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ORIGINAL PAPER

Identification of genes associated with the high-temperature fermentation trait in the *Saccharomyces cerevisiae* natural isolate BCC39850

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Abstract

The fermentative model yeast *Saccharomyces cerevisiae* has been extensively used to study the genetic basis of stress response and homeostasis. In this study, we performed quantitative trait loci (QTL) analysis of the high-temperature fermentation trait of the progeny from the mating of the *S. cerevisiae* natural isolate BCC39850 (haploid#17) and the laboratory strain CEN.PK2-1C. A single QTL on chromosome X was identified, encompassing six candidate genes (*GEA1*, *PTK2*, *NTA1*, *NPA3*, *IRT1*, and *IML1*). The functions of these candidates were tested by reverse genetic experiments. Deletion mutants of *PTK2*, *NTA1*, and *IML1* showed growth defects at 42 °C. The *PTK2* knock-out mutant also showed significantly reduced ethanol production and plasma membrane H^+ ATPase activity and increased sensitivity to acetic acid, ethanol, amphotericin B (AMB), and *β*-1,3-glucanase treatment. The CRISPR-Cas9 system was used to construct knock-in mutants by replacement of *PTK2*, *NTA1*, *IML1*, and *NPA3* genes with BCC39850 alleles. The *PTK2* and *NTA1* knock-in mutants showed increased growth and ethanol production titers at 42 °C. These findings suggest an important role for the *PTK2* serine/threonine protein kinase in regulating plasma membrane H^+ ATPase activity and the *NTA1* N-terminal amidase in protein degradation via the ubiquitin-proteasome system machinery, which affects tolerance to heat stress in *S. cerevisiae*.

Keywords *IML1* · Industrial traits · *NTA1* · *PTK2* · QTL analysis

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Introduction

Saccharomyces cerevisiae is a widely used industrial organism to produce food, beverages, biofuels, and pharmaceuticals because it is generally regarded as safe and robust to the harsh conditions used in industrial processes. However, in ethanol production at high temperatures, such as in the simultaneous saccharification fermentation process, the absence of cooling can generate heat stress, which can impair growth and fermentation efficiency. Therefore, robust *S. cerevisiae* strains with enhanced high-temperature fermentation performance are needed to meet industrial requirements.

Heat stress causes metabolic imbalances, protein misfolding, and increased membrane permeability, which lead to a reduction in the specific growth rate or loss of viability (Verghese et al. 2012; Woo et al. 2014; Godinho et al. 2021). Some wild-type *S. cerevisiae* strains are capable of efficient fermentation at temperatures above the normal range (25–35ºC), suggesting that genetic factors control high-temperature tolerance (Edgardo et al. 2008). High-temperature tolerance in *S. cerevisiae* is a complex phenotypic trait controlled by multiple genes (Parts et al. 2011; Yang et al. 2013; Caspeta et al. 2014; Liu et al. 2017; Riles and Fay 2019; Wang et al. 2019), and different methods have been employed to identify the genes involved (Caspeta et al. 2014; Wang et al. 2019; Shui et al. 2015; Li et al. 2018).

Quantitative trait locus (QTL) mapping is a powerful tool for identifying trait-associated genes, and QTLs associated with high-temperature growth and fermentation in *S. cerevisiae* have been identified (Parts et al. 2011; Wang et al. 2019). From the mapped QTLs, genes associated with high-temperature tolerance include *IRA1*, *IRA2*, *CSC1*, *VPS34*, *VID24*, *AVO1*, and *DAP1*. These genes are involved in multiple pathways in *S. cerevisiae*, such as Ras/PKA (cAMP-dependent protein kinase) signaling, calcium-permeable cation channel, vacuolar import/degradation, regulation of rapamycin complex 2 (TORC2), and ergosterol synthesis. Although several genes associated with the high-temperature tolerance in *S. cerevisiae* have been uncovered by QTL analysis, understanding of the genes controlling this trait may be incomplete owing to the limited genetic diversity of the strains used in previous studies. In particular, several genes functioning in plasma membrane composition known to be important for high-temperature tolerance have not been mapped by QTL analysis, including genes in the Pkh-Ypk (serine/threonine protein kinase) cascade (Sun et al. 2012), the ergosterol synthesis pathway genes *ERG3* and *ERG5* (Caspeta et al. 2014; Liu et al. 2017), and *OLE1* which encodes the enzyme delta-9 desaturase (Li et al. 2018). QTL mapping of high-temperature tolerant strains from diverse genetic backgrounds may lead to the discovery of new genes associated with high-temperature fermentation and a greater understanding of the genetic basis of this trait.

In this study, we preliminary assessed four wild-type *S. cerevisiae* strains (BCC39850, BCC89033, BCC63268, and SC90) originating in Thailand for their capacity to produce ethanol at high temperatures. Among these isolates, BCC39850 exhibited good potential for high-temperature fermentation. We therefore selected this yeast strain for QTL analysis by mapping the phenotypic and genotypic data from individual haploid segregants obtained from the mating of the *S. cerevisiae* isolate BCC39850 (haploid#17) and the laboratory strain CEN.PK2-1C. A single QTL containing six genes was identified and found to be associated with high-temperature fermentation. The roles of the identified genes in high-temperature fermentation were assessed through gene deletion and gene replacement.

Materials and methods

Strains, cultivation conditions, and reagents

The *S. cerevisiae* laboratory strain CEN.PK2-1C (*MATa*; *his3D1*; *leu2-3_112*; *ura3-52*; *trp1-289*; *MAL2-8c*; *SUC2*) was obtained from EUROSCARF, Frankfurt, Germany. The wild-type *S. cerevisiae* strains with high-temperature fermentation performance include BCC39850, BCC89033, and BCC63268 were obtained from the Thailand Bioresource Research Center (www.tbrcnetwork.org). The industrial *S. cerevisiae* strain SC90 was obtained from the Department of Biotechnology, Kasetsart University, Bangkok, Thailand. *Escherichia coli* DH5α (Invitrogen, Waltham, MA, USA) was used for cloning plasmid DNA. Yeast strains were grown in YPD liquid medium (20 g/L peptone, 20 g/L glucose, and 10 g/L yeast extract) and stored in YPD broth containing 20% glycerol at -70 °C. Amphotericin B (AMB) and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Haploid segregants from yeast mating and genetic data

The *S. cerevisiae* BCC39850 haploid#17 (*MATalpha*; hydrolysate toxin tolerant) was previously isolated from the wild-type isolate BCC39850 (Sornlek et al. 2024) using tetrad dissection, according to the standard micromanipulation method (Sherman and Hicks 1991). The haploid isolation was confirmed by microscopy, assessing cell morphology, sporulation ability (not sporulating), and mating type. The *S. cerevisiae* BCC39850 haploid#17 was mated with CEN.PK2-1C (*MATa;* sensitive laboratory strain) on a YPD agar

plate to generate prototrophic hybrids (F1). The yeast cells were transferred on an SC (without amino acid) agar plate, then the diploid cells were sporulated on sporulation agar plates (10 g/L potassium acetate, 1 g/L yeast extract, 0.5 g/L dextrose, 20 g/L agar). A total of 91 haploid segregants were isolated from the F1 progeny by using tetrad dissection. The genomic DNA samples of the *S. cerevisiae* BCC39850 haploid#17, CEN.PK2-1C and the haploid segregants were purified (Thermo Fisher Scientific, Waltham, MA, USA) then submitted for whole genome sequencing (MGI Tech, Guangdong, China). The single nucleotide polymorphisms (SNPs) mapping, linkage map construction and quantitative trait locus (QTL) analysis were performed according to (Sornlek et al. 2024). In brief, the quality control of raw reads was assessed (FastQC v0.11.8) and mapped to the *S. cerevisiae* S288c reference genome using minimap2. Variant calling was conducted with GATK version 4.1.4.1, using the HaplotypeCaller method (https://gatk.broadinstitute.org/hc/en-us). The genotypic data of 27,977 high-quality SNPs were determined. Functional annotation of the variants was performed using SnpEff version 3.4. QTL analysis was carried out using the R package qtl. The marker-phenotype associations and statistical significance were determined using the composite interval mapping (CIM) method. The LOD scores were calculated on the permutation tests (PT) with 1,000 iterations, which were used to establish thresholds for statistical significance ($P < 0.05$). The raw sequence read data are accessible in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA971766 (BioProject). A schematic diagram of the methodology is shown in Fig. S1 (see Additional file 1: Fig. S1).

Testing of causative gene function by gene knockout and knock-in

Candidate gene functions were tested in the CEN.PK2-1C strain background. Deletion cassettes were obtained by PCR amplification of the hygromycin deletion cassette (*hygMX6*) with 42 bp flanking homologous regions upstream and downstream of the target locus. The CRISPR-Cas9 yeast system (DiCarlo et al. 2013) was used to edit the CEN.PK2-1C genome for gene knock-in. The donor DNA templates for gene knock-in (*gea1*_M1033L, *ptk2*_V604A, *iml1*_K724E, *npa3*_K276E, and *nta1*_K341R) were synthesized by Genscript (accession numbers: OQ985400, OQ985401, OQ985402, OQ985403, and OQ985404, respectively). Donor DNAs were gel-purified (GeneJET gel extraction kit; Thermo Fisher Scientific, Waltham, MA, USA) and used to transform competent CEN.PK2-1C cells along with the corresponding gRNA plasmid and the Cas9-expressing plasmid p414-TEF1p-Cas9-CYC1t-HIS (DiCarlo et al. 2013) using the LiAc/SS Carrier DNA/PEG method, as described previously (Gietz and Schiestl 2007). Transformants were screened by colony PCR using primers that could differentiate between nonmutated and mutated genes. The PCR products were gel-purified and sequenced to verify genome editing events. All primer sequences used in this study are listed in Table S1 (see Additional file 1: Table S1).

Phenotypic assays

The primary screening for ethanol production capacity of selected wild-type *S. cerevisiae* strains (BCC89033, BCC39850, BCC63268, SC90, and CEN.PK2-1C) was performed at temperatures of 38, 40, and 42 °C in YPD medium containing 200 g/L glucose at pH 6.5. Phenotypic assays of haploid segregants and mutant strains were performed in YPD medium containing 100 g/L glucose (pH 6.5) at 42 °C with shaking at 220 rpm. To screen for the high-temperature fermentation phenotype, haploid segregants were precultured in YPD medium at 30 °C overnight. The preculture was used to inoculate 2 mL of fresh YPD in a 15 mL tube. The cells at the starting OD_{600} of 0.05 (approximately 5×10^5 cells/mL) were cultured at 42 °C with rotary shaking at 220 rpm. Cell growth (OD_{600}) and glucose consumption (g/L) were monitored at 16, 24, and 48 h. Ethanol fermentation experiments were carried out in 250 mL Erlenmeyer flasks containing 50 mL of YPD containing 100 g/L glucose (pH 6.5) and *S. cerevisiae* cells suspended at an initial density OD_{600} of 0.2 (approximately 2×10^6 cells/mL) at 42 °C. One mL samples were taken at 16, 24, and 48 h intervals for analysis of growth (OD_{600}) and ethanol production. Real-time growth monitoring was performed by microtiter plate cultivation using a Biolector system (Beckman Coulter GmbH, Krefeld, Germany) in 48 round-bottom well plates. A total of 1 mL of the YPD culture was added to each well with a starting OD_{600} of 0.05. The microtiter plate was sealed with gas-permeable film (Beckman Coulter GmbH, Krefeld, Germany) and incubated at 42 °C with shaking at 1,000 rpm and relative humidity above 80%. Biomass was monitored every 5 min for 48 h by measuring the scattered light signal reflecting the amount of biomass suspended in culture medium. Maximum specific growth rates were calculated from 0 to 28 h time points.

Ethanol and glucose analysis

The ethanol and glucose concentrations in fermentation broth obtained from ethanol fermentation experiments were measured using the Shimadzu Prominence LC-20 high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) using a refractive index detector. An Aminex-HPX-87 H column (Bio-Rad, Hercules, CA, USA) was used. The column temperature

was maintained at 65 °C with 5 mM H_2SO_4 as an isocratic mobile phase at a flow rate of 0.5 mL/min.

Spot test

Mid-log phase cell suspensions were prepared by culturing the yeast strains in YPD at 30 °C overnight. The cell density (OD_{600}) was determined, and the cell suspensions were diluted with sterile water to obtain a final OD_{600} of 1.0 (approximately 1×10^7 cells/mL). Ten-fold serial dilutions of the cell suspension were performed to obtain 10^{-1} to 10^{-5} dilutions of cell suspensions. Samples of cell suspension (5 µL) were spotted onto YPD plates or YPD plates supplemented with acetic acid (2, 4, and 5 mg/mL), ethanol (50, 120, and 150 mg/mL), and AMB (10, 15, and 20 µg/mL). Growth at 42 °C (high-temperature growth) and 30 °C (chemical tolerance) was assessed after three days of incubation.

Sensitivity to *β*-1,3-endoglucanase lysis

Cell wall integrity was determined by measuring the susceptibility of the cells to treatment with a *β*-1,3-endoglucanase cell wall-degrading enzyme. The mid-log phase cells ($OD_{600}=4–5$) were prepared from the overnight culture of *S. cerevisiae* strains in YPD at 30 °C with shaking at 220 rpm. These cells were then diluted to an OD_{600} of 2.0 (approximately 2×10^7 cells/mL) and separated from the culture medium by centrifugation. The cell pellet was resuspended in 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA, and the cells were treated with 1 unit of Zymolyase 20T (Zymo Research, Irvine, CA, USA). The OD_{600} of the cell suspension was measured at 15 min intervals for 2 h.

Plasma membrane H^+ ATPase activity assay

Plasma membrane H^+ ATPase activity was assayed as described by (Serrano 1983), with minor modifications. *S. cerevisiae* strain CEN.PK2-1C (wild-type), knock-out mutants (CEN.PK2-1C_*ptk2Δ* and CEN.PK2-1C_*nta1Δ*), and knock-in mutants with BCC39850 alleles (CEN.PK2-1C_*ptk2*^{Rep}, CEN.PK2-1C_*nta1*^{Rep}) were cultured in YPD at 30 °C with shaking at 220 rpm overnight to obtain the cell OD_{600} of 4.0, then harvested by centrifugation. The cell pellets were separated and resuspended in 0.5 mL of ice-cold extraction buffer pH 7.0 (50 mM Tris-HCl, pH 7.0, 2 mM EDTA, and 2 mM dithiothreitol). The suspension was vortexed with glass beads for 2 min. The membrane fraction was harvested by centrifugation (10,000×*g* for 5 min at 4 °C) and resuspended in sterile water (0.5 mL). Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard (Bio-Rad, Hercules, CA, USA). The membrane fraction preparations (protein concentration 0.8-1.0 mg/mL) were kept on ice for the determination of H^+ ATPase activity. The enzymatic reaction containing 0.5 mL of reaction buffer pH 7.5 (50 mM MES pH 5.7, 10 mM $MgSO_4$, 50 mM KCl, 5 mM sodium azide, and 50 mM KNO_3) and 0.02 mL of plasma membrane fraction was pre-incubated at 30 °C for 5 min. The assay was started by adding ATP (Sigma-Aldrich, St. Louis, MO, USA) to obtain a final concentration of 2 mM. Samples were taken to determine the amount of liberated inorganic phosphate at 20, 30, and 60-min intervals by the addition of 0.5 mL of molybdovanadate solution (Ueda and Wada 1970).

Data analysis

Data of OD_{600} and consumed glucose measured after 16 h of culture at 42 °C were used as phenotypic scores in QTL analysis. QTL analysis was performed as described previously (Sornlek et al. 2024). All experiments were performed independently at least three times. Analysis of variance was conducted by one-way analysis of variance (ANOVA) using Dunnett's post hoc method on the SPSS statistical package (version 18.0 for Windows, SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at $P<0.05$.

Results and discussion

Identification of QTLs associated with high-temperature fermentation trait

Four wild-type isolates of *S. cerevisiae* (BCC39850, BCC89033, BCC63268, and SC90) were identified for their capacity to produce significantly more ethanol than the laboratory strain CEN.PK2-1C at high temperature (Fig. 1a). Among these isolates, the wild-type BCC39850 was selected for this study based on its phenotype on high-temperature tolerance, mating efficiency, sporulation, and available genetic information. This isolate was previously found to be tolerant to a mixture of formulated hydrolysate toxins (furfural, acids, and phenols) and was used for QTL analysis of the toxin tolerance trait (Sornlek et al. 2024). The haploid segregants from the mating of BCC39850 haploid#17 with strain CEN.PK2-1C were re-tested for growth (OD_{600}) and glucose consumption at 16 and 24 h from cultures incubated at 42 °C (Fig. 1b). The new phenotypic data were used as phenotypic scores for QTL analysis with the previously reported genotypic data and linkage map (Fig. 1c). Based on the glucose consumption and growth OD_{600} phenotypic scores of 16 h time point data, a major QTL was identified on Chromosome X (Fig. 1d). Six candidate genes (*GEA1*,

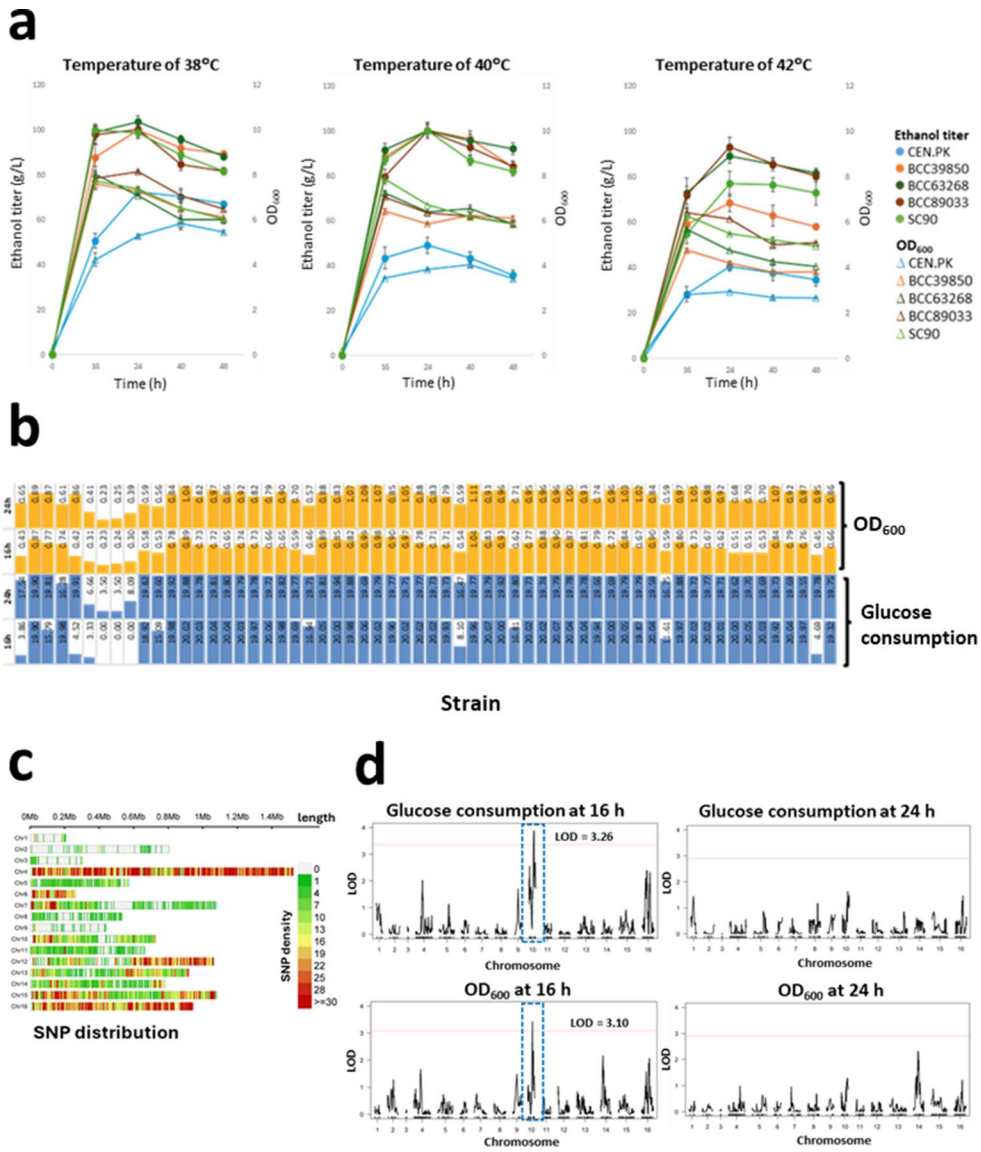

Fig. 1 Screening and phenotyping for high temperature tolerance. **a** Ethanol production of wild-type *Saccharomyces cerevisiae* strains at 38, 40 and 42 °C in YPD containing 200 g/L glucose for 24 h ($n=3$). Bars show mean values and error bars show standard deviations. CEN.PK2-1C is a wild-type laboratory strain, whereas the others are natural wild-type strains. **b** Phenotypes of selected segregants from the mating of strain BCC39850 with strain CEN.PK2-1C (glucose consumption and growth (OD_{600}) cultured in YPD at 42 °C measured at 16 h and 24 h). **c** the distributions of SNP markers over *S. cerevisiae* chromosomes (SNP density is indicated by color bars). **d** QTL mapping of 91 selected haploid segregants based on data of growth (OD_{600}) and glucose consumption at 16 h as phenotypic scores. The logarithm of the odds (LOD) were plotted against the respective chromosomal position. LOD score thresholds were determined separately for each data type by permutation test, as indicated on each panel. The additive effect results from the BCC39850 type alleles

Table 1 Genes with nonsynonymous variants in the major QTL associated with growth at 42ºC

Gene	Chr	Coordinate	Length (aa)	Affected gene	Amino acid mutation (S288c genome as ref.)	
					BCC39850 Haploid#17	CEN.PK2-1C
1	X	486586.490812	1,408	*GEA1*	M1033L	Wild-type
2	X	545787.548243	818	*PTK2*	V604A	Wild-type
3	X	553476.554850	457	*NTA1*	K341R	Wild-type
4	X	570967.572124	385	*NPA3*	K276E	Wild-type
5	X	605936.607424	not available	*IRT1*	not available	Wild-type
6	X	684567.689321	1,584	*IML1*	K724E	Wild-type

PTK2, *NTA1*, *NPA3*, *IRT1*, and *IML*) are annotated in this QTL. The BCC39850 haploid#17 parental strain possesses a non-synonymous missense SNP in all genes except *IRT1* (which is annotated as a long non-coding RNA gene), compared with the CEN.PK2-1C sequence (Table 1). The missense variants may affect the encoded proteins of these genes with a variety of annotated functions (Table 2).

At the time of writing this article, there was limited information on high-temperature fermentation associated with our currently identified gene set. The functions of these candidate genes are described as follows: *GEA1* encodes the guanine nucleotide exchange factor for ADP ribosylation factors (ARFs) and has been reported to be involved in vesicular transport between the Golgi and ER, the Golgi

Table 2 Functional description of candidate genes

No.	Gene	Short description
1	*GEA1*	Guanine nucleotide exchange factor for adenosine diphosphate ribosylation factors (ARFs); involved in vesicular transport between the Golgi and endoplasmic reticulum (ER)
2	*PTK2*	Serine/threonine protein kinase; involved in regulation of ion transport across plasma membrane
3	*NTA1*	Amidase; removes the amide group from N-terminal asparagine and glutamine residues to generate proteins with N-terminal aspartate and glutamate residues that are targets of ubiquitin-mediated degradation
4	*NPA3*	Member of the conserved Gly-Pro-Asn-loop GTPase family; has a role in transport of RNA polymerase II to the nucleus
5	*IRT1*	Long noncoding RNA that governs mating-type control of gametogenesis
6	*IML1*	GTPase-activating protein (GAP) subunit of the Iml1p/SEACIT complex (a vacuole associated protein complex)

organization, and the actin cytoskeleton organization (Gustafson and Fromme 2017). *PTK2* encodes a cytoplasmic serine/threonine protein kinase that regulates ion transport across the plasma membrane (Goossens et al. 2000; Eraso et al. 2006; Antunes and Sá-Correia 2022). *NTA1* encodes N-terminal amidase, which is a component of the N-terminal protein degradation pathways (Dougan et al. 2012; Kozlic et al. 2022). *IML1* encodes a component of the SEACIT (Npr2p, Npr3p, and Iml1p) subcomplex localized on the vacuole membrane, which inhibits the Target of Rapamycin Complex 1 (TORC1) complex (Loissell-Baltazar and Dokudovskaya 2021). *IRT1* encodes a long non-coding RNA that regulates mating-type signals and sporulation in *S. cerevisiae* (Moretto and Werven 2017). Recently, network and omics analyses of the ethanol tolerance in *S. cerevisiae* have shown that the long non-coding RNAs also function in response to the ethanol stress by interacting on their specific proteins (Wolf et al. 2023). *NPA3* encodes GPN-loop GTPase Npa3plays a critical role in the transport of RNA polymerase II to the nucleus. A reduction in GPN-loop GTPase activity was reported to increase sensitivity to DNA replication stress and defects in sister-chromatid cohesion and cell cycle progression (Mora-García et al. 2022).

Validation of candidate genes by gene deletion

To evaluate the function of QTL candidate genes affecting high-temperature fermentation, deletion mutants in the CEN.PK2-1C strain background were constructed by *hygMX6* cassette-mediated gene disruption. After selection on YPD containing 0.2 mg/mL hygromycin, integration of the deletion cassettes at target genes was validated by PCR. Five deletion mutants (CEN.PK2-1C_*gea1*Δ, CEN.PK2-1C_*ptk2*Δ, CEN.PK2-1C_*nta1*Δ, CEN.PK2-1C_*irt1*Δ, and CEN.PK2-1C_*iml1*Δ) were constructed. Gene disruption was not attempted for *NPA3*, as this gene is essential for *S. cerevisiae* viability. Real-time growth analysis of the knock-out mutants showed significantly reduced growth of CEN.PK2-1C_*ptk2*Δ, CEN.PK2-1C_*nta1*Δ, and CEN.PK2-1C_*iml1*Δ mutants. A delayed growth was also observed in CEN.PK2-1C_*gea1*Δ and CEN.PK2-1C_*irt1*Δ at the early stage compared to that of the CEN.PK2-1C strain (Fig. 2a; Table 3). Under the same growth conditions, the parental strain BCC39850 haploid #17 showed a similar growth pattern as CEN.PK2-1C_*nta1*Δ and CEN.PK2-1C_*iml1*Δ mutants. While higher ethanol production (Fig. 2b) and glucose consumption (Fig. 2c) were observed in the BCC39850 haploid#17 strain, a marked reduction in ethanol production was observed in CEN.PK2-1C_*ptk2*Δ ($P < 0.05$), but not in the other knock-out mutants. A growth reduction was observed at 42 °C but not 30 °C for CEN.PK2-1C_*ptk2*Δ by spot-test, whereas no growth defect was apparent for the other mutants (Fig. 2d).

Testing the functional effects of BCC39850 alleles by gene knock-in

The effects of protein-coding variants in the QTL candidate genes present in the BCC39850 parental strain with respect to the high-temperature fermentation phenotype were tested by gene knock-in experiments. Gene knock-ins were constructed in the CEN.PK2-1C strain background to replace the wild-type allele with the BCC39850 allele, except for the *IRT1* gene, which was not included, because it does not encode an annotated protein. The CEN.PK2-1C_*ptk2*^{Rep} and CEN.PK2-1C_*nta1*^{Rep} mutants with knock-ins of the *PTK2* and *NTA1* genes, respectively, showed significantly increased specific growth rates compared with the CEN.PK2-1C strain, whereas the *GEA1*, *IML1*, and *NPA3* knock-in mutants' specific growth rates were not different from CEN.PK2-1C (Table 3; Fig. 3a).

Data collected at the 16 h time point of fermentation revealed that the CEN.PK2-1C_*ptk2*^{Rep} and CEN.PK2-1C_*nta1*^{Rep} knock-in strains showed a significantly greater ethanol titers (Fig. 3b), glucose consumption (Fig. 3c), and ethanol productivity (Fig. 3d) than CEN.PK2-1C. Ethanol is the main metabolite produced from glycolysis-fermentation metabolism in *S. cerevisiae*. In this work, the improved ethanol production is possibly linked to the improved growth of CEN.PK2-1C_ *nta1*^{Rep} and CEN.PK2-1C_ *ptk2*^{Rep} at high-temperature. These knock-ins appeared to reach stationary phase faster than CEN.PK2-1C, which might benefit energy utilization and ethanol production at high-temperatures. Conversely, the ethanol titer and productivity profiles of CEN.PK2-1C_*iml1*^{Rep}, CEN.PK2-1C_*gea1*^{Rep} and CEN.

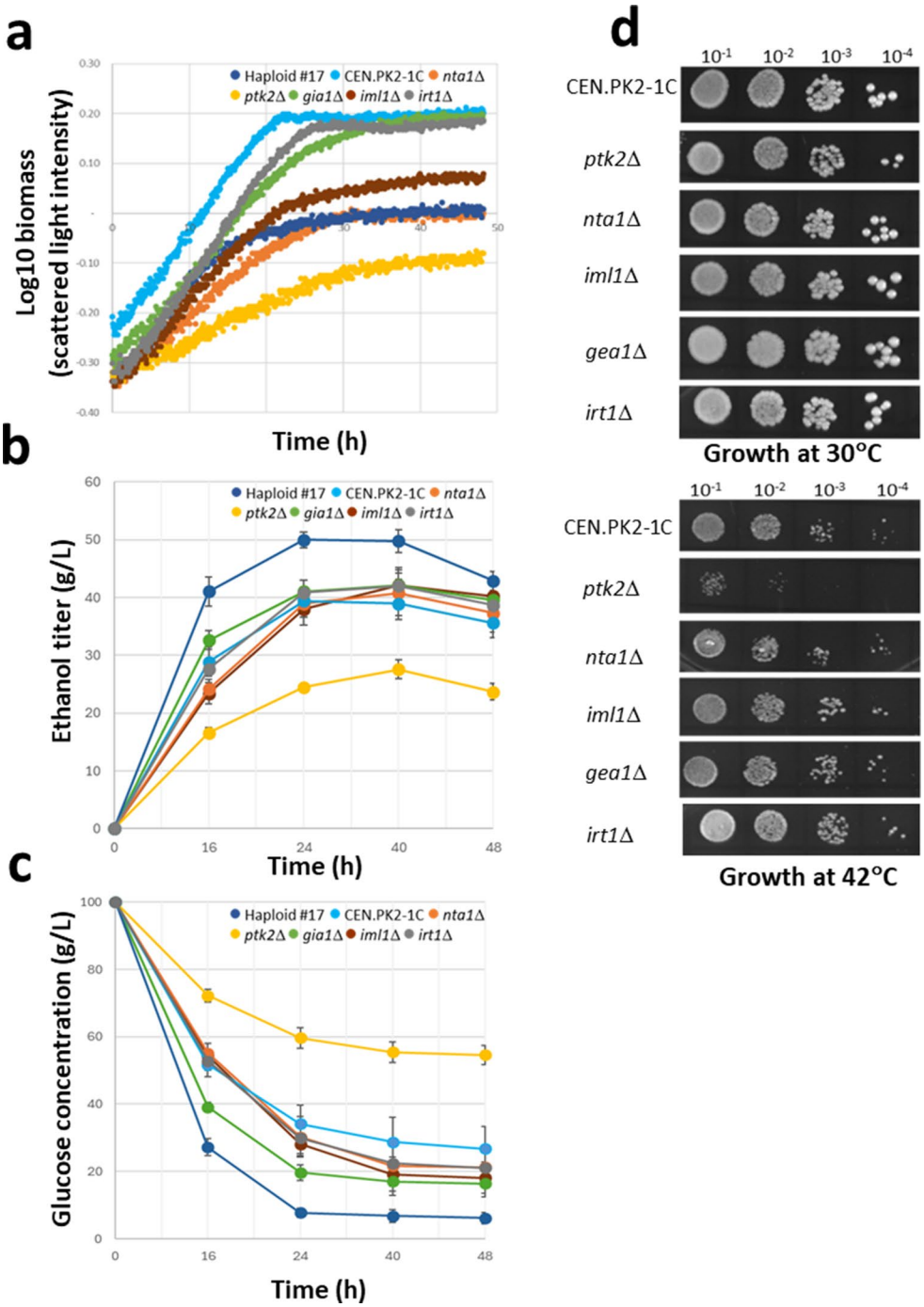

Fig. 2 Growth analysis and ethanol production of CEN.PK2-1C and knock-out mutants. **a** Biomass production, **b** Ethanol production and **c** Glucose concentration for all strains on YPD containing 100 g/L glucose at 42 °C for 48 h. Data shown in **a**, **b** and, **c** are mean values obtained from three independent experiments; error bars represent standard deviations ($n = 6$). **d** Characterization of high temperature tolerance by spot test. Growth at 30 °C (upper panel) and 42 °C (lower panel) was recorded after three days of incubation. Knock-out mutants were created in the CEN.PK2-1C strain, and the deleted genes are indicated by suffixes (*nta1*Δ, *ptk2*Δ, *gia1*Δ, *iml1*Δ, and *irt1*Δ)

PK2-1C_*npa3*^{Rep} knock-ins were not significantly different from those of CEN.PK2-1C (Fig. 3b, d).

Among the six candidate genes associated with high-temperature fermentation trait, functional relationships were demonstrated for *PTK2*, *NTA1*, and *IML1*, as deletion mutants of these genes showed a growth defect when cultured at 42ºC (Fig. 2; Table 3). We were unable to test the function of *NPA3* by knock-out, as this gene is essential. Hence, conditional mutagenesis approaches are needed to test loss-of-function mutants of this gene. Of the BCC39850 alleles tested by knock-in mutagenesis, *PTK2* and *NTA1* conferred significant improvement in high-temperature fermentation performance, although the effects were small (Fig. 3). These small effects suggest that the high-temperature fermentation trait of BCC39850 is controlled by multiple genes.

Based on the data from the deletion mutants, the roles of *PTK2*, *NTA1*, and *IML1* in thermotolerance were discussed below. During growth at elevated temperatures, the plasma membrane is the first line of defense against environmental

Table 3 Maximum specific growth rate (μMax) of *Saccharomyces cerevisiae* strains cultured at 42 °C. Means with the same letter are not significantly different ($n = 5$)

	μMax (h^{-1}) *	S.D.
CEN.PK 2–1 C	0.0209[a]	0.000
CEN.PK2-1C_*nta1*Δ	0.0175[b]	0.001
CEN.PK2-1C_*ptk2*Δ	0.0072[b]	0.001
CEN.PK2-1C_*gea1*Δ	0.0209[a]	0.003
CEN.PK2-1C_*iml1*Δ	0.0170[b]	0.001
CEN.PK2-1C_*irt1*Δ	0.0210[a]	0.001
CEN.PK2-1C_*nta1*^{Rep}	0.0226[c]	0.001
CEN.PK2-1C_*ptk2*^{Rep}	0.0229[c]	0.002
CEN.PK2-1C_*gea1*^{Rep}	0.0210[a]	0.001
CEN.PK2-1C_*iml1*^{Rep}	0.0209[a]	0.001
CEN.PK2-1C_*npa3*^{Rep}	0.0210[a]	0.000

perturbations. Heat stress causes changes in membrane fluidity, leading to the remodeling of the membrane and reorganization of membrane lipids. This perturbation also affects intracellular ion levels and pH, which are attributed to the modulation of the membrane potential (Bromberg and Weiss 2016). Therefore, it is not unexpected that genes with plasma membrane function are associated with high-temperature tolerance. *PTK2* encodes a serine/threonine protein kinase of the NPR/HAL5 family, which regulates ion transport across the plasma membrane (Eraso et al. 2006; Antunes and Sá-Correia 2022). *PTK2* kinase is located on the plasma membrane and phosphorylates plasma membrane H^+ ATPase at Ser^{899} (Eraso et al. 2006). Activation of H^+ ATPase by *PTK2* is required for nutrient uptake, osmotic balance, ion homeostasis, and stress tolerance (Goossens et al. 2000), as shown by the *PTK2* loss-of-function mutant, which is hypersensitive to acetic acid, killer toxins (K1, K28, and HM-1), and *β*-1,3-endoglucanase (Groot et al. 2001).

NTA1 encodes N-terminal amidase, which is a component of the N-terminal protein degradation pathways (Dougan et al. 2012; Kozlic et al. 2022). Proteins with N-terminal degradation signals (N-degrons) are targeted for degradation via the ubiquitin-proteasome system (UPS) machinery (Varshavsky 2019; Mehrtash and Hochstrasser 2019). N-terminal amidase converts N-terminal asparagine and glutamine residues to aspartate and glutamate, respectively, and the modified protein is targeted for UPS-mediated degradation (Kim et al. 2016). An explanation for the growth

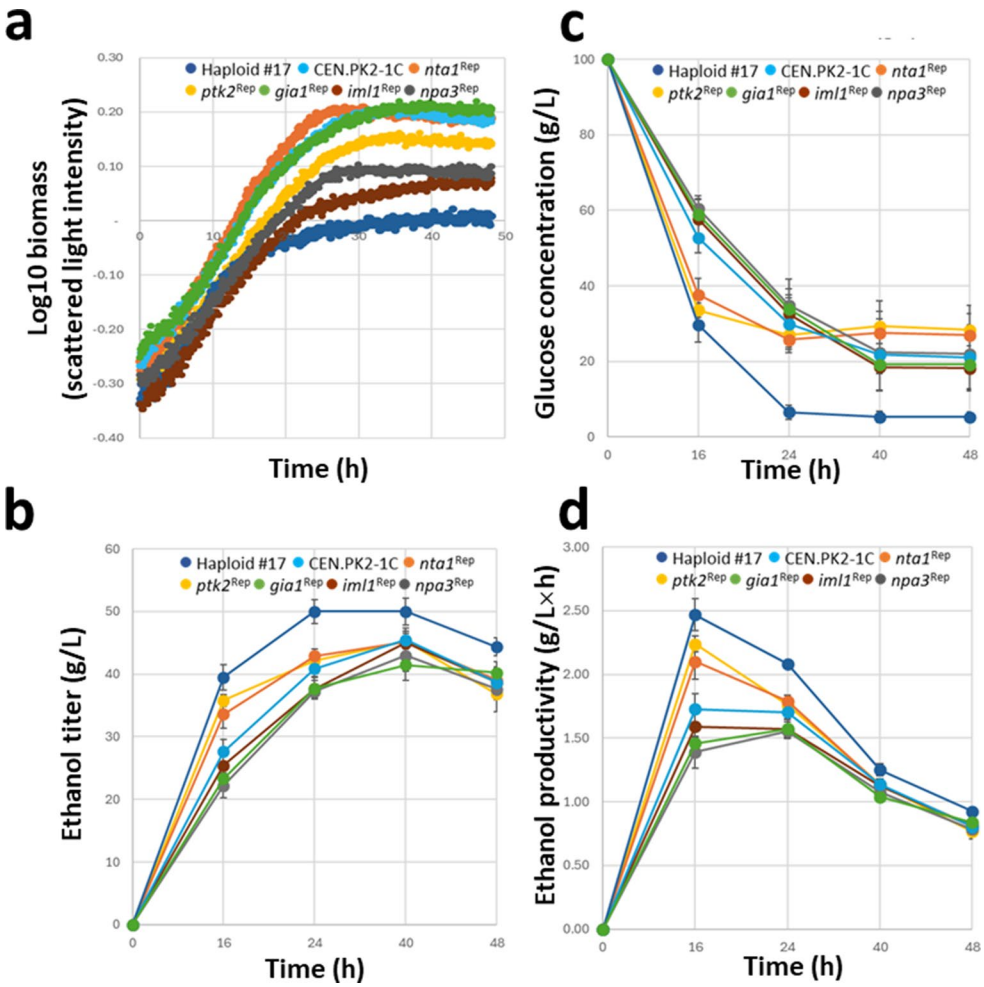

Fig. 3 Ethanol production of knock-in mutant strains. **a** Ethanol titer, **b** Glucose concentration, and **c** Ethanol productivity for CEN.PK2-1 C (wild-type) and knock-in strains with BCC39850 allele (CEN.PK2-1C_*ptk2*^{Rep}, CEN.PK2-1C_*nta1*^{Rep}, CEN.PK2-1C_*iml1*^{Rep}, CEN.PK2-1C_*gia1*^{Rep}, CEN.PK2-1C_*npa3*^{Rep}) were assessed at 42 °C. Data represent mean values obtained from three independent experiments; error bars represent standard deviations ($n = 6$)

defect of the *NTA1* knock-out mutant at 42ºC (Fig. 2a) is the inability of the mutant to degrade N-degron substrates bearing N-terminal asparagine or glutamine, leading to the accumulation of proteins normally degraded and enhanced endoplasmic reticulum stress triggered by misfolded proteins at elevated temperatures. The minor growth defect of the *NTA1* knock-out mutant (Fig. 2a) could be explained by the fact that *NTA1* functions in one degron system, among several others, in *S. cerevisiae* (Varshavsky 2019).

IML1 encodes a component of the SEACIT (Npr2p, Npr3p, and Iml1p) subcomplex localized on the vacuole membrane, which inhibits the Target of Rapamycin Complex 1 (TORC1) complex (Wang et al. 2023). TORC1 is a master regulator of growth and stress that integrates multiple signaling pathways (Mühlhofer et al. 2019). One of the major functions of TORC1 is regulation of autophagy. Deletion of any SEACIT component is associated with defective autophagy and induction of vacuolar fragmentation (Wang et al. 2023). Autophagy is upregulated in response to heat stress, particularly when yeast cells are exposed to temperatures of 42ºC or above (Mühlhofer et al. 2019). It has also been reported that the deletion of *IML1* in yeast leads to increased sensitivity to H_2O_2 (Sekiguchi et al. 2022). Therefore, the growth defect of the *IML1* deletion mutant at 42ºC (Fig. 2a; Table 3) can be explained by the inability to upregulate autophagy in response to heat stress.

Sensitivity of mutants to inhibitors, *β*-1,3-endoglucanase lysis, and assessment of plasma membrane ATPase activity

To obtain further insights into the role of QTL gene candidates in high-temperature tolerance, the mutants were assessed for their tolerance to different growth inhibitors. We replicated the findings of acetic acid and *β*-1,3-endoglucanase sensitivity from (Groot et al. 2001) and found that the CEN.PK2-1C_*ptk2*Δ strain with knock-out of the *PTK2* gene exhibited hypersensitivity to acetic acid (Fig. 4a). A small but significantly increased sensitivity to *β*-1,3-endoglucanase digestion was also observed (Fig. 4b). This deletion mutant also showed increased sensitivity to an increased concentration of ethanol compared with the CEN.PK2-1C strain (Fig. 4a). Moreover, the deletion of *PTK2* conferred sensitivity to AMB (Fig. 4a), an antifungal drug that binds ergosterol in the fungal cell membrane, leading to the formation of pores, ion leakage, and ultimately fungal cell death (Gray et al. 2012). In contrast, the sensitivities of the *NTA1* knock-out mutant (CEN.PK2-1C_*nta1*Δ) and knock-in mutants (CEN.PK2-1C_*ptk2*^{Rep} and CEN.PK2-1C_*nta1*^{Rep}) were not markedly different from those of the control (Fig. 4a and b). *PTK2* encodes a serine/threonine protein kinase involved in the activation of plasma membrane H^+ ATPase activity, which is required for the regulation of proton transportation and intracellular

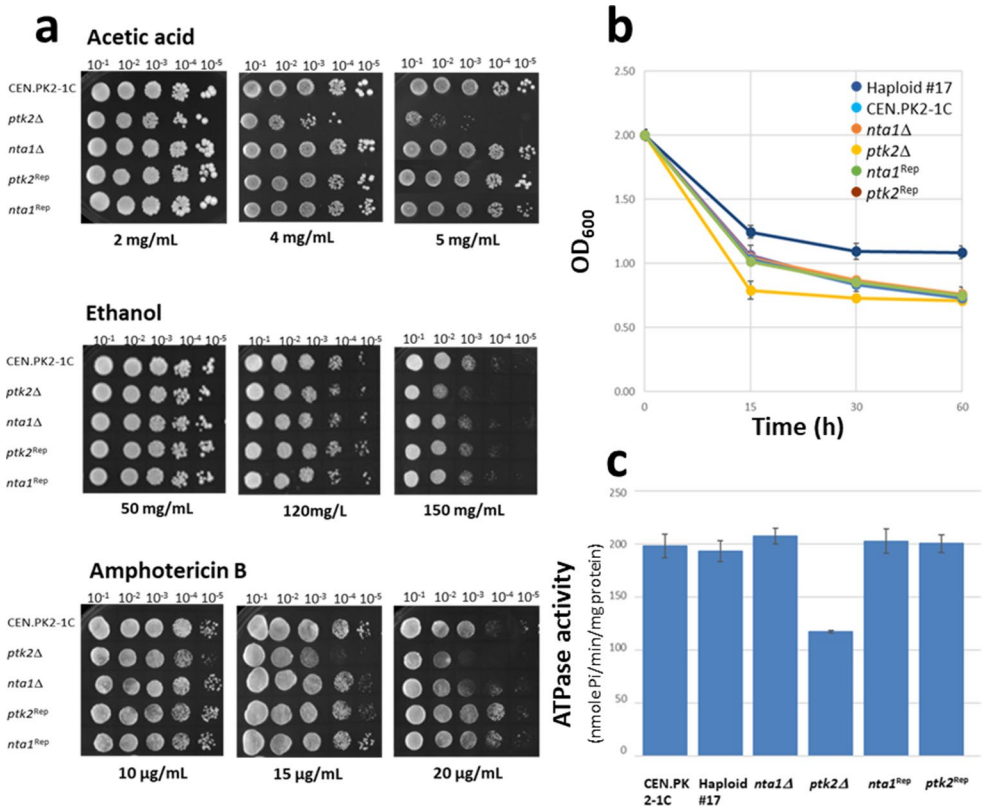

Fig. 4 Sensitivity of mutants to inhibitors, *β*-1,3-endoglucanase lysis, and assessment of plasma membrane H^+ ATPase activity **a** Spot test for sensitivity to acetic acid, ethanol, and amphotericin B. Growth was recorded after three days of incubation at 30 °C. **b** Susceptibility to *β*-1,3-endoglucanase digestion. **c** Plasma membrane H^+ ATPase activity of plasma membrane fractions (an asterisk indicates significant at 0.05 level). Data shown in **b** and **c** are mean values obtained from three independent experiments; error bars represent standard deviations ($n = 6$)

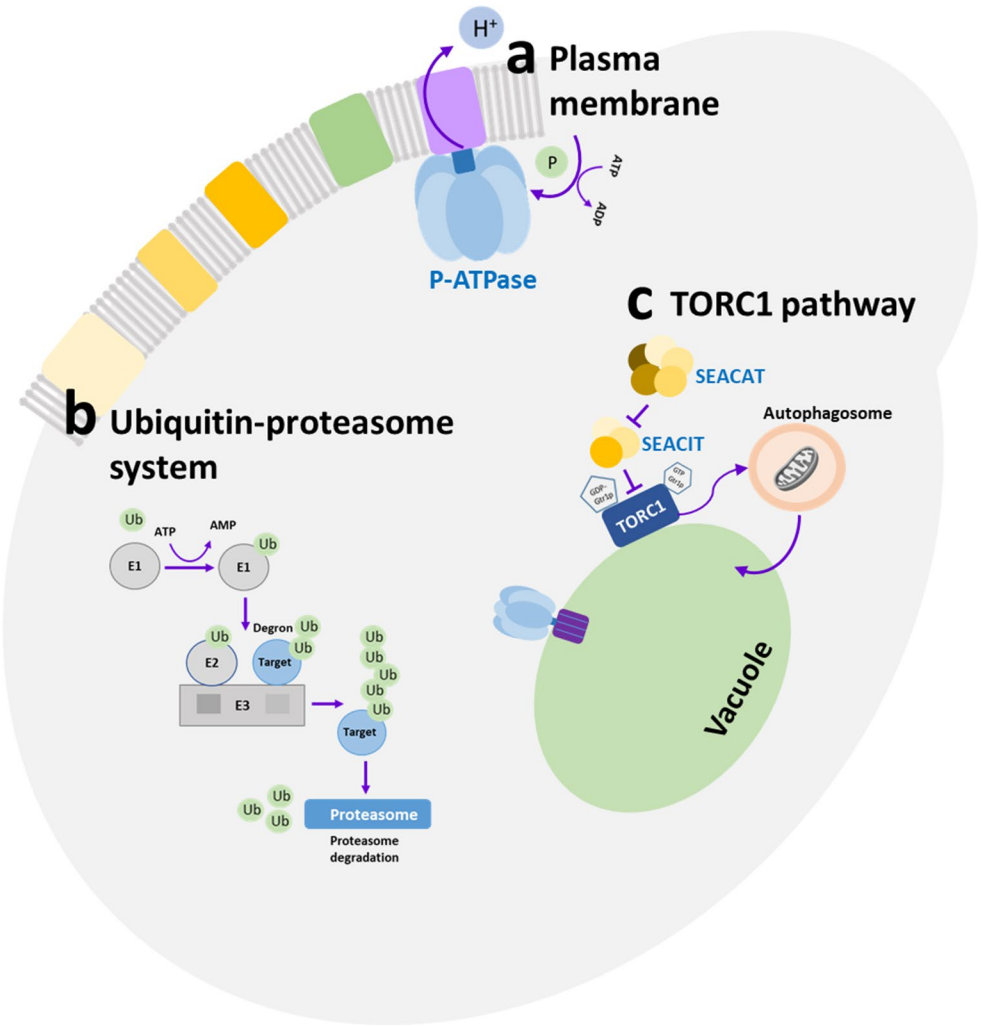

Fig. 5 Diagram of the proposed pathways involving in the high-temperature fermentation process according to the genes identified in this study **a** High-temperature stress causes changes in membrane fluidity, PTK2 kinase is located on the plasma membrane and requires for activation of H^+ ATPase to maintain osmotic balance, ion homeostasis, and high-temperature tolerance. **b** At elevated temperatures, misfolded proteins can be generated. NTA1 amidase functions to convert N-terminal asparagine and glutamine residues to aspartate and glutamate and the modified protein is targeted for UPS-mediated degradation. **c** Autophagy is upregulated in response to heat stress, a component of the SEACIT (Npr2p, Npr3p, and Iml1p) subcomplex requires for autophagy regulation via inhibition of the Target of Rapamycin Complex 1 (TORC1) complex

pH homeostasis (Goossens et al. 2000). Therefore, plasma membrane fractions obtained from the mutants were assayed for H^+ ATPase activity. Among these strains, only CEN.PK2-1C_*ptk2*Δ showed a significant reduction in H^+ ATPase activity compared with the control (Fig. 4c).

In this study, we identified a set of genes involved in high-temperature fermentation in *S. cerevisiae* through QTL mapping using the natural isolate BCC39850. We propose three possible pathways contributing to high-temperature tolerance: (1) regulation of plasma membrane H^+ ATPase activity; (2) degradation of protein substrates by the N-degron pathway, and (3) the TORC1 complex (Fig. 5). Knock-out mutants of *PTK2*, *NTA1*, and *IML1* exhibited growth defect and a reduced capacity to produce ethanol at 42ºC. Conversely, knock-in mutants with replacement of the *NTA1* and *PTK2* alleles from BC39850 showed increased growth and ethanol production at 42ºC. Confirming the contribution of these two genes to high-temperature tolerance and fermentability. This study contributes to our understanding of high-temperature tolerance in *S. cerevisiae*, which can be applied in the development of yeast strains with improved tolerance for industrial applications.

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Author contributions WS and NS conducted experiment; WS and CS analyzed data, ST, SI, WR, NR, LE, VC, ST, PS and VMS conceived and designed research; WS wrote the manuscript. All authors have read and approved the manuscript.

Data availability All data generated or analyzed during this study are included in this published article. Raw sequencing data of yeast strains that support the findings of this study have been deposited in the NCBI Sequence Read Archive (SRA) under accession PRJNA971766 (BioProject).

Declarations

Competing interests The authors declare no competing interests.

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