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Insights into the roles of exogenous phenylalanine and tyrosine in improving rapamycin production of *Streptomyces rapamycinicus* with transcriptome analysis

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Abstract

Rapamycin is an important natural macrolide antibiotic with antifungal, immunosuppressive and antitumor activities produced by *Streptomyces rapamycinicus*. However, their prospective applications are limited by low fermentation units. In this study, we found that the exogenous aromatic amino acids phenylalanine and tyrosine could effectively increase the yield of rapamycin in industrial microbial fermentation. To gain insight into the mechanism of rapamycin overproduction, comparative transcriptomic profiling was performed between media with and without phenylalanine and tyrosine addition. The results showed that the addition of phenylalanine and tyrosine upregulated the transcription levels of genes involved in rapamycin biosynthesis, precursor production, and transporters. In addition, the transcription levels of many carbohydrate metabolism-related genes were down-regulated, leading to a decrease in growth, suggesting that balancing cell growth and rapamycin biosynthesis may be important to promote efficient biosynthesis of rapamycin in *Streptomyces rapamycinicus*. These results provide a basis for understanding physiological roles of phenylalanine and tyrosine, and a new way to increase rapamycin production in *Streptomyces* cultures.

Keywords Rapamycin, *Streptomyces*, Phenylalanine and tyrosine, Transcriptome

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Introduction

Rapamycin, also known as sirolimus, is produced by *Streptomyces rapamycinicus* (formerly, *Streptomyces hygroscopicus*) and has antitumor, immunosuppressive and antifungal activities. It has attracted increasing attention for its clinical treatment and has a high market demand. However, since its discovery, the fermentation unit has been at a low level [1–4]. To meet the high demand for this compound, many efforts have been made to improve rapamycin yields through traditional random mutagenesis, optimization of the fermentation process, and engineering of regulatory genes, but with little effect [5–8].

The addition of precursors was an effective strategy to improve antibiotic production. For example, the exogenous feeding of precursors in the culture media has been reported to improve the yield of FK506 [9–11]. Exogenous proline and glutamate, precursors of streptolydigin, can significantly improve the streptolydigin production [12]. Exogenous shikimic acid, oils and lysine promoted ascomycin production [13]. These findings indicate the importance of enhancing precursor supply for increasing antibiotic production. As a secondary metabolite, rapamycin possesses a complex biosynthetic pathway. (4R,5R)-4,5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC), malonyl-CoA, and methylmalonyl-CoA are the precursors for rapamycin biosynthesis [14] (Fig. 1A). The shikimate pathway plays a crucial role in the biosynthesis of chorismate and the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). Furthermore, chorismate provide the starting unit DHCHCin rapamycin synthesis [15]. Chorismate is an intermediate metabolite of many pathways, and the addition of amino acid end products can feedback inhibit their biosynthesis, thus allowing more metabolic flux to flow to other metabolic pathways, such as DHCHC synthesis. Therefore, we speculated that the addition of aromatic amino acids might have a beneficial effect on rapamycin synthesis. On the other hand, high intracellular abundance of aromatic amino acids may influence other metabolic reactions that affect the synthesis of secondary metabolites.

In our work, we found that exogenous feeding of phenylalanine and tyrosine was very effective in increasing the rapamycin production in the high-producing *S. rapamycinicus* strain obtained by ARTP and NTG

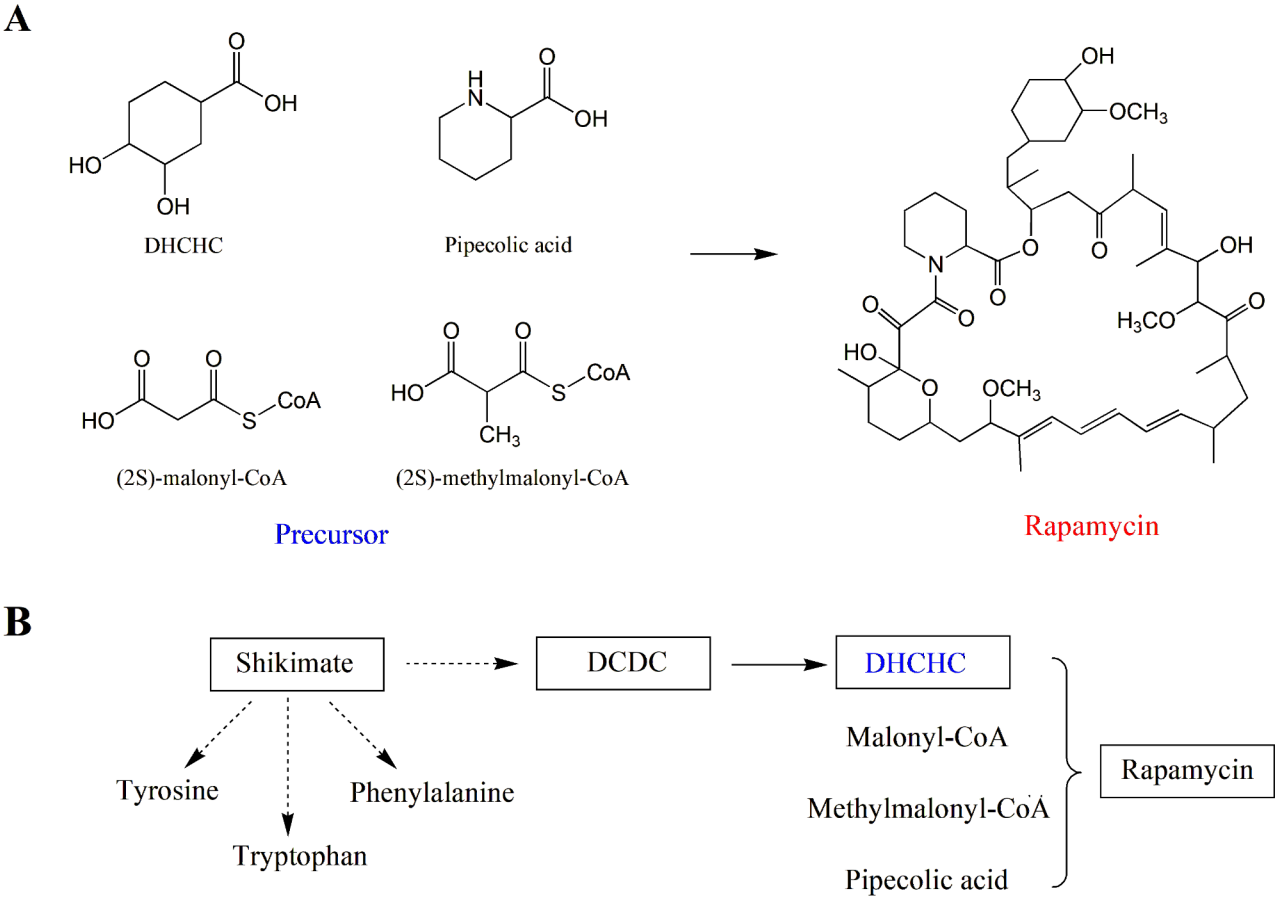

Fig. 1 **(A)** Precursors for the synthesis of rapamycin. **(B)** DHCHC is a derivative of shikimic acid

mutagenesis. We also reported the mechanism of regulation of rapamycin biosynthesis by addition of phenylalanine and tyrosine at the transcription level. This study may help us understand the relationship between phenylalanine, tyrosine and rapamycin biosynthesis, and provide a new strategy to improve rapamycin production.

Materials and methods

Strain and growth conditions

S. rapamycinicus S8-19 was cultivated at 28 °C on solid medium (0.4% glucose, 0.4% yeast extract, 1% malt extract, 2% agar, pH 7.0). For shaking flask fermentation, liquid medium (2% glucose, 1.5% soybean cake, 0.2% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.2% NaCl, 0.2% KH_2PO_4, 0.2% $CaCO_3$, pH 7.0) was used for seed cultivation. Liquid medium (4% dextrin, 2% glucose, 2.3% soybean cake, 0.5% yeast extract, 0.5% NaCl, 0.5% KH_2PO_4, 0.001% $FeSO_4{\cdot}7H_2O$, 0.0001% $CoCl_2{\cdot}6H_2O$, pH7.0) was used for fermentation culture.

RNA extraction and RNA-seq analysis

After 96 h of culture, *S. rapamycinicus* S8-19 was sampled separately from the pure culture (CK) and culture supplemented with 0.1% (W/V) phenylalanine+0.1%(W/V) tyrosine (PT). The broths were then sent to Meige Biotechnology Co. LTD. for transcriptome sequencing (using Illumina sequencing system). The transcription levels of the genes in the RNA-seq data were assessed using the FPKM (Fragments Per Kilo bases per Million fragments) method, which was calculated by using the DESeq2 software. Genes between two groups with FDR≤0.05 and $|\log_2$ (fold change)$| \geq 1$ were identified as differentially expressed genes (DEGs). DEGs were functionally classified by gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The GO terms and KEGG pathway with FDR≤0.05 were screened as significant enrichment results.

Measurement of rapamycin and packed mycelium volume (PMV)

The concentration of rapamycin was measured by HPLC as follows: 1 mL of fermentation broth was mixed with 1 mL of 95% ethanol for 60 min. Then, the mixture was centrifuged at 12,000 rpm for 1 min, and the supernatant was filtrated and subjected to HPLC analysis. HPLC was performed on Agilent series 1260 (Agilent Technologies, USA) with a C18 column (MN Nucleosil, 4.6×150 mm, 10 μm) at 50 ℃. The mobile phase was 70% acetonitrile at a flow rate of 1 mL/min, and the detection wavelength was 277 nm. The concentrations were calculated according to the standard curve of rapamycin.

The packed mycelium volume (PMV) was used to estimate the growth of *S. rapamycinicus* S8-19 as fermentation media contain insoluble components. The collected culture broths (5 mL each) were centrifuged at 4, 000 rpm for 20 min. The PMV was calculated as a percentage (%) by dividing PMV by the sample volume (5 mL). We also measured the amount of nucleic acid in the samples to get an indirect indication of bacterial growth. Total DNA was extracted using the Wizard® Genomic DNA Purification Kit, and DNA concertation was determined by absorbance measurements using the NanoDrop spectrophotometer (Thermo Scientific).

Analysis of residual glucose concentration

The concentration of residual sugar was determined using glucose determination kit (Boxbio, China) according to the procedure. 1 mL of fermentation broth was collected and centrifuged. After moderate dilution and sonication, the supernatant was used for residual sugar determination. The concentration of residual sugar was calculated by plotting the absorbance standard curve at 505 nm, D-sugar was used as standard.

Results

Exogenous addition of aromatic amino acid promotes rapamycin production

Chorismate produced by the shikimate pathway is not only the precursor of aromatic amino acids (phenylalanine, tyrosine, and tryptophan), but also the precursor of DHCHC for the synthesis of rapamycin (Fig. 1B). Here to test whether exogenous aromatic amino acid could help produce more DHCHC for rapamycin biosynthesis, we added different of aromatic amino acids to the fermentation media of *S. rapamycinicus* and measured the rapamycin titers at different times during the 7 days of fermentation by HPLC. The results showed that supplementation with any aromatic amino acid was beneficial for rapamycin production, and the addition of 1% phenylalanine resulted in the highest titer of 534.7 mg/L in all samples, which was 29.9% higher than the control. Addition of 1% tyrosine resulted in a titer of 417.7 mg/L, 11.5% higher than the control, and addition of 1% tryptophan resulted in a titer of 424.3 mg/L, 13% higher than the control (Fig. 2A).

We then combined the different aromatic amino acids (phenylalanine+tyrosine, phenylalanine+tryptophan, and phenylalanine+tyrosine+tryptophan), and found that the phenylalanine+tyrosine group showed the highest yield of 583.7 mg/L, which was 50.5% higher than the control (Fig. 2B). The above results showed that the addition of aromatic amino acids could improve the rapamycin production, and the group with phenylalanine and tyrosine showed the best effect. The results of a comparison of fermentation characteristics between with and without the addition of phenylalanine and tyrosine are shown in Fig. 2C-E and Fig. S1. We found that there

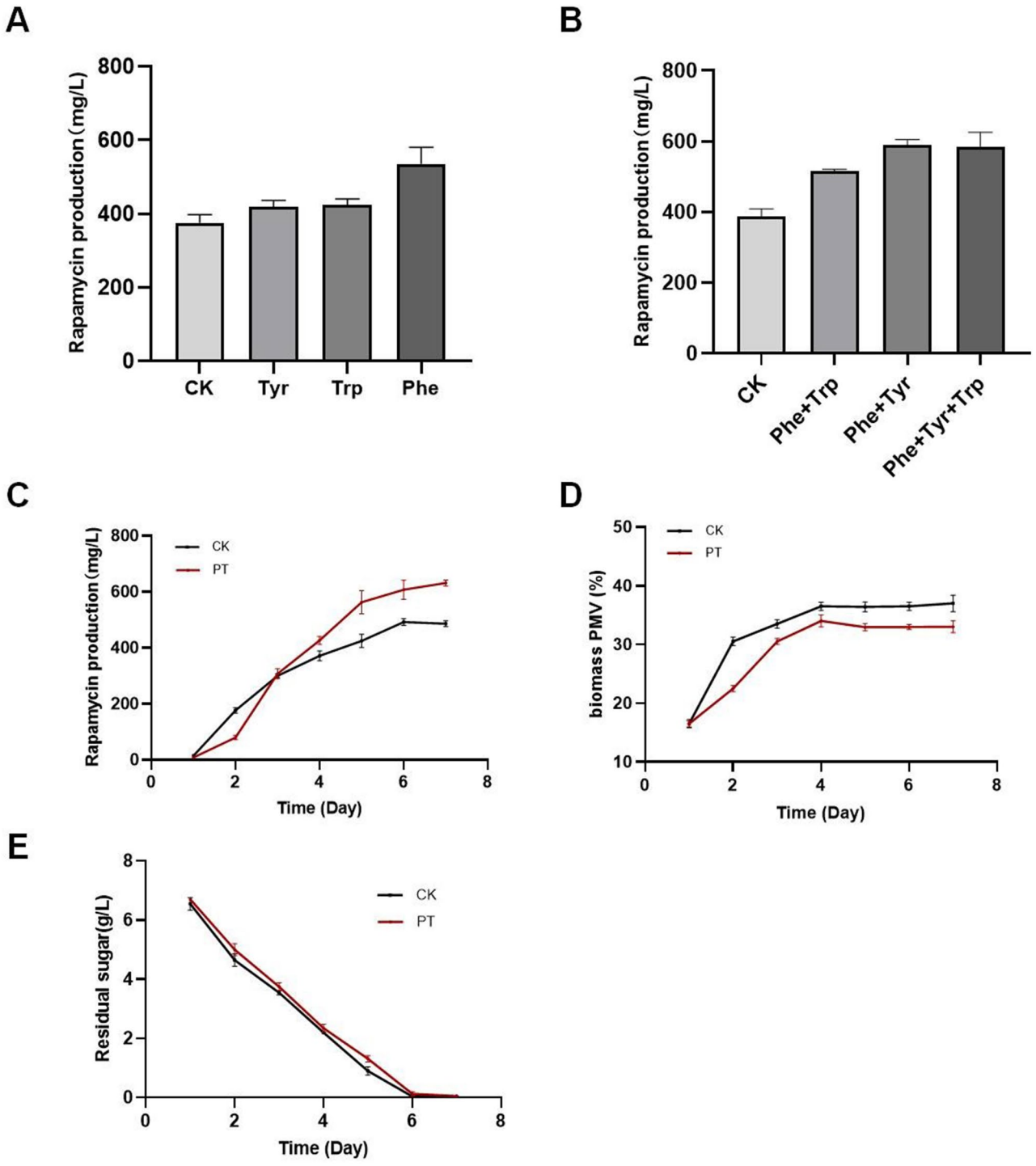

Fig. 2 Effect of aromatic amino acid addition on rapamycin production. **(A)** Comparison of different aromatic amino acids addition (phenylalanine, tryptophan and tyrosine) on rapamycin production in *S. rapamycinicus* S8-19. **(B)** Comparison of different aromatic amino acids combination on rapamycin production. Phenylalanine and tyrosine effect on (**C**) rapamycin production, (**D**) the growth curves and (**E**) residual sugar

was no significant difference in the total sugar consumption between the two groups, but the growth rate of the bacteria and the production of rapamycin showed apparent differences. In the control group (CK), the strain grew faster than that in the phenylalanine and tyrosine addition group (PT), with a maximum biomass of 37% compared to 34% in the PT group. However, the maximum yield of rapamycin in the PT group was increased to 632 mg/L under phenylalanine and tyrosine addition, compared to 392 mg/L in CK group.

Transcriptional response of *Streptomyces* to phenylalanine and tyrosine

Exogenous amino acids not only play important roles in the synthesis of related precursors, but may also act as signaling molecules to regulate expression of many important genes in metabolic pathways. To elucidate the molecular mechanism underlying the enhancement of rapamycin production in *S. rapamycinicus* S8-19 by the addition of phenylalanine and tyrosine, RNA-seq was used to analyze the transcription profiles with or without the addition of phenylalanine and tyrosine. We sampled the culture at 96 h of fermentation for RNA-seq analysis. The RNA-Seq read filtering statistics are shown in Table S1. The quality filtered reads obtained were further analyzed. The results showed that the transcription levels of 3158 genes were significantly affected by the addition of phenylalanine and tyrosine to fermentation medium (Fig. 3). Among the DEGs, the expression of 1988 transcripts was up-regulated (shown in red), while the expression of 1170 transcripts was down-regulated (shown in green) after fermentation with the addition of phenylalanine and tyrosine.

To elucidate the biological function and overall distribution of all.

DEGs regulated by phenylalanine and tyrosine, global functional analyses, gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway were performed with the clusterProfiler software. A total of 46 GO terms including 20 molecular functions, 6 cellular components, and 20 biological processes were enriched in the DEGs (Fig. 4A). By KEGG pathway analysis, it was found that DEGs mainly occurred in many metabolisms, such as carbon metabolism, amino acyl-tRNA biosynthesis, type I polyketide structures, glycine, serine and threonine metabolism, valine, leucine and isoleucine biosynthesis. In addition, we found that phenylalanine, tyrosine and tryptophan biosynthesis was also significantly enriched (Fig. 4B). All the data suggest that phenylalanine and tyrosine not only affect the biosynthesis of phenylalanine, tyrosine and tryptophan, but also globally alter the transcription of genes that regulate the biosynthesis of metabolites in *S. rapamycinicus.*

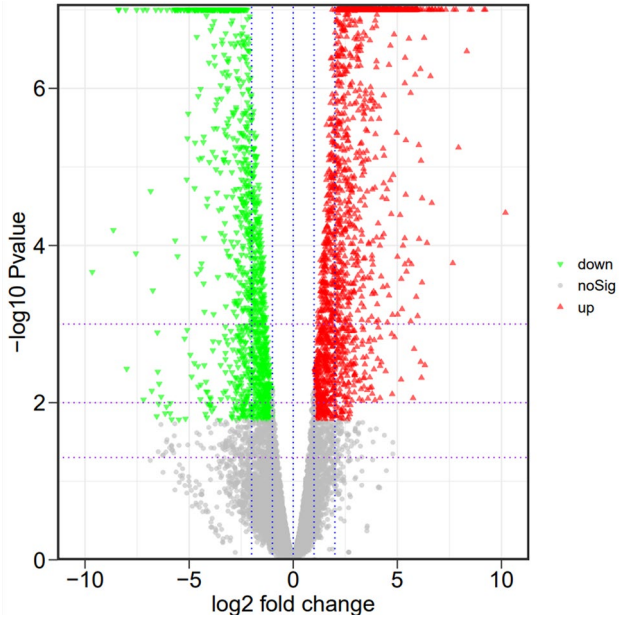

Fig. 3 Volcano plots representing up-regulated (red) and down-regulated (blue) genes in the media with (PT) and without (CK) phenylalanine and tyrosine

Effect of phenylalanine and tyrosine on expression of rapamycin gene cluster

The rapamycin biosynthetic gene cluster from *S. rapamycinicus* NRRL5491 was identified in 1995 [16]. The rapamycin gene cluster consists of three multifunctional PKS (polyketide synthase) enzymes (RapA, RapB and RapC) and one NRPS (nonribosomal peptide synthetase) enzyme (RapP) coding genes [17, 18]. In addition, *rapI*, *rapJ*, *rapM*, *rapN*, *rapO* and *rapQ* encode post-PKS tailing enzymes. *rapJ* and *rapN* encode two cytochrome P-450 monooxygenases (P450s), *rapO* encodes a homologous protein involved in the production of associated ferredoxin (Fd). *rapI*, *rapM* and *rapQ* encode three potential SAM-dependent O-methyltransferases (MTases). *orfX*, *orfW*, *orfV* may encode the ABC transporters. In addition, OrfR, OrfS, OrfH, OrfG and OrfY act as regulatory proteins. As expected, the transcriptomic data showed that the expression of most rapamycin biosynthesis genes was up-regulated in the phenylalanine and tyrosine addition group (PT) compared to the control (CK) (Fig. 5, Table S2). PKS and NRPS structural genes, pathway-specific regulatory genes and genes encoding post-PKS tailing enzymes, included in the rapamycin gene cluster, were transcriptional up-regulated. Among them, the transcription level of the NRPS gene *rapP* was up-regulated 6.3-fold. The transcription levels of PKS-related genes *rapA*, *rapB* and *rapC* were up-regulated 4.5-fold, 3.7-fold and 5.5-fold, respectively. The transcription levels of other structural genes in the gene cluster, such as *rapQ* and *rapK*, were also up-regulated. In addition, the regulatory gene *orfH* was up-regulated 2.8-fold. *orfF*, a gene associated with transport function, was up-regulated 3.6-fold, and *orfE*, a gene with unknown function was also up-regulated 4.4-fold. OrfY plays an important role as a negative regulator in rapamycin biosynthesis [19], and our results showed that the expression of *orfY* was down-regulated about 2.3-fold. Overall, these results showed that the exogenous phenylalanine and tyrosine has a significant enhancement on the expression of rapamycin gene cluster.

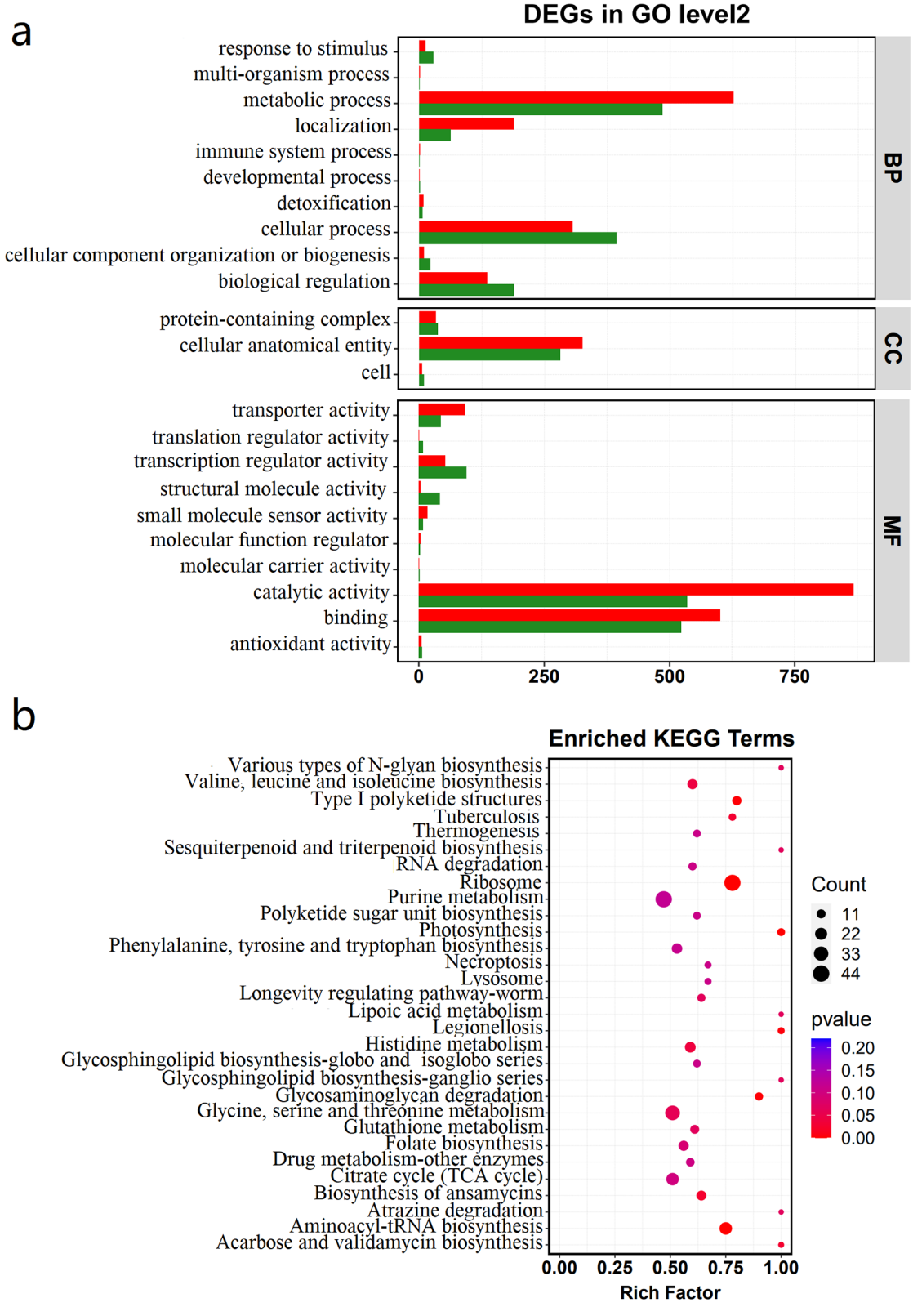

Fig. 4 GO and KEGG pathway analyses by RNA-Seq of *S. rapamycinicus* S8-19 in the media with (PT) and without (CK) phenylalanine and tyrosine

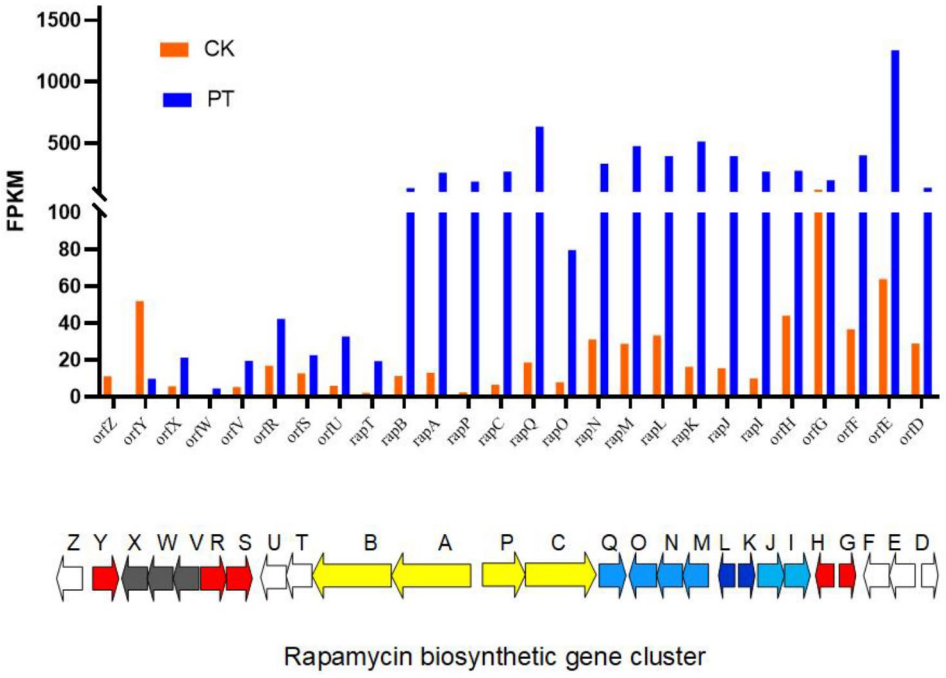

Fig. 5 FPKM values of DEGs situated in rapamycin BGC of *S. rapamycinicus* S8-19 in the media with (PT) and without (CK) phenylalanine and tyrosine

Phenylalanine and tyrosine modulate the carbohydrate metabolism and shikimate metabolic pathway

The central carbon metabolism plays a vital role in providing precursors, energy and co-factors for rapamycin production. Phenylalanine and tyrosine are intermediate metabolites related to shikimate metabolic pathway, which generates the starting unit of DHCHC for rapamycin synthesis. Chorismate is catalyzed by RapK to synthesize DCDC, which was catalyzed to produce DHCHC as rapamycin initiating unit. As shown in Fig. 6A, *rapK*, the related gene of the DHCHC biosynthetic pathway was transcriptionally up-regulated nearly 5-fold in the PT group, resulting in more conversion of chorismate to DCDC. Genes related to the competitive pathway of tryptophan biosynthesis, such as *trpA*, *trpB*, *trpC*, *trpD* and *priA* were down-regulated 1.4-3.1-fold in the PT group. In addition to help generate more DCDC for rapamycin synthesis, high intracellular levels of phenylalanine and tyrosine may also influence other metabolic reactions. Therefore, genes associated with carbohydrate metabolism and CoA precursor reservoir for rapamycin biosynthesis were also further in-depth analyzed. As shown in Fig. 6B, the results showed that the expression of DEGs related to the biosynthesis of malonyl-CoA, methylmalonyl-CoA, and L-pipecolic acid was also significantly increased, implying the accumulation of rapamycin precursors. The gene *pccB*, encoding the enzyme that catalyzes the conversion of propanol-CoA to (S)-methylmonoacyl-CoA, was transcriptionally up-regulated 3.1-fold. The genes *mcmA2* and *epi*, encoding the enzymes that catalyze succinyl-CoA to (R)-methylmonyl-CoA and (R)-methylmonyl-CoA to (S)-methylmonyl-CoA, were transcriptionally up-regulated 4.5-fold and 3.1-fold, respectively.

In addition, the gene *accA3*, encoding the enzyme that catalyzes acetyl-CoA to malonyl-CoA, was transcriptionally up-regulated 3.5-fold in the PT group compared to the control. Nevertheless, it is worth noting that the transcription level of many genes involved in glycolysis (EMP), citric acid cycle (TCA) and pentose phosphate (PPP) pathways was down-regulated (Fig. 6). The pathway genes from glucose to acetyl-CoA were significantly down-regulated at four nodes, where the transcription levels of *pfk*, *pgk*, *eno* and *aceF* were significantly down-regulated about 1.6-fold, 1.7-fold, 1.2-fold and 2.5-fold, respectively, compared to the control group. The transcription levels of genes involved in the PPP, such as G6PD gene, *gpl*, PGD gene, *rpiB*, *rpe*, *tkt*, and *tal*, were all down-regulated.

Acetyl-CoA can be synthesized from glycolytic pathway, acetate, and fatty acid in *Streptomyces*. In the PT group, we found a decrease in acetyl-CoA production from glycolytic pathway and a large increase in acetyl-CoA production by acetate and fatty acid degradation pathways. The acetate pathway related aldehyde dehydrogenase (ALDH) and acetaldehyde dehydrogenase (MhpF) encoding genes were transcriptionally up-regulated by 4.1-fold and 5.2-fold, respectively. In addition, acetyl-CoA can be produced by β-oxidation of fatty acids with abundant NADH and FADH2, and we found that the

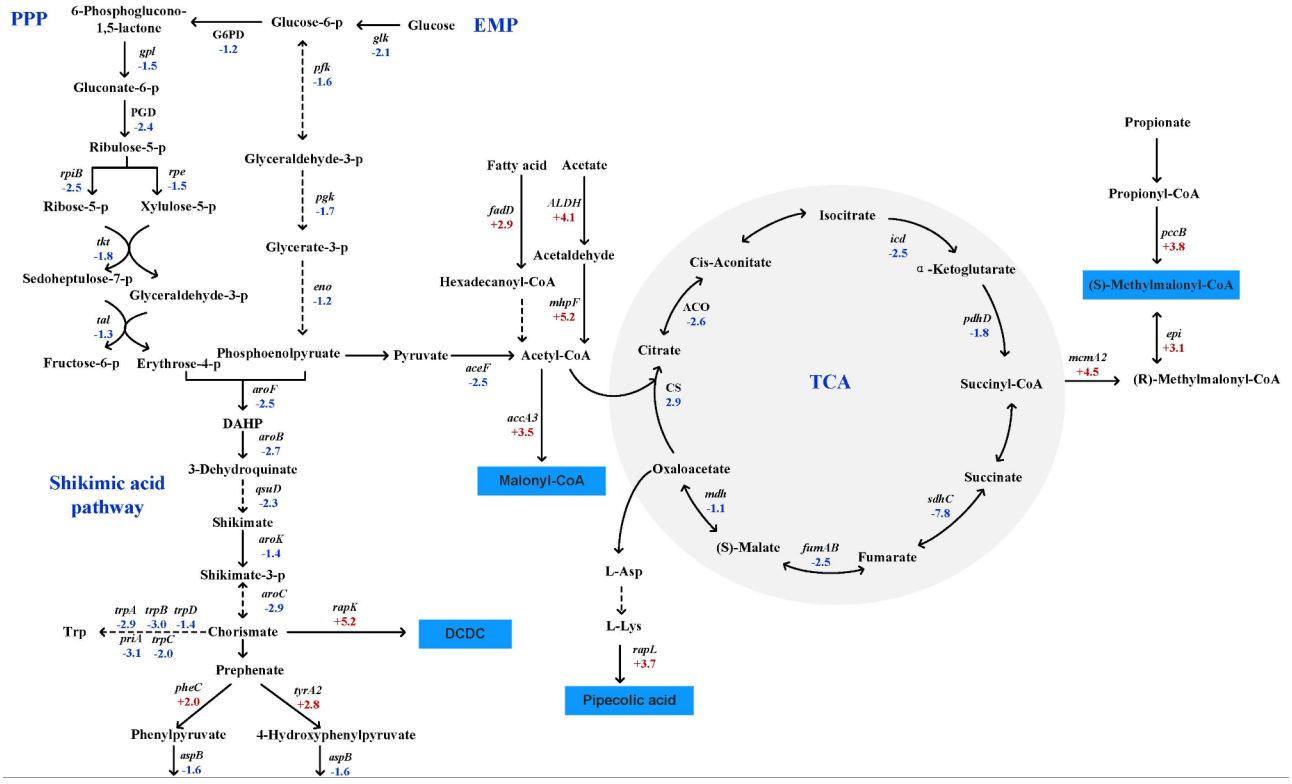

Fig. 6 Effect of phenylalanine and tyrosine on carbon metabolism in *S. rapamycinicus* S8-19

transcription levels of *fadD*, a key gene in this pathway, was up-regulated 2.9-fold. The above results demonstrated the efficient supply of acetyl-CoA from acetate and fatty acid in the PT group.

Overall, in this study, the addition of phenylalanine and tyrosine decreased the metabolism of the TCA, PPP and EMP pathways, but greatly increased the metabolism of other precursor biosynthetic pathways, which may account for the reduced biomass resulting from phenylalanine and tyrosine addition group.

Phenylalanine and tyrosine altered expression of transport genes

Transporters are the essential channels for the import of precursors and the export of antibiotics. The ABC superfamily is a well-studied class of transporter in *Streptomyces*. In the comparison of CK and PT groups, it was found that 49 genes encoding components for ABC transport systems were transcriptionally up-regulated (Fig. 7, Table S3). By the KEGG pathway analysis, the most significantly enriched ABC transport systems for transcription upregulated were sugar transporters, phosphate and amino acid transporters. The branched-chain amino acid transporter-related genes *livK*, *livH*, *livM*, *livG* and *livF* were the most up-regulated (5.8-fold, 5.6-fold, 5.8-fold, 6.8-fold and 4.4-fold, respectively). Glutamate is an amino donor for other amino acids such as tyrosine. The glutamate transport system genes, *gluA*, *gluB*, *gluC* and *gluD* were transcriptionally up-regulated 5.0-fold, 5.6-fold, 3.5-fold and 3.7-fold, respectively. In the meantime, the transcription levels of a serious of mineral and organic ion transport system genes were also up-regulated, such as phosphate transporter genes *pstA*, *pstB*, *pstC* and *pstS*, and molybdate transporter genes, *modA*, *modB* and *modC* (Fig. 7, Table S3).

Discussion

In this study, we reported that the addition of phenylalanine and tyrosine could significantly increase the yield of rapamycin. Transcriptome analysis could reveal the global changes in transcription levels, and has been used to explore the mechanisms of high-yielding of various natural products [19–21]. In addition, transcriptome analysis has been applied to identify potential targets for strain engineering [22–24]. To further understand the molecular mechanisms of rapamycin overproduction, RNA-seq analysis of transcriptional changes was performed in phenylalanine and tyrosine adding group (PT) and the control group (CK). It was found that the transcription level of rapamycin biosynthetic genes was significantly up-regulated under phenylalanine and tyrosine treatment. In addition, it was noteworthy that phenylalanine and tyrosine treatment could also up-regulate the gene expression of ABC transporters related to sugar,

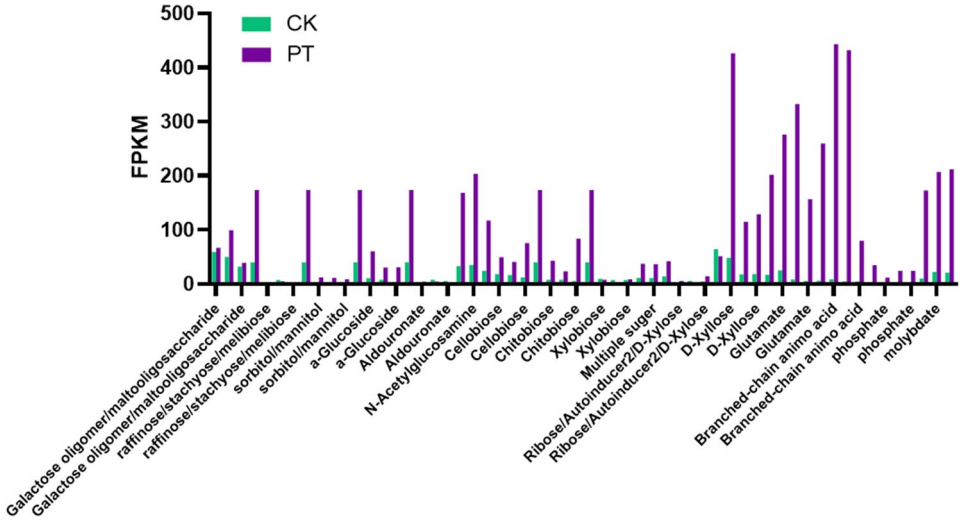

Fig. 7 Effect of phenylalanine and tyrosine on transcriptional expression of ABC transporters in *S. rapamycinicus* S8-19

phosphate, et al., which suggested that phenylalanine and tyrosine could be important ABC transporter regulators for nutrient absorption. In addition, genes related to the synthesis of rapamycin precursors, including methylmonyl-CoA, DCDC, malonyl-CoA, and L-Pipecolic acid were strongly transcriptional up-regulated.

Central carbon metabolism, including glycolysis, tricarboxylic acid cycle and the pentose phosphate pathway, is a major source of energy for cell growth, and provides precursors for the efficient production of secondary metabolites. Thus, down-regulation of the central carbon metabolism often leads to serious growth defects. On the other hand, the down-regulation of central carbon metabolism implies a redirected metabolic flux from cell biomass towards target compound production. In 2024, Wang et al. found that the enhancement of astaxanthin was due to the metabolic flux from cell biomass to astaxanthin biosynthesis by down-regulating the central carbon metabolism [24]. Interestingly, a negative correlation between cell growth and rapamycin titer was also observed in the phenylalanine and tyrosine addition group. Our results suggest that increasing rapamycin production may maintain an optimal balance between cellular biomass and the secondary metabolic synthesis.

At present, the low yield of rapamycin remains a major challenge to its widespread industrialization and commercial prospects. To address this issue, many strategies have been used to increase the production of rapamycin. For example, the yield of rapamycin has been enhanced by random mutagenesis, such as UV and NTG mutagenesis [25, 26]. In addition, fermentation conditions have been optimized using methods such as taguchi orthogonal array approach [27, 28]. Moreover, metabolic engineering and synthetic biology are now available for rational strain improvement based on metabolic and regulatory networks [29, 30]. Based on a genome-scale metabolic model (GEM), Dang et al. identified the metabolic engineering targets *pfk*, *dahP* and *rapK*. By strategically manipulating these targets, they successfully enhanced the production of rapamycin [30]. However, due to the production of secondary metabolite is complex regulated at multiple levels, from transcriptional to translational and post-translational modification, it is difficult to simulate this part of metabolism only using GEM models, which do not take these levels of regulation into account. Therefore, omics analysis such as genomics, transcriptomics and metabolomics are necessary to help investigate the biosynthesis of secondary metabolites and dissect the mechanism of antibiotic overproduction. These analyses can uncover potential clues for further strain genetic modifications. Jo et al. used comparative genomic analysis to show that a rapamycin-overproducing strain of *S.rapamycinicus* had a large deletions at both ends of its genome and a duplicated region covering the rapamycin biosynthetic gene cluster (BGC) [25]. Compared to genomic analysis, transcriptomic analysis provides a comprehensive understanding of the transcriptional landscape of an antibiotic-producing strain during fermentation. In the future, we can integrate the analysis of transcriptomic data with other analyses of omics data such as genomic and metabolic data, to better provide complementary information for the identification genes that affect the production of secondary metabolites and to facilitate the rational strain engineering work.

In conclusion, phenylalanine and tyrosine could promote rapamycin biosynthesis not only by improving the expression of rapamycin biosynthetic genes, but also of precursor and transporter related genes. These increases corresponded to decreases in the transcription levels of

genes related to central carbon metabolism. In addition, these results suggest that fine-tuning cell growth and biosynthesis is important for efficient bioproduction. These findings provide a basis for understanding physiological roles of phenylalanine and tyrosine and a new avenue for increasing rapamycin production.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02632-6.

Supplementary Material 1

Author contributions

Dongmei Xu: Data curation, Formal analysis, Methodology, Conceptualization, Writing & editing. Yaoyao Wang, Hongzhen Li and Libin Chai: Data curation, Formal analysis, Methodology. Li Feng, Bing Wang and Fengzhi Ren: Data curation, Formal analysis. Xuexia Zhang and Xuejin Zhao: Data curation, Project administration, Writing–review & editing.

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Data availability

Sequence data that support the findings of this study have been deposited in the National Library of medicine (https://www.ncbi.nlm.nih.gov/) under the accession number PRJNA1157181 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1157181).

Declarations

Competing interests

The authors declare no competing interests.

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