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Olfactory Receptor Neurons in Two Heliothine Moth Species Responding Selectively to Aliphatic Green Leaf Volatiles, Aromatic Compounds, Monoterpenes and Sesquiterpenes of Plant Origin

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Abstract

Moths of the subfamily Heliothinae are suitable models for comparative studies of plant odour information encoded by the olfactory system. Here we identify and functionally classify types of olfactory receptor neurons by means of electrophysiological recordings from single receptor neurons linked to gas chromatography and to mass spectrometry. The molecular receptive ranges of 14 types in the two polyphagous species *Heliothis virescens* and *Helicoverpa armigera* are presented. The receptor neurons are characterized by a narrow tuning, showing the best response to one primary odorant and weak responses to a few chemically related compounds. The most frequently occurring of the 14 types constituted the receptor neurons tuned to (+)-linalool, the enantioselectivity of which was shown by testing two samples with opposite enantiomeric ratios. These neurons, also responding to dihydrolinalool, were found to be functionally similar in the two related species. The primary odorants for 10 other receptor neuron types were identified as (3*Z*)-hexenyl acetate, (+)-3-carene, *trans*-pinocarveol, *trans*-verbenol, vinylbenzaldehyde, 2-phenylethanol, methyl benzoate, α-caryophyllene and caryophyllene oxide, respectively. Most odorants were present in several host and non-host plant species, often in trace amounts. The specificity as well as the co-localization of particular neuron types so far recorded in both species showed similarities of the olfactory systems receiving plant odour information in these two species of heliothine moths.

Key words: (+)-3-carene, α-caryophyllene, caryophyllene oxide, GC, GC-SCR, *Heliothis virescens*, *Helicoverpa armigera*, (3*Z*)-hexenyl acetate, host-plant selection, linalool, methyl benzoate, olfaction, 2-phenylethanol, *trans*-pinocarveol, plant volatiles, single cell recordings, terpenoids, *trans*-verbenol, vinylbenzaldehyde

Introduction

The challenge for the olfactory system in animals is to detect the large diversity of molecules released by food and plant sources and then discriminate these odours from other, less relevant ones. As shown by molecular biological studies, the olfactory information is handled not by a few receptor proteins, but by a large species-specific number present in various vertebrate and insect species (Buck and Axel, 1991; reviewed by Axel, 1995; Mombaerts, 1999, 2004; Breer, 2003; Keller and Vosshall, 2003; Hallem and Carlson, 2004). Furthermore, each type of receptor protein is expressed in distinct subsets of receptor neurons (RNs), each subset projecting in one or two specific glomeruli in the primary olfactory centre, the antennal lobe in insects and the olfactory bulb in vertebrates (reviewed by Breer, 2003; Keller and Vosshall, 2003; Mombaerts, 2004). This principle, called 'the logic of the sense of smell', indicates a relationship between the number of RN types and the number of glomeruli in the primary olfactory centres (Axel, 1995). Numerous electrophysiological studies have been performed through the years with the aim of functionally classifying olfactory RNs (e.g. Sicard and Holley, 1984; Ma and Shepherd, 2000; reviewed by Masson and Mustaparta, 1990; Shepherd, 1994; Mustaparta, 2002; Korsching, 2002). These studies have shown a large variation of molecular receptive ranges, from RNs being narrowly tuned and falling into distinct types to broadly tuned neurons with individually different molecular receptive ranges.

In heliothine moths, the molecular receptive ranges of olfactory RNs detecting insect and plant produced odorants have been described in several electrophysiological studies (reviewed by Mustaparta, 2002). Genes encoding putative olfactory receptors have also been identified in *Heliothis virescens*, where the principle of one type of gene expressed in single neurons was shown (Krieger *et al.*, 2002, 2004). In studies of the pheromone system of this species, functional tracing of the primary axons showed that each of the four identified RN types project in one specific glomerulus of the macroglomerular complex in the antennal lobe (Hansson *et al.*, 1995; Berg *et al.*, 1998). These findings were supported by optical recordings using Ca^{2+}-imaging (Galizia *et al.*, 2000; Skiri *et al.*, 2004). We have used the technique of gas chromatography linked to single cell recordings (GC-SCR) (Wadhams, 1982) in order to study plant olfactory RN types in heliothine moths (Røstelien *et al.*, 2000a,b; Stranden *et al.*, 2002, 2003a,b). Similar molecular receptive ranges of five types in three related species (*H. virescens, Helicoverpa armigera* and *Helicoverpa assulta*) were described. They were characterized by a best response to one compound defined as primary odorants and weaker responses to a few related compounds defined as secondary odorants. The primary odorants of the identified RN types were *E*-β-ocimene, geraniol, *E,E*-α-farnesene, *E*,*E*-TMTT (4,8,12-trimethyl-1,3,7,11-tridecatetraene) and (−)-germacrene D. Within one RN type, all neurons responded consistently to the same odorants, only showing variations in sensitivity. Considering the number of ordinary glomeruli (61–63) in the antennal lobe assumed to receive plant odour information in the three heliothine species (Berg *et al.*, 2002; Skiri *et al.*, 2005a), one would expect a number of at least 30 RN types.

Most heliothine species, including *H. virescens* and *H. armigera*, are polyphagous and use a broad range of plants from different families as hosts for nectar feeding and oviposition (Fitt, 1989; Matthews, 1991). This raises the question whether they detect a broad range of molecules released by the various host plant species or whether they have a restricted detection limited to some key odorants common in many plants. It has been hypothesized that some of the major pest species, like *H. armigera* and *H. virescens*, have adapted to cultivated plants since monocultures have existed for a long time. The ability of the insect to learn odours increases the utilization of abundant plants as in monocultures (West and Cunningham, 2002; Jallow *et al.*, 2004). Many behavioural studies of heliothine moths, in laboratory and field experiments, have indicated attraction or repellence to some odorants (Rembold and Tober, 1985; Tingle *et al.*, 1989, 1990; Mitchell *et al.*, 1991; Rembold *et al.*, 1991; Tingle and Mitchell, 1992; Hartlieb and Rembold, 1996; Jallow *et al.*, 1999; Bruce and Cork, 2001; De Moraes *et al.*, 2001; Mozuraitis *et al.*, 2002; Gregg and Del Socorro, 2002; Cunningham *et al.*, 2004). Thus, olfaction is important for host/food plant selection of these moths, and the identification and characterization of the olfactory RNs will help understanding their behaviour.

As an addition to the previously identified plant odour RN types in heliothine moths, we here present 14 types in *H. virescens* and *H. armigera*, of which four types recorded in both species showed similar specificity. All neurons were characterized by a narrow tuning to one primary odorant and weaker responses to a few other compounds of related structures. Minimal overlap of the molecular receptive ranges was found among the different RN types.

Materials and methods

Insects

Female *Heliothis virescens* and male and female *Helicoverpa armigera*, 3–5 days old, originated from laboratory cultures at Novartis Crop Protection, Rosental, Switzerland and at the Volcani Centre, Bet Dagan, Israel, respectively. In addition, a few *H. armigera* (females) were obtained from a laboratory culture at the Chinese Academy of Sciences, Beijing, People's Republic of China. The insects were kept at constant temperature (26.8°C) with a dark:light cycle of 14:10 h, and used in the experiments during the dark period.

Test samples

The plant substances tested were constituents of extracts, headspace samples and essential oils. Headspace samples were collected from several strains of sunflower (*Helianthus annuus*), wild (cut material) and cultivated tobacco (*Nicotiana tabacum*), tomato (*Lycopersicum esculentum*), wild briar (*Rosa dumalis*) and cotton (*Gossypium hirsutum*). In addition we used headspace samples from commercially available orange fruits, Norwegian spruce (*Picea abies*), juniper (*Juniperus communis*) (Wibe *et al.*, 1997) and maritime pine (*Pinus pinaster*) (Bichão *et al.*, 2003). The samples tested also included essential oils of cubeb pepper (*Piper cubeba* L.), ylang-ylang (*Cananga odorata*) (from Dragoco and Firmenich), eucalyptus, *Juniperus virginia* (from Dr Pagula, University of Maputo, Mozambique), clove bud (*Syzygium aromaticum*) and cinnamon (*Cinnamomum zeylanicum*). α- and β-caryophyllene isolated from cotton were provided by R.R. Heath (USDA, Gainesville, FL). Chemical reference compounds were also included in the experiments (Table 1). Headspace samples as well as reference compounds were dissolved in hexane, except for a few materials, for which a mixture of hexane and ethyl acetate (1:1) was used. Most test samples were the same as previously used (Røstelien *et al.*, 2000a,b; Stranden *et al.*, 2002, 2003a,b).

Gas chromatography linked to single cell recordings (GC-SCR)

The insect was fixed in a Plexiglas holder and immobilized by dental wax. The antennae, exposed at the top of the holder,

Table 1 Constituents of the standard mixtures most frequently used (1–4), including some compounds present in low amounts (tr, trace amounts; m, minor amounts), identified by GC-MS

Standard 1	Standard 2	Standard 3	Standard 4
p-Cymene (tr)	Borneol (tr)	α-Caryophyllene (tr)	Borneol (m)
Fenchone	Camphor	β-Caryophyllene	Camphene
1-Hexanol	(+)-3-Carene	Caryophyllene oxide (tr)	1,8-Cineol
2-Hexanol (m)	Dihydrolinalool (tr)	*racemic* β-Citronellol	Cuminaldehyde (m)
3-Hexanol (m)	(3*Z*)-Hexen-1-ol	Dihydrolinalool (tr)	*p*-Cymene
(2*Z*)-Hexen-1-ol	(2*E*)-Hexen-1-ol	*E*-Geraniol	*p*-Cymene-8-ol (m)
(2*E*)-Hexenal	*racemic* Linalool	*racemic* Linalool	(3*Z*)-Hexen-1-ol
(3*E*)-Hexenal	Myrtenal	β-Myrcene	Limonene
(3*Z*)-Hexen-1-ol	(-)-Myrtenol	(-)-Myrtenal	β-Myrcene
(3*E*)-Hexen-1-ol	*E*-Pinocarveol (tr)	1-Octanol	Myrtenal (m)
(2*E*)-Hexenyl acetate	Terpinen-4-ol	2-Phenylethanol	(-)-Myrtenol (m)
Myrtenal	α-Terpineol	β-Pinene	α-Phellandrene
Myrtenol		*E*-Pinocarveol (tr)	β-Phellandrene
		(+)-Verbenone	(-)-α-Pinene
			β-Pinene
			trans-Pinocarveol (m)
			trans-Pinocarvone (m)
			β-Pinone (m)
			Sabinene

were fastened to the wax layer by tungsten hooks. Nerve impulses from single olfactory RNs on the antenna were recorded using tungsten microelectrodes sharpened to a tip <0.3 μm (Mustaparta, 1979). The recording electrode was inserted into the base of one sensillum located at the frontal side of the flagellar segments and the indifferent electrode in contact with the haemolymph of one proximal segment.

Initially, the single RNs were screened for sensitivity to the various mixtures of plant volatiles. If a neuron responded to a sample, we tested the individual constituents separated in the gas chromatograph (GC). In the GC, a glass splitter installed at the end of the GC-column led half of the effluent into the air stream blowing over the insect antenna and the other half to the flame ionization detector. Thus, the activity of a RN and the gas chromatogram of the components separated in the GC-column were recorded simultaneously. The GC (Fisons Instruments, HRGC MEGA 2 series) was installed with two columns in parallel in the GC-oven, a polar (DB-wax, J&W Scientific) and a non-polar one (DB-5, J&W Scientific) (both columns: 30 m, i.d. 0.25 mm, film thickness 0.25 μm) as described by Røstelien *et al.* (2000b). This allowed each neuron to be tested for the same test sample with two different separation sequences. The main temperature programme used started with an initial temperature of 80°C using an increase of 6°C/min to 220°C (isothermally for 10 min). Different temperature programmes were used to adjust the resolution of closely eluting peaks, e.g. an initial temperature at 50°C (or 60°C) was used with an increase of 5°C/min. In experiments recording spikes from 2–4 neurons, a spike-analysing computer program (Autospike 32, Syntech NL, Hilversum) was employed in order to distinguish responses of the co-located neurons (Røstelien *et al.*, 2000b). Alternatively, we made analyses of spike trains using the computer program Spike 2 (CED Limited, Cambridge) as described by Stranden *et al.* (2003b).

Identification of plant volatiles

The electrophysiologically active compounds were identified by a combination of gas chromatography and mass spectrometry (GC-MS), using a Finnigan SSQ 7000 instrument connected with a Varian 3400-GC (located at the Royal Institute of Technology, Stockholm). Capillary columns were used with the same specifications as the ones used for the GC-SCR experiments (mentioned above). For a detailed description, see Røstelien *et al.* (2000a).

Table 2 Overview of the plant odour receptor neuron types, characterized by molecular receptive ranges, in *Heliothis virescens* ([v]) and/or *Helicoverpa armigera* ([a])

Active compounds	RN types 1–11											RN types I–V				
	1[v,a] (*n* = 10)	2[v,a] (*n* = 4)	3[v] (*n* = 3)	4[v,a] (*n* = 5)	5[v] (*n* = 1)	6[v] (*n* = 2)	7[v] (*n* = 2)	8[v,a] (*n* = 5)	9[a] (*n* = 1)	10[v] (*n* = 1)	11[v] (*n* = 3)	I (*n* = 26)	II (*n* = 9)	III (*n* = 14)	IV (*n* = 9)	V (*n* = 130)
Aliphatic compounds																
1-Hexanol	o	–	o	o	–	–	–	o	–	–	●	o	o	o	o	o
(2*Z*)-Hexen-1-ol	o	o	–	–	–	–	–	–	–	–	•	–	–	–	–	–
(3*Z*)-Hexen-1-ol	o	–	o	o	–	–	–	o	o	o	●	o	o	o	o	o
(3*E*)-Hexen-1-ol	o	–	o	o	–	–	–	o	–	–	•	o	o	o	o	o
(2*E*)-Hexenal	o	o	–	–	–	–	–	–	–	–	•	–	–	–	–	–
(3*E*)-Hexenal	o	o	–	–	–	–	–	–	–	–	•	–	–	–	–	–
(3*Z*)-Hexenyl acetate	o	–	o	o	–	–	–	o	o	o	●	o	o	o	o	o
(2*E*)-Hexenyl acetate	–	–	–	–	–	–	–	–	–	–	•	–	–	–	–	o
1-Heptanol	o	o	–	–	–	–	–	–	–	–	•	–	–	–	–	–
Monoterpenes (MT)																
Acyclic MT																
Dihydromyrcene	–	–	–	–	–	–	–	–	–	–	–	•	o	o	o	o
β-Myrcene	o	o	o	o	–	o tr	o	o	o	o	o	●	o	o	o	o
Z-*β*-Ocimene	–	–	–	–	–	–	–	–	–	–	–	•	o	o	o	o
E-β-Ocimene	o	o	o	o	–	–	o	o	o	–	o	●	o	o	o	o
DMNT	o	–	o	o	–	–	–	o	–	–	o	•	o	o	o	o
Dihydrolinalool	●	o	–	o	–	–	–	o	–	–	o	o	–	o	o	o
racemic Linalool	●	o	o	o	–	–	o	o	o	o	o	o	•	o	o	o
(+)-Linalool	●	o	o	o	–	–	o	o	o	o	o	o	•	o	o	o
(-)-Linalool	●	o	o	o	–	–	o	o	o	o	o	o	•	o	o	o
racemic Citronellol	o	–	o	o	–	–	–	o	–	–	–	o	●	o	o	o
Geraniol	o	–	o	o	–	–	–	o	o	o	–	o	●	o	o	o
Tetrahydrolinalool	•	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Monocyclic MT																
Terpinen-4-ol	o	o	o	o	–	–	–	•	o tr	–	–	o	o	o	o	o
Bicyclic MT																
(+)-3-Carene	o	●	o	o	–	–	–	o	–	–	o	o	o	o	o	o
cis-Verbenol[1]	–	–	–	•	–	–	–	–	–	–	–	o	o	o	o	o
trans-Verbenol[1]	o	–	o	●	–	–	–	–	–	–	o	o	o	o	o	o
Verbenone	o	–	–	•	–	–	–	–	–	–	o	o	o	o	o	o
Borneol[1,2]	o	–	–	•	–	–	–	–	–	–	o	–	–	–	–	o

Myrtenal	o	–	•	•	–	–	–	–	–	–	o	o	o	o	o	o
Myrtenol	o	–	•	●	–	–	–	–	–	–	o	o	o	o	o	o
3,6,6-Trimethyl-2-norpinanone[4]	o	–	●	o	–	–	–	o	–	–	o					
trans-Pinocarveol[1]	o	–	●	o	–	–	–	o	–	–	o	o	o	o	o	o
Sesquiterpenes																
α-Caryophyllene	o tr	o tr	o tr	o tr	●	•	–	o tr	o	–	o tr	o	o	o	o tr	o
β-Caryophyllene	o	o	o	o	o	•	o	o	o	–	o	o	o	o	o	o
Caryophyllene oxide[1]	–	–	–	–	o	●	–	o tr	o	–	o	o	o	o	–	o
Cadinene-type[1,4]	o	–	–	–	–	–	●	–	–	–	–	o	o	o	o	o
(+)-α-Copaene	–	–	–	–	–	–	o	–	o	–	–	o	o	o	o	•
β-Copaene	–	–	–	–	–	–	o	–	o	–	–	o	o	o	o	•
E-β-Farnesene	–	–	–	–	–	–	–	–	–	–	–	o	o	•	o	o
E,E-α-Farnesene	o	–	o	–	o	o	o	o	o	o	o	o	o	●	o	o
Z,E-α-Farnesene	–	–	–	–	–	–	–	–	–	–	–	o	o	●	o	o
E,E-TMTT	o	–	o	–	–	–	–	o	o	–	o	o	o	o	●	o
(-)-Germacrene D	o	o	o	o	–	–	o	o	o	o	o	o	o	o	o	●
(+)-Germacrene D	o	o	o	o	–	–	o	o	o	o	o	o	o	o	o	●
(-)-α-Ylangene	o	o	o	o	–	–	–	o	o	–	o	o	o	o	o	•
(-)-β-Ylangene	–	–	–	–	–	–	–	–	o	–	–	o	o	o	o	•
Aromatic compounds																
Cuminaldehyde[1,2]	o	–	–	–	–	–	–	•	–	o tr	o	o	o	o	o	o
p-Cymene (*MT*)	o	o	o	–	–	–	–	•	–	–	o	o	o	o	o	o
1,4-Diethylbenzene	o	o	o	–	–	–	–	●	–	–	o	o	o	o	o	o
1,3-Diethylbenzene	o	o	o	–	–	–	–	•	–	–	o	o	o	o	o	o
4-Ethylacetophenone[1]	o	–	o	–	–	–	o	●	–	–	o	o	o	o	o	o
4-Ethylbenzaldehyde[1,2]	o	–	–	–	–	–	o	●	–	–	–	–	–	–	–	o
Vinylbenzaldehyde[1,4]	o	–	–	–	–	–	o	●	–	–	–	–	–	–	–	–
Ethyl benzoate[1,3]	–	–	–	–	–	–	–	o tr	•	–	o tr	o	o	o	o	o
Methyl benzoate[1,3]	o tr	o	o tr	o tr	–	–	o tr	o tr	●	–	o tr	o tr	o tr	o tr	o tr	o tr
2-Phenylethanol[1,3]	o	–	–	o	–	–	o tr	o tr	–	●	o	o	o	o	o	o

The receptor neuron types of the present (RN types 1–11) and of previous studies [RN types I–V (Røstelien *et al.*, 2000a,b; Stranden *et al.*, 2002, 2003a,b), also characterized in *Helicoverpa assulta*] for which the odorants have been chemically identified are included. The numbers of neurons recorded within each type (*n*) is given. The identification of most compounds is based on GC-SCR, GC-MS and comparison of retention times with standards of both polar and non-polar columns, in addition to retesting with chemical standards. Fourteen compounds were not retested with standards ([1]); in addition, some are only identified by one GC-column and one ([2]) or two ([3]) GC-MS columns. A few are also lacking comparison of retention times with standards ([4]). ●, excitatory responses; o, no response; –, not tested; tr, trace amounts tested; DMNT, (3*E*)-4,8-dimethyl-1,3,7-nonatriene; *E,E*-TMTT, 4,8,12-trimethyl-1,3,7,11-tridecatetraene. The relative response strength indicated by the size of the dots (large dot ● for primary odorant and smaller ●, •, • for secondary odorants) can only be compared within each RN type.

Table 3 Plant materials containing odorants in amounts detectable by GC-SCRs from RNs of type 1–11 in *H. virescens* and *H. armigera*

Active compounds	Sunflower	Tobacco		Cotton	Wild briar	Maritime pine	Spruce	Ylang-ylang	Eucalyptus	Cubeb pepper and juniper
		c	w							
Borneol						x				
Cadinene-type		x						x		
(+)-3-Carene						x				
Cuminaldehyde	x					x	x			
p-Cymene	x					x	x			
1,3-Diethylbenzene	x	x								
1,4-Diethylbenzene	x	x								
4-Ethylacetophenone	x	x		x	x					
4-Ethylbenzaldehyde		x		x	x					
Ethyl benzoate								x		
1-Heptanol			x							
1-Hexenol			x							
(*E*2)-Hexan-1-ol			x							
(*Z*3)-Hexan-1-ol			x							
(*E*3)-Hexen-1-ol			x							
(*Z*3)-Hexenyl acetate	x		x			x				
Linalool	x	x			x	x	x		x	x
Methyl benzoate								x		
Myrtenol	x					x	x			
2-Phenylethanol					x					
trans-Pinocarveol	x					x	x		x	
Terpinen-4-ol	x	x				x	x			
Tetrahydrolinalool		x								
3,6,6-Trimethyl-2-norpinanone	x									
cis-Verbenol							x			
trans-Verbenol	x					x	x			
Verbenone							x			
Vinylbenzaldehyde		x								

X, obtained excitatory responses; c, cultivated plants; w, wild plants; open column, no response to this compound in the given sample was obtained in the examined neurons. The compounds are listed in alphabetical order.

Criteria for classification of functional RN types

The classification of different RN types is based on the following criteria according to previous studies (e.g. Wibe and Mustaparta, 1996; Wibe *et al.*, 1997; Røstelien *et al.*, 2000a,b; Barata *et al.*, 2002; Stranden *et al.*, 2002, 2003a,b; Bichão *et al.*, 2003, 2005). First, the recordings of each neuron are highly reproducible, i.e. when tested repeatedly the neurons show responses to the same compounds. Secondly, the neurons fall into distinct groups according to the molecular receptive ranges, and the neurons within one group show the same molecular receptive range and ranking of the odorant effectiveness. The classification is accentuated by the fact that neurons within a group show best response to the same primary odorant and weaker responses to the other secondary odorants. Thus, the neurons within one group are defined as one type named according to the primary odorant.

Results

The results presented in this paper are based on recordings from 38 RNs including 200 GC-SCRs. Each neuron was stimulated 1–23 times via the GC. The high reproducibility of the selective responses of the RNs to a few odorants allowed a classification of the 38 RNs into 14 distinct types according to the identified molecular receptive ranges. The primary and many of the secondary odorants were identified for 11 of the neuron types. Table 2 gives an overview of these RNs (types 1–11), for which the identified primary and secondary odorants are listed. The types 12–14, for which none of the active odorants were chemically identified, are not included in this table.

Thirty-six odorants were identified in plants and chemical standards by the use of GC-MS, and 23 of them were retested on the olfactory RNs. Seven RN types (3, 5, 6, 7, 10, 11 and 12) were found only in *H. virescens* (from which most recordings were made), five types (1, 2, 4, 8 and 14) were found in both species and two types (9 and 13) were found only in *H. armigera*. Three RN types (12, 13 and 14) responded to compounds present in trace amounts that did not allow identification by GC-MS (not included in Table 2). In many experiments, no responses were recorded to the numerous compounds tested as mixtures of plant volatiles and pheromones. The compounds eliciting responses belonged to different chemical groups, like mono- and sesquiterpenes (RN types 1–7), aromatic compounds (RN types 8–10) and oxygenated aliphatic 6-carbon compounds (RN type 11). The responses of the RNs showed the presence of the identified odorants in the various plant materials listed in Table 3. All plant odour RNs classified displayed distinct and reproducible responses with increased firing rates that followed the rise of the GC-peak of the active odorant. The decay of the responses was often slower and outlasted the GC-peak.

Olfactory RNs responding to linalool

RN type 1

Ten RNs, nine found in nine *H. virescens* and one in an *H. armigera* male, were classified as type 1 according to the consistent excitatory responses when stimulated with the acyclic oxygenated monoterpenes linalool and dihydrolinalool (Table 4). The selective responses of these neurons to linalool appeared when tested for the headspace volatiles of wild tobacco, sunflower, maritime pine, spruce sawdust, cubeb pepper, wild briar and juniper (Table 3). Figure 1A shows the results from one test with cubeb pepper, which contains larger quantities of linalool than the samples from the host plants sunflower and tobacco. The exclusive response to linalool eluting in front of the large peak of β-cubebene is shown in the recording below the gas chromatogram. Injection of a standard sample of *racemic* linalool in the DB-wax column elicited a strong response during the elution of linalool and a smaller response during the elution of dihydrolinalool, a compound that was not present in any of the biological materials tested (Figure 1B). One RN tested for different concentrations, responded in a dose-dependent manner to decadic dilutions of two samples of linalool, (−)-linalool (enantiomeric purity 97%) and (+)-linalool (enantiomeric purity 77. 5%) (Figure 1C). A slightly stronger response to the (+)- than to the (−)-sample of linalool and an intermediate response to the *racemic* mixture were obtained. Chiral columns were not available during these recordings. The same type of RN was identified in the male *H. armigera* (Figure 1E,F). This neuron showed the same relative responses to linalool and dihydrolinalool as the RNs obtained in *H. virescens* (Figure 1B). The high firing rate during the response to linalool appeared with declining spike amplitudes, which were not counted by the integrator and caused the sudden drop of the response curve in Figure 1E (marked by an asterisk). The high sensitivity of this neuron is demonstrated in Figure 1F by the strong response to the small quantity of linalool collected from the non-host maritime pine. Another linalool neuron in *H. virescens*, responding to direct stimulation with citronellol, in fact responded to linalool present as an impurity in this sample, as shown after GC-separation and identification by GC-MS (Figure 1D). Variations of the temporal response pattern were seen among the linalool responding RNs. Whereas most of them showed strong responses to small amounts of linalool (as shown in Figure 1B,E), one linalool RN displayed a low firing frequency (the maximum response rate slightly exceeding 60 impulses/s) and a slow decay of the response that far outlasted the GC-peak. The seven RNs were tested for synthetic

Table 4 Type and number (in parentheses) of responses by the 10 receptor neurons to linalool and dihydrolinalool in nine *Heliothis virescens* females and one *Helicoverpa armigera* male (cell no. 10) stimulated via the polar (DB-wax) and the non-polar (DB-5) gas chromatographic columns

Cell no.	Linalool responses		Dihydrolinalool responses	
	DB-wax	DB-5	DB-wax	DB-5
1	+ (3)	+ (6)	+ (1)	–
2	+ (4)	–	+(1)	–
3	+ (1)	–	–	–
4	+ (4)	+ (6)	–	+ (2)
5	+ (9)	+ (14)	+ (2)	+ (2)
6	+ (1)	–	+ (1)	–
7	+ (2)	–	–	–
8	+ (5)	–	–	+ (2)
9	–	+ (5)	–	–
10	+ (3)	–	+ (1)	–

The lower number of responses to dihydrolinalool is due to its presence exclusively in synthetic linalool. +, excitatory response; –, not tested.

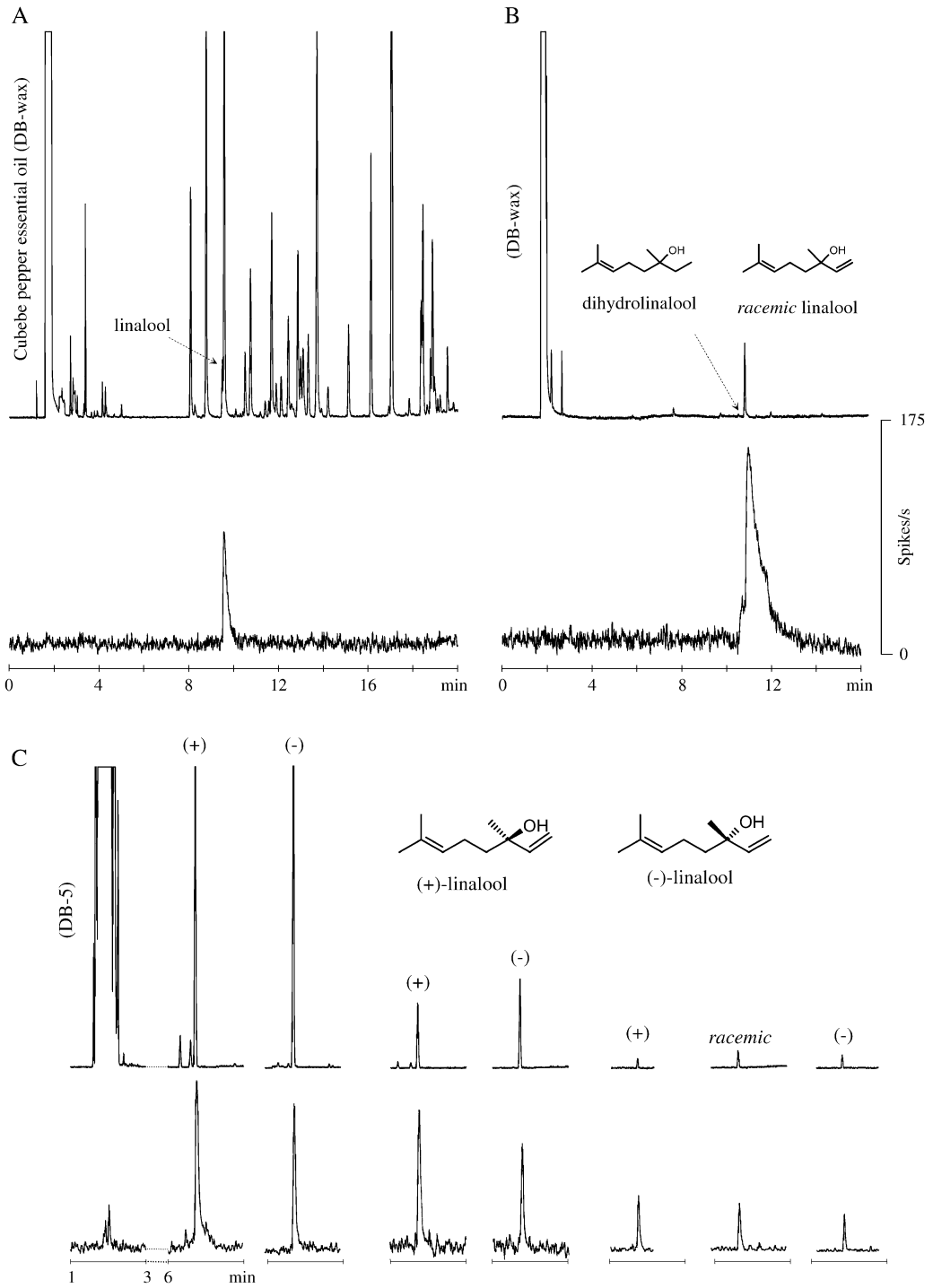

Figure 1 RN type 1 tuned to linalool. Gas chromatogram of volatiles of cubeb pepper essential oil (*Piper cubeba*) **(A)**, and of *racemic* linalool **(B)** with simultaneously recorded activity from a single RN (type 1) on the antenna of two *H. virescens* females. **(C)** Gas chromatograms of (+)-linalool (enantiomeric purity, 71.5%) and (−)-linalool (enantiomeric purity, 97%) of three decadic dilutions and the 1:1 mixture of the two linalool samples with simultaneously recorded activity of a RN (same neuron as in B). **(D)** Gas chromatogram of *racemic* citronellol and simultaneously recorded activity of one RN (type 1) of a female *H. virescens*. **(E)** Gas chromatogram of *racemic* linalool (reference compounds), and simultaneously recorded activity of RNs of type 1, 2, 4 and 8 on the antenna of a male *H. armigera*. Responses by the type 1 neuron to linalool and dihydrolinalool were obtained in addition to weak responses to two compounds, one of them tentatively identified as tetrahydrolinalool. Response by type 4 was obtained to myrtenol (a). The sudden drop of the response curve (marked by an asterisk) was caused by the declining spike amplitudes that could not be counted during the strong response to linalool. **(F)** Gas chromatogram of headspace volatiles of maritime pine (*Pinus pinaster*) and simultaneously recorded activity of four co-located RNs (same neurons as in E). Responses to linalool, myrtenol (a), (+)-3-carene (b), terpinene-4-ol (c) and borneol (d) were recorded.

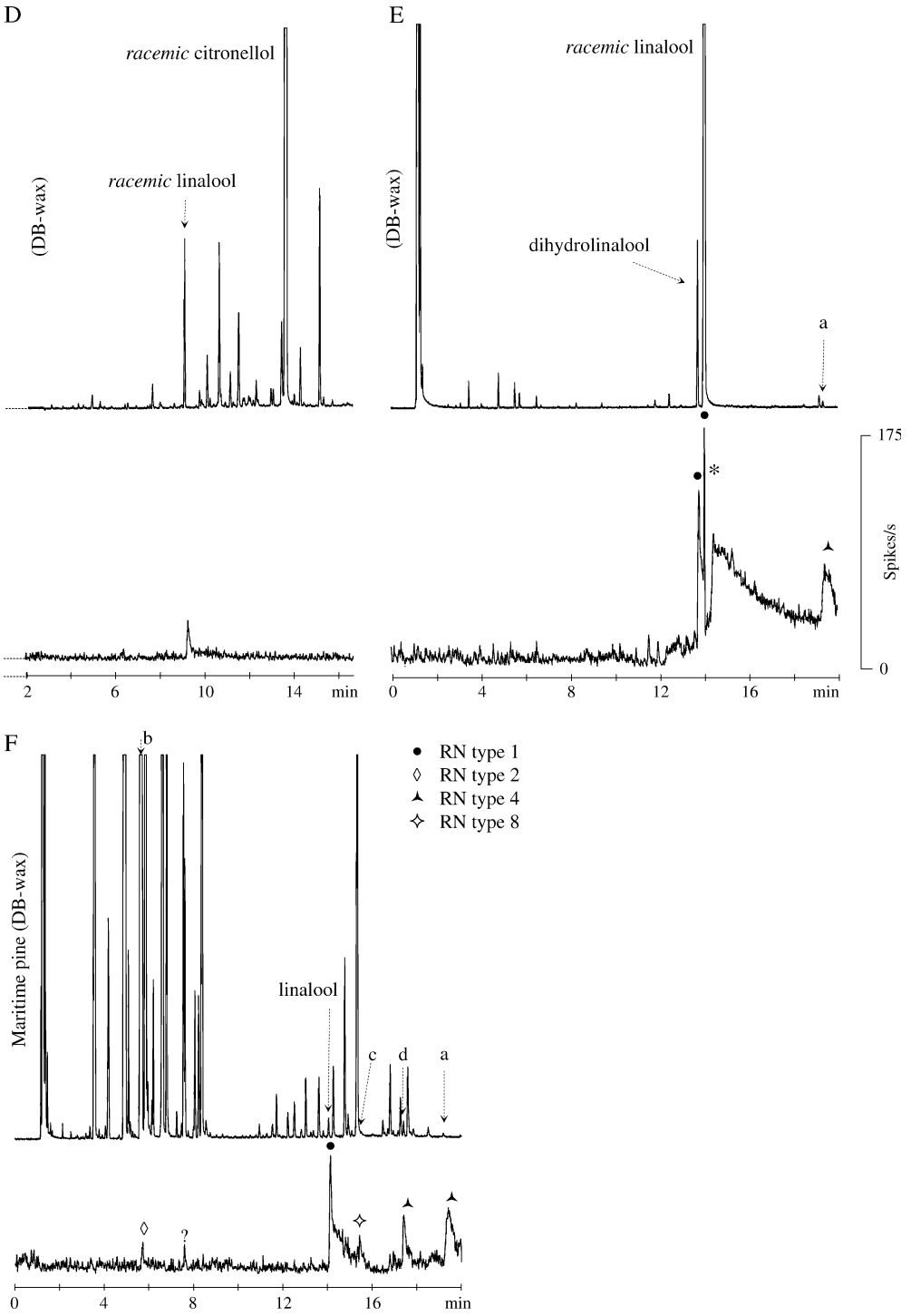

Figure 1 Continued.

linalool, also containing dihydrolinalool, and responded consistently to both compounds. The most sensitive neurons also responded weakly to two compounds present in trace amounts in the *racemic* linalool sample. One of the compounds was indicated by retention time and by mass spectra to be tetrahydrolinalool (also present in one headspace sample of cultivated tobacco plants). In a recording from another sensitive linalool neuron additional responses were obtained to trace amounts of two unidentified compounds in the standard 3 (using different column and temperature programme). In several experiments, the activity of the type 1 neurons was recorded simultaneously with activity of two

or three other neurons classified as types 2, 4 and 8 (Figures 1E,F, 2, 4 and 8). The type 1 RNs were distinguished from the other types by amplitudes and waveforms of the spikes (see Figure 4C).

Olfactory RNs responding to bicyclic monoterpenes (RN types 2–4)

RN type 2: 3-carene

Three RNs in *H. virescens* and one in a male *H. armigera* responded to the bicyclic monoterpene (+)-3-carene, and were classified as RNs of type 2. An example of one GC-SCR test is given in Figure 2. When tested for the volatiles of maritime pine the neuron responded to two compounds, of which the one eliciting the strongest response has previously been identified as (+)-3-carene (Bichão *et al.*, 2003; Almquist *et al.*, 2005). Retesting authentic materials on the same neurons confirmed the responses to (+)-3-carene. The other active compound was not identified. The response to the minor amount of linalool in Figure 2 was ascribed to the co-located RN of type 1, having smaller spike amplitudes than the (+)-3-carene RN. These neurons were tested with 23 plant samples via the GC. Interestingly, the response by neuron type 2 to (+)-3-carene was markedly reduced after the third repetition, a phenomenon that was never observed to the same extent in other recordings. In some recordings, the neuron responding to (+)-3-carene was co-located with RNs of types 1, 4 and 8. The same co-location of RNs (types 1, 2, 4 and 8) was obtained in one multiple spikes recording from a male *H. armigera* (Figure 1F).

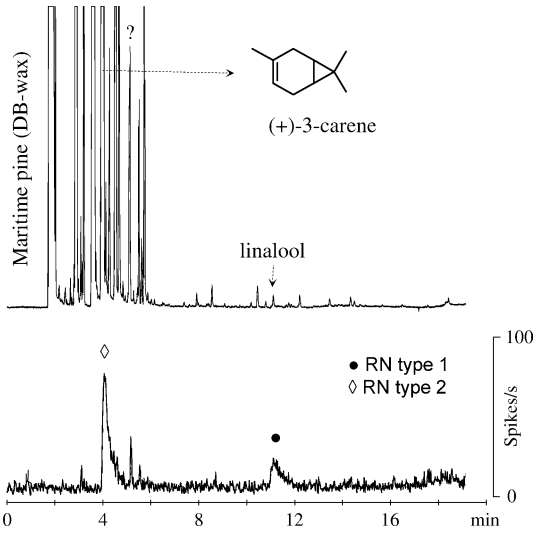

Figure 2 RN type 2 tuned to (+)-3-carene. Gas chromatogram of headspace volatiles collected from maritime pine (*Pinus pinaster*) and simultaneously recorded activity of one RN of type 2 and one of type 1 of a female *H. virescens*. The active components were identified as (+)-3-carene, linalool and one unidentified component (indicated by a question mark).

RN type 3: trans-*pinocarveol*

Three RNs of *H. virescens* responded strongest to *trans*-pinocarveol (oxygenated β-pinene) and were classified as RN type 3. Six additional compounds elicited secondary responses in neurons of this type. Examples of GC-SCRs given in Figure 3, show best response to *trans*-pinocarveol and a weaker response to a minor amount of 3,6,6-trimethyl-2-norpinanone (identified by GC-MS), when stimulated with sunflower volatiles, separated by using both column types (Figure 3A,B). Testing the same neuron for a standard containing *trans*-pinocarveol verified the response to this odorant (Figure 3C). No responses were recorded to the structurally similar hydrocarbons α- and β-pinene present in the standard. Three other components in the standard material elicited responses in this neuron. Analyses by GC-MS indicated several oxidation products of pinene (e.g. β-pinone, *cis*-pinocarveol and *trans*-pinocarvone) in the area of the three active components, so far not further identified. Weak responses to the bicyclic monoterpenes myrtenal and myrtenol were obtained in another of the three neurons tested in standard 2. No response was elicited by the structurally related bicyclic monoterpene *trans*-verbenol (present in sunflower, Figure 4), or by the large quantity of camphor, pinocamphone and isopinocamphone (present in the non-hosts maritime pine and spruce). The molecular structures of the primary and three of the secondary odorants are shown in Figure 3D.

RN type 4: trans-*verbenol*

Five RNs, four in *H. virescens* and one in an *H. armigera* male, responded strongest to the bicyclic monoterpene alcohol *trans*-verbenol and were classified as RN type 4. Additional responses by these neurons were recorded to the five similar bicyclic monoterpenes (*cis*-verbenol, verbenone, borneol, myrtenal and myrtenol, Table 2). Figure 4 shows GC-SCR during elution with two sunflower samples, containing different amounts of *trans*-verbenol. Clear dose-dependency of the neuron responses was obtained. The selectivity of this RN type was demonstrated by the similarity in structures of the primary and secondary odorants and by the absence of responses to other related molecules, like *trans*-pinocarveol (eluting before *trans*-verbenol in the sunflower blends) and α- and β-pinene (present in these and other samples). A possible enantioselectivity of this type of RNs was indicated by the markedly different response strengths to the same amounts of verbenone in two different samples (not shown). In some recordings type 4 RNs occurred together with three other RN types (1, 2 and 8), which were distinguished by analyses of spike amplitudes and waveforms. The spike amplitudes and overlay of spikes of the two neurons responding to linalool and *trans*-verbenol are shown in Figure 4C. The other responses obtained in these recordings were ascribed to RNs of type 8 (to e.g. 4-ethylacetophenone) and type 2 [to (+)-3-carene, not presented in this figure]. The presence of a similar RN type in a *H. armigera* male was indicated

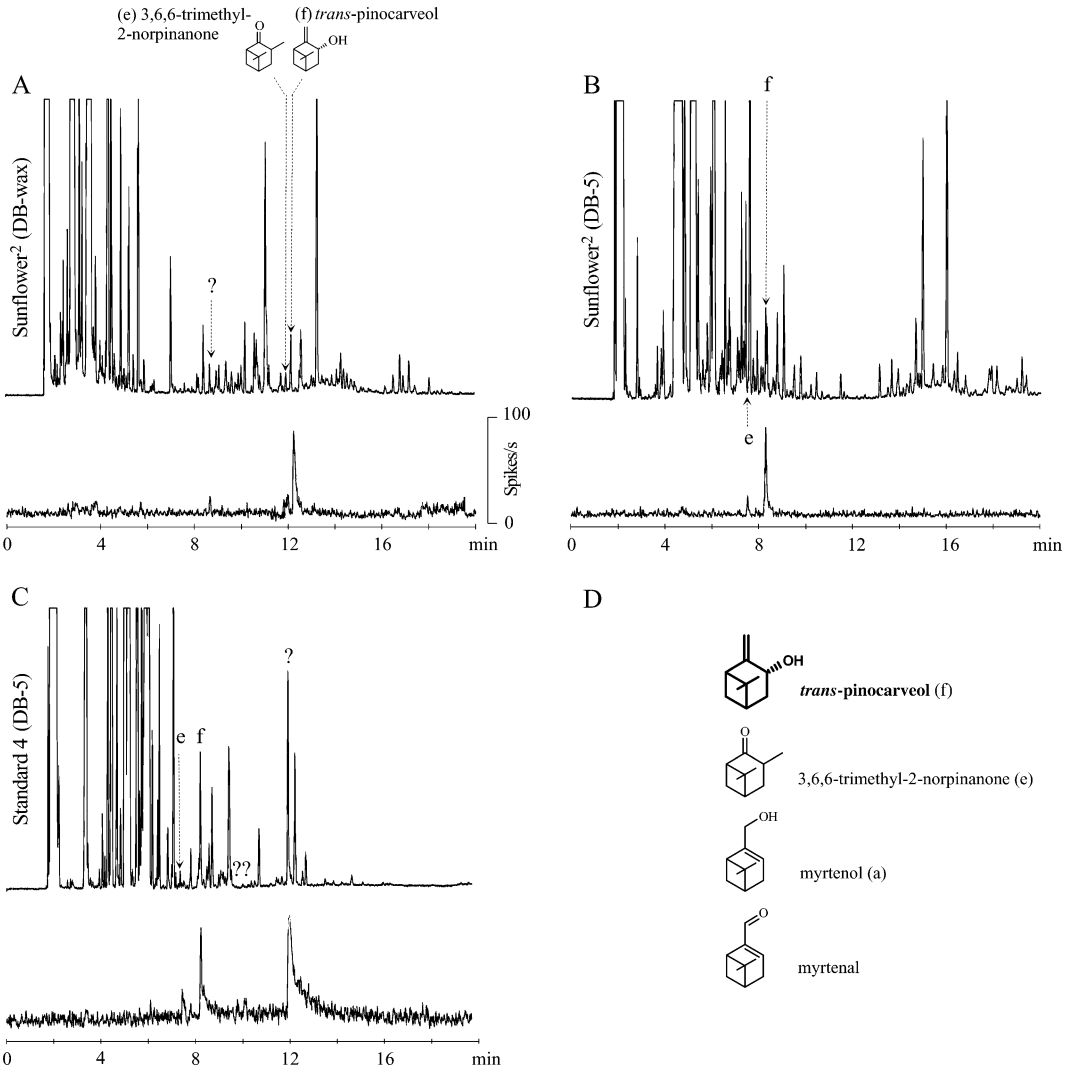

Figure 3 RN type 3 tuned to *trans*-pinocarveol. Gas chromatogram of headspace volatiles of sunflower[2] (*Helianthus annuus*) injected in the polar DB-wax column **(A)** and the non-polar DB-5 column **(B)** and simultaneously recorded activity of a RN of type 3. The strongest response is to the alcohol *trans*-pinocarveol (f); and a weaker response to 3,6,6-trimethyl- 2-norpinanone (e). **(C)** Gas chromatogram of synthetic materials in standard mixture 4 separated in the DB-5 column and simultaneously recorded activity of the RN (same as in A). Responses to *trans*-pinocarveol (f) and 3,6,6-trimethyl-2-norpinanone (e) are shown. In addition, one strong and two weaker responses were recorded to three unidentified components (indicated by question marks). **(D)** Molecular structures of the identified primary and secondary odorants of RN type 3.

by weak responses to trace amounts of *trans*-verbenol (not shown), in addition to responses to myrtenol and borneol (see Figure 1F).

Olfactory RNs responding to sesquiterpenes (RN types 5–7)

RN type 5: α-caryophyllene (humulene)

One neuron found in *H. virescens* showed a strong and long lasting response to α-caryophyllene (also called humulene) when tested for a caryophyllene sample isolated from cotton. No response by this neuron was recorded to the major compound in the sample, β-caryophyllene.

RN type 6: caryophyllene oxide

Two neurons found in *H. virescens* responded strongest to the sesquiterpene caryophyllene-oxide and were classified as RN type 6. Weaker responses by these RNs were recorded to α-caryophyllene (humulene), β-caryophyllene and two minor components, all present in the caryophyllene sample isolated from cotton.

RN type 7: bicyclic sesquiterpenes of cadinane type

Two olfactory RNs found in *H. virescens* responded exclusively to two minor components present in ylang-ylang essential oil and headspace of cultivated tobacco. GC-MS

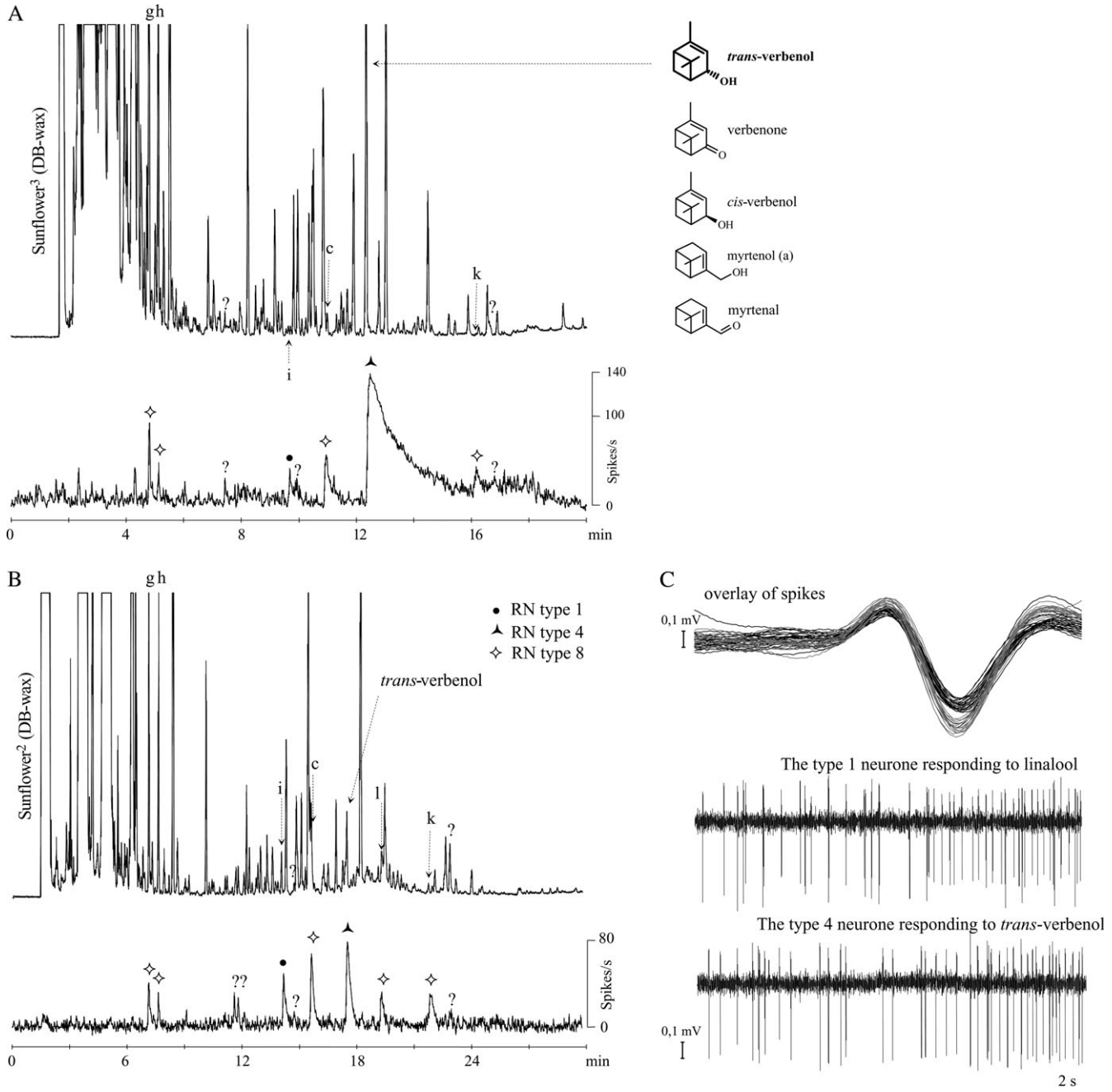

Figure 4 RN type 4 tuned to *trans*-verbenol. Gas chromatograms of headspace volatiles of two different samples of sunflower (*Helianthus annuus*) ([2] and [3]), and simultaneously recorded activities of four co-located RNs in two female *H. virescens* **(A, B)**. One RN (type 4) showed response to *trans*-verbenol, a second RN (type 1) responded to linalool (i) and a third RN (type 8) responded to *p*-cymene (g), styrene (h), terpinene-4-ol (c), 4-ethylacetophenone (k) and cuminaldehyde (l). Unidentified responses and compounds are indicated by question marks. To the right of A are shown the molecular structures of the identified primary and secondary odorants of RN type 4 (responses of the secondary odorants were not shown). **(C)** Spike sequences (2 s, amplification ×1800) of RNs of types 4 (large amplitudes) and 1 responding to *trans*-verbenol and linalool, respectively, obtained from same recording as presented in B.

analyses of these compounds in the oil suggested two sesquiterpenes. One of the odorants had a mass spectrum similar to a cadinene (Figure 5). The other compound (eluting between *E,E*-α-farnesene and γ-muurolene in the polar column) was present in too small amounts for identification. Several mixtures of cadinenes and muurolenes (among others δ-cadinene, α- and γ-muurolene) were excluded as the active odorants by comparison of retention times.

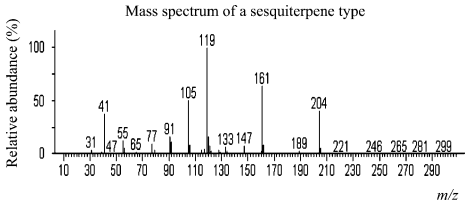

Figure 5 Mass spectrum of the odorant present in ylang-ylang essential oil, which activated neuron type 7 in *H. virescens*.

Olfactory RNs responding to aromatic volatiles (RN types 8–10)

RN type 8: vinylbenzaldehyde

Five neurons, four in *H. virescens* and one in a male *H. armigera*, were classified as RN type 8. This was based on strong responses to a compound identified by GC-MS as 4-ethylacetophenone, which is present in several of the tested plant materials (Figure 6A,B). However, the strongest response obtained by this RN type was to a compound tentatively identified by MS as a vinylbenzaldehyde isomer (Figure 6C), which was present in detectable amounts only in cultivated tobacco materials. Additional responses to at least six compounds were recorded, four of which were retested on the RNs (Table 2). The presence of the odorants in different plant materials is shown in Table 3. The primary and secondary odorants of RN type 8 are characterized by a benzene ring and a functional group of a methyl-, an ethyl- or an isopropyl ligand in the para-position. The compounds eliciting strongest responses also contained a carbonyl group (Figure 6D). Examples of GC-SCRs given in Figure 6 show responses obtained when the neurons were stimulated by an odour sample of cultivated tobacco (Figure 6A) and sunflower (Figure 6B). Together with this RN type, activities of three other RN types (1, 2 and 4) were also recorded. Co-localization of the same RN types was found in the *H. armigera* male, where the response by RN type 8 to the secondary odorant, terpinen-4-ol (Figure 1F) was obtained.

RN type 9: methyl benzoate

One RN classified as type 9 was identified in a female *H. armigera*. Figure 7 shows one GC-SCR, with a strong response to the methyl benzoate in ylang-ylang essential oil. In addition, the neuron showed weak responses to minor amounts of ethyl benzoate in the same essential oil. The neuron also responded to a compound eluting just before linalool, which could not be identified because of the small amount present. The response of this neuron to methyl benzoate was reproduced six times, whereas the responses to the two secondary odorants were repeated twice. This neuron was co-located with another neuron that did not respond to any components of the ylang-ylang oil.

RN type 10: 2-phenylethanol

One RN in *H. virescens* responded to four compounds when tested for volatiles of wild briar. All four responses were reproduced when the same sample was injected again in the same non-polar column. The compound eliciting the strongest response was identified by GC-MS and retention time as 2-phenylethanol. The three odorants eliciting secondary responses were not identified, one co-eluting with the solvent peak. The two other odorants were present in minor amounts. The mass spectrum of one indicated an aromatic compound.

Olfactory RNs responding to hexenols and hexenyl esters ('green leaf volatiles')

Type/group 11: aliphatic compounds

In three recordings with *H. virescens*, showing activity of several neurons, responses were obtained to the aliphatic 6-carbon alcohols, aldehydes and esters [1-hexanol, (2*E*)-hexen-1-ol, (2*E*)-hexenyl acetate, (3*Z*)-hexen-1-ol, (3*E*)-hexen-1-ol, (3*Z*)-hexenyl acetate, (2*E*)-hexenal, (3*E*)-hexenal and 1-heptanol (7-carbon); Figure 8C, Table 2]. Responses during the elution of hexane were recorded in all samples tested. Examples are shown in Figure 8. Spike analysis did not resolve which responses originated from each unit. Except for the cut materials of wild tobacco, containing large amounts of hexenols and hexenyl acetates, the tests with several plant materials (different strains of sunflower, cultivated tobacco and maritime pine; Table 3) elicited few responses of these neurons.

Neurons responding to unidentified compounds (types 12–14)

Several RNs showed responses to compounds eluting during the solvent peak or to unidentified minor compounds. One neuron defined as RN type 12 (co-located with a RN of type 11) in *H. virescens* responded to a compound only present in the volatiles of wild tobacco (cut materials), eluting after linalool in the non-polar column. The response was reproduced when the same tobacco sample was injected again in the same column. Another neuron defined as RN type 13 obtained in a female *H. armigera* showed weak responses during the elution of the solvent peak (hexane) and β-pinene. A few neurons, defined as RN type 14, were recorded in both species. They responded exclusively during or just after elution of the solvent peak (hexane) (the different retention times depended on the temperature programme and GC-column type).

Discussion

The present results contribute to the previous studies of heliothine moths with an additional number of functionally identified types of plant odour RNs, classified according to

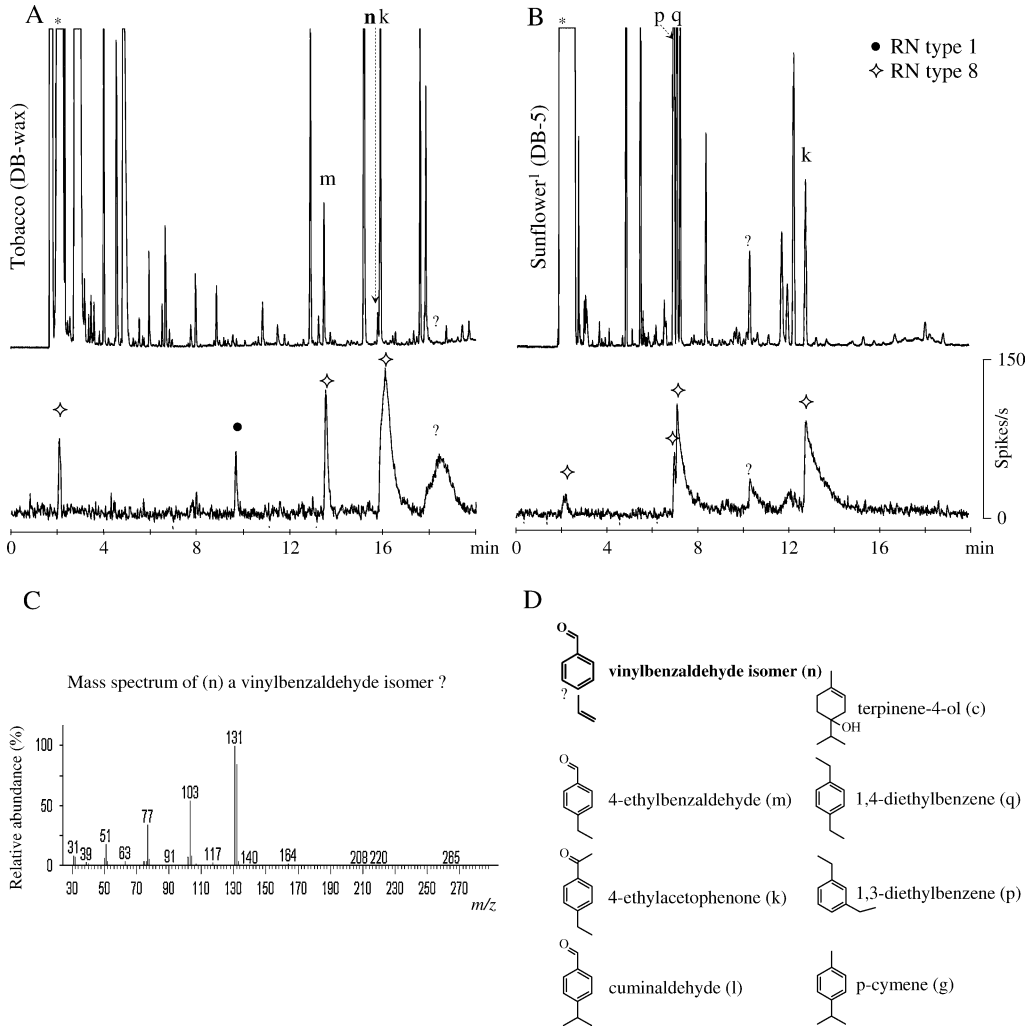

Figure 6 RN type 8 tuned to a vinylbenzaldehyde isomer. **(A)** Gas chromatogram of headspace volatiles of cultivated tobacco (*Nicotiana tabacum*), and simultaneously recorded activity of four co-located RNs (RN type 1, 2, 4 and 8) on the antenna of a female *H. virescens*. Responses to 4-ethylbenzaldehyde (m), 4-ethylacetophenone (k) and a compound tentatively identified as a vinylbenzaldehyde isomer (n, strongest response) are shown. Another co-located RN (type 1) responded to linalool. There is also a response to ethyl acetate in the solvent (marked by the asterisk). Unidentified responses and compounds are indicated by question marks. **(B)** Gas chromatogram of headspace volatiles of cultivated sunflower[1] (*Helianthus annuus*) and simultaneously recorded activities of three RNs on the antenna of a female *H. virescens*. Responses are shown to 1,3-diethylbenzene (p), 1,4-diethylbenzene (q) and 4-ethylacetophenon (k). **(C)** Mass spectra of compound (n) tentatively identified as a vinylbenzaldehyde isomer. **(D)** The molecular structures of the primary and secondary odorants of RN type 8, the vinylbenzaldehyde isomer (n), 4-ethylbenzaldehyde (m), 4-ethylacetophenon (k), cuminaldehyde (l), terpinene-4-ol (c), 1,4-diethylbenzene (q), *p*-cymene (g) and 1,3-diethylbenzene (p).

their primary and secondary odorants. With the 14 types presented here (11 with identified odorants and three defined by GC-peaks), the total number recorded so far is 19 plant odour RN types in heliothine moths. Table 2 gives an overview of the present and previous results, showing the 16 RN types for which the odorants have been identified (Røstelien *et al.*, 2000a,b; Stranden *et al.*, 2002, 2003a,b). Although the RN types 5, 9, 10 and 12 are based on recordings from a single neuron, each of them could be classified as one type because of their specific molecular receptive ranges. The reliability was shown by the high reproducibility of the responses when tested for the same or different samples containing the active odorants (except RN type 5 tested only once). The consistency of the responses recorded with this method is further demonstrated in all the other neurons recorded. This is for instance demonstrated in the present paper by the linalool neurons (RN type 1, n = 10, Table 4) and in the previous papers by the frequently occurring neurons of types I–V (Table 2). Particularly well studied are the previously reported (−)-germacrene D neurons (RN type V, n = 130) showing consistent enantioselectivity and molecular receptive ranges in the three heliothine species. The low number of many neuron types presented in this paper suggests that each of them represent a small

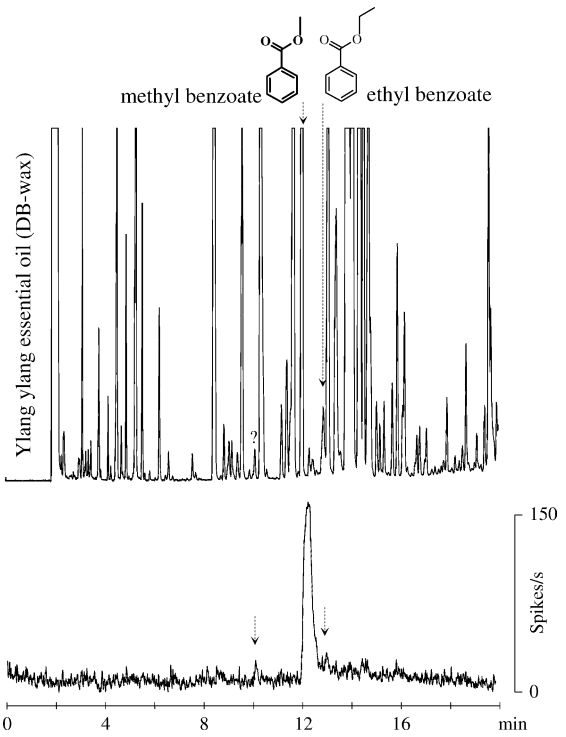

Figure 7 RN type 9 tuned to methyl benzoate. Gas chromatogram of ylang-ylang essential oil (*Cananga odorata*), and simultaneously recorded activity of a RN on the antenna of a female *H. armigera*. The strongest response was to methyl benzoate, while there were weaker responses to ethyl benzoate and an unidentified component (indicated by a question mark).

population. Further recordings will probably reveal other scattered olfactory RN types on the antenna, contributing to the understanding of how plant odour information is handled by the olfactory system of heliothine moths.

By testing volatiles obtained from many plant species of hosts and non-hosts, we have been able to screen a large number of naturally produced odorants on each RN, suggesting that the odorants identified are most probably biologically relevant. However, it is also possible that some important odorants might have been lacking in our test samples, as these polyphagous species exploit a large diversity of host plants. In addition comes the methodical constrains of the headspace collections of volatiles. For instance, the vinylbenzaldehyde isomer, which was tentatively identified as the primary odorant of the RN type 8, was only found in one particular sample of wild tobacco. The volatiles of that sample were collected with the headspace technique, using a mixture of two types of adsorbents (Porapak Q and Tenax) and eluted with a 1:1 mixture of hexane and ethyl acetate (Figure 6A,C). Thus, the presence of vinylbenzaldehyde in this particular sample might be ascribed to the different qualities of the adsorbents. This case also shows that absence of particular odorants might give the impression of a broader tuning of the RNs. The general character of the identified RN types was a narrow tuning as demonstrated by high sensitivity to one primary odorant and weak responses to a few (3–7) secondary odorants with related chemical structures. A small change at any position of the molecule resulted in reduced or no stimulatory effect. An indication of enantioselectivity was seen by RN type 1 to (+)-linalool (Figure 1) and RN type 4 to (−)-verbenone, which for (+)-linalool was demonstrated by different responses to two samples with opposite ratios of the enantiomers (Figure 1C). Tests via a chiral column showing responses to the separated enantiomers were not made in this study. The molecular receptive range of each RN type consisted of odorants within one chemical group, like short chain oxygenated aliphatic compounds (mainly with 6-carbon atoms), acyclic-, monocyclic- or bicyclic monoterpenes, sesquiterpenes or aromatic compounds. This is in accordance with results obtained in other species (compare e.g. Wibe *et al.*, 1997; Bichão *et al.*, 2003, 2005; Stensmyr *et al.*, 2001; Barata *et al.*, 2002). The classification of the neurons into distinct types correlated well with the principle that 'each neuron expresses only one type of receptor proteins', indicated in many vertebrates and invertebrates, including *H. virescens* females (Krieger *et al.*, 2002; Mombaerts, 2004). The molecular receptive ranges of the 14 RN types showed no or minimal overlap within the same chemical group. One example of overlap is (+)-linalool, being the primary odorant of RN type 1 (Figure 1), and also a secondary odorant for the previously described geraniol RN type (Table 2; Stranden *et al.*, 2003b). These results on heliothine species are in contrast to what is found in vertebrates and in some insects, where the olfactory RNs are reported as broadly tuned, commonly with overlapping molecular receptive ranges (reviewed by Masson and Mustaparta, 1990; Smith and Shepherd, 1999; Buck, 2000; De Bruyne *et al.*, 2001). This difference may reflect the low homology of the genes coding for olfactory receptor proteins in vertebrates vs. invertebrates (Breer, 2003).

An important aspect in identifying plant odorants is the variability of volatiles released by a plant species. In addition to variations of the emitted blends due to oxidative changes and circadian rhythms, the biosynthesis of volatiles is also influenced by several biotic and abiotic factors. We did not intend to study the variability, but rather collected as many constituents as possible in order to find the most effective odorants for the RNs in heliothine moths. Most of the compounds identified here as primary or secondary odorants are known as general constituents of many plant species and occurred in several of the plant materials tested (Table 3). Particularly interesting for the present study is the host plant's release of linalool (activating RN type 1), (3*Z*)-hexenyl acetate and (3*Z*)-hexenol (activating RN type/group 11) induced by feeding heliothine larvae (De Moraes *et al.*, 2001; Röse and Tumlinson, 2004). The two latter compounds released during night by tobacco plants possibly contribute to the repellence of mated *H. virescens* females. In contrast, (3*Z*)-hexenyl acetate has been found attractive to unmated *H. armigera* females when presented in

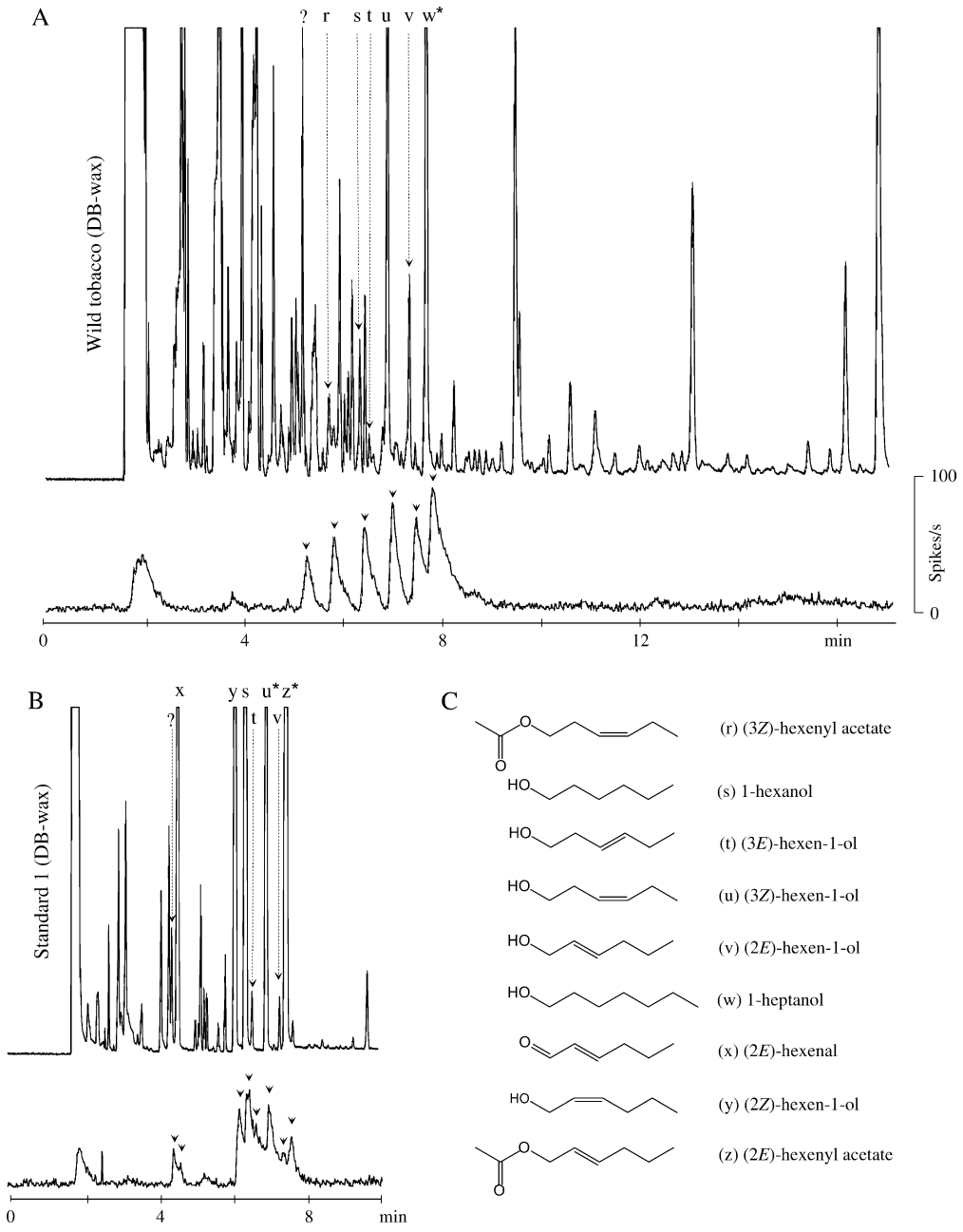

Figure 8 RN type/group 11 tuned to aliphatic 6-carbon oxygenated compounds. Gas chromatogram of headspace volatiles of wild tobacco (*Nicotiana tabacum*) **(A)** and of synthetic materials **(B)** recorded simultaneously with the activity of two to three co-located RNs (type/group 11) on the antenna of a female *H. virescens*. The identified active odorants in the tobacco mixture are (3*Z*)-hexenyl acetate (r), 1-hexanol (s), (3*E*)-hexen-1-ol (t), (3*Z*)-hexen-1-ol (u), (2*E*)-hexen-1-ol (v) and 1-heptanol (w). The compounds s, t, u and v were verified by retesting with synthetic materials. In addition, responses were identified to (2*E*)-hexenal (x), (2*Z*)-hexen-1-ol (y) and (2*E*)-hexenyl acetate (z) in the standard. Responses to hexane (solvent) were obtained in both recordings. The asterisks indicate co-elution of several compounds, i.e. the concentration of the active odorant is lower than indicated by the area of the GC-peak. The question marks indicate unidentified odorants. The individual neuron responses are indicated by arrows. **(C)** Structures of identified odorants.

olfactometer experiments (Gregg and Del Socorro, 2002). Another interesting aspect related to variability of emitted plant volatiles is that the oxidation products of a compound may activate the same RN type. The primary odorants *trans*-pinocarveol (of RN type 3) and *trans*-verbenol (of RN type 4) are major oxidation products of the common bicyclic monoterpenes β- and α-pinene, respectively (Bhattacharyya *et al.*, 1960; Devi and Bhattacharyya, 1978; Draczyñska *et al.*, 1985). Other oxidation products of the two compounds are the secondary odorants myrtenol and myrtenal activating both RN types, *cis*-pinocarveol and *trans*-pinocarvone activating RN type 3, and verbenone

(the major oxidation product of *trans*-verbenol) activating RN type 4. The circadian fluctuations of emitted compounds can be applied to e.g. benzaldehyde, methyl benzoate and 2-phenylethanol (the primary odorants of RN types 8, 9 and 10, respectively)—compounds that have a maximum release rate from tobacco flowers at night (Raguso *et al.*, 2003; Kolosova *et al.*, 2001). These aromatic compounds may attract night-flying heliothine moths, as shown, for example, by phenylacetaldehyde and 2-phenylethanol attracting unmated *H. armigera* females in olfactometer experiments (Gregg and Del Socorro, 2002). Other primary and secondary odorants identified in the present and previous studies were also shown to be attractive to mated or unmated heliothine females in various behavioural bioassays. These odorants include linalool, 3-carene, geraniol, α-caryophyllene and (−)-germacrene D, tested either as single compounds or as constituents added to blends (Rembold and Tober, 1985; Rembold *et al.*, 1991; Hartlieb and Rembold, 1996; Jallow *et al.*, 1999; Bruce and Cork, 2001; De Moraes *et al.*, 2001; Mozuraitis *et al.*, 2002; Gregg and Del Socorro, 2002).

The functional similarity of the plant odour receptor system in *H. virescens* and *H. armigera* is interesting in connection with the phylogeny of these species. The heliothine moths are considered as a monophyletic insect group, i.e. they have a common origin (Matthews, 1999). The two species living on different continents have been separated for a long time, and presumably exploited different host plant species, at least prior to the introduction of the crop hosts, which they have in common. In spite of this, they exhibit functionally similar RN types that also show similar co-localization in the sensilla demonstrated in the present study by RN types 1, 2, 4 and 8 and in the previous studies by RN types I–IV and V (Table 2; Stranden *et al.* 2003a,b). Thus, the RNs seem to be unchanged regardless of differences in the availability of host plants. The similarity may reflect the use of plant odorants that are constituents of many host and non-host plant species. The question remains which mechanisms make the heliothine species choose different host plants. This may rely on species-specific olfactory RNs not yet identified, or on differences in the central processing of odour information, if not solely based on the contact chemoreception. In addition to the innate responses, the olfactory system also has the capacity of plasticity as shown in experiments on olfactory learning and memory. The ability to learn odours has been demonstrated in heliothine moths in connection with egg laying in field studies and with feeding, i.e. appetitive learning by the use of the proboscis extension reflex (Hartlieb, 1996; Cunningham *et al.*, 1998a,b, 2004; Skiri *et al.*, 2005b). As suggested by West and Cunningham (2002) and Jallow *et al.* (2004), the learning of odours may have increased the utilization of the abundant plants of monocultures.

The results of the present study have increased the knowledge on how information about biologically relevant plant odorants is encoded in the RNs of heliothine moths, by identifying primary and secondary odorants for 14 types of RNs of which those recorded in both species show functional similarities. These results are also complementary to other studies showing the behavioural significance of some of the odorants identified. However, further electrophysiological and behavioural studies are needed. Synergistic and inhibitory effects of the odorants on the attraction of the moths, as well as the ability of the moths to learn and to discriminate the single components and mixtures are interesting objectives of further studies.

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