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Original Research Article

Transcriptome analysis reveals methanol metabolism variations for the growth damage caused by overexpression of chimeric transactivators in *Pichia pastoris*

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ABSTRACT

Methanol is a promising substrate for sustainable biomanufacturing, and *Pichia pastoris* has become a commonly used yeast for methanol utilization due to its powerful methanol metabolic pathways and methanol inducible promoter. Previous reconstruction of gene circuits highly improved transcriptional activity, but excessive expression of chimeric transactivator damaged cell growth on methanol. Here we employed transcriptome analysis to investigate the effects of chimeric transactivator overexpression on cellular metabolism and regulatory networks. The results showed that strong expression of chimeric transactivator unexpectedly downregulated methanol metabolism, especially the *alcohol oxidase 1* (*AOX1*), but without remarkable changes in expression of transcriptional factors. Meanwhile, the synthesis of peroxisomes also varied with chimeric transactivator expression. In addition, the enrichment analysis of differentially expressed genes revealed their impact on cellular metabolism. The gene expression patterns caused by different expression levels of chimeric transactivators have also been clarified. This work provides useful information to understand the transcriptional regulation of the *AOX1* promoter and methanol signaling. It revealed the importance of balancing transcription factor expression for the host improvement.

1. Introduction

Methanol is regarded as a next-generation substrate for sustainable biomanufacturing processes [1,2]. As a typical methylotrophic yeast, *Pichia pastoris* (syn. *Komagataella phaffii*) serves as a significant microbial platform for methanol biotransformation due to its powerful native methanol metabolic capacity and a series of inducible promoters responsive to methanol, e.g., particularly the *AOX1* promoter (P_{AOX1}), which is the most extensively studied and used [3]. Previous studies have elucidated the transcriptional activators and repressors of the P_{AOX1} and their related regulatory mechanisms under various carbon sources [4–10]. Based on these findings, some genetic circuits have been engineered through overexpression of transcriptional activators to augment promoter strength and enable transcriptional derepression under fermentative carbon sources [11–14]. However, it was observed that the strong expression of transcriptional activators severely affected cell growth, thereby limiting further design to increase the intensity of the P_{AOX1}-derived expression systems [5,13].

The interactions among transcription factors and promoters are crucial in determining gene transcriptional intensity. With the advancements in synthetic biology, the rational dissection and reassembly of transcription factors have emerged as potent strategies for constructing novel genetic circuits and enhancing gene transcriptional levels [15,16]. In our previous studies, we developed an artificial transcriptional signal amplification device with chimeric transactivator. It contains endogenous activation domain of transcription factor (TFAD) and specific orthogonal DNA-binding protein, to achieve high-level gene transcription, based on the elucidation of methanol regulatory mechanism of the P_{AOX1} [17,18]. Furthermore, we constructed a methanol inducible transcriptional device library covering 162 specific devices

Abbreviations: TFAD, Transcription factor activation domain; *AOX1*, *Alcohol oxidase 1*.

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[19]. However, further increased expression of the chimeric transactivators severely impaired cell growth of *P. pastoris*, despite that the removal of the endogenous DNA-binding domains should lose their interactions with promoters [19]. This phenomenon was observed for all the tested methanol inducible transactivators (Mit1, Mxr1 and Prm1) [19], limiting further development of the synthetic transcriptional systems. But the intrinsic regulatory mechanism of the residual activation domains on cell metabolism and physiology is still unknown. This study aims to involve transcriptome analysis of strains with overexpression of different chimeric transactivators to reveal their functions on cellular metabolism and physiology. With these efforts, we may understand how these chimeric transactivators affect the entire cell regulatory networks, which in turn help us find the key regulatory targets for further rational cell rewiring.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

The plasmids and strains used in this study are listed in Table S1. *Escherichia coli* was incubated at 37 °C in Low-salt-LB (LLB) medium (0.5 % yeast extract, 1 % tryptone and 0.5 % NaCl). Antibiotics (100 μg/ml ampicillin or 50 μg/ml zeocin) were added when required. *P. pastoris* was incubated at 30 °C in YPD (1 % yeast extract, 2 % tryptone and 2 % glucose) medium for cell growth. Zeocin of 100 μg/ml was added when required. For construction of plasmid pGG1LacIM1AD, the sequence of promoter P_{G1} was synthesized into plasmid pGGLacIM1AD [17] by Tsingke Biotech Co., Ltd. Then the synthetic plasmid was linearized by *Afl*II and transformed into competent cells of GS_*O5c*AG [17]. The derived strain was identified and named as G1LM.

2.2. Determination of cell growth

For growth detection of *P. pastoris* GS115 (wild-type) and engineered strains in methanol, the strains were pre-cultured in YPD medium to a log phase and then shifted to YNM medium (0.67 % YNB, 0.5 % methanol). For GS115, histidine of 50 μg/ml was supplemented. Samples were taken periodically for measurement of the OD_{600nm} values and methanol concentration. Methanol concentration was determined using an SBA-40ES biological sensor (Shandong Academy of Sciences, Shandong, China). Similar methods were performed for growth detection in glucose, glycerol and ethanol. The strains were pre-cultured in YPD medium to a log phase and then shifted to YPD medium, YPG medium (1 % yeast extract, 2 % tryptone and 2 % glycerol), and YNE medium (0.67 % YNB, 0.5 % ethanol).

2.3. RNA-Seq analysis

P. pastoris strains carrying chimeric transactivators with different TFADs, *i.e.*, GS115, FBA2LX, DAS1LX, AOX1LX, AOX1LM and AOX1LP (detailed descriptions shown in Table S1), were selected for RNA-Seq and transcriptomic data analysis. The strains were pre-cultured in YPD medium to a log phase and then shifted to YNM medium. Each strain was cultured independently in triplicate. Cells were collected at 8 h and the obtained 18 samples from 6 strains were sent to Gene Denovo Biotechnology Co., Ltd. for RNA extraction and RNA-Seq. High-quality reads were aligned onto the indexed *K. phaffii* CBS 7435 reference genome (GCA_900235035.1_ASM90023503v1) [20]. Bioinformatic analysis was performed using Omicsmart, a real-time interactive online platform for data analysis (http://www.omicsmart.com).

2.4. Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed to affirm the *MXR1AD* expression levels in the strains of GS115, FBA2LX, DAS1LX, and AOX1LX. Total RNA was isolated using RNA extraction kit (TransGen Biotech, Beijing, China), and 1 μg of DNase-treated RNA was used for the cDNA synthesis. qPCR was carried out using One-Step SYBR Enzyme Mix (GenStar, Beijing, China). The relative mRNA levels were obtained using the ΔΔCt method. The Ct value of *MXR1AD* to that of *GAPDH* in the experimental strain was normalized to that of the control strain GS115.

The primers used in RT-qPCR were listed below. RT-GAPDH F: CCAAGAGAGAGACCCTGTCA; RT-GAPDH R:TGACACCGACAACGAACATT; RT-MXR1AD F:TCCGGTGACAACGCCAAATA; RT-MXR1AD R: TGATCAGGCTGCTGGACTTG.

2.5. Statistical analysis

The relationship of the tested 18 samples were performed using the correlation analysis and PCA. AOXLP_2 was identified as significant outlier sample and excluded from subsequent transcriptome analysis. Gene expression was quantified using TPM and differential expression analysis was performed using DESeq2 (Fold change>2, Qvalue<0.05). GraphPad Prism was used for figure presentation.

3. Results

3.1. Effects of chimeric transactivators overexpression on cell growth

Previously, a methanol inducible artificial transcriptional device library was constructed using a domain-swapping strategy [19]. The device included chimeric transactivators of yeast transactivation domains and bacterial DNA-binding proteins, as well as synthetic promoters composed of protein-binding sequences and yeast core promoters. Three transactivators of P_{AOX1} (i.e., Mxr1, Mit1, Prm1) with potential DNA-binding domains excluded were employed as transactivation domains for RNA polymerase recruitment. A series of methanol inducible promoters were then used to control the chimeric transactivators, generating a comprehensive methanol inducible transcriptional device library. Nevertheless, overexpression of chimeric transactivators using strong promoter like P_{AOX1} severely damaged the growth of *P. pastoris* strains. But the intrinsic regulatory mechanism of the chimeric transactivators on cell metabolism and physiology kept unknown, even with the removal of native transcriptional DNA-binding domains.

To clarify the effects of different TFADs (i.e., Mxr1AD, Mit1AD, Prm1AD) on cell growth, we then used methanol inducible promoters of varying strengths to drive their expression and measured growth curves of different strains in methanol medium (Fig. 1). The activities of the promoters were identified in previous study [19] and ranked as follows: P_{AOX2}<P_{PEX5}<P_{FBA2}<P_{DAS1}<P_{AOX1} (Fig. S1). As for chimeric transactivators driven by the strong P_{AOX1}, all strains exhibited severe growth inhibition. Among them, the Mit1AD related overexpressing strain started growing after induction for 24 h, and the final cell concentration only reached 62.9 % relative to the wild-type (Fig. 1A). The Mxr1AD and Prm1AD related overexpressing strains started growing after approximately 48 h, and the final cell concentrations reached 69.0 % and 83.9 % relative to the wild-type respectively (Fig. 1B and C). Of note, there were also differences in the tolerance of cells to the three transactivation domains. The Prm1AD did not cause growth restriction issues when driven by promoters beyond the $P_{AOX1,}$ while the Mxr1AD only caused certain growth inhibition when driven by the promoter P_{DAS1} for which cell growth began after cultured for 20 h. The Mit1AD showed obvious repression on cell growth when using promoters of P_{PEX5} and P_{DAS1}, and the final cell concentration was lower than the wild-type. Additionally, the methanol consumption profiles of each strain were examined, which showed good correlation with their growth trends. Specifically, strains exhibiting growth impairment consumed methanol at lower rates (Fig. S1).

To determine whether overexpression of the chimeric transactivators affected cell growth on other carbon sources, we used P_{G1}, a strong mutant of the promoter P_{GAP} [21], to constitutively overexpress

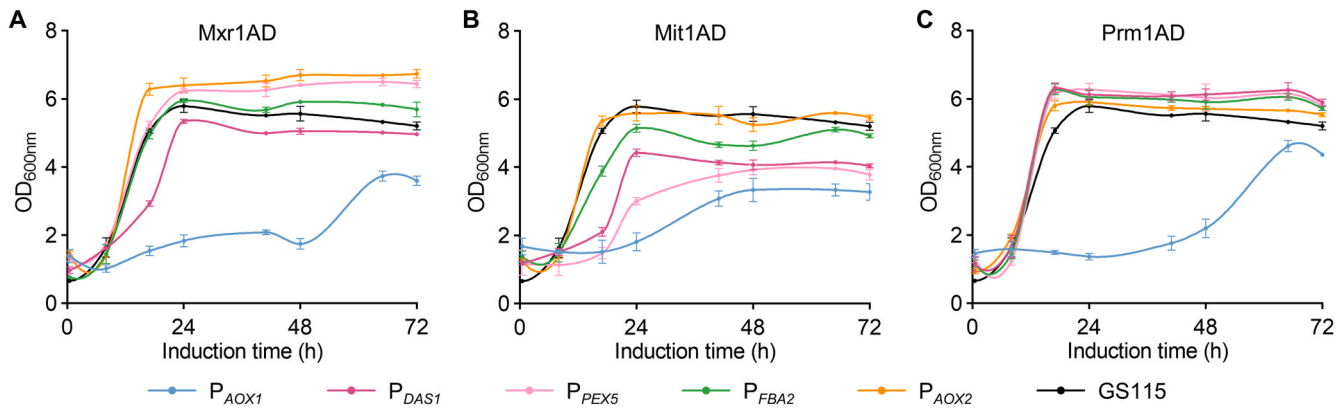

Fig. 1. Growth curves of strains overexpressing chimeric transactivators with different TFADs. (A) Mxr1AD; (B) Mit1AD; (C) Prm1AD. Different intensities of methanol inducible promoters were used to overexpress chimeric transactivators and labeled with different colors. The wild-type *P. pastoris* GS115 was used as a control.

chimeric transactivators and measured cell growth separately on glucose, glycerol, ethanol and methanol. This is because the P_{AOX1} is highly repressed by fermentative carbon sources and not suitable for this determination. The results showed that the strain harboring Mit1AD by the P_{G1} also showed growth impairment under methanol condition, while its growth was almost consistent with the wild-type under other carbon condition (Fig. S2). Therefore, growth inhibition caused by overexpression of the chimeric transactivators seemed to be methanol-dependent.

3.2. Transcriptome analysis of the chimeric transactivator-derived growth impaired strains

To elucidate the impact of transactivitor overexpression on cell growth, we selected three severely growth-impaired strains (AOX1LX, AOX1LP, AOX1LM), which were defined as AOX1TFAD strains, for transcriptome analysis using GS115 as a control. Additionally, we included the normally growing strain FBA2LX and moderately affected strain DAS2LX for comparative analysis to explore the expression patterns of genes. Principal component analysis (PCA) and sample correlation analysis (SCA) were performed to identify outlier samples

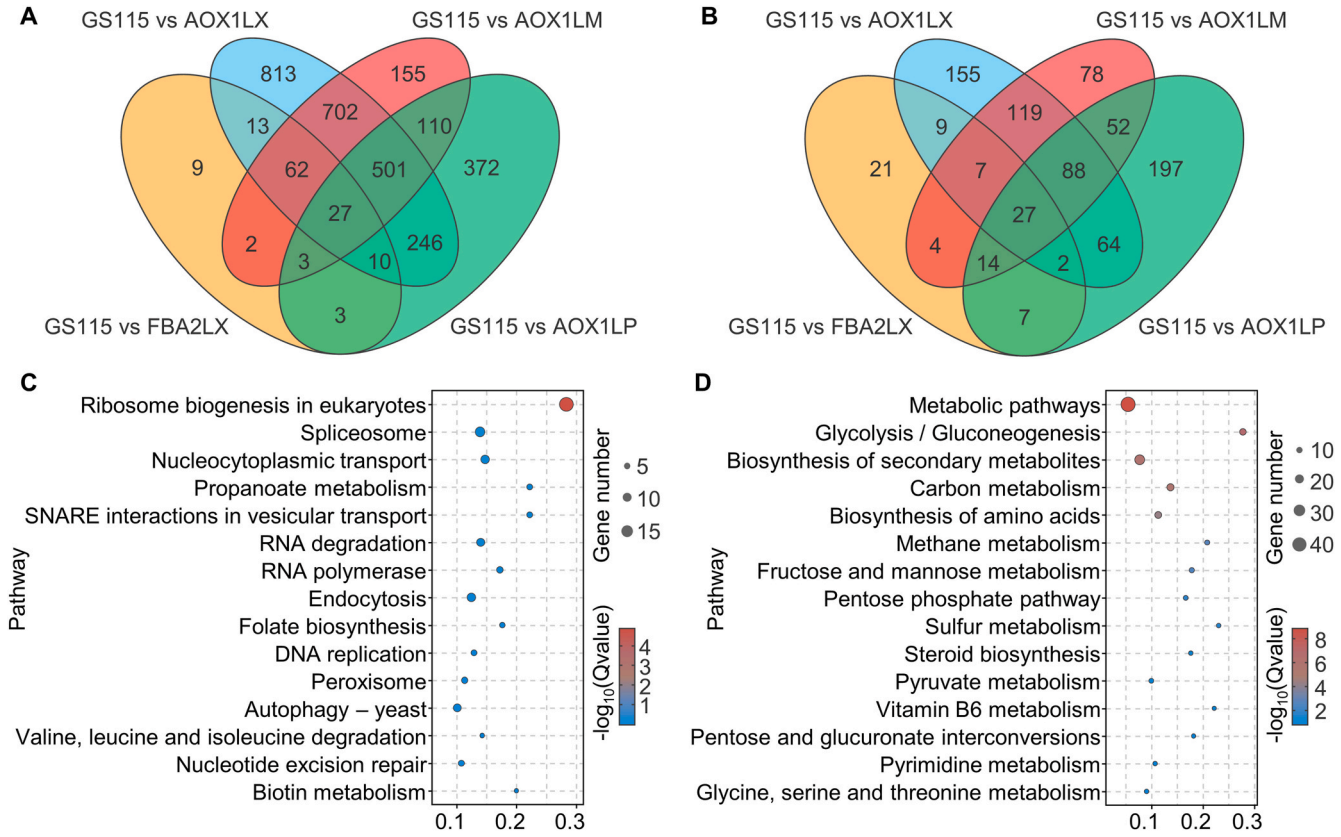

Fig. 2. Transcriptome analysis of strains overexpressing chimeric transactivators. Venn diagrams illustrating upregulated DEGs (A) and downregulated DEGs (B) between each TFAD related overexpressing strain and GS115 (Fold change>2, Q value < 0.05). KEGG enrichment analysis of 501 upregulated DEGs (C) and 88 downregulated DEGs (D), which were significantly different between GS115 and each AOX1TFAD strain but not FBA2TFAD strain. Top 15 KEGG pathways were shown according to the Q value.

(Fig. S3). The AOXLP_2 sample was regarded as the outlier and excluded to ensure the accuracy of subsequent analysis.

Transcriptome analysis revealed that the FBA2LX strain exhibited minimal differences compared to the control strain GS115, while DAS1LX showed significant differences. The three strains of P_{AOX1}-driving TFADs exhibited the largest differences, consistent with the observed growth patterns (Fig. S4). Subsequently, Venn analysis was performed to identify differentially expressed genes (DEGs) associated with cell growth impairment. The common DEGs among the control and three AOX1TFAD strains were selected except the DEGs between FBA2LX and GS115 (recognized as normal growth). The resultant DEGs set comprised 501 upregulated genes (Fig. 2A) and 88 downregulated genes (Fig. 2B). KEGG enrichment analysis was employed to analyze these DEGs. The top 15 upregulated pathways are depicted in Fig. 2C. We observed that 19 DEGs were enriched in the ribosome biogenesis in eukaryotes pathway, which may be a response to cellular stress and an adaptive mechanism to maintain normal growth and division. The other upregulated pathways also reflected a series of complex strategies adopted by yeast cells in response to growth impairment, including increasing protein synthesis, optimizing RNA homeostasis, adjusting metabolic strategies, maintaining genomic stability, and clearing damaged organelles and proteins through autophagy and degradation pathways [22,23]. Overall, these tactics collaborate in a concerted effort to facilitate yeast cell adaptation to a hostile growth environment and, where possible, to revive their growth and survival potential. The top 15 downregulated pathways are shown in Fig. 2D. Notably, 20.8 % of the genes enriched in methane metabolism were significantly downregulated, which may have severely impaired the utilization of methanol, potentially pinpointing it as a direct cause of cell growth impairment. In addition, a large number of downregulated DEGs were significantly enriched in pathways governing carbon source metabolism and energy production, potentially triggered by aberrant methanol metabolism.

3.3. Changes in methanol metabolism in growth-impaired strains

The methanol utilization (MUT) pathways and their subcellular localization within peroxisomes are pivotal determinants of methanol metabolism [24]. DEGs enrichment analysis revealed that the growth impairment of yeast cells overexpressing chimeric transactivators under methanol conditions was likely a consequence of aberrant methanol metabolism. It led to a diminished capability to harness methanol. This decline in methanol utilization may trigger a broad dysregulation of overall carbon metabolism in the cells. Analysis of the expression profiles of genes involved in MUT pathway revealed a downregulated trend in several growth-impaired strains (Fig. 3). Notably, strains of AOX1LX and AOX1LP exhibited remarkable downregulation, while AOX1LM and DAS1LX showed moderate downregulation. This pattern accorded with

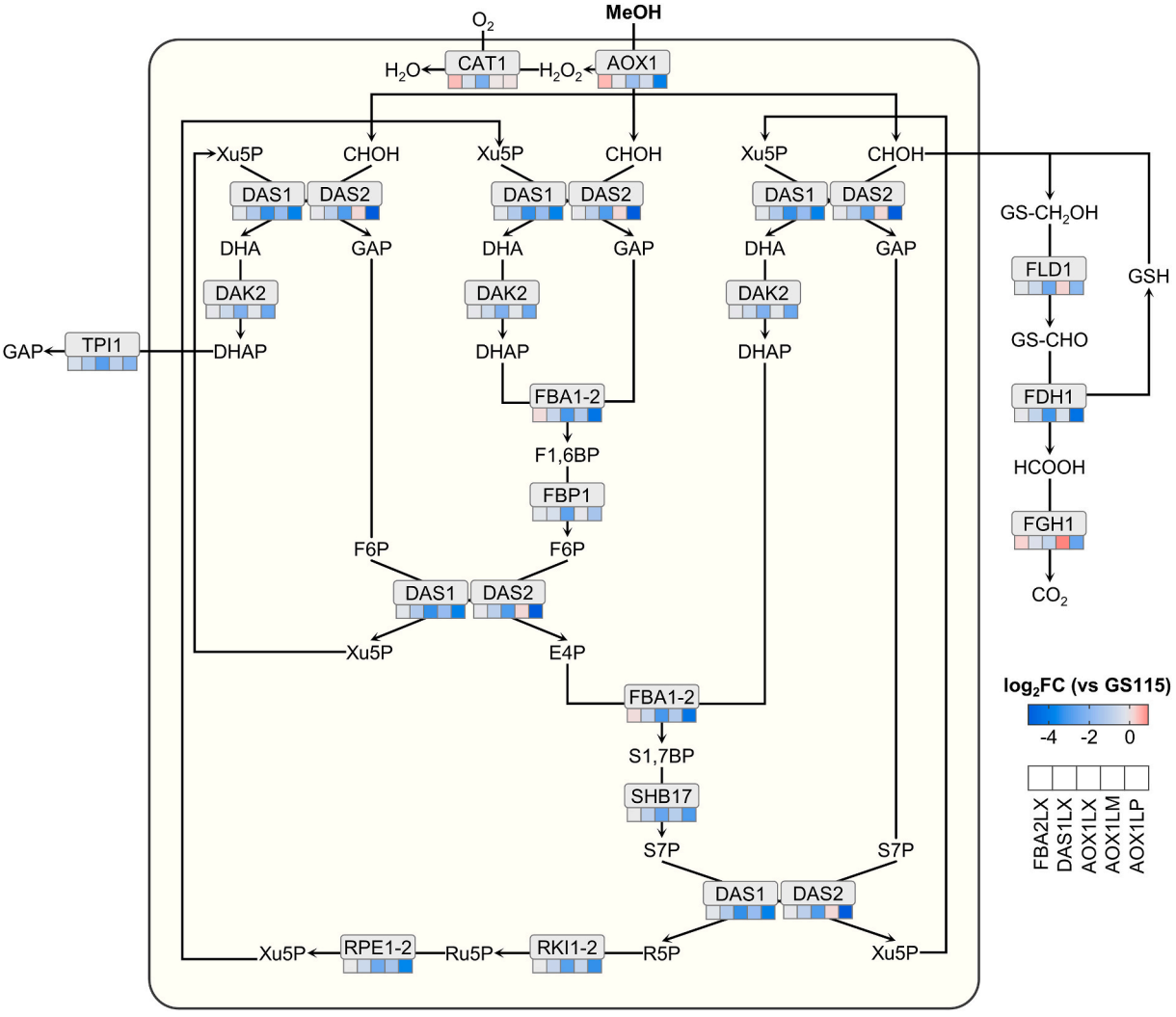

Fig. 3. Variations of MUT pathways in strains overexpressing chimeric transactivators with different TFADs. Color changes from blue to red indicated downregulation to upregulation. FC, fold change.

the growth trends of the strains, confirming that the differences in the MUT pathway are closely correlated with growth impairment. Particularly, the key genes involved in methanol assimilation metabolism, i.e., *AOX1*, *DAS1* and *DAS2*, were significantly down-regulated in strains of growth impairment. Further differential expression analysis of transcription factors of the P_{AOX1} was conducted to explore the reasons for the downregulation of *AOX1* transcription (Fig. 4). Interestingly, aside from the effects caused by overexpression of the chimeric transactivators, there were almost no significant differences consistent with the trend of *AOX1* transcription in the expression of transcriptional activators (Mxr1 [5], Mit1 [4], Prm1 [6]) and repressors (Mig1, Mig2 [10], Nrg1 [7], ROP [9]) of the P_{AOX1} in strains with growth impairment. Although the *MIT1* was significantly downregulated in Prm1AD related overexpressing strain, there was no difference in Mxr1AD related overexpressing strains. In addition, the regulatory proteins Wsc1 and Wsc3 [25] responsive to methanol signal and C4qzn3 that acted as an inhibitor of Mxr1 [8] were analyzed, and no significant expression differences were observed. These findings suggested that there should be other regulatory mechanisms which affecting gene expression of the MUT pathway.

Peroxisome is the key organelle for methanol metabolism. The generation and amount of peroxisomes directly determine functions of alcohol oxidases and methanol utilization [26,27]. Therefore, differential expression analysis was further performed on genes involved in peroxisome synthesis (Fig. 5). Similar to the MUT pathway, most peroxisomal genes were significantly downregulated in strains of AOX1LX and AOX1LP, but not remarkably different in strains of AOX1LM and DAS1LX. Accordingly, the limited synthesis and maintenance of peroxisomes is also an important reason for the weakened methanol metabolism.

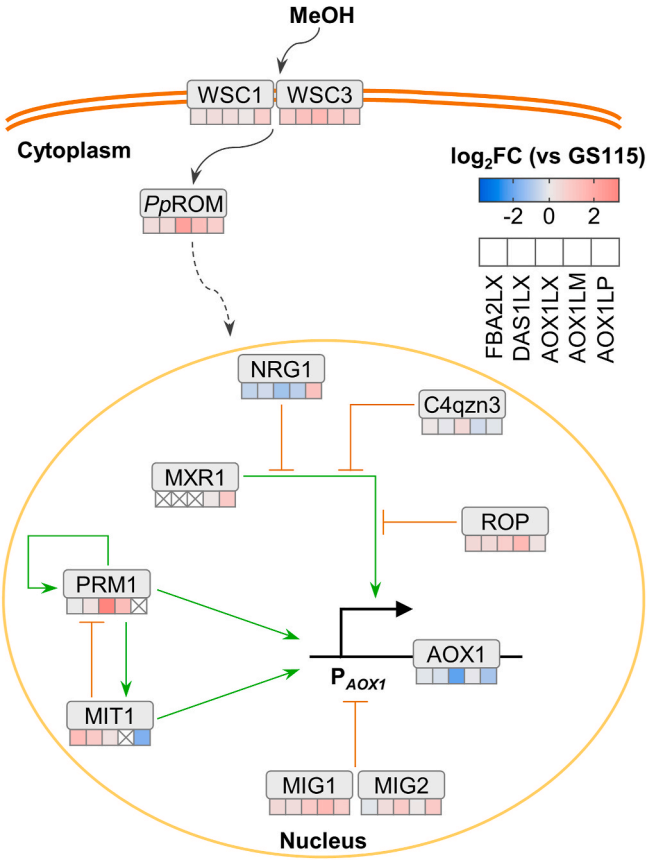

Fig. 4. Relative changes in expression levels of genes for transcriptional regulation of the P_{AOX1} promoter. The fold changes of *MXR1*, *MIT1*, *PRM1* were not shown in the corresponding TFADs related overexpressing strains due to their excessively high upregulation levels.

3.4. Analysis of gene expression patterns in strains overexpressing chimeric transactivators with Mxr1AD

As varied expression levels of the Mxr1AD dominant chimeric transactivator caused growth differences in a wide range as compared to the wild-type, we thus selected FBA2LX, DAS1LX, AOX1LX and the wild-type GS115 that with different growth behaviors and progressive Mxr1AD expression levels for the series test of cluster (STC) algorithm (Fig. S5A). The STC analysis showed that six profiles containing 3137 genes exhibited a significant statistical difference ($p < 0.05$) (Fig. S5B). Among them, 2312 genes in profiles of 10 and 19 showed an upregulated trend, and 478 genes in profiles of 9 and 0 showed a downregulated trend. Additionally, 347 genes in profiles of 18 and 12 demonstrated oscillatory changes.

Gene Ontology (GO) enrichment analysis was employed to analyze the functional classification of genes within these profiles. The results revealed that genes in profiles of 10 and 19 were predominantly involved in biological processes such as the cell cycle, chromosome segregation, and mitosis (Fig. S5C). These changes were closely related to cell proliferation and division. It may be ascribed to that the cells attempt to maintain its growth and division by accelerating the cell cycle process in response to the stress caused by increased expression of Mxr1AD. On the other hand, genes in profiles of 9 and 0 were primarily associated with biological processes related to ribosome function, protein targeting and translation initiation (Fig. S5D). These results indicated a reduction in protein synthesis and ribosomal function, which could impair the cell's ability to produce essential proteins, contributing to the growth limitations observed in the Mxr1AD related strongly expressed strains.

4. Discussion

The phenomenon of growth damage caused by overexpression of P_{AOX1}-related transactivators in *P. pastoris* has been reported in multiple studies [5,11,13]. However, the intrinsic regulatory mechanisms for this phenotype change remain unknown. Previously, it was hypothesized that the overexpression of transactivators triggers a positive feedback mechanism for the promoter, leading to cellular physiological and metabolic imbalances. In this study, chimeric transactivators with native activation domains but not DNA-binding domains were overexpressed, e.g., the Mxr1AD retaining only one-third size of the full Mxr1 protein. Nevertheless, severe growth inhibition still occurred under methanol conditions. With the transcriptome analysis, a number of genes related to methanol metabolism were significantly downregulated after overexpression of the chimeric transactivators. The results indicated that cells were able to sense different expression levels of transactivitors, further regulating transcription of genes involved in methanol metabolism and peroxisome synthesis. Notably, overexpression of Mit1AD did not show significant downregulation in the MUT pathway and peroxisome synthesis as compared to Mxr1AD and Prm1AD, indicating a differential tolerance of *P. pastoris* towards diverse TFADs. This could be attributed to distinct protein expression profiles and intracellular stabilities of different TFADs, despite that the identical promoters were used. Furthermore, this regulation exhibited a certain dose-response relationship. The subsequent recovery in cell growth (Fig. 1) may be ascribed to the influence on *AOX1* transcription, which also reduced the expression of the chimeric transactivators driven by P_{AOX1}, allowing the cells to somehow alleviate from the growth damage. It suggested that the regulation of cell growth by transactivitors appears to be reversible. This study preliminarily explored the regulatory rules and identified gene expression differences and distribution of differentially expressed genes. Accordingly, the varied metabolic pathways and physiological processes were finally clarified. It provides references and basis for decoding the molecular regulation steps by excessive expression of

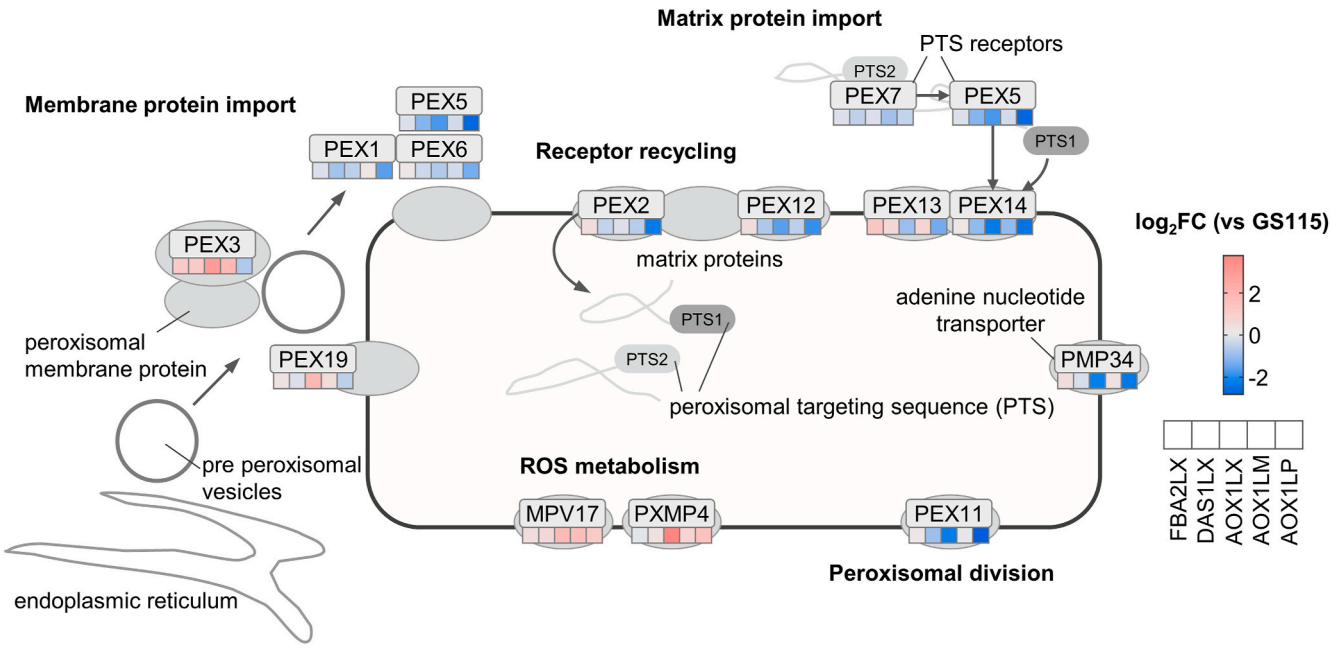

Fig. 5. Variations of peroxisome synthesis in strains overexpressing chimeric transactivators with different TFADs. Color changes from blue to red indicated downregulation to upregulation. FC, fold change.

transactivitors especially on methanol. Also, the observed metabolic and physiological variations provide candidate target for further rewiring of the *P. pastoris* host.

The overall downregulation of the MUT pathway particularly the severe decrease of the *AOX1* gene would impair methanol metabolism in cells. It seemed to be contrary to the general rule that transactivitor overexpression activating promoters. Additionally, the native transcription factors of the P_{AOX1} did not show corresponding changes with the variation of *AOX1* expression. The result indicated the existence of other unexplored mechanisms regulating the transcription of the P_{AOX1} or potential post-transcriptional regulation of *AOX1*. This potential mechanism may respond to the excessive expression levels of transcription factors. We hypothesize that the potential candidates sensing TFADs expression might be located upstream in the methanol signaling pathway, including kinases, phosphatases, or other regulatory proteins. Additionally, non-coding RNAs should also be considered as they could interact with TFADs or other regulatory proteins, affecting their activity or localization. Since the regulators directly sensing TFADs might not show transcriptional changes, some molecular interaction experiments, such as co-immunoprecipitation (Co-IP) or yeast two-hybrid screening, could be employed to identify potential protein partners of TFADs in the future work.

Methanol based sustainable biomanufacturing processes have attracted wide attention currently. For *P. pastoris*, the efficiently methanol utilization ability and intense methanol inducible promoters like P_{AOX1}, makes it an attractive host for producing various bioproducts from methanol. All the time, overexpression of transactivators has been a promising approach to enhance promoter activity and expand the transcriptional landscape, especially for the multi-copy gene expression of target proteins [14,28]. Our findings highlighted the potential caveats of this strategy. It is necessary to continue exploring underneath rules of impairment of excessive transactivator expression and breaking the intensity limitation of methanol inducible transcriptional tools. Methanol-free inducible promoters might be employed to restore expression of the key down-regulated genes involving MUT pathway and peroxisome synthesis, potentially mitigating growth inhibition. Furthermore, while the TFADs used were assumed to be activation domains of transcription factors, the potential presence of redundant structures unrelated to RNA polymerase recruitment cannot be overlooked. Reducing the size of these TFADs could help identify minimal activation domains that do not trigger cellular regulatory responses, thereby decoupling transcription factor overexpression from growth defects. Due to the incomplete annotation of the *P. pastoris* genome and the not fully elucidated regulatory mechanisms of the P_{AOX1}, this study could not decode the full mechanisms through transcriptome analysis. Additionally, the transcriptome analysis, though comprehensive, is subject to the current state of genome annotation and may not reveal post-transcriptional or epigenetic regulatory mechanisms. Future studies may benefit from a systems biology approach, integrating transcriptomic, proteomic and metabolomic data to provide a holistic understanding of cellular responses to transactivitor overexpression.

5. Conclusion

This study elucidated the effects of overexpressing chimeric transactivators on *P. pastoris* using transcriptome and phenotype analysis. Excessive expression of chimeric transactivators downregulated key genes in the MUT pathway and impaired peroxisome synthesis, leading to severe growth inhibition of *P. pastoris* cells. These insights reveal the critical need for balanced transcription factor expression in genetic rewiring and offer valuable understandings of the regulatory mechanisms governing methanol metabolism.

Data availability

Original data of transcriptome sequencing may be required from the corresponding authors for only academic studies. Other data generated or analyzed during this study are included in this article.

Ethical approval and informed consent

We declare that this paper does not report any data collected from humans or animals.

CRediT authorship contribution statement

Qi Liu: Writing – original draft, Project administration, Funding

acquisition, Formal analysis, Data curation, Conceptualization. **Ziyu He:** Formal analysis, Data curation. **Menghao Cai:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.09.008.

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