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INVESTIGATION

Genetic Basis of Variation in Heat and Ethanol Tolerance in *Saccharomyces cerevisiae*

Linda Riles and Justin C. Fay[1,2]
Department of Genetics, Washington University, St. Louis, MO 63110
ORCID ID: 0000-0002-0314-1138 (J.C.F.)

ABSTRACT *Saccharomyces cerevisiae* has the capability of fermenting sugar to produce concentrations of ethanol that are toxic to most organisms. Other *Saccharomyces* species also have a strong fermentative capacity, but some are specialized to low temperatures, whereas *S. cerevisiae* is the most thermotolerant. Although *S. cerevisiae* has been extensively used to study the genetic basis of ethanol tolerance, much less is known about temperature dependent ethanol tolerance. In this study, we examined the genetic basis of ethanol tolerance at high temperature among strains of *S. cerevisiae*. We identified two amino acid polymorphisms in *SEC24* that cause strong sensitivity to ethanol at high temperature and more limited sensitivity to temperature in the absence of ethanol. We also identified a single amino acid polymorphism in *PSD1* that causes sensitivity to high temperature in a strain dependent fashion. The genes we identified provide further insight into genetic variation in ethanol and temperature tolerance and the interdependent nature of these two traits in *S. cerevisiae*.

KEYWORDS
quantitative trait mapping
yeast
natural variation

Saccharomyces cerevisiae is widely used to make fermented foods and beverages (Sicard and Legras 2011) and is the primary organism used to produce bioethanol (Azhar *et al.* 2017). Instrumental to *S. cerevisiae*'s utility is its preference for fermentation rather than respiration in the presence of oxygen (Merico *et al.* 2007) and its tolerance to high concentrations of ethanol (Stanley *et al.* 2010). Whereas the fermentative life-style evolved long ago and is shared by many other yeast species (Hagman *et al.* 2013), *S. cerevisiae*'s ethanol tolerance, a more recent acquisition, is shared only with other *Saccharomyces* species (Williams *et al.* 2015). Thus, there is considerable interest in identifying genes and genetic variation that contribute to *S. cerevisiae*'s high ethanol tolerance.

Ethanol tolerance in yeast is a complex phenotype, as it is influenced by available nutrients and growth substrates, as well as by environmental factors such as temperature and osmotic pressure (Casey and Ingledew 1986, D'Amore *et al.* 1990). The interaction between ethanol and temperature tolerance in yeast has been of particular interest due to the similarity of the ethanol and temperature stress response and also to observations that ethanol and temperature tolerance depend on one another (Casey and Ingledew 1986, Piper 1995). One mechanism that has emerged in mediating both ethanol and temperature tolerance is the plasma membrane composition and membrane fluidity. Membrane fluidity is influenced not only by phospholipid and sterol composition, but also by ethanol and temperature (Piper 1995, Ding *et al.* 2009). Furthermore, lipid composition has been found to play an important role in both ethanol and thermal tolerance (Thomas and Rose 1979, Suutari and Laakso 1994). Both ethanol and temperature tolerance have been central to applied investigations of ethanol production by yeast; fermentation at high temperature is desirable due to reduced cooling costs, higher fermentation rates and reduced contamination, but yeast is also more sensitive to ethanol at high temperature (Banat *et al.* 1998).

Various strategies have been used to understand the genetic basis of ethanol and thermal tolerance in yeast. Screens of the yeast deletion collection have uncovered several hundred genes involved in ethanol and temperature tolerance that cover a broad range of functional categories (Fujita *et al.* 2006; van Voorst *et al.* 2006; Yoshikawa *et al.* 2009; Auesukaree *et al.* 2009; Ruiz-Roig *et al.* 2010; Nakamura *et al.* 2014). Genes have also been identified from experimental evolution with selection for high ethanol (Voordeckers *et al.* 2015) or temperature (Caspeta *et al.* 2014; Huang *et al.* 2018) tolerance. Finally, strains with different ethanol or temperature tolerance have been used to map quantitative trait loci (QTL) and the underlying genes responsible for

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[1]Present address: Department of Biology, University of Rochester, Rochester, NY 14627
[2]Corresponding Author: Department of Biology, University of Rochester, 319 Hutchison Hall, RC Box # 270211, Rochester, NY 14627-0211, 585.275.2998. Email: justin.fay@rochester.edu

these differences (Hu *et al.* 2007; Sinha *et al.* 2008; Yang *et al.* 2013). However, the majority of these genetic studies examine only ethanol or temperature tolerance, but not both.

Strains of *S. cerevisiae* are phenotypically diverse (Warringer *et al.* 2011), and genetic analysis of this diversity has advanced our understanding of quantitative traits (Liti and Louis 2012). While laboratory and industrial strains have provided important insights into genes and pathways responsible for various traits of interest, there is high genetic diversity in natural isolates (Peter *et al.* 2018), and they provide a complementary approach to identifying genes relevant to a trait of interest (Swinnen *et al.* 2012).

In this study we undertook a genetic analysis of naturally occurring variation in resistance to ethanol at high temperature. From 25 genetically diverse strains isolated from various environments, we identified a Chinese strain that was sensitive to ethanol at high temperature. Through crosses to two resistant strains, we identified two genes, *SEC24* and *PSD1* that are largely responsible for the ethanol and temperature sensitivity. Two amino acid substitutions in *SEC24* underlie the major quantitative trait locus (QTL) for ethanol tolerance at high temperature in both crosses. For temperature tolerance alone we found a single amino acid substitution in *PSD1* that underlies the major QTL in one of the crosses. Our results show that the genetic variation in tolerance to ethanol at high temperatures can be distinct from either ethanol tolerance or temperature tolerance alone.

MATERIALS AND METHODS

Yeast strains

The screen for variation in ethanol and temperature tolerance was carried out using 8 wild yeast strains from different regions of China (Wang *et al.* 2012), 15 wild strains from North America (Sniegowski *et al.* 2002; Fay and Benavides 2005; Hyma and Fay 2013), and one strain each from the Philippines and Nigeria (Fay and Benavides 2005). All strains were diploid, and the strains from China were constructed by mating two haploid versions of each strain with opposite mating types (Table S1). Forty-nine haploid strains from the Yeast Gene Deletion Collection (Winzeler *et al.* 1999) were used for non-complementation tests with HN6-α. All the strains used in this study are described in Table S1, and those commonly referenced in the text are listed in Table 1.

Phenotype assays

Strains were grown on YPD (rich medium) agar plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) supplemented with 0–10% ethanol at 25°, 30°, 37° and 40°. Ethanol was added to cooled medium immediately prior to pouring plates and used the next day after drying overnight at room temperature. Yeast strains were plated and kept wrapped in plastic film in the incubator to limit evaporation. Strains were pinned from overnight YPD microtiter plates to Singer Plus solid YPD plates, using a Singer RoTor © HDA, to a density of 384 colonies per plate. All phenotyping plates were pinned using the Singer RoTor from the Singer Plus solid medium YPD plates grown for two days to a uniform colony size at 30° to Singer Plus solid medium YPD plates with different ethanol concentrations. To avoid edge effects, the two bottom, top, left and right sides of the rectangular plates were pinned with a resistant control strain. Spot dilutions were made by diluting overnight liquid YPD cultures 1:50 with water and then making 1:10 serial dilutions.

QTL mapping

Two sets of haploid recombinant strains were generated for QTL mapping using the ethanol and temperature sensitive strain HN6-α.

Table 1 Subset of strains used in this study

Strain	Genotype
HN6-α	MATα HO:KanMX4
SD1-a	MATa HO:HygMX4
HN6	HN6-a/HN-α diploid, HO::KanMX4/HO::KanMX4
SD1	SD1-a/SD-α diploid, HO::HygMX4/HO::HygMX4
YPS163 (Oak)	MATa/α diploid, wildtype
YJF153 (Oak-a)	MATa HO:dsdAMX4, derivative of YPS163
YJF173	MATa HO::ura3-52, derivative of S288c
YJF181	MATa HO::dsdAMX4, derivative of M22 wine strain
BY4741	MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0, derivative of S288c
YJF2609	HN6-α x YJF153 recombinant, ethanol/heat sensitive, G418-
YJF2702	HN6-α x YJF153 recombinant, heat sensitive, G418-
YJF2703	HN6-α x YJF153 recombinant, heat sensitive, G418-

A set of 58 recombinants was generated from a cross between HN6-α and a haploid Oak strain derived from YPS163 (YJF153, which we refer to as 'Oak-a'), and a set of 73 recombinants was generated from a cross between HN6-α and SD1-a (Table 1). For each cross, haploid parental strains were mated and sporulated, and tetrads were dissected using a Singer System MSM 200 microscope. Recombinant strains were phenotyped by measuring the size of a single colony (mm) using a ruler and printed images after 2 days of growth at 37° on YPD and YPD supplemented with 6% ethanol (Table S2).

Recombinant strains were genotyped using RAD-seq, restriction site associated sequencing (Baxter *et al.* 2011), as previously described (Hyma and Fay 2013). DNA was extracted, digested with *Mfe*1 and *Mbo*1 and ligated to one of 48 barcoded sequencing adaptors (Table S3). Ligated products were pooled into groups of 48, size selected (150-500 bp) by gel extraction and amplified using extension primers containing one of three different indexes (Table S3). Amplified pools were quantified, pooled at equal concentrations and sequenced on an Illumina HiSeq2500. A total of 92 million reads was mapped to the S288c reference genome using BWA (v0.7.5a, Li and Durbin 2009), yielding a median of 600k mapped reads per strain. SNPs were called using the GATK unified genotyping algorithm (McKenna *et al.* 2010) and variants were filtered using vcftools (Danecek *et al.* 2011) to eliminate sites with >10% missing data, qualities less than 60 and more than two alleles. In total, 893 markers were segregating across the genome in both crosses (HN6-α x Oak-a and HN6-α x SD1-a) and were used for mapping (Data File S1). Prior work has shown that RAD-seq samples approximately 1% of the genome, and the number of markers recovered is close to that expected given the average percent difference between *S. cerevisiae* strains of 0.03% (Cromie *et al.* 2013).

R/qtl (Broman *et al.* 2003) was used for linkage analysis. The Haldane mapping function was used to estimate recombination rates, and phenotypes were mapped using the EM algorithm with a non-parametric phenotype model. Bayesian 95% confidence intervals were obtained for the quantitative trait loci (QTL) on chromosomes 9 and 14 using R/qtl.

Candidate gene complementation tests

Genes within QTL intervals were tested for complementation using two approaches: MoBY plasmid rescue and hemizygosity tests of non-complementation using the yeast deletion collection (Table S5). MoBY ORFs, Molecular Barcoded Yeast plasmids (Ho *et al.* 2009), were transformed into a heat and ethanol sensitive recombinant strain (YJF2609,

from HN6-α x Oak-a) for genes within the chromosome 9 QTL and into a heat sensitive recombinant strain (YJF2702, from HN6-α x Oak-a) for genes within the chromosome 14 QTL. Both recombinants were from the HN6-α x Oak-a cross. For tests of non-complementation, HN6-α was mated to BY4741 strains carrying single gene deletions within both the chromosome 9 and the chromosome 14 QTL regions. The colony area of HN6-α x Knockout hybrids was determined using ImageJ (Collins 2007). Images were cropped, converted to 8-bit (black and white), and then the threshold function was used to eliminate noise and clearly define colonies. Colony size was obtained for four replicate colonies from the output of the "Analyze Particles" function with size parameter of "20-infinity". A *t*-test was applied to identify knockouts that failed to significantly complement the defect.

Cloning

DNA from genes and their promoter regions was amplified by PCR and cloned into *Bam*HI/*Bgl*II cut, gel purified CEN plasmid pAG26, a gift from John McCusker (Addgene plasmid #35127) by gap repair in yeast. High copy plasmids were made by cloning Oak-a alleles of *SEC24* and *PSD1* into *Spe*I/*Xma*I cut, gel purified pRS42H (EUROSCARF #P30636, Taxis and Knop 2006) in a similar manner. Chimeric constructs were generated by PCR and gap repair of the desired parental segments. Primers used in this study are in Table S3. Allele specific restriction digests were used to confirm the constructs.

Rho° mutants

Strains were grown to saturation in the dark twice in minimal medium (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% dextrose) supplemented with 25ug/ml ethidium bromide at 30° to produce Rho° mutants, which were confirmed by streaking for single colonies on YPGly (1% yeast extract, 2% peptone, 5% glycerol, 2% agar). After this treatment mitochondrial DNA disappears (Goldring *et al.* 1970).

Site Directed Mutagenesis

PCR reactions were run using Pfu Ultra II Fusion HS DNA polymerase (Agilent Technologies, Santa Clara, CA), and pAG26 with inserts of either the Oak-a version of *SEC24* or the Oak-a version of *PSD1* as template. Site directed mutants were generated by amplifying plasmids with complementary mutant megaprimers (Tseng *et al.* 2008). XL 10-Gold Ultracompetent Cells (Agilent Technologies, Santa Clara, CA) were transformed separately with 4 PCR reactions for each mutant, and DNA from the resulting transformed strains was sequenced.

Amino acid altering mutations were generated in the Oak-a allele of *SEC24*, and mutant DNA from four independent PCR reactions was transformed into HN6-α. Phenotypes of the resulting strains were tested on YPD with and without 6% ethanol at 30° and at 37°. Three constructs from each *SEC24* mutant were sequenced, and 7 of 12 were correct.

Amino acid altering mutations were generated in the Oak-a allele of *PSD1*, and mutant DNA from four independent PCR reactions was transformed into YJF2703 (very heat sensitive). Phenotypes of the resulting strains were tested on YPD at 30° and at 37°. Three constructs from each *PSD1* mutant were sequenced, and 8 of 9 were correct.

Data availability

All strains and constructs are available upon request to the corresponding author. Supplemental Table 1: Strains used in this study. Supplemental Table 2: Recombinant strain barcode indices, sequencing and phenotypes used for QTL mapping. Supplemental Table 3: Primers used in this study. Supplemental Table 4: Summary of candidate genes tested for two QTL regions. Supplemental Table 5: Genes tested for complementation using a MoBY and/or a hemizygosity test. Supplemental Table 6: Colony size measurements using ImageJ for HN6 X KO Hemizygotes on Chromosome 14. Supplementary Figure 1: Ethanol and heat sensitivity of control strains. Supplemental Figure 2: Logarithm of the odds ratio (LOD) of a quantitative trait locus for heat and ethanol tolerance at high temperature across the genome. Supplemental Figure 3: Protein alignment of HN6, Oak and S288c for *SEC24* and *PSD1*. Supplemental Figure 4: Phenotypes of resistant wild yeast strains, Oak, Wine and S288c, containing high copy Oak allele plasmids grown at 40°. Data File S1. Genotypes and phenotypes of recombinant strains used for QTL mapping. The raw data used to call genotypes for QTL mapping has been deposited into NCBI's SRA under BioProject: PRJNA480857. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7152065.

RESULTS

To identify naturally occurring variation in temperature and ethanol resistance we screened 25 diploid strains of diverse origin (Table S1) by pinning to rich medium plates supplemented with 0–10% ethanol and growing at 37°. Heat alone was sufficient to severely restrict the growth of the HN6 diploid and had a moderate effect on three other diploid strains: HN9, SX6 and HLJ2 (Figure 1). Although most strains were able to grow at 37° on plates supplemented with ethanol, three strains from primeval forests in China, HN6, HN9 and HN14, were more sensitive to ethanol at high temperature than the others. Most strains, including the China strain SD1, and especially the North American Oak strain YPS163, tolerated growth at 37° with concentrations of ethanol as high as 6–8% (Figure 1A). None of the strains showed much variation in growth at 30° with or without 6% ethanol (Figure 1B).

HN6 (China, primeval forest), the most sensitive China strain and two heat and ethanol resistant strains, SD1 (China, persimmon) and YPS163 (North American Oak), were selected for quantitative trait (QTL) mapping. Hybrids were generated from haploid versions of HN6, SD1 and YPS163. We refer to YPS163 and its haploid derivative (YJF153) as Oak and Oak-a, respectively (Table 1). Both HN6-α x Oak-a and HN6-α x SD1-a diploid hybrids were temperature and ethanol resistant, indicating that HN6-α carries recessive temperature and ethanol sensitive alleles (Table S2). We generated 58 and 73 recombinant progeny from the HN6-α x Oak-a and HN6-α x SD1-a crosses, respectively. The 131 recombinants were phenotyped at 37° on rich medium plates with and without 6% ethanol and genotyped at 893 markers across the genome using RAD-sequencing (Methods). Controls were the haploid parental strains and the hybrids (Table S2). In contrast to the very poor growth of the homozygous diploid HN6 at 37° in the initial screens (Figure 1), HN6 showed moderate growth in later assays (see Figure 5a and Figure S1).

In both sets of recombinants, approximately 50% of the strains were able to grow at 37° with 6% ethanol, indicating a single, major effect QTL. Consistent with this finding, we identified a single QTL in both crosses for growth at 37° with ethanol on chromosome 9 (Figure 2, Figure S2). The recombinant strains showed a more continuous distribution of growth at 37° without ethanol (Table S2). We identified a single QTL on chromosome 9 for the HN6-α x SD1-a recombinants, but a different QTL on chromosome 14 for the HN6-α x Oak-a recombinants (Figure 2, Figure S2).

Complementation tests

We used two different approaches to test genes within each QTL for their effects on temperature and ethanol. Because the diploid hybrids made

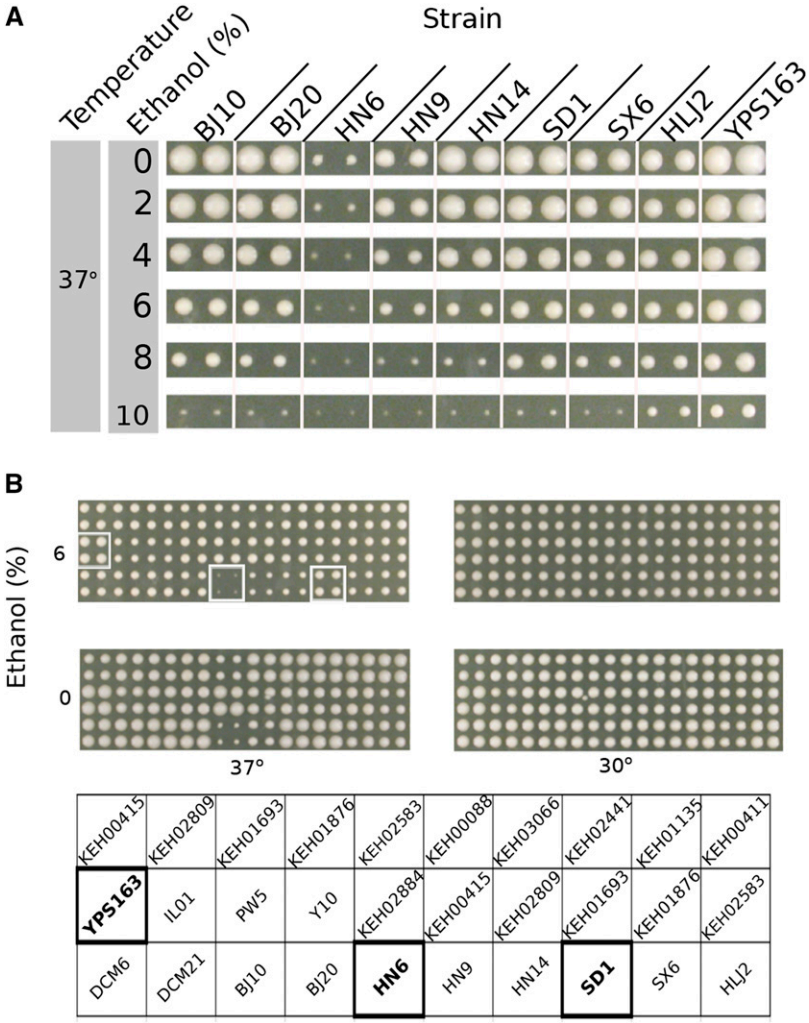

Figure 1 Ethanol and temperature dependent variation in colony size. (A) Nine diploid strains were pinned in duplicate onto rich medium plates at different ethanol concentrations (0–10%) and grown at 37°. Pictures were taken after 3 days of growth. (B) Temperature and ethanol sensitivity of twenty-five diploid *S. cerevisiae* strains. Strains were grown in quadruplicate at either 30° or 37° on rich medium (YPD) without ethanol or with 6% ethanol. The layout of the strains is shown by the names below the images and the strains chosen for further study (HN6, SD1 and YPS163) are in bold and are outlined in white.

with the Oak-a strain are temperature and ethanol resistant, we would expect that (1) addition of a causal allele on a plasmid from a temperature and ethanol resistant strain into HN6-α should increase growth, and that (2) the absence of a causal allele should reduce growth when hemizygous in an otherwise resistant diploid hybrid. For plasmid rescue experiments we used the MoBY collection of plasmids containing the coding and adjacent noncoding regions of the lab strain BY4741, a derivative of S288c, which is both temperature and ethanol resistant (Figure S1), and so presumed to carry the dominant alleles at the loci of interest. For the hemizygosity analysis we mated the sensitive strain, HN6-α, to BY4741 deletion strains. We used the plasmid and hemizygote assays rather than reciprocal hemizygosity analysis (Steinmetz *et al.* 2002) to facilitate screening the large number of genes in both regions.

Two amino acid substitutions in SEC24 cause sensitivity to ethanol at high temperature

There are 24 genes within the 56kb region on chromosome 9 associated with growth on ethanol at 37° (Table S4). Single gene deletion strains and MoBY plasmids were available for 14 genes in this region; another 8 genes had either single gene deletions or MoBY plasmids, and neither deletion strains nor plasmids were available for two genes (Table S5). None of the hybrids generated by crossing HN6-α to the deletion strains were sensitive to growth at 37° with ethanol (Figure 3A). However, a single MoBY plasmid carrying *SEC24*, an essential gene, rescued growth of a recombinant strain (YJF2609, from HN6-α x Oak-a) at high temperature with ethanol (Figure 3B). We used a recombinant strain that did not grow at 37° with ethanol rather than HN6-α (HO::KanMX4), because the recombinant did not carry the G418 resistance gene (KAN), needed to select for the MoBY plasmids. Since *SEC24* is an essential gene in S288c, it could not be tested for non-complementation using deletion strains.

To confirm *SEC24* as a causal gene we tested the HN6-α and Oak-a alleles of *SEC24* for plasmid rescue and found only the Oak-a allele rescued growth (Figure 4A). To identify causal variants within *SEC24* we first generated chimeric constructs between the HN6-α and Oak-a alleles. Plasmids bearing the HN6-α promoter region and the Oak-a coding region rescued growth at high temperature with ethanol, whereas those bearing the Oak-a promoter region and the HN6-α coding region did not (Figure 4B).

To identify causal mutations within *SEC24* we tested three nonsynonymous changes present in the coding region, all of which are near the 3′ end of the gene (Table 2, Figure S3). SIFT predictions (Sim *et al.* 2012) suggest that P786S and E839K affect gene function and that V842A will be tolerated. Here and below, the first letter refers to the amino acid in Oak and S288c (Figure S3), and the last letter refers to the

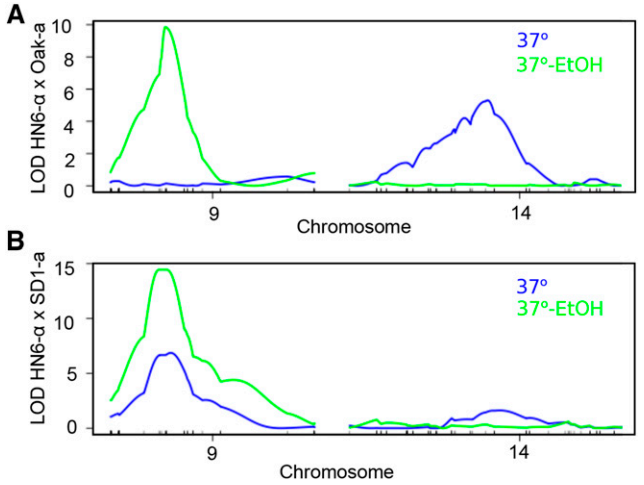

Figure 2 Quantitative trait loci for high temperature growth with and without ethanol. Logarithm of the odds ratio (LOD) of linkage across chromosomes 9 and 14, using (A) HN6-α x Oak-a recombinants and (B) HN6-α x SD1-a recombinants, grown at 37° (blue) and 37° with 6% ethanol (green).

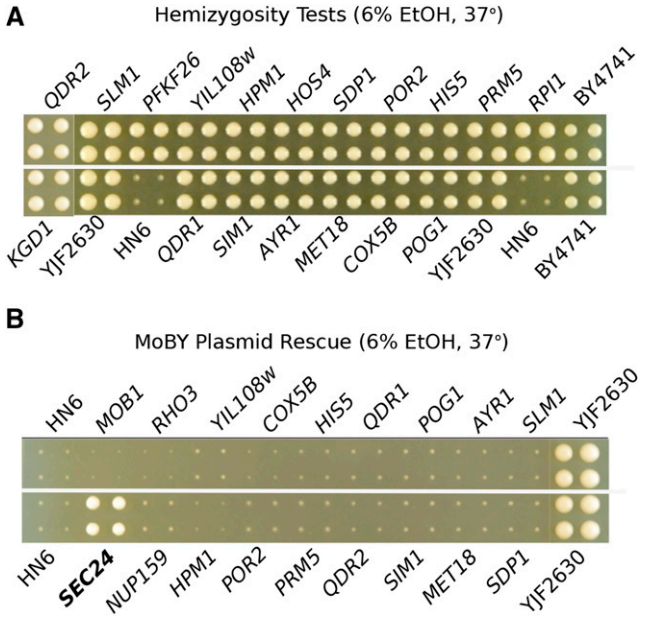

Figure 3 Complementation tests of genes within the chromosome 9 QTL identify *SEC24*. (A) HN6-α x deletion strain hybrids grown at 37° with 6% ethanol. The control hybrid HN6-α x BY4741 (YJF2630) and the parental strains HN6-α and BY4741 are also shown. There are four replicate colonies for each strain with the names of deleted genes above and below the colony images. All deletions complemented the no growth phenotype. (B) YJF2609 recombinant strains (HN6-α x Oak-a) carrying MoBY plasmids were grown at 37° with 6% ethanol, four replicate colonies per strain with genes carried by the plasmid above and below the colony images. The HN6-α and HN6-α x BY4741 (YJF2630) controls are also shown. The *SEC24* MoBY rescued the no growth phenotype of YJF2609.

HN6-α amino acid. The Fungal Orthogroups Repository (Wapinski *et al.* 2007) shows high conservation for Oak allele at P786S, less for E839K and very little for V842A.

Phenotypic tests of the three non-synonymous changes in the Oak-a allele of *SEC24* grown with 6% ethanol at 37° gave the following results: mutant P786S grew to the same extent as the positive control YJF2696; mutants E839K and V842A failed to grow. The double mutant E839K, V842A also failed to rescue growth (Figure 4C). Thus, either E839K or V842A can explain the sensitivity of HN6-α to ethanol at high temperature.

A single amino acid substitution in PSD1 causes sensitivity to high temperature

There are 39 genes within the 65kb region on chromosome 14 associated with growth at 37° without ethanol (Table S4). Single gene deletions and MoBY plasmids were available for 22 genes in this region; another 15 genes had either single gene deletions or MoBY plasmids, and only two genes were not represented by either a deletion strain or MoBY plasmid (Table S5). Three of the deletions, YNL170W, YNL169C/*PSD1* and YNL184C, showed significant effects ($P < 0.01$, *t*-test) in the hemizygous diploids (Figure 5A, Table S6), with an average ± SD of colony size of 2,735 ± 94, 3,251 ± 154 and 3,062 ± 44 pixels, respectively, compared to 4,388 ± 355 pixels in the controls (HN6-α x BY4741). Only one of the MoBY plasmids, bearing *PSD1*, rescued high temperature growth of YJF2702 (Figure 5B). The small ORF YNL170W, which had a phenotype in the hemizygosity test, has an overlap of 205bp with the 5′ end of *PSD1*, which includes G489R, the mutant responsible for the heat sensitive phenotype (see below). Plasmid rescue was conducted in the heat sensitive recombinant strain YJF2702 (from HN6-α x Oak-a) since it does not carry the KAN gene, required for maintenance of the MoBY plasmids.

To confirm that allele differences in *PSD1* affect high temperature growth, plasmids bearing the Oak-a and HN6-α alleles of *PSD1* were tested for complementation. While the Oak-a allele of *PSD1* did not rescue growth of HN6-α at 37° (Figure 6A), it was able to rescue high temperature growth of two heat sensitive recombinant strains, YJF2702 and YJF2703 (Figure 6B). Both of these recombinant strains grow at 37° on rich medium with 6% ethanol, and YJF2702 has a larger colony size (5mm) than YJF2703 (4mm). Both have the Oak-a genotype at *SEC24*.

One potential difference between the recombinant strains and HN6-α is their mitochondria.

The recombinant strains could inherit either the HN6-α or Oak-a mitochondria. Because *PSD1* functions in the mitochondria, and its deletion affects mitochondrial phenotypes (Joshi *et al.* 2012; Chan and McQuibban 2012), we tested whether (1) the heat sensitivity of HN6-α depended on the mitochondrial type and (2) whether *PSD1* rescue depended on the mitochondrial type. To test for mitochondrial effects we generated HN6-α x Oak-a hybrids with HN6-α mitochondrial DNA, Oak-a mitochondrial DNA, or no mitochondrial DNA. Only hybrids lacking a mitochondrial genome showed reduced growth, as expected for a petite mutant, and no difference was found between hybrids with HN6-α or Oak-a mitochondrial DNA (Figure 7A). To test whether rescue by the Oak-a allele of *PSD1* in the recombinant strains depends on mitochondrial type, we generated 2 recombinants (YJF2702 and YJF2703) carrying the Oak-a allele of *PSD1* on a plasmid, but lacking mitochondrial DNA (Rho^0). For both recombinants, the *PSD1* plasmid enhanced growth at high temperature in both the presence and absence of mitochondrial DNA, and the more heat sensitive recombinant (YJF2703) showed a larger effect (Figure 7B). Thus, the deficiency in the HN6-α allele of *PSD1* does not depend on the mitochondrial type or respiration conferred by mitochondrial DNA, indicating that some other locus besides mitochondria is responsible for the *PSD1* plasmid effect in recombinant strains but not in HN6-α.

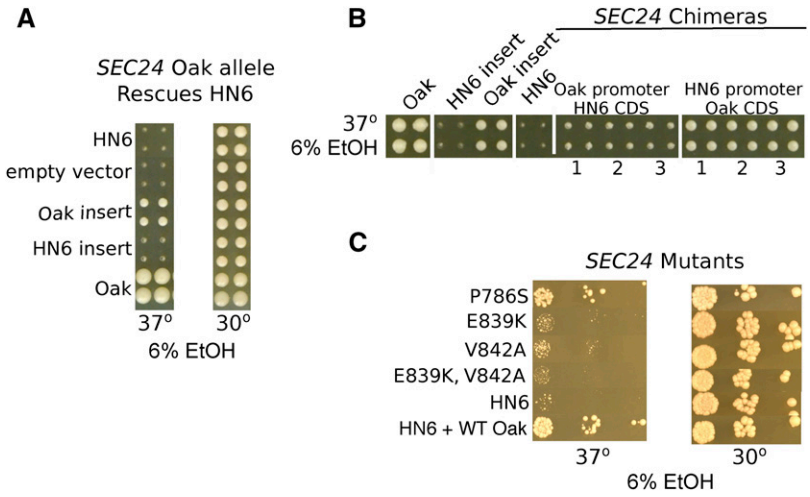

Figure 4 Two amino acid changes in *SEC24* are responsible for growth at 37° with ethanol. (A) Growth of HN6-α at 37° with 6% ethanol is rescued by plasmids bearing the Oak-a allele of *SEC24* but not the HN6-α allele of *SEC24* or the empty plasmid (pAG26), in comparison to the Oak-a strain (YJF153). Growth at 30° with 6% ethanol is shown as a control. (B) Growth of HN6-α at 37° with 6% ethanol is rescued by plasmids bearing the coding region of *SEC24* from Oak-a, relative to the Oak-a strain (YJF153). *SEC24* alleles with HN6-α promoter and the Oak-a coding region rescue the phenotype, whereas alleles with the Oak-a promoter and HN6-α coding region do not. Three transformants each are shown. The parental alleles for Oak-a and HN6-α along with the Oak-a resistant and HN6-α sensitive parental strains are controls. (C) Growth of HN6-α at 37° with 6% ethanol is rescued by plasmids bearing mutant P786S, but not by mutants E839K or V842A, either as single mutants or as the double. The controls are the parent strain HN6-α and YJF2696 (HN6-α with the WT Oak-a plasmid).

To identify causal variants in *PSD1*, we generated chimeric constructs between the HN6-α and Oak-a alleles. Plasmids bearing the HN6-α promoter region and the Oak-a coding region rescued growth of two heat sensitive recombinants (from HN6-α x Oak-a), YJF2702 and YJF2703, at high temperature, whereas those bearing the Oak-a promoter region and the HN6-α coding region did not (Figure 6C). We found three non-synonymous differences between the HN6-α and Oak-a alleles of *PSD1* (Table 2, Figure S3). According to SIFT predictions (Sim *et al.* 2012), only mutant G489R, quite close to the 3′ end of the gene, is likely to affect function; mutants G19R and S100N should be tolerated. The Fungal Orthogroups Repository (Wapinski *et al.* 2007) showed extremely high conservation for Oak allele at G489R and very little conservation in the other two.

Phenotypic tests of the three non-synonymous changes in the Oak-a allele of *PSD1* grown at 37°, gave the following results: mutant G19R and S100N rescued the heat phenotype, whereas mutant G489R failed to rescue (Figure 6D).

Expression of the Oak alleles of SEC24 and PSD1 from a high copy plasmid does not enhance tolerance to heat and ethanol or to heat alone

We tested whether expression of the Oak-a alleles of *SEC24* and *PSD1* from a high copy plasmid would further enhance tolerance of sensitive strains to heat and ethanol or to heat alone, as a consequence of presumably higher expression. HN6-α bearing the Oak-a allele of *SEC24* in high copy grew very poorly on YPD at 37° Compared with the same strain with the gene in a CEN plasmid. The CEN plasmid version of the strain showed somewhat less growth on YPD+ethanol at 37°; however, the strain with the high copy plasmid did not grow at all (Figure 8A). Although rescued by the Oak-a *PSD1* CEN plasmid, the heat sensitive strain YJF2703 bearing a high copy Oak-a allele of *PSD1* grew poorly on YPD at 37° (Figure 8B).

The effects of *SEC24* and *PSD1* on resistance to heat and ethanol and resistance to heat alone raise the possibility that increasing the activity of these genes might enhance heat and ethanol resistance in strains that carry the resistant *SEC24* and *PSD1* alleles: Oak, Wine (YJF181) and S288c (YJF173). We tested whether expression of *SEC24* and *PSD1* would improve resistance using high copy plasmids. Since 37° is a permissive temperature for the resistant Oak, Wine and S288c strains, 40° was used for phenotype testing.

The presence of high copy or CEN plasmids bearing the Oak-a alleles of *SEC24* or *PSD1* did not enhance the growth of the Oak, Wine or S288c strains grown on YPD at 40°; and there was no difference in growth between the CEN and high copy plasmids in these strains (Figure S4A). Although growth was abundant on YPD at 40° by day 3, there was little or no growth on YPD+ethanol at 40° (Figure S4B).

Table 2 Non-synonymous changes in *SEC24* and *PSD1*

Gene	Allele[1]	Conservation (%)[2]
SEC24	P786S	85
	E839K*	78
	V842A*	26
PSD1	G19R	19
	S100N	11
	G489R*	96

[1] First letter is the Oak = S288c amino acid, followed by position in Oak protein; last letter is HN6 amino acid. 22 (*SEC24*) and 13 (*PSD1*) synonymous changes were not tested. *Amino acid substitutions found to cause reduced growth.
[2] Conservation is the frequency of the Oak allele in 27 fungal species in the Orthogroups Repository.

DISCUSSION

S. cerevisiae is known for its resistance to high concentrations of ethanol (Arroyo-López *et al.* 2010). However, ethanol resistance is also known to be temperature dependent (Casey and Ingledew 1986). Prior studies have either focused on genetic variation in either ethanol or temperature stress but not both (Khatun *et al.* 2017; Kitichantaropas *et al.* 2016; Nuanpeng *et al.* 2016; Benjaphokee *et al.* 2012; Peter *et al.* 2018). In this study we mapped ethanol tolerance at high temperature and identified two large effect amino acid substitutions in *SEC24*. We also identified a smaller effect amino acid substitution in *PSD1* that caused resistance to heat alone. Our results provide new evidence for the involvement of ER to Golgi transport in ethanol tolerance at high temperature (*SEC24*) and support prior work (Jiménez and Benítez 1988) on the role of mitochondrial function in temperature tolerance (*PSD1*).

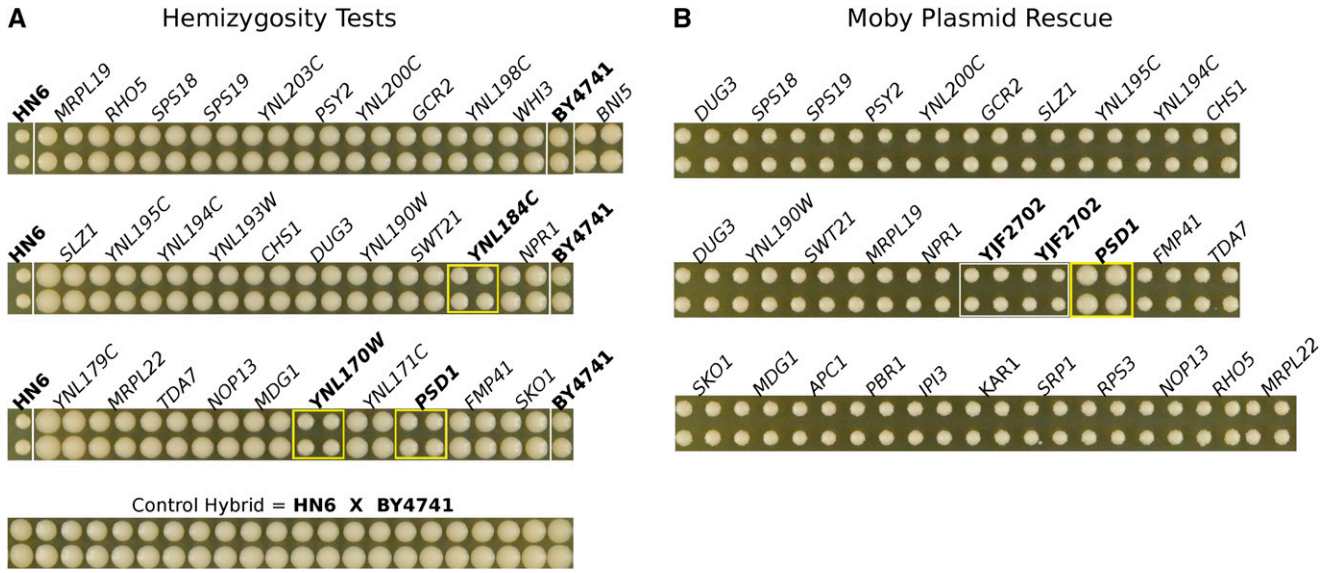

Figure 5 *PSD1* underlies the heat sensitivity on Chromosome 14. (A) Heat sensitivity of the HN6-α x Knockout hybrids, grown at 37° on YPD. The control hybrid YJF2630 (HN6-α x BY4741), HN6-α and BY4741 parental strains and three hemizygous strains that did not fully complement the heat sensitive phenotype (outlined in yellow) are labeled in bold type. Four replicate colonies are shown for each strain. (B) *PSD1* MoBY plasmid rescue of heat sensitivity of the recombinant strain YJF2702 (from the HN6-α x Oak-a cross). YJF2702 without the plasmid is shown in the second row (bold type). The strain bearing the *PSD1* plasmid is adjacent to it (outlined in yellow). Four replicate colonies are shown for each strain.

Differences in genetic background lead us to identify one gene involved in heat and ethanol tolerance (*SEC24*) and one gene involved in heat tolerance alone (*PSD1*) through quantitative trait mapping in two crosses. Both crosses shared the parental strain HN6-α, a haploid derivative of a heat and ethanol sensitive strain isolated from a primeval forest in Hainan, China (Wang *et al.* 2012). However, the two crosses differed in the other parent (Oak-a or SD1-a) and in the effects of the sensitive *SEC24* and *PSD1* alleles from HN6-α. In the HN6-α x SD1-a cross, the HN6-α *SEC24* allele conferred sensitivity to heat and even greater sensitivity to heat and ethanol combined. However, in the HN6-α x Oak-a cross, the HN6-α *SEC24* allele only caused sensitivity to heat and ethanol combined, whereas the HN6-α allele of *PSD1* caused sensitivity to heat alone. Both HN6-α defects could be rescued by plasmids carrying the corresponding alleles of *PSD1* and *SEC24* from Oak, a resistant strain collected from an oak tree in Pennsylvania, USA (Sniegowski *et al.* 2002). However, in the case of *PSD1*, rescue only occurred in HN6-α x Oak-a recombinant strains bearing the *PSD1* sensitive allele, indicating that alleles from the Oak-a background are necessary for expression of *PSD1* heat tolerance. We refined the

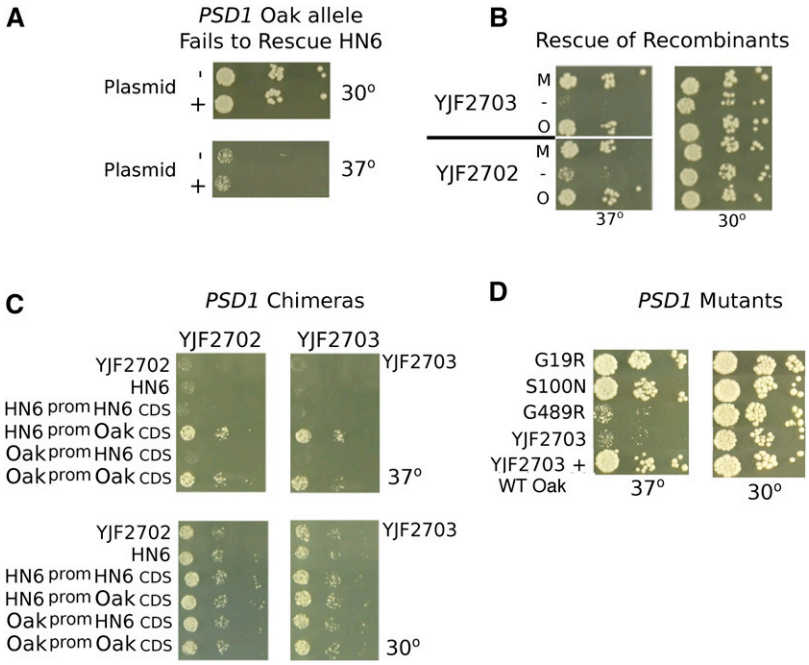

Figure 6 Rescue of the heat sensitive phenotype by Oak-a alleles of *PSD1*. (A) Spot dilutions show that the *PSD1* Oak-a plasmid fails to rescue the heat sensitive phenotype in the HN6-α parent strain grown on YPD at 37°. (B) Recombinant strains YJF2702 and YJF2703 (HN6-α x Oak-a) are rescued by the *PSD1* Oak-a plasmid (O) and by the *PSD1* MoBY (M) but not an empty vector (-). (C) YJF2702 and YJF2703 were transformed with chimeric *PSD1* plasmids, which contained either the Oak-a promoter joined to the HN6-α CDS, or the HN6-α promoter with an Oak-a CDS. The chimeric HN6-α promoter Oak-a CDS plasmid rescues the phenotype to the same extent as the control Oak-a plasmid, indicating that the CDS is responsible for rescue. The Oak-a promoter HN6-α CDS plasmid and the control HN6-α plasmid fail to rescue. (D) Mutating the three non-synonymous sites in *PSD1* shows that mutant G489R fails to rescue YJF2703 on YPD at 37°. Mutants G19R and S100N rescue heat sensitivity to the same extent as the positive control, YJF2908 (YJF2703 with the WT Oak-a plasmid).

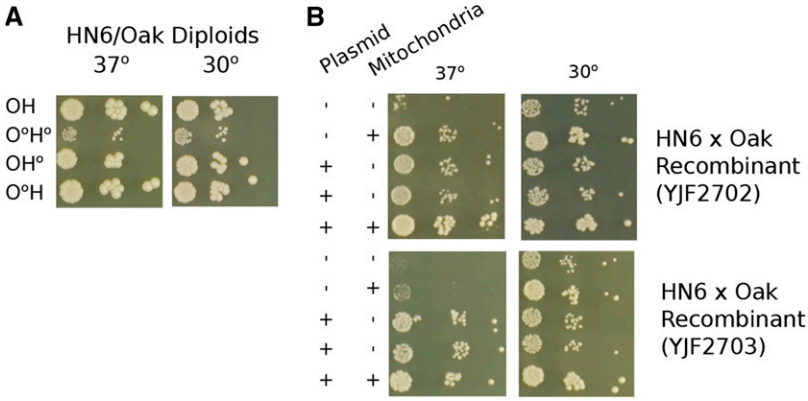

Figure 7 Mitochondrial involvement in the rescue of heat sensitive strains. (A) Spot dilutions of HN6-α/Oak-a diploids with mitochondrial DNA inherited from either parent, both parents, or no mitochondria show that strains are able to grow well at 37° with mitochondria from either parent. The O^0H diploid lacks Oak-a mitochondria; H^0O, lacks HN6-α mitochondria; O^0H^0 has no mitochondria; OH is derived from Rho^+ parents. (B) Spot dilutions of heat sensitive recombinant strains YJF2702 and YJF2703 from the HN6-α x Oak-a show that both YJF2702 and YJF2703 containing the plasmid, but had no mitochondria, grew nearly as well as the strains containing both the plasmid and mitochondria.

effects of the HN6-α alleles to a single amino acid substitution in *PSD1* and either of two substitutions in *SEC24*.

The phenotypic effects of amino acid substitutions in *SEC24* point to its importance in the combined tolerance to ethanol and high temperature. However, because *SEC24* is an essential gene it is also possible that the amino acid substitutions result in temperature sensitive alleles of *SEC24*, and that *SEC24* is not inherently involved in ethanol and heat tolerance. Our observation that *SEC24* alleles do not influence sensitivity to heat alone in one of the two crosses (HN6-α x Oak-a) does not support this later interpretation. Another potential mechanism of *SEC24* mediated sensitivity to ethanol and heat is its role in ER to Golgi transport. ER to Golgi transport is an important component of protein quality control; misfolded proteins in the ER are transported back into the cytoplasm in order to be degraded by the ubiquitin–proteasome system (Taxis *et al.* 2002). Both heat and ethanol are known to disrupt the integrity of membranes and cause proteins to denature (Casey and Ingledew 1986, Singer and Lindquist 1998), providing one explanation for *SEC24* mediated sensitivity to heat and ethanol stress. While it is not obvious why the *SEC24* allele from HN6-α is particularly sensitive to heat in the presence of ethanol, this phenotype may be mediated by defects in the transport of proteins important to heat and ethanol tolerance or to defects in the Golgi or ER membranes themselves.

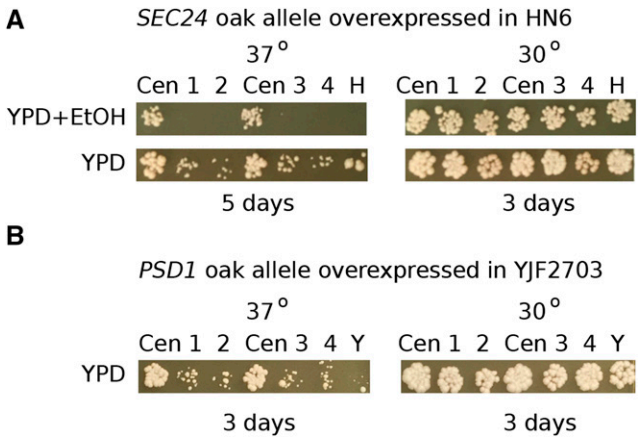

Figure 8 High copy plasmids containing Oak-a alleles of *SEC24* and *PSD1* do not enhance growth. (A) HN6-α is rescued on YPD+ethanol at 37° by the *SEC24* CEN plasmid, but the high copy version is lethal. On YPD alone HN6-α with the high copy plasmid grows very poorly at high temperature (5 days of growth). The 30° Controls are shown at 3 days of growth. There are four transformants (1 - 4) of each high copy plasmid. H = HN6, Cen = CEN plasmid. (B) A heat sensitive recombinant strain (YJF2703 from HN6-α x Oak-a) is rescued on YPD at 37° by the *PSD1* CEN plasmid, but the high copy version is deleterious. These and the 30° Controls are shown at 3 days of growth. There are four transformants (1 - 4) of each high copy plasmid. Y = YJF2703, Cen = CEN plasmid.

The mechanism by which *PSD1* affects heat sensitivity is likely related to its impact on mitochondrial membranes, but depends on other genetic factors coming from the Oak-a background. *PSD1* converts phosphatidylserine to phosphatidylethanolamine (PE), a mitochondrial phospholipid that plays an important role in mitochondrial fusion and in the maintenance of mitochondrial morphology (Joshi *et al.* 2012, Zhang *et al.* 2014). Mitochondrial function is known to be important for intrinsic heat resistance, and deletion of two genes, *CHO1* and *OPI3*, required for conversion of PE to phosphatidylcholine results in heat shock sensitivity (Jarolim *et al.* 2013). Furthermore, it has been proposed that heat induced changes in membrane fluidity influence the perception of high temperature and the expression of heat shock proteins (Carratù *et al.* 1996). While *PSD1* has not previously been identified as a gene conferring resistance to high temperatures, this may be a consequence of its dependence on unknown genetic factors segregating in the HN6-α x Oak-a recombinants. Our complementation analysis indicates that this unknown factor is not the mitochondrial DNA type.

Expression of Oak-a alleles of *PSD1* and *SEC24* from a high copy plasmid did not enhance heat or heat and ethanol tolerance, and in some instances was toxic. The lethality in sensitive strains and failure to enhance growth in resistant strains could be caused by the fact that Sec24 is one of five essential proteins that form the COPII vesicle coat. Along with Sec23, it forms the inner coat of the vesicle as a heterodimer and binds the cargo that will be transported from the rough Endoplasmic Reticulum to the Golgi apparatus (Jensen and Schekman 2011 review). High copy expression of *SEC24* might lead to an overabundance of the protein, which in turn might hinder heterodimer formation. It has been shown that overexpression of both *SEC24* and *SEC23* leads to decreased growth (Sopko *et al.* 2006) and decreased growth rate (Yoshikawa *et al.* 2011) in yeast.

High copy expression of *PSD1* was harmful to heat sensitive strains and did not enhance the growth of wild type thermotolerant strains. These results are in line with previous studies showing that overexpression decreases the vegetative growth rate (Yoshikawa *et al.* 2011). Overexpression of *PSD1* might hinder growth in yeast grown at high temperatures, as seen in our sensitive strains, since phosphatidylethanolamine is normally only expressed at high levels during growth at low temperatures (Henderson *et al.* 2013). Similar reasoning might explain

the failure of *PSD1* to enhance growth in resistant strains grown at high temperature.

In summary, the HN6-α alleles of *SEC24* and *PSD1* explain its sensitivity to heat and ethanol. However, the effects of both genes differ depending on the cross, implying that there are other differences between the Oak-a and SD1-a background that modify their effects. We conclude that ethanol and temperature tolerance are worth examining together in studies of quantitative genetic variation in *S. cerevisiae*, both to further investigate the genes and pathways involved, and to improve industrial applications of this yeast.

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