RESEARCH

Open Access

Transcriptional regulatory networks of methanol-independent protein expression in *Pichia pastoris* under the *AOX1* promoter with trans-acting elements engineering

Lei Shi¹, Jinjia Wang¹, Xiaolong Wang¹, Yuanxing Zhang^{1,2}, Zhiwei Song^{3,4}, Menghao Cai¹ and Xiangshan Zhou^{1*}

Abstract

To explore the differences in the intracellular transcriptional mechanism in carbon-derepressed and wild-type *Pichia pastoris* strains fed with three different carbon sources. RNA in carbon-derepressed ($\Delta mig1 \Delta mig2 \Delta nrg1$ -Mit1; Mut) and wild-type (WT) *P. pastoris* fed with three different carbon sources (dextrose, glycerol, and methanol) were sequenced. Differentially expressed genes (DEGs) associated with these carbon sources were obtained and clustered into modules using weighted gene co-expression network analysis (WGCNA). Signaling pathway enrichment analysis was performed using KEGG, and protein to protein interaction (PPI) network was also constructed. A total of 2536 DEGs were obtained from three intersections, and some of them were enriched in carbon sources and involved in carbon metabolism, secondary metabolisms, and amino acid biosynthesis. Two modules, MEgreenyellow (involved in protease, oxidative phosphorylation, endoplasmic reticulum protein processing, folate carbon pool, and glycerol phospholipid metabolism pathways) and ME midnightblue (involved in protease, endocytosis, steroid biosynthesis, and hippo signaling pathways) were significantly correlated with the strain type. Eight hub genes and two sub-networks were obtained from PPI network. Sub-network A enriched in proteasomes pathway while sub-network B enriched in ribosome pathway. The genes involved in carbon metabolism, secondary metabolic, and amino acid biosynthesis pathways changed significantly under different carbon sources. The changes in proteasome and ribosome activities play roles in carbohydrate metabolism in the methanol-free P_{AOX1} start-up Mut strain.

Keywords: *Pichia pastoris*, RNA-seq, Carbon sources, *AOX1* promoter

Introduction

Pichia pastoris (*P. pastoris*) is a widely used recombinant expression system for exogenous genes having prokaryotic advantages of having a simple operation and rapid growth, and also has eukaryotic cell functions of post-translational modifications of proteins. Yeast expression systems include *Saccharomyces cerevisiae* system,

methylophilic yeast expression system, and merozoite yeast expression system (van den Hazel et al. 1996). The methylophilic yeast *P. pastoris* expression system is one of the most commonly used expression systems.

The most commonly used promoter on *P. pastoris* expression system is the alcohol oxidase 1 promoter (*AOX1*), which is a carbon source-dependent promoter whose transcription expression is strongly induced by methanol, but inhibited by other carbon sources, such as glucose, glycerol, or ethanol (Liang et al. 2014). *P. pastoris* synthesizes a large number of enzymes when using methanol as the sole carbon source. However, the

*Correspondence: xszhou@ecust.edu.cn

¹ State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China
Full list of author information is available at the end of the article

use of methanol has many disadvantages as it is toxic, flammable, and volatile, which makes methanol harmful to the human body (Polupanov et al. 2012; Portnoy et al. 2011). Methanol fermentation requires large amounts of oxygen, around three to four times higher than the amount needed when the carbon source is glucose (Lin et al. 2000). It also produces hydrogen peroxide (H_2O_2) during methanol metabolism, causing oxidative stress and proteolytic degradation of some proteins (Wang et al. 2010).

Researchers have worked on modifying the expression system of *P. pastoris* by various methods which avoid the use of methanol as a carbon source (Lu et al. 2007). The most convenient method to avoid methanol dependence was to engineer other highly expressed promoters that can replace *AOX1* and work without methanol induction (Chen et al. 2007). *AOX1* promoter has several regulatory factors, such as Mig1, Mig2, Nrg1, Mit1, and Prm1. Among these factors, Mig1, Mig2, and Nrg1 inhibit *AOX1* promoter in cells grown in glucose and glycerol, while Mit1 and Prm1 activate *AOX1* promoters in cells grown in methanol (Prielhofer et al. 2013). Nevertheless, the regulatory mechanism of *AOX1* promoter in *P. pastoris* remains unclear.

An earlier study (Lin-Cereghino et al. 2006) carried out a large-scale mutation screening analysis to identify regulatory factors and to elucidate their mechanisms and the studies also not recorded how to construct an engineered strain or to verify the success of the strain construction. To understand the effects of transcriptional repressors on *AOX1* and the transcriptional differences between carbon-derepressed strain (Mut) and wild-type (WT) strain, we modified expression levels of carbon source repressors in *P. pastoris* (Wang et al. 2017). Mig1, Mig2, and Nrg1 which act as carbon source repressors were inhibited and Mit1 levels were enhanced in the carbon-derepressed strain (Wang et al. 2017). In the present study, we analyzed the transcriptomes of WT and the carbon-source derepressed strain $\Delta mig1\Delta mig2\Delta nrg1$ -Mit1 (Mut) of *P. pastoris* under different carbon sources using RNA-seq method. Differential expression genes (DEGs) analysis was applied on different carbon sources with Mut and WT strains. Weighted gene co-expression network analysis (WGCNA) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment were used to explore the effect of different carbon sources on the efficiency of gene expression. Protein-protein interaction (PPI) network analysis was used to obtain key sub-networks and hub genes with Mut and WT strains. The goal of the study was to investigate the mechanisms underlying the carbon derepression and *AOX1* promoter regulation of the methanol-free P_{AOX1} start-up $\Delta mig1\Delta mig2\Delta nrg1$ -Mit1 strain.

Materials and methods

Pichia pastoris strains and culture conditions

Pichia pastoris wild-type (WT) strain GS115 (Life Technologies, Carlsbad, CA, USA) and the genetically engineered *P. pastoris* strain $\Delta mig1\Delta mig2\Delta nrg1$ -Mit1 (Mut) (constructed and preserved in our laboratory) were used for this study. A chemostatic cultivation medium for recombinant *P. pastoris* was used to provide a stable environment to ensure the reliability and repeatability of RNA-seq analysis. In brief, Mut and WT strains (stored at -80°C) were inoculated directly into 250-mL shaking flasks containing 50 mL yeast nitrogen-base/dextrose (YND) medium (0.67% yeast nitrogen base (YNB) and 1% glucose) or into 50 mL minimal glycerol media (MGY) as the primary seed culture till $OD_{600}=2-6$, respectively, and cultivated at 30°C for 24 h with agitation (200 rpm). Then, Mut and WT seed cultures were used to inoculate into 500 mL YND or MGY medium (0.67% YNB + 1% glycerol) till $OD_{600}=2-6$, respectively, as the second seed cultures and the cultures were then cultivated for 16 h at 30°C (200 rpm). Batch cultivation was started by inoculating the secondary seeds at a concentration of 1:10 (v/v) into a fermenter containing basal salts media (BSM). Batch fermentations were carried out at 30°C , pH 5.0 (controlled by 25% ammonium hydroxide), and a minimum of 30% dissolved oxygen concentration. When carbon source in the medium was depleted and the concentration of dissolved oxygen increased sharply, the chemostat medium using glucose (20.0 g L^{-1}), glycerol (20.0 g L^{-1}), or methanol (20.0 g L^{-1}) as the sole carbon source was supplemented. The fermentation broth is drained by a peristaltic pump to maintain a constant volume of the fermentation broth and the flowrate of glucose, glycerol, or methanol. Finally, they were inoculated into a 50 mL liquid MGY medium with an initial concentration of $OD_{600}=1$, and two parallel samples were inoculated for each strain. Samples were taken when the specific growth rate reached $\mu=0.03\text{ h}^{-1}$.

RNA extraction, construction of cRNA Library and High-throughput sequencing

The detection of RNA samples was provided by Beijing Novosource Bioinformation Technology Co. RNA concentration was measured using Qubit® RNA Assay Kit with Qubit® 2.0 Fluorometer (Life Technologies, CA, USA), and RNA integrity was assessed with the RNA Nano 6000 Assay Kit by the Agilent 2100 Bioanalyzer (Agilent Technologies, USA), and only the qualified samples were used for library construction and sequencing experiments. As shown in Table 1, a total of 12 samples were used for RNA-seq analysis, including WT strains in glucose, glycerol, and methanol conditions (named as W_D, W_G, and W_M) and Mut strain in the same three

Table 1 The *P. pastoris* samples used for sequencing

	Carbon sources		
	Dextrose (D)	Glycerol (G)	Methanol (M)
Strain type			
Mut (M)			
Duplicate 1	M_D_1	M_G_1	M_M_1
Duplicate 2	M_D_2	M_G_2	M_M_2
Wild-type (W)			
Duplicate 1	W_D_1	W_G_1	W_M_1
Duplicate 2	W_D_2	W_G_2	W_M_2

Mut indicates the genetically engineered *P. pastoris* strain $\Delta mig1\Delta mig2\Delta nrg1-Mit1$

conditions (named as M_D, M_G, and M_M). Each sample was done in duplicates. Illumina HiSeq2000 (Illumina, NY, USA) high-throughput sequencing system was used to sequence RNA of the samples.

Data pre-processing

Quality control of raw reads was processed by removing reads containing adapter sequences and reads with N (information cannot be determined) bases greater than 10%. Consequently, clean reads were obtained for downstream analysis. Alignment of the paired-end clean reads to the coding sequence (CDS) of GS115 in The European Bioinformatics Institute (EMBL) database was performed using TopHat v2 (Kim et al. 2013). Based on the predicted gene model of Cufflinks (Trapnell et al. 2012), the alternative splicing event on each sample was classified and counted by Asprofile software (Florea et al. 2013). All the data obtained from reads were assembled together with Cufflinks and then compared with known gene models by Cuffcompare software to predict new transcripts. Gene expression levels were quantified as the number of reads per kilobase of exon region per million reads (RPKM).

DEG analysis

DEGs were analyzed using the R package DEseq (Anders and Huber 2010; Anders and Huber 2012). For the samples with biological repetition, the original count was corrected by DEseq and the log of the count ratio between the two groups was calculated. Finally, $\log_2$ FoldChange (FC) value was obtained. Benjamini & Hochberg's method (Benjamini and Hochberg 1995) was used for multiple testing correction and genes with an adjusted *P*-value < 0.05 was selected as the cut-off criterion for the identification of DEGs.

DEGs were analyzed for growth in different sources, including W_G vs. W_D, M_G vs. M_D, W_M vs. W_D, M_M vs. M_D, W_M vs. W_G, and M_M vs. M_G and a total of 6 groups of DEGs were analyzed. Then, we

analyzed the DEGs intersection between Mut and WT strains in the same carbon comparison, such as the intersection DEGs between W_G vs. W_D and M_G vs. M_D. Then, a total of three groups of intersection DEGs were determined. Finally, we combined these three groups of intersection DEGs together, which were thought to be genes related to carbon sources.

WGCNA network construction

Using weighted gene co-expression analysis (WGCNA) R package (Langfelder and Horvath 2008), a co-expressed network of DEGs related to their carbon sources was constructed, and further screening of the gene sets which were significantly related to their strain type, and genes which were considered to be both related to the carbon source and the strain type were selected. Two methods were used to find modules related to the strain type. First, we calculated the correlation between each module feature vector gene and strain types. Second, the mean value of the correlation between the trait and gene expression in each module was taken as the significance. The greater the significance, the more the correlation between the module and the trait. By selecting the weight value of power, the whole network was distributed as a scale-free network, and the power with structure in R^2 reached 0.8 would be generally selected. Because of batch-related effects, sample heterogeneity, and experimental conditions used, we selected power = 9 and then, based on clustering and dynamic pruning of the data (Barabási 2009), we clustered the highly correlated genes into modules. Finally, gene sets were identified significantly related to the type of strain.

Functional enrichment analysis

KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/anno_iden.php) (Xie 2011; Wu et al. 2006) software was used to analyze the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto 2000) with DEGs related to carbon sources. Then, we also analyzed the KEGG database for genes obtained from the modules of WGCNA analysis with a cut-off of *P* value < 0.05 and gene count ≥ 2 , and these pathways were significantly related to the strain type and carbon source.

Construction of protein to protein (PPI) networks

Search tool for the retrieval of interacting genes (STRING v 10.0) (Szklarczyk 2014) was used to explore interactions between proteins. *Komagataella pastoris* was selected as the reference species to predict whether there were interactions between matched protein sets. Parameter PPI score was set to 0.7 (high confidence), which requires the interacting protein nodes to be included in the above module genes. Then, the PPI network was

built by Cytoscape software (version 3.2.0) (Shannon 2003) and cytoNCA plug-in (Tang et al. 2015) (v.2.1.6, <http://apps.cytoscape.org/apps/cytonca>) was used to analyze the topological properties of the node network. The results included degree centrality (DC), Betweenness Centrality (BC), and closeness centrality (CC), and the hub proteins were obtained by ranking the network topological properties of each node. We used MCODE (Degree Cutoff=2, Node Score Cutoff=0.2, K-core=2, MaxDepth=100) (Bandettini et al. 2012) in Cytoscape to analyze the subnetworks in the PPI network. The modules with score > 5 were chosen to perform the KEGG pathway analysis using KOBAS 3.0.

Results

DEGs analysis

After data pre-processing, a total of 5381 genes in the gene matrix were obtained from 12 samples. According to the cut-off criterion used, a pairwise comparison was used to compare the three different carbon

sources (Fig. 1a). There were 399 overlapping DEGs between W_G vs. W_D and M_G vs. M_D. There were 1863 overlapping DEGs between W_M vs. W_D and M_M vs. M_D. In addition, there were 2098 overlapping DEGs between W_M vs. W_G, and M_M vs. M_G. By combining the union DEGs of the Mut strain and WT strain grown under different carbon sources, 2536 DEGs were detected as genes related to carbon sources. Bidirectional hierarchical clustering was carried out to analyze these 2536 DEGs (Fig. 1b). The results of bidirectional hierarchical clustering showed that DEG analysis only separated methanol samples from the three carbon sources, while glucose and glycerol samples were aggregated in the other branches. However, DEGs in glucose and glycerol samples were separated according to the Mut or WT types. Thus, we inferred that these genes were not only associated with carbon source treatment, but also potentially related to the strain type, laying the foundation for our next co-expression analysis.

Fig. 1 Statistics and heatmap of DEGs using three carbon sources in Δ mg1 Δ mg2 Δ nrg1-Mit1 (Mut) and wild (WT) strains. **a** DEG statistics under three carbon sources or strain types: (i) DEGs in Mut or WT strains between dextrose and glycerol groups, (ii) DEGs in Mut or WT strains between methanol and dextrose groups, (iii) DEGs in Mut or WT strains between methanol and glycerol groups. **b** DEG heatmap using three carbon sources and different strain types. Red stripes for dextrose, blue for glycerol, and yellow for methanol

The KEGG enrichment analysis results of the 2536 DEGs showed that with the change of carbon source, the genes involved in carbon metabolism, secondary metabolic pathway, and amino acid biosynthesis changed significantly (Table 2).

Screening of related modules and genes by WGCNA

To further screen genes related to strain type, we performed WGCNA analysis for the 2536 DEGs (Fig. 2). Based on the clustering and dynamic pruning method, 2536 DEGs were divided into 20 modules (the grey module was a collection of genes that cannot be aggregated into other modules) (Fig. 2a). Modules with heterogeneity coefficient < 0.2 were merged together, and finally 10 modules were obtained (Fig. 2b, c). By calculating the correlation coefficient between the module eigengene (ME) and sample traits, 4 modules (green-yellow: 122 genes, midnight-blue: 266 genes, grey60: 44 genes, light green: 41 genes) were significantly correlated with the strain type ($p < 0.05$) (Fig. 3a). Both MEgreenyellow and MEMidnightblue modules were significantly correlated with the type of strain (correlation coefficient > 0.9). Genes in the MEMidnightblue module were down-regulated in Mut vs. WT, and genes in the MEgreenyellow module were up-regulated in Mut vs. WT (Fig. 3c–d).

Functional enrichment pathway analysis

The genes in the MEgreenyellow and MEMidnightblue modules were analyzed by KEGG pathway enrichment, and the results are shown in Fig. 3b. The genes in the MEgreenyellow module were mainly enriched in proteases, oxidative phosphorylation, endoplasmic reticulum protein processing, folate carbon pool, and glycerol phospholipid metabolism pathway, while the genes in the MEMidnightblue module were enriched in proteases, endocytosis, steroid biosynthesis, and hippo signaling pathway (Fig. 3b).

In the carbon metabolism network, the expression level of *DAS1*, *TAL1*, *PKII*, and *MOX* were higher in M_G and M_D than in W_G and W_D. The expression level of *DAS1*, *TAL1*, *PKII*, *MOX*, *FLD1*, *FHD1*, *DAK*, *FBPI* in M_M were higher than in M_G and M_D. Meanwhile,

TKT, *ICL1*, *IDP2*, and *POX1* expression level in M_M was lower than that in M_G and M_D (Fig. 4).

Construction of PPI networks

We performed PPI analysis of 388 DEGs in MEgreenyellow and MEMidnightblue modules, and a total of 502 PPI edges and 155 proteins were obtained, as shown in Fig. 5a (Additional file 1: Table S1). The topological properties of PPI network were analyzed. Eight proteins (XP_002489693.1, XP_002490020.1, XP_002491845.1, XP_002492676.1, XP_002493471.1, XP_002493717.1, XP_002493779.1, and XP_002494220.1) obtained high scores in DC, BC, and CC within the list of top 30 proteins (Table 3), and these 8 proteins were deemed as hub proteins. Five sub-networks were identified by the PPI network function, of which 2 (sub-network A and sub-network B) had a score > 5 (Fig. 5b, c). Sub-network A contained 25 genes, of which 8 belonged to the MEgreenyellow module, and the other 17 genes belonged to the MEMidnightblue module. All genes in sub-network B belonged to the MEMidnightblue module. KEGG pathway enrichment was applied to sub-network A and sub-network B, and the results showed that sub-network A was enriched in the proteasome pathway, while sub-network B was enriched in the ribosome pathway.

Discussion

The commonly used and tightly regulated promoter used to drive heterologous protein expression in *P. pastoris* is the *AOX1* promoter; the gene expression of *AOX1* is repressed in carbon sources (Brierley et al. 1990). A *P. pastoris* mutant strain expressing *AOX1-lac2* fusion has been demonstrated to be able to use sorbitol, alanine, trehalose, or mannitol as the sole carbon source (Inan and Meagher 2001). The deletion of the hexose transporter PpHXT1, leading to glucose or fructose-induced expression of the *AOX1* gene and pexophagy, is associated with catabolite repression of *Aox* in *P. pastoris* (Zhang et al. 2010). We previously described a novel Mut strain of the *AOX1* promoter-based *P. pastoris* expression system, $\Delta mig1 \Delta mig2 \Delta nrg1$ -Mit1 (Wang et al. 2017). However, the mechanism underlying the catabolite and *AOX1* regulation of this expression system should be explored. In

Table 2 Five KEGG pathways related to carbon source in *P. pastoris*

Term	Gene number	P-value	Corrected P-value
Metabolic pathways	395	3.20E−06	0.000329689
Biosynthesis of secondary metabolites	179	7.32E−05	0.003657969
Biosynthesis of antibiotics	140	0.000106543	0.003657969
Biosynthesis of amino acids	83	0.000570221	0.014683197
Carbon metabolism	73	0.001622285	0.033419065

this study, we compared the gene expression patterns of the Mut strain cultured in three carbon sources (glucose, glycerol, and methanol). A total of 2536 carbon source-associated DEGs were detected from the intersection of three groups, and 388 DEGs were found to be associated with both the carbon source type and strain type (Mut or WT). Further investigation of these DEGs indicated the significant involvement of the proteasome and ribosome

pathways. Moreover, several hub genes related to AOX1 regulation and carbon sources were identified.

When the two *P. pastoris* strains (WT and Mut) were induced by different carbon sources, the DEGs were mainly associated with carbon metabolism, secondary metabolic biosynthesis, and amino acid biosynthesis. Carbon metabolism in microbial cells mainly involves glycolysis, the pentose phosphate pathway, and the three

carboxylic acid (TCA) cycle (Zhang et al. 2012). Both the TCA and glyoxylic acid cycles provide energy and precursors for microorganisms (Trapnell et al. 2014). Wang and Yan (2017) showed that when *P. pastoris* was cultured with glycerol and methanol. Genes associated with the first stage of glycolysis were expressed at the basal level. Dihydroxy acetone phosphate and dihydroxy acetone phosphate were the methanol metabolites in the peroxisome. These metabolites were then transported

through the peroxisomal membrane to the cytoplasm and entered the second stage of glycolysis. In a previous study, the growth status of yeast with glucose and methanol as carbon sources was investigated. The metabolic flow of TCA was found to have decreased by three times under methanol growth conditions, while no significant change in transcription or protein levels in the TCA cycle was observed (Rusmayer et al. 2015). These results indicate that the TCA cycle may supply different precursor

substances for other metabolic pathways when cells are grown under different carbon sources.

The glycerol transporter GT1 may participate in the process of P_{AOX1} inhibition of *P. pastoris* in a medium containing glycerol, and knockdown of this gene can eliminate the glycerol repression of P_{AOX1} (Zhan et al. 2016). In this study, we found several hub proteins in the PPI network of the 388 genes in two significant modules, including XP_002489693.1 (one of six ATPases of the 19S regulatory particle of the 26S proteasome involved in degradation), XP_002490020.1 (ubiquitin-protein ligase (E3) that interacts with Rpt4p and Rpt6p), XP_002491845.1 (metalloprotease subunit of the 19S regulatory particle of the 26S proteasome lid), XP_002492676.1 (Alpha 4 subunit of the 20S proteasome), XP_002493471.1 (Alpha 1 subunit of the 20S core complex of the 26S proteasome), XP_002493717.1 (one of six ATPases of the 19S regulatory particle of the 26S proteasome), XP_002493779.1 (Adenylate cyclase, required for cAMP production and cAMP-dependent protein kinase signaling), and XP_002494220.1 (Beta 6 subunit of the 20S proteasome). These proteins were mainly associated with the proteasome and ribosome; one of these proteins was ubiquitin ligases E3, which was

a proteolytic enzyme guiding relative protein degradation in the proteasome. The proteasome, which has many catalytic functions and can selectively degrade proteins in cells, is a polyclonal macromolecule complex widely distributed in organisms (Tomko and Hochstrasser 2011). To remove the proteins that the cell does not need for functioning as soon as possible, eukaryotic cells can increase the expression of the proteasome by upregulated transcription of genes involved in the proteasome. Ubiquitin-proteasome-linked protein degradation of the key gluconeogenic enzyme, fructose-1,6-bisphosphatase, is associated with glucose uptake and carbohydrate metabolism of *P. pastoris* (Santt et al. 2008). Ribosomes are the synthesis machine of proteins, and they are also an important site for cells to perceive the nutrition level within cells. Studies (Beilharz and Preiss 2004; Prielhofer et al. 2015) reported that the proportion of polyribosomes in total ribosomes (40s, 60s, 80s/monosomes and polysomes) increased by approximately twofold, as detected by RNA-seq sequencing, when the different carbon sources induced the expression of proteins in *P. pastoris*. When methanol was used as the carbon source, high levels of protein expression were observed. At this time, a high number of amino acids need to be

synthesized and more ribosomes need to participate in protein synthesis (Prieler et al. 2015). Therefore, we suggested that the changes in ribosomal and proteasomal activities were associated with carbohydrate metabolism and glucose-mediated as well as glycerol-mediated induction of P_{AOX1} in *P. pastoris*, indicating that the carbon derepressed expression system actually causes protein degradation and amino acid synthesis to meet the demand for heterologous protein secretion. However, the

signal transduction mechanism that induces the derepression of *AOX1* in $\Delta mig1 \Delta mig2 \Delta nrg1$ -Mit1 remains to be investigated further.

In the carbon metabolism network, several genes were up-regulated in M_G and M_D compared with W_G and W_D, such as *DAS1* and *FLD1*. *DAS1* encodes dihydroxyacetone synthase Das and *FLD1* encodes formaldehyde dehydrogenase Fld. They are all critical enzymes in the methanol utilization pathway. Both P_{DAS1} and P_{FLD1}

Table 3 Top 30 genes with higher topological properties in the protein–protein interaction network

Gene	Degree	Gene	Betweenness	Gene	Closeness
XP_002493717.1	28	XP_002490020.1	6223.7	XP_002493717.1	0.02147
XP_002489693.1	27	XP_002493779.1	6097.7	XP_002490020.1	0.02142
XP_002491845.1	26	XP_002490773.1	5526.0	XP_002489693.1	0.02136
XP_002493471.1	26	XP_002493873.1	5250.0	XP_002493873.1	0.02136
XP_002492676.1	26	XP_002493717.1	4834.5	XP_002491845.1	0.02136
XP_002494220.1	26	XP_002493265.1	4645.0	XP_002493471.1	0.02132
XP_002494177.1	26	XP_002491363.1	1818.0	XP_002492676.1	0.02132
XP_002493117.1	26	XP_002489675.1	1454.0	XP_002493779.1	0.02131
XP_002493814.1	26	XP_002490815.1	1346.0	XP_002490773.1	0.02124
XP_002491581.1	25	XP_002492039.1	1050.0	XP_002494220.1	0.02122
XP_002493329.1	25	XP_002489799.1	864.0	XP_002494177.1	0.02122
XP_002492434.1	25	XP_002489693.1	802.5	XP_002493117.1	0.02122
XP_002489540.1	25	XP_002489961.1	763.7	XP_002493814.1	0.02122
XP_002492684.1	25	XP_002491845.1	753.8	XP_002493930.1	0.02122
XP_002494172.1	25	XP_002493614.1	646.0	XP_002492940.1	0.02122
XP_002493375.1	25	XP_002492074.1	618.0	XP_002491581.1	0.02122
XP_002494106.1	25	XP_002493040.1	618.0	XP_002493329.1	0.02122
XP_002491076.1	25	XP_002493471.1	577.6	XP_002492434.1	0.02122
XP_002492927.1	25	XP_002492676.1	577.6	XP_002489540.1	0.02122
XP_002492977.1	25	XP_002493501.1	500.0	XP_002492684.1	0.02122
XP_002493941.1	25	XP_002490025.1	437.7	XP_002494172.1	0.02122
XP_002493311.1	24	XP_002492697.1	434.0	XP_002493375.1	0.02122
XP_002489719.1	24	XP_002491242.1	432.0	XP_002494106.1	0.02122
XP_002493134.1	24	XP_002491269.1	432.0	XP_002491076.1	0.02122
XP_002493007.1	22	XP_002493930.1	277.3	XP_002492927.1	0.02122
XP_002493779.1	19	XP_002492193.1	249.0	XP_002492977.1	0.02122
XP_002491334.1	15	XP_002491431.1	227.0	XP_002493941.1	0.02122
XP_002491293.1	15	XP_002494220.1	219.6	XP_002493311.1	0.02122
XP_002493265.1	15	XP_002489862.1	218.0	XP_002489719.1	0.02122
XP_002490020.1	10	XP_002492146.1	218.0	XP_002493134.1	0.02122

are regulated by methanol (Vogl and Glieder 2013). In the present study, the higher expression level of *DASL* and *FLDI* may play a critical role in carbon metabolism of the dextrose and glycerol medium. *TKT* plays an important role in the pentose phosphate acid cycle and the photo-synthetic reduced pentose phosphate cycle. In the group M_M, the expression level of *TKT* was down-regulated compared with the group M_G and M_D. This result may indicate that in the case of methanol as the sole carbon source, biotransformation efficiency of the carbon source may be lower in M_M than M_G and M_D. Further results require experimentation to verify.

Conclusion

In conclusion, with the change of the carbon source, the genes involved in the carbon metabolism pathway, secondary metabolic pathway, and amino acid biosynthesis pathway changed significantly in *P. pastoris*. Changes in

the proteasome and ribosome activities played a significant role in the carbohydrate metabolism of the methanol-free P_{AOX1} start-up $\Delta mig1\Delta mig2\Delta nrg1$ -Mit1 strain. Such an expression system could be an efficient alternative to conventional production methods using methanol as the carbon source.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s40643-020-00306-w>.

Additional file 1: Table S1. Proteins in the PPI network.

Abbreviations

WT: Wildtype; DEGs: Differentially expressed genes; PPI: Protein–protein interaction; WGCNA: Weighted gene co-expression network analysis; KEGG: Kyoto encyclopedia of genes and genomes; CDS: Coding sequence; RPKM: Reads per kilo bases per million reads; FC: Fold-change; DC: Degree centrality; BC: Betweenness centrality; CC: Closeness centrality; TCA: Three carboxylic acid.

Acknowledgements

Not applicable.

Authors' contributions

LS and MC contributed to the conception and design of the research; JW, XW, and YZ acquired data; XZ and ZS performed all the statistical analysis; LS drafted the manuscript; and MC revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Funding

None.

Availability of data and materials

All datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to publish this manuscript.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China. ² Shanghai Collaborative Innovation Center for Biomanufacturing, Shanghai 200237, China. ³ Bioprocessing Technology Institute, Singapore, Singapore. ⁴ Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore.

Received: 15 November 2019 Accepted: 16 March 2020

Published online: 25 March 2020

References

- Anders S, Huber W (2010) Differential expression analysis for sequence count data. *Genome Biol*
- Anders S, Huber W (2012) Differential expression of RNA-Seq data at the gene level-the DESeq package. *Genome Biol*
- Bandettini WP et al (2012) MultiContrast Delayed Enhancement (MCODE) improves detection of subendocardial myocardial infarction by late gadolinium enhancement cardiovascular magnetic resonance: a clinical validation study. *J Cardiovasc Magn Reson* 14:83
- Barabási AL (2009) Scale-free networks: a decade and beyond. *Science* 325(5939):412–413
- Beilharz TH, Preiss T (2004) Translational profiling: the genome-wide measure of the nascent proteome. *Brief Funct Genomic Proteomic* 3(2):103–111
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B* 57(1):289–300
- Brierley RA, Bussineau C, Kosson R, Melton A, Siegel RS (1990) Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme. *Ann N Y Acad Sci* 589:350–362
- Chen HX, Chu J, Zhang SL et al (2007) Intracellular expression of *Vitreoscilla* hemoglobin improves S-adenosylmethionine production in a recombinant *Pichia pastoris*. *Appl Microbiol Biotechnol* 74(6):1205–1212
- Florea L, Song L, Salzberg SL (2013) Thousands of exon skipping events differentiate among splicing patterns in sixteen human tissues. *F1000 Res* 2:188
- Inan M, Meagher MM (2001) Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. *J Biosci Bioeng* 92(6):585–589
- Kanehisa M, Goto S (2000) KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28(1):27–30
- Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* 14:R36
- Langfelder P, Horvath S (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* 9(1):559
- Liang S, Wang B, Pan L, Ye Y, He M, Han S et al (2014) Comprehensive structural annotation of *Pichia pastoris* transcriptome and the response to various carbon sources using deep paired-end RNA sequencing. *BMC Genomics* 9(4):511–525
- Lin J, Panigraphy D, Trinh L, Folkman J, Shiloach J (2000) Production process for recombinant human angiotensin in *Pichia pastoris*. *J Ind Microbiol Biotechnol* 24(1):31–35
- Lin-Cereghino GP, Godfrey L, de la Cruz BJ, Johnson S, Khuongsathiene S, Tolstorukov I et al (2006) Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in *Pichia pastoris*. *Mol Cell Biol* 26(3):883–897
- Lu P, Vogel C, Wang R, Yao X, Marcotte EM (2007) Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nat Biotechnol* 25(1):117–124
- Polupanov AS, Nazarko VY, Sibirny AA (2012) Gss1 protein of the methylotrophic yeast *Pichia pastoris* is involved in glucose sensing, pexophagy and catabolite repression. *Int J Biochem Cell Biol* 44(11):1906–1918
- Portnoy T, Margeot A, Linke R, Atanasova L, Fekete E, Sándor E et al (2011) The CRE1 carbon catabolite repressor of the fungus *Trichoderma reesei*: a master regulator of carbon assimilation. *BMC Genomics* 12:269
- Prielhofer R, Maurer M, Klein J, Wenger J, Kizlak C, Gasser B, Mattanovich D (2013) Induction without methanol: novel regulated promoters enable high-level expression in *Pichia pastoris*. *Microb Cell Fact* 12(1):5
- Prielhofer R, Cartwright SP, Graf AB, Valli M, Bill RM, Mattanovich D, Gasser B (2015) *Pichia pastoris* regulates its gene-specific response to different carbon sources at the transcriptional, rather than the translational, level. *BMC Genomics* 16:167
- Russmayer H, Buchetics M, Gruber C, Valli M, Grillitsch K, Modarres G, Guerrasio R, Klavins K, Neubauer S, Drexler H, Steiger M, Troyer C, Al Chalabi A, Krebichl G, Sonntag D, Zellnig G, Daum G, Graf AB, Altmann F, Koellensperger G, Hann S, Sauer M, Mattanovich D, Gasser B (2015) Systems-level organization of yeast methylotrophic lifestyle. *BMC Syst Biol* 13:80
- Santt O, Pfirrmann T, Braun B, Juretschke J, Kimmig P, Scheel H, Hofmann K, Thumm M, Wolf DH (2008) The yeast GID complex, a novel ubiquitin ligase (E3) involved in the regulation of carbohydrate metabolism. *Mol Biol Cell* 19(8):3323–3333
- Shannon P et al (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 13(11):2498–2504
- Szklarczyk D, et al (2014) STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Res*:1003
- Tang Y et al (2015) CytoNCA: a cytoscape plugin for centrality analysis and evaluation of protein interaction networks. *BioSystems* 127:67–72
- Tomko RJ Jr, Hochstrasser M (2011) Order of the proteasomal ATPases and eukaryotic proteasome assembly. *Cell Biochem Biophys* 60(1–2):13–20
- Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR et al (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 7:562–578
- Trapnell CR, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L (2014) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 9(10):2513
- van den Hazel HB, Kielland-Brandt MC, Winther JR (1996) Biosynthesis and function of yeast vacuolar proteases: review. *Yeast* 12:1–16
- Vogl T, Glieder A (2013) Regulation of *Pichia pastoris* promoters and its consequences for protein production. *New Biotechnol* 30(4):385–404
- Wang XY, Yan X (2017) The metabolic difference and mechanism of *Komagataella phaffii* in glycerol and methanol culture based on transcriptomic analysis. *Acta Microbiol Sin*:1–17
- Wang Z, Wang Y, Zhang D, Li J, Hua Z, Du G, Chen J (2010) Enhancement of cell viability and alkaline polygalacturonate lyase production by sorbitol co-feeding with methanol in *Pichia pastoris* fermentation. *Biores Technol* 101(4):1318–1323
- Wang J, Wang X, Shi L, Qi F, Zhang P, Zhang Y, Zhou X, Song Z, Cai M (2017) Methanol-independent protein expression by AOX1 promoter with trans-acting elements engineering and glucose–glycerol-shift induction in *Pichia pastoris*. *Sci Rep* 7:41850. <https://doi.org/10.1038/srep41850>

- Wu J, Mao X, Cai T, Luo J, Wei L (2006) KOBAS server: a web-based platform for automated annotation and pathway identification. *Nucleic Acids Res* 34:720–724
- Xie CEA (2011) KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res* 39:316–322
- Zhan C, Wang S, Sun Y, Dai X, Liu X, Harvey L, McNeil B, Yang Y, Bai Z (2016) The *Pichia pastoris* transmembrane protein GT1 is a glycerol transporter and relieves the repression of glycerol on AOX1 expression. *FEMS Yeast Res*. <https://doi.org/10.1093/femsyr/fow033>
- Zhang P, Zhang W, Zhou X, Bai P, Cregg JM, Zhang Y (2010) Catabolite repression of Aox in *Pichia pastoris* is dependent on hexose transporter PpHxt1 and pexophagy. *Appl Environ Microbiol* 76(18):6108
- Zhang CL, Qin ZJ, Wang GZ, Ji ZB, Wang JM (2012) Transcriptome and RNA-Seq technology. *Biotechnol Bull* 12:51–56

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen[®] journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► [springeropen.com](https://www.springeropen.com)