Synthetic Sensing Systems in *Saccharomyces cerevisiae*

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Synthetic Sensing Systems in *Saccharomyces cerevisiae*

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Dedicated to my Family

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SUMMARY

The yeast *Saccharomyces cerevisiae* is a major chemical production platform in the biotechnological industry. It is also increasingly being used as a whole cell biosensor. One method of developing such whole cell biosensors in yeast is by exploiting its mating pathway, which is normally induced by secreted pheromones leading to downstream expression of various genes. Functional expression of different recognition elements or receptors and their coupling to the yeast mating pathway can enable sensing of a variety of ligands. In this work, we have engineered a yeast strain to functionally express a heterologous human olfactory receptor gene which can be coupled to the pheromone signaling pathway, allowing yeast to detect medium chain length fatty acids, alcohols and aldehydes for the first time.

Functionally expressing heterologous olfactory receptors in yeast is a challenging task because no definitive method exists on how to express such receptors on the yeast cell surface and couple them to the downstream signaling pathway. We explore in this work how the yeast cell can selectively respond to two activating ligands via two different receptors. We also demonstrate in this work that a synthetic transcription factor can substitute for the native transcription factor in the yeast mating pathway. We believe our biosensor will not only have various uses as a versatile sensor but also aid in the design of synthetic genetic circuits.

CHAPTER 1

INTRODUCTION

A biosensor is defined as "A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds, usually by electrical, thermal or optical signals"[1]. A biosensor is thus a measurement device or system which uses a biological component as the recognition element. This recognition element of a biosensor can be whole cells, antibodies, or immobilized enzymes among others[2,3]. On activation by target analytes, biological events are converted into quantifiable electrical, optical, thermal other signals in proportion to the target concentration via a reporter system or a transducer[2,4]. The major advantages of using biosensors are their high specificity, sensitivity, and portability[5].

1.1 Whole Cell Biosensors

Whole cell biosensors have some distinct advantages that make them attractive to use as a sensor. First, numerous microorganisms exist in the natural environment and therefore allows the choice of selecting a suitable strain for a specific sensing purpose[6]. Second, the enzymes inside a cell are less prone to denaturation compared to immobilized enzyme biosensors exposed to the environment[2,7,8]. Thus whole cell biosensors have the potential to be more durable and inexpensive[7]. Third, in case of toxicity determination of a particular pollutant, whole cell biosensors, being living entities themselves, are most suitable[7]. Fourth, while analytic methods can give the amount of a dissolved pollutant, whole cell biosensors can quantify critical functional information like, if the pollutant has

effects on other secondary metabolic pathways inside the organism[6]. Fifth, whole cell biosensors are amenable to high throughput screening and can be ~1000 fold faster than analytical techniques like mass spectrometry (100 samples/day) if aided by supporting methods like fluorescence activated cell sorting[9].

There are also disadvantages of whole cell biosensors. The diffusional limitations of substrates through the cell membrane to activate the recognition element results in a slower response in whole cell biosensors than the response of immobilized enzyme-based sensors[5]. Furthermore, undesirable side-reactions that interfere with sensing may be caused by endogenous enzymes in cells[5]. Whole cell biosensors require some time for the reporter gene to be transcribed and translated which make it difficult to use such sensors for real-time monitoring[5]. In general, though biosensors have been shown to be able to be freeze-dried and then rehydrated prior to use[10], one of the main challenges in their commercial exploitation remains extending their lifetime and their durability[7].

1.2 Optical Reporter Systems

A whole cell biosensor can quantify the ligand concentration through various reporter systems like electrochemical, thermal or optical amongst others[2,4]. Though optical systems cannot be used for real time monitoring of changes in signal intensity[2,4], they offer advantages like simplicity and ease of detection while maintaining high sensitivity[4].

Whole cell biosensors using the optical reporter systems commonly incorporate reporter genes coding for elements emitting bioluminescent, fluorescent, or colorimetric endpoints. Bioluminescence is usually derived from the bacterial (Lux) or firefly (Luc) luciferase genes, fluorescence from the green fluorescence protein (GFP) gene while

colorimetric endpoints rely on the β-galactosidase (LacZ) gene[11]. Of these three different types of optical reporter systems, the use of the fluorescent reporter GFP has certain advantages over the others.

First, GFP does not need any substrate to emit light like the firefly luciferase and the LacZ reporter genes, but depends on an external light source to activate its fluorescence[11-13]. Second, fluorescent proteins in various colors are available like cyan fluorescent protein, red fluorescent protein and others which makes them amenable to use in multiple reporter systems[11-13]. Third, protein engineering has reduced the GFP fluorophore maturation time to the order of a few minutes that enables rapid readouts[13]. However, GFP based reporter systems do suffer from high fluorescence levels in the absence of inducer(s)[13].

1.3 Repressible and Inducible Optical Whole Cell Biosensors

There are two types of optical whole cell biosensors: repressible expression biosensors and inducible expression biosensors.

Repressible expression biosensors or "lights-off" biosensors[11] use a promoter that is highly active under normal growth conditions leading to a high expression in the absence of inducer(s). Under toxic conditions, this expression level is reduced and the reduction is correlated to the sample toxicity[12]. Repressible systems determine the apparent toxicity by monitoring this reduced reporter expression after a certain time of exposure. The quorum sensing bacterium *Vibrio fischerii* has been used for repressible expression systems[7]. The main disadvantage of a repressible expression system is its high levels of expression in the absence of inducer(s)[11].

Inducible expression systems or "lights on" systems have low expression levels in the absence of the inducer(s) which increases on the presence of the inducer(s)[11]. Inducible systems can be stress specific or chemical specific. Different stressors like superoxide or hydroxyl radicals, single stranded DNA etc. are responsible for inducing different stress responses in cells[14]. A stress inducible system thus helps to classify toxic compounds on the basis of the stress it induces. A chemical specific inducible system does not measure toxicity or stress but the presence of specific chemicals[14]. Inducible expression systems are much more sensitive than repressible expression systems[11] because of their low expression levels in the absence of inducer(s).

1.4 Whole Cell Biosensors with Intracellular Recognition Elements

In case of whole cell biosensors with intracellular recognition elements, the ligand of interest must permeate inside the cell and then activate the recognition element inside the cell. Thus, such a system may be limited by the diffusional and transport resistances encountered by the ligand of interest in entering inside the cell[2,15].

The most widely used intracellular recognition elements of whole cell biosensors are based on the activation of an inducible promoter by a transcription factor in response to external ligands[2,15-17]. This interaction between the target ligand and transcription factors activates or represses the expression of the reporter gene which results in a quantifiable signal change. Many such transcription factor and promoter pairs are based on natural resistance mechanisms or toxic compound metabolism[2,15-17]. For example, a whole-cell biosensor for cadmium detection was developed in *Bacillus subtilis* based on the regulatory protein CadC and the P_{CADC} promoter from *Staphylococcus sp.*[18]. The

ZntR regulatory protein and the P_{ZATAP} promoter from *Escherichia coli* were used to monitor zinc, lead and cadmium[16]. The XylR regulator protein and the P_{PU} promoter pair from the xylene degradation pathway in *Pseudomonas putida* have been used to detect organic compounds like xylene, benzene and toluene[19]. Other examples include an *E. coli* biosensor to detect L-arabinose using the AraC transcription factor and P_{BAD} promoter[20]. Transcription factors and inducible promoter pairs also exist in simple eukaryotes like *S. cerevisiae*. Examples of such promoters include P_{GAL1}, P_{MET25} and P_{CUP1}[21,22]. While P_{GAL1} and P_{CUP1} are induced by exposure to galactose and copper respectively[22,23], P_{MET25} is induced by absence of methionine[24]. While these examples demonstrate the capability of regulatory proteins to sense a wide range of novel targets, not all chemicals have known transcription factor-promoter pairs that can be utilized for their detection, limiting the use of such recognition elements[2].

Synthetic RNA switches are also used as a type of intracellular whole cell biosensor recognition element. RNA switches are a class of RNA-based sensor-regulator elements that couple a RNA sensing function, encoded in an aptamer, to a gene-regulatory function[25]. The binding of the ligand at the aptamer domain leads to a conformational change, ultimately modulating the activity of the gene-regulatory domain through splicing, transcription, translation etc. For example, an *E. coli* based whole cell biosensor was developed with an engineered RNA switch to detect the antiasthmatic drug, theophylline[26]. More recently, a modular ribozyme based device in *S. cerevisiae* was constructed that coupled aptamers recognizing theophylline or tetracycline to a hammerhead ribozyme which led to the ribozyme self-cleavage and mRNA degradation in the presence of the ligand[25]. *De novo* generation of RNA aptamers is possible and the

iterative process includes attachment of the ligand of interest to solid supports followed by affinity chromatography to identify RNA sequences that bind to the attached ligands[25,27]. Still, RNA switches suffer from a limited diversity of available parts and lack of scalable strategies to accelerate generation of new aptamers[27].

1.5 Whole Cell Biosensors with Extracellular Recognition Elements

Whole cell biosensors with extracellular recognition elements are advantageous for the detection of target molecules or ligands that cannot be easily transported to the intracellular environment[2,28]. Even in the case of ligands that can cross the cell membrane and enter the cell, the overall kinetics are significantly improved by bypassing membrane transport and using extracellular recognition elements[2,28,29]. Usually, such extracellular recognition elements or cell surface receptors are a part of various signaling cascades inside the cell that lead to expression of downstream genes on ligand activation.

Examples of such recognition elements in prokaryotes include two component histidine kinase system[30] , which consists of a histidine kinase sensor and a response regulator[30]. For example, the CusR/CusS two component system in *E. coli* can respond to copper and silver levels in the extracellular environment[31]. Surface display systems have also been developed in *E. coli*, which usually work by fusing the protein of interest (recognition element) to a carrier protein, that leads to export across the cell envelope and attachment to the cell surface[28,29]. By being displayed on the cell surface, the recognition element can access any externally added substrate and membrane penetration issues of the substrate can be overcome[28,29].

An important example of extracellular recognition component is the G-protein coupled receptor (GPCR). GPCRs are the largest and most diverse membrane protein family on the cell surface[32-35]. Located in the plasma membrane, GPCRs are seven helix transmembrane proteins[32-34,36], involved in signal transmission in the cell. The primary benefit to explore GPCRs as recognition components is the variety of its ligands[2] because of the wide diversity of GPCRs available across different species. Also, using standard biological techniques like directed mutagenesis it is now possible to engineer GPCRs, called receptors activated solely by synthetic ligands (RASSLs), that are unresponsive to endogenous ligands but can be activated by the desired small molecule ligand[37].

1.6 Olfactory Receptors

Olfactory receptors (ORs) are a sub-class of GPCRs that bind odorant ligands. ORs are located inside the nasal cavity and help animals to sense flavors and fragrances and also avoid harmful substances[38]. ORs undergo a conformational change on binding to odorants leading to an altered interaction of its intracellular loops with the α subunit of heterotrimeric G-Protein ($G_{\alpha,}$ $G_{ß}$ and G_{γ} subunits)[39]. An OR can distinguish odorants on the basis of molecular shape, size or functional group[40]. However, the perception of smell can be enhanced or decreased by presence of other odorants as well. The olfactory system often integrates responses from different olfactory cells for the purpose of olfaction[38]. Nearly 350 functional OR genes have been identified in humans which account for the whole range of our olfactory capabilities, often in a synergistic manner[39]. However, many of their odorants remain unknown and the ORs are termed as “orphan” ORs. Research on ORs and their applicability is hampered by the challenges in expressing them

heterologously. Inefficient folding in heterologous organisms lead to receptor sequestration, degradation and failure to translocate properly to its site of action, the cell membrane[36,38,41]. Various strategies, like construction of fusion proteins or chimeras[42], codon optimizing, using different G_α subunits[43,44], manipulating the temperature for expression[44] are employed in order to successfully express ORs heterologously. Functional expression of ORs also depends on successful linking of the expressed receptor to downstream signaling pathways[32-34,45]. Because of the challenges in expression and subsequent function of ORs in prokaryotes, simple eukaryotic whole cell biosensors are emerging as a desired platform for functionally expressing these receptors.

1.7 Eukaryotic Whole Cell Biosensors

The use of simple eukaryotic systems as whole cell biosensors is increasing. First, many eukaryotic genetic recognition elements like GPCRs cannot be functionally expressed in prokaryotic expression systems because of the lack of an appropriate downstream signaling pathway and their incapability to carry out post translational modifications[46]. Second, simple eukaryotes like *S. cerevisiae* share with bacteria a fast growth rate and ease of genomic manipulations[46]. Third, *S. cerevisiae* is particularly robust with a wide tolerance to pH and osmolarity which makes it a more durable biosensor[46].

1.8 Yeast as a Model System

S. cerevisiae has been long utilized in brewing and producing ethanol[47,48]. Recent scientific developments and the emergence of metabolic engineering and synthetic biology have enabled the use of yeast in production of chemicals other than ethanol[49-53]. Yeast is non-pathogenic and classified as a "generally regarded as safe" (GRAS)

organism[54]. Its genetic modification protocols are well established and its genome sequenced[55]. Unlike *E. coli*, yeast is highly robust and tolerant to a variety of industrial conditions[54,55]. It is used industrially for production of biofuels, pharmaceuticals and various chemicals including butanol, bisabolene, l-lactic acid, 1,2-propanediol, succinic acid, geraniol, farnesene, vanillin[56-61].

An important factor for the use of yeast in OR expression is its ability can carry out several post-translational modifications to the translated protein sequence which may be essential when trying to express GPCRs from mammals like humans[44]. The yeast system is also an attractive proposition because it provides a null background to study mammalian GPCRs[36,44]. This lack of competing endogenous GPCRs helps in proper ligand recognition and signal optimization of expressed heterologous GPCRs[44,62].

There are two native GPCR signaling pathways in *S. cerevisiae*: The glucose sensing pathway and the pheromone sensitive mating pathway[62]. The glucose sensing pathway mediates the activation of adenylate cyclase in response to glucose levels while the mating pathway responds to pheromones secreted by yeast cells and leads to mating. Heterologous GPCRs have been expressed in yeast using the yeast mating pathway[62].

1.9 Yeast Mating Pathway

Haploid yeast has two mating types, a and α (genotype *MATa* and *MATα*). These two cell types can mate to form a diploid yeast cell. To initiate mating, *MATα* type cells secrete a 13 residue peptide called α-Factor recognized by the Ste2 receptor in a *MATa* cell while a 12 residue peptide, the a-Factor is secreted by *MATa* type cells[62,63], which are recognized by Ste3 receptor on the *MAT α* cell. This receptor recognition leads to a

cascade of changes downstream leading to changes in expression of as much as 3% of the yeast genome[63].

Receptor stimulation first leads to the G_{α} subunit of the G protein exchanging GDP for GTP and freeing the $G_{\beta\gamma}$ heterodimer from the G protein complex. This heterodimer leads to a four level protein kinase cascade that leads to the activation of the transcription factors downstream and gene expression for mating. Two of the effectors of the $G_{\beta\gamma}$ heterodimer are Ste20 protein kinase and Ste5/Ste11 complex[63]. The $G_{\beta\gamma}$ heterodimer brings close together Ste11 and Ste20, leading to phosphorylation of Ste11 by Ste20. Ste5 acts a non-catalytic scaffold in which Ste11 and other downstream effectors Ste7 and Fus3, Kss1 are bound. Ste11 is the first member of the phosphorylation cascade (also called the mitogen-activated protein kinase kinase kinase or MAPKKK component). The phosphorylated Ste11 in turn phosphorylates Ste7 (the MAPKK component) which then phosphorylates the two MAPK components, Fus3 and Kss1[63]. These two downstream components of the phosphorylation cascade in turn activate the transcription factor Ste12 which is otherwise repressed by the Dig1/Dig2 complex. Phosphorylation of Dig1/Dig2 and Ste12 by Fus3 removes the repression and leads to activation of Ste12 transcription factor[63]. This transcription factor's binding domain has been found in nearly 100 promoters in the yeast genome most of which are genes involved in the mating pathway and cell fusion[63]. Amongst the genes upregulated are FUS1 and FIG1[63]. Another effector of the $G_{\beta\gamma}$ heterodimer is the Far1 which is involved in polarized growth of yeast cells towards the pheromone concentration gradient and leads to cell cycle arrest during the mating process. A schematic diagram of selected components of the yeast mating pathway is depicted (**Fig. 1.1**).

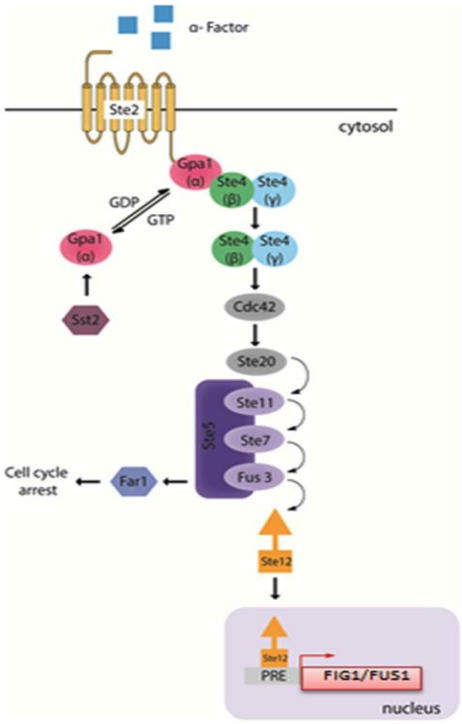


Figure 1.1. Schematic diagram of selected components of the yeast mating pathway

1.10 Expression of Heterologous GPCRs in Yeast

The first report on functional expression and signal transduction through the yeast mating pathway of a GPCR in yeast was that of a fusion protein of the β2-adrenergic receptor and the native Ste2 yeast receptor under an inducible P_{GAL1} promoter[64]. However similar results on constructing fusion GPCRs under the P_{GAL1} promoter were not observed in other cases[65]. The mammalian $G_{\alpha q}$ protein coupled muscarinic receptor was functionally expressed in yeast[66]. Deletion of the intracellular loops of the receptor increased its expression. While the shortened receptors could functionally interact with the endogenous yeast G_{α} subunit, a chimeric protein composed by replacing the last 5 amino acids in the C terminal tail of the yeast G_{α} by mammalian $G_{\alpha q}$ showed better ligand binding affinities[66]. Variations of pH and temperature have also been studied in some cases to express heterologous GPCRs in yeast. While the human β2- and α2C-adrenergic

receptors were expressed better by increasing the pH of the media from 5.5 to about 7, a heat shock at 42°C for an hour led to higher production of the mouse 5HT5A serotonin receptor[67]. Alternatively, lowering the temperature to 10-18°C led to better expression of heterologous GPCRs in yeast in some cases. This was hypothesized as a result of the induction of "cold-shock" proteins that can act as chaperones and help in proper folding of the GPCRs[68]. High sterol levels in the yeast cell membrane results from low temperature growth, which may also play a positive role in achieving higher GPCR activity[69].

The rat I7 OR and human OR 17-40 was expressed in yeast[44] by lowering the temperature to 15°C to optimize conditions. Different ligands for these receptors were also tested. The rat OR226 gene was expressed in yeast that showed sensitivity towards 2,4-dinitrotoluene (DNT)[70] by constructing a chimeric OR receptor and introducing the mammalian cAMP signaling cascade components in yeast. Protease deficient yeast cells were also utilized to prevent receptor degradation in some cases. However, an increase in the receptor levels did not correspond to an increase in receptor activity indicating not just the expression but the proper folding is critical to the function of heterologous GPCRs in yeast[65].

Considerable effort in GPCR signaling has also focused on the G_α subunit of the G protein heterotrimer. While some GPCRs can induce the signaling cascade through the endogenous Gpa1 yeast subunit[71,72], other strategies have included expressing GPCRs with a chimeric G protein with parts of both the yeast Gpa1 and mammalian G_α subunits. Though infinite number of such combinations is possible in theory, some successful ones

include replacing the last 5 C-terminal amino acids of the Gpa1 with the corresponding mammalian G_α subunit[66,73].

Recently, different chimeras of the mouse OR226 were constructed in yeast cells to test their ligand specificity to DNT using luciferase gene as the reporter[43]. It was found in this study that the mammalian G_{olf} was successful in transmitting the signal downstream in a Gpa1 null mutant. Also the human NTSR1 receptor that can bind neurotensin, an important neural modulator was recently functionally expressed in yeast using a chimeric G_α subunit[74]. Another approach is to construct fusion proteins of the GPCR and the G_α subunit. The fusion construct of a Ste2-Gpa1 chimera was able to function normally in a yeast cell[75]. However there is no one specific method to functionally express heterologous GPCRs in yeast and while some general strategies like the ones described do exist, each GPCR is unique and different and often, a combination of different strategies may be essential.

The reporter system to assess the ligand binding to GPCRs often includes a downstream promoter like P_{FUS1} or P_{FIG1} which is activated by the mating pathway transcription factor Ste12, connected to a gene like HIS3 that enables yeast growth and colony formation in a plate lacking histidine[71]. However, the slow response in the order of days has led to researchers using more sensitive and temporally efficient luciferase (Luc)[44] and green fluorescence protein (GFP).

Genetic modifications of the yeast cell are also necessary for it to act as a sensor expressing heterologous GPCRs. The FAR1 gene is always deleted in order to prevent cell cycle arrest on the pathway activation so that the cells can propagate[44,67]. Though

optional, SST2 gene deletion leads to an increased steady state signal, as the G_α subunit can remain in its GTP bound state for a longer time. The endogenous pheromone receptor gene STE2 has also been deleted in some cases[44,67].

1.11 The OR1G1 Receptor

The human olfactory receptor OR1G1 is normally expressed in the nasal epithelium of humans and is one of the many hundreds of receptors we use in order to have our sense of olfaction. This receptor was de-orphanised in 2004[76] by expressing in human embryonic kidney (HEK) cells and shown to bind to a variety of ligands which includes industrially desired chemicals and products like decanoic acid and nonanal. Medium chain fatty acids like decanoic acid can be precursors to fuels that replace gasoline[77] while nonanol is used in the manufacture of artificial lemon oil[78] . Theoretical studies predict non-polar hydrophobic interactions as the primary mode of ligand binding to the OR1G1 receptor[79].

The next chapter in this thesis describes the materials and methods section. Chapter 3 describes the results and discussions of this work. The human olfactory receptor, functionally expressed in yeast for the first time, is shown to respond to a range of ligands. We also show that a synthetic transcription factor can substitute for the native transcription factor in the mating pathway and demonstrate that one yeast cell can selectively respond to two activating ligands via two different receptors. Chapter 4 discusses the conclusions and future scope for this work.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and Chemicals

Octanoic acid (A11149), nonanoic acid (B21568), octanal (A10901) and decanal (A11656) were purchased from Alfa Aesar. Decanoic acid (D0017), dodecanoic acid (L0011), nonanal (N0296), 1-octanol (N0292), 1-nonanol (O0036) and 1-decanol (D0031) were obtained from TCI. Dodecyl aldehyde (AC36522) was purchased from Acros Organics. Dimethyl sulfoxide (DMSO) (99.9%) was obtained from J.T.Baker. α-Factor was purchased from Zymo Research. Deionised water from Millipore Water Systems was used in all cases.

Yeast extract, LB broth and LB agar were bought from EMD chemicals. Yeast nitrogen base without amino acids and agar were purchased from Becton, Dickinson and company. Peptone (bacteriological grade) was obtained from Amresco. All amino acids were bought from Sigma-Aldrich. D-Glucose (biotechnological grade) was purchased from Amresco. All restriction enzymes used in the study was bought from New England Biolabs. T4 DNA polymerase (5U/µL) and T4 DNA Ligase were purchased from Thermo Scientific and Promega respectively. High fidelity DNA polymerase was bought from BioRad.

2.2 Strains and Media

Recombinant DNA manipulations were carried out using DH10B *E. coli*. Growth media for *E. coli* transformants was LB media supplemented with 100 µg/mL ampicillin.

The *S. cerevisiae* W303 strain (*MAT*a, *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) and its derivatives were used in this study. Strain modifications were made

in the lab by the postdoctoral researcher Kuntal Mukherjee using the Delitto-Perfetto method[80]. All strains used are listed in the appendix (**Table A.2**).

2.3 Plasmid Constructions and Yeast Transformation

The plasmids used in this study are listed in Table A.1. The OR1G1 gene was codon optimized for expression in *S. cerevisiae* and commercially synthesized. $(His)_6$-OR1G1 was amplified from plasmid pCR2.1-OR1G1 using primer SB3/SB4N. The primer sequences are given in Table A.3. The insert was cloned under P_{TEF1} promoter to the plasmid pESC-His3-P_{TEF1}-P_{ADH1} (PPY111) at *BamHI*/*SacII*[81]. The sequence of OR1G1 is given in the appendix. Other plasmids used in the study were made by Kuntal Mukherjee.

The lithium acetate method was used to transform *S. cerevisiae* strains[82]. Transformants were selected on drop-out SD plates. All transformants were grown in SD media supplemented with the necessary amino acids and nucleotides that correspond to the selected markers at 30°C or 15°C for cell growth.

2.4 Flowcytometry Experiments

The desired strains were inoculated in 5mL SD His^-, Leu^- or SD Leu^- culture overnight as necessary at 30°C with shaking at 250 RPM. The next day 20 mL fresh SD dropout media was inoculated with cells to an OD_{600}= 0.06 in a 50 ml shake flasks and grown for 18 hrs with 150 RPM shaking. For experiments relating to the endogenous yeast receptor Ste2, the inoculation temperature for this step was maintained at 30°C. For the OR1G1 receptor, the inoculation temperature was kept at 15°C. If multiple flasks were incubated, all the cells were collected in one container at the end, centrifuged and resuspended in SD dropout media. These cells were then incubated at OD_{600} = 0.1 in 5mL

culture with different chemicals for 4hrs at 30°C with 150 RPM shaking. All chemicals were freshly dissolved in DMSO (1% v/v) while α-Factor was dissolved in water. Flowcytometry was done in BD LSRII flow cytometer. 10,000 cells were counted for each reading and GFP fluorescence was measured by exciting at 488 nm with 20 mW Coherent Sapphire argon laser and detection emission on the FITC channel using 515-545 nm filters. The voltage settings used were FSC: 178, SSC: 122, FITC: 600 for all experiments. The flowcytometry analysis was done using FlowJo software.

2.5 Data Analysis

Blank plasmid strains, constructed by transforming plasmids containing no reporter (GFP) gene into the desired yeast genetic background were used to calculate the corresponding strain's autofluorescence. The mean fluorescence calculated over the entire population (10,000 cells) was taken as our observed fluorescence value. Observed fluorescence values were normalized by subtracting the cell autofluorescence. The normalized non-induced fluorescence value was called the baseline. This baseline was subtracted from the normalized observed fluorescence and was termed output. The ratio of the normalized observed value to the baseline was termed fold increase in signal on stimulation.

2.6 Transfer Function Calculation

The Hill Equation was used to fit the Transfer functions to derive the biosensor performance features:

$$GFP = GFP_{max} * ([I]^n / ([I]^n + [K_m]^n))$$

where GFP_{max} is the maximum observed normalized GFP expression, [I] is the inducer concentration, K_m is the inducer concentration resulting in half-maximal induction and n

is the Hill coefficient describing biosensor sensitivity.

2.7 Toxicity Protocol

For each toxicity experiment, 800 µL of media (Leu^-, His^-) in a 24-well plate was inoculated with an overnight culture to an $OD_{600=}$ 0.1. The compound to be tested was then added after dissolving in DMSO, so that the DMSO concentration was 1% (v/v). The plate was shaken for 24 hours at 30°C using a Biotek Synergy2 microplate reader which calculated the OD_{600} at regular intervals.

CHAPTER 3

RESULTS AND DISCUSSIONS

In this work, first, we compared the characteristics of two different downstream promoters of the yeast mating pathway, P_{FUS1} and P_{FIG1}, with the endogenous Ste2 receptor of yeast and the α-Factor pheromone with the designed GFP reporter system. Next, we expressed the heterologous GPCR OR1G1 and tested its affinity towards different ligands. We also successfully substituted of the endogenous transcription factor Ste12 by an engineered eukaryotic transcription factor. Finally, we showed how both the Ste2 and OR1G1 receptors can function in the same yeast cell and respond independently to their respective ligands. Figure 3.1 gives an overview of the signaling systems that were utilized by appropriate strain modifications of yeast.

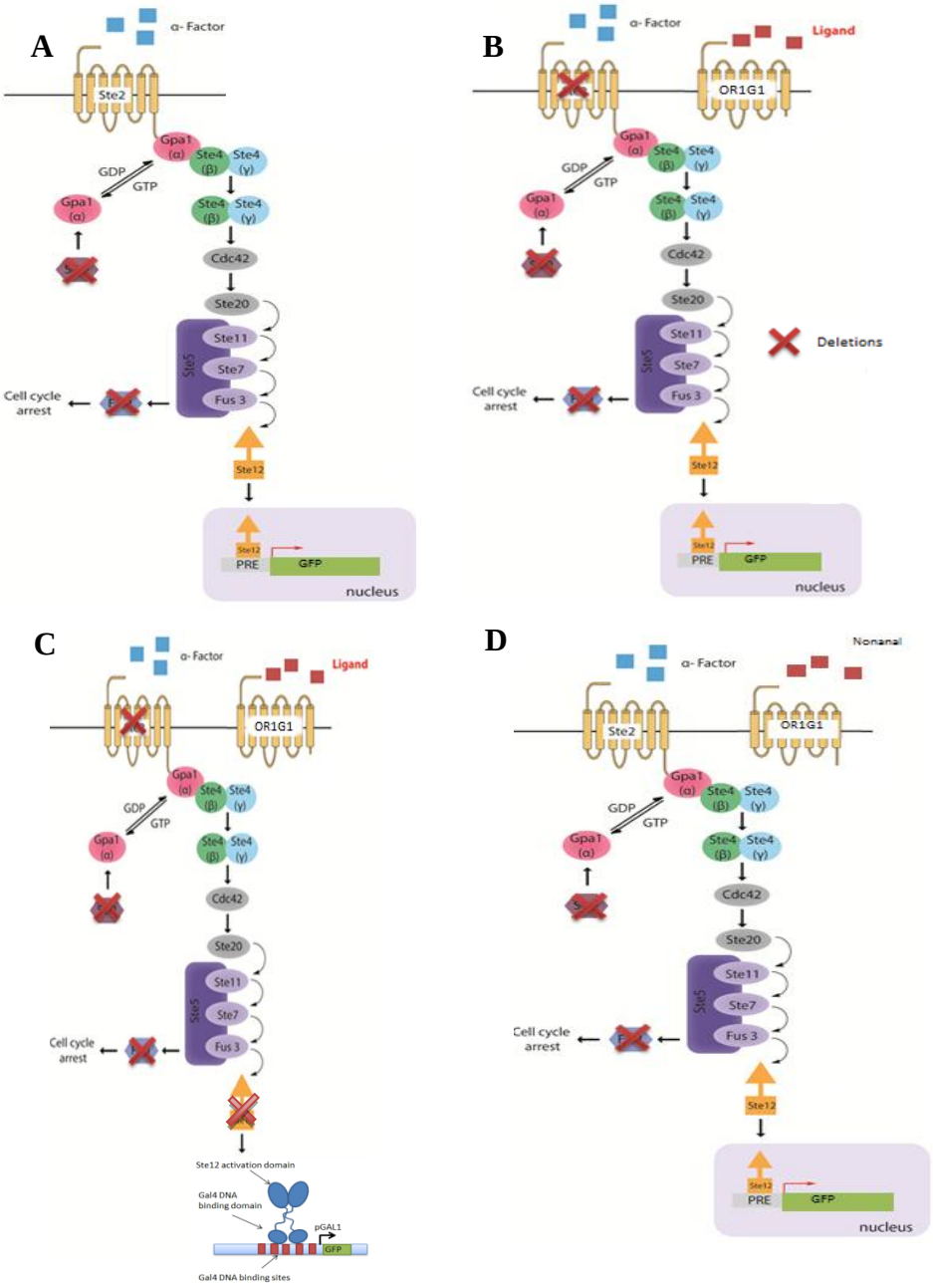


Figure 3.1. Overview of signaling systems used for A)Sensing α-Factor using strain PPY641 (W303 *far1Δ sst2Δ* strain carrying cen-P_{FIG1}-GFP) B) Sensing ligands for the OR1G1 receptor using strain PPY643 (W303 *far1Δ sst2Δ ste2Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP), C) Sensing ligands for the OR1G1 receptor with a synthetic transcription factor using strain PPY652 (W303 *far1Δ sst2Δ ste2Δ ste12Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1-P_{ADH1}-STF1 and pESC-Leu2-$P_{GAL4(5X)}$-GFP), D) Sensing α-Factor and nonanal for OR gate demonstration using strain PPY 642 (W303 *far1Δ sst2Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP)

3.1 Choice of Downstream Promoter

Most reports in literature have focused on the P_{FUS1} promoter as the downstream promoter of choice in order to express the mating pathway signal using reporters such as luciferase or green fluorescence protein[70,83]. However, there are some reports in literature that have used the P_{FIG1} promoter too for this purpose[84,85]. In 2012, the efficiencies of these two promoters were compared by expressing luciferase as a reporter of the yeast mating pathway on stimulating with α-Factor[42]. It was also concluded in the study that P_{FIG1} was the better promoter for biosensors giving higher fold increase in signal after ligand activation[42]. But in the absence of any other corroborating evidence, first a comparison of the two promoters P_{FUS1} and P_{FIG1} with the endogenous Ste2 receptor of yeast and the α-Factor pheromone was carried out in this work with the designed GFP reporter system.

Having the right promoter for the reporter system is very important. First, the promoter being used must be activated by the mating pathway transcription factor and act as a proper signal transducer so that the reporter can be transcribed. While having a strong promoter would seem to be the right way forward, by driving higher expression of the reporter, it can also have drawbacks. In sensor reporter systems, we are interested in a fold change in the expression levels after addition of the ligand. Ideally a strong promoter with very little expression of the reporter under unstimulated conditions would be ideal. Such a system would give no to very low signal when there is no ligand activating the signaling pathway.

Therefore, a comparison between two downstream promoters of the yeast mating pathway, P_{FUS1} and P_{FIG1}, was done using the endogenous Ste2 receptor and α-Factor

ligand and the results are shown in Figure 3.2. The highest levels of GFP fluorescence was observed in W303 *far1Δ sst2Δ* strain carrying pESC-P_{FUS1}-GFP (PPY638), with the fluorescence values reaching ~20000 AU. The second highest system was W303 *far1Δ sst2Δ* strain carrying pESC-P_{FIG1}-GFP (PPY639) which had fluorescence intensity values reaching ~9200 AU. The same trend of P_{FUS1} being the stronger promoter is carried over in case of expression utilizing centromeric plasmids (PPY640 and PPY641) in a W303 *far1Δ sst2Δ* strain.

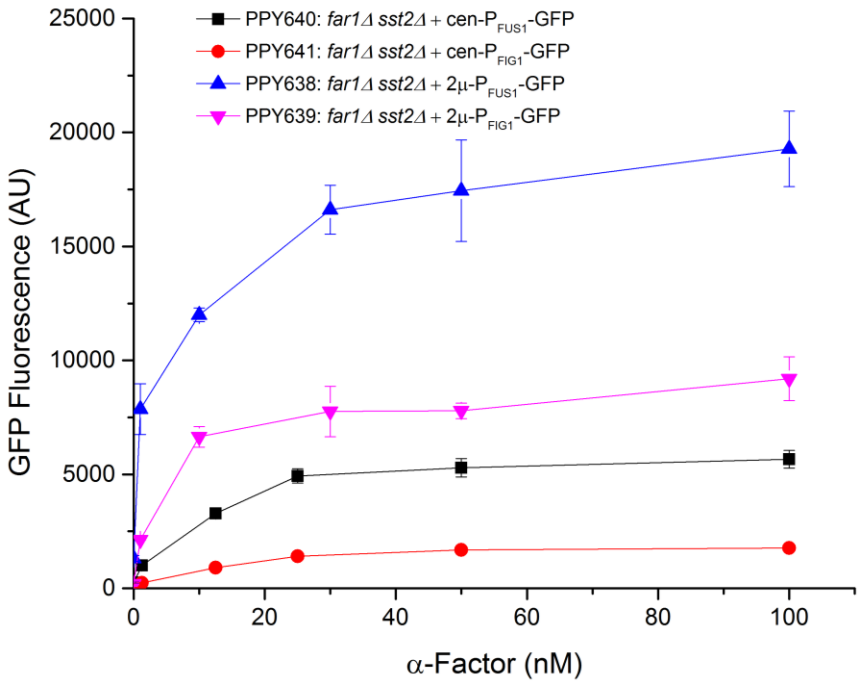


Figure 3.2. Normalized GFP Fluorescence on α-Factor Induction with P_{FUS1} and P_{FIG1} promoters of strains PPY638 (W303 *far1Δ sst2* strain carrying pESC-P_{FUS1}-GFP), PPY639 (W303 *far1Δ sst2Δ* strain carrying pESC-P_{FIG1}-GFP), PPY640 (W303 *far1Δ sst2* strain carrying cen-P_{FUS1}-GFP) and PPY641 (W303 *far1Δ sst2Δ* strain carrying cen-P_{FIG1}-GFP). All experiments were done in triplicate. Shown in the figure are the means with the standard deviations.

The maximum fold increase in signal after activation is an important characteristic of a biosensor and is calculated by dividing the maximum fluorescence on ligand induction by the fluorescence levels on no induction. Figure 3.3 shows that the maximum fold increase on α-Factor stimulation observed for the various strains tested. PPY639 and PPY641 are the strains with the highest fold increases in signal after activation and both have the P_{FIG1} promoter. This is because P_{FUS1} is a stronger promoter than P_{FIG1} but has high rates of transcription even when no inducer is present, giving overall lower fold increase in signal after activation. Therefore, we chose P_{FIG1} as the desired promoter for our sensor system in all subsequent cases.

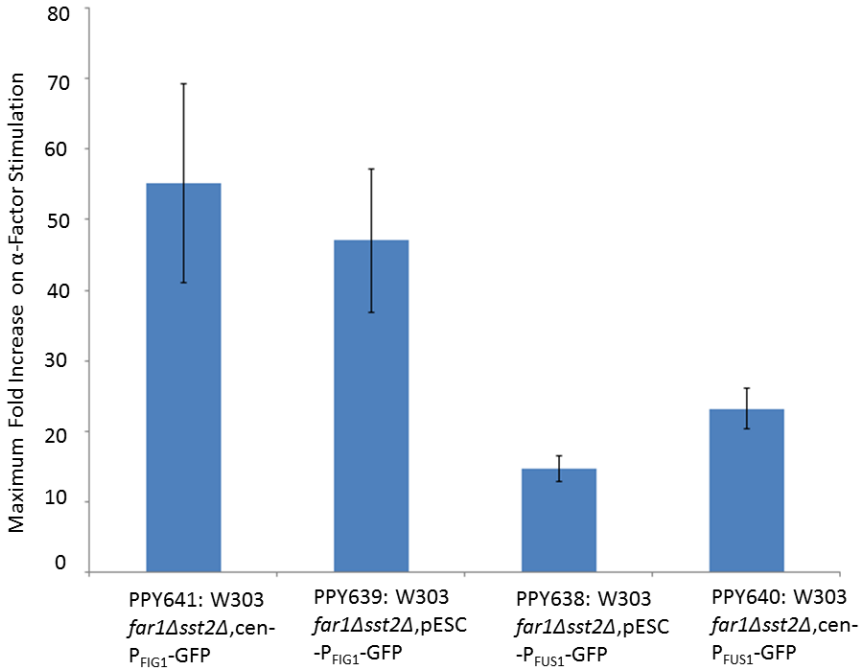


Fig 3.3. Calculated maximum fold increase of GFP fluorescence on α-Factor stimulation with different strains. Strains PPY639 (W303 *far1Δ sst2Δ* strain carrying pESC-P_{FIG1}-GFP*)* and PPY641 (W303 *far1Δ sst2Δ* strain carrying cen-P_{FIG1}-GFP) show higher fold increase in signal on activation than strains PPY640 (W303 *far1Δ sst2Δ* strain carrying cen-P_{FUS1}-GFP*)* and PPY638 (W303 *far1Δ sst2Δ* strain carrying pESC-P_{FUS1}-GFP*)*

Plasmids systems used in yeast for gene expression are mainly of two types: centromeric and 2µ. Centromeric plasmids are more stable but their copy number is low [86] while the 2µ plasmid system on the other hand has a higher copy number (30-40) but is less stable[87]. Figure 3.4 compares the efficiency of the centromeric and 2µ plasmid based reporter systems.

The W303 *far1Δ sst2Δ* strain carrying pESC-P_{FIG1} (PPY653) or cen-P_{FIG1} (PPY654) account for the cellular autofluorescence (**Fig. 3.4**). When W303 *far1Δ sst2Δ* is transformed with either pESC-P_{FIG1}-GFP (PPY639) or cen-P_{FIG1}-GFP (PPY641) in the absence of the inducer, the increase in the mean GFP fluorescence is minimal. When W303 *far1Δ sst2Δ* strain carrying pESC-P_{FIG1}-GFP (PPY639) or cen-P_{FIG1}-GFP (PPY641) is induced at saturating α-Factor concentrations, the curves show increased fluorescence.

Having a centromeric plasmid (**Fig. 3.4A**) has a more compact distribution of fluorescence with a larger percentage of cells stimulated (89.7%). A 2µ plasmid system (**Fig. 3.4B**) has a bimodal distribution with a smaller percentage of cells stimulated (61%). Based on these results, a centromeric P_{FIG1}-GFP reporter plasmid system was used in subsequent experiments. Literature reports suggest that a high copy number plasmid system of expression is best for heterologous GPCRs in yeast[44,67]. Therefore the 2µ plasmid system for heterologous GPCR expression was used for all experiments.

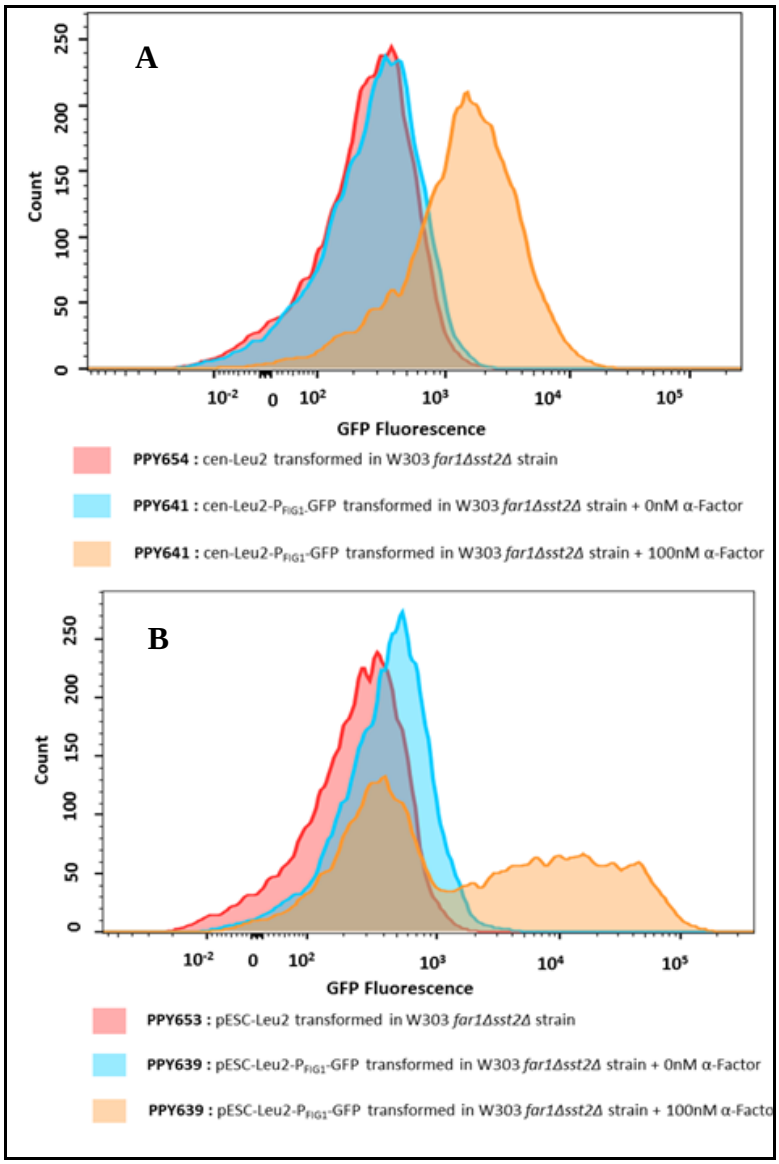


Figure 3.4. Flow cytometry histogram on α-Factor induction of A) PPY654 (W303 *far1Δ sst2Δ* strain carrying cen-Leu2), PPY641 (W303 *far1Δ sst2Δ* strain carrying cen-Leu2-P_{FIG1}-GFP) with 0nM α-Factor, PPY641 (W303 *far1Δ sst2Δ* strain carrying cen-Leu2-P_{FIG1}-GFP) with 100nM α-Factor and B) PPY653 (W303 *far1Δ sst2Δ* strain carrying pESC-Leu2), PPY639 (W303 *far1Δ sst2Δ* strain carrying pESC-Leu2-P_{FIG1}-GFP) with 0nM α-Factor, PPY639 (W303 *far1Δ sst2Δ* strain carrying pESC-Leu2-P_{FIG1}-GFP) with 100nM α-Factor. PPY654 and PPY653 account for cellular autofluorescence.

3.2 Signal Optimization using the OR1G1 Receptor

The designed GPCR expression and GFP reporter system was tested using the heterologous codon optimized human GPCR OR1G1 in yeast. The optimization of signaling using the yeast mating pathway was done using nonanal as the ligand since it has been shown to be a good ligand. The GFP fluorescence distribution on addition of nonanal to a W303 *far1Δ sst2Δ ste2Δ* strain transformed with pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP (PPY643) is shown in Figure 3.5. The protocol for GPCR expression was broken into two parts: a GPCR expression part and a ligand induction part.

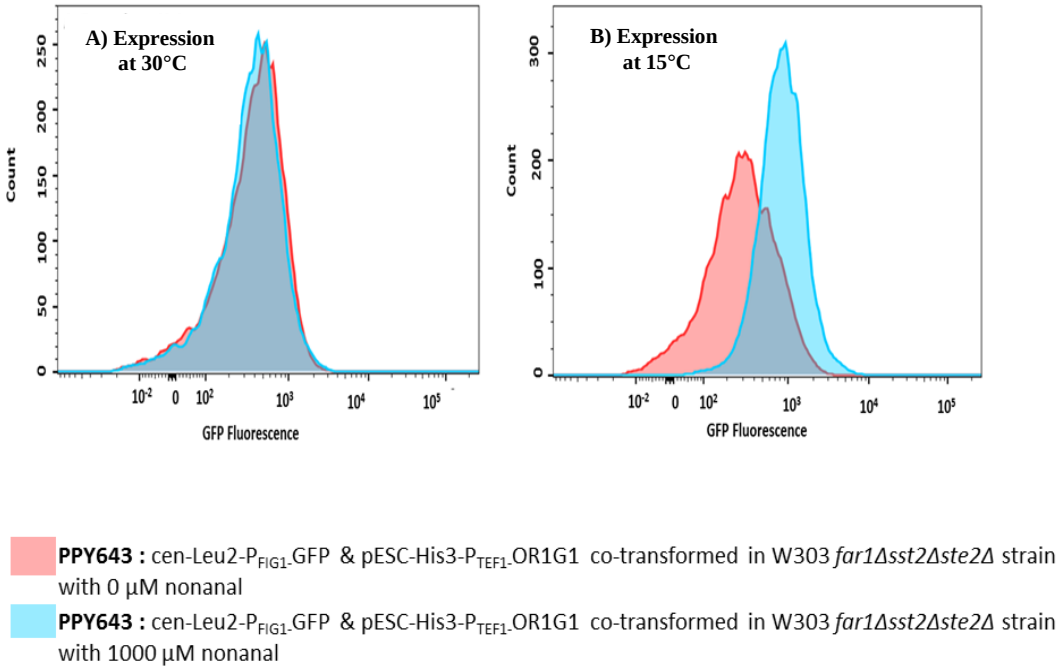


Figure 3.5. Flow cytometry histogram on nonanal induction of PPY643 (W303 *far1Δ sst2Δ ste2Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP). A) The GPCR expression temperature and the ligand induction temperature were both maintained at 30°C B) The GPCR expression temperature was lowered to 15°C while the ligand induction temperature was kept at 30°C.

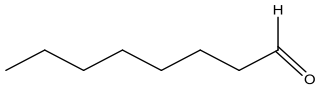
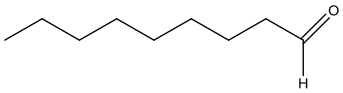
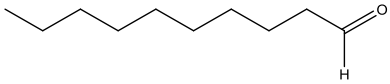
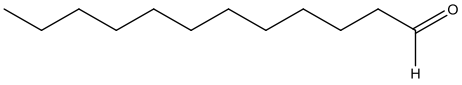
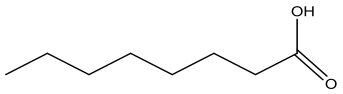
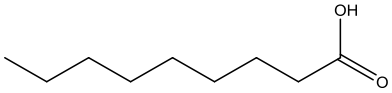
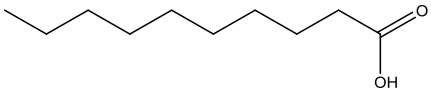
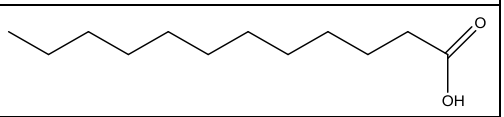
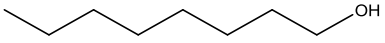
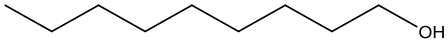
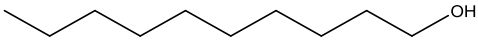
Keeping both the expression and induction temperatures at 30°C led to no change in GFP expression from the levels when no inducer was present (**Fig. 3.5A**). On lowering the temperature for GPCR expression to 15°C keeping the ligand induction temperature at 30°C, the fluorescence distribution profiles showed a significant rightward shift which meant that the ligand was successful in signaling through the mating pathway leading to induction of the GFP reporter system (**Fig. 3.5B**). It is interesting to note that, at the low induction temperature of 15°C, the GFP fluorescence in the absence of inducer seems to decrease from the corresponding levels at 30°C. However, the GFP fluorescence intensities for the same strain at two different temperatures can be different and we are interested in the relative changes in fluorescence intensities between the non-inducing and inducing states for the particular strain under the same conditions. Thus, low temperature cell growth was needed to successfully signal using the heterologous OR1G1 gene in yeast. This could be a result of cold-shock proteins that are expressed in yeast as a result of low temperatures. According to literature reports, these proteins can act as chaperones that can lead to better folding of heterologous genes and their proper expression[68,69]. Thus, for all subsequent sensing experiments done with OR1G1, the expression temperature was kept at 15°C, while the ligand induction temperature remained at 30°C.

3.3 Chemical Profiling of the OR1G1 Receptor

In order to profile the binding affinity of the OR1G1 receptor in yeast, different ligands across n-aldehydes, n-alcohols and carboxylic acids were tested. Since OR1G1 was known to bind to a few C9 and C10 organic compounds with high affinity[76] in human embryonic kidney cells, carbon chain lengths of 8-12 were tested with different

functional groups. Table 3.1 shows a list of the different ligands tested for induction with the OR1G1 receptor along with their chemical structures using a W303 *far1Δ sst2Δ ste2Δ* strain transformed with pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP (PPY643).

Table 3.1. Ligands tested for stimulation by the OR1G1 receptor in yeast

Ligand	**Structure**
Octanal	
Nonanal	
Decanal	
Dodecanal	
Octanoic Acid	
Nonanoic Acid	
Decanoic Acid	
Dodecanoic Acid	
Octanol	
Nonanol	
Decanol	

The 11 ligands shown in the Table 3.1 were tested with the OR1G1 receptor. The dose response curves of these ligands are shown in Figure 3.6. Amongst the four aldehydes (**Fig. 3.6 A**), the OR1G1 receptor is able to sense the ligands nonanal, decanal and dodecanal in a dose dependent manner, with nonanal appearing to be the best ligand. Octanal was not sensed, exhibiting no increase in GFP fluorescence across the ligand concentration range (0-1000µM). All three alcohols tested, octanol, nonanol and decanol were sensed by the receptor OR1G1 in a dose dependent manner (**Fig. 3.6 B**).

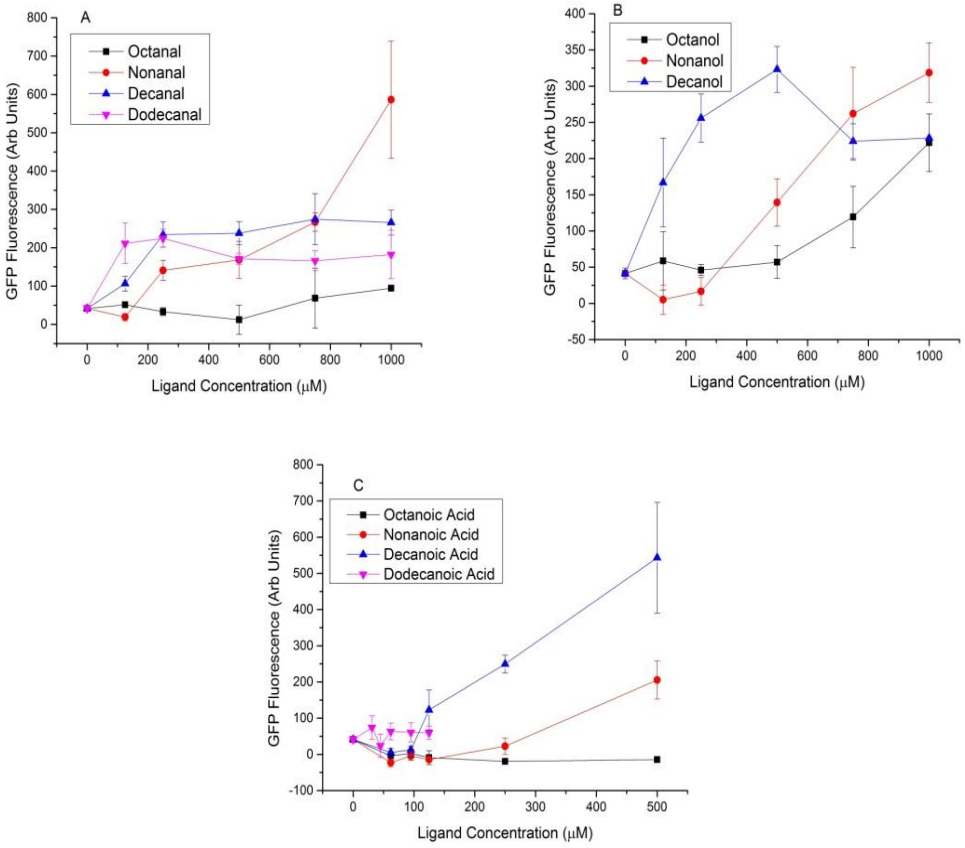


Figure 3.6. Dose dependent response curves of PPY643 (W303 *far1Δ sst2Δ ste2Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP) on induction with different A) aldehydes B) alcohol and C) acids. All experiments were done in triplicate. Shown in the figure are the means with the standard deviations.

Amongst the four acids, octanoic acid and dodecanoic acid could not stimulate the OR1G1 receptor. Decanoic acid was the best ligand among the acids while nonanoic acid was also stimulated at higher concentrations (**Fig. 3.6 C**). Since dodecanoic acid precipitates when added at a concentration above 125 µM, it was not tested above that limit. An important property of a sensor is its maximum fold increase in signal after activation. It is calculated by dividing the maximum fluorescence intensity observed on induction by the fluorescence levels on no induction. The maximum fold increase in signal after activation for all the tested ligands that gave stimulation were tabulated and are shown in Figure 3.7.

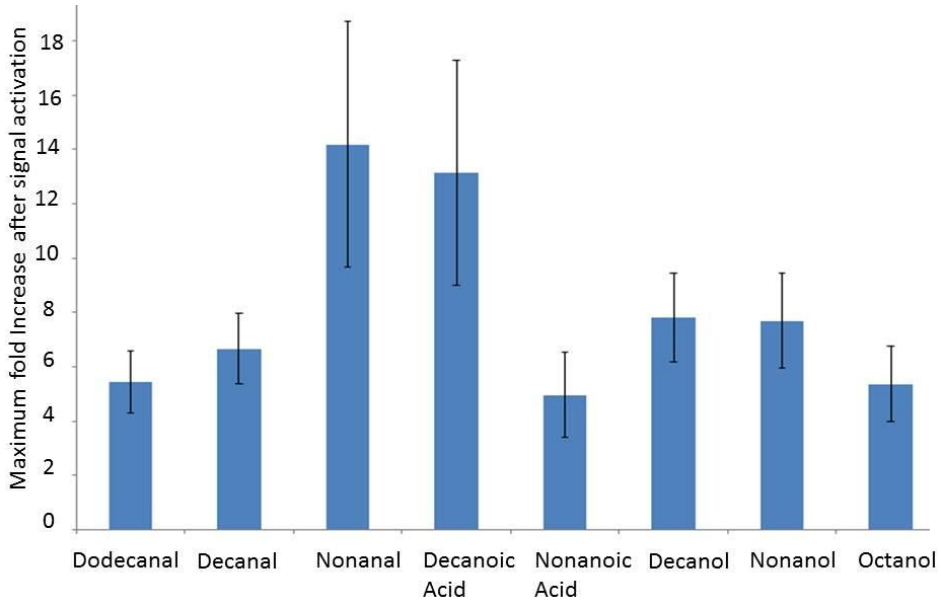


Figure 3.7. Calculated maximum fold increase of the strain PPY643 (W303 *far1Δ sst2Δ ste2Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP) on stimulation with the different ligands

The two best ligands for the OR1G1 receptor are nonanal and decanoic acid (**Fig. 3.7**). The observation in literature that OR1G1 works best with organic compounds of straight chain lengths with 9-10 carbons is upheld in this work[76]. OR1G1 was unable to sense dodecanal in human embryonic kidney cells but showed affinity towards it in this

work in yeast[76]. This may be due to the effect of expressing the receptor in a heterologous system with different pathway architecture. Notably, the expressed OR1G1 worked well with the endogenous yeast G-protein.

The dose dependent response curves obtained were fitted to a Hill equation to derive various features of the biosensor. Table 3.2 tabulates these characteristics. The dynamic range is defined as the maximum GFP fluorescence intensity observed for that ligand within the range tested. K_m is defined as the ligand concentration at half-maximal fluorescence. The sensitivity is the computed Hill coefficient which is a measure of how sensitive the sensor is towards the ligand. The OR1G1 receptor is observed to have different affinities towards the different sensed ligands, which is portrayed by the variations in the values of K_m and sensitivity.

Table 3.2 Biosensor features of the strain PPY643 (W303 *far1Δ sst2Δ ste2Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP) for different ligands

Strain Description	**Dynamic Range (GFP $_{Max}$) (AU)**	**K_m (µM)**	**Tested Range (µM)**	**Hill Coefficient**
Octanol	626	715	0-1000	5.2
Nonanol	723	548	0-1000	4.85
Decanol	727.667	120	0-1000	0.89
Nonanoic Acid	779	505	0-500	5.3
Decanoic Acid	947.667	266	0-500	3.17
Nonanal	990	764	0-1000	5.746
Decanal	680	158	0-1000	3.085
Dodecanal	629	54	0-1000	1.166

3.4 Introducing a Synthetic Transcription Factor

The transcription factor that is activated upon on stimulation of the yeast mating pathway is Ste12, which goes on to activate ~100 downstream genes that lead to cell mating and shmoo formation[63]. Replacing Ste12 by a synthetic transcription factor could lead to targeted binding of the new transcription factor around the desired reporter gene(s) and increase the observed stimulation. With this idea in mind, a synthetic transcription factor STF1 was designed as per literature reports[88]. Eukaryotic transcription factors have an activation domain and a DNA binding domain. While the STF1 retained the activation domain of the Ste12 protein, it was engineered to include the GAL4 DNA binding domain[88]. Thus, the STF1 could be stimulated by the mating pathway MAP Kinase cascade but not bind to the many different genes. It would only bind to those which had the GAL4 DNA binding sites. A synthetic promoter was engineered with five GAL4 DNA binding sites upstream of the TATA box to drive expression of the GFP and was called the $P_{GAL4(5X)}$ promoter.

The two best ligands, decanoic acid and nonanal were used to test the synthetic transcription factor based signaling system. The dose dependent response curves on stimulation of W303 *far1Δ sst2Δ ste2Δ ste12Δ* strain transformed with pESC-His3-P_{TEF1}-OR1G1-P_{ADH1}-STF1 and pESC-Leu2-$P_{GAL4(5X)}$-GFP (PPY652) are shown in Figure 3.8. Both the ligands were able to stimulate the STF1 transcription factor and lead to GFP expression from the $P_{GAL4(5X)}$ promoter (**Fig. 3.8**).

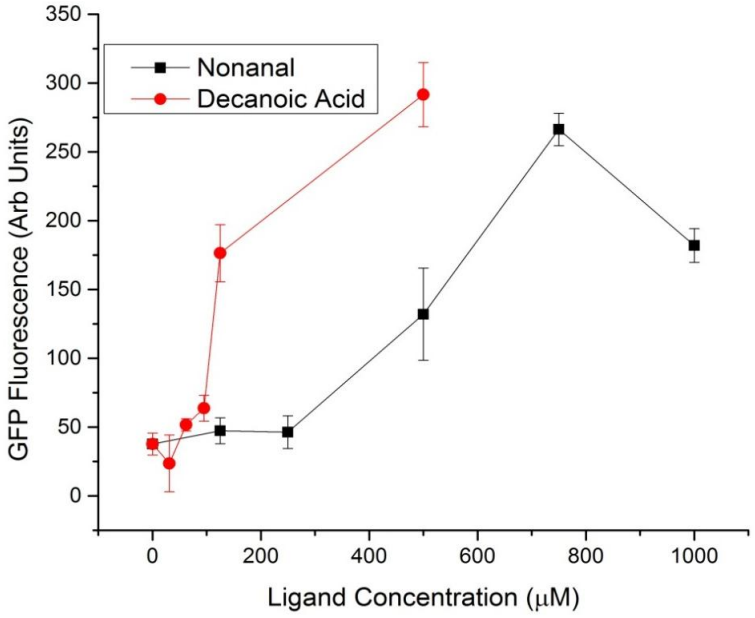


Figure 3.8. Dose dependent response of PPY652 (W303 *far1Δ sst2Δ ste2Δ ste12Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1-P_{ADH1}-STF1 and pESC-Leu2-$P_{GAL4(5X)}$-GFP) on induction with nonanal and decanoic acid. All experiments were done in triplicate. Shown in the figure are the means with the standard deviations.

3.5 Development of a GPCR-based OR Gate

A sensor system carrying both Ste2 and OR1G1 was then characterized. Such a system could sense varying ligands with its different receptors. By using an expression temperature of 15°C, both Ste2 and OR1G1 were functionally expressed in the same cell and were able to sense their respective ligands, α-Factor and nonanal (**Fig.3.9**).

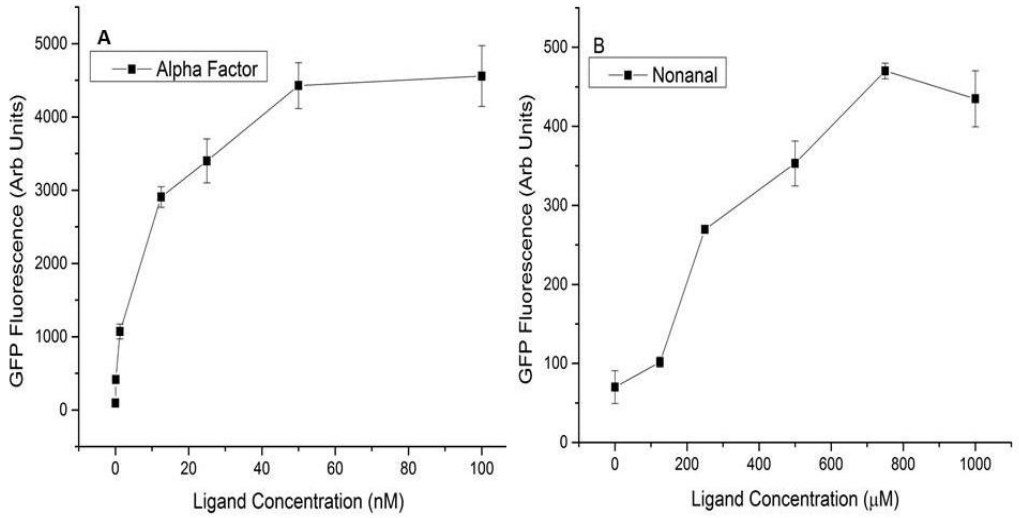


Figure 3.9 Dose dependent response of PPY642 (W303 *far1Δ sst2Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP) on induction with A) α-Factor and B) nonanal. All experiments were done in triplicate. Shown in the figure are the means with the standard deviations.

Having established that the receptors could individually sense their ligands, a question was whether the ligands could be sensed if both were present together i.e., from the viewpoint of digital electronics, whether an OR gate was functionally possible in the yeast cell. Usually in literature, the endogenous Ste2 receptor is deleted so that there is no spatial competition for binding sites between different receptors[66]. To test the dose responses of both ligands together, ten different combinations of α-Factor and nonanal were tested in a W303 *far1Δ sst2Δ* strain transformed with pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP (PPY642). The GFP fluorescence intensities observed on testing with these different combinations are plotted as a surface plot (**Fig. 3.10**).

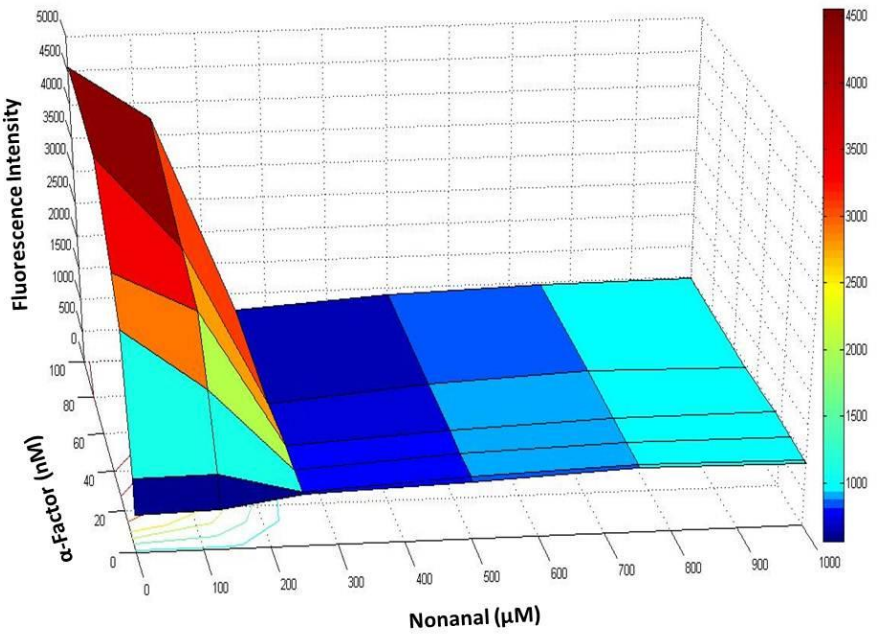


Figure 3.10. Surface plot showing fluorescence intensity on nonanal stimulation of PPY642 (W303 *far1Δ sst2Δ* strain carrying pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP)

The fluorescence intensity in the absence of either nonanal or α-Factor was taken as the baseline. Addition of either nonanal or α-Factor or their combinations is seen to lead to an increase in the fluorescence intensity from the baseline level signifying the successful function of an OR gate. However, the variation of fluorescence intensity across different combinations of nonanal and α-Factor in the W303 *far1Δ sst2Δ* strain transformed with pESC-His3-P_{TEF1}-OR1G1 and cen-Leu2-P_{FIG1}-GFP (PPY642) is intriguing. The Figure 3.10 clearly shows this non-linear dynamics and is different from what would be expected from a traditional additive OR gate where we could expect the maximum fluorescence intensities when both the ligands are saturating. Sources in literature reports cite how OR gates can be additive when both sources of stimulation are present using dual intracellular transcription factor-promoter sensing systems[89]. However

there are no literature reports on OR gates using olfactory receptors. Therefore this work shows the complex interaction between the two olfactory receptors in the same yeast cell for the first time. In this case, the GFP is expressed from the same promoter P_{FIG1} which is upregulated by the same transcription factor Ste12. As only a new receptor is introduced which signals via the same mating pathway in the yeast cell and competes for the G-Protein, the argument that the signal would not be amplified or additive can be made. Assuming that around 50% of the receptors on the surface would be OR1G1 and the rest would be Ste2, it could also be assumed that the fluorescence signal intensity should lie in between that of the two ligands when they are present alone. However, it is observed that, while the GFP fluorescence from α-Factor stimulation is much more than nonanal when the ligands are individually present, the presence of nonanal has a negative effect on the GFP stimulation when combinations of nonanal and saturating α-Factor concentrations are present. Even at 125µM nonanal concentration, the GFP fluorescence decreases significantly. At higher concentrations of nonanal starting from 250µM, the GFP fluorescence reaches values comparable to just the presence of nonanal irrespective of the α-Factor concentration in all tested combinations. It is also interesting to note while α-Factor is sensed at nanomolar concentrations, nonanal is sensed at micromolar concentrations. This difference can be due to altered receptor-ligand interactions and/or the receptor-G-protein coupling.

In order to see if the decreasing fluorescence intensities observed on nonanal addition in presence of α-Factor for the OR gate depended on the presence of the OR1G1 receptor, the strain *far1Δ sst2Δ* cen-pFIG1-GFP (PPY641) which only had the Ste2 receptor, was stimulated with saturating α-Factor and increasing concentrations of

nonanal. Figure 3.11 shows the changes in the GFP fluorescence levels of this strain for different ligand combinations.

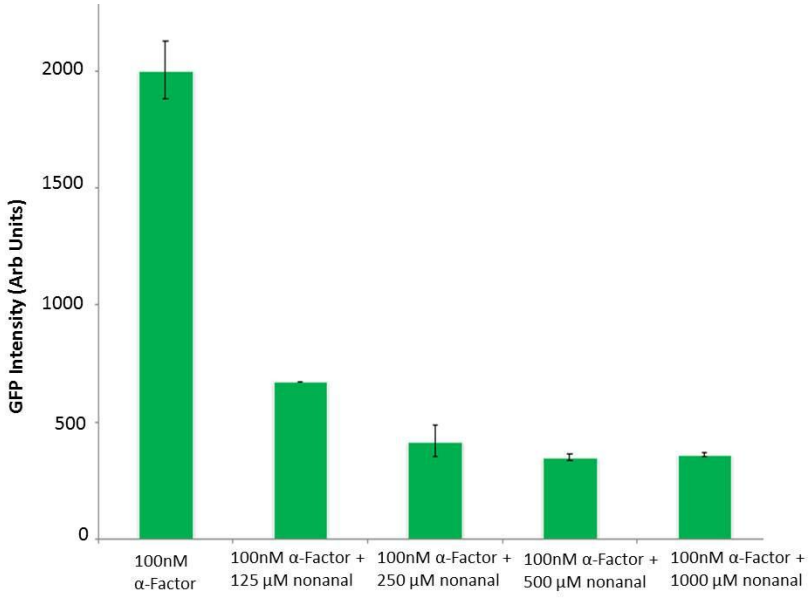


Figure 3.11. Changes in fluorescence Intensity of PPY641 (*far1Δ sst2Δ* strain carrying cen-pFIG1-GFP) on adding different nonanal concentrations with 100nM α-Factor. All experiments were done in triplicate. Shown in the figure are the means with the standard deviations.

Adding increasing amounts of nonanal in presence of saturating α-Factor in the W303 *far1Δ sst2Δ* strain transformed with cen-P_{FIG1}-GFP (PPY641) was seen to also lead to a substantial decrease in GFP fluorescence. This showed that there was interaction of nonanal with the α-Factor or the Ste2 receptor that resulted in decrease of the fluorescence intensity levels.

3.6 Chemical Toxicity

The toxicity of the ligand may also play a role towards this decrease of GFP stimulation in Figure 3.10. With this in mind, the toxicity of nonanal was tested at two different concentrations, 250µM and 1000µM (**Fig. 3.12**).

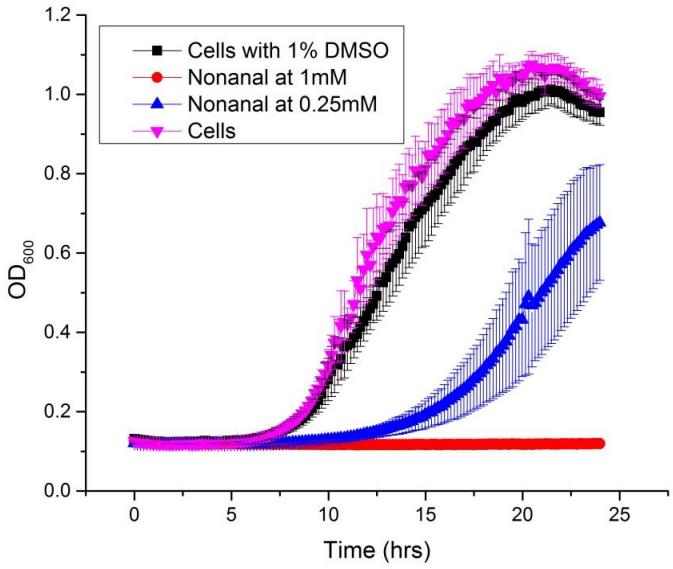


Figure 3.12. Toxicity Assay of PPY140 (W303 *far1Δ sst2Δ ste2Δ)* strain. Growth curves of PPY140 treated with nonanal at 0.25mM and 1mM are shown. All experiments were done in triplicate. Shown in the figure are the means with the standard deviations.

The control in the toxicity experiment was cells with 1% DMSO, as DMSO was used to dissolve nonanal in the experiments. The growth rates of the cells and cells with DMSO added at 1% (v/v) are not statistically significant. Also, it is evident that nonanal is highly toxic at 1mM with almost no cell growth over a 24 hour period. Even at 250µM concentration, nonanal is able to reduce the cell growth with a much longer lag phase. However, it is important to note that the effects of toxicity are seen after at least 6 hours (**Fig. 3.12**) whereas our measurements after ligand induction are completed in 4 hours while the cells are still in the lag phase of growth.

CHAPTER 4

CONCLUSIONS AND FUTURE SCOPE

In this work, the heterologous human OR1G1 receptor was expressed in the yeast *S. cerevisiae*. The reporter system for the sensor was first optimized by a comparative study between the P_{FUS1} and P_{FIG1} promoters using GFP as the reporter. It was concluded that while P_{FUS1} is stronger, its high activity even under the absence of any inducer makes it unsuitable as the promoter of choice in sensing systems. The expression conditions for the OR1G1 receptor were optimized in yeast. It was seen that decreasing the temperature down to 15°C leads to the receptor's functional expression, evident from the increased fluorescence from the downstream GFP reporter gene. Dose dependent ligand stimulation experiments were carried out in yeast strains expressing the OR1G1 receptor. It was observed that amongst the tested ligands, octanol, nonanol, decanol, nonanal, decanal, dodecanal, nonanoic acid and decanoic acid were sensed by the OR1G1 receptor. Various characteristics of the sensor towards these ligands such as the sensitivity, K_m, and dynamic range were evaluated by individually fitting the experimental data to the Hill Equation. The fold increase in signal after activation for these ligands were evaluated leading to the conclusion that decanoic acid and nonanal were the two best stimulants for this receptor in yeast. Next, a successful substitution of the endogenous transcription factor Ste12 was demonstrated by an engineered eukaryotic transcription factor STF1. It was shown that STF1 could transmit the signal through the mating pathway, though with lesser efficiency than Ste12. Finally, it was shown that both the Ste2 and OR1G1

receptors can function in the same yeast cell and respond to their respective ligands. Such a cell was able to respond to the ligands and also sensed various combinations of them, thus being the first demonstration of an OR gate in yeast using olfactory receptors. A non-additive, non-linear dynamics was observed for the OR gate with addition of nonanal having a detrimental effect on the GFP fluorescence intensity in presence of saturating α-Factor. This results from the complex interactions between the two receptors, their ligands and their common signaling pathway. Even in a yeast strain with no OR1G1 receptor, adding increasing amounts of nonanal led to a decrease in the GFP fluorescence in presence of saturating α-Factor concentration. This implied nonanal had some interaction with the α-Factor ligand or the Ste2 receptor that contributed to this decrease.

In conclusion, developing new biosensors for chemicals is an important area of research in synthetic biology. Sensors that can respond to changing levels of heterologous metabolites can not only give an analytical readout of the amount of ligand present but can be utilized to modulate cellular gene expression levels. Functionally expressing a receptor like OR1G1, which can sense a variety of industrially useful chemicals like decanoic acid and nonanal, is an important step in that direction. Yeast is now being used to produce a number of these useful chemicals *in vivo*. One can envision a positive feedback loop in yeast using the OR1G1 receptor. If yeast cells with the OR1G1 receptor are metabolically engineered to produce one of the ligands it can now sense, higher production may be feasible if the downstream reporter gene GFP is replaced by an important production gene in the pathway Other types of logic circuits like NOR, NAND and AND gates along with their various combinations have the potential to construct diverse regulatory circuits in yeast that can be used for a variety of purposes.

APPENDIX

OR1G1 sequence

MHHHHHHEGKNLTSISECFLLGFSEQLEEQKPLFGSFLFMYLVTVAGNLLIILVIIT
DTQLHTPMYFFLANLSLADACFVSTTVPKMLANIQIQSQAISYSGCLLQLYFFML
FVMLEAFLLAVMAYDCYVAICHPLHYILIMSPGLCIFLVSASWIMNALHSLLHTL
LMNSLSFCANHEIPHFFCDINPLLSLSCTDPFTNELVIFITGGLTGLICVLCLIISYTN
VFSTILKIPSAQGKRKAFSTCSSHLSVVSLFFGTSFCVDFSSPSTHSAQKDTVASV
MYTVVTPMLNPFIYSLRNQEIKSSLRKLIWVRKIHSP.

Table A.1 Table of Plasmids

	Name	Description	Reference
Plasmids			
pESC-Leu2	PPY39	Yeast shuttle vector with Leu2 marker and $P_{GAL1/GAL10}$ divergent promoter	Agilent
cen-Leu2	PPY15	Yeast centromeric shuttle vector with Leu2 marker	ATCC #
pESC-Leu2-GFP	PPY43	GFP was cloned in *BamH*I and *Hind*III site	Kuntal Mukherjee
pESC-Leu2-P_{FUS1}-GFP	PPY96	GFP was cloned under P_{FUS1} promoter	Kuntal Mukherjee
pESC-Leu2-P_{FIG1}-GFP	PPY97	GFP was cloned under P_{FIG1} promoter	Kuntal Mukherjee

pESC-His3	PPY34	Yeast shuttle vector with His3 marker and $P_{GAL1/GAL10}$ divergent promoter	Agilent
pESC-His3-P_{TEF1}-P_{ADH1} divergent	PPY111	P_{TEF1} and P_{ADH1} were cloned in opposite direction	Kuntal Mukherjee
pESC-His3-P_{TEF1}-OR1G1	PPY269	OR1G1 was cloned under P_{TEF1}	Souryadeep Bhattacharyya
cen-Leu2-P_{FUS1}-GFP	PPY389	GFP was cloned under P_{FUS1} promoter in centromeric plasmid	Kuntal Mukherjee
cen-Leu2-P_{FIG1}-GFP	PPY586	GFP was cloned under P_{FIG1} promoter in centromeric plasmid	Kuntal Mukherjee
pESC-His3-P_{TEF1}-OR1G1-P_{ADH1}-STF1	PPY595	STF1 was cloned under P_{ADH1} promoter in PPY269	Kuntal Mukherjee
pESC-Leu2-$P_{Gal4(5X)}$-GFP	PPY528	GFP was cloned under an engineered promoter containing 5 Gal4 DNA binding domains in PPY39	Kuntal Mukherjee

Table A.2 Table of Strains

Strains	**Name**	**Description**	**Reference**
W303	PPY11	MATa,leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15	ATCC® 20835
far1Δ	PPY62	W303, *far1Δ*	Kuntal Mukherjee
far1Δ, sst2Δ	PPY58	W303, *far1Δ sst2Δ*	Kuntal Mukherjee

far1Δ, sst2Δ, ste2Δ	PPY140	W303, *far1Δ sst2Δ ste2Δ*	Kuntal Mukherjee
far1Δ, sst2Δ, ste2Δ, ste12Δ	PPY161	W303, *far1Δ sst2Δ ste2Δ ste12Δ*	Kuntal Mukherjee
far1Δ, sst2Δ + PPY96	PPY638	pESC-Leu2- P_{FUS1}-GFP transformed in PPY58	Souryadeep Bhattacharyya
far1Δ, sst2Δ + PPY97	PPY639	pESC-Leu2- P_{FIG1}-GFP transformed in PPY58	Souryadeep Bhattacharyya
far1Δ, sst2Δ + PPY389	PPY640	cen-Leu2-P_{FUS1}-GFP transformed in PPY58	Souryadeep Bhattacharyya
far1Δ, sst2Δ + PPY586	PPY641	cen-Leu2-P_{FIG1}-GFP transformed in PPY58	Souryadeep Bhattacharyya
far1Δ, sst2Δ + PPY269+ PPY586	PPY642	cen-Leu2-P_{FIG1}-GFP and pESC-His3-P_{TEF1}-OR1G1 co-transformed in PPY58	Souryadeep Bhattacharyya
far1Δ, sst2Δ, ste2Δ +PPY269+PPY586	PPY643	cen-Leu2-P_{FIG1}-GFP and pESC-His3-P_{TEF1}-OR1G1 co-transformed in PPY140	Souryadeep Bhattacharyya
far1Δ, sst2Δ, ste2Δ,ste12Δ +PPY595+PPY528	PPY652	pESC-His3-P_{TEF1}-OR1G1-P_{ADH1}-STF1 and pESC-Leu2-$P_{Gal4(5X)}$-GFP co-transformed in PPY161	Souryadeep Bhattacharyya
far1Δ, sst2Δ +PPY39	PPY653	pESC-Leu2 transformed in PPY58	Souryadeep Bhattacharyya
far1Δ, sst2Δ +PPY15	PPY654	cen-Leu2 transformed in PPY58	Souryadeep Bhattacharyya

far1Δ, sst2Δ +PPY15+PPY111	PPY655	cen-Leu2 and pESC-His3 co-transformed in PPY58	Souryadeep Bhattacharyya
far1Δ, sst2Δ, ste2Δ +PPY111+PPY15	PPY656	cen-Leu2 and pESC-His3 co-transformed in PPY140	Souryadeep Bhattacharyya
far1Δ, sst2Δ, ste2Δ,ste12Δ +PPY111+PPY39	PPY657	pESC-Leu2 and pESC-His3 co-transformed in PPY140	Souryadeep Bhattacharyya

Table A.3 Table of Primers

Name	**Sequence**
SB3	ATCTAAGTTTTAATTACAAAGGATCCATGCATCACCATCACCATC
SB4N	TTAGAGCGGATCTTAGCTAGCCGCGGTTATGGGGAATGAATCTTTC

REFERENCES

1 http://goldbook.iupac.org/.

2 Park, M., Tsai, S.-L. & Chen, W. Microbial Biosensors: Engineered Microorganisms as the Sensing Machinery. *Sensors* **13**, 5777-5795, doi:10.3390/s130505777 (2013).

3 Liu, Y., Matharu, Z., Howland, M. C., Revzin, A. & Simonian, A. L. Affinity and enzyme-based biosensors: recent advances and emerging applications in cell analysis and point-of-care testing. *Anal. Bioanal. Chem.* **404**, 1181-1196, doi:10.1007/s00216-012-6149-6 (2012).

4 Mehrvar, M. & Abdi, M. Recent developments, characteristics, and potential applications of electrochemical biosensors. *Analytical Sciences* **20**, 1113-1126, doi:10.2116/analsci.20.1113 (2004).

5 Yagi, K. Applications of whole-cell bacterial sensors in biotechnology and environmental science. *Appl. Microbiol. Biotechnol.* **73**, 1251-1258, doi:10.1007/s00253-006-0718-6 (2007).

6 Liu, X., Germaine, K. J., Ryan, D. & Dowling, D. N. Whole-Cell Fluorescent Biosensors for Bioavailability and Biodegradation of Polychlorinated Biphenyls. *Sensors* **10**, 1377-1398, doi:10.3390/s100201377 (2010).

7 Bousse, L. Whole cell biosensors. *Sensors and Actuators B: Chemical* **34**, 270-275, doi:http://dx.doi.org/10.1016/S0925-4005(96)01906-5 (1996).

8 Gu, M. B., Mitchell, R. J. & Kim, B. C. Whole-cell-based biosensors for environmental biomonitoring and application. *Advances in biochemical engineering/biotechnology* **87**, 269-305 (2004).

9 Dietrich, J. A., McKee, A. E. & Keasling, J. D. in *Annual Review of Biochemistry, Vol 79* Vol. 79 *Annual Review of Biochemistry* (eds R. D. Kornberg, C. R. H. Raetz, J. E. Rothman, & J. W. Thorner) 563-590 (2010).

10 Park, S. M., Park, H. H., Lim, W. K. & Shin, H. J. A new variant activator involved in the degradation of phenolic compounds from a strain of Pseudomonas putida. *Journal of Biotechnology* **103**, 227-236, doi:10.1016/s0168-1656(03)00122-6 (2003).

11 Xu, T., Close, D. M., Sayler, G. S. & Ripp, S. Genetically modified whole-cell bioreporters for environmental assessment. *Ecological Indicators* **28**, 125-141, doi:10.1016/j.ecolind.2012.01.020 (2013).

12 Michelini, E. *et al.* Field-deployable whole-cell bioluminescent biosensors: so near and yet so far. *Anal. Bioanal. Chem.* **405**, 6155-6163, doi:10.1007/s00216-013-7043-6 (2013).

13 Close, D. M., Ripp, S. & Sayler, G. S. Reporter Proteins in Whole-Cell Optical Bioreporter Detection Systems, Biosensor Integrations, and Biosensing Applications. *Sensors* **9**, 9147-9174, doi:10.3390/s91109147 (2009).

14 Li, Y.-F., Li, F.-Y., Ho, C.-L. & Liao, V. H.-C. Construction and comparison of fluorescence and bioluminescence bacterial biosensors for the detection of bioavailable toluene and related compounds. *Environmental Pollution* **152**, 123-129, doi:10.1016/j.envpol.2007.05.002 (2008).

15 van der Meer, J. R. & Belkin, S. Where microbiology meets microengineering: design and applications of reporter bacteria. *Nature Reviews Microbiology* **8**, 511-522, doi:10.1038/nrmicro2392 (2010).
16 Ivask, A., Rolova, T. & Kahru, A. A suite of recombinant luminescent bacterial strains for the quantification of bioavailable heavy metals and toxicity testing. *Bmc Biotechnology* **9**, doi:10.1186/1472-6750-9-41 (2009).
17 Daunert, S. *et al.* Genetically engineered whole-cell sensing systems: coupling biological recognition with reporter genes. *Chemical reviews* **100**, 2705-2738, doi:citeulike-article-id:4012422 (2000).
18 Tauriainen, S., Karp, M., Chang, W. & Virta, M. Luminescent bacterial sensor for cadmium and lead. *Biosensors & Bioelectronics* **13**, 931-938, doi:10.1016/s0956-5663(98)00027-x (1998).
19 Willardson, B. M. *et al.* Development and testing of a bacterial biosensor for toluene-based environmental contaminants. *Applied and Environmental Microbiology* **64**, 1006-1012 (1998).
20 Shetty, R. S., Ramanathan, S., Badr, I. H. A., Wolford, J. L. & Daunert, S. Green fluorescent protein in the design of a living biosensing system for L-arabinose. *Analytical Chemistry* **71**, 763-768, doi:10.1021/ac9811928 (1999).
21 Cheng, T.-H., Chang, C.-R., Joy, P., Yablok, S. & Gartenberg, M. R. Controlling gene expression in yeast by inducible site-specific recombination. *Nucleic Acids Research* **28**, e108, doi:10.1093/nar/28.24.e108 (2000).
22 Labbe, S. & Thiele, D. J. Copper ion inducible and repressible promoter systems in yeast. *Expression of Recombinant Genes in Eukaryotic Systems* **306**, 145-153 (1999).
23 Eitzman, P. D. & Srienc, F. Dynamics of activation of a galactose-inducible promoter in Saccharomyces cerevisiae. *Journal of Biotechnology* **21**, 63-82, doi:10.1016/0168-1656(91)90261-s (1991).
24 Solow, S. P., Sengbusch, J. & Laird, M. W. Heterologous protein production from the inducible MET25 promoter in Saccharomyces cerevisiae. *Biotechnology Progress* **21**, 617-620, doi:10.1021/bp049916q (2005).
25 Chang, A. L., Wolf, J. J. & Smolke, C. D. Synthetic RNA switches as a tool for temporal and spatial control over gene expression. *Curr. Opin. Biotechnol.* **23**, 679-688, doi:10.1016/j.copbio.2012.01.005 (2012).
26 Thompson, K. M., Syrett, H. A., Knudsen, S. M. & Ellington, A. D. Group I aptazymes as genetic regulatory switches. *BMC Biotechnology* **2** (2002).
27 Michener, J. K., Thodey, K., Liang, J. C. & Smolke, C. D. Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways. *Metab. Eng.* **14**, 212-222, doi:10.1016/j.ymben.2011.09.004 (2012).
28 van Bloois, E., Winter, R. T., Kolmar, H. & Fraaije, M. W. Decorating microbes: surface display of proteins on Escherichia coli. *Trends Biotechnol.* **29**, 79-86, doi:http://dx.doi.org/10.1016/j.tibtech.2010.11.003 (2011).
29 Wu, C. H., Mulchandani, A. & Chen, W. Versatile microbial surface-display for environmental remediation and biofuels production. *Trends in Microbiology* **16**, 181-188, doi:http://dx.doi.org/10.1016/j.tim.2008.01.003 (2008).

30 Khorchid, A. & Ikura, M. Bacterial histidine kinase as signal sensor and transducer. *International Journal of Biochemistry & Cell Biology* **38**, 307-312, doi:10.1016/j.biocel.2005.08.018 (2006).
31 Gudipaty, S. A., Larsen, A. S., Rensing, C. & McEvoy, M. M. Regulation of Cu(I)/Ag(I) efflux genes in Escherichia coli by the sensor kinase CusS. *FEMS Microbiol. Lett.* **330**, 30-37, doi:10.1111/j.1574-6968.2012.02529.x (2012).
32 Ladds, G., Goddard, A. & Davey, J. Functional analysis of heterologous GPCR signalling pathways in yeast. *Trends Biotechnol.* **23**, 367-373, doi:10.1016/j.tibtech.2005.05.007 (2005).
33 McCusker, E. C., Bane, S. E., O'Malley, M. A. & Robinson, A. S. Heterologous GPCR expression: A bottleneck to obtaining crystal structures. *Biotechnology Progress* **23**, 540-547, doi:10.1021/bp060349b (2007).
34 Panetta, R. & Greenwood, M. T. Physiological relevance of GPCR oligomerization and its impact on drug discovery. *Drug Discovery Today* **13**, 1059-1066, doi:10.1016/j.drudis.2008.09.002 (2008).
35 Wess, J. G-protein-coupled receptors: Molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *Faseb Journal* **11**, 346-354 (1997).
36 Rosenbaum, D. M., Rasmussen, S. G. F. & Kobilka, B. K. The structure and function of G-protein-coupled receptors. *Nature* **459**, 356-363, doi:10.1038/nature08144 (2009).
37 Conklin, B. R. *et al.* Engineering GPCR signaling pathways with RASSLs. *Nature Methods* **5**, 673-678, doi:10.1038/nmeth.1232 (2008).
38 Marinissen, M. J. & Gutkind, J. S. G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends in Pharmacological Sciences* **22**, 368-376, doi:10.1016/s0165-6147(00)01678-3 (2001).
39 Ferguson, S. S. G. Evolving concepts in G protein-coupled receptor endocytosis: The role in receptor desensitization and signaling. *Pharmacological Reviews* **53**, 1-24 (2001).
40 Touhara, K. Odor discrimination by G protein-coupled olfactory receptors. *Microscopy Research and Technique* **58**, 135-141, doi:10.1002/jemt.10131 (2002).
41 Wicher, D. Functional and evolutionary aspects of chemoreceptors. *Frontiers in Cellular Neuroscience* **6**, doi:10.3389/fncel.2012.00048 (2012).
42 Fukutani, Y., Ishii, J., Noguchi, K., Kondo, A. & Yohda, M. An improved bioluminescence-based signaling assay for odor sensing with a yeast expressing a chimeric olfactory receptor. *Biotechnol. Bioeng.* **109**, 3143-3151, doi:10.1002/bit.24589 (2012).
43 Fukutani, Y. *et al.* The N-terminal replacement of an olfactory receptor for the development of a Yeast-based biomimetic odor sensor. *Biotechnol. Bioeng.* **109**, 205-212, doi:10.1002/bit.23327 (2012).
44 Minic, J. *et al.* Functional expression of olfactory receptors in yeast and development of a bioassay for odorant screening. *Febs Journal* **272**, 524-537, doi:10.1111/j.1742-4658.2004.04494.x (2005).

45 Bradford, W. *et al.* Eukaryotic G Protein Signaling Evolved to Require G Protein-Coupled Receptors for Activation. *Science Signaling* **6**, doi:10.1126/scisignal.2003768 (2013).
46 Walmsley, R. & Keenan, P. The eukaryote alternative: Advantages of using yeasts in place of bacteria in microbial biosensor development. *Biotechnol. Bioprocess Eng.* **5**, 387-394, doi:10.1007/BF02931936 (2000).
47 Legras, J.-L., Merdinoglu, D., Cornuet, J.-M. & Karst, F. Bread, beer and wine: Saccharomyces cerevisiae diversity reflects human history. *Molecular Ecology* **16**, 2091-2102, doi:10.1111/j.1365-294X.2007.03266.x (2007).
48 Sicard, D. & Legras, J.-L. Bread, beer and wine: Yeast domestication in the Saccharomyces sensu stricto complex. *Comptes Rendus Biologies* **334**, 229-236, doi:10.1016/j.crvi.2010.12.016 (2011).
49 Jain, K. K. Synthetic Biology and Personalized Medicine. *Medical Principles and Practice* **22**, 209-219, doi:10.1159/000341794 (2013).
50 Liu, D., Hoynes-O'Connor, A. & Zhang, F. Bridging the gap between systems biology and synthetic biology. *Frontiers in Microbiology* **4**, doi:10.3389/fmicb.2013.00211 (2013).
51 Malinova, V., Nallani, M., Meier, W. P. & Sinner, E. K. Synthetic biology, inspired by synthetic chemistry. *Febs Letters* **586**, 2146-2156, doi:10.1016/j.febslet.2012.05.033 (2012).
52 Stephanopoulos, G. Synthetic Biology and Metabolic Engineering. *Acs Synthetic Biology* **1**, 514-525, doi:10.1021/sb300094q (2012).
53 Zhang, L., Chang, S. & Wang, J. Synthetic biology: From the first synthetic cell to see its current situation and future development. *Chinese Science Bulletin* **56**, 229-237, doi:10.1007/s11434-010-4304-z (2011).
54 Hong, K. K. & Nielsen, J. Metabolic engineering of Saccharomyces cerevisiae: a key cell factory platform for future biorefineries. *Cell. Mol. Life Sci.* **69**, 2671-2690, doi:10.1007/s00018-012-0945-1 (2012).
55 Ostergaard, S., Olsson, L. & Nielsen, J. Metabolic engineering of Saccharomyces cerevisiae. *Microbiol. Mol. Biol. Rev.* **64**, 34-+, doi:10.1128/mmbr.64.1.34-50.2000 (2000).
56 Hong, K.-K. & Nielsen, J. Metabolic engineering of Saccharomyces cerevisiae: a key cell factory platform for future biorefineries. *Cell. Mol. Life Sci.* **69**, 2671-2690, doi:10.1007/s00018-012-0945-1 (2012).
57 Immethun, C. M., Hoynes-O'Connor, A. G., Balassy, A. & Moon, T. S. Microbial production of isoprenoids enabled by synthetic biology. *Frontiers in Microbiology* **4**, doi:10.3389/fmicb.2013.00075 (2013).
58 Kim, I.-K., Roldao, A., Siewers, V. & Nielsen, J. A systems-level approach for metabolic engineering of yeast cell factories. *Fems Yeast Research* **12**, 228-248, doi:10.1111/j.1567-1364.2011.00779.x (2012).
59 Krivoruchko, A., Siewers, V. & Nielsen, J. Opportunities for yeast metabolic engineering: Lessons from synthetic biology. *Biotechnol. J.* **6**, 262-276, doi:10.1002/biot.201000308 (2011).
60 Siddiqui, M. S., Thodey, K., Trenchard, I. & Smolke, C. D. Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools. *Fems Yeast Research* **12**, 144-170, doi:10.1111/j.1567-1364.2011.00774.x (2012).

61 Wriessnegger, T. & Pichler, H. Yeast metabolic engineering - Targeting sterol metabolism and terpenoid formation. *Progress in Lipid Research* **52**, 277-293, doi:10.1016/j.plipres.2013.03.001 (2013).
62 Versele, M., Lemaire, K. & Thevelein, J. M. Sex and sugar in yeast: two distinct GPCR systems. *Embo Reports* **2**, 574-579, doi:10.1093/embo-reports/kve132 (2001).
63 Bardwell, L. A walk-through of the yeast mating pheromone response pathway. *Peptides* **26**, 339-350, doi:10.1016/j.peptides.2004.10.002 (2005).
64 King, K., Dohlman, H. G., Thorner, J., Caron, M. G. & Lefkowitz, R. J. Control og yeast mating signal transduction by a mammalian beta-2-adrenergic receptor and Gs Alpha subunit. *Science* **250**, 121-123, doi:10.1126/science.2171146 (1990).
65 Sander, P. *et al.* Heterologous expression of the human D-2S dopamine receptor in protease deficient Saccharomyces cerevisiae strains. *European Journal of Biochemistry* **226**, 697-705, doi:10.1111/j.1432-1033.1994.tb20098.x (1994).
66 Erlenbach, I. *et al.* Functional expression of M-1, M-3 and M-5 muscarinic acetylcholine receptors in yeast. *Journal of Neurochemistry* **77**, 1327-1337, doi:10.1046/j.1471-4159.2001.00344.x (2001).
67 Minic, J., Sautel, M., Salesse, R. & Pajot-Augy, E. Yeast system as a screening tool for pharmacological assessment of G protein coupled receptors. *Current Medicinal Chemistry* **12**, 961-969, doi:10.2174/0929867053507261 (2005).
68 Kandror, O., Bretschneider, N., Kreydin, E., Cavalieri, D. & Goldberg, A. L. Yeast adapt to near-freezing temperatures by STRE/Msn2,4-dependent induction of trehalose synthesis and certain molecular chaperones. *Molecular Cell* **13**, 771-781, doi:10.1016/s1097-2765(04)00148-0 (2004).
69 Gasch, A. P. *et al.* Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell* **11**, 4241-4257 (2000).
70 Radhika, V. *et al.* Chemical sensing of DNT by engineered olfactory yeast strain. *Nat. Chem. Biol.* **3**, 325-330, doi:10.1038/nchembio882 (2007).
71 Price, L. A., Kajkowski, E. M., Hadcock, J. R., Ozenberger, B. A. & Pausch, M. H. Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway. *Molecular and Cellular Biology* **15**, 6188-6195 (1995).
72 Erickson, J. P. *et al.* Edg-2/Vzg-1 couples to the yeast pheromone response pathway selectively in response to lysophosphatidic acid. *J. Biol. Chem.* **273**, 1506-1510, doi:10.1074/jbc.273.3.1506 (1998).
73 Brown, A. J. *et al.* Functional coupling of mammalian receptors to the yeast mating pathway using novel yeast/mammalian G protein alpha-subunit chimeras. *Yeast* **16**, 11-22, doi:10.1002/(sici)1097-0061(20000115)16:1<11::aid-yea502>3.0.co;2-k (2000).
74 Ishii, J. *et al.* Microbial fluorescence sensing for human neurotensin receptor type 1 using G alpha-engineered yeast cells. *Analytical Biochemistry* **446**, 37-43, doi:10.1016/j.ab.2013.10.016 (2014).
75 Medici, R., Bianchi, E., Di Segni, G. & Tocchini-Valentini, G. P. Efficient signal transduction by a chimeric yeast-mammalian G protein alpha subunit Gpa1-Gs

alpha covalently fused to the yeast receptor Ste2. *Embo Journal* **16**, 7241-7249, doi:10.1093/emboj/16.24.7241 (1997).

76 Sanz, G., Schlegel, C., Pernollet, J. C. & Briand, L. Comparison of odorant specificity of two human olfactory receptors from different phylogenetic classes and evidence for antagonism. *Chemical Senses* **30**, 69-80, doi:10.1093/chemse/bji002 (2005).

77 Choi, Y. J. & Lee, S. Y. Microbial production of short-chain alkanes. *Nature* **502**, 571-+, doi:10.1038/nature12536 (2013).

78

79 Charlier, L. *et al.* How broadly tuned olfactory receptors equally recognize their agonists. Human OR1G1 as a test case. *Cell. Mol. Life Sci.* **69**, 4205-4213, doi:10.1007/s00018-012-1116-0 (2012).

80 Storici, F. & Resnick, M. A. in *DNA Repair, Pt B* Vol. 409 *Methods in Enzymology* (eds J. L. Campbell & P. Modrich) 329-+ (2006).

81 Li, M. Z. & Elledge, S. J. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nature Methods* **4**, 251-256, doi:10.1038/nmeth1010 (2007).

82 Schiestl, R. H., Dominska, M. & Petes, T. D. Transformation of Saccharomyces cerevisiae with nonhomologous DNA - illegitimate integration of transforming DNA into yeast chromosomes and invivo ligation of transforming DNA to mitochondrial DNA sequences. *Molecular and Cellular Biology* **13**, 2697-2705 (1993).

83 Hague, C. *et al.* Olfactory receptor surface expression is driven by association with the beta(2)-adrenergic receptor. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 13672-13676, doi:10.1073/pnas.0403854101 (2004).

84 Togawa, S. *et al.* Importance of asparagine residues at positions 13 and 26 on the amino-terminal domain of human somatostatin receptor subtype-5 in signalling. *Journal of Biochemistry* **147**, 867-873, doi:10.1093/jb/mvq022 (2010).

85 Iguchi, Y. *et al.* Control of signalling properties of human somatostatin receptor subtype-5 by additional signal sequences on its amino-terminus in yeast. *Journal of Biochemistry* **147**, 875-884, doi:10.1093/jb/mvq023 (2010).

86 Nacken, V., Achstetter, T. & Degryse, E. Probing the limits of expression levels by varying promoter strength and plasmid copy number in Saccharomyces cerevisiae. *Gene* **175**, 253-260, doi:10.1016/0378-1119(96)00171-0 (1996).

87 Mumberg, D., Muller, R. & Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**, 119-122, doi:10.1016/0378-1119(95)00037-7 (1995).

88 Pi, H. W., Chien, C. T. & Fields, S. Transcriptional activation upon pheromone stimulation mediated by a small domain of Saccharomyces cerevisiae Ste12p. *Molecular and Cellular Biology* **17**, 6410-6418 (1997).

89 Clarke, E. J. & Voigt, C. A. Characterization of Combinatorial Patterns Generated by Multiple Two-Component Sensors in E. coli that Respond to many Stimuli. *Biotechnol. Bioeng.* **108**, 666-675, doi:10.1002/bit.22966 (2011).