Effects of light and cell cycle on expression level of echr1

Fermentation kinetics supplied the extracellular cues for cell growth and rapamycin production. However, the mechanism of rapamycin production discrepancy was not still understood. To solve this, the profiles of intracellular metabolites were carried out by GC-MS to reveal key biomarkers and metabolic module as well as key enzymes in rapamycin overproduction. Samples were obtained for wild-type strain and mutant strain U2-3D9 to detect intracellular metabolites pool every 24 h (48, 72, 96 and 120h). Three parallel experiments were performed at each time point. As a result, about 86 metabolites were identified and quantified by GS-MS (Supplementary Table S1), including 29 organic acids, 25 amino acids and amides, 10 sugars and alcohols, 8 fatty acids, 7 nucleotides and 8other compounds.

Next, PCA(R2 = 0. 971, Q2 = 0. 913) and heat map were performed to analyze structure and quality of metabolomics data. In the score plot (Fig. 2a), the tight clustering of three parallel samples at each time point and clear separation between the samples of the two strains indicated that these data were suitable for further analysis of different metabolic characteristics and monitoring culture process at different phases [9]. Heat map combined with the hierarchical clustering analysis re-confirmed the above conclusion (Fig. 3). Simultaneously, PLS (using rapamycin yield as Y value, R2 (X) = 0. 594, R2 (Y) = 0. 91, Q2 = 0. 789) was performed to analyze samples from different strains and culture phases. In the score plot (Fig. 2b), the samples from mutant strain were located in the first quadrant of the coordinate axes owing to relative higher rapamycin production. In contrast, another samples from wild-type strain mainly gathered in the lower left corner of the

coordinate system. PLS analysis was consistent with PCA and heat map, suggesting the rationality of sample extraction and the reliability of data.

Moreover, PLS analysis was applied to evaluate the correlation of metabolitestorapamycin production. Accordingly, the variable importance of the projection plots (VIP) on each metabolite was generated by PLS (Fig. 2c). VIP value indicated the contribution of each metabolite to the synthesis of rapamycin. A total of 25 metabolites (VIP score > 1) ranked in the top were chosen as candidate biomarkers. Meanwhile, 25 metabolites associated with rapamycin production were also found by PLS-DA according to correlation coefficient (Fig. 2d), among them 19 metabolites were positively correlated with rapamycin production, while the rest had negative correlation with rapamycin production. Ultimately it was concluded that 22 metabolites in the analysis of PLS-DA were the same as that in the analysis of PLS (Supplementary Table S2).

To further analyze the most relevant metabolic pathway of rapamycin synthesis, MetPA integrated with KEGG pathway database was performed on the basis of 22 metabolites (see Supplementary Table S2) derived from the above analysis. The ‘ metabolome view’ shows all matched pathways according to p values by pathway enrichment analysis and pathway impact values by pathway topology analysis. In Fig. 4, Y-axis means the minus logarithm of p value calculated from the pathway enrichment analysis, X-axis represents the impact value calculated from pathway topology analysis. The p values of aminoacyl-tRNA biosynthesis (p= 5. 6×e-6), phenylalanine metabolism (p= 0. 0005), pantothenate and CoA biosynthesis (p= 0. 0005), valine, leucine and isoleucine biosynthesis (p= 0. 0008) and glycine, serine

and threonine metabolism (p= 0. 0019) were less than 0. 01 (see Supplementary Table S3), indicating that their close correlation with rapamycin synthesis. Therefore, amino acids metabolism might be a key metabolic module of rapamycin synthesis.

Analysis of amino acids metabolic modules and key biomarkers for potential regulation of rapamycin overproduction

Significant differences in the abundances of intracellular amino acids were observed between wild-type and mutant strains. In fact, amino acids are essential parts of carbon and nitrogen metabolism for maintaining cell growth and protein synthesis. In addition, amino acid catabolism is a vital source of fatty acid precursors [40] for rapamycin biosynthesis[38]. The pools of intracellular metabolites in amino acid metabolic modules containing valine, isoleucine, glutamate, arginine, ornithine, tryptamine, lysine, pipecolic acid, and saccharopine(VIP score > 1 and closely associated with rapamycin production), were compared between wild-type and mutant strains during the fermentation period in detail, as shown in Fig. 5.

Valine and isoleucine metabolismRelative abundance of valine at four time points (48, 72, 96 and 120 h) in mutant was much lower than that of wild-type strain (Fig. 5). Meanwhile, a clear negative correlation could be observed between concentrations of valine and rapamycin production, as shown in Fig. 2d. Relative abundance of isoleucine in the mutant strain was 0. 646, 0. 651, 0. 698, 0. 701, a little lower than that in wild-type strain. When cell growth entered into the stable phase, the metabolic difference between mutant and wild-type became more obvious, and relative level of

isoleucine in wild-type strain was about 2-fold of that in mutant strain. In fact, both isoleucine and valine are the precursor of methylmalonyl-CoA [22, 24]which is a key node of the carbon ï¬, ux switches from the primary branch into the secondary metabolic branch [29, 33] such asrapamycin[19]. Therefore, more isoleucine and valine in mutant strain might be used to supply methylmalonyl–CoA for rapamycin synthesis. As analyzedabove, methylmalonyl-CoA is a key node for improving rapamycin production, while valine and isoleucine metabolism are key metabolic modules related to rapamycin synthesis. This conclusion was consistent with the reports by Jung et al. [21]and Cheng et al. [6].

Glutamate, arginine and ornithine metabolismVIP values of glutamate, arginine and ornithine were 1. 3, 1. 8 and 1. 4, respectively, indicating their close association with rapamycin production. In Fig. 4, the relative abundance of glutamate in the mutant strain was 0. 25 (48 h), 0. 18 (72 h), 0. 13 (96 h), 0. 09 (120 h), while that of wild-type strain was 0. 31, 0. 21, 0. 19, 0. 11. Along with cell growth, the relative level of glutamate began to decrease, but during the whole fermentation period, relative abundance of glutamate in wild-type strain was always higher than that in the mutant strain.

The relative level of arginine in mutant strain was 0. 38 (48 h), 0. 28 (72 h), 0. 21 (96 h) and 0. 16 (120 h), while arginine was not detected in wild-type strain. Ornithine in mutant strain reached to its peak of 0. 0328 at 48 h in Fig. 5, but it cannot be detected at other time points. In the wild-type strain, relative abundance of ornithine was 0. 04 (48 h), 0. 0296 (72 h) and was not detected at another two time points. Both arginine and ornithine participate

in urea cycle[30]. In the mutant strain, more arginine was transferred into urea cycle. Thus, it might be speculated that urea cycle might be potential key metabolic module to provide nitrogen source for rapamycin synthesis.

Tryptophan metabolismTherelative abundance of tryptamine in mutant strain was approximately 50% higher than that in wild-type strain in the whole fermentation period. Tryptamine is an intermediate metabolite related toshikimate metabolic pathway which generate schorismate by the chorismate mutase. Ultimately chorismate provides a starting unit (4R, 5R)-4, 5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC) for rapamycin synthesis[15, 34]. The depletion of intracellular tryptophan or the high level of tryptamine might indicate the activation of shikimatemetabolic pathway, and more DHCHC available for rapamycin synthesis. On the other hand, high intracellular levels of aromatic amino acids could inhibit 3-deoxy-7-phospho-heptulosonate synthase, which catalyzed the rate-limiting step in shikimate production from erythrose 4-phosphate and phosphoenolpyruvate[46].

Lysine metabolism The relative level of intracellular lysine in mutant strain was 3. 92 (48 h), 2. 25 (72 h), 2. 25 (96 h) and 1. 36 (120 h), and the relative abundance of wild-type strain was 2. 08, 0. 92, 0. 91 and 0. 42, respectively. It could be seen that more lysine was quickly consumed in mutant strain to synthesize rapamycinfrom 48 h to 120 h. However, the intracellular concentration of lysine was higher in the mutant strain compared to the wild-type strain, indicating lysine play an important role inrapamycin overproduction. In addition, the relative levels of both saccharopine and pipecolic acid in mutant were lower than that in wild-type strain. Along with the fermentation process, intracellular concentrations of the two metabolites

involved in amino acids metabolism were continuously declined both in the mutant and wild-type strains.

In sum, more amino acids were used to supply precursors of rapamycin synthesis in the mutant strain, compared to that in the wild-type. As shown in Fig. 2c, these amino acids were the main contributors to differences of rapamycin production and fermentation performance. In addition, the result indicated the shortage of valine, glutamate, isoleucine, and ornithine in the synthesis of rapamycin (Fig. 2d). But lysine and rapamycin production was positively correlated since its coefficient was greater than zero, implying the addition of lysine to fermentation medium might improve the supply of intracellular lysine in the process of rapamycin synthesis. As described above, it was concluded that amino acids metabolism including lysine and arginine, valine, glutamate, isoleucine and ornithine, were key for rapamycin synthesis.

Strengthening of intracellular amino acids related to rapamycin synthesis

In order to confirm the above analysis, exogenous feeding strategy was implemented by supplementing2. 5g/L arginine, tryptophan, lysine, glutamate, isoleucine, valine and ornithine into fermentation medium at different culture phases[6, 28]. The result showed that rapamycin production was improved to different extents, as shown in Fig. 6. Addition of lysine and isoleucine at 24 h resulted in 2. 3 and 2-fold increase of rapamycin concentration, respectively. Besides, rapamycin production was improved by 1. 7 and 1. 3-fold, respectively, by adding arginine and tryptophan at 36h. Addition of ornithineat 60 h made an improvement of rapamycin by 49. 8%.

Feeding valine and glutamate at 48 h increased production to 265. 3 and 231. 5 mg/L, respectively. These results indicated that the addition of seven different amino acids was able to promoterapamycin production in varying degrees, and it was notable that addition of lysine resulted in the most significant improvement. Thereby, it was confirmed that amino acids metabolic modules were closely associated with rapamycin synthesis indeed and the closest correlation was observed between lysine and rapamycin production..