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RESEARCH ARTICLE

The transcription factor Ace2 and its paralog Swi5 regulate ethanol production during static fermentation through their targets Cts1 and Rps4a in *Saccharomyces cerevisiae*

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One sentence summary: Two cell cycle-regulated transcription factors Ace2 and Swi5 regulate ethanol production during static fermentation through their targets Cts1 and Rps4a in budding yeast.

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ABSTRACT

Saccharomyces cerevisiae is the most widely used fermentation organism for ethanol production. However, the gene expression regulatory networks behind the ethanol fermentation are still not fully understood. Using a static fermentation model, we examined the ethanol yields on biomass of deletion mutants for 77 yeast genes encoding nonessential transcription factors, and found that deletion mutants for *ACE2* and *SWI5* showed dramatically increased ethanol yields. Overexpression of *ACE2* or *SWI5* in wild type cells reduced their ethanol yields. Furthermore, among the 34 target genes regulated by Ace2 and Swi5, deletion of *CTS1*, *RPS4a*, *SIC1*, *EGT2*, *DSE2*, or *SCP160* led to increased ethanol yields, with the former two showing higher effects. Overexpression of *CTS1* or *RPS4a* in both *ace2/ace2* and *swi5/swi5* mutants reduced their ethanol yields. In contrast, deletion of *MCR1* or *HO* significantly decreased ethanol yields, with the former one showing the highest effect. Therefore, Ace2 and Swi5 are two negative regulators of ethanol yield during static fermentation of yeast cells, and both *CTS1* and *RPS4a* are major effectors mediating these two transcription factors in regulating ethanol production.

Keywords: transcription factor; ethanol fermentation; cell cycle; Ace2; Swi5; Cts1; Rps4a; cell separation

INTRODUCTION

Saccharomyces cerevisiae is the most widely used fermentation organism used for the production of ethanol. However, the ethanol concentrations at the end of fermentation are limited to be generally 10%–12% (v/v) in most distilleries all over the world. With the aid of emerging very high gravity fermentation technology, the ethanol concentration of 15% (v/v) can be achieved at the end of fermentation (Puligundla *et al.* 2011). Production of ethanol is under the control of many metabolic genes, and ethanol yield is a polygenic trait. In *S. cerevisiae*, genetic analysis of polygenic traits remains an important challenge, although genomic approaches have been successfully used to map multiple quantitative trait loci involved in ethanol tolerance, sporulation

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efficiency and acetic acid production (Swinnen *et al*. 2012; Swinnen, Thevelein and Nevoigt 2012).

The exploration of anaerobic niches and later on the competition with other microorganisms in the environment are hypothesized to be the driving forces behind the remodeling of carbon metabolism through the evolution of ethanol fermentation in yeast cells. However, the complexity of gene expression regulatory networks behind the ethanol fermentation is still not fully appreciated (Dashko *et al*. 2014). Transcription factors are proteins that bind to specific DNA sequences, thereby controlling the rate of transcription of genes. They perform this function alone or with other proteins in a complex, by promoting (as an activator) or blocking (as a repressor) the recruitment of RNA polymerase to specific genes. Phenotypic changes are often involved in alterations in transcriptional regulation. Recent advances in DNA sequencing technology and functional genomics have enabled genome-wide comparative analyses of transcriptional circuits across different yeast species, which reveals that transcription networks are remarkably plastic (Li and Johnson 2010). In recent years, introduction of novel pathways and optimization of endogenous cellular processes through metabolic engineering have been rapidly applied for the microbial production of biofuels and chemicals (Nielsen *et al*. 2013). However, the production of desirable compounds from microbes often requires simultaneous modifications of many genes, which may not be achievable by sequential gene modifications. Therefore, simultaneous alteration of many genes through one transcription factor becomes an alternate route. This has been demonstrated to be successful in both prokaryotic and eukaryotic cells through a global transcription machinery engineering approach (Alper *et al*. 2006). In addition, a number of specific transcription factors have been reported to be associated with ethanol tolerance and production. Overexpression of a truncated form of *MSN2*, encoding a stress-responsive transcriptional activator, resulted in increased tolerance to ethanol (Hong *et al*. 2010). In contrast, overexpression of a full-length *MSN2* led to an increased sensitivity to ethanol (Sasano *et al*. 2012). Gcr1 is a transcription factor that controls carbohydrate metabolism in the presence of glucose by inducing glycolytic gene expression, and *GCR1* is involved in ethanol tolerance (Teixeira *et al*. 2009, 2010).

As a model organism, the budding yeast *S. cerevisiae* is the first eukaryote with its genome sequence reported. Collections of its gene deletion mutants have been successfully used in the characterization of gene-drug and pathway-drug interactions (Zhao *et al*. 2010, 2013b; Arias *et al*. 2011; Zhang *et al*. 2013; Jiang *et al*. 2014; Xiong *et al*. 2015) as well as in the elucidation of novel regulatory mechanisms for various metabolic pathways (Fujita *et al*. 2006; Martin *et al*. 2011; Chin *et al*. 2012; Zhao et al. 2013a; Nair *et al*. 2014). In this study, we have screened 77 gene mutants for nonessential transcription factors from the diploid collection of yeast gene deletion mutants for their ethanol production performance during static fermentation, and identified Ace2 and its paralog Swi5 as negative regulators of ethanol production. Further analysis of their target genes reveals that Cts1 and Rps4a are the major effectors mediating their functions in regulating ethanol production during fermentation.

MATERIALS AND METHODS

Strains, media and primers

The wild-type (WT) *S. cerevisiae* strain BY4743 (*ura3*Δ*0/ura3*Δ*0; his3*Δ*1/his3*Δ*1; leu2*Δ*0/leu2*Δ*0*) and its isogenic deletion mutant strains were from the diploid deletion mutant library purchased from Invitrogen Inc. and used in our previous studies (Zhao *et al*. 2010, 2013b; Zhang *et al*. 2013; Jiang *et al*. 2014; Du, Cao and Jiang 2015). YPD medium contains 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract. Fermentation medium contains 100 g/L glucose, 7.5 g/L $(NH4)_2SO_4$, 3.5 g/L KH_2PO_4, 0.75 g/L $MgSO_4$ $7H_2O$, 0.1 g/L leucine, 0.02 g/L histidine and 0.02 g/L uracil. Key strains used in this study are listed in Table 1. All primers used in this study are listed in Supplementary Table 1 (Supporting Information).

In order to 'marker match' the WT strain with members of the deletion library, we constructed the WT control strain BG (BY4743 with the *kanMX4* marker of G418 resistance). Briefly,

Table 1. Key strains used in this study.

Name	Genotype	Description
WYSC4	BY4743 *HIS3*MX6	The wild type (WT) strain (**BH**) with the *HIS3*MX6 allele integrated between −216 and −107 base region upstream of one *his3* locus
WYSC7	BY4743 P_{ADH1}-*CTS1*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *CTS1* allele
WYSC8	BY4743 P_{ADH1}-*RPS4A*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *RPS4A* allele
WYSC9	BY4743 P_{ADH1}-*ACE2*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *ACE2* allele
WYSC12	BY4743 P_{ADH1}-*SWI5*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *SWI5* allele
WYSC11	BY4743 *kan*MX6	The wild type (WT) strain (**BG**) with the *kan*MX6 allele integrated between −400 and −260 base region upstream of one *his3* locus
WYSC22	*ace2*::*kanMX4*/*ace2*::*kanMX4* P_{ADH1}-*RPS4A*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *RPS4A* allele in the *ace2/ace2* deletion mutant
WYSC23	*ace2*::*kanMX4*/*ace2*::*kanMX4* P_{ADH1}-*CTS1*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *CTS1* allele in the *ace2/ace2* deletion mutant
WYSC24	*swi5*::*kanMX4*/*swi5*::*kanMX4* P_{ADH1}-*RPS4A*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *RPS4A* allele in the *swi5/swi5* deletion mutant
WYSC25	*swi5*::*kanMX4*/*swi5*::*kanMX4* P_{ADH1}-*CTS1*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *CTS1* allele in the *swi5/swi5* deletion mutant
WYSC26	**BG** P*ADH1*-*CTS1*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *CTS1* allele in the WT strain **BG**
WYSC27	**BG** P*ADH1*-*RPS4A*	The *HIS3*MX6-P_{ADH1} allele replaced the promoter of one *RPS4A* allele in the WT strain **BG**

we integrated the *kanMX4* allele between −400 and −260 base region upstream of the *his3* locus by homologous recombination with a 1626-bp PCR fragment, which was amplified with primers kanMX-F and kanMX-R from the genomic DNA of the *ace2*::*kanMX4* cells. Correct integration was confirmed by PCR with primers kanMX-DF and kanMX-DR. Similarly, to construct another WT control strain BH (BY4743 with the *HIS3*MX6 marker), we integrated the *HIS3*MX6 allele between −216 and −107 base region upstream of the *his3* locus by homologous recombination with a 1498-bp PCR fragment, which was amplified with primers HIS3MX-F and HIS3MX-R from the plasmid pFA6a-HIS3MX6-pGAL1-GFP (Longtine *et al.* 1998). Correct integration was confirmed by PCR with primers HIS3-DF and HIS3-DR.

To overexpress the *CTS1* gene under the control of *ADH1* promoter in the WT BG, the *ace2/ace2* mutant and the *swi5/swi5* mutant, from the genomic DNA of BY4743 cells, we PCR amplified the 821-bp *ADH1* promoter fragment with primers ADH1p-F and ADHp-R, and inserted it between *Bgl*II and *Pac*I sites of pFA6a-His3MX6-PGAL1-GFP (Longtine *et al.* 1998), yielding the pFA6a-P_{ADH1} plasmid that contains a *HIS3*-P_{ADH1} cassette. The insert in this plasmid was confirmed by DNA sequencing. We then PCR amplified the 2298-bp *HIS3*-P_{ADH1} cassette with primers CTS1-ADH1p-INT-F and CTS1-ADH1p-INT-R, which was introduced into the WT BG, the *ace2/ace2* mutant and the *swi5/swi5* mutant, respectively, to replace the *CTS1* promoter in these strains. This generated three strains, *BG* P_{ADH1}-*CTS1*, *ace2/ace2* P_{ADH1}-*CTS1* and *swi5/swi5* P_{ADH1}-*CTS1*. Correct integrations were confirmed by PCR with primers CTS1-DF and CTS1-DR. Similarly, we amplified the *HIS3*-P_{ADH1} cassette with primers RPS4a-ADH1p-INT-F and RPS4a-ADH1p-INT-R, and constructed other three strains, *BG* P_{ADH1}-*RPS4a*, *ace2/ace2* P_{ADH1}-*RPS4a* and *swi5/swi5* P_{ADH1}-*RPS4a*, which overexpress the *RPS4a* gene under the control of *ADH1* promoter. Correct integrations in these strains were confirmed by PCR with primers RPS4a-DF and RPS4a-DR.

Similarly, to overexpress the *ACE2*, *SWI5*, *CTS1* or *RPS4a* gene under the control of *ADH1* promoter in the WT BY4743, we PCR amplified the 2298-bp *HIS3*-P_{ADH1} cassettes with primer pairs ACE2-ADH1p-INT-F/ACE2-ADH1p-INT-R, SWI5-ADH1p-INT-F/SWI5-ADH1p-INT-R, CTS1-ADH1p-INT-F/CTS1-ADH1p-INT-R and RPS4a-ADH1p-INT-F/RPS4a-ADH1p-INT-R, respectively, and introduced them into the WT BY4743 to replace their endogenous promoters, respectively. Correct integrations were confirmed by PCR with their primer pairs ACE2-DF/ACE2-R and SWI5-DF/SWI5-DR, CTS1-DF/CTS1-DR and RPS4a-DF/RPS4a-DR.

Static fermentation model

Seed cultures were obtained by growing yeast strains in 50 ml of YPD or selective SD media overnight at 30°C. Overnight cultures were inoculated to 50 ml fermentation medium with an initial OD_{600nm} of 1.5 in 250-mL flasks, which was followed by a 8-hour ventilated shake with a speed of 220 rpm at 30°C to obtain sufficient biomass, before these aerobic cultures were switched to static cultures in the same incubator where they were kept for additional 52 h (Fig. 1). Fermentation samples of 1 ml were taken at different time points, with static cell cultures mixed well by a vigorous shake immediately before being sampled, examined for their OD_{600nm} values with a spectrophotometer (Eppendorf BioPhotometer Plus, Germany), and subsequently centrifuged at 10 000 rpm for 1 min. Cell pellets were used for measuring their biomasses (dry weights), while supernatants were pretreated with 5% TCA before being filtered with a paper filter of 0.22-μm pore size. Filtrates were used to examine ethanol and glucose concentrations by high-pressure liquid chromatography, using a Shodex SUGAR SH1011 column eluted with 5 mM H_2SO_4 at a flow rate of 1.0 ml/min and at 50°C. Ethanol and glucose were detected with a Waters 2717 refractive index detector in the Waters (Model 1515) apparatus. Data were from three independent experiments.

Determination of fermentation culture dry weight

To determine dry weights of culture samples, we used 0.45-μm membrane filters to remove the medium by filtration. The filters were washed once with deionized water and dried in an oven for 15 min as described previously (Postma *et al.* 1989).

Colony morphology and cell sedimentation assays

Colony morphology and cell sedimentation assays for yeast strains were carried out on solid and liquid YPD media, respectively, as described previously (Voth *et al.* 2005).

Semi-quantitation of band intensity

Digital signals of band intensity in RT-PCR gels were quantified and analyzed using the Bio-Rad Gel DocTM XR+ System and the Image Lab software (Version 4.0.1) (Hercules, USA) as we described previously (Xiong *et al.* 2015).

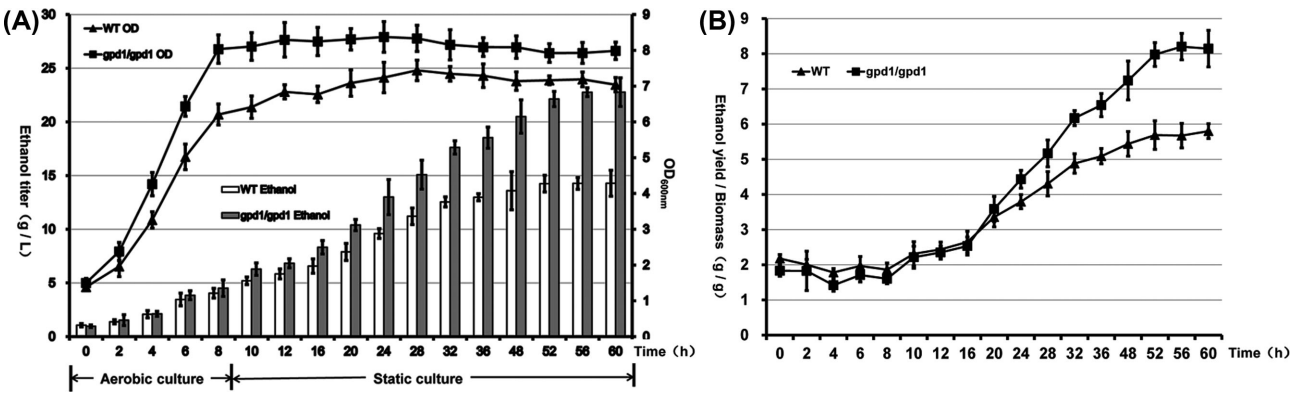

Figure 1. Static fermentation profiles of the wild type (WT) and its isogenic deletion mutant for *GPD1*. Optical density (OD) values at 600 nm (lines) and ethanol titers (bars) **(A)** as well as ethanol yields on biomass **(B)** of these two strains during aerobic and static growth phases at 30°C for indicated time.

Statistical analysis

Significant differences were analyzed by the paired-samples *t*-test with SPSS version 16.0 (USA). *P*-values of <0.05 were considered to be significant.

RESULTS

Establishment of an experimental fermentation model

A previous study showed that deletion of *GPD1*, encoding NAD-dependent glycerol-3-phosphate dehydrogenase that is the key enzyme of glycerol synthesis, reduced glycerol yield and increased ethanol yield in anaerobic batch fermentations of *S. cerevisiae* (Guo *et al*. 2011). To determine an ideal model for us to compare the ethanol fermentation capabilities of gene deletion mutants for all nonessential transcription factors, we designed an experimental fermentation model using the WT and its isogenic *gpd1/gpd1* mutant (Fig. 1; see details in 'Static fermentation model' of the Methods section). Under these fermentation conditions, both ethanol titers and yields on biomass of the *gpd1/gpd1* mutant were higher than those of the WT (Fig. 1). The ethanol yields of two strains started to differentiate at the time point of 20 h, and seemed to reach their plateaus by the time point of 52 h (Fig. 1B). This result is consistent with previous studies that deletion of *GPD1* leads to higher ethanol yields in *S. cerevisiae* (Guo *et al*. 2011; He *et al*. 2014). Therefore, our fermentation model is suitable for the objective in this study.

Genetic screen for transcription factors involved in ethanol production during fermentation

Using the phrase 'transcription factor' as a query, from the *Saccharomyces* genome database (SGD) we identified a total of 114 transcription factor genes, and 94 of them are nonessential genes. We retrieved 77 deletion mutants for these genes from our gene deletion library in the diploid BY4743 background (Supplementary Table 2, Supporting Information). Based on the fermentation model described above, we examined the ethanol production of these 77 individual gene deletion mutants. To facilitate the comparison between different mutants, we arbitrarily chose two fermentation time points of 32 and 52 h, which represent the increasing and plateau phases, respectively, of ethanol production in the WT and the *gpd1/gpd1* mutant (Fig. 1B), for measuring the biomasses and ethanol concentrations of these 77 mutants. As compared to the WT, we identified two deletion mutants for *ACE2* and its paralog *SWI5* (Dohrmann *et al*. 1992), whose ethanol yields were significantly elevated at both 32 and 52 h time points during fermentation (Fig. 2A; Data not shown).

We further characterized the fermentation profiles of these two mutants. As compared to the WT, the *ace2/ace2* mutant showed a slower growth during the aerobic growth phase, and its optical density (OD) value remained dramatically lower during the plateau phase. In contrast, the *swi5/swi5* mutant showed a similar growth rate to the WT, but its OD value remained slightly lower than that of the WT during the plateau phase (Fig. 3A). However, as compared to the WT, the ethanol yields on biomass of these two mutants remained higher during almost the entire fermentation period, with the *ace2/ace2* mutant being higher than the *swi5/swi5* mutant (Fig. 3B).

Next, we examined if overexpression of *ACE2* or *SWI5* would have an effect on ethanol fermentation. Both *ACE2* and *SWI5* are cell cycle-regulated genes (Dohrmann *et al*. 1992). We replaced the promoters of these two genes with the *ADH1* promoter in the BY4743 background, which led to elevated transcriptional expression levels of *ACE2* or *SWI5* by 1.73 and 1.63 times, respectively, as compared to that in the WT (Supplementary Fig. 1A, Supporting Information). Ethanol yields of the WT cells overexpressing *ACE2* were decreased by 40% at both 32 and 52 h, while those of the WT cells overexpressing *SWI5* were decreased by 90% and 83%, respectively, at 32 and 52 h, in comparison to those in the WT control cells (Fig. 4A). Taken together, these results indicate that Ace2 and its paralog Swi5 are negative regulators of ethanol production during static fermentation of yeast cells. In contrast, a recent work has shown that Ace2 and Swi5 are two of the 20 transcription factors, with Ace2 being the most important one, that are involved in regulating yeast response to acetic acid and furfural stresses during ethanol fermentation from lignocellulosic biomass, and that overexpression of *ACE2* improves specific ethanol productivity of yeast cells by three times in the presence of acetic acid and furfural (Chen *et al*. 2016).

Cts1 and Rps4a are major effectors for mediating functions of Ace2 and Swi5 in ethanol production

From the SGD database, Ace2 and Swi5 are known to control transcriptional expression of 76 and 127 genes, respectively (http://www.yeastgenome.org/). We tested deletion mutants of 15 genes being specific targets of Ace2, 6 genes being specific targets of Swi5, and 13 genes being common targets of Ace2 and Swi5 (Supplementary Table 3, Supporting Information; McBride, Yu and Stillman 1999). Similar to the deletion mutant of *ACE2*, the deletion mutant for *CTS1* showed significantly higher ethanol yields/biomass at both 32 and 52 h time points, while the deletion mutants for *SIC1*, *EGT2*, *DSE2*, *SCP160* and *RPS4a* showed higher ethanol yields/biomass at either 32 or 52 h time points, than those of the WT (Fig. 2B). In contrast, the deletion mutant for *MCR1* showed significantly lower ethanol yields/biomass at both 32 and 52 h time points, while the deletion mutant for *HO* showed lower ethanol yields/biomass at 52 h time point than those of the WT (Fig. 2B).

We further characterized the fermentation profile of the *cts1/cts1* mutant. As compared to the WT, the *cts1/cts1* mutant showed a similar growth rate to the WT, but its OD value remained slightly lower than that of the WT during the plateau phase (Fig. 3A). However, its ethanol yield reached higher even than those of the *ace2/ace2* and the *swi5/swi5* mutants after 52 h (Fig. 3B). Similar to deletion of *ACE2* or *CTS1*, deletion of Ace2 target gene *RPS4a* led to dramatic increase of ethanol yield at 32 h, albeit not at 52 h, time points than that of the WT (Fig. 2B). Next, we examined the effects of overexpressing *CTS1* or *RPS4a* on ethanol fermentation. We replaced the promoters of these two genes with the *ADH1* promoter in the BY4743 background, which led to increased expression levels of *CTS1* and *RPS4a* by 1.22 and 1.98 times, respectively, as compared to that in the WT BH cells (Supplementary Fig. 1A, Supporting Information). Ethanol yields of the WT cells overexpressing *CTS1* were decreased by 32% and 24%, respectively, at 32 and 52 h, while those of the WT cells overexpressing *RPS4a* were decreased by 29% and 24%, respectively, at 32 and 52 h, in comparison to that in the WT BH control cells (Fig. 4A). These results confirm that like their transcription regulator Ace2, Cts1 and Rps4a are negative regulators of ethanol production during static fermentation of yeast cells.

Genetically, overexpression of a downstream target can suppress the deletion effect of its upstream regulator. Therefore, we next examined the effect of overexpressing *CTS1* or *RPS4a*

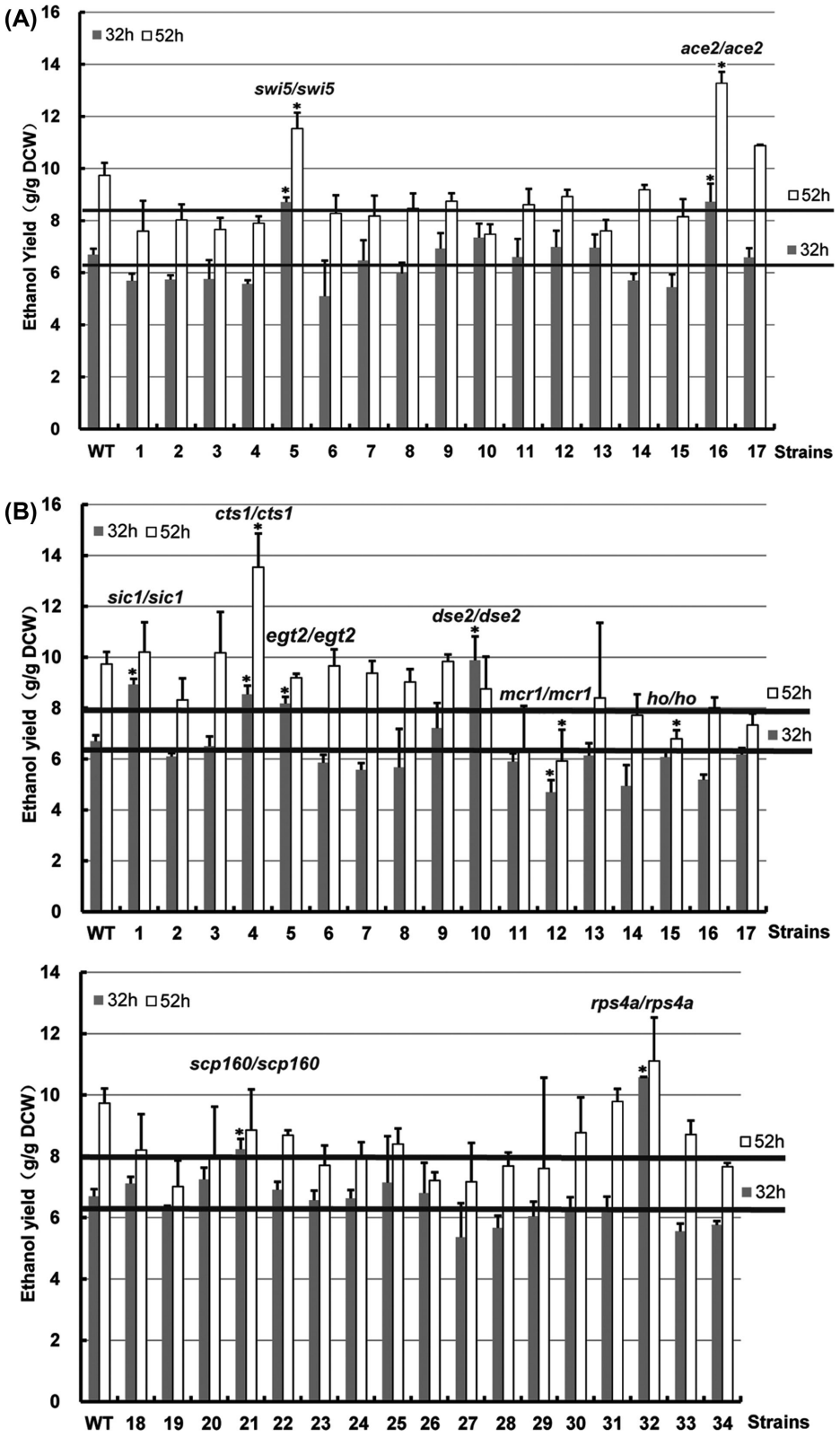

Figure 2. Ethanol yields on biomass at 32 and 52 h time points during the static fermentation. **(A)** Ethanol yields of the WT BG and its isogenic gene deletion mutants for 17 transcription factors. These mutants were numbered as in Supplementary Table 2 (Supporting Information). The arithmetic mean values of ethanol yields for the WT and all 77 mutants examined at 32 h (6.15 ± 0.32) and 52 h (8.36 ± 0.56) are indicated with two solid lines, respectively, as indicated. **(B)** Ethanol yields of the WT BG and its isogenic gene deletion mutants for 34 target genes of the transcription factor Ace2 and Swi5. These mutants were numbered as in Supplementary Table 3 (Supporting Information). The arithmetic mean values of ethanol yields for the WT and all 34 mutants examined at 32 (6.26 ± 0.43) and 52 h (7.99 ± 0.66) are indicated with two solid bars, respectively. All values were means of three independent fermentation experiments. The asterisk (*) shows statistically significant differences ($P < 0.05$) between the WT and mutants indicated. The gene names of mutants showing significant difference from the WT are shown above their corresponding bars.

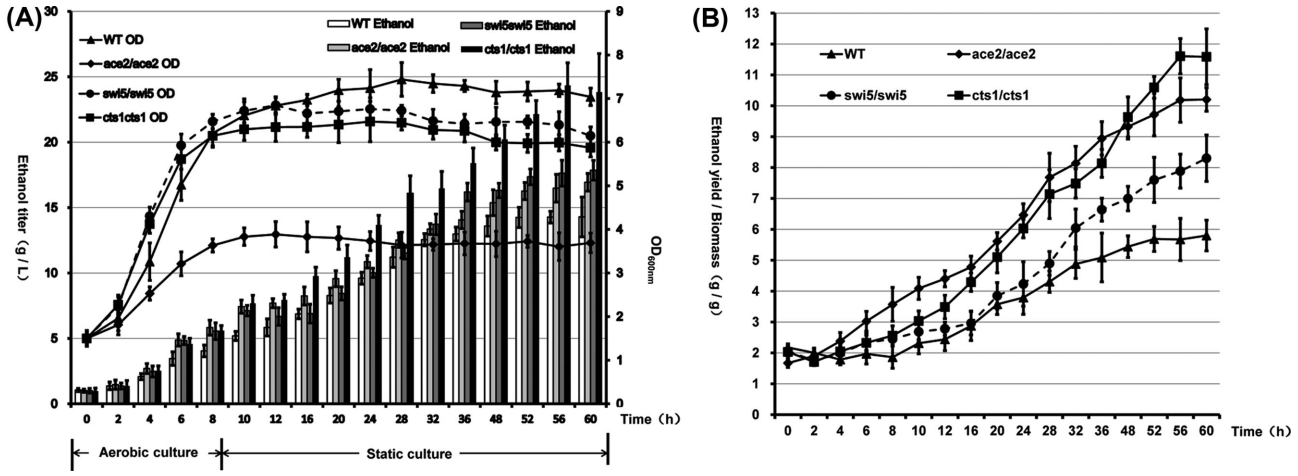

Figure 3. Static fermentation profiles of the WT and its isogenic deletion mutant for *ACE2*, *SWI5* and *CTS1*. OD_{600nm} values (lines) and ethanol titers (bars) (**A**) as well as ethanol yields on biomass, (**B**) of these four strains during aerobic and static growth phases at 30°C for indicated time.

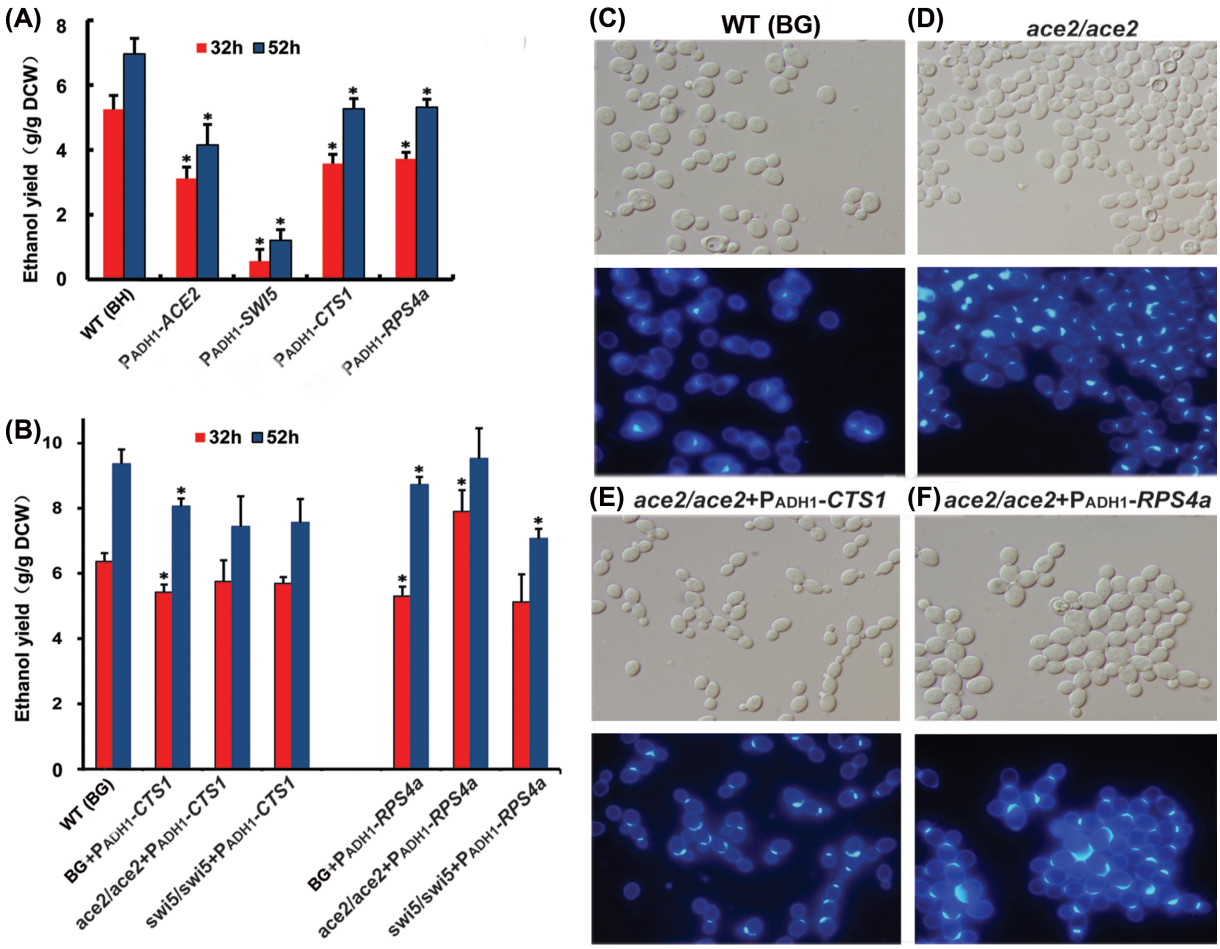

Figure 4. Effects of overexpression of *ACE2*, *SWI5*, *CTS1* or *RPS4a* on ethanol fermentation and cell morphologies. (**A** and **B**) Ethanol yields on biomass of indicated strains in the static fermentation media at 32 and 52 h post inoculation. Strains used in (A) are the WT BH (BY4743 with *HIS3* integrated at the upstream of one *his3* allele), P_{ADH1}-ACE2 (BY4743 with *HIS3*-P_{ADH1} integrated at the upstream of one *ACE2* allele), P_{ADH1}-SWI5 (BY4743 with *HIS3*-P_{ADH1} integrated at the upstream of one *SWI5* allele), P_{ADH1}-CTS1 (BY4743 with *HIS3*- P_{ADH1} integrated at the upstream of one *CTS1* allele) and P_{ADH1}-RPS4a (BY4743 with *HIS3*-P_{ADH1} integrated at the upstream of one *RPS4a* allele). Strains used in (B) are the WT BG and its isogenic deletion mutants indicated (the same as in Fig. 2A). *HIS3*- P_{ADH1} was integrated to replace endogenous promoters of *ACE2*, *SWI5*, *CTS1* or *RPS4a* in the strains indicated. The asterisk (*) shows statistically significant differences ($P < 0.05$) between the WT and mutants indicated. (**C**–**F**) Cell morphologies. Log-phase cells of the WT BG (C), the *ace2/ace2* mutant (D) and the *ace2/ace2* mutants overexpressing either *CTS1* (E) or *RPS4a* (F) under the control of *ADH1* promoter were stained with 50 μg/ml Calcofluor white for 30 min, to show the location of bud scars, before photos were taken. Upper panels were DIC images, and bottom panels were images visualized under a Nikon ECLIPSE 80i fluorescent microscope equipped with Plan fluor 100/1.3 oil objective and cube filter (EX340–380, EM435–485).

genes in the deletion mutants for *ACE2* or its paralog gene *SWI5* on ethanol yield. We replaced the promoters of *CTS1* or *RPS4a* with the *ADH1* promoter in the deletion mutants for *ACE2* or *SWI5*. This led to an increased expression of *CTS1* in *ace2/ace2* and *swi5/swi5* mutants by 2.30 and 1.22 times, respectively, and an increased expression of *RPS4a* in *ace2/ace2* and *swi5/swi5* mutants by 2.03 and 1.54 times, respectively (Supplementary Fig. 1B, Supporting Information). Static fermentation data showed that overexpression of *CTS1* also reduced the production of ethanol by 13% and 14% in the another WT BG as in Fig. 2, and completely suppressed the effect of either *ace2* or *swi5* deletion on the increase of ethanol yield, at both 32 and 52 h time points during fermentation (Fig. 4B). Similarly, overexpression of *RPS4a* decreased the production of ethanol by 14% and 7% in the WT at 32 and 52 h, respectively, and suppressed the ethanol yield of the *ace2/ace2* mutant, at the 52 h time point but not at the 32 h time point (Fig. 4B). In contrast, overexpression of *RPS4a* suppressed the ethanol yield of the *swi5/swi5* mutant at the 32 h time point, but not at the 52 h time point, during fermentation (Fig. 4B). Taken together, these data suggest that both Cts1 and Rps4a are downstream targets of Ace2 and Swi5, with Cts1 playing a bigger role than Rps4a, in mediating their regulatory functions in ethanol production.

Cts1 but not Rps4a mediates the regulatory function of Ace2 in cell separation

Previous studies have shown that deletion, genome duplication or truncation mutations of *ACE2* leads to a defect in mother-daughter cell separation (Voth *et al.* 2005; Oud *et al.* 2013). We next examined the cell morphologies of the WT and the *ace2/ace2* mutant overexpressing *CTS1* or *RPS4a*. Similar to the previous observation (Voth *et al.* 2005), log-phase growing cells of the WT showed a single-cell morphology, while cells of the *ace2/ace2* mutant showed a multicellular morphology (Fig. 4C and D). Overexpressing *CTS1* significantly, but overexpressing *RPS4a* barely, suppressed the multicellular morphology of the *ace2/ace2* mutant (Fig. 4E and F). This result suggests that Cts1 is the major downstream target of Ace2 for mediating its regulatory function in the mother-daughter cell separation after mitosis.

Deletion of *ACE2*, *SWI5*, *CTS1* or *RPS4a* increases ethanol tolerance of yeast cells

Ethanol tolerance is one of the limitation factors for ethanol yield during yeast fermentation. We next examined the ethanol tolerance of the *ace2/ace2*, the *swi5/swi5*, the *cts1/cts1* and the *rps4a/rps4a* mutants. As compared to the WT, the *ace2/ace2* mutant showed the highest ethanol tolerance, which is followed by the *swi5/swi5*, the *cts1/cts1* and the *rps4a/rps4a* mutants at lower degrees, on YPD plate containing 10% ethanol (Fig. 5A).

Cell and colony morphologies as well as cell sedimentation assays of mutants

In the W303 strain background, the *ace2* and *swi5* mutations have a defect in cell separation, resulting in clumpy clusters of cells and an altered colony morphology (Voth *et al.* 2005). Therefore, we examined the cell and colony morphologies **of** the *ace2/ace2*, the *swi5/swi5*, the *cts1/cts1*, the *dse1/dse1*, the *dse2/dse2*, the *dse4/dse4* and the *egt2/egt2* mutants in the BY4743 strain background. Consistent with the previous study (Voth *et al.* 2005), we found that the *ace2/ace2* mutant, but not other mutants, showed an altered colony morphology as compared to the WT on YPD plates (Fig. 5B), although both the *ace2/ace2* and the *swi5/swi5* mutants, and not other mutants, showed clumpy clusters of cells under the microscope (Supplementary Fig. 2, Supporting Information).

To quantify the cell separation defects, we measured the cell sedimentation rates for these mutants. As compared to the WT, the *ace2/ace2* mutant culture clears rapidly, with a

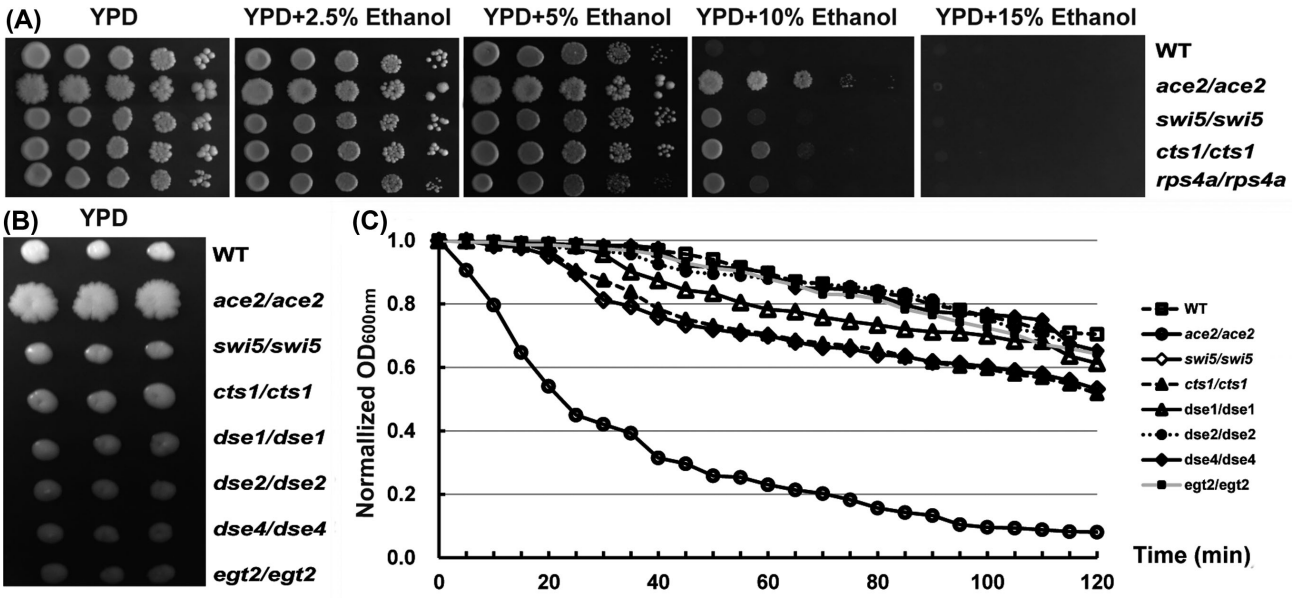

Figure 5. Ethanol tolerance, colony morphologies and cell sedimentation assays. (**A**) Ethanol tolerant phenotypes of the wild type BG (WT), the *ace2/ace2*, the *swi5/swi5*, the *cts1/cts1* and the *rps4a/rps4a* mutants on YPD plates containing indicated concentrations of ethanol. Plates were incubated at 30°C for 2 days. Colony morphologies (**B**) and cell sedimentation assays (**C**) of the wild type BG (WT), the *ace2/ace2*, the *swi5/swi5*, the *cts1/cts1*, the *dse1/dse1*, the *dse2/dse2*, the *dse4/dse4* and the *egt2/egt2* mutants. For the colony morphology assay, we inoculated three replicates for each yeast strain. For the cell sedimentation assay, the data are average of three independent experiments.

sedimentation half-time of 20 min, In contrast, there was significant decrease for the *swi5/swi5* and the *cts1/cts1* mutants at similar degrees and for the *dse1/dse1*mutant at a lower degree, but little change for the *dse2/dse2*, the *dse4/dse4* and the *egt2/egt2* mutants, in the OD until after 120 min (Fig. 5C).

DISCUSSION

Cts1 and Rps4a are shared targets of Ace2 and Swi5 during ethanol fermentation

In this study, we have identified that both Ace2 and Swi5 negatively regulate ethanol production during static fermentation of yeast cells. To our knowledge, this is the first report that cell cycle-regulating transcription factors are involved in the regulation of ethanol production during yeast fermentation. Ace2 is a transcriptional activator of a cell-cycle regulated Cts1, an endochitinase, which is required for septum destruction and cell separation after mitosis (Dohrmann *et al.* 1992; O'Conallain 1999), while its paralog Swi5 is the transcriptional activator of another cell-cycle regulated protein, the HO endonuclease (Dohrmann *et al.* 1992). However, their downstream targets Cts1 and HO appeared to show opposite functions, with the former showing a negative role and the latter showing a positive role, in regulating ethanol production during fermentation (Fig. 2B).

Although Ace2 and its paralog Swi5 are required for maximal expression of some genes, they act antagonistically at the promoters of other genes; they also regulate some genes independently (Doolin *et al.* 2001). Overexpression of *CTS1* suppresses the ethanol yield increase due to deletion of either *ACE2* or *SWI5* at both 32 and 52 h time points, while overexpression of *RPS4a* suppresses the ethanol yield increase due to deletion of *ACE2* and *SWI5* at only 52 or 32 h time points, respectively, during fermentation (Fig. 4B). These data suggest that Cts1 and Rps4a are shared targets of Ace2 and Swi5 during ethanol fermentation of yeast cells.

As compared to the WT, the *ace2/ace2* mutant has a lower biomass accumulation although its ethanol yield on biomass remains higher during the entire fermentation period (Fig. 3). Interestingly, we have shown that the *ace2/ace2* mutant has a dramatically higher ethanol tolerance than the WT, so are the *swi5/swi5*, the *cts1/cts1* and the *rps4a/rps4a* mutants with less degrees (Fig. 5A). This ethanol tolerance should at least partially contribute to the ethanol yields on biomass for these mutants. Nevertheless, the link between cell cycle and ethanol production remains to be elucidated.

Like the deletion mutant for *ACE2*, five mutants for its target genes, *CTS1*, *EGT2*, *DSE1*, *DSE2* and *DSE4* in either W303a or Σ1278b backgrounds were shown to have a defect in cell separation with different degrees (Doolin *et al.* 2001; Baladrón *et al.* 2002). However, except of deletion mutants for *ACE2*, *SWI5*, *CTS1* and *DSE1*, three mutants for Ace2 target genes, *EGT2*, *DSE2* and *DSE4* in BY4743 background did not show a defect in cell separation (Fig. 5; Supplementary Fig. 2, Supporting Information). Unlike *ACE2*, *CTS1*, *EGT2* and *DSE2*, deletion of either *DSE1* or *DSE4* did not affect the ethanol yields at both 32 and 52 h time points during yeast fermentation (No. 9 and No. 54 in Fig. 2B). Therefore, the defect in cell separation due to deletion of these genes seems not correlated to the ethanol yield of yeast cells during fermentation.

Other target genes of Ace2 involved in the ethanol fermentation

Sic1 is a cyclin-dependent inhibitor of Cdc28-Clb kinase complexes that controls G1/S phase transition, and its expression could be induced by both Ace2 and Swi5 (Toyn *et al.* 1997). Therefore, it is not surprising to note that deletion of *SIC1* increases the ethanol yield of yeast cells during fermentation as its two regulators do. *SCP160* encodes an RNA-binding and polysome-associated protein in the endoplasmic reticulum, and is involved in protein translation through interacting with Eap1, an eIF4E-binding protein (Guo *et al.* 2003; Mendelsohn *et al.* 2003). In *S. cerevisiae*, only two G proteins Gpa2 and Ras mediating two branches of a glucose-sensing pathway have been demonstrated (Conrad *et al.* 2014). The cAMP/PKA pathway mediated by Ras and Gpa2 plays a prominent role in responding to glucose levels and initiating the signaling processes that promote cell growth and division (Busti *et al.* 2010).Interestingly, signaling by activated Gpa1 requires its direct coupling to Scp160 in mating response pathway (Guo *et al.* 2003). Therefore, a similar mechanism how Scp160 is linked to ethanol production might exist in yeast cells and remains to be elucidated.

Under anaerobic conditions, yeast cells have to modify metabolic network to generate substrates (intermediates) for *de novo* reactions, such as the amino acid synthetic pathways, in a way independent of the respiratory chain and a normal Krebs cycle (Dashko *et al.* 2014). *MCR1* encodes NADH-cytochrome b(5) reductase and plays an important role in the response to oxidative damage in yeast cells (Lee *et al.* 2001). Loss of *MCR1* would lead to a higher levels of free radicals that would be an additional stress factor for yeast cells that are already exposed to ethanol stress, which might explain why there is a decreased ethanol yield at both 32 and 52 h time points for yeast cells lacking *MCR1*.

In conclusion, we have designed an experimental fermentation model using the WT and its isogenic *gpd1/gpd1* mutant. Out of 77 genes encoding nonessential transcription factors we have identified two transcription factors Ace2 and its paralog Swi5 as negative regulators of ethanol yields during static fermentation. We have further examined the ethanol fermentation yields of deletion mutants for 34 target genes of Ace2 and Swi5. Deletion of *CTS1*, *RPS4a*, *SIC1*, *EGT2*, *DSE2*, or *SCP160* shows increased ethanol yields, with the former two showing higher effects. In contrast, deletion of *MCR1* or *HO* significantly decreased ethanol yields, with the first showing the highest effect. Overexpression of *CTS1* or *RPS4a* in both *ace2/ace2* and *swi5/swi5* mutants reduced their ethanol yields. Therefore, *CTS1* and *RPS4a* are the major effectors mediating the functions of Ace2 and Swi5 in regulating ethanol production of yeast fermentation. Deletion of these two transcription factor genes (*ACE2* and *SWI5*) as well as their two target genes (*CTS1* and *RPS4a*) could be tested for their effects to improve ethanol production in industrial yeast strains. These findings improve our understanding of the regulatory mechanism of ethanol production and provide a basis for metabolic engineering of yeast cells to improve ethanol production during static high-gravity fermentation.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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