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Rational engineering approaches for establishing insect olfaction reporters in yeast

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A B S T R A C T

Insect olfaction directly impacts insect behavior and thus is an important consideration in the development of smart farming tools and in integrated pest management strategies. Insect olfactory receptors (ORs) have been traditionally studied using *Drosophila* empty neuron systems or with expression and functionalization in HEK293 cells or *Xenopus laevis* oocytes. Recently, the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) has emerged as a promising chassis for the functional expression of heterologous seven transmembrane receptors. *S. cerevisiae* provides a platform for the cheap and high throughput study of these receptors and potential deorphanization. In this study, we explore the foundations of a scalable yeast-based platform for the functional expression of insect olfactory receptors by employing a genetically encoded calcium sensor for quantitative evaluation of fluorescence and optimized experimental parameters for enhanced functionality. While the co-receptor of insect olfactory receptors remains non-functional in our yeast-based system, we thoroughly evaluated various experimental variables and identified future research directions for establishing an OR platform in *S. cerevisiae*.

1. Introduction

Olfaction is one of the primary influencers of insect behavior as certain odors and aromas can drive mating and avoidance behaviors, and the identification of food sources.[1] As insects are integral parts of the agricultural system and broader ecosystems, the study of insect olfaction has been motivated by understanding insect olfaction and how these findings can be used to promote beneficial insects and regulate pests.

While olfaction is an important sense in both mammals and insects, the molecular pathway of these senses differs greatly between these phyla. In mammals, olfactory receptors function metabotropically through G-protein coupled receptors (GPCRs), however there is some debate as to whether insect ORs can also be coupled to G proteins.[2–6] Insects also have GPCRs, recently gaining interest as drug targets for pest control,[7] but these receptors are not reported to be associated with olfaction.[8] Insect ORs are seven-transmembrane (7TM) receptors, like GPCRs, but represent a novel family of receptors.[9] Insect ORs have seven transmembrane domains, however in contrast to GPCRs, the N-terminus of the insect OR is intracellular and the C-terminus is extracellular, the inverse of GPCRs.[10] Insect ORs form heteromultimeric complexes which include the divergent OR, and four monomers of the OR co-receptor, ORCO (also referred to as Or83b in *Drosophila melanogaster*), together forming a ligand gated ion channel.[11,12] In insect olfactory signaling systems, ORCO has been identified as being critical for subcellular location and the assembly of the OR-ORCO complex that forms the ion pore in the membrane.[13] The ORCO channel is stimulated by cyclic nucleotides such as the non-naturally occurring Vanderbilt University Allosteric Agonist (VUAA1), and is conserved across insects with at least 50 % identity to other ORCOs across winged insects.[14–16]

In *D. melanogaster*, ORs are expressed from a ciliated dendrite from olfactory sensory neurons which then project into the antennal lobe in the brain.[17,18,19] Olfactory sensory neurons with the same receptor converge into specific 'glomeruli' which are used to convey further sensory information into other parts of the brain.[17,20,21] Generally, most ORs are broadly tuned to many different odorant types and one odor can interact with many different receptors.[22] OR56a in *D. melanogaster* is an example of a narrowly tuned receptor that offers a window into a specific odor-to-behavior pathway from a single receptor. Expressed in a single class of olfactory sensory neurons, OR56a only responds to geosmin which triggers an innate avoidance behavior as geosmin is associated with toxic microbes.[23]

In general, however, the deorphanization of insect ORs has been slow and primarily focused in model insect species.[24] Strategies such as the *Drosophila* 'empty neuron' system requires generation of transgenic fly lines which is time consuming and low throughput.[24,25] Interest has also arisen for insect ORs' potential for detecting volatile organic compounds (VOCs) as a diagnostic tool and in food safety.[26] Biotechnology can play an important role in this process given the novel tools being developed for insect olfactory research such as expression in liposomes and nanodiscs.[27,28] One platform that has been explored is the heterologous

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expression of insect ORs in model systems such in human embryonic kidney (HEK293) cells and in frog oocytes. A study comparing expression in *Xenopus laevis* oocytes versus transgenic *Drosophila* found that different systems provided advantages and disadvantages, but that studying the receptors *in vivo* was the most sensitive, albeit *in vitro* studies provided the potential for higher throughput despite expression challenges.[29] This trade-off provides an opportunity for the development of an expression platform compatible with expression of 7TM receptors and high-throughput screening methods. He yeast *Saccharomyces cerevisiae (S. cerevisiae)* has proven to be a functional and high-throughput chassis for the functional expression of other 7TM receptors such as GPCRs ([30,31,32,33]).

In this study, we present our endeavors towards the expression of ORCO and OR proteins in *S. cerevisiae* to create functional biosensors for the detection of odorants, and more broadly as a platform for insect olfactory receptor deorphanization. Using a multivariate approach including different OR and ORCO designs, and different calcium reporter systems we highlight the difficulties related to establishing robust proxy read-outs for olfaction in yeast cells cultivated under various conditions and time scales.

2. Methods

2.1. Cultivation of yeast and bacteria

The propagation of plasmids involved the use of the chemically competent *Escherichia coli* DH5α strain. The *E. coli* strains were cultivated in 2xYT media, which was supplemented with 100 μg/mL ampicillin. The growth temperature was maintained at 37 °C, and the cultures were agitated at 250 rpm. For yeast cultivations, a synthetic complete-dropout medium was used. The medium consisted of 6.7 g/L yeast nitrogen base without amino acids (Sigma) and 1.4 g/L yeast synthetic dropout (SD) medium supplements (Sigma), lacking uracil, histidine, leucine, and tryptophan. The pH and buffering of media was done with citric acid and disodium phosphate as previously described[34] with ammonium sulfate and urea. 2 % w/v glucose was added as a carbon source. To fulfill the auxotrophic requirements, histidine, uracil, leucine, and tryptophan were incorporated as necessary. Preculture tubes containing yeast were grown under conditions of 30 °C and 250 rpm, whereas incubation in 96-well and 24-well deep plates was carried out at 30 °C (except 25 °C growth assay) and 300 rpm. For *Komatagella phaffi* the cloning and assaying conditions were performed as previously described[35] and assayed in 5 mM $CaCl_2$ Hank's buffer.

2.2. ORCO and OR S. cerevisiae plasmid construction

The *K. phaffi ORCo* plasmid was cloned as previously described by Varela and Yadav.[35] Briefly, the *K. phaffi* vector pPICZA and PCR products of the ORCo gene were digested using *Eco*RI and *Not*I and ligated using T4 DNA ligase. The subsequent plasmid was linearized with *Sac*I for use in transformation of *K. phafii.* All other plasmids in this study were cloned using uracil specific excision reagent (USER) cloning (New England Biolabs) and the EasyClone method.[36]

Sequences that were codon optimized were sourced via UNIPROT (ORCO_DROME from *D. melanogaster*: Q9VNB5; ORCO_Anoga from *Anopheles gambiae*: Q7QCC7; Or22a from *D. melanogaster:* P81909; Or82a from *D. melanogaster*: P82986) and non-codon-optimized *D. melanogaster* ORCO sequence was sourced via FlyBase (FlyBase ID: FBgn0037324). Plasmids containing the ORCO and OR contained a Kozak sequence (AAAACA) in front of the start codon of the gene. Synthetic genes were ordered from TWIST Bioscience and IDT (Supplementary Table 1) using TWIST and IDT codon optimization algorithms. The plasmids were transformed into the chemically competent DH5α strain by heat-shocking for 45 s at 42 °C and recovered on Luria−Bertani plates supplemented with 100 μg/mL ampicillin. All plasmids sequence-verified using Sanger through Eurofins. List of plasmids constructed for this study can be found in Supplementary Table 2.

2.3. Construction of yeast strains

The 2-μm plasmids containing ORCO/OR from *D. melanogaster* and *A. gambiae* (Supplementary Table 2) were transformed into BY4741 strain using the lithium acetate/single-stranded carrier DNA/PEG method[37] alongside the CDRE plasmid expressing the calcium sensor[38] which was acquired from Addgene (Plasmid #138657) and maintained with a URA3 marker. The gCAMP6f plasmid is from.[39] For integration of the ORCO/ORs into the yeast genome, plasmids with overlap to the EasyClone genomic sites were engineered to contain the respective genetic sequence to be integrated.[40] All integrated ORCO constructs (including the mNeonGreen tagged ORCO constructs) were integrated in the XII-4 and gCAMP6f was integrated in the X-3 locus. The plasmids were *Not*I-digested for 4 h, the *Not*I enzyme was heat-inactivated, and linear fragments were integrated into yeast genomic sites with a Cas9 plasmid maintained on a histidine marker and a gRNA (leucine) plasmid targeting the respective integration site in a BY4741 strain containing the CDRE sensor maintained with a -Ura marker. Transformants were selected for on -His -Leu -Ura plates and confirmed with colony PCR. List of yeast strains constructed for this study can be found in Supplementary Table 3.

For *K. phafii* transformation, electrocompetent GS115 cells were prepared (transformation protocol: Eppendorf Protocol No. 4308,915.545–03/2004) and transformed with the *Sac*I linearized ORCO plasmid. The transformants were selected on YPD plates with 1 M sorbitol and 100 μg/mL zeocin. Resulting colonies were verified by colony PCR.

2.4. Growth curves

Strains were grown overnight in synthetic complete (SC) media with relevant drop-out with glucose. The following morning strains were back diluted 1:50 into synthetic complete media with 2 % raffinose in 2 mL. After 6 h, strains were back diluted to OD 0.2 and resuspended in SC media glucose. OD630 was taken every 10 min over 72 h in BioTek ELx808 plate reader. OD630 data was analyzed in GraphPad Prism 9. Doubling times were calculated using GraphPad Prism 9 by taking the natural log of the strains and performing a linear regression of an exponential growth with log (population) on the exponential growth part of the curve.

2.5. VUAA1 assay

Strains were started from a single colony on streak-out plates in SC media with relevant drop out in 0.5 mL in a 24-well plate. Strains were put into a shaking incubator overnight at 30 °C shaking at 300 rpm. Following overnight cultivation, strains were back diluted 1:10 in relevant SC media (for pH experiments this is when the pH 7.0 media was introduced) with relevant dropouts in 2 mL in a 24 well plate. After 9 h, strains were back diluted to OD5 using the Implen Nanophotometer. A total of 99 μl of cultivated strains at OD = 5 was aliquoted into a Greiner 96-well plate and relevant ligand was added. VUAA1 (Sigma Aldrich SML1336-5 mg) was dissolved in 100 % DMSO, aliquoted into 7 μL stocks at 200 mM and stored at −20 °C and used within 2 weeks of dissolution. Ionophore A23187 (Sigma Aldrich C7522) was diluted in DMSO and stored at 1 mM stock solution at 4 °C. Plates were incubated in BioTek Synergy Mx plate reader at room temperature for 6 h with reads for GFP (485 nm, 528 nm) and OD600 every 2 min with fast shaking. Overflow values were pruned manually, and the data was organized in Python using packages pandas, numpy, time and datetime. Data was then visualized using GraphPad Prism 9.

2.6. Plate-reader assays with K. phaffi

For assaying *K. phaffi,* assay conditions with Fluo-4-Am (Sigma Aldrich 93,596) dye were performed as previously described.[35] Three technical replicates of each colony were tested and assayed in the BioTek Synergy Mx plate reader at room temperature. ORCO from *A. gambiae* was also codon optimized for *K. phaffii* and ordered from IDT.

2.7. Flow cytometry

Cells were diluted in PBS and analyzed by a NovoCyte Quanteon™ (Agilent) flow cytometer. To measure green fluorescence, a 488 nm laser and a 525/45 BP filter were used. The cells were gated for singlets and a threshold set based on the singlet gate. FlowJo v10.8 and GraphPad Prism softwares were used to analyze the data and generate plots.

3. Results

3.1. ORCO expression in Komagataella phaffi is non-functional in presence of VUAA1

To establish a benchmark for the characterization of insect olfactory receptors in yeast, we initially aimed to reproduce previously reported results.[35] Following their protocol, we engineered *K. phaffi* strain GS115 to express ORCO from the mosquito *Anopheles gambiae* driven by the methanol-inducible *Alcohol oxidase 1* (*AOX1*) promoter and assessed calcium influx with the calcium binding dye 4-Fluo AM. Since it is expected that the method of integration can lead to different gene copy numbers, we randomly selected three different colonies from the transformed *K. phaffi* and assayed the calcium binding dye signals from the colonies with or without addition of 2 mM VUAA1. Here, for two of the ORCO-expressing *K. phaffi* colonies we observed a significant (2-way ANOVA, Col 1 $p < 0.0001$, Col 2 $p < 0.05$, $n = 3$) increase in reporter signals upon VUAA1 addition compared to cultivations without VUAA1 addition (Supplementary Fig. 1). However, we also observed a significant increase in reporter signals from strains without ORCO expression upon VUAA1 addition (2-way ANOVA, GS115 $p < 0.01$, $n = 3$)(Supplementary Fig. 1). Lastly, it should be noted that when adding 2 mM VUAA1 dissolved in 1 % DMSO we observed white precipitate in the cultures. Taken together, the significant increase in calcium binding dye signals in the control strain without expression of ORCO upon VUAA1 addition suggests that ORCO is dispensable for the changes in signals, even for the strains expressing ORCO. Also, even though a previous report[35] on this expression system used VUAA1 concentrations similar to what was used in this assay, we remain concerned about the quality of the data obtained in our data due to the VUAA1 precipitation at reported 2 mM concentrations.

Following the lack of success reproducing the previously reported ORCO data in *K. phaffi*, we shifted chassis to *S. cerevisiae* which has been successfully used to express functional 7TM receptors in our lab and others ([30,31,33,41]; N. J.[32]).

3.2. Testing and selection of calcium sensor

To establish an ORCO expression platform in *S. cerevisiae* we sought to use a genetically encoded calcium indicator (GECI), instead of the calcium dye setup, as this would allow for a cheaper and higher throughput screening in a future ORCO-OR deorphanization platform (Fig. 1). As a positive control for testing the response of GECIs to calcium influx we picked the calcium ionophore A13287 which has previously been shown to increase intracellular calcium concentration in yeast.[42]

Initially we tested a yeast-optimized gCAMP6f sensor.[39] The gCAMP6f genetically encoded sensor consists of a circularly permuted GFP which is commonly used to image activity in defined neuronal populations ([43,44]; T.-W.[45]). When exposed to ionophore, the strain engineered with the integrated gCAMP6f sensor showed significant fluorescence increase (unpaired *t*-test, $p < 0.01$, $n = 3$)(Fig. 2A and B). While the sensor worked well and validated previous reports,[39] the dynamic range was a modest two-fold (Fig. 2B). Furthermore, loading of the ligand into a 96-well format was confounded by the sensor's fast response, reaching its peak fluorescence within seconds, only to return to baseline within approximately 1.5 min (Fig. 2A). While this sensor responded well to the ionophore, we decided to test another sensor with increased time resolution.

The second sensor we tested was a calcium-dependent response element (CDRE)[38] which consisted of four repeats of a CDRE sequence upstream of a CYC1 promoter driving expression of yeGFP from a 2-μm plasmid. This sensor had a longer time resolution, with cells maintaining their elevated fluorescence for more than 4 h after ionophore addition with a peak at 2h and a dynamic output range of 10.6-fold (Fig. 2C and D). Thus, we concluded to move forward with the CDRE-based calcium sensor and sampling at 2 h post-supplementation of the ionophore. To further characterize the CDRE-based reporter, we determined the optimal conditions for the CDRE sensor expressed in *S. cerevisiae*. Here, we first tested several different cultivation media and conditions (Fig. 2E). From this we concluded that standard lab SC medium containing 1 mM of calcium chloride, provided optimal conditions for the dynamic output of the sensor (Fig. 2E), and thus we proceeded with this medium for the testing of ORCO-expressing strains.

3.3. Mitigation of VUAA1 precipitation in yeast cultivation medium

During the testing of the ORCO-expressed strain in *K. phaffi*, and again during the testing of calcium responses in *S. cerevisiae*, we noticed VUAA1 precipitation in yeast media. At a reported concentration of 0.5 mM–2 mM in 1 % DMSO we observed precipitation of VUAA1 in media with or without yeast cells present (Supplementary Fig. 2). In several studies, DMSO was used as a solvent due to previous reports documenting the use of DMSO to solubilize VUAA1.[35,46,47] While VUAA1 does not affect the fluorescence read with excitation at 485 nm and 528

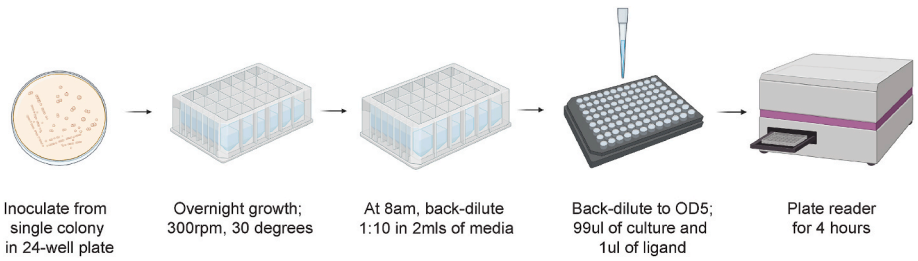

Fig. 1. Description of standard VUAA1 assay protocol for the *S. cerevisiae* strains expressing ORCO. Strains were inoculated from single colonies then grown overnight in a 24-well plate, shaking at 30 °C unless otherwise indicated. Strains were back diluted the following morning 1:10 in 2 mLs then incubated in the same conditions for 9 h. Strains were back diluted to OD5 in 1 mL using Implen Nanophotometer and added 99 μL of culture into a Greiner 96-well plate, in triplicates. A volume of 1 μL of ligand or control DMSO was added for a final concentration of 1 % DMSO. Plate was then read for 4 h with readings for yeGFP at 485 nm and 528 nm, and OD600. Shaking was set to continuous and fast and the incubator was set to 30 °C with reads every 1.5 min. Figure was designed with Biorender.

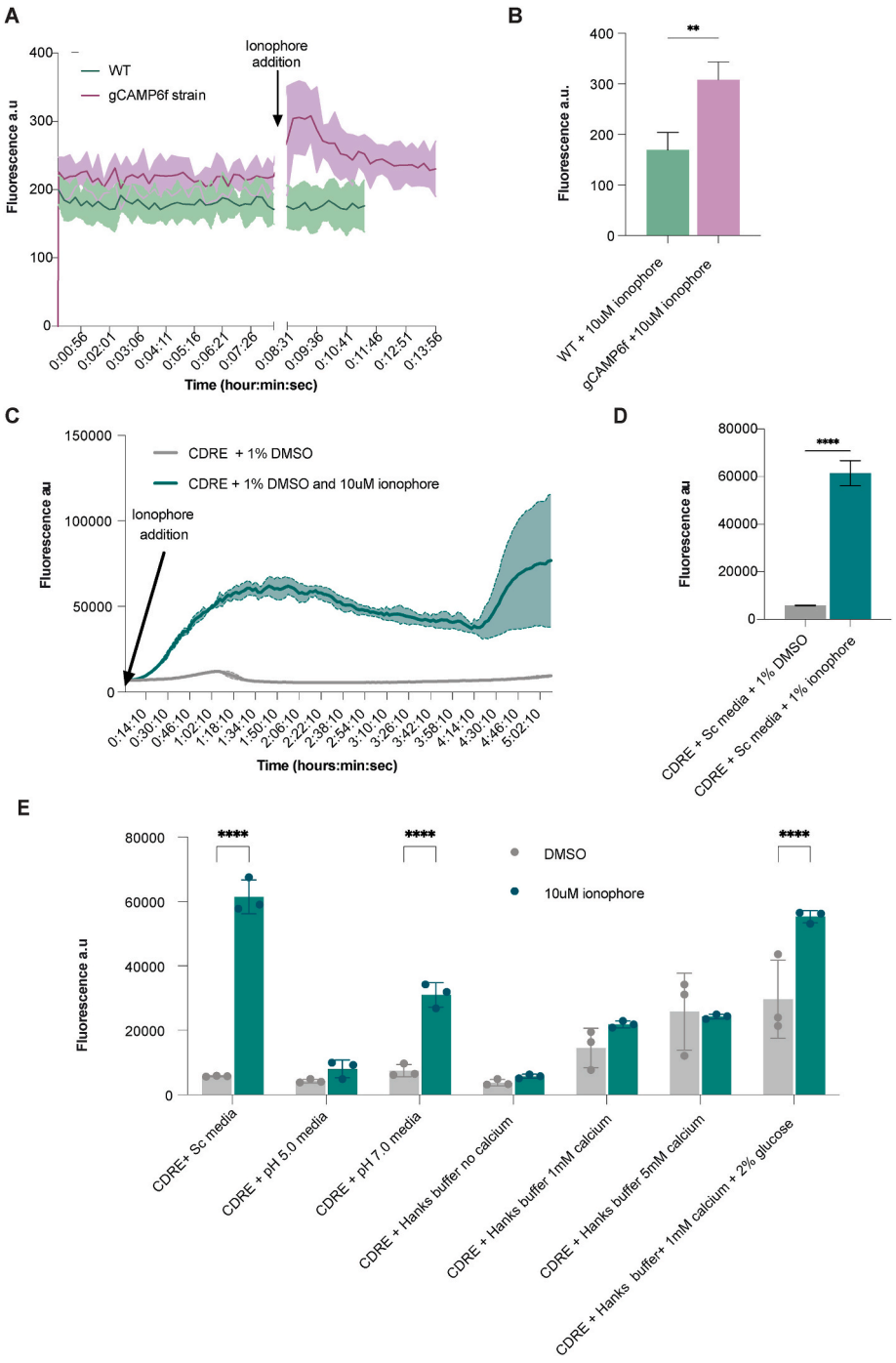

Fig. 2. Investigation of genetically encoded calcium sensors to response of 10 μM ionophore (A23187). A) Ionophore was added to culture with the gCAMP6f sensor which had been genomically integrated at the XII-4 locus.[39] The sensor responded rapidly (within 1 min of addition) and so addition of ionophore and placement back into the plate reader was completed quickly to catch the signal. **B)** Addition of ionophore caused a twofold increase in fluorescence in the gCAMP6f expressing strain with a significant increase (Unpaired *t*-test, $p < 0.01$, $n = 3$). **C)** Addition of 10 μM ionophore to plasmid based CDRE increased fluorescence overtime with a peak around 2 h which **D)** resulted in 10.6-fold increase (One-way ANOVA, $p < 0.0001$, $n = 3$) in fluorescence compared to the strain without ionophore addition. E) Different medias were tested to investigate dynamic range of the CDRE sensor in various medias such as Hank's Buffer which had been tested in[35] and pH buffered media as pH is an important factor in transmembrane receptor signaling and Hank's media with glucose as we wanted to investigate the role of glucose in affecting the sensor. Significant increase with addition of 10 μM ionophore was found in Sc media (One-way ANOVA, $p < 0.0001$, $n = 3$), pH 7.0 Sc media (One-way ANOVA, $p < 0.0001$, $n = 3$), and Hank's buffer with 1 mM calcium chloride with 2 % glucose (One-way ANOVA, $p < 0.0001$, $n = 3$). a.u = arbitrary units.

nm emission (Supplementary Fig. 2A), the precipitant is read in the absorbance 600 nm spectrum (Supplementary Fig. 2B). In our testing of VUAA1 in different media and concentrations, we determined that SC complete media (pH 5.2) had the least precipitation (Supplementary Figs. 2B–C), so we proceeded to use this medium for our assays. Furthermore, in our tests, 200 μM was the highest concentration without precipitation, and this was hence the standard concentration used in subsequent experiments. It is also of note that after one month of storage at −20 °C following manufacturer instructions, VUAA1 does not show any visible precipitation in all media tested (data not shown).

3.4. Exploring ORCO and OR designs in yeast

In the initial exploration of this project, we focused on two ORCOs. ORCO from *D. melanogaster* as this has wide interest as a model insect, and ORCO from the mosquito *A. gambaie* which had been expressed in *K. phaffii* previously, and for its interest in the mosquito's role in the spreading of malaria.[35,48] In our first attempts to functionalize ORCO in yeast, we tested the effect of expression of ORCOs under different promoter strengths. Tuning expression strength for proteins has proved beneficial for improving xylitol formation on xylan and using a strong promoter increased sensitivity of dose responses in heterologous GPCR expression.[41,49,50] Initially to test for potential impacts of varying ORCO expression we constructed expression cassettes using 3 different constitutive promoters with weak (RNR2), medium (TEF1) and strong (CCW12) expression strength cloned into 2-μm plasmids.

When ORCO was expressed in the 2-μm plasmid we observed up to 6-fold increases in fluorescence from strain designs cultivated in control media compared to a parental control strain only expressing the CDRE reporter with 1 % DMSO (Fig. 3A). Compared to the control, we observed general variation in fluorescence background with strains expressing both ORCO_Anoga or ORCO_Drome having the highest increase in background. Among the 3 promoter designs, we observed that

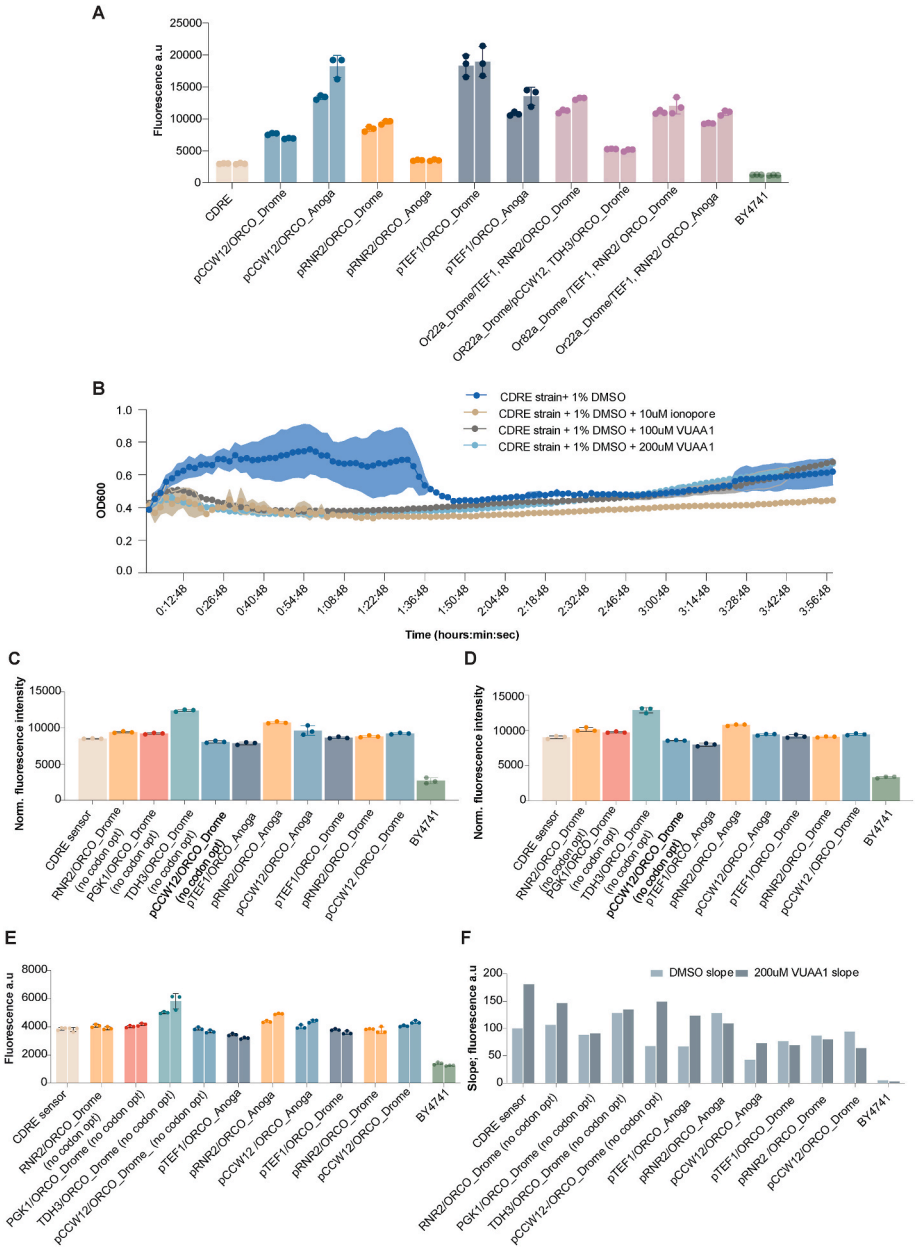

Fig. 3. Standard VUAA1 and ORCO-expressing *S. cerevisiae* assay. **A**) After 2 h exposure to 200 μM VUAA1, plasmid-based expression of ORCO has increased and varied background levels of fluorescence. For each strain, DMSO is on the left and 200 μM VUAA1 is on the right. All strains contain the CDRE plasmid and a 2-μm plasmid-based expression of ORCO, other than WT BY4741 which does not contain the CDRE plasmid. **B**) Addition of DMSO, 100 μM VUAA1, 200 μM VUAA1, and ionophore affect the OD600. OD600 and fluorescence (485 nm, 528 nm) were recorded in the BioTek SynergyMx plate reader. Due to this effect, intra-condition comparisons are made with normalized fluorescence to OD and inter-condition comparisons are made with non-normalized fluorescence a.u (arbitrary units). **C**) Fluorescence normalized to OD of the integrated ORCO-expression strains exposed to 1 % DMSO. **D**) Fluorescence normalized to OD of the integrated ORCO-expression strains exposed to 200 μM VUAA1. **E**) Fluorescence intensity of strains with genomically-integrated ORCO expression when exposed to 1 % DMSO (left bar) and 200 μM VUAA1 (right bar). **F**) The slopes of the fluorescence intensity for the first 15 min following VUAA1 or DMSO. All data visualized with GraphPad Prism 9.

expression affected fluorescence output from the reporter under control conditions and/or VUAA1 supplementation. We observed the greatest difference between DMSO and 200 μM VUAA1 condition in the pCCW12/ORCO_Anoga and pTEF1/ORCO_Anoga designs compared to the ORCO_Drome designs where we observed minimal increase or decrease in fluorescence intensity. ORCO has also been previously identified as important for localization in the cell or acting as a chaperone for the ORs.[10,51,52] To establish whether the co-expression of an OR was possibly important for the functionalization of ORCO in *S. cerevisiae,* we expressed different OR and ORCO combinations with different promoters in the 2-μm plasmid background. The OR + ORCO combination was only expressed from the 2-μm plasmid, but we did not see significant fluorescence increase in the 200 μM VUAA1 condition in the standard media assay (Fig. 3A). We observed small increases in the standard conditions in several of the OR/ORCO strains though these were not significant increases.

However, we also observed differences in OD600 with the addition of 200 μM VUAA1 (Fig. 3B). With the addition of 200 μM VUAA1 we saw an abrupt decrease (within 15 min after addition) in OD as compared to the DMSO control, possibly due to flocculation of strains due to stress from ionophore and VUAA1, though this effect of VUAA1 has not previously been reported in yeast.[53,54] With this in mind, we used the raw fluorescence arbitrary units (A.u) to compare between conditions (DMSO, VUAA1) and then normalized fluorescence a.u to OD when comparing within conditions. While the immediate addition of VUAA1 affected the OD, over time the cells continued to grow (Supplementary Fig. 3A). While the control strain BY4741 reached a higher overall optical density (OD630), the 200 μM VUAA1 and ionophore conditions had a faster doubling time (1 h 3 min and 1 h and 8 min) compared to the control (1 h 41 min) (Supplementary Fig. 3A). We also observed a slow growth phenotype in two of the strains containing 2-μm plasmids (Supplementary Fig. 3B).

3.5. Calcium reporter signals in ORCO expressing yeast strains

Moving forward, we observed that the integrated system provided decreased variation in background, increased replicability and that promoters made a substantial factor in ORCO response to 200 μM VUAA1. We chose to assay integrated ORCO strains with a great diversity of promoters to assess promoter tuning and added a non-codon optimized ORCO_Drome as codon optimization has been identified as being important in successful ORCO functionalization in other heterologous systems. Among the co-expressed ORCO/OR strains we did not see any significant increases, so we focused on the functionalization of ORCO in the *S. cerevisiae* chassis.

Across the integrated strains with the only the addition of 1 % DMSO, we observed slight variation in the background of fluorescence when normalized to OD with the highest background in pTDH3/ORCO_Drome (without codon optimization) and pRNR2/ORCO_Anoga (Fig. 3C). We also observed the increased background in the CDRE strain as compared to the wildtype yeast strain without the sensor (Fig. 3C). In the pRNR2 promoter, we observed the highest background fluorescence in the ORCO_Anoga strain while the ORCO_Drome strains were approximately the same, indicating that codon optimization did not affect the expression of ORCO from *D. melanogaster* in yeast. In the VUAA1 condition, we also saw the highest fluorescence per OD in the same strains (Fig. 3D), however no direct comparison of fluorescence per OD can be made due to the differences in effect of VUAA1 and DMSO on OD (Fig. 3B). In the fluorescence intensity we observed a modest but significant increase in the VUAA1 condition with pTDH3/ORCO_Drome as well as pTEF1/ORCO_Anoga and pCCW12/ORCO_Anoga (Fig. 3E). To further determine where the difference in these strains was due to the addition of VUAA1, we looked at the slope in the first 15 min after VUAA1/DMSO addition. While the pTDH3/ORCO_Drome strain had a slightly increased slope with VUAA1 compared to the DMSO, both TEF1/ORCO_Anoga and pCCW12/ORCO_Anoga had a decreased slope, indicating the effect of VUAA1 addition on the CDRE sensor was not apparent immediately after addition (Fig. 3F).

As 2-μm plasmids also can vary in their copy number, we also tested biological replicates of the strains and saw much greater variation in the biological replicates among the 2-μm strain compared to the integrated strains (Supplementary Fig. 4). In order to quantify some of the expression characteristics of the heterologous ORCO, we tagged the *D. melanogaster* ORCO with an N-terminal mNeonGreen fusion. Similar N-terminal fusions of ORCO have previously been constructed and found to be functional *in vivo* in *D. melanogaster*.[10] Recently, it was also shown that the N-terminal tagging of ORCO did not affect ORCO's function or localization.[55] We measured the single cell fluorescence levels by flow cytometry and compared plasmid-based and genomic expression with promoters of different strengths (Fig. 4A and B). When the fusion protein was expressed from a 2-μm plasmid, the populations displayed more heterogeneity, compared to the integrated constructs. In the integrated versions, the pTEF1 and pCCW12 promoters produced substantial shifts in the fluorescence compared to a non-ORCO expressing strain, suggesting that mNG-ORCO fusion construct is likely being expressed in the yeast cell. It also indicates that the integrated constructs achieve comparable expression levels to the plasmid-based constructs, with less cell-to-cell heterogeneity.

Overall, genome integration provides reduced background and variability in the ORCO system in both technical and biological replicates (Fig. 4). No conclusion can be made about whether ORCO from *D. melanogaster* or *A. gambiae* is more functional in the yeast platform. However, it did not appear that codon optimization was a factor in the expression of the ORCO from *D. melanogaster*, but that promoter choice affected the fluorescence background of the ORCO expressing strains. It appears that exposure to VUAA1 has an impact on the fluorescence intensity in the ORCO expressing *S. cerevisiae.* However, given the sensitivity and high background of the CDRE sensor and the factors affecting OD, it is not possible to ascertain a direct correlation between ORCO expression and calcium influx with VUAA1 addition.

3.6. Assay pH and growth temperature do not affect ORCO response to VUAA1

In further attempts to improve ORCO functionality in *S. cerevisiae*, we next investigated the role of pH and temperature. pH has been shown to play an important role in the signaling of other 7TM receptors in yeast ([30]; Nicholas J.[56]). It has also been shown that a codling moth ORCO had an increased response to 1 mM VUAA1 when expressed at higher pHs in HEK293 cells.[57] The pH of the standard yeast SC media used in these experiments is pH 5.2. When assayed in buffered pH 7.0 SC media, ORCO-expressing yeast strains we observed higher background overall in strains compared with standard SC media. When normalized to OD, all of the ORCO expressing strains had lower fluorescence background per OD than the control CDRE strain (Fig. 5A). This reduction in fluorescence when compared to the CDRE sensor only strain remains consistent in the 200 μM VUAA1 condition (Fig. 5B). When comparing the fluorescence intensity, we observe modest increases in several strains but also observed decreases in pTDH3/ORCO_Drome (without codon optimization), pRNR2/ORCO_Anoga and the CDRE only strain. While we do see modest increases in several strains in pH 7.0 media, the increased background and decreases in some strains including the control lead us to conclude that pH 7.0 does not improve ORCO functionality in *S. cerevisiae.*

Similarly, we wanted to explore if lowering the growth temperature would improve the functionality of ORCO as has been shown to be helpful in optimal protein function.[58] We tested the lowest and highest promoter strength with ORCO to determine if growth temperature at 25 °C instead of 30 °C had an effect on the functionalization. Within the DMSO control, we observed that there was similar modest increase in the CCW12/ORCO_DROME (without codon optimization) and the pRNR2/ORCO_Anoga strain which is interesting given the two different

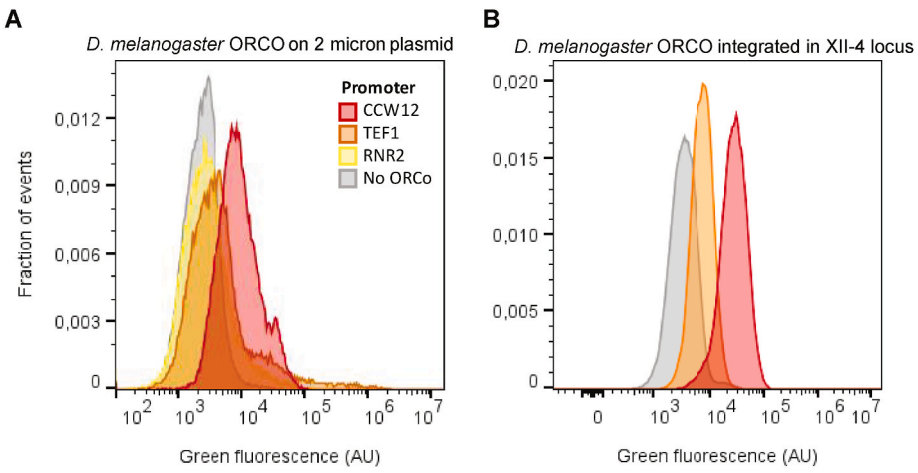

Fig. 4. Histograms comparing single cell green fluorescence profiles of *S. cerevisiae* cells expressing *D. melanogaster* ORCO from a 2-µm plasmid or genomically integrated. A) Reporter profile of yeast expressing *D. melanogaster* ORCO from a 2-µm plasmid under the control of promoters pCCW12, pTEF1, or pRNR2. **B**) Reporter profile of yeast expressing *D. melanogaster* ORCO from an integrated cassette in the XII-4 locus with a pCCW12 or pTEF1 promoters. Each sample's dataset consists of between 30,000 and 60,000 events. The plots were produced using FlowJo v10.8.

strength promoters (Fig. 5D). The other strains remained normalized fluorescence levels similar to the control. In the 200 µM VUAA1 exposure condition, we saw increase in the pRNR2/ORCO_Drome (without codon optimization) and again in CCW12/ORCO_DROME (without codon optimization) and the pRNR2/ORCO_Anoga strain (Fig. 5E). Looking at the absolute fluorescence we observed a slight increase in the pCCW12/ORCO_Drome (without codon optimization). Interestingly, compared to the same strain but with codon optimization, we see increased background fluorescence in the codon optimized strain (Fig. 5F). Overall, growth at 25 °C did not increase ORCO functionality though codon optimization did appear to influence background fluorescence in pCCW12/ORCO_Drome strains. Interestingly, the opposite effect is observed in this strain in the 30 °C growth temperature assay (Fig. 3A).

4. Discussion

A scalable and consistently functional insect olfactory receptor biosensor platform remains an elusive but very promising direction for biotechnology. While this is one of the first studies looking to express an insect olfactory receptor in yeast, yeast has previously been used to successfully express many other functional 7TM receptors. However, the presented work points to more work being needed to functionalize ORCO in yeast and overcome several hurdles before successful co-expression of ORCO and OR. While it has been reported that heterologous expression and functionalization of OR/ORCO has varied given the model organism, yeast has yet to be fully explored as a valuable and scalable chassis for OR and ORCO deorphanization.[29,59]

In some of the strains, we also observed an increase in the background of ORCO-expressing strains without the addition of VUAA1. ORCO has been shown to be leaky in mammalian cells, though it is unclear if this is the issue in the *S. cerevisiae*-based system.[60] Possibly the increased background is due to the CDRE sensor responding to non-ORCO related calcium influx. The increased background from the CDRE reporter in ORCO expressing strains could be due to a misfolded protein response as calcium homeostasis has been linked to unfolded protein response in yeast.[61] Another study found that the accumulation of misfolded proteins in the ER also resulted in an influx of calcium into the cell and that Ca2+ can also enhance protein folding.[62] As the 2-µm plasmid is a multi-copy plasmid, it is possible that ORCOs are expressed at very high levels in the cell which could be causing stress to the cell which results in an influx of calcium which is reported by the sensor. The greatest variation in the technical replicates is observed in the plasmid-based strains which further points to the importance of genome integration for insect olfactory receptors expressed in yeast. Further research would investigate the use of Ty elements as a strategy for stable multi-copy genome integration instead of the use of 2-µm plasmids or single copy integrations. Ty elements are transposable elements in yeast which have been used to integrate multiple copies of genes at different loci within the yeast genome as a strategy to vary protein production and tune expression levels in yeast (Maury et al., 2016; Sakai et al., 1990).

Another observation was that with some strains we saw a decrease in fluorescence as compared to the DMSO control when 200 µM VUAA1 was added (Figs. 3E and 5C). While VUAA1 has been shown to increase calcium flux into the cell in HEK293 and Zenopus oocytes ([63,64]; S.[65–67]), it has also been shown that the ancestral OR *Mh*OR5 from *Machilis hrabei* is inhibited by VUAA1 and does not need to be coexpressed with a coreceptor to function.[68] However, we did not see a consistent decrease with VUAA1 addition so we cannot draw this conclusion that VUAA1 is decreasing fluorescence as we also see an increase in other strains and other conditions.

One of the strategies employed in this project was the use of a genetically encoded calcium sensor. The intention of this was to provide a base strain that could be used with any combination of OR and ORCO and provided good scalability. The selected sensor, CDRE, provided a good dynamic range when 10 µM was added to the media and showed that calcium was influxing into the cell from the yeast media. While calcium binding dye could be used to titrate the amount of calcium entering the cell, we would still have the issue of the background of influx of calcium from the unfolded protein response if that is occurring in the yeast cell. While the time resolution was necessary for this sensor to be visualized on the plate reader, future optimization of another sensor could give a more dynamic picture of the effect of VUAA1 on the cell. Another of the confounding factors in this study could be the relationship between pH, glucose and calcium influx as it has been shown that increased pH increases intracellular calcium in the presence of glucose.[69] It could also be that lowering extracellular calcium from 1 mM to 0.3 mM $CaCl_2{\cdot}2H_20$ could increase the functionalization of heterologous ORCO as it has been shown to do in HEK293 cells.[60]

Further explorations would also continue to explore whether ORCO is folding correctly in the yeast cell and whether more integrated copies of ORCO would provide greater resolution. Additionally, in ORCO/OR expressed in HEK293 cells, it was found that the co-expression with RTP1S (a receptor transport protein) and the fly SNMP1 (*Drosophila* sensory neuron membrane protein 1) enhanced the functional expression of ORCO.[70] Similarly, as several GPCRs have been shown to have

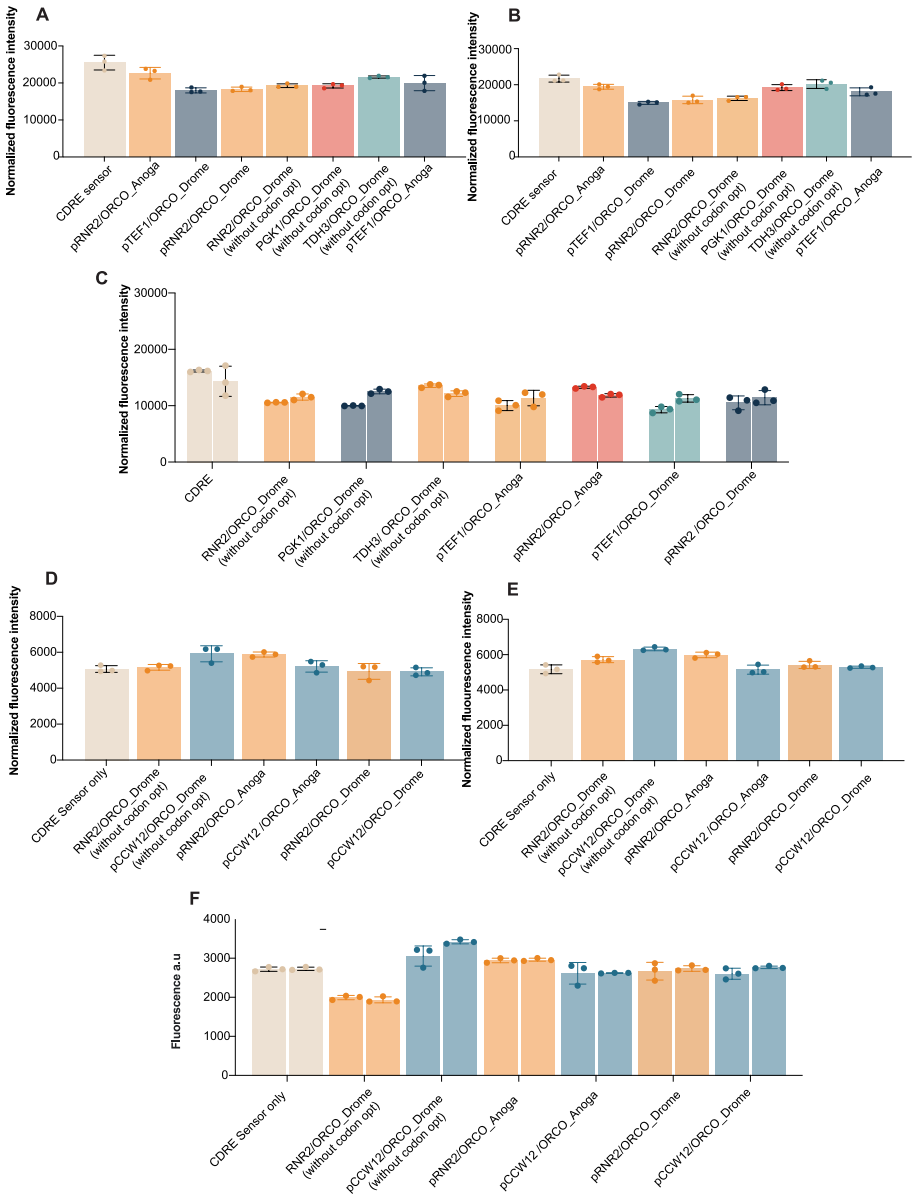

Fig. 5. pH 7.0 buffered media increases CDRE background and 25 °C growth temperature has modest effect on ORCO functionality. A) Fluorescence normalized to OD in integrated ORCO strains exposed to 1 % DMSO shows decrease in all strains compared to the control strain. **B)** Fluorescence normalized to OD in integrated ORCO strains exposed to 200 μM VUAA1 shows decrease in all strains compared to 1 % DMSO control. **C**) Left bar shows DMSO exposed strains, and the right bar shows 200 μM VUAA1 exposure. Fluorescence intensity of strains shows modest increase in some strains with VUAA1 exposure but a decrease in fluorescence in the CDRE strain when exposed to 200 μM VUAA1. **D**) Fluorescence normalized to OD in integrated ORCO strains exposed to 1 % DMSO and grown at 25 °C showed modest increase in fluorescence per OD in some strains **E**) Fluorescence normalized to OD in integrated ORCO strains exposed to 200 μM VUAA1 and grown at 25 °C showed modest fluorescence per OD increase in some strains **F**) Fluorescence intensity of strains grown at 25 °C shows slight fluorescence increase in some strains with VUAA1 exposure. Left bar shows DMSO exposed strains and the right bar shows 200 μM VUAA1 exposure. Normalized fluorescence intensity was normalized to OD. a.u = arbitrary units.

improved expression and functionalization when grown at 15 °C, this would be another avenue of exploration.[71,72] Further exploration of transport proteins, optimized expression conditions and targeting *S. cerevisiae* ER could be valuable future strategies.

Establishing *S. cerevisiae* as a platform for increased research into OR and ORCOs in heterologous systems still requires further optimization. Further steps for exploration would be exploring localization and solubility tags for ORCO and the OR. As only the ORCO from *D. melanogaster* and *A. gambiae* were used in this study, it could be interesting to see if different ORCOs are differentially expressed in yeast, despite their general conservation across winged insects. It is also a possibility that yeast membrane protein expression systems are too different from that in insects and that yeast is not a well-suited platform for insect olfactory receptor expression.

Still, there is a strong motivation to develop robust and relevant olfaction platforms. Here, establishing such a platform would allow for applications outside of basic research and into applied biotechnology. For example, the behavioral transition of the migratory locust (*Locusta migratoria*) behavior from the 'solitary' grasshopper to the 'gregarious' pest was found to be triggered by the pheromone 4-vinyl anisole (Guo et al., 2020). Of the receptors screened, 4-vinyl anisole had the strongest stimulating effect on olfactory receptor 35 (Or35), though olfactory receptor 64 (Or64) was also stimulated, to a lesser extent. This is an example of how a yeast-based rapid olfactory receptor deorphanization

platform could be very useful as the locust OR library could be expressed in yeast and screened with the pheromones to determine which pheromone interacts with which receptor, providing cheap and effective guidance in receptor identification.

As of now, the data from this research points towards ORCO being non-functional in the *S. cerevisiae* platform under present conditions. However, this research does point out several avenues of future exploration for the successful expression of ORCO and ORs in *S. cerevisiae*. As differing fluorescence background levels were observed in the strains expressing ORCO, it is possible that in addition to background calcium in the cell, ORCO is misfolding and causing stress in the cell which increases intracellular calcium.[61] As we see varying background levels depending on promoter strength but also ORCO variant, a future direction would be exploring a library of ORCO variants and mining the space for the most variant structures and expressing those in *S. cerevisiae*. Another direction would be to explore the expression of ancestral ORs which have been shown to function in the absence of ORCO, such as *Mh*OR5, and responding to a broad panel of odorants and ligands (Marmol et al., 2021). While codon optimization was considered as a factor in our study, it could be even further explored by testing alternate codon optimization algorithms. Other factors such as improper folding, poor membrane localization, or low signal-to-noise ratio in the CDRE sensor could be causes for the lack of detected ORCO functionality in *S. cerevisiae* and may warrant further investigation.

Credit

EEH-S, TS, E.D.J. and MKJ conceived the study. EEH-S and TS performed all the experiments and performed all the data analysis. EEH-S and MKJ wrote the manuscript. All authors approved the manuscript.

Declaration of competing interest

The authors declare no competing interests. Michael Krogh Jensen is an Associate Editor for Biotechnology Notes and was not involved in the editorial review or the decision to publish this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotno.2023.11.002.

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