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Engineered Quorum Sensing Using Pheromone-Mediated Cell-to-Cell Communication in *Saccharomyces cerevisiae*

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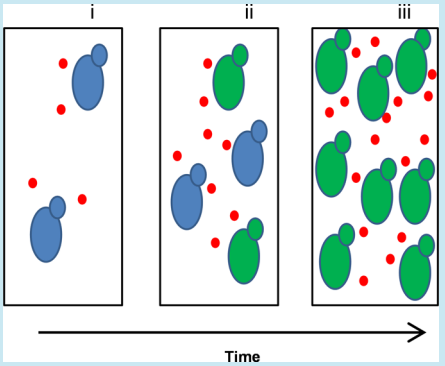
ABSTRACT: Population-density-dependent control of gene expression, or quorum sensing, is widespread in nature and is used to coordinate complex population-wide phenotypes through space and time. We have engineered quorum sensing in *S. cerevisiae* by rewiring the native pheromone communication system that is normally used by haploid cells to detect potential mating partners. In our system, populations consisting of only mating type "a" cells produce and respond to extracellular α-type pheromone by arresting growth and expressing GFP in a population-density-dependent manner. Positive feedback quorum sensing dynamics were tuned by varying α-pheromone production levels using different versions of the pheromone-responsive FUS1 promoter as well as different versions of pheromone genes (mfα1 or mfα2). In a second system, pheromone communication was rendered conditional upon the presence of aromatic amino acids in the growth medium by controlling α-pheromone expression with the aromatic amino acid responsive ARO9 promoter. In these circuits, pheromone communication and response could be fine-tuned according to aromatic amino acid type and concentration. The genetic control programs developed here are responsive to dynamic spatiotemporal and chemical cellular environments, resulting in up-regulation of gene expression. These programs could be used to control biochemical pathways for the production of fuels and chemicals that are toxic or place a heavy metabolic burden on cell growth.



KEYWORDS: *quorum sensing, α-pheromone, yeast, dynamic control, cell-to-cell communication*

In nature, gene expression and cellular metabolism are subject to multilevel regulation that is responsive to both intra- and extracellular environmental cues throughout space and time. Synthetic biology aims to engineer modular genetic control programs that resemble those found in nature in terms of their capacity to respond to a dynamic extracellular environment.[1−4] These goals are particularly relevant to the engineering of microorganisms for the production of fuels, chemicals, and pharmaceuticals. However, traditional metabolic engineering has relied upon the constitutive and static expression of enzymatic pathways to produce valuable end-products[5] without regard for the dynamic spatiotemporal and chemical environment of the cell.[1] Despite the fact that several methods are now emerging that afford dynamic, environmentally regulated control of gene expression[1,6,7] there is still a greater need for "next generation" control systems that act autonomously to regulate pathway flux toward desired products.[8]

There are several advantages gained by engineering a greater level of dynamic control over gene expression. Engineered pathways often place a metabolic burden on their host cell, as they draw cellular resources away from normal growth-based metabolism.[9] It has been demonstrated *in silico* that dynamic and biphasic use of engineered pathways can be advantageous.[10,11] This is because higher population densities are achieved before the growth-limiting metabolic burden of compound production is initiated, resulting in higher volumetric productivity. The merits of separating growth from production are also pronounced when pathway products are toxic to the host cell.[9,12] Both metabolic burden and product toxicity can be overcome by controlling gene expression at a desirable point during fermentation rather than using constitutive pathway expression. The delayed induction of such pathways is desirable but is not currently feasible in an industrial context due to the cost of chemical inducers/repressors and lack of suitability of inducible/repressible promoters.[13,14]

In nature, microorganisms sense population density by emitting intercellular signaling molecules that cause a population-wide cellular response once a critical signal concentration is reached ("quorum sensing").[15] This enables a population to delay the induction of various genetic programs that are more effective if coordinated at the population level. Our approach to implementing dynamic control of gene expression is to engineer cell-to-cell chemical communication for the population-density-dependent modulation of gene expression (quorum sensing). Engineered quorum sensing has been implemented previously for a variety of applications and has great potential to serve as a metabolic pathway control mechanism.[16]

Saccharomyces cerevisiae is one of the most widely utilized industrial micro-organisms as a result of its depth of

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characterization and genetic tractability. However, there is a distinct lack of synthetic gene circuits available for *S. cerevisiae* relative to *E. coli* and even mammalian cells.[17] The only other synthetic quorum sensing module available in yeast was engineered by using an *Arabidopsis thaliana* hormone as an intercellular signaling molecule as part of a positive feedback loop.[18] This was the first example of transferring a heterologous population-density-dependent signaling system into *S. cerevisiae*. However, there are key aspects of this system arising from the interface between the heterologous *A. thaliana* components and endogenous host metabolism that make it inappropriate for application in a metabolic engineering scenario. The integration of the heterologous plant hormone membrane receptor with endogenous yeast metabolism required that the high osmolarity glycerol (HOG) response be constitutively repressed in order to avoid non-specific HOG induction, leading to cell death. This was achieved by expressing a repressor (PTP2p) of the HOG1 protein using a galactose-inducible promoter. This requirement is undesirable in an industrial fermentation setting; furthermore, galactose is an unsuitable carbon source for industrial applications due to its high cost[19] and the low specific growth rate of yeast on galactose. This approach to the implementation of quorum sensing in yeast represents a common theme in synthetic biology where heterologous components are imported into a desired host organism[2] and highlights the challenges associated with interfacing these components with endogenous host metabolism.

An alternative to importing heterologous systems is to "rewire" existing components to achieve the desired output. *S. cerevisiae* has a cell-to-cell communication system that is used by haploid cells to detect and respond to mating partners. Briefly, haploids of opposite mating type (a or α) sense nearby potential mating partners by emitting mating-type-specific small peptide pheromones.[20] Upon pheromone binding to mating-type-specific membrane receptors at a threshold concentration, intracellular mitogen activated protein kinase (MAPK) signaling effects the mating phenotype.[20] During this process, cell cycle arrest occurs in the G1 phase, and mating-specific genes are up-regulated by approximately 100-fold. This coordinated modulation of gene expression results in the formation of membrane projections (called shmoos) to which nuclei localize prior to shmoo tip fusion between mating partners, followed by DNA exchange and the formation of diploids.[20]

The characteristics of this native communication system are highly amenable to an integrated (non-heterologous) synthetic biology approach. The interface between extracellular signaling molecules and intracellular metabolism already exists in the form of the native membrane pheromone receptor Ste2p, which rapidly enacts changes in transcription of mating-specific genes via the MAPK phosphorylation cascade and the Ste12p transcription factor. This abrogates the need to further manipulate the regulatory network to facilitate the expression of genes of interest. The pheromone-responsive MAPK network has proven to be highly plastic, with several synthetic gene networks engineered recently.[21−25] It has previously been demonstrated that mating type a (MATa) haploid yeast is capable of producing α-pheromone[26] and that cells can respond to endogenously produced pheromone when both types of pheromone membrane receptor protein (Ste2p and Ste3p) are expressed[27] and when both types of pheromone and receptor are expressed.[28] However, these studies did not explore the potential of pheromone communication in yeast as a tunable quorum sensing module.

In this work we have engineered the native pheromone cell-to-cell communication system in yeast to function as an autonomous quorum sensing module by enabling α-pheromone expression in α-pheromone-sensitive MATa haploids. This quorum sensing module affords dynamic control of gene expression that may be useful for the separation of growth from toxic biochemical production.

RESULTS AND DISCUSSION

Circuit Topology. Two pheromone-mediated communication circuits with fundamentally different topologies were constructed in *S. cerevisiae*. The first circuit is defined by a positive feedback loop where pheromone expression is up-regulated in the presence of pheromone. Positive feedback loops are widely dispersed in nature and are particularly prevalent in implementing cellular decision making where switch-like changes in gene expression are required.[29] All cells are able to respond to changes in their extracellular environment by altering gene expression, making such systems highly amenable for the engineering of dynamic control of gene expression. The second circuit we constructed therefore controls pheromone production in response to the aromatic amino acid content of the extracellular environment and has no positive feedback component under the growth conditions that were used. All circuits were constructed in the haploid MATa CEN.PK2-1c strain background, which is unable to switch mating type due to mutation at the HO locus.[30]

The first circuit is a positive feedback loop controlled by the pheromone-responsive FUS1 promoter (Figure 1a). Fus1p is a membrane protein involved in cell fusion during mating;[31] the FUS1 promoter, pFUS1, is highly induced by α-pheromone.[32] pFUS1 was linked to α-pheromone production to construct a positive feedback loop. Two different versions of the circuit were constructed by linking pFUS1 to two different mating factor α (mfα) genes. The mfα1 gene encodes a precursor peptide with four repeating units of the 13 amino acid α-pheromone peptide, while the mfα2 gene encodes a precursor peptide with only two α-pheromone units.[33] Binding of pheromone to the transmembrane G-protein coupled receptor Ste2p triggers a mitogen-activated protein kinase (MAPK) phosphorylation signal cascade that results in the derepression of the Ste12p transcription factor.[34] Ste12p activates the Far1p cyclin-dependent kinase, which is responsible for cell cycle arrest in the G1 phase and is involved in polarized growth and mating projection formation.[20] Because of this cell cycle arrest phenotype, we initially planned to delete FAR1. However, FAR1 deletion mutants did not exhibit a sustained quorum sensing response; this is consistent with previous findings, which have shown that Far1p is essential for maintaining the pheromone-response phenotype.[34] The mating phenotype is controlled by the coordinated activity of Ste12p (which up-regulates approximately 200 genes) and FAR1 (which represses a similar number of genes that are not active in the G1 phase of the cell cycle).[35] Wild type yeast strains have a secreted α-pheromone protease gene (BAR1);[36] deletion of this gene is required to avoid signal degradation.[37] The circuits were therefore constructed in a bar1Δ genetic background. A destabilized green fluorescent protein (GFP) gene codon-optimized for yeast expression (yEGFP)[38] under the control of the FUS1 promoter was integrated into the genome as a reporter of pFUS1-driven expression. The destabilized yEGFP has a half-life of approximately 30 min and is consistently

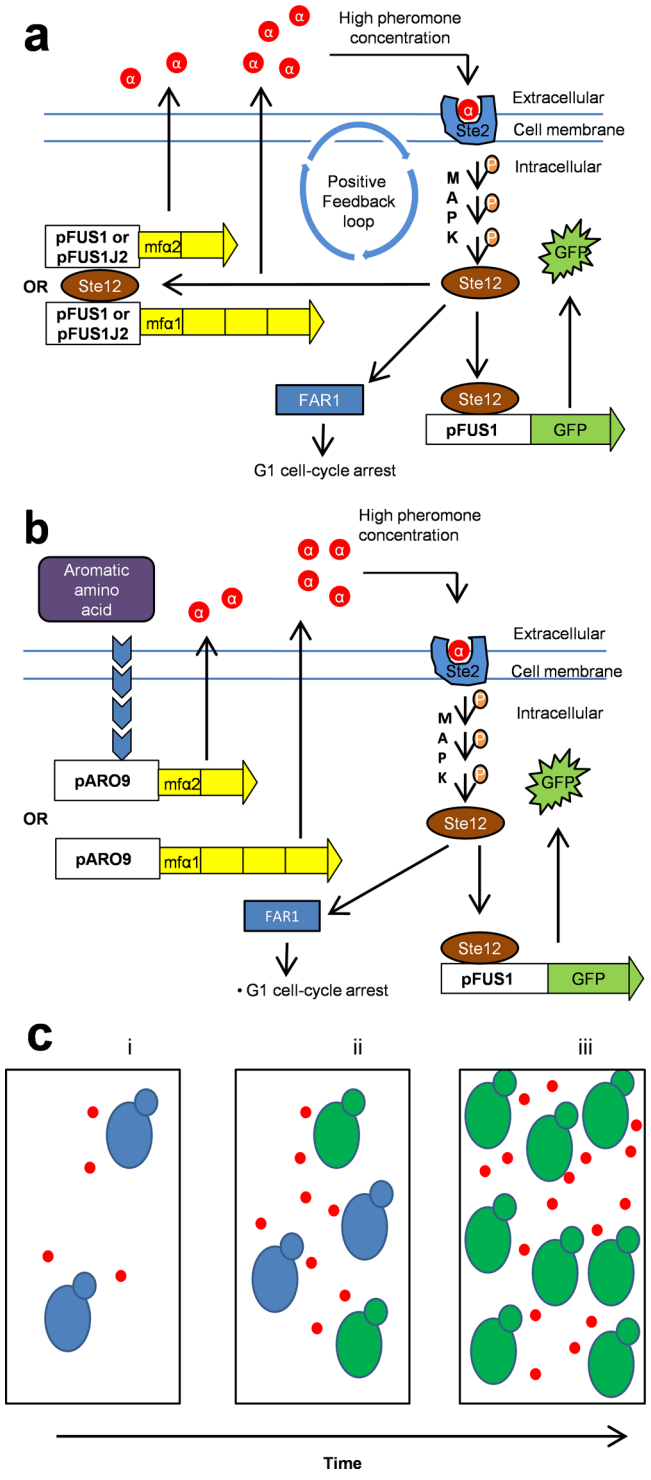

Figure 1. Pheromone communication circuit design. (a) Positive feedback quorum sensing. Pheromone production is autoinduced with positive feedback via the MAPK signal cascade in a population-density-dependent manner. The FUS1 promoter (pFUS1) controls expression of two or four α-pheromone peptides using either the mfα2 (2 peptides) or the mfα1 (4 peptides) gene. α-Pheromone secreted outside the cell binds to the Ste2 membrane receptor, which activates an intracellular mitogen activated protein kinase (MAPK) phosphorylation cascade. The Ste12 transcription factor is derepressed upon MAPK activation and promotes transcription from the pheromone-responsive FUS1 promoter (pFUS1). The pheromone response includes FAR1-mediated cell cycle arrest[46] and expression of a destabilized green fluorescent protein. (b) Aromatic amino acid responsive quorum sensing. ARO9 is up-regulated in the presence of aromatic amino acids.[40] When α-pheromone expression is controlled by the ARO9 promoter (pARO9), induction of pheromone communication can be controlled by aromatic amino acid concentrations in the growth media. As in the positive feedback quorum sensing circuit, the pheromone response is tracked via pFUS1-GFP expression in response to MAPK activation. (c) Pheromone quorum sensing concept. (i) Pheromone secreted by cells at a low population is not concentrated enough to induce GFP expression. (ii) As the population grows in a confined space (shake flask), the extracellular pheromone concentration increases, causing some of the population to switch on GFP-reporter expression (represented by green yeast cells). (iii) At a high population density, pheromone concentration is high enough to induce GFP-reporter expression in most members of the population.

unstable throughout the cell cycle, making it suitable for the detection of dynamic changes in gene expression.

In the second circuit (Figure 1b), pheromone expression is controlled by the ARO9 promoter,[39] which is up-regulated when aromatic amino acids are present in the growth medium.[40] By controlling α-pheromone production with the ARO9 promoter, the pheromone response can be tuned according to the aromatic amino acid concentration. This circuit renders quorum sensing behavior conditional upon the presence and concentration of aromatic amino acids, meaning that quorum sensing behavior can be activated when desired. Similar to the positive feedback pFUS1 system (Figure 1 a), the circuit could be tuned by expressing α-pheromone using either the mfα1 or the mfα2 gene, and MAPK-activated yEGFP was used as a reporter. This circuit was therefore designed to respond to both extracellular aromatic amino acids and population density over time. It should be noted that the ARO9 promoter is involved in the native yeast quorum-sensing response through positive feedback production of tryptophol as a communication molecule.[41] Tryptophol is an aromatic alcohol that is produced downstream of the aromatic amino acid degrading ARO9 gene. In late stationary phase, tryptophol accumulates extracellularly as a communication molecule and up-regulates transcription from the ARO9 promoter via the Aro80p transcription factor. This regulation is observed only under nitrogen starvation and very high population density conditions (neither of which were encountered in this study).

These circuits were designed so that at low population density, α-pheromone concentrations are insufficient to trigger a strong response via the intracellular MAPK signaling cascade (Figure 1 c, panel i). At a high population density, α-pheromone is more concentrated and coordinates population-wide MAPK activation and expression of genes of interest (Figure 1 c, panel iii). Circuits producing pheromone via mfα1 are expected to elicit a stronger/faster response than circuits producing pheromone via mfα2. An alternate version of the positive feedback circuit was constructed in which the mfα1 and mfα2 genes are regulated by an engineered version of the FUS1 promoter, pFUS1J2.[23] pFUS1J2 has an almost undetectable level of basal expression and a higher level of induced expression relative to the native FUS1 promoter. Circuits controlled via pFUS1J2 were expected to require a higher population density than pFUS1 to initiate the positive feedback expression of α-pheromone.

Secreted α-Pheromone Elicits a Population-Density-Dependent Cell-to-Cell Communication Response. In

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order for the α-pheromone to behave as a population density reporting molecule, it is necessary for cells to both produce the pheromone and to export it into the extracellular medium. To determine whether α-pheromone accumulates in the extracellular medium at levels significant enough to effect a biological response, sender−receiver growth arrest assays were performed (similar to the commonly used halo assay[42]). When cells engineered to produce α-pheromone were grown on solid media that had been spread with an α-pheromone-sensitive but nonproducing MATa strain (JRS09; see Table 4), growth inhibition was observed in the area surrounding test strains but not the control strain (Figure 2a,b). This indicates that the "sender" test strains are indeed producing α-pheromone and that the α-pheromone is diffusing into the medium and causing the "receiver" cells to arrest growth. One might question why, given that "sender" strains are also sensitive to α-pheromone, they do not arrest growth under these conditions. Previous results have shown that α-pheromone-producing MATa cells do not undergo a population-wide autoinduced growth arrest unless the SST2 gene (responsible for desensitization to pheromone[43]) is disrupted.[26] The fact that the "sender" strains secrete α-pheromone and still grow is consistent with these findings.

The size of the zone of clearing around the positive feedback strains (driven by pFUS1 or pFUS1J2) was similar regardless of the presence or absence of aromatic amino acids (Figure 2a,b), consistent with circuit topology (Figure 1a). There was no obvious difference in the size of the clearing zone between pFUS1 and pFUS1J2 or between the two different α-pheromone genes (encoding 2 or 4 peptide units); the semiquantitative assay is clearly not sensitive enough to distinguish between responses driven by the two promoters or the two genes.

When strains carrying the aromatic amino acid responsive circuits (driven by pARO9) were grown on solid media without tryptophan (Figure 2a), very small zones of growth inhibition were observed in the receiver growth lawn. In contrast, when the same strains were grown with tryptophan, strong growth inhibition was observed in the receiver strain (Figure 2 b). These data are consistent with previous findings that demonstrated very low levels of gene expression from the ARO9 promoter in the absence of aromatic amino acids.[40] Expression of α-pheromone from the ARO9 promoter in the presence of tryptophan is not subject to the same feedback regulation as the FUS1 promoter, which may explain the slightly larger zones of growth inhibition in the "receiver" lawn in these strains relative to the positive feedback strains (Figure 2b).

A key requirement of the engineered circuits is that they respond in a dose-dependent fashion to the appropriate inducer. This was examined using a pheromone-sensitive receiver strain that has been engineered to produce GFP in response to pheromone by placing the GFP gene under the control of the FUS1 promoter. Previously, it has been shown that the FUS1 promoter responds to α-pheromone in a dose-dependent manner.[44] This was confirmed by treating the engineered receiver strain (which does not produce α-pheromone) with varying concentrations of synthetic α-pheromone (Figure 2c). We also confirmed that deletion of the BAR1 gene in MATa haploids makes responding populations significantly more sensitive to α-pheromone, consistent with previous results.[37] Peak GFP responses were observed about 2 orders of magnitude apart: the GFP response

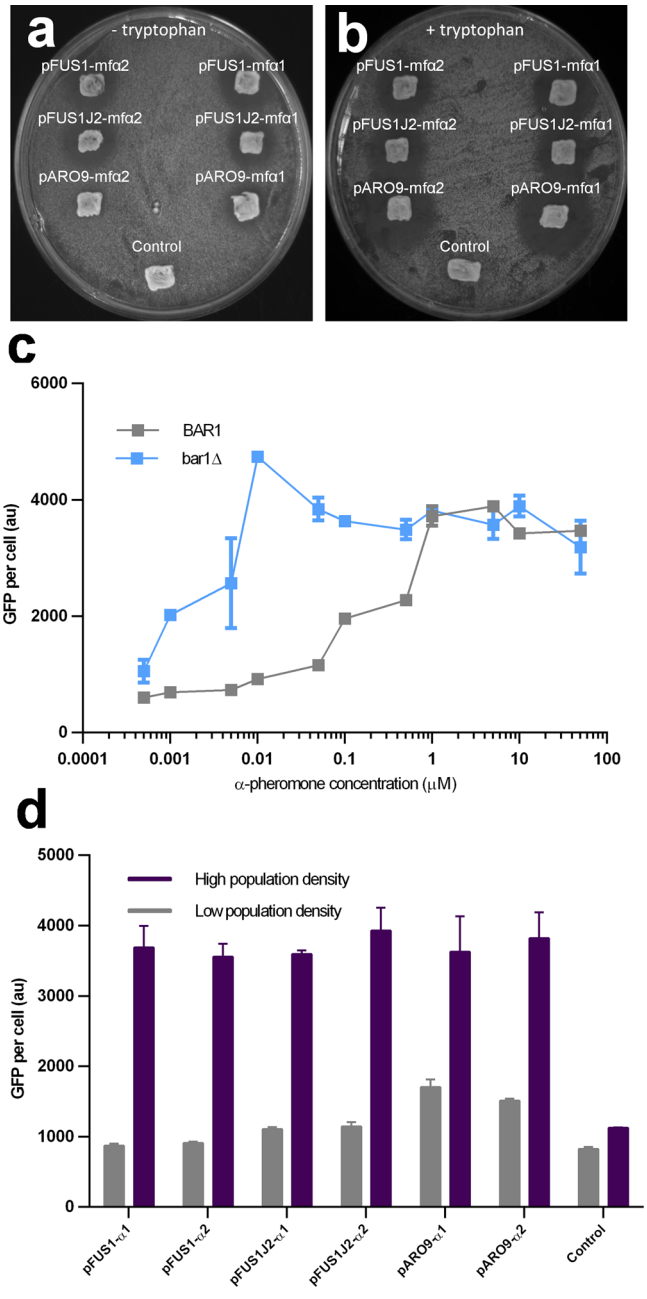

Figure 2. Sender-receiver pheromone communication. Pheromone-producing pFUS1-mfα2, pFUS1-mfα1, pFUS1J2-mfα2, pFUS1J2-mfα1, pARO9-mfα2, pARO9-mfα1, and a vector-only control strain were grown on a lawn of pheromone-sensitive MATa cells (JRS09) without tryptophan (a) or with 50 μg/mL tryptophan (b). Zones of growth inhibition around test strains indicate sender−receiver communication between pheromone-producing strains and the pheromone-sensitive lawn that arrests growth upon receiving the signal. The assay is only semiquantitative, since test strains were derived from single colonies of different sizes on solid media. Figure is representative of replicate experiments using different colonies of the same strain. (c) Strains JRS01 (BAR1) and JRS02 (bar1Δ) were grown to an OD_{660} of 1 prior to treatment with the indicated concentration of synthetic α-pheromone. Populations were incubated for 3.5 h prior to GFP fluorescence measurement. (d) A non-pheromone-producing "receiver" strain (JRS09) engineered to produce GFP in response to pheromone (via the FUS1 promoter) was treated with media conditioned by pheromone-producing "sender" strains that had been grown to low population density, unautoinduced (OD_{660} ≤ 0.2 for pARO9 strains and ≤0.07 for all others, GFP

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Figure 2. continued

fluorescence per cell ≤1500 au) or high population density, autoinduced (OD_{660} ≥ 2, GFP ≥5000 au) conditions that were defined according to strain behavior observed in quorum sensing experiments (Figure 3, Figure 6b,c). Aromatic amino acid responsive sender strains were grown with 100 μg/mL tryptophan. Receiver strains were incubated with conditioned media at a starting OD_{660} of 1 for 3.5 h prior to measuring GFP. GFP fluorescence in the control strain reflects basal activity of the FUS1 promoter. Differences in GFP fluorescence levels of conditioned media treated "receiver" populations were analyzed in biological triplicate within each "sender" strain type ($p \leq 0.05$, two-tailed two-sample t tests with unequal variance).

peaked at ∼1 μM α-pheromone in the BAR1 strain, whereas it peaked at ∼0.01 μM in the bar1Δ strain.

To confirm that the observed response elicited by the sender strains is due to a mobile molecule released into the medium and that the response is proportional to population density, the pheromone-sensitive "receiver" strains were treated with media that had been conditioned by pheromone-producing strains. According to the circuit design, the engineered strains are in a non-autoinduced state at low population density and in a maximally autoinduced state at high population density. Media was conditioned by growing pheromone-producing cells to either low population density or high population density; the cells were then removed and the conditioned media used to treat the pheromone-sensitive receiver strain that has been engineered to produce GFP in response to pheromone (driven by the FUS1 promoter JRS09; see Table 4). When medium conditioned with pheromone-producing strains growing at low population density was used to treat the receiver strain, GFP levels were slightly greater than the basal level from the FUS1 promoter (Figure 2d). This is consistent with low levels of pheromone being produced and released into the medium under these conditions. This effect was more pronounced in the aromatic amino acid responsive strains due to the presence of tryptophan in the media highly up-regulating pARO9-mediated pheromone expression even at a low population density. When medium conditioned by pheromone-producing strains at high population density was used to treat the receiver strain, significant increases in GFP were observed, consistent with much higher levels of pheromone being produced under these conditions ($p \leq 0.05$, two-tailed two-sample t tests with unequal variance). This demonstrated that strains carrying mfα1 or mfα2 genes as shown in Figure 1 produce functional α-pheromone as an extracellular communication molecule, which increases in concentration with increasing population density. This is the hallmark feature of an intercellular quorum sensing molecule.[45]

Positive Feedback Quorum Sensing. We have engineered mating type "a" strains that produce and respond to exported α-pheromone by expressing GFP and arresting growth in a population-density-dependent manner. A positive feedback loop was created by controlling α-pheromone expression with a promoter (FUS1) that has a low basal activity and is up-regulated when cells respond to pheromone (Figure 1a). We hypothesized that these strains would dynamically and autonomously control gene expression by quorum sensing with the α-pheromone.

Strains engineered with positive feedback circuits (Figure 1a) were grown in liquid media from single colonies in order to avoid quorum sensing behavior occurring in precultures affecting experimental observations. As expected, GFP reporter expression increased in parallel with increasing population density (Figure 3a). The growth curves of the mfα2- and mfα1-expressing strains were identical; however, the GFP expression activity profile of the mfα2 strain (JRS06) was slightly damped relative to that of the mfα1 strain (JRS05), with GFP accumulation starting ∼2 h later and reaching maximal expression at higher population density (Figure 3a; Table 1). This is consistent with expected behavior due to the number of α-pheromone peptides encoded by each gene. The population densities of the pheromone-producing mfα1 and mfα2 strains reached a modest OD_{660} of ∼1.3, and strains had exponential growth rates of 0.22 and 0.20 h^{-1} respectively, while the control strain reached a final OD_{660} of 6.4 and had an exponential growth rate of 0.30 h^{-1}. These differences in population growth dynamics can be explained by the fact that cells responding to pheromone arrest growth in the G1 phase of the cell cycle due to the action of the FAR1 protein.[46] A whole population growth arrest was not observed in the experimental timeframes

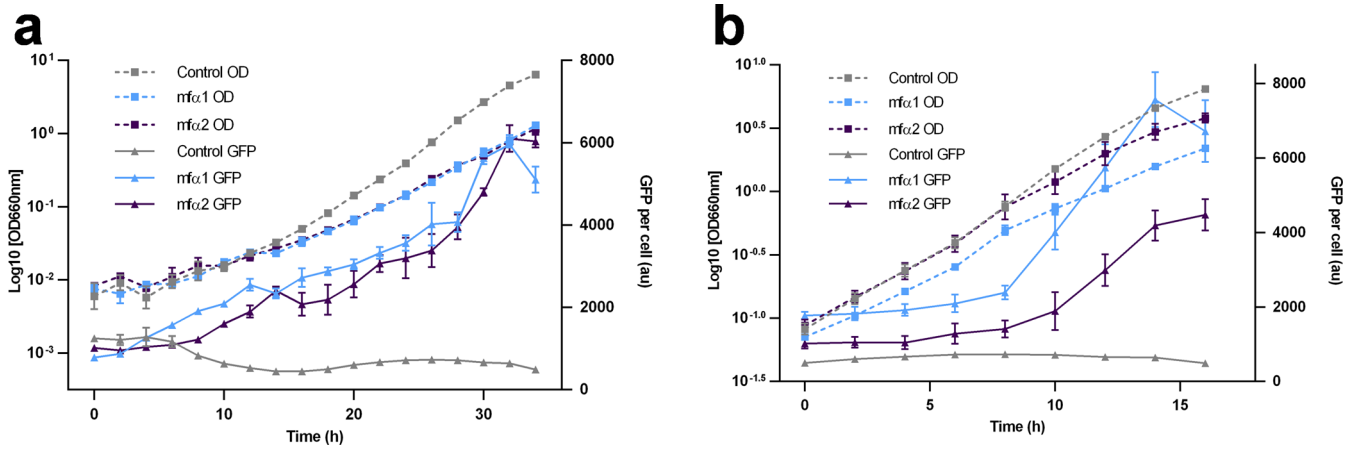

Figure 3. Positive feedback quorum sensing. (a) Pheromone-producing strains pFUS1-mfα1 (JRS05) and pFUS1-mfα2 (JRS06), as well as non-pheromone-producing control strain JRS09, were assayed for quorum sensing behavior by following population density (OD_{660}) and pheromone-responsive gene expression (pFUS1-GFP) over time. Single colonies from transformation plates were used to inoculate liquid media without preculturing. (b) Strains pFUS1J2-mfα1 (JRS07) and pFUS1J2-mfα2 (JRS08) were grown as in panel a, with behavior being observed from an OD_{660} of ∼0.07. Markers and error bars represent mean and standard deviation of biological triplicates.

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Table 1. Quorum Sensing Circuit Properties[a]

circuit/condition	quorum sensing ($OD_{660\ nm}$) initiation	quorum sensing ($OD_{660\ nm}$) saturation	Hill coefficient	response time (h)	dynamic range
pFUS1-mfα1	0.009 ± 0.001	0.550 ± 0.070	0.63 ± 0.09	24	7.6
pFUS1-mfα2	0.016 ± 0.001	0.800 ± 0.090	0.97 ± 0.12	22	6.4
pFUS1J2-mfα1	0.497 ± 0.036	1.577 ± 0.057	3.90 ± 0.60	6	7.0
pFUS1J2-mfα2	0.768 ± 0.184	3.817 ± 0.332	2.40 ± 0.27	8	4.4
pARO9-α1 induced at $OD_{660\ nm}$ of 0.183	0.183 ± 0.007	1.967 ± 0.210	0.56 ± 0.12	8	4.9
pARO9-α2 induced at $OD_{660\ nm}$ of 0.185	0.185 ± 0.003	1.847 ± 0.056	0.50 ± 0.10	8	5.2
pARO9-α2 induced at $OD_{660\ nm}$ of 0.021	0.021 ± 0.000	2.497 ± 0.150	0.59 ± 0.12	16	4.0
pARO9-α2 induced at $OD_{660\ nm}$ of 1.400	1.400 ± 0.061	4.017 ± 0.142	3.12 ± 0.79	4	5.0

[a]Quorum sensing initiation was defined as the population density at which GFP per cell first exceeds 1800 au. Quorum sensing saturation point was defined as the population density at which GFP per cell plateaus or decreases (above 4000 au). Error bars represent ±1 standard deviation of biological triplicates. Hill coefficients were determined using the four parameter variable slope dose−response model in GraphPad Prism 6, with average log normalized OD_{660} values plotted against GFP fluorescence readings in triplicate for each strain. Errors for Hill coefficients are ± standard error. Response time is defined as the time between quorum sensing initiation and quorum sensing saturation. Dynamic range was determined by dividing the mean maximum GFP by the mean minimum GFP from triplicate experiments for a given strain. "Induced" refers to the addition of 100 μg/mL of tryptophan to the media at the population density indicated.

of this study, although the growth arrest of population subsets can be inferred by comparing the population growth curves of pheromone-producing strains with those of the control strain. Whole population growth arrest has been observed in α-pheromone autoinduced populations that had the SST2 gene (which is central to the recovery from α-pheromone growth arrest[37]) deleted.[26] It is therefore likely that the continued growth of quorum sensing populations showing maximum GFP-reporter expression was due to the recovery from G1 arrest of a subset of the population via SST2-dependent mechanisms. Deletion of the SST2 gene was not practical in the design of the quorum sensing circuits created here due to the higher basal and non-specific activation of the FUS1 promoter in SST2 deleted strains.[47]

The native FUS1 promoter circuits initiated quorum sensing at very low population densities (Figure 3a). For industrial application, it is desirable to reach higher population density so that sufficient biomass is available for production of biochemicals before pathway expression is triggered. Alternate positive feedback quorum sensing strains were engineered by using the pFUS1J2 promoter[23] in place of the native FUS1 promoter for the control of α-pheromone expression. The FUS1J2 promoter has nearly undetectable basal levels of expression and higher levels of pheromone-induced expression relative to the native FUS1 promoter.[23] It was therefore expected that pFUS1J2 strains would initiate quorum sensing behavior at higher population densities than the strains engineered with the native FUS1 promoter. As for the pFUS1 circuit, reporter GFP expression (from the native FUS1 promoter) increased with increasing population density, and the growth rate was reduced relative to the control strain (Figure 3b). As expected, the FUS1J2 promoter resulted in maximal autoinduced GFP reporter expression being delayed until higher population densities were reached relative to the native FUS1 promoter carrying strains (Table 1). Similarly to the pFUS1 promoter circuits, the pFUS1J2-mfα2 circuit initiated quorum sensing at a higher population density than the pFUS1J2-mfα1 circuit (Table 1). In addition to tuning the quorum sensing "threshold" toward a higher population density response, the use of the FUS1J2 promoter also resulted in a much more "switch-like" transition between non-autoinduced and autoinduced GFP-reporter expression. The Hill coefficient, which is commonly used to describe the response dynamics of engineered regulatory systems,[29] can be used to define response cooperativity of GFP activity here. The native FUS1 promoter resulted in approximately linear GFP expression dynamics with Hill coefficients <1, while the engineered FUS1J2 promoter resulted in a much steeper, sigmoidal increase from uninduced to induced GFP expression (Hill coefficients >1) (Table 1). The FUS1J2 promoter was originally designed to convert the graded MAPK transcriptional response into a switch-like response[23] and has been used here successfully to the same effect.

A key feature of quorum sensing behavior is the requirement for a high population density to activate gene expression. However, cells are able to exhibit intermediate levels of FUS1 mediated expression in response to intermediate levels of pheromone[44] (Figure 3). Therefore, if a population initiates quorum sensing behavior at a relatively low population density, with pheromone concentration not high enough to elicit a maximal pFUS1-GFP response, then the population will have a gradual increase in quorum sensing regulated gene expression. Another important feature is that a subset of cells in a population responding to pheromone will arrest cell division in the G1 phase, thereby slowing the transition from low to high population density, contributing to the graded nature of the response. Alternatively, if the initiation of pheromone communication is delayed until a higher population density, then the effective pheromone concentration will be high enough to elicit a rapid population-wide GFP response. These important differences in circuit output can be thought of as overlaid positive (α-pheromone expression) and negative (α-pheromone diffusion at a low population density, and pheromone-induced growth arrest) feedback and are graphically represented in Figure 4. This concept explains the differences in GFP-reporter expression observed between the native FUS1 and FUS1J2 promoter strains (Figure 3). The higher level of basal expression from the native FUS1 promoter resulted in initiation of positive feedback pheromone expression at a lower population density relative to the FUS1J2 promoter, so that quorum sensing behavior was self-limiting and the transition from low to high autoinduced GFP-reporter expression was extremely gradual (Figure 3a, Table 1). This explains the negative cooperativity observed for GFP expression dynamics of the native FUS1 promoter positive feedback strains (Hill coefficients of <1; Table 1). The more

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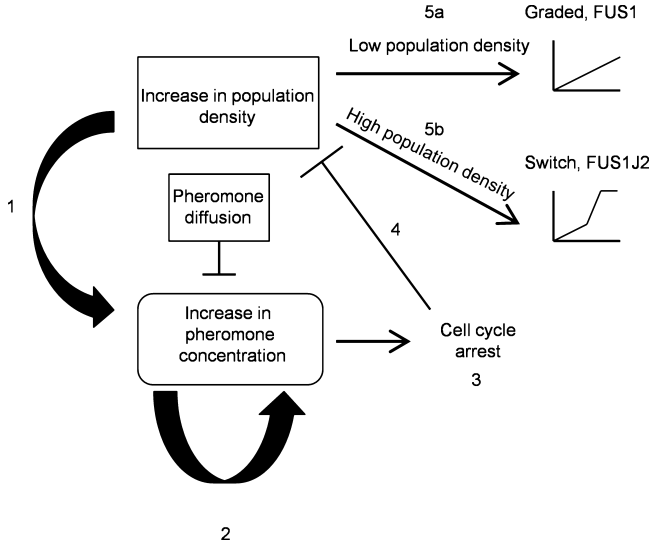

Figure 4. Model of graded signaling as a consequence of overlaid positive and negative feedback. (**1**) As populations transition from relatively low to high population density, α-pheromone concentration increases. (**2**). Increased pheromone concentration induces the expression of the α-pheromone gene from the FUS1 or FUS1J2 promoter (Figure 1a) as part of a positive feedback loop. (**3**) Cells responding to pheromone arrest growth in the G1 phase of the cell cycle. (**4**) As a proportion of a pheromone producing population undergoes cell cycle arrest, the rate of population growth decreases, delaying the attainment of high population density, and therefore a higher pheromone concentration. The output of this model is either a graded, linear increase in GFP reporter expression in the case of the native FUS1 promoter (**5a**) (Figure 3a, Table 1) or a switch-like sigmoidal increase with the FUS1J2 promoter (**5b**) (Figure 3b, Table 1). This distinction is dependent on the population density at which positive feedback pheromone expression is initiated. The native FUS1 promoter has a significantly higher level of basal expression compared to the FUS1J2 promoter,[23] meaning that expression of α-pheromone is noisier at a low population density, initiating positive feedback, α-pheromone diffusion, and growth arrest before population density and pheromone concentration are sufficient to elicit maximal GFP-reporter expression. With a lower level of basal expression the FUS1J2 promoter requires a higher population density (and pheromone concentration) to initiate positive feedback pheromone expression, meaning that the growth arrest response and pheromone diffusion do not limit the attainment of a high population density and maximal GFP-reporter response.

"switch-like" increase in GFP expression observed with the FUS1J2 promoter (Figure 3b, Table 1) is consistent with the fact that the level of basal expression is much lower than that seen with the native FUS1 promoter and the level of induced expression is higher.[23] This allowed a higher population density to be reached before the positive feedback expression of α-pheromone was initiated, meaning that maximal GFP-reporter expression could be reached without quorum sensing behavior being significantly limited by α-pheromone diffusion and the growth-arrest response. The result is positive cooperativity (Hill coefficients >1; Table 1). The interaction of engineered circuit activation with host organism physiology and growth dynamics has previously been noted for yielding nonintuitive circuit outputs[48] and is evident here also.

Positive feedback is often associated with bimodality in gene expression, where instead of a normal distribution of expression levels among cells in a population, there are two separate expression profiles.[49] Although the circuits engineered here were defined by the positive feedback regulation of α-pheromone expression and exhibited "switch-like" responses in the case of the FUS1J2 promoter, bimodality in expression of the GFP reporter was never observed (GFP populations always showed unimodal, approximately normal distributions) (Figure 5). These results are consistent with previous studies that demonstrated the unimodal, graded nature of pFUS1-regulated transcription during the pheromone response.[44,50]

Aromatic Amino Acid Induced Quorum Sensing. The quorum sensing strains that were engineered with positive feedback α-pheromone production allowed dynamic control of gene expression in response to population density. Although these responses were fine-tunable according to circuit topology, they were constitutively active. This meant that normal cultivation techniques (including preculturing regimens) could not be followed, as quorum-sensing behavior and subsequent cell-cycle arrest would occur during the preculture period. In order to analyze these strains, they had to be grown from single colonies on solid media (transformation plates) and inoculated straight into experimental shake flasks with no preculture. This situation is not useful for industrial growth of *S. cerevisiae*, as preculturing to high population densities prior to bioreactor inoculation is necessary to minimize process times, maximize product yields, and maintain fermentation reproducibility. Furthermore, quorum sensing was still triggered at relatively low population densities even in the improved pFUS1J2-driven circuit.

In order to address these problems, we implemented a greater level of user control by making pheromone expression conditional on the presence of aromatic amino acids in the growth media. The amino acid responsive quorum sensing circuits (Figure 1b) were designed so that strains could be grown in preculture without any aromatic amino acids and therefore without any quorum sensing behavior. Furthermore they were designed to allow the fine-tuning of α-pheromone communication in response to aromatic amino acid concentration and type (e.g., tryptophan, phenylalanine, or tyrosine). Population behavior could then be altered via simple modifications to the growth medium.

Amino acid concentration was expected to influence the response[40] and potentially initiate cell-cycle arrest at different population densities. Therefore, in order to understand the pheromone communication and response dynamics of these circuits, strains were grown in the absence of the inducer to a population density at which pheromone communication would elicit a response (OD_{660} of 1, as determined from the FUS1 circuits) prior to the addition of different types and concentrations of aromatic amino acids (Figure 6a). In growth medium without amino acids, only basal levels of GFP reporter were observed. This indicates that α-pheromone production is very low under these conditions, consistent with the sender–receiver growth arrest assay (Figure 2a). When quorum sensing strains were incubated with increasing concentrations of aromatic amino acids, pheromone-responsive GFP-reporter expression increased in a dose-dependent manner (Figure 6a). The dose–response dynamics were also tunable according to the type of aromatic amino acid used: GFP expression was initiated at lower concentrations of tryptophan (Trp; 0.5 μg/mL) than of phenylalanine (Phe) and tyrosine (Tyr) (5 μg/mL for both). Consistent with the background levels and pheromone dose–responses observed for the previous circuits, using mfα2 resulted in lower basal GFP expression and a slightly damped response dynamic relative to mfα1. Phe

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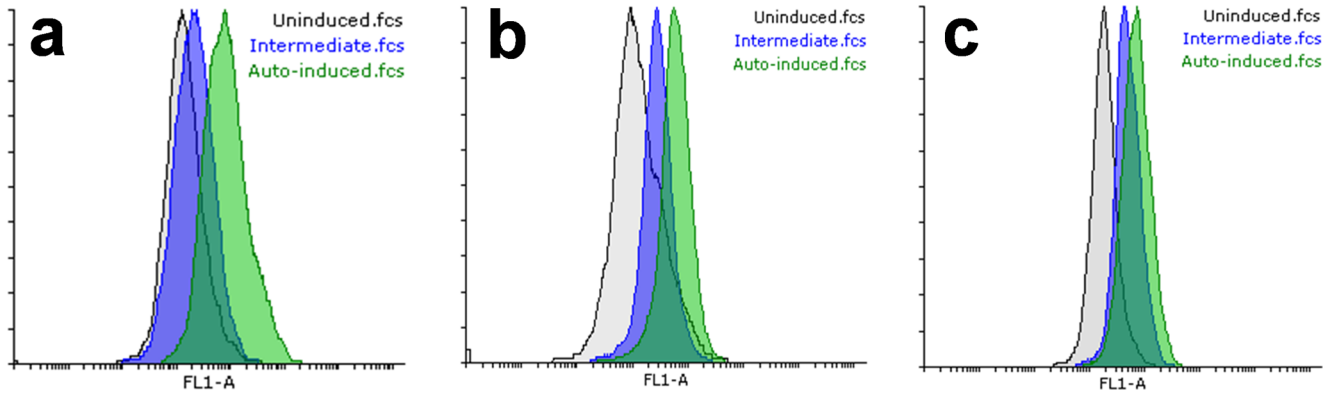

Figure 5. Graded autoinduced GFP expression. Overlayed GFP fluorescence intensity histograms from representative "uninduced" (GFP $\leq$ 1800 au), "intermediate" (GFP 3000–4000 au), and "autoinduced" (GFP $\geq$ 4500 au) time-points in fermentations of (a) strain JRS05 that produces α-pheromone using the native FUS1 promoter and the mfα1 gene, (b) strain JRS07 that produces α-pheromone using the engineered pFUS1J2 promoter[23] and the mfα1 gene, and (c) strain JRS10 that controls mfα1 expression with the ARO9 promoter. Strains expressing α-pheromone with the mfα2 gene showed the same trend. The *x*-axes (FL1-A) represent logGFP fluorescence, and *y*-axes show the cell count for each fluorescence value.

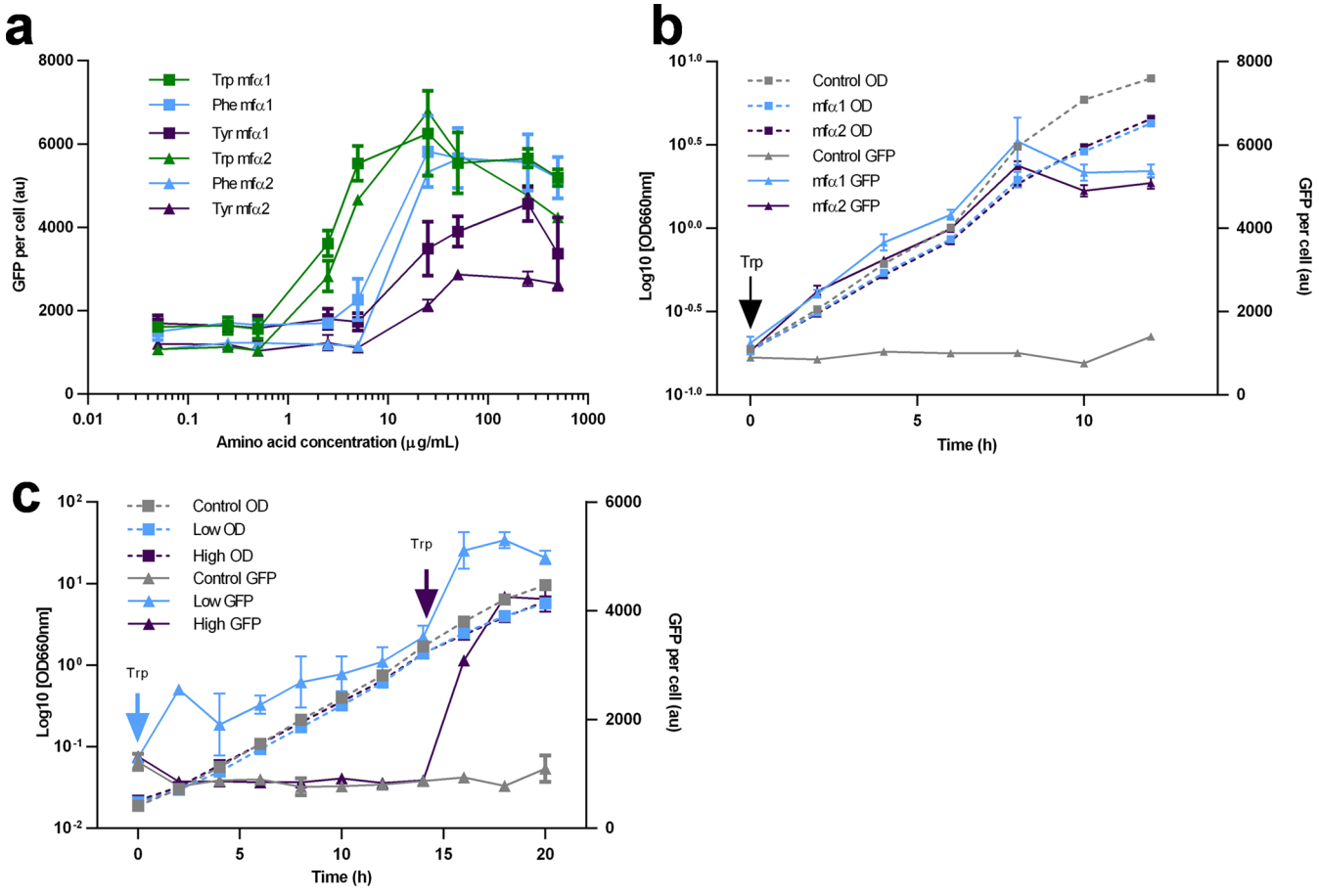

Figure 6. Aromatic amino acid induced quorum sensing. (a) Strains carrying pARO9-mfα1 or pARO9-mfα2 constructs (JRS10 and JRS11) were grown to an OD_{660} of 1 in amino acid free media (shake flask) before being treated with the indicated concentrations of tryptophan (Trp), phenylalanine (Phe), or tyrosine (Tyr) for 4 h (200 μL aliquots, 96-well plate) prior to GFP fluorescence measurement. (b) Strains pARO9-mfα1, pARO9-mfα2, and the non-pheromone-producing control strain (JRS09) were incubated with 100 μg/mL tryptophan (black arrow) after two preculture passages without any amino acids present in the media, and inoculation into the main culture at an OD_{660} of 0.18 (shake flasks). (c) Control and pARO9-mfα2 strains were inoculated into main culture at an OD_{660} of 0.02. The control strain and one lot of pARO9-mfα2 replicates were treated with 100 μg/mL tryptophan upon inoculation ("Low", blue arrow), while another set of pARO9- mfα2 replicates were treated with 100 μg/mL tryptophan after the population had grown to an OD_{660} of 1.4 ("High", purple arrow). GFP fluorescence and OD_{660} were used to measure the pheromone response and population density respectively for all strains. Markers and error bars represent mean and standard deviation of biological triplicates.

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treatment resulted in the sharpest increase in GFP-reporter expression with a stable plateau of maximal expression. In contrast, Trp and Tyr treatment resulted in more gradual GFP-reporter increases with expression declining slightly after maximal expression being reached (25 μg/mL for Trp and ~250 μg/mL for Tyr) and maximal GFP-reporter expression being lower in Tyr-treated populations (Figure 6a). Our results with respect to the response level to different aromatic amino acids are in accordance with previous results, which showed that the ARO9 system responded more strongly to Trp than to Phe and Tyr.[40] These circuits render the pheromone response inducible by aromatic amino acids, with a fine level of control over the dynamic response according to the concentration and type of aromatic amino acid.

The amino acid dose−response experiments were carried out in populations of the same density (OD_{660} of 1) and therefore represent a static response system. The aromatic amino acid responsive circuits (Figure 1b) were also used to demonstrate quorum sensing behavior in a dynamic system by addition of aromatic amino acids at low population density (Figure 6b). Although pheromone production was initiated with Trp (100 μg/mL) at low population density ($OD_{660\ nm}$ 0.185), maximal autoinduced GFP-reporter expression was not observed until an OD_{660} of 1.8 was reached, 8 h later (Figure 6 b). As with the dose−response assay (Figure 6a), pheromone expression using the mfα2 gene resulted in a slightly damped GFP-reporter expression profile relative to the mfα1 strain (Figure 6b). The quorum sensing strains showed reduced growth rates and final population densities (0.27 h^{-1}, OD_{660} of ~4) relative to the control strain (0.34 h^{-1}, OD_{660} of ~8) (Figure 6b) due to the cell-cycle arrest phenotype of cells responding to α-pheromone.[46] A whole-population growth arrest phenotype was not observed, consistent with results from the positive feedback quorum sensing strains (Figure 3).

The pARO9-α2 strain was also used to test the effect of the population density at which quorum sensing is initiated on circuit output. When quorum sensing was initiated (with 100 μg/mL Trp) at an extremely low OD_{660} of 0.02 (Figure 6c), a graded, non-cooperative GFP response profile (Hill coefficient 0.59 ± 0.12) was obtained. In a separate fermentation, quorum sensing was initiated at a 70-fold higher OD_{660} of 1.4 (Figure 6 c), resulting in the GFP expression profile being positively cooperative and switch-like (Hill coefficient of 3.12 ± 0.79). Such switch-like dynamics are commonly attainable via the use of chemical inducers such as β-estradiol[51] that act directly on the output gene. In contrast, our tryptophan induction system acts to induce pheromone communication (rather than GFP expression directly) and consequently requires that cells be in close proximity. This mechanism represents a new inducible expression system in *S. cerevisiae* that is distinct from other commonly used systems[52] due to the population-density-dependent nature of the output and the multitude of cellular changes that occur as part of the pheromone response.[20]

These results (Figure 6c) are consistent with the conceptual model that explains the differences between the FUS1 and FUS1J2 promoter strains (Figure 4) where initiation of pheromone communication at a low population density results in a graded GFP-reporter response while initiation at a high population density induces a switch-like response, although in this instance the α-pheromone diffusion effect appeared to be the driving influence behind the graded response as only a minor decrease in pARO9-mfα2 population growth rate was observed when pheromone communication was induced at a low population density. These results are also consistent with a population level "AND" logic gate where both high cell density and high aromatic amino acid concentration are required to maximally induce the pheromone response. The AND gate reinforces the population-density-dependent nature of the GFP response and exemplifies the tuneability of the pARO9-α2 strain quorum sensing behavior. Similar to the positive feedback circuits, the aromatic amino acid responsive circuits always resulted in unimodal GFP population distributions (Figure 5c).

Summary and Conclusions. The quorum sensing circuits developed in this study serve as modular genetic control programs that enable autonomous and dynamic control of gene expression. Furthermore, these programs incorporated the well-characterized MAPK-mediated pheromone response in yeast and have the potential to be interfaced with other synthetic MAPK circuits[21,23,24,53] via the incorporation of α-pheromone expression constructs. The only other quorum sensing circuit that has been engineered in *S. cerevisiae* incorporated positive feedback production of a plant hormone as an intercellular signaling molecule[18] and resulted in autoinduction being triggered at population densities similar to those observed in the native FUS1 promoter positive feedback loops created in this study. Although the plant hormone signaling system was highly orthologous to native yeast processes, interfacing it with endogenous metabolism required growth on galactose (an expensive and therefore industrially irrelevant carbon source) for strain viability. The quorum sensing circuits developed here were minimally adapted from endogenous genetic architecture in *S. cerevisiae* and reflect an emerging theme in synthetic biology that favors systems that can be integrated more seamlessly with endogenous host organism physiology.[2]

All of the circuits showed modest dynamic ranges of GFP expression with the best being about 7-fold (Table 1). This was in part due to the fact that the destabilized version of GFP that we used underestimates induction levels.[38] The yeast enhanced green fluorescent protein (yEGFP) has an approximate maturation time of 1 h and a half-life of ~7 h, making it unsuitable for measuring dynamic changes in gene expression.[38] For these reasons we chose to use a destabilized version of yEGFP that has a half-life of 30 min and never reaches full fluorophore maturation (on average) making it suitable for detecting rapid and transient changes in gene expression.[38] Even considering the effects of the destabilized GFP, the dynamic ranges that we observed are consistent with the only other synthetic quorum sensing network in yeast (~10-fold using the highly stable yEGFP[18]). A greater dynamic range would be desirable for application of these circuits in a metabolic engineering context because it would afford a greater separation of growth and production phases. The dynamic ranges of these circuits could potentially be improved for future applications by using the FUS1J2 promoter to control circuit output in place of the noisier native version of the FUS1 promoter that we used (Figure 1). While the dynamic ranges observed in the positive feedback circuits were slightly better than those obtained with the aromatic amino acid responsive circuits, the latter are far more suitable for industrial application due to the fine level of control over circuit output that is definable by environmental conditions (Table 1). Strains carrying these circuits can be grown in preculture, allowing high biomass levels to be achieved prior to inoculation of main fermentation volumes. The constitutive activation of the positive feedback circuits (there is no way to "turn them off")

Table 2. Primers Used in This Study

primer name	5′ to 3′ sequence
BAR1KOF	CGCCTAAAATCATACCAAAATAAAAAGAGTGTCTAGAAGGGTCATATACCAGCTGAAGCTTCGTACG
BAR1KOR	CTATATATTTGATATTTATATGCTATAAAGAAATTGTACTCCAGATTTCTCACTATAGGGAGACCGGCAG
BAR1DCF	AGAGATGCGTTGTCCCTGTT
BAR1DCR	ATGGTCAGAATGGGCGCTTG
yEGFPDSF	TATTTCGAATTCATGTCTAAAGGTGAAGAATTATTC
yEGFPDSR	TATTATGGATCCGATCTGCCGGTAGAGGTGTG
mfα1F	ATAATAGAATTCATGAGATTTCCTTCAATTTTTAC
mfα1R	AATAATGGATCCGATTCGATTCACATTCATC
mfα2F	TACGAATTCATGAAATTCATTTCTACCTTTCTCAC
mfα2R	ATAATAGGATCCAGAGCTCCAACCATAGTGAAC
pFUS1F	TATTATCTCGAGATCAACAACAGGGTCAGCAG
pFUS1R	TATTATGAATTCTTTGATTTTCAGAAACTTGATGG
pFUS1J2F	GAGCTCCTCGAGCCCTCCTTCAATTTTTCTG
pFUS1J2R	ATCGATGAATTCTTTGATTTTCAGAAACTTGTTGG
pARO9F	TATTATCTCGAGTTGCCGCGTGGAGACATCTG
pARO9R	TATTATGAATTCTGAGTCGATGAGAGAGTGTAATTG

preclude their application in industrial fermentations and make them difficult to handle.

The application of aromatic amino acid responsive pheromone quorum sensing for the dynamic control of metabolite production in an industrial setting would require specific retuning in each case. Therefore the quorum sensing behavior observed for the pARO9 strains (Figure 6b,c) serve only as examples of how they might be used. In theory it should be possible to scale the use of these circuits up in a bioreactor setting so that a significantly higher population density can be reached before tryptophan is used to initiate quorum sensing. However, the scale-up of synthetic gene networks has proven to be a non-trivial problem with correct circuit function being degraded in some cases.[54] The addition of purified amino acids to an industrial fermentation could be cost-prohibitive (depending on product value); this work therefore represents a "proof of concept" for the conditional control of pheromone quorum sensing. Although quorum sensing populations showed significantly reduced growth rates due to the growth arrest phenotype of cells responding to pheromone,[46] this may serve as a mechanism to decouple the population growth phase from the compound production phase. Future work will explore the potential of pheromone quorum sensing for the dynamic control of metabolic pathways that impose metabolic burden and/or toxicity limitations on population growth phases. Specifically, this work will involve applying the aromatic amino acid responsive quorum sensing circuits to the bioreactor scale production of an industrially relevant fine chemical.

METHODS

Media. Strains were grown in chemically defined liquid medium with 5 g/L ammonium sulfate and 2% glucose (w/v). Chemically defined media contained 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 4.5 mg/L $ZnSO_4{\cdot}7H_2O$, 0.3 mg/L $CoCl_2{\cdot}6H_2O$, 1 mg/L $MnCl_2{\cdot}4H_2O$, 0.3 mg/L $CuSO_4{\cdot}5H_2O$, 4.5 mg/L $CaCl_2{\cdot}2H_2O$, 3 mg/L $FeSO_4{\cdot}7H_2O$, 0.4 mg/L $Na_2MoO_4{\cdot}7H_2O$, 1 mg/L H_3BO_4, 0.1 mg/L KI, 15 mg/L EDTA, 50 μL biotin, 1 mg/L calcium panthothenate, 1 mg/L nicotinic acid, 25 mg/L myo-inositol, 1 mg/L thiamine HCl, 1 mg/L pyridoxal HCl, 0.2 mg/L *p*-aminobenzoic acid. For solid medium, 15 g/L agar was added. During strain construction Sigma dropout solution (-Ura, -Trp, -His, -Leu) was used to complement appropriate auxotrophies in agar plates (same composition as chemically defined media above), while YPD or YPG supplemented with appropriate antibiotics was used during gene deletion procedures. *E. coli* DH5α strains were grown in LB medium with kanamycin.

Strains and Plasmids. DNA manipulation and propagation were carried out using standard techniques.[55] All *S. cerevisiae* transformations were carried out using the lithium acetate method.[56] All *S. cerevisiae* genes used were amplified from CEN.PK2-1c genomic DNA extracted using a MoBio microbial DNA isolation kit. Gene deletions were performed with the reusable LoxP-KanMX-LoxP cassette as described previously.[57] All KanMX markers were removed via expression of Cre recombinase from pSH65; pSH65 was subsequently removed by growing strains without phleomycin for 48 h and patch plating colonies on YPD plates with and without phleomycin. For knockout of BAR1, primers BAR1KOF and BAR1KOR were used to amplify the LoxP-KanMX-LoxP cassette from the pUG6 plasmid (Table 3). Deletion was confirmed by PCR using primers BAR1DCF and BAR1DCR which flank the BAR1 ORF location.

Primers used in this study are shown in Table 2, and plasmids are shown in Table 3. All plasmid constructs were transformed into and maintained in *E. coli* DH5α. All promoter regions were amplified using primers that create a 5′ XhoI cut site and a 3′ *Eco*RI cut site. Similarly, all coding regions were amplified with 5′ *Eco*RI sites and 3′ *Bam*HI sites. Plasmid pTCW001 was constructed by insertion of the yEGFP-CLN2PEST-ADH1t region of the P30419 plasmid (amplified using primers yEGFPDSF and yEGFPDSR) into pRS406 at the *Eco*RI and *Bam*HI sites. A 700-bp region upstream from the FUS1 start codon was amplified from genomic *S. cerevisiae* DNA using primers pFUS1F and pFUS1R and inserted into the XhoI and *Eco*RI sites of pTCW001 to construct pTCW002 (pFUS1- yEGFP-CLN2PEST-ADH1t expression cassette). pTCW003 was constructed by inserting the coding region and 300 bp downstream of the stop codon of the mfα1 gene (amplified using mfα1F and mfα1R primers) into pRS413 at the *Eco*RI and *Bam*HI sites. pTCW004 was made the same way except the insert was the mfα2 gene and 3′ region (amplified using mfα2F and mfα2R primers). pTCW005 and pTCW006 were made via insertion of the pFUS1 promoter (amplified using pFUS1F and pFUS1R primers) 5′ of the mfα1 and mfα2 genes in pTCW003 and pTCW004, respectively, to create

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Table 3. Plasmids Used in This Study

name	details	origin
pRS404	TRP1 integrating vector	58, Euroscarf
pRS405	LEU2 integrating vector	58, Euroscarf
pRS406	URA3 integrating vector	58, Euroscarf
p30419	pFA6a-yEGFP3-CLN2$_{PEST}$-natMX6	59, Euroscarf
pTCW001	pRS406- yEGFP-CLN2PEST-ADH1t	this study
pTCW002	pRS406-pFUS1-yEGFP-CLN2PEST-ADH1t	this study
pRS413	URA3 low copy number vector	58, Euroscarf
pTCW003	pRS413-mfα1	this study
pTCW004	pRS413-mfα2	this study
pTCW005	pRS413-pFUS1-mfα1- mfα1t	this study
pTCW006	pRS413-pFUS1-mfα2- mfα2t	this study
pTCW007	pRS413-pFUS1J2-mfα1- mfα1t	this study
pTCW008	pRS413-pFUS1J2-mfα2- mfα2t	this study
pTCW009	pRS413-pARO9-mfα1- mfα1t	this study
pTCW010	pRS413-pARO9-mfα2- mfα2t	this Study
pNTI144	PFUS1J2-STE4-TADH1	23
pUG6	LoxP-KanMX-LoxP cassette	57, Euroscarf
pSH65	Galactose inducible cre-recombinase	57, Euroscarf

pFUS1-mfα1-mfα1t and pFUS1-mfα2-mfα2t expression cassettes. pTCW007 and pTCW008 were made by inserting the 500bp FUS1J2 promoter amplified from pNTI144[23] (using pFUS1J2F and pFUS1J2R primers) 5′ of the mfα1 and mfα2 genes in pTCW003 and pTCW004 to make pFUS1J2- mfα1-mfα1t and pFUS1J2-mfα2-mfα2t expression cassettes. Similarly, pTCW009 and pTCW010 was made by inserting the ARO9 promoter region (amplified with pARO9F and pARO9R) 5′ of the mfα1and mfα2 genes in pTCW003 and pTCW004 resulting in pARO9-mfα1-mfα1t and pARO9-mfα2-mfα2t expression cassettes. All plasmids were sequenced to check for PCR-mediated mutations introduced during the cloning steps.

Strains (Table 4) were constructed by transforming the plasmid components from Table 3 successively into the relevant deletion strain and selecting on appropriate dropout and/or antibiotic containing media. Yeast integrating plasmid transformants were screened for correct genomic integration as described previously.[60] Plasmids containing genes that would potentially result in pheromone production were always integrated into the relevant strain background as the last strain construction step prior to analysis. All quorum sensing strains and the control strain had all auxotrophies repaired so that (a) only the genetic differences of interest were compared and (b) only the amino acid of interest needed to be present in the growth media of aromatic amino acid responsive quorum sensing strains. Each biological replicate was derived from an individual transformant colony.

Sender−Receiver Assays. Halo assays similar to those used to determine mating type and pheromone sensitivity[42] were carried out where MATa cells engineered to produce α-pheromone were used as "senders" and non-pheromone-producing MATa cells were used as "receivers". Pheromone-sensitive bar1Δ cells (JRS09) were grown for 3 days in chemically defined liquid media and spread uniformly onto a chemically defined agar plate to act as α-pheromone receivers. After incubating the plate for 30 min at room temperature to allow for absorbance into the solid medium, approximately 1 cm^2 patches of sender strains were spread on top of the bar1Δ receiver lawn, and the plate was incubated at 30 °C for 48 h. Clearing in the receiver lawn around the sender strain patches relative to the control strain (non-pheromone-producing, JRS09) was indicative of α-pheromone production by the sender strains causing growth arrest in the receiver lawn.

For "conditioned media" experiments, pheromone-producing sender strains were grown to either low population density, non-autoinduced ($OD_{660} \leq 0.2$ for pARO9 strains and ≤ 0.07 for all others, GFP fluorescence per cell ≤ 1500 au) or high population density, autoinduced ($OD_{660} \geq 2$, GFP ≥ 5000 au) conditions. Aromatic amino acid responsive sender strains were grown with 100 μg/mL tryptophan. Pheromone-producing sender and non-pheromone-producing receiver cells were removed from media by centrifugation at 13 000g for 5 min. Receiver strains were then incubated at a starting OD_{660} of 1 for 3.5 h in media conditioned by sender strain growth prior to measuring GFP fluorescence as below.

Flow Cytometry. GFP fluorescence measurements were carried out on an Accuri C6 flow cytometer (BDbiosciences). Culture samples (0.1−2 mL as required) were sonicated for 30 s (to break cell clumps and buds) prior to the recording of

Table 4. Yeast Strains Used in This Study

name	genotype	notes	origin
CEN.PK2−1c	MATa; ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8C; SUC2	haploid MATa lab strain	Euroscarf
JRS01	CEN.PK2-1c: ura3::pRS406-pFUS1-yEGFP-CLN2PEST	expresses GFP in response to α-pheromone	this study
JRS02	CEN.PK2-1c: bar1Δ, ura3::pRS406-pFUS1-yEGFP-CLN2PEST	same as JRS01 except the α-pheromone protease BAR1 gene is deleted	this study
JRS03	CEN.PK2-1c: bar1Δ, ura3::pRS406-pFUS1-yEGFP-CLN2PEST, trp1::pRS404, leu2::pRS405	base strain	this study
JRS05	JRS03 + pRS413-pFUS1-mfα1	positive feedback quorum sensing with mfα1 gene, native FUS1 promoter	this study
JRS06	JRS03 + pRS413-pFUS1-mfα2	positive feedback quorum sensing with mfα2 gene, native FUS1 promoter	this study
JRS07	JRS03 + pRS413-pFUS1J2-mfα1	positive feedback quorum sensing with mfα1 gene, pFUS1J2 promoter	this study
JRS08	JRS03 + pRS413-pFUS1J2-mfα2	positive feedback quorum sensing with mfα2 gene, pFUS1J2 promoter	this study
JRS09	JRS03 + pRS413	control strain with auxotrophies matching "test" strains	this study
JRS10	JRS03 + pRS413-pARO9-mfα1	aromatic amino acid induced quorum sensing with mfα1 gene	this study
JRS11	JRS03 + pRS413-pARO9-mfα2	aromatic amino acid induced quorum sensing with mfα2 gene	this study

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5,000 events per sample. GFP fluorescence intensity was measured using 488 nm excitation with a 533 ± 30 nm emission filter. Populations were gated between 5×10^5 and 5×10^6 by forward scatter to exclude debris (below 5×10^5) and cell clumps (above 5×10^6). Median GFP intensity was recorded for each population. Mean GFP intensity and standard deviation were calculated from individual sample medians and reported for each strain in biological triplicate. All raw data were normalized by subtracting the autofluorescence observed from the CEN.PK2-1c base strain, which has no GFP gene. Overlay histograms were produced using Flowing Software version 2.5.

Fermentation Conditions. All time-course growth experiments were carried out in biological triplicate for each experimental strain or condition in baffled shake flasks covered in aluminum foil at 30 °C, 200 rpm, with media comprising 10% of the total shake flask volume. Positive feedback strains produced pheromone constitutively and were therefore grown from single transformant plate colonies directly into main cultures in order to avoid preculture conditions affecting quorum sensing behavior. Individual colonies that did not display quorum sensing behavior (about 1 in 6) were disregarded as background transformants not containing pheromone-producing plasmid. Amino acid inducible and other non-positive feedback strains were able to be grown in preculture without any pheromone quorum sensing. Precultures were grown to mid log phase ($OD_{660\ nm}$ of 1−3) before being diluted into the final culture volume to a starting OD_{660} of 0.2 (or as stated otherwise). Population density was measured spectrophotometrically using absorbance at 660 nm wavelength.

For dose−response experiments, precultures of indicated strains were grown as above before being divided into 200 μL aliquots in 96-well round-bottom plates (Greiner 650161) and treated with the relevant pheromone (Genscript)/amino acid/conditioned-media in triplicate for each concentration. Aliquots were incubated at 30 °C, 200 rpm, for 3.5−4 h before being analyzed on an Accuri C6 flow cytometer as specified above.

Statistical Analysis. For each time point or dose−response concentration, GFP fluorescence and OD_{660} are reported as the mean of biological triplicate cultures with error bars representing ±1 standard deviation. Population growth rates were calculated using linear regression of log normalized average OD_{660} values from triplicate fermentations for each strain (all $R^2 \geq 0.99$). Hill coefficients for time-course GFP profiles were determined (similarly to previously described[22]) using a four parameter variable slope dose−response model in GraphPad Prism 6 using the equation: $Y = GFP\text{min} + ((GFP\text{max} - GFP\text{min})) \div (1 + 10\text{^}\log GFP50 - X) \times nH))$ where Y = GFP response, X = ln OD_{660}, GFPmin = the non-autoinduced GFP per cell, GFPmax = fully autoinduced GFP, $GFP50$ = the half-maximal GFP, and nH = Hill coefficient. GFPmax was constrained to the maximum value observed for each strain in order to allow accurate fitting of the Hill equation. Hill coefficients were reported with ± standard error values generated by the curve fitting algorithm. Significant differences between conditioned media treated "receiver" GFP-reporter levels were assessed in biological triplicate for each "sender" strain using a two-tailed two-sample t test with unequal variance in Microsoft Excel.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

pFUS1, native pheromone responsive promoter; pFUS1J2, mutated version of the FUS1 promoter with lower basal and higher induced expression; GFP, green fluorescent protein; pARO9, aromatic amino acid responsive promoter

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