BRIEF REPORT

Engineering *Saccharomyces cerevisiae*-based biosensors for copper detection

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Abstract

Heavy metals, that is Cu(II), are harmful to the environment. There is an increasing demand to develop inexpensive detection methods for heavy metals. Here, we developed a yeast biosensor with reduced-noise and improved signal output for potential on-site copper ion detection. The copper-sensing circuit was achieved by employing a secondary genetic layer to control the galactose-inducible (GAL) system in *Saccharomyces cerevisiae*. The reciprocal control of the Gal4 activator and Gal80 repressor under copper-responsive promoters resulted in a low-noise and sensitive yeast biosensor for copper ion detection. Furthermore, we developed a betaxanthin-based colorimetric assay, as well as 2-phenylethanol and styrene-based olfactory outputs for the copper ion detection. Notably, our engineered yeast sensor confers a narrow range switch-like behaviour, which can give a 'yes/no' response when coupled with a betaxanthin-based visual phenotype. Taken together, we envision that the design principle established here might be applicable to develop other sensing systems for various chemical detections.

INTRODUCTION

Heavy metals are naturally occurring chemicals that are present in the environment. However, due to massive industrialization, rapid urbanization and intensive agriculture, the accumulation of heavy metals in the environment led to an imbalance in environmental and ecological impacts (Wu et al., 2016). Some of the heavy metals (Cu, Mn, Zn and Co) are essential trace elements, playing an important role in maintaining the enzyme activities. For instance copper is an essential trace element that is required for various cellular enzymes including cytochrome c oxidase, superoxide dismutase and dopamine monooxygenase (Luza & Speisky, 1996). Other heavy metals (Cd, Ag and Hg) are not essential but interfere with the cellular metabolism,

and disrupt biological functions. Nevertheless, when heavy metals are in excess amounts, they are all deleterious to living organisms (Alissa & Ferns, 2011; Briffa et al., 2020).

In the past years, techniques such as anodic stripping voltammetry (ASV) (Baldo et al., 2004), graphite furnace atomic absorption spectrometry (Bannon & Chisolm, 2001) and X-ray fluorescence spectrometry (Eksperiandova et al., 2002), have been developed for heavy-metal analysis. However, all the above detection methods rely on the use of sophisticated instruments and highly qualified staff. There is a pressing need to develop alternative and inexpensive methods that permit the on-site detection of metal ions. More recently, a microfluidic paper-based analytical device (mPAD) has been designed for quantifying metals in water, and the

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Cong Fan and Danli Zhang authors contributed equally to the experimental work.

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detection limit for copper and zinc can be as low as 0.1 ppm (Hofstetter et al., 2018; Kamnoet et al., 2021).

With the advancement of synthetic biology, there is an emerging interest in developing field-deployable 'biosensors' for water quality monitoring (Thavarajah et al., 2020). For instance, a cardiac cell-based biosensor has been employed for heavy metal detection (Liu et al., 2007). After exposure of cardiomyocytes to different heavy-metal ions, there would be characteristic changes in beating frequency, amplitude and duration, which can be monitored by the light-addressable potentiometric sensor. Nowadays, biosensors are no longer just a combination of microorganism and physical transducer for detecting specific signals (Su et al., 2011). A heavy-metal monitoring bacterial system was also reported in *Escherichia coli* (Ravikumar et al., 2012). The two-component system comprises membrane-associated sensor kinases such as ZraSR and CusSR with their cognate regulators in regulating the expression of ZraP and CusC to sense Zn(II) and Cu(II) respectively. However, the bacterial sensor had a very high background noise, and the signal output was only amplified several-fold upon the heavy-metal exposure (Ravikumar et al., 2012).

The first *Saccharomyces cerevisiae* biosensor that utilizes the copper-responsive promoter P_{CUP1} to drive the lacZ reporter for detecting Cu(II) by an amperometric method was reported two decades ago (Lehmann et al., 2000), however, the sensor could only measure the Cu(II) concentrations ranging from 0.5 to 2 mM. Since the first *S. cerevisiae* biosensor to detect Cu(II) was reported, a number of yeast biosensors for detecting heavy metals have been developed (Jarque et al., 2016). For instance, a fluorescence-based sensing system for Cu(II) was designed via the P_{CUP1}-controlled green fluorescent protein (GFP) expression (Shetty et al., 2004) or luciferase (Roda et al., 2011). More recently, P_{CUP1}-controlled expression of ADE5 and ADE7 in Δ*ade2* yeast strain to give the red pigment was also reported (Vopálenská et al., 2015). However, all the above-mentioned systems rely on the leaky CUP1 promoter, which gives relatively high background noises (Etcheverry, 1990; Romanos et al., 1992). In this study, synthetic biology design principles were applied to address the background noise commonly encountered by the CUP1-mediated sensing systems in yeast. *S. cerevisiae* was reprogrammed into a low-noise, sensitive and inexpensive device for Cu(II) detection without an additional requirement of equipment.

RESULTS AND DISCUSSION

The design of yeast biosensor with reduced noise for cu(II) detection

Although a number of yeast biosensors have been developed for the detection of heavy metals and other environmental pollutants (Jarque et al., 2016), many limitations remain with regard to their performance for on-site testing and accuracy. The copper-responsive system based on the CUP1 promoter was first reported for copper-induced expression of targeted proteins (Labbe & Thiele, 1999). However, the CUP1 promoter had a relatively high basal level because of the background copper level in the culture medium and only 20-fold of induction could be achieved upon the addition of Cu(II) (Etcheverry, 1990; Romanos et al., 1992). In *S. cerevisiae*, copper ion uptake is mediated by CTR1 and CTR3 encoded membrane-associated copper high-affinity transporters. Once inside the cell, the excess amount of copper ion is sequestrated in vacuoles (Miner et al., 2019) or by metal-binding proteins, such as metallothioneins of CUP1 and CRS5 (Pena et al., 1998). CUP1 is transcriptionally activated by Cu(II) via the copper-binding transcription factor Ace1 (Keller et al., 2005), whereas CTR1 and CTR3 are subjected to the copper-regulated transcriptional suppression mediated by the nutritional copper sensor of Mac1 (Dancis et al., 1994; Knight et al., 1996).

In this study, we reprogrammed the galactose-inducible (GAL) system into a reduced-noise and sensitive copper-sensing device. The GAL system is one of the most tightly regulated expression systems in yeast, which is subjected to glucose repression in the glucose-containing medium and de-repressed when galactose is used as the alternative carbon source (Romanos et al., 1992; West et al., 1987). GAL1, GAL7 and GAL10 mRNAs are rapidly induced >1000-fold on the addition of galactose (St John & Davis, 1981). As depicted in Figure 1A, a secondary genetic layer was introduced to control the key components involved in the GAL system, namely, the Gal4 activator and Gal80 repressor (Lohr et al., 1995). To make GAL promoters respond to copper ions, the endogenous copper-repressible promoter CTR1 from yeast was used to control the Gal80 repressor, and the Gal4 activator was put under the control of the copper-inducible promoter CUP1. The above two genetic modifications were simultaneously introduced into the yeast chromosomes by CRISPR/Cas9-mediated genome editing as previously described (DiCarlo et al., 2013).

Characterization of the performance of yeast biosensor for Cu(II) detection

Although the Gal4 activator would be expressed at a certain amount due to the leakiness of P_{CUP1}, the Gal80 repressor could keep the GAL system at its 'OFF' state since the copper sensor of Mac1 triggers the expression of Gal80 repressor under the CTR1 promoter at the nutritional copper level. As shown in Figure 1B, we successfully addressed the leaky problem for copper detection commonly encountered by other studies. BY4741-derived strain JS-CR harbouring plasmid
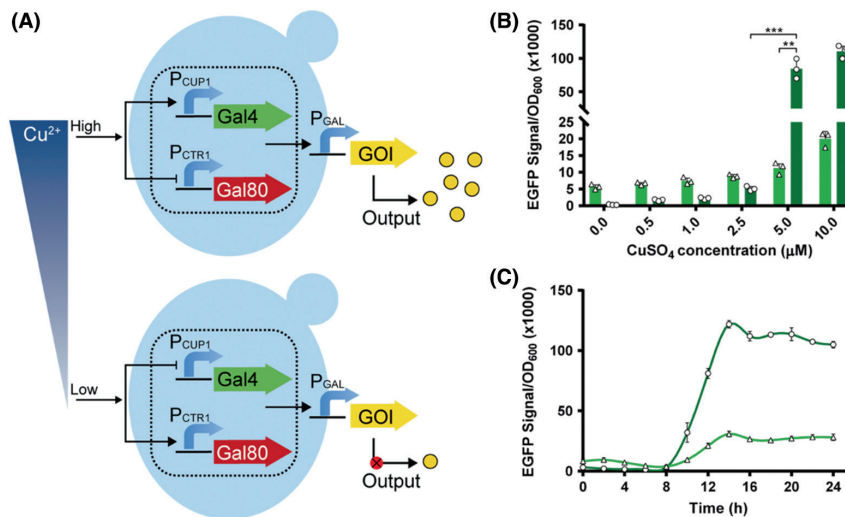
FIGURE 1 Fabrication of a reduced-noise and sensitive copper-sensing circuit. (A) Reprogramming the galactose-inducible system into a copper-sensing circuit via a layered genetic design. Gal4, the activator in the galactose-inducible system; Gal80, the repressor from the galactose-inducible system; P_{CUP1}, copper-inducible promoter; P_{CTR1}, copper-repressible promoter; P_{GAL}, galactose-inducible promoters; GOI, gene-of-interest. By introducing a layered-genetic design, the input signal from copper can be coupled with the output from galactose-inducible system. (B) EGFP signal output of BY4741 harbouring pRS425Cup1-EGFP plasmid (triangle symbols) and JS-CR harbouring pRS425Gal1-EGFP plasmid (circle symbols) in response to different concentrations of copper sulfate. Green fluorescence intensities were measured with excitation/emission at 476/512 nm. The data are collected after 24 h and represent the mean value with standard deviation. (C) Time course of EGFP levels of BY4741 harbouring pRS425Cup1-EGFP plasmid (triangle symbols) and JS-CR harbouring pRS425Gal1-EGFP plasmid (circle symbols) in response to 10 μM copper sulfate. Experiments were carried out in triplicate. Statistical analysis was performed BY use t-test (one-tailed, two-sample unequal variance; **$p < 0.01$, ***$p < 0.001$).

pRS425Gal1-EGFP (enhanced green fluorescent protein) nearly gave no appreciable EGFP signal at the nutritional copper level. When treated with 5 μM Cu(II) (~0.32 ppm), the genetic switch was turned on with a sharp increase in EGFP signal and >300-fold signal output was observed when 10 μM Cu(II) (~0.64 ppm) was added. At this moment, ~0.32 ppm Cu(II) can give a clear signal output by our yeast-based biosensor, and the detection limit is similar to the microfluidic paper-based technique (Kamnoet et al., 2021). In contrast, strain BY4741 with pRS425Cup1-EGFP (the conventional design) had a relatively high basal level of EGFP signal and only 4-fold signal output was achieved when exposed to 10 μM Cu(II) (Figure 1B). As shown in Figure 1C, time course studies of EGFP output in response to 10 μM Cu(II) revealed that the maximum output of our engineered yeast sensor could be reached around 14-h incubation. These findings confirmed that the layered genetic circuit could effectively address the leaky issue encountered by the traditional designs, and the enhanced dynamic range of our biosensor could guarantee more reliable results with less chance of false positives.

Besides, we also applied the yeast biosensor for detecting other heavy metals (Figure S1). Interestingly, we found that no appreciable amount of EGFP signal was observed upon the addition of 10 μM Fe(III), Mg(II), Mn(II), Zn(II), Co(II), Cd(II), Ag(I) or Hg(II). Previously, Cd(II) and Hg(II) were documented to trigger the repression of CTR1 and CTR3 genes (Labbe et al., 1997).

However, Cd(II) and Hg(II) require concentrations of 3 orders of magnitude greater to down-regulate the CTR3 expression. We reasoned that the synthetic circuit is at the 'OFF' state due to insufficient repression of Gal80 expression upon the exposure of 10 μM Cd(II) and Hg(II), thereby making the yeast sensor relatively specific towards the Cu(II) detection.

Copper ion detection via the betaxanthin-based colorimetric assay

We next sought to create a transformative on-site copper detection device unattainable by traditional methods. As shown in Figure 2A, we devised a betaxanthin-based colorimetric assay for potential field-deployable copper detection. Heterologous expression of CYP76AD1 (GenBank: AKH61535.1) from sugar beet *Beta vulgaris* and L-DOPA dioxygenase (DOD, GenBank: AB435372.1) from *Mirabilis jalapa* has been used for the betaxanthin production in budding yeast (DeLoache et al., 2015; Grewal et al., 2018). The monophenolase activity of CYP76AD1 can convert L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), followed by the dioxygenase activity of DOD with the ability of ring cleavage of the catechol moiety of L-DOPA, resulting in subsequent spontaneous cyclization with the α-amino group to form the betalamic acid. Betalamic acid spontaneously undergoes a Schiff base condensation reaction with amine groups via its
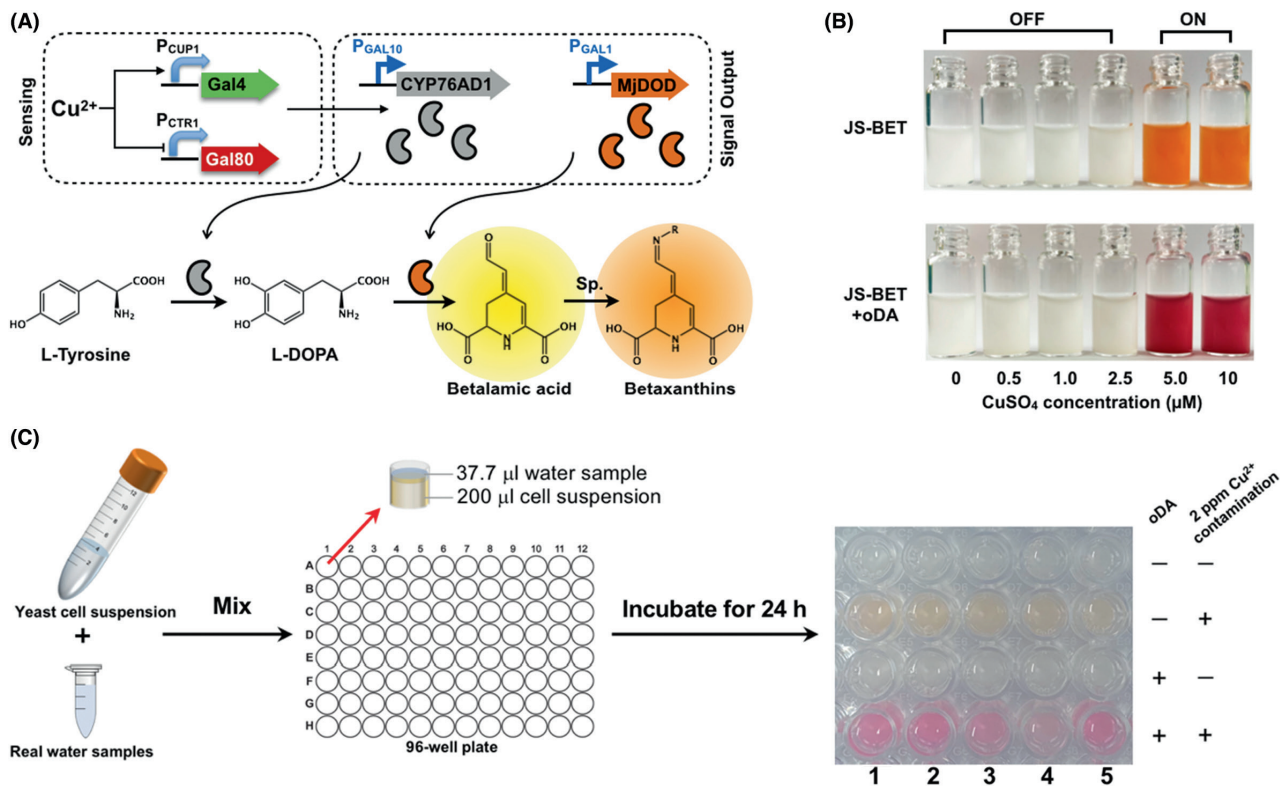
FIGURE 2 Copper ion detection by the betaxanthin-based visual phenotype. (A) Schematic description of copper detection by the product of betaxanthin in budding yeast. L-DOPA, L-3,4-dihydroxyphenylalanine; CYP76AD1, cytochrome P450 tyrosine hydroxylase from *B. vulgaris*; MjDOD, L-DOPA dioxygenase from *M. jalapa*; *Sp*., spontaneous. (B) Copper detection by betaxanthin-based colorimetric assay using strain JS-BET. Different concentrations of copper sulfate (0, 0.5, 1.0, 2.5, 5.0 and 10 μM) were added to the culture medium to mimic the copper-contaminated water. The image of betaxanthin-producing yeast was captured after 24 h cultivation. (C) Potential on-site detection of copper ion in different water samples. Different sources of water samples with or without 2 ppm copper ion contamination were tested. (1) sterile water; (2) drinking water; (3) tap water; (4) reclaimed water; (5) water from a nearby lake.

reactive aldehyde group to yield betaine with yellow to purple colour. As can be seen from Figure 2B, strain JS-BET with CYP76AD1 from *B. vulgaris* and DOD from *M. jalapa* gave noticeable orange colours when the cells were exposed to >5 μM Cu(II) (~0.32 ppm). These results indicated that the detection limit of the betaxanthin-based colorimetric biosensor can reach as low as 0.32 ppm Cu(II). The visual effect could be substantially enhanced by adding 0.5 mM amino donors such as *o*-dianisidine (oDA) (Figure 2B). Therefore, our engineered yeast sensor confers a narrow range switch-like behaviour, which can give a 'yes/no' response from the colour changes.

According to the world health organization (WHO), the Cu(II) level above 2 ppm is considered to be hazardous to humans and the environment. As a proof-of-concept, we further attempted to develop a 96-well plate-based colorimetric assay for monitoring the Cu(II) contamination in real water samples. In brief, the freeze-dried yeast cells were resuspended in the cell broth. Approximately 1:6 (volume) of water to yeast suspension were mixed together and incubated at room temperature for 24 h. As shown in Figure 2C, the control samples did not produce noticeable colours, whereas all contaminated water samples with 2 ppm Cu(II) gave the expected colour changes. Noteworthily, we found that the reclaimed water sample showed a less intense colour, indicating that there might be some other chemicals affecting the performance of our yeast sensor. Therefore, future work will be required to identify these interfering factors before the yeast biosensor can be used for practical applications.

Copper ion detection based on olfactory outputs

S. cerevisiae that capitalizes on the orthogonality and specificity of its G-protein-coupled receptor (GPCR) mating pathway has been engineered towards an 'olfactory yeast' and the resulting yeast could detect an explosive residue mimic of the odorant 2,4-dinitrotoluene (Radhika et al., 2007). More recently, an olfactory yeast biosensor that detects the hormone estradiol signal based on an odour product of isoamyl acetate was reported (Miller et al., 2020). As inspired by these 'olfactory' yeasts, we also attempted to develop odour-based olfactory outputs for Cu(II) detection.
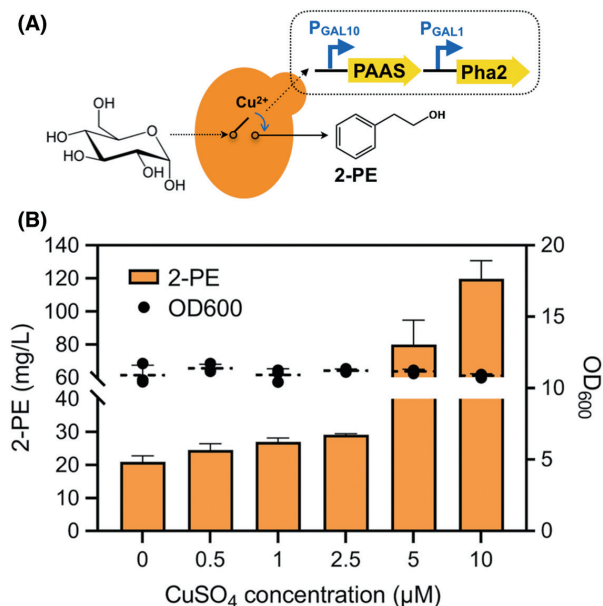
(A)

(B)

FIGURE 3 Copper ion detection based on the olfactory output of 2-phenylethanol. (A) Biosynthetic route towards 2-PE synthesis. PAAS, phenylacetaldehyde synthase from *petunia*; Pha2, prephenate dehydratase from *S. cerevisiae*. (B) the 2-PE levels in response to different concentrations of copper sulfate (0, 0.5, 1.0, 2.5, 5.0 and 10 μM). The 2-PE concentrations and OD_{600} were measured after 24 h cultivation. The black dots indicate the measured OD_{600} and the dashed lines indicate the average of OD_{600}. All experiments were performed in triplicate and the data represent the mean value with standard deviation.

(A)

(B)

FIGURE 4 Copper ion detection based on the olfactory output of styrene. (A) Biosynthetic route towards styrene synthesis. PAL2, phenylalanine ammonia lyase from *a. thaliana*; FDC1, ferulic acid decarboxylase from *S. cerevisiae*. (B) the styrene levels in response to different concentrations of copper sulfate (0, 0.5, 1.0, 2.5, 5.0 and 10 μM). The styrene concentrations and OD_{600} were measured after 24 h cultivation. The black dots indicate the measured OD_{600} and the dashed lines indicate the average of OD_{600}. All experiments were performed in triplicate and the data represent the mean value with standard deviation.

2-Phenylethanol (2-PE) is a fragrant compound that gives a rose-like smell. *S. cerevisiae* naturally synthesizes 2-PE as the fusel alcohol via the Ehrlich pathway (Huang et al., 2000). To reduce the background 2-PE level from the endogenous Ehrlich pathway, *S. cerevisiae* with Δaro10 to attenuate the Ehrlich pathway (Yuan et al., 2019) was used as the starting strain. Next, a heterologous phenylacetaldehyde synthase (PAAS, GenBank: KF500528.1) from *Petunia* (Sakai et al., 2007) and prephenate dehydratase (Pha2, GenBank: CAA86380.1) from *S. cerevisiae* were used for 2-PE production (Figure 3). As shown in Figure 3A, the background 2-PE level from the Ehrlich pathway could reach 20.93 ± 1.79 mg/L, whereas 119.69 ± 11.05 mg/L of 2-PE was obtained upon the addition of 10 μM Cu(II) for the yeast biosensor equipped with the copper-sensing genetic circuit. However, we could not clearly distinguish the samples contaminated by copper based on a blind test between the laboratory members.

To further create a reduced-noise olfactory output for Cu(II) detection, we next used the odour product of styrene that is not naturally produced by *S. cerevisiae* for a proof-of-concept study (Figure 4). Phenylalanine ammonia-lyase (PAL2, GenBank: AEE79055.1) from *Arabidopsis thaliana* and ferulic acid decarboxylase (FDC1, GenBank: QHB07990.1) from *S. cerevisiae* were used to convert L-phenylalanine into styrene
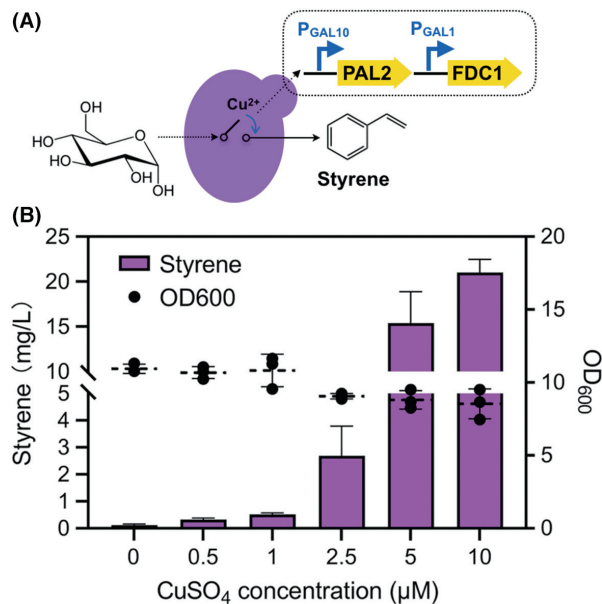
(Yuan et al., 2019). As shown in Figure 3B, the second-generation design of styrene-based olfactory output solved the problem of the background odour, and 21.0 ± 1.48 mg/L of styrene was obtained upon the addition of 10 μM Cu(II), which is above the threshold of human olfactory system. In the future, we will attempt to further improve the response time of the synthetic circuit and make the yeast sensors for real-world applications. Since many molecules can be sensed and responded by microbes, elucidating their native transcriptional regulation networks will favour the layered genetic design for the future fabrication of other biosensor systems with diverse applications.

AUTHORS' CONTRIBUTIONS

J.Y. conceived of the project and wrote the paper. J.Y. and C.F. constructed all the plasmids and strains. C.F., D.Z. and Q.M. collected the data. The authors thank the laboratory members for blind-testing all the experiments.

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CONFLICT OF INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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SUPPORTING INFORMATION

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