Noxious chemical discrimination by *Tribolium castaneum* TRPA1 channel in the HEK293 cell expression system[☆],[☆☆]

Kenji Shimomura [a],[*], Hinoki Oikawa [b], Kosuke Yamamoto [c], Takehito Terajima [a], Shunsuke Yajima [b], Motohiro Tomizawa [a]

[a] *Department of Chemistry for Life Sciences and Agriculture, Tokyo University of Agriculture, Tokyo, 156-8502, Japan*
[b] *Department of Bioscience, Tokyo University of Agriculture, Tokyo, 156-8502, Japan*
[c] *Department of Molecular Microbiology, Tokyo University of Agriculture, Tokyo, 156-8502, Japan*

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ABSTRACT

Nociception is the sensory perception of noxious chemical stimuli. Repellent behavior to avoid noxious stimuli is indispensable for survival, and this mechanism has been evolutionarily conserved across a wide range of species, from mammals to insects. The transient receptor potential ankyrin 1 (TRPA1) channel is one of the most conserved noxious chemical sensors. Here, we describe the heterologous stable expression of *Tribolium castaneum* TRPA1 (TcTRPA1) in human embryonic kidney (HEK293) cells. The intracellular Ca^{2+} influx was measured when two compounds, citronellal and L-menthol, derived from plant essential oils, were applied *in vitro* using a fluorescence assay. The analysis revealed that citronellal evoked Ca^{2+} influx dose-dependently for TcTRPA1, whereas L-menthol did not. In combination with our present and previous results of the avoidance-behavioral assay at the organism level, we suggest that TcTRPA1 discriminates between these two toxic compounds, and diversification in the chemical nociception selectivity has occurred in TRPA1 channel among insect taxa.

1. Introduction

Nociception is the sensory perception of a noxious stimulus, such as toxic chemicals and thermal and mechanical stimuli, which can potentially cause harm (He et al., 2022), facilitating escape and avoidance behavior patterns (Im and Galko, 2012). Probably because nociception is essential for survival, nociceptors are evolutionarily conserved (Sneddon, 2018). Nociception usually involves the noxious stimulus triggering sensory transduction via membrane proteins such as receptors and ion channels expressed on sensory neurons on sensillae. All animals possess nociceptors mediating thermal, chemical, or mechanical nociception, and the underlying physiological mechanisms are consistent between insects and mammals (Malafoglia et al., 2013).

Transient receptor potential (TRP) channels were initially discovered and functionally characterized as drivers of phototransduction in response to light in *Drosophila melanogaster* (Cosens and Manning, 1969; Montell and Rubin, 1989). TRP channels are nonselective cation channels that are evolutionarily conserved (Venkatachalam and Montell, 2007). The TRP channel family is divided into seven subfamilies: TRPC (Transient Receptor Potential Canonical), TRPA (Transient Receptor Potential Ankyrin), TRPV (Transient Receptor Potential Vanilloid), TRPN (Transient Receptor Potential No Mechanoreceptor Potential C), TRPM (Transient Receptor Potential Melastatin), TRPP (Transient Receptor Potential Polycystin), and TRPML (Transient Receptor Potential Mucolipin). TRP channels are the primary sensors for various types of stimuli, including smell, taste, light, gravity, and mechanical sensation (Fowler and Montell, 2013). The transient receptor potential ankyrin 1 (TRPA1) channel is a well-characterized ion channel involved in sensing noxious stimuli such as reactive chemicals, intense cold, as well as endogenous signaling associated with cell damage (Talavera et al., 2020). In adult *D. melanogaster,* the TRPA1 (dTRPA1) channel is expressed in peripheral gustatory chemosensors in the proboscis that detect reactive electrophiles (such as allyl isothiocyanate in wasabi; Kang et al., 2010), in peripheral olfactory sensillae that detect volatile compounds like citronellal (Kwon et al., 2010), and in central neurons of the brain that detect high temperatures (Hamada et al., 2008). In *D. melanogaster* larvae, the dTRPA1 is activated by menthol, lower temperatures, and high temperatures that is more sensitive when speed

of the temperature increases rapidly (Himmel et al., 2019; Luo et al., 2017). There are four dTRPA1 isoforms (dTRPA1-A-D), which showed following sensitivity: dTRPA1-C = dTRPA1-D > dTRPA1-A >> dTRPA1-B for citronellal, and dTRPA1-A > dTRPA1-D > dTRPA1-C > dTRPA1-B for menthol when transiently expressed in mammal cells (Boonen et al., 2021; Zhong et al., 2012), and, additionally, the presence of dTRPA1-independent avoidance behavioral mechanisms were suggested to both citronellal and menthol in adult fly (Kwon et al., 2010; Wang et al., 2021).

The red flour beetle, *Tribolium castaneum* (*T. castaneum*; Coleoptera: Tenebrionidae), is the first genome-sequenced beetle (Tribolium Genome Sequencing Consortium, 2008), and is widely used as a model because of its robust systemic RNA interference (RNAi)-mediated gene silencing response (Tomoyasu et al., 2008). Furthermore, *T. castaneum* is a global pest of stored food products, especially wheat and rice (Campbell et al., 2022). Several TRP channels have been found in *T. castaneum* (Matsuura et al., 2009), and *T. castaneum* TRPA1 (TcTRPA1) revealed the involvement of high temperature avoidance, but that the range of temperatures inducing a response differ between *T. castaneum* and *D. melanogaster* (Kim et al., 2015).

Essential oils derived from medicinal and aromatic plants are widely explored as sustainable insecticides and repellents (Regnault-Roger et al., 2012). Citronellal, isolated from citronella essential oils extracted from citronella grass (*Cymbopogon* spp.), and ʟ-menthol, from mint (*Mentha* spp.), evoke avoidance behavior in adult *T. castaneum* (Aggarwal et al., 2001; Zhang et al., 2011). Because both citronellal and menthol act via TRPA1 channel in *Drosophila*, TcTRPA1 mediates nociception is likely involved in the avoidance behavior to these two compounds.

Knocking down TcTRPA1 with RNAi reduced avoidance of citronellal but not ʟ-menthol in *T. castaneum* (Shimomura et al., 2022). By contrast, dTRPA1 is essential for an avoidance response (rolling behavior) to menthol in *D. melanogaster* larvae (Himmel et al., 2019). Coleoptera and Diptera diverged around 300–350 mya (Misof et al., 2014), which may account for the different chemical nociception selectivity in the TRPA1 channels in the two groups. To clarify why TcTRPA1 responded differently to its Drosophila homolog, we established heterologous stable TcTRPA1 expression in human embryonic kidney (HEK293) cells, and performed a fluorescence assay to monitor TcTRPA1 activation when these two compounds were applied *in vitro*.

2. Material and methods

2.1. TcTRPA1-expressing vector construction for mammalian cells

The full-length *TcTRPA1* cloned into a pUC19 plasmid was used as the template gene (Shimomura et al., 2022). The 5′-end gene-specific primer contained EcoRI-Kozak sequence-2XMyc-tagged and the 3′-end XbaI restriction enzyme site (Table 1). PCR was performed on a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) with KOD-plus-Neo DNA polymerase (TOYOBO Co., Ltd., Osaka, Japan). The conditions were as follows: 94 °C for 2 min, 35 cycles at 98 °C for 10 s, 58 °C for 30 s, and 68 °C for 4 min. The PCR product and pcDNA3.1(+) vector containing neomycin resistance gene for selection of stable cell line (Invitrogen Corporation, CA, USA) was double-digested using EcoRI and XbaI (New England Biolabs, Inc., MA, USA) according to the manufacturer's protocol. The purified EcoRI/XbaI-digested, 2XMyc-tagged full-length

TcTRPA1 DNA was ligated into the EcoRI/XbaI-digested pcDNA3.1(+) using Ligation high Ver.2 (TOYOBO Co., Ltd., Osaka, Japan) and used to transform ECOS Competent *Escherichia coli* (*E. coli*) DH5α cells (NIPPON GENE, Tokyo, Japan). Plasmids extracted from the *E. coli* colonies (FastGene Plasmid Mini kit, NIPPON Genetics, Co., Ltd., Tokyo, Japan) were tested using EcoRI/XbaI digestion. Large quantities of highly purified pcDNA3.1(+)/*TcTRPA1* plasmids were produced using NucleoBond Xtra Midi (MaCHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. The final purified plasmid was confirmed by sequencing.

2.2. Cell culture

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin in a 100-mm cell-culture dish at 37 °C in a 5% CO_2 incubator.

2.3. Stable cell line generation

HEK293 cells were lifted from a confluent culture using trypsin (FUJIFILM Wako Pure Chemical Corporation) and plated at a cell density of 3×10^5 cells/mL into a 100-mm cell-culture dish. The cells were incubated overnight at 37 °C in a 5% CO_2 incubator. For stable TcTRPA1 cells, 2 μg of pcDNA3.1(+)/*TcTRPA1* plasmid and 30 μL of X-treme-eGENE HP DNA transfection reagent (Sigma-Aldrich, St. Louis, MO, USA) were diluted in 1 mL DMEM and incubated at room temperature for 30 min. The mixture was then added to a 100-mm cell-culture dish containing the HEK293 cells and incubated overnight at 37 °C in a 5% CO_2 incubator. The medium was removed and replaced with fresh medium containing 500 μg/mL G-418 (FUJIFILM Wako Pure Chemical Corporation). The cells were cultured continuously for approximately 2 weeks in the presence of G-418 until antibiotic-resistant cells were established. The confluent cells were diluted and plated at a 0.5 cell/well density on a 96-well cell-culture plate. The cells were continuously grown at 37 °C in a 5% CO_2 incubator, during which the culture volumes were increased to 24, 6-well cell-culture plates and a 100-mm cell-culture dish.

2.4. Western blot analysis

Stable TcTRPA1-expressed HEK293 cells were seeded in 100-mm cell culture dishes. After the cells were confluent, the culture medium was removed from the dishes, and the cells were washed with phosphate-buffered saline (PBS) and lifted using trypsin. The cells were pelleted using centrifugation at $400 \times g$ for 5 min at room temperature and washed thrice with PBS buffer.

Cell pellets were incubated on a rotator at 4 °C for 1 h in RIPA buffer containing a protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). The cell suspension was centrifuged at $20,000 \times g$ for 15 min at 4 °C, and the supernatant was collected. The protein concentration was measured using a BCA assay kit (FUJIFILM Wako Pure Chemical Corporation). Then, 2 mg protein solution was incubated on a rotator with agarose beads conjugated with monoclonal anti-Myc-tag antibody (MBL Co., Ltd., Tokyo, Japan) for 1 h at 4 °C. After centrifugation at $2500 \times g$

Table 1
Primer sequences used in this study.

	Primer name	Primer (5′ -> 3′)
Forward	F_EcoRI_2XMyc_TcTRPA1	<u>GAATTC</u>*CACCATG***GAGCAGAAGCTGATCTCCGAGGAGGACCTGGAGCAGAAGCTGATCTCCGAGGAGGACCTG**ATGCCAAATCTAATGCATCTCCTTC
Reverse	R_XbaI_TcTRPA1	<u>TCTAGA</u>CTAAGTGCTGCTGCCTTTATTGTT

Restriction enzyme recognition sequences are underlined, the Kozak region is in italics, and 2XMyc-tag sequences are in bold.

for 1 min, the supernatant was removed, and the agarose beads were washed thrice with RIPA buffer. After removing the supernatant, SDS-PAGE sample buffer (Pharma Foods International Co., Ltd., Tokushima, Japan) was added to the agarose beads, incubated at 100 °C for 5 min to denature, and loaded onto 12.5% SDS-PAGE gel. SDS-PAGE was performed using a WSE-1165 MiniSlab gel tank (ATTO Corporation, Tokyo, Japan).

Western blot analysis was performed by transferring the proteins onto 0.45 μm polyvinylidene difluoride membrane (Immobilon, Merckmillipore, MA, USA) using Trans-blot SD Semi-dry Electrophoretic Transfer Cell (Bio-Rad, CA, USA) at 15 V for 45 min. The membrane was blocked with 5% nonfat milk powder (FUJIFILM Wako Pure Chemical Corporation) in Tris-buffered saline with Tween 20 (TBST) buffer and incubated with TBST buffer containing anti-Myc-tag mouse monoclonal primary antibody M047–3 (MBL) at 4 °C overnight. Myc-tagged TcTRPA1 was detected using an anti-mouse-IgG-horseradish peroxidase polyclonal secondary antibody 330 (MBL) with Chemi-Lumi One Ultra (Nacalai Tesque) that has ultra-high sensitivity, allowing detection in the low-femtogram range and Amersham Imager 600 (GE Healthcare, WI, USA).

2.5. Ca²⁺ influx fluorescence assay

The assay was performed using Fluo-4 Direct™ Calcium Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) without culture media removal. The confluent stable TcTRPA1-expressed HEK293 cells in a 100-mm cell-culture dish were lifted using trypsin and plated on a transparent 96-well IsoPlate-96 TC plate with a black frame (PerkinElmer Inc., MA, USA) at a density of 25,000 cells/well (50 μL). The plates were incubated overnight at 37 °C in a 5% CO_2 incubator. The next day, 49 μL Fluo-4 Direct™ Calcium Assay loading solution containing water-soluble probenecid (Thermo Fisher Scientific) was applied to each well. The cells were incubated at 37 °C in a 5% CO_2 incubator for 30 min, then incubated at room temperature for 30 min and transferred to a Varioskan LUX microplate reader (Thermo Fisher Scientific). The fluorescence was measured by exciting wells at 490 nm and detecting emission at 520 nm. Citronellal (Kanto Chemical Co., Inc., Tokyo, Japan) and *L*-menthol (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), diluted with Fluo-4 Direct™ Calcium Assay Buffer (Thermo Fisher Scientific), and 11 μL of 10X test compound solution was added to each well. Base fluorescence was measured for 30 s, and response fluorescence was measured for 120 s after compound application. Data used for dose-response analysis were obtained from the maximal response, which was approximately 30 s after compound application. The mean responses from the three wells (three biological replicates) were used to create four-parameter dose-response curves, and the EC_{50} values were estimated using GraphPad Prism data analysis software (GraphPad, Inc., CA, USA).

3. Results and discussion

We constructed a stable HEK293 cell line expressing TcTRPA1 to analyze activation by citronellal and *L*-menthol derived from essential oils. Cell lysates were immunoprecipitated and subsequently analyzed by western blotting using an anti-Myc-tag antibody to clarify TcTRPA1's recombinant expression at the protein level in HEK293 cells. The 142.6-kDa size of 2XMyc-TcTRPA1 was detected only in the lysates of the stably expressed cells but not in the lysates of mock-transfected cells (Fig. 1).

The citronellal and *L*-menthol effects on TcTRPA1 channels were determined using a Ca²⁺ influx assay (Fig. 2A). Ca²⁺ influx was determined by measuring signals from Fluo-4, a fluorescent Ca²⁺indicator. The response to citronellal was dose-dependent, with an EC_{50} of 69.67 μM (Fig. 2B). Meanwhile, *L*-menthol did not induce Ca²⁺ influx in TcTRPA1-expressing cells at concentrations ranging from 3 μM to 1 mM.
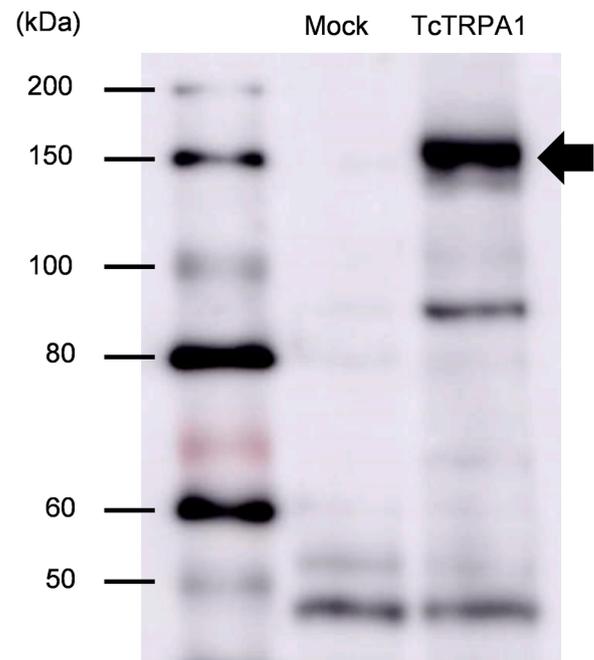

Fig. 1. *In vitro* TcTRPA1 protein stably expressed in HEK293 cells. Western blotting analysis after immunoprecipitation of 2XMyc-TcTRPA1 with anti-Myc-tag-mouse-monoclonal-antibody-agarose. The arrowhead indicated the TcTRPA1 detection (142.6-kDa), but not detected in mock-transfected supernatant.

The mock-transfected cells did not demonstrate Ca²⁺ influx in response to citronellal or *L*-menthol (Fig. 2C). TcTRPA1-expressing cells were activated by citronellal in a dose-dependent manner but not by *L*-menthol, which is consistent with a lack of response to *L*-menthol in previous whole-organism behavioral avoidance assays and RNAi knockdown studies (Shimomura et al., 2021, 2022) in *T. castaneum*.

The TRPA1 isoforms have not been characterized in *T. castaneum* (Matsuura et al., 2009), although several TRPA1 isoforms arising from alternative splicing have been reported in other insect species; four in *D. melanogaster* (dTRPA1 A-D; Zhong et al., 2012), and two or three in three mosquito genera (Li et al., 2019). All four dTRPA1s were activated by citronellal and menthol with different intensity levels in heterologous transient cell expression (Boonen et al., 2021), and the avoidance (rolling) behavior *Drosophila* larvae exhibit in response to menthol, is ablated in a homozygous mutant of *dTRPA1* (Himmel et al., 2019). Thus, TcTRPA1 does not have the *L*-menthol-responsiveness of its dipteran homologs.

However, an olfactory-receptor-mediated avoidance of essential oils-derived compounds such as citronellal and menthol is present in adult flies, suggesting the presence of a dTRPA1-independent pathway (Kwon et al., 2010; Wang et al., 2021). Knocking down odorant receptor function, or even removing the antennae, of *T. castaneum* does not ablate the behavioral response to either citronellal or *L*-menthol (Shimomura et al., 2021, 2022). Thus, TcTRPA1 appears to be the primary mechanism for detecting citronellal, and (unlike in Diptera), TcTRPA1 is not associated with *L*-menthol detection, for which TRPM channel is probably specialized (Shimomura et al., 2021). To our knowledge, this is the first molecular exploration of TRP channel function as chemical nociceptors in Coleoptera, and the first to show that coleopteran TRPA1 is not activated by *L*-menthol. Further experiments with the heterologous expression system will help clarify the breadth of TRPA1 channel nociception and help to develop new repellents for sustainable integrated pest management.
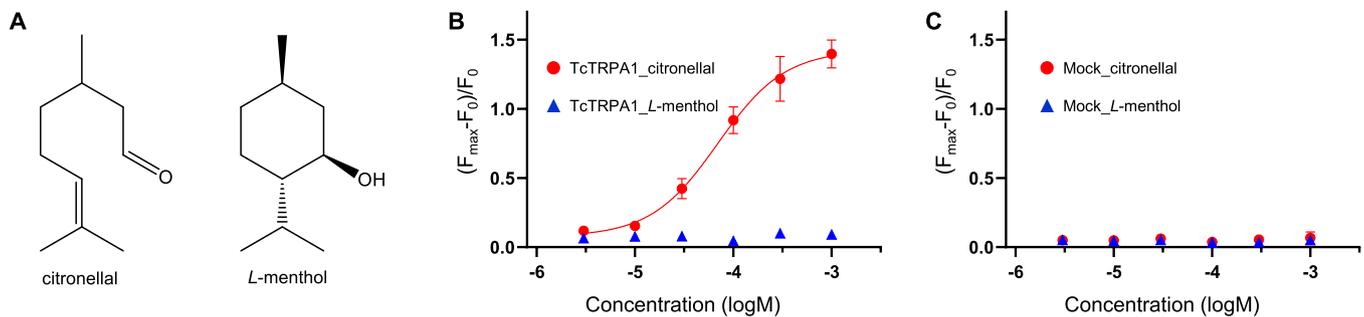
Fig. 2. (A) Chemical structure of citronellal and L-menthol used in this assay. (B) Ca^{2+} influx assay using HEK293 cells stably expressing TcTRPA1 and (C) mock-transfected HEK293 cells; dose-response curve of fluo-4 fluorescence changes induced by citronellal but not by L-menthol; the plots fitted to a four-parameter logistic model, a citronellal EC$_{50}$ of 69.67 μM calculated from the fitted curve. Data are expressed as mean ± standard deviation (SD) of the mean ($n = 3$). The y-axis represents the Ca^{2+} influx value, F$_{max}$: maximum fluorescence after compound applied, F$_0$: baseline fluorescence (See supplementary materials file Fluorescence_data.xlsx).

4. Conclusion

The TcTRPA1 channel *in vitro* can discriminate between two noxious chemical compounds, citronellal and L-menthol, accounting for TcTRPA1-mediated citronellal (but not L-menthol) described at the whole-level. The TcTRPA1-expressed cell line we described can be used for new repellent screening.

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CRediT authorship contribution statement

Kenji Shimomura: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Hinoki Oikawa:** Investigation. **Kosuke Yamamoto:** Conceptualization, Methodology, Resources. **Takehito Terajima:** Conceptualization. **Shunsuke Yajima:** Conceptualization, Supervision. **Motohiro Tomizawa:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are available in an excel file Fluorescence_data.xlsx in the supplementary materials.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2023.100066.

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