GENETICS

Specific features of the transcriptomic response to nitrogen starvation in methylotrophic yeast *Komagataella phaffii*

Anastasiya Makeeva, Anton Sidorin, Valeria Ishtuganova, Elena Sambuk, Marina Padkina, and Andrey Rumyantsev

Saint Petersburg State University, Universitetskaya nab., 7–9, Saint Petersburg, 199034, Russian Federation

Address correspondence and requests for materials to Anastasiya Makeeva, st055791@student.spbu.ru

Abstract

Non-conventional methylotrophic yeast *Komagataella phaffii* is an important production host in biotechnology and an emerging model organism. In this work, we studied *K. phaffii* response to nitrogen starvation during cultivation in media with methanol as the sole carbon source. The results were compared with a well-established model yeast *Saccharomyces cerevisiae*. Some of the observed effects of nitrogen starvation in *K. phaffii* were similar to those in *S. cerevisiae*, although this yeast does not have a metabolic pathway for methanol utilization. The effects include activation of autophagy, transport and catabolism of nitrogen-containing compounds, interconversions of amino acids, and biosynthesis of fatty acids. *K. phaffii* cells also demonstrated a specific response to nitrogen starvation including suppression of genes involved in methanol metabolism and other peroxisomal processes and activation of purine catabolism genes.

Keywords: *Komagataella phaffii, Pichia pastoris*, methylotrophy, nitrogen metabolism, nitrogen starvation, methanol metabolism, transcriptomic analysis, sulfur amino acids, purine catabolism.

Introduction

Nitrogen is one of the most crucial elements required for the biogenesis of amino acids and nitrogenous bases. The uptake of this element from the environment plays a key role in the survival of any living organism.

Yeasts, particularly *Saccharomyces cerevisiae*, can use a wide range of compounds as nitrogen sources. These include ammonium, urea, and all L-amino acids (except lysine, histidine, and cysteine). These compounds are conditionally classified as "preferred" and "non-preferred" nitrogen sources, depending on the efficacy of their utilization. Preferred nitrogen sources for *S. cerevisiae* include compounds that are readily integrated into cellular metabolism: ammonium, asparagine, glutamine, glutamate, alanine, and others. These compounds serve not only as sources of amino groups for transamination reactions and synthesis of other amino acids but also as sources of pyruvate and tricarboxylic acid (TCA) cycle intermediates (α-ketoglutarate and oxaloacetate) (Ljungdahl and Daignan-Fornier, 2012).

Less preferred nitrogen sources for *S. cerevisiae* include branched-chain and aromatic amino acids, as well as methionine. Nitrogen from these amino acids is incorporated into central metabolism through transamination steps, while their carbon skeletons are converted into non-catabolizable compounds via the Ehrlich pathway (Hazelwood et al., 2008).

The ability to utilize different substances as nitrogen sources requires permeases for their transport into the cell. In *S. cerevisiae*, more than 20 transport-

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Authors' information: Anastasiya Makeeva, PhD Student, Researcher, orcid.org/0000-0002-7181-0495; Anton Sidorin, PhD Student, Researcher; orcid.org/0000-0002-9688-9347; Valeria Ishtuganova, Researcher, orcid.org/0009-0007-6496-6073; Elena Sambuk, Professor, orcid.org/0000-0003-0837-0498; Marina Padkina, Professor, orcid.org/0000-0002-4051-4837; Andrey Rumyantsev, Researcher, orcid.org/0000-0002-1744-3890

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ers for amino acids and nitrogen-containing compounds have been identified, including both specific permeases (such as the proline transporter ScPut4p) and non-specific ones that can also function as sensors of extracellular nitrogen (such as ScGap1p) (Zhang, Du, Zhou, and Chen, 2018). The regulation of transporter activity in *S. cerevisiae* occurs at the level of transcription, post-translational modifications (such as ubiquitination), and protein localization (Bianchi, Van't Klooster, Ruiz, and Poolman, 2019).

The regulation of nitrogen metabolism in *S. cerevisiae* is provided by a complex network of signaling systems. A key component of these systems is the TOR (target of rapamycin) kinase regulatory complex. *S. cerevisiae* has two TOR kinase complexes: TORC1 and TORC2, that differ in composition, localization, and function. TORC1 is central to the regulation of numerous processes, acting as a key player in sensing intracellular nitrogen levels. The information about amino acid content from various intracellular sensors is relayed to TORC1 through the EGO, SEACIT, and SEACAT complexes. TORC1 controls a multitude of effector proteins that regulate translation, ribosome synthesis, nitrogen assimilation, amino acid synthesis, stress response genes, and autophagy. In contrast, the TORC2 complex regulates the homeostasis of the membrane, cell wall integrity, and the dynamics of the actin cytoskeleton, thereby coordinating cell growth and budding (Zhang, Du, Zhou, and Chen, 2018).

S. cerevisiae is a well-known and extensively studied model organism. However, it is now clear that this species can significantly differ in its physiology from other yeast species. For instance, *S. cerevisiae* is a Crabtree-positive organism, meaning that these yeasts primarily obtain energy through fermentation rather than respiration when growing in nutrient-rich media with glucose. Additionally, during evolution, the ancestors of *S. cerevisiae* underwent whole-genome duplication (Wolfe, 2015). The comparison of *S. cerevisiae* with other yeast species elucidates diverse regulatory mechanisms and their evolution, particularly since such comparisons can now be conducted on systemic level.

Advances in molecular biology and the development of "omics" technologies have led to the accumulation of data on the biology of other yeast species that were previously used mainly for practical purposes. One such organism is the methylotrophic yeast *Komagataella phaffii* (syn. *Pichia pastoris*). *K. phaffii* is known as an extremely efficient producer, widely used for the synthesis of recombinant proteins and various chemical compounds (Karbalaei, Rezaee, and Farsiani, 2020; Carneiro et al., 2022). The practical significance of *K. phaffii* drives the application of modern methods to study its physiological and metabolic characteristics. Consequently, the accumulation of data on the biology of these yeasts and their active use in scientific research has recently enhanced the status of *K. phaffii* as a new model organism (Bernauer, Radkohl, Lehmayer, and Emmerstorfer-Augustin, 2021).

The main feature of *K. phaffii* is the ability to use methanol as the sole source of carbon and energy. This capability is found only in a small phylogenetically related group of yeasts, including the genera *Komagataella*, *Ogataea*, *Kuraishia*, and *Candida*. The methanol metabolism pathway (MUT, Methanol UTilization) is similar among all members of this group and requires a set of unique enzymes: alcohol oxidase (AOX), dihydroxyacetone synthase (DAS), and formate dehydrogenase (FDH) (Hartner and Glieder, 2006; Yurimoto, Oku, and Sakai, 2011).

The MUT pathway begins with the oxidation of methanol to formaldehyde in peroxisomes. This reaction is catalyzed by alcohol oxidase, which in *K. phaffii* is encoded by two genes: *AOX1* and *AOX2*. The reaction produces toxic formaldehyde and hydrogen peroxide. The peroxide is neutralized by catalase activity. Formaldehyde enters both assimilation and dissimilation pathways. Part of the formaldehyde condenses with xylulose-5-phosphate in peroxisomes to form C3 compounds: dihydroxyacetone and glyceraldehyde-3-phosphate, under the action of dihydroxyacetone synthase (*DAS1,2*). Another part of the formaldehyde is oxidized to CO_2 in the dissimilation pathway with the involvement of glutathione. This branch of methanol metabolism primarily protects cells from the harmful effects of formaldehyde and generates NADH molecules (Hartner and Glieder, 2006; Berrios et al., 2022).

Methanol utilization in yeasts is accompanied by extensive proliferation of peroxisomes, where the main enzymes of the MUT pathway are concentrated. Moreover, *K. phaffii* possesses additional enzymes of the pentose phosphate (PP) pathway, localized in peroxisomes. Their genes are activated in response to methanol and are thought to have arisen from the duplication of classical PP-pathway genes (Rußmayer et al., 2015).

Methanol metabolism genes are under strict regulatory control. They are activated when methanol is the only available carbon source, and the presence of other carbon sources leads to their repression. This feature is widely used in biotechnology for controlled expression of heterologous genes and synthesis of recombinant proteins in *K. phaffii* and other methylotrophic yeasts (Karbalaei, Rezaee, and Farsiani, 2020).

However, recent studies show that the regulation of MUT genes depends not only on the carbon source but also on other environmental components. For example, the presence of methionine in the medium suppresses the expression of certain MUT genes in the presence of methanol, despite methanol being the only available carbon source for *K. phaffii* under these conditions (Ianshi-

na et al., 2023). Additionally, it has been shown that in the presence of proline as a nitrogen source in the methanol medium, MUT genes are also repressed. Moreover, proline is actively catabolized in mitochondria even in the presence of other more preferred (in classical terms) carbon (glucose, glycerol) and nitrogen (ammonium sulfate) sources (Rumyantsev et al., 2021).

The complexity of MUT-pathway regulation is further evidenced by the fact that some of the regulatory proteins of this pathway are also involved in regulating other processes. For instance, the main transcription factor of methanol metabolism genes, Mxr1p, also controls peroxisome biogenesis, as well as utilization of certain amino acids, acetate, ethanol, and fatty acids as carbon sources (Lin-Cereghino et al., 2006; Sahu and Rangarajan, 2016a; 2016b; Gupta, Krishna Rao, Sahu, and Rangarajan, 2021).

Thus, the methanol metabolism pathway in *K. phaffii* is deeply integrated into the overall cell metabolism through complex and branched regulation. Similar regulatory elements (orthologs of the corresponding proteins) are found in non-methylotrophic yeasts like *S. cerevisiae*. For example, previously mentioned Mxr1p is homologous to the *S. cerevisiae* regulatory protein Adr1, which is necessary for growth on ethanol, glycerol, and oleate (Lin-Cereghino et al., 2006). Methanol metabolism repressors KpMig1,2p and KpNrg1p also have homologs in *S. cerevisiae*, but their function in this yeast is to provide glucose repression (Wang et al., 2016; 2017).

It can be hypothesized that during evolutionary processes, when the ability for methylotrophy arose in yeasts, pre-existing regulatory blocks took control of the unique methanol metabolism pathway, ensuring its effective integration into the complex metabolic network of yeast cells.

In this work, we continue the research on gene regulation in *K. phaffii* and analyze the effects of nitrogen starvation on methanol metabolism and other cellular processes.

Materials and methods

Cultivation of yeast strains

The *K. phaffii* X-33 strain (Thermo Fisher Scientific, USA) was routinely grown on YEPD medium containing 1 % yeast extract, 2 % peptone, 2 % D-glucose and 2.4 % agar in case of a solid medium. For transcriptome analysis experiments, this strain was cultured in BMG media with glycerol to build up biomass. Cells were then transferred to media with methanol with ammonium sulfate (BMM+N) or without nitrogen source (BMM-N). The basic compositions of the media are listed in Table S1 in the supplementary materials. 2500x solutions of vitamins and trace elements were added to the media (see Table S2 in the supplementary materials).

Library preparation and sequencing

YeaStar RNA Kit (Zymo Research, USA) was used for RNA isolation from yeasts. Instead of a YR Digestion buffer, a buffer containing 1 M sorbitol, 0.1 M EDTA pH 7.4 and 0.1 % β-mercaptoethanol was used. Up to 2 % β-mercaptoethanol was additionally added to the YR Lysis buffer. Such modifications, in contrast to the methodology that was initially proposed in the kit, allowed the isolation of high-quality RNA samples from yeast cells.

The obtained RNA was treated with DNase I (Thermo Fisher Scientific, USA) and re-purified using the CleanRNA Standard kit (Evrogen, Russia). The RNA samples (300 ng each) were used for library preparation using the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Austria).

The quality of RNA and the resulting DNA fragments within the libraries were preliminarily analyzed by electrophoresis in 1 % agarose gel. The concentrations of RNA and DNA solutions were preliminarily measured using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). The exact concentrations of libraries and their final mixture were determined using a Qubit spectrofluorimeter (Thermo Fisher Scientific, USA). The quality of the final mixture of libraries was checked by capillary gel electrophoresis on an Agilent 2200 TapeStation system (Agilent, USA). Next-generation sequencing was performed at Sequencio (Moscow, Russia) in a 2×250 bp format on the Illumina NovaSeq platform.

Bioinformatic analysis of sequencing results

The Trimmomatic program (Bolger, Lohse, and Usadel, 2014) was used for filtering the reads by quality and for the removal of adapter sequences. The FastQC program (Andrews, 2010) was used to evaluate the quality of reads. The resulting reads were aligned with the reference genome of *K. phaffii* (ASM2700v1) whose sequence and annotation were taken from the NCBI (National Center for Biotechnology Information) database (De Schutter et al., 2009). Alignment was performed by the hisat-2 program (Kim et al., 2019) with standard parameters. The aligned reads were counted using the featureCounts program (Liao, Smyth, and Shi, 2014). R language version 3.6.3 (R Core Team, 2021) and DESeq2 library version 1.24.0 (Love, Huber, and Anders, 2014) were used to analyze differential gene expression.

The raw reads and the table of counts were uploaded to NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE278110). The process-

ing code was uploaded to GitHub (https://github.com/lbg-spbu/pp_nitrogen_starvation).

The results obtained are summarized in Table S3 in the supplementary materials. Genes whose adjusted p-value was less than 0.05 and the absolute estimated log-2FoldChange (logarithm of the change in the amount of expression) was greater than 0.5 were taken into further analysis. The BLAST algorithm (Camacho et al., 2009) was used to align amino acid sequences.

Functional enrichment analysis for differentially expressed genes was performed using the R programming language version 4.3.3 (R Core Team, 2021) and the clusterProfiler library version 4.10.1 (Wu et al., 2021). Using statistical methods, differentially expressed genes were grouped and assigned to specific metabolic pathways using the information from the KEGG database of annotated gene sets and metabolic pathways using the key "ppa" for the yeast *K. phaffii* (http://www.genome.jp/kegg/). Significance of enrichments was defined with p-value < 0.05. The results of the analysis were visualized using dotplot from the clusterProfiler package (Wu et al., 2021).

Gene nomenclature

For annotated *K. phaffii* MUT genes (*AOX1*, *AOX2* etc.) names were acquired from published studies, especially (Vogl et al., 2016). Other *K. phaffii* genes that are orthologs of the *S. cerevisiae* genes were found using BLAST analysis. To distinguish orthologs from different yeast species, "*Kp*" index was added to the name of *K. phaffii* genes and proteins, and "*Sc*" was added to the *S. cerevisiae* ones.

Results

The cultivation of *K. phaffii* X-33 strain was performed in two stages. First, cells were grown for 40 hours in 100 ml of BMG medium with glycerol as the only carbon source. Cells were then harvested by centrifugation and transferred into 100 ml volumes of medium with methanol — BMM+N (control with ammonium sulfate) and BMM-N (experimental samples without nitrogen source). The cells were cultured for 18 h. After that, total RNA was isolated and used for library preparation. Three control and three experimental samples were analyzed.

Transcriptome analysis showed that nitrogen starvation in the methanol containing medium alters the expression of 96 genes in the yeast *K. phaffii*. Of these differentially expressed genes (DEGs), 58 genes are activated under nitrogen deficiency and 38 are repressed. For all DEGs, we aligned the corresponding amino acid sequences with ones from the *S. cerevisiae* yeast proteome. Thus, putative orthologs of these genes in *S. cerevisiae* were identified (Table S4 in the supplementary materials). Also, a functional enrichment analysis was performed (Fig. S1 in the supplementary materials). Based on the information about the orthologs of the DEGs and the results of the enrichment analysis, groups of genes involved in various cellular processes in *K. phaffii* cells were identified and analyzed.

Nitrogen starvation influences the expression of genes encoding membrane transporters

Nitrogen starvation leads to the suppression of the *K. phaffii PAS_chr1-1_0417* and *PAS_chr1-1_0158* genes. Their orthologs in *S. cerevisiae*, *ScATO2* and *ScATO3*, encode ammonium ion exporters (Guaragnella and Butow, 2003). *K. phaffii PAS_chr2-1_0351* gene that is orthologous to *S. cerevisiae* methionine permease *ScMUP1* gene was also repressed in the absence of a nitrogen source.

On the other hand, nitrogen starvation leads to the activation of *PAS_chr2-2_0391* and *PAS_chr1-4_0394* ammonium permease genes that are orthologous to *S. cerevisiae ScMEP1* and *ScMEP2*. The expression of genes that encode amino acid transporters was also activated. These include dicarboxylic amino acid permease gene *PAS_chr4_0287* (orthologue of *ScDIP5* in *S. cerevisiae*), general amino acid permease gene *PAS_chr1-1_0030* (*ScGAP1*), lysine permease gene *PAS_chr1-1_0341* (*ScLYP1)* and high affinity proline permease gene *KpPUT4.2* (*ScPUT4*).

Nitrogen starvation induced the expression of genes involved in the transport of nucleobases and related metabolites: purine-cytosine permease gene *PAS_chr4_0514* (orthologue of *ScFCY2* in *S. cerevisiae*), uracil and allantoin permease genes *PAS_chr1-3_0059* and *PAS_chr1-1_0287* (*ScDAL4* and *ScDAL5*), hypothetic uric acid and xanthine permease gene *PAS_chr1-4_0518*. Thiamine transporter gene *PAS_chr3_0649* (*ScTHI72)* RNA levels also increased.

Interestingly, nitrogen starvation leads to the activation of *K. phaffii* genes involved in the transport of carbon sources — glucose transporter gene *KpHXT1 PAS_chr1-4_0570* and *PAS_chr4_0784* gene encoding *K. phaffii* channel-like protein, which is potentially an aquaglyceroporin KpAgp1 (Palmgren et al., 2017). Also, the expression of *PAS_chr1-1_0428* gene encoding sodium pump, involved in Na+ and Li+ efflux, was activated under nitrogen limiting conditions.

Nitrogen starvation influences the genes involved in central nitrogen metabolism

A large group of *K. phaffii* genes involved in the metabolism of nitrogen-containing compounds changed their expression under nitrogen starvation conditions in the medium with methanol (Fig. 1). A central part of

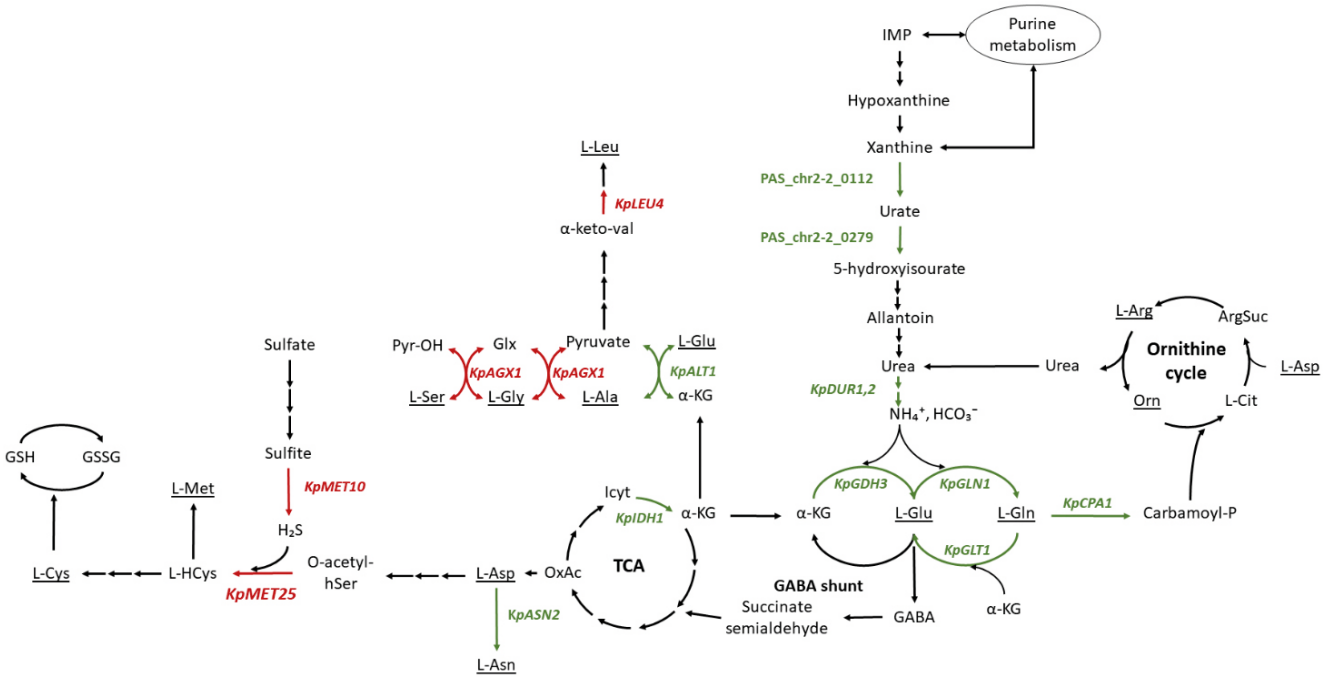

Fig. 1. Scheme representing the main components of nitrogen metabolism in *K. phaffii* (based on the data from KEGG database (Kanehisa and Goto, 2000) and the data known for *S. cerevisiae* according to SGD (Cherry et al., 2012)). Genes are placed near the reactions catalyzed by the correspondent proteins. Green arrows indicate transcription activation of the corresponding gene; red arrows indicate transcription repression. Multiple arrows indicate a set of consequent reactions.

ArgSuc — argininosuccinate, α-KG — a-ketoglutarate, GABA — γ-aminobutyric acid, Glx — glyoxylate, HCys — homocysteine, Icyt — isocytrate, L-cit — citrulline, OxAc — oxaloacetate, Orn — ornithine, O-acetyl-hSer — O-acetyl homoserine, TCA — tricarboxylic acid cycle.

nitrogen metabolism of yeast is the interconversion of glutamate, glutamine and α-ketoglutarate. In *K. phaffii* cells nitrogen starvation stimulated the expression of genes involved in the metabolism of these compounds, namely: NAD(+)-dependent glutamate synthase (*PAS_chr3_1024 — KpGLT1*, ortholog of *ScGLT1*); glutamine synthase (*PAS_chr4_0785 — KpGLN1*, ortholog of *ScGLN1*); NADP(+)-dependent glutamate dehydrogenase (*PAS_chr1-1_0107 — KpGDH3*, ortholog of *ScGDH3*); NAD(+)-dependent isocitrate dehydrogenase subunit gene (*PAS_chr4_0580 — KpIDH1*, ortholog of *ScIDH1*). In addition, the asparagine synthetase gene (*PAS_chr3_0675 — KpASN2*, ortholog of *ScASN2*), involved in the metabolism of the second pair of dicarboxylic amino acids, asparagine and aspartate, was also activated.

Lack of a nitrogen source activated the urea carboxylase/allophanate hydrolase gene (*PAS_chr4_0173 — KpDUR1,2*; ortholog of *ScDUR1,2* in *S. cerevisiae*), as well as the gene encoding the small subunit of carbamoyl phosphate synthetase (*PAS_chr4_0138 — KpCPA1*, ortholog of *ScCPA1*), which catalyzes the synthesis of citrulline, a component of the urea cycle. The putative xanthine dehydrogenase/oxidase (*PAS_chr2-2_0112*) and the putative uratoxidase (*PAS_chr2-2_0279*) genes were also induced.

The synthesis of glutamate and glutamine can also be carried out by a transaminase. The activation of this pathway in *K. phaffii* cells upon nitrogen starvation can be proposed, due to the activation of the alanine transaminase gene (*PAS_chr3_0482 — KpALT1*, ortholog of *ScALT1*). However, at the same time there was a repression of the alanine — glyoxylate transaminase gene (*PAS_chr4_0416 — KpAGX1*, ortholog of *ScAGX1*), that in *S. cerevisiae* provides transamination reactions involving such amino acids as alanine, glycine, and serine.

Genes involved in the synthesis of sulfur-containing amino acids methionine and cysteine (*PAS_chr4_0369 — KpMET10*, ortholog of *ScMET10*; *PAS_chr4_0330 — KpMET25*, ortholog of *ScMET25*) and leucine (*PAS_chr2-1_0415 — KpLEU4*, ortholog of *ScLEU4*) were repressed during nitrogen starvation.

Differential expression of some amino acid metabolism genes was accompanied by selective repression of several genes involved in protein synthesis. These included genes encoding ribosome subunit proteins: S1 (*PAS_chr4_0524*), S13 (*PAS_chr4_0456*), L3 (*PAS_chr4_0139*), S6 (*PAS_FragD_0014*), L38 (*PAS_chr1-4_0412*). The localization of the corresponding proteins in the ribosome is shown in Fig. S2. The genes of ribosome biogenesis auxiliary protein (*PAS_chr2-1_0245*), translation elongation factor EF1γ (*PAS_chr3_1071*), and chaperone Ssb2, which performs protein folding (*PAS_chr3_0731*), were also repressed.

Nitrogen starvation influences the genes involved in Ehrlich pathway

A distinct group of genes that were activated during nitrogen starvation in *K. phaffii* includes the genes of the Ehrlich pathway enzymes. This pathway in yeast involves three sequential reactions: transamination of amino acids, decarboxylation of the resulting keto acids, and their reduction to alcohols or further oxidation (Fig. 2). In *S. cerevisiae*, during the first stage BCAA (branched-chain amino acids: valine, leucine, isoleucine) and aromatic amino acids (tyrosine, tryptophan, phenylalanine), as well as methionine, are acted upon by the transaminases ScBat1p, ScBat2p, ScAro8p, and ScAro9p, which transfer amino groups to 2-oxoglutarate or other keto acids (including pyruvate). ScBat1p and ScBat2p are primarily involved in the transamination of BCAAs, whereas ScAro8p and ScAro9p are aminotransferases with broad substrate specificity. The α-keto acids formed in the transamination reactions undergo decarboxylation to produce the corresponding aldehydes. In *S. cerevisiae*, this reaction is facilitated by the decarboxylases ScPdc1p, ScPdc5p, ScPdc6p, ScAro10p, and ScThi3p. ScAro10p has broad specificity and is responsible for most of the decarboxylase activity in the Ehrlich pathway (Vuralhan et al., 2005). ScPdc1p has been shown to decarboxylate the derivatives of valine, tyrosine, phenylalanine, and isoleucine, but not leucine and methionine (Perpète et al., 2006; Pires, Teixeira, Brányik, and Vicente, 2014).

In *K. phaffii*, nitrogen starvation induces the expression of the genes encoding aminotransferase II (PAS_chr4_0147 — *KpARO9*, ortholog of *ScARO9*) and pyruvate decarboxylase 1 (PAS_chr3_0188 — *KpPDC1*, ortholog of *ScPDC1*). However, there is a decrease in the expression of the gene for poorly investigated aldehyde dehydrogenase *KpALD6-2* (*PAS_chr3_0987*, ortholog of *ScALD5*), that may be responsible for the oxidation of aldehydes to acids in the Ehrlich pathway (Fig. 2).

Nitrogen starvation influences the genes involved in carbon and lipid metabolism

The expression analysis was conducted on *K. phaffii* cells grown in a medium with methanol as the sole carbon source. The results obtained are illustrated in Fig. 3. Under these conditions, nitrogen deficiency led to a decrease in the activity of some methanol metabolism genes (*PAS_chr4_0152 — AOX2*; *PAS_chr3_0932 — FDH1)* and genes of the associated pentose phosphate pathway in peroxisomes (*PAS_chr4_0212 — RKI1-2*). Interestingly, genes *RKI1-1* and *RKI1-2* are involved in generating precursors for vitamins pyridoxine and thiamine, as well as nucleotides. The experiment showed that the repression of *RKI1-2* was accompanied by the repression of genes directly involved in thiamine synthesis (*PAS_chr3_0842 — KpTHI20*, ortholog of *ScTHI20*; *PAS_chr3_0843 — KpTHI6*, ortholog of *ScTHI6*; *PAS_chr4_0065 — KpTHI13*, ortholog of *ScTHI13*). There was also an increase in the activity of the gene encoding the low-affinity thiamine transporter (*PAS_chr3_0649 — KpTHI72*, homolog of *ScTHI72*).

Nitrogen starvation also suppressed the expression of the peroxin 6 gene (*PAS_chr1-4_0133 — KpPEX6*, ortholog of *ScPEX6*), the product of which is necessary for the recycling of the Pex5 receptor and the import of proteins containing the PTS1 (peroxisomal targeting signal 1) into the peroxisome (Grimm, Erdmann, and Girzalsky, 2016). Since all main MUT enzymes are placed in peroxisomes, the repression of genes involved in the transport of proteins into peroxisomes may also affect methanol metabolism.

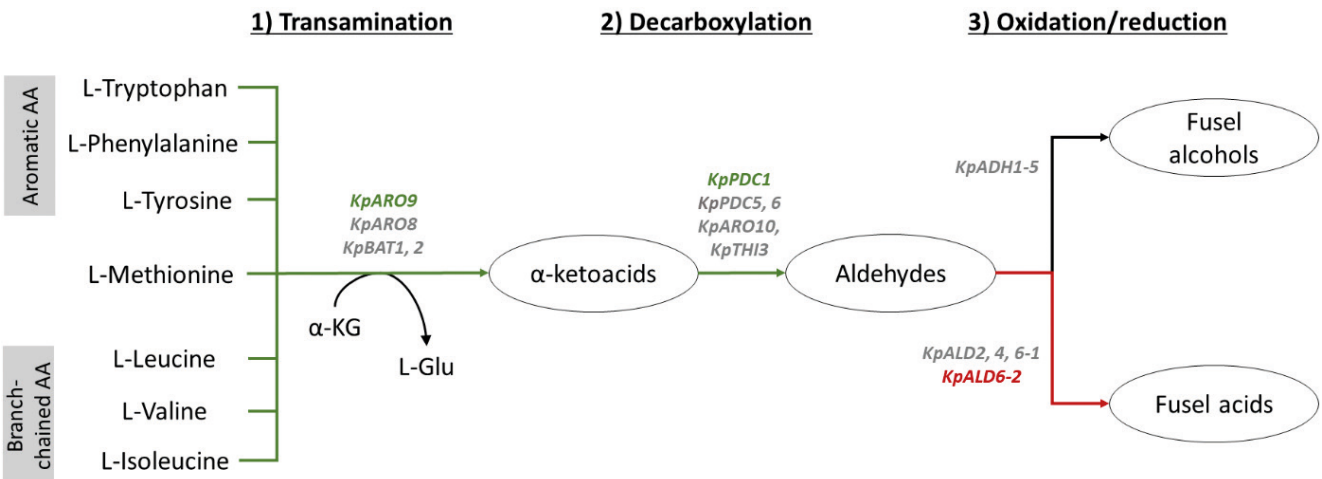

Fig. 2. Scheme representing the main components of Ehrich pathway in *K. phaffii* (based on the data from KEGG database (Kanehisa and Goto, 2000) and the data known for *S. cerevisiae* according to SGD (Cherry et al., 2012)). Genes are placed near the reactions catalyzed by the correspondent proteins. Genes activated during nitrogen starvation are indicated in green, repressed — in red. Genes that are involved in Ehrlich pathway, but which did not change the expression level during the experiment, are indicated in gray.

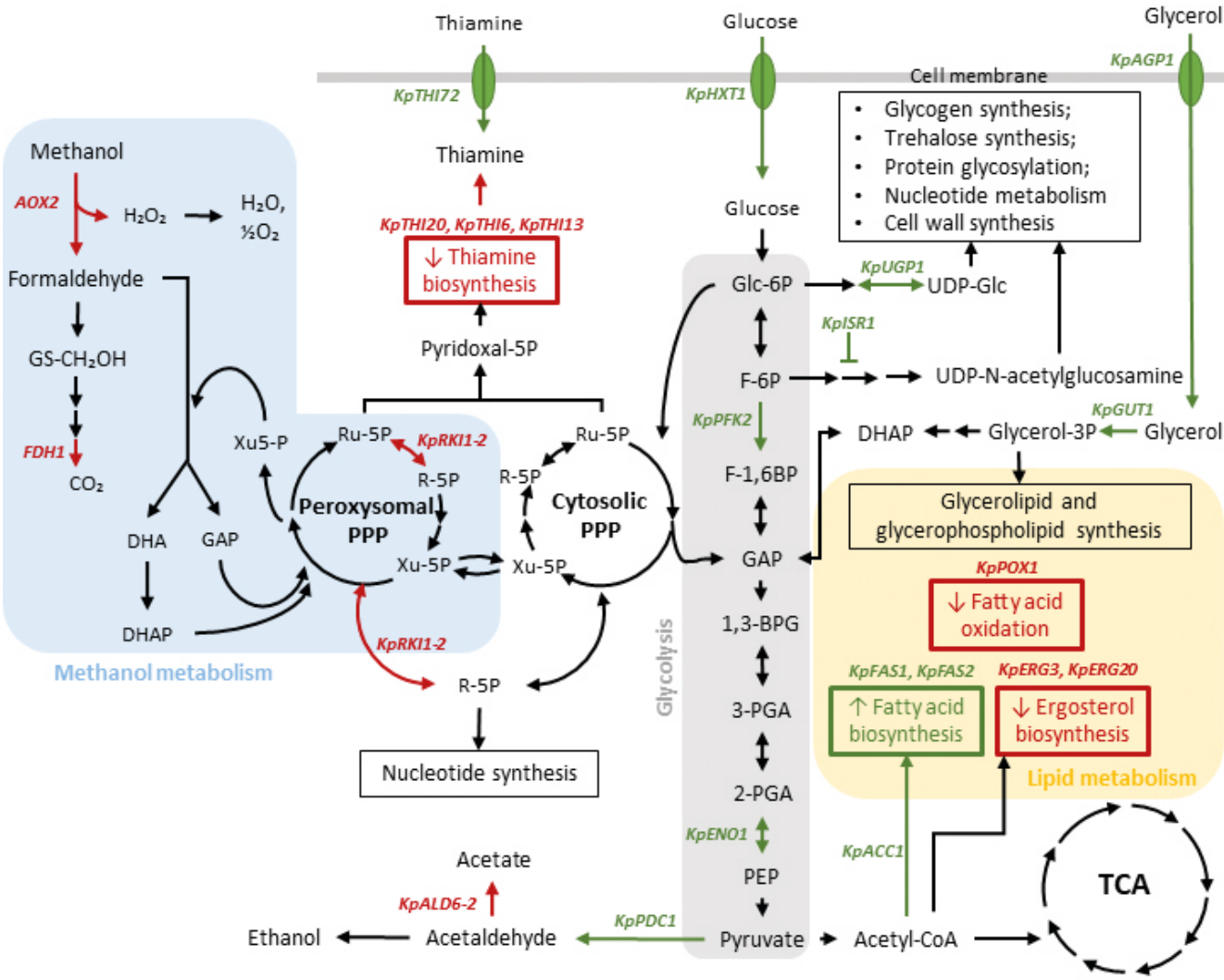

Fig. 3. Scheme representing the main components of carbon metabolism in *K. phaffii* (based on the data from KEGG database (Kanehisa and Goto, 2000) and the data known for *S. cerevisiae* according to SGD (Cherry et al., 2012)). Genes are placed near the reactions catalyzed by the corresponding proteins. Green arrows indicate transcription activation of a corresponding gene; red arrows indicate transcription repression. Multiple arrows indicate a set of consequent reactions.

1,3-BPG — 1,3-diphosphoglycerate, 2-PGA — 2-phosphoglycerate, 3-PGA-3-phosphoglycerate, DHA — dihydroxyacetone, DHAP — dihydroxyacetone phosphate, F-6P — fructose 6-phosphate, F-1,6BP — fructose 1,6-diphosphate, GAP — glyceraldehyde phosphate, Glc — glucose, Glc-6P — glucose 6-phosphate, GS — glutathione, PEP — phosphoenol pyruvate, PPP — pentose phosphate pathway, R-5P — ribose 5-phosphate, Ru-5P — ribulose 5-phosphate, TCA — tricarboxylic cycle, Xu-5P — xylulose 5-phosphate.

Alongside the suppression of methanol metabolism genes, there was an increase in the transcription of genes involved in glycerol metabolism (*PAS_chr4_0783* — *KpGUT1*, ortholog of *ScGUT1*). Additionally, the genes for glycolytic enzymes were activated: enolase (*PAS_chr3_0082* — *KpENO1*, ortholog of *ScENO1*) and the β-subunit of phosphofructokinase (*PAS_chr1-4_0047* — *KpPFK2*, ortholog of *ScPFK2*). There was also an induction of the gene for glucose-1-phosphate uridylyltransferase (*PAS_chr1-3_0122* — *KpUGP1*, homolog of *ScUGP1*), which is essential for synthesizing UDP-glucose, a component necessary for protein glycosylation and synthesis of reserve and stress-protective carbohydrates — glycogen and trehalose.

Furthermore, *K. phaffii* exhibited the activation of the kinase gene *PAS_chr4_0666*, which has the greatest similarity in the amino acid sequence to the not completely understood serine-threonine kinase Isr1 of *S. cerevisiae*. ScIsr1 is known as a negative regulator of the hexosamine biosynthesis pathway, which converts glucose into uridine-diphosphate-N-acetylglucosamine (UDP-GlcNAc) — the main precursor for protein glycosylation, GPI anchor formation, and chitin biosynthesis (Alme et al., 2020). In Fig. 3, *PAS_chr4_0666* is designated as *KpISR1*.

The differential gene expression analysis in *K. phaffii* also revealed the changes in lipid metabolism genes. Nitrogen starved *K. phaffii* cultivated in a methanol me-

dium, showed an increase in the expression of genes involved in *de novo* fatty acid biosynthesis. Primarily, these include the genes for acetyl-CoA carboxylase (*PAS_chr1-4_0249 — KpACC1*, ortholog of *ScACC1*) and fatty acid synthases (*PAS_chr1-1_0008 — KpFAS1*, ortholog of *ScFAS1*; and *PAS_chr1-4_0295 — KpFAS2*, ortholog of *ScFAS2*). At the same time, there was a decrease in the activity of the acyl-CoA oxidase gene (*PAS_chr1-4_0538 — KpPOX1*, ortholog of *ScPOX1*), involved in the β-oxidation of fatty acids.

Additionally, there was an increase in the expression of the cytochrome b5 gene (*PAS_chr4_0953 — KpCYB5*, ortholog of *ScCYB5*), necessary for the synthesis of both fatty acids and ergosterol. Conversely, the expression of some ergosterol synthesis genes decreased. For example, the C-5 sterol desaturase gene (*PAS_chr1-4_0367 — KpERG3*, ortholog of *ScERG3*), which catalyzes one of the final steps in converting episterol to ergosterol, was repressed. Similarly, the farnesyl pyrophosphate synthetase gene (*PAS_chr1-4_0314 — KpERG20*, ortholog of *ScERG20*), was downregulated. Its product catalyzes two consecutive condensation reactions leading to the formation of farnesyl pyrophosphate, an important intermediate in the biosynthesis of ergosterol, ubiquinone, dolichol, and heme.

Finally, there was an increase in the expression of the carbonic anhydrase gene (*PAS_chr4_0578 — KpNCE103*, ortholog of *ScNCE103*). This enzyme provides synthesis of bicarbonate — a key substrate for carboxylases that catalyze the formation of carbamoyl phosphate (KpCpa1,2p), malonyl-CoA (KpAcc1p), and oxaloacetate from pyruvate (KpPyc1,2p). As previously mentioned, the expression of the two carboxylase genes, *KpCPA1* and *KpACC1*, increased under nitrogen starvation in *K. phaffii*.

Nitrogen starvation influences the genes involved in autophagy

Nitrogen deficiency in *K. phaffii* stimulates the activation of genes encoding phospholipase B (*PAS_chr1-1_0465 — KpATG15*, ortholog of *ScATG15*) and carboxypeptidase (*PAS_chr3_0633 — KpATG42*, ortholog of *ScATG42*). These enzymes facilitate the degradation of the membranes and the contents of autophagosomal bodies in the vacuoles during autophagy. Carboxypeptidase ScAtg42p has recently been described in *S. cerevisiae* as a functional homolog of carboxypeptidase Y (also known as proteinase C, CPY, ScPrc1p). At least one of these peptidases is required for protein degradation and maintenance of the amino acid pool in vacuoles during nitrogen starvation in *S. cerevisiae* (Parzych, Ariosa, Mari, and Klionsky, 2018).

In addition to the activation of autophagosomal enzyme genes, nitrogen starvation in *K. phaffii* also triggers the expression of genes encoding V0c and V1c subunits of the vacuolar H+-ATPase (V-ATPase): *PAS_chr3_1040* (*KpVMA3*, ortholog of *ScVMA3*) and *PAS_chr2-1_0246* (*KpVMA5*, ortholog of *ScVMA5*). In yeast, V-ATPase plays a crucial role in maintaining the pH of intracellular compartments, particularly vacuoles, where autophagy processes occur (Nakamura, Matsuura, Wada, and Ohsumi, 1997).

Furthermore, the gene *PAS_chr2-1_0109* (orthologous to *ScFMP42/YMR221C*) was activated. ScFmp42p interacts with the protein ScAtg27p and is likely involved in the autophagy process in *S. cerevisiae* (Tarassov et al., 2008).

Nitrogen starvation influences the genes of regulatory proteins

Nitrogen starvation in *K. phaffii* led to the increased expression of the gene for an unknown transcription factor, *PAS_chr4_0626*. The protein encoded by *PAS_chr4_0626* shows the greatest similarity to the GATA factor sequence *GAT1* from *S. cerevisiae* and appears to be *K. phaffii*'s own GATA factor. ScGat1p is known as one of the main transcriptional regulators of NCR (nitrogen catabolite repression) genes in *S. cerevisiae*, along with ScGln3p, ScGzf3p, and ScDal80p, whose regulation is mediated through the TOR pathway (Zhang, Du, Zhou, and Chen, 2018).

Nitrogen-starved *K. phaffii* cells have demonstrated the induction of a general transcriptional repressor gene orthologous to *ScTUP1* (*PAS_chr3_1001*). In *S. cerevisiae*, ScTup1p is a part of several regulatory complexes, particularly Ssn6-Tup1 and Cyc8-Tup1, and is involved in regulating a vast number of cellular processes. For instance, the Ssn6-Tup1 complex, along with regulatory proteins like ScMig1p, ScRox1p, and ScNrg1p, represses specific gene groups. For example, in complex with ScMig1 it regulates glucose repression (Smith and Johnson, 2000). It is important to note that in *K. phaffii*, homologs of ScMig1p and ScNrg1p are involved in the regulation of methanol metabolism and associated processes (Wang et al., 2016; Shi et al., 2018).

The second complex, Cyc8-Tup1, in *S. cerevisiae* acts as a coactivator in the regulation of the amino acid catabolism genes in the context of nitrogen metabolism, in conjunction with the SPS amino acid sensing system (Ssy1-Ptr3-Ssy5 signaling sensor system) (Zhang, Du, Zhou, and Chen, 2018).

Additionally, in *K. phaffii* cells, the expression of the *PAS_chr3_0640* gene (orthologous to *ScTFS1*), that potentially encodes the regulatory protein KpTfs1, increased. In *S. cerevisiae*, it primarily acts as an inhibitor of autophagosomal carboxypeptidase Y and the ScIra2p, which is an inhibitor of the Ras/cAMP/PKA pathway (Chautard et al., 2004; Mima et al., 2005; Conrad et al.,

2014). Moreover, ScTfs1p interacts with numerous proteins involved in folding, translation, transcription, as well as in the tricarboxylic acid cycle and glutamate metabolism (Beaufour et al., 2012).

Besides the expression of the KpTfs1p gene, which is involved in the activation of the PKA pathway, there was an increase in the expression of the gene homologous to one of the catalytic subunits of cAMP-dependent protein kinase PKA (*PAS_chr1-4_0357*), which shows the greatest sequence similarity to the *ScTPK2* gene.

Nitrogen starvation influences the oxidative stress response and heme biosynthesis genes

During nitrogen starvation on methanol, *K. phaffii* experienced repression of the 5-aminolevulinate (ALA) synthase gene (*PAS_chr2-1_0716 — KpHEM1*, ortholog of *ScHEM1*). ScHem1 synthesizes ALA from succinyl-CoA and glycine. In yeast, this reaction is the first step in the biosynthesis pathway of tetrapyrrole compounds, which provides cells with heme necessary for the activity of catalases, peroxidases, and other heme-containing enzymes, as well as siroheme necessary for the sulfite reductase function.

Additionally, there was a repression of several genes involved in maintaining the cellular redox balance. Among them were the genes for mitochondrial peroxiredoxin (*PAS_chr1-1_0433 — KpPRX1,* ortholog of *ScPRX1*), methionine sulfoxide reductase (*PAS_chr3_0066*, ortholog of *ScMXR1*), and arabinose dehydrogenase (*PAS_chr2-1_0775 — KpARA2,* ortholog of *ScARA2*), which is involved in the synthesis of D-erythroascorbic acid. However, there was an increase in the expression of the thioredoxin gene (*PAS_chr4_0284 — KpTRX1*, ortholog of *ScTRX1*).

Discussion

Nitrogen metabolism in yeast has been extensively studied using the popular model organism, *S. cerevisiae*. To date, the key aspects and a range of compounds that these yeasts can use as nitrogen sources are well known (Godard et al., 2007; Ljungdahl and Daignan-Fornier, 2012). The response of *S. cerevisiae* cells to nitrogen starvation is also thoroughly described (Boer, de Winde, Pronk, and Piper, 2003; Li et al., 2015; Tesnière, Brice, and Blondin, 2015; Liu, Sutter, and Tu, 2021).

In this study, we investigated the transcriptomic response of another yeast species, *K. phaffii*, to nitrogen deficiency in the medium. Unlike *S. cerevisiae*, these yeasts are methylotrophic, and the analysis was conducted in methanol-containing media. Nevertheless, many of the results obtained are consistent with the known data for *S. cerevisiae*. For example, nitrogen starvation leads to the activation of autophagy genes and genes involved in the synthesis of the stress-protective carbohydrate trehalose. Also, genes controlling the central element of nitrogen metabolism in the cell (the interconversion of glutamine, glutamate, and α-ketoglutarate) are upregulated. Along with this, the transport of various nitrogen-containing compounds is enhanced, and the expression of genes for enzymes that facilitate their catabolism is increased. The export of ammonia from the cells, on the other hand, is suppressed.

The main feature that distinguishes *K. phaffii* from *S. cerevisiae* is the ability to utilize methanol. Nitrogen starvation in *K. phaffii* led to the suppression of several methanol metabolism genes. At the same time some genes involved in the metabolism of glycerol and glucose were activated, although these carbon sources were not present in the media. For example, during nitrogen starvation on methanol, glycerol kinase gene *KpGUT1* (*PAS_chr4_0783*), that provides the first stage of glycerol utilization, was activated. Previously it was shown that this gene is involved in the repression of *AOX1* by glycerol (Shen et al., 2016). The expression of *KpGUT1*, in turn, is regulated by the transcription factor KpRtg1p, which is also involved in the regulation of methanol and glutamate metabolism (Rumyantsev, Soloviev, Slepchenkov, and Sambuk, 2018; Dey, Krishna Rao, Khatun, and Rangarajan, 2018). At the same time, nitrogen starvation resulted in the increased expression of the glucose transporter gene *KpHXT1* (*PAS_chr1-4_0570*), which is also involved in the regulation of methanol metabolism. It was shown, that the deletion of this gene results in derepression of *AOX1* in response to glucose (Zhang et al., 2010). Based on these observations, it may be proposed that the activation of the glycerokinase *KpGUT1* and glucose transporter *KpHXT1* genes under nitrogen starvation conditions influence the suppression of methanol metabolism genes.

It was previously shown that the deletion of glycerol transporter gene *KpGT1* in *K. phaffii* leads to derepression of *AOX1* (Zhan et al., 2016). During nitrogen starvation, there was no significant change in the transcription of that gene, but the gene of the potential aquaglyceroporin (*PAS_chr4_0784*) was activated. It can be assumed that this aquaglyceroporin, similarly with the proteins described above, may be involved in the regulation of methanol metabolism, but this assumption requires further research.

An interesting observation was the activation of the PKA signaling pathway during nitrogen starvation, evidenced by the increased expression of *KpTFS1* gene. The PKA pathway controls the cell growth, carbohydrate metabolism, the cell cycle, and overall stress response. In *S. cerevisiae*, PKA activity promotes fermentative growth and negatively affects respiration-related traits and stationary phase transitions (e. g., carbohydrate storage, stress resistance, and other stationary states). Generally,

a PKA pathway activation signals active growth, while a decreased activity signals a transition to the stationary phase (Conrad et al., 2014). Notably, during nitrogen starvation in *K. phaffii*, cells experience stress and exhibit suppressed growth-related processes, yet the PKA pathway activation is observed.

However, it is essential to note that the PKA pathway regulates highly diverse processes in response to environmental signals, with specificity achieved through different isoforms of catalytic subunits of protein kinase A (Creamer, Hubbard, Ashe, and Grant, 2022). In *K. phaffii*, the expression of the possible *ScTPK2* ortholog encoding one of TPK isoforms was activated in response to nitrogen starvation. *S. cerevisiae* is one of the few fungal species utilizing more than two PKA catalytic subunits: ScTpk1p, ScTpk2p, ScTpk3p, while *K. phaffii* has only two *TPK* genes. In *S. cerevisiae*, the ScTpk2p isoform is involved in trehalose degradation, pseudohyphal growth, and the repression of iron uptake genes, thus regulating yeast respiratory growth (Robertson, Causton,Young, and Fink, 2000). Additionally, ScTpk2p is the only isoform containing a prion-like domain at the N-terminus, playing a crucial role in P-body and stress granule aggregation under the stress conditions (Barraza et al., 2021). ScTpk2p also partially activates the stress regulator ScMsn2p, unlike other isoforms that inhibit it, suppressing the stress response (Creamer, Hubbard, Ashe, and Grant, 2022).

However, it remains unclear what specific functions the ortholog of this isoform performs in *K. phaffii* and how they correlate with those described for *S. cerevisiae*. For instance, it is known that pseudohyphal growth (regulated by ScTpk2p) is not initiated under nitrogen deficiency in *K. phaffii* as it does in *S. cerevisiae* (De et al., 2020). Therefore, the functions of different PKA catalytic subunit isoforms in *K. phaffii* remain to be elucidated.

Additionally, it has been shown that PKA activation in *S. cerevisiae* inhibits ScAdr1p — the homolog of KpMxr1p, which regulates methanol metabolism gene expression in *K. phaffii* (Cherry et al., 1989). Thus, the activation of genes involved in regulation and functioning of the PKA pathway during nitrogen starvation on methanol in *K. phaffii* may cause the repression of methanol metabolism genes.

An important difference from *S. cerevisiae* is that *K. phaffii* yeasts possess genes for the putative xanthine dehydrogenase/oxidase and urate oxidase. These enzymes provide the catabolism of xanthine and hypoxanthine (purine degradation products) to 5-hydroxyisourate, that can be metabolized by other enzymes to ammonia. The genes of this pathway (*PAS_chr4_0173, PAS_chr2-2_0112, PAS_chr2-2_0279*) were activated during nitrogen starvation in *K. phaffii*. Meanwhile, in *S. cerevisiae*, hypoxanthine and xanthine, formed during purine degradation, are excreted from the cell rather than metabolized to uric acid and subsequent metabolites (Cultrone et al., 2005; Daignan-Fornier and Pinson, 2019). Thus, *K. phaffii* appears to be capable of using purines as nitrogen sources, unlike *S. cerevisiae*. This is further supported by the activation of the transport of purine bases into the cell in response to nitrogen starvation.

Some other elements of the transcriptional response of *K. phaffii* cells to nitrogen starvation appeared to be mosaic. For example, in addition to nitrogen metabolism, pathways for fatty acid biosynthesis and oxidation, ergosterol biosynthesis, and tetrapyrrole biosynthesis were affected during nitrogen starvation. Another interesting observation was the suppression of genes involved in transport and synthesis of sulfur-containing amino acids and leucine. Some of these effects can be explained by the imbalance of key metabolites arising under the nitrogen starvation conditions. Such metabolites include coenzyme A (CoA), S-adenosylmethionine (SAM), ATP, GTP, NAD^+, and $NADP^+$. Purine bases are important components of these compounds.

The activation of the purine catabolism genes could likely affect the pool of nucleoside triphosphates (ATP and GTP) that support a vast number of reactions in the cell. Specifically, ATP serves as a source of adenosine for the synthesis of SAM from methionine. It can be hypothesized that ATP deficiency leads to the inhibition of SAM synthesis and the accumulation of methionine in the cell (Fig. 4B). This is evidenced by the repression of the methionine-specific permease gene (*PAS_chr2-1_0351*) in response to nitrogen starvation.

In the final reaction of the methyl cycle, S-adenosylhomocysteine (SAH) is converted to homocysteine with the release of adenosine, which can be used by the cell as a nitrogen source. It is possible that active degradation of SAH with the release of adenosine during nitrogen starvation leads to the accumulation of homocysteine. The resulting imbalance between the components of the SAM cycle is partially mitigated by the cell by adjusting the level of homocysteine synthesis from O-acetylhomoserine (Fig. 4B).

The synthesis of sulfur-containing amino acids strongly depends on the synthesis of tetrapyrrole compounds in the cell, particularly siroheme, that serves as a prosthetic group for sulfite reductase. During nitrogen starvation in *K. phaffii*, the repression of the first stage of tetrapyrrole synthesis was observed (Fig. 4A). This likely leads to a decrease in the overall level of tetrapyrroles synthesis in the cell, including not only siroheme but also other heme compounds. This effect may be a consequence of reduced methanol metabolism and the associated catalase activity, which carries heme as a cofactor.

On the other hand, it should be noted that the BMM(N+) and BMM(N–) media used in the experiment differ not only in the presence/absence of am-

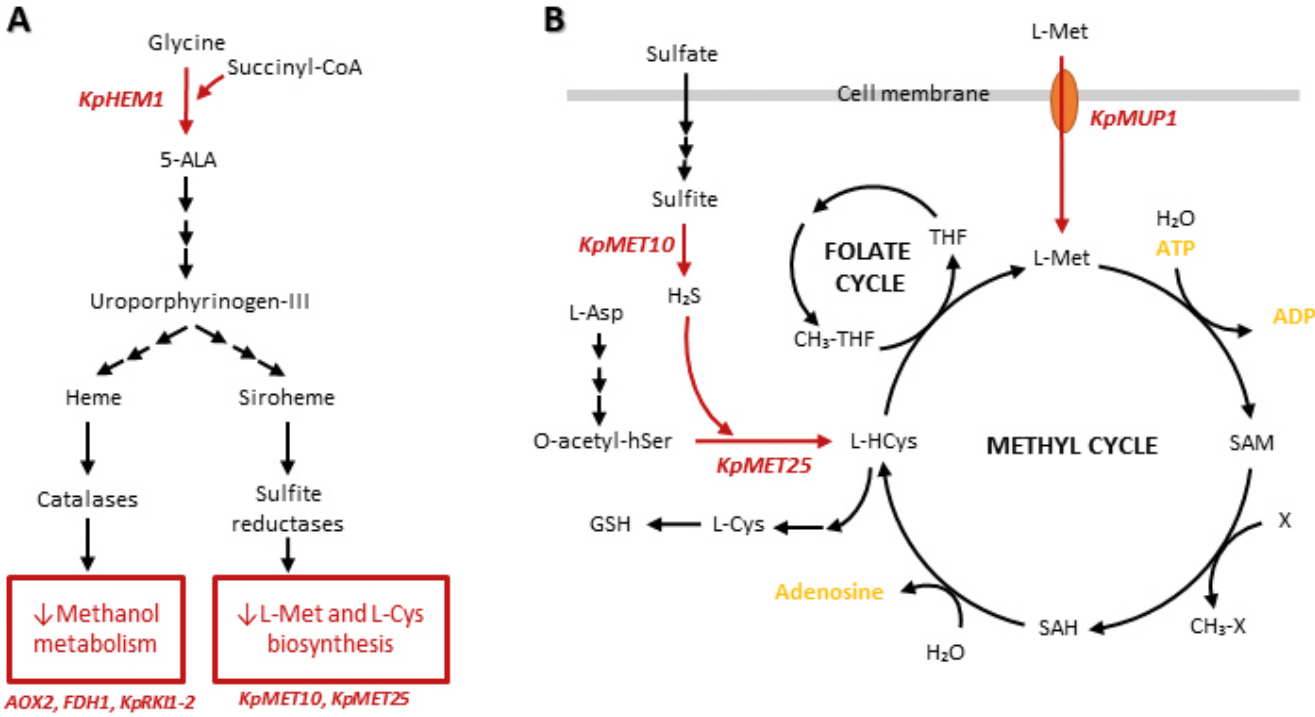

Fig. 4. A — A proposed scheme for the synthesis of tetrapyrrole compounds in *K. phaffii* under nitrogen starvation. B — A proposed scheme for methionine metabolism in *K. phaffii* under nitrogen starvation. The schemes are based on the data from KEGG database (Kanehisa and Goto, 2000) and the data known for *S. cerevisiae* according to SGD (Cherry et al., 2012). Gene names are positioned next to the reaction catalyzed by the corresponding enzyme. Genes and processes repressed during starvation are marked in red. Purine compounds whose levels are potentially reduced due to active degradation during nitrogen starvation are shown in orange. Multiple arrows represent sequences of several consecutive reactions.

5-ALA — 5-aminolevulinic acid, GSH — glutathione, L-HCys — L-homocysteine, O-acetyl-hSer — O-acetyl homoserine, SAH — S-adenosyl-homocysteine, SAM — S-adenosyl methionine, THF — tetrahydrofolate.

monia, but also in sulfate content. Yeasts growing on BMM(N–) experienced not only nitrogen starvation but also had relatively less available sulfate in the medium. This could have affected the expression of genes involved in sulfate assimilation and sulfur integration into amino acids. This interpretation must be considered, even though it does not explain the repression of the methionine permease gene.

Interestingly, methionine, cysteine, and leucine, whose transport and synthesis were suppressed in *K. phaffii* cells during nitrogen starvation, act as sensors of intracellular nitrogen and activate TORC1 (Bonfils et al., 2012; Sutter, Wu, Laxman, and Tu, 2013; Zeng, Araki, and Noda, 2024). The suppression of leucine and cysteine synthesis, as well as the suppression of methionine transport, likely contributes to a decrease in the level of these amino acids in the cell, serving as an auxiliary regulatory component, further reducing TORC1 activity during starvation.

The lack of ATP due to purine degradation, and the probable decrease in cysteine synthesis, can affect the synthesis levels of an essential cellular cofactor — coenzyme A. Acetyl-CoA is a central metabolite that performs numerous functions, serving as an energy source in the tricarboxylic acid cycle and as a structural component in anabolic reactions. Additionally, it directly participates in gene regulation through histone acetylation. The concentration of acetyl-CoA in different compartments reflects the overall energy state of the cell, balancing anabolic and catabolic processes under various conditions (Pietrocola et al., 2015). For example, the depletion of the nucleocytosolic pool of acetyl-CoA signals for autophagy induction during cell aging or starvation. Furthermore, the lack of acetyl-CoA in the nucleus and cytoplasm is more critical for autophagy regulation than TORC1 signaling pathways. Besides acetyl-CoA, other CoA derivatives involved in fatty acid metabolism, amino acid metabolism, tetrapyrrole synthesis, and TCA cycle (e. g., succinyl-CoA) are present in the cell (Leonardi, Zhang, Rock, and Jackowski, 2005).

Nitrogen starvation in *K. phaffii* yeast affects lipid metabolism and ergosterol biosynthesis. Acetyl-CoA acts as a building block for large fatty acid and terpene molecules formation. Fatty acid biosynthesis, the genes of which were activated during nitrogen starvation, requires a continuous supply of new acetyl-CoA molecules, attaching acetyl residues to the growing hydrocarbon chain and releasing CoA. At the same time, β-oxidation of fatty acids, which synthesizes acetyl-CoA, was suppressed on the transcriptomic level.

On the other hand, the changes in lipid metabolism can be explained by the fact that yeast cells initiate autophagy under nitrogen starvation, requiring a significant number of membranes to form autophagosomes. This likely leads to the increased fatty acid synthesis. It is worth noting that in the presence of methanol, *K. phaffii* cells even under optimal conditions allocate substantial resources to synthesize membrane organelles — peroxisomes, where all key steps of methanol metabolism occur (Rußmayer et al., 2015). Under nitrogen starvation on methanol, the lipid balance may shift towards autophagosome synthesis and the suppression of peroxisomes. This aligns with the observed suppression of methanol metabolism and β-oxidation of fatty acids that occur in peroxisomes.

Nitrogen starvation also suppressed the genes involved in biosynthesis of farnesyl pyrophosphate and ergosterol. The initial stages of these pathways (mevalonate synthesis reactions) require acetyl-CoA. The final reaction of mevalonate synthesis in *S. cerevisiae* is a limiting step for the entire ergosterol synthesis pathway, regulated at the transcriptional, translational and post-translational levels (Burg and Espenshade, 2011; Jordá and Puig, 2020). Ergosterol is a crucial component of cell membranes. Its reduced synthesis may relate not only to the changes in acetyl-CoA balance, but also to the overall suppression of cell growth and plasma membrane development under nitrogen starvation. This effect is further indicated by the putative suppression of N-acetylglucosamine synthesis (the main component of chitin) due to the activation of the gene, encoding the ortholog of the regulatory kinase ScIsr1p (*PAS_chr4_0666*).

Nitrogen starvation resulted in the suppression of five genes encoding ribosomal proteins. This may be a consequence of the general suppression of translation processes under nitrogen deficiency. It may be expected that other ribosome genes will also be suppressed under a longer starvation period. On the other hand, previously, when *K. phaffii* cells were cultured in the media with proline as the only nitrogen source, we also observed the suppression of four of these genes (*PAS_chr4_0524*, *PAS_chr4_0456*, *PAS_chr4_0139*, *PAS_chr1-4_0412*) (Rumyantsev et al., 2021). Both proline utilization and nitrogen starvation lead to the repression of translation elongation factor EF1γ gene (*PAS_chr3_1071*), and ribosome-associated chaperone gene (*PAS_chr3_0731*). Thus, the observed selective changes in ribosomal protein gene expression may be related to a regulated change in ribosome composition in response to nitrogen starvation or a change of nitrogen source. This may be an example of ribosome heterogeneity and ribosome composition dynamics in response to environmental factors (Genuth and Barna, 2018).

Conclusion

In this study, we investigated the transcriptomic response to nitrogen starvation in the presence of methanol in *K. phaffii* yeast. We demonstrated that nitrogen deficiency leads to the partial suppression of methanol metabolism, oxidative stress response, and some other peroxisomal processes. Concurrently, there is an enhancement of the transport and catabolism of various nitrogen-containing compounds, activation of autophagy, and central nitrogen metabolism pathways, promoting survival under nitrogen deficiency. Additionally, there are changes in the ribosome biogenesis, translation proteins, and the reduced synthesis and transport of sulfur-containing amino acids and leucine synthesis.

K. phaffii yeast is widely used in biotechnology for the synthesis of recombinant proteins and various compounds. For their cultivation, such media are used where ammonium sulfate, urea, tryptone, peptone, and amino acid mixtures can serve as nitrogen sources (Guo et al., 2012). Understanding nitrogen regulation mechanisms in these yeasts is practically important for optimizing the cultivation media and conditions.

Previous studies have shown that *K. phaffii* uses certain amino acids as complex sources of biogenic elements, providing cells with not only nitrogen but also carbon and energy (Sahu and Rangarajan, 2016b). Proline is a prime example of such an amino acid. Its presence in the medium significantly influences cell transcription, altering the expression of approximately 18.9 % of genes (Rumyantsev et al., 2021). This study highlights the response of *K. phaffii* cells specifically to nitrogen deficiency. Comparing these results with the data on *K. phaffii*'s response to proline and other amino acids will help to distinguish among the effects of these amino acids.

An important aspect of this study is that the response of *K. phaffii* yeast cells to nitrogen deficiency was examined in the presence of methanol as the sole carbon source. Our results show that nitrogen and methanol metabolism regulation in *K. phaffii* are interconnected, as nitrogen starvation causes the repression of some methanol metabolism genes. It is known that the regulatory systems of *S. cerevisiae* and *K. phaffii* are similar, involving orthologous proteins. This suggests that in *K. phaffii* the already existing regulatory system components evolved rather than new ones were formed to control methanol metabolism.

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