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Transcription level differences in *Taxus wallichiana* var. *mairei* elicited by Ce^{3+} , Ce^{4+} and methyl jasmonate

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Taxol is a precious and effective anticancer drug. Cerium and methyl jasmonate (MJ) have been shown to increase the yield of taxol in taxus cells. However, the mechanisms of cerium-mediated and MJ-mediated taxol biosynthesis remain unknown. RNA-Seq was applied to study the overall regulation mechanism of cerium and MJ on taxol biosynthesis and analyze the differences among *T. mairei* cells elicited by Ce^{3+} , Ce^{4+} and MJ on transcriptional level. Using sequence homology, 179 unigenes were identified as taxol synthesis genes. Under the condition of 100 μ M MJ, taxol synthesis genes were up-regulated. Notably, taxol synthesis genes were down-regulated expression at 1 mM Ce^{3+} and 1 mM Ce^{4+} . Differential expression genes involved in some related functions were analyzed, such as MAPK signaling pathway and plant-pathogen interaction. Sequence alignment and phylogenetic analysis of nine differentially expressed WRKYs in our data were carried out.

KEYWORDS

T. mairei, transcriptome, taxol biosynthesis, cerium, MJ

Introduction

Taxus, a genus of gymnosperms, contains at least 14 species (Hao et al., 2008). *T. mairei* is a member of taxus, which is a tall evergreen tree mainly distributed in southeast China (Wang et al., 2019). *T. mairei* can produce many chemicals with pharmaceutical properties. Among them, Taxol[®] was reported as a quite promising anticancer drug in

Abbreviations: *T. mairei*, *Taxus wallichiana* var. *mairei*; RNA-Seq, RNA sequencing; TFs, Transcription factors; FDR, False Discovery Rate; DEG, Differential Expression Gene; Nr, NCBI Non-redundant Protein.

1964 (Fang and Liang, 2005). Taxol is a plant protectant, which is a diterpenoid compound (Wu and Yuan, 2000).

At present, taxol production still depends on natural bark extraction or semi-synthesis of taxol precursors such as baccatin III (Mutanda et al., 2021). Paclitaxel drug has been in short supply for a long time and the price is high, so developing a biosynthesis method with low cost and high yield is particularly important (Wang et al., 2013; Kuang et al., 2016). The basic framework of taxol biosynthesis pathway had been established, and most of the enzymes involved in the synthesis had been cloned (Kuang et al., 2016). However, the biosynthesis pathway of taxol is unclear. Therefore, further exploration is needed to realize the continuous supply of taxol through biosynthesis. (Wen et al., 2016; Abdallah et al., 2019; Wang et al., 2021).

Elicitors can stimulate plant cells or serve as a messenger to induce the activation of the plant defense system. Elicitors not only leads the primary metabolism of the plant cells to flow in the direction of secondary metabolism but also have little influence on the culture system and finally promotes the synthesis of the target product (Mani et al., 2021).

A previous study has demonstrated that light metals such as cerium and lanthanum are very effective non-biological elicitors to promote the biosynthesis of taxol (Mithofer et al., 2004). Studies confirmed that 0.1 mM Ce⁴⁺ can effectively improve the soluble protein synthesis and cell viability of *Taxus cuspidata* cells in suspension culture and increase taxol biosynthesis and release, while 1 mM Ce⁴⁺ can significantly induce apoptosis of *Taxus cuspidata* cells (Yingjin et al., 1998; Ge et al., 2005). It was found that Ce⁴⁺ can effectively promote the hydrolysis of phosphoglyceride and the cleavage of nucleotide chain (Sun et al., 2003). The Ce³⁺ can promote the germination of pollen grains, the growth of pollen tubes and the generation of safranin in tomato callus (Chen et al., 2004). Using rare earth as a unique biological effect to improve the synthesis of taxol is of great significance for large-scale industrial production in the future. The binding of rare earth to certain molecules on the cell membrane is well known. However, how this complex further affects the transcription of defense groups needs to be further confirmed.

In addition, previous studies have reported that the addition of MJ to the suspension culture of taxus cells can significantly induce the production of taxol (Ketchum et al., 1999; Khosroushahi et al., 2006). Therefore, taxus cells with different elicitors and elicitation time can be used as a differential expression system for studying taxol biosynthesis. Moreover, compared with the transcriptome of *T. mairei* responding to MJ, the effect of cerium on the transcriptome of *T. mairei* can be better clarified.

With the advantages of high throughput, high sensitivity, low cost and wide application, RNA-seq has become the main method in the research field of transcriptome (Sharma et al., 2018; Pop-Bica et al., 2022). RNA-seq has been applied to understand various aspects of taxol synthesis. In 2010, Lee

et al. (Lee et al., 2010) reported the first transcriptome of taxus with cambium meristem cells from *Taxus cuspidata*. Li et al. (Li et al., 2012) studied the early response of *Taxus chinensis* cells elicited by methyl jasmonate (MJ) and found that a series of TFs activated by exogenous MJ, such as MYB, bHLH, ERF, AP2 and MYC, may be involved in the regulation of gene expression in taxol synthesis pathway. Yu et al. (Yu et al., 2017) studied the difference in transcription level between *Taxus media* and *T. mairei*, and proposed that the difference of taxane compounds content may be attributed to the differential expression of candidate genes involved in taxane biosynthesis pathway. However, the genome of taxus is complex, and there is still a lack of comprehensive understanding of the regulation of gene expression profile of taxol biosynthesis. At present, whole genome sequencing and related transcriptomics research are also rare, all of which indicate that the research of new generation of high-throughput sequencing technology in taxus has not yet arisen (Cusido et al., 2014).

In the present study, *T. mairei* cells elicited by Ce³⁺, Ce⁴⁺ and MJ were compared based on data generated by the same RNA-Seq method, and the regulation mechanism of cerium and MJ in taxol biosynthesis were investigated and the differences among *T. mairei* cells elicited by Ce³⁺, Ce⁴⁺ and MJ on transcriptional level were analyzed. And we expected that the biological information about the new DEGs, WRKYs and taxol synthesis-related pathways in this study would provide a basis for further research on cerium and MJ response network and regulation of taxane biosynthesis in *Taxus chinensis*.

Materials and methods

Plant materials and treatment condition

The cells used in the experiment were callus of *T. mairei*. The tender stems of *T. mairei* were disinfected with 75% ethanol and 5% sodium hypochlorite successively. The disinfected tender stems of *T. mairei* were cut into small sections and obliquely inserted into modified B5 solid medium. Callus began to grow after half a month. The cells were inoculated on modified B5 solid medium and subcultured once every 30 days at 25°C in the dark. The cells were activated in B5 liquid medium and cultured in liquid suspension at 25°C and 110 rpm in the dark. The subculture was conducted once every 10-12 d and the flask culture was conducted step by step. The specific steps were as follows: in the first stage of culture, about 3 g subcultured cells were inoculated into a 250 ml triangular flask containing 50 ml of fresh culture medium; During the second and third-stage culture, a part of the culture solution in the previous-stage culture was poured out, and fresh culture medium was supplemented to 50 ml; The fourth and fifth stages of culture were conducted in a 500 ml triangular flask with the medium changed, and the culture volume was maintained at about 150

ml. The fifth generation of liquid cells with uniform growth was sub-packaged into a 250 ml triangular flask containing 50 mL of fresh medium, and the inoculated cells weighed about 2.5-3.5 g cells (fresh weight). Subsequently, 1 mM Ce(NO₃)₃, 1 mM Ce(NH₄)₂(NO₃)₆, 100 μM MJ and an equal volume of solvent as control were added to the cultures respectively. The experiment was performed using three biological replicates for each sample. Taxanes extraction and HPLC analysis

The dried *T. mairei* cells (about 200 mg) were powdered and ultrasonicated for 10 min twice in mixed cyclohexane and methanol (1:1, v/v), then extracted three times with methylene chloride. Following centrifugation, the supernatant from the samples was filtered through a 0.2 μm polymeric filter. Filtered medium samples were used to extract taxol in a separatory funnel along with an equivalent volume of methylene chloride. Before HPLC analysis, the aqueous phase was eliminated, the methylene chloride phase was collected and allowed to evaporate at room temperature, and the remaining material was then resuspended in 1 ml of methanol and filtered through a 0.2 μm polymeric filter. The column, which was 250 mm long and 4.6 mm in diameter, was packed with Kromasil C₁₈ 5 μm and washed at a rate of 1 mL per minute with a 65:35 (v/v) combination of methanol and water. At 227 nm, the detection was made. (Yuan et al., 2001) The content of taxane was calculated by an external standard method, and the pure products of taxol, 10-deacetylbaocatin III (10-DAB) and baocatin III were taken as the reference substances.

Total RNA isolation and cDNA library construction

According to the previous report (Nims et al., 2006; Yang et al., 2006; Patil et al., 2012; Mao et al., 2018) using qRT-PCR on the time of taxol synthesis gene and some important enzyme response elicitors, we optimized the sampling time to 6h and 24h to study the changes in the transcription level of *T. mairei* cells. For the RNA-Seq, samples were taken at 6 h and 24 h after 1 mM Ce(NO₃)₃, 1 mM Ce(NH₄)₂(NO₃)₆ and 100 μM MJ addition. After the total RNA extraction (three biological replicates for each sample) and DNase I treatment, magnetic beads with Oligo (dT) were used to isolate mRNA (for eukaryotes) or by removing rRNAs from the total RNA (for prokaryotes). Mixed with the fragmentation buffer, the mRNA was fragmented into short fragments. Then cDNA was synthesized using the mRNA fragments as templates. Short fragments were purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition. After that, the short fragments were connected with adapters. After agarose gel electrophoresis, the suitable fragments were selected for the PCR amplification as templates. During the QC steps, Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System

were used in quantification and qualification of the sample library.

Illumina sequencing and *de novo* assembly

The Illumina HiSeq4000 sequencing platform was used to carry out the RNA sequencing. Transcript data of each sample was assembled from scratch using Trinity (Grabherr et al., 2011), and TGICL (Pertea et al., 2003) was used for unigenes clustering and redundancy reduction to produce unigenes and transcripts. The sequences data reported in this study were archived in the Sequence Read Archive (SRA) with the accession number SRR20217393.

Gene function annotation and differential expression analysis

Blastx was used to align unigenes sequences to the protein database NR, Swiss-Prot, KEGG and COG (Tatusov et al., 2003) (Evalue < 1e-5), and blastn was used to align unigenes to the nucleic acid database nt (Evalue < 1e-5). The protein with the highest sequence similarity was used as the function annotation information of the unigenes.

Using the results of NR annotation, GO function annotation for unigenes was obtained using Blast2GO (Conesa et al., 2005). Clean data obtained after filtration was respectively compared with the assembled unigenes through SOAPaligner v2.21 (Li et al., 2009), and the expression level of unigenes was calculated using RPKM (Mortazavi et al., 2008) (Reads per kB per Million fragments). The calculation formula is:

$$RPKM = \frac{10^6 C}{NL/10^3}$$

In this formula, RPKM (A) is the expression of Unigene A, and C is the number of reads that are uniquely aligned to Unigene A, N is the total number of reads that are uniquely aligned to all Unigenes, and L is the number of bases on Unigene A.

The level of significant gene difference between the two samples was calculated using the detection method (Audic and Claverie, 1997) described by Audic S. et al. And the level of false positive rate was controlled using FDR (False Discovery Rate) [36]. The significantly differentially expressed genes were defined as the gene with FDR ≤ 0.001 and FC > 2.

To identify the most important biochemical metabolism, signal transduction Pathways and GO functional differences in which DEGs were involved, KEGG and GO functional enrichment analysis on DEGs was performed. Using a hypergeometric test, the functional categories of pathway

and GO that were significantly enriched in differentially expressed genes were found. The level of the false-positive rate was controlled by FDR(False Discovery Rate) (Lange et al., 2003).

To eliminate the possible impact of the analysis of the significant difference in gene expression on the functional enrichment analysis of KEGG and GO, and identify the changing trends of all DEGs in the functional classifications of Pathway and GO, functional enrichment analysis on all DEGs in the KEGG and GO classifications were performed. The up-regulated genes were defined as fold change greater than 1, and the down-regulated genes were defined as fold change less than 1. The Chi-squared test was used to analyze the significance level of up-regulation or down-regulation of DEGs in the KEGG pathway and GO terms, and the level of false-positive rate was controlled by FDR(False Discovery Rate) (Lange et al., 2003).

Identification of unigenes associated with the taxol biosynthesis pathway

All unigenes were matched to known genes involved in taxol production using the Blastx program. The gene in taxol synthesis was discovered as the best alignment of unigenes with Identity > 40%, Evalue > 10 e-7, and Coverage > 80%.

A gene associated with taxol production often has many unigenes. The differential expression of this gene was converted using the formula below:

$$Diffexp = \log \left(\frac{\sum_{i=1}^n A \text{ RPKM}}{\sum_{i=1}^m B \text{ RPKM}} \right) / \log(2)$$

In this formula, Diffexp represents the differential expression of a gene related to taxol synthesis; A RPKM represents the RPKM value of the corresponding unigene in Sample case A; B RPKM represents the RPKM value of the corresponding unigene in Sample control B; n: The number of unigenes from the same gene related to taxol synthesis in Sample case A; m: The number of unigenes in the same taxol synthesis-related gene in Sample control B.

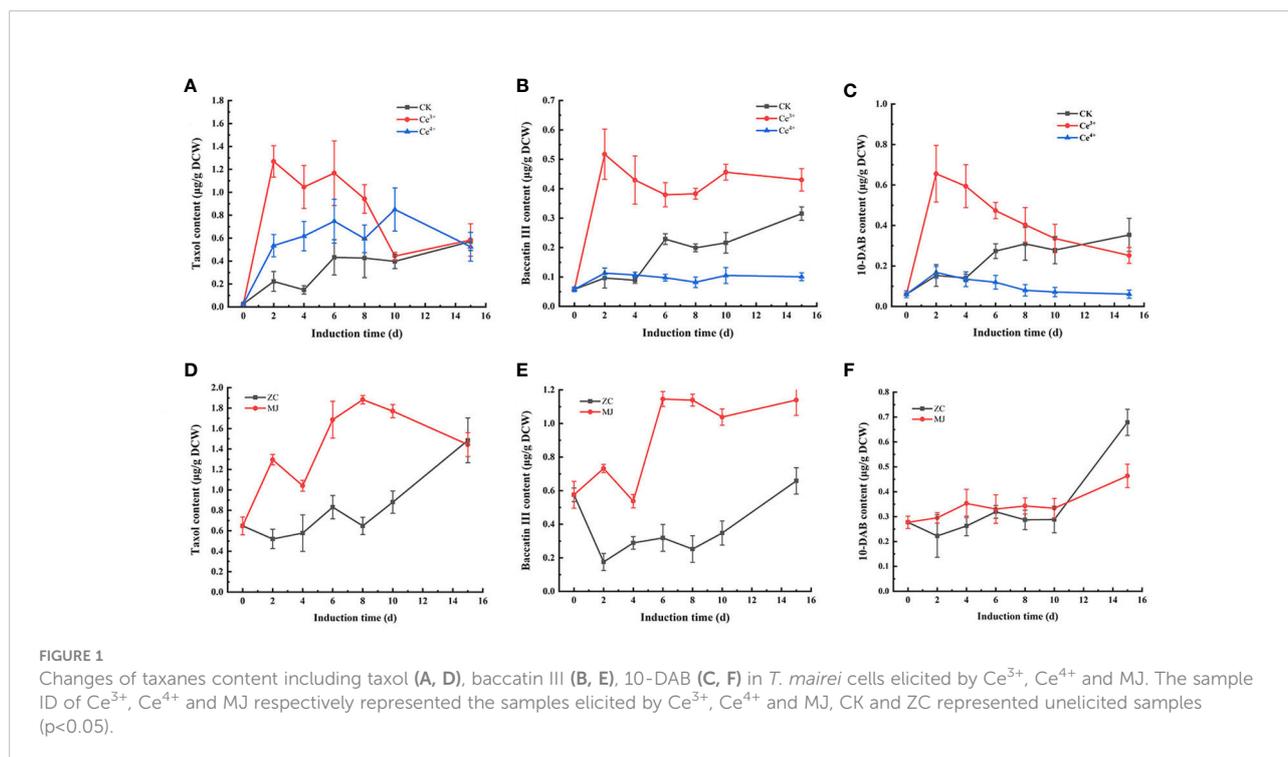
Sequence alignment and phylogenetic analyses

Using the maximum likelihood (ML) method in MEGA5 software, the WRKY phylogenetic tree was constructed and the WRKY domain sequences were aligned.

Results and discussion

Variation in the taxoid contents in elicited and unelicited *T. mairei* cells

The changes of taxol, baccatin III and 10-DAB contents in *T. mairei* cells elicited by 1 mM Ce³⁺, 1 mM Ce⁴⁺, and 100 μM MJ, respectively, were shown in Figures 1, 2. The results showed that



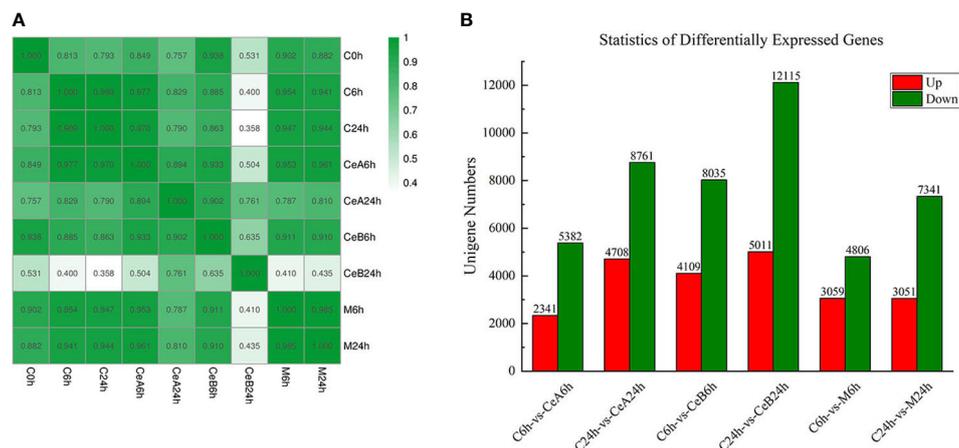


FIGURE 2

(A) Pearson correlation coefficients of all 9 samples. The expression level of each gene (the entire gene set) for each pair of samples was used to calculate the Pearson correlation coefficients, and the correlation coefficients between the two samples were visually displayed as a heat map. (B) The numbers of the up-regulated and down-regulated unigenes in the six comparisons.

Ce³⁺, Ce⁴⁺ and MJ all obviously increased the synthesis of taxol. The three systems were used as differential expression systems for studying taxol biosynthesis. Therefore, cell samples elicited by 1 mM Ce³⁺, 1 mM Ce⁴⁺, and 100 μM MJ for 6 h and 24 h were selected for transcriptome sequencing.

Transcriptome sequencing of elicited and unelicited *T. mairei* cells

The tender stem cell line of *T. mairei* was cultured in 1 mM Ce(NO₃)₃, 1 mM Ce(NH₄)₂(NO₃)₆ and 100 μM MJ elicitation system. Nine samples of elicited and unelicited cells cultured for 6 h and 24 h were selected for RNA transcription to form a library. The Illumina Hiseq4000 sequencing platform was used to generate a total of 62Gb of sequencing data, with approximate 60G of available data.

The Raw data for each sample was acquired. After base calling, preliminary quality analysis of the sequencing findings, and read filtering according to the processing guidelines, clean data can be acquired. In Table 1, quality data for each sample are presented. After filtration, each group received more than 40,000,000 reads.

The transcriptome sample data were assembled by trinity to create the contig sequence for each sample. The assembly results were then processed using sequence clustering software CDHIT for sequence splicing and redundancy removal to create a lengthy non-redundant unigene sequence. Sequence length and quantity were significant assessment factors for assembly quality. 88,326 unigenes were obtained through assembly, with an average length of 1076nt, N50 of 1811nt and GC ratio of 40.7%. The length of the unigenes was mainly distributed at 300 nt, followed by 400–1500 nt (Supplementary Material 1).

TABLE 1 Statistics of Transcriptome Sequencing Data of *T. mairei*.

Sample ID	Total Raw Reads	Total Clean Reads	Read Length	Total Clean Nucleotides (nt)	Q20 (%)	GC (%)
C0	55329508	53877612	100	5387761200	98.40	45.26
C6h	61569240	59946702	100	5994670200	98.45	45.85
C24h	46073434	44859848	100	4485984800	98.35	46.01
CeA6h	91452112	89071954	100	8907195400	98.40	45.42
CeA24h	75508994	73591328	100	7359132800	98.60	45.26
CeB6h	81491064	79391952	100	7939195200	98.45	45.81
CeB24h	58596356	57123852	100	5712385200	98.55	45.2
M6h	73107524	71158468	100	7115846800	98.25	45.55
M24h	76392090	74318888	100	7431888800	98.35	46.12

The sample ID of CeAx, CeBx and Mx respectively represented the samples elicited at different times under the conditions of Ce³⁺, Ce⁴⁺ and MJ, Cx represented unelicited samples for different times.

The functions of 51,516 unigenes were successfully annotated by NR, Swiss-Prot, KEGG, COG and GO. The number of unigenes annotated in each database was shown in [Supplementary Material 2](#).

Based on the Nr annotation results, blast2GO software was used to analyze the GO function of the gene ontology. The GO information of each gene and the GO function classification were obtained, and distribution characteristics of gene function of species at the macro level were known. Among 51400 unigenes with Nr annotations, 35031 unigenes were annotated in GO. Under the three major categories of “molecular function”, “cell component” and “participation in biological processes”, all unigenes were divided into 49 functional groups by WEGO, of which 7 categories including cell, cell part, organelle, binding, catalytic, cellular process, metabolic process were the main categories ([Supplementary Material 3](#)).

Differentially expressed gene analysis

The expression profiles of 88,326 unigenes were ultimately used for further analysis. [Figure 2A](#) showed the gene expression correlation between nine samples. The following differences were analyzed among six groups of samples: C6h-vs-CeA6h, C24h-vs-CeA24h, C6h-vs-CeB6h, C24h-vs-CeB24h, C6h-vs-M6h, C24h-vs-M24h. The statistical results of the differential

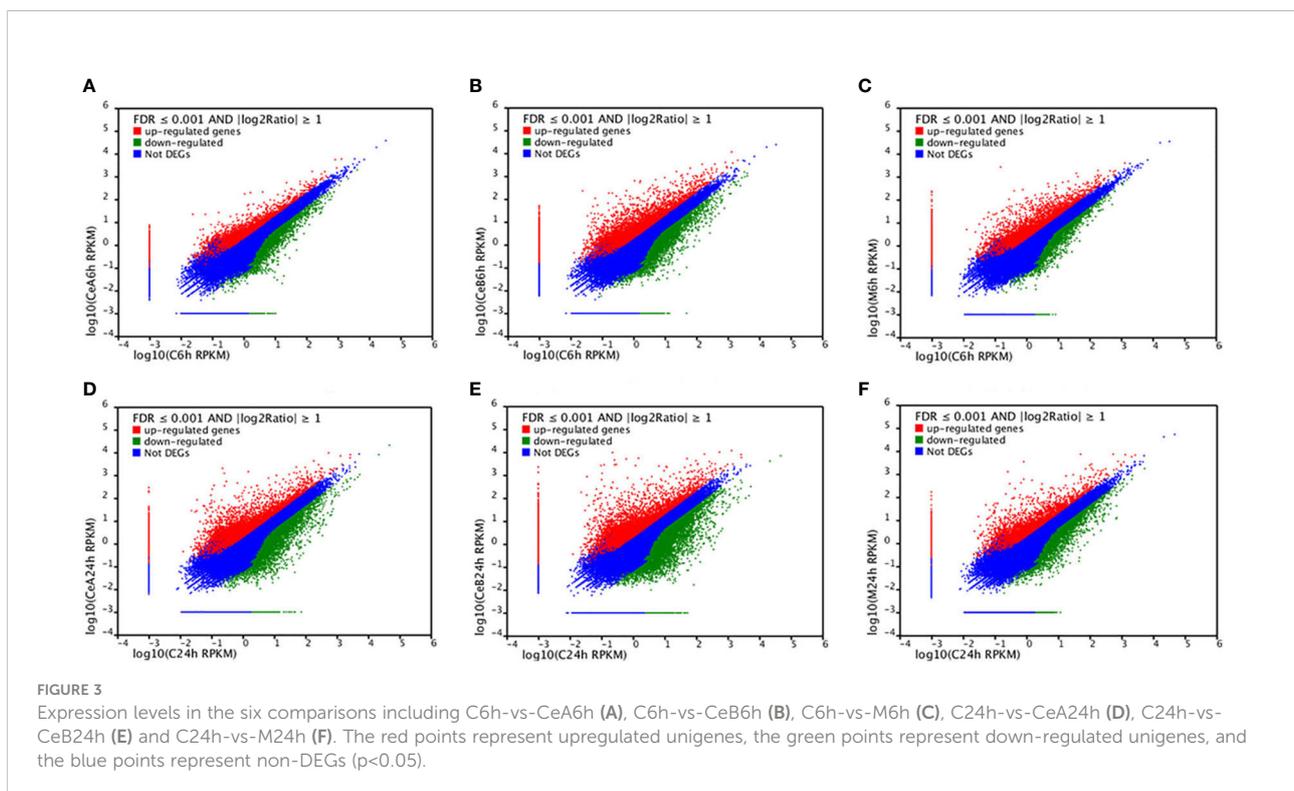
expression of unigenes in *T. mairei* under different elicitation conditions were shown in [Figure 2B](#).

[Figure 3](#) showed the changes in transcription level after elicitation of Ce^{3+} , Ce^{4+} and MJ, showing that the number of up-regulated genes and down-regulated genes increased with the treatment time prolonged.

Differential genes were significantly enriched for plant-pathogen interaction and carotenoid biosynthesis based on KEGG enrichment analysis of DEGs ([Figure 4](#)). The results showed that the metabolic pathways of plant-pathogen interaction and carotenoid biosynthesis were significantly changed under the elicitation conditions of 1 mM Ce^{3+} , 1 mM Ce^{4+} , and 100 μ M MJ in *T. mairei* cells.

The GO functional enrichment analyses of the DEGs in cellular component, molecular function and biological process were performed, respectively. The results showed that under the elicitation conditions of Ce^{3+} , Ce^{4+} and MJ, cell wall, external encapsulating structure, oxidoreductase activity, catalase activity, diterpenoid and paclitaxel biosynthesis and other cellular functions changed significantly in *T. mairei*.

The GO functional enrichment results of the differential genes showed that under the MJ elicitation, the paclitaxel biosynthesis process was significantly up-regulated and the redox process in the elicited *T. mairei* cells was significantly enhanced to provide energy supply for paclitaxel biosynthesis ([Figure 5](#), [Figure 6B](#)). However, the biosynthesis of paclitaxel was



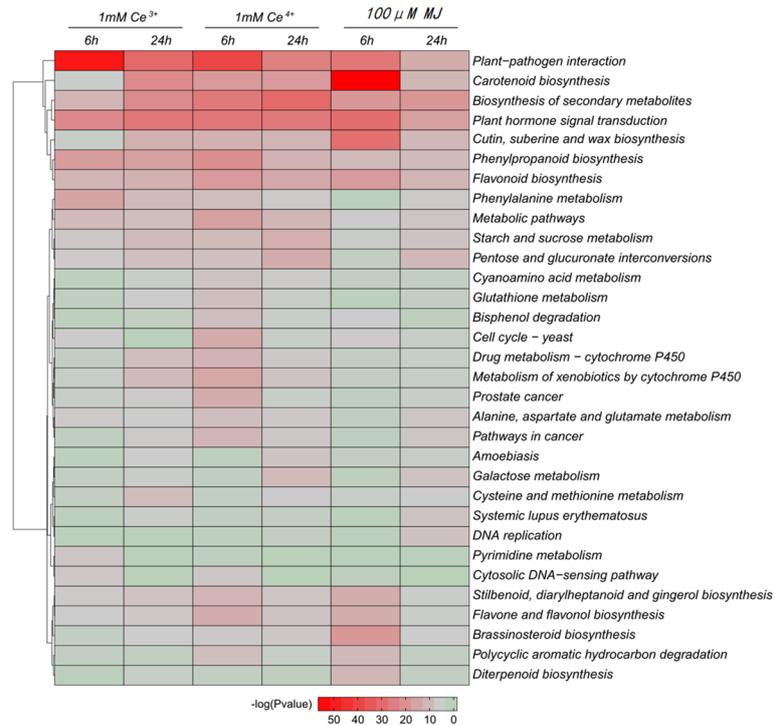


FIGURE 4 KEGG enrichment analysis of the DEGs in the six comparisons. The color indicates the significance level.

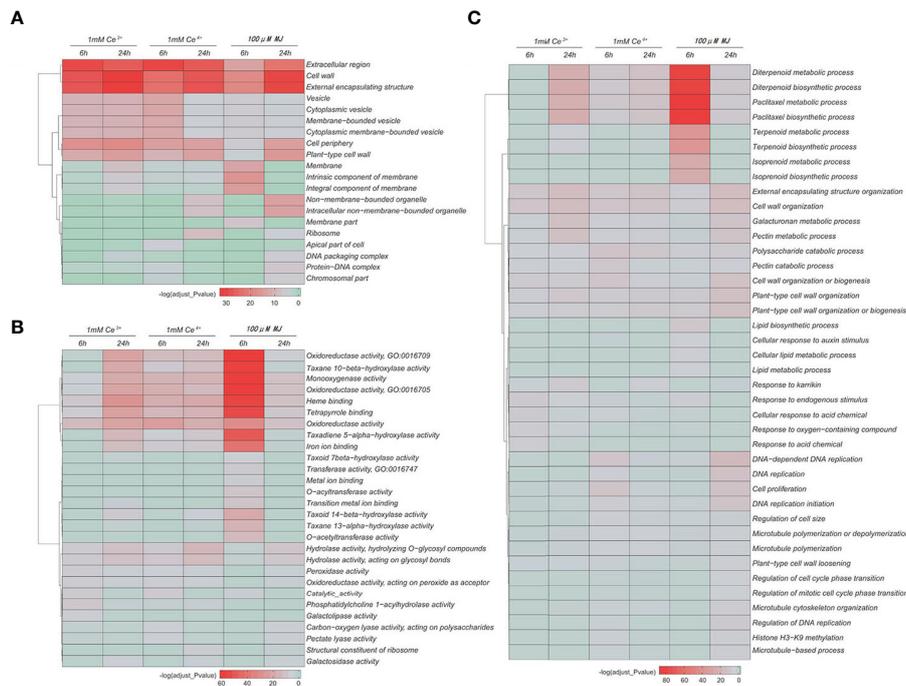


FIGURE 5 GO enrichment analysis of the DEGs in the six comparisons. (A) Cell composition (B) Molecular function (C) Biological process. The color indicates the significance level.

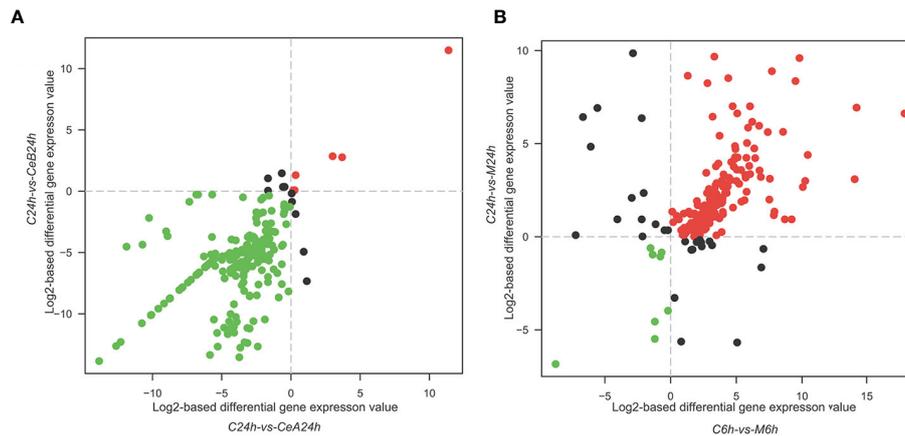


FIGURE 6

Comparison of gene expression during paclitaxel biosynthesis (GO:0042617) under different elicitation conditions. (A) The difference in gene expression of paclitaxel biosynthesis with 1 mM Ce^{3+} and 1 mM Ce^{4+} elicitation for 24 hours respectively. (B) The difference in gene expression of paclitaxel biosynthesis with 100 μ M MJ elicitation for 6 and 24 hours.

significantly down-regulated at 1 mM Ce^{3+} and 1 mM Ce^{4+} elicitation (Figure 5, Figure 6A).

DEGs involved in taxol biosynthesis

A total of 179 unigenes were identified as 12 taxol synthase coding genes by homologous comparison analysis. To elucidate the changes of DEGs in the taxol biosynthesis pathway after elicited by Ce^{3+} , Ce^{4+} and MJ, differential enrichment analyses on gene expression of each gene were performed, and the results were shown in Figure 7A. The transcriptional expression changes of genes in taxol biosynthesis pathway were shown in Figure 7B. Gene expression levels of elicited cells showed obvious regularity compared with unelicited cells. Interestingly, the taxol synthesis genes were up-regulated under the MJ elicitation condition, but the taxol biosynthesis genes were down-regulated under the 1 mM Ce^{3+} and 1 mM Ce^{4+} elicitation conditions. It might be that Ce^{3+} and Ce^{4+} could significantly induce the *T. mairei* cells defensive response leading to apoptosis, and the expression of taxol synthesis gene in *Taxus chinensis* cells responded very quickly to cerium. A previous study (Yang et al., 2006) found that in the cell apoptosis elicited by Ce^{4+} , an ERK-like kinase was rapidly activated at 5 min of elicitation, and continuously activated within 120min. After 240 min of elicitation, ERK-like was down-regulated and maintained until 48 h. Additionally, O_2^- was rapidly elicited by Ce^{4+} . The first burst of O_2^- appeared in 3.7–4 h, and the second burst of O_2^- appeared in 7 h. However, the specific mechanism of this interesting phenomenon need more study.

Taxol is a phytoalexin produced by the defensive reaction of taxus cells when infected by pathogenic bacteria, and the synthesis of taxol will be affected by the external environment and the cell state (Yuan et al., 2002). KEGG Pathway enrichment of the differential genes indicated that the plant-pathogen interaction pathway in *T. mairei* was significantly up-regulated after elicitation by Ce^{3+} and Ce^{4+} . Hypersensitive response (HR) induced by the non-affinity interaction of plant pathogens is a typical programmed cell death model (Yang et al., 2006).

Responsive genes involved in plant-pathogen interaction

The KEGG Pathway enrichment of DEGs indicated that plant-pathogen interaction pathway in *T. mairei* was significantly up-regulated after elicited by Ce^{3+} , Ce^{4+} and MJ. Plant-pathogen interaction pathway was therefore analyzed. Figure 8 shows the differential expression of proteins in the plant pathogen interaction pathway in C6h-vs-CeA6h, C6h-vs-CeB6h and C6h-vs-M6h.

The genes encoding CDPK were significantly up-regulated in C6h-vs-CeA6h and C6h-vs-CeB6h. The receipt and decoding of calcium signals were significantly regulated by the calcium-dependent protein kinase (CDPK), a class of key calcium signal receptors. Plant physiological activities such as growth and development, stress responses, and hormone signaling all depend on CDPKs. (Xiao et al., 2017; Li et al., 2022)

The genes encoding CNGCs were significantly up-regulated in C6h-vs-CeA6h and C6h-vs-M6h and down-regulated in C6h-

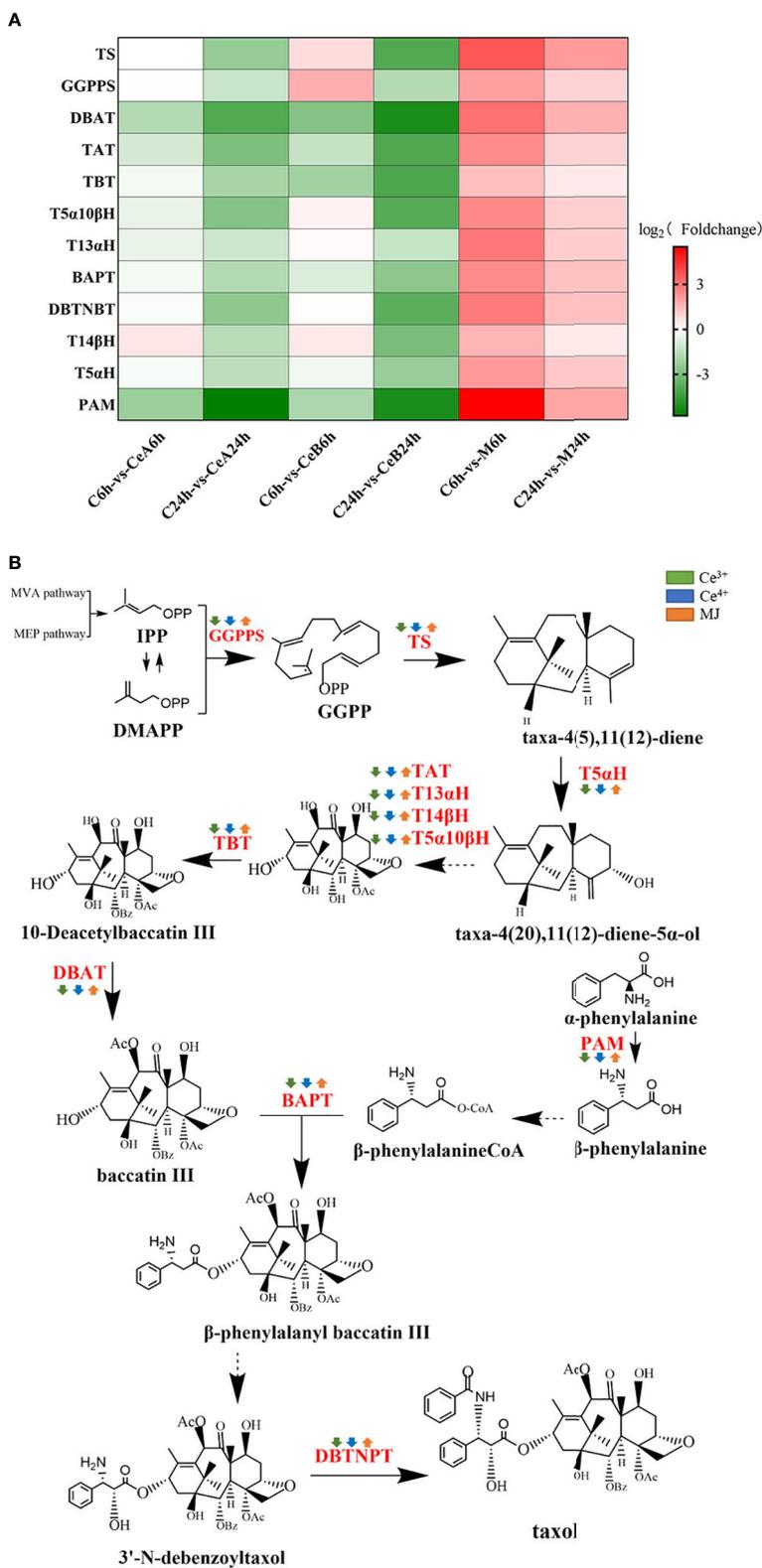


FIGURE 7
(A) Comparison of DEGs in taxol biosynthesis pathway. The color density indicated \log_2 (Foldchange). Red indicated the upregulation of gene expression and green indicated the downregulation of gene expression. **(B)** DEGs assigned to the taxol biosynthesis pathway. Green is elicited by Ce^{3+} , blue is Ce^{4+} , orange is MJ. The up arrows indicate the upregulation of gene expression; the down arrows indicate the downregulation of gene expression.

vs-CeB6h. A class of non-selective cation channels called cyclic nucleotide-gated channels (CNGCs) was triggered by the direct binding of cyclic nucleotides like cAMP and cGMP to regulate cellular communication. These channels were mostly permeable to Ca^{2+} . Plant growth, pathogen defense, and thermotolerance were all impacted by the CNGC family. (Kakar et al., 2017; Zhao et al., 2022)

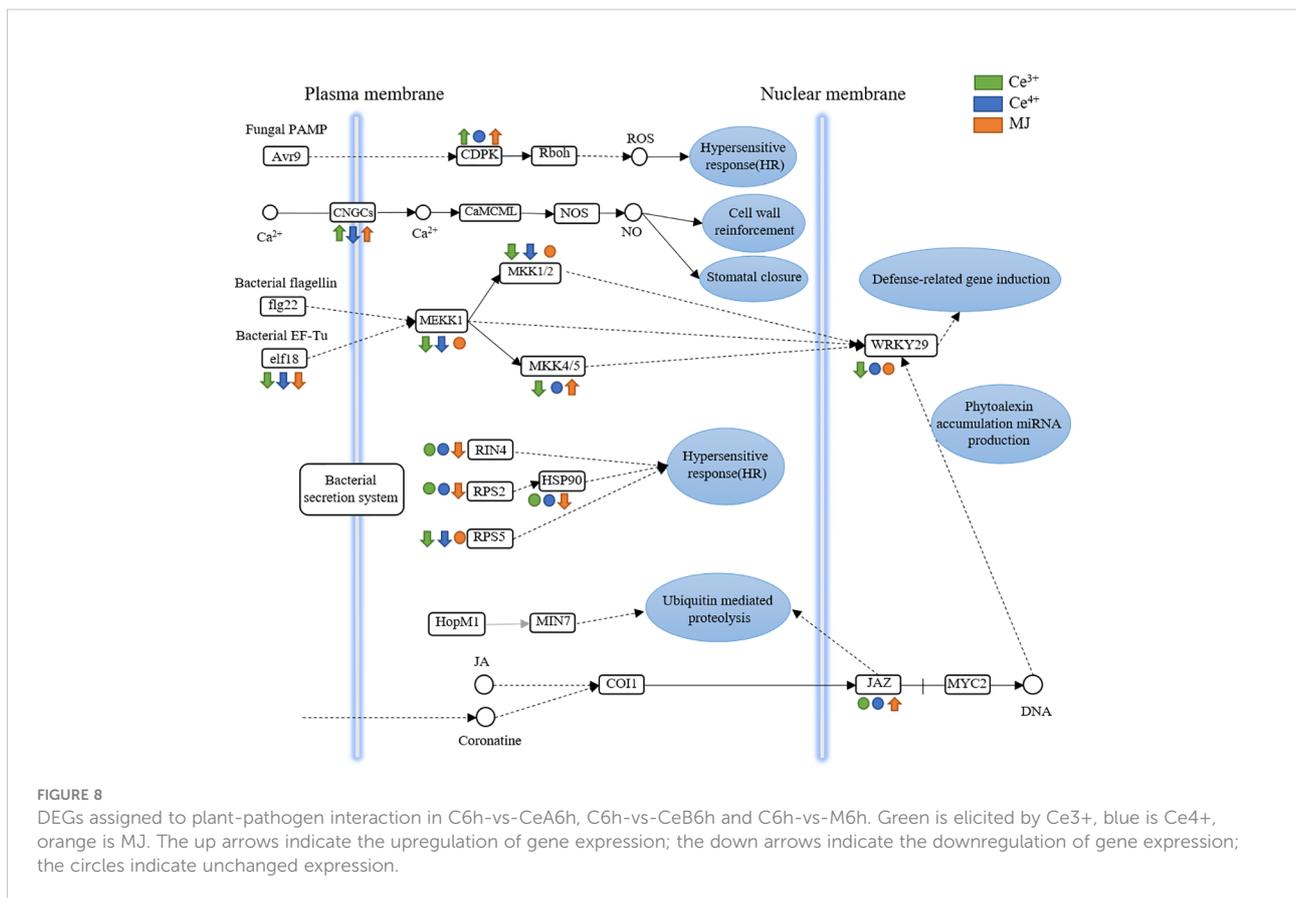
The genes encoding elf8, MKK and MEKK were significantly down-regulated in C6h-vs-CeA6h and C6h-vs-CeB6h. These signals were transmitted into the nucleus and to transcription factors (WRKY29). The down-regulation of WRKY29 indicated the genes related to immune defense was down expression. The evolutionarily conserved MAPK signaling cascade, which was crucial for plant growth and development as well as various stress responses, was mainly composed of mitogen-activated protein kinase kinases (MKK). (Li et al., 2020)

The genes encoding JAZ were significantly up-regulated in C6h-vs-M6h. The primary repressors in the jasmonate signaling system were proteins from the Jasmonate ZIM-domain (JAZ) family, which were essential for plant growth, defenses, and stress responses. (Thines et al., 2007; Gfeller et al., 2010) Therefore, MJ might promote the expression of the JAZ-encoding gene and inhibit the JA signal transduction pathway. There were multiple regulators involved in the sensitive and

dynamic process of JA recognition, signal transduction, and transcript reprogramming of downstream genes (Chini et al., 2007). Previous studies revealed the key role of JAZ protein in the biosynthesis of specific plant metabolites such as alkaloids, artemisinin and tanshinone. In *Artemisia annua*, AaJAZ8 negatively regulated artemisinin biosynthesis, the first line of defense against malaria, in response to JA elicitation (Ma et al., 2018). In *Salvia miltiorrhiza*, the overexpression of SmJAZ3 and SmJAZ9 reduced the content of tanshinone (Shi et al., 2016). Therefore, the JAZ gene had a wide range of roles in regulating adaptation to environmental challenges and in regulating the development and specific metabolism of different plants.

Responsive genes involved in MAPK signaling pathway

Studies confirmed that 0.1 mM Ce^{4+} can effectively improve the soluble protein synthesis and cell viability of *Taxus cuspidata* cells in suspension culture and increase taxol biosynthesis and release, while 1 mM Ce^{4+} can significantly induce apoptosis of *Taxus cuspidata* cells (Yingjin et al., 1998; Ge et al., 2005). Taxol is a kind of plant protectant. Rare earth can stimulate taxus cells to improve taxol synthesis, indicating that rare earth can



enhance the defensive response of taxus. (Yuan et al., 2002) Studies showed that Ce^{4+} induced *Taxus cuspidata* cells to activate O_2^- generate through the NADPH oxidase pathway, which mediated the MAPK pathway or NO signaling molecule, and activated the downstream caspase-like enzyme through the signaling cascade (caspase-like family or mitochondrial pathway). The activation of three nucleases was a downstream event of signal transduction process, regulated by caspase3-like or other signaling molecules, and finally led to apoptosis. (Yuan, 2006)

There are varieties of MAPK pathways in plant cells. Each pathway is related and independent, and they play an important role in cell signal transduction (Sharma et al., 2021). In plants, the MAPK cascade is intertwined with other signal transduction pathways to form a molecular interaction network (Andreasson and Ellis, 2010).

Figure 9 showed the differential expression of proteins in the MAPK pathway in C6h-vs-CeA6h, C6h-vs-CeB6h and C6h-vs-M6h. Genes encoding MKP, PPP3C and MLK3 were significantly up-regulated and genes encoding MEKK1, MEKK2/3, LZK, DAXX, ASK1, TAK1, etc. were significantly down-regulated in C6h-vs-CeB6h. Besides, *T. mairei* cells elicited by Ce^{3+} and MJ showed the same gene expression trend like that elicited by Ce^{4+} .

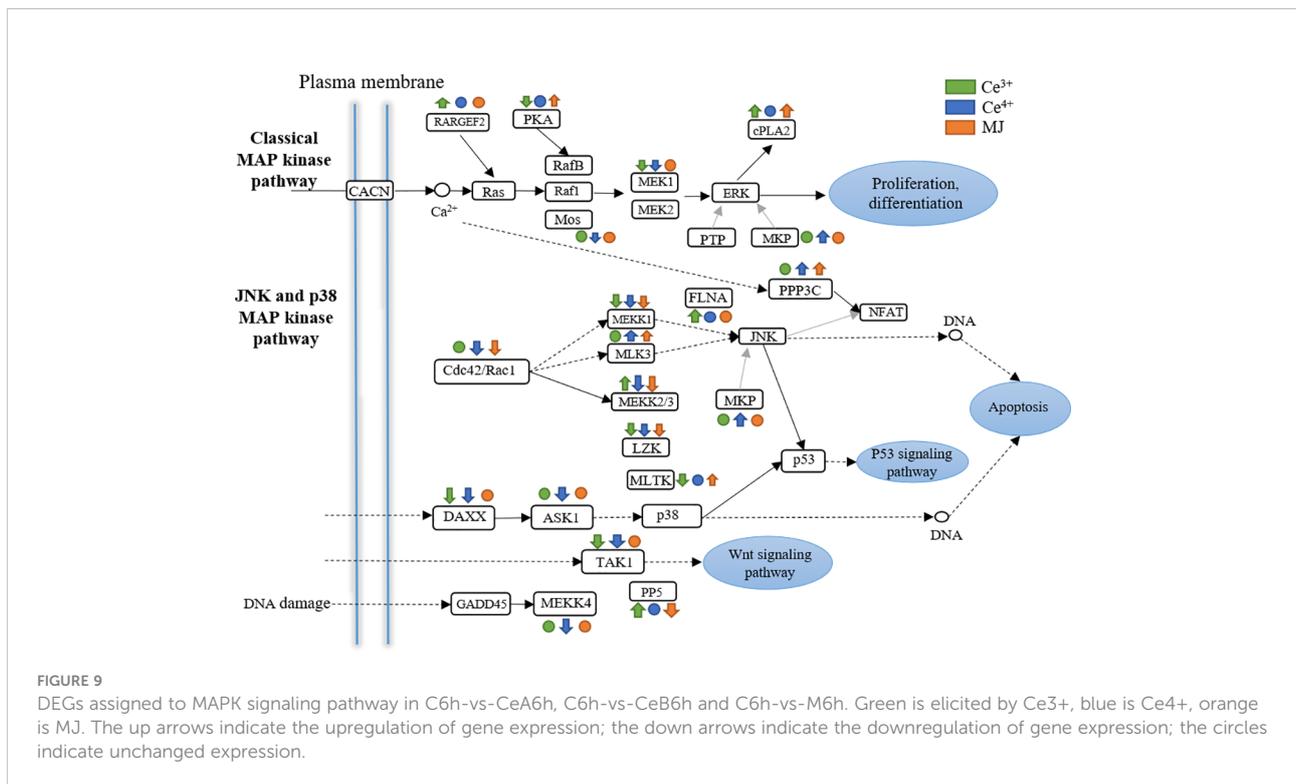
MEKK1 protein is a key kinase activator for cell stress response. Activation of MEKK1 can stimulate a variety of reactions, including mitogen-activated protein (MAP) kinase

and cell migration (Filipcik et al., 2020). MEKK2 and MEKK3 belong to the MEKK/STE11 subfamily that is widely expressed and effective activators of the NF- κ B and MAPK pathways (Zhang et al., 2006). The Death-domain associated protein 6 (DAXX) is an evolutionarily conserved and widely expressed multifunctional protein, which is involved in many cellular processes, including transcription, cell proliferation, cell cycle regulation, Fas-induced apoptosis and many other events. In the nucleus, DAXX interacts with transcription factors, epigenetic modifiers, and chromatin-remodeling proteins (Bogolyubova and Bogolyubov, 2021). TAK1 functions as a mediator in the signaling pathway of transforming growth factor-beta (TGF-beta) superfamily members (Yamaguchi et al., 1995).

Diverse cellular functions in plants, including growth, development, biological and abiotic stress responses, are regulated by this network. For instance, MAPK in plants can target and regulate transcriptional factors including bZIP, MYB, MYC and WRKY under stress conditions (Zhang and Zhang, 2022).

Regulation of the expression of WRKY

By stimulating the co-expression of many genes in the secondary metabolic pathway, TFs contribute significantly to the control of secondary metabolite production and accumulation (Hong et al., 2013). It has been reported that several transcription



factor families, including WRKY, bHLH, and AP2/ERF, are involved in the production and accumulation of taxol (Dai et al., 2009; Li et al., 2013; Lenka et al., 2015; Zhang et al., 2015).

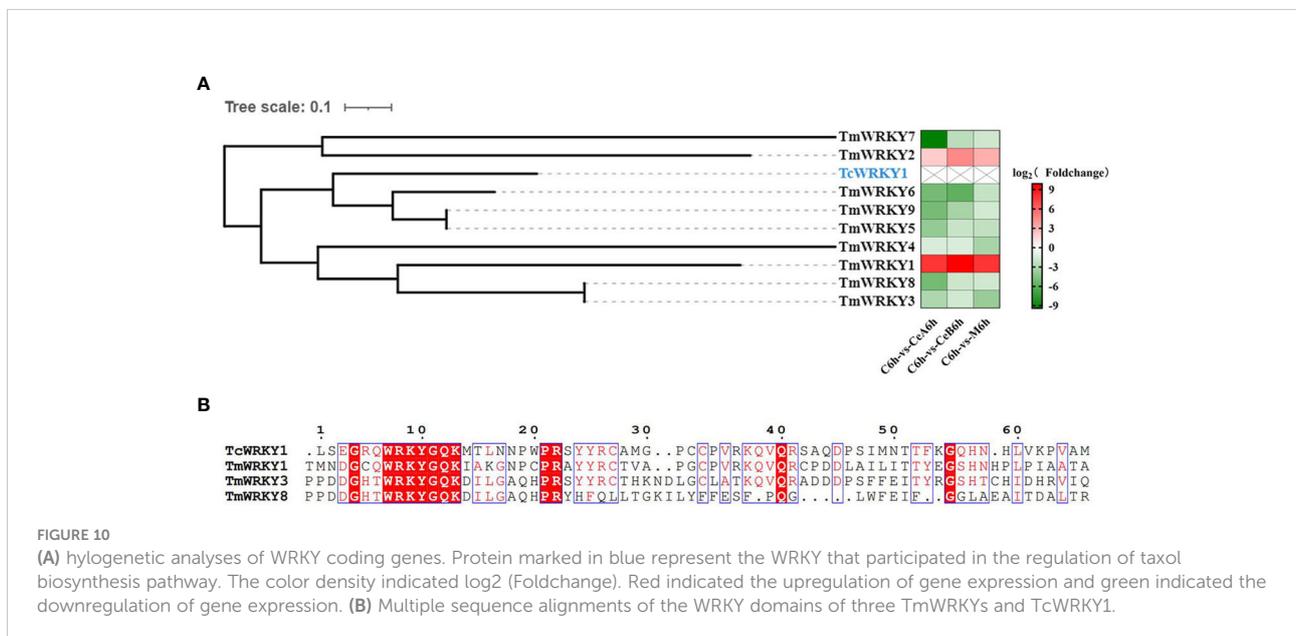
The conservative WRKYGQK motif in the WRKY domain is whence WRKY got its name (Wu et al., 2005). A group of plant-specific transcription factors (TFs) known as WRKY is crucial for pathogen defense, non-biological signaling, plant hormone signaling, and the control of secondary metabolism (Zhang et al., 2018). For example, AaWRKY1 regulated the amorpha-4,11-diene synthase, which was involved in the biosynthesis of artemisinin in *Artemisia annua* (Dongming Ma et al., 2009). GaWRKY1 regulated the sesquiterpene synthase CAD1 ((+)- δ-cadinene synthase -A) gene, which was involved in the regulation of the gossypol pathway in plant cotton (Xu et al., 2004). A study has found that TcWRKY1 can specifically interact with the promoter of the DBAT gene in paclitaxel biosynthesis. The other two WRKY TF, TcWRKY8 and TcWRKY47, significantly increased the expression levels of several genes related to taxol biosynthesis (Li et al., 2013). All these results indicated that the WRKY factor played an important role in taxol biosynthesis.

The transcriptome data showed that 83 unigenes were annotated to encode putative WRKY in Plant Transcription Factor Database. Sequence alignment and phylogenetic analysis of 9 differentially expressed WRKYs in the data (Supplementary Material 4) with TcWRKY1, which were known to be involved in taxol synthesis, revealed some homology of their nucleotide sequences (Figure 10A). Compared with control, 7 of the 9 genes encoding WRKY in three elicitation groups were all down-

regulated, and other s were up-regulated. Additionally, the WRKY domain sequences alignments of three TmWRKYs and TcWRKY1 were shown in Figure 10B.

Conclusion

The transcriptome of *T. mairei* cells unelicited and elicited by cerium and MJ were studied using Illumina sequencing technology. Analysis of the annotated unigenes revealed significant transcriptional complexity in *T. mairei* cells and provided more information for *T. mairei* cells response to cerium and MJ. After elicited by cerium and MJ, the downstream signal transduction stimulate the channels of MAPKs and other signal pathway components through superoxide anion. Then key TFs related to defense, such as WRKY, were triggered. TFs activated downstream defense pathways, such as JA signal transduction pathway and plant-pathogen interaction pathway, which finally increased taxol biosynthesis. Notably, Ce³⁺, Ce⁴⁺ and MJ can significantly increase taxol biosynthesis and release in *T. mairei*. However, taxol synthase coding genes had higher transcription levels by adding MJ, while Ce³⁺ and Ce⁴⁺ generally reduced the transcription levels of these genes. This might be that cerium could significantly induce the plant defensive response of taxus cells, leading to cell apoptosis. In conclusion, our transcriptome data will serve as an important public information platform for the comprehensive understanding of the cerium and MJ response networks and regulatory patterns, as well as the



molecular mechanisms of cerium-mediated and MJ-mediated taxol biosynthesis in *T. mairei*.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, SRR20217393.

Author contributions

Conceptualization and design: NH, W-JG, S-TL, and P-PH. Methodology and formal analysis: NH, S-TL, Y-RL, and P-PH. Validation: S-TL, NH, JL and W-JG. Writing original draft preparation: NH, W-JG, JL, and P-PH. Plant cell culture: NH, JZ, Y-JW, H-HX and M-YL. Supervision and project administration: P-PH. Writing - review and editing: All authors. All authors contributed to the article and approved the submitted version.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.1040596/full#supplementary-material>

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