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Discovery and engineering of a 1-butanol biosensor in *Saccharomyces cerevisiae*

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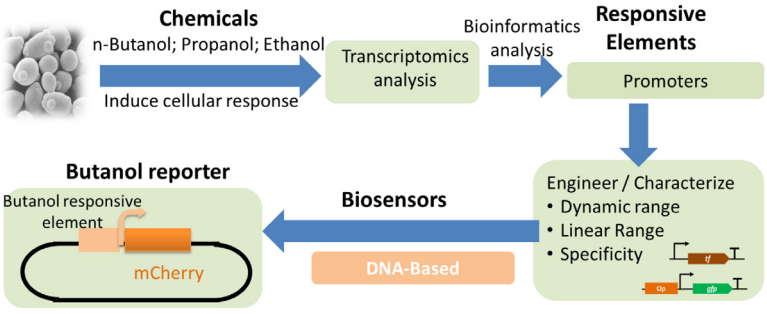
HIGHLIGHTS

- A universal method was adopted to effectively identify biosensors for any metabolite.
- Transcriptomic changes were analysed when *S. cerevisiae* was exposed to alcohols.
- Promoters were identified that responded specifically to 1-butanol.
- The promoter-based biosensors could distinguish varied 1-butanol production levels.

GRAPHICAL ABSTRACT



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ABSTRACT

The present study aimed to develop a universal methodology for the discovery of biosensors sensitive to particular stresses or metabolites by using a transcriptome analysis, in order to address the need for *in vivo* biosensors to drive the engineering of microbial cell factories. The method was successfully applied to the discovery of 1-butanol sensors. In particular, the genome-wide transcriptome profiling of *S. cerevisiae* exposed to three similar short-chain alcohols, 1-butanol, 1-propanol, and ethanol, identified genes that were differentially expressed only under the treatment of 1-butanol. From these candidates, two promoters that responded specifically to 1-butanol were characterized in a dose-dependent manner and were used to distinguish differences in production levels among different 1-butanol producer strains. This strategy opens up new opportunities for the discovery of promoter-based biosensors and can potentially be used to identify biosensors for any metabolite that causes cellular transcriptomic changes.

1. Introduction

Over the past decade, metabolic engineering and synthetic biology have been applied to engineer recombinant microorganisms to produce a broad range of products, including fuels, natural products, pharmaceuticals, and chemicals (Liao et al., 2016; Nielsen and Keasling, 2016). Recent advances in bioinformatics, synthetic biology and systems biology have contributed greatly to the design and engineering of microbial cell factories. To enhance the perfor-

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mance of these cellular factories, novel biosensor devices have been designed, built, and tested. Biosensors can sense and respond to desired products or important intermediates in engineered pathways, and have been increasingly used in the field of metabolic engineering, including high-throughput screening (Dietrich et al., 2012; Pfleger et al., 2007; Skjoedt et al., 2016), dynamic pathway control (Dahl et al., 2013; Xu et al., 2014; Zhang et al., 2012), metabolite concentration measurement and imaging (Mustafi et al., 2014; Paige et al., 2012; Rogers and Church, 2016), and adaptive evolution (Chou and Keasling, 2013; Leavitt et al., 2017; Mahr et al., 2015). Biosensor mediated strain engineering has been reviewed recently (Rogers et al., 2016; Zhang et al., 2015).

In nature, cells are evolved with a variety of sensing mechanisms, such as riboswitches, transcription factors, enzymes, or metabolite-responsive promoters. However, only a few biosensors have been well characterized to date, which significantly limited their applications. The majority of the successful biosensor applications have been developed for *Escherichia coli*. Even though *Saccharomyces cerevisiae* has several advantages over *E. coli* as a host for the industrial production of a variety of value-added chemicals due to its genetic tractability, robustness, and resistance to phage infection, there is a distinct lack of engineered control elements for sensing and regulating endogenous or heterologous pathways. To date, there are limited examples of biosensors developed in yeast for detecting and regulating metabolite concentrations.

Increasing concerns about depleting crude reserves have renewed interests in the biological production of 1-butanol, due to its desirable fuel properties and its role as a platform chemical. *E. coli* producer strains have been engineered in a number of different ways by metabolic engineering and have been shown to produce high levels of 1-butanol (Dellomonaco et al., 2011; Huffer et al., 2012; Peralta-Yahya et al., 2012). Additionally, engineered *S. cerevisiae* strains have also shown some promise for overproduction of 1-butanol (Shi et al., 2016b; Si et al., 2014), although current production level of 1-butanol in *S. cerevisiae* is currently lower than that in *E. coli*. *In vivo* metabolite biosensors would enable the construction of better yeast producer strains via dynamic control of cell metabolism. Furthermore, researchers can use the biosensors as high-throughput screening tools to select for high butanol-producing clones. However, biosensors for 1-butanol and other short-chain alcohols are currently limited. To date, there is only one report on the detection of 1-butanol using transcription factor-promoter pairs in *E. coli* (Dietrich et al., 2012). The biosensor was successfully applied to optimize 1-butanol biosynthesis in engineered *E. coli*, identifying a variant with a 35% increase in 1-butanol specific productivity. Unfortunately, this biosensor has a broad response for other short (branched) alcohols such as 2-methyl-1-butanol, and may not work well in yeast.

High-throughput genomics technologies can be applied to identify and build novel promoter-based biosensors, which may then be deployed for various purposes, including preventing the build-up of toxic intermediates in producer strains engineered with heterologous biosynthetic pathways. When intermediate metabolites accumulate to toxic levels, native stress responses are turned on to cope with the assault, but cell growth and production titer are often affected. One universal stress response is the change of promoter activity. Based on this mechanism, it allowed the identification of novel promoters responding to cadmium via genome wide gene expression profiling of the methylotrophic yeast *Hansenula polymorpha* exposed to cadmium (Park et al., 2007). In *E. coli*, researchers performed whole-genome transcriptome analysis to identify promoters that responded to farnesyl pyrophosphate (FPP), and then used these promoters to control accumulation of FPP (Dahl et al., 2013). Recently, the use of transcriptional analysis helped to identify a low pH-inducible promoter, P*gas*, in *Aspergillus niger* (Yin et al., 2017). Similarly, upon the proteomic analysis of *E. coli* in response to extracellular threonine, novel threonine promoters were discovered and utilized in screening of threonine hyper-producers (Liu et al., 2015). Besides the omics methods, a phenylalanine-responsive promoter was screened from a promoter library (Mahr et al., 2016).

To address the lack of a 1-butanol biosensor, transcriptome analysis was performed to identify 1-butanol responsive elements for use in biosensor devices based on cellular stress responses. Specifically, *S. cerevisiae* was exposed to high concentrations of various short-chain alcohols and Illumina RNA sequencing (RNA-seq) was performed to profile the transcriptome of the cells. Then the genes were determined that were differentially expressed in the presence of each alcohol, and candidate genes were shortlisted to those that responded only to 1-butanol. Promoters of these candidates were further tested and engineered for their specificity and sensitivity to 1-butanol. Finally, two optimized promoters demonstrated the possibility to screen different mutant strains for increased production of 1-butanol.

2. Materials and methods

2.1. Strains

All plasmid construction was performed with *E. coli* strain DH5α as described by Sambrook and Russell (Sambrook and Russell, 2001). *S. cerevisiae* strain HZ848 (*MATα ura3Δ ade2-1 his3-11,15 leu2-3,112 can1-100 trp1-1*) was used as the host strain in this study. The 1-butanol producing strain, the *adh1Δ* strain, was adopted from a previous study (Shi et al., 2016b). The method used for yeast transformation was the standard LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007).

2.2. Media and growth conditions

E. coli cells were cultured at 37 °C and 200 rpm in Luria-Bertani (LB) containing 100 mg/L ampicillin when needed.

For transcriptome and quantitative RT-PCR analyses, *S. cerevisiae* strains were cultivated in YPAD medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate and 2% glucose) at 30 °C with 250 rpm agitation in baffled shake-flasks. When cells reached early log phase, 1-butanol, 1-propanol, or ethanol were added at the concentration of 0, 0.4%, or 1% (v/v), and samples were collected 0.5 h and 2.5 h after the addition of alcohols.

To test for response to 1-butanol and other alcohols, *S. cerevisiae* strains were cultured at 30 °C and 250 rpm in YPAD or synthetic dextrose (SD) medium containing 20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids (YNB-AA) (Formedium, Hunstanton, UK), and the appropriate amino acid drop out mix (CSM-LEU, Formedium LTD). 1-Butanol or another alcohol (1-propanol or ethanol) was added after 3 h cultivation with the final concentration at 0%, 0.2%, 0.5%, or 1% (v/v). After 20 h, cells were collected for the measurement of fluorescence intensity.

To produce 1-butanol, *S. cerevisiae* strains were cultivated in YPAD medium at 30 °C with 250 rpm agitation in baffled shake-flasks for micro-anaerobic growth. For micro-anaerobic fermentation, 5 mL cultures were grown in Bellco 18 × 150 mm anaerobic glass tubes sealed with rubber stoppers and aluminum crimps (Chemglass, Vineland, NJ). Vacuum was applied through a syringe needle for 20 min, and sterile nitrogen was then added to create micro-anaerobic conditions. After 96 h, cells were collected for the measurement of fluorescence intensity and 1-butanol concentration.

2.3. Transcriptome analysis

Total RNA was isolated from *S. cerevisiae* using standard phenol-chloroform extraction methods and the RNA quality was checked using the Agilent Bioanalyzer (RIN $\geq$ 8). The RNA samples were stored at −80 °C until further processing. To generate stranded RNA-seq libraries, poly(A)+ RNA was firstly isolated using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA) and then the libraries were made using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA), both according to manufacturer's instructions. Subsequently, the libraries were sequenced on the HiSeq Rapid instrument (Illumina) to generate paired end 100 bp reads. Every sample was sequenced to a depth of around 10–15 million reads. The sequencing data were processed using a standard RNA-seq analysis pipeline at the Genome Institute of Singapore. Briefly, the sequencing reads were mapped to the reference genome of *Saccharomyces cerevisiae* (sacCer2) using BWA (-aln setting) and the expression levels (Fragments Per Kilobase of transcript per Million mapped reads or FPKMs) were calculated using Cufflinks (Trapnell et al., 2010) with all annotated Ensembl genes. Statistical significance (P-values) were calculated using the Student's *t*-test. Subsequently, Gene Ontology (GO) analysis was performed on the list of significant genes ($P < 0.05$) for each chemical treatment using DAVID Functional Annotation Tool (Huang et al., 2009) with the functional annotation clustering performed with GOTERM_BP_DIRECT only. Motif searches were then performed on the promoter sequences (1000 bp upstream of annotated start sites) of the significant genes using SCOPE with all the three in-built algorithms (BEAM, PRISM, and SPACER) (Carlson et al., 2007; Chakravarty et al., 2007).

2.4. Plasmid construction

Plasmid pRS425 harboring a *LEU2* expression cassette was adopted as the backbone plasmid. Linearized plasmid pRS425, the *mCherry* gene, and the *TEF1* terminator were obtained by PCR. Plasmid cloning was performed by Gibson Assembly Cloning Kit (New England Biolabs) following the manufacturer's instructions to assemble the linearized plasmid pRS425, the *mCherry* gene, and the *TEF1* terminator, resulting in plasmid pSS-mCherry. Plasmid pSS-mCherry was linearized by *Sac*II/*Bam*HI and was adopted as the backbone plasmid for cloning in the selected candidate promoters.

The selected candidate promoters were amplified by PCR based on information from Saccharomyces Genome Database (http://www.yeastgenome.org/). Then, each of the amplified candidate promoter was assembled into the linearized backbone pSS-mCherry plasmid.

The mCherry expression cassette controlled by $P_{YDL167C\text{-}T3}$ or $P_{YIL104C\text{-}T4}$ was introduced into the plasmid pSS99 (Shi et al., 2016a) for constructing pSS142 and pSS143, respectively. The ADH7 and ARO10* expression cassettes were obtained from plasmid pRS423-ADH7-ARO10* (Shi et al., 2016b) and inserted into *Sma*I site of plasmid pSS142 and pSS143 to form pSS153 and pSS154, respectively.

All primers were synthesized by Sigma Aldrich (Singapore). QIAprep Spin Plasmid Mini-prep Kits (Qiagen, Valencia, CA) were employed to prepare plasmid DNA from *E. coli*. All restriction enzymes were obtained from New England Biolabs (Ipswich, MA).

2.5. Analytical methods

Yeast cells containing plasmids with different promoters were cultured in the corresponding media as described above. Aliquots of 80 μL cell culture were transferred to a Corning 96-well, clear bottom fluorescence plate (ThermoFisher Scientific, Rockford, IL). The excitation and emission wavelengths for GFP and mCherry fluorescence signals were measured at 488–520 and 580–620 nm, respectively, using a Tecan Infinite M200 Pro mciroplate reader (Tecan Group Ltd., Männedorf, Switzerland). The fluorescence intensity was normalized to cell density that was determined by measuring the absorbance at 600 nm using the same microplate reader.

Samples for 1-butanol measurement were collected after 96 h of cultivation, and centrifuged at 12,000 rpm for 5 min. The supernatant was filtered and stored at −80 °C prior to analysis to minimize evaporation. The concentration of 1-butanol was measured using a Shimadzu GC–MS QP 2010 (Shimadzu Corporation, Kyoto, Japan) and HP-INNOWAX column (Agilent Inc., Palo Alto, CA) as described elsewhere (Shi et al., 2016b).

2.6. Statistical analysis

All experiments were conducted in biological triplicates unless otherwise stated. Student's *t*-test was performed to estimate whether the influence of a variable is significant. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Transcriptome analysis of S. cerevisiae's response to various alcohols

The transcriptome of *S. cerevisiae* was analysed by RNA-seq when the cells were treated with one of three different short chain alcohols, namely 1-butanol, 1-propanol, and ethanol. The yeast cells were grown to early log phase, before each alcohol was added to a final concentration of 0%, 0.4%, or 1% (v/v). The addition of 1-butanol and 1-propanol resulted in an obvious toxicity to *S. cerevisiae*, while the addition of ethanol caused less stress to *S. cerevisiae*, which is consistent with a previous study (González-Ramos et al., 2013). After 0.5 h or 2.5 h, samples were collected for RNA extraction and library construction. Clustering analysis of the sequencing data revealed that overall, all the 0.5 h samples grouped together with the control samples, indicating that longer treatment duration was required to induce major changes in the transcriptome (Fig. 1A). Hence, the subsequent analyses were focused on the 2.5 h chemical treatment datasets. Unexpectedly, however, all the samples that were treated with ethanol even for 2.5 h also clustered together with the control samples, although cells that were exposed to 1-propanol or 1-butanol for 2.5 h formed a separate distinct cluster. Consequently, much fewer genes were differentially expressed upon ethanol treatment compared to 1-propanol or 1-butanol treatment ($P < 0.05$, Student's *t*-test) (Fig. 1B). Additionally, it was found that most of the genes that were differentially expressed in the presence of an alcohol were up-regulated. Strikingly, Gene Ontology (GO) analysis revealed that many of the differentially expressed genes were involved in non-coding RNA processing functions, including tRNA modifications and rRNA maturation, suggesting a role for post-transcriptional gene regulation in the yeast's response to short chain alcohols.

Next the promoter regions (1000 bp upstream of the start codon) of the differentially expressed genes were searched for motifs. Genes up-regulated upon 1-butanol or 1-propanol treatment and genes down-regulated upon 1-propanol treatment were further investigated, since each of the three categories contained at least 100 significant genes, which allowed the identification of *cis*-regulatory elements confidently. For the 1-butanol-induced up-regulated genes, three distinct motifs were identified, including a

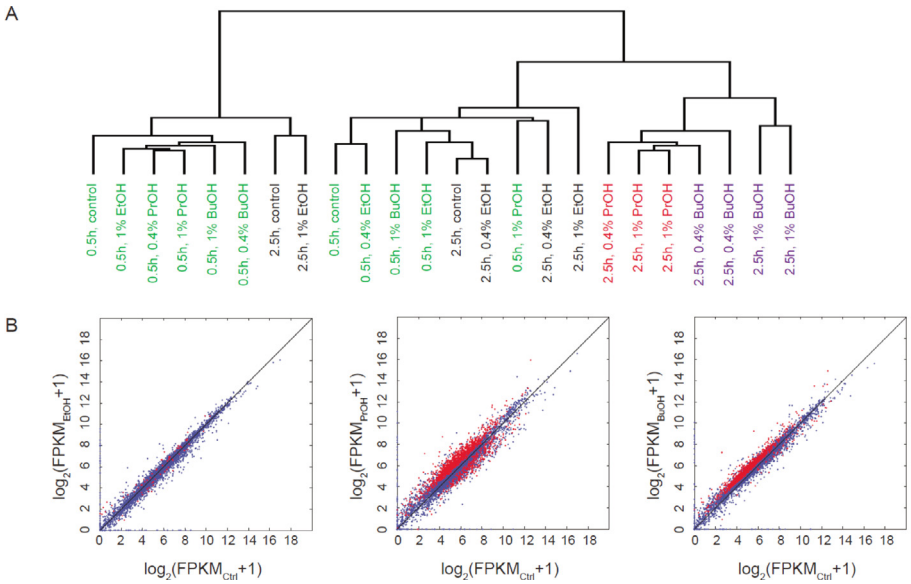

Fig. 1. Transcriptome analysis of *S. cerevisiae*'s responses to 1-butanol (BuOH), 1-propanol (PrOH), or ethanol (EtOH). For each chemical, two different concentrations (0.4% and 1%) and two different timepoints (0.5 h and 2.5 h) were tested. (A) Hierarchical clustering of the RNA-seq data. All the 0.5 h samples (in green) clustered with the control samples. Additionally, the samples that were treated for 2.5 h with 1-butanol (in purple) or 1-propanol (in red) formed a separate distinct cluster. (B) Expression levels (FPKMs) of cells that were untreated versus cells that were treated with ethanol (left), 1-propanol (middle), or 1-butanol (right). Blue dots indicate the insignificant genes, while red dots indicate the significant genes ($P < 0.05$, Student's *t*-test). Overall, there were more genes that were significantly up-regulated than down-regulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

repetitive 10 bp sequence GYTGYTGYTG and a bipartite motif CGATG-N_9-AAA. For the 1-propanol-induced up-regulated genes, three putative *cis*-regulatory elements were identified, including one motif that is rich in A's and T's (AAAATTTTTCA) and another motif that contains multiple GA-repeats (GATGAGATGAG). Lastly, for the 1-propanol-induced down-regulated genes, three novel motifs were also identified, including a motif that is rich in A's and G's (AAAAAGGAAA) and another motif that contains multiple GAC-repeats (GACGACGACGACGA).

3.2. Identification and confirmation of 1-butanol responsive biosensors

The goal of the present work was to develop biosensors for 1-butanol in *S. cerevisiae*. From the RNA-seq data, 435 genes were identified that were differentially expressed only upon treatment with 1-butanol but not with ethanol or 1-propanol. Subsequently, 50 genes were randomly selected for further analysis. These genes were separated into two groups (Table 1). In Group A, the 21 genes contained in their promoters a motif sequence that had been identified (GYTGYTGYTG or CGATG-N_9-AAA). Based on the RNA-seq analysis, the expression changes for these genes ranged from 1.5-fold to 2.8-fold. Quantitative RT-PCR experiments were performed on 8 randomly selected genes and it validated the expression changes in all of them, thereby providing confidence in the genome-wide transcriptome analysis. In Group B, the 29 genes did not contain a motif in their promoters but showed at least a 3-fold change in expression upon 1-butanol treatment.

Reporter assays were performed to evaluate whether the promoters of the 50 selected genes (Table 1) may be used in biosensor devices for 1-butanol. Each promoter (from 1 bp to around 1000 bp upstream of the translation start codon) was fused to a gene encoding the mCherry fluorescent protein. A constitutive promoter, *TEF1* promoter (P_{TEF1}) (Blazeck and Alper, 2013), was fused to mCherry (P_{TEF1}-mCherry) and adopted as a positive control, while a promoterless construct (Empty-mCherry) was adopted as a negative control. The gene cassette contained the promoter of interest, *mCherry*, and the TEF1-terminator. The resultant reporter cassettes were introduced into *S. cerevisiae* to evaluate promoter strength and response for 1-butanol.

Here, YPAD medium was used to conduct transcriptomic analysis. However, when the signal of mCherry was tested, strains grown in YPAD gave a high background. SC-LEU medium was chosen here to test the 1-butanol response due to its low background signal, enabling effective discrimination in 1-butanol response in a liquid culture assay format. Besides, SC-LEU medium is a defined minimal medium, which excluded the interference from the unclear composition of rich YPAD medium.

The engineered strains were grown to early exponential growth phase, before 1% 1-butanol was added to the culture medium to test whether the various constructs could respond properly to 1-butanol (Fig. 2). As expected, the positive control, P_{TEF1}-mCherry, showed a high expression of mCherry, while the negative control, Empty-mCherry, showed a weak expression of mCherry. Neither of the two control strains showed a response to 1-butanol. Importantly, it was found that four different reporter cassettes, namely $P_{YLR056W}$-mCherry, $P_{YDL167C}$-mCherry, $P_{YIL104C}$-mCherry, and $P_{YPL272C}$-mCherry, exhibited a significant increase in fluorescence upon exposure to 1% 1-butanol.

Here, only 8% (4/50) selected targets showed a positive response to 1-butanol. A possible explanation for the false positive is that the changes at the mRNA level could be transient and different transcripts may have varying half-lives. A transient upregulation at the mRNA level may not cause an increase on the protein expression. The reduction in false positives can be achieved by cross analysis of multiple omics data or using a priori biological knowledge to exclude unlikely relationships.

3.3. Evaluation and characterization of 1-butanol responsive biosensors

Four promoters were identified as indicators of 1-butanol concentration. However, many cellular stress responses are not stressor specific as cell responses to macromolecular damage without regard to the type (Kültz, 2005). The response from 1-butanol

Table 1
Candidate genes selected from transcriptome analysis.

Candidate gene[a]	MOTIF	Fold-change[b]
Group A		
YLR056W	gttgttgctg	2.8
YHR100C	cgatgcgcattggtaaa	2.5
YPR015C	gttgttgtcg	2.2
YNR032C-A	gctgttgttg	2.0
YDL167C	cgatgcccagtggaaaa	1.9
YNL218W	cgatgacctaccttaaa	1.8
YLR014C	cgatgagcttcataaaa	1.8
YOL125W	gttgttgttg	1.7
YGR159C	gctgttgctg	1.7
YNL023C	cgatgagctctgtgaaa	1.7
YJL011C	cgatggcattgaaaaaa	1.7
YHR170W	cgatgagcttcaaaaaa	1.6
YGL016W	cgatgaggagacaaaaa	1.6
YBL060W	cgatgacctttcttaaa	1.6
YML082W	cgatgccgtctggaaaa	1.6
YIL104C	cgatggtgatgaaaaaa	1.6
YIL103W	cgatgcattaacagaaa	1.6
YMR239C	cgatgagctcatgaaaa	1.6
YMR312W	cgatgagcagaatgaaa	1.6
YPR190C	cgatgagcttttgaaaa	1.5
YJL183W	gttgttggtg	1.5
Group B		
YLR466C-B	–	12
YMR316C-B	–	7.8
YMR046W-A	–	4.6
YGR122C-A	–	4.5
YGR161W-A	–	0.16
YJR140W-A	–	29.8
YAR047C	–	11.5
YOR102W	–	6.2
YCR087W	–	5.9
YOL106W	–	5.9
YDR034C-A	–	5.1
YHL037C	–	5.0
YOL164W-A	–	4.1
YOR237W	–	3.9
YCR081C-A	–	3.7
YHR153C	–	3.5
YJL114W	–	3.5
YPL272C	–	3.4
YJL077C	–	3.4
YLR261C	–	3.4
YBR190W	–	3.3
YLL053C	–	3.3
YOR105W	–	3.3
YJR097W	–	3.3
YBL071C	–	3.3
YIL014C-A	–	3.2
YEL050W-A	–	3.2
YAR053W	–	3.1
YGR175C	–	3.0

[a] Gene name was adopted as systematic names.
[b] The value is the fold-change of gene expression for the samples collected after 2.5 h treatment of 1-butanol (1%).

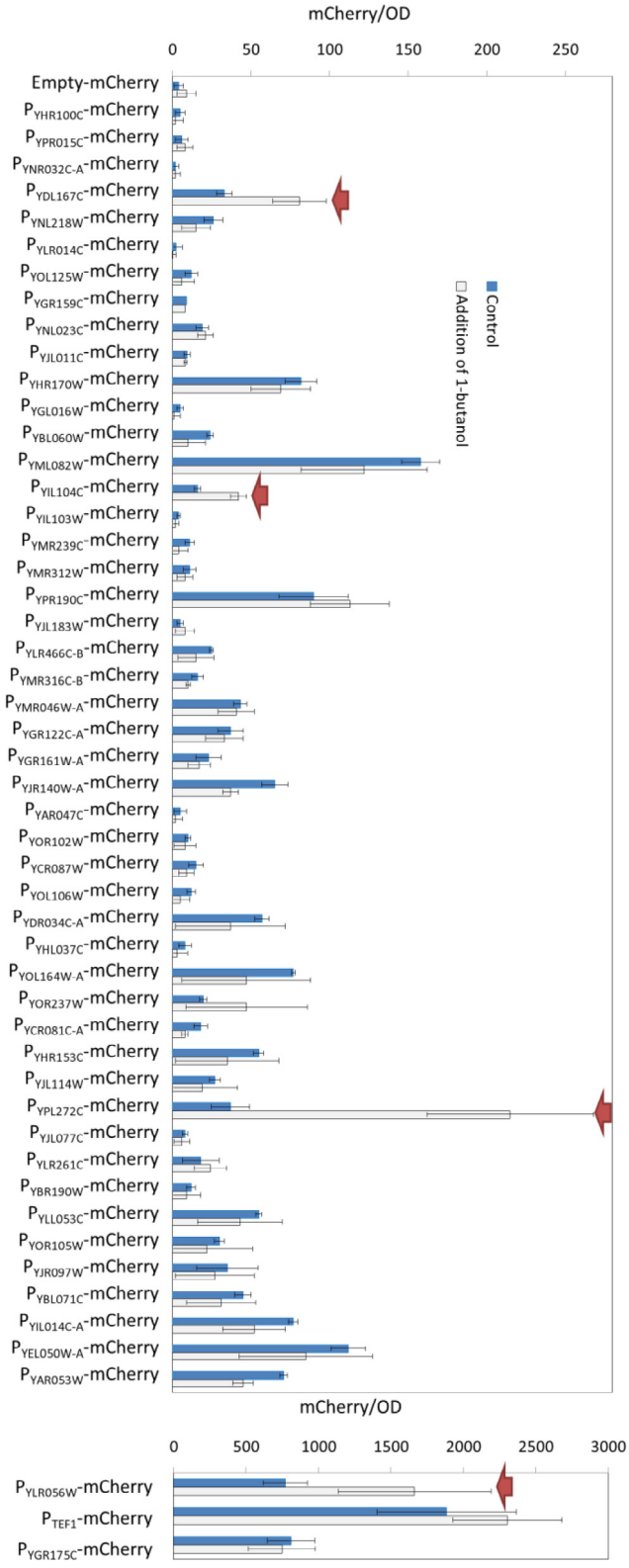

Fig. 2. Measurements of *S. cerevisiae*'s response to 1-butanol (1%) using a plate reader. The mCherry fluorescence reporter was controlled by the promoters of selected genes as listed in Table 1. The promoter of each candidate gene was chosen to be around 1 kb upstream of its coding sequence. *S. cerevisiae* strains were cultured in SC-LEU medium and collected 20 h after the addition of 1-butanol. Four selected promoters were indicated with arrows. Data represent mean ± s.d. of 3 biological replicates.

may not be specific as other short-chain alcohols may introduce similar damage to the cell and cause the same response (Brynildsen and Liao, 2009). Ideal biosensors should have a good specificity. To determine their dose response and specificity, the four candidate promoters, together with the two controls (P_{TEF1}-mCherry and Empty-mCherry), were tested for their response to different short-chain alcohols 1-butanol, 1-propanol, and ethanol at various concentrations (0%, 0.2%, 0.5%, and 1% (v/v)) (Fig. 3). It was found that the expression of mCherry controlled by two promoters, $P_{YDL167C}$ and $P_{YIL104C}$, were responding specifically to 1-butanol in a dose-dependent manner. $P_{YLR056W}$ and $P_{YPL272C}$ showed a response to both 1-butanol and 1-propanol. It is interesting to see that $P_{YPL272C}$ gave a higher and much more sensitive response to 1-propanol, showing its potential to be a 1-propanol sensor. Similar results were obtained in independent flow cytometry experiments. The response tests to different alcohols are not always consistent with the RNA-seq data, indicating there may

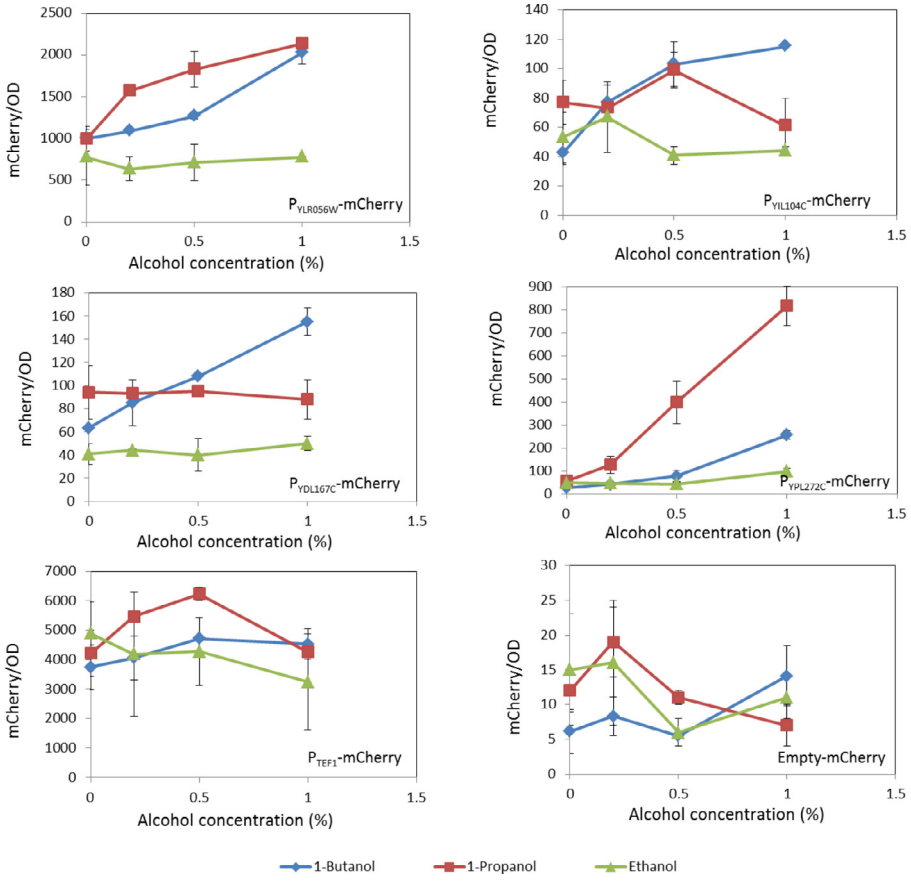

Fig. 3. Dose–response tests to different short-chain alcohols (1-butanol, 1-propanol, and ethanol) using a plate reader. *S. cerevisiae* strains were cultured in SC-LEU medium and collected 20 h after the addition of alcohols. Four selected promoters were indicated with arrows. Data represent mean ± s.d. of 3 biological replicates.

be false positives found by RNA-seq. Since ethanol is the major by-product in *S. cerevisiae* (Shi et al., 2016b), the specific response to 1-butanol would result in low crosstalk *in vivo*. For the first time, the results found that there is a specific response to the 1-butanol for promoter $P_{YDL167C}$ and $P_{YIL104C}$. Therefore, it is likely that $P_{YDL167C}$ and $P_{YIL104C}$ can work as 1-butanol responsive promoters and will not respond to ethanol produced *in vivo*.

Notably, both $P_{YDL167C}$ and $P_{YIL104C}$ were from Group A and contained novel *cis*-regulatory elements that had been identified. However, when the motif sequences were deleted from the promoters, no significant change was observed in 1-butanol response, suggesting that there may be redundant genetic regulatory mechanisms acting on the promoters.

YDL167C, also known as *NRP1*, encodes a putative RNA binding protein of unknown function. However, this gene was shown to be involved in yeast resistance to a series of cellular stresses (de Graaf et al., 2009; Outten and Culotta, 2005; Teixeira et al., 2009). *YIL104C*, also known as *SHQ1*, is required for the assembly of box H/ACA small nucleolar ribonucleoprotein complexes (snoRNPs) and thus for pre-rRNA processing, and can respond to hypoxia (Dastidar et al., 2012). Changes of snoRNPs level are also capable of participating in the regulation of cellular response to external stress and controlling activation of apoptosis (Lin et al., 2015; Michel et al., 2011). These results suggest Nrp1p and Shq1p are important for cell adaptation to environment pressure and support the concept that 1-butanol induces its expression.

Promoters of smaller size can give advantages both in the construction of expression vector and chromosome integration. Besides, unwanted regulation may exist in the unnecessary areas. Therefore, the minimal promoter fragments that were sufficient to drive reporter expression in response to 1-butanol were determined. By systematically shortening $P_{YDL167C}$ and $P_{YIL104C}$, it was found that truncated version 3 of $P_{YDL167C}$ ($P_{YDL167C\text{-}T3}$, 569 bp) and truncated version 4 of $P_{YIL104C}$ ($P_{YIL104C\text{-}T4}$, 489 bp) contained enough sequence to sense 1-butanol (Fig. 4). Deleting any more sequences from these truncated promoters resulted in a loss of response to 1-butanol. Interestingly, although they are both unresponsive to varying concentrations of 1-butanol, $P_{YDL167C\text{-}T4}$-mCherry (489 bp) showed a relatively weak expression of the reporter but $P_{YIL104C\text{-}T5}$-mCherry (408 bp) showed a relatively high expression of the reporter. Hence, it appears that the 489–569 bp stretch of sequence in $P_{YDL167C}$ may contain a binding site of a 1-butanol responsive activator, while the 408–489 bp stretch of sequence in $P_{YIL104C}$ may contain a binding site of a 1-butanol responsive repressor instead. The maximum concentration of 1-butanol that can be measured by the biosensors were determined. Since strains of *S. cerevisiae*, in general, were not able to tolerate and grow in 2% 1-butanol (Knoshaug and Zhang, 2009), the two truncated promoters ($P_{YDL167C\text{-}T3}$ and $P_{YIL104C\text{-}T4}$) were tested for their response to an exogenous addition of 1-butanol up to 2%. The results showed the two truncated promoters responded to 1-butanol in a dose-dependent manner up to 1.25% 1-butanol. However, the final OD dropped below 0.1 when 1-butanol was added with a concentration above 1%.

Previous studies have shown that promoters could be engineered for further improvement (Blazeck and Alper, 2013; Rajkumar et al., 2016; Zhang et al., 2016). However, these works require the information on transcription factor binding data and transcriptional regulation. The two promoters, $P_{YDL167C\text{-}T3}$ and $P_{YIL104C\text{-}T4}$, were identified and behaved like "black boxes" without any known metabolite-responsive mechanism, and it would be hard to engineer them for improved performance on 1-butanol

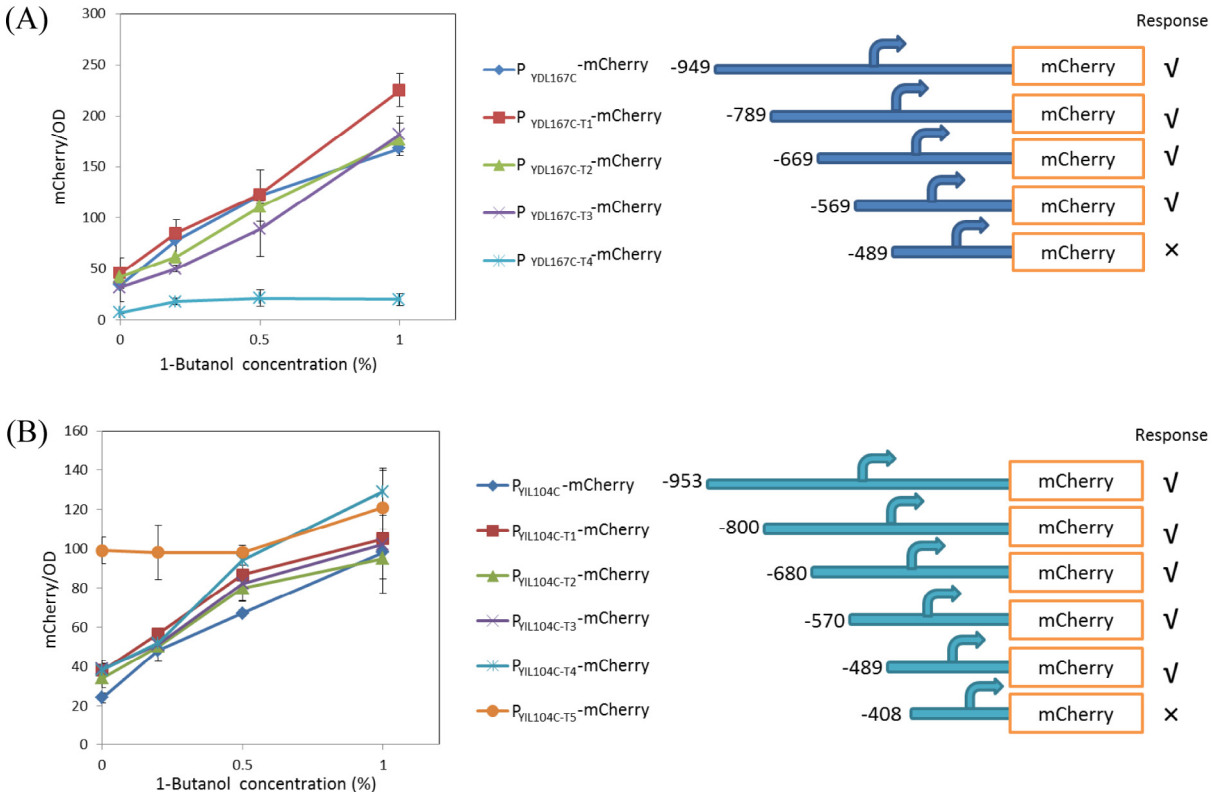

Fig. 4. Dose–response tests of truncated promoters of (A) YDL167C and (B) YIL104C to 1-butanol using a plate reader. *S. cerevisiae* strains were cultured in SC-LEU medium and collected 20 h after the addition of 1-butanol. Data represent mean ± s.d. of 3 biological replicates.

response. In the future, promoter engineering using error-prone PCR or other random mutagenesis methods can be further explored to improve the sensitivity of these two promoters.

3.4. Application of the two selected promoters

Two reporter cassettes, $P_{YDL167C\text{-}T3}$-mCherry and $P_{YIL104C\text{-}T4}$-mCherry, may be deployed in various yeast producer strains that are grown in different culture media. Hence, the two promoters, $P_{YDL167C\text{-}T3}$ and $P_{YIL104C\text{-}T4}$, were tested for their response to 1-butanol in SC-LEU medium and in YPAD medium. Although both promoters gave higher fluorescence readouts in YPAD medium than in SC-LEU medium, they were able to sense 1-butanol in a dose-dependent manner in both culture media.

Next, it was sought to demonstrate that the biosensor devices could distinguish between yeast strains that produced different titers of 1-butanol. Two distinct 1-butanol producer strains have been previously constructed. The *S. cerevisiae* strain *adh1*Δ can produce up to 120 mg/L, while strain COM can produce up to 835 mg/L of 1-butanol in YPAD medium (Shi et al., 2016b). Hence, the reporter cassettes, $P_{YDL167C\text{-}T3}$-mCherry and $P_{YIL104C\text{-}T4}$-mCherry, were transferred into the two *S. cerevisiae* producer strains as well as the original HZ848 wildtype strain. The titers of 1-butanol were measured to ensure that the biosensors were not affecting alcohol production in the transformed yeast cells (Fig. 5A). It was found that both the biosensors were able to distinguish between the strains HZ848 and COM and also between the strains *adh1*Δ and COM successfully (Fig. 5A). Besides, as shown in Fig. 5B, to evaluate the performance of the biosensors in FACS sorting, mixed strains (strain *adh1*Δ and strain COM) with the reporter cassettes, $P_{YDL167C\text{-}T3}$-mCherry or $P_{YIL104C\text{-}T4}$-mCherry, were sorted by FACS, with a threshold of top 1% of the mean fluorescence values. The selected colonies were then identified by PCR analysis. The initial mixing ratio of strain *adh1*Δ and strain COM was 1000 to 1, as measured by OD, and PCR analysis of 50 colonies from the initial mixture plated on agar did not detect any COM colony. After three rounds of sorting, the fluorescence of sorted strain mixture was markedly higher compared to the initial strain mixture (Fig. 5B), and nearly 40% or 35% of sorted cells with the reporter cassettes $P_{YDL167C\text{-}T3}$-mCherry or $P_{YIL104C\text{-}T4}$-mCherry were identified as strain COM from 50 colonies, respectively. The results indicate that both of this two biosensors were able to effectively differentiate between different 1-butanol producers and sort the hyper-producing strains.

A new biosensor must be engineered or discovered for every chemical of interest. Currently, most biosensors have been developed using transcription factors (Dietrich et al., 2012; Qian and Cirino, 2016; Wang et al., 2016), and this method is limited to the availability of previously well-characterized transcription factors. A more efficient workflow needs to be established for biosensor design. Another strategy reported was to develop biosensors based on stress responses. Without known transcription factors, omics analysis was used to identify promoters showing stress responses to cadmium (Park et al., 2007), FPP (Dahl et al., 2013), low pH (Yin et al., 2017), and threonine (Liu et al., 2015).

In this study, a general strategy for discovery of biosensors with high specificity was developed based on stress responses in *S. cerevisiae*. As proof of concept, two 1-butanol sensors were identified using this strategy, and they did not show any response to other short-chain alcohols, ethanol and 1-propanol. The identified promoters are the only biosensors for 1-butanol in yeast, and they can be used to differentiate 1-butanol production among different yeast strains, providing a tool for high throughput combinatorial engineering or screening to improve1-butanol production in yeast using genome-scale engineering methods such as RNA interference-assisted genome evolution, RAGE (Si et al., 2015, 2017).

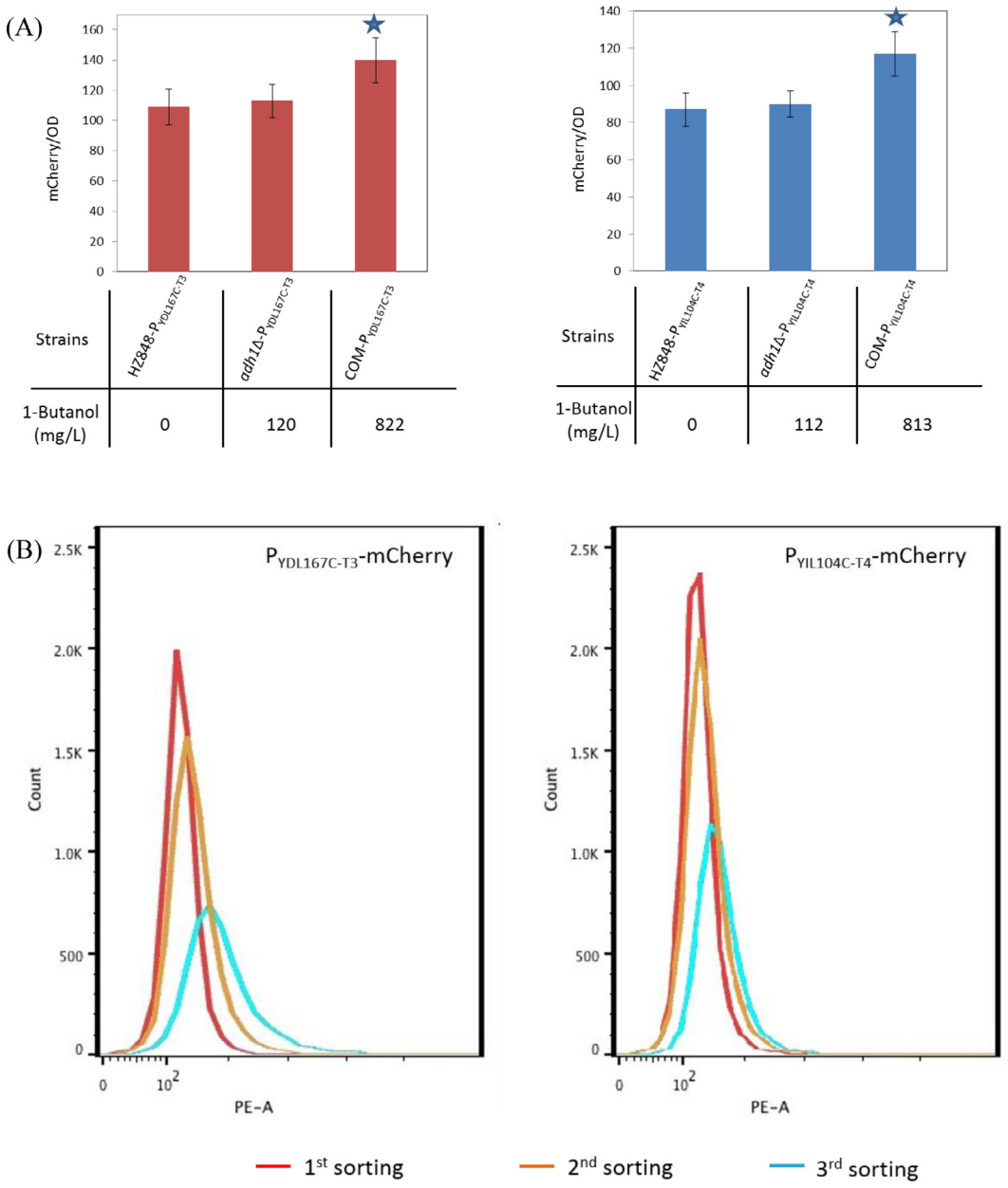

Fig. 5. Biosensor validation in strains with varied ability to produce 1-butanol. (A) Fluorescence intensities were measured using a plate reader. Host strain HZ848 does not produce 1-butanol; strain *adh1*Δ can produce 120 mg/L 1-butanol; strain COM can produce 835 mg/L 1-butanol. *S. cerevisiae* strains were cultured in SC-LEU medium and collected 96 h after the start of fermentation. Data represent mean ± s.d. of 3 biological replicates. Solid pentagram (★) indicates a result that was significantly different compared to the other two strains ($P < 0.05$, Student's *t*-test). (B) FACS results of mixed strains (strain *adh1*Δ and strain COM) with the reporter cassettes, $P_{YDL167C\text{-}T3}$-mCherry or $P_{YIL104C\text{-}T4}$-mCherry. The initial mixed ratio of strain *adh1*Δ and strain COM is 1000 to 1. Strains exhibiting the top 1% of the fluorescence values were sorted by flow cytometry using the Becton Dickson FACSAria flow cytometer (Franklin Lakes, NJ, USA). The screened strains were then cultivated on agar plate at 30 °C overnight for PCR and in liquid medium for next round of sorting. After three rounds of sorting, the histograms revealed that, with the introduction of the reporter cassettes ($P_{YDL167C\text{-}T3}$-mCherry or $P_{YIL104C\text{-}T4}$-mCherry), the fluorescence of sorted strain mixture was markedly higher compared to initial strain mixture.

High specificity is a highly desirable trait for a biosensor. For example, when 1-butanol is produced in *S. cerevisiae*, the main by-product is ethanol (Shi et al., 2016b; Si et al., 2014). Ethanol and 1-butanol can cause a similar transcriptional change in *E. coli* (Clarke and Voigt, 2011). Promoters sensing 1-butanol may also respond to ethanol. Thus, it will be important to identify a biosensor with high specificity to 1-butanol. The method described here should be suitable for the discovery of molecules that can generate stresses to the cell, as one universal stress response is the change in promoter activity. Researchers can subsequently use these stress-response promoters to develop specific biosensors.

4. Conclusion

The work described here demonstrated the general use of transcriptome analysis to identify native *S. cerevisiae* promoters that respond to specific chemicals, in this case 1-butanol. Through extensive characterization and engineering, two promoters were identified that could be used to specifically drive the expression of mCherry upon exposure to 1-butanol in a dose-dependent manner and to differentiate the levels of 1-butanol production among different producer strains. In the future, the promoters can be integrated into an existing 1-butanol producer strain to improve the

production yield of 1-butanol using genome evolution methods such as RAGE.

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Appendix A. Supplementary data

The complete list of strains and plasmids, more transcriptome analyses and butanol response tests could be found in supplementary materials, which can be seen on the website of Bioresource Technology.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.06.114.

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