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Effect of interfering TOR signaling pathway on the biosynthesis of terpenoids in *Salvia miltiorrhiza Bge*

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ABSTRACT

The TOR (Target of Rapamycin) signaling pathway, which takes TOR kinase as the core, regulates the absorption, distribution, and recycling of nutrients by integrating metabolic network and other signaling pathways, thus participating in the plant growth-defense trade-off. While terpenoids play an important role in plant growth, development, stress response, and signal transduction. The effect of the TOR signaling pathway on terpenoid biosynthesis in plants has yet to be studied in detail. In this study, the tissue culture seedlings of *Salvia miltiorrhiza* were treated with the TOR inhibitor AZD8055. The results show that the roots of the control group had begun to grow on the 8th day, while the seedlings treated with AZD8055 had no rooting signs. Combined with the expression changes of genes related to the TOR signaling pathway in the first 8 days, samples on the 3rd, 6th, and 8th days were selected for RNA-Seq analysis. Through RNA-Seq analysis, a total of 50,689 unigenes were obtained from the samples of these three periods, of which 4088 unigenes showed differential expression. The function enrichment and time-series analysis of differentially expressed genes (DEGs) showed that the main influence of the TOR signal pathway on plant growth-related processes was gradually transmitted with treatment time after TOR was inhibited. Pathway enrichment analysis of DEGs showed that the genes in the biosynthesis of terpenoids, such as diterpenoid and carotenoid biosynthetic pathways, could be regulated. Compared with other stages, DEGs related to terpenoid biosynthesis were mainly regulated in the S2 stage. In addition, the genes involved in terpenoid skeleton biosynthesis was also considerably enriched in the S2 stage, according to the results of gene set enrichment analysis (GSEA) of unigenes. Inhibition of the TOR signaling pathway may affect the biosynthesis of terpenoid signaling molecules, inhibit gibberellin's biosynthesis, and promote abscisic acid's biosynthesis. This study has discussed the effect of interfering with the TOR pathway on terpenoid biosynthesis in *S. miltiorrhiza* from the perspective of omics and provides new insight into the interaction between the terpenoid biosynthesis pathway and the growth-defense trade-off of medicinal plants.

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Introduction

Plant growth and development are affected by a variety of environmental factors. In order to

respond to these environmental factors, plants have evolved sophisticated stress tolerance strategies to cope with a myriad of biotic and abiotic stresses. Due to limited nutrients, plants achieve growth-defense trade-offs by regulating metabolism and various signaling pathways to allocate energy to growth or defense responses[1,2]. Target of Rapamycin (TOR) signaling pathway regulates plant growth and development by regulating nutrient absorption, distribution, and recycling through interaction with metabolic networks and other signaling pathways and is crucial for environmental adaptation and stress response[3–7]. The serine/threonine kinase protein TOR is the core component of the TOR signaling pathway, and in plants, it exists mainly in the form of the growth regulatory complex TORC1[3]. TOR can activate the TOR signaling pathway by sensing upstream environmental stress and nutritional signals, directly or indirectly triggering downstream signals[8]. In the past, the research on TOR kinase and TOR signaling pathway mainly focused on model plants such as Arabidopsis thaliana, but the related research in medicinal plants was not reported.

The study of the TOR signaling pathway in plants depends on TOR inhibitors to a great extent. TOR inhibitors can inhibit the activity of TOR, thus destroying the function of TOR and interfering with the TOR signaling pathways. Rapamycin is the first inhibitor of TOR, which can inactivate the TOR protein by forming a ternary complex with FRB domain of the TOR protein FKBP 12 (FK506 binding protein 12), thus effectively inhibit TORC1 activity in yeast and animals. There are great differences in the sensitivity of plants to rapamycin. Tomato is very sensitive to rapamycin[9], but most plants, such as Arabidopsis, potato, and cotton are not sensitive to rapamycin due to the structural change of FKBP 12 protein because it cannot form a ternary complex[3]. AZD8055 (AZD) is an ATP-competitive TOR inhibitor (asTORis) that inhibits the activity of TOR protein by targeting the ATP-binding domain of the TOR protein complex and competing with ATP for the site of

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the binding domain[10]. And AZD has been proved to inhibit the activity of TOR in many plants, so it is widely used in the study of the TOR signaling pathway in plants[11–15]. However, whether *S. miltiorrhiza* is sensitive to rapamycin and AZD has not been confirmed.

Terpenoids are important metabolites produced by plants, which can regulate plant growth by participating in primary and secondary metabolic pathways and help plants cope with biotic and abiotic stress[16]. All terpenoids are derived from a common five-carbon building block, isopentenyl diphosphate (IPP), and its allylic isomer dimethylallyl diphosphate (DMAPP), which are the core of the terpenoid biosynthetic pathway and are produced via the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway[17]. Then, IPP and DMAPP form FPP (farnesyl diphosphate), GPP (geranyl diphosphate), and GGPP (geranylgeranyl diphosphate), which are direct precursors of terpenes. Finally, under the action of different terpene synthases, the precursors are subsequently transformed into various terpenoids[17,18]. Gibberellins (GAs), Abscisic acid (ABA) are terpenoid signaling molecules involved in plant growth, development, and stress response[19]. The terpenoid biosynthesis pathway regulates their biosynthesis and signals transduction pathways. In addition, terpenoids are important components in many medicinal plants, such as tanshinone in *S. miltiorrhiza* and paclitaxel production in *Taxus spp*. Therefore, it is of great significance to study the biosynthesis pathway of terpenoids for the research of medicinal plants.

In recent years, the TOR signaling pathway has been widely studied as an important regulator in plant growth-defense trade-offs. However, the role of TOR signaling in the biosynthesis of terpenoids has not been described. *S. miltiorrhiza* is a plant with a unique efficacy and high medicinal value. The research on terpenoids of *S. miltiorrhiza* mainly focuses on the synthesis pathway of terpenoids, and the effects of other signaling pathways on the biosynthesis of terpenoids need further study.

Taking the tissue culture seedlings of *S. miltiorrhiza* as the research material, treated with rapamycin and AZD8055 as TOR inhibitors, respectively, and comparing the tissue culture seedlings of *S. miltiorrhiza* sensitivity to rapamycin and AZD8055, choose the best one. Transcriptome sequencing was used to compare the gene expression changes of *S. miltiorrhiza* tissue culture seedlings after AZD treatment for different time, and to explore the influence of TOR signaling pathway on terpenoid biosynthesis pathway. It has been found that the TOR signaling pathway can regulate the biosynthesis of terpenoids, especially the synthesis of terpenoid hormones, and thus participate in the regulation of plant growth and defense.

Materials & methods

Plant material and growth conditions

S. miltiorrhiza tissue culture seedlings (hereinafter refer to as tissue culture seedlings as seedlings) were obtained from healthy plants of *Salvia miltiorrhiza Bge*. It was introduced from the medicinal botanical garden of Chengdu University of Traditional Chinese Medicine. The explants were obtained from healthy plants of *S. miltiorrhiza*, and the callus was obtained after sterilization. The obtained cluster buds were transferred to a 1/2 MS solid rooting medium to induce aseptic seedlings of *S. miltiorrhiza*[20]. The same developmental stage, similar leaf number and similar height plants were used in all of the experiments. All growth experiments were performed at 25°C, 12 h (light)/12 h (dark) photoperiod treatment, and 6000 light intensity.

TOR inhibitor treatment

To determine whether TOR inhibitors work on *S. miltiorrhiza*, rapamycin, and asTORis inhibitor AZD8055 (AZD) were selected to treat *S. miltiorrhiza* seedlings. Rapamycin and AZD were dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM concentration master mix, respectively, stored at −20°C and diluted into the medium at the concentration ratio when used. Different TOR inhibitor treatment groups were created using the rapamycin and AZD at a concentration of 10 μM; DMSO containing the same dose of inhibitor at a concentration of 10 μM was used as a vehicle control group (DMSO at a concentration of 1 μL/mL), and medium alone was utilized as the blank control group. *S. miltiorrhiza* seedlings were pre-cultured for two weeks, and selected uniform *S. miltiorrhiza* seedlings were treated and cultured under the above conditions for 15 days. Meanwhile, to verify the sensitivity of *S. miltiorrhiza* seedlings to AZD, 0 μM, 1 μM, 2 μM, and 10 μM were set as different concentration treatment groups AZD, and uniformly grown *S. miltiorrhiza* seedlings were selected and cultured for 15 days.

Growth measurements

Plants were photographed and growth parameters were recorded after experiment, four plants were randomly selected from each group to measure, the number of new leaves and roots, the length of roots were recorded as growth indicators. And the length of roots was measured by ImageJ, and each root was measured three times, and the average value was obtained.

RNA sample collection

S. miltiorrhiza seedlings were treated and cultured in the AZD treatment group at 2 μM concentration and the blank group without AZD addition, starting 24 h after treatment, and samples were collected for eight consecutive days, snap frozen in liquid nitrogen and stored in a −80°C refrigerator for use in subsequent RT-qPCR experiments, and RNA-Seq analysis. Whole plants on days 3, 6 and 8 of growth treatment were selected for RNA-Seq analysis. Three biological replicates were available for each sample.

RNA extraction, library preparation, and sequencing

According to the manufacturer's instructions, total RNA was extracted from the tissue using Trizol Reagent kit (Invitrogen), and genomic DNA was removed using DNase I (TaKara). Total RNA was verified for purity and integrity by 1% agarose

gel electrophoresis and 2100 Bioanalyser (Agilent Technologies). After the total RNA was extracted, mRNA was enriched by Oligo (dT) beads, according to the polyA selection method. Then double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA). According to Illumina's library construction protocol, synthetic cDNA was subjected to end-repaired, phosphorylated, and 'A' base addition. cDNA target fragments were screened by 2% Low Range Ultra Agarose followed by PCR amplified. The paired-end RNA-seq sequencing library was sequenced (2×150 bp read length) using an Illumina NovaSeq 6000 sequencer, performed at Majorbio Bio-pharm Biotechnology Co., Ltd (Shanghai, China).

De Novo assembly and annotation

The raw data were filtered using fastp (https://github.com/OpenGene/fastp)[21]. Then clean data were used to do a de-novo assembly with Trinity (http://trinityrnaseq.sourceforge.net/)[22]. All the assembled transcripts were searched against the NCBI protein non-redundant (NR, http://ftp.ncbi.nlm.nih.gov/blast/db/), SwissPort. (http://web.expasy.org/docs/swissprot_guideline.html), Pfam (http://pfam.xfam.org/), Clusters of Orthologous Groups of proteins (COG, http://www.ncbi.nlm.nih.gov/COG/), GO (http://www.geneontology.org) and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) databases using BLASTX to identify the proteins that had the highest sequence similarity with the given transcripts to retrieve their function annotations and a typical cutoff E-values less than 1.0×10^{-5} was set.

Differential expression analysis and functional enrichment

The expression level of each gene was calculated according to the transcripts per million reads (TPM) method. RSEM (http://deweylab.biostat.wisc.edu/rsem/)[23] was used to quantify gene abundances. Differential expression analysis between the two groups was performed using the DESeq2[24], DEGs with |log2 (foldchange)| ≥1, and P-value≤0.05 were considered to be significantly different expressed genes (DEGs). In addition, functional-enrichment analysis including GO (Gene Ontology, http://www.geneontology.org) and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) were performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways at P-adjust≤0.05 compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were carried out by Goatools (https://github.com/tanghaibao/Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do)[25]. Then, the Log2FC values of three different groups of DEGs in different periods were clustered by using Mufzz (DOI: 10.18129/B9.bioc.Mfuzz)[26] and ClusterGVis (https://github.com/junjunlab/ClusterGVis)[27] as well as time-series analysis to obtain the trend of relative expression in the three stages.

Gene Set Enrichment Analysis (GSEA)

Gene Set Enrichment Analysis was performed using the GSEA (Version 3.0) provided by the Massachusetts Institute of Technology[28]. Based on the KEGG database, the unigenes were used as the gene set and the gene expression matrix was obtained. Normalized enrichment score (NES) and false discovery rate (FDR, p-adjust) were used to quantify enrichment magnitude and statistical significance, respectively.

RT-qPCR and validation of RNA-Seq data

Total RNAs were extracted from *S. miltiorrhiza* seedlings and treated with RNase-free DNase I (Foregene CO., LTD., Chengdu, China). And Validation using the same RNA samples as for RNA-seq sequencing. The reverse transcription reaction is performed using 800 ng of the total RNA samples with RT Easy™II Kit (Foregene CO., LTD., Chengdu, China). The cDNA products were used as templates for quantitative PCR. The qPCR reaction was carried out with the Real-Time PCR Easy™-SYBR Green I (Foregene CO., LTD., Chengdu, China) in an FQD-48A Real-Time PCR System (Bioer, China). All the primers used for RT-qPCR are designed on Primer 6 software and listed in Table S1. The *SmActin* gene was used as the internal control, and the $2^{-\triangle\triangle}$CT method calculated the relative expression levels[29]. Three biological replications and technical replications were implemented for each sample to ensure reproducibility and reliability.

Statistical analysis

R (version 4.2.2) and GraphPad Prism 9 were used for the basic statistical analysis.

Results

Effects of TOR inhibitors rapamycin and AZD on the growth of *S. miltiorrhiza*

In order to evaluate whether TOR inhibitor rapamycin (RAP) and AZD can affect the growth of *S. miltiorrhiza*, the seedlings of *S. miltiorrhiza* were cultured in the medium containing these two TOR inhibitors (10 μM) for 15 days. The results showed that there was no obvious difference in the growth and rooting of *S. miltiorrhiza* seedlings treated with 10 μM RAP and DMSO solvent control and blank control (Figure 1A), and there was no significant difference in the number of new leaves, the number of roots, and the length of roots among the three groups (Figure 1B). However, the seedlings treated with 10 μM AZD showed obvious growth inhibition, which showed smaller and weaker, no rooting, and the number of new leaves was significantly lower than the other three groups (Figure 1).

These results indicated that the seedlings were not sensitive to RAP at 10 μM concentration, but AZD at 10 μM concentration significantly altered the growth and development of *S. miltiorrhiza* seedlings. In addition, DMSO at the concentration of 1 μL/mL had no inhibitory effect on the growth of *S. miltiorrhiza* seedlings. This observation was consistent with the findings of previous studies in Arabidopsis[30].

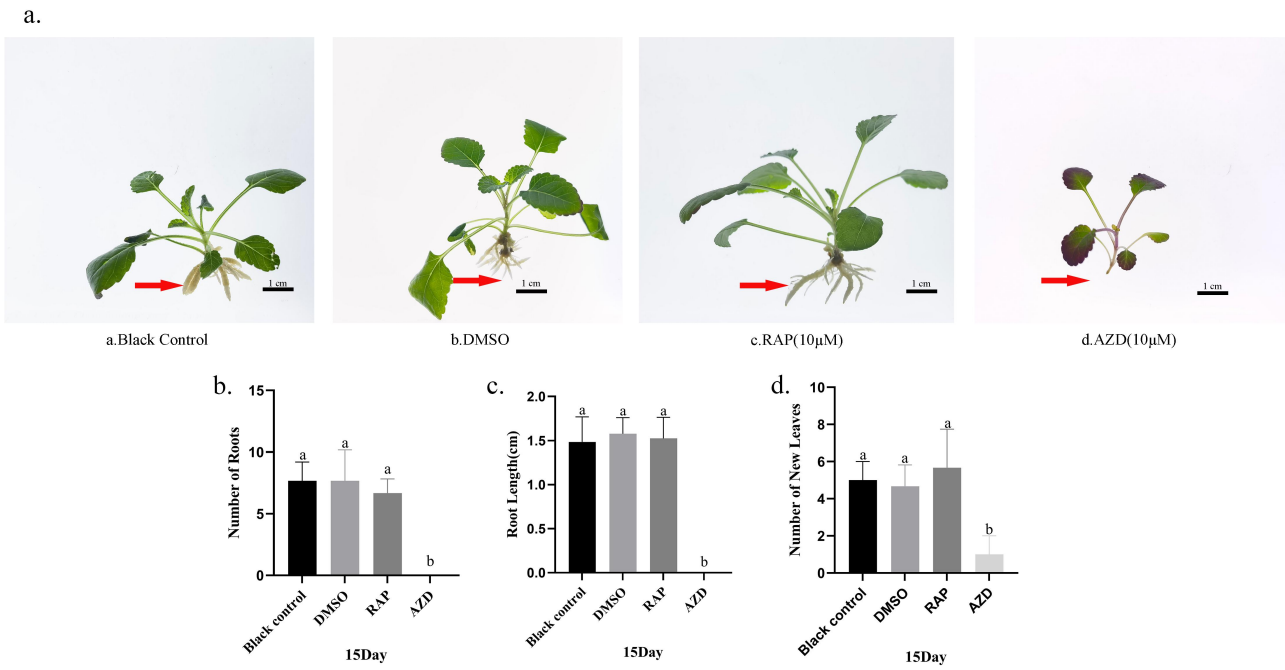

Figure 1. Effect of different TOR inhibitor on the growth of *S. Miltiorrhiza* seedlings. (A) The seedlings of *S. Miltiorrhiza* were grown in the medium containing blank control, DMSO solvent control, 10μM Rap and 10μM AZD, for 15 days. The red arrow points to the root where the difference is obvious. (B) The average number of new leaves, the number of roots and the length of roots of the seedlings measured after 15 days of growth in the above medium. Data are mean±standard error of 4 replicate samples. Different lowercase letters indicate statistically significant differences between treatments according to Tukey's multiple range test.

Screening of AZD concentration

S. miltiorrhiza seedlings were cultured in different doses of AZD for 15 days to find a suitable concentration for the inhibition research, growth in medium containing different concentrations of AZD is shown in the figure (Figure 2). The growth of *S. miltiorrhiza* seedlings appeared to be inhibited by 0.5 μM AZD, and the number of new leaves, the length, and number of roots were reduced, and the inhibition effect was enhanced with the increase of AZD concentration. Compared with the normal seedlings, the seedlings treated with 2 μM AZD showed obvious growth inhibition. The number and length of roots and the number of new leaves

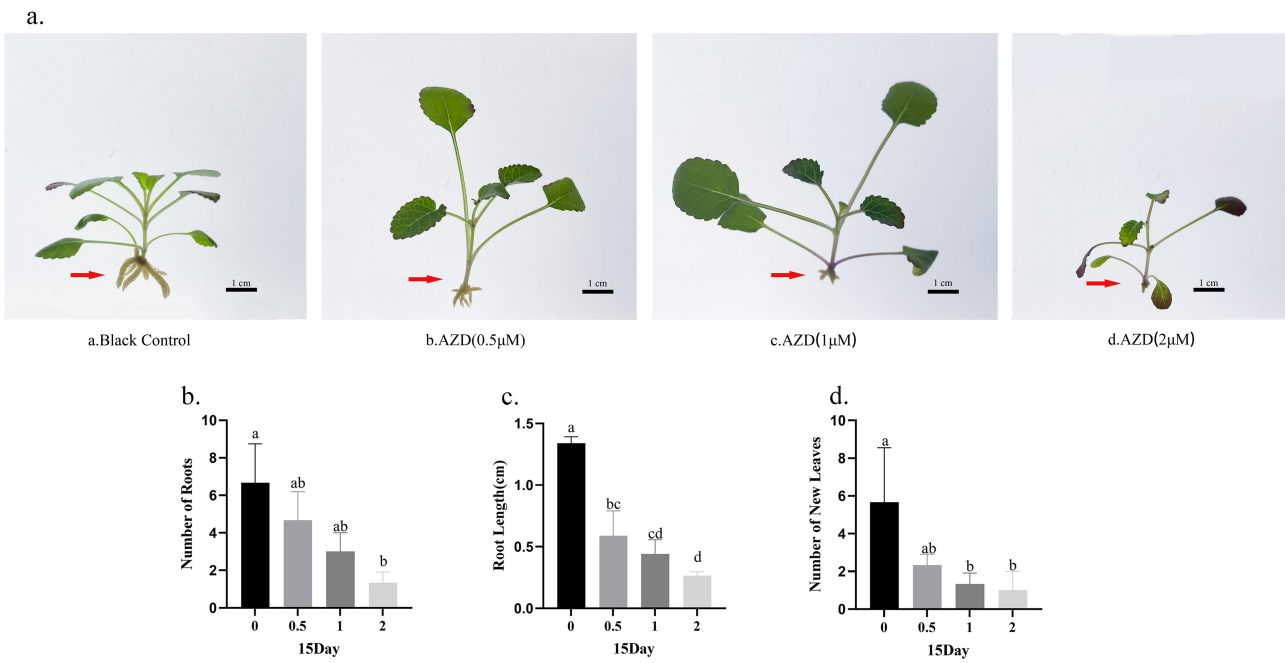

Figure 2. Effect of different concentrations of AZD8055 on the growth of *S. Miltiorrhiza* seedlings. (A) The seedlings of *S. Miltiorrhiza* were grown for 15 days in a medium containing different concentrations of AZD (0μM,0.5μM,1μM,2μM). The red arrow points to the root where the difference is obvious. (B) The average number of new leaves, the number of roots and the length of roots of the seedlings measured after 15 days of growth in the above medium. Data are mean±standard error of 4 replicate samples. Different lowercase letters indicate statistically significant differences between treatments according to Tukey's multiple range test..

were significantly lower than those of the control (0 μM), and the leaves and stems turned red, suggesting that the seedlings had stress response (Figure 2B). Therefore, the inhibitory effect of a concentration of 2 μM meets the requirements of this study, and 2 μM was selected as the AZD concentration for subsequent experiments.

The seedlings of *S. miltiorrhiza* were cultured in the medium containing AZD at a concentration of 2 μM. It was found that the seedlings of the control group generally began to take root on the eighth day, while the seedlings of the treatment group showed no signs of rooting (Figure 3A). Then, the expression of the TOR-related genes *SmTOR*, *SmRAPTOR-1, SmLST8–1*, and the downstream effector *SmS6K–1* was detected during the 8-day treatment, and it was found that the expression of these genes on the 3rd, 6th, and 8th day was different from that on other days (Figure 3B, Table S2A). It is speculated that there may be an obvious stress response during this time. Therefore, samples from the 3rd, 6th, and 8th days were selected for subsequent RNA-Seq experiments, and the control group and a treatment group were set up in each period, and three biological replicates were set up at each sampling points. A total of 18 samples were sequenced for transcriptome.

Transcriptome analysis of inhibiting TOR

RNA-Seq sequencing results showed that each sample obtained more than 7.53 Gb of Clean Data for assembly, with the Q20 base percentage above 97.41% and the Q30 base percentage above 92.88%. A total of 50,689 unigenes were obtained, with an average GC content of 41.97%. The average length of unigenes was 1170bp, and the N50 length was 2019bp. The transcriptome data had good integrity. All quality inspection data are given in Supplementary Tables S3–S5.

All unigenes obtained in this transcriptome sequencing were compared with six major databases (NR, Swiss-Prot, Pfam, COG,

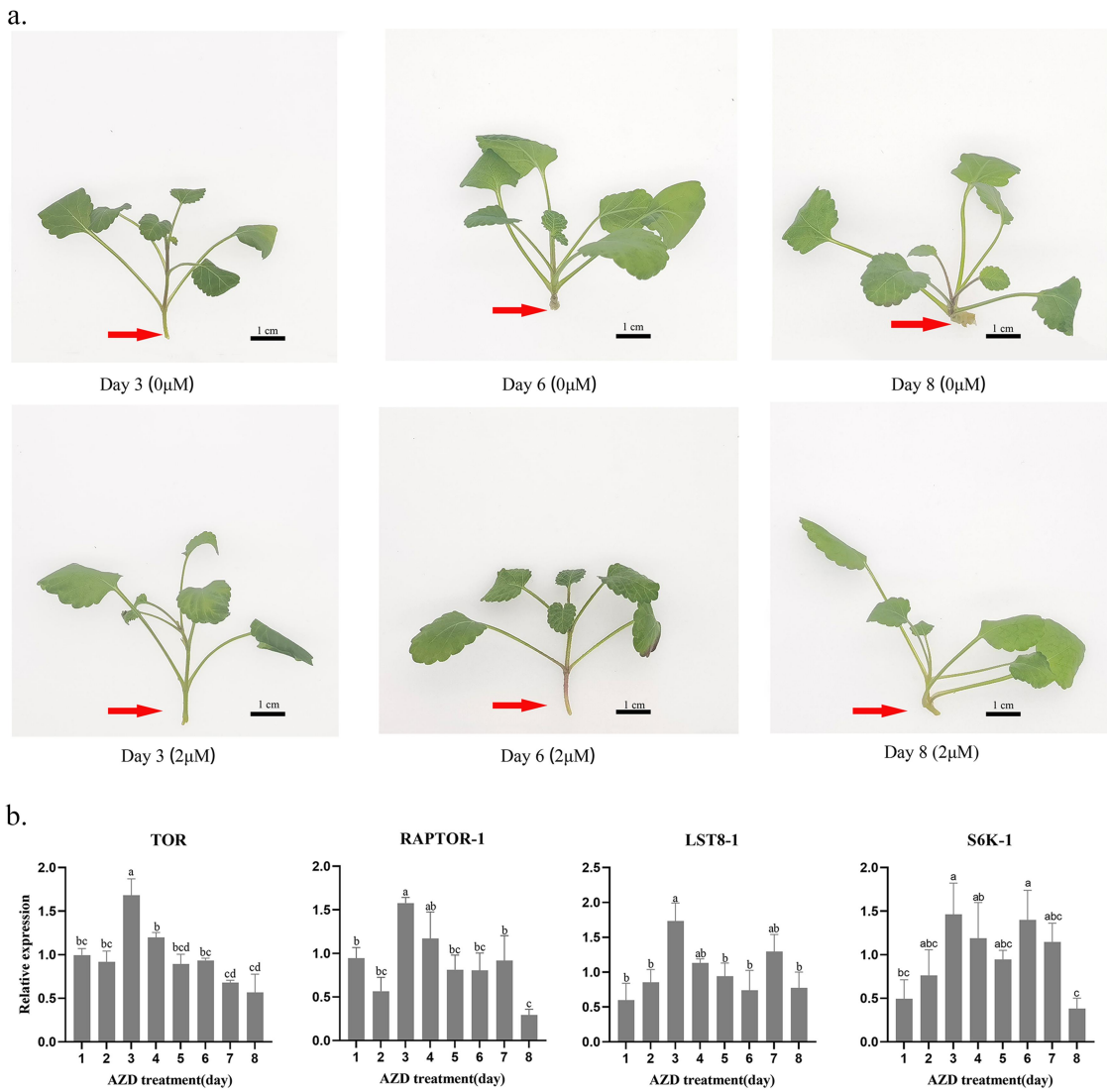

Figure 3. Effect of continuous treatment with 2 μM concentration of AZD8055 on the growth of *S. Miltiorrhiza* seedlings. (A) Phenotypes of *S. Miltiorrhiza* seedlings cultured in medium containing 2μM AZD and blank medium at different treatment times. The red arrow points to the root where the difference is obvious. (B) Relative expression of TOR-related genes in *S. Miltiorrhiza* seedlings within eight days after treatment with the TOR inhibitor AZD. Data are means±standard deviation from three biological replicates. Different lowercase letters between samples denote significant differences according to one-way ANOVA and Tukey's test ($p<0.05$)..

GO, and KEGG databases), and 27,930 unigenes (55.14%) were annotated in at least one of the databases (Table S6). Calculate the Pearson correlation coefficient of three biological replicates of each group of samples according to the expression amount to verify the consistency of biological replicates. The results show that the correlation of all biological replicates is greater than 0.818 (Figure S1).

Fifteen Unigenes were selected to detect their relative expression levels at S1, S2, and S3 and compared with the results of RNA-Seq (Figure S2A, Table S2B). Meanwhile, the results of the linear regression analysis showed that they were significantly correlated ($R^2 = 0.8396$) (Figure S2B). Therefore, the transcriptome data obtained by RNA-seq in this experiment proved accurate and reliable.

Analyses of differential gene expression were performed on RNA-seq data from the S1, S2, and S3 stages. A total of 4088 DEGs (Figure 4A) were identified in S1, S2, and S3, among which 1627 DEGs were identified in S1, 593 genes were up-regulated, and 1034 genes were down-regulated; 1813 DEGs were generated in the S2 stage, 815 genes were up-regulated, and 998 genes were down-regulated; A total of 1671 DEGs were generated in S3, 646 genes were up-regulated, and 1025 genes were down-regulated. The Venn diagram of DEGs produced in three stages showed that 148 genes were differentially expressed in three stages (Figure 4B). The finding shows that S2 had the greatest number of differentially expressed and up-regulated genes.

In order to understand the expression changes of the DEGs after TOR inhibition, time-series analysis was performed by using $\log_2$FC values of all DEGs produced in the three stages. It was found that the expression of these DEGs was very different in the three stages (Figure 4C). According to the results of cluster analysis and time-series analysis, the relative expression trends of 4088 DEGs in the three stages can be roughly divided into three categories: the Cluster1, which contains 1304 DEGs, has relatively higher expression in S2 and S3 stages with the increase of inhibition time; the Cluster2, which expression level decreased significantly at S3 stage, contained 1419 DEGs; and the Cluster3, which had the lowest relative expression level in S2 stage, contained 1365 DEGs.

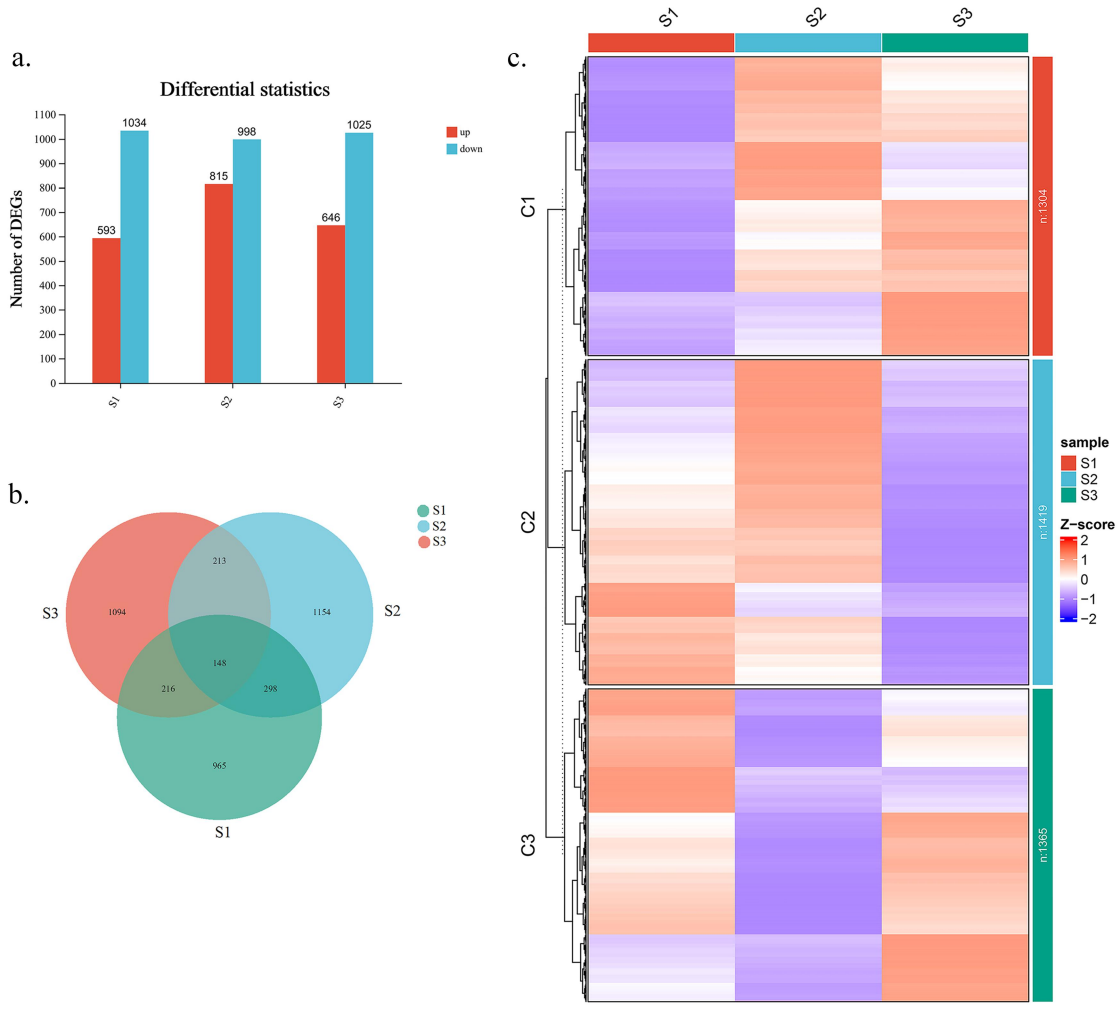

Figure 4. Statistical and time series analysis of differentially expressed genes (DEGs). (A) The number of differentially expressed genes in the three stages. (B) The common and unique DEGs among the three stages. Numbers represent the DEGs. (C) DEGs are clustered by time series analysis. The heat map is made according to their log2FC values, C1, C2 and C3 represent the three clusters of DEGs after clustering.

Differential gene expression regulation after TOR inhibition

GO enrichment analysis was carried out to understand the potential function of the DEGs obtained in the three stages. The GO terms of the top 20 enrichment degrees in the three stages were obtained (Figure 5A, Table S7). In the S1 stage, the top GO results showed that DEGs are mainly involved in the biological processes, including the pectin catabolic process, polysaccharide catabolic process, microtubule-based movement, mitotic cell cycle process, and cell cycle process. In the

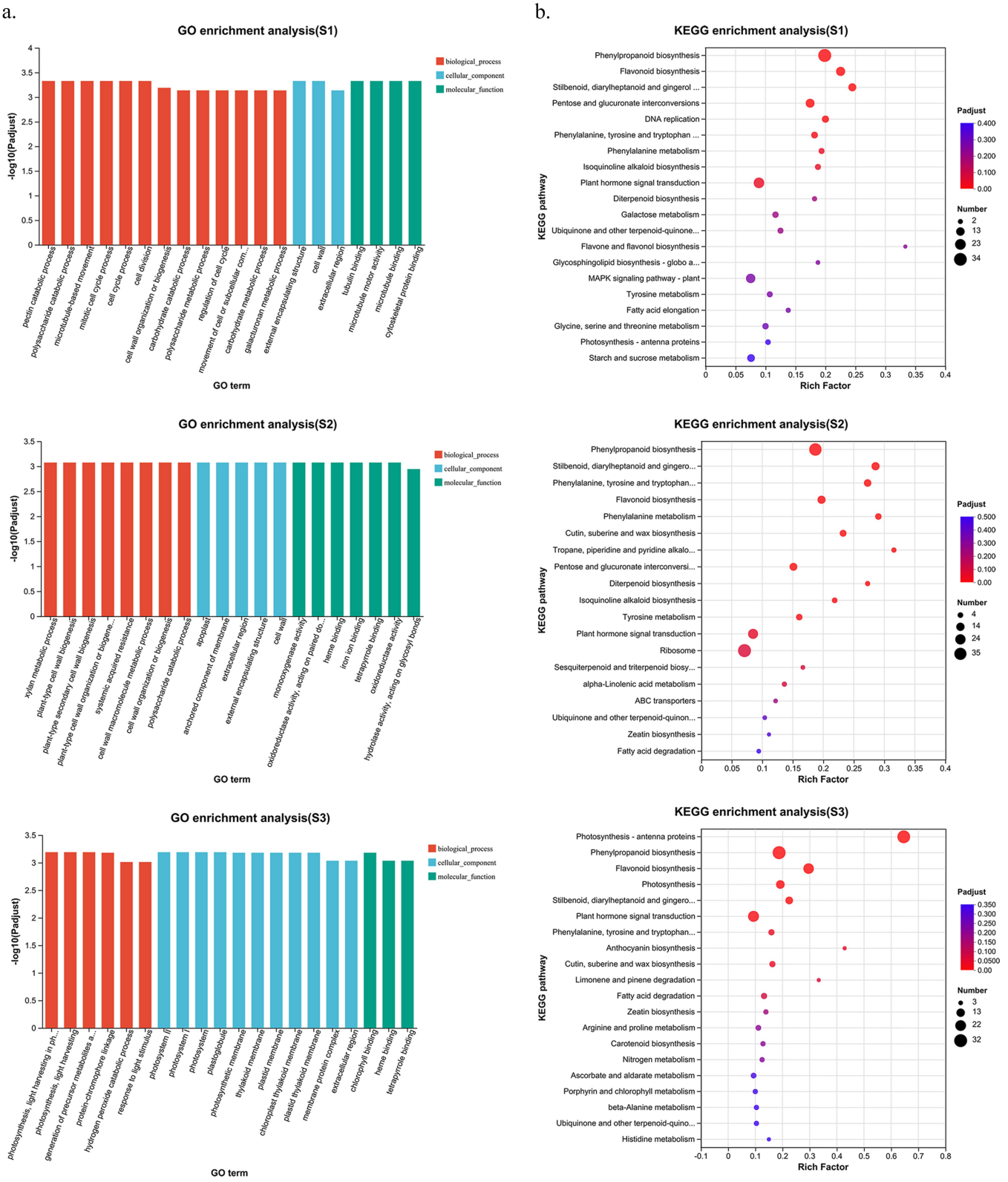

Figure 5. Functional analysis of DEGs. (A) GO enrichment analysis of DEGs in the three stages. (B) KEGG pathway enrichment analysis of DEGs at the three stages.

S2 stage, DEGs are mainly involved in the biological processes, including the xylan metabolic process, plant-type cell wall biogenesis, plant-type secondary cell wall biogenesis, plant-type cell wall organization or biogenesis, and systemic acquired resistance. In the S3 stage, the top GO results showed that DEGs are mainly involved in photosynthesis-related biological processes, including photosynthesis, light harvesting in photosystem I, photosynthesis, light harvesting, and the generation of precursor metabolites and energy.

In order to further understand the biological functions of the DEGs, a KEGG enrichment analysis was performed. Based on the KEGG database, the pathway analysis of DEGs generated in the S1, S2, and S3 stage were conducted, and the biological pathways with the enrichment degree in the top 20 in the three stages are shown in Figure 5B (Table S8). The results show that the pathways related to the biosynthesis of secondary metabolites, such as Phenylpropanoid biosynthesis, Flavonoid biosynthesis and Phenylalanine, tyrosine, and tryptophan biosynthesis were significantly enriched in the three stages. In addition, biological pathways involved in growth were also significantly enriched in the three stages. Such as pentose and glucuronate interconversions and DNA replication (enriched in the S1 stage), ABC transporters and ribosomal biological processes (enriched in the S2 stage), and photosynthesis-related pathways (enriched in the S3 stage). The signal pathways involved in the plant immune mechanism also showed obvious enrichment. The MAPK signaling pathway was mainly enriched in the S1 stage, and plant hormone signal transduction showed different degrees of enrichment in three stages.

Regulation of Terpenoid Biosynthetic Pathway after Inhibiting TOR Activity

GO enrichment and KEGG enrichment showed that many terpene biosynthesis pathways were found to be regulated. GO enrichment showed that diterpenoid biosynthetic and metabolic processes were significantly enriched mainly in the S2 stage. KEGG enrichment showed that diterpenoid biosynthesis, sesquiterpene and triterpene biosynthesis, zeatin and carotenoid biosynthesis, limonene, and pinene degradation pathways are the most obviously regulated terpenoid biosynthesis pathways after the TOR signaling pathway was disturbed. They show different degrees of regulation at S1, S2, and S3 stages. A total of 25 DEGs were identified from these pathways that showed significant enrichment at least in one stage, and the relative expression of these DEGs in the three phases is shown in Figure 6.

Terpene synthases (TPSs) are the key enzymes responsible the biosynthesis of terpenes. A total of 11 TPSs involved in

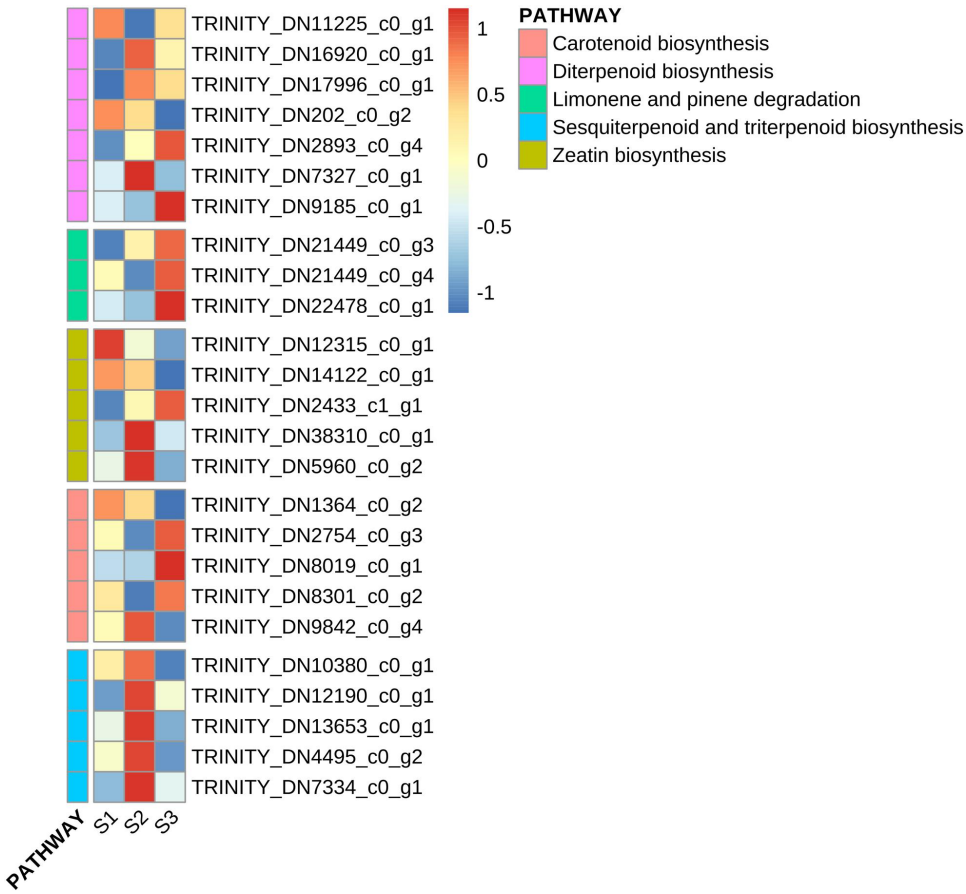

Figure 6. Heat map of expression trends of terpenoid biosynthesis pathway-related DEGs enriched by KEGG at the three stages. The heatmap shows the expression trends of DEGs involved in diterpene biosynthesis, carotenoid biosynthesis, Limonene and pinene degradation, and Zeatin biosynthesis at the three stages. The normalized Log2FC values were used for the heatmap.

terpenoid biosynthesis were identified from DEGs and showed differential expression (Table S9). The cytochrome gene family encompasses oxidases and cytochrome P450 (CYP450) monooxygenases, involved in many oxidation reactions and can be closely involved in terpene biosynthesis by controlling the functional modification of terpenes. Most P450s involved in terpene modification belong to a wide range of CYP 71 and 76 families. From the DEGs that displayed differential expression following TOR inhibition, 60 P450s genes were found (Table S10), of which 26 belonged to CYP 71 and 76 families. These include CYP 76AH3 and CYP 76AK1, which are closely related to the synthesis of tanshinone.

GSEA analysis of biological pathways regulated after inhibition of TOR

In order to further explore the regulation of the biosynthesis pathway of *S. miltiorrhiza*, especially the regulation of terpenoids biosynthesis pathway, GSEA analysis of unigenes was carried out based on the KEGG database. Pathway enrichment was mapped by the standardized enrichment fraction (NES) at the three stages (Figure 7, Table S11).

The GSEA analysis results show that ribosomal biological processes and photosynthesis-related pathways are obviously enriched in the three stages, and photosynthesis-related pathways are the main biological processes with down-regulation in the three stages. In addition, the biological process of DNA replication is mainly enriched and down-regulated at the S1 stage, according to DEGs and GSEA analysis. Besides, we discovered that the autophagy and the RNA degradation process was enriched and up-regulated in the S1 and S3 stage (Figure 7).

For the terpene biosynthesis pathway, we found that the results of GSEA supplemented the results of the DEGs pathway enrichment analysis. In addition, in GSEA analysis, the terpenoid backbone biosynthesis was significantly enriched and upregulated at the S2 stage. The relative expression changes of unigenes enriched in terpenoid skeleton synthesis pathway in three periods are shown in Figure 8. The phenylpropane pathway and the tyrosine-derived pathway were also found to be significantly enriched in the three stages in DEGs enrichment analysis and GSEA analysis. And

we screened 17 genes from DEGs that can be annotated to the key enzyme genes of the salvianolic acid synthesis pathway. The relative expression of the 17 DEGs in three stages are shown in Figure 9.

Effects on Transcription Factor (TF) families

Transcription factors (TF) are a class of proteins that bind to gene promoters and regulate gene expression at different levels. They are very important in regulating the development and growth of plants and adapting to the environment. The DEGs generated in the three stages revealed 140 TFs with membership in 28 transcription factor families (Table S12). The highest number of TFs were annotated to the MYB/MYB-related family, with 38 TFs, 18 TFs to the AP 2-EREBP family, and 11 TFs to the bHLH family (Figure 10A). The number of TFs displaying significant differential expression in the S1, S2, and S3 stages are represented in Figure 10B. The number of TFs showing significant differential expression is the largest in the S2 stage, and the bHLH, AP2-EREBP and WRKY transcription factors involved in coping with adversity stress mainly show significant differential expression in the S2 stage. MYB/MYB-related TFs showed significant differential expression in all three stages and participate in regulating metabolism and signaling pathways.

Discussion

Rapamycin is an inhibitor of the TOR kinase, which inhibits the TOR kinase by binding to the FRB domain of TOR protein and forming a ternary complex with FKBP12. The majority of plants, including Arabidopsis thaliana, rice[31], and cotton[14], are considered to be rapamycin-insensitive based on currently available studies. That is, rapamycin treatment has no impact on their growth and development. Because the structure of the FKBP12 protein in these plants has been changed, it cannot bind rapamycin and form a ternary complex with the TOR

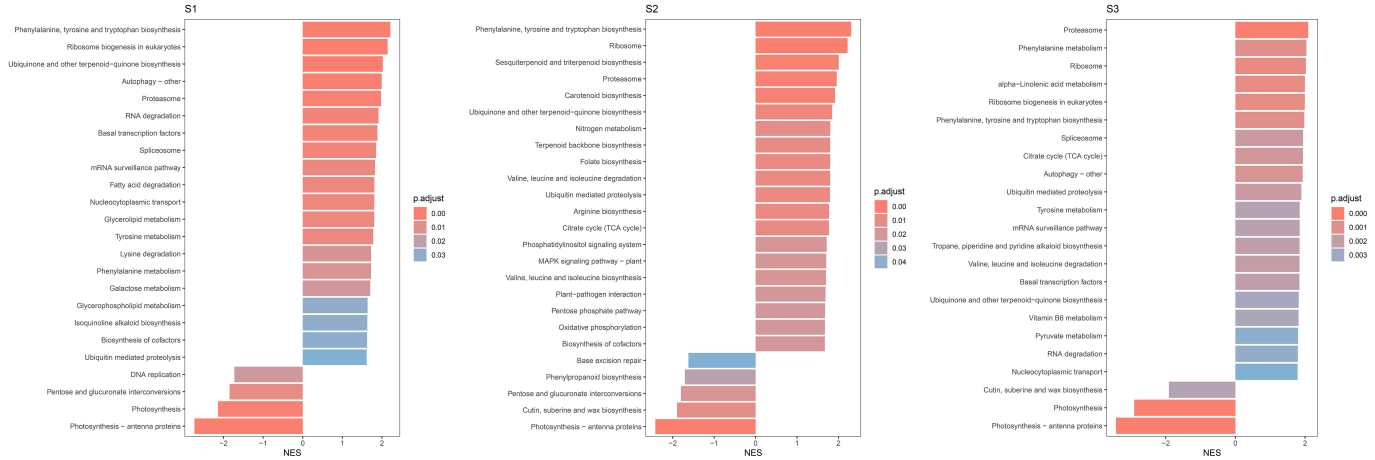

Figure 7. Pathway enrichment in GSEA analysis. Pathway enrichment of GSEA analysis at the three stages. Negative values of NES indicate the down-regulation of pathway expression, and positive values indicate the up-regulation of pathway expression.

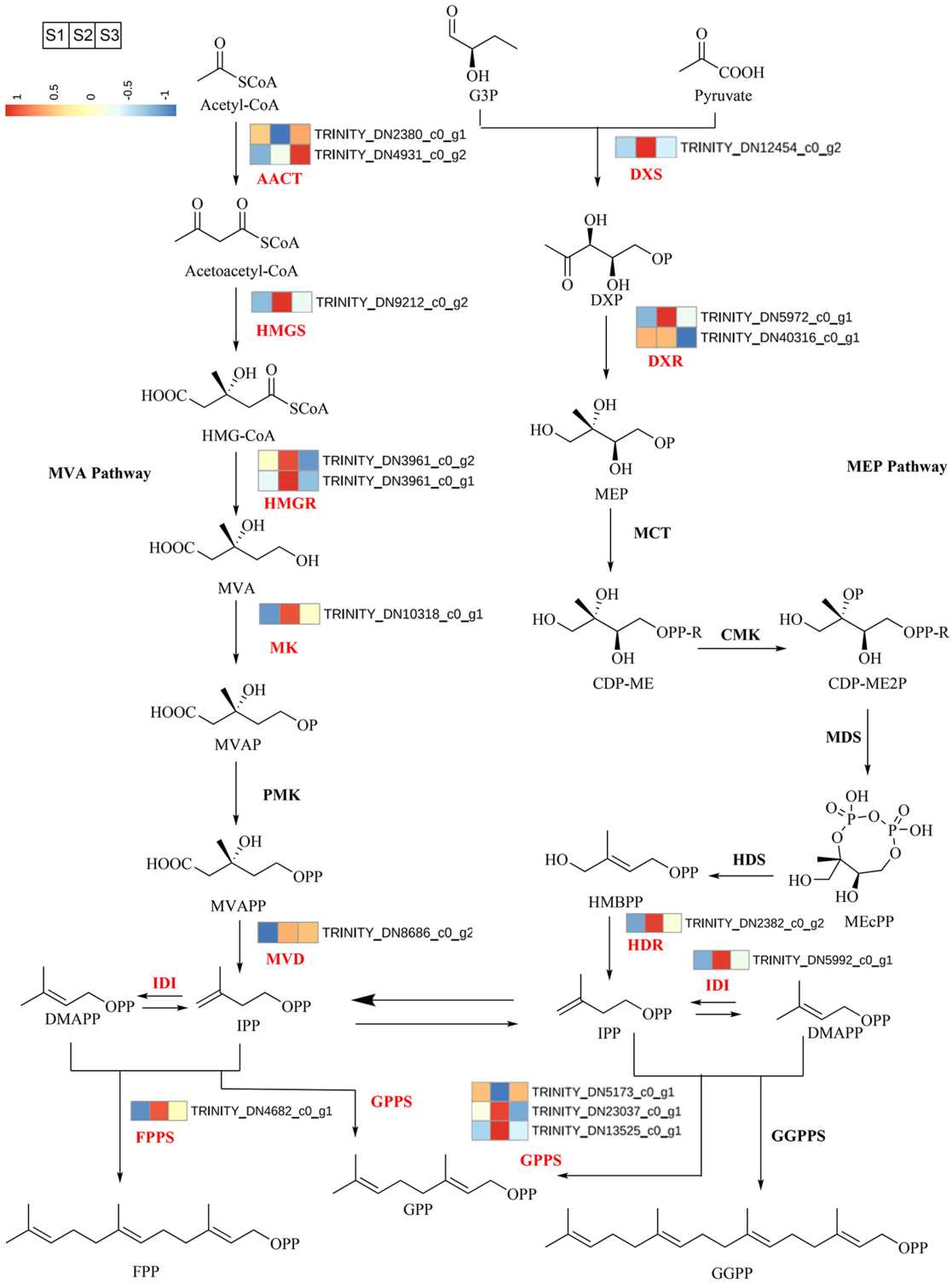

Figure 8. The relative expression levels of the genes enriched in the terpene skeleton synthesis pathway were analyzed by GSEA in the three stages. Solid arrows represent single biosynthetic steps. The color blocks represent the relative expression of the gene at the three stages according to the heatmap. Normalized Log2FC values were used for the heatmap. AACT: acetyl-CoA C-acetyltransferase, HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase, HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase, MK: mevalonate kinase, PMK: 5-phosphomevalonate kinase, MDC: mevalonate pyrophosphate decarboxylase, DXS: 1-deoxy-D-xylulose-5-phosphate synthase, DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase, MCT: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, MDS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, HDS: 4-hydroxy-3-methylbut-2-enyl diphosphate synthase, HDR: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, IDI: isopentenyl pyrophosphate isomerase, FPPS: farnesyl diphosphate synthase, GPPS: geranyl diphosphate synthase, GGPPS: geranylgeranyl diphosphate synthase.

kinase[3]. But not all plants are insensitive to rapamycin. Rapamycin causes a severe reaction in the tomato[9]. Rapamycin will greatly slow down the growth and development of tomatoes, and it will have a similar inhibitory effect as asTORis treatment of tomatoes. As an efficient asTORis. The results show that, like most plants, such as

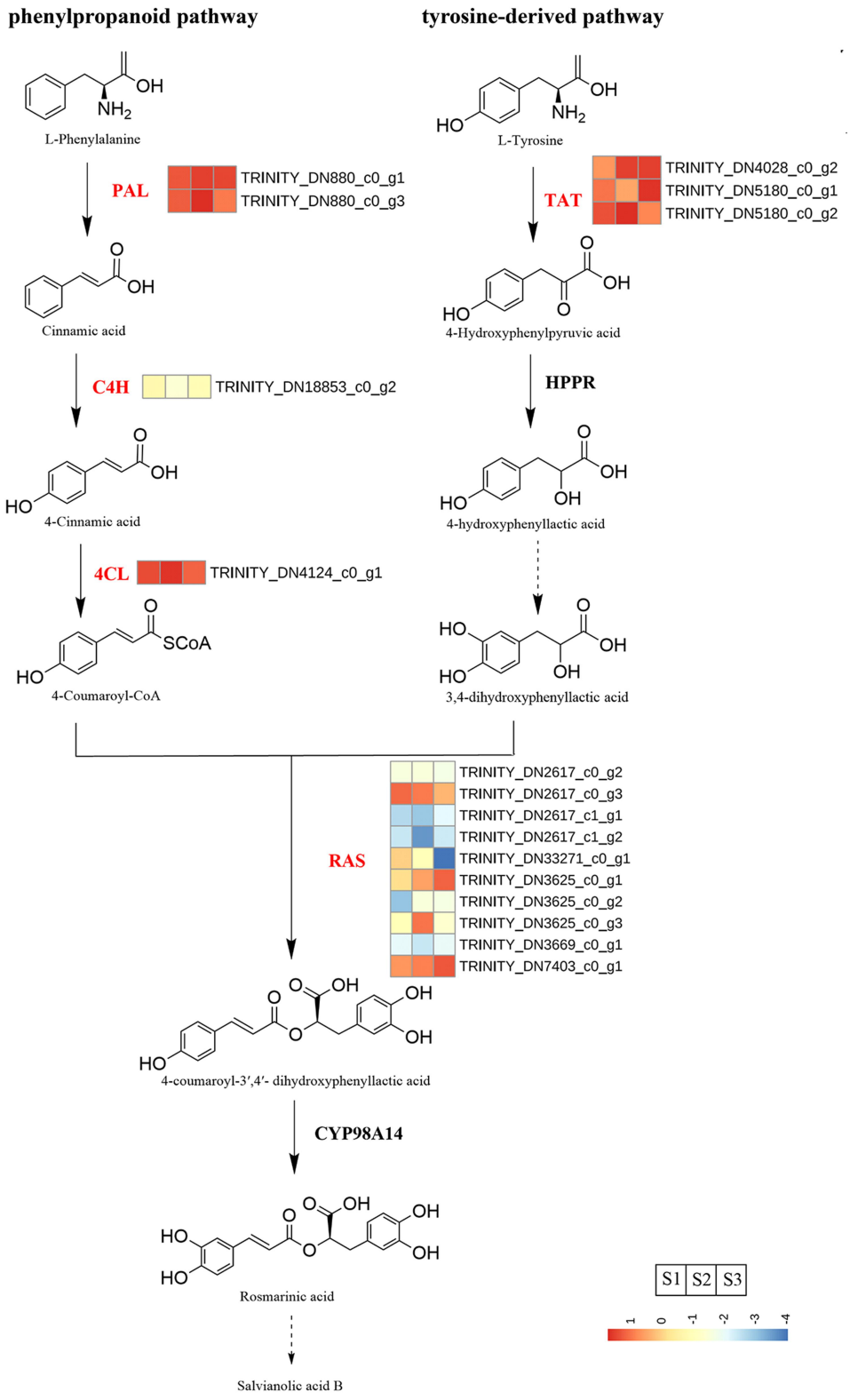

Figure 9. Relative expression of salvianolic acid biosynthesis DEGs in the three stages. Solid arrows represent single biosynthetic steps. Dashed arrows represent multiple steps. The color blocks represent the relative expression of the gene at the three stages according to the heatmap. Log2FC values were used for the heatmap. C4H: cinnamate 4hydroxylase, PAL: phenylalanine ammonia lyase, 4CL: 4-coumarate: coenzyme A ligase, RAS: rosmarinic acid synthase; TAT: tyrosine aminotransferase, HPPR: p-hydroxyphenylpyruvate reductase.

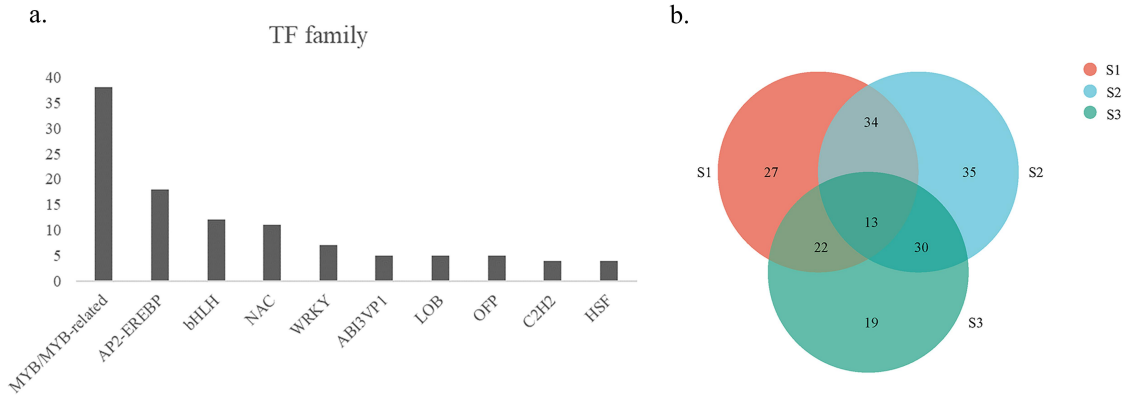

Figure 10. The summary of TFs information in DEGs. (A) Top 10 TFs Families in DEGs. (B) The common and unique TFs among the three stages. Numbers represent the TFs.

Arabidopsis thaliana, *S. miltiorrhiza* is insensitive to rapamycin (Figure 1), but AZD, as a potent asTORi, can effectively inhibit the growth and development of *S. miltiorrhiza* seedlings (Figure 2).

Previous studies in many plants, such as Arabidopsis thaliana, have shown that interfering with TOR signaling pathway by inhibiting the TOR activity can affect biological processes such as cell growth and photosynthesis and regulate the synthesis, transport, and metabolism of nutrients[12,15,32,33]. By analyzing the differential expression of the three stages of biological processes after inhibiting TOR activity, it was found that interfering with the TOR signaling pathway may affect the biological processes related to the growth and defense of *S. miltiorrhiza*. Moreover, the influence of the TOR signaling pathway on plant biological processes may be gradually regulated, and signals are transmitted from near to far. It is mainly reflected in the influence on growth-related biological processes. At the S1 stage, mitosis-related biological processes, DNA replication and other biological processes are first regulated, and the DEGs related to cell wall formation and isoprene metabolism are significantly regulated at the S2 stage. Photosynthesis-related biological processes are regulated in all three stages. With the continuous interference of the TOR signaling pathway, more biological processes are regulated, and the photosynthesis-related biological processes are most obviously regulated in the S3 stage. Autophagy is a highly conservative process for plant to adapt to nutritional conditions, and it is regulated in the S1 and S3 stage. Therefore, it is speculated that properly inhibiting TOR activity may promote the autophagy of *S. miltiorrhiza* and enhance the recycling of nutrients. In addition, SnRK1, as an important central factor in plant response to stress, can form negative feedback regulation with TOR under energy deficiency and participate in the regulation of autophagy[34]. The negative regulatory mechanism of SnRK1 and TOR in *S. miltiorrhiza* needs further study.

After the inhibition of the TOR signaling pathway, many terpenoid biosynthesis pathways were significantly regulated. Terpenoids can play an important role in plant growth maintenance as primary metabolites, such as chlorophyll and carotenoids in photosynthetic pigments. Terpenoids can also act as an important secondary metabolite, optimizing the interaction between plants and their environment[35,36]. Terpene skeleton biosynthesis is essential for synthesizing terpenoids, providing the core precursor for downstream terpenoids through the synthesis of isoprene[17]. The relative expression of key enzyme genes for the manufacture of the terpene skeleton considerably increased after AZD treatment inhibited TOR of *S. miltiorrhiza* (Figure 8), may facilitate the biosynthesis of the isoprene unit and downstream terpenoids, and thus be involved in regulating plant growth and stress response processes. TPSs are a key enzyme for synthesizing terpenoids, and their gene expression can reflect the synthesis of different terpenoids in plants. According to TPSs, known to be involved in the modification and synthesis of specific terpenoids in *S. miltiorrhiza*[37], a total of 11 TPS show differential expression, thus affecting the biosynthesis of terpenoids downstream. In a word, the TOR signaling pathway can be interfered by inhibiting TOR activity, thus may affect the biosynthesis of terpenoid precursors, intermediate diphosphate precursors, and terpenoids.

As terpenoid plant hormones, GA and ABA are synthesized by diterpenoid biosynthesis pathway and carotenoid biosynthesis pathway, respectively. Moreover, GA and ABA can regulate the growth and development response and produce the defense response to stress[19,38]. Interfering with the TOR signal pathway by inhibiting TOR activity in *S. miltiorrhiza* may affect the biosynthesis of terpenoid hormones, especially GA and ABA. Ent-kaurenoic acid oxidase (KAO), GA20-oxidases (GA20oxs), and GA30-oxidases (GA3oxs) are all key oxidases involved in GA biosynthesis, among which the GA20oxs gene is easily affected by abiotic stress factors, and these oxidases play a positive role in GA biosynthesis pathway and promote GA biosynthesis[19,39]. The diterpenoid biosynthesis pathway showed obvious differential expression mainly in the S2 stage, with the key oxidase genes *SmKAO1* (TRINITY_DN2893_c0_g4), *SmGA20ox2* (TRINITY_DN9185_c0_g1), *SmGA3ox2* (TRINITY_DN11225_c0_g1) were enriched into the diterpenoid biosynthesis pathway in S2 stage and showed obvious down-regulation. Meanwhile, they also showed different down-regulation in S1 and S3 stages, which may lead to the inhibition of GA biosynthesis.

The biosynthesis of ABA is catalyzed by various enzymes through the carotenoid pathway with β-carotene as the precursor. According to the DEGs enrichment analysis and GSEA analysis, after the TOR signaling pathway was interfered by TOR activity inhibition, the carotenoid biosynthesis pathway was obviously up-regulated and differentially expressed. In addition, 9-cis-epoxycarotenoid dioxygenase (NCED), as the key rate-limiting enzyme of ABA biosynthesis, is up-regulated and can promote ABA biosynthesis, and it is found that the application of AZD to inhibit TOR can significantly increase the transcription level of NCED3 in Arabidopsis[40–42]. The results show that *SmNCED3* (TRINITY_DN8301_c0_g1) was up-regulated at S1, S2, and S3 stages, may promote ABA biosynthesis by up-regulating the expression level of NCED3. In addition, studies have shown that ABA can inhibit the TOR signaling by activating SnRK2 under stress conditions[43]. After inhibiting the TOR activity of *S. miltiorrhiza*, *SmSnRK2 (TRINITY_DN1723_c0_g2)* was found to be significantly up-regulated in S2 and S3 stages, indicating that SnRK2 may be activated at this time, thereby increasing the inhibitory effect on TOR. Therefore, interfering with TOR signaling pathway by inhibiting TOR activity in *S. miltiorrhiza* may lead to the inhibition of GA biosynthesis pathway and the promotion of ABA biosynthesis, which is consistent with the study of the effect of the TOR signaling pathway on plant hormone biosynthesis[4].

Phenolic compounds are synthesized through the shikimic acid/phenylpropanoid pathway and participate in metabolism and biological processes as important secondary metabolites produced in plants, playing a key role in the acclimation process of plants to abiotic stress. It mainly includes phenolic acids, flavonoids, and stilbenes. Salvianolic acid is an important phenolic acid compound in *S. miltiorrhiza*. Its precursors 3,4-dihydroxyphenyllactic acid 9 and 4-coumaroyl-CoA separately are synthesized mainly through the phenylpropanoid biosynthesis pathway and the tyrosine derivation pathway and then modified by several enzymes involved in the RA branch pathway to produce various salvianolic acids[44,45]. After AZD inhibited TOR activity of *S. miltiorrhiza*, several key genes of the salvianolic acid synthesis pathway were significantly up-regulated at S1, S2, and S3 stages, which may promote the synthesis of phenolic acid secondary metabolites, such as the biosynthesis of salvianolic acid precursors 3,4-dihydroxyphenyl lactic acid and 4-coumaroyl coenzyme A and salvianolic acid (Figure 9). The TOR signaling pathway plays an important role in plant growth and defense response by integrating the metabolic network and regulating primary and secondary metabolic responses[46,47]. The inhibition of TOR activity in *S. miltiorrhiza* to disturb the TOR signaling pathway may produce a wide range of regulation of primary and secondary metabolic pathways in *S. miltiorrhiza*.

TFs play a key role in regulating plant growth-defense trade-offs[1]. After the TOR signaling pathway was disturbed, many transcription factors in *S. miltiorrhiza* showed different expression in response. These TFs are mainly from MYB/MYB-related, bHLH, AP2-EREBP, WRKY, and NAC TFs families, and show different degrees of differential expression at the three stages. These TFs have been shown to play an important role in the synthesis of secondary metabolites by regulating plant hormone signaling pathways, participating in plant defense signal transduction, responding to stress factors and participating in plant growth-defense trade-offs[48–50]. Most of these TFs involved in plant defense signal transduction were significantly differentially expressed at the S2 stage, the S2 stage may be the key stage for TFs to regulate the synthesis of secondary metabolites and transmit defense signals after the TOR signaling pathway is inhibited. Some TFs in these TF families have been shown to be involved in the regulation of tanshinone and salvianolic acid - biosynthesis.[44,50]. Such as *SmWRKY2* (TRINITY_DN5552_c0_g1)[51], which positively regulates tanshinone synthesis by activating the *SmCPS* gene of the diterpene synthesis pathway, *SmMYB98* (TRINITY_DN7853_c0_g1)[52] which promotes the accumulation of tanshinone and salvianolic acid, and *SmMYB36* (TRINITY_DN4675_c0_g1)[53] which participates in the regulation of primary and secondary metabolism and promotes the biosynthesis of tanshinone were significantly up-regulated at the S2 stage. At the same time, *SmMYB111* (TRINITY_DN1058_c2_g3)[54], which positively regulates salvianolic acid biosynthesis, was significantly up-regulated at all three stages. The inhibition of TOR activity by AZD may affect the synthesis and accumulation of tanshinone and salvianolic acid by affecting the expression of transcription factors, but the specific regulatory mechanism needs further experimental demonstration and research.

Conclusions

In this study, the results show that inhibition of TOR activity could firstly affect the growth and development-related processes of *S. miltiorrhiza* by disturbing the TOR signaling pathway and then therefore to affect the biosynthesis pathway of terpenoids in *S. miltiorrhiza* after a certain inhibition time, especially regulating the biosynthesis of terpenoid phytohormones, which participated in the growth and development and defense response of *S. miltiorrhiza*. These results increase our understanding of the relationship between the TOR signaling pathway and the biosynthesis of terpenoids, especially terpenoid phytohormones, and may provide new directions for studying plant stress response mechanisms.

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Disclosure statement

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