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Manipulating cell flocculation-associated protein kinases in *Saccharomyces cerevisiae* enables improved stress tolerance and efficient cellulosic ethanol production

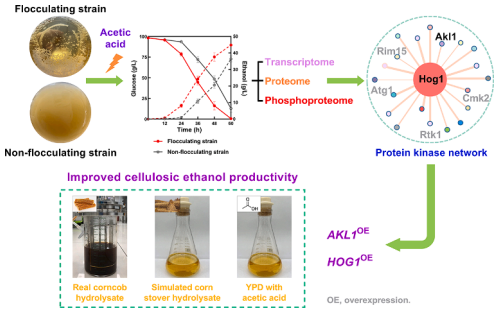
Pei-Liang Ye, Xue-Qing Wang, Bing Yuan, Chen-Guang Liu, Xin-Qing Zhao *

State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

HIGHLIGHTS

- Flocculation leads to profound changes of protein kinases under acetic acid stress.
- Phosphoproteome analysis identified key kinases responded to acetic acid stress.
- Overexpression of HOG1 and GPX1 reduced ROS content and improved stress tolerance.
- Overexpression of protein kinase Akl1 enhanced ethanol fermentation using corn cob.

GRAPHICAL ABSTRACT



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ABSTRACT

Cell self-flocculation endows yeast strains with improved environmental stress tolerance that benefits bioproduction. Exploration of the metabolic and regulatory network differences between the flocculating and non-flocculating cells is conducive to developing strains with satisfactory fermentation efficiency. In this work, integrated analyses of transcriptome, proteome, and phosphoproteome were performed using flocculating yeast *Saccharomyces cerevisiae* SPSC01 and its non-flocculating mutant grown under acetic acid stress, and the results revealed prominent changes in protein kinases. Overexpressing the mitogen-activated protein kinase Hog1 upregulated by flocculation led to reduced ROS accumulation and increased glutathione peroxidase activity, leading to improved ethanol production under stress. Among the seven genes encoding protein kinases that were tested, *AKL1* showed the best performance when overexpressed, achieving higher ethanol productivity in both corncob hydrolysate and simulated corn stover hydrolysate. These results provide alternative strategies for improving cellulosic ethanol production by engineering key protein kinases in *S. cerevisiae*.

* Corresponding author.
E-mail address: xqzhao@sjtu.edu.cn (X.-Q. Zhao).

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1. Introduction

Challenges of climate change and environmental deterioration due to consumption of fossil fuels demand efficient production of second-generation biofuels using lignocellulosic biomass as a feedstock (Liu et al., 2019). Nevertheless, inhibitors present in lignocellulosic hydrolysate lead to growth inhibition and impaired fermentation efficiency (Kumar et al., 2020). The inhibitors mainly include acetic acid, furfural, 5-Hydroxymethylfurfural (5-HMF), and others (Wang et al., 2018). Among the inhibitors, acetic acid is commonly present in lignocellulosic hydrolysate, and is highly toxic for fermenting microbial strains. Acetic acid disrupts the intracellular pH homeostasis, affects the function of the cell wall and plasma membrane, and induces oxidative stress and programmed cell death (Ndukwe et al., 2020). Therefore, developing acetic acid tolerant strains by manipulating the related key target genes is of great importance for efficient lignocellulosic biorefinery.

Yeast cell flocculation is an asexual, reversible, and calcium-dependent cell aggregation, which is widely used for cost-effectively separating biomass from fermentation medium (Zhao & Bai, 2009). The self-flocculating yeast strain SPSC01 has been used for industrial continuous ethanol fermentation (Xu et al., 2005). Flocculation endows yeast cells with improved tolerance to ethanol and acetic acid stresses (Cheng et al., 2017; Lei et al., 2007). Furthermore, flocculation was also reported to achieve faster ethanol production using dilute acid spruce hydrolysate, and stronger flocculation led to more efficient detoxification of furfural (Westman et al., 2014). In the *FLO5*-induced flocculating strains, flocculation also improved wine fermentation performance and thermotolerance, suggesting the importance of flocculation in yeast stress resistance (Di Gianvito et al., 2018; Vergara-Álvarez et al., 2019). Flocs can form a compact structure and protect cells from environmental stresses. However, physical shielding cannot fully explain the protective mechanisms of cell flocculation. In the previous studies, flocculation has been reported to cause changes in transcription, increased cell surface hydrophobicity, and enhanced plasma membrane ATPase activities (Di Gianvito et al., 2018; Lei et al., 2007; Smukalla et al., 2008; Westman et al., 2014). However, it still remains unclear how flocculation affects response to stresses in yeast cells.

Manipulation of multiple genes, such as *RTC1*, *ACS1*, *RCK1*, *PMA1*, *CCW12*, and *ADY2*, has been revealed to endow yeast cells with improved acetic acid resistance (Chen et al., 2022; Kong et al., 2021; Lee et al., 2017; Oh et al., 2019; Qin et al., 2020; Zhang et al., 2017). To further identify other key genes, transcriptome and proteome analyses, ZFP-TF library, and random mutagenesis have been employed (Almeida et al., 2009; Li et al., 2020; Ma et al., 2015). However, studies of improving yeast robustness by manipulating protein kinases are still limited. Several signaling pathways, including retrograde response (RTG), target of rapamycin (TOR), and Ras–cAMP–PKA, were reported to regulate acetic acid programmed cell death (AA-PCD) (Guaragnella & Bettiga, 2021). Membrane phosphoproteome analysis has been employed to study the early response of *S. cerevisiae* to acetic acid stress (1 h exposure to 50 mM acetic acid at pH 4.0), and involvement of protein kinase Hrk1 which regulates various transporters was identified to be involved in acetic acid stress response (Guerreiro et al., 2017). However, so far, no reports on integrated analyses of multi-omics data have been performed to study adaptive responses to acetic acid stress and breed robust yeast strains.

In this study, transcriptome, proteome, and phosphoproteome were performed to explore key stress response proteins by comparing flocculating and non-flocculating strains under acetic acid stress. Several key protein kinases were identified that could be manipulated to improve yeast stress tolerance and enhance cellulosic ethanol fermentation. The results in this work provide a basis for efficient cellulosic ethanol production using industrial yeasts.

2. Materials and methods

2.1. Strains, media, and materials

The plasmids and microbial strains used in this study are listed (see supplementary material). The flocculating yeast host strain *S. cerevisiae* strains SPSC01 and its mutant PLY01 (SPSC01-ΔFLO1) (Tang et al., 2019), as well as the engineered yeast strains developed in this study, were cultured in YPD medium containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. Antibiotics, either 300 μg/mL G418 or 500 μg/mL hygromycin B were added to select transformants. For yeast two-hybrid system strains, SD medium (6.7 g/L yeast nitrogen base without amino acid, 20 g/L glucose, and 0.6 g/L DO supplement-His/Leu/Trp/Ura) with 20 mg/L uracil and 20 mg/L histidine was used for selection of transformants. When performing fermentation experiments, the fermentation medium (100 g/L glucose, 4 g/L yeast extract, and 3 g/L peptone) was used, and different concentrations of inhibitors (acetic acid, furfural, NaCl, and H_2O_2) were supplemented for the stress tolerance test. *Escherichia coli* DH5α was used to propagate plasmid and cultivated in Luria-Bertani medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl). Antibiotic of 100 μg/mL ampicillin or 50 μg/mL kanamycin was added into LB medium for selection of transformants.

2.2. Construction of deletion and overexpression strains

All the engineered strains were constructed using CRISPR-Cas9 based genome editing (Tang et al., 2019). The deletion or overexpression donor-cassettes were amplified by PCR using SPSC01 genome as a template. The primers for donor-DNA amplification and gRNA construction are listed (see supplementary material). The plasmid Cas9-G418 was introduced into SPSC01 or PLY01 to yield strain SPSC01-Cas9 and PLY01-Cas9, firstly. The specific gRNA protospacer sequence targeting genes were fused to parent plasmid gRNA_clone, followed by transforming the newly constructed gRNA plasmids into strain SPSC01-Cas9 or PLY01-Cas9 with corresponding donor-DNA. The correct transformants were verified by PCR and sequencing.

2.3. Transcriptomic, proteomic and phosphoproteomic analysis

Yeast strains of SPSC01 and PLY01 were cultured in the presence of 5.0 g/L acetic acid stress until the exponential growth phase, and then cells were collected and washed with distilled water twice for multi-omics analysis. Total RNA was extracted from yeast cells through the HiPure Yeast RNA Kit (Magen Biotechnology, China). RNA sequencing and transcriptomic analysis were performed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd, China. For proteomic analysis, frozen samples were ground in liquid nitrogen, tagged with TMT (Tandem Mass Tags) label, and subsequently analyzed by LC-MS/MS. In addition, phosphopeptides were enriched from samples using titanium dioxide beads (TiO_2) before being analyzed by LC-MS/MS in phosphoproteomic analysis. Both proteomic and phosphoproteomic analyses were performed by Shanghai Lu-Ming Biotech Co., Ltd, China. Cells from two replicates of SPSC01 or PLY01 under acetic acid stress were detected in the transcriptome. For proteome and phosphoproteome analyses, three biological replicates of SPSC01 or PLY01 were used for analysis.

The raw transcriptomic data have been deposited to NCBI Sequence Read Archive (SRA) with the dataset identifier SUB10330244. Mass spectrometry proteomic data and phosphoproteomic data are available from the PRIDE database under accession numbers PXD028214 and PXD028217.

2.4. Growth evaluation and ethanol fermentation

The growth of engineered yeast strains was evaluated in shake flasks and by Bioscreen C (Bioscreen, Finland). The strains were firstly cultivated overnight and transferred into fresh YPD medium. When seeds

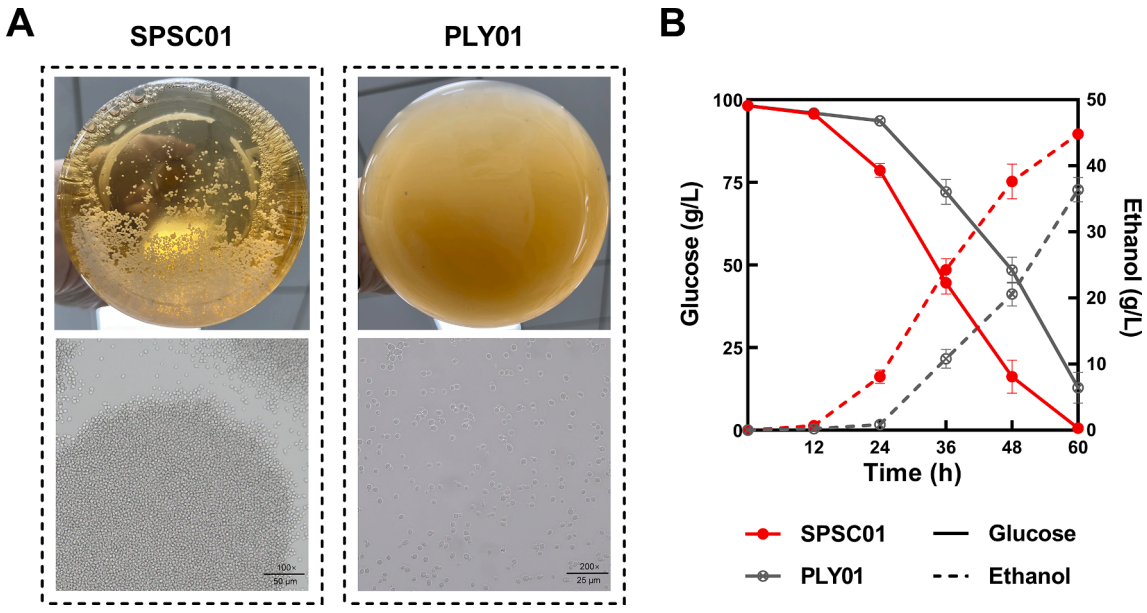

Fig. 1. Improved acetic acid tolerance by yeast cell flocculation. A, Observation of flocculating strain SPSC01 and non-flocculating strain PLY01 (SPSC01-ΔFLO1). Upper panel, photograph of the shake flask culture; lower panel, images under optical microscope. B, Ethanol fermentation of the two strains in the presence of 5.0 g/L acetic acid. Data are averages from at least three replicates, and error bars represent SD.

grew to the exponential phase, cells were collected by centrifuging at 3,000 g for 3 min, washed with sterile distilled water, and inoculated in fermentation medium with an initial OD_{600} of 0.1 in a microtiter plate. In Bioscreen C, cell growth (OD_{600}) of the yeast strains was measured and recorded every 0.5 h. The culture conditions of strains were controlled at 30 °C and medium speed shaking. For the stress tolerance test, 4 g/L furfural or 10 mM H_2O_2 was added to the culture medium.

Batch fermentation of yeast strains was performed in flasks and bioreactors. Ethanol fermentation properties were firstly evaluated in 250 mL flasks containing 100 mL medium. The fermentation was performed at 30 °C, 150 rpm without pH adjustment. The inoculation method was consistent with previously described. For flocculating strain SPSC01, cells were deflocculated before inoculation following the procedure of: 1) centrifuged at 3,000 g for 3 min to remove the medium; 2) washed twice with sterile 0.1 M sodium citrate buffer (pH 4.5); 3) resuspended in 0.1 M sodium citrate buffer (pH 4.5). Inhibitors (5.0/7.5 acetic acid, 0.8 M NaCl, or 15 mM H_2O_2) were supplemented into the fermentation medium to evaluate the growth and ethanol production performance of strains under stress. Fermentation medium with simulated corn stover hydrolysate inhibitor mixture (containing 4.33 g/L acetic acid, 0.34 g/L formic acid, 0.53 g/L furfural, and 0.36 g/L 5-HMF) was also used for fermentation performance assessment (Zhang et al., 2015). These inhibitors were present in the medium throughout the cultivations. When performing experiments in bioreactors, 1.2 L fermenters (16-Unit Parallel Bioreactor, T&J Bio-engineering, China) were used, containing 500 mL fermentation broth, operating at 30 °C, 150 rpm, 0.08 vvm, without pH adjustment. Corncob hydrolysate, containing 199.6 g/L glucose, 19.2 g/L xylose, 1.5 g/L acetic acid, and other unknown components, was directly served as the medium in bioreactors without pH adjustment. Corn steep liquor was supplemented into the hydrolysate at 20 g/L for supplying sufficient nitrogen source.

2.5. Analysis of fermentation performance

Samples of culture broth were collected within an interval of 12 h until glucose was consumed completely. The concentrations of glucose, ethanol, acetic acid, glycerol, and other inhibitors in fermentation broth or hydrolysate were detected via high-performance liquid chromatography (HPLC) system (Waters Alliance e2695 HPLC, Waters, USA) with a Bio-Rad Aminex® HPX-87H column. The elution condition was controlled at 50 °C, 4 mM sulfuric acid, and 0.6 mL/min flow rate.

2.6. Detection of antioxidant capacity

Multiple parameters of antioxidant capacity of *S. cerevisiae* strains, including reactive oxygen species (ROS), glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD), and glutathione, were measured by corresponding assay kits (Beyotime, China) according to the manufacturer's instructions, respectively. Protein assay was determined with a BCA Protein Quantification Kit (Vazyme, China).

2.7. Yeast two-hybrid interaction assays

Haploid yeast strain AH109 (Clontech, Japan) was used for yeast two-hybrid interaction assays. Target gene sequences were fused into initial plasmids pGADT7 or pGBKT7 (Clontech, Japan) through ClonExpress® Ultra One Step Cloning Kit (Vazyme, China). The primers for plasmids construction and amplification of target gene sequences are listed (see supplementary material). For the construction of yeast two-hybrid strains, bait and prey plasmids were simultaneously introduced into parent strain AH109 using the lithium acetate method mentioned above and selected on SD medium lacking leucine and tryptophan (SD-LT). AH-AMcm2-BMcm10 and AH-A-B were used for positive control and negative control, respectively. Strains were activated in fresh SD-LT and spotted on solid SD-LT plates. Plates were incubated at 30 °C for 3–5 days.

2.8. Statistical analysis

All experiments were independently performed at least three times. The results were expressed as mean and standard deviation (SD). Statistical analysis was performed using Student's *t*-test with a significant level of p-value < 0.05.

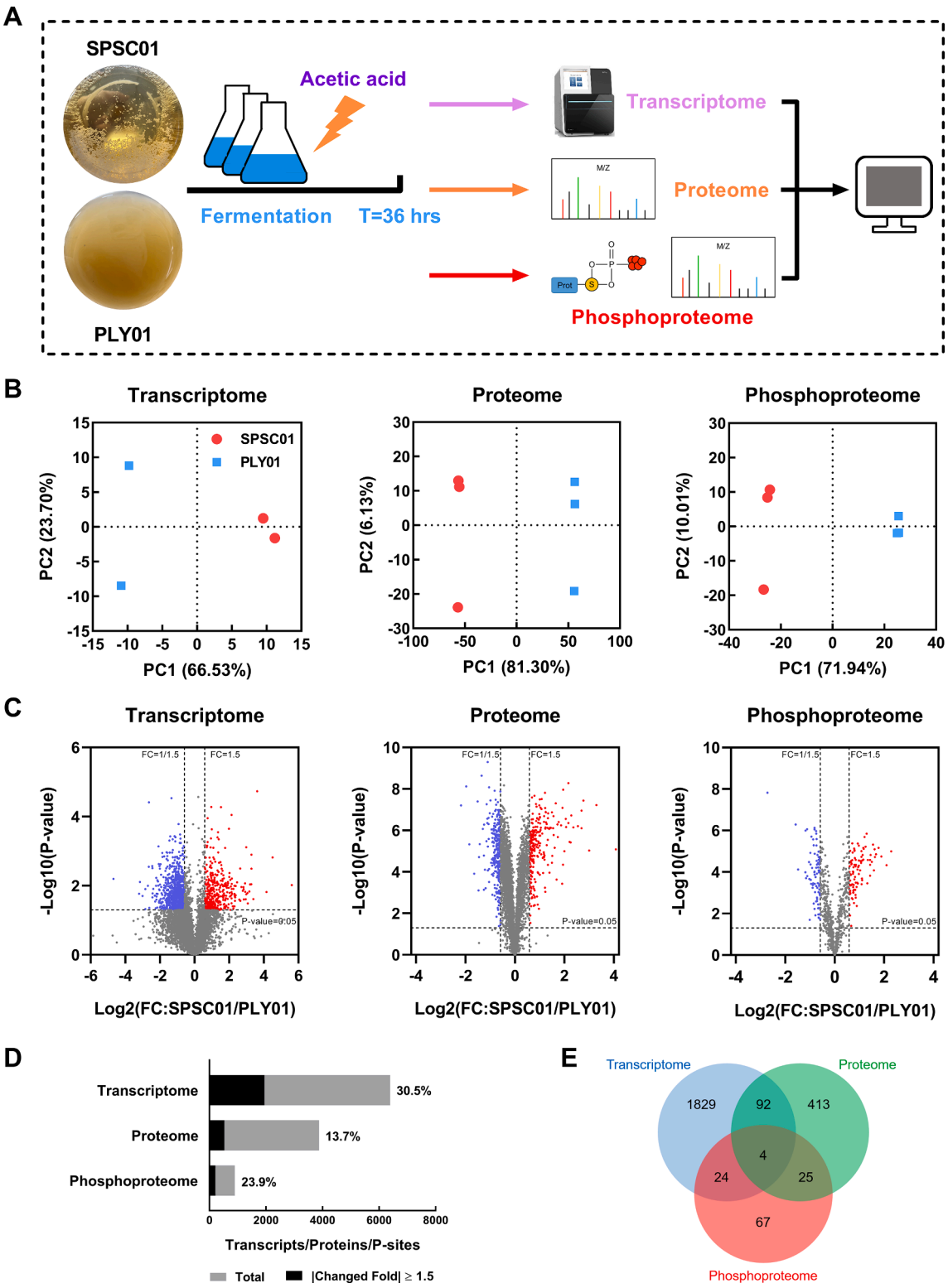

Fig. 2. Differential global gene transcription, protein expression, and protein phosphorylation in SPSC01 and PLY01, revealed by corresponding omics analyses under acetic acid stress. A, Scheme for the experimental design. B, PCA analysis of the transcriptomic, proteomic, and phosphoproteomic data. The first principal component (PC1) and second principal component (PC2) are shown. Three plots represent PCA analysis of transcriptomic, proteomic, and phosphoproteomic data, respectively. Each dot represents an individual sample. C, Volcano plot of quantitative transcriptomic, proteomics, and phosphoproteomics data. The transcripts/proteins/P-sites with |changed fold| (SPSC01/PLY01) $\geq$ 1.5 (p-value $<$ 0.05) were selected as significant change. Three plots represent volcano plot of transcriptomic, proteomic, and phosphoproteomic data, respectively. Red dots represent significant up-regulation; Blue dots represent significant down-regulation. D, Change ratio of genes, proteins, and P-sites in corresponding omics data. Black bars represent the number of transcripts/proteins/P-sites with |changed fold| (SPSC01/PLY01) $\geq$ 1.5 (p-value $<$ 0.05), whose ratio to total is shown on the right side of the bar. E, Venn diagram shows unique and shared transcripts/proteins/P-sites significantly regulated (p-value $<$ 0.05) in the corresponding omics data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

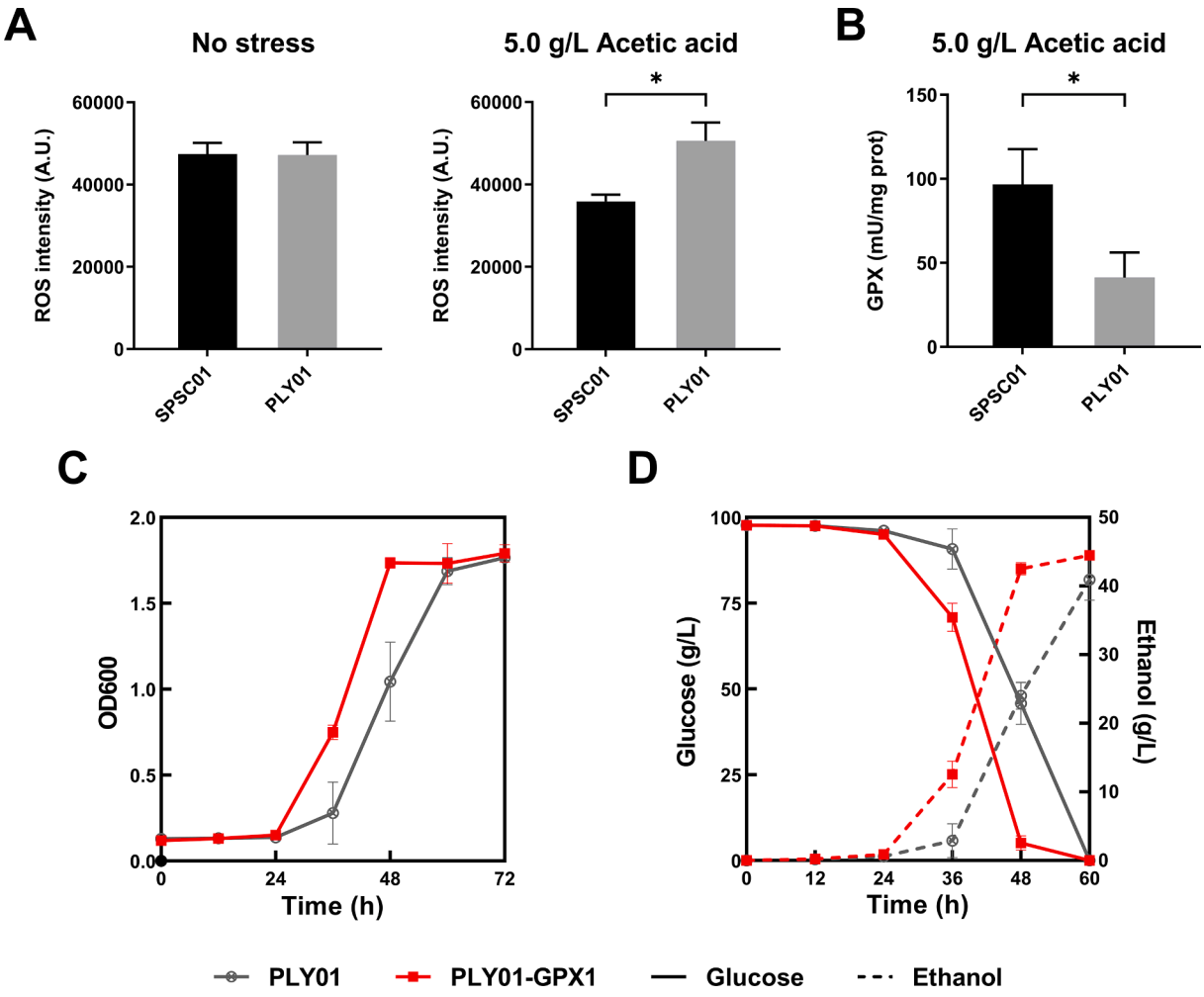

Fig. 3. Improved antioxidant capacity endows cells with higher resistance to acetic acid. A, ROS accumulation in SPSC01 and PLY01 with or without 5.0 g/L acetic acid stress during ethanol fermentation. B, GPX activity of SPSC01 and PLY01 in the presence of 5.0 g/L acetic acid stress. C and D, growth and ethanol fermentation performance of PLY01 and PLY01-GPX1 in the presence of 5.0 g/L acetic acid stress. "*" represents p-value < 0.05. Data are averages from at least three replicates, and error bars represent SD.

3. Results and discussion

3.1. Flocculation improved acetic acid tolerance

Previous studies revealed that cell flocculation endowed yeast cells with improved acetic acid stress tolerance (Cheng et al., 2017). The phenotype of the non-flocculating mutant PLY01 (SPSC01-ΔFLO1) was compared with that of the parent strain SPSC01 (Fig. 1A). The fermentation performance with strains SPSC01 and PLY01 under 5.0 g/L acetic acid stress was tested. Non-flocculating strain PLY01 showed slower glucose consumption and ethanol productivity than flocculating strain SPSC01 (Fig. 1B). These results are different from the previous studies in that flocculation did not affect tolerance to carboxylic acids (Westman et al., 2014), possibly due to different culture conditions and strain genetic background. Besides, oxygen-limited condition was used here, different from the anerobic condition in the previous study (Westman et al., 2014).

3.2. Multi-omics analysis revealed tremendous intracellular physiological changes

In the previous studies, global gene transcription of the flocculating and the non-flocculating yeast cells was analyzed using Affymetrix S98 chips under stress-free condition (Smukalla et al., 2008). Transcription analysis was performed under acetic acid stress here, and multiple protein kinases-encoding genes were found to be enriched in the differential transcripts, which were not reported previously (Smukalla et al., 2008). The results strongly indicate the involvement of protein kinase-mediated regulatory network affected by yeast flocculation for regulation of stress resistance. The transcriptomic results prompted a deeper investigation of proteomic and phosphoproteomic changes to explore key protein kinases that may be responsible for improved stress tolerance by cell flocculation (Fig. 2A).

Principal component analysis (PCA) of transcriptomic, proteomic, and phosphoproteomic data was performed to confirm the similarities of biological replicates and the distinctions between SPSC01 and PLY01 (Fig. 2B). The PCA analysis revealed that flocculation caused tremendous differences in genes expression, protein abundance, and protein phosphorylation. Specifically, flocculation led to great changes of 1949 genes, 532 proteins, and 214 phosphosites with the |changed fold| (SPSC01/PLY01) ≥ 1.5 (p-value < 0.05) (Fig. 2C and D). More altered genes were found in transcription (30.5%), in contrast to those in protein expression (13.7%) and P-sites in phosphorylation (23.9%). These results suggest that yeast flocculation has a great impact on not only gene transcription but also protein expression and protein phosphorylation. Meanwhile, the three omics data shared marginal common changes (Fig. 2E), suggesting the importance of exploring in-depth mechanisms using data obtained by integrated multi-omics analyses.

Table 1
Ethanol production performance of SPSC01, PLY01, and gene-overexpression strains under various conditions.

Parameter	Fermentation medium with 5.0 g/L acetic acid in the flask									
	SPSC01	PLY01	PLY01-AKL1	PLY01-ATG1	PLY01-CMK2	PLY01-GPX1	PLY01-HAL5	PLY01-HOG1	PLY01-RIM15	PLY01-RTK1
t (h)	60	60	60	60	60	48	60	48	60	60
E_p (g/L)	44.8	40.9	44.0	42.6	43.7	44.5	39.4	43.8	42.7	43.1
X (g(DCW)/L)	–	3.04	3.48	2.99	3.04	3.42	3.05	3.70	3.15	3.14
μ (h^{-1})	–	0.0504	0.0557	0.0570	0.0574	0.0575	0.0530	0.0600	0.0572	0.0552
q (g/L/h)	0.784	0.527	0.763	0.636	0.695	0.885	0.554	0.913	0.842	0.786
$Y_{E/S}$ (g/g)	0.456	0.425	0.458	0.443	0.454	0.455	0.409	0.444	0.444	0.448
Y (%)	89.3	83.2	89.6	86.7	88.8	89.0	80.1	87.0	86.9	87.6
	Fermentation medium with hydrolysate-simulated inhibitor mixture in the flask									
t (h)	96	96	72	96	96	96	96	84	96	84
E_p (g/L)	44.8	43.7	43.3	44.1	44.1	43.7	43.8	43.1	43.2	44.6
X (g(DCW)/L)	–	3.47	3.98	3.71	3.85	3.68	4.03	3.99	3.87	3.28
μ (h^{-1})	–	0.0275	0.0385	0.0323	0.0310	0.0314	0.0334	0.0334	0.0328	0.0321
q (g/L/h)	0.516	0.297	0.573	0.464	0.378	0.412	0.452	0.513	0.446	0.531
$Y_{E/S}$ (g/g)	0.449	0.437	0.434	0.442	0.442	0.438	0.439	0.432	0.433	0.447
Y (%)	87.9	85.6	84.9	86.4	86.4	85.8	85.9	84.5	84.7	87.4
	Corncob hydrolysate in bioreactor									
t (h)	–	96	84	–	–	–	–	96	–	–
E_p (g/L)	–	89.6	89.6	–	–	–	–	89.2	–	–
X (g(DCW)/L)	–	3.94	4.14	–	–	–	–	4.10	–	–
μ (h^{-1})	–	0.0244	0.0293	–	–	–	–	0.0282	–	–
q (g/L/h)	–	0.962	1.066	–	–	–	–	1.016	–	–
$Y_{E/S}$ (g/g)	–	0.459	0.459	–	–	–	–	0.457	–	–
Y (%)	–	89.8	89.8	–	–	–	–	89.4	–	–

t, fermentation time; E_P, ethanol produced; X, cell density; μ, specific growth rate; q, ethanol productivity; $Y_{E/S}$, ethanol yield, g (ethanol)/g (glucose); Y, ethanol yield based on the theoretical value of 0.511 g (ethanol)/g (glucose). On account of the uneven distribution of flocs in flask medium, the X (cell density) of SPSC01 was not recorded. For fermentation in corncob hydrolysate, only PLY01, PLY01-HOG1, and PLY01-AKL1 were tested. "PLY01-AKL1", "PLY01-ATG1", …, and "PLY01-RTK1" represent corresponding gene overexpression strains, respectively. Data are averages from at least three replicates.

The differentially phosphorylated sites were specifically checked in the phosphoproteome. Multiple changed protein kinases associated with intracellular signal transduction were found. Particularly, the phosphorylation level of stress-related proteins (including Akl1, Gcn2, Gpd1, Hal5, Hsp30, Pan1, Mrh1, Ptk2, Tpo4, etc.) changed significantly. Other phosphorylation events with unknown functions were also discovered. Compared to the previous report on membrane phosphoproteomic (Guerreiro et al., 2017), no overlapping phosphosites of proteins were identified in the current study, maybe due to the difference between short-term exposure and long-term adaptation to acetic acid stress.

3.3. Various strategies defended cells from acetic acid stress, and the antioxidant capacity played an important role

To better understand the great differences between SPSC01 and PLY01 in the presence of acetic acid, functional annotation analysis of changed genes, proteins, and phosphosites were performed to identify enriched Gene Ontology (GO) categories. Among all the categories, the glycolytic process (GO:0006096), which was reported to be strongly repressed under acetic acid stress in the previous study (Dong et al., 2017), was significantly improved by cell flocculation in transcriptome and proteome. Specifically, the abundance of Hxk1, Tdh1, Pgk1, and Eno1 was 2.71, 5.13, 1.87, and 3.21-fold higher, respectively, in the flocculating yeast SPSC01 when compared to that of PLY01. In addition, the steroid metabolic process (GO:0008202) was also enriched. In the previous study, ergosterol biosynthesis was significantly up-regulated with zinc sulfate addition under acetic acid stress, which is associated with improved growth and fermentation (Zhang et al., 2017). The results in this study suggest that flocculation may also exert function in regulating steroid metabolism under acetic acid stress.

In addition, antioxidant capacity-related processes, consisting of oxidation–reduction process (GO:0055114), oxidoreductase activity (GO:0016491), cellular response to oxidative stress (GO:0034599), and cell redox homeostasis (GO:0045454), were enriched in both transcriptome and proteome. In coniferyl aldehyde-resistant and iron-resistant *S. cerevisiae* strains, similar antioxidant capacity-related GO categories were also reported to be enriched, indicating that these GO categories could respond to multiple stresses (Balaban et al., 2020; Hacısalihoğlu et al., 2019). Acetic acid causes oxidative stress and ROS production in 15 min (Giannattasio et al., 2013; Guaragnella et al., 2007). It has been proved that decreased ROS accumulation led to enhanced resistance to acetic acid through metabolic engineering, including expressing endogenous lycopene pathway or overexpressing *RCK1*, *PMA1*, or *OLE1* (Lee et al., 2017; Nasution et al., 2017; Oh et al., 2019; Pan et al., 2018). Generally, improved CAT activity was the reason for decreased ROS accumulation in acetic acid-tolerant strain. Therefore, the antioxidant capacity of SPSC01 and PLY01 was evaluated W/O acetic acid stress (Fig. 3A). SPSC01 exhibited significantly less ROS level than PLY01 in the presence of 5.0 g/L acetic acid, which declared enhanced antioxidant capacity in flocculating strain and was consistent with the data in omics analysis. However, SPSC01 showed decreased CAT activity no matter acetic acid stress is present or not (data not shown). The expression of *CTT1* in transcriptome and the abundance of Ctt1 in proteome were analyzed, which was the major catalase in *Saccharomyces cerevisiae*. Even though the expression of *CTT1* was up-regulated in SPSC01 compared to PLY01 (4.30-fold), the abundance of this protein was reduced (0.67-fold). The results suggest that there might be other mechanisms to regulate translation, stabilization, and degradation of catalase Ctt1 when other antioxidant pathways can offset its function. The activity of SOD and GPX, the level of glutathione, and the ratio of GSH/GSSG were further detected. However, the similar results of glutathione and GSH/GSSG did not reveal the reason for the efficient scavenging of ROS in SPSC01. Strangely, no activity of SOD was found in several repeated experiments. In contrast, improved activity of GPX (2.34-fold) was discovered in flocculating strain SPSC01 (Fig. 3B), accompanied by an increase of *GPX1* mRNA (2.92-fold) under acetic acid stress. Similarly, improved transcriptional level of *GPX1* was also found in silver-resistant and nickel-resistant *S. cerevisiae* strain, indicating the important role of *GPX1* in response to stresses (Küçükgöze et al., 2013; Terzioğlu et al., 2020). The *GPX1*-overexpressing strain PLY01-GPX1 was subsequently constructed to verify the function of Gpx1 in resisting acetic acid stress through replacing the native *GPX1* promoter into constitutive strong promoter *TEF1p*. Strain PLY01-GPX1 showed a higher specific growth rate (14.1% up) and ethanol

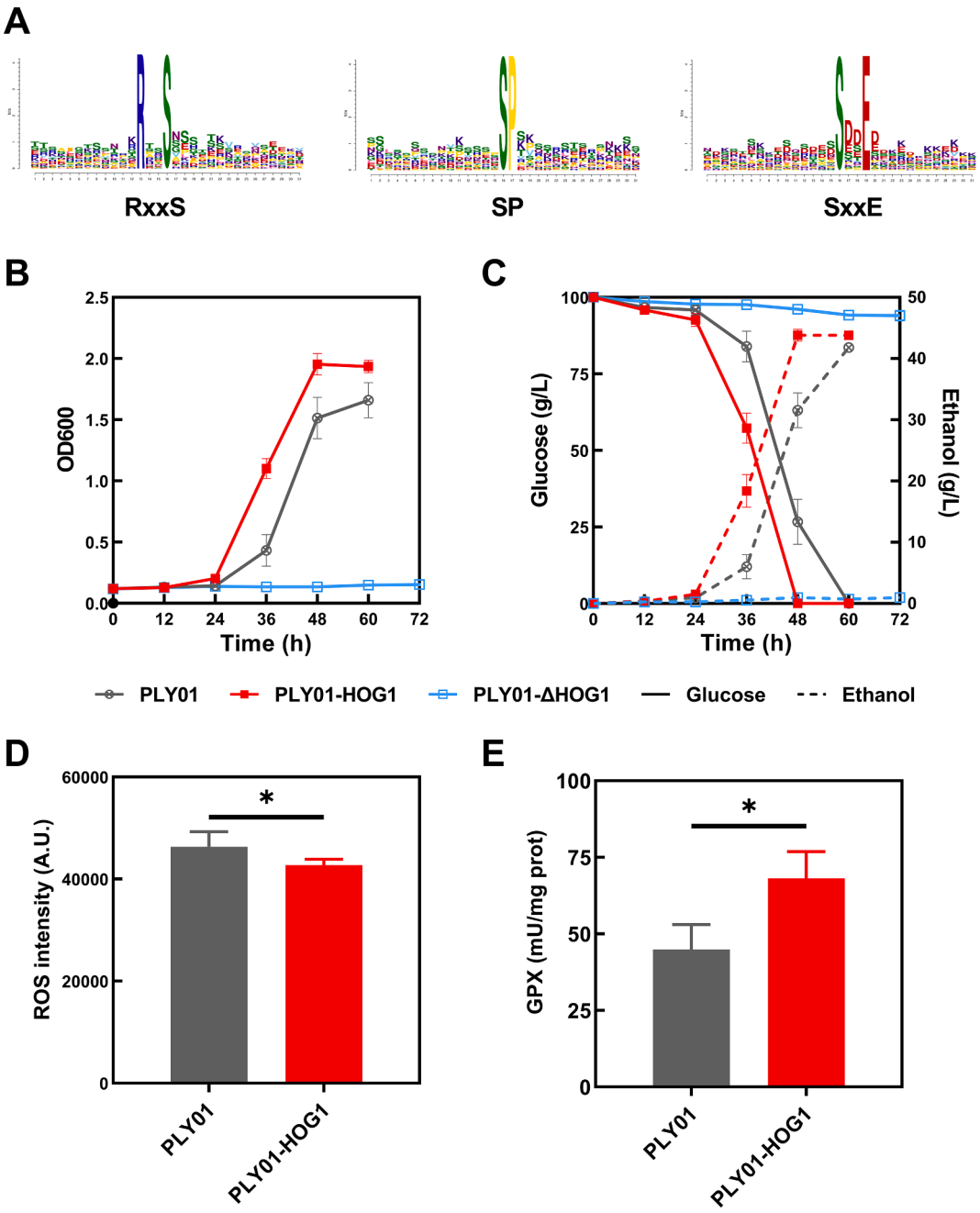

Fig. 4. Hog1 is the key regulator in response to acetic acid. A, Enriched phosphorylated residues motifs in phosphoproteomic data obtained by comparing of SPSC01 and its non-flocculating mutant PLY01, detected via MOMO (https://meme-suite.org/meme/tools/momo). B and C, growth and fermentation performance of parent strain PLY01, *HOG1* overexpression strain PLY01-HOG1, and *HOG1* deletion strain PLY01-ΔHOG1 in the presence of 5.0 g/L acetic acid stress. D and E, ROS accumulation and GPX activity were tested in PLY01 and PLY01-HOG1. "*" represents p-value < 0.05. Data are averages from at least three replicates, and error bars represent SD.

productivity (67.9% up) in the presence of 5.0 g/L acetic acid compared to its parent strain PLY01 (Table 1, Fig. 3C and D). These results demonstrate that enhanced antioxidant capacity plays an important role in defending yeast cells from acetic acid stress. Furthermore, it is the first time to discover that improved activity of GPX contributed to yeast acetic acid stress resistance and efficient ethanol production.

3.4. *Hog1 acted as a master regulator and improved the activity of GPX*

Stress-activated protein kinases (SAPKs) play a crucial role in response to stress (de Nadal & Posas, 2015). Besides GO analysis of phosphoproteome, enriched P-site motifs were identified, which helped dig out preferred phosphosites and the core protein kinases in the signaling network. The most significantly enriched P-site motifs, including RxxS, SP, and SxxE, are shown in Fig. 4A. In a previous study, RxxS and SP (S/T-P) motifs, which were Hog1-specific, were enriched in response to hyperosmotic stress (Romanov et al., 2017). SxxE motif was preferred by casein kinase 2, which was associated with cell growth and proliferation (Maiti et al., 2003). In the previous studies, Hog1 was found to respond to acetic acid stress through directly phosphorylating the plasma membrane aquaglyceroporin Fps1, mediating *CTT1* expression, and resisting AA-PCD (de Nadal & Posas, 2010; Guaragnella et al., 2019; Mollapour & Piper Peter, 2007). Furthermore, numerous changed mRNA, proteins, and phosphosites in omics data have been found to be

Table 2
Differential expression and phosphorylation of the selected protein kinases.

Protein kinases	Description	Changed fold in the proteome	Changed fold in the phosphoproteome
Akl1	Ser/Thr protein kinase, regulates endocytosis and actin cytoskeleton organization.	1.36	1.07, 1.11, 1.41, 1.60, 1.67, 1.69, 1.72, 1.84
Atg1	Ser/Thr kinase, required for vesicle formation in autophagy and the cytoplasm-to-vacuole targeting (Cvt) pathway.	1.73	0.97, 0.93, 0.93
Cmk2	Calmodulin-dependent protein kinase, negatively controls the calcium/ calcineurin signaling pathway.	1.58	2.53
Hal5	Nutrient-responsive protein kinase, whose overexpression increases sodium and lithium tolerance, whereas gene disruption increases cation and low pH sensitivity and impairs potassium uptake.	1.23	1.80
Rim15	Protein kinase that is involved in cell proliferation in response to nutrients.	1.29	–
Rtk1	Putative protein kinase, abundance increases in response to DNA replication stress.	1.28	1.96, 2.12

Notes: Multiple numbers in "Changed fold in the phosphoproteome" represent the phosphorylation level of various sites in the same protein. "-" represents not detected in the phosphoproteome.

regulated by the HOG pathway. The abundance of Hog1 was also 1.29-fold up-regulated in the proteome. It implies that Hog1 may play a central role in flocculation-associated improved stress tolerance.

To further confirm the assumption, the *HOG1* overexpression strain and the *HOG1* deletion strain from PLY01 were constructed, respectively, which were termed PLY01-HOG1 and PLY01-ΔHOG1. Consistent with the previous studies, deletion of gene *HOG1* led to significantly reduced tolerance to acetic acid stress and little growth in 72 h (Gutmann et al., 2021; Mollapour & Piper Peter, 2007; Mollapour & Piper, 2006) (Fig. 4B and C). Notably, overexpression of *HOG1* exhibited better fermentation performance (ethanol productivity, 0.913 g/L/h) compared to PLY01 (ethanol productivity, 0.527 g/L/h) under acetic acid stress (Table 1, Fig. 4B and C). To the best of authors' knowledge, this is the first time to report that overexpression of *HOG1* improves acetic acid tolerance in *S. cerevisiae*. Also, the ROS accumulation and activity of GPX were tested in PLY01 and PLY01-HOG1, except for PLY01-ΔHOG1 that barely grew. Overexpression of *HOG1* induced significantly higher activity of GPX and efficient scavenging of ROS (Fig. 4D and E). Hog1 was reported to be dispensable for the expression of *GPX1* under Ca^{2+}-induced osmotic stress (Ohdate et al., 2010). The findings in this study provide new evidence that Hog1 is involved in the regulation of Gpx1 when yeast cells are exposed to acetic acid.

In addition, fermentation experiments under 0.8 M NaCl or 15 mM H_2O_2 were performed using the *HOG1* mutant strains and the control strain. Interestingly, PLY01-HOG1 showed the best performance under 15 mM H_2O_2, suggesting that overexpression of *HOG1* did increase the intracellular antioxidant capacity in *S. cerevisiae*. However, the growth and ethanol production of PLY01 and PLY01-HOG1 were similar under 0.8 M NaCl stress, despite the strong repression in PLY01-ΔHOG1.

3.5. Other protein kinases are involved in acetic acid stress

It was reported that Hog1 phosphorylates plasma membrane aquaglyceroporin Fps1 and facilitates degradation of Fps1 to repress the entry of undissociated acetic acid (Mollapour & Piper Peter, 2007). However, the abundance of Fps1 in SPSC01 was almost the same as it in PLY01 (0.96-fold), despite a slightly higher phosphorylation level (1.25-fold). These results cannot explain the reason for obviously improved tolerance to acetic acid in SPSC01. Therefore, Hog1 is supposed to acts as a master regulator and control other kinases in the signaling network to combat acetic acid stress. Six possibly related key protein kinases were selected for further study, whose abundance or phosphorylation level has changed significantly (Table 2). Corresponding overexpression strains were constructed, consisting of PLY01-AKL1, PLY01-ATG1, PLY01-CMK2, PLY01-HAL5, PLY01-RIM15, and PLY01-RTK1. Subsequently, the fermentation performance of these strains under 5.0 g/L acetic acid stress was evaluated. Overexpressing key protein kinase genes in PLY01 enhanced acetic acid tolerance and ethanol productivity, except for *HAL5* (Table 1, Fig. 5A and B). Among them, the best performance was observed in PLY01-RIM15 (59.8% higher ethanol productivity compared to PLY01). According to the previous work, Rim15 was one of the putative substrates of Hog1 (Romanov et al., 2017). It

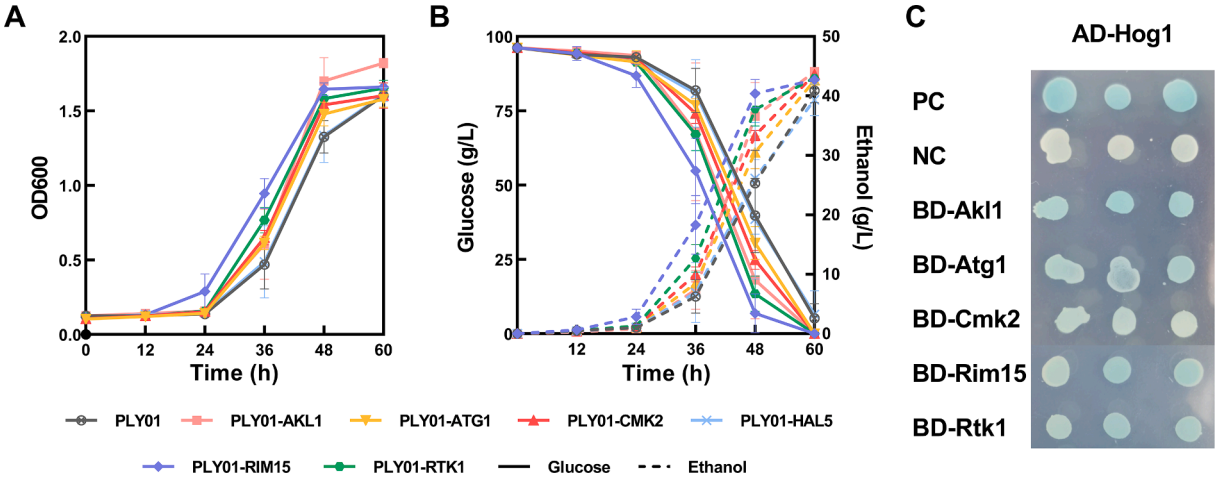

Fig. 5. Multiple protein kinases interact with Hog1 and contribute to acetic acid resistance. A and B, cell growth and ethanol fermentation performance using the recombinant yeast strains overexpressing six selected protein kinase genes and the control strain PLY01 in the presence of 5.0 g/L acetic acid. C, Hog1 was verified to interact with selected protein kinases *in vivo* through yeast two-hybrid system. PC, positive control. NC, negative control. AD, *GAL4* activation domain. BD, *GAL4* DNA binding domain. AD and BD were fused to Hog1 or target protein kinases, respectively.

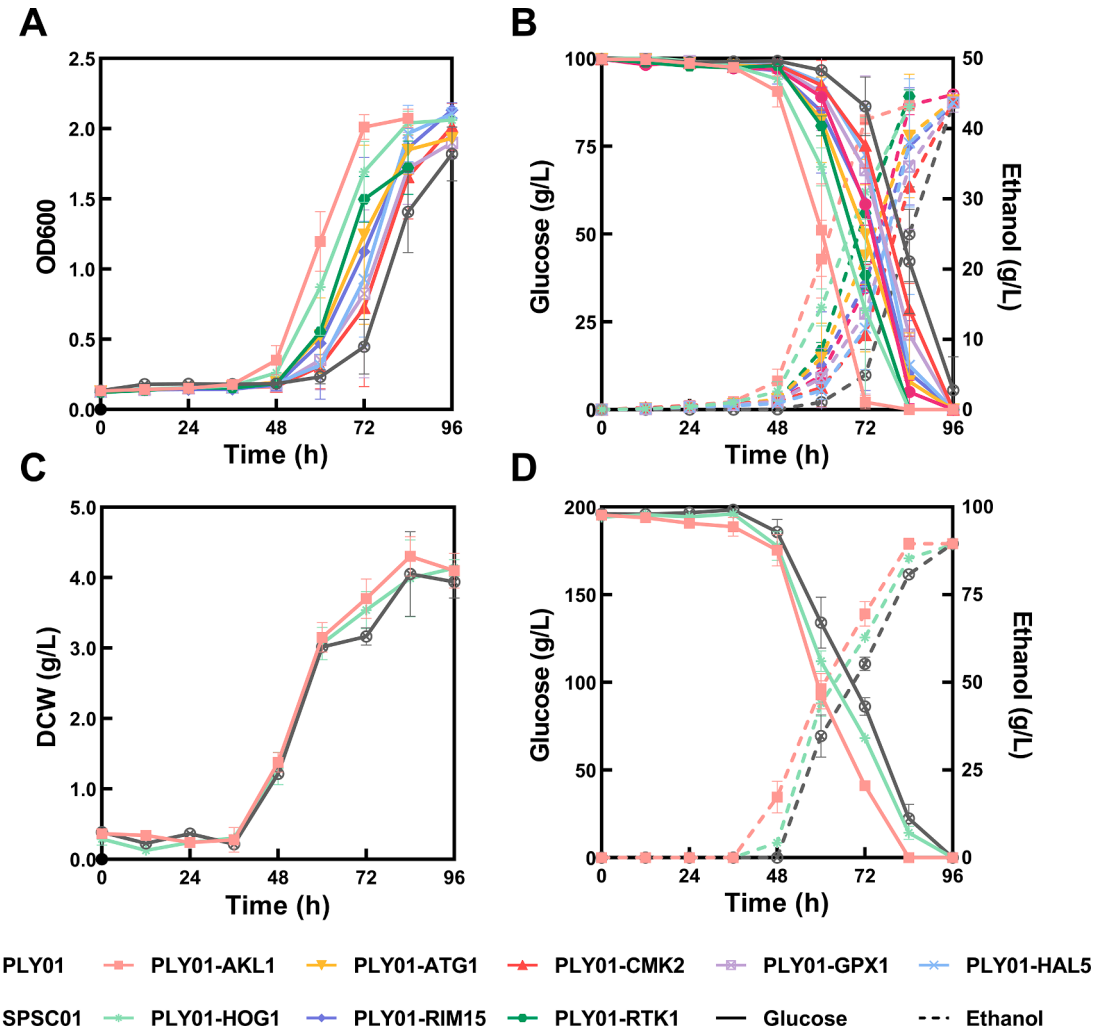

Fig. 6. Overexpression of key protein kinases and antioxidant stress-related genes improves cellulosic ethanol fermentation. A and B, growth and ethanol fermentation of the gene-overexpression strains in fermentation medium with simulated corn-stover hydrolysate inhibitor mixture. C and D, growth and ethanol fermentation of PLY01, PLY01-HOG1, and PLY01-AKL1 using corncob hydrolysate.

suggests that Hog1 may directly activate Rim15 and other protein kinases for further regulation of cellular function under stress. Yeast two-hybrid interaction assays were performed using different kinases, and the results confirmed the interactions between Hog1 and the selected protein kinases (Fig. 5C). These findings indicate that Hog1 might act as a master regulator of the signaling network to facilitate other protein kinases and work together with them to defend yeast cells from acetic acid stress.

The tolerance of the overexpression strains of *AKL1*, *ATG1*, *CMK2*, *HAL5*, *RIM15*, and *RTK1* to other stresses, including 1.0 M NaCl, 4.0 g/L furfural, and 10 mM H_2O_2, was tested. Overexpression of the protein kinase genes led to discrepant growth under various stresses (see supplementary material). It was worth noting that overexpression of *RIM15* significantly increased ethanol yield by 6.4% in the presence of 1.0 M NaCl through reduced production of glycerol, which benefited the production of biofuels using lignocellulosic biomass. Except for PLY01-ATG1, other overexpression strains showed better growth in the presence of 10 mM H_2O_2, indicating the importance of these protein kinases in the resistance to oxidative stress. However, for furfural, the improvement was not obvious.

3.6. Improved fermentation efficiency by overexpression of the key protein kinase genes using lignocellulosic hydrolysate

To confirm that whether improved multiple stresses tolerance by protein kinases was beneficial for high-efficiency biorefinery, the fermentation of these overexpression strains in the medium with corn stover hydrolysate-simulated inhibitor mixture was assessed, consisting of different concentrations of acetic acid, formic acid, furfural, and 5-HMF (Zhang et al., 2015). Flocculating strain SPSC01 and strains overexpressing seven protein kinase genes or one antioxidant gene *GPX1* showed better fermentation performance compared to PLY01 (Fig. 6A and B). Notably, the specific growth rate and ethanol productivity of PLY01-AKL1 increased by 40.0% and 92.9% respectively, which reduced the fermentation time by 24 h, and greatly improved the production efficiency (Table 1). Akl1 is a downstream kinase of TORC2-Ypk1 signaling and phosphorylates Pan1 to negatively regulate endocytosis in response to stress (Bourgoint et al., 2018; Roelants Françoise et al., 2017). The phosphorylation level of Pan1 was up-regulated in phosphoproteome (1.59-fold), which is also phosphorylated by Hog1 under osmotic stress (Reiter et al., 2012). Based on the result in Fig. 5C, besides direct phosphorylation of Pan1 by Hog1, Hog1 might phosphorylate Akl1, and then Pan1 was activated by Akl1. Through these two pathways, Hog1 regulated the phosphorylation level of Pan1 in response to various stresses. Furthermore, PLY01-HOG1 and PLY01-

RTK1 also reduced the fermentation time by 12 h. The tolerance of *HOG1* overexpressing strain to multiple stresses in mimetic corn stover hydrolysate was foreseeable since Hog1 played a crucial role in flocculation-associated stress tolerance. Rtk1 is a putative protein kinase and is limitedly studied. It might interact with casein kinase 1/2, which is associated with Hog1 (Breitkreutz et al., 2010). In this study, Rtk1 was confirmed to interact with Hog1 (Fig. 5C), suggesting that Rtk1 might be directly phosphorylated by Hog1.

Based on the excellent fermentation performance in the medium with inhibitor mixture, PLY01-AKL1 and PLY01-HOG1 were selected for fermentation assessment using corncob hydrolysate, with PLY01 as the control. PLY01-AKL1 exhibited the highest ethanol productivity (10.8% higher than the control strain) and reduced the fermentation time by 12 h (Table 1, Fig. 6C and D). In summary, these results indicate that overexpression of *AKL1* is a viable strategy for developing robust industrial yeast strains for cellulosic ethanol production.

4. Conclusion

Integrated analysis of multi-omics data revealed changes in expression of multiple protein kinases and their protein phosphorylation status by yeast cell flocculation under acetic acid stress. Overexpression of the protein kinase Hog1 and the detoxifying enzyme gene *GPX1* improved stress tolerance. Subsequently, overexpressing genes of key protein kinases that interacted with Hog1 enhanced acetic acid tolerance and fermentation performance using corncob hydrolysate. Especially, up to 40.0% higher specific growth rate and 92.9% higher ethanol productivity were achieved through overexpressing the protein kinase gene *AKL1*. These results provide alternative strategies to improve cellulosic ethanol production by manipulating key protein kinases.

CRediT authorship contribution statement

Pei-Liang Ye: Investigation, Methodology, Writing – original draft. **Xue-Qing Wang:** Investigation. **Bing Yuan:** Investigation. **Chen-Guang Liu:** Writing – review & editing. **Xin-Qing Zhao:** Supervision, Funding acquisition, Project administration, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.126758.

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