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ORIGINAL ARTICLE

Characterization and application of a putative transcription factor (SUT2) in *Pichia pastoris*

Yankun Yang[1,2] · Yating Zheng[1,2] · Pengcheng Wang[1,2] · Xiang Li[1,2,3] · Chunjun Zhan[1,2,4,5] · Robert J. Linhardt[6] · Fuming Zhang[6] · Xiuxia Liu[1,2] · Jinling Zhan[1,2] · Zhonghu Bai[1,2]

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Abstract

Pichia pastoris is able to metabolize methanol via a specific MUT (methanol utilization) pathway. Based on the powerful AOX1 (Alcohol Oxidase 1) promoter, the *P. pastoris* expression system has become one of the most widely used eukaryotic expression systems. The molecular mechanisms of methanol metabolic regulation remain unclearly understood, so it is important to identify and develop new transcriptional regulators. Our previous studies suggested that the expression of *SUT2* could be induced by methanol but is repressed by glycerol, which indicates that SUT2 may be involved in methanol metabolism through an unknown mechanism. *SUT2* encodes a putative transcription factor-like protein harboring a Gal4-like Zn_2Cys_6 DNA-binding domain in *Pichia pastoris*, and its homolog in *Saccharomyces cerevisiae* regulates sterol uptake and synthesis. This study shows that the overexpression of SUT2 promoted the expression of AOX1 and increases ergosterol content in cells. Furthermore, via truncation of the putative *SUT2* promoter at diverse loci, the − 973 base pair (bp) to − 547 bp region to the ATG was shown to be the core element of the inducible promoter P_{SUT2}, which strongly responds to the methanol signal. The transcriptional start site of *SUT2*, "A" at the 22nd bp upstream of ATG, was determined with 5′-rapid amplification of cDNA ends. A forward-loop cassette was constructed with *MXR1* (Methanol Expression Regulator 1, a positive transcription factor of P_{AOX1}) promoted by P_{SUT2}, enabling moderate elevation in the expression level of Mxr1 and high activity of P_{AOX1} without damaging cellular robustness further boosting the production of heterologous proteins. The P_{AOX1}-driven expression of enhanced green fluorescent protein in this novel system was improved by 18%, representing a promising method for extrinsic protein production. SUT2 may play roles in methanol metabolism by participating in sterol biosynthesis. P_{SUT2} was characterized as a novel inducible promoter in *P. pastoris* and a P_{SUT2}-driven MXR1 forward-loop cassette was constructed to enhance the P_{AOX1} activity, laying a foundation for further development and application of *P. pastoris* expression system.

Keywords *Pichia pastoris* · Methanol utilization (MUT) pathway · *SUT2* · Promoter · Transcriptional regulation · Forward-loop cassette

Introduction

The methylotrophic yeast, *Pichia pastoris* (*P. pastoris*), is a widely used host for heterologous protein production, with favorable properties, such as growth to high cell density and high capacities for protein secretion (Vogl and Glieder 2013). *P. pastoris* was able to metabolize methanol via a specific MUT (methanol utilization) pathway (Ozimek et al. 2005; Couderc and Baratti 2014). AOX1 (Alcohol oxidase 1) is the first and rate-limiting enzyme of the MUT pathway and P_{AOX1} (Promoter of *AOX1*) is the key to the efficient expression of heterologous proteins. AOX1 accounts for about 5% of the total mRNA and 30% of total soluble proteins in the cell using

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✉ Yankun Yang
yangyankun@jiangnan.edu.cn

Extended author information available on the last page of the article

methanol as the sole carbon source (Vogl and Glieder 2013). Nevertheless, cells grown on repressing carbon sources (e.g., glucose, glycerol, ethanol) show complete lack of AOX activity (Inan and Meagher 2001). This tight regulation is exerted on the transcriptional level, as *AOX* mRNA is undetectable in cells grown on repressing carbon sources (Ellis et al. 1985; Cregg et al. 1989; Koutz et al. 1989). However, the methanol induction mechanism of AOX1 is still unclear, so it is important to find, identify and develop new transcriptional regulators involved in methanol metabolism.

In a previous study, we found that the expression of PAS_chr1-4_0516 (homologous to *SUT2* in *Saccharomyces cerevisiae*) and *MIT1* could positively respond to a change in carbon source giving high expression levels in methanol-containing medium (Li et al. 2018). *SUT2* reportedly encodes a hypothetical transcription factor that is a member of the Zn_2Cys_6 family in *S. cerevisiae* (Rutzler et al. 2004; Joshua and Hofken 2017). S.cSUT2 regulates sterol uptake under anaerobic conditions along with S.cSUT1, and also controls adaptations to anaerobic growth, sterol biosynthesis, as well as filamentation and mating (Ness et al. 2001). However, it is unknown whether the function of the *P.pSUT2* gene is related to ergosterol.

The correlation between SUT2 and the MUT pathway was studied via detecting the expression level of SUT2 and AOX1 in strains exhibiting different expression level of AOX1 and SUT2, respectively, to characterize the function of SUT2 in *P. pastoris*. We investigated the inducer of the *SUT2* gene and the relationship between the function of SUT2 and ergosterol. Moreover, the *SUT2* promoter was isolated and characterized to study the regulatory elements in response to methanol metabolism. Over the past decades, some inducible promoters have been developed in *P. pastoris*, including P_{FLD1}, P_{PHO89}, and P_{THI11}. However, the number of strong promoters available for heterologous protein production is still limited and additional inducible promoters are needed (Shen et al. 1998; Ahn et al. 2009; Stadlmayr et al. 2010). This study adds a new inducible promoter in *P. pastoris* system. A forward-loop cassette was constructed with *MXR1* promoted by P_{SUT2} that enhances the P_{AOX1} activity to apply P_{SUT2} for optimization of the *P. pastoris* expression system. Future work is planned to identify the core promoter region and use this novel promoter for the high-yield expression of pharmaceutical and industrial proteins.

Materials and methods

Strains and cultivation

P. pastoris GS115, a histidine auxotrophic, was purchased from Invitrogen (Carlsbad, CA, USA) and used as a host for gene expression experiments. *P. pastoris* was cultured with shaking at 30 °C in 1% yeast extract, 2% peptone, and 2% glucose (YPD); glycerol BMGY medium (0.25%) or 1% methanol BMMY medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 3 g L^{-1} K_2HPO_4, 11.8 g L^{-1} KH_2PO_4, 13.4 g L^{-1} YNB, 4×10^{-4} g L^{-1} biotin, 2.5 mL L^{-1} of glycerol or 10 mL L^{-1} of methanol); 0.25% glycerol MG or 1% methanol MM medium (13.4 g L^{-1} YNB, 0.04 g L^{-1} L-histidine, 4×10^{-4} g L^{-1} biotin and 2.5 mL L^{-1} of glycerol or 10 mL L^{-1} of methanol). *Escherichia coli* strain DH5α, Top10 (TransGen, Beijing, China) was used as the host for the construction of plasmids and grown in LB medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) at 37 °C. Ampicillin or kanamycin was added to LB medium at a final concentration of 50 μg mL^{-1}; G418 and Zeocin were added to the YPD medium at final concentrations of 0.3 g L^{-1} and 0.1 g L^{-1}, respectively. Electroporation was performed to transform *P. pastoris*. Other essential molecular operations followed the standard protocol as previously described (Zhan et al. 2016).

Plasmid and strain construction

Plasmids were generated by standard techniques. The strains used in this study are listed in Table 1, and the primers and plasmids are listed in the Supplementary Tables S1 and S2, respectively.

sut2Δ strain

The GS115 strain with *SUT2* deletion was constructed by homologous recombination with the G418 resistance gene *KAN* as the marker. First, the upstream region of *SUT2* was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with genomic DNA as the template. Next, the *KAN* sequence together with its own promoter and terminator (1556 base pair (bp)) was amplified by PCR using the plasmid pMD19-T-GT1-del (previously constructed in our lab) as the template and the primers KAN-F and KAN-R. The PCR-amplified fragments SUT2-up and KAN were digested with the corresponding enzymes and then inserted into the *Hin*dIII/*Sma*I-digested pMD19-T vector to yield pMD19-SUT2up-KAN. The downstream region of *SUT2* was amplified with the primers SUT2-down-F and SUT2-down-R to yield a 0.6-kb fragment. The fragment was digested with *Sma*I/*Sac*I and cloned into pMD19-SUT2up-KAN digested with the same enzymes to construct the plasmid pMD19-SUT2-del. The deletion cassette was amplified with pMD19-SUT2-del as the template and SUT2-up-F/SUT2-down-R as primers, and subsequently transformed into wild type. G418-resistant transformants were isolated on YPD solid (YPDS) medium supplemented with 0.3 mg mL^{-1} G418.

Table 1 Strains used in this study

Strain	Genotype	Source or reference
E. coli		
Trans5α	F-ϕ80d lacZ_M15 Δ(lacZYA-argF) U169 end A1 recA1 hsdR17 (rk–, mk +) supE44λ-thi-1 gyrA96 relA1 phoA	TransGen Biotech
P. pastoris		
GS115	Wilde-type Mut$^+$, *his4*	Invitrogen
SUT2Δ	GS115, deficiency of *SUT2*; Selection of G418	This study
SUT2-GS115	Selection of Zeocin^R-resistant expression vectors	This study
KM71	*His4*, deficiency of *AOX1*, *arg4*	Invitrogen
AOX1-GS115	Selection of Zeocin^R-resistant expression vectors	This study
pSM-pAE-GS115	Selection of G418 in MD medium	This study

SUT2-overexpression strain

SUT2 was amplified by PCR using genomic DNA as the template and primers SUT2-F and SUT2-R. The *SUT2* fragment was inserted into vector pGAPZ B at *Eco*RI/*Xho*I sites to yield pGAPZB-SUT2. Finally, pGAPZB-SUT2 was digested with *Avr*II and transferred into wild type, yielding the *SUT2*-overexpression strain.

AOX1-overexpression strain

Following the above method, we constructed the *AOX1*-overexpression strain.

Construction of P_{SUT2} libraries

The P_{AOX1} of pAE was removed by PCR with pAE_F/R as primers to construct the EGFP expression vector pSE (Supplementary Fig. S1). Then, the putative *SUT2* promoter, at the − 2000 bp to − 1 bp region to the ATG start codon (the negative sign used in this study means relative to the ATG start codon), was amplified by PCR with genomic DNA as the template. Primers used for this PCR (S2000-F and S2000-R) carried restriction sites for *Bgl*II and *Kpn*I, respectively. After digesting and ligating both sequences with specific enzymes, we obtained pS(2000)E (Fig. S1). Then, vectors that contained truncated versions of the promoter were constructed to identify the key regulatory regions of P_{SUT2}.

Forward-loop cassette to increase the intensity of P_{AOX1}

P_{SUT2} (2 kb) and *MXR1* (1.2 kb) were amplified by PCR using genomic DNA as the template and primers pS2000-F/R and Mxr1-F/R, respectively. Two fragments were ligated by fusion PCR with primers pS2000-F and Mxr1-R. The product, which was digested with *Bam*HI, was inserted into pPIC9K digested with *Oli*I and *Bam*HI to yield pSMxr. Finally, pSM was digested with *Sal*I, and transferred into the pAE-GS115 strain, yielding the *MXR1*-overexpression strain under the control of $P_{SUT2(2000)}$, pSM-pAE-GS115.

Reverse transcription (RT)-PCR

Cells were collected for RT-PCR and total RNAs were extracted by the standard protocol for the GeneJET RNA Purification Kit (Thermo Fisher Scientific). The transcript levels were normalized by the endogenous reference gene *ACTIN1* in each sample and calculated by the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

Transcription start site (TSS) identification and sequence analysis of the *SUT2* promoter

The TSS of P_{SUT2} was identified using 5′-rapid amplification of cDNA ends (RACE). Total RNAs were reverse-transcribed with 5′-RACE_R1 using Goldenstar™ RT6 cDNA Synthesis Kit (Tsingke) at 42 °C for 50 min, followed by 85 °C for 5 min. The cDNA was purified with GeneJET PCR Purification Kit (AXYGEN), and poly C was added to the 5′-end using terminal deoxynucleotidyl transferase (TaKaRa). The obtained product was used as the template for PCR with the universal primer 5′-RACE_F and the gene-specific primer 5′-RACE_R2/R3. Through sequencing, the 5′-terminal nucleotide of the RACE-PCR products that was identical with the genomic sequence was designated as the TSS.

The online program BDGP (https://www.fruitfly.org/seq_tools/splice.html) was used to predict the TSS in the 747 bp upstream region of *SUT2*. The online program Tfsitescan (https://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) was used to search for transcription factor-binding sites (TFBS) in the cloned 2000-bp upstream region of *SUT2*.

EGFP fluorescence measurement

EGFP expression was analyzed based on its fluorescence using an Enspire 2300 microplate reader (Perkin Elmer,

USA) with an excitation wavelength of 488 nm and an emission wavelength of 507 nm. The fluorescence value was calculated by subtracting the blank value.

Sterol analysis

Strains were cultivated for biomass and sterol analyses. After obtaining the OD_{600} value, the samples were centrifuged (3000× *g* for 5 min at 4 °C) and the yielded biomass was washed twice with distilled water. The cells were then frozen at − 80 °C and freeze dried. Dried cells were weighed before sterol analysis. Ten milligrams of dried yeast cells were suspended in methanol:1-propanol (1:1) and homogenized using an Ultra Turrax homogenizer for 5 min. After centrifugation, the supernatant was transferred to high-performance liquid chromatography (HPLC) vials. The samples were analyzed by HPLC with a SYMMETRY C18, 4.6 × 250-mm column (Waters, MA, USA). A mobile phase of methanol was used at 0.8 mL min^{-1}, and the assay was run at 30 °C. Reference sterol was obtained from Sangon Biotech.

Results

Expression characteristics of *P. pastoris SUT2*

We performed RT-PCR to determine the transcript abundance of *SUT2* under different culture conditions to further verify our previous RNA-sequencing results (Li et al. 2018). The wild-type strain was cultured in synthetic medium with glycerol or methanol as the sole carbon source for 24 h or 48 h. The expression of *AOX1* was consistent with previous reports (Couderc and Baratti 2014; Capone et al. 2015) describing *AOX1* repression by glycerol and marked induction by methanol (Fig. 1). The expression level of *SUT2* was four- to fivefold higher than that of *ACTIN1*, which is often constitutively expressed at high levels. Of note, when *P. pastoris* is grown on methanol, the relative mRNA level of *SUT2* is one-third that of *AOX1*. We conclude that *SUT2* is inductively expressed at high levels when grown in methanol.

Expression of *SUT2* in strains with different expression levels of *AOX1*

Three strains, *aox1Δ* strain (KM71), wild-type and AOX1-overexpression strain, were cultured with methanol as sole carbon source to study the effect of *AOX1* expression on *SUT2*. Compared with the wild-type strain (Fig. 2), at 36 h, the expression level of *SUT2* decreased remarkably in *aox1Δ* strains and increased in the AOX1-overexpression strains. At 48 h, the expression level of *SUT2* in wild-type strains was similar with that of AOX1-overexpression strains, which was not significant, probably because AOX1 protein has been accumulating, the total expression level of AOX1 protein is getting closer at 48 h. These results indicated that there was a positive correlation between *SUT2* and *AOX1*.

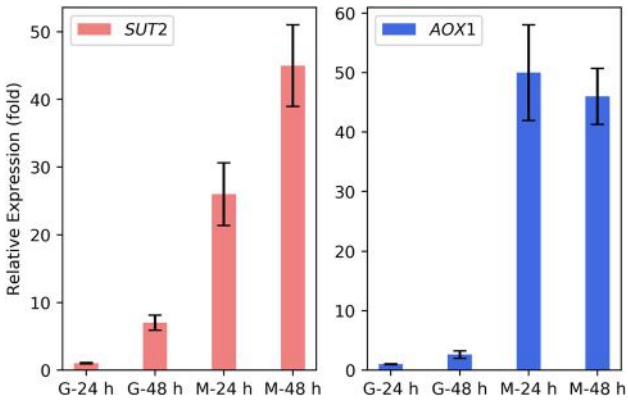

Fig. 1 Relative transcription levels of *AOX1* and *SUT2* in different carbon sources. Error bars represent the standard deviation of three biological replicates. The independent sample t-test was used to determine significance. *G* glycerol, *M* methanol

Expression of *SUT2* in different strains induced by formaldehyde/methanol

Two strains, wild type and *aox1Δ*, were cultured with methanol or formaldehyde to identify the inducer of P_{SUT2}. The cells were collected and transcription levels of *SUT2* and *AOX1* were measured using RT-PCR. Comparison of various inducers at each concentration showed nearly identical induction profiles of the *SUT2* promoter in the two strains, in which the induction efficiency of methanol was higher than that of formaldehyde (Fig. 3). For wild type, the *SUT2* transcription level under 5-mM methanol was 4.2-times higher than that under 5-mM formaldehyde, and the *SUT2* transcription level under 1.4-mM methanol was 6.9-times higher than that under culture with formaldehyde. For *aox1Δ*, the *SUT2* transcription level under 5-mM methanol was 2-times higher than that under 5-mM formaldehyde, and the *SUT2* transcription level under 1.4-mM methanol was 6.1-times higher than that under formaldehyde. These results suggest that methanol is the main inducer of the *SUT2* promoter.

Expression levels of *AOX1* in strains with different expression levels of *SUT2*

Three strains, *sut2*Δ, wild-type, and SUT2-overexpression strain, were cultured with methanol as the sole carbon source, and the transcription levels of *SUT2* and *AOX1* were detected by RT-PCR (Fig. 4). The transcription level of *AOX1* in the *sut2*Δ strain was about 24% lower than that of the wild type, and the transcription level of *AOX1* in the

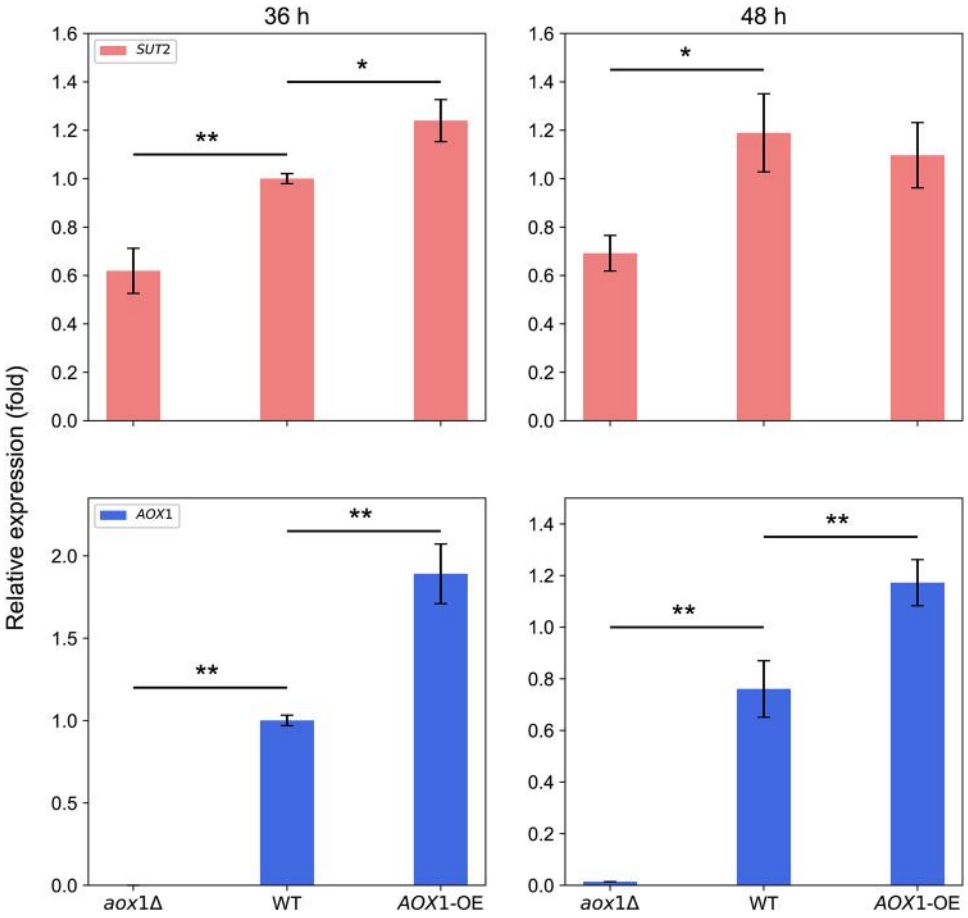

Fig. 2 Transcriptional level of SUT2 in wild type, *aox1*Δ, and AOX1-overexpression strain. Error bars represent the standard deviation of three biological replicates. Pink indicated SUT2, and blue represented AOX1. *$p < 0.05$; **$p < 0.01$

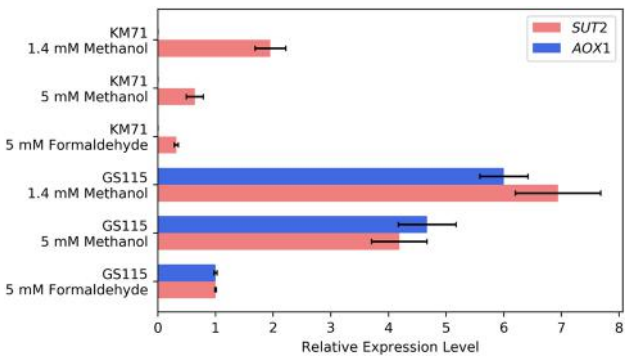

Fig. 3 Comparison of relative transcription levels of *AOX1* and *SUT2* in wild type and *aox1Δ* with methanol and formaldehyde as inducers. In the case of formaldehyde, it is impossible to collect data in a saturated regime because of the toxic effect of a high concentration of formaldehyde on cell growth. Therefore, the transcription level of the strains under the condition of 5-mM formaldehyde has been plotted in the diagram. Error bars represent the standard deviation of three biological replicates

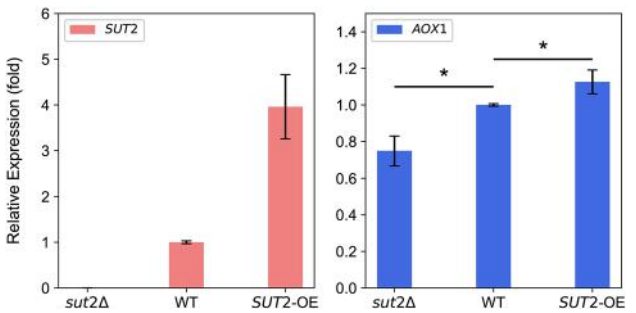

Fig. 4 Transcription level of *SUT2* and *AOX1* in wild-type, *sut2*Δ, and SUT2-overexpression strain. Pink indicated SUT2, and blue represented AOX1. Error bars represent the standard deviation of three biological replicates. *$p < 0.05$

SUT2-overexpression strain was 12% higher than that of the wild type.

We also tested the expression levels of *AOX1* in the three strains followed the standard protocol as previously described (Li et al. 2018). One activity unit was defined as the amount (μmol) of chromogenic substance produced by the catalytic reaction per minute with 1 mg of the enzyme. The results showed that at first 24 h, there was no significant difference in the activity of the AOX among these strains in glycerol medium (Fig. 5). With the induction of methanol at 24 h, the AOX activity of the overexpression strain was 20% higher than that of the wild-type strain at 48 h, and the activity of the defective strain was 13% higher than that of

the wild-type strain. These results demonstrate that overexpression of SUT2 can improve the expression of *AOX1* in a methanol-induced environment, although the effect of SUT2 deficiency is not clear.

Relationship between the function of *SUT2* and ergosterol

Three strains *sut2Δ*, wild-type, and SUT2-overexpression strain were cultured with MM or MG. The content of ergosterol in the cells was detected by HPLC. The extracellular ergosterol was increased by ~ 35–50% (Fig. 6), while the change of the intracellular ergosterol was slightly. It may be because the internal environment is stable, the ergosterol content is relatively constant. However, during the whole growth process, the contents of ergosterol showed an increasing trend from *sut2Δ* to wild-type to SUT2-overexpression strain. There is a significant difference between SUT2-overexpression strain at 60 h and *sut2Δ* at 36 h ($p = 0.0286 < 0.05$). Overall, the content of ergosterol in *P. pastoris* was positively correlated with the expression level of SUT2.

TSS identification and sequence analysis of the *SUT2* promoter

The TSS of *SUT2*, which is important for promoter activity, was identified by 5′-RACE analysis. Amplified products for the two pairs of primers were, respectively, detected by agarose gel electrophoresis. There was a band above 100 bp for the primers 5′-RACE_F and 5′-RACE_R2, and a band about at 100 bp for the primers 5′-RACE_F and 5′-RACE_R3 (Fig. 7a, b). These two bands were isolated and the products were re-amplified by PCR and cloned into pMD19-T, and 20 colonies were sequenced. The 5′-ends of six of the 20 clones were located at the site of − 22 bp (Fig. 7c); whereas, the 5′-ends of the remaining clones were closer to ATG.

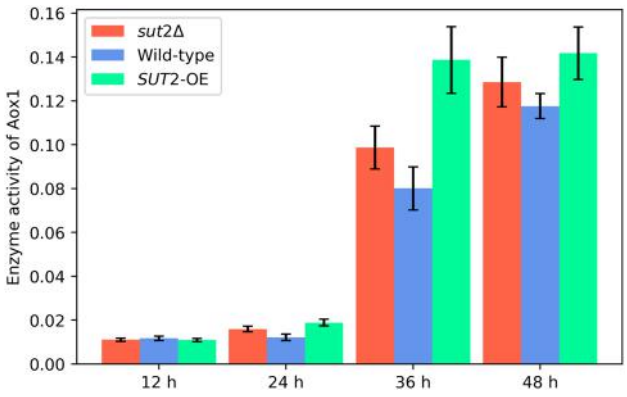

Fig. 5 Enzyme activity of Aox in wild-type, *sut2*Δ, and SUT2-overexpression strain. For the first 24 h, the strains were cultured in BMGY and then washed twice with PBS (pH 7.4). Subsequently, the cells were shifted into BMMY for continued culture until 48 h. The samples were harvested at 12 h, 24 h, 36 h, 48 h. Blue indicated wild-type strain, orange represented *sut2*Δ strain and green represented SUT2-overexpression strain. Error bars represent the standard deviation of three biological replicates

The 747-bp region, which is the interval between *SUT2* and its upstream gene *RNG*, was used to search for the TFBS. The putative TATA box was found at the region from − 55 to − 49 bp and the putative CAAT box was found at the region from − 110 to − 103 bp (Fig. 7c). We infer that the TSS is the "A" at − 22nd bp, based on the predicted result of the position of the TSS and core elements in the *SUT2* promoter, and the considerable number of transformants containing this fragment.

Identification of regulatory upstream sequences in P_{SUT2}

The putative P_{SUT2}, at − 2000 bp to − 1 bp, was constructed to initiate *EGFP* and was truncated at sites − 973 bp, − 747 bp, − 547 bp, − 460 bp and− 247 bp, respectively (Fig. 8). The different truncated promoters showed varying activities in methanol (Fig. 8), whereas barely changed in glycerol (Fig. S2). These results showed that the region from− 973 to − 547 bp contains positive methanol-regulatory sites. Thus, mutations or deletions in this region could cause a decrease in promoter activity.

Forward-loop conversion cassettes could transform existing P_{AOX1}-based expression strains into more efficient methanol induction systems

The transcriptional activating factors Mxr1, Mit1, and Prm1 can be induced by methanol. Therefore, enhancing their expression may lead to a higher expression level of P_{AOX1} and even de-repression in glucose or glycerol. Based on the regulatory mode of P_{AOX1}, Mxr1 was modified by overexpression with P_{SUT2} to construct a positive feedback loop in *P. pastoris*. When Mxr1 was initiated by P_{SUT2}, it could be induced by methanol, which would subsequently promote the expression of AOX1. This process, which induced by methanol, could increase the expression of Mxr1 initiated by P_{SUT2} repeatedly, further inducing P_{AOX1} (Fig. 9a). The pSM-pAE-GS115 strain was cultured in glycerol and methanol respectively, then the fluorescence value of these strains was detected, and the highest expression values of EGFP per strain were compared. The fluorescence expression per unit of OD_{600} was 118% that of the wild type (Fig. 9b).

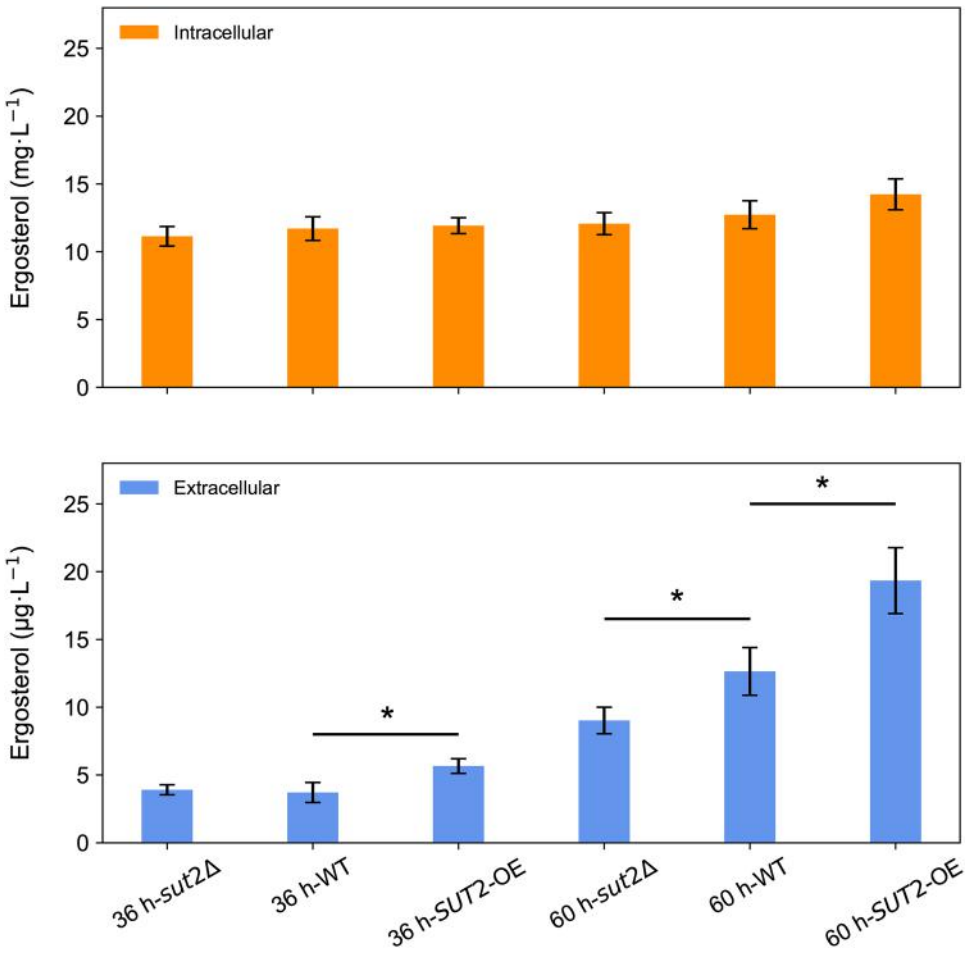

Fig. 6 Sterol analysis was carried out in wild-type, *sut2*Δ, and SUT2-overexpression strain. All characterizations were done on strains cultivated in shake flasks at 30 °C in minimal medium with methanol. Samples for intracellular and extracellular sterol analysis were taken at 36 h and 60 h. Error bars represent the standard deviation of three biological replicates. $^{*}p < 0.05$

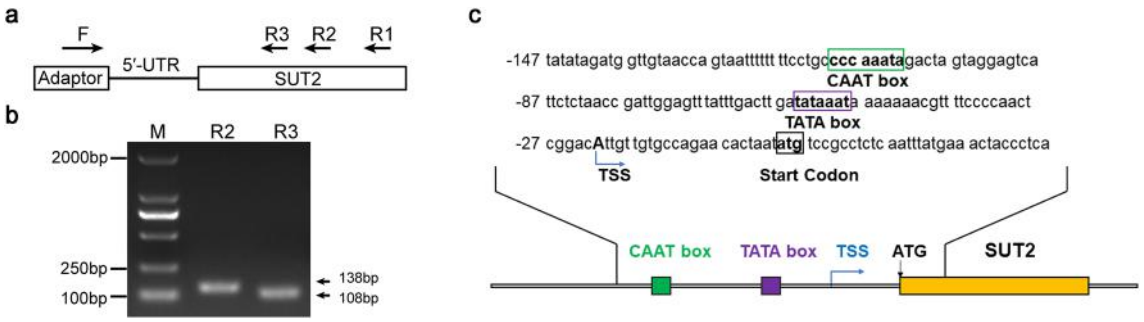

Fig. 7 Characterization of P_{SUT2}. **a** A schematic representation of the primer design for 5′-RACE assays. Only part of the ORF of *SUT2* is shown. **b** The analysis of 5′-RACE products. PCR with two pairs of primers was used to generate *SUT2* 5′-RACE products that were subjected to agarose gel electrophoretic separation, with arrows corresponding to the amplicons. **c** Partial nucleotide sequences around the inferred TSS and predicted core elements of the *SUT2* promoter region. The CAAT box and the TATA box are indicated in green and blue boxes, respectively

Discussion

Based on our previous study (Li et al. 2018) and the current work, we believe that SUT2 indirectly participates in the MUT pathway. SUT2 is highly expressed in a glycerol de-repression and methanol environment, which is similar to *AOX1*. A deficiency of AOX1 can reduce the transcriptional level of *SUT2* in a methanol-containing medium. Given that SUT2 is induced mainly by methanol rather than formaldehyde, these results collectively suggest that

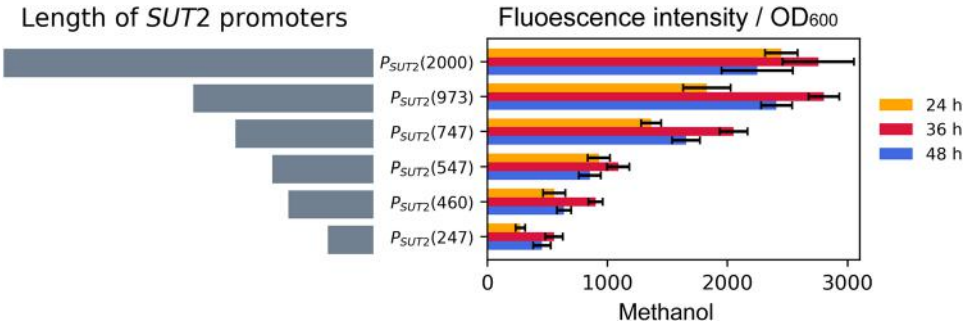

Fig. 8 Identification of regulatory sequences in the P_{SUT2}. (Left) Schematic of systematic truncations were made to the putative P_{SUT2}. (Right) Changes in truncated promoter strength were tested with cells cultured in methanol. Error bars represent the standard deviation of three biological replicates

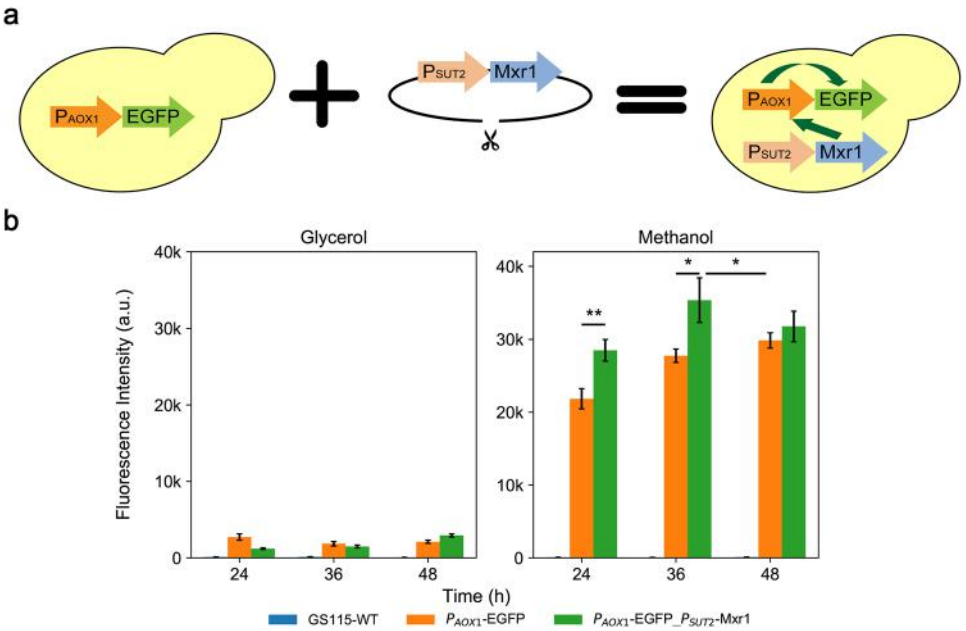

Fig. 9 Construction and detection of forward-loop strain. **a** *P. pastoris* strain bearing *EGFP* under control of P_{AOX1} was transformed with conversion cassettes (linearized plasmids) containing MXR1 under control of P_{SUT2} resulting in higher expression level strain. **b** Fluorescence value (per biomass) of the aforementioned strains in the medium with glycerol or methanol as the carbon source. Error bars represent the standard deviation of three biological replicates. a.u., normalized to OD_{600}; $^{*}p < 0.05$; $^{**}p < 0.01$

the expression of SUT2 might be triggered by the process of methanol oxidation by alcohol oxidase.

SUT2 overexpression can promote the expression of AOX1, and the loss of SUT2 would result in a reduction in the transcriptional level of *AOX1*. This indicates that SUT2 may positively, but indirectly, regulate the expression of AOX1. The function of SUT2 was further studied by detection of ergosterol levels in strains with different levels of *SUT2* expression. The results showed that in methanol-containing medium, overexpression of *SUT2* led to a slight, but reproducible, increase in the ergosterol content. We can infer that P.pSUT2 is indeed functionally correlated with sterol biosynthesis, which is similar to other yeasts grown under aerobic conditions, such as *S. cerevisiae* (Ness et al. 2001; Joshua and Hofken 2017).

Wriessnegger et al. (2007) found that ergosterol is the most abundant sterol of *P. pastoris* peroxisomal membranes. When peroxisome proliferation is induced by growth on methanol, alcohol oxidase is the predominant peroxisomal protein detected (Gould et al. 1992). Therefore, ergosterol is clearly essential for the existence of AOX1 in cells as it participates in peroxisome proliferation. In addition, ergosterol can be catalytically converted to ergosterol glucoside by sterol glucosyltransferase encoded by the *P.pATG26* gene, which is involved in the vacuole-dependent selective degradation of peroxisomes (pexophagy) in response to glucose or ethanol (Oku et al. 2003; Stasyk et al. 2003). We conjecture that ergosterol is essential for the formation and stability of the peroxisome. Thus, we propose that *P.pSUT2* is involved in methanol metabolism by participating in sterol biosynthesis, but the mechanism by which this occurs still needs to be elucidated (Fig. 10).

Additionally, the promoter of the *SUT2* gene was characterized for the presence of regulatory elements in response to methanol, and these results were applied to optimize the expression system in *P. pastoris.* Our data suggest that the TSS is the "A" at the 22nd bp upstream of ATG, which matched with the predicted start site. According to the relative positions and conserved sequences of the core elements in the promoter, we predict the positions of the TATA box

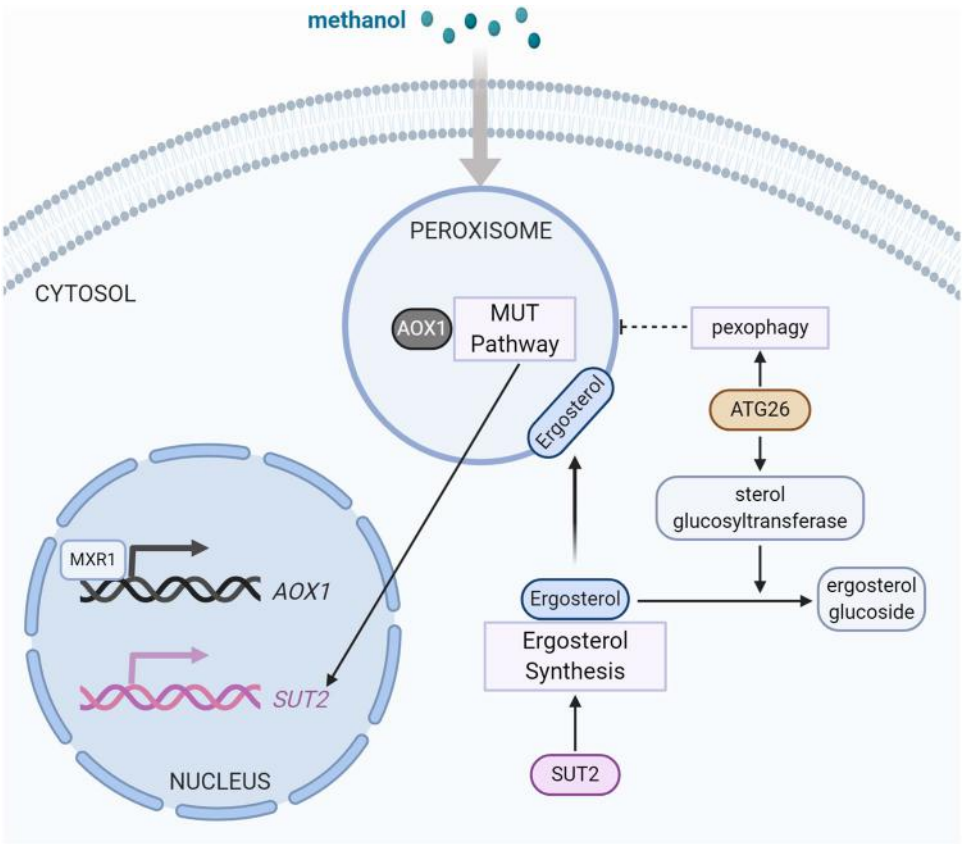

Fig. 10 *SUT2* is likely to be involved in methanol metabolism by participating in sterol biosynthesize. Solid lines indicate existing experimental evidence that has been reported; dash lines demonstrate relationships that have not been investigated in *P. pastoris*

and CAAT box of P_{SUT2}. The regulatory regions of P_{SUT2} in response to methanol, identified as the region from − 973 to − 547 bp in this study, can be further accurately determined by an electrophoretic mobility-shift assay, DNA pull-down assay, and other in vitro experiments. The regulatory region can be used as a sensor in other promoters for inducing a response to methanol. Based on the lack of available inducible promoters in *P. pastoris*, we characterized the promoter of the *SUT2* gene as a potential inducible promoter. Moreover, P_{SUT2} was applied to construct a positive feedback loop by initiating Mxr1. This represents a novel inducible promoter for initiating the transcription factor in methanol metabolism, which can improve the expression of AOX1. In addition, when cultured in glycerol and other carbon sources, this cycle is blocked, which is beneficial for the accumulation of biomass at the initial stage of fermentation.

In summary, we found that SUT2 could be induced by methanol but be repressed by glycerol in *P. pastoris*. This study shows that SUT2 may play roles in methanol metabolism by participating in sterol biosynthesis. It lays the foundation for further analysis of methanol metabolism mechanism. Further experiments are needed to verify the relationship between ergosterol synthesis and methanol metabolism. Moreover, the SUT2 promoter is a novel inducible promoter in *P. pastoris*. It was isolated and characterized to study the regulatory elements in response to methanol metabolism. And a P_{SUT2}-driven MXR1 forward-loop cassette was constructed which could be applied for optimization of the *P. pastoris* expression system in future.

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Compliance with ethical standards

Conflict of interest All the authors declared that they have no conflicts of interest to this work.

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

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Affiliations

Yankun Yang[1,2] · Yating Zheng[1,2] · Pengcheng Wang[1,2] · Xiang Li[1,2,3] · Chunjun Zhan[1,2,4,5] · Robert J. Linhardt[6] · Fuming Zhang[6] · Xiuxia Liu[1,2] · Jinling Zhan[1,2] · Zhonghu Bai[1,2]

[1] Key Laboratory of Carbohydrate Chemistry and Biotechnology, School of Biotechnology, Jiangnan University, Wuxi 214122, China

[2] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China

[3] Molecular Systems Biology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

[4] Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi 214122, China

[5] Joint BioEnergy Institute, Emeryville, CA, USA

[6] Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA