

RESEARCH ARTICLE



Transcriptome analysis of $\Delta mig1\Delta mig2$ mutant reveals their roles in methanol catabolism, peroxisome biogenesis and autophagy in methylotrophic yeast *Pichia pastoris*

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Abstract

Two catabolite repressor genes (*MIG1* and *MIG2*) were previously identified in *Pichia pastoris*, and the derepression of alcohol oxidase (AOX) expression was realized in $\Delta mig1$ or $\Delta mig1\Delta mig2$ mutants grown in glycerol, but not in glucose. In this study, genome-wide RNA-seq analysis of $\Delta mig1\Delta mig2$ and the wild-type strain grown in glycerol revealed that the expression of numerous genes was greatly altered. Nearly 7% (357 genes) of approximately 5276 genes annotated in *P. pastoris* were significantly upregulated, with at least a two-fold differential expression in $\Delta mig1\Delta mig2$; the genes were mainly related to cell metabolism. Approximately 23% (1197 genes) were significantly downregulated; these were mainly correlated with the physiological characteristics of the cell. The methanol catabolism and peroxisome biogenesis pathways were remarkably enhanced, and the genes *AOX1* and *AOX2* were upregulated higher than 30-fold, which was consistent with the experimental results of AOX expression. The Mig proteins had a slight effect on autophagy when cells were grown in glycerol. The expression analysis of transcription factors showed that deletion of *MIG1* and *MIG2* significantly upregulated the binding of an essential transcription activator, Mit1p, with the *AOX1* promoter, which suggested that Mig proteins might regulate the *AOX1* promoter through the regulation of Mit1p. This work provides a reference for the further exploration of the methanol induction and catabolite repression mechanisms of AOX expression in methylotrophic yeasts.

Keywords *Pichia pastoris* · RNA-seq · Mig · Alcohol oxidase · Catabolite repression

Introduction

The methylotrophic yeast *Pichia pastoris* has been developed as an effective expression system for various heterologous proteins (Cereghino and Cregg 2000; Gasser et al. 2013). This organism is especially attractive as it possesses a strong and tightly regulated methanol-inducible promoter of the *alcohol oxidase 1* gene (*AOX1*) (P_{AOX1}) and grows to reach high cell densities (Li et al. 2007; Vogl and Glieder 2013). P_{AOX1} is induced only in response to methanol, but repressed in alternative carbon sources, such as glucose or glycerol (Hartner and Glieder 2006).

In methylotrophic yeasts, a diauxic cell usually appears in a mixture of glycerol/methanol or glucose/methanol without the repression of the methanol-utilizing enzymes, such as alcohol oxidase (AOX), catalase (CAT), and dihydroxyacetone synthase (DAS) (Egli et al. 1980; Inan and Meagher 2001). As previously reported, *AOX1* expression in *P. pastoris* is almost undetectable in glycerol (Hartner and

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Glieder 2006; Inan and Meagher 2001), but exhibits ~3–30 and 60–80% derepression, respectively, in *Candida boidinii* and *Hansenula polymorpha* (Hartner and Glieder 2006; Sasano et al. 2010). Although the phenomenon of catabolite repression in yeasts has been known for a long time, there are only a few reports that describe the regulatory mechanism of the repression of AOX synthesis in methylotrophic yeasts (Leão-Helder et al. 2004; Stasyk et al. 2008; Zhang et al. 2010). As previously reported, some aspects of catabolite repression are mediated by a Cys2His2 zinc-finger transcription factor, Mig1p/CreA, which specifically binds to DNA with a GC-rich consensus sequence and a flanking AT sequence, conserved in most eukaryotic species (Kulmburg et al. 1993; Mathieu and Felenbok 1994; Nehlin and Ronne 1990; Nehlin et al. 1991). In *Saccharomyces cerevisiae*, Mig1p functions to repress the transcription of genes under conditions of glucose sufficiency, such as those genes encoding enzymes for the utilization of the sugars galactose, sucrose, or maltose (Carlson 1999; Schüller 2003). In the methanol-utilizing yeast *H. polymorpha*, the researchers developed conditions mimicking the molecular pathway of catabolite repression of the conventional yeast *S. cerevisiae* (the so-called Snf1-Mig1 pathway) (Carlson 1999; Gancedo 1998) to identify and study the role of transcriptional repressors HpMig1p and HpMig2p in catabolite repression. The repression pathway for AOX in this yeast partially relies on the two Mig1p homologs, irrespective of the carbon source (glucose, sucrose, or ethanol) (Stasyk et al. 2007). *P. pastoris* and *H. polymorpha*, which are regarded to be belonging to the same taxonomic position, were identified to have methanol-assimilating capabilities (Hazeu et al. 1972). The methanol utilization pathway and the regulation of alcohol oxidase genes are similar in both strains (Hartner and Glieder 2006; Yurimoto et al. 2011). Thus, the regulation of catabolite repression of P_{AOX1} in *P. pastoris* may also be related to Mig1p-mediated transcriptional repression. We therefore cloned two Mig1p homologs (designated Mig1p and Mig2p) in *P. pastoris* (Wang et al. 2017). Gene knock-out analysis showed that the double-deletion of *MIG1* and *MIG2* allowed the expression of alcohol oxidase under glycerol (Wang et al. 2017), which was similar to findings in *H. polymorpha* (Stasyk et al. 2007).

However, it is unknown how these Mig proteins affect global cell metabolism, especially methanol catabolism and the closely related peroxisome biosynthesis. The next-generation sequencing technology for RNA (RNA-seq) is a useful and cost-efficient tool for transcriptome analysis (Marguerat and Bähler 2010), which offers better dynamic range, detects very subtle changes in gene expression, characterizes the alternative splicing of mRNAs, and detects novel transcripts (Ozsolak and Milos 2010; Wilhelm and Landry 2009). Thus, RNA-seq based transcriptome analysis was used in this study to plot the genetic and metabolic variations of the

$\Delta mig1 \Delta mig2$ mutant grown in glycerol with P_{AOX1} derepression in comparison with the wild type strain, which may help us clarify the regulatory roles of Mig proteins.

Materials and methods

Strains, growth conditions and microbial techniques

The strains used in this study were listed in Table S1. The cells were grown on standard liquid or solid media at 30 °C as previously described (Zhang et al. 2010). The concentration of methanol in medium was 0.5% (v/v) while that of any other carbon source was 1% (w/v) unless indicated otherwise. Cell density was determined by measuring absorbance at 600 nm. Construction of strains of $\Delta mig1$, $\Delta mig2$ and $\Delta mig1 \Delta mig2$ were described previously (Wang et al. 2017).

Analysis of cell growth and AOX activity

Cells of each strain were preincubated overnight in YNB medium supplemented with glucose (1%, v/v) and transferred into fresh YNB medium supplemented with various carbon sources (1% glucose, 1% glycerol, 0.5% methanol, 1% glycerol + 0.5% methanol) at an initial OD₆₀₀ of 0.03. Preparation of crude cell extracts and assays of AOX activity were carried out as described previously (Zhang et al. 2010). The AOX activity was detected since 12 h culture of cells on various carbon sources (Zhang et al. 2010) to collect enough cells for analysis.

Strains and culture conditions for RNA-seq

The following *P. pastoris* strains were used for RNA-seq analysis: wild type GS115 and $\Delta mig1 \Delta mig2$ mutant (Wang et al. 2017). *P. pastoris* cells were grown in yeast nitrogen base (YNB) with adequate auxotrophic requirements containing 1% glucose or 1% glycerol as carbon source in batch cultures. For transcriptome analysis, cells were pre-cultured in glucose at 30 °C prior to a shift to fresh media containing glycerol as sole carbon source. Four independent glucose-grown cultures (two parallel samples for each strain) were used to inoculate into fresh medium containing glycerol. As previously reported, transcription of genes involved in methanol catabolism varied significantly even after culture in glucose or glycerol for only 2 h. Thus the glycerol cultures (initial OD₆₀₀ of 1.0) grown for 4 h, which led to comparative cell density to cells grown for 12 h in the cell growth and AOX activity analytic experiments, were used for RNA isolation.

RNA preparation

Total RNA was extracted using Ribopure Yeast kit (Ambion, Austin, TX) and treated for 30 min at 37 °C with RNase free DNase I (Ambion) for removing residual genomic DNA. The quality and quantity of the purified RNA were determined with a Nanodrop ND-1000 spectrophotometer (LabTech, USA) by measuring absorbance at 260 nm/280 nm (A_{260}/A_{280}). RNA integrity was further verified by agarose gel electrophoresis.

Preparation of cDNA library for RNA-seq

The cDNA libraries were prepared according to the manufacturer's instructions (Illumina). Poly (A) mRNA was isolated from the total RNA samples with oligo (dT) magnetic beads (Invitrogen). To avoid priming bias when synthesizing cDNA, the mRNA was firstly fragmented before cDNA synthesis. The purified mRNA was fragmented into small pieces by the RNA fragmentation kit (Ambion) and applied as template for first-strand cDNA synthesis using random hexamer primers and reverse transcriptase (Invitrogen). The second-strand cDNA was synthesized using RNase H (Invitrogen) and DNA polymerase I (New England Biolabs, USA). The Illumina Genomic DNA Sample Prep kit (Illumina, USA) was used to generate 95-bp paired-end (PE) cDNA libraries. The libraries were loaded onto flow cell channels for sequencing on the Illumina GA IIx system. A total of four paired-end cDNA libraries of *P. pastoris* cells were constructed for each of the test groups. Duplicate technical replicates were performed for each group.

Mapping reads to reference genome and annotated genes

The 95-bp raw PE reads (400 bp insert size), generated by the Illumina GA IIx system, were firstly processed by the FASTX-Toolkit to remove the reads containing sequencing adapters and reads of low quality (phred quality < 5) and then aligned the remaining reads using the EMBL *P. pastoris* GS115 coding sequence database as reference by the Burrows–Wheeler Aligner's Smith–Waterman Alignment (BWA) program (Li and Durbin 2010).

Data deposit

The original sequencing is deposited in SRA (Short Read Archive) with the accession number SRP094666 in the National Center for Biotechnology Information database (NCBI, <http://www.ncbi.nlm.nih.gov>).

Normalized expression level of gene by RNA-seq

The gene expression level by RNA-seq was normalized by the number of reads per kilobase of exon region per million mapped reads (RPKM) using the formula described by Mortazavi et al. (2008). The cut-off value for determining gene transcriptional activity was determined based on a 95% confidence interval for all RPKM values of each gene. Two biological replicates of each sample were used for differential gene expression analysis between the $\Delta mig1\Delta mig2$ mutant and WT strain. A 0.81–0.88 Pearson correlation coefficient was obtained for biological replicates.

Differential expression analysis

The R package DEGseq (Wang et al. 2010) was used to identify differentially expressed genes in different strains with a ratio of RPKM in $\Delta mig1\Delta mig2$ to WT, higher than 2.0 or lower than 0.5 ($\log_2(\text{ratio}) \geq 1$ or ≤ -1) and p value < 0.05 [cut-off at 5% false discovery rate (FDR)]. FDR (Benjamini and Hochberg 1995) was performed using the default parameters to obtain an adjusted p -value between the $\Delta mig1\Delta mig2$ group versus the WT group. Then the resulting expression intensity values were analyzed by the MA plot-based method, as described by Wang et al. (2010).

Gene ontology and KEGG pathway analysis

The ORF-encoded proteins were categorized according to the *P. pastoris* genome gene ontology (GO) annotations from NCBI (<http://www.ncbi.nlm.nih.gov/>), UniProt (<http://www.uniprot.org/>) and the GO (<http://www.geneontology.org/>). The “elim Fisher” algorithm (Alexa et al. 2006) was used for GO enrichment test because it can iteratively remove the genes mapped to significant GO terms from more general (higher level) GO terms so that the significant GO terms will not be overshadowed by the general GO terms. GO categories with a p value < 0.01 were reported.

Pathway analysis was used to find out the significant pathway of the differential genes. Pathway annotations of RNA-seq genes were downloaded from KEGG (Kyoto Encyclopedia of Genes and Genomes) (<http://www.genome.jp/kegg/>). A Fisher exact test was used to find the significant enrichment pathway. The resulting p values were adjusted using the FDR algorithm (Benjamini and Hochberg 1995). Pathway categories with a FDR < 0.05 were reported. Enrichment provides a measure of the significance of the function: as the enrichment increases, the corresponding function becomes specific, which helps us to find those more significant pathways in the experiment. The enrichment was given by: $\text{enrichment} = (n_g/n_a) / (N_g/N_a)$ where n_g is the number of differential genes within the particular pathway, n_a is the total number of genes within the same pathway, N_g

is the number of differential genes which have at least one pathway annotation, and N_a is the number of genes which have at least one pathway annotation in the entire RNA-seq.

Validation of RNA-seq analysis by quantitative real-time PCR

Real-time quantitative PCR (qPCR) was performed to validate gene transcription levels determined by RNA-seq. The *P. pastoris* GS115 samples for qPCR were as same as those for Illumina sequencing. Total RNA (1 µg) was reversely transcribed into cDNA using PrimeScript™ RT-PCR Kit (TaKaRa). Each 20 µl reaction solution contained primers (optimal concentration), SYBR Premix Ex Taq (TaKaRa, Dalian, China) and cDNA templates (1/100 dilution), and was performed on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers for qPCR (Table S2) were designed using Primer Express software (Applied Biosystems, Foster City, CA), with predicted products in the 100- to 300-bp size range. The cycle threshold values (CT) were determined and the relative fold differences were calculated by the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008) using *ACT1* as the endogenous reference genes (Vandesompele et al. 2002). Samples were run in triplicate in a 96-well plate and each experiment was repeated three times. Two experiments of independently cultivated yeast were performed to confirm the reproducibility of the results.

Results

Regulation of cell growth and catabolite repression of AOX expression by *P. pastoris* Mig1p, Mig2p

In our previous study (Wang et al. 2017), two genes coding for transcription repressors, *MIG1* (PAS_chr4_0334) and *MIG2* (PAS_chr1-4_0526), were identified via the *P. pastoris* genome database (<http://bioinformatics.psb.ugent.be/webtools/bogas/overview/Picpa>) (De Schutter et al. 2009). To determine the functions of Mig1p and Mig2p, the $\Delta mig1$, $\Delta mig2$, and $\Delta mig1\Delta mig2$ strains were constructed (Wang et al. 2017), and derepression of alcohol oxidase (AOX) expression was realized in $\Delta mig1$ or $\Delta mig1\Delta mig2$ grown in glycerol, but not in glucose. However, as this previous work focused on the metabolic engineering of *P. pastoris* for the development of a methanol-free expression system, our main interest was whether the derepression of AOX expression was realized; consequently, the bioinformatic analysis of Mig proteins and the regulation of cell growth and derepression of AOX expression by Mig proteins were not systematically analyzed.

Phylogenetic analysis revealed that Mig1p and Mig2p are members of the subgroup of transcriptional repressors that contain selected homologs of Mig proteins and the fungal CreA protein. Interestingly, the distance of the branch between *P. pastoris* Mig1p and *H. polymorpha* Mig1p (HpMig1p) (Stasyk et al. 2007) is short, as is that between PpMig2p and *H. polymorpha* Mig2p (HpMig2p) (Stasyk et al. 2007) (Fig. S1A), which implied that the two putative transcriptional repressors may play similar roles in catabolite repression in *P. pastoris* as HpMig1p and HpMig2p in *H. polymorpha*. The translated protein sequences of *P. pastoris* Mig1p and Mig2p have 444 and 454 amino acid residues, respectively. The amino acid sequences contain conserved Mig1p/CreA/CRE1 analogs from different microorganisms, but also have some distinctive features. Both Mig proteins contain two Cys2His2 zinc-finger motifs, which harbor a putative phosphorylation sequence for the cAMP-dependent protein kinase located in the second zinc-finger region (Fig. S1B). *P. pastoris* Mig2p contains a stretch rich in glutamine, near amino acids 159 and 186 (Fig. S1C), which appears in several proteins that are presumed to regulate gene expression (Schultz and Carlson 1987), but similar regions cannot be found in Mig1p. Moreover, an additional conserved region was found in yeast Mig proteins (Fig. S1D): the so-called C-terminal “effector domain” (Ostling et al. 1996) existed in Mig1p and Mig2p, although the protein sequence differed in Mig1p and Mig2p (LPSLSL and LPPIRSL, respectively) (Fig. S1E).

The lack of *MIG1* strongly damaged cell growth, especially in the presence of glucose and glycerol, whereas the deficiency of *MIG2* resulted in only mild damage (Fig. 1). The double deletion of *MIG1* and *MIG2* caused more severe effects, with severe inhibition of cell growth in various carbon sources (Fig. 1). To gain further insight into the repressive effects of Mig1p and Mig2p triggered by rich carbon sources, we assayed the AOX activity in the mutants. A glucose concentration of 1% highly repressed AOX expression during the whole culture process, regardless of whether *MIG1* or *MIG2* genes were deleted (Fig. 1a). However, $\Delta mig1$ resulted in modest AOX expression during the late phase; and interestingly, $\Delta mig1\Delta mig2$ showed a strong derepression of AOX expression in 1% glycerol compared with the WT, in which AOX expression was almost completely repressed (Fig. 1b). Catabolite repression by glycerol was further relieved when the cells were cultivated in 1% glycerol supplemented with 0.5% methanol (glycerol + methanol) (Fig. 1c, d). For both the $\Delta mig2$ and $\Delta mig1\Delta mig2$ mutants grown in glycerol and glycerol + methanol, the AOX activity reached a high level and then decreased gradually to almost zero before 40 h (Fig. 1b, d). This was different to strains cultured with methanol as the sole carbon source, in which the AOX activity remained at a high level for up to

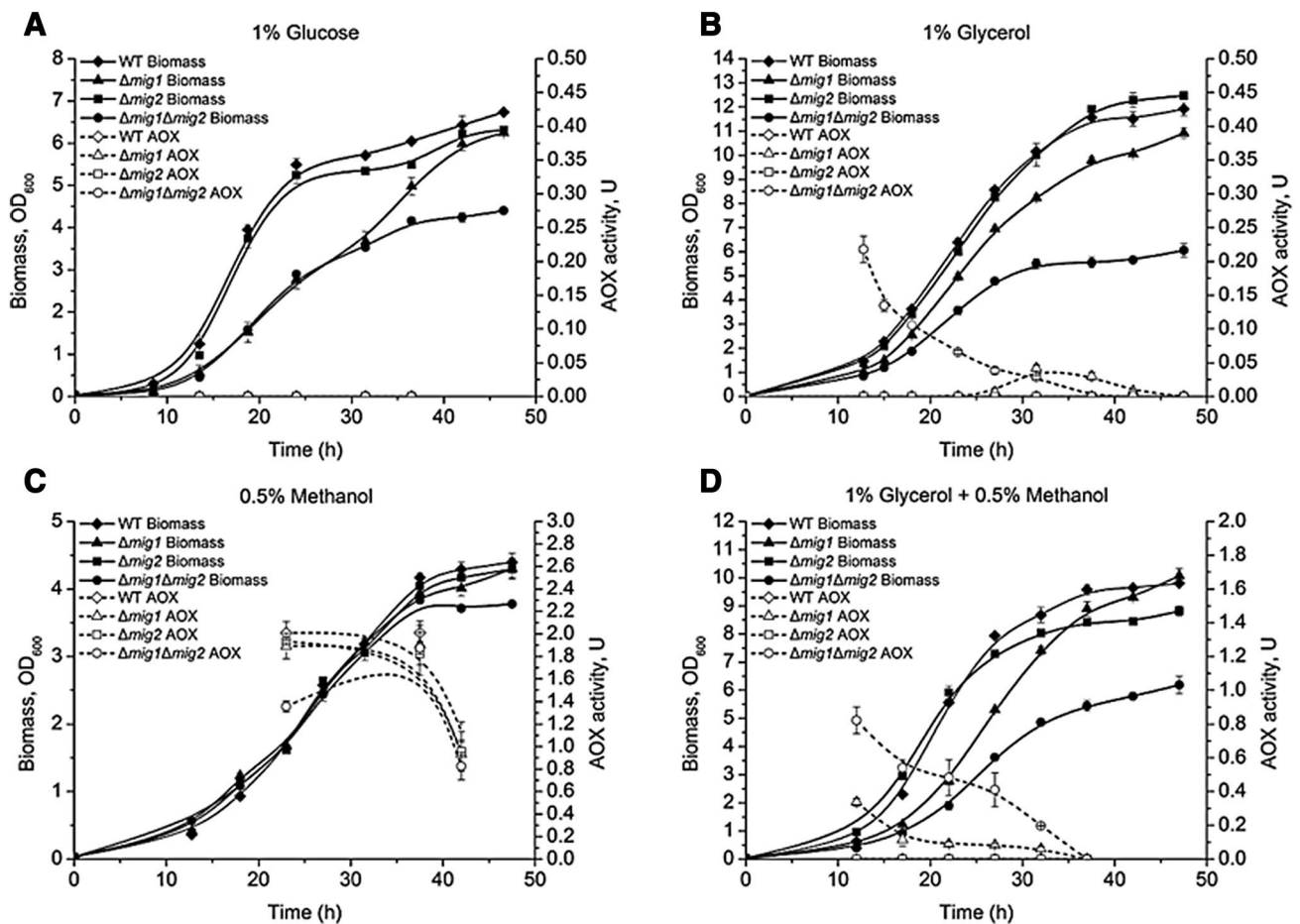


Fig. 1 Relative expression levels of *P. pastoris* (wild type and mutants) alcohol oxidase during growth on various carbon sources: **a** 1% glucose; **b** 1% glycerol; **c** 0.5% methanol; **d** 1% glycerol+0.5% methanol

40 h, despite the sharp decrease later caused by methanol depletion.

Summary of RNA-seq data sets

To explore how Mig proteins affected the $\Delta mig1\Delta mig2$ mutant, cultured in glycerol showing P_{AOX1} derepression, and its related physiological process in comparison with their effect on the wild type strain, transcriptome analysis based on RNA-seq was conducted on the wild type (W) and $\Delta mig1\Delta mig2$ double-deletion mutant (S) grown in glycerol. The ORFs in the RNA-seq dataset for differential expression analysis involved 5277 of the mapped or unmapped ORFs in the reference genome. Each group contained two biological replicates, which showed good correlation (Fig. S2A), and the data were combined for further analysis. In total, 26,247,210 reads were obtained and a close agreement between the biological replicates was confirmed by the Pearson correlation coefficients of 0.952 (W) and 0.962 (S) (Table S3).

Transcriptional comparative analysis reveals differentially expressed genes between $\Delta mig1\Delta mig2$ and wild type

To assess differences more accurately between transcriptome data in $\Delta mig1\Delta mig2$ versus wild type, the data were analyzed by the DEGseq package in R software (Wang et al. 2010). A two-fold or greater change in expression and p value < 0.05 (cut-off at 5% FDR) was determined as significant. An MA-plot of differentially expressed genes is shown in Fig. S2B. A comparative analysis of the RNA-seq data revealed that 1554 genes were differentially expressed and 357 genes were upregulated by at least two-fold when $AOX1$ and $AOX2$ were increased by more than 30-fold. GO analysis revealed that the genes upregulated in the double-mutant strain were mainly involved in cell metabolism (Fig. 2a), whereas the downregulated genes appeared to be mainly associated with cell physiology processes (Fig. 2b). In addition, KEGG metabolic pathway analysis indicated that the genes upregulated in double-mutant strain were

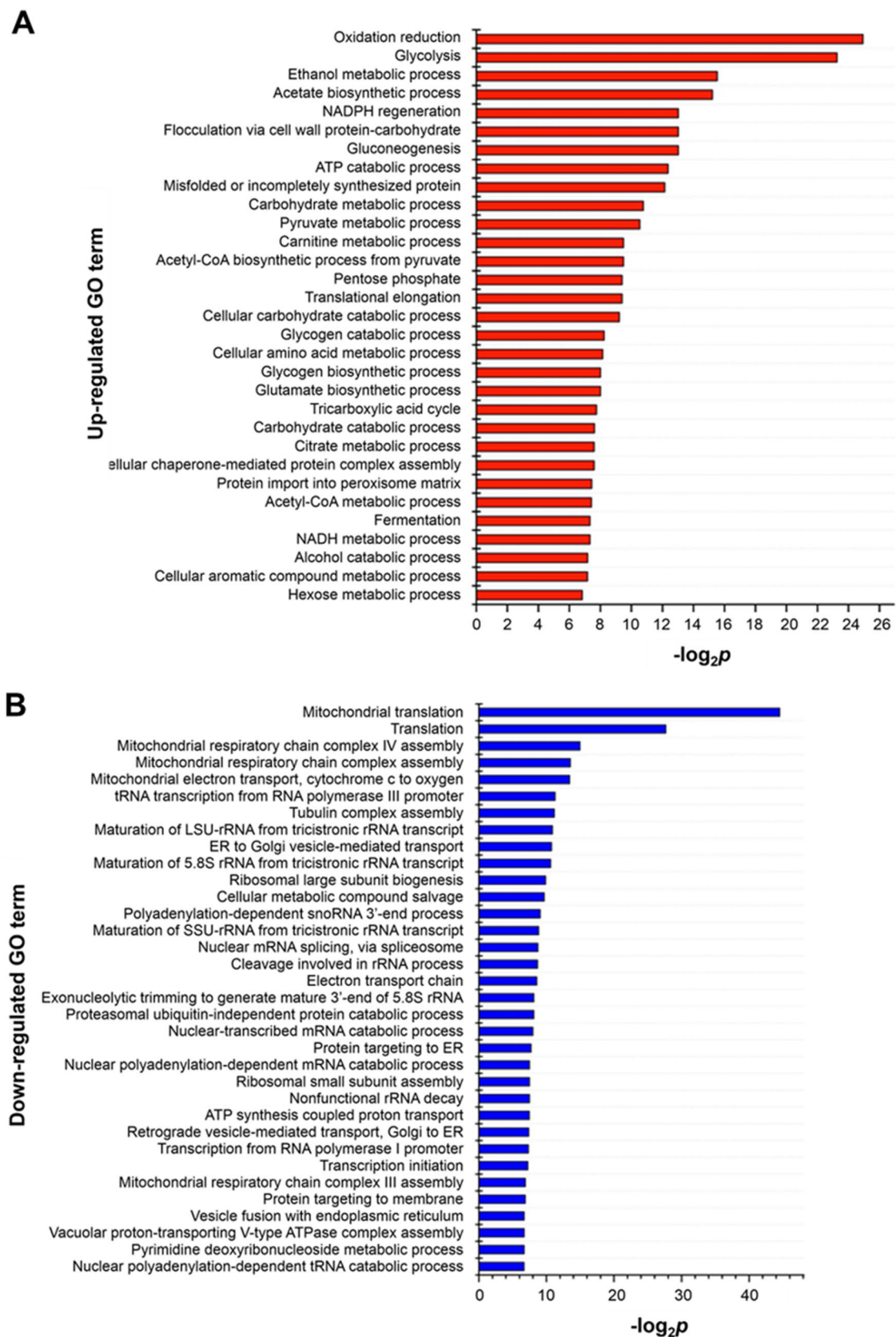


Fig. 2 Significantly upregulated (a) and downregulated (b) genes and their assigned gene ontology (GO) categories in *P. pastoris*

located specifically in the pathways of the metabolic processes: biosynthesis of secondary metabolites; glycolysis and gluconeogenesis; pyruvate metabolism; glyoxylate and dicarboxylate metabolism; and the citrate cycle. Conversely, the downregulated genes were mainly associated with ribosomes, biosynthesis of secondary metabolites, and oxidative phosphorylation (Fig. 3). Generally, KEGG pathway analysis showed similar results to the GO analysis; that is, substance metabolism in the cell was enhanced, whereas DNA and protein synthesis and cell division were reduced by the deletion of *MIG1* and *MIG2* grown in glycerol.

Enhancement of methanol catabolism and peroxisome biogenesis pathways in $\Delta mig1\Delta mig2$ mutant

In Fig. 4, an overview of the methanol metabolism (Van Der Klei et al. 2006), including a ratio of RPKM in $\Delta mig1\Delta mig2$ mutant to that in WT, was presented. In peroxisomes, methanol was oxidized to formaldehyde and hydrogen peroxide by AOX, which was 32.6 times upregulated at the transcriptional level, which also supported the AOX expression results shown in Fig. 1b. Hydrogen peroxide was detoxified by catalase (CAT) to water and oxygen (1.5-fold upregulation). Formaldehyde can be further metabolized via two

different routes: dissimilation via formaldehyde dehydrogenase (FLD1, 1.2-fold upregulation), *S*-formyl glutathione hydrolase (FGH, 0.9-fold upregulation), and formate dehydrogenase (FMD, 2.4-fold upregulation) to CO_2 , generating NADH and CO_2 , or assimilation via the peroxisome-borne enzyme dihydroxyacetone synthase (DHAS, 6.2-fold upregulation) to generate cell constituents. DHAS is part of the xylulose-5-phosphate cycle and catalyzes the formation of two C3-molecules (dihydroxyacetone and glyceraldehyde-3-phosphate) from one C1 (formaldehyde) and one C5 (xylulose-5-phosphate) compound. Thus, methanol catabolism was highly upregulated by the deletion of *MIG1* and *MIG2* in *P. pastoris* grown in glycerol.

AOX synthesis is closely related to peroxisome biogenesis (Distel et al. 1988; Ozimek et al. 2005). *PEX* genes control the development and function of the peroxisomes (Sibirny 2016). Clear upregulations were observed for *PEX1* (2.6-fold), *PEX5* (2.6-fold), *PEX6* (3.2-fold), *PEX14* (2.3-fold), all of which were correlated with the import of peroxisomal matrix enzymes (AOX, DHAS, and CAT), as shown by peroxisome biogenesis in KEGG (Fig. 5). Additionally, a peroxisome membrane protein- encoded gene, *PMP47B*, was also highly upregulated (2.75-fold). Moreover, most of the *PEX* genes were moderately increased in $\Delta mig1\Delta mig2$ grown in glycerol (Table S4).

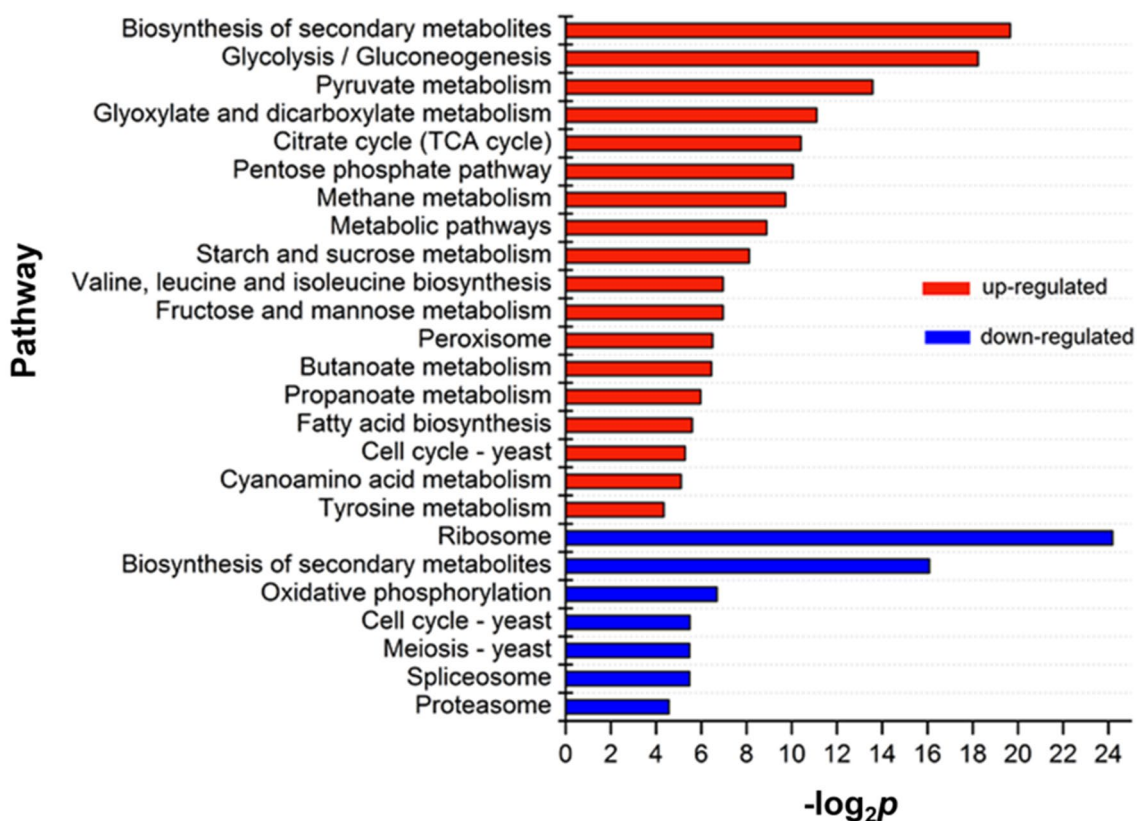


Fig. 3 Statistically significant KEGG classifications of differentially expressed genes in *P. pastoris*

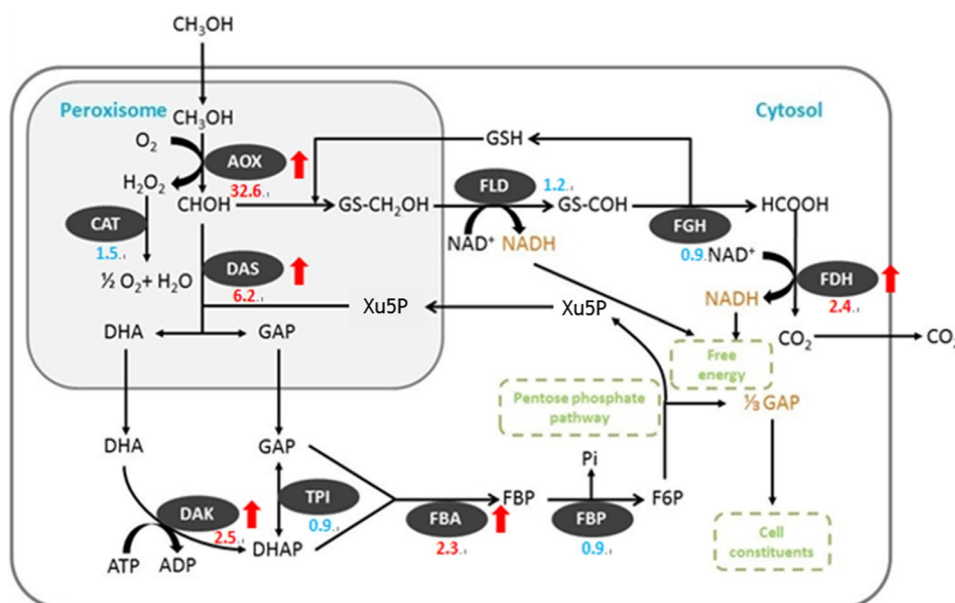


Fig. 4 Significantly differentially expressed genes in the methanol utilization pathway in *Pichia pastoris*. The schematic diagram was adapted from Van Der Klei et al. (2006). *AOX* alcohol oxidase, *FLD* formaldehyde dehydrogenase, *FGH* S-formylglutathione hydrolase, *FDH* formate dehydrogenase, *CAT* catalase, *DAS* dihydroxyacetone synthase, *DAK* dihydroxyacetone kinase, *TPI* triosephosphate isomerase, *FBA* fructose-1,6-bisphosphate aldolase, *FBP* fructose-1,6-bisphosphatase, *DHA* dihydroxyacetone, *GAP* glyceraldehyde-

3-phosphate, *DHAP* dihydroxyacetone phosphate, *F1,6BP* fructose-1,6-bisphosphate, *F6P* fructose-6-phosphate, *Pi* phosphate, *Xu5P* xylulose-5-phosphate, *GSH* glutathione. Red numbers indicate the fold- change of the upregulated genes in the $\Delta mig1 \Delta mig2$ strain compared with the WT strain, and blue numbers indicate the fold- change of downregulated genes in the $\Delta mig1 \Delta mig2$ strain compared with the WT strain. (Color figure online)

Fig. 5 Significantly differentially expressed genes related to the peroxisome biosynthesis pathway in *Pichia pastoris*. The pathway is referred to that of *Saccharomyces cerevisiae* from KEGG pathway database. Red numbers indicate the fold change of upregulated genes in $\Delta mig1\Delta mig2$ strain compared with the WT strain and blue numbers indicate the fold change of downregulated genes in the $\Delta mig1\Delta mig2$ strain compared with the WT strain. (Color figure online)

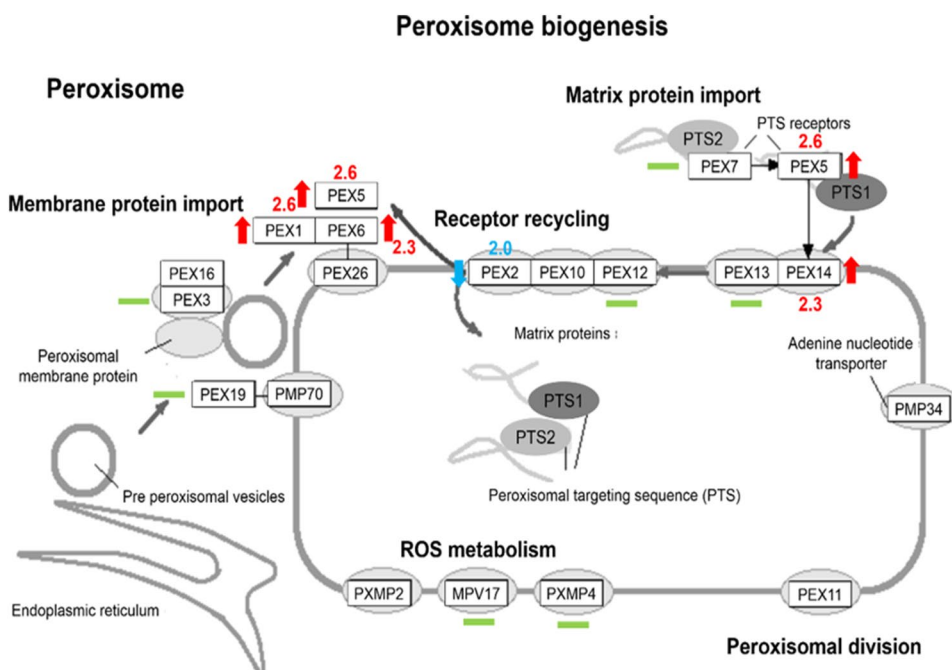


Table 1 Differentially expressed genes related to transcription factors in *P. pastoris*

Locus ID	Description	Log2 Fold change	P value
PAS_chr4_0601	Fungal AP-1-like factor	1.41	1.10E−79
PAS_chr1-1_0165	Activating transcription factor	1.34	7.30E−02
PAS_chr1-1_0033	bZIP factor	1.10	3.57E−03
PAS_chr1-4_0407	bHLH factor	−1.37	3.82E−09
PAS_chr2-2_0153	Regulatory factor X	1.27	1.56E−146
PAS_chr1-3_0126	GATA-binding protein	1.72	3.82E−09
PAS_chr1-4_0658	GATA-binding protein	1.10	3.82E−09
PAS_chr4_0626	GATA-binding protein	1.86	1.10E−79
PAS_chr1-1_0113	Replication factor A1	−1.13	3.09E−01
PAS_chr2-1_0206	COMPASS component SPP1	−2.75	3.82E−09
PAS_chr1-3_0099	Metallothionein expression activator	1.01	3.82E−09
PAS_chr4_0487	Mxr1, positive transcription factor of P _{AOXI}	−1.21	1.10E−79
PAS_chr1-4_0526	Mig2	−	−
PAS_chr4_0334	Mig1	−	−
PAS_chr2-1_0757	Transcriptional regulatory protein CAT8	2.17	0.00E+00
PAS_chr3_0759	Transcriptional regulatory protein LEU3	−1.46	3.82E−09
PAS_chr1-4_0401	Transcriptional activator protein UGA3	−1.24	0.00E+00
PAS_chr2-1_0029	Arginine metabolism regulation protein II	−1.20	3.82E−09
PAS_chr2-1_0582	Heme activator protein 1	1.38	3.82E−09
PAS_chr2-2_0301	Cellular nucleic acid-binding protein	−1.11	3.82E−09
PAS_chr1-4_0308	Homeobox protein cut-like	−2.84	1.56E−146
PAS_chr2-1_0784	Homeobox protein YOX1/YHP1	−1.63	3.82E−09
PAS_chr1-4_0286	Forkhead transcription factor HCM1	−1.41	3.82E−09
PAS_chr2-1_0026	Heat shock transcription factor	−1.06	3.82E−09
PAS_chr2-1_0583	Myb-like DNA-binding protein REB1	1.67	3.82E−09
PAS_chr1-4_0102	Myb-like DNA-binding protein FlbD	1.02	3.82E−09
PAS_chr1-3_0252	Myb-like DNA-binding protein RAP1	−1.01	3.82E−09
PAS_chr3_0092	TEA domain family member	1.23	3.82E−09
PAS_chr3_0968	MADS-box transcription factor	1.06	4.98E−19
PAS_chr4_0093	MADS-box transcription factor	1.63	0.00E+00
PAS_chr1-4_0183	Transcription initiation factor TFIID	−2.62	0.00E+00
PAS_chr1-1_0018	Structure-specific recognition protein 1	1.05	3.82E−09
PAS_chr2-1_0017	HMG box factor	−1.11	1.53E−03
PAS_chr4_0937	Transcription factor STE12	−1.04	3.82E−09
PAS_chr2-2_0362	Meiosis-specific transcription factor NDT80	−1.20	1.10E−79
PAS_chr4_0203	Prm1, positive transcription factor of P _{AOXI}	1.27	7.74E−18
PAS_chr3_1242	Nrg1, negative transcription factor of P _{AOXI}	−1.09	6.66E−02
PAS_chr3_0836	Mit1, positive transcription factor of P _{AOXI}	2.38	0.00E+00

Identification of all differentially expressed genes was based on *P* value < 0.05. The absolute value of “Fold change” is the magnitude of up or down-regulated for each gene. Fold change ≥ 2 indicates up-regulated and ≤ −2 indicates down-regulated in $\Delta mig1 \Delta mig2$

Effects of *MIG1* and *MIG2* double deletion on autophagy

The adaptation of *P. pastoris* cells to methanol or other carbon sources requires a major rearrangement of the cellular architecture. Several *ATG* (autophagy related gene) genes (*ATG1*, *ATG5*, *ATG12*, *ATG16*, *ATG27*, and *ATG28*) were downregulated by more than two-fold, but only one gene

(*ATG26*) was upregulated by more than two-fold (Table S5), which indicated that autophagy in the $\Delta mig1 \Delta mig2$ mutant was ameliorated to a certain extent and proved that Mig proteins may also act as a regulator of autophagy in *P. pastoris*. Interestingly, other pexophagy related genes, *ATG30* (Farré et al. 2008), *ATG25* (Monastyrsky et al. 2005), and *ATG9* (Chang et al. 2005), were not clearly different between the wild-type strain and the $\Delta mig1 \Delta mig2$ mutant. Additionally,

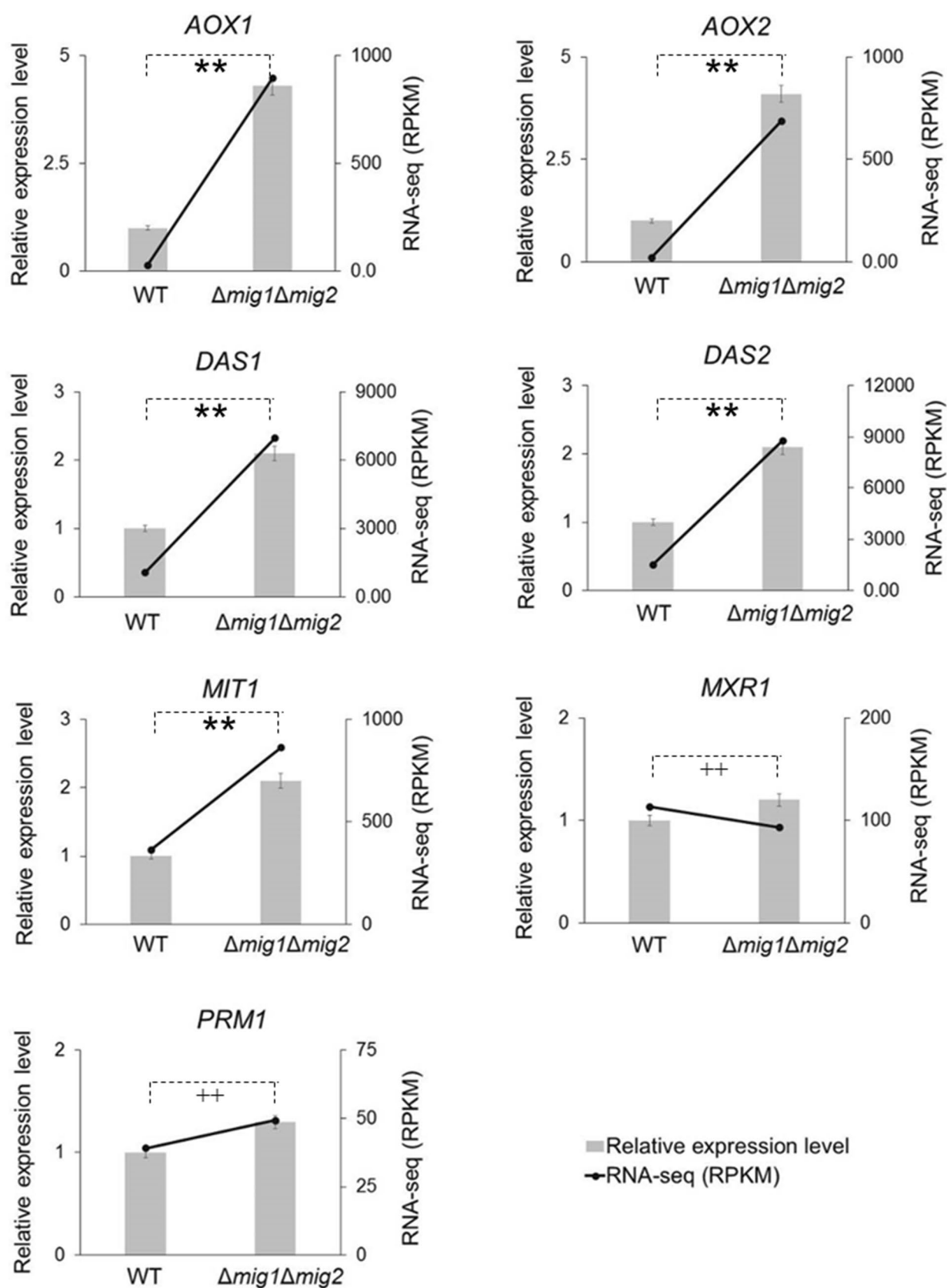


Fig. 6 Quantitative RT-PCR validations for RNA-seq data in *Pichia pastoris*. A total of seven genes were selected for the quantitative qRT-PCR experiments. *AOX1* (PAS_chr4_0821), *AOX2* (PAS_chr4_0152), *DAS1* (PAS_chr3_0832), and *DAS2* (PAS_chr3_0834) were used for the methanol metabolism pathway; *MIT1* (PAS_chr3_0836), *PRM1* (PAS_chr4_0203), and *MXR1* (PAS_chr4_0487) were transcription factors. One-way analysis of variance (Origin 7.0, OriginLab Corporation, USA) was used to determine the significant difference of gene transcription in quantitative RT-PCR experiments between the wild type and $\Delta mig1\Delta mig2$ strains. The significance levels were at $*p < 0.05$, $**p < 0.01$, and $^{++}p > 0.05$

ATG8, which is related to various selective and non-selective macroautophagic processes, and *ATG11*, which is involved in selective macroautophagy (Carlson 1999; Carmona et al. 2002), also showed no clear differences in either strain grown in glycerol. Overall, Mig1p and Mig2p only slightly affected autophagy when the cells were grown in glycerol.

Effects of *MIG1* and *MIG2* double deletion on expression of transcription factors

As transcription repressors, Mig1p and Mig2p might function as transcription factors for the direct regulation of P_{AOX1} , or the indirect regulation of the expression of transcription factors of P_{AOX1} . Therefore, we analyzed the variation in expression of the transcription factors in the KEGG database (http://www.genome.jp/kegg-bin/get_htext#A1) in $\Delta mig1\Delta mig2$. As shown in Table 1, two genes (2.17-fold for PAS_chr2-1_0757; 2.38-fold for PAS_chr3_0836) were significantly upregulated, whereas three genes (2.75-fold for PAS_chr2-1_0206; 2.84-fold for PAS_chr1-4_0308; 2.62-fold for PAS_chr1-4_0183) were significantly downregulated. Among them, the PAS_chr3_0836 gene encodes a critical transcription activator, Mit1p, of P_{AOX1} in *P. pastoris*, which has been previously verified by our group (Wang et al. 2016). In addition, the deletions of *MIG1* and *MIG2* did not significantly influence the two other identified transcription factors of P_{AOX1} , Mxr1 (Lin-Cereghino et al. 2006) and Prm1 (Sahu et al. 2014). The possible transcription factor binding sites of P_{AOX1} and P_{MIT1} were then analyzed by the online software AliBaba2.1 (<http://gene-regulation.com/pub/programs/alibaba2/index.html?>), which works by the construction of the matrices on the fly from TRANSFAC 4.0 sites (<http://gene-regulation.com/pub/databases.html#transfac>). The scanning results indicated that P_{MIT1} holds two Mig1-binding motifs (5'-ATCTTCCCCGCC-3'; 5'-CCTCCCCACA-3'), which contain conserved regions with the reported Mig1/CreA binding sites in *S. cerevisiae* and *Aspergillus nidulans* (Kulmburg et al. 1993; Mathieu and Felenbok 1994; Nehlin and Ronne 1990; Nehlin et al. 1991). However, P_{AOX1} showed no Mig1-binding motifs by the online promoter scanning. Thus, Mig proteins probably repress P_{AOX1} through the negative regulation of Mit1p

expression. Moreover, by BLAST searching, we found that the PAS_chr3_0757 gene encodes a protein homolog of the transcription activator, Cat8 in *S. cerevisiae*, which encodes a transcription activator for the expression of a glucose-repressible alcohol dehydrogenase (Hedges et al. 1995; Walther and Schüller 2001). In *S. cerevisiae*, Mig1p also suppressed Cat8 (Hedges et al. 1995). However, the deletion of this gene did not affect AOX expression in *P. pastoris* (data not shown). As for the three downregulated genes, their specific relationship with AOX expression could not be identified with our present knowledge. Thus, Mig proteins may have a broad function in gene regulation related to carbon catabolism and other biological processes in *P. pastoris*.

Validation of unigenes and gene expression profiling

In order to experimentally validate the reliability of gene expression obtained by RNA-seq data, seven genes were selected and their transcription levels were confirmed through the measurement of the relative mRNA levels by using quantitative RT-PCR with the primers listed in Table S2. Our results, shown in Fig. 6, indicated that the data from qRT-PCR were predominantly consistent with those of RNA-seq, which confirmed the validation of the RNA-seq data and certified the reliability of the RNA-seq data. The differences in the relative fold-change in transcription between qRT-PCR and RNA-seq were mainly ascribed to the biases of the different primers.

Discussion

The methylotrophic yeast *P. pastoris* has been widely used for various heterologous proteins because of its powerful protein expression ability driven by P_{AOX1} . However, the transcription regulation networks of P_{AOX1} regarding methanol induction and catabolite repression have not been fully elucidated.

As a transcription factor, Mig1p has been identified to participate in catabolite repression in *S. cerevisiae* (Carlson 1999; Schüller 2003). Moreover, in the methanol-utilizing yeast *H. polymorpha*, the repression pathway for AOX is only partially reliant on the two Mig1p homologs, irrespective of the carbon source (glucose, sucrose, or ethanol) without glycerol (Stasyk et al. 2007). *P. pastoris* and *H. polymorpha* were identified to have similar methanol-assimilating capabilities and regulation mechanisms of the respective alcohol oxidase genes, despite the strict repression of alcohol oxidase genes in *P. pastoris* (Egli et al. 1980; Hazeu et al. 1972). Therefore, the roles of *MIG1* and *MIG2* were explored with regard to its regulation on P_{AOX1} in *P. pastoris*.

As glycerol is commonly used in *P. pastoris* fermentation, the roles of *MIG1* and *MIG2* in the repression of AOX expression in glycerol were considered in the presence of glucose, which was different from the study conducted in *H. polymorpha* (Stasyk et al. 2007). The double deletion of *MIG1* and *MIG2* damaged cell growth in glucose and glycerol (Fig. 1), which was also in accordance with the results in *H. polymorpha* (Stasyk et al. 2007). This might be attributable to the downregulation of the cellular physiological processes reflected in the GO and KEGG analysis results (Figs. 2, 3). Conversely, although the double deletion of *MIG1* and *MIG2* caused the derepression of AOX expression in glucose in *H. polymorpha*, it was strictly repressed in *P. pastoris*, which indicated that catabolite repression was stricter in *P. pastoris*. However, AOX expression was largely derepressed in $\Delta mig1\Delta mig2$ mutants grown in glycerol.

To completely comprehend the global metabolic changes in $\Delta mig1\Delta mig2$, RNA-seq was applied to generate two high-resolution transcriptional profiles of the WT and $\Delta mig1\Delta mig2$ strains grown with glycerol as the sole carbon source. This provided information on the induction and repression of metabolic genes, as well as non-metabolic genes. Consistent with the AOX activity detected in glycerol medium (Fig. 1b), the methanol catabolism pathway was markedly strengthened, with the *AOX1* level increased by more than 30 times (Fig. 4). The results illustrated that Mig proteins highly repressed P_{AOX1} . Two possible modes may be involved in this process: either by Mig proteins directly bound to P_{AOX1} ; or the repressed expression of positive transcription factors of P_{AOX1} or the activated expression of the negative transcription factors of P_{AOX1} . As the expression of several enzymes other than AOX, i.e., dihydroxyacetone synthase (DAS), formate dehydrogenase (FDH), dihydroxyacetone kinase (DAK), fructose-1,6-bisphosphate aldolase (FBA), were significantly and synchronously upregulated, Mig proteins were more likely to act as an upstream global regulator to control the expression of transcription factors of P_{AOX1} and the promoters of other upregulated genes in the methanol catabolism pathway. To investigate the possibility that Mig proteins regulated the expression of the transcription factors of P_{AOX1} , the variations in the expression of the transcription factors between WT and $\Delta mig1\Delta mig2$ were analyzed (Table 1). Two significantly upregulated genes and three downregulated genes coded for the transcription factors were identified. As previously reported, Mit1p was a critical transcription activator of P_{AOX1} (Wang et al. 2016). Unexpectedly, Mit1p expression was significantly increased in the $\Delta mig1\Delta mig2$ mutant. For another upregulated gene (PAS_chr3_0757), the deletion did not derepress AOX expression (data not shown); thus, it was excluded as a regulator of P_{AOX1} . Moreover, as for the three downregulated genes, we could not identify a specific relationship between them and AOX expression based on our current knowledge.

In *S. cerevisiae*, Mig1p was verified to bind to the promoter of *GAL4*, which codes for the transcription activator Gal4 of the *GAL* genes (Nehlin et al. 1991). Moreover, the online analysis of the transcription factor binding sites predicted that P_{MIT1} contained two Mig1-binding motifs, similar to the Mig1/CreA binding sites in *S. cerevisiae* and *A. nidulans* (Kulmburg et al. 1993; Mathieu and Felenbok 1994; Nehlin and Ronne 1990; Nehlin et al. 1991). Therefore, it could be inferred that Mig1p and Mig2p probably act as transcription repressors of genes, whose transcription was activated by Mit1p. This in turn leads to repression of AOX expression. However, this speculation requires further experiments for verification.

In addition to the enhanced methanol catabolism pathway, the peroxisome biogenesis pathway was also strengthened. As methanol was not used solely in the glycerol medium and AOX was exactly a peroxisomal enzyme in *P. pastoris* and other methylotrophic yeast it was likely that peroxisome biogenesis was regulated by AOX expression, but not through the direct methanol induction process; however, this requires further study for clarification. In addition, the autophagy in $\Delta mig1\Delta mig2$ appeared to be relieved to some extent, which indicated that Mig proteins might also function in the regulation of autophagy in glycerol in *P. pastoris*. However, pexophagy was apparently unaffected as the related genes *ATG30*, *ATG25*, *ATG8*, and *ATG11* showed no clear changes in different strains grown in glycerol.

Generally, our data show comprehensive information on the role of *P. pastoris* *MIG1* and *MIG2* in the derepression of P_{AOX1} and its related biological processes in *P. pastoris*. This study provides a useful reference for the further exploration of the methanol induction and catabolite repression mechanisms of P_{AOX1} , and the future specific and systematic experimental studies of the regulatory mechanisms proposed in this work will allow us to further clarify their roles.

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Compliance with ethical standards

Conflict of interest Lei Shi declares that she has no conflict of interest. Xiaolong Wang declares that he has no conflict of interest. Jinjia Wang declares that she has no conflict of interest. Ping Zhang declares that she has no conflict of interest. Fei Qi declares that he has no conflict of interest. Menghao Cai declares that he has no conflict of interest. Yuanxing Zhang declares that he has no conflict of interest. Xiangshan Zhou declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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