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Characterization of olfactory receptor neurons for pheromone candidate and plant volatile compounds in the clover root weevil, *Sitona lepidus*

Kye Chung Park [a,*], Mark McNeill [b], C. Rikard Unelius [a,c], Hyun-Woo Oh [d], David M. Suckling [a]

[a] The New Zealand Institute for Plant & Food Research, Lincoln 7608, New Zealand
[b] AgResearch Limited, Private Bag 4749, Christchurch 8140, New Zealand
[c] Faculty of Health and Life Sciences, Linnaeus University, 391 82 Kalmar, Sweden
[d] Korea Research Institute of Bioscience & Biotechnology, Daejeon 305-806, South Korea

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ABSTRACT

Antennal olfactory receptor neurons (ORNs) for pheromone and plant volatile compounds were identified and characterized in male and female clover root weevil, *Sitona lepidus* (Gyllenhal), using the single sensillum recording technique with five pheromone-related compounds, and 40 host and non-host plant volatile compounds. Overall, seven different types of olfactory sensilla containing specialized ORNs were identified in each sex of *S. lepidus*. Among them, three different types of sensilla in the males and two types in the females housed ORNs specialized for pheromone-related compounds. The ORNs in males were specialized for 4-methyl-3,5-heptanedione or one or more of four stereoisomers of 5-hydroxy-4-methyl-3-heptanone. In contrast, female sensilla did not contain ORNs sensitive to 4-methyl-3,5-heptanedione while they contained ORNs sensitive to and specialized for the stereoisomers of (4*S*,5*S*)-5-hydroxy-4-methyl-3-heptanone. In addition to the pheromone-related ORNs, four types of olfactory sensilla contained ORNs responsive to plant volatile compounds in male *S. lepidus*, and five types in females. Most of the ORNs identified in *S. lepidus* showed a high degree of specificity to specific volatile compounds although some of the active compounds showed overlapping response spectra in the ORNs across different types of sensilla. The most active plant volatile compounds were the four green leaf volatile compounds, (*E*)-2-hexenol, (*Z*)-2-hexenol, (*Z*)-3-hexenol and (*E*)-2-hexenal, and isomers of two monoterpenols, (±)-linalool and (±)-α-terpineol, all eliciting strong responses from relatively large numbers of ORNs in male and female *S. lepidus*. Our study indicates that *S. lepidus* has a set of highly sensitive and selective ORNs for pheromone and plant volatile compounds. Further work is needed to elucidate the behavioral implications of these findings.

1. Introduction

Olfactory receptor neurons (ORNs) on the insects' antennae play an essential role in detecting and discriminating important odorants for locating their mate and food. Phytophagous insects have species-specific sets of antennal ORNs and many of these ORNs are specialized for detecting a narrow range of volatile compounds (Barata et al., 2002; Larsson et al., 2001; Stensmyr et al., 2001). Often, the profiles of ORNs are directly related to the behavioral activities of their corresponding active compounds (Ache and Young, 2005; Bargmann, 2006). In pheromone communication, a number of sex-specific ORNs in insect antennae are specialized for detecting sex pheromone or its behavioral antagonist compounds (Baker, 2008; Baker et al., 2004; Larsson et al., 1999). In host location, the presence of ORNs specialized for detecting kairomones released from host and non-host plants has been reported in a wide range of insect groups (Andersson et al., 2012; Larsson et al., 2001; Olsson et al., 2006). Sensory physiological and olfactory receptor protein studies indicate that the profiles of the ORNs in insects are species-specific (Ache and Young, 2005; Andersson et al., 2012). Furthermore, the specialization of ORNs plays critical parts of species-isolation and host-specificity in insects (Andersson et al., 2009; Baker, 2008).

Electrophysiological recording can be used to identify the active compounds on the specific ORNs and to examine the response spectra of the ORNs. Single sensillum recording (SSR) measures action potentials from individual ORNs in olfactory sensilla, providing valuable information on the specificity and sensitivity of each ORN to different volatile compounds. The information obtained can often be used to develop effective synthetic semiochemical baits to manage the behavior of insects. Indeed, synthetic blends of volatile compounds identified by the headspace sampling of host plants and subsequent electrophysiological recordings have

* Corresponding author. Tel.: +64 3 977 7351; fax: +64 3 977 7341.
E-mail address: kpark@plantandfood.co.nz (K.C. Park).

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been proven to be behaviorally active in various insects (Bruce and Pickett, 2011; Olsson et al., 2009).

Sitona (Coleoptera: Curculionidae) is a relatively large genus of weevils, containing around 100 species. *Sitona* weevils are phytophagous, feeding exclusively on plants of the family Fabaceae, with their larvae being subterranean feeders (Danthanarayana, 1967). Several species of *Sitona* are important agricultural pests of legumes (Petrukha, 1970). The clover root weevil, *Sitona lepidus*, is of European origin but has spread to North America and New Zealand, being a destructive pest of white and red clover in temperate grasslands (Barratt et al., 1996; Bright, 1994; Murray and Clements, 1994). *Sitona* species exhibit considerable species-specificity in host selection. For example, *Sitona lineatus* feeds and oviposits around pea (*Pisum sativa*), bean (*Vicia faba*) and vetch (*Vicia sativa*) (Landon et al., 1997), *S. regenstenensis* feeds on broom (*Cytisus scoparius*) (Danthanarayana, 1969), *Sitona discoideus* and *Sitona humeralis* feed on lucerne (*Medicago* spp.) (Aeschlimann, 1984; Phillips and Barratt, 2004), *Sitona crinitus* attacks lentils (*Lens* species) (El-Bouhssini et al., 2008), and *S. lepidus* feeds on clover (*Trifolium* spp.) with a preference for white clover (*Trifolium repens*) (Johnson et al., 2004; Phillips and Barratt, 2004). It is likely that olfactory cues are involved in host location in *Sitona* weevils, as shown in behavioral observations (Hardwick and Harens, 2000). Therefore, it could be hypothesized that different species of *Sitona* would have species-specific sets of ORNs for discriminating between their host plants and non-host plants. However, it is not yet well understood how *S. lepidus* locate their host plants and mates, and what kind of olfactory cues are involved.

A diketone, 4-methyl-3,5-heptanedione, is an aggregation pheromone in *S. lineatus* (Blight et al., 1984; Blight and Wadhams, 1987; Toshova et al., 2009). Recently, it has been reported that the males of another *Sitona* species, *S. discoideus*, produce a sex-specific monoketone, (4*S*,5*S*)-5-hydroxy-4-methyl-3-heptanone, as well as the diketone (Unelius et al., 2013). A diastereomer of this monoketone, (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone, has been known as an aggregation pheromone compound in several weevil species in the related genus *Sitophilus*, Curculionidae (Phillips et al., 1985; Schmuff et al., 1984; Walgenbach et al., 1987). It is common in insects that taxonomically related species share structurally similar compounds for inter- and intra-specific olfactory communication (Cossé et al., 1998; Domingue et al., 2007). It appears that some *Sitona* species share 4-methyl-3,5-heptanedione as a common compound for their pheromone communication, since field studies showed several species were attracted to traps baited with this compound in Hungary and Bulgaria (Toshova et al., 2009; Toth et al., 1998). In this case, species-specific sets of ORNs for this and other pheromone compounds would facilitate differentiating semiochemical signals among different *Sitona* species, as in other groups of insects. The strong preference of *S. lepidus* to white clover (Johnson et al., 2004) also indicates that this weevil uses chemical signals to locate its host plant.

In this study, we have investigated the profiles of antennal ORNs in male and female *S. lepidus*, using the SSR technique, with a hypothesis that *S. lepidus* has a specialized intra- and inter-specific peripheral olfactory sensory system for mate and host location. Specialized ORNs for pheromone-related and plant-volatile compounds have been identified and their response profiles characterized.

2. Materials and methods

2.1. Insects

Adults of *S. lepidus* used in the experiments were collected from *T. repens* stands in the Canterbury region of New Zealand. The age of the weevils at the time of collection was indeterminable, but at least 7 days old. Males and females were distinguished based on the shape of the ventrite (Bright, 1994) and kept in separate containers with fresh-cut *T. repens*.

2.2. Microscopy

Adult males and females of *S. lepidus* were individually fixed in PBS-buffered 2.5% paraformaldehyde–glutaraldehyde solution in distilled water for at least 2 days. For scanning electron microscope (SEM) observation the fixed antennae of six males and five females were mounted on aluminum stubs and gold-coated with a sputter coater (SC502, Polaron). The antennae were then observed with a SEM (FEI Quanta 250 FEG). For light microscopy (LM) and transmission electron microscopy (TEM) the fixed antennae from one male and one female were further fixed with 1% osmium tetroxide in water, embedded in epoxy resin (Epon 812), sectioned at 1 μm thick for LM or at <150 nm thick for TEM with an ultra-microtome (Leica, Austria). The sections for LM were stained with methylene blue and the sections for TEM with uranyl acetate and lead citrate. The sensilla on the antennae of *S. lepidus* were observed with a light microscope (Elipse Ci-L, Nikon, Japan) and a transmission electron microscope (CM20, Philips, the Netherlands).

2.3. Test compounds and odor presentation

Five pheromone-related compounds (4-methyl-3,5-heptanedione and the four stereoisomers of (4*S*,5*S*)-5-hydroxy-4-methyl-3-heptanone) (Fig. 1) and 40 host or non-host plant volatile compounds were used as stimuli in the single sensillum recording (SSR) (Table 1). At least 12 of the plant volatile compounds investigated for SSR activities are present in red and white clover (Buttery et al., 1984; Figueiredo et al., 2007; Kigathi et al., 2009), the host plant of *S. lepidus*. The rest of the test compounds are common volatiles across many plant species. The source, purity and presence in clovers of the test compounds are shown in Table 1. The plant volatile compounds were purchased from commercial

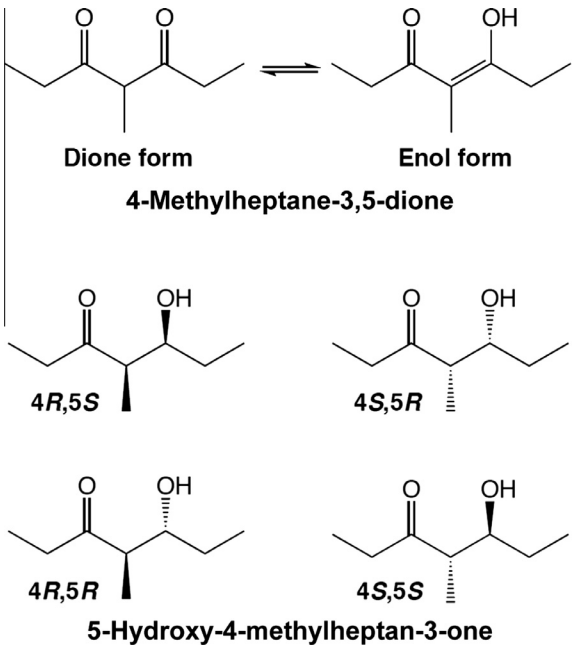

Fig. 1. A diketone, 4-methylheptane-3,5-dione, and the four isomers of a monoketone, 5-hydroxy-4-methylheptan-3-one, pheromone-related compounds used in our study. Note that the diketone is in equilibrium with an enol form (ca 10% enol).