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Transcriptomic Analysis of Aromatic Amino Acid Biosynthesis Under Nitrogen Starvation in *Saccharomyces cerevisiae*

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Abstract

Yeast *Saccharomyces cerevisiae* has been a preferred organism for scientific research on many sensing and signaling processes. Its ability to adapt to changes in reproductive conditions, especially its fermentative properties have made this yeast species an industrially important species. Because of that *S. cerevisiae* has been genetically modified to create many kinds of beneficial chemicals from biomass. *S. cerevisiae* cells have the ability to detect the quality of nitrogen sources in their environment. And It has many regulatory mechanisms to utilize alternative nitrogen sources after the favored sources have been depleted. And proline is the least desired nitrogen source for many laboratory yeast strains. Usage of proline results in the slowest growth rates. The yeast *S. cerevisiae* can only get cellular nitrogen from tryptophan, tyrosine, or phenylalanine. The major products of this catabolism are tryptophol, phenylethanol, and tyrosol, which are elements of the mixture of alcohols known as fusel oil and this makes yeast *S. cerevisiae* important also in the production of seconder metabolites. Next Generation Sequencing allows deep analysis of gene expression. The effect of growth in media containing poor nitrogen source, proline, on the aromatic amino acid biosynthetic pathway was observed using NGS technologies in this work. According to the transcriptomic analysis results, we found that 14 genes are responsible for aromatic amino acid biosynthesis and 12 genes (*ARO1, ARO2, ARO4, ARO7, ARO8, ARO9, PHA2, TRP1, TRP2, TRP3, TRP4,* and *TYR1*) were down-regulated, while 2 genes (*ARO3* and *TRP1*) were up-regulated. In addition, *ARO1, ARO2, ARO4, ARO7, ARO9, PHA2, TRP1, TRP2, TRP3, TRP4,* and *TYR1* genes have showed 1.5-fold or more expression change, the fold difference of three genes (*ARO3*, *ARO8*, and *TRP5*) was not significantly important.

Keywords: Aromatic amino acids, Proline, *Saccharomyces cerevisiae*, Transcriptome

1. Introduction

Living organisms must be able to adapt to changes in their nutritional environment to survive, and as a result, they have evolved defenses to deal with the changing circumstances efficiently. Various important nutrient-signaling pathways allow yeast cells to perceive the nutrients in their environment and coordinate broad responses including cell growth and stress tolerance [1]. For scientific research on

nutrient-sensing and signaling processes, the yeast *Saccharomyces cerevisiae* has been a preferred organism.

S. cerevisiae cells have the ability to detect the quality of nitrogen sources in their environment, allowing them to use preferred nitrogen-containing substances over non-preferred ones or to express pathways for using alternative nitrogen sources after the favored sources have been depleted [2].

Preferred nitrogen sources, or nitrogen compounds, which are those that can be quickly converted into the primary amino acid precursors, ammonia, glutamate, and glutamine, activate the NCR (Nitrogen Catabolite Repression) pathway. Genes involved in using alternative and less preferable nitrogen sources, such as proline, urea, and allantoin, are repressed by NCR [3]. Nitrogen catabolite repression (NCR) is a mechanism which is responsible for the regulation of genes involved in poor nitrogen source utilization. It is known that *S. cerevisiae* yeast cells regulate the expression of nitrogen catabolite pathways with four regulator proteins called Gln3, Gat1, Dal80, and Deh1 [4, 5].

The TOR (Target of Rapamycin) pathway, collaborating with other signaling pathways, is a key mediator of signals derived from nutrients and organizes cell development. The TOR protein kinases, which were initially found in yeast, are the key players in this signaling cascade. It is thought that TOR is triggered by amino acid-derived signals and up and down regulates a variety of anabolic and catabolic activities, such as translation, transcription, ribosome biogenesis, actin deposition to regions of active cell development, protein degradation, mRNA destabilization, and autophagy [6]. TOR also directly regulates NCR by controlling Gln3 cytoplasmic retention via Ure2 [7]. Gln3 is a transcriptional activator and is involved in the positive regulation of NCR. In a nitrogen-limited environment, Gln3 migrates to the nucleus and the NCR-responsive genes are upregulated, whereas in a nitrogen-rich environment, Gln3 remains in the cytoplasm and the transcription of NCR-responsive genes is minimal. Nitrogen limitation and lack of glutamine induce Gln3p activation [8].

The quality of the nitrogen source provided during nutritional upshift is the only factor affecting TORC1 activity. Collectively, the level of TORC1 activation and the nitrogen quality are correlated. Preferred nitrogen sources, as opposed to poor nitrogen sources, generally promote and maintain TORC1 activity [9]. And proline is the least desired nitrogen source for many laboratory yeast strains. Usage of proline results in the slowest growth rates. However, yeast cells have evolved a regulatory circuit that allows them to utilize proline in the environment when preferred nitrogen sources are not available [10].

Aromatic substances are synthesized in *S. cerevisiae* through the aromatic amino acid biosynthetic pathway (AAP) [11]. This tightly controlled route is crucial to yeast metabolism and feeds multiple other pathways such as quinone, folate and Ehrlich pathways. The center of AAP is the shikimate pathway that consists of two branches: tryptophan, and tyrosine and phenylalanine [12]. The yeast *S. cerevisiae* can only get cellular nitrogen from tryptophan, tyrosine, or phenylalanine. The major products of this catabolism are tryptophol, phenylethanol, and tyrosol, which are elements of the mixture of alcohols known as fusel oil [13].

In order to obtain desired products from biomass, the yeast *S. cerevisiae* offers various benefits as a cell factory because of its durability and strong resistance to stress in fermentative processes. Because of that *S. cerevisiae* has been genetically modified to create many kinds of beneficial chemicals [14].

The purpose of this paper is to contribute previous data about aromatic amino acid biosynthetic pathways using NGS (Next Generation Sequencing) which allows deep analysis of gene expression. It is observed that the effects of growth in media containing poor nitrogen source, proline, on the aromatic amino acid biosynthetic pathway. According to the obtained results, *ARO7*, *ARO9*, *PHA2*, *TYR1*, and *TRP4* genes

were down-regulated in yeast cells transferred from the medium containing strong nitrogen, ammonium sulfate, to the medium containing poor nitrogen, proline, while only the *TRP1* gene was up-regulated.

2. Material and Methods

2.1. Yeast strain and growth condition

S. cerevisiae yeast strain (BY4741: *MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0*) was used in this study. The BY4741 strain is a standard haploid *S. cerevisiae* strain whose genome has been fully sequenced and is known to contain no mutations in genes related to the metabolic pathways [15]. The *S. cerevisiae* yeast strain BY4741 was obtained from the EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis) collection at the Institute of Microbiology, University of Frankfurt. *S. cerevisiae* yeast cells were grown overnight in YNBD (0.17% yeast nitrogen base (w/o amino acids and ammonium sulfate) + 0.5% ammonium sulfate + 20 mg/l histidine + 60 mg/l leucine + 20 mg/l methionine + 100 mg/l Uracil + 2% dextrose) medium at constant temperature (30°C) and shaking (120 rpm). The overnight culture was transferred to a fresh YNBD medium with an OD_{600} value of 0.2 and grown under the same conditions until the logarithmic phase (OD_{600}: 0.8-1.0). Yeast cells that reached the logarithmic phase were divided into two groups. After washing, yeast cells in the first group were transferred to fresh YNBD medium containing 0.5% ammonium sulfate and yeast cells in the other group were transferred to fresh YNBD medium containing 0.1% proline. Both cultures were incubated for 4 hours at a constant temperature (30 °C) with shaking (120 rpm). After incubation, yeast cells were precipitated and washed with sterile distilled water and used for total RNA isolation.

2.2 Total RNA isolation, cDNA synthesis and RNA sequencing

Total RNA isolation of yeast *S. cerevisiae* cells grown on different nitrogen sources was performed using the yeast specific RiboPure™ RNA Purification Kit, Yeast (Invitrogen, AM1926) RNA isolation kit as recommended by the manufacturer. Total RNA isolated from yeast cells was quantified using Qubit® RNA Assay kit. In addition, Agilent® RNA 6000 Pico Kit was used to determine RNA Integrity Number (RIN) values and RNA samples were stored in -80 °C deep freezer for further use. Dynabeads® mRNA DIRECT™ Micro Purification Kit (Catalog No: 61021, Invitrogen) was used to separate and enrich mRNA molecules from isolated total RNA. mRNA samples were separated from total RNA using the polyA binding method. All mRNA samples were equalized to 330 ng/µl and polyA RNAs were fragmented using the Ion Total RNA-Seq Kit v2 (Catalog No: 4475936, Ion Torrent) and the number of mRNAs obtained was quantified using the Qubit® RNA Assay kit. The mRNA samples of yeast cells grown in different nitrogen sources were sent to MedSanTek (Istanbul, Turkey) for cDNA library construction and RNA sequencing. RNA sequencing of yeast strains was performed using the Ion S5 XL (Thermo Fisher Scientific, Ion torrent, A27214) Next Generation Sequencer. After RNA sequencing, the result files with ".bam" extension were obtained for later analysis.

2.2 Data Analysis

The Galaxy platform is a free, publicly available web-based data analysis program that analyzes next-generation sequencing (such as RNA-Seq) data online. The ".bam" files sent by MedSanTek were analyzed using the Galaxy online analysis platform (https://usegalaxy.org). mRNA counts and differential expressed genes were carried out. For this purpose, IonXpressRNA_001_rawlib.basecaller.bam and

IonXpressRNA_002_rawlib.basecaller.bam files of yeast cells grown in ammonium sulfate, IonXpressRNA_003_rawlib.basecaller.bam and IonXpressRNA_004_rawlib.basecaller.bam files of yeast cells grown in proline were uploaded to Galaxy platform. The ".bam" files were converted to ".fastq" format using the "bedtools Convert from BAM to FastQ (Galaxy Version 2.27.1) tool within the Galaxy platform [16]. Before analyzing RNA sequence reads, read quality was examined using the FastQC High Throughput Sequence QC Report (v0.11.9) tool.

In order to perform RNA Seq analysis on the Galaxy platform, ".fastq" files were aligned using HISAT2 alignment tool (Galaxy Version 2.2.1+galaxy0) and the yeast (*Saccharomyces cerevisiae*, sacSer3) reference genome, and binary alignment map (BAM) files were obtained. The ``.bam" files were converted to sequence alignment map (SAM) format using Bam to Sam (Galaxy Version 2.0.1) on the Galaxy platform and transcript counts were carried out using the featureCounts tool (Galaxy Version 2.0.1+galaxy2) [17, 18, 19]. The differentially expressed genes were determined by using the DESeq2 (Differential Gene Expression Analysis Tool) tool on the galaxy platform. Log_2FC, p-value and standard deviation were calculated. Results with p-value less than 0.05 were considered significant [20]. The genes related to the aromatic amino acid biosynthesis in *S. cerevisiae* were downloaded from *Saccharomyces* Genome Database (SGD) and analyzed. Gene ontology analysis of differentially expressed genes was carried out by using ShinyGO 0.76.3 online tool. The expression levels of genes responsible for aromatic amino acid biosynthesis were determined using whole transcriptome data of yeast cells grown on different nitrogen sources.

3. Results

The expression levels of genes related to aromatic amino acid biosynthesis under nitrogen starvation were investigated in *S. cerevisiae* yeast cells. To investigate the effect of proline treatment on aromatic amino acid biosynthesis, transcriptome analysis in *S. cerevisiae* cells was carried out in nitrogen starvation and differentially expressed genes were determined (data not shown). According to the obtained results, 44% of the nitrogen metabolism-related genes were down-regulated, while 55% of those were up-regulated. In this study, the genes related to aromatic amino acid biosynthesis were also analyzed. The features of these genes were downloaded from *Saccharomyces* Genome Database (SGD) and were given in Table 1. It was defined that 14 genes are liable for aromatic amino acid biosynthesis, and *ARO1* and *ARO8* possess more than two processes.

In *S. cerevisiae* yeast cells, differentially expressed genes involved in aromatic amino acid metabolism after proline treatment were given Figure 1. Differentially expressed genes (DEGs) were detected using the criterion of |log2 (Fold Change) |> 1 and q-value 0.05 for a significant gene expression difference. In addition, according to the fold difference results of differentially expressed genes, genes with a change of 1.5 fold or more were considered to have a significant change.

As a result of transcriptome analysis of genes related to aromatic amino acid biosynthesis, it is showed that two genes (*ARO3* and *TRP1*) were up-regulated, while 12 genes (*ARO1, ARO2, ARO4, ARO7, ARO8, ARO9, PHA2, TRP1, TRP2, TRP3, TRP4,* and *TYR1*) were down-regulated. In addition, *ARO1, ARO2, ARO4, ARO7, ARO9, PHA2, TRP1, TRP2, TRP3, TRP4,* and *TYR1* genes have showed 1.5-fold or more expression change, the fold difference of three genes (*ARO3, ARO8,* and *TRP5*) was not significantly important.

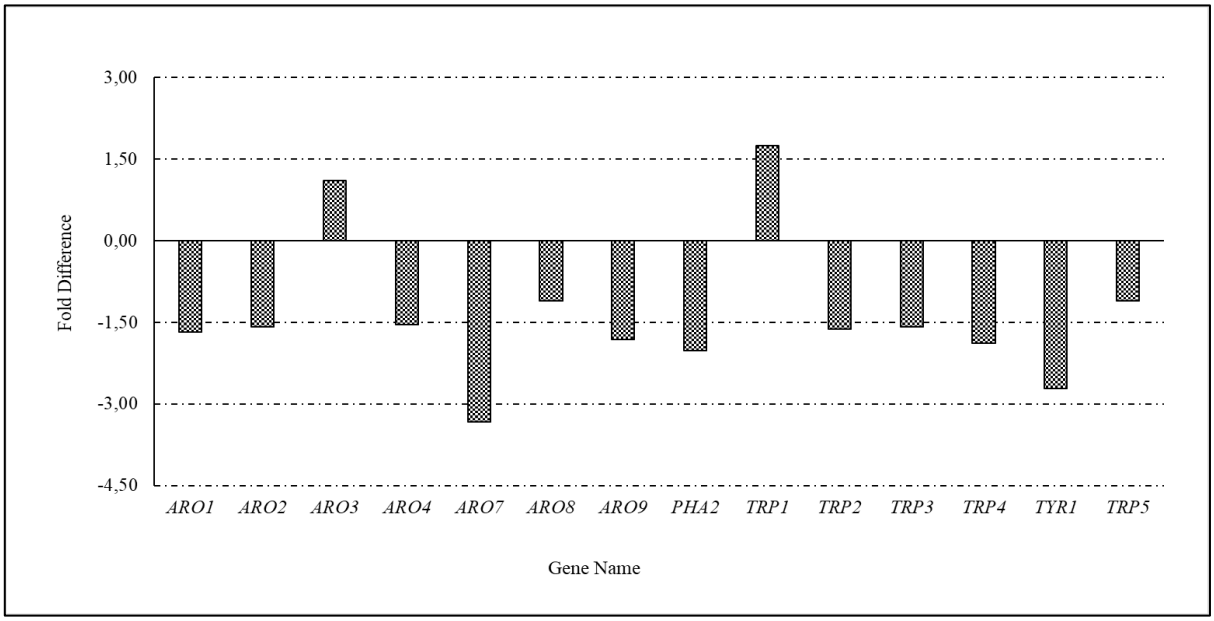
In this study, the gene ontology and pathway analysis were carried out by using ShinyGO 0.76.3 online bioinformatics tool. According to the analysis of GO enrichment for biological processes, all the aromatic

amino acid biosynthesis related genes were responsible for 15 different processes. As a result, it was found that all genes (*ARO1, ARO2, ARO3, ARO4, ARO7, ARO8, ARO9, PHA2, TRP1, TRP2, TRP3, TRP4, TRP5* and *TYR1*) control 6 metabolic processes included in the organic acid biosynthetic process, small molecule biosynthetic process, carboxylic acid metabolic process, oxoacid metabolic process, organonitrogen compound biosynthetic process, aromatic amino acid family metabolic process.

Table 1. The properties of genes related to aromatic amino acid biosynthesis in *S. cerevisiae*

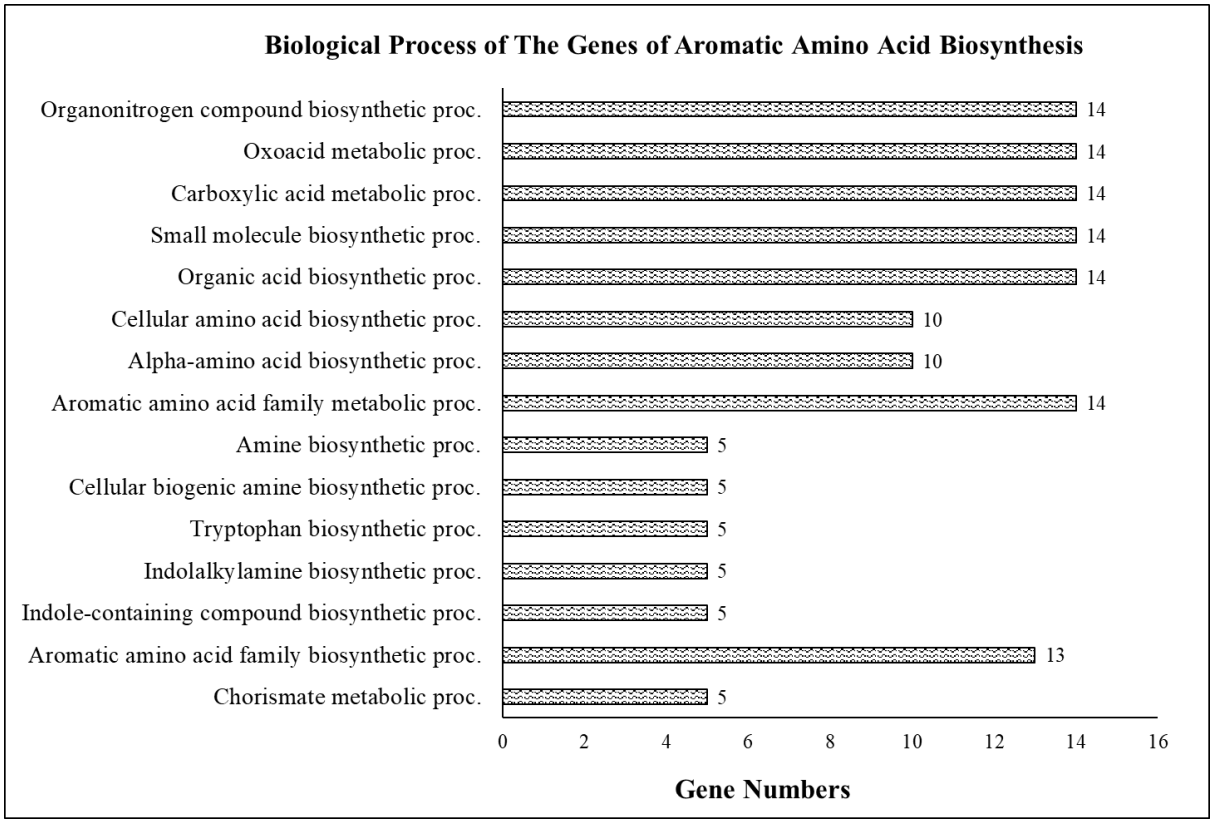
Gene Name	Gene ID	Reaction EC	Enzymatic Activity
ARO1	*YDR127W*	4.2.3.4	3-dehydroquinate synthase
		4.2.1.10	3-dehydroquinate dehydratase
		1.1.1.25	Shikimate dehydrogenase
		2.7.1.71	Shikimate kinase
		2.5.1.19	3-phosphoshikimate 1-carboxyvinyltransferase
ARO2	*YGL148W*	4.2.3.5	Chorismate synthase
ARO3	*YDR035W*	2.5.1.54	3-deoxy-D-arabine-heptulosonate-7-phosphate synthase
ARO4	*YBR249C*	2.5.1.54	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase
ARO7	*YPR060C*	5.4.99.5	Chorismate mutase
ARO8	*YGL202W*	2.6.1.5/2.6.1.27/2.6.1.57	Aromatic amino acid aminotransferase
		2.6.1.1/2.6.1.9/2.6.1.27/2.6.1.57	Aromatic amino acid aminotransferase
ARO9	*YHR137W*	2.6.1.58	Aromatic amino acid aminotransferase
PHA2	*YNL316C*	4.2.1.51	Prephenate dehydratase
TRP1	*YDR007W*	5.3.1.24	N-(5'-phosphoribosyl)-anthranilate isomerase
TRP2	*YER090W*	4.1.3.27	Anthranilate synthase
TRP3	*YKL211C*	4.1.3.27	Anthranilate synthase
		4.1.1.48	Indole-3-glycerol phosphate synthase
TRP4	*YDR354W*	2.4.2.18	Anthranilate phosphoribosyl transferase
TRP5	*YGL026C*	4.2.1.20	Tryptophan synthetase
TYR1	*YBR166C*	1.3.1.13	Prephenate dehydrogenase (NADP+)

Figure 1. Transcriptional changes of genes responsible for aromatic amino acid biosynthesis after proline treatment.



The aromatic amino acids biosynthetic process is controlled by 13 genes (*ARO1, ARO2, ARO3, ARO4, ARO7, ARO8, PHA2, TRP1, TRP2, TRP3, TRP4, TRP5* and *TYR1*) and alpha-amino acid biosynthetic process and cellular amino acid biosynthetic process are controlled by 10 genes (*ARO7, ARO8, ARO9, PHA2, TRP1, TRP2, TRP3, TRP4, TRP5* and *TYR1*). The obtained results were given in Figure 2.

Figure 2. GO analysis results of the aromatic amino acid biosynthesis related genes according to the biological process



The analysis of gene ontology for the molecular function was performed using ShyniGO 0.76.3 online analysis tool. According to the obtained results, the aromatic amino acid biosynthesis related genes were

responsible for 19 different processes. These results showed that 7 genes (*ARO1, ARO3, ARO4, ARO8, ARO9, TRP3,* and *TRP4*) were responsible for transferase activity, 6 genes (*ARO1, ARO2, TRP2, TRP*3, *TRP5*, and *PHA2*) were responsible for lyase activity. The obtained results were given in Figure 3.
KEGG (Kyoto Encyclopedia of Genes and Genomes) is a database to understand the cellular function and biological uses. This database is obtained from molecular knowledge, particularly comprehensive datasets constructed by genome sequencing and high-throughput analysis technologies [21]. In this study, KEGG pathway analysis of aromatic amino acid biosynthesis related genes was studied by using ShyniGO 0.76.3 online analysis tool. According to the KEGG analysis results, all genes (*ARO1*, *ARO2*, *ARO3*, *ARO4*, *ARO7*, ARO8, *ARO9*, *PHA2*, *TRP1*, *TRP2*, *TRP3*, *TRP4*, *TRP5*, and *TYR1*) responsible for aromatic amino acid biosynthesis were found to be involved in secondary metabolite biosynthesis (sce01100) and metabolic pathways (sce01100), and 13 genes (*ARO1*, *ARO2*, *ARO3*, *ARO4*, *ARO8*, *PHA2*, *TRP1*, *TRP2*, *TRP3*, *TRP4*, and *TRP5*) are responsible for amino acid biosynthesis pathways (sce01230). It was also found that 12 genes (*ARO1*, *ARO2*, *ARO3*, *ARO4*, *ARO8*, *ARO9*, *PHA2*, *TRP1*, *TRP2*, *TRP3*, *TRP4*, and *TRP5*) are responsible for phenylalanine, tyrosine, and tryptophan biosynthesis (sce01230) (Figure 4).

Figure 3. GO analysis results of the aromatic amino acid biosynthesis related genes according to the molecular function

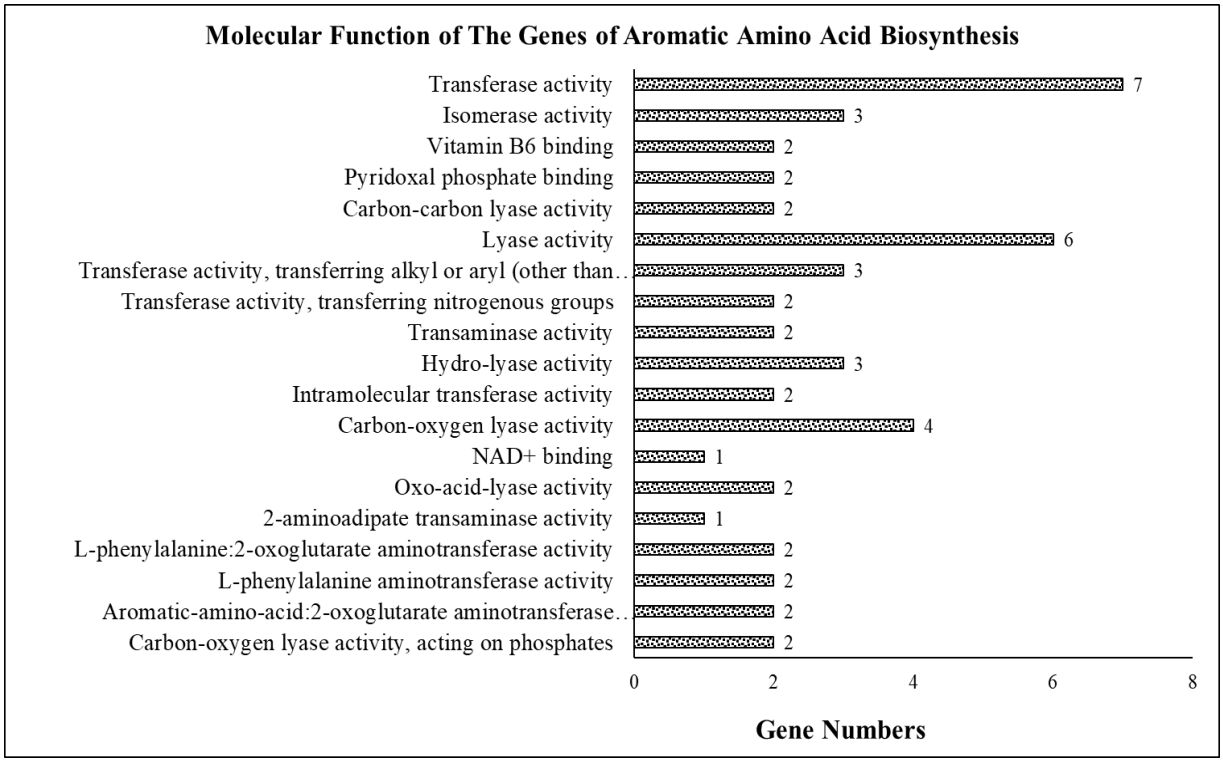
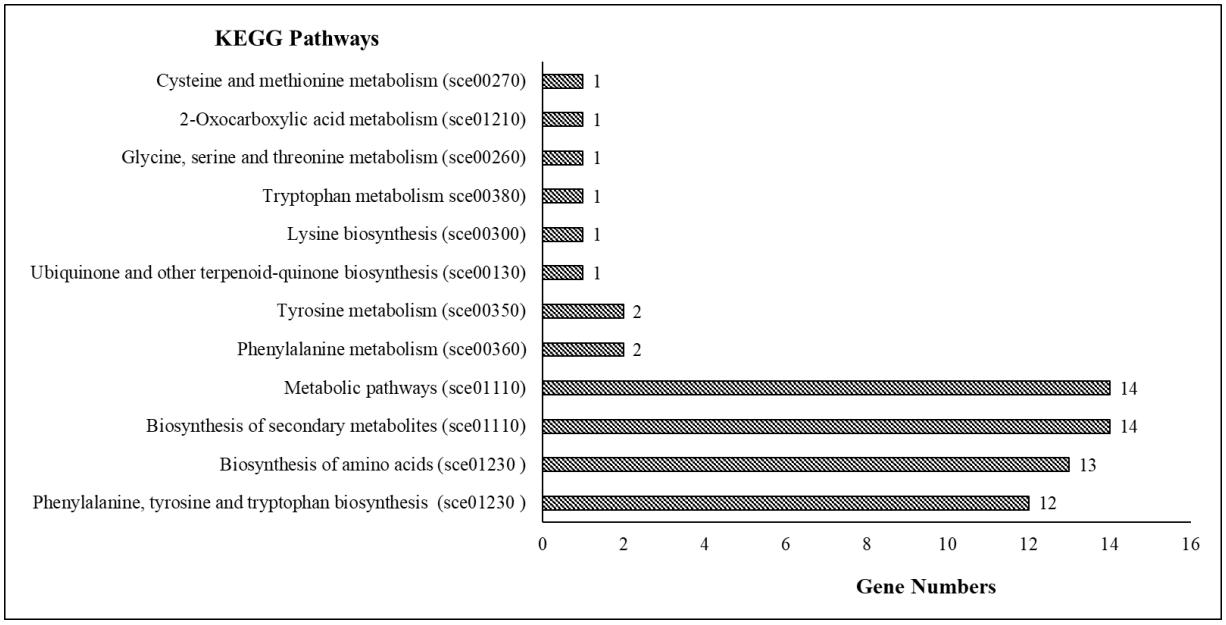


Figure 4. KEGG Pathway analysis of the aromatic amino acid metabolism related genes



Discussion

Transcriptional regulation is a more significant process which permits any organism responding to intracellular and extracellular signals. Thanks to the responses, organisms can regulate cellular activities [22]. A previous study showed that *S. cerevisiae* yeast cells encode 12 genes for 15 of the 17 reactions that are responsible for three aromatic amino acid biosynthesis [11]. In this study, encoding 14 genes were found, and these genes are responsible for 16 enzymatic activities (Table 1). According to the results, the aromatic amino acids biosynthesis related genes were generally down-regulated, except *ARO3* and *TRP1*. It is known that Nrg1p and Nrg2p are DNA-binding proteins which are responsible for the transcriptional repression of various genes that include DNA binding sites [23]. According to the *in-silico* promotor analysis via Yeastract Database, it is found that *ARO1, ARO2, ARO4, ARO7, ARO9, PHA2, TRP3,* and *TRP4* genes contain Nrg1p and Nrg2p binding sites (data not shown). Therefore, these genes may have been down-regulated. Iraqui et al. (1999) revealed that *ARO9* gene expression level was not affected directly by general nitrogen transcriptional factors, while *ARO9* gene expression was down-regulated after proline treatment in the current study [13]. According to the *in-silico* promotor analysis, it is also found that the promotor region of the *ARO9* gene possesses an Nrg2p binding site. In yeast, the transcription factor, Cbf1p, regulates several genes related to amino acid biosynthesis, respiration, and lipid biogenesis [24]. Cbf1p is a chromatin remodelling enzyme and is responsible for changing nucleosome position. Thus, other factors can bind to promotor upstream elements [25]. In this study, It is found that the gene promotor sequence of *TPR1* which is up-regulated has a Cbf1p binding site, and this binding site is inside the nucleosome structure. In the poor nitrogen source, the nucleosome position in the promotor region of *TRP1* may have been changed and therefore, *TRP1* gene expression level may have been increased.

4. Conflict of Interest

The authors declare no conflict of interest.

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