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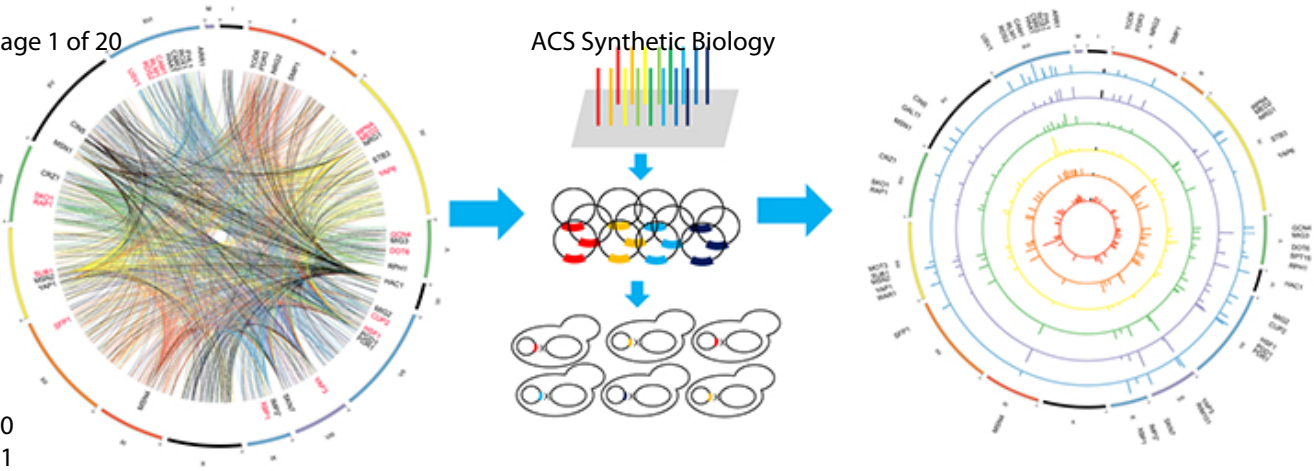

Transcriptional regulatory networks involved in C3-C4 alcohol stress response and tolerance in yeast

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Abstract

Alcohol toxicity significantly impacts the titer and productivity of industrially produced biofuels. To overcome this limitation, we must find and use strategies to improve stress tolerance in production strains. Previously we developed a multiplex navigation of global regulatory networks (MINR) library that targeted 25 regulatory genes that are predicted to modify global regulation in yeast under different stress conditions. In this study, we expanded this concept to target the active sites of 47 transcriptional regulators using a saturation mutagenesis library. The 47 targeted regulators interact with more than half of all yeast genes. We then screened and selected for C3-C4 alcohol tolerance. We identified specific mutants that have resistance to isopropanol and isobutanol. Notably, the WAR1_K110N variant improved tolerance to both isopropanol and isobutanol. In addition, we investigated the mechanisms for improvement of isopropanol and isobutanol stress tolerance, and found that genes related to glycolysis play a role in tolerance to isobutanol, while changes in ATP synthesis and mitochondrial respiration play a role in tolerance to both isobutanol and isopropanol. Overall, this work sheds light on basic mechanisms for isopropanol and isobutanol toxicity, and demonstrates a promising strategy to improve tolerance to C3-C4 alcohols by perturbing the transcriptional regulatory network.

Keywords: Transcriptional regulatory network; Isobutanol; Isopropanol; Yeast; Alcohol tolerance

Renewable transportation fuels such as bioethanol are needed to combat global climate change.[1] Higher-carbon alcohols such as isopropanol or isobutanol can be blended with gasoline at 16%, compared to ethanol which is blended at 10%, resulting in a gasoline blend with lower greenhouse gas emissions that is still compatible with current engines and fuel transportation/storage systems.[2,3] However, C3-C4 alcohols are toxic to the yeast *Saccharomyces*

cerevisiae which limits titer and productivity during industrial fermentation of these products.[4,5] Tolerance to alcohols is a complex phenotype, and studies have shown that multiple genetic modifications are required to improve tolerance to high ethanol levels.[6–8] Therefore, new methods are needed to discover and utilize mutations that improve stress tolerance.

Previous studies have identified some mechanisms responsible for improved tolerance to ethanol and higher-carbon alcohols. General adaptation to alcohol stress (both ethanol and C3-C4 alcohols) can result in changes to nutrient transport,[9,10] membrane lipid metabolism/composition,[11,12] translation and protein degradation,[13,14] mitochondrial activity,[15] and antioxidative capacity[16] in yeast. In addition to modifying the yeast cells, the media can also be modified to improve alcohol tolerance. Supplementation of growth medium with KCl and KOH to increase both extracellular potassium and pH leads to increased tolerance to multiple alcohols.[17] While some mechanisms of tolerance are the same for ethanol and higher-carbon alcohols, other tolerance mechanisms for higher-carbon alcohols are different from those identified for ethanol. For example, knocking down the Hsp70 family of heat shock proteins results in increased tolerance to isobutanol,[18] but has no effect on ethanol tolerance.[19] Deletion of *GCN2*, a protein kinase involved in the activation of the eIF2 translation initiation factor, or *GCN4*, which activates the transcription of amino acid biosynthetic genes, enhances resistance to 1-butanol[20]. Overexpression of the transcription factor *MSS11,* which regulates invasive growth and starch degradation also enhances isobutanol tolerance.[21] Recently, Kuroda et al. found the pentose phosphate pathway (PPP) and *GLN3*, a transcriptional activator of nitrogen catabolite repression, play a role in isobutanol tolerance in yeast.[22] Deletion of the *GND1* or *ZWF1* genes, both involved in the PPP, leads to sensitivity to isobutanol but not to ethanol, whereas deletion of *GLN3* results in increased tolerance to branched-chain alcohols. Thus, it is necessary to study tolerance to higher carbon alcohols such as isopropanol or isobutanol in addition to ethanol tolerance to fully understand tolerance mechanisms in yeast.

To engineer such complex traits, a large mutational space needs to be explored in order to find an ideal phenotype. Adaptive laboratory evolution (ALE) and other random mutagenesis methods have been applied for stress tolerance studies, but these methods are only able to sample a small fraction of the total possible mutational landscape with a mutation rate of 10^{-10} - 10^{-9} per base pair per replication.[23] Forward engineering methods, such as global transcriptional machinery engineering (gTME), identified that mutations in specific regulatory proteins can lead to improvements in desired phenotypes,[24–26] but the number of genes affected or regulated by the mutant is much smaller than that of the entire yeast genome. To better enable the engineering of complex phenotypes like tolerance, there is a need to understand how all regulators interact in a cell and to use this knowledge to construct a comprehensive and responsive regulatory network.

Most cells use transcription factors (TFs) to mediate changes in cellular function in order to adapt to changing environments. Approximately 200 TFs have been characterized or predicted in *S. cerevisiae*, resulting in ~4000 interactions between regulators and promoter regions[27]. To better understand complex interactions between regulatory proteins (such as regulatory feedback loops), the identification of transcriptional changes in response to environmental conditions, as

well as high resolution mapping of TF binding sites is necessary. CRISPR/Cas methods have been reported for genome engineering from a single gene to whole genome scales.[28–32] Existing approaches, such as CRISPR–Cas9- and homology-directed-repair (HDR)-assisted genome-scale engineering (CHAnGE) and CRISPR-enabled trackable genome engineering (CREATE),[33,34] enable trackable editing of yeast genomes in a massively multiplex manner. Based on these studies, we previously developed the multiplex navigation of global regulatory networks (MINR) technique, which incorporates CRISPR-Cas9 genome editing, barcoding, and targeted mutagenesis of 25 transcriptional regulators to identify mutants that improved ethanol tolerance.[35] In this study, we expanded this concept and constructed a library containing over 83,000 mutations to perform saturation mutagenesis of the active sites of 47 transcriptional regulators that interact with more than half of all yeast genes. We then screened and selected for C3-C4 alcohol tolerance. The mutants identified with improved tolerance shed light on different mechanisms to improve stress tolerance in yeast.

Results and Discussion

Transcriptional regulator library construction

We generated a genome-wide transcriptional regulatory network for stress tolerance by choosing 47 regulators that are involved in cellular stress responses including those involved in hydroxymethylfurfural (HMF) tolerance,[36] osmotic tolerance,[37] ethanol tolerance,[8] the glucose response,[38] the salt stress response,[39] and the alkaline pH stress response,[40] among others (Table S1). In total, the transcriptional regulatory network included interactions between targeted regulators and more than 3000 genes in the yeast genome involved in multiple molecular functions and biological processes (e.g. metabolism). We used the CREATE method[35] to construct a saturation mutagenesis library with tens of thousands of members targeting the DNA binding domains of these transcriptional regulators. This high resolution mutational library perturbs the transcriptional regulatory network and thus should reprogram metabolism. Following target phenotype selection and high-throughput sequencing, the total number of 83,020 specific mutations can be mapped for each trait.

The libraries were constructed using previously described methods (Fig. 1 and Methods). Briefly, 83,020 oligonucleotide cassettes were designed and synthesized on a microarray. Each cassette contains a gRNA targeting a specific region of a regulator and a homology directed repair template that codes for the desired mutation in that regulator. The gRNAs also serve as the barcodes for tracking the frequency of individual cassettes (i.e. mutations) by next generation sequencing (NGS) pre- and post-selection. All of the cassettes for each regulator were grouped into a sub-library, for a total of 47 sub-libraries. Each sub-library was amplified out of the pool of all cassettes by PCR using sub-library specific priming regions. Each sub-library was cloned and transformed into *S. cerevisiae* BY4741 separately. To determine genome editing efficiency, 50 colonies were randomly picked from each of six randomly selected sub-libraries, and both the

plasmid barcodes and the targeted genomic sites were sequenced (Fig. S1). The editing efficiency was 60 to 98% and mutational diversity was 50 to 80% in these sub-libraries (Fig. S1). Non-edited colonies were found to have editing cassettes with mutations in the spacer region, which arise during oligo synthesis and cloning, and likely account for the variability in editing efficiency due to deficiencies in gRNA targeting[29] (Fig. S2). Thus, to ensure adequate coverage for each sub-library, each selection included 10-times more independent clones than the total number of designs in the sub-library. Sub-libraries that showed improved growth during selections were subjected to NGS both pre- and post-selection. NGS analysis of four sub-libraries from pre-selection showed that 57%-97% of the designed mutations were present (Fig. S3).

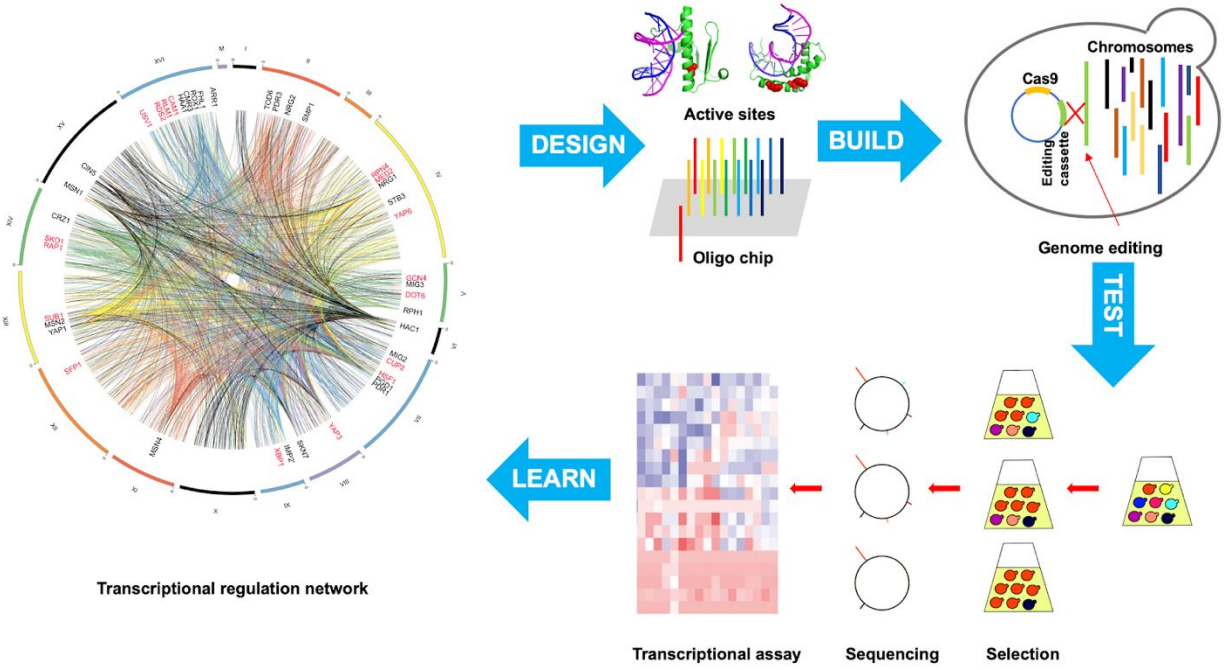

Figure 1

Engineering C3-C4 alcohol tolerance using the transcriptional regulator library

Growth selections for each sub-library were done in the presence of different concentrations of isopropanol and isobutanol (Fig. 2a). After selection, the regions of the editing cassettes containing the barcodes were sequenced and all of the mutations that resulted in improved growth in isopropanol or isobutanol were mapped (Fig. 2a). Mutations in 13 of 47 regulators significantly improved cell growth under either isopropanol or isobutanol stress compared to the non-targeting control (*S. cerevisiae* BY4741 expressing a gRNA plasmid that does not target the genome) (Fig. 2a). To verify that the mutants isolated during the population level screens do confer a growth advantage, we re-introduced the significantly enriched variants ($P < 0.05$) from these 13 genes into the parental strain, and tested them with different concentrations of isopropanol and isobutanol.

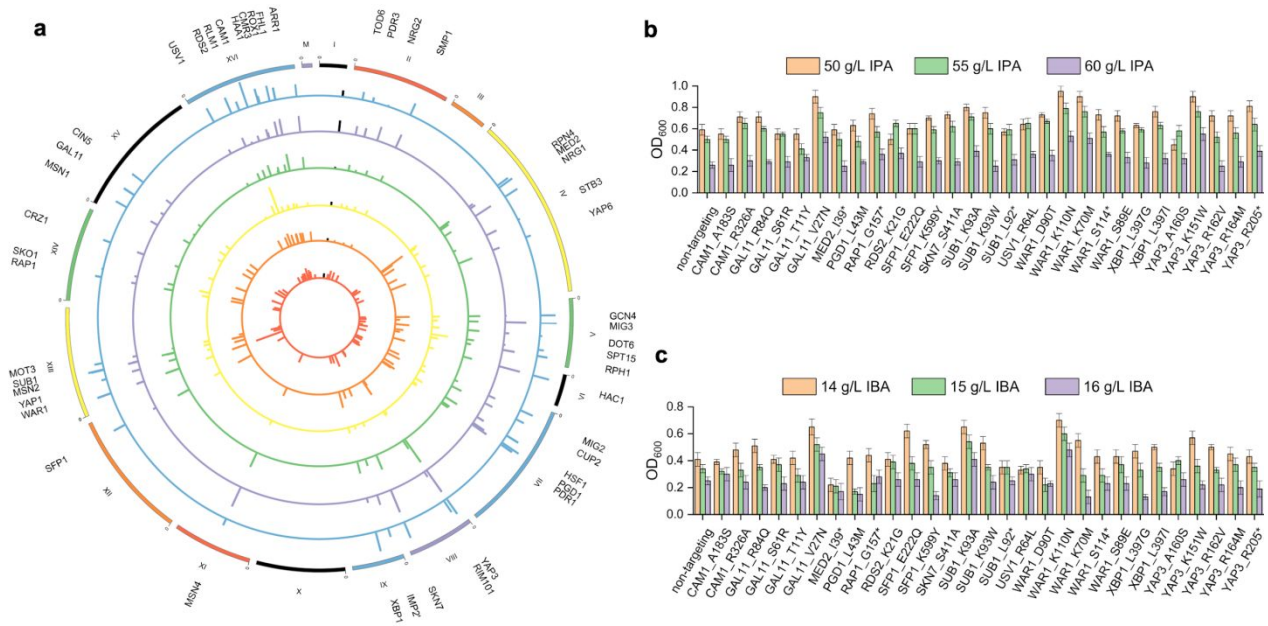

Figure 2

For isopropanol verification, most of the reconstructed variants grew similarly to, or faster than the non-targeting control under 50 - 60 g/L isopropanol (Fig. 2b). We observed that the mutants with >50% increased cell growth were WAR1_K110N, WAR1_K70M, YAP3_K151W, and GAL11_V27N. WAR1 encodes a transcription factor that is a member of the C6 zinc finger class, containing a DNA binding domain also known as the Zn2Cys6 binuclear zinc cluster or zinc knuckle.[41] War1p's principal target is PDR12 that encodes a plasma membrane transporter.[41] The K110N and K70M mutations in the DNA binding domain could therefore affect the active transport of compounds into and/or out of the cell by perturbing expression of target genes. *YAP3* encodes an AP-1 type transcription factor, has a basic leucine zipper domain (bZIP), and activates genes that have a Yap recognition element (YRE; 5'-TTAC/GTAA-3') in their promoter regions.[42] The K151W mutation was located in the bZIP domain, which could alter the cellular response to endoplasmic reticulum (ER) stress, cell stress response, and the unfolded protein response.[43] GAL11 (also called MED15) encodes a component of the tail module of the mediator transcriptional regulation complex, and is necessary for transcription by RNA polymerase II (RNAPII) in almost all cases.[44] The V27N mutation in the KIX domain could affect RNA metabolism by directly interacting with transcriptional activators Gal4p and Gcn4p.[45]

In 14-16 g/L isobutanol, most of the reconstructed variants grew similarly to, or faster than the non-targeting control. We observed that the mutants with >50% increased cell growth were WAR1_K110N and SUB1_K93A (Fig. 2c). *SUB1* encodes a stress-response transcription reinitiation factor that plays both positive and negative roles in transcription initiation. The K93A mutation is located in the DNA-binding domain, and could therefore alter Sub1p binding to single-stranded DNA, and DNA-dependent processes including replication, DNA repair, and chromatin organization.[46] Interestingly, WAR1_K110N improved cell growth under both isopropanol and

isobutanol stress (Fig. 2b, 2c), which indicates that there may be universal stress response mechanisms affected by this mutation.

Genome-wide transcriptional analysis of isopropanol tolerant mutants

To gain an understanding of how the identified mutations could lead to isopropanol tolerance, we performed RNAseq[47] for the top tolerance variants (WAR1_K110N, WAR1_K70M, YAP3_K151W, and GAL11_V27N) under 50 g/L isopropanol in order to analyze changes in global gene expression. Changes in expression of genes involved in RNA modification, mRNA processing, mitochondrial respiration, stress response, cytoplasmic translation, transport, and ATP synthesis were associated with isopropanol tolerance in all the tested variants (Fig. 3).

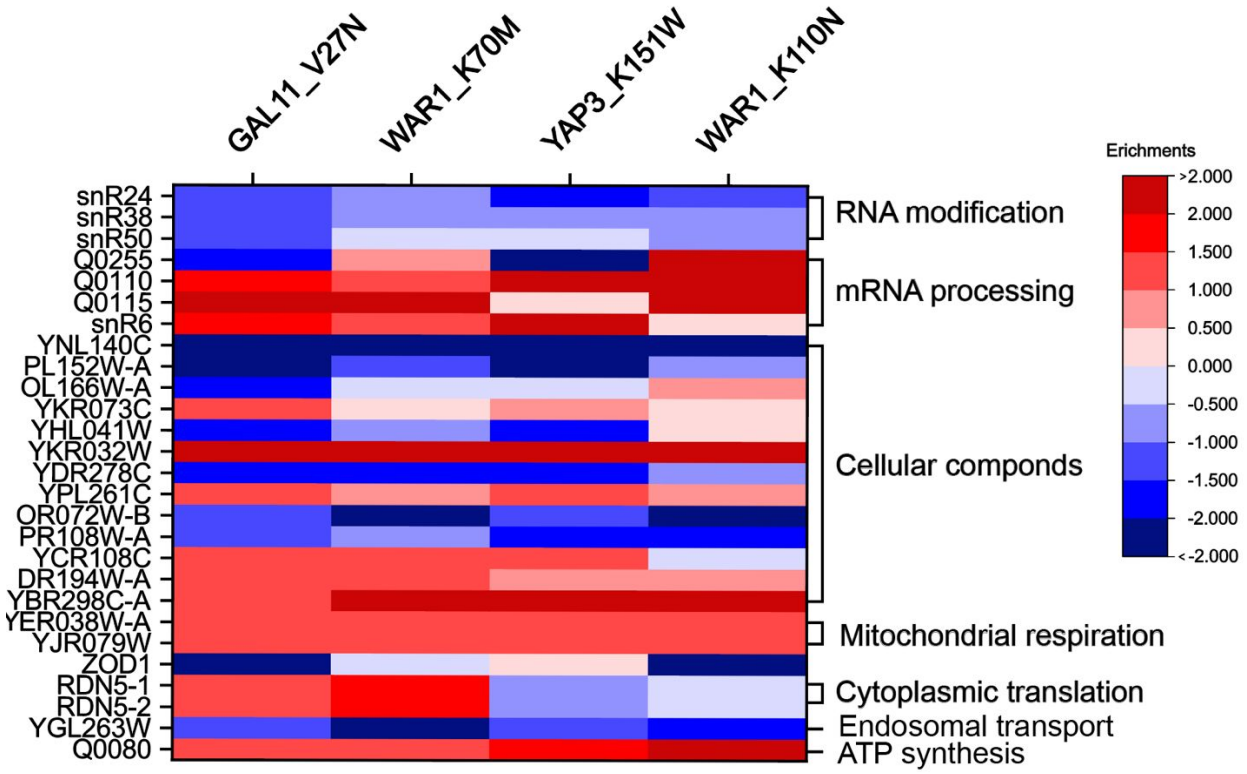

Figure 3

Under isopropanol stress, RNA metabolism was significantly affected in the tolerant variants. The C/D box small nucleolar RNA genes, *snR24*, *snR38*, and *snR50*, involved in RNA modification and rRNA processing[48] were downregulated in all the variants. Furthermore, *Q0110*,[49] *Q0115*,[50] and *snR6*,[48] which are involved in RNA splicing and mRNA processing, were up-regulated in all these mutants. Previous studies implicated that these transcriptional changes may improve RNA stability under stress conditions.[51]

Energy metabolism in the mitochondria is vital for sustaining cell life under stress conditions.[52] Under 50 g/L isopropanol, *YER038W-A* and *YJR079W*, genes involved in

mitochondrial respiration,[53,54] were upregulated in all the mutants. Moreover, the ATP synthase gene, *Q0080*,[55] was also upregulated. The WAR1 gene regulates plasma membrane transport, and decreased cellular transport has also been implicated as a mechanism to improve stress resistance.[56] *YGL263W* encodes an endosomal protein involved in turnover of plasma membrane proteins and was down-regulated in WAR1_K110N and WAR1_K70M, which has been shown to decrease cell transport and potentially improve stress resistance.[57]

Genome-wide transcriptional analysis of isobutanol tolerant mutants

We examined the transcriptional changes for the two most tolerant strains (WAR1_K110N and SUB1_K93A) under 14 g/L isobutanol stress. Under isobutanol stress, 129 genes were significantly up- or down-regulated in the isobutanol-tolerant strains compared to the non-targeting control strain. We observed that the largest class of genes induced by isobutanol were related to RNA modification and biosynthesis (Fig. 4a and Fig. S4). Sub1p interacts with the carboxyl-terminal domain (CTD), and plays several roles during transcription.[58] Genes related to RNA modification were significantly up-regulated in SUB1_K93A, which indicates that the K93A mutation in *SUB1* leads to an increase in RNA metabolism under isobutanol stress.

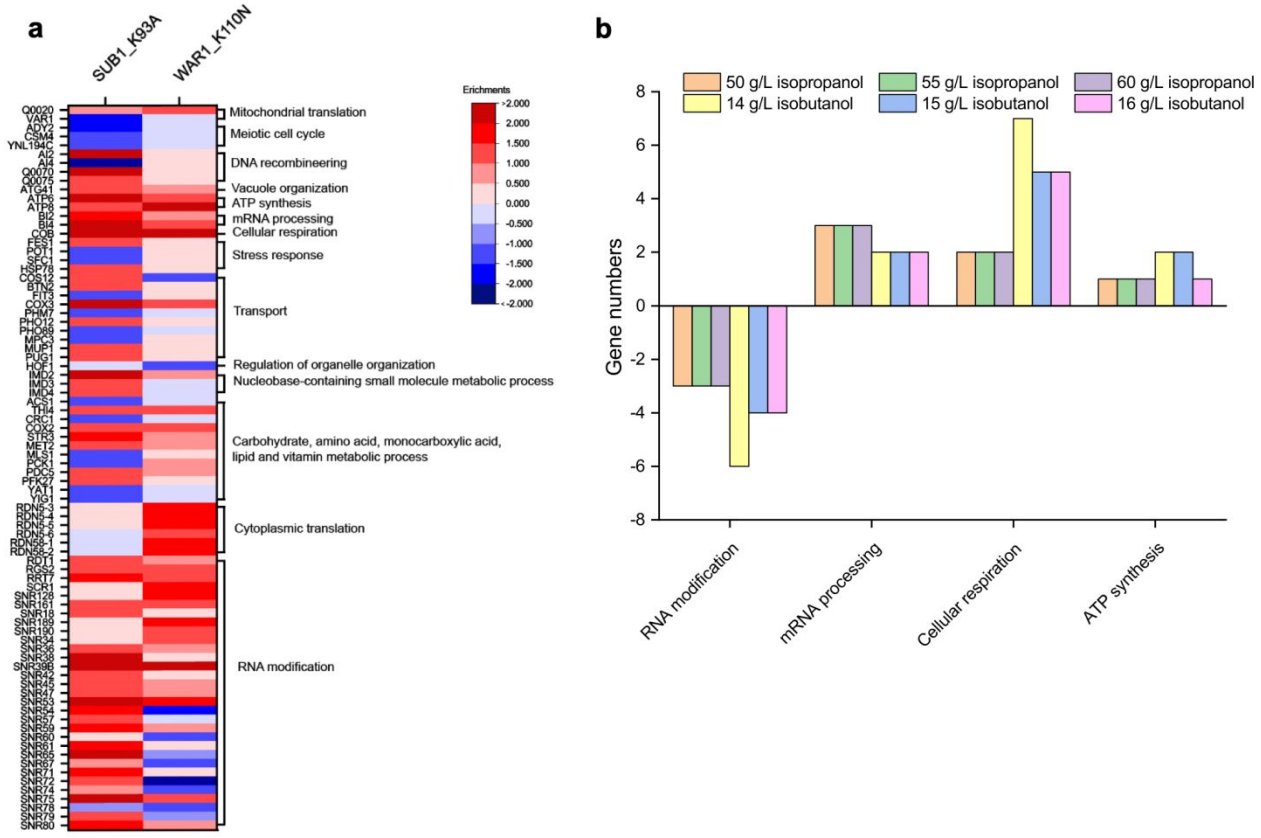

Figure 4

Essentially, cells need energy for cell growth and related metabolic activity. The upregulation of *AI2*,[60,61] *BI2*,[62] *BI4*,[63] *COB*,[64] *COX2*,[65] and *COX3*[66] genes involved in cellular

respiration, was observed in all the mutants (Fig. 4a). Furthermore, the ATP synthase genes *ATP6*[67] and *ATP8*[68] were upregulated under 14 g/L isobutanol, which could further improve ATP synthesis (Fig. 4a). The upregulation of cellular respiration and ATP synthase genes may be an effort to counteract increased ATP utilization rates due to overall reduced ATP synthesis or impairment of the plasma membrane. In addition, Ghiaci and coworkers applied proteome analysis to investigate the transcriptional changes in their isobutanol adapted mutant, *JBA-mut*.[15] They found that 21 out of 34 up-regulated proteins are predicted to be mitochondrial proteins, including 12 proteins, such as ATP-synthase and cytochrome c1, that are components of the respiratory chain. These results showed that cellular respiration and ATP synthesis are good targets for improving isobutanol tolerance.

Heat shock proteins (HSPs) can assist in the folding or refolding of proteins that are denatured due to stress conditions.[69,70] The gene encoding HSP78 was upregulated in both the SUB1_K93A and WAR1_K110N variants under isobutanol stress (Fig. 4a). Similarly, Ghiaci and coworkers found that the small HSP (Hsp42p) was upregulated in their JBA-mut strain under 1.9% isobutanol in rich media.[15] Conversely, Crook and coworkers knocked down the Hsp70 family of HSPs to increase isobutanol tolerance.[18] Although *HSP78* from the Hsp100 family and *HSP42* from the Hsp60 family interact with Hsp70 family proteins,[70,71] the transcriptional changes of *HSP78* and *HSP42* are different from the Hsp70 family proteins in isobutanol tolerant yeast.

The WAR1_K110N variant confers resistance to both isopropanol and isobutanol

During the verification of tolerant strains, the WAR1_K110N variant grew faster than the non-targeting control under different concentrations of both isopropanol and isobutanol, which indicated an universal mechanism in this mutant for C3-C4 alcohol resistance. We compared genes with significant transcription change in WAR1_K110N under 50 g/L isopropanol and 14 g/L isobutanol (Fig. 4b). The results showed that, when compared to the non-targeting control, genes related to RNA modification, mRNA processing, cellular respiration, and ATP synthesis exhibit a similar transcriptional change under both alcohols. We further investigated transcription of these genes with increased alcohol concentration (55 and 60 g/L isopropanol as well as 15 and 16 g/L isobutanol). The results showed that there were still similar trends for different groups of genes (Fig. 4b). Strains with increased tolerance can potentially increase the productivity of the target compound.[72] Although some identified mutations could also reduce the cells' ability to grow under other conditions unrelated to product toxicity[73] or fail to affect product formation, tolerant mutants should be suitable as host strains for further optimization of C3-C4 alcohol production.

Changes in expression of glycolytic genes which are significant for alcohol tolerance

Cell growth rate has a positive correlation with the rate of glycolysis, which is frequently used as a metabolic model for cell growth.[74,75] Under isobutanol stress, upregulation of *PFK27* and *PDC5*, as well as downregulation of *MLS1* in the SUB1_K93A mutant might suggest

accelerated conversion of glucose to acetyl-CoA, which could increase the rate of glycolysis (Fig. 5a). We further tested the strains with down-regulation of these genes on isobutanol resistance using CRISPR interference (CRISPRi).[76,77] The strains with down-regulated *PFK27* and *PDC5* showed sensitivity to isobutanol (Fig. 5b), but the strains with down-regulated *MLS1* had a comparable cell growth rate as the non-targeting control (Fig. 5b), which indicated upregulation of glycolysis genes is indeed involved in tolerance to isobutanol. To determine how large of a role of the PFK27 and PDC5 genes individually play in isobutanol tolerance, strains overexpressing these genes could be constructed and tested for isobutanol tolerance. In addition, these genes may be good targets for overexpression when constructing alcohol tolerant yeast strains. Conversion of acetyl-CoA in the mitochondria is an additional limitation for metabolic engineering in *S. cerevisiae*.[78] The upregulated *PDC5* gene in SUB1_K93A could also further improve cytoplasmic acetyl-CoA synthesis, which is beneficial for products using acetyl-CoA as a precursor. *PCK1* gene encodes phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis, which can further improve metabolic flux towards acetyl-CoA. Under isobutanol stress, *PCK1* gene was down-regulated in SUB1_K93A, and slightly up-regulated in WAR1_K110N. Thus, we also tested the effect of down-regulated *PCK1* on isobutanol resistance, and the results showed the constructed strain with down-regulated *PCK1* had a comparable cell growth rate as the non-targeting control (Fig. 5b).

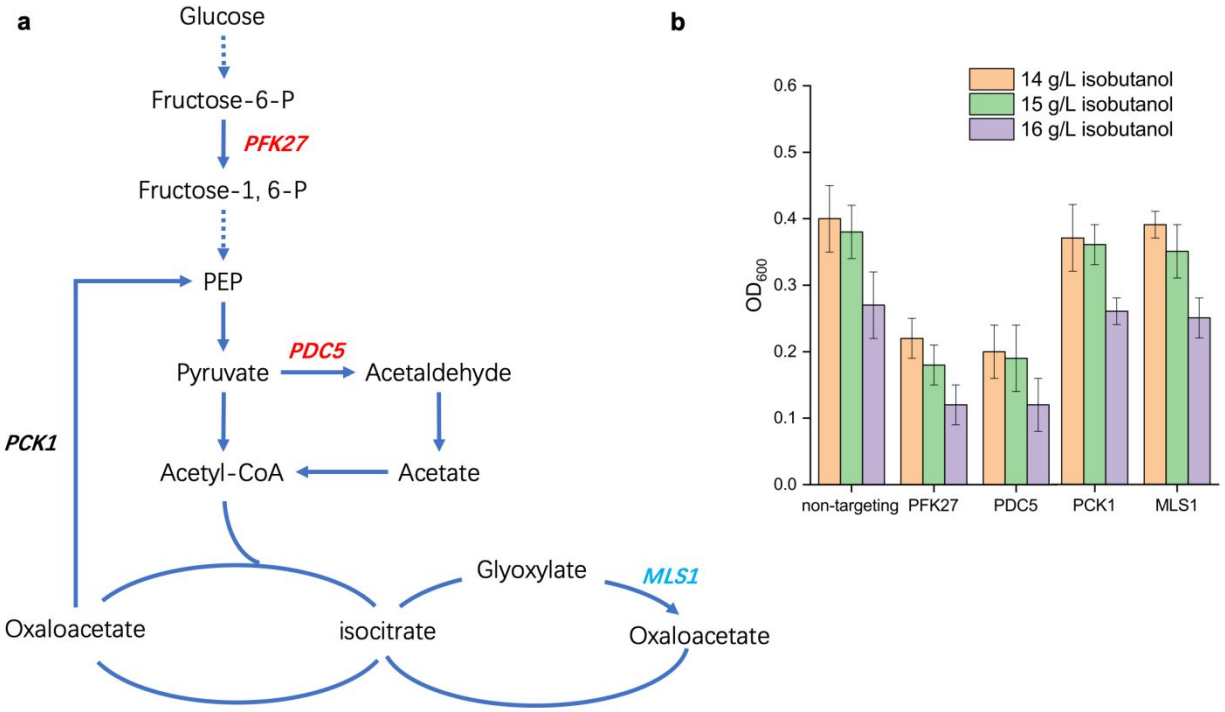

Figure 5

The WAR1_K110N variant exhibited increased tolerance to both isopropanol and isobutanol. We previously showed that the transcription of genes related to glycolysis and PPP under ethanol stress was significantly increased in ethanol-tolerant mutants.[35] Kuroda and

coworkers found that deletion of some enzymes in the PPP decreased isobutanol tolerance, but did not affect ethanol tolerance.[22] For WAR1_K110N, genes related to PPP did not have significant transcriptional change, but genes related to glycolysis were up-regulated under isobutanol selection. Thus, we further tested the WAR1_K110N variant for ethanol tolerance. Under 6% ethanol, the cell growth rate of WAR1_K110N was not significantly higher than the non-targeting control (Fig. S5). Overall, these results suggest that changes in expression of glycolytic genes which are significant for alcohol tolerance, but the toxicity caused by isopropanol and isobutanol has a unique mechanism that differs from the ethanol-induced stress response in yeast.

In summary, we constructed a regulatory network library involving 47 transcriptional regulators that interact with more than half of all yeast genes. Then, we tested this library under different concentrations of isopropanol and isobutanol, and identified a series of isopropanol and isobutanol tolerant strains in a single round of experiments. Furthermore, we found that single point mutations can have large effects on tolerance to alcohols, which also reveals potentially new genotype-phenotype interactions under stress conditions. We identified a variant, WAR1_K110N, that exhibited higher tolerance to both isopropanol and isobutanol but not ethanol. In addition, we identified several previously unknown mechanisms for conferring tolerance to C3-C4 alcohols. We show that increased expression of genes related to glycolysis is important and specific to isobutanol tolerance, and changes in ATP synthesis and mitochondrial respiration play a role in tolerance to both isopropanol and isobutanol tolerance. Therefore, modifying the transcriptional regulatory network can provide information about the mechanisms of the stress response and increasing resistance to isopropanol and isobutanol. Furthermore, the verified tolerant strains can be used as more robust yeast chassis strains for C3-C4 alcohol production.

Methods

Library design and construction

The targeted sites are shown in Table S1, and were either designed in our the previous study[35] or were regions/residues identified from the literature and related databases, such as Saccharomyces Genome Database (SGD) (https://www.yeastgenome.org/), SMART (http://smart.embl-heidelberg.de/), Gene3D (http://gene3d.biochem.ucl.ac.uk/), PDB (http://www.rcsb.org/pdb), SUPERFAMILY (http://supfam.org/SUPERFAMILY/), and Pfam (http://pfam.xfam.org/).

The oligo pools were amplified using primers in Table S2. The amplification process used was 2-step PCR, and the conditions were shown as follows: 98°C/30 s; 10 cycles of 98°C/15 s, 60°C/20 s and 72°C/60 s; 10 cycles of 98°C/15 s and 72°C/60 s; and 72°C/5 min. The PCR products were purified (QIAquick PCR Cleanup Kit, Qiagen., USA). The backbone (derived from pMINR-ADE2 (Table S3)) was amplified using primers in Table S2. The PCR conditions were shown as follows: 98°C/30 s; 30 cycles of 98°C/10 s, 61°C/30 s and 72°C/5 min; and 72°C/2 min.

After amplification, the backbone was incubated with DpnI and then gel extracted (QIAquick Gel Extraction Kit, Qiagen, USA).

The purified library oligos and backbone were assembled using standard Gibson assembly followed by electroporation into *E. cloni* cells (Lucigen Corporation, Middleton, WI, USA). Then, library plasmids were extracted (QIAprep Spin Miniprep Kit, Qiagen, USA)

The transcriptional regulatory library construction and selection

Yeast were transformed with library plasmids using the high-efficiencyLiAc/SS carrier DNA/PEG method.[79] After overnight culture, we centrifuged the cells and resuspended them in 1 mL SC-Ura medium. 1 uL of culture was plated for coverage calculation. The rest of the cells were used for selection. For selections, cells were diluted to an initial OD_{600} of 0.05 and were serially diluted twice during selection. Selections were performed for 48-96h. in SC-Ura medium with different concentrations of isopropanol (50, 55, and 60 g/L) and isobutanol (14, 15, and 16 g/L). After selection, plasmids were purified from yeast (Yeast Plasmid Miniprep II kit, Zymo Research, Irvine, CA, USA) for analysis by Illumina paired-end sequencing.

RNAseq

RNA samples were prepared using a Qiagen RNeasy Mini Kit, and the pooled sample was sequenced using an Illumina MiSeq V3 150-cycle kit. RNAseq data were analyzed using the following workflow in KBase[81]: align reads to reference genome using HISAT2, assemble transcripts using StringTie, and create differential expression matrix using DESeq2.

Mutant reconstruction and verification

Each editing cassette (Table S3) was synthesized as a gBlock (IDT). The cassettes were cloned into the editing plasmid by Gibson assembly, followed by transformation into the parental yeast strain and growth tests as described for the pooled selections. The cell growth test for the WAR1_K110N variant under 6% ethanol used the same workflow as indicated above for growth selections. For the CRISPRi experiments, we replaced the sequence of Cas9 to dCas9 in editing plasmid, and the workflow is the same as indicated above.

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Author Contributions

R.L., L.L. and R.T.G. developed the concept. R.L., L.L., and C.A.E., all aided in the design of experiments. The experiments were done by R.L., L.L., and E.F.F. The data analysis was done by R.L. and L.L. The paper was written by R.L., L.L., E.F.F., and C.A.E.

Notes

The authors declare no competing financial interest.

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Supporting Information

The information for Illumina paired-end sequencing, oligos for library construction and sequencing, the data for library construction, the transcription data for library variants, and verification of various strains.

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Figure Legends

Figure 1 The workflow for enhancement of C3-C4 alcohol tolerance in yeast using the transcriptional regulation network. Design: The oligos for the library (each containing a donor homology arm) are computationally designed and synthesized as pools. Each sub-library contains the designs for one targeted regulator. The new targeted regulators are labeled in red. Build: Editing vectors are cloned in multiplex by PCR amplifying library subpools followed by Gibson assembly into the backbone plasmid. Yeast expressing Cas9 are then transformed with the editing plasmids, resulting in high efficiency genome editing. Test: After selection, the frequency of each plasmid before (X_i) and after (Y_i) selection is determined by PCR amplification and next-generation sequencing. An enrichment score (E_i) is determined for each mutant by calculating $\log_2$ of the ratio of the frequency of the initial and final time points. Learn: Enrichment scores are used to rapidly identify beneficial variants at the single nucleotide or amino acid scale in parallel at thousands of loci.

Figure 2 Transcriptional regulator libraries for improved C3-C4 alcohol tolerance. (a) Enrichment at all targeted genes under different concentrations of isopropanol and isobutanol. From inner to outer, the selections used were 50 g/L, 55 g/L, and 60 g/L isopropanol and 14 g/L, 15 g/L, and 16 g/L isobutanol. **(b-c)** The verification of positive mutants under different concentrations of (b) isopropanol and (c) isobutanol.

Figure 3 Heat map showing genes with greater than a 2-fold (E_j >1 and <-1) transcriptional change in all four variants compared to their values in the non-targeting control under isopropanol stress (50 g/L isopropanol). The transcription change (E_j) was calculated as follows:

$$E_i = log_2(\frac{Y_i}{X_i})$$

where X_j is the number of transcripts in the non-targeting control and Y_j is the number of transcripts in each mutant.

Figure 4 Genome-wide transcriptional analysis of isobutanol tolerant mutants. (a) Heat map showing genes with greater than a 2-fold (E_j >1 and <-1) transcriptional change in two variants compared to their values in the non-targeting control under isobutanol stress (14 g/L isobutanol). **(b)** The number of genes in each functional category for the WAR1_K110N mutant under different concentrations of isopropanol and isobutanol. The gene numbers for down-regulated and up-regulated genes are shown as negative and positive, respectively. The transcription change (E_j) was calculated as follows:

$$E_i = log_2(\frac{Y_i}{X_i})$$

where X_j is the number of transcripts in the non-targeting control and Y_j is the number of transcripts

in each mutant.

Figure 5 Involvement of glycolysis in isobutanol tolerance in yeast. (a) The glycolytic pathway is shown with gene names for each enzyme. Genes shown in red or blue were upregulated and downregulated under isobutanol stress, respectively. Genes with no significant change in transcription are shown in black. **(b)** Isobutanol tolerance verification for the strains with targeted genes downregulated using CRISPRi technology.