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BIOTECHNOLOGY and BIOENGINEERING

Improved Galactose Fermentation of *Saccharomyces cerevisiae* Through Inverse Metabolic Engineering

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ABSTRACT: Although *Saccharomyces cerevisiae* is capable of fermenting galactose into ethanol, ethanol yield and productivity from galactose are significantly lower than those from glucose. An inverse metabolic engineering approach was undertaken to improve ethanol yield and productivity from galactose in *S. cerevisiae*. A genome-wide perturbation library was introduced into *S. cerevisiae*, and then fast galactose-fermenting transformants were screened using three different enrichment methods. The characterization of genetic perturbations in the isolated transformants revealed three target genes whose overexpression elicited enhanced galactose utilization. One confirmatory (*SEC53* coding for phosphomannomutase) and two novel targets (*SNR84* coding for a small nuclear RNA and a truncated form of *TUP1* coding for a general repressor of transcription) were identified as overexpression targets that potentially improve galactose fermentation. Beneficial effects of overexpression of *SEC53* may be similar to the mechanisms exerted by overexpression of *PGM2* coding for phosphoglucomutase. While the mechanism is largely unknown, overexpression of *SNR84*, improved both growth and ethanol production from galactose. The most remarkable improvement of galactose fermentation was achieved by overexpression of the truncated *TUP1* (t*TUP1*) gene, resulting in unrivalled galactose fermentation capability, that is 250% higher in both galactose consumption rate and ethanol productivity compared to the control strain. Moreover, the overexpression of t*TUP1* significantly shortened lag periods that occurs when substrate is changed from glucose to galactose. Based on these results we proposed a hypothesis that the mutant Tup1 without C-terminal repression domain might bring in earlier and higher expression of *GAL* genes through partial alleviation of glucose repression. mRNA levels of *GAL* genes (*GAL1*, *GAL4*, and *GAL80*) indeed increased upon overexpression of *tTUP*. The results presented in this study illustrate that alteration of global regulatory networks through overexpression of the identified targets (*SNR84* and t*TUP1*) is as effective as overexpression of a rate limiting metabolic gene (*PGM2*) in the galactose assimilation pathway for efficient galactose fermentation in *S. cerevisiae*. In addition, these results will be industrially useful in the biofuels area as galactose is one of the abundant sugars in marine plant biomass such as red seaweed as well as cheese whey and molasses.

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KEYWORDS: glucose repression; gene target identification; genomic library; truncated transcriptional activator; small nuclear RNA

Introduction

Currently, biofuels are produced predominantly from food crops such as corn and sugarcane in the case of ethanol, and

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palm oil and soybean in the case of biodiesel. Using food crops for the production of biofuels has caused a controversy of ''fuel versus food.'' Due to the abundance and sustainability of feedstocks, a lot of research efforts are placed on commercializing the production of biofuels from terrestrial non-food crops such as switchgrass and Miscanthus, and agricultural and forestry residues (corn stover and bagasse). Another potential source of biofuels is marine plant biomass such as macroalgae and microalgae. Macroalgae has several attributes that would make it an attractive renewable source for the production of biofuels. First, production yields of marine plant biomass per unit area are much higher than those of terrestrial lignocellulosic biomass. Second, marine biomass can be depolymerized relatively easily compared to lignocellulosic biomass as it does not contain recalcitrant lignin and cellulose crystalline structures. Third, the rate of carbon dioxide fixation by marine biomass is much higher than by terrestrial biomass, making it an attractive option for sequestration and recycling of carbon dioxide (Packer, 2009). Unlike lignocellulosic biomass, the major sugar in the red seaweed is galactose. Unfortunately, ethanol yield and productivity from galactose are significantly lower than those from glucose (Ostergaard et al., 2000b) though *Saccharomyces cerevisiae* is inherently capable of fermenting galactose into ethanol.

Galactose utilization in *S. cerevisiae* is mediated by the Leloir pathway where galactose is phosphorylated to galactose-1-phophate by galactokinase (*GAL1*) and then isomerized to glucose-1-phosphate by galactose-1-phosphate uridyltransferase (*GAL7*). Phosphoglucomutase and phosphomannomutase (*GAL5*/*PGM2*) convert glucose-1-phosphate into glucose-6-phosphate which can be shunted both to the glycolytic pathway and the pentose phosphate pathway (Boles et al., 1994; Hofmann et al., 1994; Tiwari and Bhat, 2008). Galactose utilization of *S. cerevisiae* has been extensively studied as a model system for understanding transcriptional control mechanisms of gene regulation in eukaryotic cells (Douglas and Hawthorne, 1964; Ideker et al., 2001; Lohr et al., 1995). Gal4 and Gal80 are main transcriptional regulators of the *GAL* genes which are repressed very tightly in the presence of glucose (Johnston et al., 1994). Binding of the transcriptional activator Gal4 on upstream activation sites (UAS) of the *GAL* genes is required for induction of the *GAL* genes whereas the transcriptional repressor Gal80 inhibits the function of Gal4 by binding tightly to Gal4 (Leuther and Johnston, 1992; Wu et al., 1996). When glucose is present, a Mig1-Tup1-Ssn6 protein complex binds to the upstream repression sites (URS) of the *GAL4* and *GAL*1 genes, resulting in a tight repression of the *GAL* genes (Keleher et al., 1992; Nehlin et al., 1991; Treitel and Carlson, 1995). In addition to the aforementioned transcriptional regulators, *GAL6* is known to negatively regulate the *GAL* genes (*GAL1*, *GAL2*, and *GAL7*) by affecting mRNA degradation of the *GAL* gene transcripts (Ostergaard et al., 2001).

Saccharomyces cerevisiae utilizes galactose much slowly compared with glucose. Two different approaches were successfully applied to improve galactose fermentation. The first approach improved galactose utilization through engineering of the regulatory network controlling the expression of the *GAL* genes. Ostergaard et al. (2000a) were able to increase metabolic flux through the galactose utilization pathway by deleting three negative regulatory genes (*GAL6*, *GAL80*, and *MIG1*) and overexpressing *GAL4*. The resulting strain showed a 19% increase in specific galactose uptake rate (3.57 vs. 3.01 mmol gal/g cell h) and a 154% increase in specific ethanol production rate (4.19 vs. 2.71 mmol ethanol/g cell h) compared with the parental strain. The second approach was to overexpress *PGM2*, a metabolic gene coding for phosphoglucomutase. *PGM2* had been identified as a highly expressed gene in the strain harboring the engineered regulatory network (*ΔGAL6*, *ΔGAL80*, and *ΔMIG1* plus overexpression of *GAL4*) through DNA microarray study (Bro et al., 2005). Overexpression of *PGM2* led to a 70% increase in galactose uptake rate compared to the parental strain. These results suggest that metabolic flux through galactose utilization pathway can be increased either by engineering regulatory network or perturbation of a single metabolic enzyme.

Identification of gene targets that are responsible for desired phenotypes using genome-wide perturbation libraries has been demonstrated (Santos and Stephanopoulos, 2008a). Genomic libraries on multi-copy plasmids and transposon knockout libraries have been employed for gene targeting in the context of many beneficial phenotypes such as improved xylose assimilation in yeast (Jin et al., 2005), higher lycopene accumulation in *Escherichia coli* (Alper et al., 2005; Jin and Stephanopoulos, 2007), enhanced polyhydroxybutyrate accumulation in *Synechocystis* sp (Tyo et al., 2006), and higher L-tyrosine production in *E. coli* (Santos and Stephanopoulos, 2008b). These genetic tools enable perturbed targets to be traceable and transferable. In this study, we applied an inverse metabolic engineering approach to improve galactose fermentation in *S. cerevisiae*: we first obtained transformants of higher galactose utilization capability by introducing a genome-wide library into the wild type strain and then characterized the screened transformants to identify the targets of modified genes. Based on the inverse metabolic engineering approach, we have identified three gene targets whose overexpression elicited improved galactose utilization; (1) *SEC53* coding for phosphomannomutase which exhibits also phosphoglucomutase activity, (2) *SNR84* coding for a small nuclear RNA, and (3) a truncated form of *TUP1* coding for a global repressor of transcription.

Materials and Methods

Strains and Plasmids

Saccharomyces cerevisiae CEN.PK2-1D (*MATα ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8^C SUC2*) (Stark and Stansfield, 2007; Van Dijken et al., 2000) and *S. cerevisiae*

L2612 (*MATα leu2-3 leu2-112 ura3-52 trp1-298 can1 cyn1 gal*$^+$) (Cho et al., 1999) were used for galactose fermentation experiments. *S. cerevisiae* SC288C was used for constructing genomic libraries. *E. coli* DH5α (F^- *recA1 endA1 hsdR17* [r_K^- m_K^+] *supE44 thi-1 gyrA relA1*) (Invitrogen, Gaithersburg, MD) was used for gene cloning and manipulation.

Media and Culture Conditions

Yeast strains were routinely cultivated at 30°C in YP medium (10 g/L yeast extract, 20 g/L Bacto peptone) with 20 g/L glucose. To select transformants using a *TRP1* selectable marker, yeast synthetic complete (YSC) medium containing 6.7 g/L yeast nitrogen base plus 20 g/L glucose, 20 g/L agar, and CSM-Leu-Trp-Ura (Bio 101, Vista, CA). For ethanol fermentation, yeast cells were grown in 50 mL of YSC medium containing galacotse and/or glucose in a 250 mL flask.

Construction of *S. cerevisiae* Genomic Library

Saccharomyces cerevisiae genomic DNA was isolated from the strain S288C (*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1*). The genomic DNA was then partially sheared with sonication. The DNA fragments in the size range from 2 to 5 kb were isolated and ligated into the blunt-ended *BamH*I site of the pRS424 plasmid (Christianson et al., 1992). While the genomic library constructed using the S288C strain offers faster and easier identification of gene targets because its genomic sequence has been determined, there is a chance that the mutated genes (especially *gal2* coding for galactose permease) in the genome of the S288C strain might have not been captured in our search.

Construction of *PGM2* Overexpression Strain and *TUP1* Knockout Strain

To construct *PGM2* overexpression vector, pRS424-P_{TEF}-*PGM2*, the ORF of *PGM2* was amplified by PCR with the primers, 5_PGM2: 5′-GGCGGATCCA TGTCATTTCA AATTGAAACG-3′ and 3_PGM2: 5′-GGCCTCGAGT TAAGTACGAA CCGTTGGTT. The amplified DNA fragment was cleavaged by *BamH*I and *Xho*I, and then ligated into the *BamH*I and *Xho*I site of pRS424TEF. Deletion of *TUP1* was performed as described by Brachmann et al. (1998). A linear DNA cassette was prepared by PCR with a pair of primers containing 40 bp DNA sequences of 5′ upstream and 3′ downstream of the *TUP1* using the pRS424 plasmid as a template for amplifying *TRP1*. The resulting linear DNA cassette was transformed into *S. cerevisiae* CEN. PK2-1D cell using Yeast EZ-transformation Kit (BIO 101). The *TUP1* deletion (*Δtup1*) mutant was selected in YSC medium containing uracil and leucine.

mRNA Abundance Measurement by RT-PCR

Yeast cells from the cultures were harvested at different time points (12 and 48 h), and mRNAs were extracted by RNAeasy kit (Qiagen, Valencia, CA). Using the mRNA as a template, cDNA was constructed using random oligonucleotides and the Reverse Transcription System Kit (Promega, Madison, WI). The abundances of mRNAs coding for *GAL* genes were measured by amplifying the genes using the corresponding cDNAs as templates for PCR. The primers used for the amplification are listed in the Table I. An expression level of *TRP1* was used as a loading control.

In vitro Phosphoglucomutase (PGM) Activity Measurement

Phosphoglucomutase activities were assayed at 30°C by monitoring the reduction of NAD^+ in a 100 μL reaction mixture at 340 nm using a spectrophotometer (Molecular device spectra M2, Palo Alto, CA). The reaction mixture contained 6.5 mg/mL NAD trihydrate, 40 mg/mL glucose-1-phosphate dipotassium dehydrate, 0.5 mg/mL glucose-1,6-diphosphate, tetracyclohexylammonium salt, 150 μ/mL glucose-6-phosphate dehydrogenase in imidazole buffer. The protein contents of cell extracts were determined by the method of Bradford assay.

Table I. Oligonucleotide sequences of primers used for RT-PCR experiments.

Target mRNA	Primer sequence	Amplicon size (bp)
TRP1	Forward ATGTCTGTTATTAATTTCACAG	675
	Reverse CTATTTCTTAGCATTTTTGA	
MIG1	Forward ATGCAAAGCCCATATCCAAT	1,515
	Reverse TCAGTCCATGTGTGGGAAGG	
GAL1	Forward ATGACTAAATCTCATTCAGA	1,587
	Reverse TTATAATTCATATAGACAGC	
GAL4	Forward ATGAAGCTACTGTCTTCTAT	2,646
	Reverse TTACTCTTTTTTTGGGTTTG	
GAL80	Forward ATGGACTACAACAAGAGATC	1,308
	Reverse TTATAAACTATAATGCGAGA	

Analytical Methods

Glucose and galactose concentrations were determined by high-performance liquid chromatography (Waters, Milford, MA) with a Sugar-PKA1 column (Waters). Ethanol concentration was determined by gas-chromatography with an HP-1 column (Agilent, Wilmington, DE). Cell growth was monitored by determining the optical density at 600 nm (A_{600}). One A_{600} unit was equivalent to 0.3 g cell/L for *S. cerevisiae*.

Results

Screening of Transformants Exhibiting Improved Galactose Fermentation Capability

After introducing the *S. cerevisiae* genomic library on a multi-copy plasmid into *S. cerevisiae* CEN.PK2-1D, three different selection methods were employed to isolate transformants that exhibited improved galactose fermentation. First, we plated the transformants into big agar-plates (150 mm in diameter) containing galactose as a sole carbon source. Each plate contained about 300–500 colonies for visual screening. About 30,000 colonies were screened visually to identify fast-growing transformants. In the first round of screening, 96 fast-growing colonies were selected and tested for improved galactose fermentation in various culture conditions (20 g/L of glucose and 20 g/L of galactose, 40 g/L of galactose, or 100 g/L of galactose). Two plasmids from fast-growing colonies (#1-2 and #1-4) were independently isolated after confirming the positive effects of the plasmids by re-transformation into different parental strains. Sequence analysis of the isolated plasmids revealed that both plasmids contained *SEC53* (Fig. 1A). It was quite impressive that the two randomly picked colonies out of the 96 colonies contained the same *SEC53* insert. *SEC53* encodes for phosphomannomutase which converts mannose-1-phosphate into mannose-6-phosphate. However, the enzyme is reported to possess phosphoglucomutase activity as well (Boles et al., 1994). The beneficial effects of *SEC53* overexpression might be caused by the enhanced activity of phosphoglucomutase catalyzing glucose-1-phosphate into glucose-6-phosphate which has been already suggested as a rate-limiting step in galactose utilization (Bro et al., 2005).

Second, we performed serial subcultures of transformants ten times using the liquid medium containing galactose in order to enrich fast-growing transformants. The serial transfer was practiced by inoculating old cultures into fresh cultures every 24 h. Initial cell densities were adjusted to the same cell density (A_{600} of 0.1) during the transfers. We observed very rapid increases in cell density and ethanol production after the second transfer. These rapid increases may be caused by induction or adaptation effects due to transition from glucose to galactose since the initial culture was grown on glucose. During the serial transfers, we observed gradual increases in cell density and ethanol production until the fourth transfers (Fig. 1B). This suggests that fast-growing transformants on galactose were enriched. However, cell densities and ethanol concentrations varied after the fourth transfer until the serial transfers were finished. After the tenth transfer, 96 single colonies were isolated randomly and their capabilities of galactose fermentation were investigated. In addition, the reproducibility of improved galactose fermentation after re-transformation of the isolated plasmids from the outperforming colonies was checked. Two colonies (#2-3 and

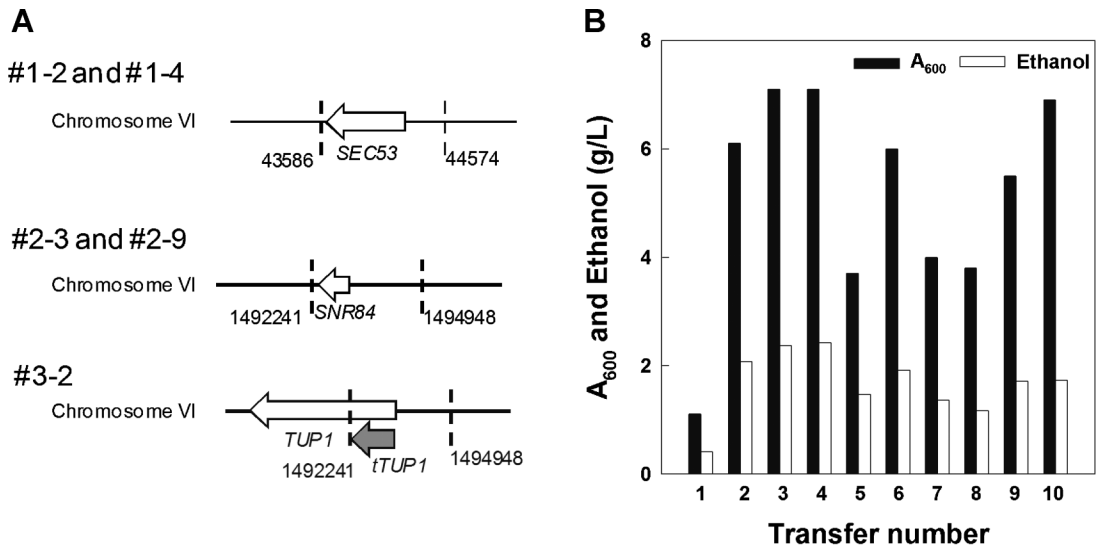

Figure 1. Screening from genome-wide perturbation libraries for improved galactose fermentation. (**A**) Genomic coordinates of the identified targets from three different screening methods. (**B**) Enrichment of fast-growing transformants on galactose through serial subcultures.

#2-9) showed both better growth and ethanol production from galactose compared with the control strain. The isolated plasmids from the two selected transformants were sequenced in order to identify the inserts. Interestingly, #2-3 and #2-9 colonies harbored an identical plasmid containing a C-terminal region of *HLR1*, an N-terminal region of *RBA50*, and the full length *SNR84* (Fig. 1A). *SNR84* encodes an H/ACA box small nuclear RNA (snoRNA) and is known to be involved in pseudouridylation of ribosomal RNA. Although it is currently unknown how the amplification of the small nuclear RNA affects galactose utilization, the identification of the same insert from two best galactose-fermenting transformants suggests that the beneficial effect of *SNR84* amplification on galactose metabolism is significant.

Third, a combined method of the above mentioned methods was employed by screening fast-growing colonies on agar plate containing 40 g/L of galactose after serial transfer of the culture three times on a liquid medium containing 100 g/L of galactose. We decided to perform serial transfer three times because we observed good enrichment patterns from our previous enrichment experiments (Fig. 1B). Forty eight colonies exhibiting bigger colony sizes on the agar plates were selected and subjected to the further selection procedure as described before. Finally, two colonies were selected (#3-2 and #3-40). The plasmid isolated from #3-2 did not contain any intact open reading frame but it contained a promoter region and an N-terminal region of *TUP1* which is one of global transcriptional regulators in yeast. Detailed sequence analysis revealed that the insert was truncated at the 280 amino acid residue by the stop codon adjacent to the ligation site of genomic DNA fragments. Notably, the same insert containing *SNR84* which had been identified in #2-3 and #2-9 was also identified from the plasmid from #3-40. The multiple identification of the same target (*SNR84*) from different screening methods suggests that *SNR84* plays an important role in galactose utilization in yeast.

Improved Growth and Fermentation on Galactose by the Amplification of *SNR84*

Among the identified targets, *SNR84*, coding for a small nuclear RNA, was identified by the two different selection methods. These results suggest that the function of *SNR84* is closely involved in galactose metabolism. The physiological function of *SNR84* has not been reported in *S. cerevisiae*. We investigated fermentation profiles of transformants harboring extra copies of *SNR84* in a multi-copy plasmid in various conditions (20 g/L of glucose, 40 g/L of galactose, 100 g/L of galactose, or a mixture of 20 g/L of glucose and 20 g/L of galactose). While the amplification of *SNR84* resulted in almost identical fermentation profiles in a glucose-containing medium, it consistently increased the galactose uptake and ethanol production rates significantly in galactose-containing media compared with the control stain.

Several representative fermentation profiles are shown in Figure 2. For the high cell density fermentation (initial $A_{600} \sim 20$), the transformant overexpressing *SNR84* consumed 40 g/L of galactose in 20 h (Fig. 2B) while the transformant harboring the control plasmid (pRS424) consumed the same amount of galactose in 28 h (Fig. 2A), which was a moderate improvement in galactose utilization. As a result, volumetric ethanol productivity from galactose increased by 30% (0.6 vs. 0.9 g ethanol/L h). Next, galactose fermentation by the transformant overexpressing *SNR84* was compared with that by the control strain under the low cell density conditions. Low cell density fermentation is better suited to assess cells' capacity of fermentation while high cell density fermentation is more relevant to assess process viability. We observed a more dramatic effect with *SNR84* overexpression in the moderate cell density (Fig. 2C and D) fermentation. Under the low cell density conditions, ~40% of galactose remained unconsumed after 28 h in the fermentation with the control strain, whereas all of galactose was completely consumed by the same time in the fermentation with the SNR84 transformant. In summary, the amplification of *SNR84* significantly increases the galactose consumption rate and ethanol productivity compared with the control strain using galactose as a sole carbon source or as a mixture with glucose.

Improved Growth and Fermentation on Galactose by Overexpressing a Truncated Form of *TUP1*

During the evaluation of fermentation characteristics of the many selected strains under various conditions, we found that the #3-2 strain exhibited not only improved galactose fermentation but also significantly lowered the degree of glucose repression compared to the control strain; i.e., the #3-2 strain exhibited a faster transition from glucose to galactose metabolism. The plasmid from the #3-2 strain contained a truncated form of *TUP1*. Tup1 is a general transcriptional co-repressor which coordinates glucose repression with Mig1 and Ssn6 (Keleher et al., 1992; Treitel and Carlson, 1995). Two main *GAL* genes (*GAL1* and *GAL4*) whose gene products play important roles in induction of the galactose utilization pathway are repressed by the Mig1-Tup1-Ssn6 complex. Tup1 possesses a C-terminal repression domain at amino acid residues from 288 to 389 (Zhang et al., 2002). Therefore, the truncation at the amino acid residue of 280 might have resulted in a mutant *TUP1* without the C-terminal repression activity. Consequently, leaky expression of *GAL1* and *GAL4* genes might be caused through the action of the dominant negative mutant Tup1 (Fig. 3A).

The transformant with the truncated *TUP1* (t*TUP1*) in a multi-copy plasmid showed drastically improved galactose fermentation compared with the control (Fig. 3B–E). The ethanol production rates were drastically increased (1.33 vs. 0.68 g ethanol/L h) by overexpression of t*TUP1* (Fig. 3B vs. Fig. 3C). The positive effect by overexpression of t*TUP1* was

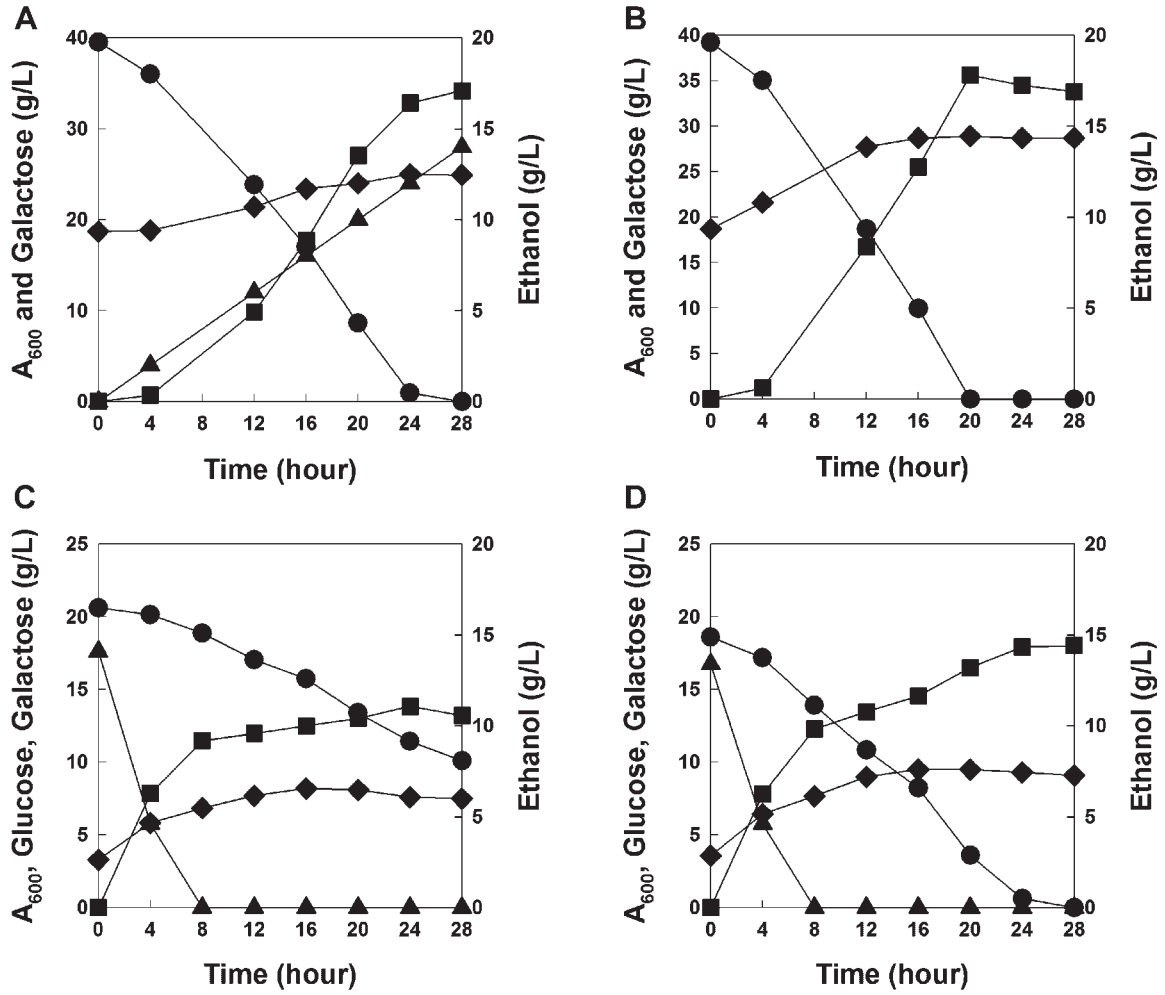

Figure 2. Galactose fermentation profiles by *S. cerevisiae* CEN.PK with the control plasmid (**A** and **C**), and the transformant with the isolated plasmid containing *SNR84* (**B** and **D**). (A and B): 40 g/L of galactose; (C and D): mixture of 20 g/L of glucose and 20 g/L of galactose. Symbols: cell density (◆), ethanol (■), glucose (▲), and galactose (●).

also observed in the fermentation experiments using a mixture of glucose and galactose (Fig. 3D vs. Fig. 3E). Interestingly, the transition period from glucose to galactose fermentation was significantly shortened in the transformant with t*TUP1*, which suggests that glucose repression was alleviated by the overexpression of t*TUP1*. As a result, the transformant consumed both sugars (glucose and galactose) almost three times faster than the parental strain (8 vs. 24 h).

In order to elucidate the putative action mechanism of t*TUP1*, we performed galactose fermentation experiments with a *TUP1* knockout mutant (*Δtup1*). The *Δtup1* mutant grew faster than the parental strain and the transformant with t*TUP1* (Fig. 4A). However, the knockout mutant produced much smaller amounts of ethanol from galactose (Fig. 4B). These results suggest that deletion of *TUP1* is not beneficial for ethanol production from galactose and certain amounts of intact Tup1 is necessary for effective galactose fermentation. We speculate that a certain degree of glucose repression is required for optimal ethanol production through inhibition of the respiratory pathway as exemplified by the Crabtree effect. Tup1 is known to be involved in the repression of as many as 3% genes of *S. cerevisiae* including glucose repressible genes (DeRisi et al., 1997; Smith and Johnson, 2000). The detrimental effects by the deletion of *TUP1* is probably due to the nature of Tup1 participating in the control of expression of a tremendous number of genes involved in ethanol production. It is plausible to postulate that the mutant Tup1 (encoded by t*TUP1*) competes with the functional Tup1 for binding on the Ssn6-Mig1 complex, and the lack of C-terminal repression domain in the mutant Tup1 might prevent the mutant Tup1-Ssn6-Mig1 complex from repressing genes located at the downstream regions. This competitive inhibition by the mutant Tup1 will reduce the formation of the functional Tup1-Ssn6-Mig1 complex, leading to partial alleviation of glucose repression (Fig. 5A).

In order to test our hypothesis, we performed RT-PCR experiments to check whether or not the expression levels of

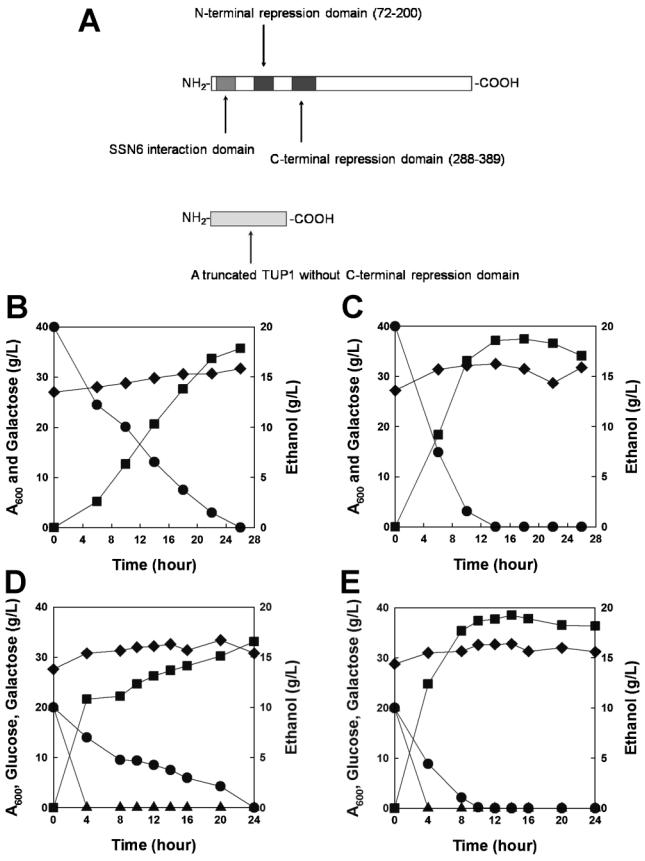

Figure 3. Improved ethanol fermentation from galactose by overexpression of t*TUP1*. (**A**) Illustration of the truncated amino acid residue in the context of the known functional domains of *TUP1*. Galactose fermentation profiles by *S. cerevisiae* CEN.PK with the control plasmid (**B** and **D**) and transformants overexpressing t*TUP1* (**C** and **E**). (B and C): 40 g/L of galactose; (D and E): mixture of 20 g/L of glucose and 20 g/L of galactose. Symbols: cell density (◆), ethanol (■), glucose (▲), and galactose (●).

glucose-repressible *GAL* genes (*GAL1*, *GAL4*, and *GAL80*) and *MIG1* (a glucose repressor) are affected by overexpression of t*TUP1*. The mRNA levels of *GAL1* and *GAL80* were significantly higher in the transformant expressing t*TUP1* than the control strain during galactose fermentation (Fig. 5B–D). Though lower than those of *GAL1* and *GAL80*, the expression level of *GAL4* was also higher in the transformant expressing t*TUP1* than the control. The higher mRNA expression levels of *GAL1* and *GAL4* are consistent with our expectations that the Ssn6-Mig1-the mutant Tup1 complex is no longer effective as a repressor of *GAL1* and *GAL4*. In contrast to our expectations, the expression levels of *MIG1* were consistently higher in the transformant expressing t*TUP1* than the control. Despite the high level of *MIG1*, the expression levels of *GAL1* and *GAL4* are higher since the expression of *GAL1* and *GAL4* is no longer controlled by the Ssn6-Mig1-Tup1 complex (Fig. 5A). These results support our hypothesis that t*TUP1* improves galactose fermentation through the mechanism that alleviates the repression of *GAL* genes. We also observed that the improved galactose utilization by overexpression of t*TUP1* resulted in an improved ethanol production rather than improved cell growth. We also investigated the effects of overexpression of t*TUP1* on the utilization of other sugars (maltose, mannose, and fructose). However, we did not observe any beneficial or detrimental effects on sugar utilization rate, cell growth rate, and ethanol production rate by the overexpression of t*TUP1* when we used those three sugars as a sole carbon source (Fig. 6). These results suggest that the competition between the truncated Tup1 and wild type Tup1 for the formation of the repression complex with Mig1 and Ssn6 might resulted in selective de-repression of *GAL* genes although the complex binds to many other genes.

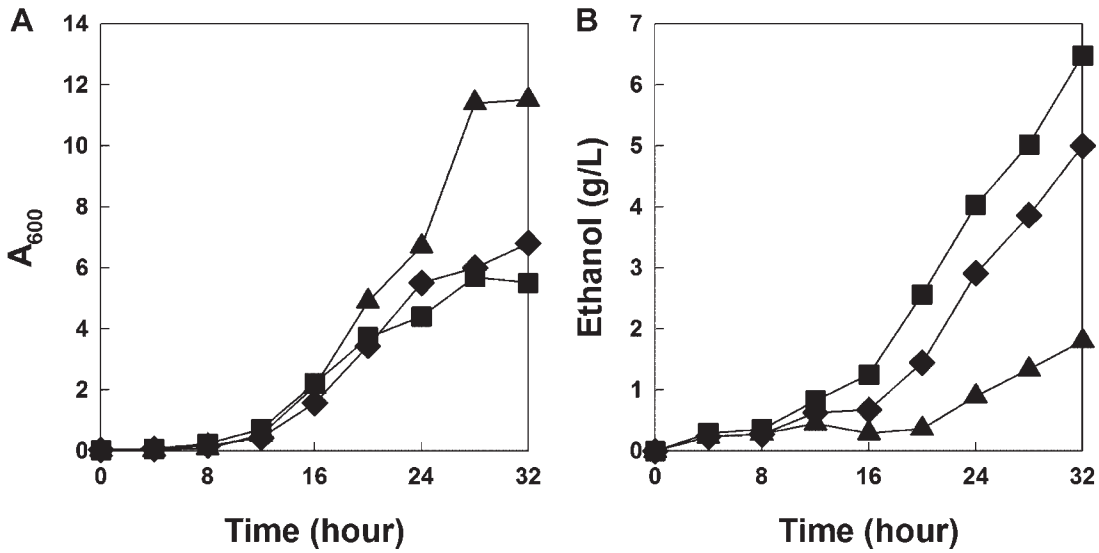

Figure 4. Comparison of cell growth (**A**) and ethanol production (**B**) of the *Δtup1* mutant (▲), the transformant overexpressing t*TUP1* (■), and the control strain (◆).

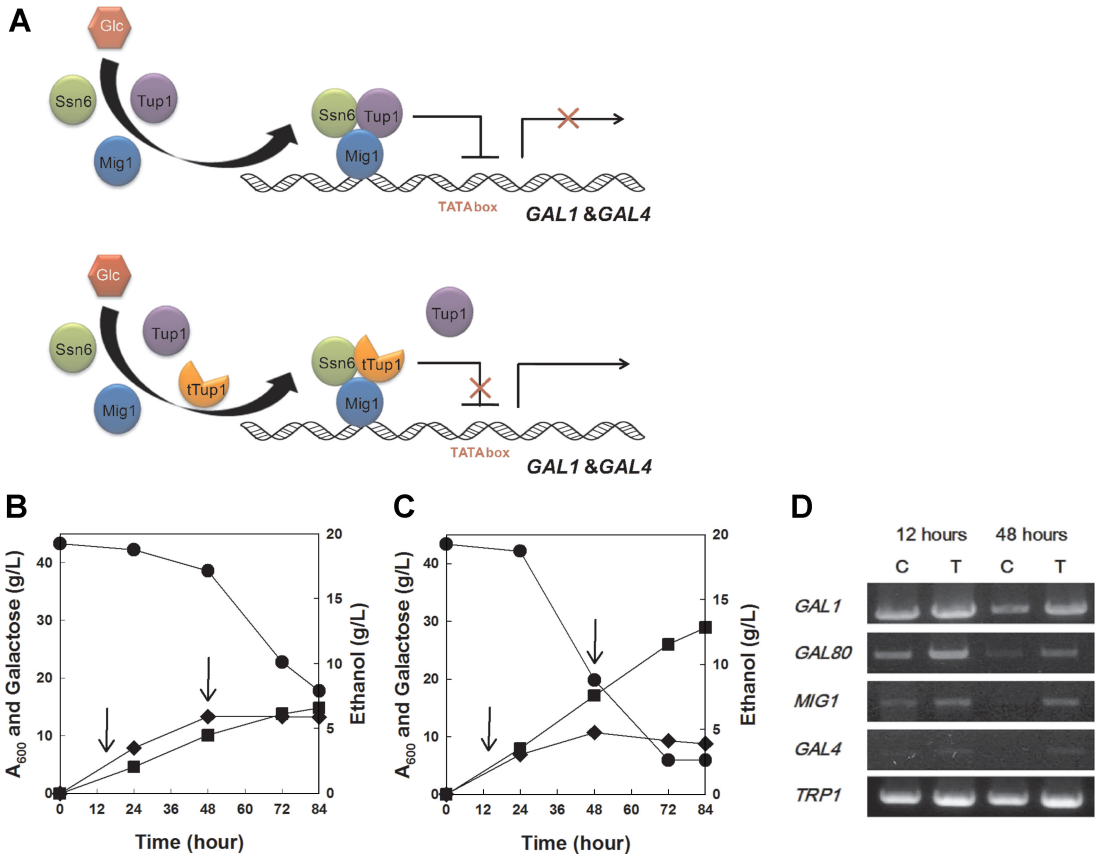

Figure 5. Putative action mechanism of t*TUP1* on the improvement of ethanol fermentation from galactose (**A**). Validation of the proposed mechanism of t*TUP1* for improved galactose utilization through up-regulation of *GAL* genes during galactose fermentation (**B**: fermentation profiles of the control strain, **C**: fermentation profiles of the t*TUP1* overexpressing mutant, and **D**: comparison of mRNA levels of *GAL* genes (*GAL1*, *GAL4*, and *GAL80*)) and a glucose repressor (*MIG1*) in the transformant overexpressing t*TUP1* (T), and the control strain (C). Symbols: cell density (◆), ethanol (■), and galactose (●). *TRP1* gene was chosen as a control gene. mRNAs were isolated from the samples noted by the arrows. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

Finally, we investigated whether or not there are synergistic effects between the previously identified gene target (*PGM2*) and new targets (*SNR84* and t*TUP1*) presented in this study on galactose fermentation (Fig. 7). Overexpression of *PGM2* in a multi-copy plasmid under the control of TEF promoter improved galactose fermentation as previously reported (Bro et al., 2005) (Fig. 7B) compared to the control strain (Fig. 7A). Nevertheless, we observed a slightly better improvement in galactose fermentation by simultaneous overexpression of the *SNR84* and t*TUP1* (Fig. 7C and D). These results suggest that synergistic improvement of galactose fermentation may not be induced by the combinatorial perturbations of the identified targets.

Discussion

Manipulation of metabolic fluxes either in endogenous or heterologous metabolic pathways through genetic and environmental perturbations has been applied to many biotechnological applications. However, identification of the genes which are highly related with desired metabolic phenotypes has been regarded as a bottleneck of metabolic engineering. The inverse metabolic engineering approach (Bailey et al., 1996; Jin et al., 2005; Santos and Stephanopoulos, 2008a; Tyo et al., 2007) enabled us to identify endogenous gene targets for improving galactose utilization in *S. cerevisiae*. One metabolic enzyme (*SEC53*), a small nuclear RNA (*SNR84*), and a truncation mutant of a global transcriptional factor *TUP1* were identified as beneficial genetic perturbations for galactose fermentation when the targets are introduced into *S. cerevisiae* using a multi-copy plasmid. However, none of the targets are directly associated with both the galactose metabolic pathway and the known regulatory, or metabolic networks of galactose metabolism. Although the implementation of the identified targets into *S. cerevisiae* caused physiological and metabolic changes in galactose metabolism, we did not observe such changes in glucose metabolism, which suggests that action mechanisms of the identified targets in this study are selective for improved galactose fermentation. As shown in the current study and previous studies (Jin et al., 2005;

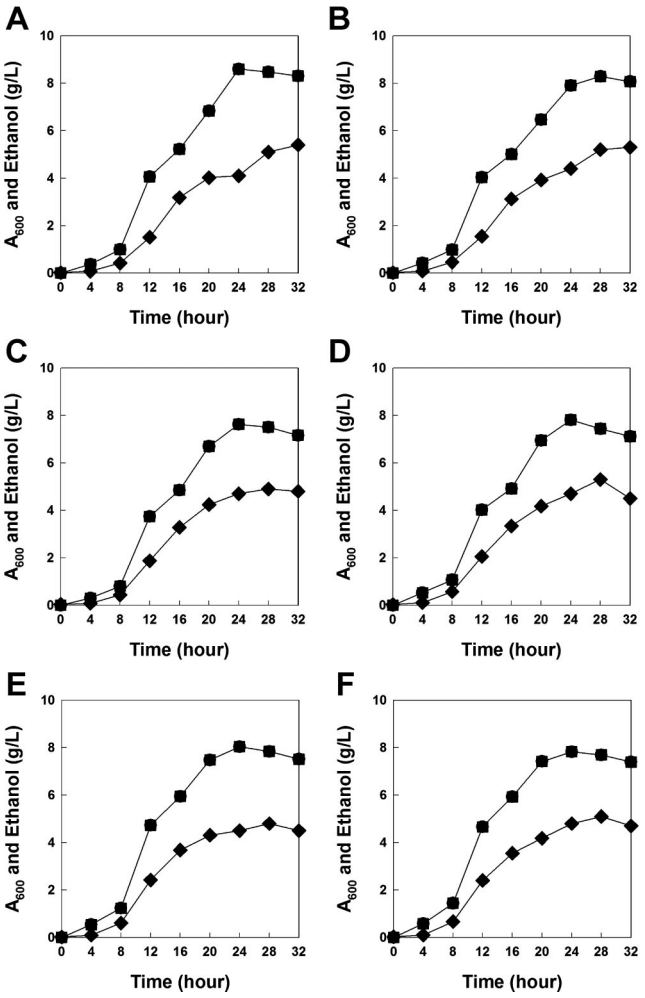

Figure 6. Comparison of cell growth and ethanol production of the transformant overexpressing t*TUP1* (**A**, **C**, and **E**) and the control strain (**B**, **D**, and **F**) using various carbon sources a sole carbon source. Maltose (20 g/L) (A and B), 20 g/L of mannose (C and D), and 20 g/L of fructose (E and F). Symbols: cell density (◆) and ethanol (■).

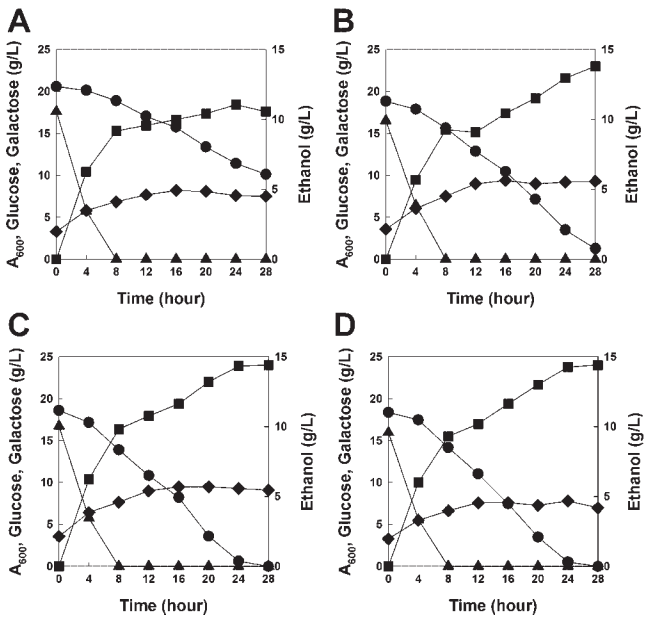

Figure 7. Glucose and galactose fermentation profiles by *S. cerevisiae* CEN.PK with the control plasmid (**A**), and transformants with a *PGM2* overexpression cassette (**B**), the isolated plasmids containing *SNR84* (**C**), and a truncated *TUP* (**D**). Symbols: cell density (◆), ethanol (■), glucose (▲), and galactose (●).

Santos and Stephanopoulos, 2008a; Tyo et al., 2007), the inverse metabolic engineering approach is effective to discover novel targets which not only exploited for improvement of intended phenotypes, but also generate new understanding or at least hypothesis on how the metabolism of interest is controlled.

Among the identified targets from this study, *SEC53* can be classified as a confirmatory target because it may share the similar action mechanisms with *PGM2*. *PGM2* was already identified as an overexpression target through DNA microarray studies of two mutants generated by the perturbation of the *GAL* gene regulatory network (*GAL1* overexpression and *Δgal6 Δgal80 Δmig1*) (Bro et al., 2005). The protein coded by *SEC53* was reported to exhibit similar biochemical activities of *PGM2* and overexpression of *SEC53* is known to complement *pgm1*/*pgm2* mutants (Boles et al., 1994). Therefore, the beneficial effects of *SEC53* overexpression on galactose metabolism would be caused by the similar mechanism of *PGM2* overexpression. We measured in vitro phosphoglucomutase activities of the transformants overexpressed with the identified targets after growing on different carbon sources (Table II). Although we observed a small increase in PGM activity by the overexpression of *SEC53*, the overexpressions of *PGM2*, *SNR84*, and t*TUP1* resulted in significant increases in PGM activity. This overlap validates our methodology in identifying gene targets that improve galactose fermentation using a genomic library. Similar approaches have been successfully applied to identify gene targets eliciting many phenotypes of biotechnological importance, such as improved xylose utilization (Jin et al., 2005), higher tolerance to ethanol and isobutanol (Hong et al., 2010) and enhanced lycopene production (Jin and Stephanopoulos, 2007; Kang et al., 2005). All of the studies were able to discover novel genetic perturbations and to confirm existing or known genetic perturbations responsible for desired phenotypes. These suggest that the

Table II. In vitro phosphoglucomutase activities in a control strain and the transformants with the identified target genes harvested after growth in media containing glucose, galactose, a mixture of glucose and galactose.

	In vitro PGM activity (mU/mg)		
Strain	Glucose	Galactose	Glucose + Galactose
Control	12.01 ± 0.44	14.98 ± 0.05	11.00 ± 0.08
SEC53	12.31 ± 0.21	16.05 ± 0.84	13.97 ± 1.15
PGM	15.25 ± 0.21	19.43 ± 0.36	18.24 ± 0.88
SNR84	15.07 ± 0.70	21.72 ± 0.52	16.30 ± 0.16
t*TUP1*	20.44 ± 0.24	31.47 ± 0.07	29.70 ± 0.88

inverse metabolic engineering approach based on genomic library is robust and capable of discovering new gene targets.

Known genetic perturbations for improved galactose fermentation in yeast may be classified to two types. The first type improves ethanol production (yield and rate) at the expense of cell growth. Deletion of *GAL80*, *MIG1*, *GAL6*, and overexpression of *GAL4* are examples of genetic perturbations in this category. The second type improves both ethanol production and cell growth on galactose. The overexpressions of *PGM2, SEC53*, *SNR84*, and a truncated *TUP1* are the examples of gene targets in this second category. Actually, we did not observe any cell growth defects accompanied by the improved ethanol production in all experiments performed with newly identified targets. This makes sense because we selected the targets based on cell growth rather than ethanol production on galactose.

An unexpected but very interesting finding in this study was that a truncated form of *TUP1* was identified as an overexpression target through our selection even though we intended to identify full-length genes using a genomic library. Most of transcriptional activators are composed of a binding domain and an effecter domain. As such, genomic libraries generated using random fragmented DNAs can encode mutants which might exert altered control of gene expression through truncation of either binding or effector domains. Especially, the truncation at the C-terminal region would not affect expression of the truncated protein since its promoter region is intact. Therefore, it can often result in generating a mutant transcriptional factor which displays a totally different transcriptional control as in the case of the truncated *TUP1* presented in this study. Identification of *SNR84* as an overexpression target for improved galactose fermentation also is an intriguing finding. Although we do not fully understand the how overexpression of *SNR84* enhances galactose metabolism, the improvement of galactose fermentation by the *SNR84* overexpression was quite impressive (Fig. 6C). These unexpected findings suggest that alteration of regulatory networks through gene targets (t*TUP1* and *SNR84*) which are not associated directly with galactose metabolism is as effective as the overexpression of a rate limiting metabolic enzyme (*PGM2*) in the galactose metabolic pathway for efficient galactose fermentation in *S. cerevisiae*.

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