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Deletion of the *GCW13* gene derepresses Gap1-dependent uptake of amino acids in *Pichia pastoris* grown on methanol as the sole carbon source

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ABSTRACT

In *Pichia pastoris*, most of the Glycosylphosphatidylinositol (GPI)-anchored proteins are of unknown function. Gcw13, one of these GPI-anchored proteins, was found to exert an inhibitory effect on the growth of the histidine auxotrophic *P. pastoris* strain GS115 on methanol as the sole carbon source. To investigate the biological function of Gcw13, RNA sequencing (RNA-Seq) was performed to compare the difference of gene expression between GS115 and *GCW13*-deletion strain D13. RNA-Seq analysis showed that, in strain D13, the expression of genes involved in the methanol utilization pathway or peroxisome biogenesis was not changed, and a high proportion of genes involved in the biosynthesis of amino acids were down-regulated, whereas *GAP1*, which encodes a general amino acid permease, was significantly up-regulated. Besides, the intracellular concentrations of various amino acids were significantly higher in D13 than that in GS115. We also observed that deletion of *GCW13* resulted in more Gap1 presented on the cell surface and more active uptake of the toxic proline analogue L-azetidine-2-carboxylate acid (AzC). These results suggest that Gcw13 suppresses the expression of *GAP1* and facilitates the endocytosis of Gap1 on methanol, resulting in decreasing Gap1-dependent uptake of amino acids in *P. pastoris*, which might contribute to the poor growth of GS115 on methanol.

1. Introduction

Glycosylphosphatidylinositol (GPI)-anchored proteins are widely found in eukaryotic cells. Proteins that are to be GPI-anchored have a GPI attachment signal peptide at the C-terminus. The signal peptide is recognized, cleaved and replaced by pre-assembled GPI by the action of a GPI transamidase resides in the endoplasmic reticulum (ER) [1]. The process of GPI anchoring is highly conserved, occurring in protozoa, yeasts, fungi, plants and animals [2] and GPI-anchored proteins share structural features that allows their identification using genome-wide approach [3]. Some GPI-anchored proteins have homologs in other organisms and their biological functions can be inferred by BLAST search, whereas the functions of those with no BLAST hits are hard to interpret.

The structural complexity and sequence diversity of GPI-anchored proteins suggest a rich spectrum of biological functions, but not many of them have been confirmed experimentally. Most of the known GPI-anchored proteins are identified as enzymes, structural components, surface antigens, adhesion molecules, and receptors, *etc.* [4]. GPI-anchored proteins also appear to be involved in the process of signal transduction. They are enriched in lipid raft microdomains, the “hot spots” for signaling processes through many cellular receptors. The GPI-anchored proteins deliver signals by coalescence of microdomains through cross-linking, which generates threshold levels of activation signals through clustered microdomain associated the dual acylation of the Src family protein tyrosine kinases (PTKs) [4]. In yeasts, GPI-anchored proteins are either covalently incorporated into the cell wall network or attached to the plasma membrane. Besides the above mentioned functions, many cell wall-localized GPI-anchored proteins are involved in cell wall remodeling, cell separation, mating, or entry into stationary phase [5–7].

Pichia pastoris is a species of methylotrophic yeast that can grow to very high cell densities in simple, inexpensive media, making it an excellent expression system for the production of heterologous proteins [8]. Heterologous GPI-anchored proteins are used as the “anchor” in *P. pastoris* in cell surface display technology for the

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construction of whole-cell catalysts [9]. However, native GPI-anchored proteins from *P. pastoris* are little studied. In our previous study, we have identified 50 GPI-anchored proteins, and for 68% of which, BLAST search showed no significant similarities to any protein sequence of known function in the GenBank database [10].

In this study, we found that deletion of *GCW13*, a gene encoding one of the GPI-anchored proteins we identified in the histidine auxotrophic *P. pastoris* strain GS115, resulted in a markedly increased growth on methanol as the sole carbon source. Deletion of *GCW13* alters the expression of Gap1 and the intracellular amino acid profile in GS115. Thus, we sought to investigate the function of Gcw13 in the regulation of the Gap1-dependent uptake of amino acids in *P. pastoris*.

2. Materials and methods

2.1. Strains and growth conditions

All the strains and plasmids used in this study are listed in Table 1. ALL primers used in this study are listed in Table S1. *P. pastoris* strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose), BMGY (1% yeast extract, 2% peptone, 1.34% YNB, 1% glycerol) or BMMY (1% yeast extract, 2% peptone, 1.34% YNB, 2% methanol) at 30 °C. Strains were also grown on BMMY plates with or without 100 μg/mL L-azetidine-2-carboxylic acid (AzC). *Escherichia coli* DH5α cells were grown in LB medium at 37 °C. Bleomycin (100 μg/mL for yeast and 25 μg/mL for *E. coli*), was added to the medium if required.

2.2. Plasmid construction

Plasmid pA01 was constructed by replacing the *AOX1* promoter region of the plasmid pAOX1-eGFP reported previously [11] with the fragment containing full-length *GAP1* gene and its native promoter region. Briefly, the vector fragment (without the AOX1 promoter region) was amplified from pAOX1-eGFP using primers vp1 and vp2. The fragment containing full-length *GAP1* gene and its native promoter region was amplified from GS115 genomic DNA using primers GAP1-1 and GAP1-2 which introduced 15–20 bp homologous ends. The two fragments were joined together to generate the plasmid pA01 using Seamless Assembly Cloning Kit according to the manufacturer's protocol (Clone Smarter Technologies Inc. US).

2.3. Construction of strains

Deletion of *GCW13* gene in *P. pastoris* GS115 was achieved by Cre/loxP system as previously described [12]. The lox71-Cre-ZeoR-lox66 (CORE) cassette, which contains a P_{AOX1}-driven Cre recombinase gene (*Cre*) and P_{TEF1}-driven bleomycin resistant gene (*ZeoR*), was amplified from pPICZC plasmid [12], with lox71 and lox66 sites introduced by primers P3 and P4, respectively. The upstream and downstream homologous regions of *GCW13* were amplified from GS115 genomic DNA, with the 30–40 bp of overlap with the CORE cassette, using primers p5/p6wp and p7p/p8, respectively. The three overlapping fragments were then fused together by overlapping extension PCR to form the "up-CORE-down" knock-out cassette. The knock-out cassette was transformed into GS115 and bleomycin resistant colonies were selected and transferred to liquid BMMY for transient expression of Cre recombinase which caused the recombination of the CORE cassette, thereby excising the Cre-ZeoR cassette and resulting in a double-mutant lox72 site.

The *GCW13* complement strain was also constructed using the Cre/loxP system, by replacing the double-mutant lox72 site in the D13 genome with a knock-in cassette "up-*GCW13*-CORE-down". The knock-in cassette was obtained by fusing the following three fragments: the upstream homologous region of *GCW13* with the full-length *GCW13* gene amplified from GS115 genomic DNA by primers p5/p6, the CORE cassette and the downstream homologous region of *GCW13*.

For the construction of strains GS115pA01 and D13pA01, which express Gap1-eGFP fusion protein driven by that native promoter of *GAP1*, plasmid pA01 was linearized by *Nde* I and transformed into GS115 and D13 respectively.

HIS4 complement strains were constructed by transforming *HIS4* fragment amplified from pPIC9K by primers HIS4-1/HIS4-2 into strains GS115 and D13, respectively.

2.4. RNA isolation and RNA sequencing (RNA-Seq)

For RNA sequencing, total RNA was extracted from strains GS115 or D13 grown in BMMY for 12 h using the hot acidic phenol method [13]. The DNA contamination was eliminated using RNase-free DNase I (Tiangen Biotech, China) in a reaction at 37 °C for 30 min and followed by a heat deactivation step as recommended by the manufacturer. Poly(A)+ mRNA was selected using NEB Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). The mRNA libraries were constructed using the NEB Next Ultra RNA library prep kit for Illumina (New England Biolabs) following the manufacturer's protocol. Sequencing was performed on an Illumina HiSeq X Ten sequencer for 150 cycles. The high-quality reads that passed the Illumina filter were subjected to the subsequent bioinformatics analysis. The adapter sequences were trimmed from the reads. The reads were mapped to the reference sequence database (*Komagataella phaffii* RNA assembly ASM2700v1) using the hyper-accurate mapping algorithm FANSe3 in the NGS analysis platform "Chi-Cloud" (http://www.chi-biotech.com). Splice variants were merged. Gene expression levels were quantified using the RPKM method [14]. Genes with at least 10 reads were considered quantifiable genes. Differential expressed genes (DEGs) were analyzed using the edgeR package (version 3.12.0) [15] considering at least 2-

Table 1
Strains and plasmid used in this study.

Strain/plasmid	Relevant characteristics	Reference/source
GS115	Wild type, *his4*	Invitrogen
D13	Derived from GS115, *GCW13*Δ::loxP, *his4*	This study
C13	Derived from D13, *GCW13*-loxP, *his4*	This study
GS115pA01	Derived from GS115, *his4*, *GAP1*-eGFP (*BleoR*	This study
D13pA01	Derived from D13, *GCW13*Δ::loxP, *his4*, *GAP1*-eGFP (*BleoR*	This study
GS115H	Derived from GS115, *HIS4*	This study
D13H	Derived from D13, *GCW13*Δ::loxP, *HIS4*	This study
pPICZC	Zeor, carrying Cre-ZeoR cassette	[12]
pA01	Derived from pPICZA, PGAP1 instead of PAOX1, carrying GAP1, Zeor	This study

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