Original Research Article

Overexpression of arginase gene *CAR1* renders yeast *Saccharomyces cerevisiae* acetic acid tolerance

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ABSTRACT

Acetic acid is a common inhibitor present in lignocellulose hydrolysate, which inhibits the ethanol production by yeast strains. Therefore, the cellulosic ethanol industry requires yeast strains that can tolerate acetic acid stress. Here we demonstrate that overexpressing a yeast native arginase-encoding gene, *CAR1*, renders *Saccharomyces cerevisiae* acetic acid tolerance. Specifically, ethanol yield increased by 27.3% in the *CAR1*-overexpressing strain compared to the control strain under 5.0 g/L acetic acid stress. The global intracellular amino acid level and compositions were further analyzed, and we found that *CAR1* overexpression reduced the total amino acid content in response to acetic acid stress. Moreover, the *CAR1* overexpressing strain showed increased ATP level and improved cell membrane integrity. Notably, we demonstrated that the effect of *CAR1* overexpression was independent of the spermidine and proline metabolism, which indicates novel mechanisms for enhancing yeast stress tolerance. Our studies also suggest that *CAR1* is a novel genetic element to be used in synthetic biology of yeast for efficient production of fuel ethanol.

1. Introduction

Increasing greenhouse emissions coupled with rising reliance on fossil energy have compelled policy makers to promote the production of biofuels, such as bioethanol, from lignocellulose biomass using budding yeast *Saccharomyces cerevisiae* [1–3]. However, growth and metabolism repression by inhibitors present in lignocellulose hydrolysate is one of the limiting factors for economic cellulosic ethanol production [4–8]. Acetic acid is a common inhibitor generated at large quantity during the pretreatment of lignocellulose biomass. It is toxic to the yeast cell and inhibit cellulosic ethanol production due to at least the following three reasons: Firstly, the protons dissociated from acetic acid in the cytosol lead to cytoplasmic acidification, thereby inhibiting important metabolic processes [9,10]; secondly, acetic acid can severely inhibit the activities of transporters and metabolic enzymes [9–12]; and thirdly, acetic acid can induce oxidative stress [13]. Due to the fact that

multiple genes are involved in acetic acid tolerance [14], It is necessary to reveal new genes conferring to improved acetic acid tolerance to develop efficient yeast cell factories for bio-conversion of lignocellulosic biomass.

Great efforts have been made to render yeast tolerant to acetic acid by systematic manipulating key genes involved in stress tolerance. For example, enhancement of acetic acid tolerance in *S. cerevisiae* was achieved by the overexpression of transcriptional factors such as Znf1p [15], Ppr1p [13,16] and Haa1p [17], the overexpression of protein kinases such as Hog1 [18] and Kic1p [19], the overexpression of a histone modification enzyme Set5p [13], elevated pression of the eukaryotic translation factor eIF5A [20], as well as the deletion of acetic acid importer Ady2p [21,22]. On the other hand, protective agents or metal ions were known to exert beneficial effects to yeast cells under stress conditions [23,24]. Among the protective amino acids, it was found that the presence of proline improved growth of *S. cerevisiae* in the presence

of acetic acid stress [25–28]. In our previous studies, we demonstrated that the presence of zinc sulfate exerted protection for *S. cerevisiae* towards acetic acid stress [29], and using a zinc-finger-based artificial transcription factor can improve the acetic acid tolerance of *S. cerevisiae* [30]. Furthermore, we also found by integrative transcriptomic and proteomic analysis that elevated expression of arginase (Car1p), which catabolizes arginine to ornithine and urea [31], at both mRNA (by 2.34 folds) and protein (by 1.23 folds) levels associated with the zinc sulfate supplementation during continuous ethanol fermentation. Considering that arginine can be converted to proline, we assume that overexpression of *CAR1* may exert protective effects towards *S. cerevisiae* by improving stress resistance.

In this study, we overexpressed *CAR1* in *S. cerevisiae* and evaluated its effect on acetic acid tolerance. The engineered strain exhibited improved growth and ethanol fermentation performance under acetic acid stress compared to the control strain. We also examined the effects of *CAR1* overexpression on transcription of amino acid biosynthesis genes and the intracellular amino acid content. Our work suggested novel roles and mechanisms of yeast *CAR1* gene, and also provide a viable strategy to enhance cellulosic ethanol production efficiency by overexpressing arginase.

2. Material and methods

2.1. Strains and culture medium

Escherichia coli DH5α was used for plasmid propagation. The laboratory strains *S. cerevisiae* S288c and the industrial strain *S. cerevisiae* 4126 were used for this work. All strains were listed in Table 1.

Luria-Bertani medium (NaCl 10 g/L, yeast extract 5.0 g/L and tryptone 10 g/L) was used for *E. coli* culture, in which 100 μg/mL Ampicilin was supplemented for transformant selection and plasmid propagation. *S. cerevisiae* strains were cultured in the YPD medium composed of glucose 20 g/L, peptone 20 g/L and yeast extract 10 g/L. For solid agar plates, 20 g/L Bacto agar was added into the YPD medium, and G418 was supplemented at 300 μg/mL for screening the transformant of *S. cerevisiae*. Fermentation medium composed of 4 g/L yeast extract, 3 g/L peptone and 100 g/L glucose was used for ethanol fermentation by *S. cerevisae* strains. Acetic acid tolerance was exerted on by adding 5 g/L acetic acid into the YPD agar medium and liquid media, and the initial pH of the media was measured at ~3.5.

2.2. Construction of recombinant yeast strains

All primers used in this study were listed in Table S1. The open reading frame (ORF) of *CAR1* was amplified by polymerase chain reaction (PCR) using the genomic DNA extracted from *S. cerevisiae* S288c

as the template. Then the ORF of *CAR1* was cloned into the pHO vector [32] at the *Xma*I and *Pac*I restriction sites, yielding the *CAR1* overexpression plasmid with the *PGK1* promoter and the *CYC1* terminator. The recombinant plasmid was named pHO-CAR1, and the sequence was confirmed by DNA sequencing at Sangon Biotech, Shanghai, China. Finally, both the empty vector pHO and the *CAR1*-overexpressing vector pHO-CAR1 were linearized by *Not*I and transformed into *S. cerevisiae* 4126 and *S. cerevisiae* S288c by electroporation as previously described [33]. After screening and verification of the gene integration using diagnostic PCR, the recombinant yeast strain with *CAR1* overexpression based on *S. cerevisiae* 4126 was designated as 4126-CAR1, while the strain transformed with the empty vector was named as 4126-HO.

2.3. Yeast culture and ethanol fermentation

To compare the fermentation performance of *S. cerevisiae* strains, batch fermentation was carried out in 250 mL flasks with 100 mL fermentation medium, and the initial optical density at 600 nm (OD_{600}) was adjusted to about 0.2 for inoculation with all experiments. All flasks were sealed with rubber plugs for CO_2 to be released. After inoculation, cells were incubated in a rotatory shaker operating at 30 °C and 150 rpm. To test acetic acid tolerance for yeast strains during the fermentation, 5.0 g/L acetic acid was added into the fermentation medium before inoculation. Fermentation medium without acetic acid addition served as the control. To test its effect on the fermentation, ornithine was added into the fermentation medium at 0.1, 1.0 and 10.0 mM, respectively. All fermentation experiments described above were conducted without pH control.

2.4. Assessment for yeast growth, cell viability and acetic stress tolerance

Yeast growth was determined by detecting OD_{600} for the culture with a spectrophotometer (Multiskan GO, ThermoFisher Scientific, Finland). Yeast cell viability was determined by counting the colony forming units (CFUs), which was normalized to that detected for the control: yeast cells cultured at their growth phase without stressful conditions. Both the sample and its control were diluted serially to same cell densities (OD_{600}) suitable for counting CFU, and CFU for the control was designated as 100%. Acetic acid stress tolerance of yeast cells was evaluated on agar plates by spot assay according to reference [13].

2.5. Analysis for ethanol fermentation

The fermentation broth of 1 mL was centrifuged at 10,000×*g* at room temperature for 2 min to remove yeast cells. The supernatant was further filtrated with 0.22 μm membrane and diluted properly for analysis by high performance liquid chromatography (HPLC) with 20 μL sample uploaded. Glucose, glycerol, acetic acid, ethanol and furfural in the fermentation both were analyzed with high performance liquid chromatography (HPLC, Waters e2695, Waters, MA, USA) equipped with Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA), the Refractive Index Detector (RID, Waters 2414, Waters, MA, USA) and a UV/Vis Detector (Waters 2498, Waters, MA, USA). The operating temperatures for the column and RI were 65 °C and 50 °C, respectively, and 4 mmol/L H_2SO_4 was used as the mobile phase.

2.6. Real-time quantitative PCR analysis

Reverse transcription quantitative PCR (RT-qPCR) was performed to determine the relative expression of mRNAs. *S. cerevisiae* cells cultured in the fermentation medium were grown to their exponential phase, and then harvested by centrifuging at 10,000×*g* for 5 min at 4 °C. Total RNA was purified from the yeast cells using the Spin Column Yeast total RNA Purification Kit (Sangon, Shanghai, China) following the manufacturer's instruction, which was then reversely transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China)

Table 1
Yeast strains used in this study.

Strain	Description	Source
S. cerevisiae S288c	Laboratory yeast strain for research	Donated by Prof. Jens Nielsen at Chalmers University, Sweden
S. cerevisiae 4126	An industrial yeast strain for ethanol fermentation	Donated by Mrs Jana Otrubo at University of Waterloo, Canada
S. cerevisiae 4126-CAR1	*S. cerevisiae* 4126 carrying HO::KanMX-P_{PGK1}-*CAR1*-T_{CYC1}	This study
S. cerevisiae 4126-HO	*S. cerevisiae* 4126 carrying HO::KanMX	This study
S. cerevisiae S288c-CAR1	*S. cerevisiae* S288c carrying HO::KanMX-P_{PGK1}-*CAR1*-T_{ADH1}	This study
S. cerevisiae S288c-HO	*S. cerevisiae* S288c carrying HO::KanMX	This study

for the PCR reaction using iQ™ SYBR® Green Supermix and CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Shanghai, China). Relative gene expression levels were determined by the $2^{-\Delta\Delta Ct}$ method [34] using *ALG9* as the internal control. All the primers for RT-qPCR were listed in Supplementary Table S1.

2.7. Detection of reactive oxygen species accumulation

Yeast cells were collected at the log phase (~OD_{600} 1.0) by centrifugation and washed twice with 0.1 M PBS (pH 7.0), followed by re-suspending in the PBS containing 10 μM 2′, 7′-dichlorofluorescein diacetate (DCFH-DA). The reactive oxygen species (ROS) accumulation in yeast cells was measured and calculated following the methods described in the previous work [29].

2.8. Determination of intracellular ATP concentration

The intracellular ATP concentration was determined via luminescence method using the ATP Assay Kit (Beyotime, Nanjing, Jiangsu, China) following the manufacturers' instructions. Briefly, yeast cells were harvest by centrifugation at 4 °C and $10,000 \times g$ for 2 min. Then cell pellets were washed twice and immediately lysed using the lysis buffer under low temperature provided by ice. After removing cell debris by centrifugation again at 4 °C, crude cell extract was collected and protected with ice for determining intracellular ATP concentration immediately. The luminescence was detected with the Multi-Mode Detector (Enspire 2300, PerkinElmer, USA). The whole process was completed within 1 h, and the final ATP concentration was normalized with the protein concentration of the crude cell extract determined by the BCA protein assay kit (Beyotime, Nanjing, Jiangsu, China).

2.9. Plasma membrane integrity assay

Yeast cell membrane integrity was determined as described previously [35], with minor modifications. Briefly, 1 mL of yeast cells cultured to exponential-phase were harvested, and washed twice with 0.1 M PBS (pH 7.0). Then, the cells suspended in 1 mL PBS were treated with 15 μM propidium iodide (PI) for 20 min in dark. After washing twice with PBS, cell pellets were re-suspended in 1 mL PBS. The PI fluorescence intensity (excitation, 485 nm; emission, 635 nm) of 200 μL cell suspension was measured by a Multi-Mode Detection (Enspire 2300, PerkinElmer, USA). The final fluorescence intensity of each sample was normalized with cell concentration (OD_{600}).

2.10. Determination of intracellular amino acid compositions

The intracellular amino acid compositions in *S. cerevisiae* cells were determined by High Speed Amino Acid Analyzer (AAA) (Hitachi L-8900, Tokyo, Japan). Yeast cells growing at their exponential phase were harvested from fermentation broth by centrifuging at 4 °C, $3000 \times g$ for 5 min. After being washed twice in sterilized distilled water, cell pellets were suspended in 1 mL 0.1 M HCl. Then 500 μL glass beads with diameter of 0.5 mm were added into the suspension, which was vortexed vigorously for 10 min in a metal bath pre-cooled at −20 °C to disrupt the cells. After being centrifuged at $12,000 \times g$ and 4 °C for 5 min, 800 μL supernatant was well mixed with 400 μL 16.7% (w/v) sulfosalicylic acid and incubated at 4 °C for 1 h. The mixture was centrifuged at $15,000 \times g$ and 4 °C for 30 min, and the supernatant was collected and neutralized with 1 M NaOH. The supernatant was filtered by 0.22 μm filter before being analyzed. The final amino acid content was normalized by dry cell weight (DCW). The DCW of each sample was determined with twice washed cells incubated at 45 °C for over 48 h.

2.11. Determination of the concentration of spermidine

Dansyl chloride derivatization based HPLC method was adopt for the detection of polyamines, which was modified from a method described previously [36]. In brief, for derivatization, 0.125 mL of saturated $NaHCO_3$ solution and 0.25 mL of dansyl chloride solution (5 mg/mL in acetone) were added to 0.25 mL of sample (dilute samples whenever needed with minimal medium). The samples for determination of the concentration of spermidine was prepared with the powder dried under vacuum from the crude cell extract was prepared with the same method as that for the determination of intracellular amino acid compositions. For each sample, 500 μL of the crude cell extract solution was dried under vacuum, and then re-suspended and diluted with minimal medium whenever needed. After being incubated at 40 °C for 1 h in dark with occasional shaking, the derivatization reaction was stopped by adding 0.275 mL of methanol into the reaction mixture. Then samples filtered through a 25-mm syringe filter (0.45 μm Nylon) were used for HPLC detection. The following chromatographic condition are used: C18 (100 mm × 4.6 mm; i. d., 2.6 μm; Phenomenex Kinetex), excitation wavelength 340 nm, emission wavelength 515 nm, sample injecting 1.5 μL, column temperature 40 °C, detector sensitivity was set at level 7, acquisition starts at 4.0 min. The mobile phase is water and methanol with the speed of 1 mL/min. The elution program is as follows: 50%–65% methanol (0–5 min), 65%–75% methanol (5–7.5 min), 75%–87.5% methanol (7.5–9.5 min), 87.5%–100% methanol (9.5–10.5 min), 100% methanol (10.5–11.5 min), 100%–50% methanol (11.5–13.5 min), 50% methanol (13.5–16 min).

2.12. Statistical analysis

All experiments were performed in triplicates. The results of RT-qPCR, antioxidant enzyme activities, ATP content, and amino acid compositions were expressed as mean values and standard deviations. Statistical analysis was performed using the *t*-test at the significance: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3. Results

3.1. CAR1 overexpression enhances acetic acid tolerance of S. cerevisiae

Previous studies showed that *CAR1* is regulated by its native promoter comprising multiple regulatory elements that can response to environmental signals [37]. To enable constitutive expression of *CAR1*, the *PGK1* promoter was used to drive its overexpression in this study. After recombinant strains were obtained and verified, their acetic acid tolerance was evaluated and compared with the control strain (Fig. 1).

Spot assay showed improved acetic acid tolerance in the *CAR1*-overexpressing strain *S. cerevisiae* 4126-CAR1 (4126-CAR1) when comparing with the wild type strain *S. cerevisiae* 4126 (4126-WT) and the strain carrying the empty vector *S. cerevisiae* 4126-HO (4126-HO). Without acetic acid stress, no significant difference was observed in the growth of these strains. Similar results were observed when *CAR1* overexpression was engineered in *S. cerevisiae* S288c (Fig. S1A).

Ethanol fermentation was quantitatively evaluated for these strains in the presence of 5.0 g/L acetic acid, and 4126-CAR1 showed improved growth with over 30% higher cell density (Fig. 1B−E). As a result, both glucose consumption and ethanol production of the *CAR1* overexpressing strain were facilitated with less glycerol produced, and consequently ethanol productivity was doubled, with ethanol yield increased by 23.5% and 27.3%, respectively compared with 4126-HO and 4126-WT (Table 2). Similar results were observed when *CAR1* overexpression was engineered in *S. cerevisiae* S288c (Figs. S1B–E). Since no significant difference was detected between 4126-WT and 4126-HO, we selected 4126-HO as the only control strain for the following studies. Similar results were observed for ethanol fermentation from the hydrolysate of corn stover pretreated with dilute acid (Fig. S2). The acetic acid concentrations of 4126-WT, 4126-HO, 4126-CAR1 decreased as the fermentation was completed.
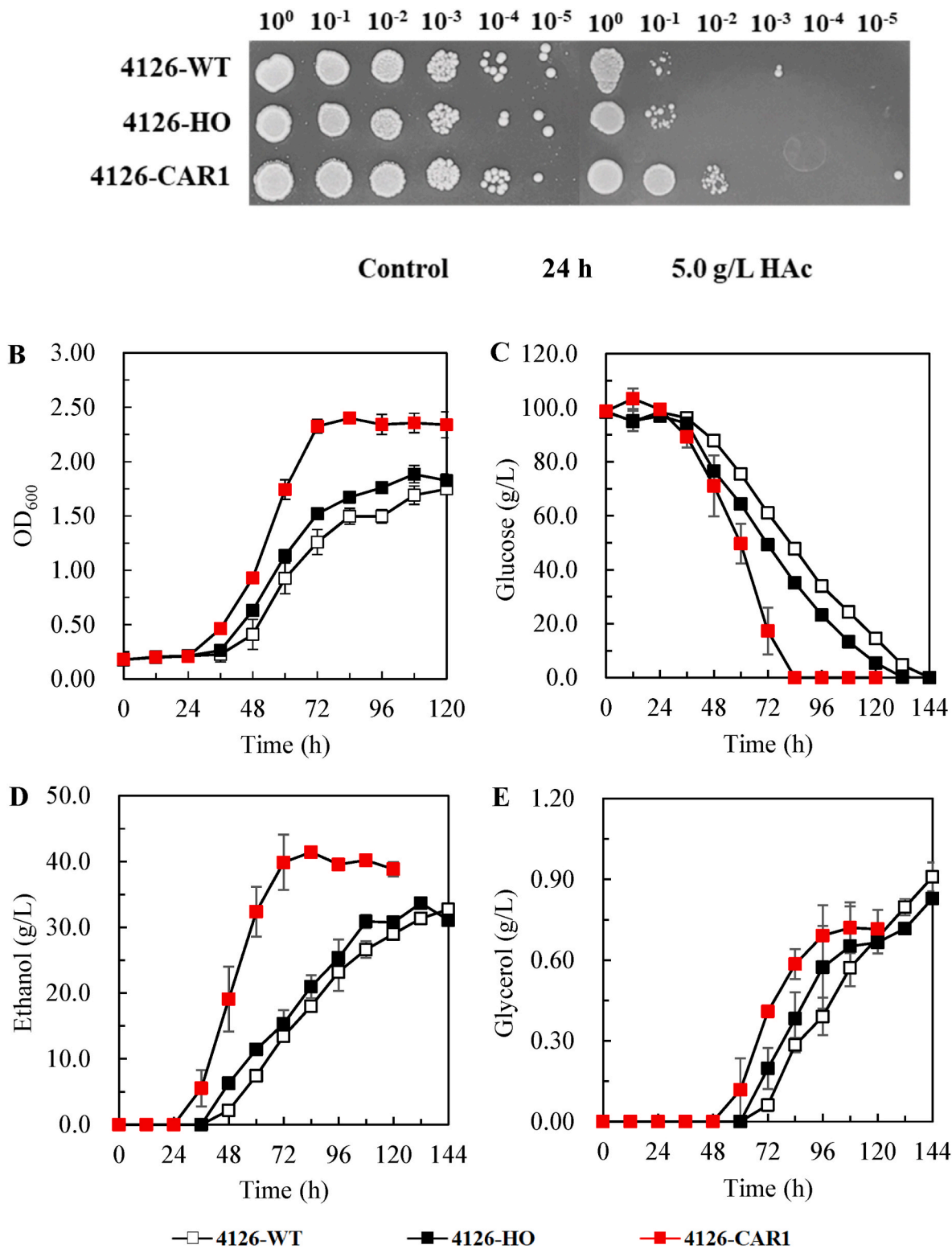
Fig. 1. Impact of *CAR1* overexpression on the growth of *S. cerevisiae* 4126 strains on YPD agar plates supplemented with 5.0 g/L acetic acid to test their tolerance to the inhibitor and without acetic acid supplementation as the control (A), and ethanol fermentation by *S. cerevisiae* 4126 engineered with *CAR1* overexpression using YPD medium composed of 4 g/L yeast extract, 3 g/L peptone, 100 g/L glucose and 5 g/L acetic acid (B–E). The data for ethanol fermentation were expressed as the means for triplicate with standard deviations. 4126-WT, *S. cerevisiae* 4126; 4126-HO, *S. cerevisiae* 4126 engineered with the empty plasmis; 4126-CAR1, *S. cerevisiae* 4126 engineered with *CAR1* overexpression, respectively.

Table 2

Ethanol fermentation performance of *S. cerevisiae* 4126-WT, 4126-HO and 4126- CAR1 in the presence of 5.0 g/L acetic acid.

Yeast strain	Fermentation time (h)	Ethanol production (g/L)	Ethanol yield (g/g)	Ethanol productivity (g/L/h)
4126-WT	144	32.73 ± 0.15	0.33 ± 0.002	0.23 ± 0.002
4126-HO	132	33.68 ± 0.02	0.34 ± 0.000	0.26 ± 0.003
4126-CAR1	84	40.86 ± 0.58	0.42 ± 0.006	0.49 ± 0.021

3.2. CAR1 overexpression affects intracellular amino acid composition

We are interested in whether such a *CAR1* overexpression led to the changes of amino acid metabolism in *S. cerevisiae* under acetic acid stress, and therefore examined the intracellular amino acid compositions for those strains under conditions with and without such a stress. Totally 26 amino acids and their derivatives were analyzed (Table S2), and the statistical analysis indicated that 24 of them were significantly affected by the *CAR1* overexpression (Fig. 2E). While the content of isoleucine, serine, histidine, asparagine and methionine was substantially decreased in *S. cerevisiae* 4126-CAR1 compared to that detected for *S. cerevisiae* 4126-HO under the acetic acid stress condition, no such an impact was observed for the *CAR1* overexpression strain when no acetic acid stress was applied, suggesting a differential regulation on amino acid metabolism under the stressful condition. We also compared the global amino acid profile of the control strain with or without acetic acid stress, and found that it was different from the case observed in the *CAR1*-overexpressing strain. Therefore, we speculate that *CAR1* overexpression might affect the overall amino acid metabolism of *S. cerevisiae* under acetic acid stress conditions.

Analysis of intracellular amino acids revealed that ornithine was increased in *S. cerevisiae* 4126-CAR1 under the acetic acid stress condition (HAc-CAR1) compared to that detected with the control strain (HAc-HO), but the accumulation of arginine and proline was decreased by 27.3% and 50.6%, respectively, making the total amino acids reduced as high as 25.9% (34.38 mg/gDCW v.s. 46.42 mg/gDCW (Fig. 2A−D and Table S2). On the other hand, no significant difference was observed in ornithine, arginine and total amino acids between *S. cerevisiae* 4126-CAR1 (Ctr-CAR1) and *S. cerevisiae* 4126-HO (Ctr-HO) when no acetic acid stress was applied, and the content of proline was decreased by the overexpression of *CAR1*.

The results highlighted in Fig. 2 were detected for yeast cells cultured in the rich medium supplemented with yeast extract and peptone for laboratory research, and amino acids were available for potential uptake by the yeast cells, which might affect the profile of their intracellular amino acids. We therefore performed culture using the minimal medium without amino acids to rule out such a concern (Fig. S3 and Table S3). The same profiles were obtained, confirming that the effect of *CAR1* overexpression on yeast acetic acid tolerance was independent on the uptake of amino acids.

Notably, under the acetic acid stress condition, ornithine content in *S. cerevisiae* 4126-CAR1 (HAc-CAR1) increased by about 60.0% when compared with that in the control strain (HAc-HO). However, unexpectedly, the contribution of increased ornithine content to the growth of *S. cerevisiae* was negligible (Fig. S4A, B and E), despite the fact that the supplementation of ornithine at 10 mM increased the intracellular ornithine content by 442% (Fig. S4C), respectively. The cell viability was not significantly affected by supplementation of ornithine at 10 mM as well (Fig. S4F). We therefore conclude that *CAR1* overexpression in *S. cerevisiae* improves acetic acid tolerance through mechanisms other than affecting ornithine content. We did not find significant changes in acetic acid concentration when comparing the *CAR1* overexpressing strain with that of the control strain. Therefore, the improved growth is not related to elevated acetyl-CoA by acetic acid utilization.

3.3. Transcriptional profiling of genes involved in ornithine, arginine and proline metabolism influenced by CAR1 overexpression

Since *CAR1* encodes arginase for arginine degradation, we first quantified the relative transcription of genes related to arginine catabolism. As can be seen in Fig. 3A and S5A, no matter whether or not acetic acid stress was applied, the transcription of *CAR1* was enhanced in *S. cerevisiae* 4126-CAR1, which perturbed the expression of genes involved in arginine catabolism. Since arginine degradation is the major upstream pathway for ornithine synthesis, the *CAR1* overexpression enhanced ornithine anabolism, and consequently intracellular accumulation of ornithine was observed, no matter whether the acetic acid stress was applied (Table S2).

When acetic acid stress was applied, the overexpression of *CAR1* in 4126-CAR1 was compromised, which consequently affected transcription of other genes. Comparing 4126-CAR1 with 4126-HO, the transcription of ornithine decarboxylase gene *SPE1* and mitochondrial ornithine acetyltransferase encoding gene *ARG7* was significantly enhanced, while the expression of ornithine transaminase gene *CAR2* and ornithine carbamoyltransferase (OTCase) encoding gene *AGR3* were down-regulated. Although the transcription of P5C reductase encoding gene *PRO3* was enhanced, it wouldn't contribute substantially to ornithine catabolism to proline due to the down-regulation of *CAR2*. As a result, the accumulation of proline was suppressed since about 50% lower proline was detected in 4126-CAR1 than that in 4126-HO.

3.4. Acetic acid tolerance rendered by CAR1 overexpression is independent on the spermidine metabolism

Previous studies demonstrated that the elevation of polyamine spermidine level in *S. cerevisiae* could improve tolerance to various lignocellulose-derived inhibitors [36,38]. Moreover, another study revealed that yeast cells could cope with stress by activating polyamine metabolism [39,40]. In this study, the expression level of *SPE1*, the enzyme responsible for the initial reaction in polyamine synthesis, was quantified in this study. The results showed that *SPE1* transcription was upregulated by 4.43-fold in 4126-CAR1 compared to 4126-HO. (Fig. 3A). As mentioned earlier, ornithine is the essential substrate for polyamine biosynthesis. In this study, we measured the ornithine content in *S. cerevisiae* 4126-CAR1 (HAc-CAR1) and found that it was approximately 60.0% higher than that in the control strain (HAc-HO). Based on this observation, we hypothesized that the increased production of polyamines, particularly spermidine, could be the underlying mechanism for acetic acid tolerance. We quantified the spermidine content of 4126-CAR1 and 4126-HO under acetic acid stress. Contrary to our expectation, spermidine content in 4126-CAR1 (0.45 mg/gDCW) was marginally lower than that in 4126-HO (0.77 mg/gDCW) (Fig. 3B). These results indicated that the enhanced acetic acid tolerance by *CAR1*-overexpression was not related to the elevated spermidine level. Therefore, we proposed that a novel mechanism, distinct from the previously reported spermidine-independent pathway [36,38], was involved in acetic acid resistance upon *CAR1* overexpression.

3.5. CAR1 overexpression increase ATP content and enhance cell membrane integrity

In response to acetic acid stress, cytoplasmic membrane proton-pumping ATPases and vacuolar proton-pumping ATPase are employed
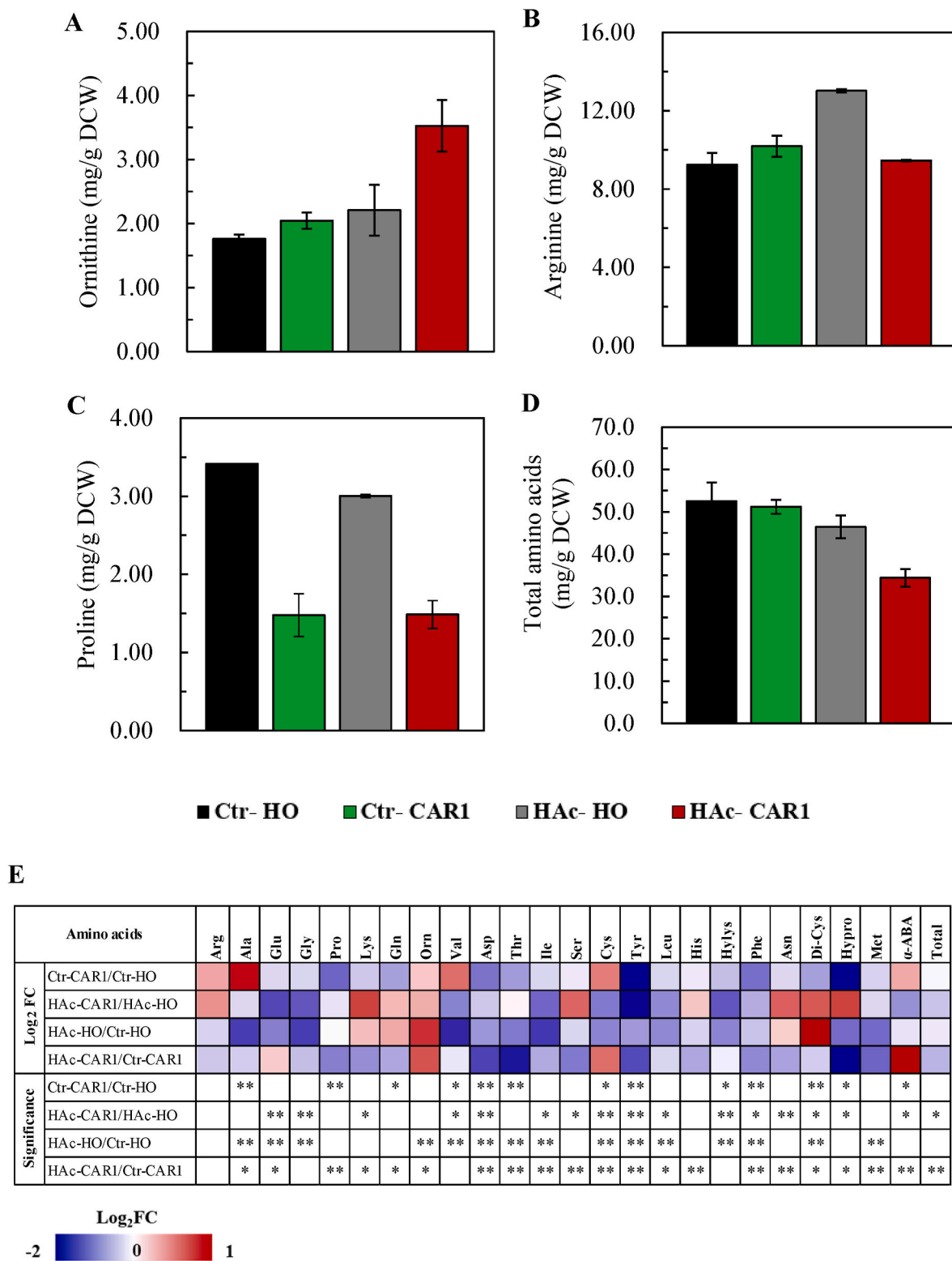
Legend: ■ Ctr- HO   ■ Ctr- CAR1   ■ HAc- HO   ■ HAc- CAR1

E

	Amino acids	Arg	Ala	Glu	Gly	Pro	Lys	Gln	Orn	Val	Asp	Thr	Ile	Ser	Cys	Tyr	Leu	His	Hylys	Phe	Asn	Di-Cys	Hypro	Met	α-ABA	Total
Log2 FC	Ctr-CAR1/Ctr-HO																									
	HAc-CAR1/HAc-HO																									
	HAc-HO/Ctr-HO																									
	HAc-CAR1/Ctr-CAR1																									
Significance	Ctr-CAR1/Ctr-HO		**			**		*		*	**	**			*	**			*	**		**	*		*	
	HAc-CAR1/HAc-HO			**	**		*			*	**		*	*	**	**	*		**	*	**	*	*		*	*
	HAc-HO/Ctr-HO		**	**	**				**	**	**	**	**		**	**	**		**	**		**		**		
	HAc-CAR1/Ctr-CAR1	*	*			**	*	*	*		**	**	**	**	**	**	*	**		**	**	*	*	**	**	**

Log₂FC   -2 ■■■ 0 ■■■ 1

Fig. 2. An overview on the profile of amino acid metabolism (A, B, C and D) and the heat map (E) highlighting the fold changes of intracellular amino acids. Ctr-CAR1 and Ctr-HO represent *S. cerevisiae* 4126 engineered with *CAR1* overexpression and the empty plasmid cultured in the YPD medium without acetic acid supplementation, and HAc-CAR1 and HAc-HO represent those two recombinat strains cultivated Detailed data for amino acids are available in Table S2. Abbreviations: Ala, alanine; Arg, arginine; Asn, asparaginate; Asp, asparate; Cys, cysteine; Di-Cys, dicyseine; Gln, glutamine; Glu, glutamate; Gly, glycine; His, histidine; Hylys, hydroxylysine; Hypro, hydroxyproline; Ile, isoleusine; Lys, lysine; Leu, leucine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, thronine; Tyr, tyrosine; Val, valine; α-ABA, α-aminobutyric acid. Yeast cells were harvested at their exponential growth phase for the analysis with triplicate under the statistic significance $*p < 0.05$ and $**p < 0.01$.
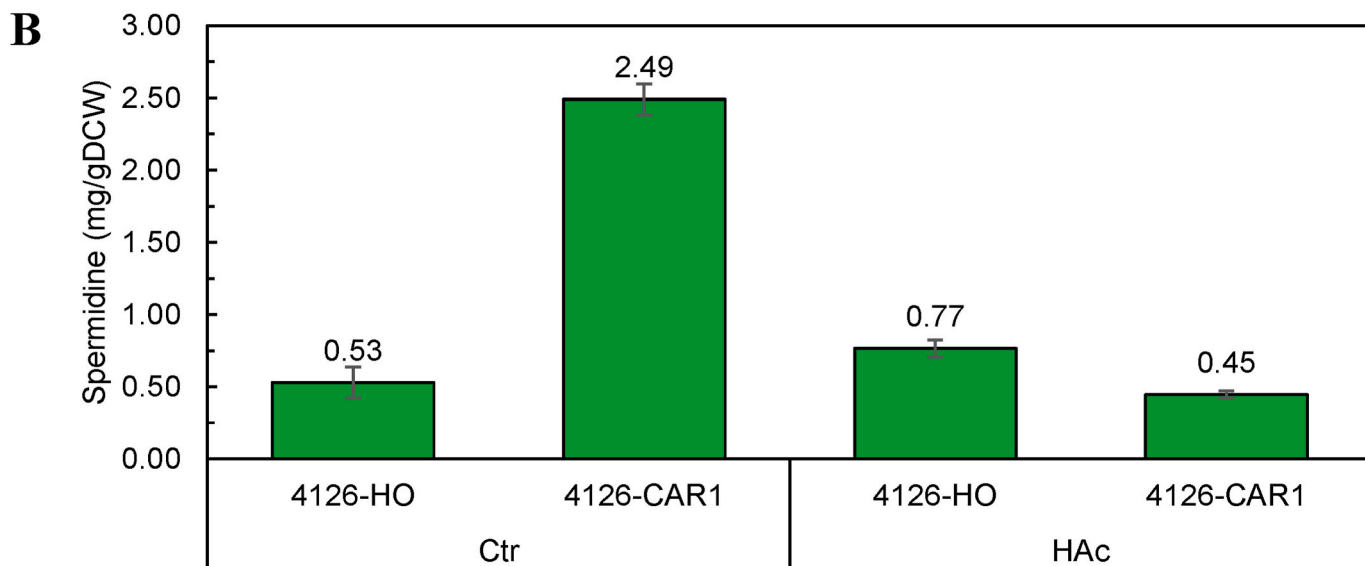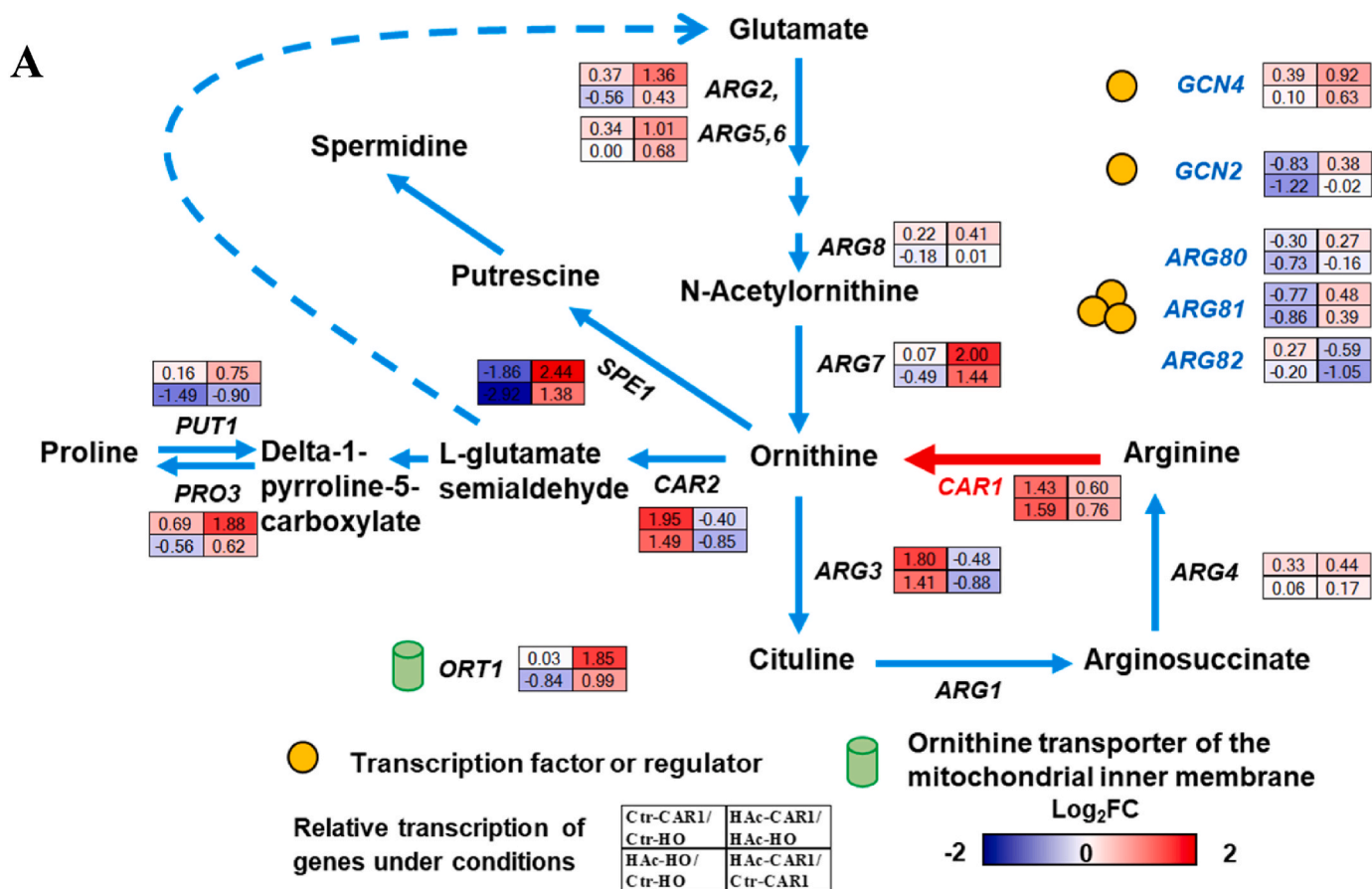
Fig. 3. Transcription of genes related to the arginine metabolism (A) and intracellular spermidine contents of the recombinant strains (B). Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using the expression of *ALG9* as the internal control, which is highlighted by the fold change (Log$_2$FC). Pathways for arginine metabolism was constructed based on information from the KEGG Pathway (http://www.genome.jp/kegg/). Intracellular spermidine contents of the recombinant strains were determined dansyl chloride derivatization based HPLC method as described in the Material and Method section. Yeast cells grew to exponential-phase in fermentation medium were harvest and used for the determination of transcription and intracellular spermidine contents.

to pump protons out at the expense of ATP consumption [41]. Hence, it is of great significance to maintain relatively high ATP content to resist acetic acid stress. The intracellular ATP content in *S. cerevisiae* 4126-CAR1 was 3.23-fold higher than that detected in *S. cerevisiae* 4126-HO under the acetic acid stress condition (Fig. 4A), indicating that

the *CAR1* overexpression might be conducive to maintain the high level of intracellular ATP in *S. cerevisiae*.

Cell membranes are important barrier against environmental stresses. It was reported that good membrane integrity contributed to maintaining cell activity under acetic acid conditions [22,42]. Plasma
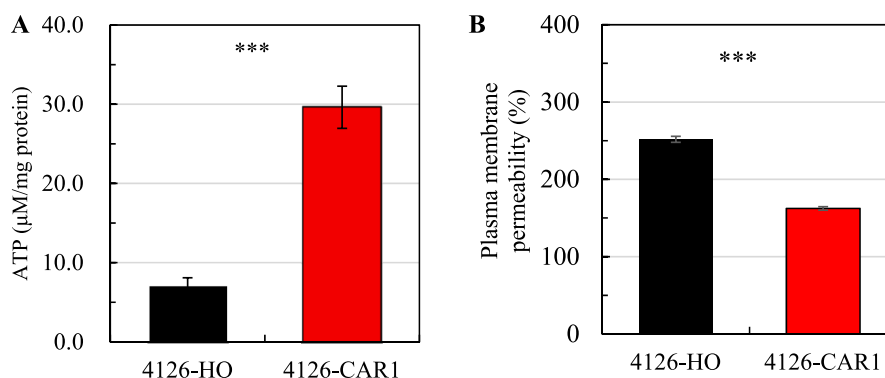
A

B

Fig. 4. Intracellular ATP (A) and plasma membrane permeability (B) for *S. cerevisiae* 4126-CAR1 and *S. cerevisiae* 4126-HO. The yeast cells were harvested at their exponential growth phase at 36 h and 48 h, respectively, with their OD_{600} of 0.46 and 0.63 for the analysis with triplicate under the statistical significance ***$p < 0.001$.

membrane permeability characterized by the propidium iodide uptake significantly decreased in *S. cerevisiae* 4126-CAR1 compared to that detected with *S. cerevisiae* 4126-HO under the acetic acid stress condition (Fig. 4B), suggesting a less damage in its cell membranes. The transcription of genes involved in ergosterol biosynthesis was quantified to explore molecular mechanism underlying this phenomenon, and the expression of *ERG3*, *ERG4*, *ERG6* and *ERG28*, was significantly up-regulated in *S. cerevisiae* 4126-CAR1 compared to that observed in *S. cerevisiae* 4126-HO regardless of the presence of acetic acid (Fig. S5C). Therefore, the *CAR1* overexpression in *S. cerevisiae* enhanced ergosterol biosynthesis either directly or indirectly, which consequently improved its cell membrane integrity [43].

4. Discussion

In this study, we observed remarkable improved acetic acid tolerance by *CAR1* overexpression in *S. cerevisiae*, which provides alternative strategy for yeast strain development for efficient cellulosic biorefinery. Previous studies mostly focused on the effect of individual amino acids on the stress tolerance of *S. cerevisiae* [25,44–46]. However, we observed significant decrease of total amino acid content by *CAR1* overexpression under acetic acid stress, which was not observed in the non-stressed condition. Our report is the first to reveal decreased total amino acid content under stress condition by overexpression a single gene *CAR1*. It was reported that single gene deletion influences the global amino acid metabolome of yeast cells [47], and our experimental results further revealed different changes of amino acid composition under stress condition comparing with stress free condition.

Our current studies also revealed that overexpression of *CAR1* regulates acetic acid tolerance through mechanisms different from reported ones. Both arginine and proline are known protectants for yeast cells to tolerate various stresses. It was reported that improved intracellular arginine increased ethanol tolerance of *S. cerevisiae* YS58 [46], and increased proline improved tolerance to multiple lignocellulose-derived inhibitors including furfural, acetic acid and phenol [45]. Decreased ornithine as well as proline content was observed when metabolomics analysis was performed to study the adaptation of an industrial yeast strain in a mixture of inhibitors containing acetic acid, furfural and phenol [48]. Although ornithine metabolism is closely related to arginine and proline, significantly decreased arginine and proline contents comparing to that detected in the control strain were observed in *S. cerevisiae* 4126-CAR1 under the acetic acid stress condition in this work, suggesting different mechanisms from that of proline in regulating acetic acid tolerance by *CAR1* overexpression.

It was suggested that the accumulation of spermidine in *S. cerevisiae* enhanced tolerance to multiple lignocellulose-derived inhibitors [36, 38]. However, spermidine content in *CAR1* overexpression strain decreased by 58.4% when compared with the control strain in the presence, indicating a mechanism significantly different from the previous studies [36,38]. Intracellular accumulation of amino acids is a dynamic balance between their uptake from medium and metabolism through synthesis and degradation, particularly when rich medium with various amino acids is used. In order to address such a concern, both *S. cerevisiae* 4126-CAR1 and *S. cerevisiae* 4126-HO were cultured with the minimal medium without amino acids supplemented (Table S3). Although data with individual amino acids are different, we confirmed that the *CAR1* overexpression still showed beneficial effects on growth of *S. cerevisiae* under acetic acid stress. These results suggest that the effects of *CAR1* overexpression is not dependent on uptake of amino acids in the culture medium, at least under the conditions employed in this study.

Consistent with the changes in amino acid contents, changes in transcription of genes involved in amino acid translocation as well as arginine metabolism also showed significant differences, suggesting regulation of amino acid metabolism gene expression by *CAR1*. Expression of genes for arginine metabolism is regulated by the multimeric complex composed of ArgR encoded by *ARG80*, *ARG81* and *ARG82* with their expression regulated by the transcription factor encoded by *GCN4* that can be activated by a protein kinase encoded by *GCN2* [44]. The expression of these genes was also regulated in *S. cerevisiae* 4126-CAR1 under the acetic acid stress condition to affect its amino acid metabolism. In addition, amino acid metabolism regulated at post-translational level also contributed to the equivalence of intracellular amino acid content [44]. More efforts are needed to elucidate the direct mechanism relating to modulation of amino acid by *CAR1* overexpression for improved acetic acid tolerance in the future.

Compared to the control, a higher intracellular ATP content was observed in *S. cerevisiae* 4126-CAR1. Under acid stress, yeast cells pump both the proton and the anion out of the cell at the expense of ATP, and studies have found that increasing acid stress resulted in higher cellular ATP levels [49], suggesting that higher ATP levels may increase yeast tolerance to acid stress. Our results are consistent with this hypothesis. Since biosynthesis of arginine, proline is energy-consuming, we thus propose that at least the partial mechanism underlying improved acetic acid tolerance in the *CAR1* overexpressing strain is related to repressing biosynthesis of certain amino acids to elevate its intracellular ATP level, which benefits tolerance to acetic acid as reported [13,49]. In addition, we also observed improved integrity for cell membranes by *CAR1* overexpression, which added evidence in the effect of *CAR1* in modulating membrane lipid metabolism. Although it is difficult to distinguish direct impacts from indirect ones, our results reveal possible link between overexpression of *CAR1* and metabolism of total amino acid, ATP and lipid to maintain high viability of *S. cerevisiae* under acetic acid stress, and therefore improve its ethanol fermentation performance.

In conclusion, we confirmed that *CAR1* overexpression in *S. cerevisiae* improve its tolerance to acetic acid and thereby boosts ethanol production. Our results also suggest that the underlining mechanism are different from the known ones related to proline and spermidine, and might related to decreased total amino acid metabolism for the engineered strain, particularly the regulation on its biosynthesis of amino acids for reducing their intracellular accumulation properly to save energy in the form of ATP. Nevertheless, follow-up studies warrant elucidating the direct mechanism for the improved stress tolerance. Our studies also suggest that *CAR1* is a novel genetic element to be used in synthetic biology of yeast for efficient production of fuel ethanol.

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CRediT authorship contribution statement

Liang Xiong: carried out the major part of the experiments and drafted the manuscript, designed the experiments. **Ya-Ting Wang:** revised the manuscript. **Ming-Hai Zhou:** included some additional experiments and refined the manuscript. **Hiroshi Takagi:** were involved in the data analysis and discussion, revised the manuscript. **Jiu-Fu Qin:** designed the experiments, were involved in the data analysis and discussion, revised the manuscript. **Xin-Qing Zhao:** conceived the study, designed the experiments, were involved in the data analysis and discussion, revised the manuscript.

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.synbio.2024.05.013.

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