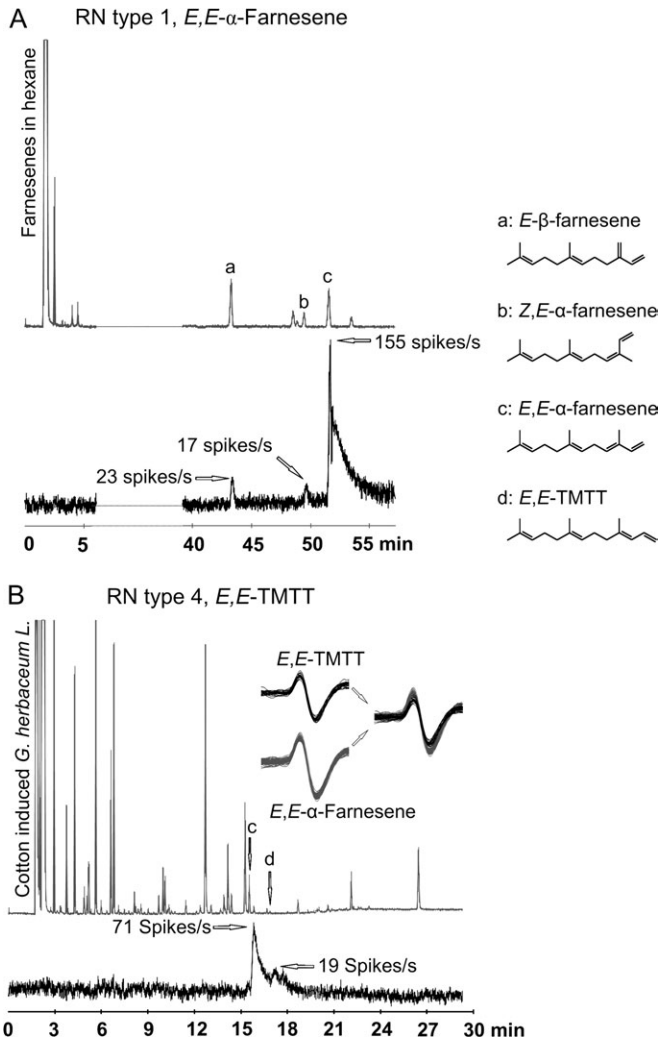


Figure 2 Gas chromatogram of a sample (upper trace) and the simultaneous recording of a RN (trace below) **(A)** Injection of a mixture of farnesenes and the RN of type 1 responding to 3 compounds: *E*-β-farnesene (secondary odorant), *Z*,*E*-α-farnesene (secondary odorant), and *E*,*E*-α-farnesene (primary odorant). The structural similarity of the farnesene isomers is shown to the right. **(B)** Injection of a headspace sample of induced cotton (*Gossypium herbaceum* L.) and the RN of type 4 responding to the compound *E*,*E*-TMTT (trace amount). The colocated RN of type 1 responded to *E*,*E*-α-farnesene present in the sample. (inset) Separation of RN types by analysis of spike amplitude and waveform.

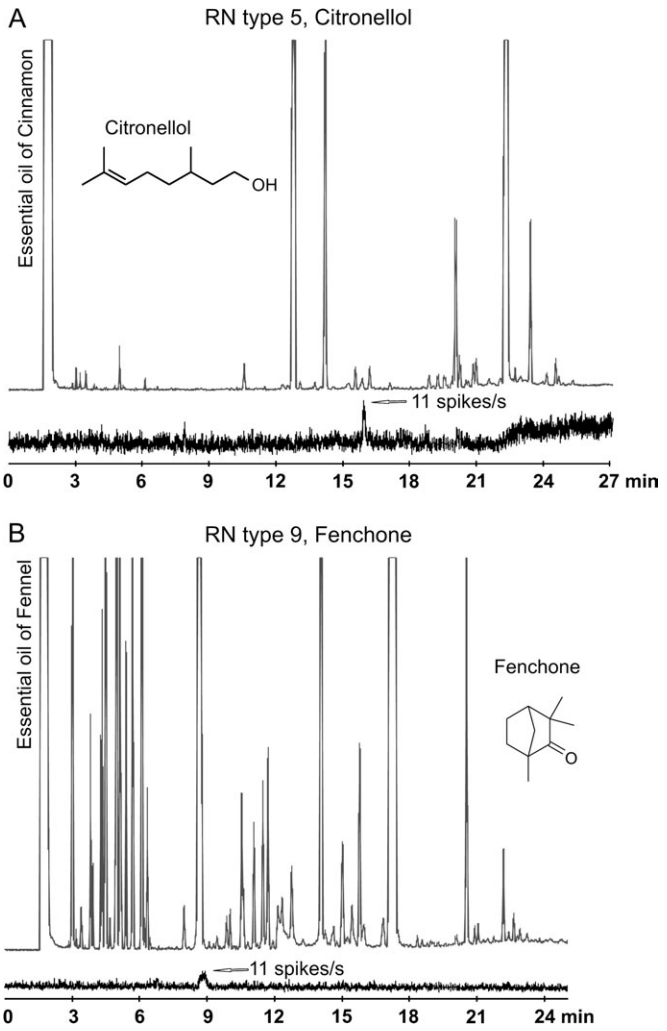


Figure 3 Gas chromatograms (upper trace) and simultaneously recorded activity of a RN (trace below). **(A)** Injection of essential oil of cinnamon and the response of a RN type 5 at the retention time of citronellol. **(B)** Injection of an essential oil of fennel and the response of a RN of type 9 to the eluent of fenchone.

acetate. Two RNs, responding best to this odorant, were classified as RN type 11. Stimulation of these neurons was performed 15 times via the GC, showing secondary responses to hexyl propanoate and (2*E*)-hexenyl acetate present in standards 6 and 7, respectively. The narrow tuning appeared by responses only to the 3 compounds out of more than 40 structurally similar odorants present in the tested standards 2, 5, 6, and 7. Figure 4 shows responses of one of the RNs when tested for 3 standards that contained the 3 active compounds. The samples containing the primary odorant at the same concentrations elicited a nearly identical response, exemplifying the reproducibility of the result.

RN type 12: (2E)-hexenal

A RN responded when screened with cartridges containing headspace samples of *A. thaliana*, standard 2, and standard 7. Injection of the standards in the GC showed that this RN responded to (2*E*)-hexenal. In addition to this response, a second compound (trace amount) present in the headspace sample of *A. thaliana* also activated the RN. However, the small amount of the compound did not allow identification by GC–MS. The RN was stimulated 8 times via the GC, showing a narrow tuning by exclusively responding to the 2 odorants of the numerous volatiles present in the test mixtures (headspace sample of *A. thaliana*, standards 2, 5, 6, and 7). Figure 5 shows response by the RN to (2*E*)-hexenal when stimulated with standard 2 via the GC. The response

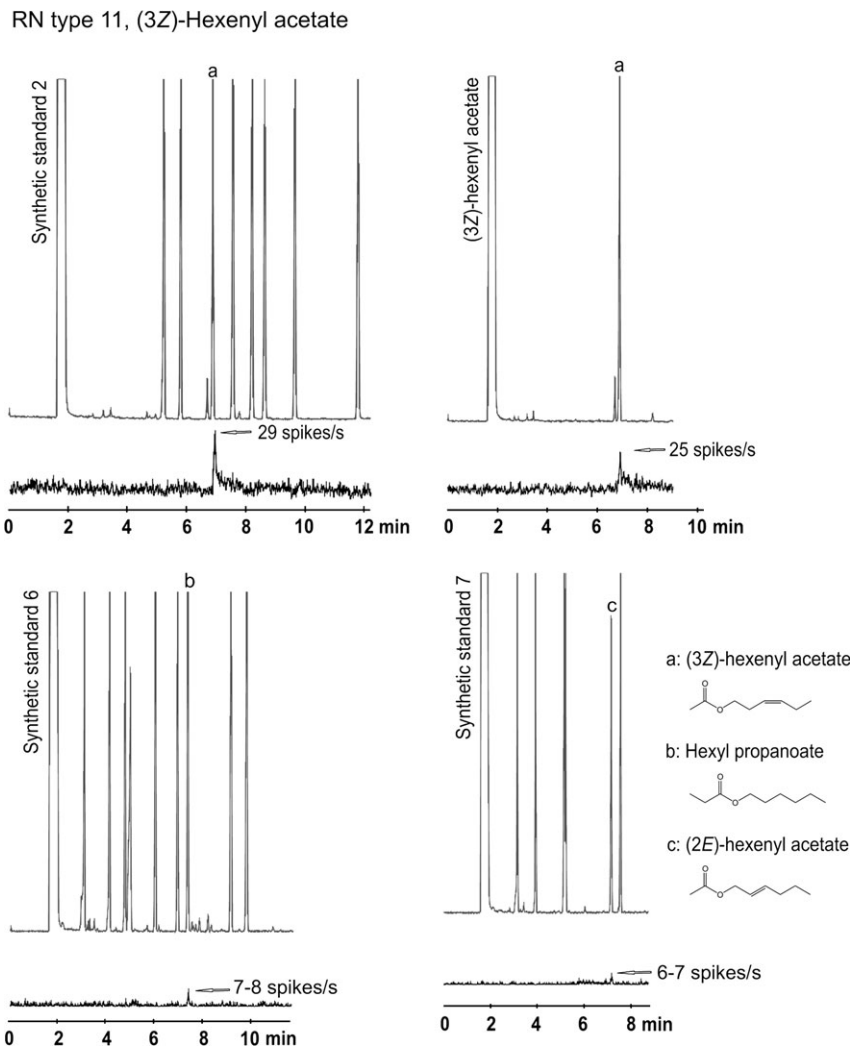


Figure 4 Gas chromatograms of synthetic standards 2, 6, 7, and (3*Z*)-hexenyl acetate (upper trace) and simultaneous recording of activity of a single RN (trace below) of type 11. The RN responded at the retention times of (3*Z*)-hexenyl acetate (primary odorant) in 2 different samples. The RN type also responded at the retention times of (2*E*)-hexenyl acetate and hexyl propanoate (secondary odorants). (right, below) The molecular structures of the 3 compounds are shown.

frequency increased with increasing concentration of the GC peak, then rapidly decayed, and stopped firing for 10 s after the GC peak (Figure 5A). This unusually long-lasting cessation was repeatedly obtained in this RN after stimulation with (2*E*)-hexenal via the GC.

RN type 14: 3-octanone

Two RNs classified as type 6 were activated by 3-octanone (Figure 5B). These RNs appeared in the same recordings as another RN type responding to linalool (type 10), previously presented in Ulland et al. (2006). The RN type 14 showed no responses to the numerous other compounds including those present in the headspace samples and standards (Table 2). Separation of the spikes originating from RN type 14 and the linalool RN type was based on analysis of spike waveforms and amplitudes.

Aromatics

RN type 15: p-methylanisole

A RN type colocated with RN type 10 responded during stimulation with cartridges containing the essential oil of ylang-ylang. Stimulation via the GC with ylang-ylang showed one response at the retention time of *p*-methylanisole. The response to this compound was verified by stimulation via the GC with synthetic *p*-methylanisole. Injection of the isomers *o*- and *m*-methylanisole showed no activation of the RN. This RN type found in 2 recordings was classified as RN type 15. The 2 RNs showed relatively weak responses of about 20 spikes per second when stimulated via the GC with 0.5 μg of *p*-methylanisole. It is possible that this compound is a secondary odorant, whereas the primary odorant was not present among the numerous tested volatiles.

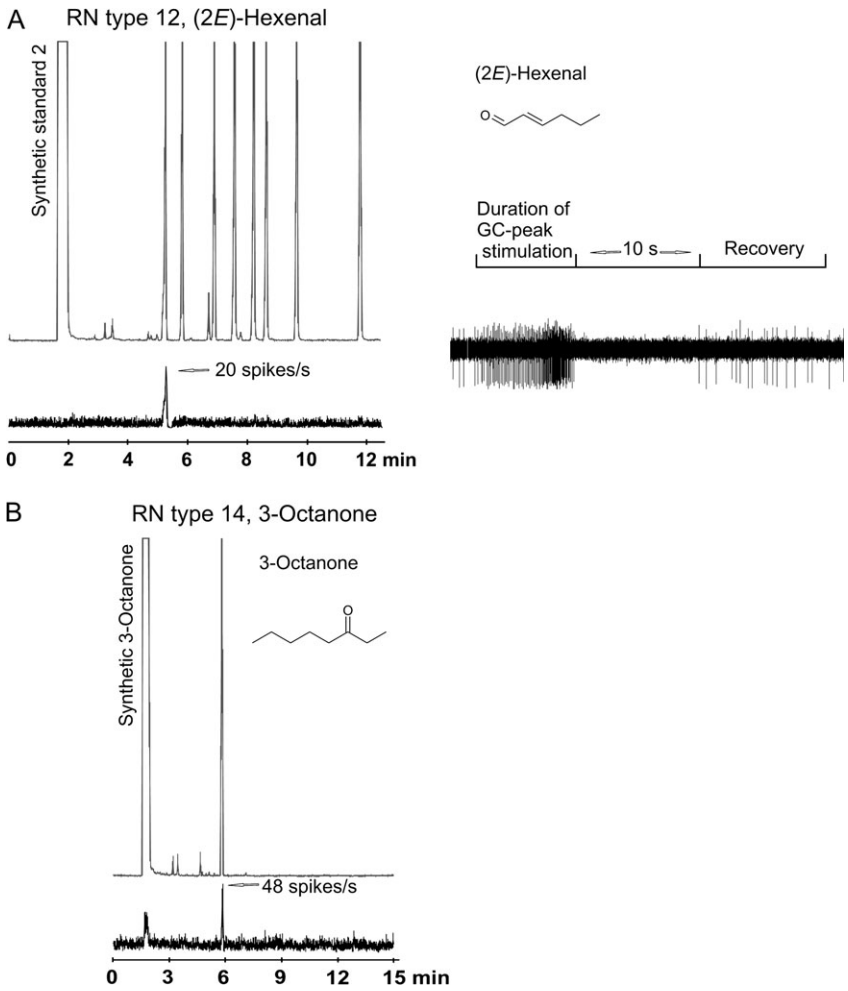


Figure 5 Gas chromatogram of a sample (upper trace) and the simultaneous recording of a RN (trace below) **(A)** Injection of standard 2 and the response of RN type 12 to (2*E*)-hexenal. The RN showed an increased firing rate after stimulation with (2*E*)-hexenal and a rapid decay at stimulus (GC peak) offset followed by a spike cessation. (right) A trace of the recorded spike activity shows the response characteristics of the RN. **(B)** The RN of type 14 responded to the stimulation with 3-octanone.

RN type 16: o-methylanisole

Screening with cartridges containing essential oil of ylang-ylang and a blend of synthetic *o*-, *p*-, and *m*-methylanisole activated 2 RNs. One of them responded only to *o*-methylanisole, whereas the other responded in addition to *p*-methylanisole. The responses by the 2 RNs were weak: 10–20 spikes per second to 0.05–0.5 μg of *o*-methylanisole when stimulated via the GC. Based on the recordings obtained so far, we classified these RNs as type 16. Due to the limited data, it was difficult to further determine the specificity of these neurons.

RN type 17: anethole

Two RNs responded when stimulated with cartridges containing essential oils of clove bud, cinnamon, and peppermint. Injection of the essential oils of fennel and clove bud in the GC showed that the neurons were activated by the aromatics anethole (Figure 6A) and eugenol (Figure 6B), with the strongest response to anethole. However, the responses were elicited by high concentrations of both compounds, somewhat stronger response to anethole present in largest amounts. Unfortunately, the neuron was not tested for the same concentration in order to prove that anethole has the strongest effect.

RN type 18: indole

In 3 experiments, a RN responded to indole when tested by direct stimulation via cartridges with 0.1 μg of synthetic indole. Injection in the GC column of standard 4 containing indole elicited responses to this compound only in one of the 3 neurons. The lack of responses in the other 2 neurons might be due to elution problems of indole in the GC column. None of the 3 RNs showed responses to the other compounds present in standard 4. The 3 RNs were classified as type 18.

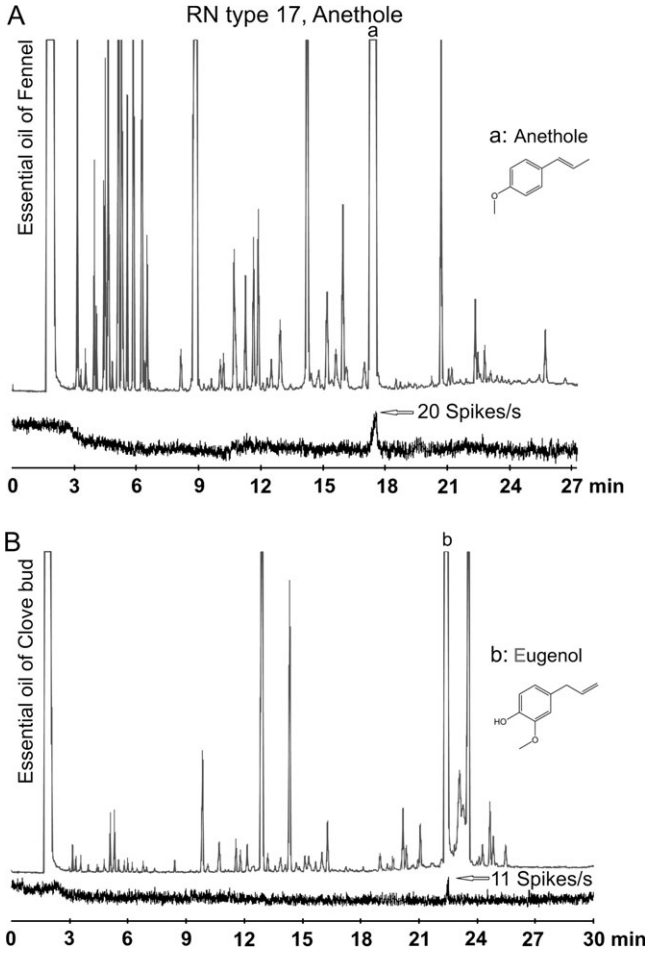


Figure 6 Gas chromatograms of essential oils (upper trace) and simultaneously recorded activity of a RN (trace below) of type 17. **(A)** The RN responded to anethole (primary odorant) present in the essential oil of fennel. **(B)** Injection of clove bud essential oil and the response of the RN type 17 to eugenol (secondary odorant).

Iso- and thiocyanates

RN type 20: butyl thiocyanate

A single neuron classified as RN type 20 responded to the compound butyl thiocyanate. However, only a weak response was obtained to the relatively large amounts of the GC-eluted compound. This RN did not show responses to the other compounds present in standard 10.

RN type 21: phenyl isothiocyanate

In the screening tests, a cartridge containing standard 10 elicited a response of one RN. Injection of the standard in the GC showed that the RN responded to phenyl isothiocyanate. This RN type was classified as RN type 21. The response was reproduced 3 times by injection of this standard in the GC. The selectivity of the neuron was demonstrated by no responses to the other 6 compounds present in standard 10 or to the other compounds present in the test material used for screening.

RN type 10: (R)-(–)-linalool; RN type 13: green leaf volatiles; RN type 19: methyl salicylate

These 3 RN types have previously been described in detail by Ulland et al. (2006, 2008). They are included in Table 2, in order to give an overview of the RN types recorded in *M. brassicae*.

Discussion

Together with 3 previously described RN types, the present 19 functional types of olfactory RNs in *M. brassicae* give information about which plant odorants that are biologically relevant and how the 21 RN types may be tuned to these odorants. The high reproducibility of the results was demonstrated by repeated responses of a RN to the same odorants when stimulating via the GC with headspace samples, essential oils, and synthetic compounds. Thus, the responses of RNs only appearing once (RN type 2, 7–10, 13, 20, and 21) can be considered reliable, indicating that these RNs belong to a particular type. Their rare appearance may reflect a small number of this population of RNs on the antennae. Other RNs, appearing up to 12 times and showing the same molecular receptive ranges as well as ranking of the odorants according to stimulatory effects, have been classified as one type. The consistency of the MRR within one type is in accordance with the findings in molecular biological studies in vertebrates and insects, that one RN expresses only one type of receptor proteins (Clyne et al. 1999; Störtkuhl and Kettler 2001; Wetzel et al. 2001; Krieger et al. 2002; Keller and Vosshall 2003; Hallem and Carlson 2004; Mombaerts 2004).

The aim of the present study was to identify among hundreds of plant-produced compounds as many as possible of the relevant odorants and RN types of *M. brassicae*. Thus, we tested via the GC headspace samples of host plants like *B. napus* and *B. oleracea*, as well as of nonhost plants and essential oils like ylang-ylang. In addition to giving information about the active compounds, the results also give the important information of which odorants the RNs do not respond to. The 21 RN types so far found are only a part of the expected number. We may assume a number in the range of 30–60, by considering the number of glomeruli (67 ± 1) in the antennal lobe of this species (Rospars 1983). As shown in molecular biological studies of vertebrates and the fruit fly, RNs of each type project in only one or two specific glomeruli (Gao et al. 2000; Vosshall et al. 2000). In moths, like *H. virescens*, functional tracing RNs of the pheromone system have also shown projection of the same RN type in one specific glomerulus (Berg et al. 1998). Whether the RNs of the plant odor system in moths project in one or two glomeruli remains to be shown. Thus, in future studies along this line, we expect to find more

RN types tuned to other plant odorants. The question is why the number yet obtained is so small. Probably, relevant odorants have been lacking in the test materials because we have recorded more than 70 RNs on the antennae of *M. brassicae* that did not show responses to any of the odorants in the tested materials. It could be that the volatiles in the cut and intact plant material were not trapped in the headspace due to the selectivity of the adsorbents and solvents used, but it is more likely that the odorants were not present in the plants of *Brassica* spp. and *A. thaliana* ecotypes. We should not ignore that missing plant-produced compounds might be due to other aspects of collecting volatiles because headspace of whole plants and macerated tissues differs (Tollsten and Bergström 1988). Another important aspect is induction of volatiles by feeding larvae. In retrospect, we see that it would have been important to test samples from plants with feeding larvae for obtaining induced compounds, a topic for future experiments. Nevertheless, 21 RN types have been obtained, 19 types in this study. The RNs showed a narrow tuning to the active odorants, most of them responded strongly to a primary odorant (RN types 1–8, 10–14, and 18–19) and weaker to a limited number of secondary odorants (RN types 1, 3, 7–8, 10–11, 13, and 19). The results on narrow tuning to one primary odorant are similar to results obtained in heliothine moths and the weevils investigated with the same method of GC-SCR as used in the present study (e.g., Wibe et al. 1997; Bichão, Borg-Karlson, Araújo, and Mustaparta 2005; Røstelien et al. 2005). Some of the RNs (RN types 9, 15–17, and 20–21) described here showed a low sensitivity to the active odorants identified from essential oils and synthetic compounds. Although we have tested numerous odorants present in the headspace samples, essential oils, and synthetic standards, it is likely that the primary odorants at least for some of these neurons (like RN types 9 and 15) were lacking, meaning that the responses might have been to secondary odorants. Future recordings will probably add data to the MRRs of these RNs. Another possibility is that RNs with low sensitivity, like those responding to isothiocyanates, in fact detect these typical host-produced compounds at high concentrations, contributing at a close range to host attraction (Rojas 1999a).

Among the 21 RN types classified in *M. brassicae*, some showed similar specificity as RNs previously found in other species, whereas others were novel types, previously not described. Noteworthy are the RN types tuned to odorants that are typical for the host plant genus *Brassica*, like thiocyanates. Surprisingly, only 2 RNs (types 20 and 21) responded to butyl thiocyanate and phenyl isothiocyanate, respectively. They may belong to a small population of RNs on the antennae. In an earlier study, Rojas (1999a) showed relative weak electro antennogram (EAG) responses to isothiocyanates in *M. brassicae*, indicating either responses of a small population of RNs or that these compounds might not be the relevant isothiocyanates. The role of a small population of RNs may not be unimportant but rather give significant messages about the host plant whether it is suitable or unsuitable for oviposition. Other novel RN types found in this study by the use of GC-SCR include RNs tuned to compounds that are more common in many plant species, like *E*,*E*-farnesol (RN type 2), 1,8-cineol (RN type 6), γ-terpinene (RN type 7), and 3-octanone (RN type 14).

In our laboratory, the use of the same test protocols in the various studies allows us to compare RN types across species of moths and weevils. We can compare the RN types not only based on their primary odorant but also on the basis of secondary odorants. Some RNs in *M. brassicae* have the same primary odorants as RN types in heliothine moths (types 1, 3, 4, 8, and 13) as well as the weevils *Anthonomus rubi* (types 10, 11, 15, and 19) and *Pissodes notatus* (type 9). Whereas some of the RN types with the same primary odorants in *M. brassicae* and heliothines also respond to the same secondary odorants (types 1, 3, and 4), others differ in their responses to the secondary odorants (types 8 and 13) (Bichão et al. 2003; Bichão, Borg-Karlson, Wibe, et al. 2005; Røstelien et al. 2005). Fewer similarities were found between RN types in *M. brassicae* and the weevil *A. rubi*. Here the secondary odorants differed or the primary odorants in one species were found as secondary odorant in the other species. Interestingly, RN types tuned to the sesquiterpenes *E*,*E*-α-farnesene, *E*-β-ocimene, and *E*,*E*-TMTT (RN types 1, 3, and 4 in *M. brassicae*, respectively) are previously described in 3 heliothine species (*H. virescens*, *Helicoverpa armigera*, and *Helicoverpa assulta*). In addition, the *E*,*E*-α-farnesene RNs show the same ranking of the response strength to the different isomers, *E*,*E*-α-, *E*-β- and *Z*,*E*-α-farnesene. Similarity between the RNs of these moth species was also found for the *E*-β-ocimene RN type, responding weaker to β-myrcene, and the *E*,*E*-TMTT RN that did not respond to other compounds. Another aspect of these RN types in the 4 moth species is their colocation in the same sensillum, as they appeared in the same recordings. The fourth colocated RN tuned to *E*,*E*-farnesol in *M. brassicae* was different in the 3 heliothine moth species where it was tuned to geraniol. The presence of similar RN types and colocation in the same sensillum across different insect species implies a conservation or reappearance independent of the evolution of host plant range.

In addition to provide knowledge about the peripheral olfactory coding mechanisms, the present results also indicate which odorants might be behaviorally important in *M. brassicae*. In principle, all primary odorants should be tested on the behavior, which is a challenging task considering possible attractive or repulsive effects as well as ratio specificity. As a beginning, single primary odorants might first be tested for attractive or repulsive effects, which has been done for methyl salicylate (Ulland et al. 2008). The results of these field experiments indicated that methyl salicylate inhibits oviposition of mated *M. brassicae* females when added to dispensers on natural as well as artificial plants. Thus, methyl salicylate should not be added to mixtures with compounds

for testing attraction. Other behavioral studies of *M. brassicae* have previously been carried out in wind tunnel experiments (Rojas 1999a, 1999b; Rojas et al. 2000). Here attraction to compounds that elicited EAG responses as well as attraction to undamaged and damaged host plants was tested. Interestingly, mated *M. brassicae* females oriented toward (3*Z*)-hexenyl acetate, α-terpinene, 1,8-cineol, chrysanthenone, camphor, and (2*E*)-hexenal, which include 3 of the primary odorants (RN types 6, 11, and 12) identified in the present study. Altogether, these behavioral studies form a valuable start for future tests with blends of the volatiles identified as primary odorants. In principle, the aim is to determine which odorant mixture has the best attractive effect and which odorants are repellent and may inhibit oviposition by female *M. brassicae*.

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