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Doctoral Thesis

UNVEILING SENSORY MECHANISMS FOR THE CONTROL OF TWO INSECT PESTS: FROM BEHAVIOR TO MOLECULAR INTERACTIONS

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Abstract

Pest control strategies targeting insect olfaction represent a promising venue for control of tortricid insects (Lepidoptera: Tortricidae). Among tortricids, the grapevine moth *Lobesia botrana* (Denis and Schiffermüller) and the codling moth *Cydia pomonella* (L.) are serious pests for worldwide production of fruit crops. We employed several approaches to the olfactory system, from electrophysiological and behavioral studies in the grapevine moth, to bioinformatic and molecular studies of olfactory sensory proteins in the codling moth. At the receptor level, we studied both the Olfactory Receptors (ORs), the most common class of sensory proteins mediating detection of odors in insect antennae, and the Transient Receptor Potential (TRP) channels, a novel family of receptor, that recently were also found in the antennae of lepidopterous species. We demonstrated electrophysiological and behavioral responses of the grapevine moth to volatiles emitted by a non-host, *Perilla frutescens*, previously known to activate TRPs in the rat, *Rattus norvegicus*. In the codling moth, we characterized a novel TRP channel (TRPA pyrexia-like) and we confirmed activation of its human orthologue to the same non-host compounds active on the olfactory system of the grapevine moth. ORs were heterologously expressed *in vivo* and *in vitro*, for identification of their ligands among host and non-host plant volatiles and pheromones (deorphanization). Among several ORs of codling moth, we deorphanized a candidate pheromone receptor (PR) to plant synergists, an OR to non-host volatiles and another PR candidate to a pheromone antagonist of the insect. Our study thus opens for refinement of existing pest control, or novel applications. The behavioral response of the grapevine moth to volatiles from a non-host plant, and the identification of a novel TRP channel in the codling moth may have perspectives for application in agriculture, targeting the somatosensory system of these tortricids. The evolutionary implications of the responses of the human orthologue of TRPA pyrexia-like to volatiles active on the grapevine moth olfactory system could imply a large degree of conservation of the receptor function. In the codling moth, identification of synergist and antagonist ligands for candidate PRs and deorphanization of an OR to non-host plant volatiles suggest a possible role of these receptors in reproductive and ecological isolation. This could lead to further refinement of existing semiochemical-based control techniques, by enabling a better understanding of mate- and host-finding in this species.

Keywords

Chemical Ecology, Tortricid pests, *Lobesia botrana*, *Cydia pomonella*, Olfaction, Insect behavior, Non-host plants, Volatile compounds, TRP channels, Olfactory Receptors, Pheromones, Synergists, Semiochemicals

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Introduction and aims

Tortricid moths (Lepidoptera: Tortricidae) include some of the world's most devastating insect species for agricultural products. They represent a large family of over 10.350 species described and many of these are economically important pests for crop production. Among tortricids, we focused our research on two model species, which are also two of the main organisms threatening agricultural economy in Trento Province, Northern Italy: the grapevine moth *Lobesia botrana* (Denis & Schiffermüller) and the codling moth *Cydia pomonella* (L.) (Figure 1).

Figure 1 The grapevine moth *Lobesia botrana* (left) and the codling moth *Cydia pomonella* (right). Respective damages on grape and apple (below). Photos by Umberto Salvagnin, Claudio Ioriatti and Alberto Maria Cattaneo.

L. botrana is the major insect pest of commercial viticulture in the Palearctic and Nearctic regions (Avidov and Harpaz, 1969; Bae and Komai, 1991; Varela et al., 2010; Zalom et al., 2014), and *C. pomonella* is one of the key pests of apple orchards worldwide (Witzgall et al., 2008; codling moth information support system, http://ipmnet.org/codlingmoth/). Furthermore, being polyphagous insects, these tortricids are even serious pests for other fruit crops. For instance, *L. botrana* larvae are known to feed on up to 40 plant species belonging to 27 families (Ioriatti et al., 2011); *C. pomonella* is one of the key pests of apple, pear and walnut, causing also serious damages in other fruit crops (plums), or in non-hosts fruits proximal to infested orchards (Pest notes - University of California). For both species, control still largely relies on insecticide applications, even though semiochemically-based management methods, such as the sex pheromone mating disruption, have shown to be effective (Anfora et al., 2008;

Witzgall et al., 1999; 2008). There is thus great interest in identifying new attractant or repellent semiochemicals, in order to develop alternative control strategies.

The insect olfactory system is hence an attractive target for the control of fruit pests like tortricids: olfaction is an essential sensory modality, allowing them to find food, hosts and sexual partners (Knight and Light, 2001; Witzgall et al., 1993; 1996; 2001). In insects, odorants like several plant compounds are detected mostly by olfactory sensory neurons (OSNs) that innervate specialized sensilla on the ventral side of their antennae. On the plasma membrane of OSNs, the detection of odorants is mediated mostly by olfactory receptors (ORs), representing key elements of insect olfactory systems (Clyne et al., 1999). ORs are specific sensory proteins highly divergent within and between insect species, and they work together with a co-receptor (Orco) (Sato et al., 2008), an insect OR unique, in that it is highly conserved (Krieger et al., 2003; Larsson et al., 2004). Orco and ORs constitute heteromeric complexes of unknown stoichiometry but comprising at least one variable odorant-binding subunit (OR) together with the co-expressed universal integral part of Orco (Benton et al., 2006). Within ORs, a male-biased receptor clade is represented by pheromone receptors (PRs), which mostly detects pheromones (Jacquin-Joly and Merlin, 2004; Ihara et al., 2013; Leal, 2013). However, in moths, odorant receptors clading as PRs were recently reported to respond to non-pheromone compounds (Jordan et al., 2009). The number of ORs expressed in proper subsets of OSNs and their compound-specificity determine the range of odorants an insect can detect. The quality, intensity and temporal pattern of odorant stimuli are encoded by OSNs and processed within the brain (Hansson and Anton, 2000). Although insect ORs can be activated by odorants working like G-protein coupled receptors (Krieger and Breer, 1999; Jacquin-Joly and Merlin, 2004) or like ligand-gated cation channels (Sato et al., 2008), current findings revealed OR activation to be mediated by cellular-signaling cascades (Sargsyan et al., 2011; Getahun et al., 2013). Multiple molecular mechanisms are at the base of signal transduction in insect olfactory systems (Sakurai et al., 2014) and the complete series of molecules involved in these mechanisms remain partially unknown.

However, tortricids use olfaction to search for oviposition substrates providing food sources for the offspring. Indeed, many experimental results confirmed plant volatile compounds to play a relevant role in the three events of the tortricid host-plant selection process: host finding, landing on the proper plant organ, egg-laying stimulation (Witzgall et al., 2005; Anfora et al., 2009). Plant volatiles have also been shown to enhance male attraction to female sex pheromone (Light et al., 1993; Light et al., 2001; Yang et al. 2004). At the light of these evidences, the identification of plant-derived olfaction-active compounds, able to interfere with the insect pest behavior, would be an ecologically safe approach to set-up new management strategies. Accordingly, the huge diversity of plant secondary metabolites (volatile or non-volatile) appears to be a rich source of molecules suitable for these kinds of agricultural applications.

Apart from ORs, recent investigations revealed possible involvements in insect olfaction of a novel family of sensing-related transmembrane proteins, known as Transient Receptor Potential (TRP) channels (Liedtke, 2007). TRPs constitute homo and hetero tetrameric complexes on the plasma membrane of sensory neurons and of single cells of many eukaryotic organisms (Denis and Cyert, 2002; Zhou et al., 2003; Clapham, 2003; Eichinger et al., 2005; Martinac et al., 2008; Nilius et al., 2011; Fowler

and Montell, 2013; Ihara et al., 2013). TRPs enable sensing of external environment by multiple activations modalities elicited by chemical or physical stimulations (Liedtke, 2007). Several natural compounds (e.g. capsaicin, allyl-isothiocyanates, menthol, carvacrol) commonly found in food plants and spices (chilly-pepper, mustard, peppermint, oregano) are known to activate mammalian TRPs (Caterina et al., 1997; Jordt et al., 2004; Bautista et al., 2007; Xu et al., 2006). Activation of TRP channels by these compounds induces action potential on trigeminal nerves (Story et al., 2003) which elicits the so-called somatosensory sensation: a combination of chemosensory and physical perceptions such as hotness, tingling, freshness or cooling (Tominaga et al., 1998). Among TRPs involved in somatosensory sensations, the ion channel TRPA1 was demonstrated to interact with noxious chemicals from mustard (Jordt et al, 2004), it was recently reported to bind multiple types of compounds from somatosensory plants (Nilius and Flockerzi, 2014), and to play a key role in the perception of nociceptive cold (Bandell et al., 2004). Sensitivity to noxious chemicals of exogenous origins make TRPA1 a critical and druggable element to control nociception. Furthermore, demonstrations of mammalian TRPA1 involvements in inflammation (Trevisani et al., 2007) and irritation mechanisms (Bessac and Jordt, 2008) make it a potential candidate for further pharmacological applications and aim current attempts in the design of innovative drugs from natural ligands of the receptor (Bassoli et al., 2013). Within numerous food plants producing compounds active on TRPs, *Perilla frutescens* L. (Lamiales: Lamiaceae), original from Japan (commonly known as *shiso*) and Korea (commonly known as *kaennip*), was recently demonstrated to activate TRPA1 (Bassoli et al., 2009). Secondary metabolites from *P. frutescens* such as perillaldehyde (PA) and perillaketone (PK) were found to strongly elicit rat TRPA1 response in a recombinant HEK293 cells expression system. Despite its wide use in Asian cousin, *P. frutescens* has not been investigated exhaustively, except applications in traditional Chinese medicine (Yu et al., 1997). Although, the reported food and pharmacological properties of this plant among its different varieties (Nitta et al., 2006) have currently renewed the economic importance of its cultivations (Ito, 2008).

Somatosensory plants have always been used for both their peculiar and interesting gustative sensations (i.e. capsicum, garlic, pepper, mint etc.) but they have been also used in agriculture for their known ability to have a defensive role against herbivorous predators and to repel insects, nematodes, worms and other infestants (Leung and Foster, 1996, Barnard et al., 1999). The growing molecular knowledge about the role of TRPs is addressing the use of specific natural compounds and their analogues as insecticides, and it is opening new routes to control insects' perception system for agriculture. Indeed, past investigations revealed TRPs being expressed in the antennae of the lepidopterous species *Manduca sexta*, being potentially involved in pheromone transduction (Ackermann, 2004). Investigations that are more recent revealed TRP being expressed and located at the basis of pheromone sensing tricoidea sensilla of *Spodoptera littoralis* (Chouquet et al., 2009). This opened a completely new perspective for understanding mechanisms of odor perception and processing in lepidopterous fruit pests, and as a consequence, for setting up innovative insect control strategies targeting TRP channels. Among insect TRPs, the role of TRPA1 in thermoreception has been demonstrated in *Anopheles gambiae* providing a basis for targeting mosquito heat responses as a mean toward reducing malaria transmission (Wang et al., 2009). TRPA1 belongs to the insect TRPA subfamily (*TRPA1*, *painless*, *pyrexia*, *water witch*), which contrary from the unique representative in mammals (Clapham, 2003) evolved as an asset of multiple members (Matsuura et al., 2009; Fowler and Montell, 2013). Insect TRPAs are reported for sensing

thermal stimuli (Viswanath et al., 2003; Lee et al., 2005; Sokabe and Tominaga, 2009) but also gravity (Sun et al., 2009), hygrosensation (Liu et al., 2007) and they are involved in insect nociception (Tracey et al., 2003). Furthermore, activation of insect TRPAs to aversive chemicals from food plants and spices (Al-anzi et al., 2006; Kwon et al., 2010; Kang et al., 2010) and similar findings for TRPAs of moths (Wei et al., 2015) represent potentials to target these sensory proteins for the development of innovative pest control strategies, based on somatosensation.

Based on this background, the main goal of my thesis was to shed light to some unknown processes of the insect perception. In particular our studies were aimed at characterizing the function of important sensory receptors expressed in the antenna of the selected model insects, which are also key pests for our agriculture, making use of the most recent and groundbreaking technologies. The long-term perspective is to accelerate the research towards the set-up of new environmentally friendly pest control methods based on the interference with the insect sensory systems.

Specific aims were:

1. To study the behavioural and physiological responses of *L. botrana* to somatosensory-active volatiles emitted by the non-host plant *Perilla frutescens* in order to confirm previous hypothesis in one of our model species and support the following molecular analyses.
2. To identify and characterize TRPs expressed in the antenna of *C. pomonella*, for whom genomic and molecular data had been recently provided in my host laboratory.
3. To deorphanize the most relevant ORs expressed in the antenna of *C. pomonella*. Identification of these receptors and cloning of their full-length coding sequence was at the base of their functional expression by two heterologous methods, in *Drosophila* OSNs and Human Embryonic Kidney (HEK293T) cells, with the aim to identify their main ligands, both agonists and antagonists.

Main methods and results

1. We have demonstrated that volatile compounds from the non-host *P. frutescens*, such as perillaldehyde and isoegomaketone, were detected by both male and female *L. botrana* by electrophysiological experiments (GC-EAD), and induced behavioral effects in both sexes. Females showed enhanced oviposition on a combination of perillaldehyde and host odors, as compared to host odors alone, while virgin male insects showed greater behavioral activity in Y-tube bioassays with isoegomaketone, compared to solvent-only assays, while showing no preference for or against the compound. These compounds have been used in the further experiments and could be the basis for novel control efforts for tortricid pest species.

2. Taking advantage from a previous *C. pomonella* antennal transcriptome analysis, we have found out the expression of five different TRP candidates. One of these TRPs was characterized as belonging to the TRPA subfamily, already found across other Lepidoptera and Hymenoptera species and hypothesized to be involved also in thermal perception. Retro transcription PCR, genomic, and transcriptome analysis of this TRPA, showed the existence of alternative splice forms in different body parts of the insect, with differences between males or females. Based on findings for homologous of

other insects, the role of these splice forms may be possibly involved in different thermal sensing, depending on their expression and combinations in sensory organs.

3. We expressed the human-TRPA1 in the Human Embryonic Kidney (HEK293T) cell system in order to screen activation by the *P. frutescens* essential oils, as well as single synthetic derivatives of natural ligands from this plant. We confirmed sensitivity of human TRPA1 to *P. frutescens* and we demonstrated possible competitive antagonism between *Perilla* compounds isoegomaketone and perillaketone, but not between *Perilla* compounds and main ligands of the receptor, such as allyl-isothiocyanates.

4. The *C. pomonella* antennal transcriptome has allowed the identification and characterization of some significant ORs. By means of heterologous expression in *Drosophila* OSNs, we targeted expression of CpomORs in both the T1 neuron of tricoidea sensilla, normally hosting pheromone receptors, and in the ab3A neuron of basiconic ab3 sensilla, normally hosting olfactory receptors. Screening known pheromones and synergists of the codling moth, we have identified pear ester, normally emitted by host plants, to be the main ligand of the PR-candidate CpomOR3. A candidate PR of the codling moth was deorphanized towards a host plant compound, which role was previously reported to synergize mating behavior. We reported a molecular evidence of the role of a plant volatile enhancing host-finding but also mate-finding. We demonstrated mechanism of action for plant compounds stimulating male attraction by direct activation of pheromone receptors.

5. Targeting expression in *Drosophila* ab3A of the codling moth CpomOR19, and of its homologous receptor from the Noctuid moth *Spodoptera littoralis* Boisduval, SlitOR19, we identified both receptors responding to non-host indanones. Interestingly, although these two species differ by taxonomic position and with respect to their host plants and feeding habits, they share a receptor responding to the same compounds. Higher affinity for analogs of alkyl-2-indanones and lack of response for similar indanones, proved the requirements of particular molecular features for these ligands to activate OR19s, such as the presence of a 5-carbon ring, a keto-group in position 1 and an alkyl-group in position 2. Indanones (such as 1-indanone) are found in *Spodoptera* larval frass, but they are also present in the root extract of tropical plants, in decaying wood fungi, and in filamentous marine cyanobacteria, and they are also emitted by fern (*Pteridium aquilinum*). Despite findings suggest indanones deter moth oviposition in conspecific females, the ecological role of these compounds is still not known. The identification in two closely related species of the receptors for indanones, allows better understanding of their ecology towards *C. pomonella* and *S. littoralis*, in terms of sensing of non-hosts but possibly also in oviposition deterrence.

6. By means of heterologous expression in HEK293T cells, we functionally characterized CpomOrco and for the first time, we deorphanized a candidate pheromone receptor of the codling moth, CpomOR6, to a pheromone compound, (E,E)-8,10-dodecadien-1-yl acetate (codlemone acetate). Codlemone acetate is a strong pheromone antagonist of the codling moth, despite being not the main pheromone of *C. pomonella*. Lack of activation to codlemone acetate when tested on CpomOR6 using expression in *Drosophila* T1 neuron, demonstrated HEK293T to be a promising alternative strategy to study olfactory receptors of the codling moth. Indeed, functional expression of CpomOR3, we previously validated by

both OSN-based methods in *Drosophila*, was also successfully undertook in the HEK293T system, confirming activation by pear ester. Comparing HEK with *Drosophila*, we validated response of this PR-candidate also for the analogous of pear ester, methyl-(E,Z)-2,4-decadienoate, which activation in both systems suggested different insect sensing when compared to pear ester.

Chapter I

Response of the European Grapevine Moth *Lobesia botrana* to somatosensory-active volatiles emitted by the non-host plant *Perilla frutescens*

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Response of the European grapevine moth *Lobesia botrana* to somatosensory-active volatiles emitted by the non-host plant *Perilla frutescens*

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Abstract. The European grapevine moth *Lobesia botrana* Denis & Schiffermüller (Lepidoptera: Tortricidae) is a major pest on grapes worldwide. Attempts to develop control methods for this pest based on grape kairomones demonstrate limited success and studies indicate that a major limiting factor is overlap between synthetic kairomones and background odours in the vineyard. Behaviourally active compounds from non-host plants may thus represent an effective alternative for monitoring and control methods. Extracts from food plants (i.e. from capsicum, garlic and peppermint, which elicit the so-called somatosensory sensation) are traditionally used in agriculture for the control of pest insects. Among those plants, *Perilla frutescens* L. (Lamiales: Lamiaceae), native of Asia, contains compounds activating sensory ion channels in mammals, which are known to be involved in the perception of somatosensory compounds and are expressed in tortricid moth antennae. In the present study, in search of non-host volatiles with potential application in pest control, essential oil metabolites isolated from *P. frutescens* are screened for biological activity on the olfactory system of *L. botrana*. The compounds (*S*)-(−)-perillaldehyde and isoegomaketone, which are released from different *P. frutescens* varieties, are identified by gas chromatography-coupled electroantennographic detection. In a dual-choice oviposition test, females show a preference for a combination of host odours and perillaldehyde, preferring this over a host-plant odour bouquet alone. In Y-tube olfactometer assays, virgin males show a higher level of activity in the presence of isoegomaketone, even if not significantly responsive to the compound.

Key words. Electroantennography, grapevine moth, isoegomaketone, oviposition bioassay, perillaldehyde, Y-tube olfactometer.

Introduction

Olfaction is an essential sensory attribute of insects, enabling them to avoid predators, as well as to search for food, mates and suitable substrates for oviposition (Ache & Young, 2005). The olfactory system is thus an attractive target for the control of insect pests, and the identification of plant-derived olfaction-active compounds that interfere with insect pest behaviour thus represents a promising approach for developing novel environmentally friendly management strategies. The great diversity of plant secondary metabolites (volatile and non-volatile) appears to comprise a rich source of molecules that could be screened in search of suitable candidates for such control methods.

Volatiles from certain food plants and spices (e.g. cinnamon, mustard, garlic, peppermint, wasabi, lemongrass) are known to activate a specific family of sensory-related transmembrane proteins, termed transient receptor potential (TRP) channels, across species and phyla (Caterina *et al.*, 1997; Bandell *et al.*, 2004;

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Jordt *et al.*, 2004), which elicit the so-called somatosensory sensation, a combination of chemosensory and physical perception (e.g. heat, cold, tingling) (Tominaga *et al.*, 1998; Story *et al.*, 2003; Bautista *et al.*, 2007). These plants are also documented for their ability to repel insects (Leung & Foster, 1996; Barnard, 1999). Volatiles with somatosensory properties (e.g. isothiocyanate, citronellal, menthol) emitted from plants are demonstrated to interact with antennal TRPs (Al-Anzi *et al.*, 2006) and to repel *Drosophila melanogaster* (Kwon *et al.*, 2010). In addition, recent experiments demonstrate the expression of TRP channels in the antennae of *Spodoptera littoralis* (Lepidoptera: Noctuidae), at the base of olfactory sensilla (Chouquet *et al.*, 2009).

Among plants producing somatosensory-inducing compounds, *Perilla frutescens* L. (Lamiales: Lamiaceae) produces compounds activating TRPs in rat (*Rattus norvegicus*): TRPA1 (Bassoli *et al.*, 2013) and TRPM8 (Bassoli *et al.*, 2009). *Perilla frutescens* is a food plant commonly used in Asian cuisine, especially in Korea (known as *kaennip*) and in Japan (known as *shiso*). Varieties of *P. frutescens* are characterized by their chemical composition, and are referred to as chemotypes. Among the most common are the PA-type, containing 1,8-*p*-menthadiene-7-al, also known as (*S*)-(−)-perillaldehyde (PA) as the major volatile component of the essential oil; the PK-type, containing 3-(4-methyl-1-oxopentyl)furan (perillaketone; PK); and the IK-type, containing various amounts of 3-(4-methyl-1-oxo-2-pentenyl)furan (isoegomaketone; IK), together with PK (Nitta *et al.*, 2006).

Non-host plants such as *P. frutescens*, with putative ligands for TRP channels, may thus provide novel compounds with the potential for developing new pest control strategies. With this aim, the biological activity of essential oil metabolites isolated from *P. frutescens* are screened on the olfactory system of the grapevine moth *Lobesia botrana* (Denis et Schiffermüller) (Lepidoptera: Tortricidae). *Lobesia botrana* is one of the most economically important insect pests in viticulture, and is a suitable model organism as a result of the detailed knowledge available regarding how it detects host-plants by olfaction. Apart from grape (*Vitis vinifera*), *L. botrana* is able to develop on a wide range of cultivated and wild plants, although the spurge flax *Daphne gnidium* (Myrtales: Thymelaeceae) is considered to be its original host (Maher & Thiéry, 2006). *Lobesia botrana* is reported to occur in Japan (Bae & Komai, 1991), although it is never found to be associated with *P. frutescens*, despite systematic studies of the phytophagous insect fauna associated with this economically important plant (Yanagida *et al.*, 1996; Itoh *et al.*, 2003). At present, the most common control strategy for *L. botrana* is insecticide application. To avoid the development of resistance, formulations of synthetic insecticides are being continually changed and modified. Chemical control strategies are also frequently combined with biological, microbiological and pheromone mating disruption methods in integrated pest management approaches, which are aimed at minimizing pesticide use and slowing the development of resistance (Ioriatti *et al.*, 2011). Accordingly, the identification of novel non-host compounds would enable new possibilities for the control of *L. botrana*.

In the present study, the activities of essential oils extracted from two *P. frutescens* varieties for *L. botrana* are investigated. Gas chromatograph-coupled electroantennographic detection (GC-EAD) is used to identify compounds that are antennally active to both male and female insects. Behavioural activity of *Perilla* compounds is tested using dual-choice oviposition assays for female insects and Y-tube olfactometer assays for males.

Materials and methods

Insects

Lobesia botrana adults used in experiments came from a laboratory colony maintained at Fondazione Edmund Mach, San Michele all'Adige, Trento, Italy. To avoid inbreeding, the laboratory colony is refreshed each year by adding wild larvae collected from Trento vineyards. Larvae were allowed to develop in clear plastic boxes ($35 \times 20 \times 6\,cm^3$), placed in a growth chamber under an LD 16 : 8 h photocycle at $22 \pm 1\,°C$ and $65 \pm 2\%$ relative humidity (RH), and were provided with a semi-synthetic diet (Tasin *et al.*, 2011) *ad libitum* until their pupation. Adults were also kept under an LD 16 : 8 h photocycle at $22 \pm 1\,°C$ and 65% RH. For oviposition bioassays, adults were allowed to emerge in plastic boxes ($30 \times 30 \times 30\,cm^3$). To ensure mating, virgin 24-h-old females were placed in plastic containers (length 5 cm, diameter 2 cm) singly, along with one male of the same age. After one night, containers were inspected for eggs. Only females laying one to 10 eggs were selected for oviposition experiments and these females were considered to be mated. Mated females had never been exposed to plant odours prior to experiments and each female insect was used only once. For electrophysiological and Y-tube olfactometer experiments, insects were allowed to emerge in glass jars (height 30 cm, diameter 15 cm) with moist paper at the bottom, a net lid and access to sucrose solution ($400\,mg\,mL^{-1}$ in water). Virgin adults were kept alone, whereas mated adults were kept with an adult of the same age and opposite sex, in plastic jars (height 10 cm, diameter 5 cm), with moist paper at the bottom and access to sucrose solution. Males were used in Y-tube olfactometer assays within 2–5 days after emerging.

Preparation of essential oils

The *P. frutescens* plants used in the present study were cultivated in an open field. The samples with the highest concentration of perillaldehyde (in PA-type plants) and isoegomaketone and perillaketone (in IK-type plants) were collected from May to October 2011, which covers the entire period of the year during which the plants produce these compounds at the altitude and under the climatic conditions in which the plants were cultivated (Fondazione Minoprio, province of Como, Italy; 380 AMSL). For each sample, 20–30 g of leaf material was subjected to steam distillation. Distillates were extracted with

dichloromethane (CH_2Cl_2) and evaporated under vacuum to produce essential oils. Chemical composition of essential oils was verified by direct or reverse-phase thin-layer chromatography (TLC) using TLC silica gel plates (60778/25EA; Fluka, Switzerland) with fluorescent indicator at 254 nm, as needed, and analyzed by high-performance liquid chromatography (HPLC) (Dynamax Pump, Varian Inc., Palo Alto, California; Altima C18 column, WR Grace & Co., Columbia, Maryland, pi = 132 bar, flow 70–100% MeOH, UV-detector 254 nm). The presence and relative amounts of perillaldehyde, perillaketone and isoegomaketone were estimated by retention time and comparison with authentic samples. As reference compounds, a commercial sample of (*S*)-(−)-perillaldehyde was obtained from Sigma-Aldrich (St Louis, Missouri); for perillaketone and isoegomaketone, natural samples were used that had been isolated and purified previously from perilla leaves (Bassoli *et al.*, 2009, 2013). The PA-type essential oils showed a perillaldehyde content higher than 96%; IK-type essential oils contained PK and IK in variable amounts depending on the time during the growing period when plant samples were collected. The relative percentage of IK ranged from 23% (at 168 days of growth) to 83% (at 39 days of growth), as estimated by HPLC. For bioassays, a mixture of IK-type essential oils from multiple crops harvested throughout the season was used, resulting in a composition of 56% PK and 44% IK.

GC-EAD

The *P. frutescens* essential oil extracts were diluted in HPLC-grade pure hexane (Sigma Aldrich; 99.9%) to achieve a total concentration of all compounds of 1 μg μL^{-1}. Electrophysiological experiments were performed using 2–5-day-old female and male adults (testing both virgin and mated) in a GC-EAD set-up. A single antenna was detached from the head of the insect at the scape using fine forceps, and the apical segment of the flagellum was removed. The antenna was inserted between two conical glass capillary adaptors, adjusted to a suitable shape using an electrode puller (model PP-830; Narishige, Japan), filled with Kaissling Solution (Kaissling, 1987) and integrated in the electroantennographic circuit.

A 1-μL injection of 1 μg μL^{-1} total concentration of essential oil compounds was performed on a 5890 GC (Hewlett-Packard, Palo Alto, California), with a polar Innowax column (30 m × 0.32 mm; J & W Scientific, Folsom, California), programmed from 60 °C (hold 3 min) at 8 °C min^{-1} to 220 °C (hold 7 min) and interfaced with the EAG apparatus. The split of outlet from GC column was a 1 : 1 ratio between the flame ionization detector and the mounted antenna, according to instrument settings. Electroantennographic responses to essential oil compounds were recorded, with multiple repetitions for PA essential oil and IK essential oil dilutions on virgin and mated females and males. A compound was considered electrophysiologically active when it elicited at least three antennal responses that could clearly be differentiated from background noise. Compounds that were electrophysiologically active were subjected to further testing for behavioural activity.

Plant volatile blends

To produce a lure based on host volatiles that would be attractive to female *L. botrana*, host compounds previously identified to elicit a female response were formulated in a control blend. The control blend adopted for oviposition bioassays was composed of compounds emitted by the original host plant *D. gnidium*, as well as *V. vinifera*. This blend contained six volatiles identified from the headspace of *D. gnidium* shoots, flowers and the headspace of unripe *V. vinifera* grapes [(*E*)-linalool oxide (furanoid), (*Z*)-linalool oxide (furanoid), linalool, (*E*)-β-caryophyllene, (*E*,*E*)-α-farnesene and methyl salicylate]. Five additional compounds [ethyl benzoate, (*E*)-linalool oxide (pyranoid), (*Z*)-linalool oxide (pyranoid), benzothiazole and (*Z*)-3-hexenylbenzoate] that enhance oviposition in *L. botrana* and are specific to *D. gnidium* (Tasin *et al.*, 2010) were also added to the control blend. Compounds were added in ratios matching those identified in headspace of host plants (Tasin *et al.*, 2010). The control blend was diluted in HPLC-grade pure hexane (Sigma Aldrich; 99.9%) to a total concentration of 1 μg μL^{-1}, and solvent was tested in preliminary assays as a blank stimulus. For dual-choice oviposition bioassays, compounds were loaded in red rubber septa (20 mm straight plug stopper; Wheaton Industries Inc., Millville, New Jersey). For the 'host' stimulus, 10 μL of the host-plant blend was applied to each septum, for a total loading of 10 μg of host compounds.

In oviposition experiments, host compounds alone (host) were compared with a combination of hosts compounds with doses of *Perilla* samples (host + *Perilla*). Application of perillaldehyde was based on electrophysiological response of female insects. Two variants of the host + *Perilla* treatment were tested: either 10 μL of 1 μg μL^{-1} *P. frutescens* essential oil dilution from perillaldehyde chemotype plants (PAEO) or 10 μL of 1 μg μL^{-1} commercial perillaldehyde lure from Sigma-Aldrich (CAS 18031-40-8) were added to host lures. To confirm the amount of perillaldehyde, and to check the quality of samples, 1 μg μL^{-1} dilutions of *P. frutescens* essential oil, as well as commercial perillaldehyde lure, were analyzed by GC (see Supporting information, Fig. S1).

In Y-tube olfactometer bioassays, the application of isoegomaketone was based on the electrophysiological response of male insects. The essential oil from isoegomaketone chemotype plants was tested at a dilution of 1 ng μL^{-1} in HPLC-grade hexane. Dual-choice experiments were carried out, comparing a septum loaded with 100 μL of IK essential oil solution (for a total amount of 0.1 μg) with a septum loaded with 100 μL of solvent, as a control.

Oviposition bioassays

Mated *L. botrana* females were used in bioassays to determine their oviposition preference, comparing a host blend with a combination of the host blend with PA lures. A dual-choice oviposition assay, including a blank, was performed as described previously (Tasin *et al.*, 2011). Oviposition assays were conducted in cylindrical cages (length 45 cm, diameter 25 cm),

made of metallic net (2-mm mesh), kept under an LD 16 : 8 h photocycle at 25 °C and 60 ± 2% RH. Each cage contained two transparent plastic cones (height 13 cm, bottom diameter 6.1 cm, top diameter 8.8 cm), located 7 cm below the cage top, 10 cm from sides of the cage and separated by 25 cm. Each plastic cone had 30 holes (inner diameter 1.5 ± 0.2 mm) to allow diffusion of volatiles. The control (host) septum and the test (host + *Perilla*) septum were prepared as described above, and placed in a fume hood for 2 h to permit solvent evaporation, after which they were placed separately in the two plastic cones, randomizing their location at every trial, to avoid position effect. At dusk, one mated female was released into the centre of each cage. After 72 h, the female was removed, and the eggs laid on the plastic cone were counted, and the percentage of the total number of eggs laid on each glass was calculated. Oviposition trials were repeated until $n \geq 15$.

Y-tube olfactometer bioassays

The responses of virgin and mated males to *P. frutescens* volatiles were tested in a glass Y-tube olfactometer (stem length 40 cm, arm length 40 cm; diameter 7 cm; side arms at 60 cm), as described previously (Mazzoni *et al.*, 2009). Each arm was connected to a glass flask (diameter 15 cm), with a constant flow of charcoal-filtered air (0.2 ± 0.02 m s^{-1}) at a temperature of 23 ± 2 °C and 60 ± 2% RH. Light intensity was adjusted to achieve a rising gradient (10–20 ± 3 lux) from the beginning of the stem to the glass flasks. Behaviour was recorded 10 min after releasing the insect into the olfactometer. Insects were scored according to the distance that they had travelled in the Y-tube at the end of the recording time. Insects were scored as choice when they reached each arm, limited to the part more than 10 cm downstream from the centre of the Y-fork. To provide a more accurate description of the behaviour for insects stopped halfway, they were scored as pre-choice when they stopped on the first 10 cm of the arms, as well as the 10 cm preceding the Y-fork in the stem. Insects that did not move into either of these parts were scored as non-choice.

To ensure that no bias was introduced by residual solvent, the response of virgin and mated males to the solvent alone was tested. HPLC-grade hexane was loaded in the rubber septa and attached to a 2-cm Petri dish, which was inserted in one of the glass flasks and connected to the instrument. An empty septum was inserted in the other glass flask. For this initial trial, 20 repetitions were carried out. Septa with and without IK essential oil were immediately attached to 2-cm Petri dishes, inserted in glass flasks and connected to instrument, to allow the solution to evaporate inside the instrument and enhance the spread of plant odourants. Septa were refreshed every three or four replications. To avoid position effects, the arm location was randomized every five replications. This trial was repeated 75 times. As a further validation of the bioassay, an additional randomized trial using just hexane was performed ($n = 75$). For statistical analysis, choices in the Y-tube olfactometer were scored as active, whereas non-choice and pre-choices were scored as non-active.

Statistical analysis

All statistical tests were performed using the KYPLOT, version 5.0 (Kyenslab Inc., Japan). To determine significant differences in the number of eggs laid in the female dual-choice oviposition bioassay, a chi-square test was used. This test was also used to validate any significant difference between the two treatments. To determine significances in Y-tube olfactometer assays, a chi-square test in a contingency table was used with Yates' correction.

Results

GC-EAD

The results of the GC-EAD experiments showed a clear response to peaks of essential oils from PA-type and IK-type *P. frutescens* in female and male insects (Fig. 1).

When PA essential oil was injected into the GC-EAD, an antennal response to PA [main peak, retention time (RT) 17.24 min] was observed both in virgin and mated females (Fig. 1, left). A response to PA was also observed in virgin males but, in contrast, the injection of PA essential oil elicited no response in mated males.

Injection of IK essential oil gave a completely different response for the two main components (Fig. 1, right). Application of perillaketone (RT 17.05 min) elicited no response from virgin or mated insects of either sex, whereas responses to isoegomaketone (RT 19.05 min) were recorded from the antennae of virgin and mated males, with a notably higher response for virgin males than for mated ones. By contrast, isoegomaketone elicited no response in females.

According to HPLC estimation of quantity, smaller peaks relating to minor components of essential oil represent < 4% of the total content of PA essential oil and < 1% of the total content of IK essential oil, respectively. In GC-EAD experiments, none of these minor components elicited a clear and repeatable response on insect antennae.

Oviposition bioassays

Perilla compounds were shown to have a significant effect on female oviposition choice. Combinations of PA essential oil or synthetic perilladehyde with the host odour lure resulted in a significant increase in female oviposition rates (Fig. 2). When 10 µg of PA essential oil was combined with host plant odours, female insects showed approximately a 10-fold (9.6–90.4%) enhancement of oviposition choice, whereas the addition of synthetic perilladehyde gave a four-fold (19.9–80.1%) enhancement of oviposition choice, compared with host plant odours alone. This difference in oviposition enhancement between synthetic perilladehyde and PA essential oil extract was not statistically significant ($\chi^2 = 3.44$, d.f. = 1, $P > 0.05$).

Y-tube olfactometer bioassays

Virgin males showed significant activation in the presence of IK essential oil, moving from a non-choice zone to the choice

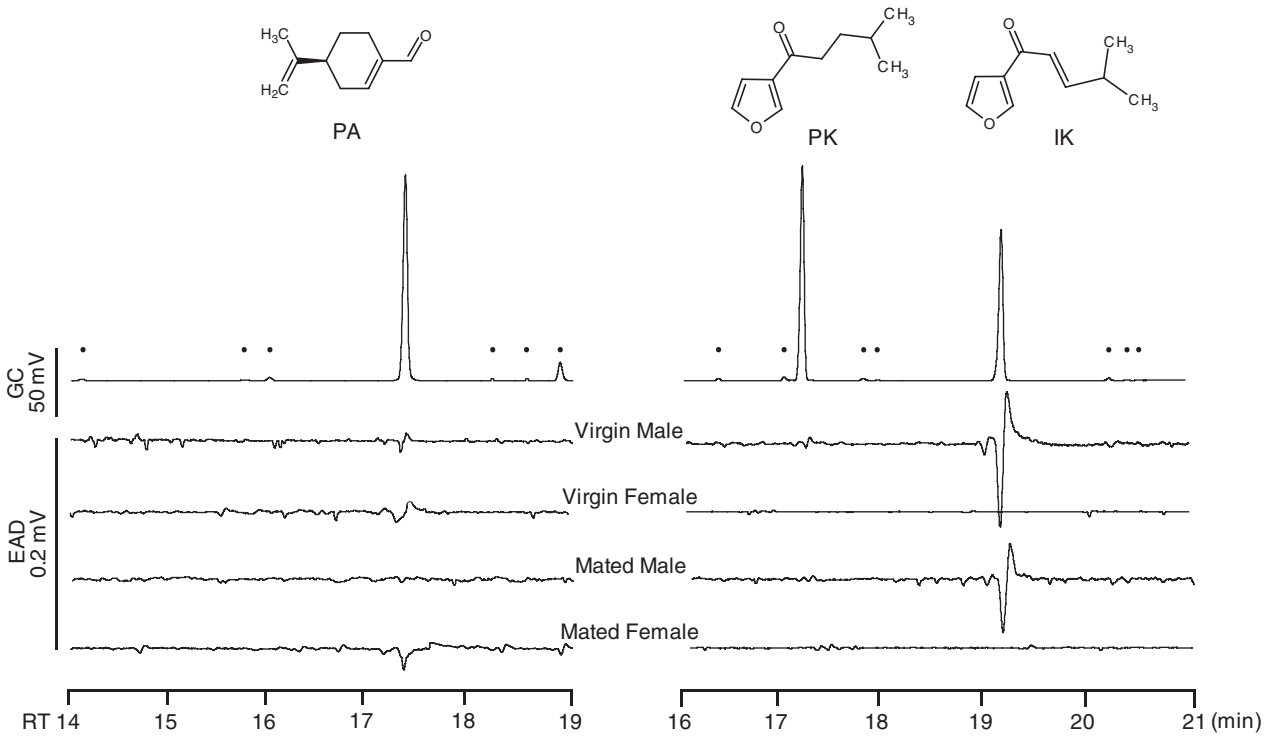

Fig. 1. Gas chromatograph-coupled electroantennographic detection (GC-EAD) traces of *Perilla frutescens* essential oils tested against male and female European grapevine moth *Lobesia botrana*. The responses of females to *S*-(−)-perillaldehyde (PA) and males to isoegomaketone (IK) were identified. Peaks (GC 50 mV) represent the main constituents of essential oils; depolarizations (EAD 0.2 mV) represent antennal responses. Smaller peaks (dots) related to minor components of essential oils were detected in the GC track of both PA essential oil and IK essential oil. RT, retention time.

zone (36 out of 75) when a dose 0.1 µg of IK essential oil was present, compared with experiments where only solvent was present (23 out of 75) ($\chi^2 = 4.02$, d.f. = 1, $P = 0.045$). Mated males showed no significant activation (31 out of 75) when a dose of 0.1 µg of IK essential oil was present, compared with experiments where only solvent was present (19 out of 75) ($\chi^2 = 3.63$, d.f. = 1, $P > 0.05$) (Fig. 3). However, neither virgin, nor mated males showed any significant preference for or against IK essential oil ($\chi^2 = 0.03$, d.f. = 1, $P > 0.05$ and $\chi^2 = 0.13$, d.f. = 1, $P > 0.05$, respectively).

Discussion

The present study shows that compounds from the non-host plant *P. frutescens* are detected by both female and male *L. botrana* and induce behavioural effects in both sexes. Females show enhanced oviposition on a combination of *S*-(−)-perillaldehyde and host odours compared with host odours alone, whereas virgin male insects show greater behavioural activity in Y-tube bioassays with isoegomaketone compared with solvent-only assays, at the same time as showing no preference either for or against the compound.

In GC-EAD tests with essential oil extracts from different *P. frutescens* varieties, *S*-(−)-perillaldehyde elicits an electrophysiological response from both virgin and mated females. A response to *S*-(−)-perillaldehyde is also observed in virgin males but, in contrast, the injection of PA essential oil elicits no response in antennae of mated males. On the other hand, isoegomaketone gives a response on antennae from both virgin and mated males. As validated by previous electrophysiological studies on *L. botrana*, where the tendency of more frequent responses to non-host plant compounds in mated females and unmated males compared with unmated females and mated males is reported (Masante-Roca *et al.*, 2002), the present results suggest that unmated males are generally more responsive than mated ones to compounds from *Perilla* plants. Potentially, such differences could be part of the explanation for the behaviour observed after mating. The electrophysiological data form the basis for selecting *P. frutescens* essential oils to assess in behavioural assays.

Compounds from *P. frutescens* PA-type essential oil cause enhanced egg-laying in mated females when added to a host-odour bouquet based on *V. vinifera* and *D. gnidium*. The addition of synthetic *S*-(−)-perillaldehyde increases oviposition to levels not significantly different from those achieved with PA essential oil (Fig. 2), suggesting that *S*-(−)-perillaldehyde is the main, perhaps the only, active compound in PA essential oil. However, because *S*-(−)-perillaldehyde is the main constituent of both samples, with only minor impurities (see Supporting information, Fig. S1), the effects of minor components at low concentrations could be masked. However, the lack of a GC-EAD response to any of these compounds would appear to argue against this (Fig. 1). Altogether, this suggests that

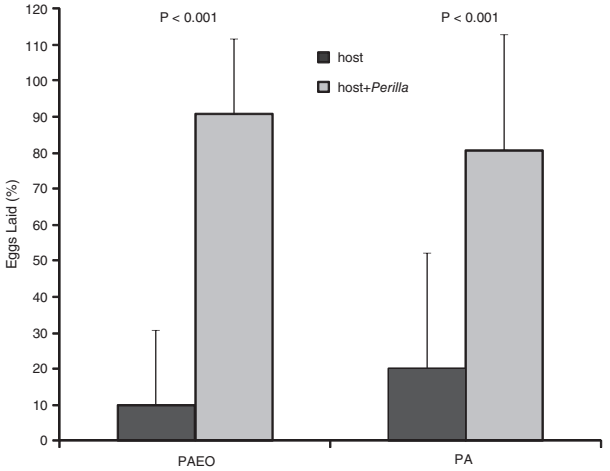

Fig. 2. Comparison of percentages of eggs laid in a dual-choice oviposition bioassay for *Lobesia botrana*. The percentage of eggs laid on host blend alone (host), was compared with the percentage laid on host blend with *Perilla* compounds (host + *Perilla*), testing either essential oil extract (PAEO) or synthetic perillaldehyde (PA). Significant enhancement of oviposition choice was observed by the addition of *Perilla* compounds to host blend (PAEO: $\chi^2 = 63.7$, d.f. = 1, $P < 0.001$; PA: $\chi^2 = 35.0$, d.f. = 1, $P < 0.001$). No significant difference was observed between host blend with PAEO and host blend with PA ($\chi^2 = 3.44$, d.f. = 1, $P > 0.05$).

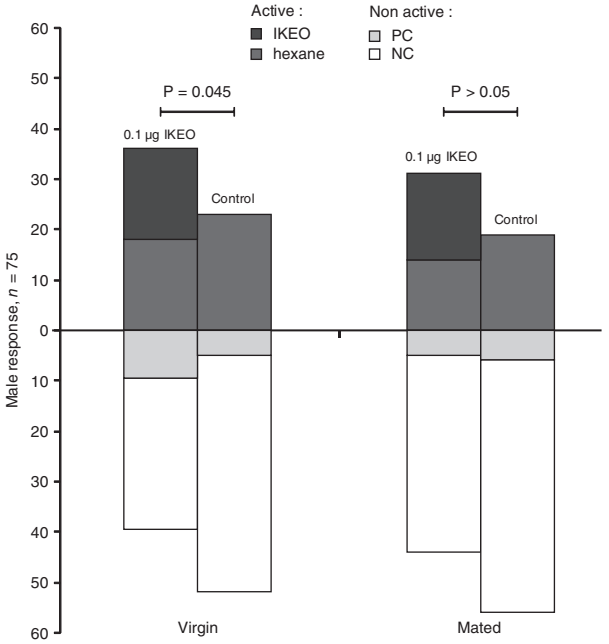

Fig. 3. Behavioural responses of virgin and mated male *Lobesia botrana* to isoegomaketone. Responses to essential oil extract (0.1 µg of IKEO) and solvent control alone (control) were compared in a Y-tube olfactometer. For insects that were scored as active (above 0 in the figure), no significant preference for the arm with isoegomaketone (IKEO) or the arm with the solvent (hexane) was found in virgin (left) or mated males (right), tested with 0.1 µg of IKEO. However, a significantly greater proportion of active virgin males, compared with control, was scored moving from no choice (NC) and pre-choice (PC) areas into either arm of the olfactometer ($P = 0.045$). Mated males showed no significant activation ($P > 0.05$).

S-(−)-perillaldehyde is the most likely active compound as an oviposition stimulant for *L. botrana*.

Repeated electrophysiological responses to isoegomaketone are recorded from both virgin and mated males (Fig. 1), whereas no responses are detected from female antennae, despite multiple attempts (data not shown). One possible explanation for this would be that isoegomaketone is only detected by males, although it is also possible that the olfactory neurones detecting the compound are too rare in females to cause a detectable depolarization of the antenna, as indicated by a study comparing GC-coupled single sensillum recordings to GC-EAD in the beetle *Hylobius abietis* (Wibe, 2004). When *L. botrana* behaviour is tested in Y-tube olfactometer bioassays, males show no significant preference for or against IK essential oil (Fig. 3). However, compared with control experiments, the presence of IK essential oil results in significantly increased activation, with more virgin males leaving the no-choice and pre-choice areas of the Y-tube. There is accumulating evidence that plant volatiles synergize or modulate the behavioural responses to pheromones in lepidopteran species (Landolt & Phillips, 1997; Reddy & Guerrero, 2004) and the ability of IK to interfere with only male *L. botrana* opens the possibility of eventual synergism of IK with *L. botrana* pheromones.

The importance of plant odours for behaviour in *L. botrana* is well documented. Volatiles emitted from grape in all phenological stages guide female insect oviposition choice (Tasin *et al.*, 2005). Other grape volatiles, representing only a fraction of the bouquet emitted by grape [(*E*)-β-caryophyllene, (*E*)-β-farnesene and (*E*)-4,8-dimethyl-1,3,7-nonatriene at 100 : 78 : 9 ratio], elicit anemotactic behaviour at remarkably low doses: females are attracted at release rates of only a few nanograms per minute, at levels almost as low as those known for the attraction of male moths to the female sex pheromones (Tasin *et al.*, 2006). Furthermore, other odourants released by inflorescences and grape berries [limonene, 4,8-dimethyl-1,(*E*)-3,7-nonatriene, (±)-linalool, (*E*)-caryophyllene, (*E*,*E*)-α-farnesene and methyl salicylate] elicit or deter female oviposition, depending on their ratio (Anfora *et al.*, 2009). Host odours from grape also affect male behaviour, where different compounds, including (*E*)-β-caryophyllene, (*Z*)-3-hexenyl acetate, 1-hexanol or 1-octen-3-ol, enhance male attraction to female sex pheromones, whereas (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*Z*)-3-hexenol or methyl salicylate only affect the initial male behavioural responses (von Arx *et al.*, 2012). However, apart from grape, many studies report on the extreme polyphagy, the generalist feeding adaptation and the plasticity of *L. botrana*. Its larvae are known to feed on up to 40 plant species belonging to 27 families, generally growing in warm and dry environments of the Mediterranean basin (Ioriatti *et al.*, 2011).

Non-hosts present in the native range of *L. botrana* are also shown to elicit both electrophysiological and behavioural responses. Compounds from tansy *Tanacetum vulgare* (Asterales: Asteraceae), a sympatric non-host for *L. botrana*, are active at the olfactory level of the insect (Gabel, 1992), with essential oil fractions eliciting electrophysiological responses

(Gabel *et al.*, 1994). Further studies report such essential oil fractions attracting females but reducing oviposition and mating behaviour (Gabel & Thiéry, 1994), although, according to more recent findings, the attraction of the moth to leaves and flowers of tansy is not observed when male and female *L. botrana* are tested in wind tunnel (Masante-Roca *et al.*, 2007). Using GC-EAD, β-thujone and thujyl alcohol are identified as active compounds emitted from tansy flowers (Masante-Roca *et al.*, 2002). Apart from tansy, *P. frutescens* is thus far the only non-host plant that emits compounds active on the olfaction of *L. botrana*, showing attractiveness in terms of stimulation to female oviposition, whereas most of the non-host volatiles are identified as a result of their disruptive or repellent influence in insect host location (Zhang *et al.*, 2013).

The identification of volatiles from *P. frutescens*, active on the olfactory system of *L. botrana* and known to elicit cross-sense somatosensory perceptions, has the potential to unravel unknown mechanisms of insect-sensing. Significant responses in oviposition and Y-tube olfactometer assays, suggest that *S*-(−)-perillaldehyde and isoegomaketone are potentially behaviourally active compounds on mated females and virgin males, respectively.

Moreover, the identification of compounds from a non-host plant in geographical regions where it is not sympatric with the target pest opens the possibility for avoiding overlap with the background odours if adopted in novel applications for the management of insect pest populations.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/phen.12067

Figure S1. Gas chromatography (GC) tracks of perillaldehyde essential oil and synthetic perillaldehyde. Comparison of GC-tracks (GC 50 mV) of perillaldehyde essential oil (PAEO, upper track, 1-μL injection of 1 μg μL^{-1}) and synthetic *S*-(−)-perillaldehyde (PA, lower track, 1 μL-injection of 1 μg μL^{-1}). The synthetic *S*-(−)-perillaldehyde (main peak in the middle) can be distinguished from peaks of impurities (arrows). Comparison indicated only a slight difference in the amount of S-(−)-perillaldehyde (retention time 17.24 min) in the PAEO sample (area = 1046) compared with the synthetic PA sample (area = 903.1). The minor difference in amount of synthetic PA, motivated by the presence of several peaks at lower and higher retention time in the sample, absent in the PAEO solution and likely related with impurities as by-products from chemical synthesis, was judged to be irrelevant.

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Chapter II

The novel insect TRPA *pyrexia-like* is expressed in the codling moth *Cydia pomonella* and it is spliced in multiple variants

The novel insect TRPA *pyrexia-like* is expressed in the codling moth *Cydia pomonella* and it is spliced in multiple variants

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Abstract

Transient Receptor Potential (TRPs) channels are an ancient family of cation channels, working as metabotropic triggers responding to environmental cues. In insects, TRPs have been reported to be involved in perception of physical and chemical stimuli, the latter representing potential targets for applications in pest management. From an antennal transcriptome generated by next generation sequencing, we characterized five candidate TRPs in the fruit pest *Cydia pomonella* L. (Lepidoptera: Tortricidae). The coding DNA sequence of one of these was extended to full length, and phylogenetic investigation revealed it orthologous to the *Bombyx mori* TRP *pyrexia-like* (*pyr-l*), a novel member of the insect TRPA group with unknown function. Reverse transcription PCR revealed the existence of five alternate splice forms of *CpPyr-l*. Identification of a novel TRPA and its splice forms in codling moth antennae open investigation of their possible sensory role.

Introduction

Transmembrane cation channels from the Transient Receptor Potential (TRP) family are key for multiple sensory modalities, including vision, hearing, chemosensation, thermosensation and mechanosensation (Liedtke, 2007; Fowler & Montell, 2013), thus allowing the animals to achieve vital behaviors like avoidance of noxious temperatures (Tracey et al., 2003) or detection of heat emitted from hosts (Wang et al., 2009). TRPs have been divided into seven subfamilies, of which four (TRPC, TRPV, TRPA and TRPN) play a role in sensory systems (Fowler & Montell, 2013). Most insects appear to possess around a dozen TRP genes, approximately half the number of genes found in most mammals (Matsuura et al., 2009). However, there are reported cases where single insect TRP channels are responsible for detecting multiple sensory stimuli. For example, the *Drosophila* TRPV channel Nanchung (Nan) is essential for hearing (Kim et al., 2003; Gong et al., 2004) and hygrosensation (Liu et al., 2007). In *Drosophila*, some channels of the TRPC subfamily also function both in vision (Hardie & Minke, 1992; Niemeyer et al., 1996) and in cold-avoidance (Rosenzweig et al., 2008). Furthermore, the *Drosophila* TRPN channel NompC is associated with mechanosensation (Walker et al., 2000) as well as hearing (Eberl et al., 2000; Göpfert et al., 2006).

In contrast to mammals, in which only one TRPA channel has been identified (Clapham, 2003), insects appear to have an expanded TRPA subfamily, with four or five genes per species (Matsuura et al., 2009).

Like other TRP subfamilies, insect TRPAs appear to be versatile. For example, several *D. melanogaster* TRPA channels (dTRPA1, Pyrexia, Painless) detect different ranges of temperature and are involved in thermotaxis (Viswanath et al., 2003; Lee et al., 2005; Sokabe & Tominaga, 2009), but Pyrexia and Painless are also involved in negative geotaxis, by sensing gravity (Sun et al., 2009). A fourth TRPA channel, Water witch (Wtrw), is involved in hygrosensation (Liu et al., 2007). Interestingly, insect TRPA channels are also involved in chemosensation (Kwon et al., 2010; Kang et al., 2010). Notably, the *Drosophila* TRPA Painless, initially identified as a nociceptive heat sensor (Tracey et al., 2003), was later found to detect allyl-isothiocyanates found in wasabi (Al-anzi et al., 2006), and sugars (Xu et al., 2008). Plants emitting compounds active on TRPs are usually repellent to insects, via the activation of their olfactory systems (Leung & Foster, 1996; Barnard, 1999). In a more recent study, compounds emitted by the plant *Perilla frutescens* L. (Lamiales: Lamiaceae) were reported to be active on rat TRPA1 (Bassoli et al., 2013) and TRPM8 (Bassoli et al., 2009), and we recently demonstrated that *P. frutescens* compounds are detected by the olfactory system of the tortricid pest *Lobesia botrana* (Cattaneo et al., 2014). Furthermore, TRPA1 in the crop pest moth *Helicoverpa armigera* also detects repellent chemicals (Wei et al., 2015), which indicates that members of the insect TRPA subfamily represent potential targets for pest control strategies.

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a major pest of commercial crops such as apple, pear and walnuts in Palearctic and Nearctic regions (Pest notes, University of California, 2011). Whereas olfaction-based pest control methods have been developed (Ridgway et al., 1990; Witzgall & Arn, 1997), a better understanding of the molecular mechanisms of the olfactory process in this species may lead to the identification of new targets for olfactory disruption. In that search, we have previously sequenced the antennal transcriptome of this moth, and notably identified candidate olfactory and pheromone receptors (Bengtsson et al., 2012), functionally identifying two of them (Bengtsson et al., 2014, Gonzalez et al., 2015). In this paper, we identify five candidate TRPs belonging to the TRPA and TRPC subfamilies. Among the TRPAs, we have characterized *CpPyr-l*, an orthologue of the *B. mori pyrexia-like* gene. By performing RACE-PCR and searching a preliminary genome obtained by shotgun sequencing, we obtained the full-length coding sequence of the gene. We investigated its expression in male and female adult body parts by reverse transcription (RT)-PCR. This led to the identification of alternative splice forms with different expression patterns among genders and body parts, which were verified by intron/exon prediction using a genomic overview based on gDNA and RNA-sequencing.

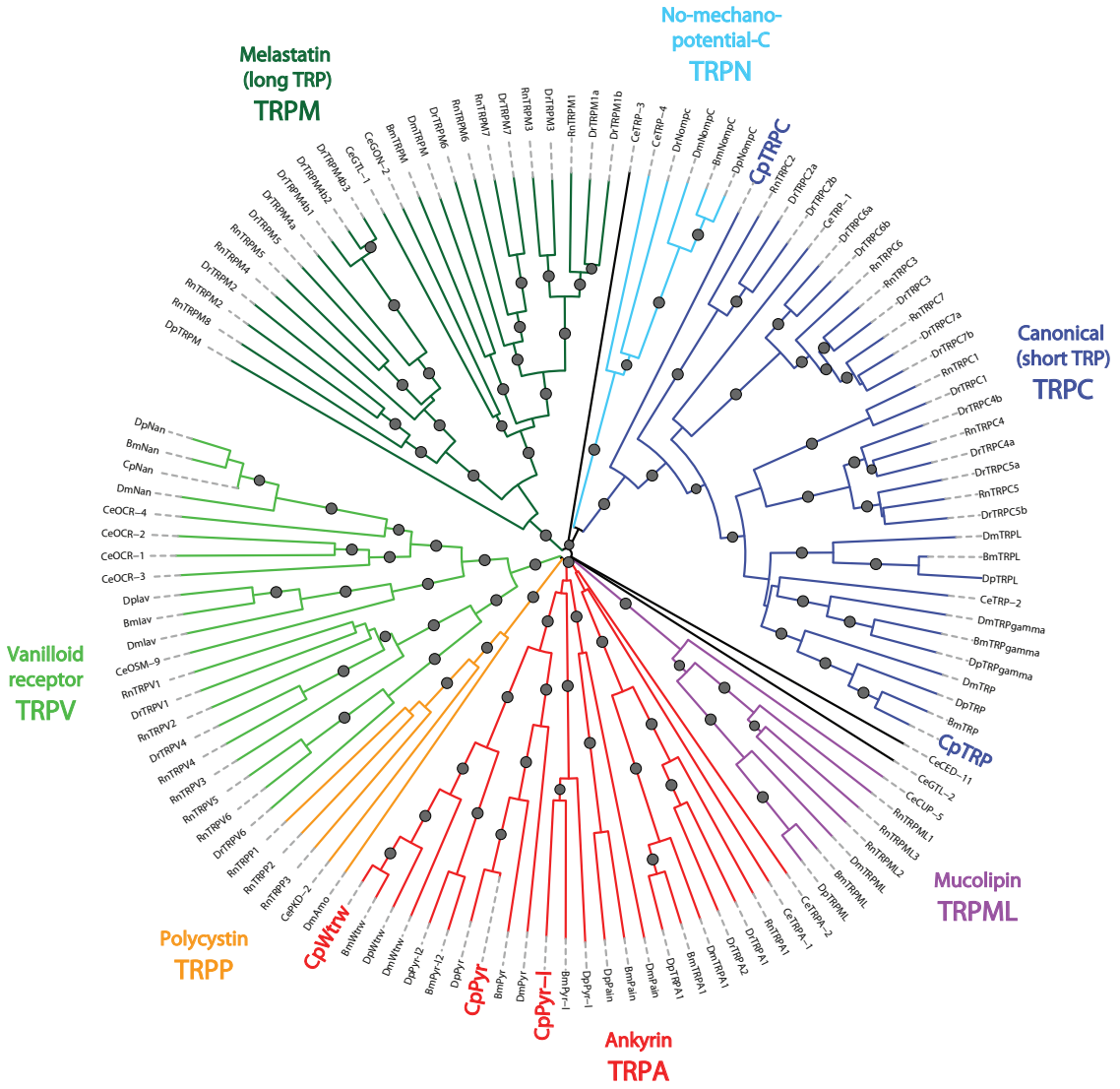
Results

Antennal repertoire of C. pomonella TRPs

Using BLAST search on the *C. pomonella* antennal transcriptome (for details, see Bengtsson et al., 2012), we identified the partial sequences of five candidate TRPs that were judged to be incomplete at both 5' and 3' parts because of the lack of start and stop codons in the open reading frame. A phylogenetic analysis revealed that these candidates belong to the TRPA and TRPC subfamilies (Figure 1). Interestingly, one of the candidate TRPs (*CpPyr-l*) appears to be an orthologue of a novel TRPA channel recently found in *B. mori* [LOC101739591, unplaced scaffold: NW_004581748.1 (498382..519338)] called *pyrexia-like* (*BmPyr-l*). A further two TRPA candidates we identified (*CpPyr* and *CpWtrw*) are expected to be the

orthologues of the *D. melanogaster* TRPAs *pyrexia* (*Pyx*) and *Water witch* (*Wtrw*), respectively. Among the TRPC subfamily, one TRP candidate we identified (*CpTRP*) is an orthologue of *DmTRP*, but the other (*CpTRPC*) does not present clear orthology relationships within the subfamily. The previously described *CpNan* (Nguyen et al., 2013) is part of the TRPV subfamily, and is an orthologue of the *D. melanogaster Nanchung* gene.

Figure 1 Neighbor-joining tree of metazoan candidate TRPs. C. pomonella candidate TRPs identified by transcriptome analysis in bold. Cp: *C. pomonella* L.; Rt: *Rattus norvegicus* Berkenhout; Dr: *Danio rerio* F. Hamilton; Ce: *Caenorhabditis elegans* Maupas; Dm: *Drosophila melanogaster* Meigen; Bm: *B. mori* L.; Dp: *Danaus plexippus* L.; circles: bootstrap values >80. Accession numbers are given in Table S1.



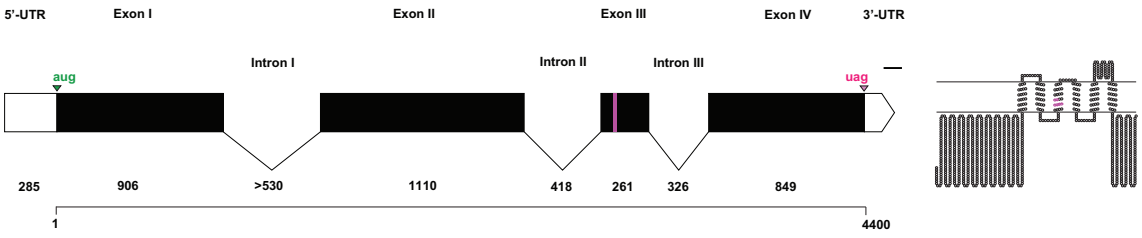
Assembly of the open reading frame of the codling moth pyrexia-like TRP

Since the reported mRNA length of the *B. mori pyrexia-like* orthologue (3764 bp, XM_004926128.2) was more than three times longer than our *CpPyr-l* TRP contig (1012 bp), which also lacked of start and stop codons in frame, we judged the latter to be incomplete at both 5' and 3' ends. In an attempt to extend the sequence to full length, we performed 5' and 3'- RACE-PCR. A partial coding sequence (CDS) of 1677 bp for the *CpPyr-l* TRP was generated by merging a 703 bp 5'-RACE-PCR product and a 432 bp 3'-RACE-

PCR product with the 1012 bp contig, generating a 1776 bp partial transcript with an expected stop codon but without any candidate start codon. This was used as a template to query non-annotated *C. pomonella* sequences from genome and transcriptome data (unpublished): BLAST on genome returned 1073 additional base pairs upstream of the RACE-extended template CDS. This extension included an intron of an approximate length of 530 bp, starting 543 bp upstream of the template. On transcriptome, we extended the 5' with 1734 additional base pairs, including a 5'UTR of 285 bp and a start codon in frame within 1449 bp. To confirm the stop codon, we extended the sequence 4166 bp from the 3' end by RACE-PCR, but no additional stop codon in frame with the partial CDS appeared. The full sequence of *CpPyr-l* TRP has been submitted to Genbank (accession number KU130118).

Sequencing and preliminary assembly of the *CpPyr-l* genomic locus and comparison with antennal RNA-seq returned an overview of intron/exon boundaries within the *CpPyr-l* locus. Overall, the locus of the *CpPyr-l* gene (Figure 2) can provide a full-length CDS of 3126 bp. The locus is constituted of four exons separated by three medium-sized introns. Interestingly, RNA-seq but not preliminary genomic assembly revealed 15 additional nucleotides (5'-CTCCATCGGCCTGGC-3') within the third exon, positioned between nucleotide 3033 and 3047 counted from ATG, indicating that their origin to be mRNA editing. For the 1041 translated amino acid sequence of *CpPyr-l*, the TMHMM 2.0 model predicted six transmembrane domains (between amino acids 619 and 641; 654 and 676; 691 and 713; 720 and 738; 753 and 775; 826 and 848), an N-terminal cytoplasmic region (from 1 to 618), and a C-terminal cytoplasmic region (from 849 to 1041) as would be expected for TRPs.

Figure 2 Graphical overview of the *CpPyr-l* genomic locus. Introns and exons (left), topology representation of the translated polypeptide (right). (Left) white rectangles: UTRs; black rectangles: exons; lines: introns; magenta rectangle: additional nucleotides from mRNA-editing (5'-CTCCATCGGCCTGGC-3'); green arrowhead and letters: start-codon; magenta arrowhead and letters: stop-codon; numbers: bp-lengths of UTRs, exons and introns; scale bar: 100 bp. (Right) magenta amino acids: additional amino acids translated from edited mRNA (Nt-GSIGLA-Ct).

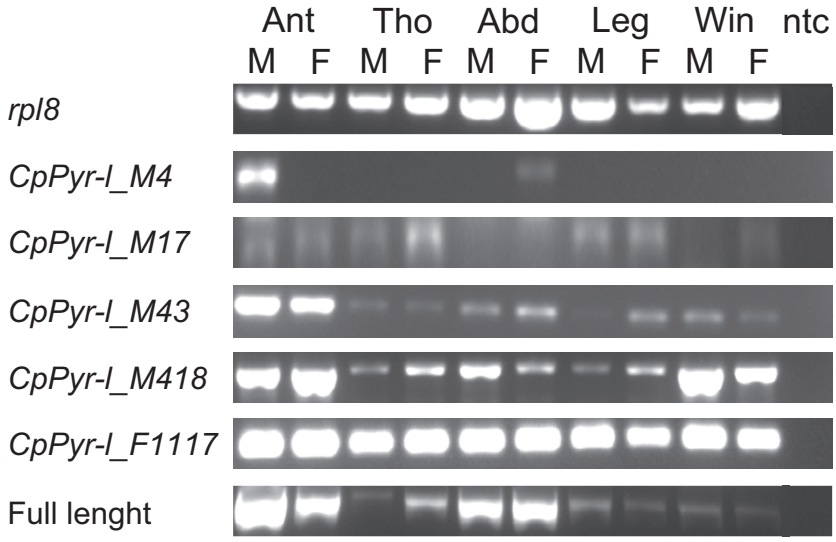


RT-PCR on adult body parts and confirmation of CpPyr-l splice forms

PCR of *E.coli* colonies transformed with pDONR221 containing the expected full-length CDS of *CpPyr-l*, and also revealed several positive clones containing what appeared to be different size transcripts of *CpPyr-l* (data not shown). Sequencing of plasmids purified from positive clones revealed eleven rearrangements of the CDS, suggesting possible generation of splice forms from the original *CpPyr-l*. RT-PCR on adult male and female body parts confirmed the existence of five out of the eleven potential splice forms (*CpPyr-l_M4*, *CpPyr-l_M17*, *CpPyr-l_M43*, *CpPyr-l_M418* and *CpPyr-l_F1117*) in at least two cDNA samples.

Sequences of these five splice forms have been submitted to Genbank (accession numbers KU130119, KU130120, KU130121, KU130122, and KU130123 respectively). Lack of amplification for six out of the eleven potential splice forms in all cDNA samples (*CpPyr-l_M41*, *CpPyr-l_M415*, *CpPyr-l_M417*, *CpPyr-l_F1111*, *CpPyr-l_F1115*, *CpPyr-l_F1124*) means that at this point, we cannot confirm their existence (Figure 3).

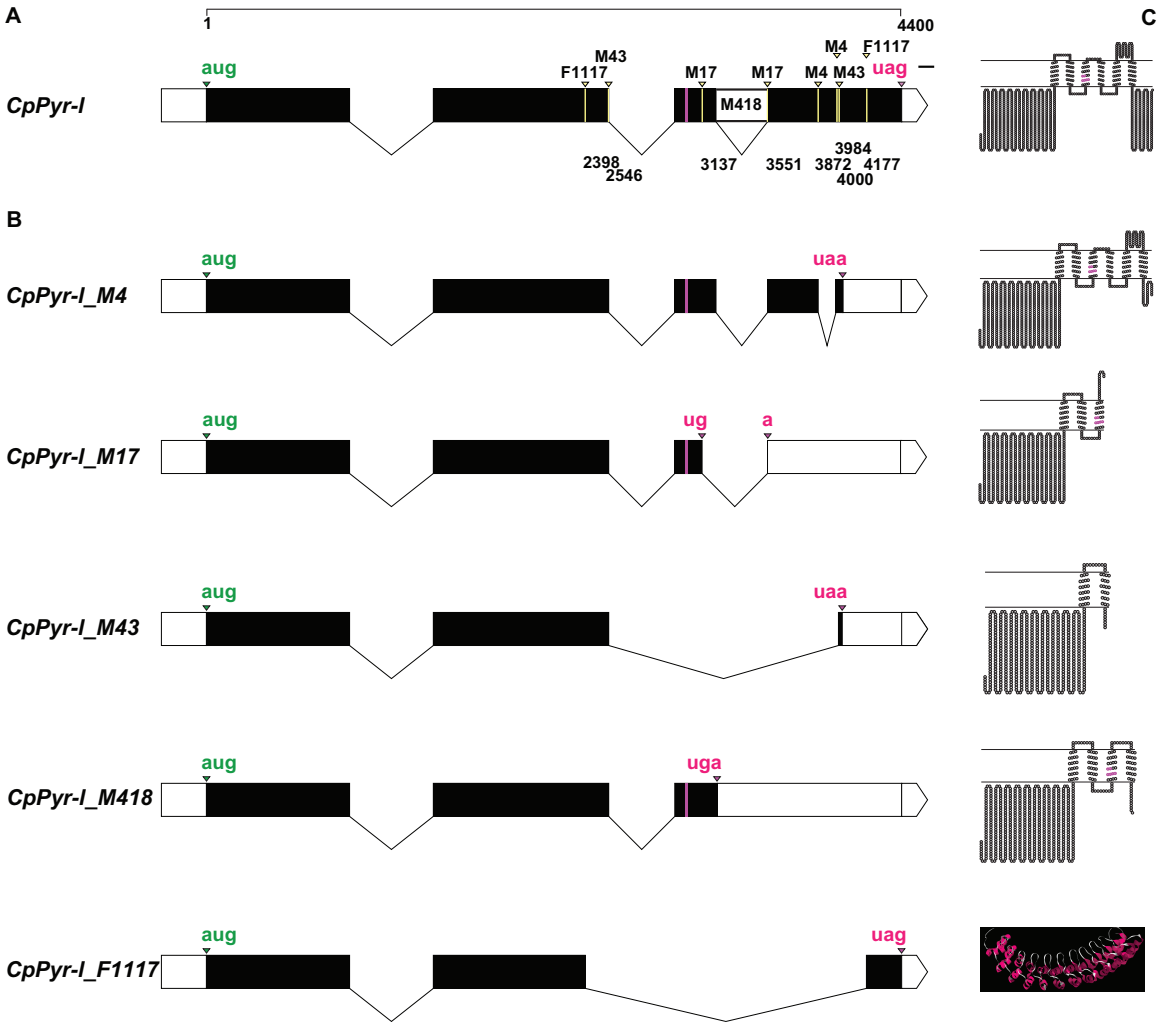
Figure 3 Validation of *CpPyr-l* splice forms in male and female body parts by RT-PCR. For comparison, the full-length *CpPyr-l* gene was also included. The housekeeping gene *rpl8* was used as a positive control in all body parts. Ant: antennae; Tho: thorax; Abd: abdomen; Leg: legs; Win: wings; ntc: no template control; M: male; F: female.



Genome and transcriptome data was used to verify positions of splicing sites expected for the generation of splice forms previously confirmed by RT-PCR (*CpPyr-l_M4*, *CpPyr-l_M17*, *CpPyr-l_M43*, *CpPyr-l_M418* and *CpPyr-l_F1117*, Figure 4 A-B). The *CpPyr-l_M4* splice form is generated by the excision of a short 112 bp fragment within the fourth exon, between positions 3872 and 3984. This splicing is responsible for the termination at a premature UAA stop codon, shortening the C-terminal domain of the transmembrane protein. The downstream splicing site generating *CpPyr-l_M17* form is expected to be in coincidence with the 3' end of the third intron, at position 3551. Interestingly, the premature excision of 88 bp upstream of the third intron at position 3138 affects the open reading frame to generate a premature UGA-stop codon by combination of nucleotides U3136, G3137 and A3552 located on splicing sites boundaries. This prematurely terminates translation and it is potentially responsible for the generation of a truncated polypeptide having only three transmembrane domains, compared to six translated from the full CDS. The *CpPyr-l_M43* splice form is generated by excision of a 1454 bp fragment between position 2546 at the end of the second exon, in coincidence with the second intron, and position 4000 in the fourth exon. The excision shortens the CDS and modifies the open reading frame to an anticipated termination at a candidate UAA stop codon, at position 4022. Sequencing of the pDONR221 clone of *CpPyr-l_M418* splice form revealed an extra CDS region, which genomic overview revealed to correspond with the third un-spliced intron. The presence of the third intron in *CpPyr-l_M148* likely makes it an incomplete splice form, the CDS of which is characterized by a premature termination due to an alternative candidate UGA stop codon located in the

sequence of the un-spliced intron. Possible splicing sites in the second and fourth exons at positions 2398 and 4177 respectively, determines excision for a 1779 bp fragment, which could generate the *CpPyr-l_F1117* splice form. This candidate splice form lacks most of the coding sequence between exon II and exon IV, which codes for transmembrane domains, and it makes the potential translated protein soluble. Since a topological transmembrane representation was not possible, we reported a 3D prediction of the tertiary structure.

Figure 4 Graphic representation of introns, exons and topology of the translated polypeptides, of the full-length *CpPyr-l* variant and verified splice forms. A, Introns and exons for the full-length *CpPyr-1* variant. White rectangles: 5'-UTR, M418 additional region, 3'-UTR; black rectangles: exons; lines: introns; magenta rectangle: additional nucleotides from mRNA-editing; green arrowheads and letters: start codon; magenta arrowheads and letters: stop codon; yellow arrowheads and bars: splicing sites of RT-PCR verified splice forms (abbreviations indicated above arrowheads); numbers: splicing sites positions, counted from the start-codon; scale bar: 100 bp. B, Introns and exons for *CpPyr-1* splice form variants (*CpPyr-l_M4*, *CpPyr-l_M17*, *CpPyr-l_M43*, *CpPyr-l_M418* and *CpPyr-l_F1117*). C, Topology of the translated polypeptides of the full length CpPyr-l and of splice form variants *CpPyr-l_M4*, *CpPyr-l_M17*, *CpPyr-l_M43* and *CpPyr-l_M418*. Magenta: amino acids translated from edited-mRNA; 3D-prediction (rastop): CpPyr-l_F1117.



Discussion

Contrary from what is known for the only one TRPA subunit expressed in mammals (Nilius & Flockerzi, 2014; Macpherson et al., 2007; Bandell et al., 2004; Jordt et al., 2004; Story et al., 2003), multiple subunits of insects TRPAs constitute a subfamily of several sensors responding to different types of stimuli (Wei et al., 2015; Wang et al., 2011; Kang et al., 2010; Wang et al., 2009; Sun et al., 2009; Liu et al., 2007; Lee et al., 2005; Tracey et al., 2003). Currently, the implication in sensory modalities of additional insect TRPA members is still ignored, since only genome sequencing has unveiled their existence (Suetsugu et al., 2013; Honeybee genome consortium, 2006) and functional studies have not yet been conducted. Although the discovered functionalities of known insect TRPAs are giving significant contributions to understand their roles in nociceptive and thermal sensing (Braun, 2012), unveiling the existence of new members and their functional characterizations may represents the new frontier to better understand sensory mechanisms of insects.

In a screen of the antennal transcriptome of the tortricid pest *C. pomonella*, we identify a novel member of the recently identified group of insect TRPAs currently reported as Pyrexia-like and related to the better-known Pyrexias. We also demonstrate the existence of different splice forms for *CpPyr-l*, which appears to have sex- and body part-specific expression patterns. Apart from CpPyr-l, we characterized four additional candidate TRPs, belonging to the two main TRP subfamilies, TRPA and TRPC. Phylogenetic analysis indicated that the candidate TRPs belong to several orthology groups, i.e. Pyrexia, Pyrexia-like and Water witch (TRPAs), TRP and TRPC (TRPC) (Figure 1). While we did not find all the TRPs canonically involved in thermal sensing (TRPA1, Painless, Nanchung), we did find TRPs previously reported to be involved in sensing of heat (Pyrexia), cold (TRP), as well as hygroscopic sensing (Water witch). This suggest the existence of thermal and hygroscopic modalities in codling moth antennae, as recently demonstrated in *Drosophila* (Gallio et al., 2011; Liu et al., 2007), hymenoptera (Ruchty et al., 2010) and reported long ago for other insects (Altner and Loftus, 1985).

In *Drosophila*, Pyrexia-expressing neurons appear to be widely distributed throughout the fly body, and are most likely involved in detection of high temperatures (Lee et al., 2005). We observed a similar pattern of body-wide expression for the *C. pomonella pyrexia*, where RT-PCR indicated that it was expressed in all body parts, except male wings and female antennae. Similarly, *CpPyr-l* and *CpWtrw* were expressed in almost all body parts (Figure S1).

Apart from the *B. mori* locus LOC101739591 to which we report *CpPyr-l* to be the orthologue, another *pyrexia-like* locus is located on an unplaced scaffold: LOC101742191 [NW_004581694.1 (1024969..1028744)]. While the former transcribes two splice variants (XM_004926128.2 and XM_012690360.1), without any bibliography being currently available, the latter transcribes only one variant (XM_004923230.2) also known as *BmTRPA4* (Sato et al., 2014; Suetsugu et al., 2013). Apart from reported negative results from injection of *BmTRPA4*-RNAi in *B. mori* eggs to study induction of diapauses (Sato et al., 2014) little is known regarding the function of this Pyrexia-like. It could be speculated that it has a function similar to the closely related Pyrexia (Suetsugu et al., 2013), which is involved in temperature sensing (Lee et al., 2005) but also in the regulation of circadian clocks (Wolfgang et al., 2013). On the other

hand, its characterization as a TRPA member suggests that it may be involved in sensing humidity (Liu et al., 2007) or possibly somatosensory compounds (Al-anzi et al., 2006). Functional studies of Pyrexia in *D. melanogaster* demonstrated different temperature sensitivities for different Pyrexia isoforms and for their combinations (Lee et al., 2005). When co-expressed, immunostaining showed both isoforms to be co-localized, suggesting a possible hetero-tetramerization of the quaternary structure of the channel, with a possible functional role in sensing specific temperature ranges. While speculative, a possible explanation for the existence of multiple Pyrexia-like splice forms in *C. pomonella* could be a combinatorial-based system for thermal sensing, similar to that observed for Pyrexia in *D. melanogaster*.

Evolutionary studies suggest that the TRPA *pyrexia* has been subject to duplication events, with subsequent differentiation (Matsuura et al., 2009). For example, the TRPA member *water witch* (*Wtrw*) is reported to be the result of a retrotransposition event from *pyrexia*. Undergoing further retrotranspositions in the common ancestor of holometabolous insects, *HSTRPA* in Hymenoptera and *water witch-2* (*Wtrw2*) in Lepidoptera differentiated from *Wtrw*. Our phylogenetic investigation (Figure 1) suggests that the Lepidoptera *pyrexia-like* derived from an ancestral separation occurring before the one separating *pyrexia* and *water witch*. A similar path may have led to the evolution of a further TRPA member identified in Hymenoptera and Coleoptera as *TRPA5* (Matsuura et al., 2009). The orthologue of *TRPA5* in *Apis mellifera* L. (Hymenoptera: Apidae) (locus Pyx2 on LG4) was also identified in *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) (locus LOC657486 on ChLG4), and it could be hypothesized that this gene has been retained in Hymenoptera and Coleoptera, but not in other insect groups. Like *B. mori*, current annotations report also *A. mellifera* to have two separate loci for *pyrexia-like* (LOC102656691 on LG2 and LOC102654980 on LG5), suggesting a more variable group of TRPAs for Hymenoptera. Indeed, since Hymenoptera miss *TRPA1*, they could potentially compensate in heat sensing using other genes, like *HSTRPA* (Matsuura et al., 2009), or perhaps further TRPAs, e.g. *TRPA5* and *pyrexia-like*. The identification of *pyrexia-like* genes in both Lepidoptera and Hymenoptera could indicate a separation event for *pyrexia-like* and *pyrexia* in Hymenoptera and Lepidoptera, analogous to how *TRPA5* and *pyrexia* have separated in Hymenoptera and Coleoptera.

Our data pointed to mRNA editing by the identification of 15 additional nucleotides within the coding region of the third transmembrane domain of CpPyr-l. Current findings report mRNA editing occurring for K^+ channels in multiple organisms, including insects (Holmgren & Rosenthal, 2015). For instance, in *Drosophila*, editing generates multiple isoforms. Their frequency varies between different parts of the adult's anatomy (Ingleby et al., 2009), having a variety of functional effects, including changes to activation, deactivation and inactivation kinetics, and some small shifts to the channel voltage sensitivity (Ryan et al., 2008). Pyrexia is known to be a thermal-gated K^+ channel (Lee et al., 2005) and the relatedness of *CpPyr-l* with *pyrexia* support our mRNA-editing findings for *CpPyr-l*. These post-translational modifications as well as their expression plasticity we observed in RT-PCR follow a general pattern of regulation of TRP function at multiple post-transcriptional levels (Voolstra & Huber, 2014) and raise the question of potential functional effects. In addition, topology predictions indicated that certain CpPyr-1 splice forms lack most of the voltage-sensing domain (TM1-TM4), possibly leading to a shift in function (Figure 4, C). For CpPyr-l_M43, most of the voltage-sensing domain was lacking (TM2-TM6), while for CpPyr-l_M418, TM5 and TM6 were missing, which form the central cation-conducting pore. Expression patterns of the full length

CpPyr-l and variants *CpPyr-l_M43* and *CpPyr-l_M418* were mostly similar, with the exception of a high expression in the wings for *CpPyr-l_M418* (Figure 3). Genomic overview further confirmed the existence of these splice forms. Indeed, the position of the upstream splicing site for *CpPyr-l_M43* corresponds with the intron/exon boundary between exon II and intron II and *CpPyr-l_M418* is the un-spliced variant generated by lack of intron III excision (Figure 4, B).

CpPyr-l_M17 and *CpPyr-l_F1117* splice forms were observed in most body parts. Transmembrane and 3D-predictions of the CpPyr-l_F1117 splice form confirmed it is a soluble ankyrine-repeat module, missing all transmembrane domains. Transmembrane proteins of eukaryote organisms like TRPAs are associated with the cytoskeleton by protein/protein interactions in the cytosolic side of the plasma membrane, involving their ankyrine domains and spectrin/actine complexes (Baines, 2010). Even if *in vitro* expression and purification of heterologous ankyrines has been reported and has successful biotechnological applications (Binz et al., 2003), the existence and possible functionalities of soluble ankyrines in biological systems have not been reported. In insects, it has been demonstrated that epigenetic regulation occurring by DNA methylation may regulate expression of specific genes by causing widespread and diverse changes in alternative splicing (Li-Byarlay et al., 2013; Foret et al., 2012). Occurring in all body parts without specific pattern, it is possible that *CpPyr-l_F1117* and/or *CpPyr-l_M17* splicing aims at silencing translation of the original CDS, by generating non-functional forms.

Other splice forms may translate proteins functioning as TRPA sensors in the codling moth. For instance, among splice forms, *CpPyr-l_M4* appears to be expressed only in male antennae and in the female abdomen. The existence of this splice form is confirmed by the identification of its downstream splicing site proximal with the one identified for *CpPyr-l_M43*, as validated by genomic overview (see above). Transmembrane predictions indicate that this splice form retains six transmembrane domains. Only a shorter re-arranged C-terminal distinguished it from the original full-length Pyrexia-like. Although the N- and C-terminal regions are reported to be important to mediate control of channel gating (Hoffman et al., 2002) and activations of thermal-TRP-channels (Brauchi et al., 2006), this CpPyr-l_M4 form may be the best candidate functional channel, together with the full-length CpPyr-1. CpPyr-l_M4 preserves a complete ankyrine-repeats N-terminal, required for TRPA-activation (Macpherson et al., 2007), and a six-TM structure, including TM5, TM6 and their connecting loop forming the central cation-conducting pore. Whereas the full length CpPyr-l is possibly involved in a general transduction pathway because of its wide expression in all insect body parts, CpPyr-l_M4 may be involved in more specific sensing function in male antennae and the female abdomen, possibly the ovipositor.

In conclusion, we report here the first identification of a Pyrexia-like TRP in the codling moth. This novel insect TRPA derives from an ancestral separation occurring before the one separating *pyrexia* and *water witch* in Lepidoptera. CpPyr-l is closely related to the thermal TRP Pyrexia and it is expressed in antennae and body parts of the codling moth. We demonstrate a high degree of post-transcriptional versatility in its locus, with multiple alternative spice forms and RNA-editing. This opens for investigation of possible roles in sensing, e.g. of temperature, which is speculated to be mediated in a combinatorial fashion by multiple splice forms of insect TRPAs.

Experimental procedures

Dissection, nucleic acid extraction

C. pomonella pupae were obtained from a laboratory rearing (Andermatt Biocontrol, Grossdietwil, Switzerland), and adults were allowed to emerge in cages kept at 23 °C, 70 ± 5% RH, 16 h : 8 h light : dark cycle and fed with 10% sugar solution. As previously reported (Bengtsson et al., 2014) dissection of 2-3 day old female and male insects was performed using sharp forceps: antennae were removed at the base of the pedicel, and legs at the coxa. For thorax samples, head, wings, legs and abdomen were removed. Wings were removed at their base, and the abdomen removed at the connection to the thorax. All body parts were immediately flash-frozen using liquid nitrogen, and thereafter kept at -80 °C. RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany), that included a DNase step to remove genomic DNA contamination. A gDNA sample was extracted from one male and one female adult insect using the DNeasy kit (Qiagen) following the recommended protocol. Body-part RNAs and gDNA were quantified using Nanodrop (Nanodrop 8000 UV-vis Spectrophotometer, Thermo Scientific Wilmington, DE, USA).

cDNA library construction, and bioinformatics

Male and female contigs previously obtained (Bengtsson et al., 2012) were analyzed through bioinformatics, in search of candidate TRPs. Tblastn searches were performed using available amino acid sequences of Lepidoptera and other insect TRPs. Contigs presenting similarity to TRP genes were further assembled using Cap3 (http://pbil.univ-lyon1.fr/cap3.php). Open reading frames (ORFs) were searched and translated to amino acid sequences using ExPASy (http://www.expasy.org/translate/), and Blastx on the Genbank non-redundant database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to verify their annotation. TRP sequences were studied by sequence alignment using MAFFT version 6 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Toh, 2010). For *CpPyr-l* and its splice forms, transmembrane domains were predicted from translated sequences using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). Topology configurations were predicted with TOPO 2.0 (http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py). For the candidate *CpPyr-l_F1117* splice form, which was predicted to lack transmembrane segments, we predicted the tertiary structure of the related polypeptide using the Proteus structure prediction server 2.0 (http://www.proteus2.ca/proteus2/). Among proposed 3D-models, the model 1N11.pdb (Protein Database, http://www.rcsb.org/pdb/home/home.do) related with the D34-region of a human ankyrin, was chosen being reported by the server to be the best candidate (e-value = 2.0 E-31) to represent the 3D structure of the CpPyr-l_F1117 protein. To perform 3D-representation, Rastop 2.2 was used (available for the public domain at http://www.geneinfinity.org/rastop/).

Phylogenetic investigation of CpomTRPs

TRP sequences of *Rattus norvegicus* Berkenhout, *Danio rerio* F. Hamilton, *Caenorhabditis elegans* Maupas and *Drosophila melanogaster* Meigen were downloaded from their proper genome browsers (http://rgd.mcw.edu/; http://zfin.org/; http://www.wormbase.org/; http://flybase.org/). In addition to *C. pomonella* TRP sequences, Lepidoptera TRP sequences were searched in two other species (the silk moth *B. mori* L. and the monarch butterfly *D. plexippus* L.) using NCBI-blast

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the amino acid sequences of *D. melanogaster* TRPs as a query. The partial amino acid sequence of a *C. pomonella* TRP-subunit, previously identified by BAC-FISH mapping on the Z-chromosome and reported to be the CpNan TRPV-candidate (Nguyen et al., 2013) was also included in the dataset. The 121 amino acid sequences were aligned using MUSCLE (Edgar, 2004), and the neighbor-joining tree was built using the BioNJ algorithm with Poisson correction of distances, as implemented in SeaView v.4 (Gouy et al., 2010). Node support was assessed using a bootstrap procedure based on 1000 replicates. Figure 1 was created using the iTOL web server (Letunic & Bork, 2007).

Rapid Amplification of cDNA Ends (RACE) PCR of CpPyr-l

To extend *CpPyr-l* by RACE-PCR in 5' and in 3' direction, 5' and 3' cDNAs were created from antennal RNA using First Choice RLM-RACE kit (Ambion, Life technologies, Grand Island, NY USA) and SMARTer kit (Clontech, Mountain View, CA, USA). Amplifications were conducted according with the recommended protocols. Primers were designed by hand using existing contig data as reference. Thermodynamic features were checked by Oligoevaluator (Sygma Genosys, http://www.oligoevaluator.com), and putative oligodimerization was checked by oligo analyzer (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx). Primer melting temperatures were estimated using the salt-adjusted algorithm on the Oligocalc website (ww.basic.northwestern.edu/biotools/OligoCalc.html). For primers, the goal was a GC% 40-60, Tm < 70 °C, and to create a product with at least 150 bases of overlap with existing contig data. However, in some cases, it was necessary to compromise on one or several of these conditions (Table 1).

Table 1 Sequences and estimated Tm for primers.

Primers	**Sequence**	**Tm (°C)**
RACE primers (5' or 3')		
5'_CpPyr-l_1	AGCGGAACTGGATCATGAAG	64.3
5'_CpPyr-l_2	GAGATGGTGATGGCTGCAGGAAGGAGGG	65.0
3'_CpPyr-l-1	CAGGAAAACCAAGATGGAGGCACG	66.9
3'_CpPyr-l-2	GAGACGCCATTTTAGACAAAGCTCAAGCTC	63.5
CDS extension primers (Fw or Rv)		
Fw_CpPyr-l	*att*B1-ATGGCAGCTTTATCAGGCGGCG	65.8
Rv_CpPyr-l	*att*B2-TTATTTACTTAACTTACTTTCTAATCTTAACAA	61.4
Sequencing primers (Fw or Rv)		
M13 Fw	GTAAAACGACGGCCAGT	52.4
M13 Rv	CAGGAAACAGCTATGACC	53.8
Seq1-Fw	ATGATGGAGAGACTCCAATCCATTC	64.1
Seq2-Fw	ATGGGCTGGTTCCCTTTACATACAG	65.8
Seq3-Fw	TGCTGGCATGGTTAGAGATG	58.4

Starting from RLM-RACE 5'-cDNA, 5' *CpPyr-l* was extended using the 5'_CpPyr-l_1 gene-specific primer together with the 5' RACE Outer primer, supplied with the kit. Amplification was performed with the

supplied thermostable DNA polymerase using a temperature program of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, Tm of the primer for 30 sec, 72 °C for 3 min, and a final elongation of 72 °C for 7 min. An aliquot of 1.0 µL of the reaction mix was used as template to perform the nested amplification using the 5'_CpPyr-l_2 gene specific primer together with 5' RACE Inner primer, supplied with the kit.
Starting from SMARTer-RACE 3'-cDNA, PCR amplification of 3'*CpPyr-l* was performed using the 3'_CpPyr-l-1 primer combined with the Universal primer A mix supplied with the kit. Amplification was performed with Advantage 2 polymerase (Clontech) using a temperature program of 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 sec, Tm of gene specific primers for 1 min, 68 °C for 90 sec, and a final elongation of 68 °C for 7 min. To perform the nested amplification, the 3'_CpPyr-l-2 gene-specific primer was combined with the Nested Universal Primer A, also supplied with the kit.
PCR products were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized using a Gel Doc XR (Bio-Rad, Hercules, CA, USA). Relevant bands were excised and purified using the QIAquick Gel extraction kit (Qiagen). Quantification was conducted using a Nanodrop 3300 Fluorospectrometer (Thermo Scientific) with the PicoGreen® dsDNA reagent kit (Molecular Probes, Life Technologies). Samples were sequenced (Sanger sequencer, 3730xl Applied Biosystems, Life Technologies) using gene specific primers. Alignment of amplicon sequences from RACE-PCR amplifications was performed using Multalin (Corpet, 1988). The 5' and 3' sequenced regions were assembled with existing contig data to generate a partial CDS-template of 1776 bp. Despite being partial, this sequence was checked using the online tool ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi).

Querying genome and transcriptome assemblies.
In order to identify the full-length CDS of the *CpPyr-l* TRP, we used Blast on preliminary assemblies of a whole shotgun sequenced genome and an Illumina sequenced transcriptome (which are still under analysis and will be published elsewhere). We blasted the assemblies using the 1776 bp *CpPyr-l* TRP from RACE-PCR as query in tblastn searches. Scaffolds that passed a threshold of e-30 were mapped against the query and manually assembled by hand using BioEdit into a single scaffold that contained the putative full length CDS. The six genome scaffolds and the four transcripts that matched our RACE-PCR template are available for download and inspection at https://www.researchgate.net/profile/Omar_Rota-Stabelli. Sequencing and preliminary assembly of the *CpPyr-l* genomic locus and comparison with antennal RNA-seq returned an overview of intron/exon boundaries within the *CpPyr-l* locus. The final CDS provided by the locus was checked using the online tool ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Identification of candidate CpPyr-l splice forms
Total RNAs extracted from male and female antennae were converted to cDNA using the RT-for-PCR kit (Clontech). The full length CDS was amplified with Fw_CpPyr-l and Rv_CpPyr-l, and *att*B regions were attached (*att*B1 forward region: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACA-3'; *att*B2 reverse region: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3', Gateway Technology, Invitrogen, Life technologies), suitable for cloning into pDONR221. Amplification was performed with Phusion (New England Biolabs, Ipswich, MA, USA) using a temperature program of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 58 °C for 15 sec, 68 °C for 3 min and 10 sec, and a final elongation step of 68 °C for 4 min. A 4.0 µL PCR volume was mixed with 1.0 µL BP-clonase (Gateway Technology, Invitrogen)

and 150 ng pDONR221, to be incubated 4 hours at 25 °C. A 2.0 µL volume of the reaction was used to transform TOP10 competent cells, 50 µL of which were plated on 50 µg/mL Kanamycin selective media and incubated overnight.

Colonies were screened by picking individual colonies from plates and dissolving it in 50 µL of LB-medium, which was incubated for 2 hours at 37 °C and 225 rpm. PCR were conducted using 1.0 µL of this culture using the Fw_CpPyr-l and Rv_CpPyr-l primers and amplifying with GoTaq Green Master Mix (Promega, Fitchburg, WI, USA). Amplifications were conducted with a temperature program of 95 °C for 15 minutes, followed by 35 cycles of 95 °C for 30 sec, 55 °C for 15 sec, 72 °C for 3 min 10 sec, and a final elongation step of 72 °C for 4 min. Samples were analyzed as described above. Cultures giving clear bands were grown at 37 °C and 225 rpm overnight in selective LB media with 50 µg/mL Kanamycin, after which plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen). Quantification was conducted using Nanodrop 3300 Fluorospectrometer with PicoGreen® dsDNA reagent kit. Samples were sequenced using Sanger sequencer and universal M13-primers, as well as Fw_CpPyr-l, Rv_CpPyr-l, and other primers designed on the CDS (Table 1).

Alternative splice forms were verified by reverse-transcription (RT) PCR on cDNA samples from insect body parts, followed by sequencing of amplified bands. Positions of intron/exon boundaries within the *CpPyr-l* locus were compared with splicing site positions of verified splice forms. Graphical intron/exon representation of splice forms was done using the online tool Exon-Intron Graphic Maker (http://wormweb.org/exonintron).

Reverse Transcription (RT)-PCR

To investigate transcripts of *CpPyr-l* and its splice forms, RT-PCRs were performed on cDNA samples prepared from antenna, thorax, abdomen, leg and wing total RNAs using the RT-for-PCR kit (Clontech). Amplifications were performed using the GoTaq Green Master Mix (Promega), splice form-specific forward primers designed based on Sanger sequencing data of pDONR221 clones (Table 2) and the reverse primer Rv_CpPyr-l, previously used to amplify the final assembled CDS (Table 1).

Table 2 List of forward primers designed to verify the existence of candidate *CpPyr-l* splice forms.

Splice forms	**Forward primers**	**Tm (°C)**	**RT-PCR verified splice forms**
CpPyr-l_M4	TAGACTTGCAAAACAATTTG	50.2	*
CpPyr-l_M17	AAGTTTGGCTCCATCGGCC	59.5	*
CpPyr-l_M41	ACTGACGGCCCTAAGAAATTC	59.5	
CpPyr-l_M43	CTCGTATTGATTCAGGAAAAC	54.4	*
CpPyr-l_M415	TGGAAGAAGTTTTAGACTTGC	55.4	
CpPyr-l_M417	TTTATCTACGTTTGTGGCGTT	55.4	
CpPyr-l_M418	TAGTTTTAGGTACCTATAAGC	53.0	*
CpPyr-l_F1111	TTAGTTGAGAGTTTCCTAACT	53.4	
CpPyr-l_F1115	TTGTTGCTAAAAGATGGCGCC	59.5	
CpPyr-l_F1117	TTTTACACTATTATAGCCATT	49.0	*
CpPyr-l_F1124	TGAATACTTGGAAGAAGTTTA	51.7	

Except for CpPyr-l_M17 and CpPyr-l_M418 cases, all forward primers were designed to overlap splicing sites, the position of which were identified by sequencing pDONR221-clones and comparing these to the sequence of the final assembled CDS. For the final assembled CDS form, parallel amplifications were conducted using the Fw_CpPyr-l and Rv_CpPyr-l primers. Positive control of cDNA synthesis consisted of amplification of the housekeeping gene *rpl8* (rpl8_Fw: 5'-GAGTCATCCGAGCTCARMGNAARGG-3'; rpl8_Rv: CCAGCAGTTTCGCTTNACYTTRTA; Tm = 54°C). A temperature program with an initial 5-min step at 95 °C, and then 45 cycles of 95 °C for 1 min, primer melting temperature for 1 min, 72 °C for 3 min 10 sec, and a final 7-min step at 72 °C was used. Each PCR reaction was repeated at least three times and controls consisted of no template PCRs. All PCRs were performed in parallel on a genomic DNA (gDNA) template. No amplification or amplifications of larger size products were observed in most cases, revealing that no significant gDNA contamination occurred in our cDNA preparations. Amplifications were analyzed on a 1.5% agarose gel, stained with ethidium bromide, and visualized using a Gel Doc XR (Bio-Rad). Product identity was confirmed by direct sequencing (Sanger) following gel extraction (QIAquick Gel Extraction Kit, Qiagen). RT-PCR from male and female body parts cDNAs was compared to verify the presence of candidate splice forms.

For the other TRP candidate RT-PCRs, primers were designed based on partial 454-contigs (Table S2). In addition to *rpl8*, the codling moth olfactory co-receptor (*CpOrco*, Bengtsson et al., 2012) was used as an antenna positive control. RT-PCRs were conducted as above, except for minor adjustments of the temperature settings, using a 72 °C extension for 1 min 45 sec. To verify the identity of the amplicons, bands were gel-purified and Sanger sequenced using gene specific primers.

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Chapter III

Activation of the human Transient Receptor Potential cation channel TRPA1 to somatosensory compounds from the Asian food plant *Perilla frutescens*

Activation of the human Transient Receptor Potential cation channel TRPA1 to somatosensory compounds from the Asian food plant *Perilla frutescens*

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Abstract

Several compounds from food plants and spices are very well known for their somatosensory properties, targeting the chemestetic receptor TRPA1. TRPA1 activation is normally involved in sensing of taste and smell, but also of cold, nociception, and irritation mechanisms, to demonstrate properties of TRPA1-active compounds for potential applications as flavours or drugs. Performing expression in HEK293T cells we studied human TRPA1 channel activation to compounds of the Asian food plant *Perilla frutescens* (L.) and their synthetic derivatives previously demonstrated to activate the rat-orthologous. We reported some *P. frutescens* compounds being partial agonists of the channel [EC50 (μM): PK-16 (107.66) > PA (160.47) > ASA (210.92) > PK (349.92)] while others are potential competitive antagonists (PK vs IK). Our findings provide important insight into the functional properties of natural ligands from *P. frutescent* for agrifood applications and open new frontiers for possible design of new drugs from their synthetic derivatives.

Highlights

- *Compounds derived from the food plant Perilla frutescens mediate human TRPA1 channel activity*
- *The compounds appear to be partial agonists of the channel*
- *The potency sequence (EC50, μM) is PK-16(107.66) > PA(160.47) > ASA(210.92) > PK(349.92)*
- *Comparison with last findings revealed different activation modalities for rat TRPA1 to these compounds and no inhibition for human TRPA1 between these compounds and allyl-isothiocyanates*
- *PK and IK Compounds from the P. frutescens PKIK-type variety are potential competitive antagonists on human TRPA1*

Keywords

Transient Receptor Potential, TRPA1 ion channel, Human Embryonic Kidney 293T cells, Calcium imaging, *Perilla frutescens*, somatosensory compounds

Introduction

Transient Receptor Potential (TRP) is an ancient family of transmembrane proteins, working as cation channels of the plasma membranes of sensory neurons and of single cells of many eukaryotic organisms. In mammals, 28 TRPs have been identified (Clapham, 2003; IUPHAR/BPS Guide to PHARMACOLOGY - http://www.guidetopharmacology.org/) and they are distinguished from other chemosensory molecules because of their associations to multiple sensing modalities.

Mammalian TRPs are divided into six subfamilies, among which, TRPA, TRPV and TRPM have been reported to respond to physical stimuli but also chemical stimuli, such as several compounds from food plants and spices (Liedtke and Heller, 2007). For example, TRPV1 (sensitive to high temperature) and TRPM8 (sensitive to low temperature) were also demonstrated to interact with compounds associated with high or low temperature perception, such as capsaicin from chilly pepper (Caterina et al., 1997) and menthol from peppermint (Bautista et al., 2007) respectively. Among TRPs, the mammalian TRPA1 channel was demonstrated to be involved in noxious cold-sensing (Bandell et al. 2004) and further identified to be the somatosensory receptor of mustard oil derived from *Sinapis* ssp. (Brassicales: Brassicaceae) (allyl-isothiocyanate) and phytocannabinoids from *Cannabis sativa* L. (Rosales: Cannabaceae) (tetra-hydro-cannabinol) (Jordt et al., 2004).

Like mustard oil, several more ligands exist in food plants, spices and food products derived from plants and are reported to activate TRPA1 receptors across species and phyla. For instance, allycil and diallyl disulfide from garlic (Bandell et al., 2004), cinnamaldehyde from cinnamon (Hinman et al., 2006), resveratrol from red wine (Yu et al., 2013) and several more natural ligands from the essential oil content of common plants or from food (Nilius and Flockerzi, 2014) trigger Ca^{++} movement in cell-based systems where TRPA1s are heterologously expressed. The mammalian TRPA1 channel is abundantly expressed in the somatosensory system, including trigeminal neurons (Story et al., 2003). Compounds active on these receptors induce action potential on trigeminal nerves to elicite the so-called somatosensory sensation: a combination of chemosensory and physical perceptions (Tominaga et al., 1998).

Among food plants, the Asian *Perilla frutescens* L. (Lamiales: Lamiaceae), known as *kaennip* in Korea and as *shiso* in Japan, is commonly used in traditional Eastern cusine. Despite its wide use as food, properties of *P. frutescens* has not been investigated exhaustively. Application of extracts of this plant are described in traditional Chinese medicine, for the treatment of atopic dermatitis and for other anti-inflammatory and anti-allergic properties (Yu et al., 1997) aiming the current renewed economic importance and cultivation interests for this species among its different varieties (Ito, 2008). Varieties of *P. frutescens* are characterized by their chemical composition, and are referred to as chemotypes. Recently, monoterpens emitted by different chemotypes of this plant, like S-(-)-1,8-*p*-menthadiene-7-al (perillaldehyde) from PA-type varieties and 3-(4-methyl-1-oxopentyl)furan (perillaketone) from PK-type varieties, were reported to activate rat TRPA1 (rTRPA1), and slight inhibit rTRPM8 (Bassoli et al., 2009). Further experiments revealed that synthetic derivatives of PK appear to be more potent than their natural variant (Bassoli et al., 2013).

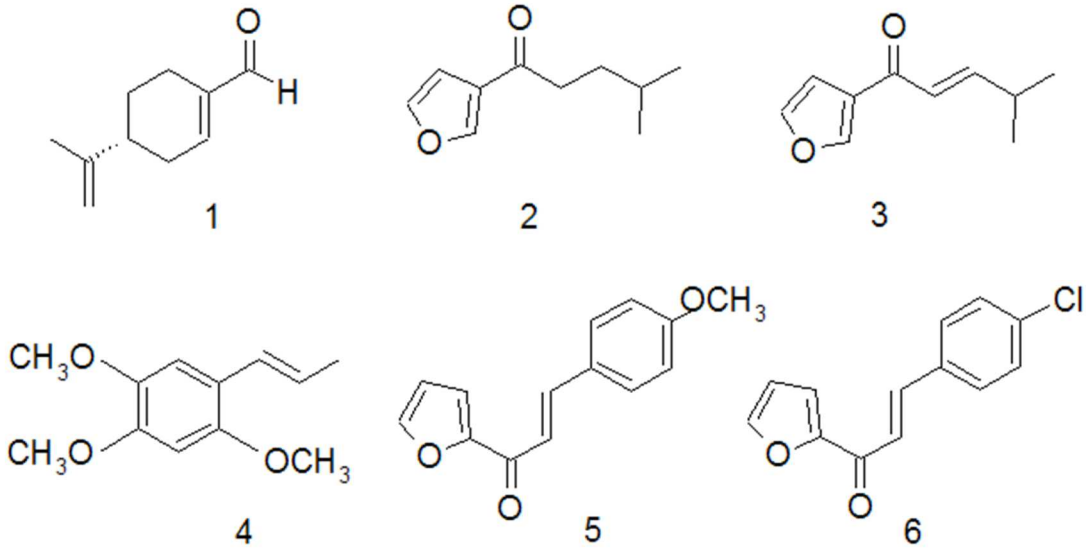
Here we show that human TRPA1 channel orthologue (hTRPA1) is sensitive to *Perilla* derived compounds. Among chemotypes of *P. frutescens*, PA and PK-types activated the receptor. Further tests,

demonstrated activation of the receptor also to the phenylpropanoid 1,2,4-trimethoxy-5-[(E)-prop-1-enyl]benzene, (α-asarone, ASA, Wang et al., 2014) identified as an additional somatosensory compound of the essential oil content of *Perilla* (Bassoli and Borgonovo, unpublished). Testing another variety, containing various amounts of 3-(4-methyl-1-oxo-2-pentenyl)furan (isoegomaketone, IK) together with PK, kwon as PKIK-type (Nitta et al., 2006), we observed possible competitive antagonism between PK and IK compounds. Further experiments on rTRPA1, recently demonstrated synthetic derivatives from PK to be even more potent than their natural variant and being strong antagonists of main ligands from mustard oil (Bassoli et al., 2013). To validate activation of the human orthologue, among synthetic derivatives we tested 3-(4-Methoxy-phenyl)-1-furan-2-yl-propenone (PK-16) and 3-(4-Chloro-phenyl)-1-furan-2-yl-propenone (PK-18), reported to be strong agonists of rTRPA1. The compounds tested appear to be partial agonists of the channel with the potency sequence of EC50 (μM) PK-16 (107.66) > PA (160.47) > ASA (210.92) > PK (349.92). Our study shed lights on human TRPA1 activation modalities for *P. frutescens* natural ligands, which further demonstrates their somatosensory properties. Activation of the human TRPA1 to synthetic derivatives of *P. frutescens* compounds supports possible design of innovative drugs from these natural ligands with the aim to target somatosensory TRP channels.

Results

Many of the HEK293T cells transfected with hTRPA1 generated transient calcium signal upon application of MO. The amplitude of the responses depended on MO concentration and overall sensitivity of the cells to the agonist was well correlated with BFP expression suggesting functional expression of hTRPA1 channel. The parameters of concentration dependence for the MO were estimated to be MO_{EC50} = 85.25 ± 14.99 μM with Hill coefficient 2.90 ± 1.30 μM and were consistent with previous estimates (nan]i[on, application note). Having confirmed functional expression of hTRPA1 channels we then screened a panel of natural compounds from *P. frutescens* (PA, PK and ASA) including two synthetic derivatives previously suggested as rTRPA1 channel modulators (PK-16, PK-18) (Figure 1).

Figure 1 Structures of compounds derived from *P. frutescens*. **1**, S-(-)-1,8-*p*-menthadiene-7-al (perillaldehyde, PA); **2**, 3-(4-methyl-1-oxopentyl)furan (perillaketone, PK); **3**, 3-(4-methyl-1-oxo-2-pentenyl)furan (isoegomektone, IK); **4**, 1,2,4-trimethoxy-5-[(E)-prop-1-enyl]benzene (α-asarone, ASA); **5**, 3-(4-Methoxy-phenyl)-1-furan-2-yl-propenone (synthetic derivative, PK-16); **6**, 3-(4-Chloro-phenyl)-1-furan-2-yl-propenone (synthetic derivative, PK-18).

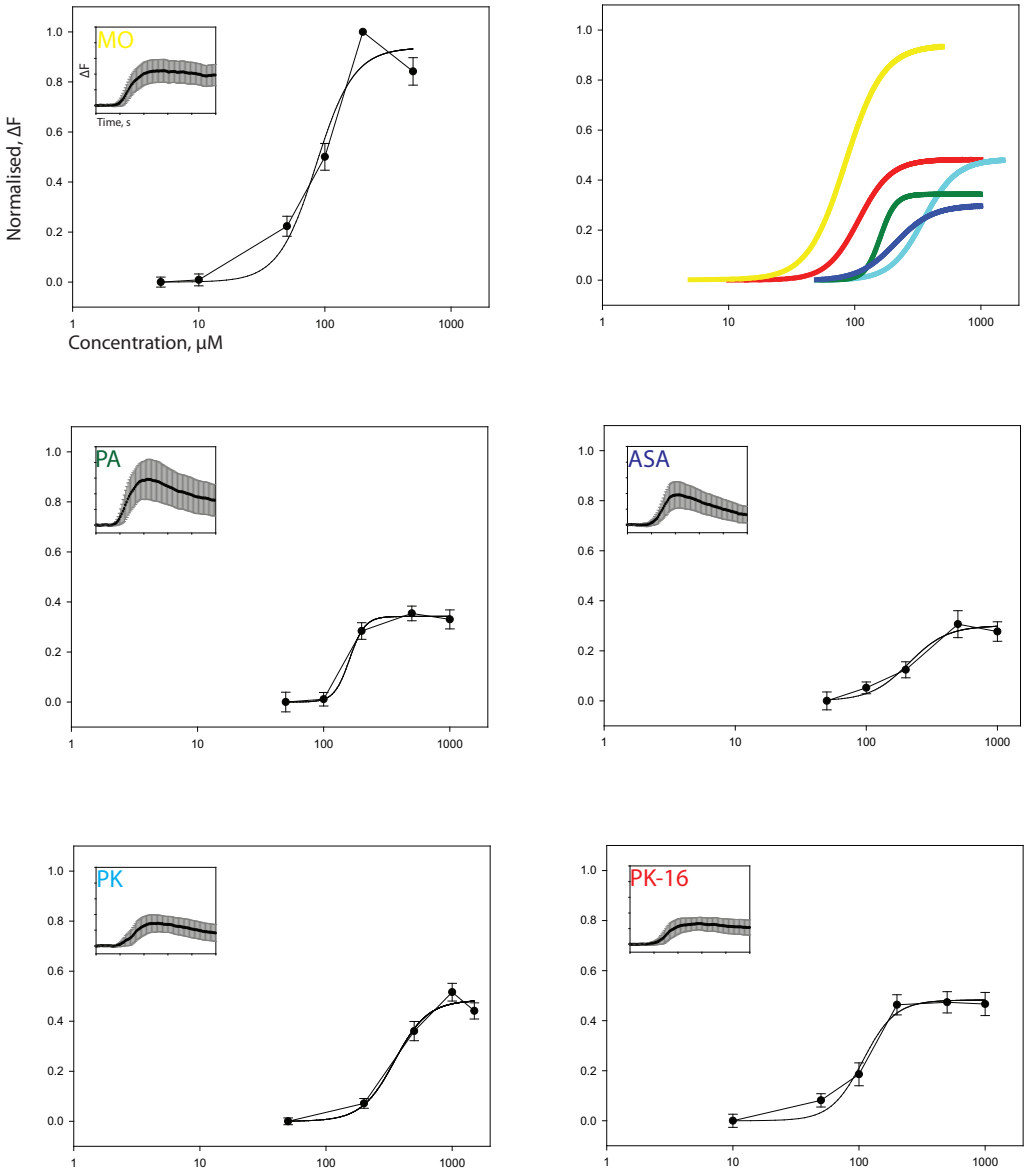


The responses to MO at close to saturating concentration (200 µM) were used for normalization and comparison between agonists tested in further experiments.

Main compounds of *P. frutescens* essential oils (PA, PK, and ASA) and the PK synthetic derivative PK-16 were able to activate hTRPA1 channel mediated calcium responses.

However, all compounds evoked substantially smaller calcium responses in comparison to MO (saturating concentrations of agonists, Figure 2). The dose response relationship of the hTRPA1 channel response to PA yielded an EC50 of 160.47 ± 9.12 µM with a normalized to MO peak amplitude of 0.33 ± 0.04 (n = 76 – 164). Similarly, PK activated the hTRPA1 channel dependent calcium response much less robustly than MO, with a normalized amplitude of 0.44 ± 0.03. The concentration-dependence yielded an EC50 of 349.92 ± 53.01 µM (n = 78 – 151). Similarly, concentration dependences for ASA and PK-16 yielded EC50s of 210.92 ± 36.43 µM and 107.66 ± 10.71 µM and normalized amplitudes of 0.28 ± 0.04 (n = 52 – 139) and 0.47 ± 0.05 (n = 16 – 114), respectively.

Figure 2. Effects of some *Perilla* derived compounds on human TRPA1 channel activity. The HEK cells expressing hTRPA1 channels generate calcium signal in response to application of the compounds (insets). The effects were concentration dependent and reversible. Graphs show the concentration dependences of the compounds expressed as a function of normalized mean peak fluorescence intensity change (ΔF) versus drug concentration. In all cases, the responses were individually normalized to the saturating concentration of MO (200 µM). Error bars represent standard error of mean. Data were fit with the Hill equation (solid lines). Insets represent average calcium response elicited by a drug application obtained in a single experiment. Grey color depicts standard deviation. Top right panel, summary plot of the concentration dependences.



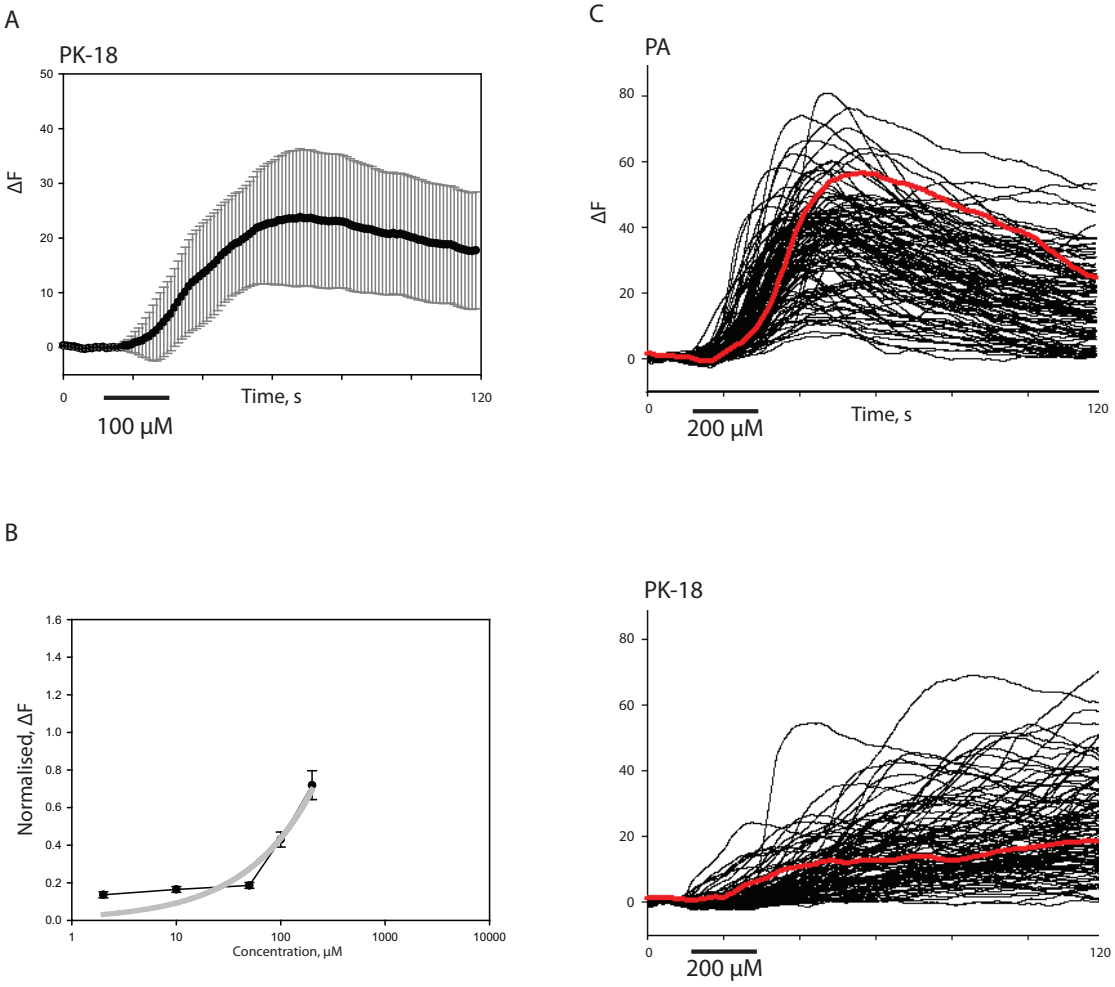
Overall, the potency sequence of the compounds was PK-16>PA>ASA>PK with the synthetic derivative PK-16 and its natural analog PK being the most and the least potent ligands respectively (Table 1).

Table 1 Comparison of potencies (EC50) calculated for *P. frutescens* samples between human and rat TRPA1, according with our findings and Bassoli et al. (2013). MO: mustard oil, as reference.

Sample	**Human TRPA1 (μM)**	**Rat TRPA1 (μM)**
MO	85.25 ± 14.99	2.5 ± 0.7
PA	160.47 ± 9.12	40.7 ± 7.63
PK	349.92 ± 53.01	21.9 ± 1.93
PK-16	107.66 ± 10.71	20.9 ± 2.27
ASA	210.92 ± 36.43	Inactive

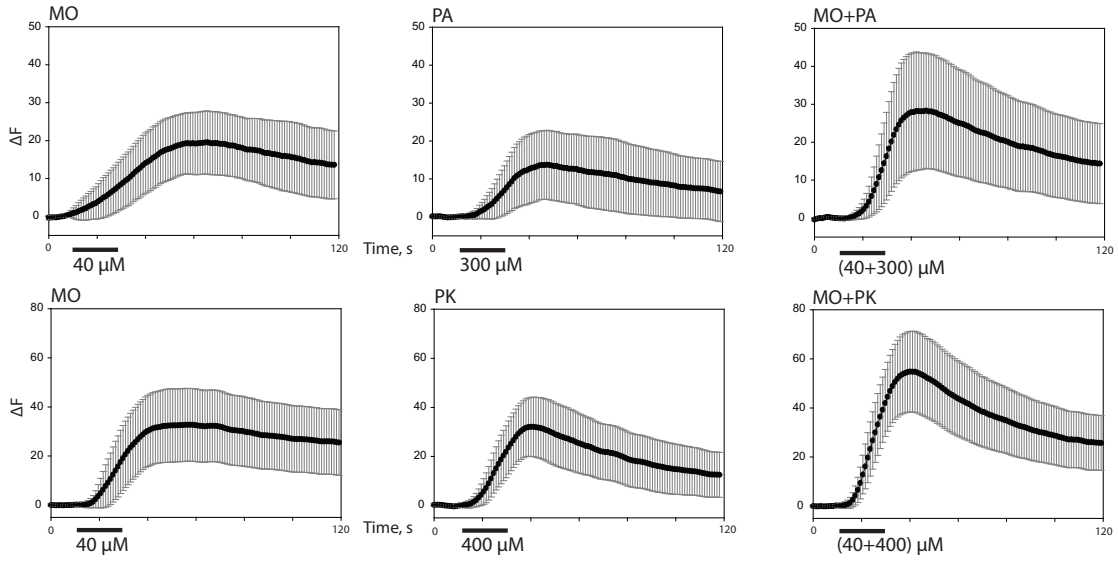
Unlike other agonists, the responses to the synthetic PK-derivative, PK-18, were relatively slow, incoherent and mostly irreversible (Figure 3). This property as well as low solubility of PK-18 [1.2 E-4 mol/L, LogP = 3.48; Scifinder, 2015; Chemical Abstracts Service: Columbus, OH, 2015; CAS Registry Number 111042-59-2 (accessed Nov 12, 2015); calculated using ACD/Labs software, version 11.02; ACD/Labs 1994-2015] made the compound impractical in terms of obtaining experimental data and interpretation.

Figure 3. Effects of PK-18 on calcium signal mediated by hTRPA1 expressed in HEK cells. A, Typical average reponse generated by PK-18 (100 µM, n = 83). Grey bars depict standard deviation. B, PK-18 concentration dependence. Limited by a solubility threshold of the drug [1.2 E-4 mol/L (Scifinder, 2015; Chemical Abstracts Service: Columbus, OH, 2015; CAS Registry Number 111042-59-2 (accessed Nov 12, 2015); calculated using ACD/Labs software, version 11.02; ACD/Labs 1994-2015)]. Data represent normalized mean ± SEM. C, Comparison of the calcium responses of the same set of cells (n = 104) to two different *Perilla* compounds PA (top panel) and PK-18 (bottom panel). Note: reponses to PK-18 are slow and incoherent. Horizontal black bars below traces mark timing of the drug pulse application (20s); red track within traces mark the simple straight line (SigmaPlot 11).



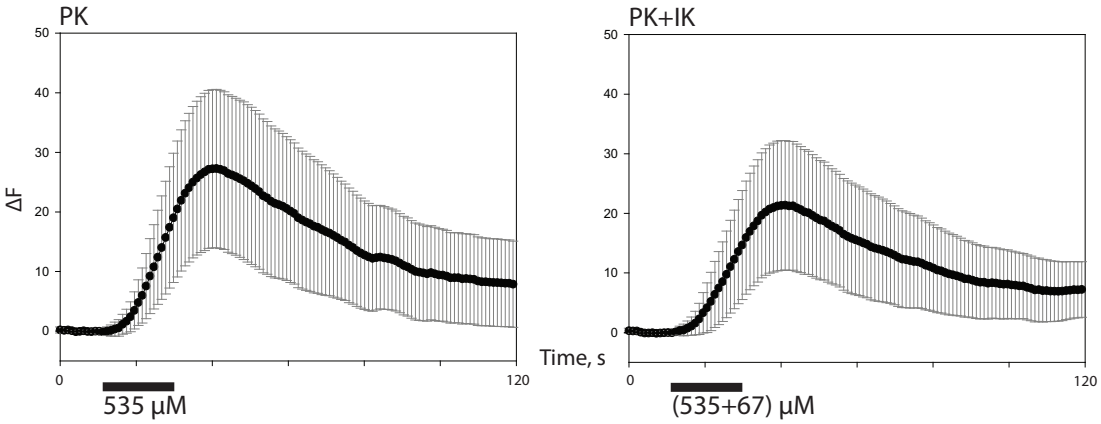
The relatively low efficacy of the compounds tested may suggest that they are partial agonists of the hTRPA1 channel. To probe whether these compounds could promote the hTRPA1 channel desensitisation (Bassoli et al., 2013) and/or inhibit the channel activity somewhat competing with MO, we used following experimental paradigm. The hTRPA1 channel mediated calcium signal was initially activated by MO (40 µM), then by high responsive concentration proximal to saturation of an agonist (PA or PK), followed by combined application of MO and an agonist (PA or PK). Each compound was tested in the individual series of experiments. Amplitudes of the calcium responses were mean ± SEM (Figure 4). In both experimental sets, the combination of ligands (MO + PA or PK) generated greatest responses providing lack of antagonistic effect.

Figure 4. Effects of PA and PK on MO activated hTRPA1 channel. hTRPA1 channel mediated calcium signal was initially activated by MO (40 µM, left panels), then by high responsive doses of an agonist (PA: 300 µM, n = 111, or PK: 400 µM, n = 86, middle panels) followed by combined application of MO and an agonist (PA or PK, right panels). Amplitudes of the calcium responses were (mean ± SEM): MO, 40 µM 19.48 ± 8.30; PA, 300 µM 13.73 ± 9.10; MO, 40 µM + PA, 300 µM 28.33 ± 15.22; MO, 40 µM 32.73 ± 14.82; PK, 400 µM 32.08 ± 12.12; MO, 40 µM + PK, 400 µM 54.82 ± 16.41. Note: in both experimental sets the combination of ligands (MO + PA or PK) generated greatest responses. Horizontal black bars below traces mark timing of the drug pulse application (20s).



Comparing responses of hTRPA1 elicited by PKIK-type sample (535 µM PK and 67 µM IK) with PK-type sample (535 µM PK), ~ 22% mean ± SEM decreasement of the response elicited by PKIK was observed (Figure 5). Single cell normalized responses analyzed by T-test gave significant difference between the two samples (P = 1.13 E-05; t = -4.49; dof = 83).

Figure 5. Effects of PK and PKIK on hTRPA1 channel. The hTRPA1 channel mediated calcium signal was initially activated by PK (535 µM, n = 84, left panel), then by PKIK dose containing the same PK amount (535 µM PK + 67 µM IK, right panel) Amplitudes of the calcium responses were (mean ± SEM) PK, 535 µM 27.27 ± 13.30; PK, 535 µM + IK, 67 µM 21.36 ± 10.86. Horizontal black bars below traces mark timing of the drug pulse application (20s).



Discussion

Apart from the main natural ligands of mammalian TRPA1s (Jordt et al., 2004; Macpherson et al., 2007), novel molecules from additional food plants and spices were identified (Hinman et al., 2006; Xu et al., 2006; Hata et al., 2012; Yu et al., 2013; Nilius and Flockerzi, 2014) unveiling properties of their food sources for innovative applications in agrifood and pharmaceutical industries (Vriens et al., 2008; Eid et al., 2008; Holzer, 2011). Indeed, apart from taste (Roper, 2014) and olfaction (Zufall, 2014), the role of TRPs is mostly documented in chemestetic modalities associated with thermal sensing (Bautista et al., 2007; Caterina et al., 1997), nociception (Bandell et al., 2004; Story et al., 2003; Tominaga et al., 1998), irritation (Bessac and Jordt, 2008) and inflammation mechanisms (Trevisani et al., 2007). This inspired our efforts for the identification of novel somatosensory molecules for agrifood applications (Bassoli et al., 2009) and possible development of innovative drugs derived from chemical modifications of natural ligands of TRPA1 (Bassoli et al., 2013).

Activation of hTRPA1

A number of natural compounds from the Asian food plant *P. frutescens* and a synthetic derivative can mediate the activity of human TRPA1 channel. The potency sequence of the compounds, PK-16>PA>ASA>PK, was different as compared with the effects of the same compounds on rat TRPA1 channel activity (Table 1). Specifically, rTRPA1 is almost equally susceptible to PK-16 and PK, while hTRPA1 is more sensitive to PK-16 than PK.

Our earlier findings (Bassoli et al., 2013) also suggest that some *Perilla* derived compounds could promote the rTRPA1 channel desensitisation and/or inhibit the channel activity possibly competing with MO. Here we show that potentially the strongest antagonists, PA and PK, did not reduce calcium signal activated by MO. Instead, the hTRPA1 channel mediated calcium signal was proportionally augmented in the presence of saturating concentrations of these coumpounds.

Significant differences observed could be explained by both different experimental approaches used in the studies and, most likely, by species-specific heterogeneity in pharmacological properties of the rat and human versions of the channels. Yet further analysis of structure/function correlation of these TRPA channel orthologues is necessary for identifying critical functional domains/residues determining the biophysical and pharmacological heterogeneity.

Another compound that we identified as a partial agonist of hTRPA1 is α-asarone (ASA, ASA_{EC50} = 210.9 ± 36.4 μM); interestingly, this compound is not active on the rat orthologue (Table 1). ASA is a phenylpropainoid normally present in trans- and beta-types in essential oils from ginger species within *Asarum* (Piperales: Aristolochioaceae) and it was also found in *Perilla* (Bassoli and Borgonuovo, unpublished data).

Agonists of TRPA1 channel are usually divided into two main categories based on their mechanism of activation: electrophiles and non-electrophyles. Electrophiles such as allyl-isothiocyanates (Jordt et al., 2004) and α-β unsaturated aldehydes (Macpherson et al., 2007; Trevisani et al., 2007), activate TRPA1 channels by covalent modifications, condensing α-β-unsaturated bonds with nucleophilic mercapto-groups of cysteine residues of the receptor in a Michael addition. Non-electrophiles, such as carvacrol from oregano (Xu et al., 2006), activate TRPA1 channels by interacting with the channel without covalent modifications. According to molecular features of ligands tested here (Figure 1), PA does not have a ketone group and ASA lacks of α-β-unsaturated bonds suggesting that both compounds may be involved in non-covalent interaction with the channel rather than in covalent channel modifications on N-terminal cysteines mediated by Michael addition, as hypothesized for the electrophilic carbonyl of PK (Bassoli et al., 2009, 2013).

Different potencies characterizing different compounds from *P. frutescens* chemotypes and possible differences in their interaction modes with the TRPA1 channels suggest the importance of chemotype mapping of this plant (Nitta et al., 2006), which have always been at the base of different use of its varieties in food (Ito et al., 2008) and medicine (Yu et al., 1997).

Despite the common use of *P. frutescens* in Asian cousine, aversive properites are reported for its main monoterpenes. PK is known since long ago to induce lung toxicity in mammals like horses (Breeze et al., 1984) and sheeps (Abernathy et al., 1992) and despite being deposited as a sweetner (Maire and Piggot, 1991) PA was recently reported to have a degree of allergenicity and it is currently restricted by IFRA (Tisserand and Young, 2014; http://www.ifraorg.org/). On the other hand, inhibitory activities on lung inflammation were recently demonstrated for PK and PA together with phenylpropanoids of *P. frutescens* (allyltetramethoxybenzene, caffeic acid, dillapiole, elemicin, myristicin, nothoapiole, rosmarinic acid and methyl ester) (Lim et al., 2014). While the molecular target/s for these compounds have not been identified yet, there are evidences implicating TRP channels including TRPA1 in physiological and pathophysiological reactions associated with irritation, respiratory depression and neurogenic lung inflammation (Trevisani et al., 2007; Bessac and Jordt, 2008). Whether a possible role of *P. frutescens* compounds in activation or inhibition of inflammation mechanisms in mammals is still controversial, verified activation of hTRPA1 to natural ligands from this plant like the phenylpropanoid α-asarone and monoterpens PK and PA may contribute for a better understanding of this molecular interaction in inflammation mechanisms. Thus, our findings may provide a useful insight into the role

of TRPA1 channels and their potential agonists in irritation process/es in general and pulmonary inflammation in particular.

Competitive antagonism between compounds from PKIK-type P. frutescens

Major essential oil components of the PKIK-type *P. frutescens* variety (Nitta et al., 2006) are perillaketone and various amounts of a saturated variant of PK, isoegomaketone (3-(4-methyl-1-oxo-2-pentenyl)furan; Figure 1). Isoegomaketone is a *P. frutescens* monoterpen known for antitumor properties (Cho et al., 2011). Despite its molecular targets have not been identified yet, IK was recently reported to activate rTRPA1 channels (IK_{EC50} = 7.6 ± 0.2 μM) being even more potent than *P. frutescens* derived natural ligands (Bassoli et al., 2013). In our experiments calcium signal activated by the PKIK-type variety was lower (~78%) than calcium signal activated by similar concentration of PK alone (Figure 5). While speculative in the context of current study, the lower calcium signal generated in response to PKIK may suggest that ligands, PK and IK, are competitive agonists of hTRPA1 channel.

Activation of hTRPA1 to PK synthetic derivatives

Consistent with the data published previously, the synthetic PK-derivative PK-16 is the most potent compound tested (EC50 = 107.66 ± 10.71 μM). On the other hand, responses of the synthetic PK-derivative PK-18 were relatively slow, incoherent and mostly irreversible and its low solubility made the compound impractical in terms of obtaining experimental data and interpretation. Both PK and its synthetic analogs PK-16, PK-18 have ketone group that can potentially serve as an electrophilic target for the attack of nucleophilic residues of the channel. Futher structure-function relationship analysis is necessary to understand what structural features can account for different potencies of the compounds and different kinetic parameters of their effects.

Conclusions

Testing human TRPA1, we reported two natural terpenoids (PA and PK) and a synthetic derivative from PK (PK-16) to activate the receptor. Among these compounds, performing dose/response experiments we demonstrated higher sensitivity of the receptor towards the synthetic PK-16 and the natural PK. Interestingly, we report hTRPA1 responding to a phenylpropanoid (ASA), normally synthesize by *Asarum* plants, but also identified in the essential oil content of a novel chemotype of *P. frutescens* (*var. acutifolia*).

Contrary from past findings on rat TRPA1, tests of *P. frutescens* compounds such as PA and PK on human TRPA1 reported no inhibitory activity towards the main ligand allyl-isothiocyanate. We rather suggest competitive antagonism between two monoterpens (PK and IK) emitted by a specific chemotype of this plant, the PKIK-type. Significant decreasement of PK-response when tested with IK reported, despite indirectly, possible binding of IK on the human TRPA1. The hTRPA1 activation by natural ligands from *P. frutescens* represent additional findings for the reported use of this food plant triggering multiple mechanisms of chemestetic sensations by targeting TRP-channels, according with the reported roles of these receptors in mammalian taste (Roper, 2014) and olfaction (Zufall, 2014). Together with recent findings on rat (Bassoli et al., 2013), activation of the human TRPA1 orthologue by synthetic

ligands derived from PK, being more potent than the natural ligand, make of this plant a source of molecules as potential templates for the synthesis of new drugs targeting TRP channels.

Experimental procedures

Preparations of essential oils and compounds

P. frutescens was grown at the Fondazione Minoprio (Minoprio - Vertemate (CO) Italy, 380 AMSL) during 2010. The leaves were collected between May and October and freezed, then mixed and submitted to steam distillation to obtain the essential oils with a constant average composition. Essential oils from *P. frutescens* PA-type, PK-type and PKIK-type were prepared as previously reported (Bassoli et al., 2009, Cattaneo et al., 2014). Asarone was isolated as the main component of essential oil obtained from dry leaves of *P. frutescens var. acuta* (*soyeop*) bought at the medicinal plants market in Seoul, Korea in 2008. The essential oil has an average yield of 0.25 g/100 g dry leaves. The chemical structure of this metabolite was determined by NMR and GC-MS investigations and was consistent with those described in the literature for α-asarone (Nitta et al., 2006) and with an authentic sample. For the in vitro assays a commercial sample of asarone (Sigma Aldrich, St. Louis, MO, USA, >97% GC) was used. PK derivatives 2-propen-1-one, 1-(2-furanyl)-3-(4-methoxyphenyl)-,(2*E*)- (PK-16) and 2-propen-1-one, 3-(4-chlorophenyl)-1-(2-furanyl)-,(2E)- (PK-18) were prepared according with Bassoli et al., 2013. Structures of *Perilla* derived compounds are reported in Figure 1.

Heterologous expression and transient transfection

HEK293T cells were grown in HEK cell media at 37 °C with 5% CO_2 [Dulbecco's modified Eagle's medium (DMEM) enriched with 10% heat-inactivated fetal bovine serum (MP Biomedicals, Solon, OH, USA), 2 mM L-glutamine, and 100 µg/mL Penicillin/Streptomycin (Invitrogen, Life Technologies, Grand Island, NY USA)].

For transient expression, semi-confluent HEK293T cells were grown in 35-mm dishes and transfected with pcDNA3/TO plasmid DNA (0.25 µg aliquot, Invitrogen, Carlsbad CA, USA) carrying the coding sequence of hTRPA1 (generously provided by Drs. Gunter Gisselmann and Hanns Hatt). For parallel control of the channel expression in HEK cells a separate plasmid (an aliquot of 0.67 µg, pEBFP-Nuc, Clontech) carrying the coding sequence of a blue fluorescent protein (BFP) under the regulation of the same promoter for hTRPA1 (CMV) was co-transfected. Transfection aliquots were combined in 100 µL DMEM, mixed with 3.0 µL Calfectine (SignaGen Laboratories, Rockville MD, USA) and incubated 20 minutes to be dropped on HEK cells covered by 1.0 mL of HEK cell media. After incubation in the transfection media/mix for 10-18 hours, the transfection mix was replaced with 1.0 mL fresh HEK cell media and cells were incubated for 8 additional hours. Cells were then split in 35-mm plastic Petri dishes and allowed to recover for up to 4 hours prior calcium imaging experiments. Measurements were performed within 44 to 72 hours after the beginning of the transfection protocol.

To estimate transfection efficiency, a parallel transfection was conducted using the positive control vector pcDNA5/TO/LACZ (Invitrogen) and staining with 0.1% XGal according with Leonhardt/Cardoso protocol (Leonhardt and Cardoso, 1997). LACZ transfected preparations were compared with non-transfected preparations, to validate staining for the majority of cells (Figure S1).

Calcium imaging

HEK293T cells co-transfected with pcDNA5/TO/hTRPA1 and pEBFP-Nuc DNA, were incubated for 30min-2h at room temperature in 0.5-1.0 mL HEK cell solution (mM: 140 NaCl, 5.0 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.5) including the fluorescent calcium indicator Fluo-4AM (Invitrogen) at 5.0-15 µM prepared with 0.2-0.06% Pluronic F-127 (Invitrogen). After incubation, the buffer was removed and cells were rinsed with 4.0 mL HEK Ca^{++} solution (mM: 140 NaCl, 2.0 $CaCl_2$, 10 HEPES, pH 7.5). Cells were placed on the stage of an inverted microscope (Olympus IX-71) equipped with a cooled CCD camera (ORCA R2, Hamamatsu). Two gravity fed perfusion contours were used. First contour was continuously washing cells with HEK Ca^{++} solution (~250 µL/min). Second was used for stimulation and/or application of the compounds tested. Switch between the perfusion channels and regulation of pulse duration were controlled by a multi-channel rapid solution changer (RSC-160, Bio-Logic) and Clampex 9 software (Molecular Devices). Stimulus duration was 10 seconds when cells were stimulated with mustard oil and 20 seconds when stimulated with other compounds. More complex stimulation protocols are specified in the Results.

Calcium imaging experiments were carried out under the control of Imaging Workbench 6 software (INDEC Systems). Stored time series image stacks were analyzed off-line using Imaging Workbench 6, Clampfit 10.5, SigmaPlot 11 or exported as TIFF files into ImageJ 1.42 (available from public domain at http://rsbweb.nih.gov/ij/index.html). Continuous traces of multiple responses were compensated for slow drift of the baseline fluorescence when necessary. All recordings were performed at room temperature (22-25°C).

Stimulus

Aliquots of stimulus solutions were prepared in different volumes of HEK Ca^{++} solution depending from experimental needs, diluting stock solutions of our samples previously dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich).

Mustard oil (MO, Sigma Aldrich), PK, PA, ASA, PK-16 and PK-18 samples were dissolved in DMSO in the order of millimolar (mM) to prepare stimulus in HEK Ca^{++} solution in the order of micromolar (µM). PKIK was dissolved in DMSO in the order of milligram/mL (mg/mL) to prepare stimulus in HEK Ca^{++} solution in the order of microgram/mL (µg/mL). For the latter, concentrations of PK and IK compounds were calculated according with their respective percentage content in the essential oil sample.

According to HPLC based estimations, PKIK-type essential oil from *P. frutescens* contains a mixture of PK as the major component and IK as the secondary metabolite; the relative content of the two compounds changes during growing of the plant (Bassoli and Borgonovo, unpublished). For experiments reported here, we used a mixture of samples collected during all the harvesting season. The amount of PK and IK in the sample determined by HPLC is 88% and 11% respectively. In our experiments, we used *P. frutescens* samples diluted to a final PK dose of ~535 µM (high responsive dose of PK proximal to saturation). Thus, this PK dose in PKIK-type essential oil contained ~67 µM IK.

Generation of dose/response relationships

Dose/response relationships were generated for pure compounds (ASA, PK-16, PK-18) and *P. frutescens* essential oils predominantly containing only one compound, as estimated by HPLC [PK-type (PK > 98%) and PA-type (PA > 98 %)]. Different concentration ranges were chosen for different compounds/samples, depending on compound concentration eliciting the minimal detectable response and the stimulus saturating concentration. To estimate and, if necessary, correct the system sensitivity to excitation light intensity and to the mechanical disturbance of the cells potentially caused by perfusion system, we performed a control tests before each individual experiments using HEK Ca^{++} solution without stimulus. The allyl-isothiocyanate (MO, 200 µM) was used as a positive control and as a reference for normalization the experimental data. To minimize rundown of the calcium responses especially to saturating stimuli the consecutive stimuli were applied every 45-60 min and multiple Petri dishes were used for different doses. To estimate EC50s, the normalized dose/response data were approximated by a modified Hill equation.

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Chapter IV

A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester

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A predicted sex pheromone receptor of codling moth *Cydia pomonella* detects the plant volatile pear ester

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Plant volatiles mediate host discrimination and host finding in phytophagous insects. Understanding how insects recognize these signals is a current challenge in chemical ecology research. Pear ester, ethyl (*E,Z*)-2,4-decadienoate, is a powerful, bisexual attractant of codling moth *Cydia pomonella* (Lepidoptera, Tortricidae) and strongly synergizes the male response to female-produced sex pheromone. We show here that the codling moth odorant receptor (OR) CpomOR3 is dedicated to detecting this plant volatile. Heterologous expression of CpomOR3 in *Drosophila* T1 trichoid and ab3A basiconic sensilla, followed by a screening with codling moth pheromone compounds and known plant volatile attractants, confirms that CpomOR3 binds to pear ester. Although CpomOR3 does not respond to any of the pheromone components tested, a phylogenetic analysis of lepidopteran chemosensory receptor genes reveals a close relationship of CpomOR3 with pheromone receptors (PRs) in moths. This corroborates the interaction of ecological and social chemosensory cues during premating communication. The finding that a plant volatile compound, pear ester, is a specific ligand for a PR-like lepidopteran receptor adds to our understanding of insect-plant interactions and emphasizes the interaction of natural and sexual selection during the phylogenetic divergence of insect herbivores.

Keywords: olfaction, odorant receptor, heterologous expression, semiochemical, sex pheromone, plant volatile, insect control

INTRODUCTION

Interactions between plants and insects shape many terrestrial ecosystems, and the primary mode of communication between plants and insects is chemical. Plant volatile chemicals mediate recognition of adult food sites, adequate oviposition sites and larval host plants (Bruce and Pickett, 2011) and accordingly play a prominent role in premating reproductive isolation and phylogenetic diversification of insect herbivores (Dres and Mallet, 2002; Smadja and Butlin, 2009; Matsubayashi et al., 2010). Decoding the plant volatile signatures that enable insects to discriminate between host and non-host plants is a long-standing research challenge in chemical ecology (Dethier, 1947, 1982; Ehrlich and Raven, 1964).

The identification of behaviorally active plant volatiles is a delicate and tedious task since plants release a large suite of volatiles, with no apparent correlation between the relative abundance of these compounds and their behavioral role in associated insects. Moreover, a behavioral response is frequently elicited by compound blends, where single compounds can often be exchanged with no apparent loss of activity (Bengtsson et al., 2006; Tasin et al., 2006, 2010; Pinero et al., 2008; Riffell et al., 2009; Cha et al., 2011; Schmidt-Busser et al., 2011; Thoming and Knudsen, 2014). This makes it particularly difficult to determine which plant volatiles encode host finding in phytophagous insects. In comparison, the identification of insect sex pheromones is facilitated by the production of few compounds in dedicated glands in one sex, together with a strong, distinctive behavioral response in the other.

The larvae of codling moth, *Cydia pomonella* (Lepidoptera, Tortricidae), feed on apple, pear, and walnut. The main sex pheromone compound codlemone, (*E,E*)-8,10-dodecadien-1-ol, was identified long ago (Roelofs et al., 1971; Beroza et al., 1974), but it is still open to question which compounds evoke attraction of egg-laying codling females to the plant host. Plant odorants obviously account for host attraction in codling moth, and several compounds from apple fruit and foliage elicit a strong antennal response. However, these compounds produce only a rather weak behavioral response (Bengtsson et al., 2001; Coracini et al., 2004; Hern and Dorn, 2004; Witzgall et al., 2005).

The strongest known kairomonal attractant is a pear ester, ethyl (*E,Z*)-2,4-decadienoate (Jennings et al., 1964; Berger and Drawert, 1984; Willner et al., 2013), which attracts codling moth adult males and females, as well as larvae (Knight and Light, 2001; Light et al., 2001; Light and Knight, 2005). This makes pear ester a versatile tool for sustainable insect control. It is used to monitor the seasonal abundance of codling moth (Knight and Light,

2012; Knight et al., 2013), as well as to enhance population control by mating disruption, in blends with codlemone (Knight et al., 2012). More recently, a microencapsulated formulation of pear ester has been developed for disruption of larval orientation and host finding (Light and Beck, 2012; Knight and Light, 2013).

Pear ester has been identified by screening codling moth antennal response to a wide range of apple and pear volatiles, followed by field trapping (Light et al., 2001; Light and Knight, 2005). Its biological significance is, however, not entirely clear, since it is found mainly in pear and only in some apple cultivars (Jennings et al., 1964; Berger and Drawert, 1984; Willner et al., 2013). The association of codling moth with cultivated apple is, on the other hand, recent and the response to pear ester may stem from an evolutionarily ancient host plant of codling moth.

Given the difficulties associated with completely assessing the pool of plant volatiles produced by the various host plants of codling moth, it is sensible to also investigate the response of single odorant receptors (ORs), many of which are likely dedicated to the perception of plant volatiles. ORs interface insects with their odor environment by binding odorants, and are expressed in olfactory sensory neurons (OSNs), which transmit olfactory information to the brain. The number of ORs expressed on the antenna and their compound-specificity determines the range of odorants an insect can detect. General ORs are tuned to environmental odors including plant volatiles, while pheromone receptors (PRs), a male-biased receptor clade, respond mainly to sex pheromones (Jacquin-Joly and Merlin, 2004; Ihara et al., 2013; Leal, 2013).

An emerging technique, which is quickly becoming an integral part of the toolbox for identification of behaviorally relevant plant odorants, is the functional characterization ("deorphanization") of ORs, following expression in heterologous expression systems. The OR repertoire of *Drosophila* has been studied exhaustively (Hallem et al., 2004; Kreher et al., 2005; Hallem and Carlson, 2006) and current research aims at other insect groups. For moths, a number of ORs and PRs have been identified and functionally characterized, using various heterologous expression systems, including human embryonic kidney (HEK) cells (Grosse-Wilde et al., 2007), *Xenopus* oocytes (Sakurai et al., 2004; Nakagawa et al., 2005; Jiang et al., 2014), Sf9, a cell line derived from fall armyworm *Spodoptera frugiperda* ovaries (Jordan et al., 2009), and *Drosophila* OSNs (Syed et al., 2010; Montagné et al., 2012), which is an *in vivo* antennal expression approach.

Expressing ORs in single *Drosophila* neurons comprises two main advantages. The biochemical environment of *Drosophila* OSNs endogenously provides odorant binding proteins (OBPs) and Orco, a canonical receptor conserved across insects (Krieger et al., 2003; Jones et al., 2005; Leal, 2013), which may enhance response sensitivity and specificity of the expressed OR, compared with non-insect cell lines. In addition, electrophysiological techniques, namely single sensillum recordings (SSRs) are well established for *Drosophila* sensilla.

Two main systems are available for expression and deorphanization of ORs in *Drosophila* OSNs, the "empty neuron" (ab3A) in ab3 basiconic sensilla, which lacks its native OR (Dobritsa et al., 2003) and the *Or67d*^{GAL4} knock-in mutant line in trichoid T1 sensilla (Kurtovic et al., 2007). While the empty neuron system has been used mainly to functionally characterize general odorant receptors, pheromone receptors may respond more strongly when expressed in T1 rather than in ab3A (Syed et al., 2010; Montagné et al., 2012).

We have previously identified 43 candidate OR protein sequences in the antennal transcriptome of codling moth, five of which cluster within the conserved pheromone receptor clade of lepidopteran PRs (Bengtsson et al., 2012).

We here show that CpomOR3, belonging to the PR clade, is strictly tuned to pear ester. This result emphasizes the biological significance of pear ester (Light et al., 2001) and shows that the PR clade contains co-evolving receptors for sex pheromones and for host odorants. This corroborates the modulation of male sexual behavior by host plant odorants in codling moth (Trona et al., 2010, 2013), and adds to our understanding of the evolution of sexual communication and olfaction-driven speciation in insect herbivores.

METHODS

INSECTS, DISSECTION, AND RNA EXTRACTION

Cydia pomonella pupae were obtained from a laboratory rearing center (Andermatt Biocontrol, Grossdietwil, Switzerland), and adults were allowed to emerge in cages kept at 23°C, 70 ± 5% relative humidity and a 16 h:8 h light:dark cycle, and fed with 10% sugar solution. For dissections, 2–3 day old female and male insects were used. Using sharp forceps, antennae were removed at the base of the pedicel, and legs at the coxa. For thorax samples, head, wings, legs, and abdomen were removed. Wings were removed at their base, and the abdomen removed at the connection to the thorax. All body parts were immediately flash-frozen using liquid nitrogen, and thereafter kept at −80°C. RNAs were extracted using the RNeasy kit (Qiagen, Hilden, Germany).

RAPID AMPLIFICATION OF cDNA ENDS (RACE)-PCR

RACE-PCR was performed to obtain the complete open reading frame (ORF) for *CpomOR3*. A cDNA library for extension in the 5′ direction was created using the SMARTer kit (Clontech, Mountain View, CA, USA) on male antennal RNA. For the PCR reaction, the Advantage 2 kit (Clontech) was used, with a temperature program of 95°C for 2 min, then 30 cycles of 95°C for 1 min, 65°C for 90 s, 68°C for 2 min and a final elongation of 68°C for 7 min. A gene-specific primer (5′-CCCTAGAGCTTCGGTGTCCAATGTAGAGC-3′) was used together with the Universal primer mix (Clontech). The PCR product was analyzed by electrophoresis on an agarose gel, and the relevant band excised and purified by the Gel extraction kit (Qiagen). It was then cloned into the pGEM®-T Easy plasmid (Promega, Fitchburg, WI, USA), with which TOP10 cells were transformed (Invitrogen, Life Technologies, Carlsbad, CA, USA). Plasmids were subsequently purified using the Miniprep kit (Qiagen). Purified plasmids were quantified by nanodrop (Nanodrop 8000 UV-Vis Spectrophotometer, Thermo Scientific, Wilmington, DE, USA) and then Sanger sequenced (3730xl Applied Biosystems, Life Technologies) using the forward and reverse M13 universal primers. Transmembrane domains were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/

TMHMM/), on sequence translated to protein using ExPASy (http://web.expasy.org/translate/).

PHYLOGENETIC ANALYSIS

Amino acid sequences of CpomORs clustering in the candidate PR clade (Bengtsson et al., 2012) were included in a dataset together with sequences of candidate PRs from the following Lepidoptera: *Antheraea polyphemus* (Forstner et al., 2009), *Bombyx mori* (Nakagawa et al., 2005), *Danaus plexippus* (Zhan et al., 2011), *Diaphania indica* (Mitsuno et al., 2008), *Epiphyas postvittana* (Jordan et al., 2009), *Heliconius melpomene* (Heliconius Genome Consortium, 2012), *Helicoverpa armigera* (Liu et al., 2012), *Heliothis virescens* (Grosse-Wilde et al., 2007; Wang et al., 2010), *Manduca sexta* (Grosse-Wilde et al., 2010), *Mythimna separata* (Mitsuno et al., 2008), *Ostrinia furnacalis* (Miura et al., 2010; Leary et al., 2012), *O. nubilalis* (Wanner et al., 2010; Leary et al., 2012), *O. scapulalis* (Miura et al., 2009, 2010), *Plutella xylostella* (Mitsuno et al., 2008; Sun et al., 2013), *Spodoptera exigua* (Liu et al., 2013) and *S. littoralis* (Legeai et al., 2011; Montagné et al., 2012). Sequences from *B. mori* (BmorOR6) and *H. melpomene* (HmelOR5, 6, and 7) were also included in the dataset as external groups, since they belong to the sister group to the PR clade (Poivet et al., 2013). The CpomOR1 sequence was not included in the dataset because of its short length (only 101 amino acid residues). The 74 amino acid sequences were aligned using the online version of MAFFT v.7 (Katoh and Standley, 2013), with the G-INS-i algorithm (Katoh et al., 2005) and default parameters.

Phylogenetic reconstruction was performed using the maximum likelihood method. The LG+I+G+F substitution model (Le and Gascuel, 2008) was determined as the best-fit model of protein evolution by ProtTest 2.4 (Abascal et al., 2005) following Akaike information criterion. Rate heterogeneity was set at four categories, and the gamma distribution parameter and the proportion of invariable sites were estimated from the dataset. Tree reconstruction was performed using PhyML 3.0 (Guindon et al., 2010), with both SPR (Subtree Pruning and Regrafting) and NNI (Nearest Neighbor Interchange) methods for tree topology improvement. Node support was estimated using a bootstrap procedure based on 100 replicates, and nodes supported by a bootstrap value below 70% were collapsed. The figure was created using the iTOL web server (Letunic and Bork, 2011) and Adobe Illustrator.

REVERSE TRANSCRIPTION (RT)-PCR FOR *CPOMOR3* EXPRESSION ANALYSIS

cDNAs were synthesized from RNAs extracted from different tissues using the RT-for-PCR kit (Clontech), following the recommended protocol. Integrity of cDNAs was tested by PCR, using degenerate primers for *RPL8* (Forward primer 5′-GAGTCATCCGAGCTCARMGNAARGG-3′; Reverse primer 5′-CCAGCAGTTTCGCTTNACYTTRTA-3′) and GoTaq Green Master Mix (Promega) with an annealing temperature of 54°C. PCR reactions to screen for expression of *CpomOR3* in different tissues used GoTaq Green Master Mix, and consisted of an initial 5-min step at 94°C, and then 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final 7-min step at 72°C. Gene specific primers (GSP) for *CpomOR3*, 5′-AGATGAAGAGTATCGGAATTGCATGG-3′ (forward) and 5′-CCAACTGGGATCATGCCACAAGC-3′ (reverse), were used, giving a product of 436 bp. Product identity was confirmed by direct sequencing, following gel extraction (QIAquick Gel Extraction Kit, Qiagen). Each PCR reaction was repeated three times and control consisted of a no template PCR. PCR was performed in parallel on *C. pomonella* genomic DNA templates, extracted from larvae using PureLink Genomic DNA kit (Invitrogen). No amplification or amplification of larger size bands was observed, revealing specific cDNA amplification at the expected size. Products were analyzed on a 1.5% agarose gel and visualized after staining with ethidium bromide using a Gel Doc XR (Bio-Rad, Hercules, CA, USA).

HETEROLOGOUS EXPRESSION OF PUTATIVE ORs IN *DROSOPHILA MELANOGASTER*

The complete ORF encoding CpomOR3 was amplified by PCR (forward primer 5′-ATGTTTAGTTATGAAAATGAAGACAGC-3′, reverse primer 5′-TCAAGTCATTTCTTCAGTAGAGGT-3′), with antennal cDNA created by the RT-for-PCR kit (Invitrogen) as a template. The purified PCR product was then cloned into the PCR8/GW/TOPO plasmid (Invitrogen). The cassette with the insert was then transferred from the TOPO/GW/PCR8 plasmid to the destination vector (pUASg-HA.attB, constructed by E. Furger and J. Bischof, kindly provided by the Basler group, Zürich), using the Gateway LR Clonase II kit (Invitrogen). The integrity and orientation of the insert was confirmed by sequencing. A transformant *UAS-CpomOR3* line was generated by BestGene (Chino Hills, CA, USA), using the PhiC31 integrase system. Briefly, recombinant pUASg-HA.attB-CpomOR3 plasmids were injected into embryos of a *D. melanogaster* line containing an attP insertion site within the second chromosome (genotype y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP'}ZH-51C), leading to non-random integration. To drive expression of *CpomOR3* in OSNs housed in T1 sensilla, the transformant *UAS-CpomOR3* line was crossed to the *Or67d*^{GAL4} strain (kindly provided by Barry Dickson) to generate a double homozygous line *w*$^{+}$;*UAS-CpomOR3*;*Or67d*^{GAL4}. To verify insertion of the *UAS-CpomOR3* construct into the genome, gDNA was extracted and used as template in PCR with primers for the full ORF of *CpomOR3*.

Additionally, to compare the similarity of results between expression sites (trichoid and basiconic sensilla) male flies with the genotype *w*;*UAS-CpomOR3*/CyO;+/+ were mate paired with female flies of the genotype *w;delta-Halo/Cyo;Dmel-UAS-OR22a-Gal4*. This cross drove ectopic expression of *CpomOR3* in the A neuron of the ab3 sensilla, which also expressed the endogenous DmelOR22a receptor in the same neuron. SSR recordings in parental flies from the cross confirmed the absence of any response from DmelOR22a to pear ester (data not shown).

SINGLE SENSILLUM RECORDINGS

The *D. melanogaster* line expressing *CpomOR3* in T1 OSNs, along with the flies expressing *CpomOR3* in ab3A OSNs were tested by SSRs. In all cases, flies were restrained as described in Stensmyr et al. (2003). Briefly, flies were immobilized in 100 µl pipette tips with only the top half of the head protruding. The left

antenna was pushed onto a piece of double-adhesive tape, and held firm by a capillary pressing down from above. Sensilla were contacted with tungsten electrodes (diameter 0.12 mm, Harvard Apparatus Ltd, Edenbridge, United Kingdom) electrolytically sharpened in a saturated KNO_3 solution. A DC-3K micromanipulator equipped with a PM-10 piezo translator (Märzhäuser Wetzler GmbH, Wetzler, Germany) was used to gently maneuver the recording electrode into the base of a sensillum. The reference electrode was inserted through the eye using a DC-3K Rachts PM-10 piezo micromanipulator (Märzhäuser Wetzler GmbH, Wetzler, Germany). The signal from the OSNs was registered and amplified 10 times with a probe (INR-02, Syntech, Hilversum, the Netherlands), and transferred to a computer through an IDAC-4-USB (Syntech) interface, where it was visualized and analyzed with the software Autospike v. 3.4 (Syntech). A constant flow of 0.65 m/s of charcoal-filtered and humidified air was delivered through a glass tube with its outlet approximately 15 mm from the antenna. Stimuli were presented to the insect by inserting a stimulus pipette through a hole in the glass tube, and blowing an air puff of 2.5 ml during 0.5 s through the pipette into the air stream, using a stimulus controller (Syntech SFC-1/b).

Table 1 | Synthetic compounds tested on CpomOR3.

Compound	Biological activity	Source	CAS	Purity (%) (GCMS)
(*E,E*)-8,10-Dodecadienol	Main pheromone component of *C. pomonella*	IRCHA, gift from Prof Heinrich Arn	33956-49-9	98.6 (isomeric purity: 80.1 E,E; 13.6 E,Z; 0.9 Z,E; 5.4 Z,Z)
(*E,Z*)-8,10-Dodecadienol	Synergist for attraction of males of *C. pomonella*	Gift from Prof Rickard Unelius, University of Kalmar, Sweden	33956-50-2	99.8 (isomeric purity: 95.0 E,Z; 0.0 Z,E; 1.5 E,E; 3.5 Z,Z)
(*Z,E*)-8,10-Dodecadienol	Synergist for attraction of males of *C. pomonella*	Gift from Prof Rickard Unelius, University of Kalmar, Sweden	33956-51-3	99.5 (isomeric purity: 84.0 Z,E; 9.9 E,E; 1.7 E,Z; 4.4 Z,Z)
(*Z,Z*)-8,10-Dodecadienol	Antagonist for attraction of males of *C. pomonella*	Gift from Prof Rickard Unelius, University of Kalmar, Sweden	39616-21-2	94.25 (isomeric purity: 77.7 Z,Z; 11.3 Z,E; 2.9 E,E; 8.1 E,Z)
(*E,E*)-8,10-Dodecadienol acetate	Synergist for attraction of males of *C. pomonella*	Bedoukian Inc	53880-51-6	96.2
(*E*)-8-Dodecenol	Minor pheromone component of *C. pomonella*	Voerman, Pherobank	42513-42-8	97
(*E*)-9-Dodecenol	Minor pheromone component of *C. pomonella*	Farchan Labs Inc	35237-62-8	99.7
(*E*)-10-Dodecenol	Minor pheromone component of *C. pomonella*	Voerman, Pherobank	35237-63-9	99.7
1-Dodecanol	Minor pheromone component of *C. pomonella*	Fluka	112-53-8	98.1
(*E*)-β-Farnesene	Synergist for *C. pomonella*	Bedoukian	18794-84-8	98.6
Butyl hexanoate	Synergist for *C. pomonella*	Bedoukian	626-82-4	97.7
Ethyl-(*E,Z*)-2,4-Decadienoate	Synergist for *C. pomonella*	Aldrich	3025-30-7	98.2
(*Z,E*)-9,12-tetradecadienyl acetate	Main pheromone component of *Spodoptera littoralis*	Pherobank	30507-70-1	94.8
4,8-Dimethyl-1, (*E*)-3,7-non-atriene	Antagonist for female attraction of *S. littoralis*	Gift from Prof Wittko Franke, University of Hamburg, Germany	51911-82-1	95
3,7-Dimethyl-1, (*E*)-3,6-octatriene	Antagonist for female attraction of *S. littoralis*	SAFC	3779-61-1	95.4

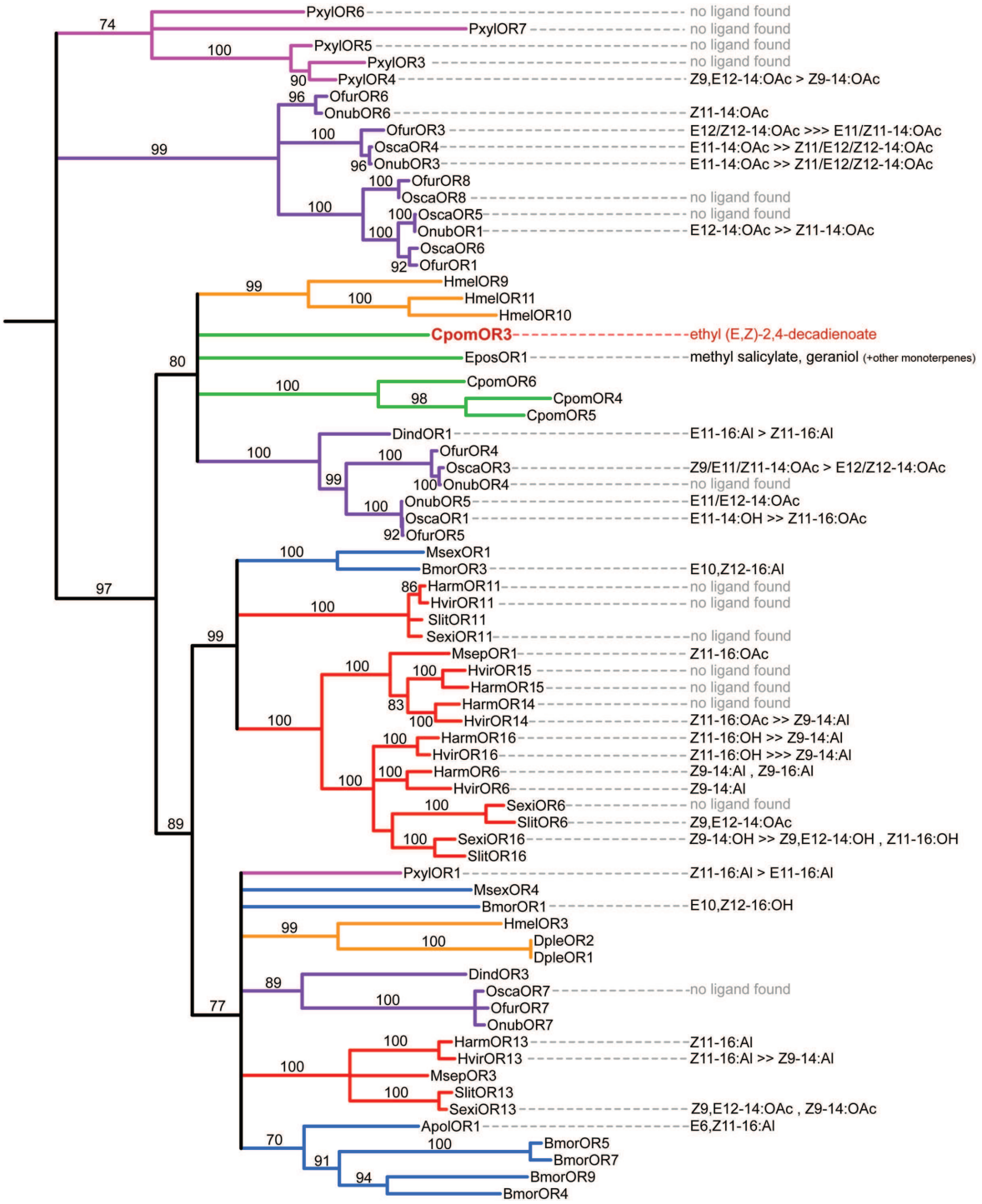

FIGURE 1 | Maximum likelihood tree of lepidopteran candidate PRs, highlighting their corresponding ligand(s). Branch colors represent different lepidopteran lineages: blue for Bombycoidea, red for Noctuoidea, orange for Papilionoidea, purple for Pyraloidea, green for Tortricoidea, and pink for Yponomeutoidea. The outgroup (containing BmorOR6 and HmelOR5, 6, and 7) has been removed from the figure. Functional data has been compiled from the literature (see references in the Methods section). "No ligand found": OR did not respond to any tested pheromone component. Cpom, *Cydia pomonella*, Apol, *Antheraea polyphemus*, Bmor, *Bombyx mori*, Dple, *Danaus plexippus*, Dind, *Diaphania indica*, Epos, *Epiphyas postvittana*, Hmel, *Heliconius melpomene*, Harm, *Helicoverpa armigera*, Hvir, *Heliothis virescens*, Msex, *Manduca sexta*, Msep, *Mythimna separata*, Ofur, *Ostrinia furnacalis*, Onub, *O. nubilalis*, Osca, *O. scapulalis*, Pxyl, *Plutella xylostella*, Sexi, *Spodoptera exigua*, Slit, *S. littoralis*.

SYNTHETIC COMPOUNDS AND ODOR STIMULI

An array of pheromone compounds for *C. pomonella* and related species (Witzgall et al., 1996), as well as known pheromone synergists (El-Sayed, 2014), were tested on CpomOR3 (**Table 1**). Combinations of the *C. pomonella* main pheromone compound, codlemone, with the synergists were also tested, as they have previously been shown to create distinct activation patterns in the antennal lobe, the primary olfactory center, compared to either compound alone (Trona et al., 2013). Purity of compounds was estimated by GC-MS.

Stimuli were prepared by applying compounds to 1.5 × 1 cm pieces of filter paper that were placed in disposable glass Pasteur pipettes (VWR International, Stockholm, Sweden). Truncated 1 ml pipette tips were put on the wide end of the Pasteur pipettes, to reduce evaporation of the test compound(s). Compounds were diluted in hexane (redistilled from 95%, Labscan, Dublin, Ireland). A volume of 10 µl of a 1 µg/µl solution was applied to filter papers for a total amount of 10 µg per stimulus. The same dilution procedure was used in dose-response experiments, except that compounds were diluted to concentrations ranging from 0.1 ng/µl to 10 µg/µl in decadic steps, to achieve different concentrations when 10 µl of the diluted compound were applied to the filter paper in the stimulus pipette. Control stimuli with only solvent were also prepared. Fresh stimuli were prepared before each recording session, and kept at −18°C until the start of the recording session, to avoid evaporation. Only complete recording sessions of the entire set of test stimuli were evaluated, and only one screening or dose response session was performed from a single sensillum per individual. A total of 16 screenings were performed, while for dose response experiments, 10 replicates were performed.

Responses were quantified by counting the number of spikes for 500 ms starting from the onset of response (as determined by the earliest response for the recording session), subtracting the number of spikes during the 500 ms before response, and doubling this value to get the response in Hz (spikes/s). Responses of T1 sensilla to different pheromone and pheromone synergist compounds were compared using ANOVA with repeated measures, while responses to different doses of pear ester with the two types of sensilla evaluated were compared with Two-Way ANOVA. All statistical analyses were performed using SPSS Version 19.0 (IBM Corp., Armonk, NY, USA).

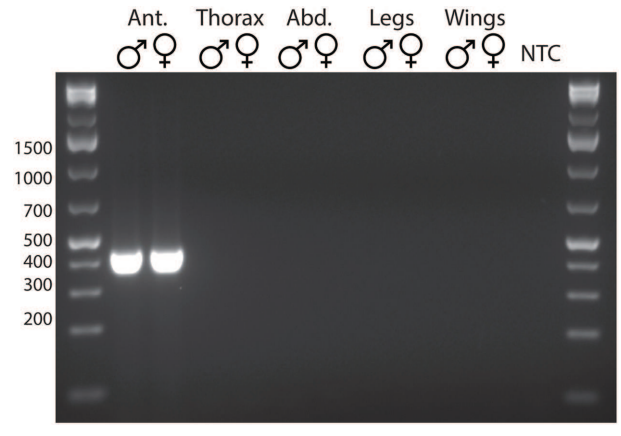

FIGURE 2 | Reverse transcription PCR showing antennal specific expression of *C. pomonella* OR3 in both sexes. Ant., antennae, Abd., abdomen, NTC, no template control.

RESULTS

CLONING OF THE OPEN READING FRAME OF *CPOMOR3* AND SEQUENCE ANALYSIS

The partial *CpomOR3* sequence (Bengtsson et al., 2012), judged to be complete at the 3′ end based on the presence of a stop codon, but not at the 5′ end, was extended by 5′ RACE-PCR. Merging the sequence of the 1096 bp 5′RACE-PCR product we obtained together with the previous sequence led to a 1281 bp transcript, containing the complete ORF of *CpomOR3*, confirmed by alignment of the deduced protein with other lepidopteran ORs. The full ORF sequence for *CpomOR3* was further amplified and sequenced to verify the absence of chimera. The full sequence has been submitted to Genbank (accession number KJ420588). The TMHMM2.0 model predicted 6 transmembrane domains for CpomOR3. CpomOR3 exhibits a mean sequence identity of 34.3% with other PRs, with a maximum identity of 41.4% with *Diaphania indica* OR1. Alignment with lepidopteran candidate PRs did not reveal any notable feature of CpomOR3, apart from a serine residue—also present in other tortricid sequences—located within the final transmembrane domain (position 296), instead of the glycine residue found in all the other lepidopteran PR sequences.

PHYLOGENY OF LEPIDOPTERAN CANDIDATE PRs

A maximum likelihood phylogeny was built from a large dataset containing CpomOR3 to 6—the putative *C. pomonella* PRs (Bengtsson et al., 2012)—and 70 other candidate PR full-length sequences. In this tree (**Figure 1**), the candidate PRs grouped within five large sub-clades within the PR clade. All the sequences from tortricid moths (*C. pomonella* and *E. postvittana*, green branches), including CpomOR3, clustered within one of these five clades (supported by a bootstrap value of 80), albeit the exact relationships between CpomOR3 and the other receptors of this clade were not resolved due to low bootstrap support values (to reflect lack of support, nodes with a bootstrap value lower than 70 were collapsed). Even if the CpomOR1 sequence was not part of this dataset because of its short length, it also clustered in the same clade during previous analyses, as a sister group to EposOR1 (data not shown). All the PR candidates from *C. pomonella* characterized to date thus have a relatively recent common origin, in spite of their low sequence identity levels.

TISSUE-RELATED EXPRESSION OF CpomOR3

Reverse transcription PCR showed a clear expression pattern for *CpomOR3*, with strong expression in antennae, but not in other body parts (**Figure 2**). Moreover, there was no sex-specific expression of *CpomOR3*, as it appeared to be expressed in antennae of both males and females.

RESPONSE SPECTRUM OF CpomOR3 TO PUTATIVE LIGANDS

Single-sensillum recordings from transformed *Drosophila* line expressing CpomOR3 in T1 OSNs revealed that these neurons only responded to pear ester (41 spikes/s, $N = 16$) out of 15 compounds. Six different mixtures of different combinations of pheromone components and plant compounds were also tested, and only the one that contained pear ester and codlemone elicited a significant response (**Figure 3**). No synergy between these two compounds was observed (Bonferroni *post-hoc* test). Dose response experiments established the threshold of response to pear ester to be at 10 μg for both trichoid T1 and ab3A OSNs (**Figure 4**).

DISCUSSION

CpomOR3 IS TUNED TO THE PLANT VOLATILE PEAR ESTER

Electrophysiological recordings from *Drosophila* basiconic ab3 and trichoid T1 sensilla, housing OSNs heterologously expressing CpomOR3, demonstrate that CpomOR3 is tuned to pear

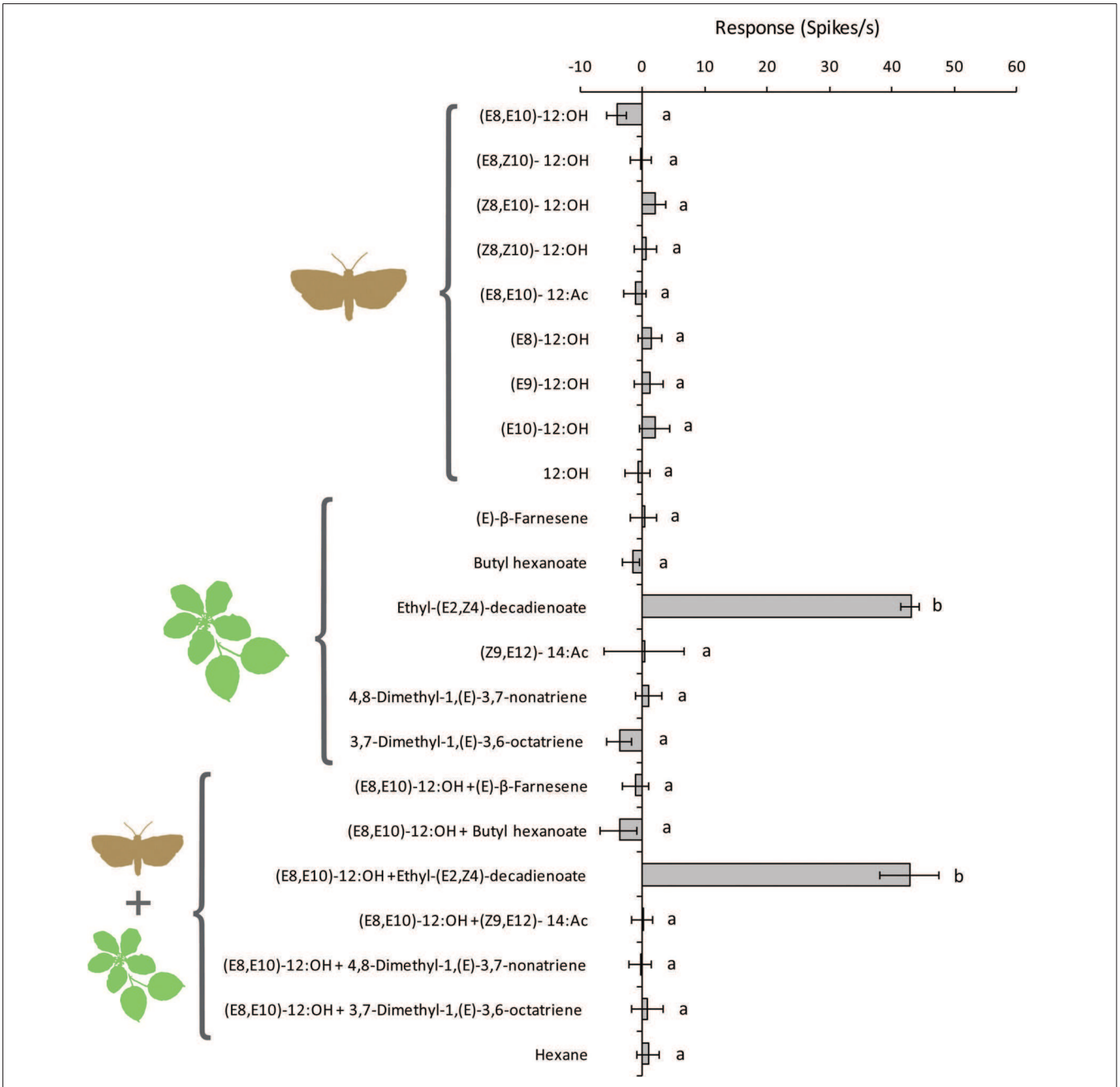

FIGURE 3 | Response (in Hz) of *D. melanogaster* T1 OSNs expressing CpomOR3 to stimulation with known *C. pomonella* pheromone components, plant-related synergists, and combinations of the main pheromone component codlemone, with plant synergists. Letters denote subgroups with a statistically significant separation (Repeated measures ANOVA with Bonferroni *post-hoc* test, $n = 16$).

ester, ethyl (*E,Z*)-2,4-decadienoate (**Figures 3**, **4**). Reverse transcription PCR suggests that CpomOR3 is expressed without sex bias in the antennae of both males and females (**Figure 2**). This finding matches the behavioral evidence, since pear ester is a bisexual codling moth attractant (Light et al., 2001; Light and Knight, 2005). The existence of a dedicated receptor corroborates the significance of pear ester for host plant detection in codling moth males and females, and contributes to current research aiming at a complete identification of codling moth host plant attractants.

Results from these heterologous expression studies confirm previous recordings obtained from codling moth antennae, showing presence of OSNs responding to pear ester (De Cristofaro et al., 2004; Ansebo et al., 2005). However, a spatially tight arrangement of sensilla on codling moth antennae renders it difficult to obtain replicated recordings from the same sensillum type, and to differentiate between responses from co-localized OSNs in the same sensillum, or even from OSNs in adjacent sensilla (Lee and Baker, 2008). This further demonstrates the appreciable addition of heterologous OR expression in *Drosophila* to the toolbox for identification of behaviorally relevant plant odorants.

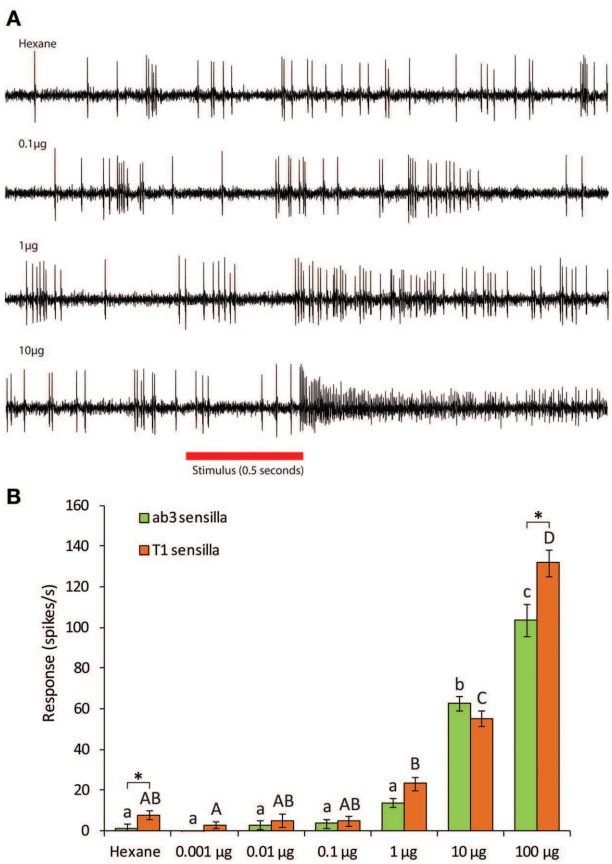

FIGURE 4 | (A) Traces of single sensillum recordings from *D. melanogaster* T1 sensilla expressing CpomOR3 to pear ester at different doses. **(B)** Dose-dependent response of CpomOR3 to pear ester in different types of sensilla. Bars of the same color followed by different letters indicate subgroups with statistically significant differences. Asterisks denote significant differences among different types of sensilla for the dose indicated (Two-Way ANOVA with Tukey's *post-hoc* test, $p < 0.05$, $n = 10$).

Intracellular recordings of axons of OSNs projecting to the antennal lobe (AL), the olfactory center of the insect brain, and functional imaging of AL glomeruli, receiving input from OSNs expressing the same ORs, support our finding that pear ester activates a dedicated olfactory channel and that interaction of pear ester with other compounds, including the sex pheromone codlemone, takes place in the AL, and not at the periphery (**Figure 3**; Trona et al., 2010, 2013).

CpomOR3 BELONGS TO THE PHEROMONE RECEPTOR CLADE

CpomOR3 belongs to the conserved clade of lepidopteran pheromone receptors (**Figure 1**), although it binds to pear ester only and to none of the pheromonal compounds produced by *C. pomonella* females or closely related *Cydia* species (Witzgall et al., 1996, 2001). CpomOR3 was almost equally sensitive when expressed in trichoid T1 and basiconic ab3 sensilla, except at the highest dose of pear ester (**Figure 4**). Interestingly, the pheromone receptors BmorOR1 of silkmoth *B. mori* and SlitOR6 of cotton leafworm moth *S. littoralis* were more sensitive when expressed in T1 than in ab3 sensilla (Syed et al., 2010; Montagné et al., 2012). This indicates that T1 sensilla, containing an important PR partner, the sensory neuron membrane protein (Benton et al., 2007), are more adapted for correct PR functioning, whereas plant odorant ORs function equally well in T1 or ab3.

In addition, the demonstration that an OR clustering in the PR clade is a plant odorant receptor offers an explanation for the lack of a response of orphan lepidopteran PRs to pheromone compounds (Wang et al., 2010; Liu et al., 2013). Phylogenetic analysis confirms that the lepidopteran PR clade contains another co-evolved receptor for plant compounds, EposOR1, from another tortricid species, the light brown apple moth *E. postvittana*. The strongest ligand for EposOR1 is a common plant compound, methyl salicylate (Jordan et al., 2009), which has a behavioral effect in many insects (**Figure 1**; El-Sayed, 2014). With the currently available sequence and functional data, phylogenetic analysis cannot resolve if EposOR1 and CpomOR3 have a single ancestor, or if two unique evolutionary events gave rise to these plant volatile receptors within the PR clade (**Figure 1**). However, both CpomOR3 and EposOR1 belong to the same clade, which notably also contains the four other *C. pomonella* candidate PRs (Bengtsson et al., 2012; Garczynski et al., 2012). Further studies, using both pheromones and plant volatiles, will help to understand the functional divergence of the PR clade.

INTERACTION BETWEEN PEAR ESTER AND CODLING MOTH PHEROMONE

The finding that a codling moth PR is tuned to pear ester is remarkable. It corroborates the interaction between pear ester and codlemone, which may play an important role in codling moth premating communication and reproductive isolation (Trona et al., 2013).

Axons of OSNs expressing the same OR or PR genes converge onto the same glomerulus in the antennal lobe (AL). Since each OR corresponds to a glomerulus in the AL, it follows that new

glomeruli arise during OR repertoire expansion. Indeed, closely related ORs with high sequence similarity are often expressed in OSNs that project to neighboring glomeruli in the AL (Couto et al., 2005; Masse et al., 2009; Ramdya and Benton, 2010; Cande et al., 2013).

Accordingly, the architecture of the codling moth AL lends support to the hypothesis that the OR genes for pear ester and codlemone, the codling moth sex pheromone, are closely related—the glomeruli dedicated to pear ester and codlemone are adjacent glomeruli in the codling moth AL, where stimulation with a blend of codlemone and pear ester produces a very strong synergistic effect (Trona et al., 2010, 2013). Although the PR for codlemone has not yet been found, we can reasonably assume that it belongs to the PR clade, which contains the putative pheromone receptors CpomOR1, and CpomOR4 through 6 (**Figure 1**; Bengtsson et al., 2012).

Chemosensory receptor genes arise by gene duplication and progressively diverge following adaptive changes. In *Drosophila*, phylogenetically related chemosensory genes on a chromosome tend to be located closely together on a chromosome (Nei et al., 2008; Sanchez-Gracia et al., 2009). Physically neighboring chemosensory genes restrict genetic recombination and thus become a combined target for selection. Tight physical linkage between host performance and preference genes, leading to assortative mating through habitat choice, has been first discovered in pea aphids (Hawthorne and Via, 2001; Smadja et al., 2012). Key traits that are associated via linkage and which combine ecological and sexual selection are particularly powerful during phylogenetic divergence (Servedio et al., 2011; Merrill et al., 2012; Safran et al., 2013).

In codling moth, chemosensory receptor genes encoding host preference and mate recognition, tuned to the plant volatile pear ester and sex pheromone, are expected to be associated to facilitate host adaptation and reproductive isolation in concert. This hypothesis can be tested after the receptor gene for codlemone has been found.

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Chapter V

A conserved odorant receptor detects the same 1-indanone analogs in a tortricid and a noctuid moth

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A Conserved Odorant Receptor Detects the Same 1-Indanone Analogs in a Tortricid and a Noctuid Moth

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Odorant receptors (ORs) interface animals with airborne chemical signals. They are under strong selection pressure and are therefore highly divergent in different taxa. Yet, some OR orthologs are highly conserved. These ORs may be tuned to odorants of broad importance, across species boundaries. Two widely distributed lepidopteran herbivores, codling moth *Cydia pomonella* (Tortricidae) feeding in apples and pears, and the African cotton leafworm *Spodoptera littoralis* (Noctuidae), a moth feeding on foliage of a wide range of herbaceous plants, both express a receptor ortholog, OR19, which shares 58% amino acid identity and 69% amino acid similarity. Following heterologous expression in the empty neuron system of *Drosophila melanogaster*, we show by single sensillum recordings that CpomOR19 and SlitOR19 show similar affinity to several substituted indanes. Tests with a series of compounds structurally related to 1-indanone show that 2-methyl-1-indanone, 2-ethyl-1-indanone, 3-methyl-1-indanone, and 1-indanone elicit a strong response from both ORs. A keto group in position 1 is essential for biological activity and so are both rings of the indane skeleton. However, there is an important difference in steric complementary of the indane rings and the receptor. Methyl substituents on the benzene ring largely suppressed the response. On the other hand, alkyl substituents at position 2 and 3 of the five-membered ring increased the response indicating a higher complementarity with the receptor cavity, in both CpomOR19 and SlitOR19. Our results demonstrate a conserved function of an odorant receptor in two moths that are phylogenetically and ecologically distant. It is conceivable that a conserved OR is tuned to signals that are relevant for both species, although their ecological roles are yet unknown. Our finding demonstrates that functional characterization of ORs leads to the discovery of novel semiochemicals that have not yet been found through chemical analysis of odorants from insects and their associated host plants.

Keywords: ***Cydia pomonella***, ***Spodoptera littoralis***, **olfaction, olfactory receptor, 1-indanone, orthologous genes, structure activity relationships, functional characterization**

INTRODUCTION

Perception of olfactory cues plays a fundamental role in insect life, and the olfactory system has evolved through adaptations to new environments, host, plant, and mate-finding signals (Bergstrom, 2008; Smadja and Butlin, 2009; Hansson and Stensmyr, 2011). Several studies have shown that the family of odorant receptor (OR) genes, which encode for proteins that detect and discriminate odorants, is highly divergent among insect taxa and even among closely related species (Jacquin-Joly and Merlin, 2004; Su et al., 2009; Engsontia et al., 2014; Depetris-Chauvin et al., 2015). This suggests that olfactory systems have evolved rapidly to enable perception of relevant odor signals. Selection drives the evolution of genes that facilitate host and mate finding, whereas behaviorally redundant OR genes are no longer expressed (Sánchez-Gracia et al., 2009; Hansson and Stensmyr, 2011; Suh et al., 2014; Andersson et al., 2015). Consequently, the insect OR repertoire is expected to be tuned to odor cues of ecological relevance, as indicated in the functional comparison between the OR repertoire of the vinegar fly, *Drosophila melanogaster,* and the malaria mosquito *Anopheles gambiae*, which shows little overlap (Hill et al., 2002; Carey et al., 2010; Suh et al., 2014; Karner et al., 2015). Orthologous ORs are of particular interest since may be tuned to odorants that are behaviorally and ecologically relevant across species (Bohbot et al., 2011).

Insect ORs identified so far generally show a low level of sequence conservation between species, ranging from 20 to 40% amino acid identity (Rützler and Zwiebel, 2005; Bohbot et al., 2007; Martin et al., 2011; Engsontia et al., 2014). A striking exception is the OR co-receptor, ORco, which shares 60–90% amino acid identity across different insect orders (Krieger et al., 2003; Larsson et al., 2004). A plausible reason for this conservation may lie in its function: ORco is an obligate co-receptor that forms a complex with ligand-selective ORs and is required for trafficking to olfactory neuron dendrites in all insects (Larsson et al., 2004; Jones et al., 2011). Apart from ORco, conserved ligand-selective ORs have been identified in closely related species. The OR2/OR10 clade of the mosquitoes *Aedes aegypti* and *An. gambiae* share 69% of amino acid identity and both respond strongly to indole, an important host signal for both species (Bohbot et al., 2011). Within Lepidoptera, several examples of conserved function for orthologous receptors have been reported, especially within the pheromone receptor family (de Fouchier et al., 2014; Jiang et al., 2014). There are clusters of ORs, however, that share high amino acid identity across species but whose function has not yet been elucidated; for example, OR18, a highly conserved receptor in six noctuid species, with an average of 88% amino acid identity (Brigaud et al., 2009).

A number of lepidopteran OR gene repertoires have been described, following genome and transcriptome sequencing (Jordan et al., 2009; Grosse-Wilde et al., 2011; Montagné et al., 2012, 2014; Cao et al., 2014; Gu et al., 2014; Liu et al., 2014; Corcoran et al., 2015; Yang et al., 2015; Zhang et al., 2015a,b). In our own transcriptome sequence analyses of the antennae of the codling moth (*Cydia pomonella*: Tortricidae; Bengtsson et al., 2012) and the cotton leafworm (*Spodoptera littoralis*: Noctuidae; Legeai et al., 2011; Jacquin-Joly et al., 2012; Poivet et al., 2013) we have identified one OR (OR19) with relatively high sequence similarity in both species. In *S. littoralis*, SlitOR19 was shown to be narrowly tuned to 1-indanone (de Fouchier et al, unpublished). We have compared the responses of SlitOR19 and its homolog CpomOR19 to 1-indanone, and its analogs, showing a similar response spectrum for these receptor orthologs in the codling moth and the African cotton leafworm. A qualitative structure-activity study of these receptors leads toward a better comprehension of the effect of amino acid sequence differences on OR tuning.

MATERIALS AND METHODS

Phylogenetic and Sequence Analysis

The previously described CpomOR19 amino acid sequence (Bengtsson et al., 2012) was used as a query in BLASTp search on the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Among hits, putative ORs belonged to lepidopteran species only (*C. pomonella, S. littoralis, Bombyx mori, Heliothis virescens, Helicoverpa armigera, Helicoverpa assulta, Manduca sexta,* and *Danaus plexippus*). Sequences of the putative ORs retrieved were aligned with MAFFT, using the FFT-NS-2 algorithm with default parameters. A maximum likelihood tree was constructed with MEGA6 using the JTT+F algorithm with a bootstrap consensus inferred from 1000 replicates and Poisson correction of distances (Tamura et al., 2013).

The membrane topologies and transmembrane domains of CpomOR19 and SlitOR19 were predicted with five different prediction models-TMHMM (https://www.cbs.dtu.dk/services/TMHMM/), METSAM-SVM (http://bioinf.cs.ucl.ac.uk/psipred/), TOPCONS (http://topcons.cbr.su.se/), RHYTHM (http://proteinformatics.charite.de/rhythm/), and TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html).
From these, we selected the model that best fitted the OR characteristic structure (seven-transmembrane domains and extracellular C-terminus) and illustrated it with Protter (Omasits et al., 2014).

Heterologous Expression of Putative ORs in *Drosophila melanogaster*

The complete open reading frames (ORFs) encoding CpomOR19 and SlitOR19, from start codon to stop codon, were amplified by PCR, (CpomOR19: forward primer 5′-ATGTTTAGTTATGAAAATGAAGACAGC-3′, reverse primer 5′-TCAAGTCATTTCTTCAGTAGAGGT-3′; SlitOR19: forward primer 5′-ATGAAAAACCATTACATCTTGAA-3′, reverse primer 5′-TTACGAAGTTTGCGCATAAAAC-3′), using antennal cDNA synthetized with the RT-for-PCR kit (Invitrogen) as a template. For cloning of OR19 homologs, total RNA was extracted from 100 dissected antennae of mixed male and female 2–3 day old adult moths of each species. For extractions Trizol reagent (Invitrogen) was used according to manufacturer's standard protocol. After extraction, total RNA was purified via spin column purification with the RNeasy Mini Kit (Qiagen) according to manufacturer's standard protocol. Total RNA was used as template for first

strand cDNA synthesis with the RevertAid H minus Reverse Transcriptase kit, according to manufacturer's standard protocol. ORF sequence from start codon to stop codon of OR19 was PCR amplified from the cDNA. The purified PCR products were then cloned into the PCR8/GW/TOPO plasmid (Invitrogen), after which One Shot TOP10 cells were transformed (Invitrogen), and plated for overnight growth on Spectinomycin selective lysogeny broth (LB) growth plates. Colonies were assayed for the presence of the relevant insert in the correct orientation by PCR using either the forward gene specific primer (GSP) together with the M13 reverse primer, or the reverse GSP together with the M13 forward primer. Plasmids were purified by Miniprep (Qiagen), and then sequenced to confirm the presence and integrity of the OR inserts. The cassettes with the inserts were then transferred from the PCR8/GW/TOPO plasmid into the destination injection vector (pUASg-HA.attB) constructed by E. Furger and J. Bischof, kindly provided by the Basler group, Zürich (Bischof et al., 2007), using the Gateway LR Clonase II kit (Invitrogen). The destination vector with the correct insert (as confirmed by sequencing) was transformed into One Shot TOP10 cells (Invitrogen). Resultant colonies were cultured in 20 ml of LB media with Ampicillin and purified by Midiprep (Qiagen); the integrity and orientation of the inserts was confirmed by sequencing. Transformant UAS-CpomOR19 and UAS-SlitOR19 lines were generated by BestGene (Chino Hills, CA, USA) and Fly Facility (Clermont-Ferrand, France), respectively, using the PhiC31 integrase system. Briefly, recombinant pUASg-HA.attB-CpomOR19 and –SlitOR19 plasmids were injected into embryos of a *D. melanogaster* line containing an attP insertion site within the third chromosome (genotype y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb), leading to non-random integration; the transgenes were then crossed into the Δhalo mutant background. To drive expression of CpomOR19 and SlitOR19 in OSNs housed in the ab3 basiconic sensilla, the described transgenic lines were crossed with Δhalo; OR22a-Gal4 mutant *D. melanogaster* (Dobritsa et al., 2003; Hallem et al., 2004).

Single Sensillum Recordings

Flies expressing either CpomOR19 or SlitOR19 in the A neuron of ab3 basiconic sensilla were tested by single sensillum recordings (SSRs). Flies were restrained as described in Stensmyr et al. (2003). Briefly, flies were trapped inside 100 µl pipette tips with only the top half of the head protruding. A glass capillary was used to push the left antenna onto a piece of double-sided adhesive tape placed on a piece of glass. Both the pipette tip and the piece of glass with the antennae were mounted and fixed with dental wax on a microscope slide. Tungsten electrodes (diameter 0.12 mm, Harvard Apparatus Ltd., Edenbridge, UK), were electrolytically sharpened with a saturated KNO_3 solution, and used to penetrate the eye and the sensilla of the flies. The recording electrode (introduced at the base of the sensilla) was maneuvered with a DC-3K micromanipulator equipped with a PM-10 piezo translator (Märzhäuser Wetzler GmbH, Germany). The reference electrode was manually inserted through the eye. The signal from the olfactory sensory neurons (OSNs) was amplified 10 times with a probe (INR-02, Syntech, Hilversum, the Netherlands), digitally converted through an IDAC-4-USB (Syntech) interface, visualized and analyzed with the software Autospike v. 3.4 (Syntech).

During the recording sessions, a constant flow of 0.65 m/s of charcoal-filtered and humidified air was delivered through a glass tube with the outlet 15 mm apart from the antenna. The panel of odorant stimuli was presented to the insect by blowing air through pipettes inserted in a lateral hole of the glass tube delivering the constant charcoal-filtered humidified air. The air puff was controlled with a stimulus controller (Syntech SFC-1/b) and consisted of a flow of 2.5 ml of air during 0.5 s.

Synthetic Compounds and Odorant Stimuli

To determine ligands detected by CpomOR19, initially a panel with a wide range of synthetic compounds was tested (**Table 1**). The list of compounds included general plant odors previously tested for deorphanization of SlitOR19 (de Fouchier et al, unpublished), codling moth pheromone components (Arn et al., 1985), and microbial odorants (Witzgall et al., 2012). Compounds were diluted in redistilled hexane (LabScan), acetone (Sigma-Aldrich), or paraffin oil (Merck) to a concentration of 10 µg/µl. Stimuli were prepared by applying 10 µl (100 µg) of the diluted test compounds to 1.5 × 1 cm pieces of filter paper placed inside disposable glass Pasteur pipettes (VWR International, Stockholm, Sweden). Pipette tips were placed on the end of the Pasteur pipettes to decrease evaporation of compounds. Control pipettes with only solvent (hexane, acetone, and paraffin oil) were also prepared.

To investigate structural activity relationships between 1-indanone and selected analogs, a second odorant panel was tested for flies expressing either CpomOR19 or SlitOR19 (**Figure 1**). Compounds eliciting significant response in comparison to the solvent were used for dose response experiments, the concentration of the test compounds ranged from 1 ng to 100 µg in decadic steps applied to the filter paper in the stimulus pipette. Comparisons between receptor-activating compounds were made after correction for differences in vapor pressure (Bengtsson et al., 1990).

Fresh filter papers were prepared before each recording session, and kept at −18°C until the start of the recording session. Only complete recording sessions of the entire set of test stimuli were evaluated, and only one screening or dose response session was performed per individual fly and on a single sensillum.

SSR responses were quantified by counting the number of spikes for 500 ms starting from the onset of the response (as determined by the earliest response for the recording session), subtracting the number of spikes during 500 ms before response. Five whole-panel screenings for ligands of CpomOR19 were performed, screenings of the panel of structurally related compounds were done five times for CpomOR19 and SlitOR19. For dose response experiments, eight replicates were carried out at each dose for each receptor.

Responses of CpomOR19 and SlitOR19 to the panel of structurally related compounds and dose response experiments

TABLE 1 | Responses of *D. melanogaster* flies expressing CpomOR19 to synthetic compounds tested at 100 μg on filter paper.

Compound class	Compound	Chemical purity (%)	Source	Spike frequency[a]
HYDROCARBONS				
Monoterpenes	α-Pinene	98	Aldrich	+
	β-Pinene	99	Fluka	+
	β-Myrcene	95	Fluka	
	β-Ocimene	90	Safc	
	3-Carene	95	Aldrich	
Sesquiterpenes	α-farnesene	99	Bedoukian	
	α-Copaene	98	Bedoukian	
	α-Humulene	98	Aldrich	+
	β-Caryophyllene	98.5	Aldrich	
Homoterpenes	TMTT[b]	98	Aldrich	+
	DMNT[c,d]	95		
ALCOHOLS				
Aliphatics	1-Hexanol	98	Aldrich	++
	1-Heptanol	99	Aldrich	
	1-Octanol	99.5	Aldrich	
	1-Nonanol	99.5	Aldrich	
	1-Tetradecanol	99	Fluka	+
	(Z)-3-Hexenol	98	Aldrich	+
	(E)-2-Hexenol	96	Aldrich	+
	Butyl alcohol	99.5	Sigma	+
	(E)-3-Hexen-1-ol	97	Aldrich	+
	Codlemone[e]	98.6		
	1-Dodecanol	98	Fluka	+
	(E)-9-Dodecenol	99	Farchan Labs Inc	+
Aromatics	Thymol	99.5	Aldrich	
	Carvacrol	98	Aldrich	+
	Eugenol	98	Aldrich	
	Estragol	96	Sigma	
Monoterpenes	Geraniol	98	Aldrich	
	Citronellol	95	Aldrich	
	± Linalool	97	Aldrich	+
Sesquiterpenes	*(E,E)*-Farnesol	95	Aldrich	+
	± Nerolidol	98	Aldrich	+
Diterpenes	Phytol	99	Aldrich	
ALDEHYDES				
Aliphatics	*(E)*-2-Hexenal	98	Aldrich	+
	Nonanal	95	Aldrich	
	Decanal	99	Aldrich	
Aromatics	Phenyl acetaldehyde	98	Aldrich	
	Benzaldehyde	99.5	Aldrich	
ETHERS				
Aromatics	Benzyl methyl ether	98	Aldrich	
ESTERS				
Aliphatics	*(Z)*-3-Hexenyl acetate	98	Aldrich	
	Butyl butyrate	99	Aldrich	
	Methyl hexanoate	99	Aldrich	+
	Hexyl butyrate	98	Aldrich	
	Methyl jasmonate	98	Aldrich	
	Propyl hexanoate	99	Aldrich	+
	Pear ester	98	Aldrich	+
	Isoamyl acetate	95	Aldrich	+
	Isobutyl acetate	99	Aldrich	+
	Codlemone acetate	97	Bedoukian	+
	Hexyl propionate	97	Aldrich	+
	Butyl acetate	99	Aldrich	+
Aromatics	Methyl salicylate	99	Sigma	+
	Methyl benzoate	99	Aldrich	+
	2-Phenylethyl acetate	99	Aldrich	
KETONES				
Aliphatics	Geranyl acetone	96	Aldrich	+
	(Z)-Jasmone[d]	98		
	2-Heptanone	98	Aldrich	+
	Sulcatone	98	Aldrich	+
Aromatics	Acetophenone	99	Acros	+
	1-indanone	99	Aldrich	+++
ACIDS				
Aliphatics	Acetic acid	99	Aldrich	
OTHERS				
	Indole	99	Aldrich	

[a] *Spike frequency (Hz) is used as measure of response strength: 1–10 Hz (+), 11–49 Hz (++), >50 Hz (+++).*
[b] *(E,E) 4,8,12-trimethyltrideca-1,3,7,11-tetraene.*
[c] *(E)-4,8-dimethyl-1,3,7-nonatriene.*
[d] *Gift from Prof. Wittko Francke.*
[e] *Gift from Prof. Heinrich Arn.*

were compared with Two-way ANOVA with repeated measures, followed by LSD *post-hoc* test. All statistical analyses were performed using SPSS Version 19.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Phylogeny and Sequence Analysis

Comparison of protein sequences of putative orthologs from different lepidopteran species showed that the receptors OR21 and OR22 of *B. mori*, along with OR19 of *S. littoralis*, *H. virescens,* and *C. pomonella* cluster within one group (**Figure 2**). Among these sequences, SlitOR19 shared the highest amino acid identity (58%) with CpomOR19, while the others share 42–55% (**Figure 3A**). According to receptor topology prediction (OCTOPUS algorithm, TOPCONS), the main differences between the two sequences were observed in the putative extracellular C-terminus which SlitOR19 has a four residues shorter sequence, along with the addition of residues in two regions, one located in the fourth transmembrane domain (M) and the other in the third intracellular loop (RPKSAP). However, most of the non-conservative point mutations correlated to substitutions in

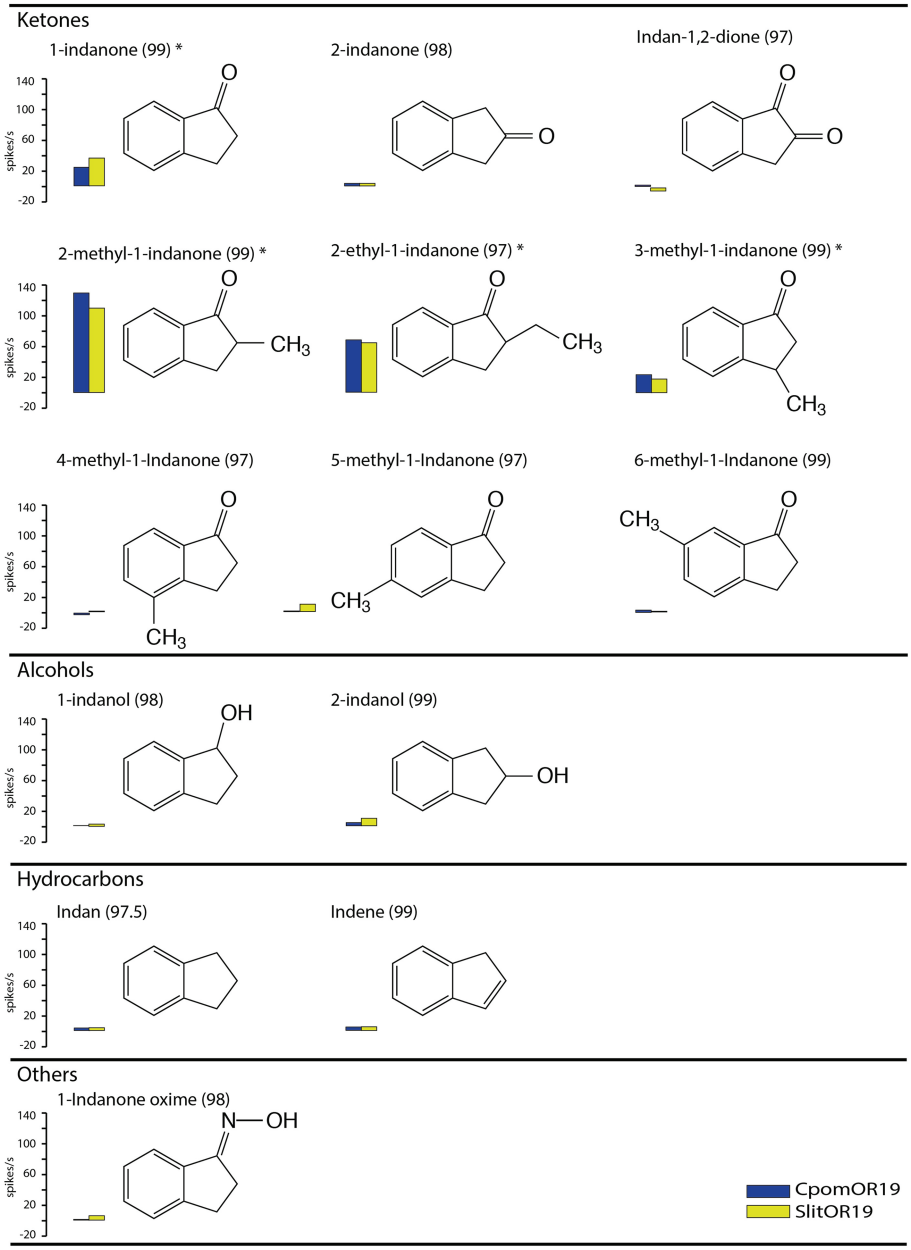

FIGURE 1 | Response profiles of CpomOR19 and SlitOR19 to 1-indanone and structurally related compounds at 100 μg on filter paper. Asterisks denote significant differences between the response elicited by the indicated compound and the solvent at $P < 0.05$ (Two-way ANOVA with repeated measures, LSD *post-hoc* test, $n = 5$). Chemical purity is shown in brackets, compounds were purchased from Aldrich.

the first transmembrane region and in the cytoplasmic side (loop 2), while only a few mutations are predicted to be located on the extracellular side (**Figure 3B**).

Selectivity of CpomOR19 toward Putative Ligands

SSR recordings from ab3A OSN of *D. melanogaster* that expressed CpomOR19 showed that of 64 stimuli tested at the maximum dose of 100 μg loaded on filter paper, only 1-indanone elicited a strong electrophysiological response (>50 Hz; **Table 1**, **Supplementary Figure 1**).

Effect of Chemical Structure on Specificity and Sensitivity of CpomOR19 and SlitOR19

When tested at the maximum dose of 100 μg, the responses of CpomOR19 and SlitOR19 did not differ significantly between them for any of the indanone analogs tested. Besides 1-indanone, both ORs responded to three of the other 13 compounds tested.

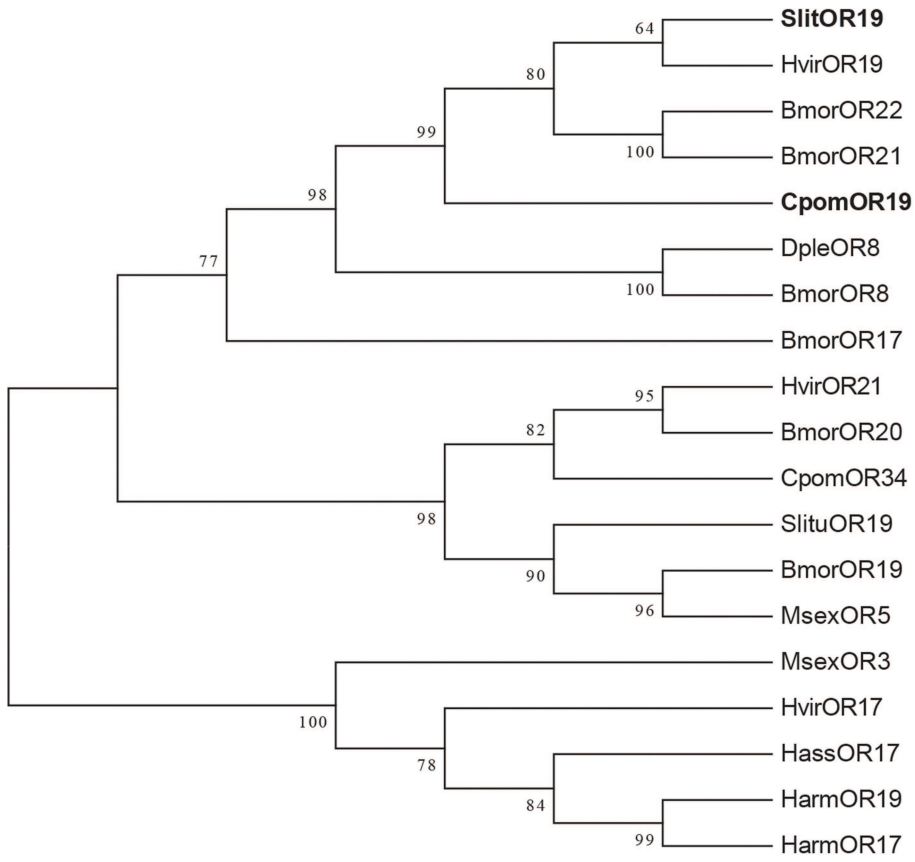

FIGURE 2 | Maximum-likelihood tree of the sequences of *Cydia pomonella* CpomOR19 and homologs from other lepidopteran species. The distance tree is calculated by MEGA6 based on sequence alignment using MAFFT. Branch support is shown for values above 60%. *C. pomonella* (Cpom), *B. mori* (Bmor), *S. littoralis* (Slit), *S. litura* (Slitu), *H. virescens* (Hvir), *D. plexippus* (Dple), *M. sexta* (Msex), *H. assulta* (Hass), *H. armigera* (Harm).

The strongest responses were elicited by 2-methyl-1-indanone and 2-ethyl-1-indanone, followed by 1-indanone and 3-methyl-1-indanone (**Figure 1**).

Dose-response experiments also revealed that both CpomOR19 and SlitOR19 had a lower threshold for 2-methyl-1-indanone and 2-ethyl-1-indanone, reacting to lower amounts of these than to 1-indanone and 3-methyl-1-indanone (**Figure 4**). For 2-methyl-1-indanone, 1 μg on the filter paper was sufficient to elicit a significant response in comparison to the solvent and with correction for differences in vapor pressure taken into account, 2-ethyl-indanone elicited above-threshold responses at quantities below 1 μg. The only significant discrepancy between the two receptors was observed in CpomOR19 that responded more strongly to 3-methyl-indanone than SlitOR19 at a dose of 10 μg.

DISCUSSION

Codling moth *C. pomonella* (Lepidoptera, Tortricidae) and African cotton leafworm *S. littoralis* (Lepidoptera, Noctuidae) share two orthologous ORs with conserved function, CpomOR19 and SlitOR19 (**Figures 1**, **2**). Furthermore, SlitOR19 and CpomOR19 are expressed in adults of both sexes of *S. littoralis* and *C. pomonella* (Bengtsson et al., 2012; Poivet et al., 2013). This is an intriguing finding: in addition to taxonomic position (Kristensen et al., 2007), the two species differ with respect to host plant and feeding habit. *C. pomonella* larvae mine in apple and pear fruit, or in walnuts, whereas *S. littoralis* feeds on the leaves of a very wide range of herbaceous plants (Salama et al., 1971; Bradley et al., 1979). The occurrence of receptors with conserved function and their similar expression patterns likely reflect a role of one or more substituted indanone compounds in the behavioral ecology of these two species.

Structurally and Functionally Conserved ORs

Sequence similarity is not a reliable indicator of OR function. However, our results show that the response profiles of CpomOR19 and SlitOR19, with 58% amino acid identity, are virtually the same: both respond to 1-indanone and structurally related compounds (**Figures 1**, **3A**). Similarly, pheromone receptors from heliothinae moths, HarmOR14b, HassOR16 and HvirOR6, with amino acid identities between 53 and 65%, all responded to (*Z*)-9-tetradecenal (Jiang et al., 2014). In contrast,

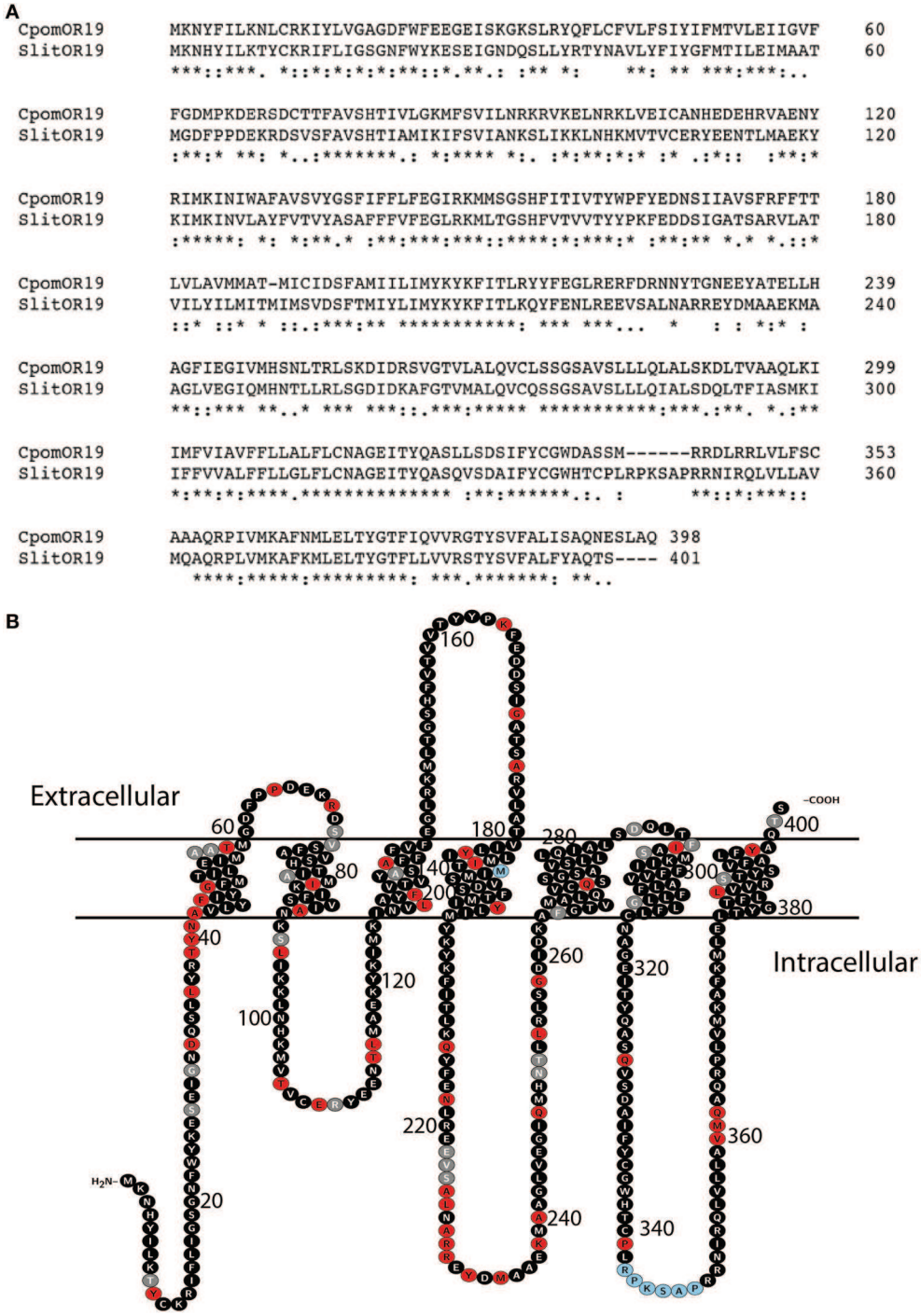

FIGURE 3 | (A) Amino acid alignment of CpomOR19 and SlitOR19. Amino acid sequence differences are indicated as highly (:) and moderately (.) conservative substitutions and non-conservative substitutions (blanks), while asterisks indicate identity across both sequences. **(B)** Putative protein topology of SlitOR19 and its differences with CpomOR19. Gray dots indicate moderately conservative substitutions, red dots indicate non-conservative substitution of residues and light blue dots indicate addition of residues in SlitOR19 as compared to CpomOR19.

a single mutation is enough to change the specificity of a sex pheromone receptor between two species of *Ostrinia* (Leary et al., 2012), demonstrating that minor changes in amino acid sequences can lead to conformational changes in membrane proteins that have profound effects on OR function, specificity and sensitivity (Curran and Engelman, 2003; Hopf et al., 2015).

For CpomOR19 and SlitOR19, most of the non-conserved mutations were found on the first transmembrane region

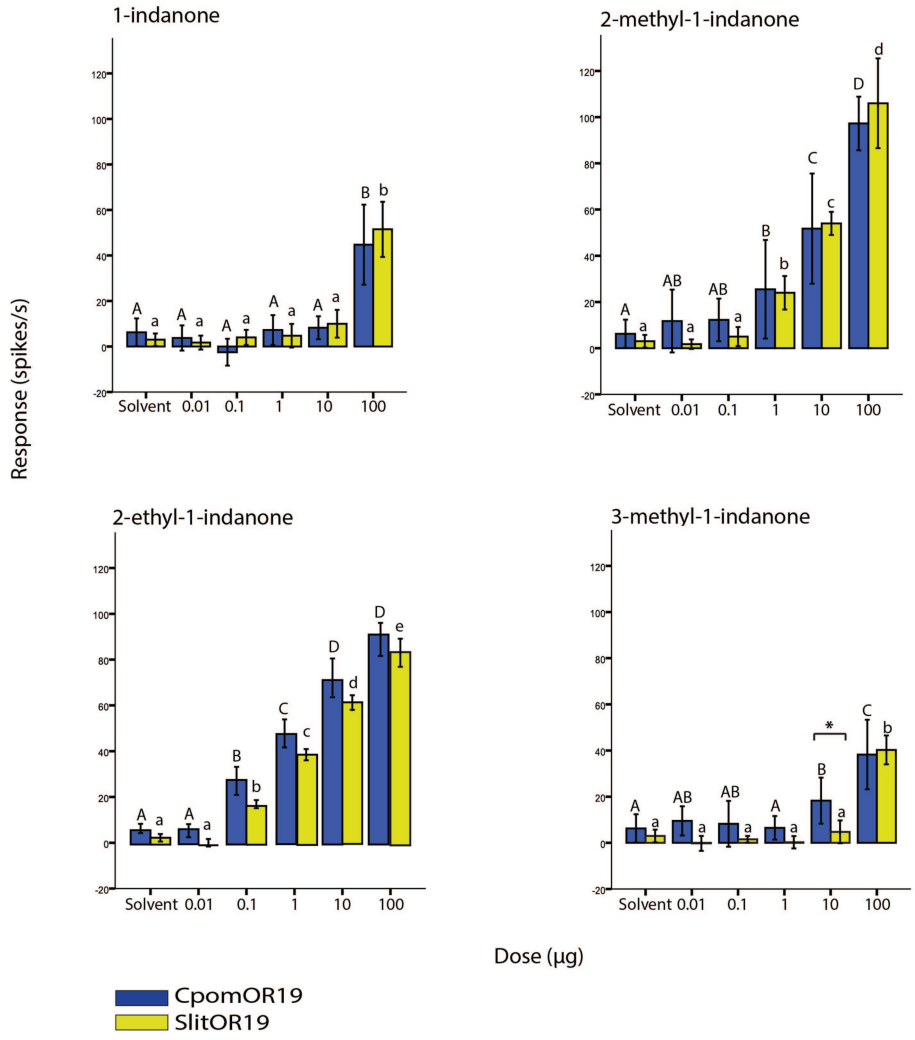

FIGURE 4 | Dose-dependent responses of CpomOR19 and SlitOR19 to 1-indanone and structurally related compounds. Responses to 2-ethyl-1-indanone are adjusted to account for differences in vapor pressure. Bars of the same color followed by different letters indicate subgroups with statistically significant differences. Asterisk denotes significant differences between species for the dose indicated at $P < 0.01$ (Two-way ANOVA with repeated measures, LSD *post-hoc* test, $n = 8$).

and on the intracellular loop 2 of the predicted proteins (**Figure 3B**). Hopf et al. (2015) showed that the N-terminus tail, the extracellular loop 2 and the intracellular loop 3, are kept under strong evolutionary constraint, indicating their functional importance in receptors of *D. melanogaster*. Point mutations within the third and sixth transmembrane regions can affect the sensitivity and selectivity of ORs, as demonstrated by Steinwender et al. (2015) for the pheromone receptor OR7 of *Ctenopseustis oblicuana* and *Ctenopseustis herana,* and may drive speciation events. In CpomOR19 and SlitOR19, these regions show only minor changes, except a deletion of the final four residues of the C-terminus sequence of SlitOR19. However, this deletion did not affect OR tuning, compared with CpomOR19. In contrast, Hill et al. (2015) recently demonstrated that a deletion of the C-terminus in one of the two paralogous ORs in the mosquito *Culex quinquefasciatus* has a profound effect on enantiomeric selectivity. The specific mechanisms governing OR functions remain, however, unknown. It therefore cannot not be excluded that non-conservative mutations concern even functional sites: amino acid interactions, which appear to strongly affect functional properties, may restore receptor tuning.

CpomOR19 and SlitOR19 are Tuned to 1-Indanones

Among the first panel of odorants 1-indanone elicited the strongest response (**Table 1**). Ensueing tests with a number of structurally related 1-indanone analogs showed that the affinity of both ORs to 2-methyl-1-indanone and 2-ethyl-1-indanone was even higher (**Figures 1**, **4**).

Analysis of the molecular receptive range of CpomOR19 and SlitOR19 provides insight into their interaction with odorant ligands. For both ORs, the nature and position of the functional group and the presence and position of methyl and ethyl substituents all affected receptor-ligand interactions.

A carbonyl group in position 1 is required for biological activity, as demonstrated by the lack of response toward alcohols, hydrocarbons and an imine. This is in agreement with Liljefors et al. (1984), showing that the functional group plays an essential role in successful ligand-OR interactions. Acetophenone, a substance which interacts with the receptor through both the carbonyl group and the benzene ring at the same position in space as 1-indanone, did not elicit an OR response. We therefore deduce that the five-membered ring of the indane skeleton is required for biological activity. Finally, a complete lack of response to indan-1,2-dione indicates that the polarity and electron distribution of the additional keto-group intervene and prevent the molecule from binding to the OR. By introducing alkyl substituents as space-probes at different positions of the indane structure, we were able to characterize the degree of complementarity between this part of the substrate and the receptor. A similar approach was taken by Jönsson et al. (1992) to study the interaction of a moth sex pheromone with its receptor cell. Addition of a methyl and ethyl group to the second carbon of the five-membered ring increases the response. This suggests the alkyl group interacts with a complementary receptor site within the OR, that could consist of a hydrophobic "pocket." Our results also indicate that the addition of methyl space-probe groups to the benzene ring (4-, 5- and 6-methyl-1-indanone) decreased biological activity. We hypothesize that these additions caused repulsive, steric interference between the analog and a complementary receptor site of the OR.

Earlier analyses of the molecular receptive range of ORs by electrophysiological recordings from native olfactory sensory neurons (OSNs) support our findings. For example, Stranden et al. (2003) demonstrated structural-activity relationships in the electrophysiological responses of three heliothine moths to the sesquiterpene germacrene D. The selective response of these OSNs to germacrene D was defined by the ten-membered ring system, the position of three double bonds and the position of the isopropyl group. Research on pheromone receptors of the moth *Agrotis segetum* has also shown that changes in shape and bulkiness, length, position of the double bond or nature of the functional group of the (*Z*)-5-decenyl acetate molecule (one of the three pheromone components of this species), have an effect, direct or indirect, on the interaction of the molecules with the receptor binding sites. Here, the acetate group, the double bond and the terminal alkyl chain are the three molecular parts which are most likely responsible for the selectivity of the receptor (Bengtsson et al., 1987, 1990; Jönsson et al., 1991).

The response to the indanone analogs was overall similar for CpomOR19 and SlitOR19, although significant differences were observed in dose-response relationships to 3-methyl-indanone (**Figure 4**). This response shift may be due to residue substitutions. Further experiments, for example including ORs with induced point mutations, are required to reveal the basis of these differences.

The Ecological Role of Indanes is Yet Unknown

Semiochemicals are natural compounds which elicit a behavioral response, and which activate dedicated ORs at low concentrations (Bohbot and Dickens, 2012). *Spodoptera* larval frass, which deters oviposition in conspecific females, contains 1-indanone (Klein et al., 1990; Anderson et al., 1993), but we were unable to corroborate presence of 1-indanone or any other indane in frass collections of *S. littoralis* reared on several diets (data not shown). Indanone is found in roots of tropical plants (Okpekon et al., 2009), decaying wood fungi (Rukachaisirikul et al., 2013), and filamentous marine cyanobacteria (Nagle et al., 2000), which are probably not relevant for *S. littoralis* or *C. pomonella*. However, our results indicate that one or several indanone analogs are ligands for CpomOR19 and SlitOR19, but the source of these compounds and their behavioral and ecological roles are yet to be elucidated.

Pterosins are a group of natural compounds, composed of modified 2-methyl-1-indanones (Syrchina and Semenov, 1982). Pterosins are produced by the fern *Pteridium aquilinum* and are known to be toxic and show anti-feeding effects in various insects (Jones and Firn, 1979). These compounds make good candidates for ligands of CpomOR19 and SlitOR19 since they are similar in structure to 2-methyl-1-indanone, which elicited one of the strongest responses in our screening. Unfortunately we were unable to test pterosins, because they are not commercially available and we did not screen plants producing them. To our knowledge, pterosins are not produced by other plants and ferns are not commonly found in *C. pomonella* and *S. littoralis* habitats, but structurally similar compounds may occur in their host or non-host plants. Further research on plant or insect chemical profiles, together with behavioral studies of substituted indanes, is needed to identify the natural, key ligands for OR19 and to decipher their ecological relevance.

The olfactory and behavioral responses of codling moth and cotton leafworm to host and non-host plants have been studied thoroughly (Bäckman et al., 2001; Bengtsson et al., 2001, 2014; Witzgall et al., 2005; Trona et al., 2010, 2013; Saveer et al., 2012; Binyameen et al., 2013, 2014; Borrero-Echeverry et al., 2015). Our study accentuates that analytical chemistry of current, known host plant associations provides an incomplete pool of compounds for the identification of the ligands mediating insect olfactory behavior. Our comparison of an ortholog OR in *C. pomonella* and *S. littoralis* validates functional characterization of OR repertoires as an alternative approach, leading to a more complete description of the olfactory system.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2015.00131

Supplementary Figure 1 | Response profile of CpomOR19 to synthetic compounds tested at 100 μg on filter paper (mean ± SE, $n = 5$).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter VI

Functional characterization of candidate pheromone receptors of the insect pest *Cydia pomonella* by heterologous expression in HEK293T cells and *Drosophila* OSNs

Functional characterization of candidate pheromone receptors of the insect pest *Cydia pomonella* by heterologous expression in HEK293T cells and *Drosophila* OSNs

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Abstract

Investigation of functional mechanisms of insect olfactory receptors (ORs) opens for novel control strategies based on interference with insect communication. In a recent study, we identified candidate pheromone receptors of the codling moth *Cydia pomonella* L. (CpomPRs), one of the major agricultural pest worldwide, among which we demonstrated CpomOR3 responding to the *Cydia*-kairomone pear ester (ethyl-(E,Z)-2,4-decadienoate). By heterologous expression in Human Embryonic Kidney cells (HEK293T), here we determined another CpomPR, CpomOR6, responding to (E,E)-8,10-dodecadien-1-yl acetate, a strong antagonist of *C. pomonella* male attraction to its pheromone. Using HEK cells as well ab3 basiconic sensilla of *Drosophila melanongaster*, we further confirmed CpomOR3 response spectrum to pear ester and to its analogue methyl-(E,Z)-2,4-decadienoate. To our knowledge, this is the first study to compare and demonstrate the feasibility of both heterologous expression and deorphanization methods.

Introduction

Within the insect order of Lepidoptera, tortricids represent one of the most economically important group of pest for crops cultivations worldwide.

Among tortricids, the codling moth *Cydia pomonella* (L.) is a key pest in apple, pear and walnut orchards both in Palearctic and Nearctic regions. The codling moth is distributed over all continents except Antarctica, and has one to five generations per year, with higher number of generations in warmer climates (codling moth information support system, http://ipmnet.org/codlingmoth/). Damage to the crop can be extensive, from 20 to 90% of fruits depending on the host species, and as a polyphagous insect, *C. pomonella* can target apple, pear, and walnuts, but at times also plums and other cultivated fruits in proximity of infected orchards (Pest notes, University of California, 2011). These features make the codling moth one of the most notorious pests among tortricids representing the best model to study this insect family.

The codling moth, like most insects, relies on olfaction, to search for food and mates, and to find suitable substrates for oviposition (Witzgall et al. 1999; 2005). It is thus of great general interest to understand mechanisms of odor perception, in order to exploit them for setting up new control methods of insect pests. A successful application of olfactory-based control of insect pests is that of pheromone-based mating disruption. Mating disruption is one of the most efficient approaches for the control of tortricids and in particular for the management of *C. pomonella* L. (Ridgway et al. 1990; Witzgall et al. 2008).

In insects, odorants are detected by olfactory sensory neurons (OSNs) that innervate specialized cuticular sensilla, mostly found on the antennal surface. The detection of odors is mediated by expression of specific olfactory receptors (ORs) working together with the olfactory co-receptor (Orco) as heteromeric complex of unknown stoichiometry but comprising at least one variable odorant-binding subunit (OR) together with the co-expressed universal integral part of Orco on the plasma membrane of OSNs (Benton et al. 2006).
Orco is an insect OR but is unique in that it is highly conserved in insect species, whereas conventional ORs are highly divergent within and between species, and it is expressed in most OSNs, whereas conventional ORs are expressed only in specific subsets of OSNs (Vosshall et al. 2000; Krieger et al. 2003; Nakagawa et al. 2005). Requirements of Orco for OR function are proved from disrupted behavioral and electrophysiological response to odorants when the gene codifying the Orco subunit is inactivated (Larsson et al. 2004).

The number of ORs expressed in their proper subset of OSNs and their compound-specificity determine the range of odorants an insect can detect. The quality, intensity and temporal pattern of odorant stimuli perception are encoded by OSNs and processed within the brain (Hansson and Anton, 2000). General ORs are tuned to environmental odors including plant volatiles, while pheromone receptors (PRs), a male-biased receptor clade between ORs, respond mainly to sex pheromones (Jacquin-Joly and Merlin, 2004; Ihara et al. 2013, Leal et al. 2013, Trona et al. 2013). However, in moths, odorant receptors clading as PRs may respond to non-pheromone compounds (Bengtsson et al. 2014, Jordan et al. 2009).

Earlier studies suggested that insect ORs and PRs that transduce chemical signals into electrical signals were GPCRs, functioning via a heterotrimeric G-protein-mediated second messenger cascade (Krieger and Breer, 1999; Jacquin-Joly and Merlin, 2004). However, a more recent physiological analysis of ORs provided evidences of ORs and PRs functioning as heteromeric odorant-gated ion channels together with Orco (Sato et al. 2008). Coutrary from findings of ORs as ionotropic receptors, last findings suggested that insect ORs might function as metabotropic receptors since second messengers activating protein kinase C, modulate responses to odorants through the phosphorylation of Orco (Sargsyan et al. 2011; Getahun et al. 2013).
An initial transcriptome analysis of the codling moth antenna reveled 43 putative olfactory receptors, of which five clustered in the so-called pheromone-receptor clade (Bengtsson et al. 2012). Nevertheless, Walker et al. (Walker, W.B.; Gonzalez, F.; Garczinsky, S.; Witzgall, P. The chemosensory receptors of codling moth *Cydia pomonella* - expression in larvae and adults. Submitted for publication, 2015),

revised and increased the list of total putative ORs to 58, of which 12 are grouped in the PR-clade. In their study, they compared the newly available transcriptome from other tortricids and adjusted the nomenclature of the previously predicted CpomORs, renaming the previously predicted PR candidates CpomOR1, CpomOR3, CpomOR4, CpomOR5 and CpomOR6, to CpomOR6, CpomOR3, CpomOR1, CpomOR2 and CpomOR4 respectively.

We recently performed heterologous expression of CpomORs in *Drosophila melanogaster* OSNs both by means of the $Or67^{GAL4}$ line in trichoid T1 sensilla (Kurtovic et al. 2007), and by the *δ-halo* mutant line lacking Or22a/b that is normally expressed in ab3A neurons (empty neuron) in basiconic sensilla (Dobritsa, 2003). By single sensillum recordings (SSR) from OSNs expressing one of these candidate pheromone receptors, CpomOR3, we showed that it responded strongly to ethyl-(E,Z)-2,4-decadienoate [pear ester, (E,Z)-ED, Bengtsson et al. 2014]. Surprisingly, either no pheromonal compounds emitted by *C. pomonella* females or closely related species within the genus *Cydia* (Witzgall et al. 1996, 2001) were found to activate CpomOR3. Sensing of a synergist for a CpomPR-candidate and not of pheromones suggested involvement of CpomOR3 in activation of synergic effects to pheromones when binding pear ester. Support came from previous findings of the proximity in the codling moth Antennal Lobe (AL) of glomeruli activated by (E,Z)-ED with glomeruli activated by codlemone, the main pheromone compound of *C. pomonella* (Roelofs et al. 1971). Such findings identified a strong activation of these glomeruli when (E,Z)-ED is sensed together with codlemone (Trona et al. 2010, 2013). Indeed, volatile compounds emitted from host-plants, such as pear ester, are known to enhance male attraction to female sex odors of codling moth (Light et al. 1993; Light et al. 2001; Yang et al. 2004) and variations in the proportion of the same volatiles released from apple, pear and walnut, was demonstrated to greatly affect oviposition (Witzgall et al. 2005).

Apart from heterologous expression in *D. melanogaster*, functional characterization of candidate pheromone receptors from other tortricids, such as *Ctenopseustis obliquana* and *Ctenopseustis herana*, has recently been obtained using a heterologous expression system based on Human Embryonic Kidney cells (Steinwender et al. 2015). Moreover, a variety of similar methods of PRs deorphanization, including the use of *Xenopus* oocytes (Sakurai et al. 2004; Nakagawa et al. 2005; Mitsuno et al. 2008; Miura et al. 2010; Wanner et al. 2010; Wang et al. 2011) and other HEK cell types (Grosse-Wilde et al. 2007; Forstner et al. 2009) have been successfully explored.

Even though part of these studies (Grosse-Wilde et al. 2007; Forstner et al. 2009) report successful activation of moth PRs coupled to the inositol trisphosphate cascade activated by mouse Gα15 (Offermanns and Simon, 1995), PRs from tortricids expressed in a cell-based system can also be activated when co-expressed with Orco (Steinwender et al. 2015). Among multiple sensory modalities for activation of insect olfactory receptors (Sakurai et al. 2014), Orco plays a foundamental role (Larsson et al. 2004; Benton et al. 2006; Sargsyan et al. 2011; Getahun et al. 2013). Co-expression of PRs with Orco may represent a promising strategy for a better understanding of molecular and physiological mechanisms at the base of insect olfactory systems.

In our study, we functionally expressed the codling moth olfactory co-receptor (CpomOrco) and to build upon previous results in *C. pomonella*, we used a cell-based method (HEK293T) to characterize two

odorant receptors from the so-called PR clade, co-expressed with CpomOrco. We contrast these results with functional findings obtained with the *D. melanogaster* system based on targeting expression of CpomPRs both in tricoid T1 sensilla and in the empty neuron (*δ-halo*). These findings represent an important breakthrough for the deorphanization of codling moth olfactory receptors, that is an essential step for understanding the mechanisms of insect attraction to biologically relevant odors and, consequently, for exploiting and setting up innovative insect control strategies based on the interference with olfactory communication.

Results

Rapid amplification of cDNA ends (RACE) PCR of the pheromone receptor CpomOR6

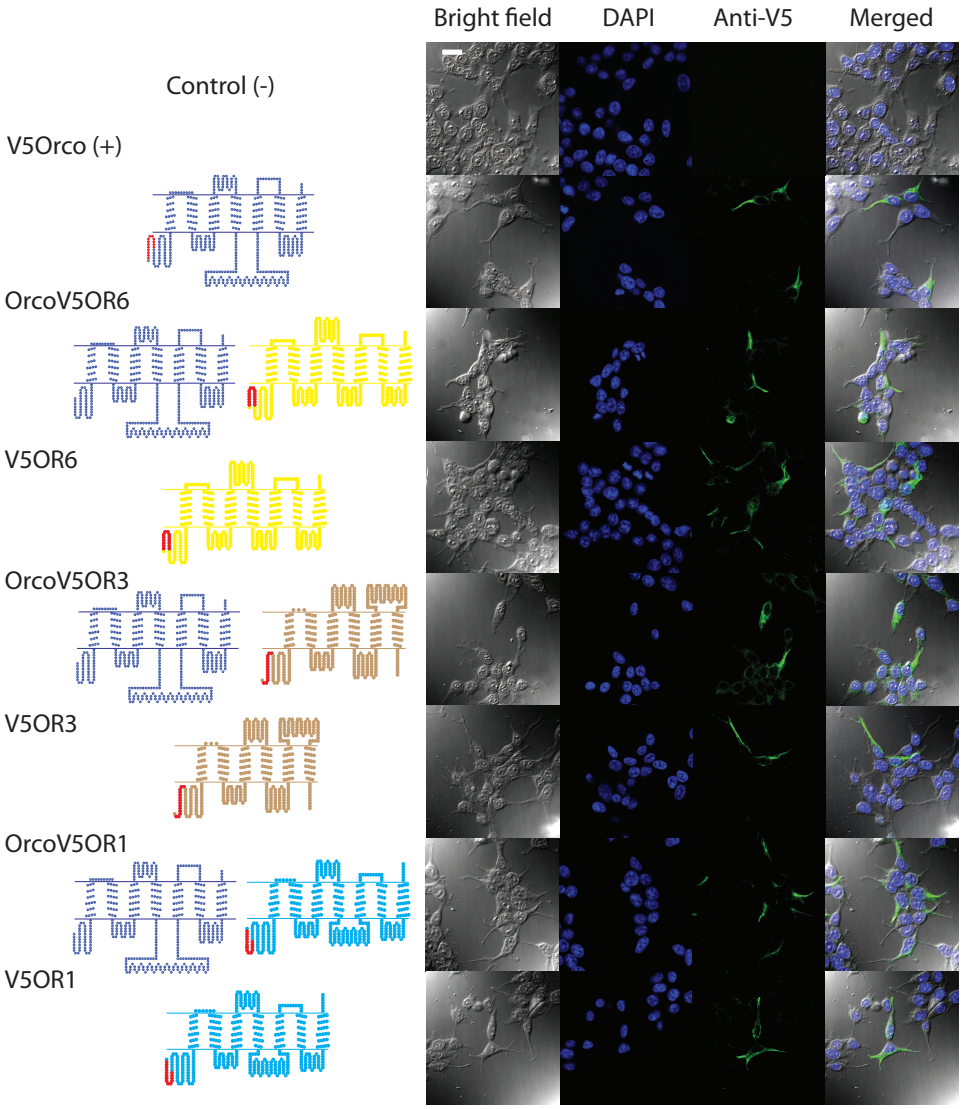
The partial sequence of CpomOR6 was judged to be incomplete at 5' because of lack of start codon in frame. In an attempt to extend the sequence to full length, we performed 5' RACE-PCR. Merging the sequence consensus of a 1191 bp 5'RACE-PCR product together with the partial contig sequence of 306 bp, led to a transcript containing a complete ORF of 1248 bp. The full sequence has been submitted to Genbank (JN836671).

To confirm the correct identification of CpomOR6 as an olfactory receptor, the number of transmembrane domains was predicted using TMHMM 2.0 and TMPred. Both algorithms predicted the seven transmembrane domains expected for an olfactory receptor and intracellular localization for the N-terminal, which is typical of the seven-transmembrane topology of insect olfactory receptors (Lundina et al. 2007). Like CpomOR6, TMHMM 2.0 and TMPred predictions revealed CpomOR1 likewise exhibit seven transmembrane topology and orientation expected for insect ORs, while six transmembrane topology and orientation were predicted for CpomOR3 as already reported (Bengtsson et al., 2014). Transmembrane orientations were estimated using TOPO2 (Figure 1, left).

Immunohistochemistry of olfactory receptors

Immunohistochemical experiments were performed using V5-CpomOrco transfected cells as a positive control (Figure 1) because of the functional expression of CpomOrco (Figure 2). Non-transfected cells were used as a negative control. Clear staining of the plasma membrane in HEK cells using the V5 Epitope Tag Antibody DyLight 488 conjugate (E10/V4RR) for the V5-tags translated on the N-terminal region indicated correct expression of the heterologously expressed olfactory receptors. Comparison of HEK-cells transfected with ORs and Orco, and HEK-cells transfected with only ORs, showed clear labeling of the plasma membrane in both, indicating that CpomORs were correctly expressed and targeted with and without CpomOrco.

Figure 1 Immunohistochemistry. Left: topological representation of codling moth olfactory receptors (TOPO2): dark blue: CpomOrco; yellow: CpomOR6; brown: CpomOR3; light blue: CpomOR1; red: N-terminal V5-tag. Right: Confocal microscopy analysis (bright field, DAPI, Anti-V5, Merged) of expression and targeting of olfactory receptors for HEK293T: co-transfections of CpomOrco+V5ORs DNA (OrcoV5OR6, OrcoV5OR3, OrcoV5OR1) and transfections with only V5OR DNA (V5OR6, V5OR3, V5OR1). White bar: 20 µm. Blue: nuclei; Green: plasma membrane staining.



Study of physiological properties of homomeric and heteromeric codling moth olfactory co-receptor

To study physiological properties of CpomOrco, activation of the receptor was initially investigated on transfected HEK293T stimulated with 250 µM Acetamide,*N*-(4-ethylphenyl)-2-[[4-ethyl-5-(3-pyridinyl)-4*H*-1,2,4-triazol-3-yl]thio]- (VUAA1) in comparison with stable transformed HEK-cells expressing Orco of *Anopheles gambiae* (AgamOrco) (Figure 2, A). Response to VUAA1 was recorded using calcium imaging and the fluorescence variation of responsive cells was compared between stable preparations expressing AgamOrco and transient preparations transfected with CpomOrco, or CpomOrco+OR1. As expected, kinetic of response analysis suggested higher sensitivity for stable transformed cells expressing AgamOrco alone, but when CpomOrco was co-expressed with OR1, apparent higher sensitivity to the ligand by faster recovery to the basal fluorescence compared with CpomOrco, suggested the capability of our system to express functionally both codling moth receptors. As part of these experiments, we tested CpomOrco transfected cells stimulating with 250 µM VUAA1. Calcium imaging revealed sincronised fluorescence response testing cells within 80-second trials (Figure 2, B2). Comparing amplitudes of the calcium responses (Figure 2, B3) with whole-cell patch-clamp

action potential from a single cell of the same responsive cluster (Figure 2, B4) we confirmed activation of the codling moth olfactory co-receptor when stimulated with VUAA1.

To investigate on different activation modalities between CpomOrco homomeric and CpomOrco+OR heteromeric complexes, we performed initial experiments comparing responses to VUAA-compounds between different preparations. Sensitivities between CpomOrco and CpomOrco+ORs to VUAA-compounds were tested performing dose-response experiments to VUAA1 and the analogous acetamide,2-[[4-ethyl-5-(4-pyridinyl)-4*H*-1,2,4-triazol-3-yl]thio]-*N*-[4-(1-methylethyl)phenyl]- (VUAA3) (Figure 2, C1-2). Dose/response experiments showed different sensitivities for these compounds for different preparations expressing CpomOrco rather than CpomOrco+ORs (Figure 2, C2). CpomOrco transfections revealed less sensitivity to VUAA compounds than CpomOrco+ORs co-transfections. VUAA1 tests revealed less sensitivity for CpomOrco than CpomOrco+OR1, +OR3 and +OR6. VUAA3 tests revealed less sensitivity for CpomOrco than CpomOrco+OR6, +OR1 and +OR3. Different sensitivies between CpomOrco and CpomOrco+ORs may suggest functional expression of CpomORs in co-transfected preparations. Interestingly, although solubility constant of VUAA1 and VUAA3 are reported as 36 µM and 17 µM respectively (Scifinder, 2015; Chemical Abstracts Service: Columbus, OH, 2015; CAS Registry Number 525582-84-7 and 585550-72-7; accessed Nov 12, 2015), even unsoluble aliquotes up to 1000 µM were not able to saturate our system. This suggested CpomOrco to be possibly even more active to higher concentrations of VUAAs. For this motivation, EC50s to VUAA compounds are not reported.

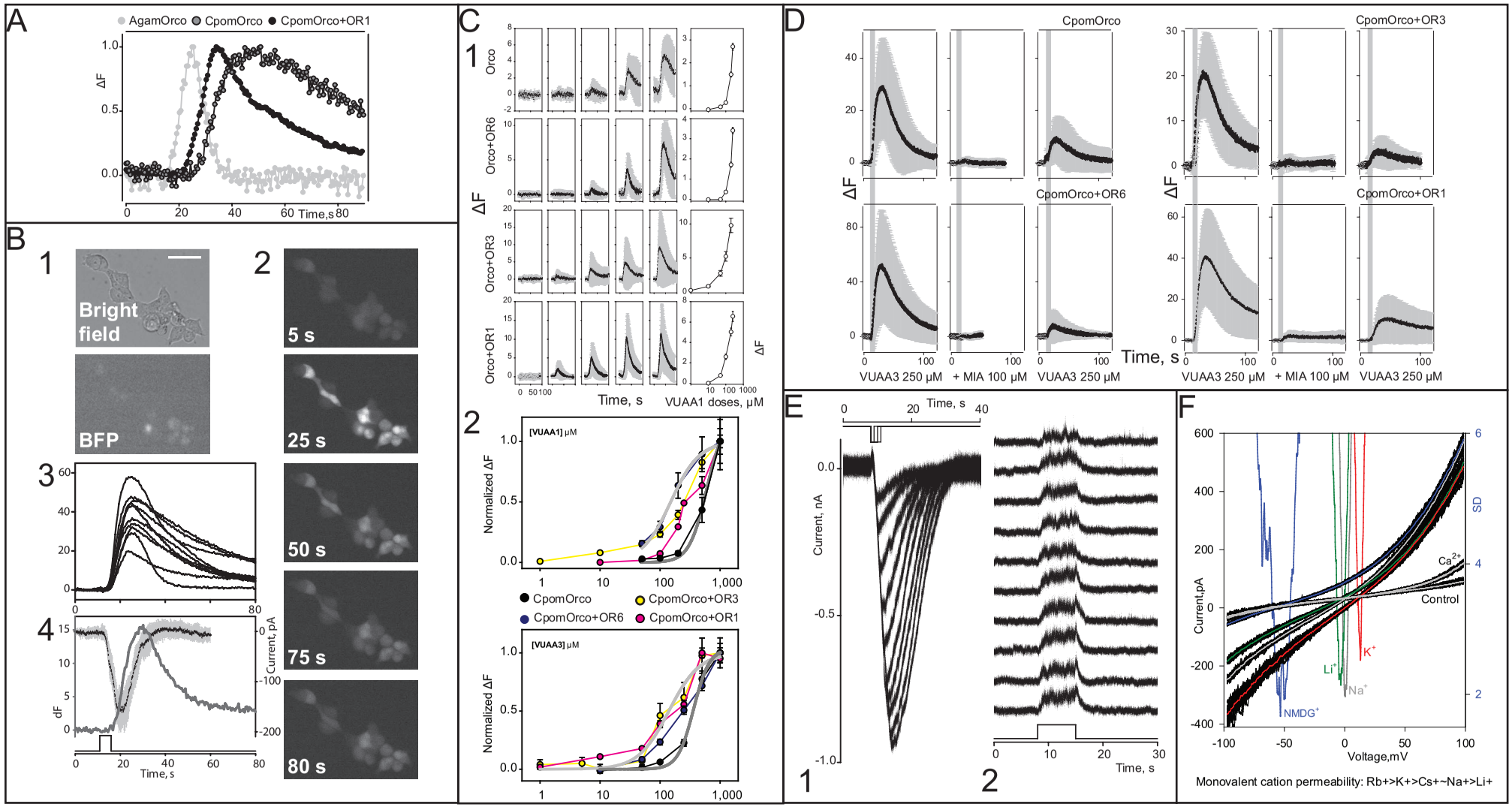
To test physiological inactivation of the olfactory co-receptor, we performed inhibitory experiments using the amiloride derivative 5-(N-methyl-N-isobutyl)amiloride (MIA). We confirmed CpomOrco sensitivity to the MIA-inhibitor validating inactivation to 250 µM VUAA3 response after incubation with 100 µM MIA (Figure 2, D). Comparing CpomOrco transfected cells and cells transfected with CpomOrco+ORs, inhibition was observed in any case.

With the aim to investigate on a possible ionotropic rather than a metabotropic activation modality for CpomOrco, we performed whole cell and outside-out patch-clamp recordings (Figure 2, E1-2) measuring VUAA3-evoked integral and unitary currents on HEK293T cells transfected with CpomOrco+OR1 when stimulated with 250 µM.

Whole-cell patch-clamp recordings demonstrated low conductance and flickering gating kinetics and repeatable increased response to 100 ms increased applications of VUAA3 stimulus (Figure 2, E1). The same result was validated for unitary currents on HEK293T cells performing outside-out patch-clamp recordings (Figure 2, E2). This finding suggested ionotropic activation of the CpomOrco+OR1 complex.

To study monovalent cation permeability properties for codling moth Orco+OR complexes, we measure the shift in reversal potentials of the channel currents (Vr values, voltage axis) induced by stimulation with 250 µM VUAA3 on a CpomOrco+OR1 preparation (Figure 2, F). When Na^+ buffer was replaced randomly by other buffers containing different monovalent cations (Rb^+; K^+; Cs^+; Na^+; Li), we validated different reversal potentials following the sequence $Rb^+ > K^+ > Cs^+ \sim Na^+ > Li^+$. This suggested K^+ to be the most permeable monovalent cation for OR-complexes of the codling moth, followed by Na^+, in olfactory sensory neurones of the insect.

Figure 2 Functional expression and electrophysiological studies of CpomOrco. A, Amplitudes of the calcium responses to 250 µM VUAA1 stimulus between CpomOrco and CpomOrco+OR1 transient transfected HEK293T and AgamOrco stable transfected HEK293. B, Activation of HEK293T cells expressing CpomOrco by VUAA1 stimulus (250 µM): (1) bright field and BFP positive cells (Bar: 20 µm); (2) time scale of activation before stimulus (5 s) and after stimulus (25-50-75-80 s). (3) Amplitudes of the calcium responses measured by calcium imaging, after stimulus. (4) Amplitude track of one cell and integral current track measured by whole-cell patch-clamp recording of the same cell, after stimulus. C, Response to VUAA1 of CpomOrco and CpomOrco+ORs transfected cells. (1) Dose-response means of HEK293T cells expressing CpomOrco, CpomOrco+OR6, CpomOrco+OR1 (VUAA1 doses: 10-50-100-200-250 µM), and CpomOrco+OR3 (VUAA1 doses: 1-10-50-100-250 µM). Grey color depicts standard deviation. (2) Normalized dose/response curves to VUAA1 and VUAA3. VUAA1 doses: for CpomOrco and CpomOrco+OR6, 50-100-200-500-1000 µM; for CpomOrco+OR3, 1-10-50-100-200-500-1000 µM; for CpomOrco+OR1, 10-50-100-200-250-500-1000 µM. VUAA3 doses: for CpomOrco and CpomOrco+OR6, 10-50-100-250-500-1000 µM; for CpomOrco+OR3, 1-5-10-50-100-250-500-1000 µM; for CpomOrco+OR1, 1-10-50-100-250-500-1000 µM. D, Inhibitory experiments with MIA: amplitude means to 250 µM VUAA3 stimulus before (left) and after (middle) incubation with 100 µM MIA inhibitor. Recovery of 250 µM VUAA3 response after MIA-washing (right). Vertical bars: stimulus. E, 250 µM VUAA3-evoked integral (1) and unitary currents (2) recorded from HEK293T expressing CpomOrco+OR1. Ordinate: whole-cell current, nA. Abscissa: time, s. F, Permeation of monovalent cations through CpomOrco+OR1 complex. Black lines: whole-cell current-voltage characteristics; colored lines: averaged current traces; parabola-shaped lines: standard deviation (SD) for each point in the average current trace; right-hand axis: SD values of the average current function; left-hand axis: current (pA); x-axis: reversal potential.


A
AgamOrco
CpomOrco
CpomOrco+OR1
ΔF
Time,s
B
1
Bright field
BFP
2
5 s
25 s
50 s
75 s
80 s
3
4
dF
Current, pA
Time, s
C
1
Orco
Orco+OR6
Orco+OR3
Orco+OR1
ΔF
Time, s
VUAA1 doses, μM
2
[VUAA1] μM
[VUAA3] μM
Normalized ΔF
CpomOrco
CpomOrco+OR3
CpomOrco+OR6
CpomOrco+OR1
D
CpomOrco
CpomOrco+OR3
CpomOrco+OR6
CpomOrco+OR1
ΔF
Time, s
VUAA3 250 μM
+ MIA 100 μM
VUAA3 250 μM
E
Time, s
Current, nA
1
2
F
Current,pA
Voltage,mV
SD
Ca2+
Control
K+
Li+
Na+
NMDG+
Monovalent cation permeability: Rb+>K+>Cs+~Na+>Li+

Functional expression and deorphanization of codling moth olfactory receptors

HEK293T expressing CpomOrco+OR6, CpomOrco+OR3 and CpomOrco+OR1 were tested for response to a library of codling moth pheromones and synergist compounds, previously reported to be active on the insect (Table 1). Additional compounds, e.g. plant volatiles, volatiles from fermentation and commercial drugs, were also tested (Table S1).

Table 1 Main codling moth pheromones and synergists.

compound	MW (g/mol)	Solubility (M)	LogP	Boiling point (°C at 760 mmHg)	CAS	Source	Reference
(-)-β-caryophyllene	204.35	3.40E-08	6.416±0.248	268.4±10.0	87-44-5	Sigma	*53*
(*E*)-β-farnesene	204.35	1.50E-08	6.139±0.304	272.5±20.0	18794-84-8	Bedoukian	*2 & 53*
(E,E)-8,10-dodecadien-1-yl-acetate (codlemone acetate)	224.34	3.20E-04	5.061±0.223	314.7±11.0	53880-51-6D	Bedoukian Inc	*2*
(*E,E*)-8,10-dodecadienol (codlemone)	182.30	2.50E-04	4.096±0.204	270.7±9.0	76600-88-9	Fluka	*2*
(E,E)-α-farnesene	204.35	1.00E-08	6.304±0.316	279.6±20.0	502-61-4	Bedaukian	*53*
(*Z*)-3-hexenol	100.16	0.14	1.697±0.206	156.5±0.0	928-96-1	Aldrich	*53*
(*Z*)-3-hexenyl acetate	142.20	0.025	2.400±0.228	174.2±19.0	3681-71-8	Gift from Prof Peter Witzgall[2]	*53*
1,8-p-menthadien-7-al (perillaldehyde)	150.22	6.10E-03	3.053±0.335	238.0±29.0	2111-75-3	Gift from Prof Angela Bassoli[7]	*8*
1-dodecanol	186.33	5.00E-05	4.914±0.177	258.0±3.0	112-53-8	Sigma Aldrich	*2*
3-(4-methyl-1-oxopentyl)furan-(perillaketone)	166.22	2.10E-03	2.851±0.318	224.4±13.0	553-84-4	Gift from Prof Angela Bassoli[7]	*8*
butyl hexanoate	172.26	2.30E-03	3.842±0.205	206.8±8.0	626-82-4	Bedoukian	*2*
ethyl-(E,Z)-2,4-decadienoate [(E,Z)-ED]	196.29	1.00E-03	4.454±0.229	264.7±9.0	3025-30-7	Aldrich	*2 & 53*
Linalool	154.25	6.70E-03	2.795±0.263	198.5±0.0	78-70-6	Firmenich	*53*
methyl salicilate	152.15	0.021	2.523±0.240	222.0±0.0	119-36-8	Fluka	*53*
methyl-(E,Z)-2,4-decadienoate [(E,Z)-MD]	182.26	2.30E-03	3.944±0.229	246.0±9.0	4493-42-9	Gift from Prof Peter Witzgall[2]	*22*
nonanal	142.24	2.30E-03	3.461±0.223	190.8±3.0	124-19-6	Aldrich	*53*
(E)-β-ocimene	136.23	2.00E-05	4.418±0.275	175.2±10.0	3779-61-1	Fluka	*53*

In tests with CpomOR6, we observed clear activation in response to stimulation with codlemone acetate (Figure 3, A1-2). Subsequent EC50 estimations indicated codlemone acetate EC50 = 51.84 ± 13.21 µM (n = 68, Figure 3, A2), however amplitude at saturating concentrations (18.91 ± 10.31) was only ~28% of the positive control amplitude (69.71 ± 27.29; Figure 3, A1-2). Interestingly, compared to the positive control, we observed a long lasting codlemone acetate activation of HEK293Ts, which led to a delayed

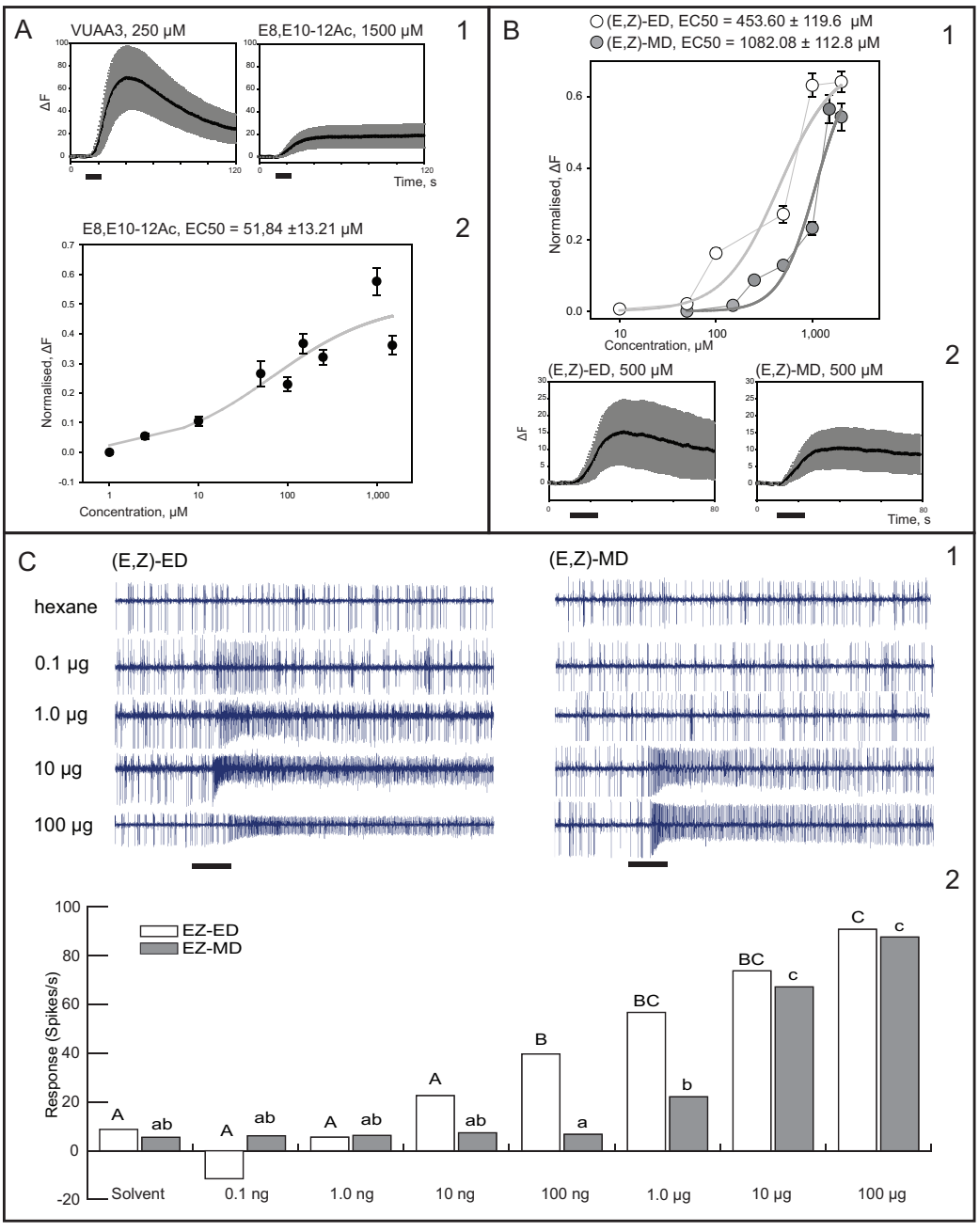
recovery after stimulation. This necessitated long intervals between recordings, in order to allow cells to recover completely after stimulus.

According with our previous investigations in *Drosophila* OSNs (Bengtsson et al. 2014) we tested activation of CpomOR6 expressed in T1 sensilla towards the same library of synthetic pheromones, synergists and combination of synergists with the main pheromone of *C. pomonella* (codlemone, (E,E)-8-10-dodecadien-1-ol) (Table S2). No significant response was recorded for any of the compounds we tested (spikes/s = 1.02, n = 3), neither for combinations (spikes/s = 0.56, n = 3). In addition, applications of a dose of codlemone up to 100 ng revealed no response of the receptor (spikes/s = 2.67, n = 3). Testing (E,E)-8-10-dodecadien-1-yl-acetate very low response was recorded (spike/s = 1.33, n = 3). Between our heterologous expression methods, we confirmed CpomOR6 to be activated only when expressed in HEK293T.

In a previous study, we demonstrated that the predicted pheromone receptor CpomOR3 responded to ethyl-(E,Z)-2,4-decadienoate, (E,Z)-ED (Bengtsson et al. 2014), and here we confirm this result in HEK293T. Furthermore, we show that an analogous ester emitted by pear, methyl-(E,Z)-2,4-decadienoate [(E,Z)-MD, Knight and Light, 2001], also activates CpomOR3 (Figure 3, B1-2). EC50 estimations ($EC50_{HEK\text{-}(E,Z)\text{-}ED}$ = 453.60 ± 119.6 µM; $EC50_{HEK\text{-}(E,Z)\text{-}MD}$ = 1082.08 ± 112.8 µM) and dose/response plots (Figure 3, B1) suggested lower CpomOrco+OR3 specificity for (E,Z)-MD than (E,Z)-ED. Furthermore, longest delay in recovery after HEK293T stimulation with (E,Z)-MD was observed (Figure 3, B2).
For the dose/response of (E,Z)-ED when CpomOR3 was heterologously expressed in *Drosophila* ab3A-neurons, a repeated measures ANOVA determined that different doses of the compound elicited significant differences in OR3 ($F(7, 91) = 42.17$, $P < 0.001$). Post hoc tests using the Bonferroni correction revealed that CpomOR3 needed a minimum concentration of 100 ng of (E,Z)-ED to elicit a response significantly different from the solvent ($p = 0.026$). On the other hand, for the dose/response of (E,Z)-MD a repeated measures ANOVA determined that different doses of (E,Z)-MD also elicited significant differences in CpomOR3 ($F(7, 84) = 41.68$, $P < 0.001$). Post hoc tests using the Bonferroni correction revealed that OR3 needed a minimum concentration of 10 µg of (E,Z)-MD to elicit a response significantly different from the solvent ($p = 0.020$).
Application of different heterologous expression systems confirmed CpomOR3 sensitivity to both (E,Z)-ED and its analogous (E,Z)-MD, with higher sensitivity for (E,Z)-ED rather than (E,Z)-MD was validated.

Although dose/response experiments with VUAAs suggested functional expression of CpomOR1 when co-transfected with CpomOrco in HEK293T (Figure 2, C), testing compounds of Table 1 and Table S1 on CpomOR1 revealed no ligands activating the receptor.
Testing CpomOR1 expressed in *Drosophila* T1 OSN to pheromones and synergists (spikes/s = 0.61, n = 5) and their combinations with codlemone (spikes/s = 0.00, n = 5) (Table S2), no evident response was revealed. In addition, applications of a dose of codlemone up to 100 ng was unable to activate the receptor (spikes/s = -2.40, n = 5).

Figure 3 Functional expression of codling moth olfactory receptors. A, Functional expression of CpomOrco+OR6 in HEK293T. (1) Amplitudes of the calcium responses (mean ± SEM) to 250 µM VUAA3 positive control (69.71 ± 27.29 at time = 40 s; left) and to 1500 µM (E,E)-8,10-codlemone acetate (18.91 ± 10.31 at time = 40 s; right); n = 68. Black bar: stimulus. (2) Normalized dose/response plot to codlemone acetate. B, Functional expression of CpomOR3 in HEK293T. (1) Normalized dose/response of (E,Z)-ED (white) and the analogous (E,Z)-MD (grey). (2) Comparison of CpomOrco+OR3 amplitudes of the calcium responses to 500 µM pear ester (15.07 ± 9.48 at time = 30 s; left) and to 500 µM methyl ester (10.40 ± 5.91 at time = 30 s; right); n = 151. Black bar: stimulus. C, Functional expression of CpomOR3 in *Drosophila* ab3 basiconic sensilla. (1) Comparison between SSR-spikes related with CpomOR3 responses to (E,Z)-ED (left) and (E,Z)-MD (right) at 0.1-1.0-10-100 µg doses. Black bar: stimulus. (2) Dose/response thresholds of spikes/s of ab3A-neurones expressing CpomOR3, stimulated with different doses of (E,Z)-ED (white, n = 13) and (E,Z)-MD (grey, n = 13).



Discussion

Functional characterization of CpomOR6 as a pheromone receptor

In our study, we demonstrate that the predicted pheromone receptor OR6 in *C. pomonella*, first identified by transcriptome screening (Bengtsson et al. 2012), detects a minor pheromone component found both in *C. pomonella* and related species, codlemone acetate ((E,E)-8,10-dodecadien-1-yl-acetate, Figure 3). To our knowledge, this is among the first successful deorphanizations of an insect pheromone receptor using the HEK heterologous expression system (Steinwender et al. 2015; Grosse-Wilde et al. 2007; Forstner et al. 2009). Among insect olfactory receptors, receptors for pheromone appear especially difficult to functionally characterize (Sun et al. 2013), as illustrated by our own experiments, where CpomOR6 did not produce any response to codlemone acetate when expressed in T1 sensilla in *D. melanogaster*. The T1 sensilla are known to be involved in pheromone detection in *D. melanogaster*, and are the preferred choice compared to the *δ-halo* system for suspected pheromone receptors, as previous studies have showed PRs to exhibit greater sensitivity when expressed in T1 (Montagné et al. 2012).

Of the first 43 ORs initially identified in *C. pomonella* (Bengtsson et al. 2012), five were predicted to be possible pheromone receptors. Among these, there appeared to be robust male expression for CpomOR6 and CpomOR1 in particular, and hence, both could be possible candidate receptors for codlemone. However, despite the structural similarities between codlemone and codlemone acetate, response to codlemone (5.7 ± 3.64 at time = 40 s; Figure S1) appeared relatively reduced and slow to consider the compound as a possible ligand. Although (E,E)-codlemone acetate elicits an evident response (18.91 ± 10.31 at time = 40 s) lower amplitude than the positive control suggest other isomers, such as (E,Z)-, (Z,E)- and (Z,Z)- codlemone acetates to be possible candidate ligands. Further investigations have to be undertaken to validate this hypothesis. Interestingly, activation of CpomOR6 by (E,E)-codlemone acetate led toward much longer activation of the receptor than activation with VUAA3. This may suggest efficient binding of the ligand and its difficult release from the olfactory receptor.

Apart from the codling moth, in which codlemone acetates are known to be strong pheromone antagonists (Hataway et al. 1974), all four geometric isomers of codlemone acetate are reported to be pheromone compounds in tortricid species. Most tortricids of the Eucosmini and Grapholitini tribes of the subfamily Olethreutinae use codlemone acetate isomers as their main sex pheromones (Witzgall et al. 1996). In the pear moth, *C. pyrivora*, as well as in the pea moth, *C. nigricana*, geometric isomers of (E,E)-8,10-dodecadien-1-yl acetate are powerful attraction antagonists (Witzgall et al. 1993, 1996; Makranczy et al. 1998). Isomer blends of (E,Z) with (E,E)-8,10-dodecadien-1-yl acetate attract males of the North American filberworm *Cydia latiferrana*, pest of acorns, walnuts and hazelnuts (Chambers et al. 2011, Davis et al. 1984). Different European pheromone races of *Cydia splendana* prefer (E,Z)+(E,E) isomers blends or (E,E)+(Z,E) isomers blends of codlemone acetate, depending from their geographical distribution in the continent (South Sweden, or South France, Switzerland and Hungary) where *C. splendana* pests only oak or oak and chestnut (Bengtsson M. et al. 2014). These evidences report how closely related species to *C. pomonella*, some of which also pest plants in the same host-range (e.g. *C. pyrivora*, *C. latiferrana*), use codlemone acetate as a main pheromone component. While speculative, a

possible explanation of the existence of the codlemone acetate receptor in *C. pomonella* may be as a remanence of the former ancestor of the insect. However, conserving a receptor dedicated to detect other species may be important for reproductive isolation. Otherwise, since the pheromone is also emitted by moths within the same host range, their detection may facilates host finding for *C. pomonella*. The arise of a receptor specialized for the detection of a main pheromone compound like codlemone, may likely represent a step towards allopatric speciation of the codling moth.

In *C. pomonella*, SSR results indicated that codlemone acetate isomers are detected by two types of OSNs located in sensilla trichodea on male antennae (Bäckman et al. 2000). One main type of receptor neurons are mostly responsive to the main pheromone of the codling moth: codlemone [(E,E)-8,10-12OH], and tenfold less to codlemone geometric isomers [(Z,E);(E,Z);(Z,Z)], while they are even less responsive to (E,E)-codlemone acetate and other codlemone acetate geometric isomers [(Z,E);(E,Z);(Z,Z)]. A second type of receptor neuron detects all geometric isomers of codlemone acetate, with the (E,E) isomer eliciting the strongest response, and no response for codlemone or any of its geometric isomers. Potentially, CpomOR6 could thus be the receptor found on the second type of receptor neurons, which has a matching response spectrum, responding only to codlemone acetate, and not to codlemone. We consider the existence of a further pheromone receptor, responding mainly to codlemone, and potentially with a secondary, weaker response to codlemone acetate, to be highly likely.

Investigation of the expression and mode of action of Orco

Given the fundamental role of Orco (Larsson et al. 2004; Benton et al. 2006; Sargsyan et al. 2011; Getahun et al. 2013) and hypothesis of ORs functioning with Orco as heteromeric ligand-gated ion channels (Sato et al. 2008), we studied the activity of Orco towards known ligands for this receptor, to validate its functional expression. High-throughput screening on AgamOrco expressing HEK293 cells revealed a particular class of synthetic compounds (VUAAs) able to interact with Orco (Jones et al. 2011). After confirming the activity of VUAA1 in our assay towards the AgamOrco receptor in stable transfected cells, we compared the activity with that of CpomOrco, and CpomOrco+OR1 (Figure 2, A). All cell lines responded to VUAA1, and dose/response with this compound as well as the commercial derivative VUAA3, showed that the saturation point was not reached for any of the lines (CpomOrco, CpomOrco+OR6, CpomOrco+OR3, or CpomOrco+OR1). We speculate that this might be due to the known artifact, where HEK293T, if transient-transfected, are known to be less sensitive then stable-transfected. As we observed polymerization of VUAAs when compounds were diluted at 1000 µM, higher concentrations were not tested. However, validation using left-shift of dose/response plots indicated that our system expressed CpomOR6, CpomOR3 and CpomOR1 functionally when transfected with CpomOrco. Furthermore, immunohistochemistry indicated CpomOrco as well as the CpomORs expressed and targeted correctly (Figure 1).

Whole-cell and outside-out patch-clamp recordings of cell lines with CpomOrco+OR1 showed multiple responses to continuous application of VUAA3, which indicates that CpomOrco+ORs function as ionotropic receptors. Monovalent cation permeability studies confirmed higher permeability for Rb^+. While we identified Rb^+ to be the most permeant monovalent cation of the CpomOrco+OR1 complex,

we note that Rb^+ gradient is not commonly established in biological systems. Interestingly, reports have found high concentrations of K^+ (~200 mM) in the sensillum lymph of moths (Zufall et al. 1991), which we report to be the most permeable monovalent cation for CpomOrco+OR1 after Rb^+, and we suggest that it is likely to be the best candidate for the codling biological system.

Early studies on OSNs of *D. melanogaster* demonstrated DmelOrco (OR83b) to be required as a chaperon to target ORs to plasma membranes of OSNs (Larsson et al. 2004). In contrast, results from immunohistochemistry indicate that for our HEK293T system, ORs are expressed and targeted to plasma membranes of both when independently transfected, and when co-transfected with CpomOrco. However, while correctly expressed and targeted, ORs without CpomOrco appeared non-functional, as we observed in a separate test of (E,Z)-ED on cells transfected with CpomOR3 alone (Figure S2). This concords with patch-clamp results (Figure 2, E1-2), which also indicate in the codling moth that CpomOrco and OR together constitute a functional cation channel required for cation permeation.

Comparison of HEK293T and D. melanogaster methods for heterologous expression

In a previous study, CpomOR3 was shown to respond to pear ester (E,Z)-ED, a non-pheromone compound (Bengtsson et al. 2014). Expression in the HEK293T system validated this response, and furthermore led to the identification of a secondary ligand, the analogous methyl-(E,Z)-2,4-decadienoate [(E,Z)-MD]. Testing HEK293T expressing CpomOR3 to (E,Z)-ED rather than (E,Z)-MD we confirmed evident differences in the sensitivity for these two compounds (453.60 ± 119.6 μM and 1082.08 ± 112.8 μM respectively, Figure 3, B1) and in activation and recoveries of the response after stimulation (Figure 3, B2), which may suggests compound-specific recognition modalities for the codling moth receptor in the presence of these two ligands. Further evidences for this possibility rised from SSR-tests on CpomOR3 expressed in ab3 sensilla, revealing significant differences between dose effects for (E,Z)-ED and (E,Z)-MD (Figure 3, C2). Taking validations from both heterologous systems, we may assume the codling moth to be able to distinguish between these two odorants. This is consistent with our previous observations of different sensitivities reported for CpomOR19 when expressed in ab3 basiconic sensilla responding to different types of alkyl-1-indanones (Gonzalez et al. 2015). The difference between these two compounds in terms of one carbon of the alkyl group, may determine different binding to the receptor, perhaps related to polarity and interaction with the allosteric-site of the protein.

Recent findings reported adjacent glomeruli dedicated to (E,Z)-ED and to codlemone in the codling moth AL producing very strong synergic effects when stimulated with a blend of codlemone and (E,Z)-ED (Trona et al. 2010, 2013). Interaction of (E,Z)-MD with the same receptor binding (E,Z)-ED, may suggest a similar effect at the neurological level in the codling moth for (E,Z)-MD if potentially combined with codlemone. Taking the possibility of a compound specific recognition modality, we would expect different neurological and behavioral effects towards sensing of (E,Z)-ED rather than (E,Z)-MD**.**

In contrast to results with CpomOR3, CpomOR6 failed to elicit any response to codlemone acetate when expressed in *Drosophila* sensilla trichodea (hosting pheromone-binding proteins, PBPs). While the underlying reason(s) as to why CpomOR3 but not CpomOR6 produces response when expressed in

Drosophila olfactory sensilla is unknown, it may be speculated that it could involve different physical properties of the ligands we identified binding these receptors. For instance, the boiling point of codlemone acetate (314.7±11.0 °C), which correlates with its volatility, is the highest among pheromones and synergists we tested. Indeed, boiling points of ethyl and methyl esters, active on the *Drosophila* ab3 basiconic sensilla, are significantly lower [(E,Z)-ED = 264.7±9.0 °C; (E,Z)-MD = 246.0±9.0 °C]. Furthermore, taking vapour pressure of these compounds (mm Hg, 25 °C - modified Grain method, http://www.thegoodscentscompany.com/), codlemone acetate (0.001) compared with esters [(E,Z)-ED = 0.01; (E,Z)-MD = 0.028], is expected to be less volatile of about 10 and 28 times, respectively. Difficult volatility of codlemone acetate when applied on *Drosophila* antennae for SSR recording, may compromise activation of the sensory neuron expressing CpomOR6, when stimulation is performed. Instead, by HEK293T cell system, no influences in phase of applications of this stimulant are expected since this method is based on fluid perfusion.

In both contexts, weak activation of CpomOrco+OR6 in HEK293T when compared with its positive control (Figure 3, A1) and absent activation in *Drosophila* olfactory sensilla may suggest requirements of a specific metabotropic machinery. Further accessory proteins of *C. pomonella*, which are not expressed in our heterologous systems, may be required for signal transduction as suggested by recent findings in metabotropic regulations for the activation of insect olfactory receptors (Sargsyan et al. 2011; Getahun et al. 2013). Potentially, this may also explain the lack of response of CpomOR1 in either heterologous system. Another potential pitfall here is of course a lack of relevant ligands, despite our testing with an extensive panel of pheromone compounds and synergists. Whole cell and outside-out patch-clamp recordings on HEK293T expressing CpomOrco+OR1 suggested an ionotropic activation of Orco+OR subunits (Figure 2, E1-2). As mentioned above (Getahun et al. 2013) intrinsic regulations may be at the base of activation of CpomOR1. Accessory proteins of the transduction machinery, e.g. G-protein and Phospholipase C (Gp+PLC), rather than G-protein and Adenilate Cyclase (Gp+AC) may be required for signal transduction after ligand binding on the olfactory receptor, since the existence of alternative metabotropic models for the activation of insect ORs (Sakurai et al. 2014).

Conclusions & perspectives

Starting from RACE-PCR, we completed, cloned and heterologously expressed the coding sequence of the candidate pheromone receptor CpomOR6, and led to its deorphanization.

Comparing two heterologous expression systems, we validated activation of the receptor by the main pheromone antagonist of the codling moth, codlemone acetate, when expressed in HEK293T rather than in the T1 OSN of *D. melanogaster*. Our findings report for the first time the activation of a pheromone receptor candidate of the codling moth for a pheromone compound, despite belonging to a minority content of the bouquet emitted by females of *C. pomonella*. These evidences, confirmed the nature of CpomOR6 as a pheromone receptor.

Identification of the receptor for codlemone acetate allows better understanding of pheromone sensing mechanisms in the codling moth. For instance, CpomOR6 sensing to codlemone acetate and not to codlemone is in accordance with earlier studies reporting activation of a second type of sensory neurons in male antennae, with specific response to this compound (Bäckman et al. 2000). Furthermore, being

codlemone acetate a main antagonist to codlemone, identification of CpomOR6 activation represents the starting point to validate molecular and neurological properties of its sensing, opening future design for control strategies based on mating disruption in the orchard. Further benefits to applications of mating disruption will rise when the receptor of codlemone will be deorphanised.

Using the same methods, we heterologously expressed CpomOR3, which we previously reported to respond to pear ester (Bengtsson et al. 2014), and we validated activation of this receptor by the main ligand (E,Z)-ED and its analogous (E,Z)-MD.
Testing both heterologous systems, we observed different sensitivity of the receptor, suggesting distinct recognition modalities for the two compounds by the codling moth, according with higher sensitivity for (E,Z)-ED rather than (E,Z)-MD. In any case, activation of CpomOR3 to both compounds when expressed by both heterologous systems confirms the capability of HEK293T and *Drosophila* OSNs to target deorphanization of PR-candidates of our tortricid model, which still represents a complex task in the study of insect sensory proteins.

HEK293T was demonstrated to be a successful alternative of heterologous expression for deorphanization of codling moth olfactory receptors, we up to now performed targeting expression in T1 and *δ-halo* OSNs of *Drosophila* (Bengtsson et al. 2014; Gonzalez et al., 2015). Furthermore, expression of the codling moth Orco in HEK293T allowed the validation of ionotropic activation modalities for the co-receptor, demonstrating the method to be also a functional tool for molecular and physiological studies of activation of insect ORs.

Experimental Procedures

Insect dissection and RNA extraction

C. pomonella pupae were obtained from a laboratory rearing (Andermatt Biocontrol, Grossdietwil, Switzerland), and adults were allowed to emerge in cages kept at 23 °C, 70 ± 5 % RH and 16 h : 8 h light/dark cycle, and were fed 10% sugar solution. For dissections, 2-3 day old female and male insects were used. Using sharp forceps, antennae were removed at the base of the pedicel and immediately flash-frozen using liquid nitrogen, and thereafter kept at -80 °C. RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany), that included a DNase digestion to eliminate genomic DNA contamination. Antennal RNA was quantified using Nanodrop (8000 UV-vis Spectrophotometer, Thermo Scientific, Wilmington, DE, USA).

Rapid amplification of cDNA ends (RACE) PCR

While the full length sequences of CpomOrco, CpomOR3, and CpomOR1 were previously reported (Bengtsson et al. 2012, 2014), RACE PCR was performed to obtain the complete open reading frame of CpomOR6.
Libraries of cDNA were created from antennal RNA using the SMARTer kit (Clontech, Mountain View, CA, USA). Primers sequences were designed by hand using existing contig data as reference, and thermodynamical features were checked by Oligoevaluator (Sygma Genosys,

http://www.oligoevaluator.com/). Putative oligodimerization was checked by oligo analyzer (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx), and melting temperatures were estimated using the salt-adjusted algorithm of the Oligocalc website (http://www.basic.northwestern.edu/biotools/OligoCalc.html).

For primers, the goal was a GC% 40-60, Tm < 70 °C, and to create a product with at least 150 bases of overlap with existing contig data. The designed sequence of the 5'_OR6 primer, which successfully extended the CDS of CpomOR6, is reported in Table 2.

Table 2 Cloning primers.

OR6 5'-RACE Primer	**Sequence**	**Tm (°C)**
5'_OR6	CCCATGGTACTGCATATACTTCATCACCGAGACG	65.42
CDS-primers		
Fw_Orco	ATGATGGGTAAAGTGAAATCTCA	57.60
Rv_Orco	TTACTTCAGTTGTACTAACACCATGA	61.70
Fw_OR6	ATGCAGACAAAAAGGCAAACCAG	61.00
Rv_OR6	TTAGTCTGCGAATGTGGCTAGC	61.00
Fw_OR3	ATGTTTAGTTATGAAAATGAAGACAGC	60.80
Rv_OR3	TTAAGTCATTTCTTCAGTAGAGGT	58.30
Fw_OR1	ATGTCTTTGAAAAGCCGTGTTTGG	62.00
Rv_OR1	TTACCCCTCAGCAGCGAAAG	60.50

SMARTer RACE PCR was performed using an adjusted version of the supplied protocol. Supplied thermostable DNA polymerase was used with a temperature program of 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 1 minute, 65.42 °C for 90 seconds, 68 °C for 2 minutes, and a final elongation of 68 °C for 7 minutes. The 5'_OR6 primer was combined together with Universal primer A mix supplied in the kit, with 2% DMSO per reaction volume added.

PCR products were analyzed by electrophoresis on 1.5% agarose gel. Bands were visualized after staining with ethidium bromide using a Gel Doc XR (Bio-Rad, Hercules, CA, USA). Relevant bands were excised and purified by the Gel extraction kit (Qiagen). Quantification was performed using a Nanodrop 3300 Fluorospectrometer (Thermo Scientific) using the PicoGreen® dsDNA reagent kit (Molecular Probes catalog # P-11496). Samples were direct sequenced (Sanger sequencer, 3730xl Applied Biosystems, Life Technologies) using gene specific primers. The 5' sequenced region was assembled with existing contig data and the candidate CDS was identified using the online tool ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Total RNA extracted from male and female antennae were submitted to full-length cDNAs synthesis using RT-for-PCR kit (Clontech), and the full length CDS was amplified (primers Fw_OR6 and Rv_OR6 in Table 2) and was sequenced to confirm that the assembly was correct. To confirm the identity of the sequence as an olfactory receptor, the nucleotide sequence was converted to amino acids using the ExPASy translate tool (http://web.expasy.org/translate/), after which transmembrane domains were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and TMPred

(http://www.ch.embnet.org/software/TMPRED_form.html). The Topology of the transmembrane protein was verified using TOPO2 (http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py). The completed sequence of OR6 has been deposited in Genbank, JN836671.

Cloning of olfactory receptors into pcDNA5/TO

In order to produce amplicons suitable for cloning into pDONR221 (Invitrogen Life technologies, Grand Island, NY, USA), we located *att*B regions suitable for BP-clonase-recombination upstream of the CDS primer sequences (*att*B1 forward region: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACA-3'; *att*B2 reverse region: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3', Gateway Technology, Invitrogen). CDS primers (Table 2) were designed to amplify full-length CpomOR sequences (Genbank database accession numbers, CpomOrco: JN836672; CpomOR6: JN836671; CpomOR3: KJ420588; CpomOR1: JN836674.1). For forward primers, a *NotI* restriction site (5'-GCGGCCGC-3') followed by the HEK-cell optimized 5'-CACC-3' Kozak sequence (Dr. Richard Newcomb, personal communication) and the gene-specific forward sequence, were located downstream *att*B1 sequence. For reverse primers, an *ApaI* restriction site (5'-GGGCCC-3') followed by the reversed-stop codon (5'-TTA-3') and the gene-specific reverse sequence, were located downstream *att*B2 sequence. To create V5-N-terminal variants suitable for immunohistochemical experiments, 42 nucleotides (5'-GGCAAGCCTATCCCTAATCCTCTGCTGGGCCTGGACAGCACC-3') coding for 14 additional amino acids of a V5-epitope tag (Nt-GKPIPNPLLGLDST-Ct) were added to the forward primer between the start codon and the rest of the gene-specific forward sequence.

A temperature program of 94 °C for 5 minutes was followed by 35 cycles of 94 °C for 1 minute, Tm of the primer for 1 minute, 68 °C for 2 minutes, and a final elongation step of 68 °C for 7 minutes. A 4.0 µL PCR volume was mixed with 1.0 µL BP-clonase (Gateway Technology, Invitrogen) and 150 ng of pDONR221 (Invitrogen), and was incubated for 4 hours at 25 °C. Of this reaction volume, 2.0 µL was used to transform TOP10 competent cells (Invitrogen). After transformation, 50 µL of the reaction was plated on 50 µg/mL Kanamycin selective media and incubated overnight at 37 °C.

Colonies were sampled, and to start cultures they were diluted in 50 µL LB-media, to be grown for 2 hours at 37 °C and 225 rpm. Colony PCR was performed to confirm inserts, using 1.0 µL culture from single colony-volumes with the M13FW universal primer and the relevant reverse OR-primer, with GoTaq Green Master Mix for PCR (Invitrogen). Amplifications were conducted with a temperature program of 95 °C for 15 minutes, followed by 35 cycles of 95 °C for 45 seconds, 55 °C for 1 minute, 72 °C for 2 minutes, and a final elongation of 72 °C for 7 minutes. Colony PCR samples were analyzed by electrophoresis on 1.5% agarose gel and visualized after staining with ethidium bromide using a Gel Doc XR (Bio-Rad). Cultures producing relevant bands in colony PCR were grown at 37 °C and 225 rpm overnight in 5.0 mL selective LB media with 50 µg/mL Kanamycin. The pDONR221 plasmids containing OR-genes were purified using miniprep kit (Qiagen). Plasmid quantification was performed using Nanodrop (8000 UV-vis Spectrophotometer), and samples were direct sequenced (Sanger sequencer, 3730xl) using M13 universal primers.

A 2.0 µg aliquote of each pDONR221/CpomOR and pDONR221/V5-CpomOR DNA was digested overnight at the limit of star activity, in a reaction volume with 0.5X FastDigest *NotI* and *ApaI* added

(Thermo Scientific), following the recommended protocol. Reaction volumes were run on 1.5% agarose gel and visualized after staining with ethidium bromide and digested bands were purified by Gel extraction kit (Qiagen). Quantification was conducted using Nanodrop 3300 Fluorospectrometer with PicoGreen® dsDNA reagent kit. From the purified bands, 50 ng of the reaction was combined with 50 ng pcDNA5/TO (previously digested and purified), 1.0 U T4 DNA ligase and 1X of the supplied reaction buffer (Thermo Scientific), which was incubated 2 h at room temperature for ligation.

Of this reaction volume, 2.0 µL was used to transform TOP10 competent cells. Colony PCR was performed to screen positive colonies, and colonies selected for correct inserts were amplified, vector extracted and purified by miniprep, and confirmed by sequencing (Sanger sequencer, 3730xl). In order to perform heterologous expression, pcDNA5/TO/CpomORs and pcDNA5/TO/V5-CpomORs were scaled up using GeneJet Plasmid Midiprep Kit (Qiagen).

Heterologous expression in HEK293T and transient transfection

HEK293T cells were grown in HEK cell media [Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (MP Biomedicals, Solon, OH, USA), 2 mM L-glutamine, and 100 µg/mL Penicillin/Streptomycin (Invitrogen)] at 37 °C with 5% CO_2.

To test transient expression of CpomORs for calcium imaging or patch-clamp recording experiments, 35-mm petri dishes containing semi-confluent HEK293T cells were transiently transfected. To transfect HEK cells with CpomOrco, we used 1.0 µg of pcDNA5/TO/CpomOrco DNA. In order to promote HEK-cell expression of the olfactory receptor (Carpentier et al. 2007), we used double aliquots for pcDNA5/TO/CpomOR DNAs (2.0 µg), combined with 1.0 µg of pcDNA5/TO/CpomOrco for co-transfections (CpomOrco+ORs). To report expression for calcium imaging experiments, 1.0 µg of a separate plasmid DNA (pEBFP-Nuc, Clontech) carrying the coding sequence for a blue fluorescent protein (BFP) was used. In patch-clamp recordings, 1.0 µg of a separate plasmid DNA (pXOOM, Clontech) carrying the coding sequence for a green fluorescent protein (GFP) was used to report expression. In order to report candidate OR-expressing cells, expression of both fluorescent reporter genes was under the regulation of the same promoter for CpomOR genes (CMV). Transfection DNAs were dissolved in 100 µL sterile DMEM, mixed with 3.0 µL Calfectine (SignaGen, Rockville, MD) and incubated 20 minutes before dropping on HEK cells covered with 1.0 mL fresh media, following the recommended protocol. To estimate transfection efficiency, a parallel transfection was conducted using the positive control vector pcDNA5/TO/LACZ (Invitrogen) and staining with 0.1% XGal according with Leonhardt/Cardoso protocol (*Leonhardt* and Cardoso, 1997). LACZ transfected preparations were compared with non-transfected preparations, to validate staining for the majority of cells.

Transfections were conduced overnight. HEK cell media was replaced with 1.0 mL fresh media to incubate cells at 37 °C for up to 6 hours, at which point part of the cell culture was spread in the middle of a 35-mm plates as individual cells or small clusters. After 12 hours of incubation at 37 °C 5% CO_2, cells were rinsed at the sides with 2.0 mL fresh HEK media. Cells were allowed to recover for at least 1 hour prior to calcium imaging.

Immunohistochemistry

To study membrane localization of olfactory receptors in HEK293T, cells were rather overnight transfected with pcDNA5/TO/V5-CpomOrco or co-transfected with pcDNA5/TO/CpomOrco combined with pcDNA5/TO/V5-CpomORs. To compare heterologous expression of olfactory receptors alone, further transfections were prepared for pcDNA5/TO/V5-CpomORs without CpomOrco DNA. Since the functional expression of CpomOrco, V5-CpomOrco was considered as a positive control. Non-transfected HEK cells were used as a negative control.

After growth, cells were split into 12 well-plates, each containing a single 12 mm cover slip, previously sterilized with ethanol and 10 minutes UV-light/side, and coated with matrigel matrix (Corning, Tewksbury, MA) diluted 1:40 in DMEM.

After overnight growth at 37 °C 5% CO_2, cover slips were washed gently with room temperature Hank's Balanced Salt Solution 1X (HBSS, Invitrogen) and incubated 15 min on ice soaked in ice-cold 100% methanol (Sigma Aldrich, St. Louis, MO, USA).

After incubation, methanol was removed, and cover slips were washed twice with HBSS 1X and stained overnight at 4 °C with V5 Epitope Tag Antibody, DyLight 488 conjugate (E10/V4RR) (Thermo Scientific) diluted 1:100 in staining solution (Zhuang and Matsunami, 2008).

After staining, cover slips were washed twice with HBSS 1X, and placed on microscope slides (Fisher Scientific, Pittsburg, PA) with one drop of DAPI-fluoromont-G (Southern Biotech, Birmingham, AL), for analysis by confocal microscopy.

Confocal microscopy

Samples were analyzed with Leica TCS-SP5 Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany) using HCX PL APO CS 63.0x1.20 WATER UV lens, 1.33 refraction index.

Scanner settings were calibrated with PinHole (m): 133.6 µm; PinHole (airy): 1.2; Zoom: 1.7. Images were taken step sizing the size-depth, optimizing the number of section by halving the numbers provided by the system.

Hardware was set to have all lasers active (405 Diode, UV; Argon, Visible; DPSS 561, Visible; and HeNe 633, Visible) with Argon, Visible at 29%.

In order to distinguish nuclei fluorescence from antibody-labeled plasma membrane extrusions, DAPI was exited using pre-set DAPI parameters, calibrating Laser Line UV (405) at 27% and all other Laser Line at 0%. Emission PMT was calibrated between 417 and 496 nm, Gain: 693 nm, Offset: 0, Transmission: 504, Offset: 0.

To detect DyLight Antibody with excitation/emission rate 493/518 nm, pre-set FITC parameters were adopted, calibrating Laser Line visible (488) at 76% and all other Laser Line at 0%. Emission PMT was calibrated between 500 and 560 nm, Gain: 808 nm, Offset: 0, Transmission: inactive.

All parameters were adjusted by the company-provided software (Leica Microsystems LAS AF TCS MP5). Images were analyzed using the same software and elaborated using ImageJ 1.42 (available from public domain at http://rsbweb.nih.gov/ij/index.html).

Calcium imaging

To test activation of olfactory receptors, CpomOrco or CpomOrco +ORs HEK293T cells were incubated 1 hour at room temperature in 0.5-1.0 mL HEK cell Ringer supplied with Magnesium (mM: 140 NaCl, 5.0 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.5) and containing the fluorescent calcium indicator Fluo - 4AM (Invitrogen) at 5.0-15 µM prepared with 0.2-0.06% Pluronic F-127 (Invitrogen). After incubation, the buffer was removed and cells were rinsed with 4.0 mL fresh HEK Ca^{++}Ringer (mM: 140 NaCl, 2.0 $CaCl_2$, 10 HEPES, pH 7.5), and placed on the stage of an inverted microscope (Olympus IX-71) equipped with a cooled CCD camera (ORCA R2, Hamamatsu). Cells were continuously superfused with Ca^{++}Ringer using two gravity fed perfusion contours. The stimulating contour washing the cells (~250 µL/min) was switched rapidly to the stimulus contour using a multi-channel rapid solution changer (RSC-160, Bio-Logic) under the software control of Clampex 9 (Molecular Devices). Fluorescence imaging was performed using Imaging Workbench 6 software (INDEC Systems). Stored time series image stacks were analyzed off-line using Imaging Workbench 6, Clampfit 10.5, SigmaPlot 11 or exported as TIFF files into ImageJ 1.42. Continuous traces of multiple responses were compensated for slow drift of the baseline fluorescence. All recordings were performed at room temperature (22-25 °C).

1. Dose/response to VUAA-compounds. To study activation properties of the codling moth olfactory co-receptor, pure Acetamide,*N*-(4-ethylphenyl)-2-[[4-ethyl-5-(3-pyridinyl)-4*H*-1,2,4-triazol-3-yl]thio]- (VUAA1), CAS 525582-84-7 (Glixx Laboratories, Southborough, MA) and Acetamide,2-[[4-ethyl-5-(4-pyridinyl)-4*H*-1,2,4-triazol-3-yl]thio]-*N*-[4-(1-methylethyl)phenyl]- (VUAA3), CAS 585550-72-7 (Molport, Riga, Latvia), were diluted in dimethyl sulfoxide (Sigma Aldrich) to a final concentration of 100 mM.

Dose/response experiments using VUAA1 were performed testing activation at 50, 100, 200, 500, 1000 µM for CpomOrco and CpomOrco+OR6, at 1.0, 10, 50, 100, 200, 500, 1000 µM for CpomOrco+OR3 and at 10, 50, 100, 200, 250, 500, 1000 µM for CpomOrco+OR1 testing different HEK-cells samples. Amplitudes of the calcium responses were determined using Clampfit 10.5, and responses were normalized to the response recorded at 1000 µM VUAA1.

Dose/response experiments with VUAA3 were performed similarly, testing activation 10, 50, 100, 250, 500, 1000 µM for CpomOrco and CpomOrco+OR6, at 1.0, 5.0, 10, 50, 100, 250, 500, 1000 µM for CpomOrco+OR3 and 1.0, 10, 50, 100, 250, 500, 1000 µM for CpomOrco+OR1 testing different HEK-cells samples. Amplitudes of the calcium responses were determined using Clampfit 10.5, and responses were normalized to the response recorded at 1000 µM VUAA3.

2. Inhibitory experiments. To study inhibitory properties of the codling moth olfactory co-receptor, 5 seconds stimulus using 250 µM VUAA3 was tested on HEK cells expressing CpomOrco alone or co-expressing CpomOrco+ORs. After stimulations, cells were superfused for 10 minutes with 100 µM of the amiloride derivative inhibitor 5-(N-methyl-N-isobutyl)amiloride (MIA), CAS 2609-46-3 (Sigma Aldrich) diluted in HEK Ca^{++}Ringer and supplied by a separate gravity fed perfusion contour. The response to 250 µM VUAA3 was recorded after incubation with the inhibitor. The inhibitor solution

was substituted with fresh HEK Ca^{++}Ringer and cells were washed for 1 hour before further stimulation with 250 µM VUAA3 for 5 seconds to record the recovered response to the ligand.

3. Screening of candidate ligands. In order to screen CpomOR6, CpomOR3 and CpomOR1 for response, HEK cells expressing CpomOrco+OR6, CpomOrco+OR3 and CpomOrco+OR1 were stimulated with an array of compounds including insect pheromone compounds and plant volatile synergists, previously reported to be active on the olfactory system and on behavior in the codling moth (Witzgall et al. 2005). Activation for other compounds we recently tested on CpomOR3 by heterologous expression in *Drosophila* OSNs (Bengtsson et al. 2014) was also investigated. Non-host plant volatiles from the Asian food plant *Perilla frutescens*, which we previously reported to be detected by the grapevine moth (Cattaneo et al. 2014), were also tested. The additional pear-emitted compound methyl-(E,Z)-2,4-decadienoate, analogous of pear ester and previously reported to be active on codling moth larvae (Knight and Light, 2001) was also tested (Table 1). A further set of additional compounds was employed, including compounds common among plants and fruits, as well as fermentation volatiles, and other volatiles (Table S1). Physical parameters were collected from Scifinder (Scifinder, 2015; Chemical Abstracts Service: Columbus, OH, 2015; accessed Nov 12, 2015) and Chemspider (http://www.chemspider.com/). Compounds were diluted in dimethyl sulfoxide or ethanol (Sigma Aldrich) depending on their solubility constant, and stimulations were optimized applying 100 µM of each compound for 10 seconds, ending each experiment with a stimulation with 100 µM of VUAA1 as positive control for 5 seconds.

4. Dose/response of CpomORs. To estimate the dose/response relationships on CpomOR6, we performed a dose/response experiment testing (E,E)-8,10-dodecadien-1-yl-acetate (codlemone acetate) at concentrations from 1.0, 2.5, 10, 50, 100, 150, 250, 500, to 1000 µM, on different petri dishes.
A similar dose/response experiment was performed for CpomOR3, stimulating with a set concentrations of ethyl-(E,Z)-2,4-decadienoate ranging from 10, 50, 100, 500, 1000, to 2000 µM and methyl-(E,Z)-2,4-decadienoate ranging from 50, 150, 250, 500, 1000, 1500, to 2000 µM on different petri dishes.
For both receptors, amplitudes of the calcium responses were determined by Clampfit 10.5, and normalized to the response recorded for 250 µM VUAA3.

Patch-clamp electrophysiology
For ionotropic investigation of the activation of the olfactory co-receptor, VUAA3-evoked integral and unitary currents were recorded from whole HEK-cells expressing CpomOrco+OR1. An initial stimulus intensity of 250 µM VUAA3 was modulated by increasing the duration of the compound pulse in 100-ms increments. Time between successive sweeps was set to 40 s, holding potential at +50 mV, current scale at 5 pA and stimulus duration of 7 s. Solutions for electrodes were NaCl 140 mM, EGTA 0.5 mM, Hepes 10 mM, pH 7.4, while bath solutions were NaCl 140 mM, $CaCl_2$ 1.0 mM, $MgCl_2$ 0-1.0 mM, KCl 5.0 mM, Hepes 10 mM, pH 7.4.
Unitary currents were recorded from outside-out patches excised from HEK cells and evoked by stimulation with 250 µM VUAA3. Holding potential was set at +50 mV, current scale at 5.0 pA and

stimulus duration of 7 s. Solutions for electrodes were KCl 140 mM, EGTA 2.0 mM, Hepes 10 mM, pH 7.4, while bath solutions were NaCl 140 mM, EGTA 2 mM, Hepes 10 mM, pH 7.4.

For monovalent cation permeability studies, whole-cell current-voltage characteristics were generated from CpomOrco+OR1-expressing HEK cells using series of 15 ms step at -100 mV followed by a 150 ms voltage ramp (linear change in voltage ~0.67 mV/ms). From -100 mV to +100 mV, current-voltage (CV) was applied from a holding potential of -50 mV. The interval between sweep starts was 1.0 s. In order to determine the reversal potentials of currents, we averaged current traces and the standard deviation for each point in the average current trace was calculated. To better visualize the position of the minimum of the function SD curves of the average, current function were scaled separately. After obtaining 50-200 CV characteristics in symmetrical NaCl 140 mM, cells were exposed in a random order to one of the following solutions: LiCl 140 mM; KCl 140 mM; RbCl 140 mM; CsCl 140 mM. Series of 50-200 ramps were obtained for every cation. The corresponding reversal potentials were estimated based on position of the minimum of standard deviation for the average current trace. Currents were activated by 250 μM VUAA3.

Heterologous expression of CpomORs in Drosophila melanogaster

The complete ORFs encoding CpomOR6, CpomOR3 and CpomOR1 were amplified by PCR using proper CDS-primers (Table 2), with antennal cDNA created by the RT-for-PCR kit (Invitrogen) as a template. Purified PCR products were then cloned into the PCR8/GW/TOPO plasmid (Invitrogen). Cassettes with inserts were then transferred from their TOPO/GW/PCR8 plasmids to the destination vector (pUASg-HA.attB, constructed by E. Furger and J. Bischof, kindly provided by the Basler group, Zürich), using the Gateway LR Clonase II kit (Invitrogen). The integrity and orientation of inserts was confirmed by sequencing (Sanger sequencer, 3730xl). Transformants *UAS-CpomOR6*, *UAS-CpomOR3* and *UAS-CpomOR1* lines were generated by Best Gene (Chino Hills, CA, USA), using the PhiC31 integrase system. Briefly, recombinant pUASg-HA.attB-CpomOR6 and CpomOR1 plasmids were injected into embryos of a *D. melanogaster* line containingan *attP* insertion site within the third chromosome (genotype y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb), leading to non-random integration. To drive expression of *CpomORs* in OSNs housed in T1 sensilla, transformants *UAS- CpomOR6* and *CpomOR1* lines were crossed to the *Or*67*d*GAL4 strain (kindly provided by Barry Dickson) to generate double homozygous lines *w*+; *UAS- CpomOR*; *Or*67*d*GAL4. Additionally, to drive expression of CpomOR3 in OSNa housed in ab3 basiconic sensilla, the transgene was crossed into the *δ-halo* mutant background OR22a-Gal4 mutant *D. melanogaster* (Dobritsa et al. 2003; Hallem et al. 2004). To verify insertion of *UAS-CpomORs* constructs into the genome, gDNA was extracted and used as template in PCR with primers for the full ORF of *CpomORs* (Table 2).

Single Sensillum Recordings

CpomOR1 and CpomOR6 expressed in T1 trichoid sensilla, and CpomOR3 expressed in the A neuron of ab3 basiconic sensilla, were tested through single sensillum recordings (SSR). As described by Stensmyr et al. (2003), flies of 3-8 days old were inmmobilized in 100 μL pipette tips with only the top half of the head protruding. The left antenna of each insect was gently pushed with a glass capillary

against a double-sided adhesive tape placed on a piece of glass. This piece of glass along with the pipette tip were fixed with dental wax on a microscope slide. Electrolytically sharpened tungsten electrodes (Harvard Apparatus Ltd, Edenbridge, United Kingdom) were used to penetrate the insect´s body. The reference electrode was manually inserted in the right eye of the fly, while the recording electrode was maneuvered with a DC-3K micromanipulator equipped with a PM-10 piezo translator (Märzhäuser Wetzler GmbH, Wetzler, Germany) and inserted at the base of the determined sensilla. Signals coming from the olfactory sensory neurons were amplified 10 times with a probe (INR-02, Syntech, Hilversum, the Netherlands), digitally converted through an IDAC-4-USB (Syntech) interface, and visualized and analyzed with the software Autospike v. 3.4 (Syntech). A constant flow of 0.65 m/s of humidified air (charcoal-filtered) was delivered through a glass tube to the antenna. The panel of odorants was given to the insect by inserting pipettes containing a piece of filter paper with the correspondent stimulus in a lateral hole of the glass tube and puffing a flow of 2.5 mL of air during 0.5 seconds through the pipette. For CpomOR1 and CpomOR6 the panel of odorants was prepared by applying 10 μL of a solution of 1.0 μg/μL of the compounds in Table S2, for a total amount of 10 μg per stimulus. In the case of CpomOR3, a similar dilution process was used for the dose/response experiments of (E,Z)-ED and (E,Z)-MD. Compounds were diluted from concentrations ranging from 0.01 ng/μL to 10 μg/μL in decadic steps, allowing reaching concentrations from 100 pg to 100 μg per stimulus when 10 μL of the dilution was applied in the piece of filter paper. In all cases, to characterize the intensity of the response, spike frequency was calculated by subtracting the spikes recorded 0.5 s before the stimulus from the number of spikes recorder 0.5 s after the stimulus and multiplied by 2 to get the response in spikes/s. The number of spikes were corrected accounting for differences in vapor pressure (Bengtsson et al. 1990). Dose/response experiments between of pear ester and its analogue were compared with two-way ANOVA with repeated measures, followed by LSD *post-hoc* test.

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Conclusions and perspectives

Wine grape and apple production are two of the major agricultural sectors worldwide. The grapevine moth *L. botrana* is key pest of grapes in the Palaearctic and Nearctic regions causing indirect damage to wine grape by larval populations of carpophagous generations inducing botrytis and acid rot to bunches. The codling moth *C. pomonella* is the economically most important pest on pome fruit worldwide. The control of these moths is still currently relying mainly on insecticides but an effective management is hampered by a high degree of variability in their population densities within vineyards and apple orchards and the lack of adequate monitoring and population modelling tools. An increased level of public concern about the environmental impact of pest management in these agroecosystems have led to the development of more low impact, biotechnical methods, applicable also in organic agriculture, such as pheromone mating disruption, attract-and-kill, push-and-pull (Witzgall et al., 2008; Ioriatti et al., 2011). However, such techniques often show drawbacks and failures and therefore additional research is needed to improve all the semiochemical-based control methods and hasten their adoption for pest control.

Previous studies at Fondazione Edmund Mach indeed demonstrated that it is still worthwhile investigate both the molecular and physiological mechanisms of pheromone-based techniques and the effect of plant volatiles on the pheromone response with new technological approaches since this may optimize their application (Anfora et al., 2005, 2008, 2009; Bengtsson et al., 2012, 2014; Trona et al., 2013). This knowledge has been the background and the starting point of my thesis, taking into account that only an integrated and multidisciplinary approach can face the challenge of understanding and ultimately controlling these insects.

All the mentioned methods are based on the interference with the neurophysiological mechanisms involved in olfactory intraspecific (pheromones) and interspecific (kairomones and host plant metabolites) insect communication, both at peripheral and brain level confirming that insect senses are an attractive target for the control of insect pests. Indeed, olfaction, taste and nociception are essential sensory modalities for insects, allowing them to avoid lethal substances or predators, to find food, hosts and sexual partners or to choose oviposition substrates and food sources for the offspring. Unveiling the communication mechanisms in these species would therefore be the base from which to develop innovative methods of interference. The huge diversity of plant secondary metabolites (volatile or non-volatile), as those identified in *P. frutescens*, appears to be a rich source of molecules suitable for these kinds of control applications.

Accordingly, our studies shed light to some unknown processes of the perception in *L. botrana* and *C. pomonella*. In particular we characterized the function of important sensory receptors expressed in the antenna of the selected insects making use of the most recent and groundbreaking technologies from electrophysiological and behavioral assays, to bioinformatic and molecular characterization of receptor proteins.

At the receptor level, we studied both the Olfactory Receptors (ORs), the most common class of sensory proteins mediating detection of odors in insect antennae, and the Transient Receptor Potential (TRP) channels, a novel family of receptor. We demonstrated electrophysiological and behavioral responses of the grapevine moth to volatiles emitted by the non-host, *P. frutescens*, previously known

to activate TRPs in the rat, *Rattus norvegicus*. In the codling moth, we characterized a novel TRP channel (TRPA pyrexia-like) and we confirmed activation of its human orthologue to the same non-host compounds active on the olfactory system of the grapevine moth. ORs were heterologously expressed in vivo and in vitro, for identification of their ligands among host and non-host plant volatiles and pheromones (deorphanization). Among several ORs of codling moth, we deorphanized a candidate pheromone receptor (PR) to a plant synergist, an OR to non-host volatiles and another PR candidate to a pheromone antagonist of the insect.

With these approaches we advocate to open up new venues to develop control strategies that target the sensory pathways of these pests. We also speculate that the technologies set-up during this study and the results obtained with our model organisms may offer new opportunities for addressing some longstanding questions in the field of insect biology with a practical outcome.

The long-term perspective is hence to accelerate the research towards the set-up of new environmentally friendly pest control methods based on the interference with the insect sensory systems. The reduction in insecticide use should improve the quality of life for growers, consumers, as well as public living around the wine-growing areas so reducing the conflict between agricultural and urban world.

Apart from agricultural applications, comparison of molecular, physiological and behavioural experiments between vertebrates and invertebrates will ultimately lead to expand our understating of the animal olfactory systems in general with possible new biotechnological applications. As a mode of fact, the mechanisms that enable sensory discrimination are remarkably similar across species and even phyla and several principles of organization are evolutionary conserved from invertebrates to mammals. OR and TRP active compounds that have been identified in the course of the research have therefore several possible applications also in food, pharmaceutical, herboristic and cosmetic industry. For their implications in some type of cancer, in nociception and in endocannabinoid system they could also have a strong impact in medicine research.

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Supplementary material

Chapter I

Figure S1. Gas chromatography (GC) tracks of perillaldehyde essential oil and synthetic perillaldehyde. Comparison of GC-tracks (GC 50 mV) of perillaldehyde essential oil (PAEO, upper track, 1-µL injection of 1 µg µL−1) and synthetic *S*-(−)-perillaldehyde (PA, lower track, 1 µL-injection of 1 µg µL−1). The synthetic *S*-(−)-perillaldehyde (main peak in the middle) can be distinguished from peaks of impurities (arrows). Comparison indicated only a slight difference in the amount of S-(−)-perillaldehyde (retention time 17.24 min) in the PAEO sample (area=1046) compared with the synthetic PA sample (area=903.1). The minor difference in amount of synthetic PA, motivated by the presence of several peaks at lower and higher retention time in the sample, absent in the PAEO solution and likely related with impurities as by-products from chemical synthesis, was judged to be irrelevant.

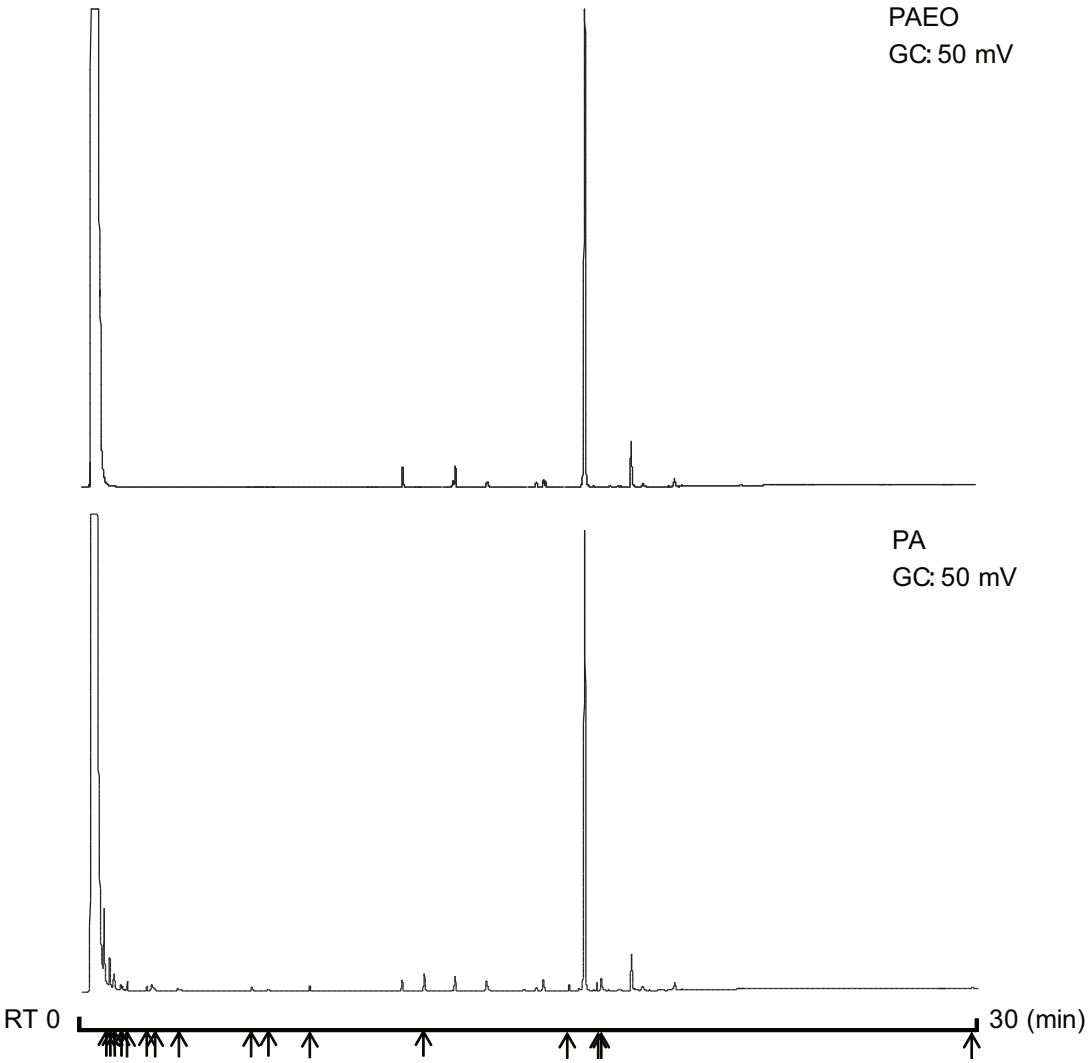


Table S1 List of accession numbers of TRP sequences used for phylogenetic investigation.

Subfamily	Sequence name	Accession	Source
TRPA	BmWtrw	NP_001296536	GenBank
	DpWtrw	EHJ69686	GenBank
	DmWtrw	CG31284	Flybase
	DpPyr-l2	EHJ68880	GenBank
	BmPyr-l2	NP_001296502	GenBank
	DpPyr	EHJ76008	GenBank
	BmPyr	NP_001296484	GenBank
	DmPyr	CG17142	Flybase
	BmPyr-l	XP_004926185	GenBank
	DpPyr-l	EHJ78201	GenBank
	DpPain	EHJ76831	GenBank
	BmPain	NP_001296553	GenBank
	DmPain	CG15860	Flybase
	DpTRPA1	EHJ74088	GenBank
	BmTRPA1	XP_012551534	GenBank
	DmTRPA1	CG5751	Flybase
	DrTRPA2	ZDB-GENE-050106-1	Zfin
	DrTRPA1	ZDB-GENE-050105-6	Zfin
	RnTRPA1	1303284	rgd
	CeTRPA-1	CE42588	Wormbase
	CeTRPA-2	CE18081	Wormbase
TRPML	DpTRPML	EHJ66521	GenBank
	BmTRPML	XP_004932903	GenBank
	DmTRPML	CG8743	Flybase
	RnTRPML2	NP_001034094	NCBI
	RnTRPML3	NP_001012059	NCBI
	RnTRPML1	NP_001099373	NCBI
	CeCUP-5	CE45023	Zfin
	CeGLT-2	CE40563	Zfin
	CeCED-11	CE00409	Zfin
TRPC	BmTRP	XP_012551652	GenBank
	DpTRP	EHJ65374	GenBank
	DmTRP	CG7875	Flybase

	DpTRPgamma	EHJ68691	GenBank
	BmTRPgamma	XP_012547133	GenBank
	DmTRPgamma	CG5996	Flybase
	CeTRP-2	CE32915	Wormbase
	DpTRPL	EHJ65372	GenBank
	BmTRPL	XP_004922702	GenBank
	DmTRPL	CG18345	Flybase
	DrTRPC5b	ZDB-GENE-091112-24	Zfin
	RnTRPC5	619787	rgd
	DrTRPC5a	ZDB-GENE-040812-1	Zfin
	RnTRPC4	621276	rgd
	DrTRPC4b	ZDB-GENE-120329-1	Zfin
	DrTRPC1	ZDB-GENE-070830-1	Zfin
	RnTRPC1	619783	rgd
	DrTRPC7b	ZDB-GENE-140129-2	Zfin
	DrTRPC7a	ZDB-GENE-091113-40	Zfin
	RnTRPC7	628820	rgd
	DrTRPC3	ZDB-GENE-140129-1	Zfin
	RnTRPC3	61973	rgd
	RnTRPC6	619788	rgd
	DrTRPC6b	ZDB-GENE-081030-19	Zfin
	DrTRPC6a	ZDB-GENE-040724-114	Zfin
	CeTRP−1	CE33009	Wormbase
	DrTRPC2b	ZDB-GENE-050712-3	Zfin
	DrTRPC2a	ZDB-GENE-130530-602	Zfin
	RnTRPC2	628819	rgd
TRPN	DpNompC	EHJ73805	GenBank
	BmNompC	XP_012546363	GenBank
	DmNompC	CG11020	Flybase
	DrNompC	ZDB-GENE-030728-7	Zfin
	CeTRP−4	CE42788	Wormbase
	CeTRP−3	CE03452	Wormbase
TRPM	DrTRPM1b	ZDB-GENE-070424-31	Zfin
	DrTRPM1a	ZDB-GENE-070112-1372	Zfin
	RnTRPM1	1597140	rgd
	DrTRPM3	ZDB-GENE-060531-95	Zfin
	RnTRPM3	1304888	rgd
	DrTRPM7	ZDB-GENE-021115-2	Zfin

	RnTRPM7	620053	rgd
	RnTRPM6	1309942	rgd
	DrTRPM6	ZDB-GENE-111212-1	Zfin
	DmTRPM	CG44240	Flybase
	DpTRPM	EHJ78405	GenBank
	BmTRPM	XP_012551960	GenBank
	CeGON−2	CE30390	Wormbase
	CeGTL−1	CE33754	Wormbase
	DrTRPM4b3	ZDB-GENE-121214-115	Zfin
	DrTRPM4b2	ZDB-GENE-061214-2	Zfin
	DrTRPM4b1	ZDB-GENE-061214-3	Zfin
	DrTRPM4a	ZDB-GENE-090302-3	Zfin
	DrTRPM5	ZDB-GENE-060503-736	Zfin
	RnTRPM5	1310620	rgd
	RnTRPM4	620244	rgd
	DrTRPM2	ZDB-GENE-061214-4	Zfin
	RnTRPM2	1311889	rgd
	RnTRPM8	620762	rgd
TRPV	DpNan	EHJ73092 + EHJ68701	GenBank
	BmNan	XP_004923070	GenBank
	DmNan	CG5842	Flybase
	CeOCR−4	CE40877	Wormbase
	CeOCR−2	CE17232	Wormbase
	CeOCR−1	CE41127	Wormbase
	CeOCR−3	CE40872	Wormbase
	DpIav	EHJ71463	GenBank
	BmIav	XP_004925321	GenBank
	DmIav	CG4536	Flybase
	CeOSM−9	CE20445	Wormbase
	RnTRPV1	628841	rgd
	DrTRPV1	ZDB-GENE-030912-8	Zfin
	RnTRPV2	3965	rgd
	DrTRPV4	ZDB-GENE-030912-7	Zfin
	RnTRPV4	69337	rgd
	RnTRPV3	1564531	rgd
	RnTRPV5	620636	rgd
	RnTRPV6	69335	rgd
	DrTRPV6	ZDB-GENE-040624-12	Zfin

TRPP	RnTRPP1	NP_001244281	NCBI
	RnTRPP2	1559992	rgd
	RnTRPP3	NP_001099822.1	NCBI
	CePKD−2	CE38663	Wormbase
	DmAmo	CG6504	Flybase

Table S2 List of forward and reverse primers designed to validate the expression of candidate Cp-TRPs.

Gene	**Forward primer**	**Reverse primer**	**Tm (°C)**
CpPyr-l	ATGGGCTGGTTCCCTTTACATACAG	TTATTTACTTAACTTACTTTCTAATCTTAACAA	61.4
CpPyr	TACCCAGCGTTCCAACTACC	CATGAGAGCAGCGAACTGAA	63.2
CpWtrw	TAGCCGGTTACTCCACCATC	AAAACAGGGGAGGGTCATTC	63.2
CpTRP	ATTCCCTCAGGCACTCACAA	CATGAAAGCTGGAAGGCTGT	64.3
CpTRPC	GGGAGACCAAGTCAACGGTATGC	GATGCGTTCAGTGTACGTGTGC	65.4
CpOrco	CCGGAGCCCACTGATATAGA	CCTCAGAACCGTCGTACCAT	64.3

Figure S1 Reverse Transcription PCR of candidate Cp-TRP channels (*CpPyr-l*, *CpPyr*, *CpWtrw*, C*pTRP*, *CpTRPC*) in male and female *C. pomonella* body parts (Antennae, Thorax, Abdomen, Legs, Wings). Ntc: non-template control; M: male; F: female; *CpOrco*: Antennal control; *rpl8*: positive control.

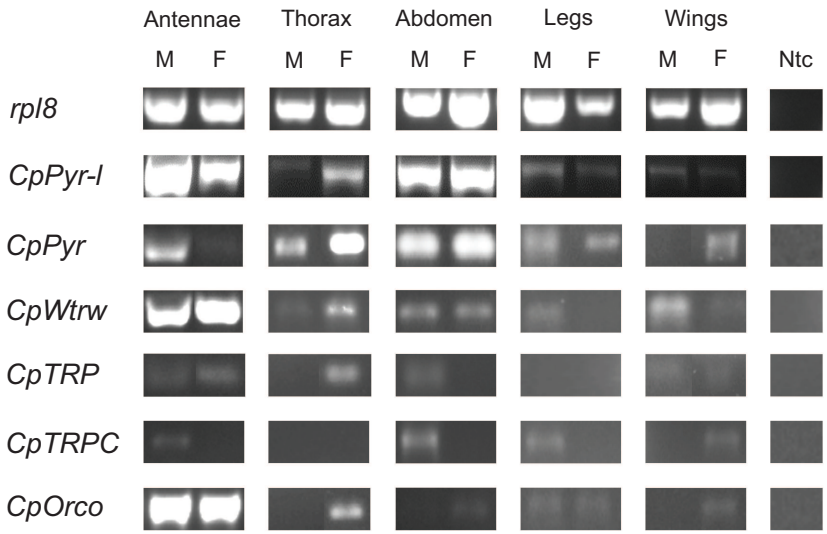


Figure S1 Monitoring of the transfection efficiency by a colorimetric protocol. Comparison between pcDNA5/TO/LACZ transfected HEK293T cells and non-transfected cells after 10 minutes, 1 hour, 2 hours, 3 hours staining 0.1 % XGal, according with *Leonhardt* and Cardoso, 1997. Bar: 100 µm.

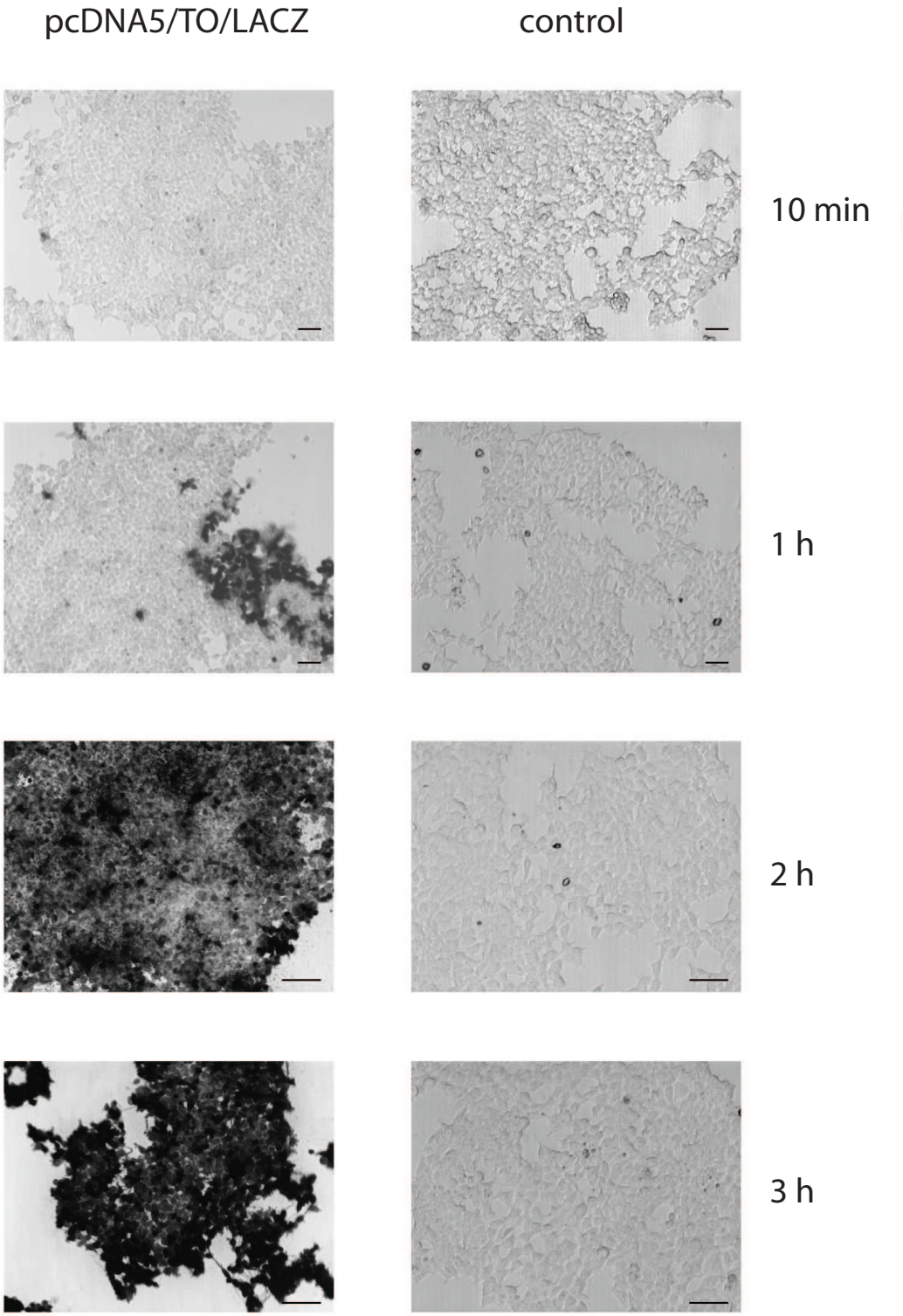


Figure S1 Response profile of CpomOR19 to synthetic compounds tested at 100µg on filter paper (mean ± SE, *n* = 5).

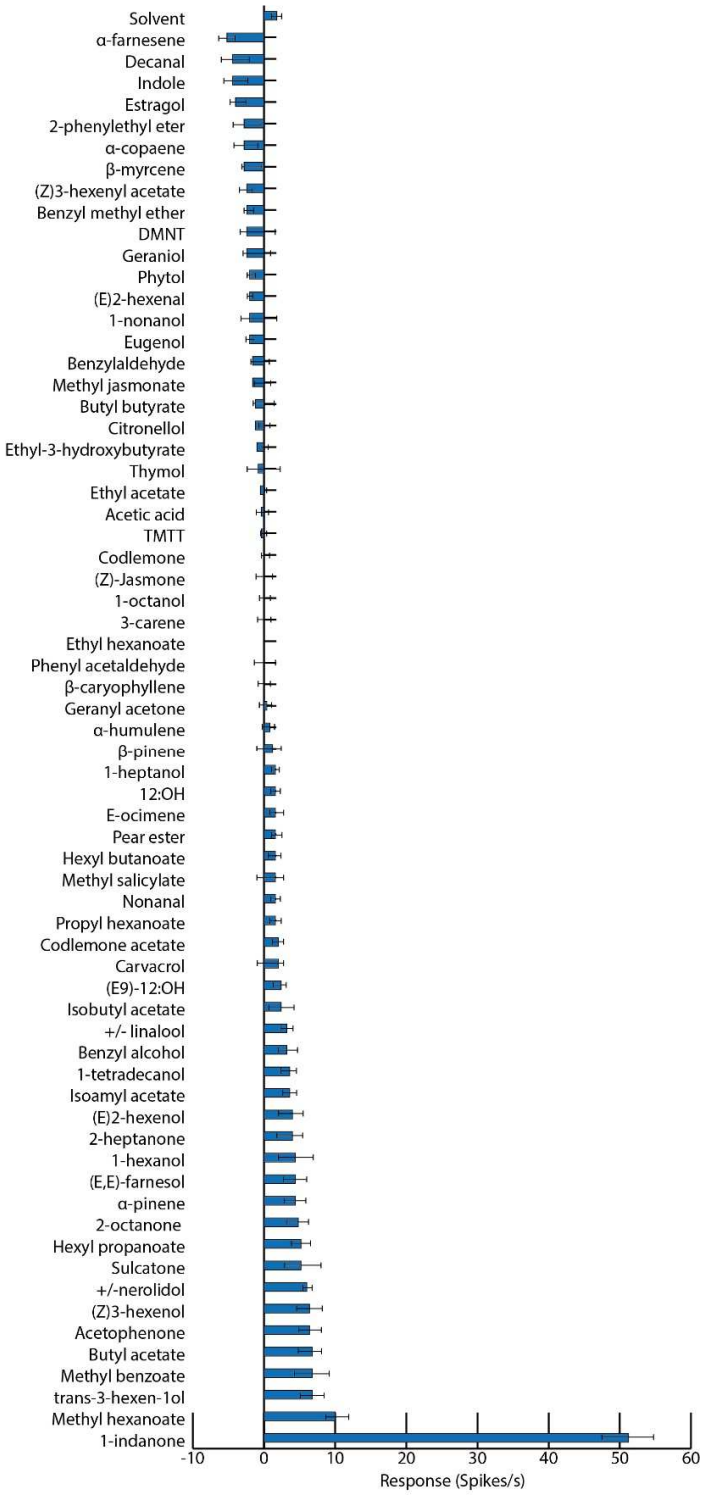


Table S1 Additional list of compounds tested on CpomOrco+ORs in HEK293T.

Compounds	MW (g/mol)	Solubility (M)	LogP	CAS	Source
(-)-carvone	150.22	7.80E-03	2.268±0.334	6485-40-1	MBI lab
(-)-α-pinene	136.23	6.50E-05	4.321±0.237	80-56-8	Fluka
(+)-carvone	150.22	7.80E-03	2.268±0.334	2244-16-8	MBI lab
(+)-nootkatone	218.33	2.40E-04	3.765±0.275	4674-50-4	Givaudan
(E)-2-hexenal	98.14	0.09	1.790±0.281	505-57-7	MBI lab
(E)-2-hexenol	100.16	0.14	1.655±0.212	928-95-0	MBI lab
(Z)-3-hexenol	100.16	0.14	1.697±0.206	928-96-1	Safc
(Z)-3-hexenyl acetate	142.20	0.025	2.400±0.228	3681-71-8	Safc
(Z)-jasmone	164.24	3.90E-03	2.020±0.337	488-10-8	MBI lab
+/-nerolidol	222.37	2.10E-05	4.682±0.295	3790-78-1	Aldrich
+/-phytol	296.53	8.10E-10	8.230±0.255	150-86-7	Aldrich
1-heptanol	116.20	0.029	2.367±0.177	111-70-6	Aldrich
1-indanone	132.16	4.60E-03	1.419±0.329	83-33-0	Aldrich
1-nonanol	144.25	2.70E-03	3.386±0.177	143-08-8	Fluka
1-octanol	130.23	9.00E-03	2.876±0.177	111-87-5	Sigma Aldrich
1-octen-3-ol	128.21	0.018	2.519±0.220	3391-86-4	MBI lab
1-pentanol	88.15	0.24	1.348±0.176	71-41-0	MBI lab
1-pentene-3-ol	86.13	0.46	0.991±0.220	616-25-1	MBI lab
1-tetradecanol	214.39	2.70E-06	5.933±0.178	112-72-1	Fluka
2-butanone,4-(4-hydroxyphenyl)-(raspberry ketone)	164.20	0.031	1.309±0.212	5471-51-2	Givaudan
2-propen-1-one,1-(2-furanyl)-3-(4-methoxyphenyl)-,(2*E*)-(PK analogue 16)	228.24	5.10E-04	2.752±0.338	137444-58-7P	Gift from Prof Angela Bassoli[7]
2(3H)-furanone,5-butyldihydro-4-methyl-(whiskey lactone)	156.22	0.011	1.968±0.280	39212-23-2	MBI lab
2,5 dimethyl pyrazine	108.14	6.02	0.687±0.315	123-32-0	MBI lab
2-butyl acetate	116.16	0.081	1.648±0.212	105-46-4	MBI lab
2-ethylfuran	96.13	0.029	2.300±0.241	3208-16-0	MBI lab
2H-pyran, tetrahydro-4-methyl-2-(2-methyl-1-propen-1-yl)-, (2R,4R)-(trans-rose oxide)	154.25	7.40E-03	3.186±0.265	5258-11-7	Givaudan
2H-pyran, tetrahydro-4-methyl-2-(2-methyl-1-propen-1-yl)-,(2S,4R)-(cis-rose oxide)	154.25	7.40E-03	3.186±0.265	3033-23-6	Givaudan

2-heptanone	114.19	0.044	1.996±0.193	110-43-0	MBI lab
2-hexylpyridine	163.26	0.045	3.766±0.188	1129-69-7	MBI lab
2-isobutyl-3-methoxypyrazine	166.22	0.05	2.547±0.377	24683-00-9	MBI lab
2-isobutylthiazole	141.23	0.058	1.715±0.222	18640-74-9	MBI lab
2-methyl-1-butanol	88.15	0.34	1.192±0.187	137-32-6	MBI lab
2-methylbutyl acetate	130.18	0.034	2.158±0.212	624-41-9	MBI lab
2-methylbutyraldehyde	86.13	0.086	1.267±0.227	96-17-3	MBI lab
2-octanone	128.21	0.018	2.506±0.193	111-13-7	MBI lab
2-phenylethanol	122.16	0.16	1.504±0.186	60-12-8	MBI lab
2-propen-1-one,3-(4-chlorophenyl)-1-(2-furanyl)-,(2*E*)-(PK analogue 18)	232.66	1.20E-04	3.482±0.339	111042-59-2P	Gift from Prof Angela Bassoli[7]
3-Isothiocyanato-1-propene (Mustard oil)	99.15	3.23E-03	1.9±0.1	55-06-7	Aldrich
3-methyl-1-butanol	88.15	0.34	1.192±0.187	123-51-3	MBI lab
3-methyl-1-pentanol	102.17	0.12	1.702±0.188	589-35-5	MBI lab
3-methyl-2-butenal	84.12	0.31	1.190±0.316	107-86-8	MBI lab
3-octanone	128.21	0.018	2.506±0.193	106-68-3	MBI lab
3-pentanone	86.13	0.26	0.977±0.192	96-22-0	MBI lab
4-ethyl guaiacol (4-ethyl-2-methoxyphenol)	152.19	0.014	2.434±0.224	2785-89-9	Givaudan
4-isopropyl phenol	136.19	0.015	2.986±0.200	99-89-8	Givaudan
4-*tert*-butyl cyclohexanol	156.27	0.01	3.092±0.213	98-52-2	Givaudan
4-tert-butyl cyclohexanone	154.25	5.70E-03	2.630±0.264	98-53-3	Givaudan
4-tert-butyl phenol	150.22	6.30E-03	3.397±0.214	98-54-4	Givaudan
4-vinyl guaiacol (2-methoxy-4-vinylphenol)	150.17	0.015	2.573±0.249	7786-61-0	Givaudan
6-methyl-5-hepten-2-ol	128.21	0.041	2.057±0.236	1569-60-4	MBI lab
6-methyl-5-hepten-2-one	126.20	0.052	1.947±0.238	110-93-0	MBI lab
acetic acid	60.05	3.28	-0.322±0.184	64-19-7	Sigma Aldrich
acetophenone	120.15	0.02	1.674±0.217	98-86-2	Fluka
acetyl eugenol	206.24	1.40E-03	2.710±0.240	93-28-7	Givaudan
amylbutyrate	158.24	5.40E-03	3.333±0.205	540-18-1	MBI lab
anisole b-cyclocitral	108.14	0.03	2.170±0.203	100-66-3	MBI lab
b-cyclocitral	152.23	6.50E-03	3.100±0.319	432-25-7	Safc
benzaldehyde	106.12	0.02	1.452±0.242	100-52-7	Sigma Aldrich
benzoic acid	122.12	0.046	1.559±0.206	65-85-0	MBI lab
benzothiazole	135.19	0.27	1.899±0.297	95-16-9	MBI lab

benzyl acetate	150.17	0.017	1.998±0.224	140-11-4	MBI lab
benzyl alcohol	108.14	0.043	1.055±0.206	100-51-6	Aldrich
benzyl methyl ether	122.16	0.05	1.843±0.239	538-86-3	Aldrich
b-ionone	192.30	1.20E-3	3.589±0.275	79-77-6	MBI lab
bourgeonal	190.28	4.50E-04	3.486±0.245	18127-01-0	MBI lab
butanol	74.12	0.65	0.839±0.176	71-36-3	MBI lab
butyl acetate	116.16	0.073	1.804±0.205	123-86-4	MBI lab
butyl butanoate	144.21	0.013	2.823±0.205	109-21-7	Aldrich
camphor	152.23	6.90E-03	2.089±0.300	464-49-3	MBI lab
carvacrol	150.22	6.40E-03	3.162±0.205	499-75-2	Aldrich
cineole (1,8-) eucalyptol	154.25	5.90E-03	2.795±0.267	470-82-6	MBI lab
cineole 1,4-	154.25	4.40E-03	2.496±0.266	470-67-7	MBI lab
cinnamaldehyde	132.16	0.023	1.900±0.283	14371-10-9	MBI lab
cis-2-penten-1-ol	86.13	0.4	1.146±0.212	1576-95-0	MBI lab
citral	152.23	0.011	3.127±0.359	5392-40-5	MBI lab
citronellal	154.25	2.90E-03	3.297±0.259	106-23-0	MBI lab
citronellol	156.27	3.00E-03	3.239±0.235	106-22-9	MBI lab
cyclodecanone	154.25	0.013	2.929±0.252	1502-06-3	MBI lab
cycloheptanecarbaldehyde	126.20	0.012	2.394±0.225	4277-29-6	MBI lab
cyclohexanone	98.14	0.15	0.821±0.251	108-94-1	MBI lab
cyclopentanecarboxaldehyde	98.14	0.041	1.339±0.225	872-53-7	MBI lab
d-decalactone	170.25	5.60E-03	2.469±0.278	705-86-2	MBI lab
decanal	156.27	9.80E-04	3.970±0.223	112-31-2	Sigma
dihydro eugenol (2-methoxy-4-propylphenol)	166.22	6.00E-03	2.943±0.224	2785-87-7	Givaudan
4-Allylanisole (estragol)	148.20	4.30E-03	3.088±0.223	140-67-0	Aldrich
ethyl vanillin	166.17	9.80E-03	1.718±0.272	121-32-4	Givaudan
eugenol	164.20	0.011	2.403±0.236	97-53-0	Aldrich
farnesol	222.37	1.90E-05	4.828±0.309	4602-84-0	Aldrich
fennaldehyde	178.23	3.50E-03	2.023±0.250	5462-06-6	Givaudan
geraniol	154.25	5.90E-03	2.942±0.271	106-24-1	Aldrich
geranylacetone	194.31	2.30E-03	3.834±0.268	3796-70-1	MBI lab
hedione	226.31	1.80E-03	2.653±0.272	24851-98-7	MBI lab
helional	192.21	8.10E-04	1.982±0.343	1205-17-0	MBI lab
heliotropin	150.13	4.40E-03	1.050±0.302	120-57-0	Givaudan
heptanal	114.19	0.013	2.442±0.223	111-71-7	MBI lab
heptyl butyrate	186.29	1.00E-03	4.352±0.206	5870-93-9	MBI lab
hexanal	100.16	0.031	1.932±0.223	66-25-1	MBI lab
hexanol	102.17	0.086	1.858±0.177	111-27-3	Acros

hexyl 2-methylbutanoate	186.29	1.10E-03	4.196±0.212	10032-15-2	Safc
hexyl acetate	144.21	0.013	2.823±0.205	142-92-7	MBI lab
hexyl alcohol	102.17	0.086	1.858±0.177	111-27-3	MBI lab
hexyl hexanoate	200.32	4.40E-04	4.861±0.206	6378-65-0	Safc
isoamylacetate	130.18	0.034	2.158±0.212	123-92-2	MBI lab
isobutyl acetate	116.16	0.081	1.648±0.212	110-19-0	MBI lab
isoeugenol	164.20	7.30E-03	3.081±0.248	97-54-1	Givaudan
isomenthone	154.25	5.50E-03	2.755±0.260	491-07-6	MBI lab
isopentyl acetate	130.18	0.034	2.158±0.212	123-92-2	Fluka
isosafrol	162.19	5.90E-04	3.904±0.349	120-58-1	Givaudan
isovaleraldehyde	86.13	0.086	1.267±0.227	590-86-3	MBI lab
isovaleric acid	102.13	0.23	1.051±0.193	503-74-2	MBI lab
isovaleronitrile	83.13	0.11	1.039±0.199	625-28-5	MBI lab
lilial	204.31	2.20E-04	3.839±0.249	80-54-6	MBI lab
limonene, R(+)	136.23	2.50E-05	4.552±0.241	5989-27-5	Aldrich
linalool	154.25	6.70E-03	2.795±0.263	78-70-6	Aldrich
liral	210.31	3.10E-03	2.424±0.256	31906-04-4	MBI lab
menthone (-)	154.25	5.50E-03	2.755±0.260	89-80-5	MBI lab
methional	104.17	0.26	0.436±0.323	3268-49-3	MBI lab
methyl benzoate	136.15	0.02	2.124±0.204	93-58-3	MBI lab
methyl diantilis	182.22	0.039	1.571±0.265	5595-79-9	Givaudan
methyl eugenol	178.23	4.30E-03	2.655±0.243	93-15-2	Givaudan
methyl valerate	116.16	0.073	1.804±0.205	624-24-8	MBI lab
methyl-iso-eugenol	178.23	3.60E-03	3.049±0.239	93-16-3	Givaudan
methyl atratate, evernyl (mousse cristal)	196.20	0.01	2.843±0.336	4707-47-5	Givaudan
n-butyl acetate	116.16	0.073	1.804±0.205	123-86-4	MBI lab
nonanedeioic acid (azelaic acid)	188.22	0.046	1.196±0.197	123-99-9	MBI lab
octanal	128.21	5.40E-03	2.951±0.223	124-13-0	MBI lab
octanoic acid	144.21	0.015	2.735±0.184	124-07-2	MBI lab
orivone 4-(tert.-pentyl)-cyclohexanone	168.28	2.40E-03	3.140±0.264	16587-71-6	Givaudan
p-cymene	134.22	9.90E-05	4.014±0.189	99-87-6	MBI lab
pentanal = valeraldehyde	86.13	0.077	1.423±0.222	110-62-3	MBI lab
pentanol	88.15	0.24	1.348±0.176	71-41-0	MBI lab
pentyl acetate (amyl acetate)	130.18	0.030	2.314±0.205	628-63-7	MBI lab
phenyl acetaldehyde	120.15	0.016	1.760±0.224	122-78-1	Aldrich
phenylacetaldehyde	120.15	0.016	1.760±0.224	122-78-1	MBI lab
phenylethylamine	121.18	0.085	1.435±0.189	64-04-0	MBI lab
prenyl acetate	128.17	0.069	2.017±0.274	1191-16-8	MBI lab

propan-2-ol (isopropanol)	60.10	2.34	0.173±0.187	67-63-0	MBI lab
propyl acetate	102.13	0.18	1.295±0.205	109-60-4	MBI lab
propyl hexanoate	158.24	5.40E-03	3.333±0.205	626-77-7	Safc
pyrazine	80.09	12.5	-0.002±0.232	290-37-9	MBI lab
pyrollidine	71.12	2.15	0.085±0.242	123-75-1	MBI lab
thymol	150.22	5.80E-03	3.252±0.205	89-83-8	Sigma
trans-2-heptenal	112.17	0.037	2.300±0.282	18829-55-5	MBI lab
trans-2-hexenal	98.14	0.09	1.790±0.281	6728-26-3	Aldrich
trans-2-pentenal	84.12	0.22	1.281±0.281	1576-87-0	MBI lab
trans-2-hexen-1-ol	100.16	0.14	1.697±0.206	928-97-2	Sigma Aldrich
triethylamine	101.19	0.31	1.647±0.222	121-44-8	MBI lab
tropional	192.21	8.10E-04	1.982±0.343	1205-17-0	Givaudan
undecanal	170.29	4.20E-04	4.480±0.223	112-44-7	MBI lab
vanillin	152.15	0.024	1.208±0.272	121-33-5	Givaudan
vanillyl acetone (zingerone)	194.23	0.024	1.168±0.237	122-48-5	Givaudan
α-humulene	204.35	1.10E-08	6.592±0.249	6753-98-6	Fluka
γ-decalactone	170.25	4.20E-03	2.451±0.278	706-14-9	MBI lab

Table S2 Responses of CpomOR6 and CpomOR1 expressed in *Drosophila* T1 sensilla to pheromones, synergists and their combinations.

Replicate	**Compounds (10 µg/stimulus)**	**CpomOR6 (Spikes/s)**	**CpomOR1 (Spikes/s)**
1	Blank	4	4
	Hexane	4	-4
	(*E8,E10*)-12:OH	0	6
	(*E8,Z10*)- 12:OH	4	-2
	(*Z8,E10*)- 12:OH	-2	0
	(*Z8,Z10*)- 12:OH	6	-2
	(*E8,E10*)- 12:Ac	-6	2
	(*E8*)-12:OH	4	-2
	(*E9*)-12:OH	-2	-4
	(*E10*)-12:OH	8	-2
	12:OH	0	-2
	(*E*)-β-Farnesene	10	0
	Butyl hexanoate	4	4
	Ethyl-(*E2,Z4*)-decadienoate	2	-2

	(*Z9,E12*)- 14:Ac	-2	2
	4,8-Dimethyl-1,(*E*)-3,7-nonatriene	6	2
	3,7-Dimethyl-1,(*E*)-3,6-octatriene	2	2
	(*E8,E10*)-12:OH +(E)-β-Farnesene	2	-2
	(*E8,E10*)-12:OH + Butyl hexanoate	-4	2
	(*E8,E10*)-12:OH +Ethyl-(E2,Z4)-decadienoate	8	6
	(E8,E10)-12:OH +(Z9,E12)- 14:Ac	2	2
	(*E8,E10*)-12:OH + 4,8-Dimethyl-1,(E)-3,7-nonatriene	0	4
	(*E8,E10*)-12:OH + 3,7-Dimethyl-1,(E)-3,6-octatriene	4	2
	(E8,E10)-12:OH (100 ng)	8	-4
2	Blank	-4	-6
	Hexane	8	0
	(*E8,E10*)-12:OH	-2	2
	(*E8,Z10*)- 12:OH	0	-2
	(*Z8,E10*)- 12:OH	2	0
	(*Z8,Z10*)- 12:OH	-8	0
	(*E8,E10*)- 12:Ac	2	4
	(*E8*)-12:OH	-6	4
	(*E9*)-12:OH	6	0
	(*E10*)-12:OH	0	-2
	12:OH	6	4
	(*E*)-β-Farnesene	0	4
	Butyl hexanoate	4	-4
	Ethyl-(*E2,Z4*)-decadienoate	-10	2
	(*Z9,E12*)- 14:Ac	18	-4
	4,8-Dimethyl-1,(*E*)-3,7-nonatriene	-4	-6
	3,7-Dimethyl-1,(*E*)-3,6-octatriene	-2	8
	(*E8,E10*)-12:OH +(E)-β-Farnesene	10	2
	(*E8,E10*)-12:OH + Butyl hexanoate	-6	-2
	(*E8,E10*)-12:OH +Ethyl-(E2,Z4)-decadienoate	6	-2
	(E8,E10)-12:OH +(Z9,E12)- 14:Ac	-4	-4
	(*E8,E10*)-12:OH + 4,8-Dimethyl-1,(E)-3,7-nonatriene	-6	-2
	(*E8,E10*)-12:OH + 3,7-Dimethyl-1,(E)-3,6-octatriene	-2	2

	(E8,E10)-12:OH (100 ng)	-8	2
3	Blank	2	-2
	Hexane	2	0
	(*E8,E10*)-12:OH	2	0
	(*E8,Z10*)- 12:OH	4	0
	(*Z8,E10*)- 12:OH	8	6
	(*Z8,Z10*)- 12:OH	-8	0
	(*E8,E10*)- 12:Ac	8	4
	(*E8*)-12:OH	4	0
	(*E9*)-12:OH	0	2
	(*E10*)-12:OH	-6	0
	12:OH	-6	2
	(*E*)-β-Farnesene	2	2
	Butyl hexanoate	6	2
	Ethyl-(*E2,Z4*)-decadienoate	-2	2
	(*Z9,E12*)- 14:Ac	-6	2
	4,8-Dimethyl-1,(*E*)-3,7-nonatriene	4	2
	3,7-Dimethyl-1,(*E*)-3,6-octatriene	-4	0
	(*E8,E10*)-12:OH +(E)-β-Farnesene	0	0
	(*E8,E10*)-12:OH + Butyl hexanoate	-2	-6
	(*E8,E10*)-12:OH +Ethyl-(E2,Z4)-decadienoate	-2	-2
	(E8,E10)-12:OH +(Z9,E12)- 14:Ac	-2	-4
	(*E8,E10*)-12:OH + 4,8-Dimethyl-1,(E)-3,7-nonatriene	2	0
	(*E8,E10*)-12:OH + 3,7-Dimethyl-1,(E)-3,6-octatriene	4	-2
	(E8,E10)-12:OH (100 ng)	8	2
4	Blank	-	-4
	Hexane	-	-8
	(*E8,E10*)-12:OH	-	-12
	(*E8,Z10*)- 12:OH	-	-2
	(*Z8,E10*)- 12:OH	-	8
	(*Z8,Z10*)- 12:OH	-	4
	(*E8,E10*)- 12:Ac	-	2
	(*E8*)-12:OH	-	0
	(*E9*)-12:OH	-	4
	(*E10*)-12:OH	-	-4

	12:OH	-	-2
	(*E*)-β-Farnesene	-	0
	Butyl hexanoate	-	0
	Ethyl-(*E2,Z4*)-decadienoate	-	2
	(*Z9,E12*)- 14:Ac	-	-10
	4,8-Dimethyl-1,(*E*)-3,7-nonatriene	-	4
	3,7-Dimethyl-1,(*E*)-3,6-octatriene	-	2
	(*E8,E10*)-12:OH +(E)-β-Farnesene	-	-6
	(*E8,E10*)-12:OH + Butyl hexanoate	-	2
	(*E8,E10*)-12:OH +Ethyl-(E2,Z4)-decadienoate	-	-8
	(E8,E10)-12:OH +(Z9,E12)- 14:Ac	-	8
	(*E8,E10*)-12:OH + 4,8-Dimethyl-1,(E)-3,7-nonatriene	-	0
	(*E8,E10*)-12:OH + 3,7-Dimethyl-1,(E)-3,6-octatriene	-	-2
	(E8,E10)-12:OH (100 ng)	-	-8
5	Blank	-	6
	Hexane	-	-4
	(*E8,E10*)-12:OH	-	0
	(*E8,Z10*)- 12:OH	-	-2
	(*Z8,E10*)- 12:OH	-	-4
	(*Z8,Z10*)- 12:OH	-	12
	(*E8,E10*)- 12:Ac	-	4
	(*E8*)-12:OH	-	2
	(*E9*)-12:OH	-	-2
	(*E10*)-12:OH	-	2
	12:OH	-	2
	(*E*)-β-Farnesene	-	2
	Butyl hexanoate	-	-8
	Ethyl-(*E2,Z4*)-decadienoate	-	10
	(*Z9,E12*)- 14:Ac	-	0
	4,8-Dimethyl-1,(*E*)-3,7-nonatriene	-	-8
	3,7-Dimethyl-1,(*E*)-3,6-octatriene	-	4
	(*E8,E10*)-12:OH +(E)-β-Farnesene	-	4
	(*E8,E10*)-12:OH + Butyl hexanoate	-	6
	(*E8,E10*)-12:OH +Ethyl-(E2,Z4)-decadienoate	-	-12
	(E8,E10)-12:OH +(Z9,E12)- 14:Ac	-	0

	(*E8,E10*)-12:OH + 4,8-Dimethyl-1,(E)-3,7-nonatriene	-	4
	(*E8,E10*)-12:OH + 3,7-Dimethyl-1,(E)-3,6-octatriene	-	10
	(E8,E10)-12:OH (100 ng)	-	-4

Figure S1 Comparison between amplitudes of the calcium responses (mean ± SEM) to VUAA3 250 µM (69.71 ± 27.29 at time = 40s; left), codlemone 1500 µM (5.7 ± 3.64 at time = 40s; E8,E10-12OH, middle) and codlemone acetate 1500 µM (18.91 ± 10.31 at time = 40s; E8,E10-12Ac, right); n = 68. Grey color depicts standard deviation.

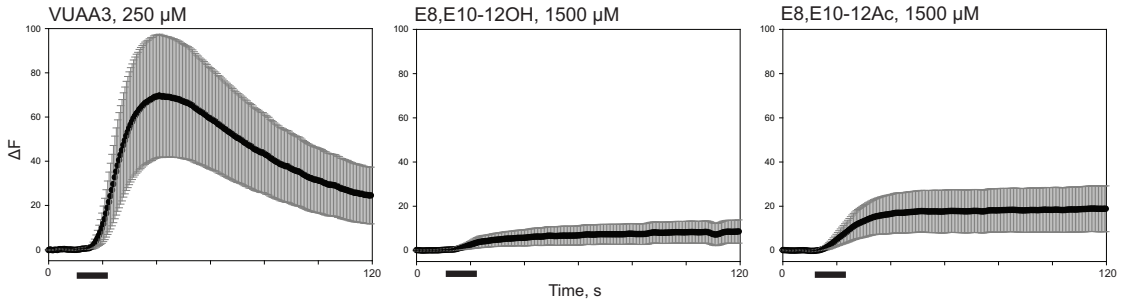
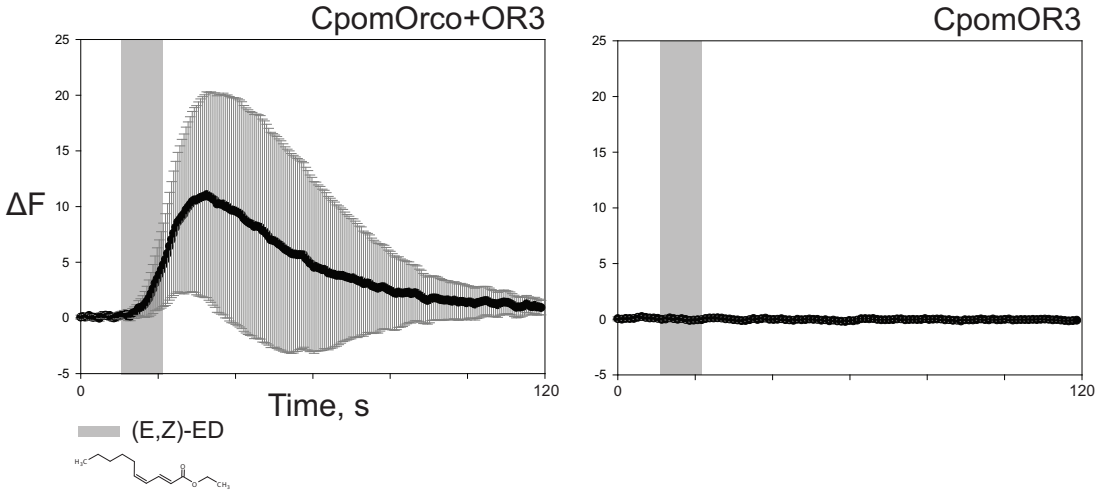


Figure S2 Comparison between amplitudes of the calcium responses (mean ± SEM) of CpomOR3 transfected HEK cells when co-transfected with CpomOrco (CpomOrco+OR3, n = 69) and without co-transfection (CpomOR3, n = 69). Stimulation was performed for 10 seconds using 250 µM (E,Z)-ED (Vertical bar). Grey color depicts standard deviation. Black bars: stimulus (10 s).



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With the aim to share some last thoughts with the ones who red this dissertation up to this point, I want to confide that the more I learnt how to become a scientist, the more I learnt how to become a man. I suggest to all readers, especially if young scientists, to never give up when doing science, but especially to never waive of themselves for science but rather to make of science part of themselves. Somato-sense your life, smell it and taste it, and do your best to leave a contribution to the next.

we were granted a lifetime to learn how to live forever

Alberto Maria Cattaneo