RESEARCH ARTICLE

Molecular quantification of *Saccharomyces cerevisiae* α-pheromone secretion

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Abstract

Saccharomyces cerevisiae yeast cells court each other by producing an attractive sex pheromone specific to their mating type. Cells detect the sex pheromone from potential mates using a well-defined intracellular signalling cascade that has become a model for studying signal transduction. In contrast, the factors contributing to the production of pheromone itself are poorly characterized, despite the widespread use of the *S. cerevisiae* α-pheromone secretion pathway in industrial fungal protein expression systems. Progress in understanding pheromone secretion has been hindered by a lack of a precise and quantitative pheromone production assay. Here, we present an ELISA-based method for the quantification of α-pheromone secretion. In the absence of pheromone from the opposite mating type, we found that each cell secretes over 550 mature α-pheromone peptides per second; 90% of this total was produced from *MF*α1. The addition of **a**-pheromone more than doubled total α-pheromone secretion. This technique offers several improvements on current methods for measuring α-pheromone production and will allow detailed investigation of the factors regulating pheromone production in yeast.

Introduction

Pheromones play a crucial role in mating across many taxa. In the budding yeast *Saccharomyces cerevisiae*, these pheromones allow haploid cells to locate appropriate mating partners, coordinate the morphological, physiological and genetic changes required for conjugation between mating types and provide a basis for mate choice (Jackson & Hartwell, 1990; Kurjan, 1993; Rogers & Greig, 2009). *Saccharomyces cerevisiae* exhibits a bipolar mating system, and each mating type produces a different pheromone. *MAT*α cells secrete a 13 amino acid peptide (α-pheromone) via the classical secretory pathway, while *MAT***a** cells secrete a 12 amino acid peptide (**a**-pheromone) transported across the plasma membrane by the ATP-binding cassette protein Ste6 (Kuchler *et al.*, 1989). Most research has focused on α-pheromone because of interest in manipulating the classical secretory pathway for fungal expression systems (Cereghino & Cregg, 2000; Kjeldsen, 2000), as well as the practical difficulties arising from the hydrophobicity and extensive post-translational modifications of **a**-pheromone.

*MAT*α cells express α-pheromone from two distinct loci: *MF*α1 and *MF*α2. The pheromone is initially translated as a preproprotein consisting of a signal region, a pro-region containing multiple glycosylation sites, and a variable number of pheromone repeats. Post-translational processing occurs in both the endoplasmic reticulum, where the signal region is removed and the pro-region glycosylated, and the Golgi apparatus, where the mature pheromones are released from the proprotein by the combined action of Kex1, Kex2 and Ste13 (Fuller *et al.*, 1988). The two α-pheromone encoding loci differ in several ways. *MF*α1 contains between two and six pheromone repeats, all encoding identical mature peptides with the sequence WHWLGLKPGQPMY. In contrast, all known *MF*α2 sequences contain two repeats: one identical to the *MF*α1-encoded peptide and one containing two substitutions resulting in a mature peptide with the sequence WHWLNLRPGQPMY (α′-pheromone, Singh

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et al., 1983). *MF*α1 is transcribed at much higher rates than *MF*α2, and results of semi-quantitative assays indicate that the majority of mature pheromone is produced from this locus (Kurjan, 1985).

Pheromones induce morphological, behavioural and transcriptional changes in cells of the opposite mating type through the pheromone response pathway (reviewed in Wang & Dohlman, 2004). High pheromone concentrations inhibit cell division by inducing cell cycle arrest. This response provides the basis for a widespread qualitative measure of pheromone production called a 'halo' assay; pheromone spotted onto a lawn of cells of the opposite mating type produces a zone of growth inhibition proportional to the amount of pheromone spotted. While halo assays are simple and effective for identifying mutations resulting in extreme secretion defects (Zhao *et al.*, 2007; Banuelos *et al.*, 2010; Gharakhanian *et al.*, 2011; Ricarte *et al.*, 2011; Corbacho *et al.*, 2012), a more sensitive and quantifiable technique is required to improve not only our understanding of the fine-scale genetic and physiological determinants of secretion (c.f. Idris *et al.*, 2010), but also the evolutionary forces shaping intercellular signalling (Krakauer & Pagel, 1996).

We have developed an ELISA-based technique for measuring α-pheromone production in *Saccharomyces sensu stricto* yeast. This technique produces quantitative, highly reproducible results. Here, we measure α-pheromone production, and changes in cell number, of wild-type and pheromone production mutants in the presence and absence of **a**-pheromone. We provide direct measures of total pheromone secretion rate per cell, the absolute contributions of the *MF*α1 and *MF*α2 loci, and the effect of **a**-pheromone induction on α-pheromone production.

Materials and methods

Yeast culture

All strains were isogenic with s288c and were cultured in liquid YEPD medium (1% yeast extract, 2% peptone and 2% glucose) at 30 °C on an orbital shaker. Strains used in this study are listed in Table 1.

α-Pheromone quantification

Cultures were grown overnight in 5 mL YEPD. To ensure all strains were growing exponentially at the start of the experiment, 400 μL of each overnight culture was diluted in 5 mL fresh YEPD and incubated for 3 h. At the end of this period, cells were counted by OD_{600}, washed three times in water to remove any secreted pheromone and resuspended in 5 mL YEPD at a concentration of 1×10^7 cells mL^{-1}.

Each strain was resuspended in three tubes: one 0-h control and two experimental treatments: +**a**-pheromone and −**a**-pheromone. Immediately after resuspension, 1 mL of the control was transferred to a microtube and centrifuged at 12 000 ***g*** for 2 min. Cell pellets were washed three times in water and fixed in 1 mL 4% paraformaldehyde in 1 × PBS and stored at 4 °C until counting. The supernatant was removed to a 10 000 MWCO Hydrosart Vivaspin 2 column (Sartorious Stedim Biotech) and spun for 12 min at 4000 ***g*** in a swing-bucket centrifuge to remove any remaining cells. Hydrosart columns (but not PES columns, 0.45 μm PVDF filters or 0.45 μm cellulose ester filters) effectively eliminated materials (cells, proteins, etc.) that compete for binding sites on the ELISA plate without removing appreciable levels of α-pheromone. The flow through from the columns was transferred to 96-well Maxisorp Immunosorp plates (Nunc) in triplicate 100 μL samples. Experimental treatments were subjected to the same protocol following a 60-min incubation period. A 60-min incubation period was long enough to produce a strong signal in the assay but short enough to prevent cells from becoming refractory to the **a**-pheromone (c.f. Achstetter, 1989).

A standard curve was made on each plate using doubling dilutions of synthetic α-pheromone (96% purity; T6901 Sigma-Aldrich) dissolved in YEPD and ranging in concentration from 1.00 μg mL^{-1} to 1.95 ng mL^{-1}. To correct the standard curve for the loss of pheromone during filtration, the 1.00 μg mL^{-1} sample was filtered as above and serially diluted into filtered YEPD. Filtered YEPD was included as a blank control. Two additional wells containing 1.00 μg mL^{-1} synthetic α-pheromone were including as controls for non-specific binding of the

Table 1. Strains used in this study

Name	MFα type	Full genotype
WTα	*MF*α1 *MF*α2	*MAT*α *ura3 his3 leu2 met15*
α1 producer	*MF*α1 *mf*α2	*MAT*α *ura3 his3 leu2 met15 mf*α*2::KANMX*
α2 producer	*mf*α1 *MF*α2	*MAT*α *ura3 his3 leu2 met15 mf*α*1::NATMX*
Null producer	*mf*α1 *mf*α2	*MAT*α *ura3 lys2 his3 leu2 met15 mf*α*1::KANMX mf*α*2::KANMX*
WT**a**	*MF*α1 *MF*α2	*MAT***a** *ura3 his3 leu2 met15*
Halo tester		*MAT***a** *ura3 his3 leu2 met 15 sst2::KANMX bar1::URA3*

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secondary antibody. All standards, blanks and controls were plated as duplicate 100 μL samples.

ELISAs were performed according to standard protocols. Briefly, plates were incubated overnight at 4 °C with shaking, blocked for 1 h with 300 μL per well 1% BSA in PBST (1 × PBS containing 0.01% Tween 20), incubated for 1 h in 100 μL 300 ng mL^{-1} custom-made polyclonal rabbit anti-α-pheromone (GenScript, see below) in 0.5% BSA in PBST and then for 1 h in 100 μL of a 1 : 5000 dilution of HRP-conjugated goat anti-rabbit IgG (4050-05; Southern Biotech) in 0.5% BSA in PBST. Following each step, plates were washed 4× with 300 μL per well PBST using an ELx50 microplate strip washer (Biotek). Colorimetric reactions were carried out in 100 μL 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma-Aldrich), stopped with 50 μL 2 N HCl and quantified by measuring absorbance at 450 nm using a Tecan Infinite M200 PRO microplate reader equipped with MAGELLAN 7.0 software.

Absorbance readings were corrected for background absorbance by subtracting the mean value of the blank replicates from the mean value of the experimental (or standard) replicates. α-pheromone concentrations of experimental samples were calculated from the synthetic α-pheromone standard curve specific to each plate. All strains in a single biological replicate were measured on the same plate, and a total of four biological replicates were measured. All negative controls (secondary antibody only and 0-h controls) showed no detectable α-pheromone (all values were within three standard deviations of the blanks - data not included).

a-Pheromone treatment

All strains were tested under two experimental conditions: +**a**-pheromone and −**a**-pheromone. Synthetic **a**-pheromone, a kind gift from Mark D. Distefano (see Mullen *et al.*, 2011 for details), was dissolved in methanol prior to use. Pilot experiments testing the upregulation of α-pheromone secretion in the presence of various **a**-pheromone concentrations (10, 1, 0.1 and 0.01 μg mL^{-1}) revealed that 0.1 ng mL^{-1} was sufficient to induce the maximal response. This concentration was used in all subsequent experiments. At the beginning of the 60-min incubation period, 5 μL **a**-pheromone dissolved in methanol was added to the +**a**-pheromone treatment, while 5 μL methanol was added to the −**a**-pheromone treatment.

Cell counts

Cells were counted using a Z1 Coulter particle counter (Beckman Coulter) equipped with a 100 μm aperture tube. For each fixed cell sample, three aliquots were diluted 250-fold in Isoton II diluent (Beckman Coulter) and each aliquot measured three times. The double median was used as a measure of cell concentration. The rate of pheromone production was calculated using the following formula:

$$\text{Secretion rate} = \frac{\ln \frac{N_{(t)}}{N_0} \times P_{(t)}}{t(N_{(t)} - N_0)}$$

Where $N_{(t)}$ is the cell number at time t, N_0 is the initial cell number, t is the duration of the incubation and $P_{(t)}$ is the amount of pheromone measured at time t. When no change in cell number was observed, the final pheromone amount was simply divided by the cell number.

Custom antibodies and peptides

A polyclonal anti-α-factor antibody was raised in rabbits against the synthetic peptide CWHWLQLKPGQPMY and affinity purified by a commercial supplier (Genscript). The α′ peptide (sequence WHWLNLRPGQPMY) was synthesized by the same source to a purity of 94% and dissolved in water prior to use.

Halo assays

Approximately 2000 halo tester cells were spread on YEPD plates (YEPD containing 2.5% agar) and allowed to dry for 1 h. Filtered α-pheromone supernatants were prepared as described above and 10 μL spotted onto a sterile filter disk placed on the lawn of tester cells. Plates

Fig. 1. Quantification of α-pheromone production and cell number for different *Saccharomyces cerevisiae* strains. (a) Production of α-pheromone by different strains. Bars represent mean α-pheromone molecules secreted per cell s^{-1} over four replicates (± SE) during the 60-min pheromone collection period. Open bars: methanol treated; closed bars: **a**-pheromone treated. Statistical analysis was performed using general linear modelling ($F_{8,12} = 75.77$, $P < 0.0001$) followed by Tukey HSD pairwise comparisons; strains producing no detectable pheromone were not included in the analysis. Bars marked with different letters are significantly different. (b) Change in cell number during the 60-min pheromone collection period. Bars represent mean change over four replicates (± SE), expressed relative to starting number of cells (mean ± SE = 8.31 × 10^6 cells mL^{-1} ± 0.08 × 10^6 cells mL^{-1}). Open bars: methanol treated; closed bars: **a**-pheromone treated. Each level of **a**-pheromone treatment was analysed using a separate general linear model (without **a**-pheromone, $F_{12,27} = 30.34$, $P < 0.0001$; with **a**-pheromone, $F_{12,27} = 13.80$, $P < 0.0001$). (c) Halos produced by different strains with (+) and without (−) treatment with 100 ng mL^{-1} **a**-pheromone.

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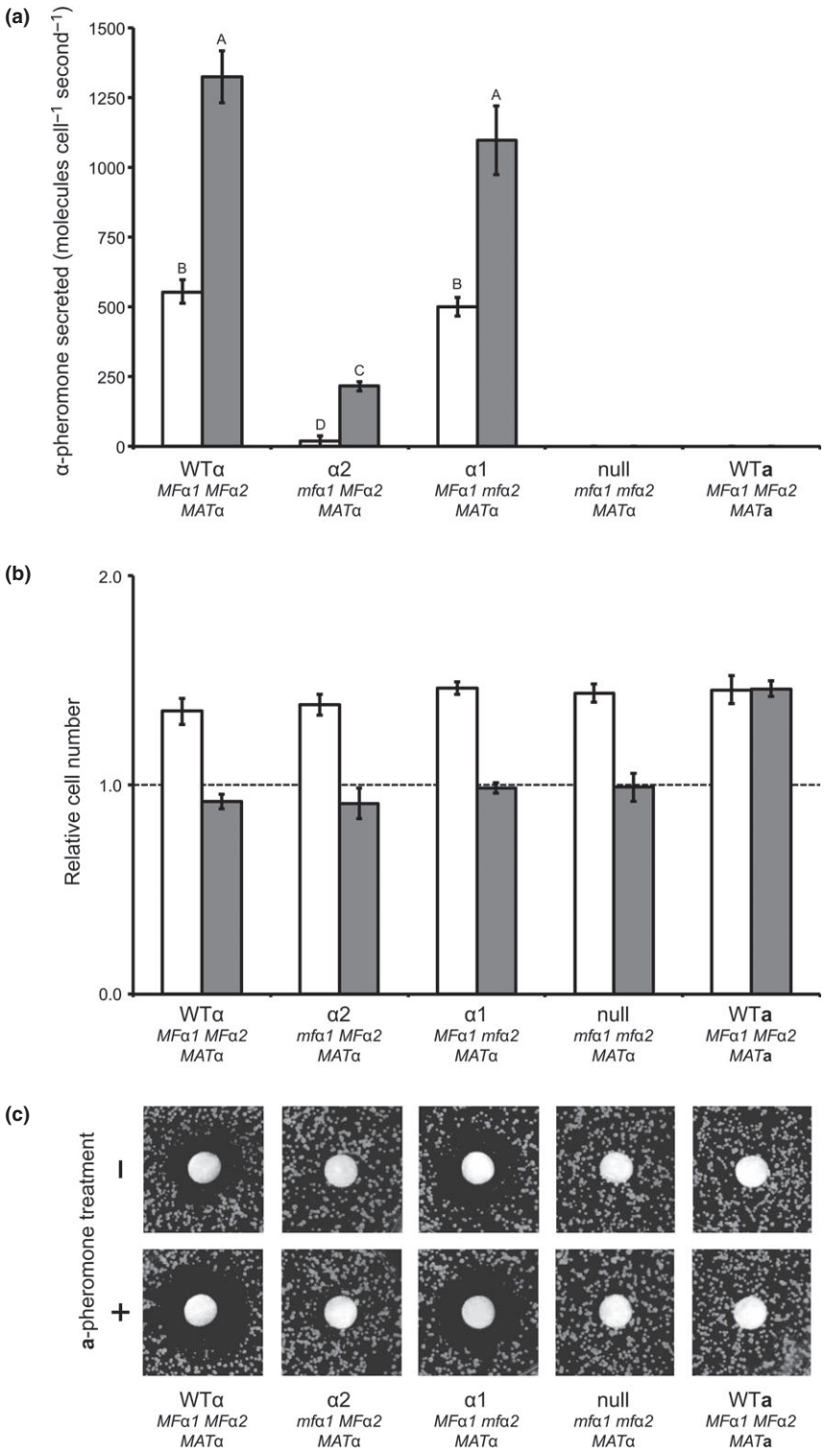

were allowed to grow for 48 h prior to photographing. All α-pheromone-secreting strains were measured on the same plate and the controls on a separate plate. Each plate included a synthetic α-pheromone control.

Statistical analysis

Both pheromone secretion and cell number were analysed using general linear models in JMP 7.0 (SAS Institute). Cell

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numbers were analysed separately for the two different **a**-pheromone treatments. Each model included four replicates at each of two time points (initial and final) for the five different strains resulting in a sample size of 40 measurements. Factors included replicate (as a blocking variable), time, strain and the strain × time interaction. Pheromone secretion (natural logarithm transformed and adjusted for cell number) was analysed in a single model including replicate (as a blocking variable), **a**-pheromone treatment, strain and the strain × **a**-pheromone interaction as predictors. Only final pheromone concentrations were included in the model as initial levels were undetectable. Strains that failed to produce detectable amounts of pheromone were excluded from the model. Pairwise comparisons were Tukey HSD tests.

Results

We tested α-pheromone secretion in five different strains: WTα (both *MF*α1 and *MF*α2 functional), α1 producer (only *MF*α1 functional), α2 producer (only *MF*α2 functional), a null producer (neither *MF*α1 nor *MF*α2 functional), and a WT**a** control (both loci functional, but silent in *MAT***a** cells). Each strain was assayed four times. Overall, the vast majority of the α-pheromone secreted was encoded by *MF*α1 (Fig. 1a, strain $F_{2,12} = 203.46$, $P < 0.0001$). The average pheromone secretion (across **a**-pheromone treatments) of the α1 producer was not significantly different from the WTα producer, while both of the strains secreted significantly more than the α2 producer (Tukey HSD pairwise comparisons between strains). The assay was highly specific; no pheromone was detected in either the null producer or the WT**a** control.

In the absence of **a**-pheromone, each cell of the WTα strain secreted a mean ± SE of 554.51 ± 41.32 molecules of pheromone s^{-1} (Fig. 1a). Under these conditions, the α1 producer secreted 90.13% of the total WTα production; the level of pheromone secretion by the α2 producer was at the limit of detection, averaging only 3.54% of the WTα level. Cell number increased significantly during the 60-min incubation period (time $F_{1,27} = 300.04$, $P < 0.0001$), but no significant difference was detected between strains in either overall cell number (strain $F_{4,27} = 1.55$, $P = 0.2162$) or in growth rate (time × strain $F_{4,27} = 0.81$, $P = 0.5308$).

The addition of synthetic **a**-pheromone to the culture medium induced higher secretion in all α-producing strains (**a**-pheromone $F_{1,12} = 150.26$, $P < 0.0001$); the average induction across these strains was 2.48-fold. No difference in the response to **a**-pheromone was detected between strains (strain × **a**-pheromone $F_{2,12} = 1.20$, $P = 0.3335$). The relative contribution of the *MF*α1 locus was slightly lower in the presence of **a**-pheromone; the α1 producer accounted for 83.05% of the WTα level. The higher total α-pheromone production in this treatment allowed more accurate measurement of secretion by the α2 producer, which accounted for 16.99% of the WTα total. Pheromone production by the α1 and α2 producers summed to 100.04% of the WTα level.

The addition of **a**-pheromone had a second effect; it completely stopped the growth of all *MAT*α strains, while exhibiting no effect on the growth of the WT**a** control (Fig. 1b). On average, there was no change in cell number over time (time $F_{1,27} = 3.86$, $P = 0.0598$). However, change in cell number was strain-dependent (strain $F_{4,27} = 16.55$, $P < 0.0001$; strain × time $F_{4,\ 27} = 15.39$, $P < 0.0001$), with *post hoc* pairwise comparisons showing that the cell number of only the *MAT***a** strain changed significantly over time.

To validate the results of the ELISA, we visualized α-pheromone concentrations using halo assays. The zones of growth inhibition provided qualitative support for the ELISA-based measures (Fig. 1c).

Finally, we tested whether our assay was capable of detecting the α′ peptide, produced from the second repeat of *MF*α2. We quantified synthetic α′-pheromone at two different concentrations (100 and 50 ng mL^{-1}) relative to synthetic α-pheromone standards using the protocol described above. The magnitude of the α′-pheromone response was similar to an equivalent concentration of synthetic α-pheromone (mean = 78%).

Discussion

We have described a sensitive quantitative assay for measuring α-pheromone secretion in the budding yeast *S. cerevisiae*. We have used this assay to provide the first direct measurements of the amount of pheromone secreted by haploid *S. cerevisiae* cells, the relative contributions of the *MF*α1 and *MF*α2 loci, and the induction of α-pheromone secretion in response to **a**-pheromone.

Previous studies have estimated α-pheromone secretion by the response of *MAT***a** cells to it, usually by measuring the zone of *MAT***a** cell growth inhibition around a source of α-pheromone on an agar plate. Using such halo assays, Kurjan (1985) estimated that the amount of α-pheromone secreted from *MF*α2 was only 0.2% of the total produced by *MF*α1 and *MF*α2 together. We found that the proportion of pheromone produced from *MF*α2 was considerably higher, equivalent to roughly 10% of the total in uninduced cells and 17% of the total in cells induced by the presence of **a**-pheromone. Achstetter (1989) used halo assays to estimate that α-pheromone secretion was induced three- to fourfold by the presence of **a**-pheromone. He observed a similar induction (3.6 ×) of invertase activity when *MAT*α cells expressing a recombinant MFα1-Suc2

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fusion protein under the *MF*α1 promoter from a high copy-number plasmid were exposed to **a**-pheromone. Both of these values are higher than the 2.48-fold increase in total α-pheromone secretion we measured in response to **a**-pheromone in the current study. Recently, Gonçalves-Sá & Murray (2011) examined the ability of cultured *MAT*α cell-free supernatants to induce chemotropic mating projections (shmooing) in *MAT***a** cells. By comparing their results to standard synthetic α-pheromone preparations, they estimated the secretion rate of WTα cells (in the absence of **a**-pheromone) to be approximately 700 molecules per cell s^{-1}. This estimate is in close agreement with our observed mean ± SE value of 554.51 ± 41.32 molecules per cell s^{-1}.

Measurement of α-pheromone production based on the pheromone response of *MAT***a** cells has several drawbacks. Assays based on growth inhibition are highly sensitive to variation in temperature, incubation time, medium depth and composition, and the number of *MAT***a** cells plated. Furthermore, most halo assays measure the amount of pheromone secreted over the course of 1 or 2 days by a concentrated patch of *MAT*α cells (Kurjan, 1985; Zhao *et al.*, 2007; Banuelos *et al.*, 2010; Gharakhanian *et al.*, 2011; Ricarte *et al.*, 2011; Corbacho *et al.*, 2012); any difference in growth rate between strains will have a large effect on halo size (Smith & Greig, 2010). Using the activities of recombinant proteins as proxy measures for α-pheromone secretion allows more precise quantification, but may not accurately reflect the normal behaviour of the nonmanipulated system. Cell differentiation into shmoos is also quantifiable, but is a threshold trait: in WT**a** cells, the difference between an α-pheromone concentration that induces no effect and a concentration that induces a maximal effect is extremely small (Malleshaiah *et al.*, 2010), limiting the dynamic range of any assay. Furthermore, distinguishing shmoos from vegetative cells is labour-intensive and error-prone.

The ELISA-based assay described here overcomes all of these limitations. First, it allows direct quantification of α-pheromone concentration eliminating any bias caused by the use of indirect reporters such as *MAT***a** response or recombinant proteins. Second, the high sensitivity of the assay allows any effect of differences in growth rate between strains to be either minimized (by collecting pheromone over short time periods) or eliminated altogether (by inhibiting cell growth by treatment with **a**-pheromone). Third, the assay can be optimized to accurately quantify a wide range of α-pheromone concentrations.

A sensitive assay that allows direct quantification of α-pheromone production will complement existing strategies such as pulse-chase radiolabeling to allow detailed investigations of secretion in the *S. cerevisiae* model system. The contribution of genes outside of the specific α-pheromone processing pathway to secretion remains poorly understood, as does the interaction between different pathways (Idris *et al.*, 2010). Furthermore, this assay will allow comparison of pheromone signals in wild yeast populations (all members of *Saccharomyces sensu stricto* produce similar pheromones), providing a means to determine the relevance of pheromone production to yeast ecology and evolution.

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Authors' contribution

D.W.R. and E.M. contributed equally to this work.

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