BMC Biology

RESEARCH

Open Access

Unveiling the thermotolerance mechanism of *Pichia kudriavzevii* LC375240 through transcriptomic and genetic analyses

Yanhua Qi[1,2†], Qijian Qin[1,2†], Jiayin Ma[3], Bin Wang[1,2*], Cheng Jin[1,3*] and Wenxia Fang[1,2*]

Abstract

Background Thermotolerance is a critical trait for yeasts employed in industrial settings, and the utilization of unconventional yeasts has gained notable attention in recent years. However, the mechanisms underlying thermotolerance in unconventional yeasts, particularly *Pichia* spp., remain insufficiently elucidated.

Results This study focuses on the thermotolerance of a non-traditional yeast strain *Pichia kudriavzevii* LC375240, renowned for its remarkable thermotolerance. Through transcriptomic analysis of both short-term and long-term heat shock exposures, we uncovered an intricate regulatory response in *P. kudriavzevii*. During long-term heat treatment, the yeast exhibited elevated expression of genes involved in the tricarboxylic acid (TCA) cycle and suppressed expression of genes in the pentose phosphate pathway (PPP). Additionally, long-term heat treatment led to an upregulation of heat shock proteins (HSPs) and an increase in trehalose, glutathione (GSH), and superoxide dismutase (SOD) levels, along with a reduction in the intracellular NADPH/$NADP^+$ ratio and pyruvate content. These changes collectively contribute to the thermotolerance of *P. kudriavzevii*. CRISPR-Cas9-mediated knockout experiments further highlighted the critical roles of HSPs, antioxidases, and the trehalose metabolic pathway in the yeast's response to high temperatures.

Conclusions Taken together, this study demonstrates that *P. kudriavzevii* adapts to thermal stress through a combination of enhanced TCA cycle, reduced PPP, increased HSPs, trehalose, GSH, and SOD levels. These findings provide a comprehensive understanding of the molecular mechanisms underlying thermotolerance in *P. kudriavzevii*.

Keywords Thermotolerance, Non-traditional yeasts, *Pichia kudriavzevii*, Transcriptomic analysis, Heat shock proteins

Background

Saccharomyces cerevisiae, renowned for its historical role in producing various foods and beverages like bread, beer, and wine, has been a staple for centuries [1]. Through evolution, *S. cerevisiae* has acquired advantageous traits for food and beverage fermentations, as well as for first-generation bioethanol production, showcasing its ability to thrive in low oxygen conditions and resist high ethanol concentrations [2–5]. However, the demands of second-generation bioethanol production necessitate heightened tolerance to diverse harsh fermentation conditions, including osmotic stress, heat stress, ethanol stress, and exposure to inhibitory compounds

†Yanhua Qi and Qijian Qin contributed equally to this work.

*Correspondence:
Bin Wang
bwang@gxas.cn
Cheng Jin
jinc@im.ac.cn
Wenxia Fang
wfang@gxas.cn
[1] Institute of Biological Science and Technology, Guangxi Academy of Sciences, Nanning, Guangxi 530007, China
[2] State Key Laboratory of Non-food Biomass Energy Technology, Nanning, Guangxi 530007, China
[3] State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

present in lignocellulosic hydrolysates, such as weak acids, furan aldehydes, and phenolic compounds [6–9].

Beyond its role in bioethanol production, *S. cerevisiae* serves as a predominant player in various bioprocesses and protein expression systems, producing a multitude of bioproducts [10–12]. Nevertheless, limitations such as hyperglycosylation, poor secretion, incorrect folding of heterologous proteins, and relatively low robustness against osmotic and temperature stresses in industrial applications persist [13]. Consequently, further optimization is essential for *Saccharomyces*-mediated bioethanol production, bioprocesses, and protein expression. Ongoing research is actively exploring the natural diversity of *S. cerevisiae* to identify strains with enhanced stress and inhibitor tolerance. Additionally, superior *S. cerevisiae* strains are being developed through evolutionary engineering and targeted genetic modification, aiming to overcome existing limitations and improve its applicability in industrial settings [5].

Utilizing non-conventional yeast species with inherent tolerance to challenging environmental conditions presents an alternative strategy. This category encompasses various candidates, including *Yarrowia lipolytica* (known for salt and oxidative stress tolerance), *Ogataea polymorpha* (exhibiting heat tolerance), *Pichia pastoris*, *Pichia stipitis*, *Kluyveromyces marxianus* (recognized for extreme thermotolerance) and *Kluyveromyces lactis*, each noted for their adaptability to diverse harsh environments and suitability for different bioprocesses [12–16]. Furthermore, these non-conventional yeasts are recognized for their efficient mass production capabilities and can serve as safe and authentic sources for producing various pharmaceutical proteins [12, 17].

In addition to extensively studied non-conventional yeasts such as *Y. lipolytica*, *O. polymorpha*, *P. pastoris*, and *K. lactis*, recent reports highlight several strains of *P. kudriavzevii* capable of thriving and producing high levels of ethanol at elevated temperatures [18–24]. Certain strains of *P. kudriavzevii* also exhibit multi-stress tolerance, including resistance to acid, ethanol, thermo, and salt [25, 26]. A noteworthy finding from our previous study is that the newly isolated *P. kudriavzevii* LC375240 demonstrates thermotolerance at high temperatures of 37 and 42 °C. Impressively, this strain exhibits robust growth and ethanol productivity with strong tolerance to various stresses, such as acetic acid, furfural, formic acid, H_2O_2, and high concentrations of ethanol at 42 °C [27]. These distinctive traits suggest a highly promising application of *P. kudriavzevii* LC375240 in industrial settings. Given this, to delve into the molecular mechanism of the thermotolerance exhibited by *P. kudriavzevii* LC375240, this study conducted transcriptomic analysis, genetic, and biochemical validations by subjecting the strain to short- and long-term heat treatments.

Results

Heat treatment triggered global gene expression in *P. kudriavzevii*

The yeast strain *P. kudriavzevii* LC375240 has been previously identified for its exceptional tolerance to a variety of stressors, including high temperature, acetic acid, furfural, formic acid, H_2O_2, and high concentrations of ethanol [27]. This study aimed to unravel the molecular mechanism underlying thermotolerance in *P. kudriavzevii* by analyzing global gene expression profiles using RNA sequencing (RNA-seq). To achieve this, *P. kudriavzevii* LC375240 was cultivated at 37 °C in fresh medium until the mid-exponential phase ($OD_{600}\approx0.8$, approximately 4 h). The culture was then subjected to either a short-term heat shock (SHS) at 42 °C for 0.5 h or a long-term heat treatment (LHT) at 42 °C for 2 h. Differential gene expression analysis was conducted with three biological replicates to ensure high consistency (Additional file 1: Fig. S1A). Applying a false discovery rate (FDR) of ≤ 0.01 and $|\log_2 FC| \geq 1$, a total of 1409 differentially expressed genes (DEGs) were identified during SHS, with 763 downregulated genes (Additional file 2: Table S1) and 646 upregulated genes (Additional file 2: Table S2) (Fig. 1A and Additional file 1: Fig.S1B). Under LHT, 2659 DEGs were identified, consisting of 1277 downregulated genes (Additional file 2: Table S3) and 1382 upregulated genes (Additional file 2: Table S4) (Fig. 1A and Additional file 1: Fig. S1B).

Notably, 955 DEGs exhibited differential expression between SHS and LHT, encompassing 358 downregulated (Additional file 2: Table S5) and 597 upregulated genes (Additional file 2: Table S6) (Fig. 1A and Additional file 2: Fig. S1B). These findings highlight the significant impact of temperature and exposure duration on gene expression. A Venn diagram revealed that 162 genes were uniquely regulated under SHS, 848 genes under LHT, and 65 genes showed differential expression between SHS and LHT (Fig. 1B), suggesting that LHT induced more extensive changes in gene expression. Moreover, KOG function classification unveiled that the most significant changes occurred in genes related to translation, ribosomal structure, and biogenesis, with approximately 200 DEGs identified under both SHS and LHT (Fig. 1C). Additionally, pathways with over 50 DEGs, such as translation, ribosomal structure and biogenesis, and post-translational modification, showed significant variations between SHS and LHT (Fig. 1C). In summary, exposure to SHS and LHT triggered global changes in gene expression in *P. kudriavzevii*, activating or deactivating distinct

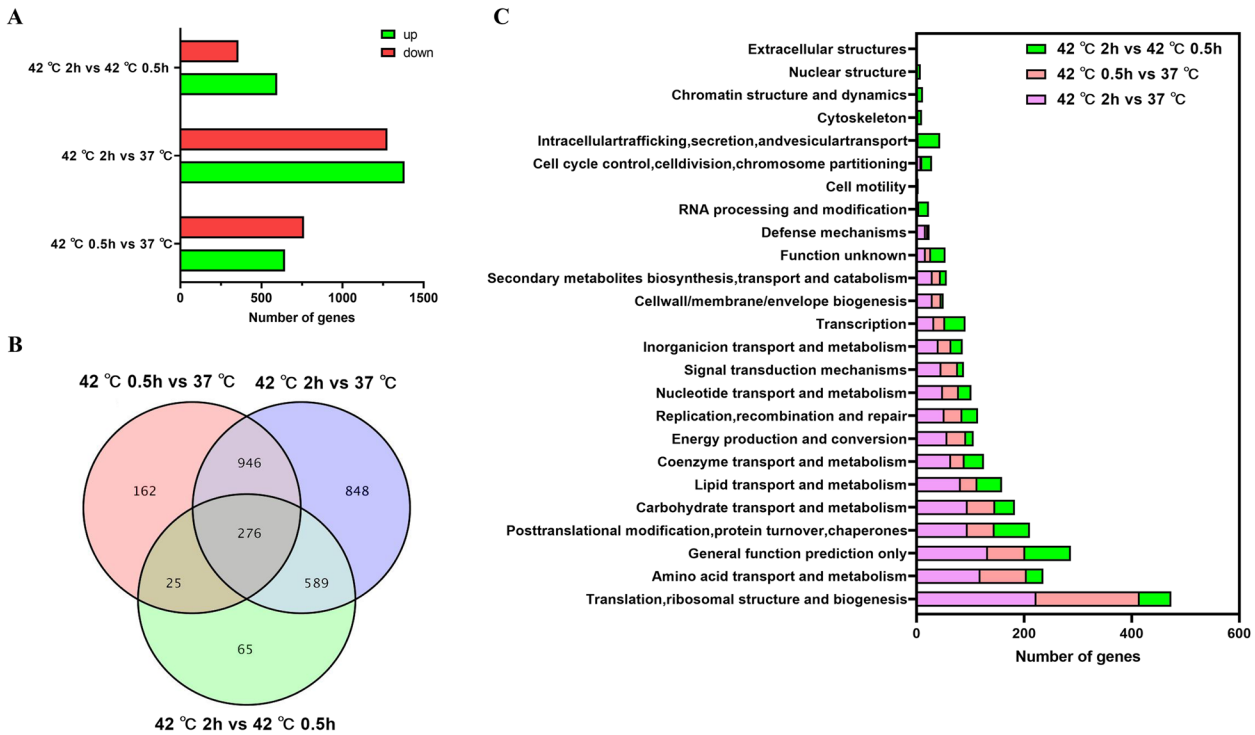

Fig. 1 Differential gene expression in *P. kudriavzevii* under long or short heat treatment. **A** Statistics of the differentially expressed genes. **B** Venn diagram showing the co-regulated genes of *P. kudriavzevii* under different temperature. **C** KOG function classification of the DEGs

functional pathways and revealing the yeast's adaptive responses to heat treatment.

Metabolic adaptations of *P. kudriavzevii* to heat treatment: enhanced TCA cycle and altered redox homeostasis

The TCA cycle is a cornerstone of central carbon metabolism, providing the primary energy source for aerobic organisms. RNA-seq analysis revealed that the majority of genes associated with the TCA cycle were upregulated in *P. kudriavzevii* under LHT. In contrast, SHS resulted in minimal changes in TCA cycle gene expression, except for the upregulation of *lsc1* (Table 1). To assess whether there is an increase in carbon fluxes within the TCA cycle, we measured the intracellular pyruvate levels at various temperatures. As shown in Fig. 2A, intracellular pyruvate content remained unchanged under SHS compared to growth at 37 °C and significantly reduced under LHT. This reduction is likely attributed to the increased energy demand at elevated temperatures, which accelerates the TCA cycle and depletes pyruvate. These results underscore the critical role of the TCA cycle in meeting the heightened energy requirements of *P. kudriavzevii* during heat treatment, particularly under prolonged exposure.

NADPH, primarily generated by the pentose phosphate pathway (PPP), is vital for reducing oxidized glutathione (GSSG) and maintaining redox homeostasis [28, 29]. Transcriptomic analysis indicated a downregulation of PPP genes during heat shock, with a significant decrease in the expression of 6-phosphogluconate dehydrogenase, the rate-limiting enzyme in the PPP, particularly under LHT (Table 2). Consistent with these findings, the intracellular NADPH/$NADP^+$ ratio was significantly lower under heat shock conditions compared to growth at 37 °C (Fig. 2B).

Glutathione reductase converts GSSG and NADPH to reduced glutathione (GSH), a key antioxidant. Measurements showed elevated GSH levels under heat shock, especially during LHT (Fig. 2C). Interestingly, total thiol content remained unchanged (Fig. 2D), while protein thiols increased significantly (Fig. 2F), likely due to oxidative stress from high temperatures, which can oxidize protein thiol groups. In summary, these findings suggest that heat shock in *P. kudriavzevii* induces a lower NADPH/$NADP^+$ ratio and elevated GSH levels, reshaping redox homeostasis. Additionally, the activation of the TCA cycle and suppression of the PPP pathway under heat treatment underscore the yeast's metabolic flexibility in responding to prolonged thermal challenges.

Table 1 Differentially expressed genes in TCA cycle upon heat shock

ID	Name	Log_2FC	Regulated	Annotation
42 °C 0.5 h vs 37 °C				
EVM0002137	*lsc1*	1.5952104	Up	Succinyl-CoA synthetase alpha subunit
42 °C 2 h vs 37 °C				
EVM0000971	*pcka*	5.3745075	Up	Phosphoenolpyruvate carboxykinase (ATP)
EVM0002875	*pyc*	3.2252527	Up	Pyruvate carboxylase
EVM0004091	*acna*	2.7306367	Up	Aconitate hydratase
EVM0002256	*glta*	3.2250419	Up	Citrate synthase
EVM0002137	*lsc1*	3.7014484	Up	Succinyl-CoA synthetase alpha subunit
EVM0000479	*lsc2*	1.5526949	Up	Succinyl-CoA synthetase beta subunit
EVM0001734	*mdh2*	3.8520108	Up	Malate dehydrogenase
EVM0003487	*mdh2*	2.0422207	Up	Malate dehydrogenase
EVM0004003	*mdh2*	3.8640342	Up	Malate dehydrogenase
EVM0003832	*idh3*	1.9739856	Up	Isocitrate dehydrogenase (NAD^+)
EVM0000875	*idh3*	2.3464930	Up	Isocitrate dehydrogenase (NAD^+)
EVM0004363	*idh3*	1.5641766	Up	Isocitrate dehydrogenase (NAD^+)
EVM0004556	*sdh2*	1.3259453	Up	Succinate dehydrogenase
EVM0002949	*sdh2*	−1.97126207	Down	Succinate dehydrogenase

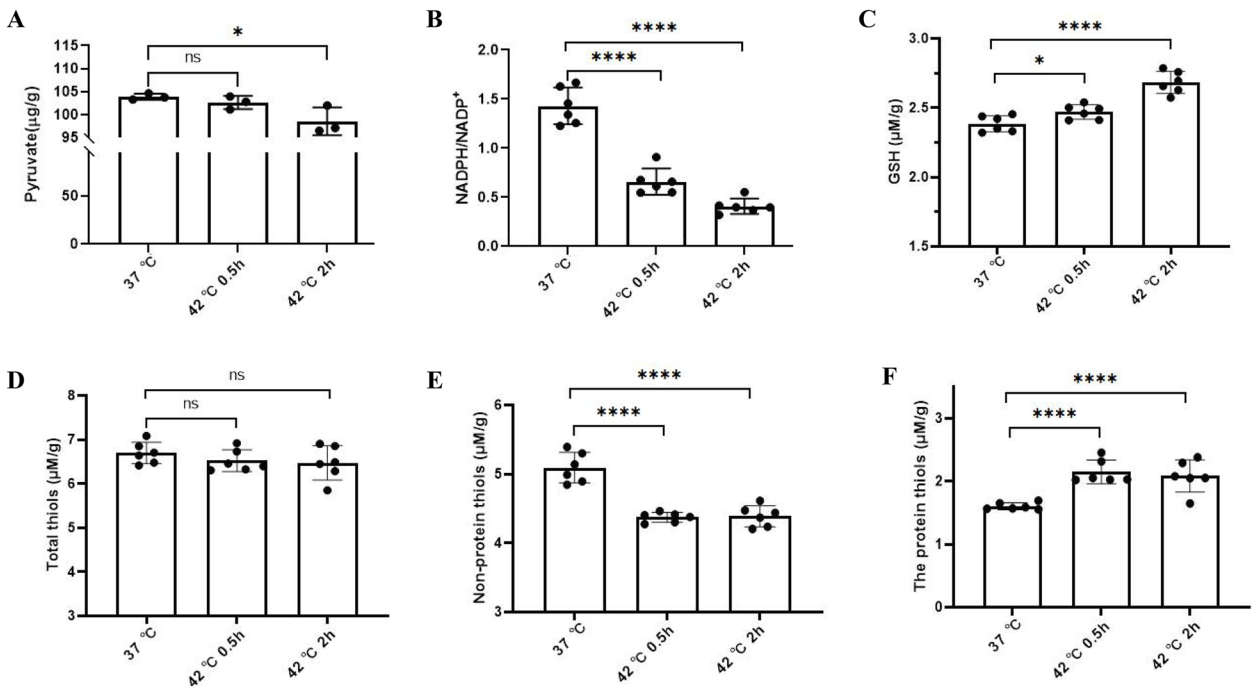

Fig. 2 Intracellular pyruvate, NADPH/$NADP^+$ ratio, GSH, total thiols, non-protein thiols and protein thiols in *P. kudriavzevii*. **A** The content of pyruvate. **B** NADPH/$NADP^+$ ratio. **C** The content of GSH. **D** The content of total thiols. **E** The content of non-protein thiols. **F** The content of protein thiols. Values are the average of six independent experiments ± *SD*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

Comprehensive metabolic reprogramming in *P. kudriavzevii* under LHT

LHT induced differential expression of genes across multiple metabolic pathways (Fig. 3), including the TCA cycle, PPP, trehalose metabolism, steroid biosynthesis, pyrimidine metabolism, purine metabolism, glycerophospholipid metabolism, and aminoacyl-tRNA biosynthesis. Notably, most genes associated with the TCA cycle and trehalose metabolism were upregulated (Tables 1 and 4), reflecting their critical roles in meeting

Table 2 Differentially expressed genes in PPP pathway upon heat shock

ID	Name	Log_2FC	Regulated	Annotation
42 °C 0.5 h vs 37 °C				
EVM0004270	*rpia*	−2.68021140	Down	Ribose 5-phosphate isomerase A
EVM0000404	*prsa*	−1.64089848	Down	Ribose-phosphate pyrophosphokinase
EVM0001894	*prsa*	−1.45331325	Down	Ribose-phosphate pyrophosphokinase
42 °C 2 h vs 37 °C				
EVM0001415	*pgls*	−1.13694238	Down	6-phosphogluconolactonase
EVM0003113	*pgd*	−1.57422204	Down	6-phosphogluconate dehydrogenase
EVM0004435	*rpe*	−1.7659663	Down	Ribulose-phosphate 3-epimerase
EVM0004270	*rpia*	−2.48690958	Down	Ribose 5-phosphate isomerase A
EVM0000824	*talb*	−1.02132482	Down	Transaldolase
EVM0000404	*prsa*	−1.53125485	Down	Ribose-phosphate pyrophosphokinase
EVM0001894	*prsa*	−1.41849464	Down	Ribose-phosphate pyrophosphokinase
EVM0002559	*prsa*	−1.48571351	Down	Ribose-phosphate pyrophosphokinase
EVM0004971	*rbsk*	3.47225864	Up	Ribokinase

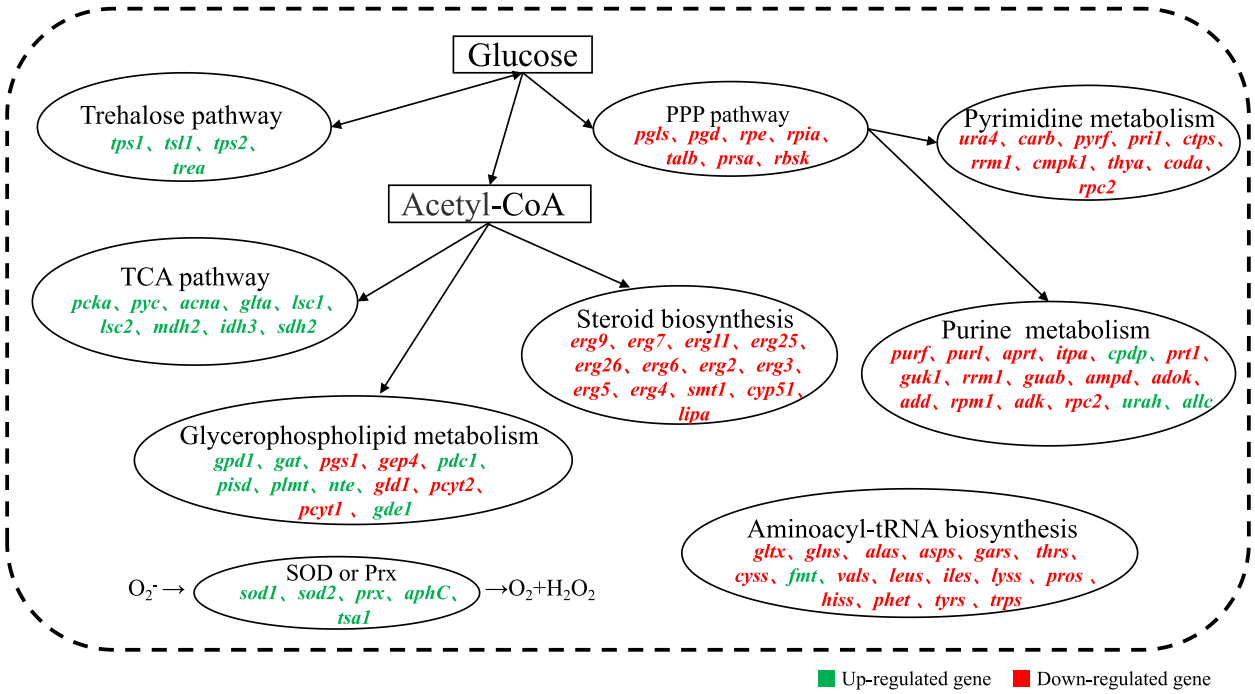

Fig. 3 Metabolic regulatory networks governing thermotolerance in *P. kudriavzevii* under long-term heat treatment (LHT). Genes in various metabolic pathways are depicted in green or red, denoting upregulation or downregulation, respectively

the heightened energy demands and maintaining stress tolerance under high-temperature conditions. In contrast, genes involved in steroid biosynthesis, pyrimidine metabolism, purine metabolism, and aminoacyl-tRNA biosynthesis pathway were predominantly downregulated (Additional file 2: Tables S7-S11). Conversely, genes involved in glycerophospholipid metabolism displayed a mix of upregulation and downregulation (Additional file 2: Table S8). These findings highlight the complex metabolic adaptations of *P. kudriavzevii* under LHT, demonstrating the necessity for fine-tuned regulation of multiple pathways to enhance thermotolerance and ensure survival under high-temperature conditions.

Heat shock proteins drive adaptation to thermal stress

Heat shock proteins (HSPs) play a pivotal role in protecting organisms from thermal stress by preventing protein aggregation, refolding damaged proteins, clearing irreversibly aggregated proteins, and enhancing the thermal stability of soluble proteins [30, 31]. Consistent with these roles, *P. kudriavzevii* exhibited significant upregulation of HSP-related genes under heat treatment. Specifically, during SHS, three HSP genes—*sti1* (Hsp90 co-chaperone), *hsp78*, and *hsp10*—were upregulated. Under LHT, additional genes, including *hsf1* (heat shock factor), *ssa2* (Hsp70 family), *sse1* (Hsp110 family), and *ssc1*, showed increased expression (Table 3). Quantitative real-time reverse transcription PCR (qRT-PCR) confirmed that all seven HSP genes exhibited significantly higher expression levels under LHT compared to 37 °C (Fig. 4A), reflecting the increased demand for HSPs in facilitating adaptation to prolonged high-temperature environments.

Among the HSPs, Hsp70 is particularly critical in responding to heat shock. It functions as both a sensor for Hsf1-mediated cytoprotection [32, 33] and a facilitator of protein folding and resolubilization of aggregated proteins under stress conditions [34]. The interaction between Hsf1 and Hsp70 forms a two-component feedback loop that regulates the heat shock response in yeast [33]. Notably, Hsp70 is a direct target of Hsf1, and upon heat shock, the dissociation of Hsp70 from Hsf1 activates Hsf1, leading to increased *hsp70* expression [33, 35]. Hsp90's activity is intricately linked to Hsp70, as it relies on Hsp70 chaperones for remodeling and activation of proteins [36]. Additionally, Hsp70 and Hsp110 families collaborate in the degradation of misfolded proteins through the ubiquitin-proteasome system [37]. Based on

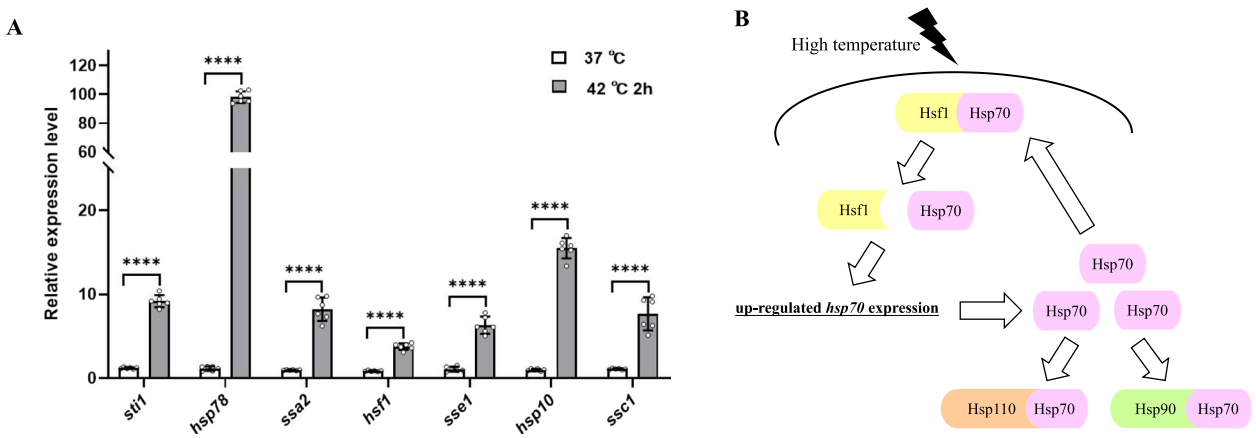

Fig. 4 Expression of HSPs in *P. kudriavzevii* under LHT. **A** Transcription levels of HSP genes at 37 or 42 °C. qRT-PCR analysis was performed as described in the "Methods" section, utilizing *act1* as an internal standard. Six independent experiments were performed and results are presented as mean ± *SD*. ****, $p < 0.0001$. **B** Model illustrating the role of HSPs in response to high temperatures

Table 3 Heat shock proteins with upregulated gene expression level under heat shock stress

ID	Name	log_2FC	Regulated	Annotation
42 °C 0.5 h vs 37 °C				
EVM0000198	*sti1*	1.682096	Up	hsp90 family protein Sti1
EVM0000521	*hsp78*	2.278416	Up	heat shock protein Hsp78
EVM0003787	*hsp10*	1.748119	Up	heat shock protein Hsp10
42 °C 2 h vs 37 °C				
EVM0000198	*sti1*	2.4487666	Up	hsp90 family protein Sti1
EVM0000521	*hsp78*	5.1647458	Up	heat shock protein Hsp78
EVM0001440	*ssa2*	2.6322678	Up	hsp70 family protein Ssa2
EVM0001793	*hsf1*	1.5004229	Up	heat shock transcription factor Hsf1
EVM0002817	*sse1*	1.7379203	Up	hsp110 family protein Sse1
EVM0003787	*hsp10*	3.5868202	Up	heat shock protein Hsp10
EVM0004039	*ssc1*	2.8340037	Up	heat shock protein Ssc1

the elevated expression of heat shock-related proteins, we propose a model for the role of HSPs in the heat shock response (Fig. 4B). In this model, Hsf1 and molecular chaperones establish a negative feedback loop to regulate Hsp70 expression. Upon heat treatment, activated Hsp70 interacts with Hsp90 to refold damaged proteins and collaborates with Hsp110 to enhance Hsp70-dependent protein folding rates. This coordinated response enables *P. kudriavzevii* to maintain protein homeostasis and survive under high-temperature conditions.

Trehalose biosynthesis and catabolism in *P. kudriavzevii* contribute to heat treatment adaptation

Trehalose, a recognized storage carbohydrate in yeast, plays a pivotal role in stabilizing biological membranes, proteins, and nucleic acids under stress conditions [30]. Transcriptome analysis data revealed that genes associated with trehalose biosynthesis (*tps1*, *tsl1*, and *tps2*) and catabolism (*nth1*) were upregulated under both SHS and LHT conditions, with a notably higher expression observed during LHT (Table 4). This upregulation encompassed crucial enzyme subunits, including trehalose-6-phosphate synthase Tps1, trehalose-6-phosphatase Tps2, and the regulatory subunits Tsl1 and Tps3. Additionally, the gene encoding neutral trehalase (Nth1), which catalyzes the conversion of trehalose to glucose and facilitates the return to normal growth following thermal stress [38], was also upregulated under heat shock stress.

To assess the impact of these gene expressions on trehalose metabolism, we measured intracellular trehalose content using the anthrone-sulfuric acid method. As shown in Fig. 5A, the trehalose levels significantly increased under both SHS and LHT compared to growth at 37 °C. These results suggest that *P. kudriavzevii* enhances trehalose production as part

Table 4 Differentially expressed genes in trehalose metabolic pathway upon heat shock stress

ID	Name	Log_2FC	Regulated	Annotation
42 °C 0.5 h vs 37 °C				
EVM0002081	*tps1*	1.949210795	Up	Trehalose 6-phosphate synthase
EVM0003762	*tsl1*	1.820512506	Up	Trehalose synthase complex regulatory subunit
EVM0004248	*tps2*	1.969955351	Up	Trehalose 6-phosphate synthase/phosphatase
EVM0004304	*nth1*	2.551869191	Up	Alpha-trehalase
42 °C 2 h vs 37 °C				
EVM0002081	*tps1*	4.003381138	Up	Trehalose 6-phosphate synthase
EVM0003762	*tsl1*	3.119510827	Up	Trehalose synthase complex regulatory subunit
EVM0004248	*tps2*	2.376911055	Up	Trehalose 6-phosphate synthase/phosphatase
EVM0004304	*nth1*	3.186980852	Up	Alpha-trehalase

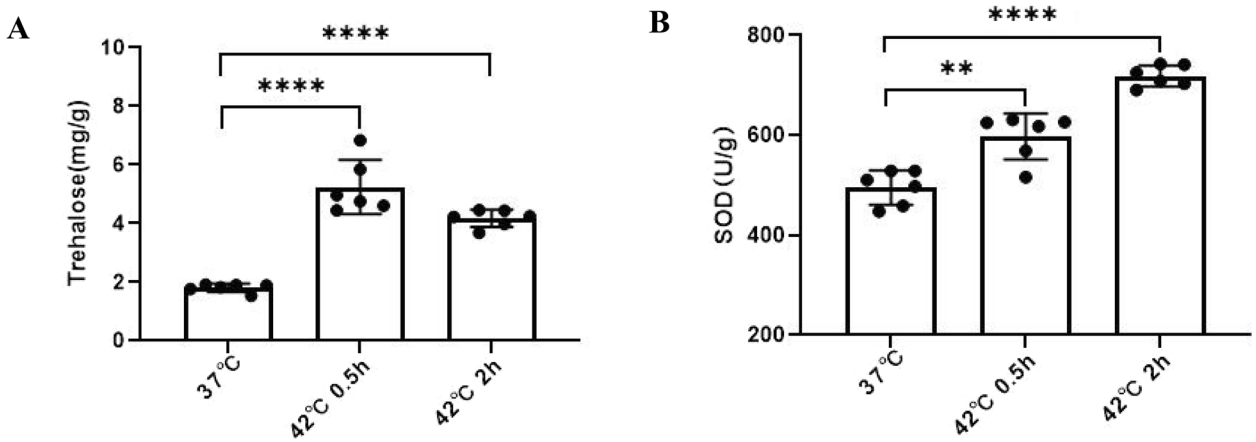

Fig. 5 Intracellular metabolites analysis of *P. kudriavzevii* cultivated at 37 or 42 °C. **A** Measurement of trehalose content was conducted using visible spectrophotometry after extraction with trichloroacetic acid; **B** SOD activity was evaluated by assessing its inhibition of the reduction of nitroblue tetrazole (NBT) under light. Values are presented as mean ± *SD* from six independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

of its thermal protective mechanism against high temperatures.

Activation of antioxidant defense mechanisms in *P. kudriavzevii* under heat treatment

Heat shock stress is known to accelerate the production of reactive oxygen species (ROS), potentially leading to oxidative damage in yeast cells [39]. In *S. cerevisiae*, oxidative damage stands out as a prominent secondary consequence of heat shock stress, prompting the activation of multiple antioxidant defense mechanisms. These include enzymatic antioxidant defenses such as superoxide dismutases (SOD) (cytoplasmic Sod1 and mitochondrial Sod2), catalases (peroxisomal catalase A and cytosolic catalase T), and peroxiredoxin (Prx), collectively serving as primary protective mechanisms against oxidative damage [30, 40, 41].

Based on RNA-seq analysis, the upregulation of *sod1* and *sod2* transcription in *P. kudriavzevii* was observed under both SHS and LHT conditions (Table 5). Strikingly, the transcripts of the *sod2* were significantly higher under LHT compared to SHS, suggesting an increased demand for mitochondrial Sod2 to counteract reactive oxygen species (ROS) during prolonged heat treatment. Furthermore, an in-depth analysis of SOD activities revealed that enzymatic activities were markedly higher under LHT than SHS, emphasizing the significance of elevated SOD levels under conditions of extended heat treatment (Fig. 5B). Additionally, our findings revealed that four peroxiredoxins were upregulated under LHT, whereas only one showed increased expression under SHS. This disparity underscores *P. kudriavzevii*'s reliance on SOD and peroxiredoxins to maintain intracellular redox environment under elevated temperatures, particularly during prolonged heat treatment.

Contribution of heat shock proteins, superoxide dismutases, and trehalose metabolism to thermotolerance in *P. kudriavzevii*

Through transcriptome analysis, we identified a set of robustly upregulated genes, including heat shock proteins (*hsp78* and *hsp10*), superoxide dismutases (*sod2*), and related proteins in trehalose metabolism (*tps1*, *tsl1*, and *nth1*), which may play a crucial role in responding to heat treatment. To assess the impact of these genes on heat treatment, we employed CRISPR-Cas9 technology to construct in-frame deletion mutations, resulting in five mutants (Δ*hsp78*, Δ*hsp10*, Δ*sod2*, Δ*tsl1*, and Δ*nth1*), with the exception of *tps1*. The thermotolerance of these mutants was compared to the wild-type strain (Δ*ura3*) at both 37 and 42 °C (Additional file 1: Fig. S2), where no significant growth differences were observed.

In a subsequent experiment, cultures of the five mutants and the Δ*ura3* strain, all adjusted to equal cell densities, were incubated at 55 °C for varying durations. As shown in Fig. 6, there was no significant difference in percent survival between Δ*hsp10* and the Δ*ura3* strain after incubation at 55 °C for up to 15 min. In contrast, the percent survival of the other four mutants was dramatically reduced compared to the Δ*ura3* strain. These results indicate that heat shock proteins, superoxide dismutases, and trehalose metabolism significantly contribute to thermotolerance in *P. kudriavzevii.*

Discussion

Non-conventional yeasts have gained recognition as highly significant chassis strains for thermotolerance in industrial applications [24]. While research on thermotolerance has been extensively conducted using transcriptome and proteome analyses in *K. marxianus* [42, 43], the molecular mechanisms of stress response in *Pichia* species remain poorly understood. This study aims to unravel the molecular mechanism of thermotolerance

Table 5 Expression of superoxide dismutase genes during heat shock stress

ID	Name	log_2FC	Regulated	Annotation
42 °C 0.5 h vs 37 °C				
EVM0001194	*sod2*	1.308473183	Up	Superoxide dismutase [Mn]
EVM0002820	*prx*	1.529593265	Up	Peroxisomal peroxiredoxin
42 °C 2 h vs 37 °C				
EVM0002560	*sod1*	1.022187449	Up	Superoxide dismutase [Cu–Zn]
EVM0001194	*sod2*	3.354683529	Up	Superoxide dismutase [Mn]
EVM0002820	*prx*	2.022500341	Up	Peroxisomal peroxiredoxin
EVM0003456	*aphc*	2.094914174	Up	Glutathione peroxidase-like peroxiredoxin
EVM0003786	*tsa1*	1.636383225	Up	Peroxiredoxin Tsa1
EVM0004105	*prx*	1.111130565	Up	Peroxisomal peroxiredoxin

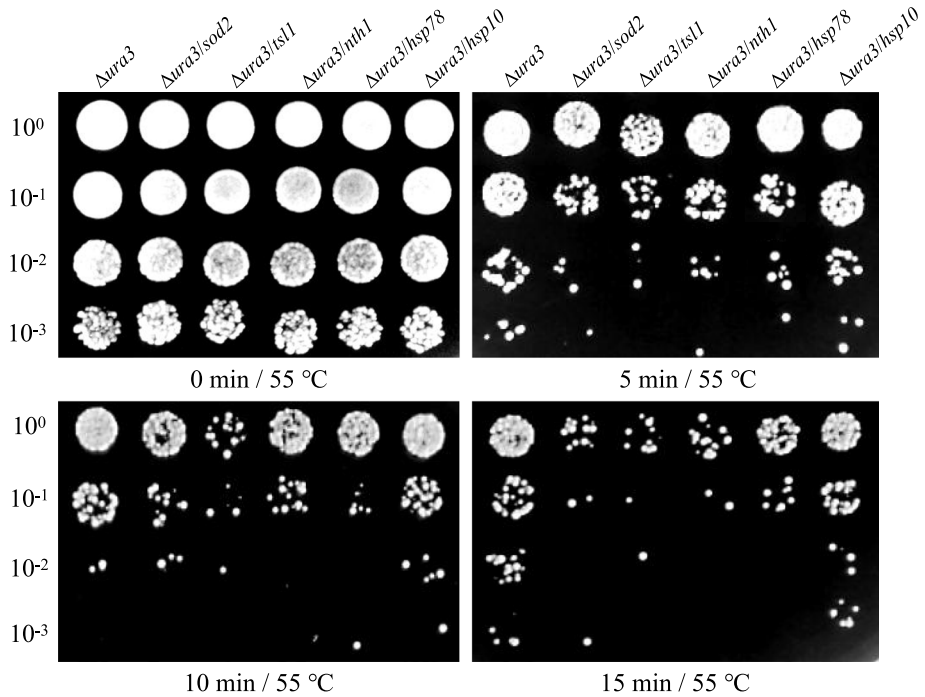

Fig. 6 Heat sensitivity of thermotolerance-related gene deletion mutants. Cells in the mid-log phase were subjected to high temperatures of 55 °C for heat shock durations of 0, 5, 10, and 15 min. Subsequently, the cells were serially diluted and inoculated onto YPD plates, followed by incubation at 30 °C for 2 days

in *P. kudriavzevii* through a comprehensive approach, encompassing genetic analyses, transcriptome profiling, and metabolic analysis. Our findings reveal significant differential gene expression in response to heat shock, particularly under LHT. Heat shock profoundly influences the expression of genes involved in multiple metabolic pathways, including the trehalose pathway, ergosterol synthesis pathway, TCA cycle, and PPP. Although a similar regulatory pattern of metabolic pathways is observed between SHS and LHT, more genes and more significant differences are evident under LHT. This implies that the molecular mechanisms involved in adapting to short-term and long-term heat treatment are distinct in *P. kudriavzevii*.

It has been demonstrated that HSPs serve as crucial elements in conferring resistance to high temperatures, activating silent proteins, or repairing damaged proteins in yeast cells [44–46]. Chamnipa et al. identified key genes, such as *ssq1* and *hsp90*, playing a critical role in thermotolerance in *P. kudriavzevii* [24]. In our study, the gene expression levels of several HSPs were upregulated, consistent with qRT-PCR results. While only three HSPs were upregulated upon SHS, seven HSPs were upregulated under LHT. This implies that *P. kudriavzevii* cells require a heightened expression of HSPs to adapt to LHT, particularly the transcription factor *hsf1*, known for regulating the expression of *hsp70*. Functioning as a vital molecular chaperone in yeast cells, Hsp70 participates in critical cellular processes, including protein folding and translocation across membranes [47, 48]. Given its ability to interact with multiple proteins, a proposed regulatory mode of the Hsp70 chaperone is depicted (Fig. 4B). Additionally, Hsp78 and Hsp10, chaperons found in the mitochondrial matrix, exhibit differential roles in temperature sensitivity of *S. cerevisiae* [49, 50]. In *P. kudriavzevii*, only Hsp78 plays a crucial function in cellular heat shock tolerance, while Hsp10 does not, distinguishing it from *S. cerevisiae* and underscoring the unique role of Hsp78 in thermotolerance in *P. kudriavzevii*.

Trehalose, known as a thermal protecting agent in *K. marxianus* and *S. cerevisiae*, plays a role in shielding cells from damage induced by heat stress [51]. In *P. kudriavzevii*, the expression of the genes in trehalose pathway and intracellular trehalose were significantly increased upon heat shock. It is interesting to note that a higher content of trehalose was produced in *P. kudriavzevii* under SHS. As the *nth1* gene that encodes neutral trehalase Nth1 was also upregulated in *P. kudriavzevii*, it is likely that the relatively lower trehalose content in *P. kudriavzevii* under long-term heat treatment was due to degradation catalyzed by Nth1. And then, we revealed that trehalose metabolic pathway played an important

role in responding to high temperatures by the construction of deletion mutants. However, the deletion mutant of *tps1* gene was not obtained in selective media containing trehalose as the sole carbon source, implying its essentiality. The *tps1* gene, encoding a trehalose-6-phosphate synthase, play a different role in different yeast species, such as its deletion eliminated the yeast ability to grow on glucose as the sole carbon source in *S. cerevisiae*, but not in *K. lactis* [52]. Based on our results, it is clear that *P. kudriavzevii* uses trehalose as a thermal protecting agent and the defect of trehalose metabolic pathway has great influence on resistance of cells to thermotolerant stress.

Previous studies have showed that mutation of the *erg3* and *erg5*, the genes encoding sterol dehydrogenases, enhances thermotolerance and higher ergosterol content in yeast cells [53, 54]. However, in this study, we found that the expression of the genes in ergosterol synthesis pathway was downregulated whereas ergosterol content was not changed in *P. kudriavzevii* (Additional file 2: Table S12 and Additional file 1: Fig. S3). On the other hand, the *gpd1*, a key gene for glycerol synthesis, was upregulated in *P. kudriavzevii* under LHT (Additional file 2: Table S8), which suggests a role of glycerol as a thermal protectant in *P. kudriavzevii*.

In addition to HSPs, trehalose and SOD, we also found that most genes involved in TCA cycle was upregulated in *P. kudriavzevii* under LHT, a response similar to that observed in *S. cerevisiae*. In contrast, genes involved in PPP pathway were significantly suppressed under LHT. This contrasts with findings in *K. marxianus*, where TCA cycle gene expression is suppressed at temperatures ranging from 30 to 45 °C [42, 55], and no significant changes in PPP-related gene expression in either *S. cerevisiae* or *K. marxianus* under heat stress [42, 56]. As the TCA cycle and PPP pathway are two basic metabolic pathways to supply NADH, NADPH and nitrogen metabolism [57], we propose that *P. kudriavzevii* enhances energy production via these pathways during LHT to facilitate the degradation of damaged proteins and DNA repair. These responses differ markedly from those observed in other yeast species, highlighting distinct long-term adaptations to heat treatment in *P. kudriavzevii*.

The Iswi chromatin remodelers, including ATPases Isw1 and Isw2, are crucial for regulating nucleosome positioning and are typically associated with transcriptional repression [58]. These complexes influence gene expression by contributing to chromatin organization. In the context of thermotolerance, transcriptomic analysis revealed significant downregulation of the *isw2* gene under both short-term and long-term heat treatment conditions (Additional file 2: Table S1 and Table S3). This downregulation suggests that *P. kudriavzevii* may modulate chromatin structure to promote the transcriptional activation of heat-responsive genes, enabling an adaptive response to elevated temperatures.

Conclusions

In conclusion, our study explored the response of *P. kudriavzevii* to heat-induced environmental stress. We found that *P. kudriavzevii* effectively resists thermal stress through several adaptive mechanisms, including enhancement of the TCA cycle, modulation of the PPP pathway, and upregulation of heat shock proteins (HSPs), trehalose, glutathione (GSH), and superoxide dismutase (SOD) activity. These findings provide a comprehensive understanding of the mechanisms underlying the thermotolerance of *P. kudriavzevii*, offering a theoretical foundation for the rational engineering of more thermotolerant *Pichia* strains for industrial applications.

Methods

Strains and medium

The yeast strain of *Pichia kudriavzevii* LC375240 strain was preserved from our lab [27]. The *P. kudriavzevii* strain was pre-cultured overnight in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) at 37 °C.

Global transcriptional analysis

The *P. kudriavzevii* strains were grown in fresh medium to the mid-exponential phase (OD_{600}≈0.8) at 37 °C for 4 h and then continued to cultivate it at 42 °C for 0.5 or 2 h, and collected by suction filtration at 37 or 42 °C. The cells were washed with RNA-free water and send to the BioMarker company (Beijing, China) for sample extraction and sequencing. Total RNA was isolated from *P. kudriavzevii* strains and quantity and quality was evaluated on a Nanodrop spectrophotometer ND-8000 and Agilent 2100 bioanalyzer respectively. The sample was subjected to RNA sequence in an Illumina NovaSeq6000 platform. And the FASTQ files associated with this project have been deposited in the NCBI database under accession number PRJNA793071.

Real-time quantitative reverse transcription PCR (qRT-PCR)

The cDNA was synthesized from total RNA of returned samples using HiScript III 1st Strand cDNA Synthesis kit (Vazyme) according to the manufacturer's protocol. The qRT-PCR was performed with different primer sets using cDNA as templates and the primers for qRT-PCR are listed in Additional file 2: Table S13, *act1* of *P. kudriavzevii* as an internal control. Relative expression level was calculated with *act1* as control.

Intracellular metabolite assays

The *P. kudriavzevii* strains were grown to the mid-exponential phase (OD_{600}≈0.8) at 37 °C and transferred it to a new fresh medium for 0.5 or 2 h at 42 °C, and then collected and washed using distilled water, ground with liquid nitrogen and tested intracellular metabolites. Pyruvate and NADPH/$NADP^+$ were measured using kits (Pyruvate: BC2205, Solarbio, Beijing, China; NADPH/$NADP^+$, QYS-231039, QIYBio, Shanghai, China). Total glutathione was extracted and used for the measurement of thiol content using Ellman's reagent (DTNB), with spectrophotometric readings taken at 412 nm [59]. The concentrations of total thiols and non-protein thiols were determined according to the method of Sedlak and Lindsay (1968) [60], using Elman's reagent [DTNB]. Protein thiol content was calculated as the difference between total thiols and non-protein thiols. Anthrone-sulfuric acid method was used in the study to measure the intracellular trehalose [61, 62]. Measurement of superoxide dismutase activity is referred to the article from Spitz et al. [63]. Extraction and measurement of ergosterol is referred to the article from Arthington-Skaggs et al. [64].

Construction of thermotolerance-related gene mutants in *P. kudriavzevii*

The thermotolerance-related gene mutants in *P. kudriavzevii* were constructed by CRISPR-Cas9 technology. For construction of deletion mutant, 1000-bp upstream and 1000-bp downstream fragments flanking *ura3* were amplified using the total DNA and then formed a 2000-bp donor DNA by fusion PCR. The gRNA was cloned onto optimized pHTX1-Cas9-AOXTT-Zeocin plasmid to express the CRISPR/Cas9 [65], and then the recombinant plasmid and donor DNA were introduced into the strain by electroporation method. The cells were screened for YPD plates containing 100 μg/mL Zeocin and 1 mg/mL 5-FOA. The obtained *ura3* deletion mutant was further confirmed by PCR and named Δ*ura3*. Next, *ura3* gene was cloned onto pHTX1-Cas9-AOXTT-Zeocin plasmid and other deletion mutants were constructed using Δ*ura3* in the same way, and the deletion mutant was screened for SD plates and further confirmed by PCR.

Heat shock assay

The yeast cells were cultured in YPD medium until they reached an optical density at 600 nm (OD_{600}) of 1 and then shifted to 55 °C in a water bath at different time (0, 5, 10 and 15 min). Subsequently, 3 μL aliquots of 10^0, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were applied onto YPD plates after serial dilution. These plates were then incubated at 30 °C for a duration of 2 days, and the growth response was evaluated.

Abbreviations

SHS	Short-term heat shock
LHT	Long-term heat treatment
DEGs	Differentially expressed genes
TCA	Tricarboxylic acid cycle
PPP	Pentose phosphate pathway
HSPs	Heat shock proteins
GSH	Glutathione
SOD	Superoxide dismutase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-025-02159-1.

Additional file 1: Fig. S1 Differential expression analysis under varying temperature in *P. kudriavzevii*. A, Heat map corresponding to each sample under different temperature; B, Volcano plots of differentially expressed genes for 42 °C 0.5 h vs 37 °C, 42 °C 2 h vs 37 °C or 42 °C_2 h vs 42 °C_0.5 h. Fig. S2 The growth of thermotolerance-related gene deletion mutants and wild-type strain at 37 °C and 42 °C. A, Mid-log phase yeast cells were inoculated onto YPD plates and incubated at 42 °C for 2 days; Kinetic growth curves in YPD at 37 °C (B) and 42 °C (C). Fig. S3 Ergosterol content in *P. kudriavzevii* at 37 °C or 42 °C. Values are the average of six independent experiments ± SD. ns, no significant difference.

Additional file 2: Table S1. The down-regulated genes during SHS. Table S2. The up-regulated genes during SHS. Table S3. The down-regulated genes during LHT. Table S4. The up-regulated genes during LHT. Table S5. The down-regulated genes between SHS and LHT. Table S6. The up-regulated genes between SHS and LHT. Table S7. Steroid biosynthesis with regulated gene expression level during LHT. Table S8. Glycerophospholipid metabolism with regulated gene expression level during LHT. Table S9. Purine metabolism with regulated gene expression level during LHT. Table S10. Pyrimidine metabolism with regulated gene expression level during LHT. Table S11. Aminoacyl-tRNA biosynthesis with regulated gene expression level during LHT. Table S12. Ergosterol synthesis pathway with regulated gene expression level during heat shock stress. Table S13. The primers in this study.

Acknowledgements

We sincerely thank the editor and reviewers for their invaluable feedback, insightful comments, and strong support of this study. Their expertise and constructive suggestions have greatly improved the quality of our work.

Authors' contributions

W.F., B.W. and J.C. conceived the study. Y.Q., J.M. and Q.Q. carried out the experiments. W.B. and J.C. performed bioinformatics analysis. W.F. and Y.Q. wrote the manuscript with the input from all other authors. All authors read and approved the final manuscript.

Funding

This work was supported by Natural Science Foundation of Guangxi Province (2023GXNSFBA026057), Science and Technology Major Project of Guangxi (Guike AA24206051), National Key Research and Development Program of China (2021YFA0910600), Guangxi Science and Technology Base and Talent Special Project (AD23026030), and Research Start-up Funding of Guangxi Academy of Sciences (2021YBJ706).

Data availability

The authors confirm that all data generated or analysed during this study are included in this published article, its supplementary information files are publicly available. The FASTQ files associated with this project have been deposited in the NCBI database under accession number PRJNA793071.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors read and approved the final manuscript.

Competing interest
The authors declare no competing interests.

Received: 31 July 2024 Accepted: 13 February 2025
Published online: 23 February 2025

References

1. Dequin S, Casaregola S. The genomes of fermentative *Saccharomyces*. C R Biol. 2011;334:687–93. https://doi.org/10.1016/j.crvi.2011.05.019.
2. Querol A, Fernandez-Espinar MT, del Olmo M, Barrio E. Adaptive evolution of wine yeast. Int J Food Microbiol. 2003;86:3–10. https://doi.org/10.1016/s0168-1605(03)00244-7.
3. Piškur J, Polakova S, Merico A, Compagno C, Piskur J, Rozpedowska E. How did *Saccharomyces* evolve to become a good brewer? Trends Genet. 2006;22:183–6. https://doi.org/10.1016/j.tig.2006.02.002.
4. Conant GC, Wolfe KH. Increased glycolytic flux as an outcome of whole-genome duplication in yeast. Mol Syst Biol. 2007;3:1–12. https://doi.org/10.1038/msb4100170.
5. Steensels J, Verstrepen KJ. Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. Annu Rev Microbiol. 2014;68:61–80. https://doi.org/10.1146/annurev-micro-091213-113025.
6. Taylor MP, Mulako I, Tuffin M, Cowan DA. Understanding physiological responses to pre-treatment inhibitors in ethanologenic fermentations. Biotechnol J. 2012;7:1169–81. https://doi.org/10.1002/biot.201100335.
7. Almeida JR, Modig T, Petersson A, Hän-Häerdal B, Lidén G, Gorwa-Grauslund MF. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol. 2007;82:340–9. https://doi.org/10.1002/jctb.1676.
8. Basso LC, Basso TO, Rocha SN. Ethanol production in Brazil: the industrial process and its impact on yeast fermentation. In: Bernardes MA, editor. Biofuel production-recent developments and prospects. Rijeka: InTech. 2011; p. 85–100. https://doi.org/10.5772/17047.
9. Radecka D, Mukherjee V, Mateo RQ, Stojiljkovic M, Foulquié-Moreno MR, Thevelein JM. Looking beyond *Saccharomyces*: the potential of non-conventional yeast species for desirable traits in bioethanol fermentation. FEMS Yeast Res. 2015;15:fov053. https://doi.org/10.1093/femsyr/fov053.
10. Herrmann GF, Krezdorn C, Malissard M, Kleene R, Paschold H, Weuster-Botz D, Kragl U, Berger EG, Wandrey C. Large-scale production of a soluble human β-1,4-galactosyltransferase using a *Saccharomyces cerevisiae* expression system. Protein Expr Purif. 1995;6:72–8. https://doi.org/10.1006/prep.1995.1010.
11. Lü JL, Zhou PJ. Ethanol production from microwave-assisted FeCl3 pretreated rice straw using free and immobilized cells of *Trichoderma viride* and *Sacharomyces cerevisiae*. J Energy Eng. 2017;143:04016043. https://doi.org/10.1061/(ASCE)EY.1943-7897.0000396.
12. Rebello S, Abraham A, Madhavan A, Sindhu R, Binod P, Bahuleyan AK, Aneesh EM and Pandey A. Non-conventional yeast cell factories for sustainable bioprocesses. FEMS Microbiology Lett. 2018;365(21). https://doi.org/10.1093/femsle/fny222.
13. Malak A, Baronian K, Kunze G. Blastobotrys (Arxula) adeninivorans: a promising alternative yeast for biotechnology and basic research. Yeast. 2016;33:535–47. https://doi.org/10.1002/yea.3180.
14. Geijer C, Ledesma-Amaro R, Tomás-Pejó E. Unraveling the potential of non-conventional yeasts in biotechnology. FEMS Yeast Res. 2022;22(1):foab071. https://doi.org/10.1093/femsyr/foab071.
15. Wagner JM, Alper HS. Synthetic biology and molecular genetics in non-conventional yeasts: current tools and future advances. Fungal Genet Biol. 2016;89:126–36. https://doi.org/10.1016/j.fgb.2015.12.001.
16. Madhavan A, Jose AA, Binod P, Sindhu R, Sukumaran RK, Pandey A, Castro GE. Synthetic biology and metabolic engineering approaches and its impact on non-conventional yeast and biofuel production. Front Energy Res. 2017;5:8. https://doi.org/10.3389/fenrg.2017.00008.
17. Stöckmann C, Scheidle M, Dittrich B, Merckelbach A, Hehmann G, Melmer G, Klee D, Büchs J, Kang HA, Gellissen G. Process development in *Hansenula polymorpha* and *Arxula adeninivorans*, a reassessment. Microb Cell Fact. 2009;8:22. https://doi.org/10.1186/1475-2859-8-22.
18. Yuangsaard N, Yongmanitchai W, Yamada M, Limtong S. Selection and characterization of a newly isolatedthermotolerant *Pichia kudriavzevii* strain for ethanolproduction at high temperature from cassava starch-hydrolysate. Anton van Leeuw. 2013;103:577–88. https://doi.org/10.1007/s10482-012-9842-8.
19. Prillinger H, Molnár O, Eliskases-Lechner F, Lopandic K. Phenotypic and genotypic identification of yeasts from cheese. Anton Van Leeuw. 1999;75:267–83. https://doi.org/10.1023/a:1001889917533.
20. Chu Y, Li M, Jin J, Dong X, Xu K, Jin L, Qiao Y, Ji H. Advances in the application of the non-conventional yeast *Pichia kudriavzevii* in food and biotechnology industries. J Fungi (Basel). 2023;9(2): 170. https://doi.org/10.3390/jof9020170.
21. Daniel HM, Vrancken G, Takrama JF, Camu N, De Vos P, DeVuyst L. Yeast diversity of Ghanaian cocoa bean heap fermentations. FEMS Yeast Res. 2009;9:774–83. https://doi.org/10.1111/j.1567-1364.2009.00520.x.
22. Koh JH, Suh HJ. Biological activities of thermo-tolerant microbes from fermented rice bran as an alternative microbial feed additive. Appl Biochem Biotechnol. 2009;157:420–30. https://doi.org/10.1007/s12010-008-8311-3.
23. Isono N, Hayakawa H, Usami A, Mishima T, Hisamatsu M. A comparative study of ethanol production by *Issatchenkia orientalis* strains under stress conditions. J Biosci Bioeng. 2012;113:76–8. https://doi.org/10.1016/j.jbiosc.2011.09.004.
24. Chamnipa N, Thanonkeo S, Klanrit P, Thanonkeo P. The potential of the newly isolated thermotolerant yeast *Pichia kudriavzevii* RZ8-1 for high-temperature ethanol production. Braz J Microbiol. 2018;49:378–91. https://doi.org/10.1016/j.bjm.2017.09.002.
25. Hisamatsu M, Furubayashi T, Karita S, Mishima T, Isono N. Isolation and identification of a novel yeast fermenting ethanol under acidic conditions. J Appl Glycosci. 2006;53:111–3. https://doi.org/10.1002/yea.3662.
26. Kitagawa T, Tokuhiro K, Sugiyama H, Kohda K, Isono N, Hisamatsu M, Takahashi H, Imaeda T. Construction of a beta-glucosidase expression system using themultistress-tolerant yeast *Issatchenkia orientalis*. Appl Microbiol Biotechnol. 2010;87:1841–53. https://doi.org/10.1007/s00253-010-2629-9.
27. Ndubuisi IA, Qin Q, Liao G, Wang B, Moneke AN, Ogbonna JC, Jin C, Fang W. Effects of various inhibitory substances and immobilization on ethanol production efficiency of a thermotolerant *Pichia kudriavzevii*. Biotechnol Biofuels. 2020;13:91. https://doi.org/10.1186/s13068-020-01729-5.
28. Pocsi I, Prade RA, Penninckx MJ. Glutathione, altruistic metabolite in fungi. Adv Microb Physiol. 2004;49:1–76. https://doi.org/10.1016/S0065-2911(04)49001-8.
29. Cunha JT, Romaní A, Costa CE, Sá-Correia I, Domingues L. Molecular and physiological basis of *Saccharomyces cerevisiae* tolerance to adverse lignocellulose-based process conditions. Appl Microbiol Biotechnol. 2019;103(1):159–75. https://doi.org/10.1007/s00253-018-9478-3.
30. Gao L, Liu Y, Sun H, Li C, Zhao Z, Liu G. Advances in mechanisms and modifications for rendering yeast thermotolerance. J Biosci Bioengineer. 2016;121(6):599–606. https://doi.org/10.1016/j.jbiosc.2015.11.002.
31. Parsell DA, Taulien J, Lindquist S. The role of heat-shock proteins in thermotolerance. Philos Trans R Soc Lond B Biol Sci. 1993;339(1289):279–85. https://doi.org/10.1098/rstb.1993.0026.
32. Wang Y, Gibney PA, West JD, Morano KA. The yeast Hsp70 Ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds. Mol Biol Cell. 2012;23:3290–8. https://doi.org/10.1091/mbc.E12-06-0447.
33. Krakowiak J, Zheng X, Patel N, Feder ZA, Anandhakumar J, Valerius K, Gross DS, Khalil AS, Pincus D. Hsf1 and Hsp70 constitute a two-component feedback loop that regulates the yeast heat shock response. eLife. 2018;7:e31668. https://doi.org/10.7554/eLife.31668.
34. Mayer MP, Bukau B. Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci. 2005;62:670e684. https://doi.org/10.1007/s00018-004-4464-6.
35. Solís EJ, Pandey JP, Zheng X, Jin DX, Gupta PB, Airoldi EM, Pincus D, Denic V. Defining the essential function of yeast hsf1 reveals a compact

transcriptional program for maintaining eukaryotic proteostasis. Mol Cell. 2016;63:60–71. https://doi.org/10.1016/j.molcel.2016.05.014.
36. Kravats AN, Hoskins JR, Reidy M, Johnson JL, Doyle SM, Genest O, Masison DC, Wickner S. Functional and physical interaction between yeast Hsp90 and Hsp70. Proc Natl Acad Sci U S A. 2018;115(10):E2210–9. https://doi.org/10.1073/pnas.1719969115.
37. Kandasamy G, Andréasson C. Hsp70-Hsp110 chaperones deliver ubiquitin-dependent and -independent substrates to the 26S proteasome for proteolysis in yeast. J Cell Sci. 2018;131(6):jcs210948. https://doi.org/10.1242/jcs.210948.
38. Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G. Insights on the evolution of trehalose biosynthesis. BMC Evol Biol. 2006;6:1411–21. https://doi.org/10.1186/1471-2148-6-109.
39. Auesukaree C. Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. J Biosci Bioeng. 2017;124(2):133–42. https://doi.org/10.1016/j.jbiosc.2017.03.009.
40. Li JR, Yu P. Expression of Cu, Zn-superoxide dismutase gene from *Saccharomyces cerevisiae* in *Pichia pastoris* and its resistance to oxidative stress. Appl Biochem Biotechnol. 2007;136:127–39. https://doi.org/10.1007/BF02685943.
41. Hiltunen JK, Mursula AM, Rottensteiner H, Wierenga RK, Kastaniotis AJ, Gurvitz A. The biochemistry of peroxisomal beta-oxidation in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev. 2003;27:35–64. https://doi.org/10.1016/S0168-6445(03)00017-2.
42. Fu X, Li P, Zhang L, Li S. Understanding the stress responses of *Kluyveromyces marxianus* after an arrest during high-temperature ethanol fermentation based on integration of RNA-Seq and metabolite data. Appl Microbiol Biotechnol. 2019;103(6):2715–29. https://doi.org/10.1007/s00253-019-09637-x.
43. Li P, Fu X, Chen M, Zhang L, Li S. Proteomic profiling and integrated analysis with transcriptomic data bring new insights in the stress responses of *Kluyveromyces marxianus* after an arrest during high-temperature ethanol fermentation. Biotechnol Biofuels. 2019;12:49. https://doi.org/10.1186/s13068-019-1390-2.
44. Mayer MP, Le Breton L. Hsp90: Breaking the symmetry. Mol Cell. 2015;58:8–20. https://doi.org/10.1016/j.molcel.2015.02.022.
45. Röhl A, Rohrberg J, Buchner J. The chaperone Hsp90: changing partners for demanding clients. Trends Biochem Sci. 2013;38:253–62. https://doi.org/10.1016/j.tibs.2013.02.003.
46. Schopf FH, Biebl MM, Buchner J. The HSP90 chaperone machinery. Nat Rev Mol Cell Biol. 2017;18(6):345–60. https://doi.org/10.1038/nrm.2017.20.
47. Zuiderweg ER, Hightower LE, Gestwicki JE. The remarkable multivalency of the Hsp70 chaperones. Cell Stress Chaperones. 2017;22(2):173–89. https://doi.org/10.1007/s12192-017-0776-y.
48. Marszalek J. Mitochondrial Hsp70-function and evolution. Postepy Biochem. 2016;62(2):69–76.
49. Leonhardt SA, Fearson K, Danese PN, Mason TL. HSP78 encodes a yeast mitochondrial heat shock protein in the Clp family of ATP-dependent proteases. Mol Cell Biol. 1993;13(10):6304–13. https://doi.org/10.1128/mcb.13.10.6304-6313.1993.
50. Höhfeld J, Hartl FU. Role of the chaperonin cofactor Hsp10 in protein folding and sorting in yeast mitochondria. J Cell Biol. 1994;126(2):305–15. https://doi.org/10.1083/jcb.126.2.305.
51. Matsumoto I, Arai T, Nishimoto Y, Leelavatcharamas V, Furuta M, Kishida M. Thermotolerant yeast *Kluyveromyces marxianus* reveals more tolerance to heat shock than the brewery yeast *Saccharomyces cerevisiae*. Biocontrol Sci. 2018;23(3):133–8. https://doi.org/10.4265/bio.23.133.
52. Gomes AMV, Orlandi ACAL, Parachin NS. Deletion of the trehalose tps1 gene in Kluyveromyces lactis does not impair growth in glucose. FEMS Microbiol Lett. 2020;367(10):fnaa072. https://doi.org/10.1093/femsle/fnaa072.
53. Caspeta L, Chen Y, Ghiaci P, Feizi A, Buskov S, Hallström BM, Petranovic D, Nielsen J. Biofuels altered sterol composition renders yeast thermotolerant. Science. 2014;346(6205):75–8. https://doi.org/10.1126/science.1258137.
54. Liu G, Chen Y, Færgeman NJ, Nielsen J. Elimination of the last reactions in ergosterol biosynthesis alters the resistance of *Saccharomyces cerevisiae* to multiple stresses. FEMS Yeast Res. 2017;17(6). https://doi.org/10.1093/femsyr/fox063.
55. Lertwattanasakul N, Kosaka T, Hosoyama A, Suzuki Y, Rodrussamee N, Matsutani M, Murata M, Fujimoto N, Suprayogi, Tsuchikane K, Limtong S, Fujita N, Yamada M. Genetic basis of the highly efficient yeast Kluyveromyces marxianus: complete genome sequence and transcriptome analyses. Biotechnol Biofuels. 2015;18:8:47. https://doi.org/10.1186/s13068-015-0227-x.
56. S.Ye Y, Zhu Y, Pan L, Li L, Wang X, Lin Y. Gaining insight into the response logic of Saccharomyces cerevisiae to heat shock by combining expression profiles with metabolic pathways. Biochem Biophys Res Commun. 2009;385(3):357–62. https://doi.org/10.1016/j.bbrc.2009.05.071.
57. Zhang B, Ren L, Zeng S, Zhang S, Xu D, Zeng X, Li F. Functional analysis of PGI1 and ZWF1 in thermotolerant yeast *Kluyveromyces marxianus*. Appl Microbiol Biotechnol. 2020;104(18):7991–8006. https://doi.org/10.1007/s00253-020-10808-4.
58. Smolle M, Venkatesh S, Gogol MM, Li H, Zhang Y, Florens L, Washburn MP, Workman JL. Chromatin remodelers Isw1 and Chd1 maintain chromatin structure during transcription by preventing histone exchange. Nat Struct Mol Biol. 2012;19(9):884–92. https://doi.org/10.1038/nsmb.2312.
59. Monteiro G, Kowaltowski AJ, Barros MH, Netto LE. Glutathione and thioredoxin peroxidases mediate susceptibility of yeast mitochondria to Ca(2+)-induced damage. Arch Biochem Biophys. 2004;425(1):14–24. https://doi.org/10.1016/j.abb.2004.03.005.
60. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. 1968;25(1):192–205. https://doi.org/10.1016/0003-2697(68)90092-4.
61. Trevelyan WE, Gammon JN, Wiggins EH, Harrison JS. Studies on yeast metabolism. II. Synthesis of cell carbohydrates during glucose fermentation and its inhibition by azide. Biochem J. 1952;50(3):303–10. https://doi.org/10.1042/bj0500303.
62. Tan HG, Mei YJ, Guan FM, Wang RM. Determination of trehalose content by anthrone-sulphuric acid colorimetric method. Modern Food Science and Technology. 2006;1:125–8. https://doi.org/10.7506/spkx1002-6630-200908048.
63. Spitz DR, Oberley LW. An assay for superoxide dismutase activity in mammalian tissue homogenates. Anal Biochem. 1989;179(1):8–18. https://doi.org/10.1016/0003-2697(89)90192-9.
64. Arthington-Skaggs BA, Lee-Yang W, Ciblak MA, Frade JP, Brandt ME, Hajjeh RA, Harrison LH, Sofair AN, Warnock DW, Candidemia Active Surveillance Group. Comparison of visual and spectrophotometric methods of broth microdilution MIC end point determination and evaluation of a sterol quantitation method for in vitro susceptibility testing of fluconazole and itraconazole against trailing and nontrailing Candida isolates. Antimicrob Agents Chemother. 2002;46(8):2477–81. https://doi.org/10.1128/AAC.46.8.2477-2481.2002.
65. Weninger A, Hatzl AM, Schmid C, Vogl T, Glieder A. Combinatorial optimization of CRISPR/Cas9 expression enables precision genome engineering in the methylotrophic yeast *Pichia pastoris*. J Biotechnol. 2016;235:139–49. https://doi.org/10.1016/j.jbiotec.2016.03.027.

Publisher's Note