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Modulation of *Drosophila suzukii* type 1 tyramine receptor (DsTAR1) by monoterpenes: a potential new target for next generation biopesticides

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ABSTRACT

This study proposes a biochemical and molecular model for the interaction between the *Drosophila suzukii* type 1 tyramine receptor (DsTAR1) and monoterpenes.A preliminary molecular and functional characterization of DsTAR1 cDNA revealed that a 1.8 kb long ORF codes for a 600 amino acid polypeptide featuring seven transmembrane domains, as expected for a GPCR. A stable HEK 293 cell line expressing DsTAR1 was tested for responsiveness to tyramine (TA) and octopamine (OA). In intracellular calcium mobilization studies, TA led to a concentration-dependent increase in $[Ca^{2+}]_i$ ($pEC_{50} \sim 6.40$), completely abolished by pre-incubation with the antagonist yohimbine 1 μM. Besides, in dynamic mass redistribution (DMR) studies, TA evoked a positive DMR signal in a concentration-dependent manner ($pEC_{50} \sim 6.80$).The recombinant cell line was then used to test three monoterpenes (thymol, carvacrol and α-terpineol) as putative ligands for DsTAR1. The terpenoids showed no agonist effects in both DMR and calcium mobilization assays, but they increased the potency of the endogenous ligand, TA, acting as positive allosteric modulators. Moreover, expression analysis on adults *D. suzukii*, exposed for 24, 72 or 120 h to a sublethal concentration of the three monoterpenes, showed a downregulation of *DsTAR1*.This evidence has led to hypothesize that the downregulation of *DsTAR1* might be a compensatory mechanism in response to the positive allosteric modulation of the receptor induced by monoterpenes.Therefore, these findings might be useful for the development of a new generation of biopesticides against *Drosophila suzukii*, targeting TAR1.

1. Introduction

The Spotted Wing Drosophila (*Drosophila suzukii*, Matsumara, 1931) is a phytophagous pest native of Asia, that prefers ripe fruits where the females lay the eggs through a robust ovipositor (Mitsui et al., 2006; Rota-Stabelli et al., 2013). The fruits damaged by larvae developing inside lose their commercial value (Lee et al., 2011). Furthermore, the oviposition wounds allow for secondary infections by other insects and pathogens, including fungi, yeast and bacteria (De Camargo and Phaff, 1957; Louise et al., 1996).

Since *D. suzukii* arrival in Europe and North America in 2008 (Walsh et al., 2011; Cini et al., 2012; Asplen et al., 2015), numerous chemical compounds have been tried as insecticides, including organophosphates (malathion, diazinon, dimethoate), pyrethroids (permethrin, pyrethrin, zeta-cypermethrin), neonicotinoids (acetamiprid, imidacloprid, thiamethoxan), diamides (cyantraniliprole, chlorantraniliprole) and spinosyns (spinosad, spinetoram) (Bruck et al., 2011; Cuthbertson et al., 2014; Profaizer et al., 2015; Shawer et al., 2018). Spinosad, a biopesticide produced by the metabolism of *Saccharopolyspora spinosa*, is the most effective chemical tool against *D. suzukii* (Van Timmeren and Isaacs, 2013). Unfortunately, cases of low susceptibility and/or resistance in some populations in North America have recently been described (Grees and Zalom, 2018).

In the last few years, essential oils (EOs) have received a growing interest: they are natural, volatile and complex compounds accumulated by aromatic plants as secondary metabolites. Indeed, since the 80's it is known that they can be used in insect pest control (Regnault-Roger, 1997). EOs are characterized by two predominant components with different biosynthetic origins, phenylpropanoids and terpenes (monoterpenes and sesquiterpenes) (Pichersky and Gang, 2000). Terpenes are molecules made from the combination of several 5-carbon-base (C_5) units called isoprene. Phenylpropanoids in plants are syn-

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thesized from the amino acids tyrosine and phenylalanine. Their structure is characterized by a phenylic unit (C_6) bound to a propanoic unit (C_3). Phenylpropanoids occur less frequently than terpenes and they serve as essential components of a number of structural polymers (Regnault-Roger et al., 2012).

The terpenes composition of EOs is variable between different plant species but monoterpenes are usually more abundant, accounting up to the 90% of them (Bakkali et al., 2008). Typically, they exert their toxic effect by reducing or disrupting invertebrate growth at several life stages (Konstantopoulou et al., 1992). For instance thymol, a constituent of the essential oil from the plant *Thymus vulgaris L.*, is a monoterpene known for its pesticide action against numerous arthropods and is widely used to control *Varroa destructor* (Calderone et al., 1997).

As far as *Drosophila suzukii* is concerned, several EOs, and their major terpenic components, showed toxic activity toward the pest, including the EO from *Mentha piperita* (menthol), *Perilla frutescens* (perilla aldehyde) and *Thymus zygis* (thymol and carvacrol) (Park et al., 2016).

The precise mechanism of action of EOs is still unclear. It is thought that several terpenes can interact with P450 cytochromes, an enzymatic class involved in the insecticide detoxification processes (Jensen et al., 2006). Some monoterpenes, for example thymol, cause neuronal degeneration through direct binding to GABA receptors (Priestley et al., 2003). Other terpenes, such as linalool and 1,8-cineole, inhibit acetylcholinesterase (Mills et al., 2004) while eugenol and geraniol inhibit neuronal activity (Price and Berry, 2006). Moreover, several monoterpenes have been shown to interact with the octopaminergic/tyraminergic system, analogous to the adrenergic system present in the vertebrates. The biogenic amines tyramine (TA) and octopamine (OA), present in traces in the vertebrate nervous system, are important neurochemical modulators in invertebrates (David and Coulon, 1985). Their biosynthetic pathway begins with the decarboxylation of tyrosine to TA by tyrosine decarboxylase. Thereafter, TA is hydroxylated into OA by tyramine β-hydroxylase (Roeder, 2005). Several studies, initially focused on OA and then extended to TA, have shown that both these amines are neurotransmitter controlling numerous insect physiological processes such as reproduction (Clark and Lange, 2003; Donini and Lange, 2004; Da Silva and Lange, 2008), locomotion (Saraswati et al., 2004; Fox et al., 2006), immune functions (Baines and Downer, 1994; Adamo, 2009) and smell and related learning (Kutsukake et al., 2000; Pophof, 2002; Farooqui et al., 2003; Schwaerzel et al., 2003). In most cases, OA and TA exert their action by interacting with and activating the corresponding receptors, which are G Protein-Coupled Receptors (GPCRs). These receptors are classified into five main groups based on their structure: α-adrenergic-like receptors (OctαR also known as OAMB or OA1), β-adrenergic-like receptors (OctβR, also known as OA2), tyramine receptors type 1 (TA/OA or TAR1) and tyramine receptors type 2 and 3 (TAR2 and TAR3), although TAR3 was identified only in *D. melanogaster* (Bayliss et al., 2013; Wu et al., 2014).

Several studies have revealed that natural molecules with insecticidal activity, such essential oils, can interact with octopaminergic (Enan, 2001; Enan, 2005a) and with tyraminergic receptors in *D. melanogaster* (Enan, 2005b). In particular, TAR1 can be stimulated by several monoterpenes in *D. melanogaster* as well as in *Rhipicephalus microplus* (Enan, 2005b; Gross et al., 2017), suggesting that these natural molecules might exert their insecticidal activity through the interaction with TAR receptors.

TAR1 has been characterized in several insects (Saudou et al., 1990; Blenau et al., 2000; Ohta et al., 2003; Rotte et al., 2009; Wu et al., 2013; Gross et al., 2015; Hana and Lange, 2017; Ma et al., 2019) and demonstrated to be involved in important physiological processes such as olfactory response, control of metabolism (obesity) and locomotor activity (Kutsukake et al., 2000; Roeder, 2005; Li et al., 2017).

This paper describes the structural and functional features of TAR1 (DsTAR1) from *Drosophila suzukii* and its sensitivity to three monoterpenes, thymol, carvacrol and α-terpineol. These information might therefore help to shed some light on the possible use of monoterpenes as biopesticides against this pest.

2. Materials and methods

2.1. Insects and reagents

Drosophila suzukii flies were reared on an artificial diet with a photoperiod of 16 h light: 8 h dark, at a temperature of 23 ± 1 °C.

Endothelin-1 (ET-1), tyramine hydrochloride, octopamine hydrochloride, yohimbine hydrochloride, brilliant black, Bovine Serum Albumin (BSA), probenecid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *p*-menth-1-en-8-ol (α-terpineol), *p*-cymene (carvacrol) and 3-hydroxy *p*-cymene (thymol) were all obtained from Sigma-Aldrich (St Louis, USA). Pluronic acid and fluorescent dye Fluo-4 AM were purchased from Thermo Fisher Scientific. All compounds were dissolved in dimethyl sulfoxide (10 mM) and stock solutions were kept at −20 °C until use. Serial solution were made in the assay buffer (Hanks' Balanced Salt solution (HBSS)/HEPES 20 mM buffer, containing 0.01% BSA and 0.1% DMSO).

2.2. Isolation and cloning of the full-length Drosophila suzukii tyramine receptor (DsTAR1)

Sequence alignment by BLASTN performed with the orthologous gene *DmTAR1* (Accession: X54794) from *D. melanogaster*, suggested that the putative transcript XM_017071090 predicted in the *D. suzukii* genome project (Accession: PRJNA325161) might code for the putative DsTAR1 (Accession: XP_016926579).

Total RNA was extracted from six adult flies using High Pure RNA Tissue Kit (Roche, Switzerland), quantified in a micro-volume spectrophotometer Biospec-Nano (Shimadzu, Japan) and analysed by 0.8% *w/v* agarose gel electrophoresis. One μg of RNA was treated with DNase I (New England Biolabs, USA) and used for the synthesis of cDNA, carried out with the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific). For amplification of the full *DsTAR1* open reading frame (ORF), specific primers were designed based on the annotated *DsTAR1* sequence (Table 1). High fidelity amplification was achieved using Herculase II Fusion DNA Polymerase (Agilent, USA) and a touchdown thermal profile: predenaturation at 95 °C for 3 min, followed by 5 cycles at 95 °C for 20 s, 70–60 °C for 30 s (minus 2 °C/cycle), 68 °C for 2 min, 30 cycles at 95 °C for 20 s, 60 °C for 30 s, 68 °C for 2 min and a final extension at 68 °C for 5 min. PCR product was gel puri-

Table 1
Primer use in this study.

Primers	Primer sequence
cDNA cloning	
DsTAR1-For	5′-TTCCGTCCGCCATTCAACC-3′
DsTAR1-Rev	5′-TCAATTCAGGCCCAGCAGC-3′
Quantitative RT-PCR	
Fw-*DsTAR1*-RT	5′-GCAGTCCTCGTCCACCTG-3′
Rev-*DsTAR1*-RT	5′-TTAAGGGACGTCTGCTCGTC-3′
AK-Fw	5′-CTACCACAACGATGCCAAGA-3′
AK-Rev	5′-AAGGTCAGGAAGCCGAGA-3′
TBP-Fw	5′-CCACGGTGAATCTGTGCT-3′
TBP-Rev	5′-GGAGTCGTCCTCGCTCTT-3′
PKA-Fw	5′-CGGAGAACCTGCTAATCGAC-3′
PKA-Rev	5′-CCATTTCGTAGACGAGCACA-3′

fied used Wizard SV Gel and PCR Clean-Up System (Promega, USA), cloned into pJET 1.2/blunt vector (Thermo Fisher Scientific) and transformed into *E.coli* SIG10 5-α Chemically Competent Cells (Sigma–Aldrich). Positive clones were selected using LB broth agar plates with 100 μg/ml ampicillin. Plasmid was then extracted and verified by DNA sequencing (BMR Genomics, Italy). The sequence, named *DsTAR1*, was deposited in GenBank with the accession number MK405664.

For expression in Human Embryonic Kidney (HEK 293) cells, the open reading frame of *DsTAR1* was excised from pJET 1.2 vector and inserted into the pcDNA 3.1 (+) Hygro vector using *NotI* and *XbaI* restriction sites.

2.3. Multiple sequence alignment and general bioinformatics analysis

Multiple protein sequence alignments between the deduced amino acid sequence of DsTAR1 and other type 1 tyramine receptors sequences were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and BioEdit Sequence Alignment Editor 7.2.6.1. Phylogenetic neighbour-joining analysis was performed by MEGA software (version 7) with 1000-fold bootstrap resampling. The *Drosophila melanogaster* GABA B receptor (GABABR) was used as an outgroup to root the tree.

2.4. Expression in HEK 293 and stable line creation

HEK 293 cells were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagles medium high glucose (D-MEM) supplemented with 10% fetal bovine serum (Microtech, Italy). To prevent bacterial contamination, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) were added to the medium. The cells were transfected with pcDNA 3.1 (+)/DsTAR1 using lipofectamin 2000 (Invitrogen, USA). Stably transfected cells were selected with Hygromycin B 100 μg/ml supplemented medium. After two weeks, the resistant colonies were treated with trypsin and separately propagated in 24-well plates. These individual cell lines were analysed for the stable integration of the recombinant DNA by RT-PCR. The clonal cell line most efficiently expressing DsTAR1 was chosen for these studies.

2.5. Calcium mobilization assay

When confluence was reached, cells were seeded at a density of 50,000 cells per well, total volume of 100 μl, into poly-D- lysine coated 96-well black, clear-bottom plates. After 24 h incubation at normal cell culture condition, the cells were incubated with HBSS 1× supplemented with 2.5 mM probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. After that, the loading solution was removed and HBSS 1× supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μM brilliant black was added. Cell culture and drug plates were placed into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA) and fluorescence changes were measured after 10 min of stabilization at 37 °C. On-line additions were carried out in a volume of 50 μl/well after 20 s of basal fluorescence monitoring. To facilitate drug diffusion into the wells the present studies were performed at 37 °C with three cycles of mixing (25 μl from each well moved up and down 3 times). The fluorescence readings were measured every 2 s for 120 s.

2.6. Dynamic mass redistribution assay

For DMR measurements the label-free EnSight Multimode Plate Reader (Perkin Elmer, MA, US) was used. When confluence was reached, cells were sub-cultured as required using trypsin/EDTA and used for experiments. Cells were seeded into Enspire TM -LC 384-wells fibronectin-coated plates and cultured 20 h to form a confluent monolayer in the cell culture medium. Cells were seeded at a density of 20,000 cells/well/30 μl. The day of the experiment cells were manually washed twice and maintained with the assay buffer (Hank's Balanced Salt Solution (HBSS) 1× with 20 mM HEPES, 0.01% Bovine Serum Albumin) for 90 min before DMR experiment.

Agonism protocol: a 5 min baseline was first established, followed by adding compounds manually in a volume of 10 μl and recording compounds triggered DMR signal for 60 min.

Antagonism/modulation protocol: antagonists / modulators were added manually 30 min before reading the 5 min baseline. After baseline establishment, TA or ET-1 were injected and DMR signal was recorded for 60 min. The antagonist/modulator properties of ligands were measured by assessing the concentration-response curve to TA and ET-1 in the absence and in presence of a fixed concentration of antagonist/modulator. All the experiments were carried out at 37 °C.

2.7. Data analysis and terminology

All data were elaborated using Graph Pad Prism 6.0 (La Jolla, USA). Concentration-response curves were fitted using the four parameters log logistic equation:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max}\text{-Baseline})}{\left(1 + 10^{(LogEC_{50} - Log[compound]) * Hillslope}\right)}$$

Data are expressed as mean ± SEM of n experiments performed in duplicate and were analysed using one- or two-way analysis of variance (ANOVA) followed by Dunnett's or Turkey's test for multiple comparison. Agonist potency was expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. Antagonists / modulators potencies were assayed at single concentrations against the concentration-response curve to TA.

2.8. Monoterpenes exposure bioassay

A 15 cm × 2 cm petri dish was used to expose adults *D. suzukii* to monoterpenes. 8 ml of a 1% agar and 5% sucrose solution were placed on the bottom of the petri. Monoterpenes (lipophilic in nature) stock solutions were prepared at a standard concentration of 10 mg/ml in acetone to ensure complete solubilization and stored at −20 °C. Dilutions to 1 mg/l final concentration were then made in water for each monoterpene, this concentration being close to the LD_{50} of all three terpenes tested (Kim et al., 2016; Park et al., 2016). A similar solution of water and acetone was used as negative control. 300 μl of the diluted compounds were then used to soak a 12 cm diameter paper disc positioned in the petri dish on top of the agar/sucrose gel.

Thirty adult flies (fifteen males and fifteen females 3–5 days-old) were placed inside the petri dish. The insects were incubated for 24, 72 or 120 h at a photoperiod of 16 h light: 8 h dark, at 23 ± 1 °C. The effect of monoterpenes exposure on *DsTAR1* mRNA levels was evaluated, by RT-qPCR, after each time point.

2.9. Quantitative real-time PCR analysis

Total RNA was extracted from *Drosophila suzukii* samples at various developmental stages (1st to 3rd instar larvae, pupae, adult males and females), different tagmas (head, thorax and abdomen, dissected from adults) or adult flies subjected to the monoterpene exposures using High Pure RNA Tissue Kit (Roche, Switzerland). The three tagmas of *D. suzukii* (head, thorax and abdomen) were dissected out in a RNA preservation medium (20 mM EDTA disodium (pH 8.0), 25 mM sodium citrate trisodium salt, 700 g/l ammonium sulphate, final pH 5.2).

The thorax presented all associated appendix (wings and legs). One μg of RNA was treated with DNase I (New England Biolabs) and used for cDNA synthesis, carried out with iScript Reverse Trascription Supermix (Bio-Rad, USA), according to the manufacturer's instructions. Real time PCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad) in a 12 μl reaction mixture containing 0.8 μl of total cDNA obtained from one μg of RNA, 6 μl Sybr Green Mix (SIGMA), 0.4 μl forward primer (10 μM), 0.4 μl reverse primer (10 μM) and 4.4 μl of nuclease free water. Thermal cycling conditions were: 95 °C for 2 min, 40 cycles at 95 °C for 15 s and 60 °C for 20 s. After the cycling protocol, a melting-curve analysis from 55 °C to 95 °C was applied. In expression analysis on tissues and development stages of *D. suzukii*, *DsTAR1* was quantified utilizing the relative quantification method (Larionov et al., 2005). After monoterpenes treatment *DsTAR1* and *PKA* (accession number: NW_016019885.1) expression levels were quantified using the qBase+ algorithm (Hellemans et al., 2007). AK and TBP were used as reference housekeeping genes in both expression studies for the normalization (Zhai et al., 2014). Gene-specific primers (Table 1) were used and for each sample three independent biological replicates, made in triplicate, were performed.

3. Results

3.1. cDNA cloning and molecular characterization of DsTAR1

The ORF of *DsTAR1* is 1803 bp long and codes for a 600 aa polypeptide with a predicted molecular mass of 64.31 kDa (Fig. 1).

In terms of genomic structure *DsTAR1* appears to be encoded by four different exons (Supplementary fig. S1, panel A), separated by three long introns. The same genomic organization is observed in the

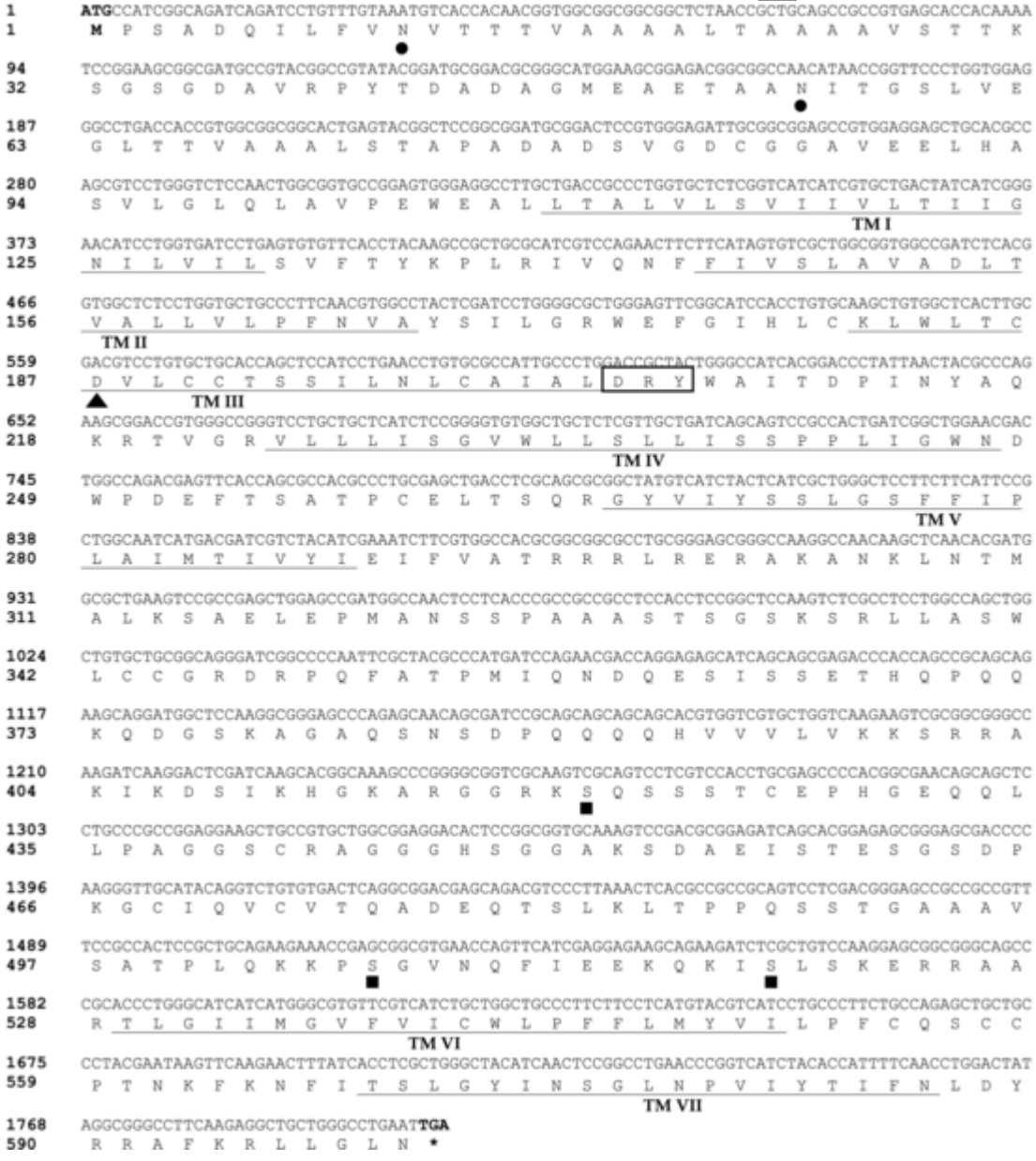

Fig. 1. Nucleotide sequence of the tyramine receptor open reading frame cloned from *Drosophila suzukii* and deduced amino acid sequence. Prediction of the DsTAR1 transmembrane segments (underlined and numbered from I to VII) was obtained with TMHMM v. 2.0 software. After the third transmembrane domain there is the DRY motif (highlighted with a black box) important for the stabilization of GPCRs between inactive and activate conformation. Potential sites for N-linked glycosylation (predicted with NetNGlyc 1.0 server) are shown with a dot (●) and potential sites for PKA or PKC phosphorylation (predicted with NetPhos 3.1 server) are shown with a square (■). The black triangle (▲) represents an aspartic acid in TM3 (D_{187}) highly conserved in TAR1 family.

D. melanogaster counterpart coding for type 1 tyramine receptor (Chromosome 3L – NT_037436.4).

The analysis of the expected TAR1 polypeptide by TMHMM v. 2.0 software predicted the existence of seven transmembrane domains, a typical feature of GPCRs (Fig. 1). Hydropathy profile analysis, according to the Kyte and Doolittle method (Kyte and Doolittle, 1982), further confirmed the presence of seven transmembrane helices, along with an eighth domain located close to the amino-terminal end of the polypeptide (Supplementary fig. S1, panel B). This extra domain has been found in other biogenic amine receptors, in particular in the TAR1s from *D. melanogaster* (Saudou et al., 1990) and *B. microplus* (Baxter and Barker, 1999). This extra domain might be a cleavable signal sequence or leader peptide, a sequence that plays a key role during the first steps of the intracellular transport of G Protein-Coupled Receptors (Rutz et al., 2015). In *DsTAR1* sequence there are two asparagine residues, located in the N-terminal domain before the first transmembrane domain, that form the classic motif N-X-S/T for N-glycosylation sites (Nørskov-Lauritsen and Bräuner-Osborne, 2015). Furthermore, several serines were identified as putative phosphorylation sites, targeted by Protein kinase C or Protein kinase A, in the intracellular loops, especially in the loop between TM V and TM VI. The aspartic acid in TM III (D^{187}), indicated by a black triangle in Fig. 1, is conserved in all the members of the TAR1 family, because it interacts with the amino group of TA, the principal agonist of these receptors (Ohta and Ozoe, 2014).

The amino acid sequences of several insect biogenic amine receptors were used for multiple sequence alignment and to construct a neighbour-joining phylogenetic tree with MEGA 7 server. The results indicate that DsTAR1 clusters in the family of TAR1s, phylogenetically close to its orthologue from *D. melanogaster* (Fig. 2).

Multiple sequence alignment between DsTAR1 amino acid sequence and TAR1s from *D. melanogaster*, *P. regina* and *C. suppressalis* revealed a similarity in the transmembrane domains, while less conserved regions were found in the N-terminal region of the proteins and in the intracellular loop between the 5th and 6th transmembrane segments (Fig. 3). The highest protein identity was found, as expected, with *Dm*TAR1 (*Drosophila melanogaster* TAR1) with more than 93% sequence identity (Fig. 3).

3.2. Expression pattern of DsTAR1

Tissue localization analyses of the receptor were performed by RT-qPCR to understand the function of *DsTAR1* in *D. suzukii*.

Total RNA was extracted from different development stages (first to third larvae, pupae and adult) and from three different tagmas (head, thorax and abdomen).

The analysis revealed that *DsTAR1* was expressed in all developmental stages of *D. suzukii*, with high levels found in the first instar larvae and in the adult males (Fig. 4, panel A). Furthermore, in adults, the expression levels in whole males were about twice than in females. The head of males and females accumulated higher levels of *DsTAR1* mRNA than the two other tagmas. In particular, the heads of males accumulated much higher mRNA levels of *DsTAR1* in comparison to the female counterparts. Interesting, the male abdomens showed a significant difference in the *DsTAR1* expression levels as compared to females, suggesting a possible role of TA in male reproductive system (Fig. 4, panel B).

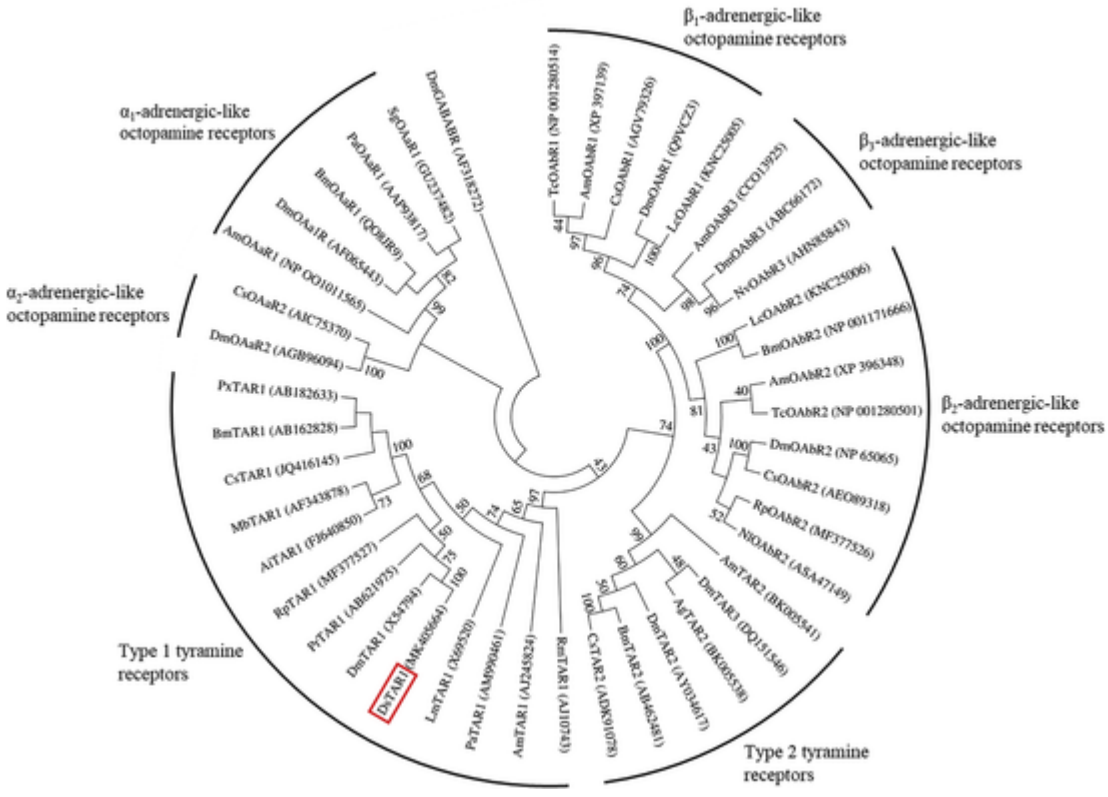

Fig. 2. Phylogenetic relationships resulting from the neighbour joining analysis of DsTAR1 (highlighted with a box) and other insect amine receptors. The values shown at the nodes of the branches are the percentage bootstrap support (1000 replications) for each branch. *Drosophila melanogaster* GABA-B receptor (DmGABABR) was chosen as outgroup. Dm, *Drosophila melanogaster*; Ds, *Drosophila suzukii*; Pr, *Phormia regina*; Rp, *Rhodnius prolixus*; Px, *Papilio xuthus*; Cs, *Chilo suppressalis*; Bm, *Bombyx mori*; Ai, *Agnotis ipsilon*; Mb, *Mamestra brassicae*; Pa, *Periplaneta americana*; Lm, *Locusta migratoria*; Am, *Apis mellifera*; Rm, *Rhipicephalus microplus*; Sg, *Schistocerca gregaria*; Ag, *Anopheles gambiae*; Tc, *Tribolium castaneum*; Nv, *Nilaparvata lugens*; Lc, *Lucilia cuprina*.

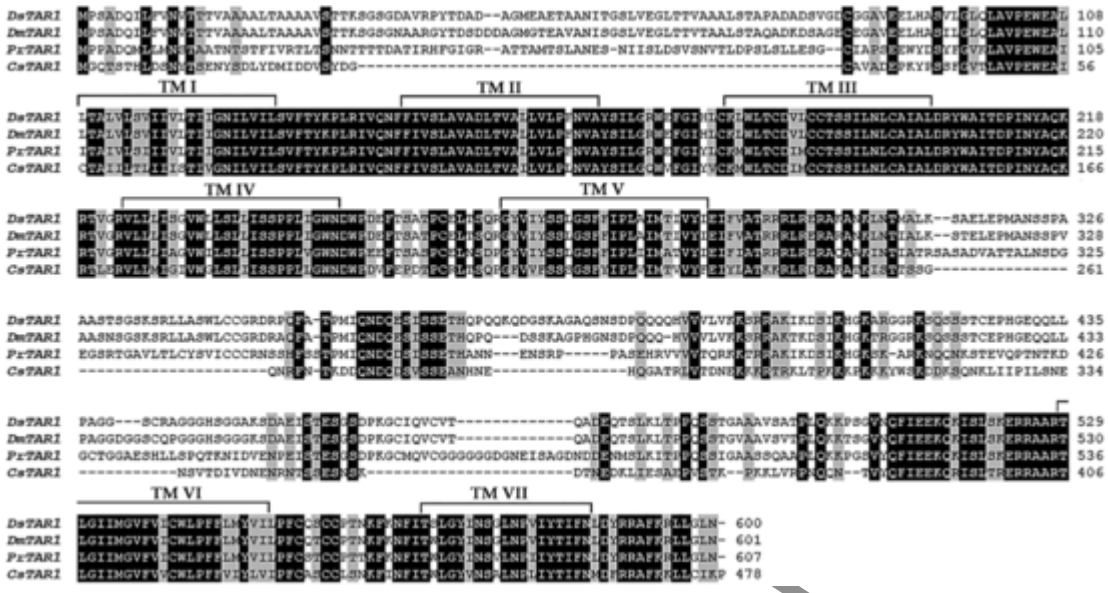

Fig. 3. Amino acid sequence alignment of DsTAR1 with orthologous receptors from *D. melanogaster* (DmTAR1), *P. regina* (PrTAR1) and *C. suppressalis* (CsTAR1). The putative seven transmembrane domains (TM I-VII) are indicated with a black line. Identical residues are highlighted black while conservative substitutions are shaded.

3.3. Pharmacological data

To confirm the function of DsTAR1 as TA receptor and its possible interaction with monoterpenes, the cloned cDNA was expressed in HEK 293 cells and tested for the ability to respond to TA and OA, like other TAR1s studied so far in insects, as well as to several monoterpenes. The responses were evaluated with two different assays, the calcium mobilization assay and the dynamic mass redistribution (DMR) assay, the latter being a label free technique that has been previously demonstrated to be useful for investigating the functional profile of G Protein-Coupled Receptors (Grundmann and Kostenis, 2015).

In the calcium mobilization assay performed on HEK 293_{DsTAR1} cells, TA evoked the release of intracellular calcium in a concentration-dependent manner with pEC_{50} and E_{max} values of 6.35 (6.07–6.62) and 105 ± 15% over the basal values, respectively (Fig. 5, panel A). On the contrary, OA did not stimulate any intracellular calcium release when tested in the concentration range 10 μM - 100 pM (data not shown). ET-1, chosen as external control (Atwood et al., 2011), was able to increase in HEK 293_{DsTAR1} cells the intracellular calcium mobilization with a potency value of 7.02 (6.88–7.15) and maximal effects of 152 ± 12% (Fig. 5, panel B). Yohimbine, tested as agonist up to 10 μM on the DsTAR1, did not elicit the intracellular calcium mobilization (data not shown). Yohimbine 1 μM was also tested as antagonist against TA and ET-1. The molecule was able to rightward shift the concentration response curve to TA (Fig. 5, panel A), while the same concentration did not significantly affect the concentration response curve to ET-1 (Fig. 5, panel B). A pA_2 of 7.87 was calculated for yohimbine against TA assuming a competitive type of antagonism. In wild type HEK 293 cells, TA and OA were completely inactive (data not shown), while ET-1 stimulated calcium mobilization in a similar manner observed in HEK 293_{DsTAR1} cells (data not shown).

The same cell lines were then treated with three monoterpenes (α-terpineol, carvacrol and thymol) to evaluate their possible agonist effect. α-terpineol did not elicit $[Ca^{2+}]_i$ mobilization in any cell line (Supplementary fig. S2, panel C and F), while carvacrol and thymol stimulated $[Ca^{2+}]_i$ mobilization only at 100 μM in both HEK 293 wild type and DsTAR1 transfected cells (Supplementary fig. S2, panels A, B, D and E).

DMR assays revealed that TA is able to evoke a positive concentration dependent signal (Fig. 6, panel A) in HEK 293_{DsTAR1} while OA can elicit an intracellular Ca^{2+} release only at 10 μM (data not shown). In these experiments, TA showed a potency value of 6.87 (6.46–7.28) and maximal effect of 164 ± 25 pm (Fig. 6, panel C). The DsTAR1 antagonist yohimbine, tested as agonist up to 1 μM did not modify per se the DMR signal (data not shown). However, yohimbine 1 μM was able to rightward shift the concentration response curve to TA without changing the agonist maximal effect with a pA_2 value of 7.24 (6.56–7.92) (Fig. 6, panel B and C). ET-1 elicited concentration-response curves with or without yohimbine 1 μM (Supplementary fig. S3). In wild type HEK 293 cells, TA and OA were completely inactive while ET-1 evoked a concentration-dependent DMR response (pEC_{50} 7.86 (7.46–8.27) and maximal effects of 440 ± 27 pm). The effects of the highest concentrations of the two agonists tested in the two HEK 293 cell lines are summarized in Table 2. The DMR analyses also confirmed that the three monoterpenes tested do not act as agonist of DsTAR1. Two (100 and 10 μM) or four (up to 0.1 μM) different monoterpenes concentrations were tested on HEK 293 wild type or DsTAR1 stably transfected cells, respectively. All three terpenes at the highest concentration appeared to be able to stimulate a DMR signal in HEK 293_{DsTAR1} comparable to the one observed in HEK 293 wild type cells. Lower concentrations were, on the other hand, completely inactive in both cell types (Supplementary fig. S4).

Recently, several monoterpenes have been also shown to act as modulators of TAR1 from *Rhipicephalus (Boophilus) microplus*, increasing the in vitro TA response (Gross et al., 2017).

To investigate the possible antagonist/modulatory action of the three monoterpenes toward DsTAR1 in DMR experiments, HEK 293_{DsTAR1} cells were pre-incubated for 30 min with 10 μM, 1 μM or 0.1 μM of each terpene.

Upon the addition of TA (10 μM–10 pM) all three monoterpenes, at 10 and 1 μM, were able to increase TA potency (Fig. 7; Table 3). In particular, α-terpineol 1 μM was able to shift the concentration response curve to TA by 5 folds. The higher potency was observed for the endogenous agonist with a pEC_{50} of 7.44 (7.28–7.59) in HEK 293_{DsTAR1} pre-incubated with the monoterpene as compared to 6.81 (6.66–6.89) in the control. (Table 3). All three monoterpenes, in the same concentration ranges, did not modify the concentration response curve to ET-1 (Data not shown).

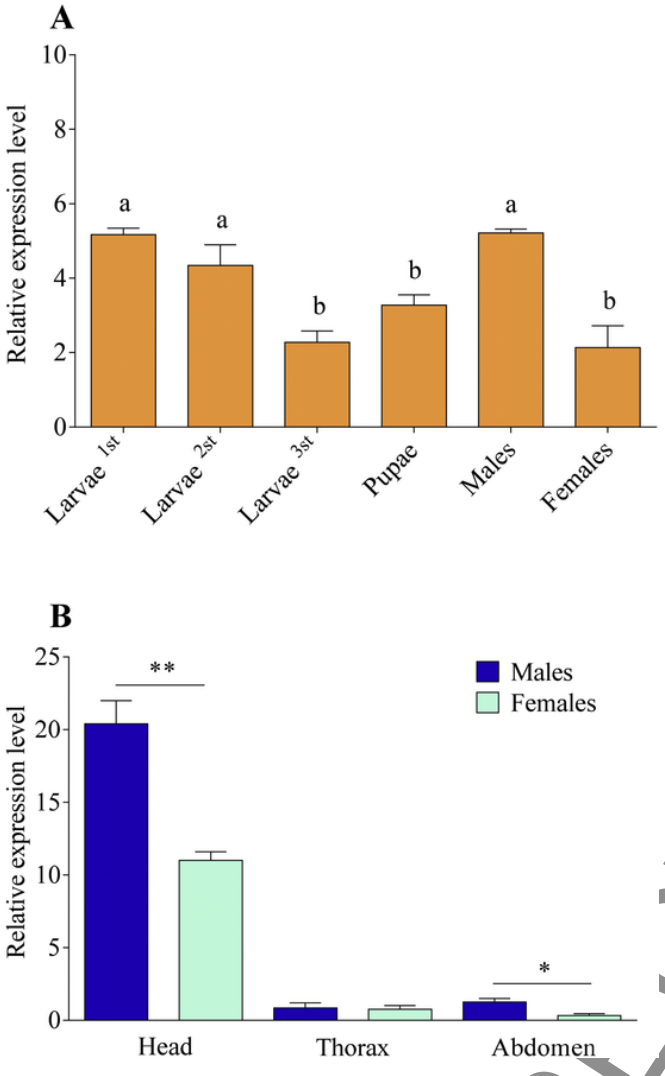

Fig. 4. mRNA expression levels of *DsTAR1* gene in *D. suzukii*. (A) Expression of *DsTAR1* gene in different development stages (first to third larvae, pupae and adult). *DsTAR1* in first larvae was used as a comparator for the LSD (Least Significant Difference) statistical analysis. (B) Expression of *DsTAR1* in tagmas (head, thorax and abdomen) on female and male adult flies. Data represent means ± SEM of three independent experiments performed in triplicate. * $p < .05$ ** $p < .01$ according to Student's *t*-test. Arginine kinase (*AK*) and TATA Box Protein (*TBP*) were used as housekeeping genes.

Taken together these experiments confirm that, as predicted by structural analysis, DsTAR1 is a functional type 1 tyramine receptor sensitive to TA. In both calcium mobilization and DMR assays, TA stimulated DsTAR1 causing significant changes in $[Ca^{2+}]_i$ mobilization and dynamic mass redistribution. Furthermore, DMR experiments revealed that the three monoterpenes do no act as DsTAR1 agonists but rather as positive allosteric modulators of the receptor.

3.4. Expression of DsTAR1 and PKA genes after monoterpenes exposure

To evaluate the effect of the exposure to monoterpenes on the expression levels of *DsTAR1* and *PKA* genes, adult flies of *D. suzukii* were exposed to 1 mg/l of carvacrol, α-terpineol and thymol and the mRNA levels analysed by qPCR. The concentration tested was close to the LD_{50} of each monoterpene (Kim et al., 2016; Park et al., 2016).

The exposure induced an interesting downregulation of *DsTAR1* gene expression. Significant differences were observed for α-terpineol at 24 h (5.2-fold lower than control), for thymol at 24 h (3.5 fold lower than control) and 72 h (12.9 fold lower than control) and for carvacrol at 120 h (5.2 fold higher than control) (Fig. 8, panel A). On the other hand, the mRNA levels of *PKA* (chosen as internal unrelated control) were not significantly altered by any treatment (Fig. 8, panel B). This gene was chosen as an internal control, given propensity of TAR1 to couple with G_i proteins. These G proteins, in fact, have PKA as the last transduction effector.

4. Discussion and conclusions

This study describes the first molecular and functional characterization of DsTAR1, a type 1 tyramine receptor from the phytophagous *D. suzukii*.

The structural analysis of DsTAR1 predicted amino acid sequence revealed many features shared with other insect type 1 tyramine receptors. The hydropathy profile revealed the typical organization of a GPCR with seven transmembrane segments along with an additional region also found in *D. melanogaster* TAR1 (Saudou et al., 1990). This putative TM VIII, located at the beginning of the amino-terminal end, is not unusual in these receptors. In several studies, it was postulated that this short amino acid sequence might be a signal peptide, necessary for the correct vesicular transport of the protein (Saudou et al., 1990; Baxter and Barker, 1999). At position 187 in DsTAR1 predicted amino acid sequence there is an aspartic acid residue highly conserved among TAR1s, believed to interact with the amine group of TA. This binding is strengthened through weak interactions with three serine residues present in TM V: S^{271}, S^{272} and S^{275} in DsTAR1 (Ohta et al., 2004). Amino acid sequence alignment revealed similarity between DsTAR1 and other insect type 1 tyramine receptors, especially with *D. melanogaster* TAR1. The two proteins differ only for 44 differ-

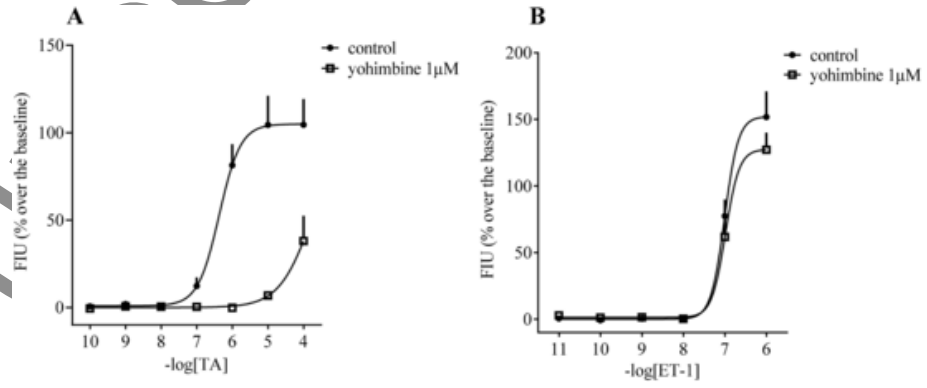

Fig. 5. Concentration-response curves, by calcium mobilization assay, to TA (A) or ET-1 (B) in the absence (control) and in presence of 1 μM yohimbine in DsTAR1 transfected HEK293 cells. Data represent means ± SEM of four separate experiments performed in duplicate.

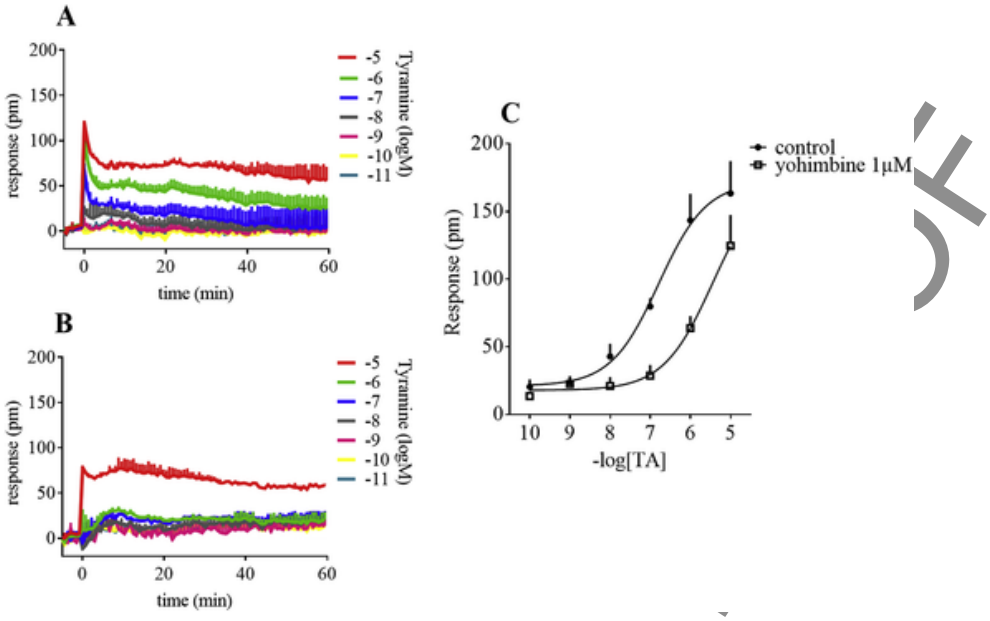

Fig. 6. Baseline corrected DMR traces of TA in the absence (panel A) and in presence (panel B) of 1 µM yohimbine and concentration-response curve to TA (panel C) in the absence (control) and in presence of 1 µM yohimbine, in HEK293$_{DsTAR1}$ cells. Data are the means ± SEM of four experiments performed in duplicate.

Table 2
Effects of the highest concentrations tested for the ligands in HEK 293 and HEK 293$_{DsTAR1}$ cells.

	HEK 293 wt E_{max} (pm ± SEM)	HEK 293$_{DsTAR1}$ E_{max} (pm ± SEM)
Buffer	6 ± 14	-8 ± 15
TA 10 µM	-2 ± 33	164 ± 25 *
ET-1 1 µM	440 ± 27 *	263 ± 37 *

* $p < .05$ vs buffer according to one-way ANOVA followed by the Dunnett's test for multiple comparisons.

ent residues, localized in the N-terminus and in the intracellular loop between TM V and TM VI. Interestingly, these two regions are the least conserved among all TAR1 sequences analysed, therefore suggesting a role in defining the different molecular and functional characteristics of the receptors.

To shed some light on the role that DsTAR1 plays in *D. suzukii*, an expression profile analysis of the receptor was performed. RT-qPCR revealed that *DsTAR1* is significantly more expressed in male adult flies than in females. *DsTAR1* mRNA accumulates especially in the head of males as compared to female adult flies and to the other two tagmas (thorax and abdomen). The significant difference between the male and female abdomens is an interesting observation that suggests a possible role of TA in male reproductive system. A recent study in *D. melanogaster* has indeed shown that this receptor is mainly expressed in the brain and in the male reproductive organs (El-Kholy et al., 2015). This expression profile is in accordance with other studies on type 1 tyramine receptors that have described a higher expression in brain and nerve cords, while the mRNA is almost absent in other districts or organs (Rotte et al., 2009; Wu et al., 2013; Ono and Yoshikawa, 2004). Furthermore, TAR1 mRNA has been described in other districts located in the head, such as the antennas and the maxillary palps, suggesting a role for type 1 tyramine receptors in neurons responsible for olfactory and taste responses (Kutsukake et al., 2000). The marked difference in expression between adult males and females suggests that *D. suzukii* type 1 tyramine receptor is probably involved in the control of male specific functions such as the development and function of male reproductive organs and the search for female partners through specific behavioral or physiological processes (El-Kholi et al., 2015; Hana and Lange, 2017).

Structural data suggest that DsTAR1 codes for a TA receptor. To confirm its function the cDNA was cloned, stably expressed in HEK 293 cells and tested in functional studies for its sensitivity to TA and OA. Moreover, the pharmacological effects of yohimbine were also investi-

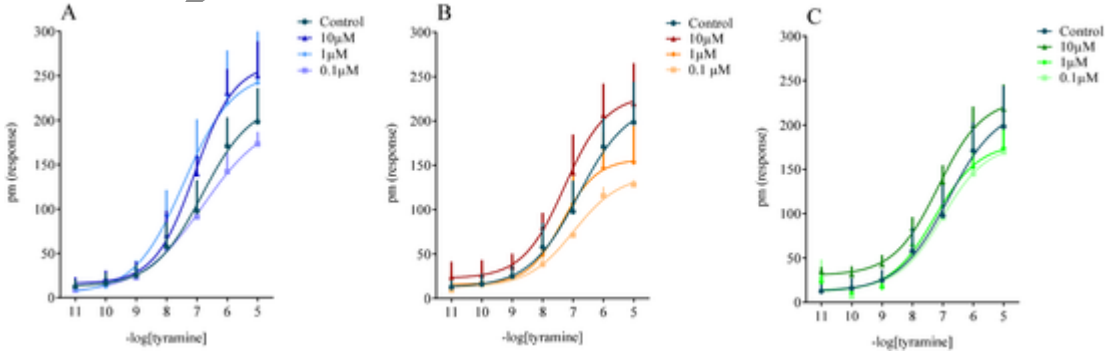

Fig. 7. Concentration-response curves, by DMR assays, to TA after recording in HEK 293$_{DsTAR1}$ cells pre-incubated with 10 µM, 1 µM or 0.1 µM of carvacrol (panel A), α-terpineol (panel B) or thymol (panel C). Data are means ± SEM of at least three separate experiments made in duplicate.

Table 3
pEC_{50} and E_{max} values of TA, in HEK 293_{DsTAR1} cells, after a pre-incubation with buffer (control) or 10 μM, 1 μM or 0.1 μM of monoterpenes.

Chemical	Concentration	TA	
		pEC_{50} ± SEM	E_{max} (pm ± SEM)
	Control	6.81 ± 0.07	223 ± 25
Carvacrol	10 μM	7.15 ± 0.06 *	230 ± 14
	1 μM	7.28 ± 0.09 *	179 ± 26
	0.1 μM	6.95 ± 0.13	184 ± 17
α-terpineol	10 μM	7.13 ± 0.09 *	261 ± 22
	1 μM	7.44 ± 0.06 *	253 ± 33
	0.1 μM	6.86 ± 0.16	198 ± 5
Thymol	10 μM	7.23 ± 0.11 *	232 ± 21
	1 μM	7.37 ± 0.08 *	156 ± 22
	0.1 μM	6.79 ± 0.16	152 ± 13

* $p < .05$ vs control according to one-way ANOVA followed by the Dunnett's test for multiple comparisons.

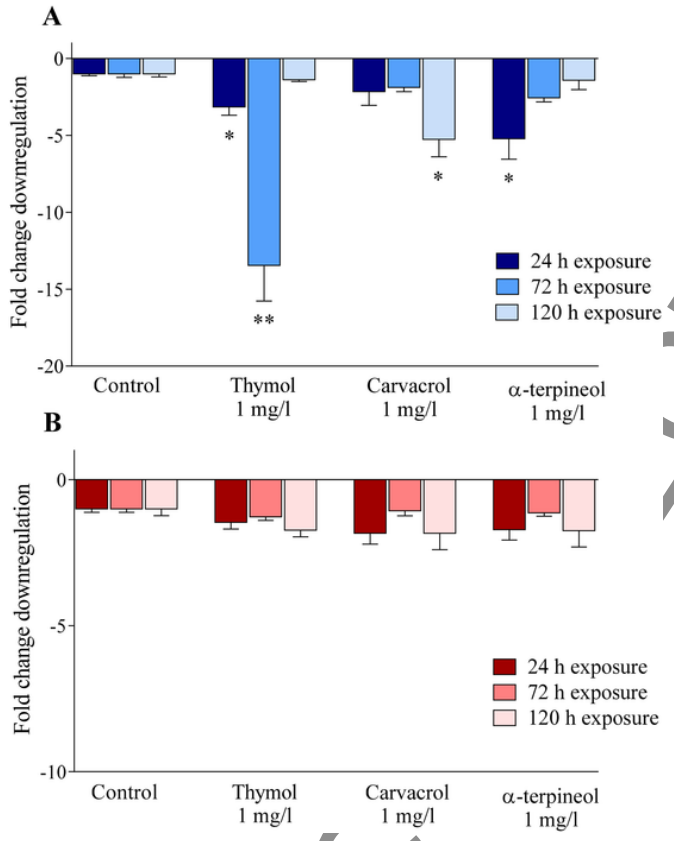

Fig. 8. *DsTAR1* (A) and *PKA* (B) expression levels in *D. suzukii* adult flies after 24, 72 or 120 h of continuous exposure to monoterpenes. Data represent means ± SEM of three independent experiments performed in triplicate. $*p < .05$ $**p < .01$ vs control according to one-way ANOVA followed by Dunnett's test for multiple comparisons. Arginine kinase (*AK*) and TATA Box Protein (*TBP*) were used as housekeeping genes.

gated since this compound has been reported to act as a TAR1 antagonist in previous studies (Gross et al., 2015; Hana and Lange, 2017).

The *D. suzukii* cloned receptor was functionally studied using two different pharmacological assays. In the calcium mobilization assay, TA was able to increase the intracellular calcium mobilization with a pEC_{50} of 6.35 while OA was inactive. The effect of TA was sensitive to the antagonist yohimbine similarly to the orthologous type 1 tyramine receptor of *Drosophila melanogaster* (Saudou et al., 1990; Enan, 2005a).

The *D. suzukii* receptor was further studied by DMR assay, which is based on an optical biosensor technology. This recently developed analysis does not employ labelled molecules and therefore it monitors integrated receptor signaling responses including those mediated by GPCRs (Ferrie et al., 2011; Tran et al., 2012; Carter et al., 2014; Grundmann and Kostenis, 2015). DMR has never been applied to type 1 tyramine receptors before.

In the DMR assay, TA activated DsTAR1 with a pEC_{50} of 6.87 while OA showed a signal only at 10 μM concentrations. Yohimbine was able to rightward shift the dose-response curve to TA showing a competitive type of interaction and a pA_2 value of 7.24.

Collectively, the receptor cloned from *D. suzukii* displays the pharmacological profile expected for a member of the TAR1 family in terms of rank order of potency of agonists, i.e. TA > OA and sensitivity to the selective and competitive antagonist yohimbine, that displayed nanomolar potency. These features were detected not only at the level of the calcium pathway but also, for the first time, in terms of dynamic mass redistribution. Therefore the functional data presented here not only confirm the structural analysis on the predicted protein but expand the biochemical knowledge on TAR1 receptors as well.

Studies carried out on TAR1 have shown that monoterpenes interact with the receptor, either by acting as agonists (*Drosophila melanogaster* Enan, 2005b) or as modulators *Rhipicephalus* (*Boohilus*) *microplus* (Gross et al., 2017). Therefore, HEK 293 cells stably expressing DsTAR1 were used to verify whether these biochemical interactions could also be observed in *Drosophila suzukii*.

When tested as agonists in DMR experiments, thymol, carvacrol and α-terpineol were not able to generate pharmacological responses attributable to the interaction with DsTAR1. Similar results were obtained in the calcium mobilization assay, where monoterpenes were tested on both HEK wild type and HEK 293_{DsTAR1} cells at 1, 25 and 100 μM, that is the same concentration range tested on TAR1 from *D. melanogaster* in a similar calcium mobilization assay (Enan, 2005b). Thymol and carvacrol stimulated a release of $[Ca^{2+}]_i$ but only at 100 μM, and this signal was detected in both HEK 293 wild type and stably transfected DsTAR1 cells. However, α-terpineol was inactive at all concentrations tested (100, 25 and 1 μM) and on both cell lines.

These monoterpenes are known to elicit calcium release in many different tissues and cell types and are agonists for many different ion channels and receptors (Magyar et al., 2002; Krizaj et al., 2003; Szentesi et al., 2004; Vogt-Eisele et al., 2007; Sarkozi et al., 2007). Therefore, it is not surprising that they could induce a remarkable elevation in $[Ca^{2+}]$ levels and an intense DMR signal in both stably transfected and wild type HEK 293 cells at high concentration. On the other hand, at lower concentrations (25 μM) all monoterpenes were unable to stimulate the release of $[Ca^{2+}]$ in HEK 293 stably expressing DsTAR1, contrary to what observed by Enan (Enan, 2005b) for the type 1 tyramine receptor from *D. melanogaster*. These functional differences observed between the two phylogenetically close receptors might be structurally connected to the 44 amino acids that differentiate the amino acid sequences of the two receptors (as judged by alignment between DmTAR1 and DsTAR1). Unfortunately, there are no structural studies that might shed some light on how amino acid changes in these regions could be directly related to a different receptor sensitivity. Therefore, the structural reasons of the different sensitivity of DmTAR1 and DsTAR1 to monoterpenes remain to be assessed.

A possible modulatory action of the three monoterpenes on DsTAR1 was investigated by DMR assay. Pre-treatment of the transfected DsTAR1 cells with 10 or 1 μM of the three monoterpenes promoted an increase in agonist potency; the larger effect was obtained with 1 μM α-terpineol that produced a 5-fold increase in TA potency.

It has been reported that carvacrol can induce a positive allosteric modulation on type 1 tyramine receptor from *Rhipicephalus* (*Boophilus*) *microplus*, allowing the terpene to stabilize and enhance the pharmacological activity of TA, through a conformational change in the receptor (Gross et al., 2017). Therefore, the monoterpenes might interact with DsTAR1 with a similar biochemical mechanism.

Furthermore, a similar modulatory effect was observed by Gross in 2015 on the RmTAR1 (Gross et al., 2015) after treatment with a metabolite of the amitraz insecticide (BTS-27271) that caused an increased TA response when tested in vitro at 10 μM. This similarity in mechanism might suggest that monoterpenes modulate DsTAR1 comparable to that of an insecticide molecule.

The three monoterpenes were also tested on whole insects at sublethal concentrations to reveal possible effects on *DsTAR1* transcription. A transcriptional downregulation of the receptor was noticeable for all treatments, which was not observed for the *PKA* control gene, thus ruling out a generalized downregulation. This monoterpene-induced transcriptional effect might be either exerted directly on *DsTAR1* or by means of an adjustment carried out on the regulatory pathway of the receptor.

Taken together, one might hypothesize that the downregulation of *DsTAR1* represents a compensatory mechanism in response to the enhanced DsTAR1 signaling due to positive allosteric modulatory effect of monoterpenes. Furthermore, it will be fundamental to understand how the downregulation of DsTAR1 could interfere with the normal *Drosophila suzukii* behavior and physiology.

In conclusion, the present data contribute to widen the existing knowledge about the role of TA and particularly DsTAR1 in insect physiology. Moreover, the identification of a role for monoterpenes in DsTAR1 action, through a set up and validated in vitro system, will allow the pharmacological quest for biomolecules that may possibly act as innovative biopesticides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2020.02.015.

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