Title: Evolutionary engineering improves tolerance towards jet fuel replacements in yeast

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Abstract: Monoterpenes are liquid hydrocarbons with applications ranging from flavor and fragrance to replacement jet fuel. Their toxicity, however, presents a major challenge for microbial synthesis. Here we evolved limonene tolerant yeast and sequenced six strains across the 200 generation evolutionary time course. Mutations were found in the tricalbin proteins Tcb2p and Tcb3p. Genomic reconstruction in the parent strain showed that truncation of a single protein ($tTcb3p^{1-989}$), but not its complete deletion, was sufficient to recover the evolved phenotype improving limonene fitness by nine-fold. $tTcb3p^{1-989}$ increased tolerance towards two other monoterpenes (β-pinene and myrcene) by 11- and 8-fold, respectively, and tolerance towards the bio-jet fuel blend AMJ-700t (10% cymene, 50% limonene, 40% farnesene) by 4-fold. $tTcb3p^{1-989}$ is the first example of successful engineering of phase tolerance and creates opportunities for the production of the highly toxic C_{10} alkenes in yeast.

51

52 **Introduction**

53 Monoterpenes are ten-carbon (C_{10}) terpenes derived from two five-carbon (C_5) isoprene units
54 (22). Traditionally used in the flavor and fragrance industry, monoterpenes, such as *d*-
55 limonene, are now also being sought after as precursors for light end components of "drop-
56 in" jet fuels (7). With the ability to convert simple sugars into a variety of enantiomerically
57 pure monoterpene products (16, 27, 35), microbial synthesis has the potential to overcome the
58 low yields, impurities and high-cost associated with conventional chemical synthesis or
59 extraction from biological tissues (10). The C_{15} sesquiterpene, farnesene, is currently
60 produced via yeast fermentation for diesel markets (34). Monoterpenes and sesquiterpenes
61 are both derived from the mevalonate pathway indicating that yeast has the potential to
62 provide sufficient carbon, redox power and energy to produce commercial quantities of
63 monoterpenes. Unlike sesquiterpenes, however, monoterpenes are highly toxic. In *S.*
64 *cerevisiae*, for example, limonene halts growth at 60 mg/l (7). As maximizing titers is an
65 absolute requirement for cost effective production (19), toxicity is a critical challenge facing
66 microbial monoterpene production and biotransformation (23).

67

68 Being oils, monoterpenes are sparingly soluble in water and cause phase rather than
69 molecular toxicity. Molecular toxicity is caused by water-soluble compounds partitioning
70 into the plasma membrane and interfering with membrane properties (38). Molecular toxicity
71 is proportional to a solvents aqueous concentration and is at its maximum at a solvents
72 solubility point (i.e., at membrane and water saturation) (6). Phase toxicity occurs beyond a
73 solvents solubility and is defined as inhibition due to a distinct second phase (7). The
74 molecular mechanisms of phase toxicity remain poorly understood, but we demonstrated in *S.*
75 *cerevisiae* that limonene interferes with cell wall integrity rather than membranes (6).

Accordingly, rational strategies for alleviating toxicity, such as manipulation of membrane fluidity or expression of membrane-bound solvent pumps, have failed to resolve this problem (20).

In the past decade, adaptive laboratory evolution (ALE) (9, 32) has proven to be a successful technique for identifying biological solutions to combat alcohol (2, 8) and organic acid (21, 43) toxicity. Here we used ALE to identify genetic targets to alleviate phase toxicity. We describe a mutation in a single protein (tTcb3p$^{1\text{-}989}$) that can serve as a tool for the production of monoterpenes beyond their inhibitory limits.

Materials and Methods

Strains, media and chemicals

The *Saccharomyces cerevisiae* strain S288C (*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1*) was used as the parental strain and provided by the Australian Wine Research Institute (AWRI), Adelaide, South Australia, Australia. A list of all strains used in this study can be found in Table S3. Knockout strains (Δ*tcb3 and* Δ*tcb2*) were derived from the BY4741 strain deletion collection (17). Genotypes for Δ*tcb3* and Δ*tcb2* strains are: MATa*; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; tcb3Δ::kanMX4* and similarly for Δ*tcb2* MATa*; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; tcb2Δ::kanMX4.* All chemicals were purchased from Sigma-Aldrich and were of analytical-grade. AMJ-700t was prepared in a 1 ml stock by mixing 50% v/v limonene, 10%v/v cymene and 40% v/v farnesene. Unless otherwise noted, synthetic media (CBS) consisted of 2 g/l sucrose, 5 g/l $(NH_4)_2$ SO_4, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, and vitamins and trace metals described in (7). YPD solid medium consisted of 10 g/l yeast extract, 10 g/l polypeptone and 20 g/l sucrose. LM is YPD medium containing 300 mg/l

limonene. CBS^+ media was CBS but consisted of 5 g/l sucrose with the addition of 20 mg/l uracil, 50 mg/l L-histidine, 50 mg/l L-methionine and 100 mg/l L-leucine.

Adaptive evolution by serial batch passage

The parental strain (S288C) was transferred from a glycerol stock stored at -80°C and grown on solid YPD medium at 30ºC. A single colony of the parent strain was used to inoculate 10 ml of CBS media and this pre-culture was grown overnight until the mid-exponential growth phase. The appropriate volume from the pre-culture was used to inoculate 22 ml of CBS media in 250 ml Teflon™-lined screw-top baffled shake flasks to a target OD_{660} = 0.2. The initial volume of limonene added to the culture at inoculation was 1.0 µl (38 mg/l). Limonene was directly added to the culture aseptically using a 10 µL glass-syringe (Gerstel, Mulheim, Germany) immediately after inoculation. The culture was then incubated at 30°C at 200 rpm (Orbit 25 mm, Multitron, Infors, Bottmingen, Switzerland) until the cell density reached mid-exponential phase (OD_{660} ~ 1-2, corresponding to circa 3 doubling times) before they were transferred. For the next round, the appropriate volume of the previous limonene-challenged culture was used to inoculate fresh medium (22 ml) to a target OD_{660} = 0.2. After several transfers (2-4) at a given limonene load, the amount of limonene was increased (between 0.2 – 0.5 µl (7.6-19 mg/l) of additional limonene). After 52 daily sequential passages the final limonene load was 5.8 µl (222 mg/l). After each passage, 0.5 ml of culture (in the presence of limonene) was added to 0.5 ml of 40% v/v glycerol solution and the samples were stored at -80°C.

Screening mutants

Mutants were screened by streaking frozen glycerol stocks out onto solid LM medium plates. The largest single colonies (8-12) were subjected to a second competitive growth screen in

Applied and Environmental Microbiology

liquid CBS medium containing 95 mg/l of limonene. Isolates were pre-cultured in CBS medium (10 ml) overnight at 30ºC (200 rpm) and the appropriate volume of the pre-culture was used to inoculate 22 ml of CBS to an OD_{660} = 0.2 in 250 ml Teflon™-sealed screw-top shake flasks. Then 2.5 µl of limonene (95 mg/l) was added immediately after inoculation and growth was monitored for 12 h using a Libra S4 spectrophotometer at 660 nm. The mutant with the highest specific growth rate was chosen for tolerance testing.

Tolerance test

The evolved and un-evolved strains were evaluated for tolerance to each toxic compound as described in (7). Briefly, cultures were grown until mid-exponential phase (~ OD_{660} = 1.0) and then dosed with varying amounts of solvent. The optical density was evaluated for the ensuing 6 h. The minimum inhibitory concentration (MIC) is defined as the amount of solvent required to inhibit the specific growth rate by approximately 50% compared to that of the control strain with no solvent present. For fitness tests, 3.6 µl (138 mg/l) of limonene was added to the inoculum (22 ml at OD_{660} = 0.2) and growth was monitored as described above. The ratio of the OD_{660} at 12 and 0 h was used as a relative fitness metric for solvent tolerance (3). CBS medium containing 5 g/l sucrose was used in all tolerance tests (CBS^+ medium was used for the Δ*tcb3* strain and fitness in the reconstructed tTcb3p, TB516, S288C and BY4741 strains was also tested in CBS^+ medium, Table S7). All tolerance tests were performed in biological triplicate for each compound and strain.

DNA isolation

All DNA was isolated according to Otero *et al.* (31) with the following modifications. A total of 5 ml of culture was harvested in mid-log (OD ~ 3) (16,000 rcf, 25°C), washed twice with distilled water, pelleted, and re-suspended in 800 µl of lysis buffer (1% SDS, 50 mM EDTA,

0.1 M TrisHCl, pH 7.5). The suspension was transferred to FastPrep screw cap tubes containing 1 g of 0.5 µm acid washed beads and 100 µl of 5 M NaCl. To lyse the cells, four bead-beating cycles (30 s of beating followed by 1 min on ice) were carried out in a mechanical bead beater (John Morris Scientific, Pty Ltd., Sydney, Australia). The suspension was then harvested (16,000 rcf, 4°C) for 15 min, and the resulting clear liquid (circa 800 µl) was transferred to new 1.5 ml extra-clean tubes (Mo Bio, Carlsbad, CA). Next, 777 µl of a chloroform:isoamyl alcohol (24:1) mixture was added and incubated at 25°C for 30 min with gentle rocking. The mixture was then pelleted for 15 min (16,000 rcf, 25°C) and the top, aqueous layer was transferred to a new extra-clean tube and mixed with 70% v/v isopropanol and centrifuged (16,000 rcf, 25°C) for 6 min. The isopropanol suspension was decanted and the pellet was re-suspended in 70% ethanol. The ethanol suspension was centrifuged (16,000 rcf, 25°C) for 6 min and the pellet was allowed to dry for 45 min. The pellet was re-suspended in 50 µl of DNA and RNA-free water containing 100 µg/ml RNAse A and incubated at room temperature for 30 min.

Genome sequencing

Paired-end (100 bp) genomic DNA libraries were generated using TruSeq DNA Sample Preparation Kits according to the manufactures instructions (Illumina, San Diego, CA). Genomic DNA was sheared to a target insert size of 300-400 base pairs using a Cavaris ultrasonicator (model M220, Cavaris Inc., Woburn, MA). Whole genome re-sequencing was performed by the Queensland Centre for Medical Genomics (Institute for Molecular Bioscience, University of Queensland, Australia) using an Illumina HiSEQ 2500 system (HiSEQ Control Software 2.0.5, RTA 1.17.20) following the standard rapid sequencing workflow of cluster generation and sequencing by synthesis. Initially, libraries were loaded onto an Illumina cBot instrument for template hybridization and initial extension steps using

the TruSEQ Rapid Duo cBot Sample Loading Kit (cat# CT-402-4001). Flowcells were then moved to the HiSEQ 2500 instrument to complete cluster generation and sequencing using TruSeq Rapid Cluster Kit - Paired-End (cat# PE-402-4001) and TruSeq Rapid SBS Kits – 200 Cycle (cat# FC-402-4001) following the manufacturer's publications. Samples were loaded at a concentration of 6pM and a total of 209 cycles of sequencing were completed consisting of 2x 101bp reads and a single 7bp index sequence.

For mutation analysis, the default settings in Bowtie2 (v 2.0.2) and samtools (v 0.1.18) were used in the alignment and mapping (24). The input paired-end reads (101 bp) were trimmed based on quality (3 bases from the start and 23 bases from the end) to produce 75 bp reads for each sample. The results from the mapping were used to identify single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELS) between the mutant and reference strain. The initial sequencing results and mutations, which were identified according to the default software settings mentioned above, are listed in Table S1. The mutations in Table S1 were further filtered and alignments were viewed using the Integrative Genomics Viewer (IGV) software (36). A mutation was only called if it met the following criteria: the mutation occurred in a coding region and SNPs and INDELS were filtered using a Phred-like consensus quality >30. For SNPs, the coverage depth was ≥ 10 and >95% of the base pair reads harbored the mutation. For INDELS, some INDELS were filtered out if the number of reads with INDELS was less than the number of reads without INDELS. For example, initially 39 mutations were identified in evolved strain TB302, however, after filtering only 10 mutations occurred in coding regions and 2 of them met the filtering criteria. Annotation and prediction of the effects caused by the mutations was carried out using the program snpEff (v 3.1) (13). The data was run against the ensemble build (EF4.68) of the S288C reference genome and used as input in the snpEff database.

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200

201 **Genomic reconstruction**

202 Introduction of the mutations, *PDR3* (Q763L) and *TCB3* (frame shift at 989), into the
203 parental strain (S288C) was carried out using the recyclable dominant marker cassette
204 *amdSYM* (39). Throughout the text, the reconstructed S288C strain harboring the truncation
205 mutation at position 989 in *TCB3* is referred to as tTcb3p. The details of the construction and
206 primers used can be found in the supplemental material (Table S2 and Table S8 for SNP
207 reconstruction efficiency). A gain-of-function assay was performed for all reconstructed
208 strains. As described above, the relative fitness towards limonene, cymene, β-pinene,
209 myrcene and AMJ-700t was investigated for the parental strain, evolved (TB516) and
210 reconstructed strains.

211

212 **Fluorochrome staining and flow cytometry**

213 For S288C and tTcb3p strains, cell viability and cell wall damage were assessed using the
214 vital staining dye propidium iodide (PI) and cell wall binding dye calcofluor white (CFW).
215 Briefly, cells were treated with an inhibitory amount of limonene (138 mg/l) during midlog
216 growth phase (20 g/l CBS media). After 2 h of treatment, cell viability (described in (7)) and
217 cell wall staining (described in (6)) were tested in biological triplicate.

218

219 **Transcriptomics**

220 Total RNA was isolated from S288C and tTcb3p cultures during mid-exponential growth
221 phase. For control (no limonene) and limonene challenged (107 mg/l) cultures as described
222 previously (6) approximately 10 ml of culture was quickly pelleted (17,572 rcf x *g*) for 2 min
223 at 4°C and then immediately re-suspended in 2 ml of RNA*later*® solution (Life
224 Technologies, Carlsbad, CA) and stored at 4°C. The RiboPure-Yeast kit (Ambion, Life

225 Technologies, Carlsbad, CA) was used for total RNA extraction according to manufacturer's
226 instructions except bead beating was used to lyse the cells as previously described in (6). The
227 RNA quality was assessed using an Agilent 2100 Bioanalyzer and RNA 6000 Nano kit
228 according to manufacturer's methods (Agilent, Santa Clara, CA). For the microarrays
229 (Affymetrix yeast genome 2.0), samples were labeled with 100 ng of total RNA starting
230 material and labeled using the Affymetrix IVT Express Labelling Assay, according to the
231 manufacturer's instructions (Santa Clara, CA). For hybridization, 4 µg of fragmented biotin
232 labelled cRNA was hybridized to each array at 45°C for 16 hours at 60 rpm in an Affymetrix
233 hybridization oven. Arrays were washed according to the manufacturer's instructions using
234 an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned using an Affymetrix
235 S3000 scanner. The entire microarray processing procedure was performed at the Ramaciotti
236 Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia).
237 Analysis of differentially expressed genes and gene set analysis (GSA) was carried out in R
238 using the default values in Piano (42). All analysis was performed in biological triplicate.
239
240 **Cell wall proteomics**
241 The total cell wall extracts were isolated for the parental strain S288C and tTcb3p$^{1-989}$
242 cultures during mid-exponential growth phase. Cultures were inoculated to a starting OD_{660} =
243 0.1 in 22 ml of CBS (5 g/l sucrose) and allowed to grow until OD_{660} = 1.0 in the absence and
244 presence of limonene challenge (107 mg/l). Approximately 20 ml of culture was pelleted
245 (4,025 rcf for 5 min at room temperature), washed with sterile water, snap frozen by quickly
246 submerging the sample in dry ice for 2 min and stored at -80°C. Cell wall protein extraction
247 followed the method previously described in (37). Briefly, yeast cells were lysed with a bead
248 beater, insoluble cell wall material was stringently washed, N-glycans were released with
249 EndoH and deglycosylated proteins covalently linked to the cell wall polysaccharide were

digested with trypsin. Peptides were desalted with C18 ZipTips (Millipore) and detected by LC-ESI-MS/MS with a Prominence nanoLC system (Shimadzu) and TripleTof 5600 mass spectrometer with a Nanospray III interface (AB SCIEX). For information dependent acquisition (IDA), analyses were performed as described (4). Identical LC conditions were used for SWATH-MS, with an MS-TOF scan from an *m/z* of 350-1800 for 0.05 s followed by high sensitivity information independent acquisition with 26 *m/z* isolation windows with 1 *m/z* window overlap each for 0.1 s across an *m/z* range of 400-1250. The overall SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra) method was previously described in (18) and has been successfully used for proteomic quantification in yeast and other tissues without the use of wild-type vs. wild-type statistical comparisons (26, 40). Collision energy was automatically assigned by the Analyst software (AB SCIEX) based on *m/z* window ranges. Proteins were identified from IDA data using ProteinPilot (AB SCIEX) as described (4). False discovery rate analysis using ProteinPilot was performed on all searches. Peptides identified with greater than 99 % confidence and with a local false discovery rate of less than 1 % were included for further analysis. *S. cerevisiae* cell wall proteins identified in this discovery data were used as the spectral library for analysis of SWATH MS/MS data using the SWATH processing script within PeakView 1.2 (AB SCIEX). Fragment ion peak area was used for quantification. The software package MSstats (11) was used for statistical analysis in R. Statistical significance cutoff was set at $p < 0.05$. All data was performed in biological triplicate.

Sequencing, transcriptome and proteome data is available online (http://pathway.aibn.uq.edu.au/S288c/).

Results

Evolved mutants show improved tolerance towards limonene

Serial batch passaging was used to isolate limonene-tolerant yeast strains. The parental S288C strain underwent 52 daily sequential transfers under limonene stress. The limonene load increased from 38 mg/l to 222 mg/l. Tolerant strains were isolated as single colonies on plates during the adaptive evolution process and tested for limonene tolerance. Two strains were isolated between 100-200 generations and 4 strains were isolated at 200 generations. Two tests were used to examine the improved fitness. The minimum inhibitory concentration (MIC in Table 1 and Fig. S1) and relative fitness (Fig. 1) were measured for each strain and compared to the parent. Relative fitness was defined as the ratio of the cell density (OD660) at 12 hr to 0 hr under limonene exposure. All of the evolved strains showed at least a 1.9-fold improvement in the MIC (Table 1) and at least a 9-fold improvement in the relative fitness for limonene (Fig. 1). The maximum relative fitness score (10.9 ± 1.6) was achieved at 120 generations (strain TB302) and did not significantly improve for later generations (160-200 in Fig. 1). In the absence of limonene, the maximum fitness score was 23.4 ± 0.4. For all strains and all tolerance tests, cell viability remained above 90% after 12 h of incubation (Table S1). These results show that adaptive evolution employing serial batch transfer under constant limonene challenge was successful for isolating limonene-tolerant phenotypes.

Identification of mutations by whole-genome sequencing

Six resistant strains were isolated across the evolutionary time course in order to identify targets for genetic engineering. Mutations were identified by whole-genome re-sequencing

and each genome was compared with that of the parental strain, which was also sequenced and contained no mutations. For each evolved strain, we first identified all mutations occurring in coding regions. A summary of the sequencing results are listed in Table S1 and all of the mutations for each strain are listed in Table 1. The selection criteria for mutation calling is described in the Materials and Methods section. All resistant strains had a mutation (Q763L) in *PDR3*, while all strains with maximum fitness scores harbored an INDEL in either *TCB2* (deletion of amino acids 314-316, strain TB302 at 120 generations) or the closely related gene *TCB3* (frameshift at base-pair 2966, resulting in the truncation of the protein to 989 amino acids) (Fig. 1). Considering that all of the other mutations found (Fig. 1 and Table 1) varied between each of the evolved strains, we assumed these to be passenger mutations arising from genetic drift and decided to focus our engineering efforts on the *PDR3* and *TCB3* mutations only. The genome data is available for download at http://pathway.aibn.uq.edu.au/S288c/.

Genomic reconstruction of the limonene-tolerant phenotype

We next constructed single- and double- mutations for *PDR3* (Q763L) and *TCB3* (frame shift 989) genes in the parental strain and investigated their relative fitness in a gain-of-function assay. A gain-of-function assay examines if a mutation confers the phenotype by reconstructing the mutation in the parent strain as opposed to evaluating the phenotype by knocking out genes systematically in an evolved strain. While a single base change in $pdr3^{Q763L}$ showed no significant improvement in fitness compared to the parental strain, the mutation in $tcb3^{1\text{-}989}$ (leading to truncated protein $tTcb3p^{1\text{-}989}$) increased limonene fitness by 9-fold and showed no statistical difference in comparison to the evolved strain (Fig. 2). The double mutant, $pdr3^{Q763L}$ and $tcb3^{1\text{-}989}$, resulted in a relative fitness score of 11.5 ± 0.5, which is also statistically insignificant compared to the fitness score achieved by the evolved

mutants or the single *TCB3* mutant (Fig. 2). The reconstructed double mutant, carrying both the *TCB3* and *TCB2* mutations (*tcb3*$^{1\text{-}989}$, *tcb2*$^{1\text{-}316}$), showed no additional increase in fitness compared to the single tTcb3p$^{1\text{-}989}$ mutant (Table S7). This data revealed that the mutation in tTcb3p$^{1\text{-}989}$ alone could confer limonene tolerance.

Given the *TCB3* mutation encoded a truncated version of Tcb3p, we questioned whether this mutation resulted in a nonfunctional protein and asked if limonene resistance was due to effective loss of Tcb3p. A *TCB3* knock-out strain, however, showed no enhanced tolerance and matched that of the S288C fitness (Δ*tcb3* in Fig. 2). This demonstrates that, despite encoding a truncated protein, the evolved tTcb3p is a gain-of-function mutation. This result further highlights the advantage of ALE over other approaches, where traditional screening of knock-out libraries would not have identified *TCB3* as a viable target.

Next, we questioned what the potential biological implications were of truncating Tcb3p. Normally, Tcb3p resides exclusively in the cortical endoplasmic reticulum (cER) tethering the cER to the plasma membrane (PM) (28). It was recently demonstrated, however, that a truncated version of Tcb3p, lacking the C2 lipid binding domains (S288C Tcb3p in Fig. 2b), caused Tcb3p to untether from the PM and co-localize in the perinuclear endoplasmic reticulum (nER) and the cER. We investigated if limonene resistance was associated with this dual localization property. We created a similarly truncated version of Tcb3p at position 531, which resulted in limonene tolerance (*tcb3*$^{1\text{-}531}$ in Fig. 2a). This data strongly indicates that the gain of function and observed resistant phenotype may be linked to the ability of tTcb3p to reside in both the cER and nER.

Alternatively, limonene resistance may also be due to the loss of the tricalbin complex. Tcb2p forms a heterocomplex with Tcb3p (and Tcb1p) by binding to the C-terminal portions of tricalbins 1 and 3 (14). With the C-terminus of Tcb3p absent in both truncated versions (position 989 or 531) we questioned if limonene resistance was associated with the breakdown of the Tcb2p-Tcb3p tricalbin complex. A full knock-out of Tcb2p was effective at increasing limonene resistance (Fitness of Δ*tcb2* = 7.86 ± 1.27 in Fig. 2). In addition to the dual localization property of Tcb3p in the ER, the absence of the tricalbin complex may also contribute to the resistance mechanism.

Changes to the cell wall integrity, transcriptome and proteome

We previously demonstrated that limonene damages the cell wall (6). Hence, we explored if the tTcb3p$^{1-989}$ mutation improved the structural integrity of the cell wall during limonene stress. Cell wall integrity was measured using calcofluor white (CFW) which is a fluorochrome that binds to cell wall polysaccharides (33). Cells with damaged walls show increased sensitivity and a higher mean fluorescence intensity (MFI) when stained with CFW (6). Shown in Fig. 3c, 59 ± 6% of S288C cells showed substantial cell wall damage after limonene exposure. This was a ten-fold increase in number of damaged cells compared to the untreated control (6 ± 1% in Fig. 3b). In contrast, the mutant cells harboring the single mutation tTcb3p$^{1-989}$ showed a much smaller increase in cell wall damage after limonene treatment, with a two-fold increase in number of cells damaged (24 ± 1% vs. 11 ± 1%, Fig. 3e and f). In addition, loss of cell wall integrity affected cytokinesis and defects in cell separation were found to be higher in S288C treated cells compared to the tTcb3p$^{1-989}$ cells (Fig. S3). These results demonstrate that the tTcb3p$^{1-989}$ protein has the ability to preserve cell wall integrity during limonene treatment.

In wild-type strains, limonene exposure causes a substantial transcriptional response (6). When challenged with limonene, the total number of differentially expressed genes (29 genes; see Table S4) was substantially lower for the tTcb3p$^{1-989}$ strain compared to S288C (453 genes reported in (6)). This is partly explained by the large number of regulated genes associated with growth defects in S288C (6). The mutant tTcb3p$^{1-989}$ showed very small perturbations to growth when challenged with limonene and thus the smaller gene list was not surprising (Fig. S2). However, roughly 30% of the up-regulated gene list is associated with cell wall repair (Table S4). In yeast, the cell wall signaling pathway (CWI) is responsible for responding to cell wall damage (25). The CWI cascade was highly up-regulated in S288C (6), but was not induced in mutant cells (Fig. 4). In tTcb3p$^{1-989}$ cells, limonene stress induced genes associated with cell wall maintenance, which are not part of the CWI signaling pathway. *ECL1*, *SMP1*, *TIR2*, and *YPL277C* were up-regulated in the mutant but not in S288C and only three up-regulated genes were shared between the strains (*YGP1*, *FIT2*, *FIT3*). Several genes involved in iron ion homeostasis were also up-regulated (*SIT1, HMX1, ARN1, FRE5, TIS11*). This response was similarly observed for S288C exposed cells (6). Global transcript analysis was also performed in the absence of limonene to investigate expression patterns directly caused by the *TCB3* mutation (tTcb3p$^{1-989}$ mutant compared to S288C gene expression). However, no genes were differentially expressed.

We also characterized the impact tTcb3p$^{1-989}$ had directly on cell wall protein abundance during limonene shock. For untreated cells, Ygp1p was the only protein induced in the mutant compared to S288C (Table S5). The cell wall proteins Plb1p, Plb2p, Ecm33p, Tos1p, Cis3p and Uth1p were found to be lower in abundance in the mutant vs. S288C (Table S5). During limonene challenge, cell wall proteins Pbl2p, Cis3p, and Ccw14p were induced in the mutant but absent in the parental strain. Gas3p, Yap7p and Cwp1p were the only common

regulated cell wall proteins found during limonene stress (Fig. 4). Taken together, these data demonstrate that both the *TCB3* mutation and limonene affect protein secretion or cell wall biosynthesis, but in different ways. The underlying mechanisms of this, however, remain unresolved.

Tolerance towards other terpenes

To test if tTcb3p$^{1-989}$ also conferred tolerance towards other terpenes, the tolerance assays were repeated with a range of compounds. Fitness towards β-pinene and myrcene increased significantly (11- and 8-fold, respectively) compared to S288C (Fig. 5) and the reconstructed strain matched or improved the fitness compared to the evolved strain (TB516). The mutant showed no fitness increase for the aromatic monoterpene cymene (Fig. 5) as well as other aromatics (xylene, benzene and styrene in Table S6). A four-fold improvement was observed (Fig. 5) in the tolerance against a terpene jet fuel precursor mixture (AMJ-700t). AMJ-700t contained by volume 50% limonene, 10% cymene and 40% farnesene. After a single hydrogenation step, AMJ-700t can be converted to the paraffin jet fuel mixture AMJ-700 (50% limonane, 10% cymene and 40% farnesane), which was successfully used in a demonstration flight during Rio+20 in June 2012 (1).

Discussion

Strains with improved fitness towards limonene and other toxic terpenes were identified through adaptive evolution employing serial batch passaging (Fig. 1 and Fig. 5). In typical ALE approaches, the final strain is sequenced and compared against the parent strain (3). Here, we re-sequenced genomes across the evolutionary time course and constructed a spatiotemporal representation of the environmentally selected mutations. This enabled us to more precisely judge which mutations were potentially significant. For example, if TB511

419 was the only final strain sequenced then six potential gene targets would have had to been
420 examined. Alternatively, we captured mutations in *TCB2/3* and *PDR3* that occurred early on
421 in ALE (Fig. 1). When analyzing the final evolved strains (TB511-519) this information was
422 useful in deciphering which genes to focus on for strain development.
423
424 A mutation in the transcriptional activator for the pleiotropic drug resistance network, *PDR3*,
425 for example, was found in every evolved strain (Fig. 1). Up-regulation of ABC transporters
426 during limonene stress has previously been observed, but overexpression of these failed to
427 improve limonene tolerance (20). Similarly, we observed that a genomic reconstruction of the
428 mutation ($pdr3^{Q763L}$) in S288C did not significantly improve fitness (Fig. 2a). The
429 transporters may address low levels of molecular toxicity, while failing to address the major
430 issue of phase toxicity.
431
432 Mutations in tricalbin [TRI (three) CA (calcium) L (lipid) BIN (binding)](14) proteins Tcb2p
433 or Tcb3p were found in all evolved strains with maximum fitness scores (Fig. 1). A mutation
434 in *TCB2* was found early (120 generations, strain TB302 in Fig. 1), while an INDEL causing
435 truncation at position 989 of *TCB3* was found at later points. Genomic reconstruction of *tcb3-*
436 *989* in S288C revealed that the limonene resistant phenotype can be achieved by this single
437 point mutation. The correlation of genotype to phenotype is generally a complex problem
438 (15). To our knowledge, no previous report has described a single point mutation that confers
439 tolerance towards toxic next-generation biofuels in yeast.
440
441 While the specific role of the *TCB3* mutation remains unresolved, this work shows that it is
442 involved in preventing cell wall damage. Limonene stress causes reduced cell wall intergrity
443 in S288C (Fig. 3c) and strongly induces the cell wall integrity signaling pathway (Fig. 4). In

444 the reconstructed strain, limonene did not severely affect cell wall integrity (Fig. 3f) and there
445 was no activation of CWI signaling during limonene shock (Fig. 4). Limonene caused
446 different and in several instances opposite effects on the cell wall proteome, while fewer
447 genes involved in cell wall maintenance and biogenesis were affected in the tTcb3p$^{1\text{-}989}$
448 variant (Fig. 4). The mutation may directly enhance cell wall resilience, e.g., by increasing
449 the degree of crosslinking. For example, Cis3p and Ccw14p serve as structural stabilizers
450 covalently linking cell wall polysaccharides (29, 30) and were upregulated in the mutant but
451 not the S288C (Fig. 4). Enhanced wall protection was evident after investigating budding
452 patterns during limonene stress (Fig. S3).
453 In addition to biofuel applications, this work has revealed new insights into the biological
454 function of tricalbin proteins, which have homologues to synaptotagmins and SNARE
455 proteins in mammalian cells (41). Little is known about the biological function of tricalbins
456 other than that they can tether the ER to the plasma membrane (28, 41). This study links
457 tricalbins to the regulation of cell wall integrity, which has not been previously described.
458 The data also shows that when tricalbins act as a heterodimer compared to a free protein
459 different biological behavior is observed *in vivo*. This work serves as a foundation for future
460 studies to explore the mechanism and functional implications of this distinction between
461 complexed and non-complexed tricalbins.
462
463 Commercial-level biological production of monoterpenes depends on solving the toxicity
464 problem. Currently, monoterpene olefin production in yeast is still in the developmental stage
465 and titers have yet to reach inhibitory levels (60-200 mg/L). As strain engineering will lead to
466 better performing strains, phase toxicity will become a major constraint. Monoterpenes
467 passively diffuse in and out of cells (5, 12), which means that high endogenous cellular
468 production will drive transport to the extracellular environment increasing the solvent phase

and enhancing phase toxicity effects. This is the first example of successful engineering of phase tolerance and the first report of a single point mutation that confers tolerance to a range of toxic next-generation biofuels. Given the simplicity of the Tcb3p mutation, our work provides a straightforward strategy that can be tailored to any yeast strain to increase tolerance with direct applications to biocatalysis or biotransformation of toxic alkenes.

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Competing Financial Interests Statement

The authors declare no competing financial interests.

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643

644 Tables:

645 **Table 1. Summary of limonene MICs and mutations found in evolved strains**
646

Strain	Gen	MIC[a] (mg/l)	FC[b]	Mutation	Gene	Function
S288C	0	69	-	-	-	-
TB302	120	130	1.9	SNP (Q763L)	*PDR3*	Transcriptional activator of the pleiotropic drug resistance network
				INDEL (deletion E314,V315,P316)	*TCB2*	ER protein located in the mother and daughter bud, involved in ER-plasma membrane tethering
TB405	160	122	1.8	SNP (Q763L)	*PDR3*	See above
				INDEL (frame shift 989)	*TCB3*	ER protein located in the mother and daughter bud, involved in ER-plasma membrane tethering
				INDEL (frame shift 192)	*TSA1*	Thioredoxin peroxidase
				SNP (Q192G)		
				SNP (D380H)	*UBP11*	Ubiquitin-specific protease
TB511	200	138	2.0	SNP (Q763L)	*PDR3*	See above
				INDEL (frame shift 989)	*TCB3*	See above
				INDEL (frame shift 192),	*TSA1*	See above
				SNP (Q192G)		

				SNP (W580S)	*KSP1*	Serine/threonine protein kinase
				SNP (nonsense Q1014*)	*PSE1*	Karyopherin/importin that interacts with the nuclear pore complex
				SNP (L84S)	*RPL30*	Ribosomal 60S subunit protein
TB516	200	138	2.0	SNP (Q763L)	*PDR3*	See above
				INDEL (frame shift 989)	*TCB3*	See above
				INDEL, SNP (Q192G)	*TSA1*	See above
				SNP (R159T)	*CDC34*	Ubiquitin-conjugating enzyme, regulates cell cycle progression by targeting key substrates for degradation
TB517	200	145	2.1	SNP (Q763L)	*PDR3*	See above
				INDEL (frame shift 989)	*TCB3*	See above
				SNP (nonsense Q1014*)	*PSE1*	See above
				SNP (L84S)	*RPL30*	See above
TB519	200	138	2.0	SNP (Q763L)	*PDR3*	See above
				INDEL (frame shift 989)	*TCB3*	See above
				SNP (G826R)	*SIP3*	Transcription cofactor, acts through interaction with DNA-bound Snf1p
				SNP (L127F)	*YMR102c*	Protein of unknown function

[a] The minimum inhibitory concentration (MIC) is defined as the amount of limonene required to inhibit the specific growth rate by approximately 50% compared to growth rate with no solvent present for each strain.

[b] Fold change (FC) is the ratio of the MIC for the evolved and S288C strain.

Figure legends

Figure 1. Summary of the evolved strains' relative fitness and genetic mutations. Relative fitness is defined as the ratio of the cell density (OD_{660}) at 12 and 0 h during limonene exposure (138 mg/l). Error bars represent one SD above the mean for biological replicates (n = 3). The location of each mutation can be found in Table 1.

Figure 2. (a) Relative fitness towards limonene for reconstructed mutations. Relative fitness is defined in Fig 1. The evolved strain score represents the average fitness score for all evolved strains (TB302-519). Beneficial mutations in *PDR3* and *TCB3* were constructed in the parent strain (S288C). Error bars represent one SD above the mean for biological replicates (n = 3). Significance was determined by one-way ANOVA to correct for multiple comparisons relative to the S288C (****$p < 0.001$, ns, not significant; $p > 0.05$). **(b)** Diagram of S288C and mutant $tTcb3p^{1\text{-}989}$. The amino acid length is indicated; TMD, transmembrane domain; SMP, synaptotagmin-like-mitochondrial lipid-binding protein; C2, lipid binding domain.

Figure 3. Histograms representing the variation in fluorescence emitted from S288C and $tTcb3p^{1\text{-}989}$ mutant cells. (**a** and **d**) unstained cells; (**b** and **e**) unexposed control cells stained with calcofluor white (CFW); (**c** and **f**) cells stained with CFW after 2 h of limonene exposure. The mean fluorescence intensity (MFI) and percentage of damaged cells (Pop %) are reported for biological replicates (n = 3, ± SD).

Figure 4. Cell wall transcriptome and proteome response during limonene stress (+/- limonene treatment). Venn diagram representing overlap and differences in cell wall gene expression (up-regulated genes only) and protein abundance for S288C and $tTcb3p^{1\text{-}989}$ cells. Up-regulated cell wall genes from the S288C were used from Brennan et al. (6). Up-regulated

genes (mutant) and cell wall proteins (S288C and tTcb3p$^{1-989}$ mutant) were analyzed in this study. Transcriptomic data are italicized genes (e.g., *MTL1*) and proteomic data is represented as unitalicized proteins (e.g., Sag1p).

Figure 5. Relative fitness towards other terpenes. S288C, evolved (TB516), and the reconstructed tTcb3p$^{1-989}$ strains were subjected to relative fitness assays for β-pinene (198 mg/l), myrcene (361 mg/l), cymene (140 mg/l) and AMJ-700t (225 mg/l). AMJ-700t contains by volume 10% cymene, 50% limonene, 40% farnesene. Controls represent no exposure. Relative fitness is defined in Fig 1. Error bars represent one SD from the mean for biological replicates (n = 3).