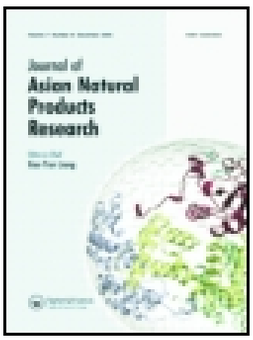

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The MVA pathway genes expressions and accumulation of celastrol in *Tripterygium wilfordii* suspension cells in response to methyl jasmonate treatment

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ABSTRACT

Celastrol is an important bioactive triterpenoid in traditional Chinese medicinal plant, *Tripterygium wilfordii*. Methyl Jasmonate (MJ) is a common plant hormone which can regulate the secondary metabolism in higher plants. In this study, the mevalonate (MVA) pathway genes in *T. wilfordii* were firstly cloned. The suspension cells of *T. wilfordii* were elicited by MJ, and the expressions of MVA pathway genes were all enhanced in different levels ranging from 2.13 to 22.33 times of that at 0 h. The expressions were also enhanced compared with the CK group separately. The accumulation of celastrol in the suspension cells after the treatment was quantified and co-analyzed with the genes expression levels. The production of celastrol was significantly increased to 0.742 mg g^{-1} after MJ treatment in 288 h which is consistent with the genes expressions. The results provide plenty of gene information for the biosynthesis of terpenoids in *T. wilfordii* and a viable way to improve the accumulation of celastrol in *T. wilfordii* suspension cells.

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Celastrol; methyl jasmonate; mevalonate pathway; *Tripterygium wilfordii*; triterpenoids

1. Introduction

Celastrol is one of the bioactive triterpenoids in the traditional Chinese medical plant, *Tripterygium wilfordii* (Leigongteng). The compound has been demonstrated to be anti-cancer [1], antioxidant [2], and anti-inflammatory [3] and was recently gaining importance for treatment of cancer and inflammation. Many researches are interested in the relative mechanisms and clinical application of the compound. Moreover, recent research found that celastrol is a promising therapeutic for Gaucher disease [4] and a leptin sensitizer for the treatment of obesity [5,6].

Celastrol is a triterpenoid secondary metabolic product in *T. wilfordii* and was derived from the natural plants traditionally. However, the content of the compound in natural plants was far below the quantity demanded. Therefore, it is more convenient to produce the

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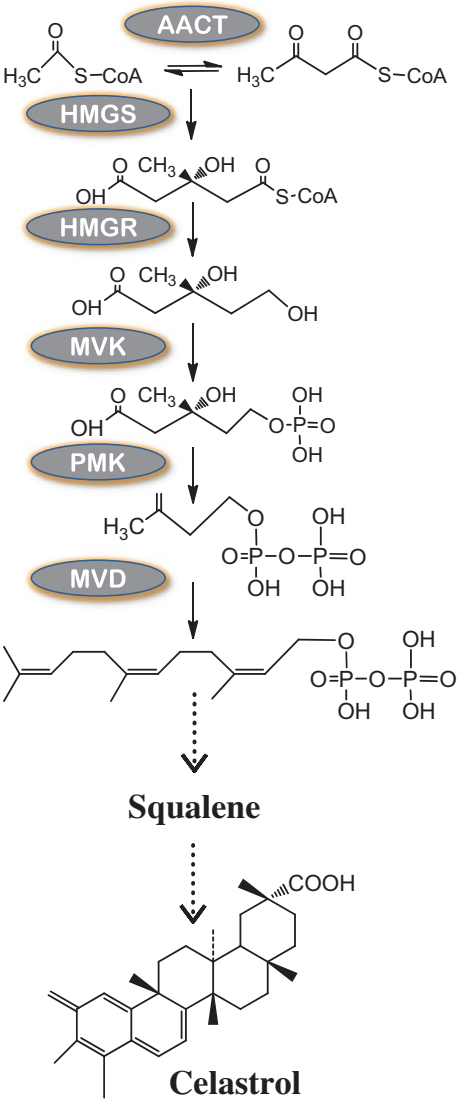

Figure 1. Biosynthesis pathway of celastrol.

compound through tissue cultivation or synthetic biology strategies [7]. The biosynthesis pathway of celastrol can be divided into three periods: the biosynthesis of terpenoids backbone, the formation of squalene, and the specific catalysis in *T. wilfordii* to produce celastrol. The mevalonate pathway has six steps and the enzymes including acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and diphosphomevalonate decarboxylase (MVD). The MVA pathway provides isopentenyl pyrophosphate (IPP) for triterpene biosynthesis. IPP was catalyzed to form farnesyl diphosphate (FPP). FPP was then catalyzed to form squalene by squalene synthase (SQS) [8,9],

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Table 1. MVA pathway genes in *T. wilfordii*.

Gene	Accession number	Full length (bp)	ORF site (bp)	Amino acid residue (aa)	pI	MW (kDa)
TwAACT2	KR260988	1529	125–1537	410	6.55	42.54
TwHMGR1	KM983112	2396	91–1830	584	6.37	61.60
TwHMGR2	KM983113	2132	78–1844	516	6.53	63.24
TwMVK	KR260989	1283	44–1207	345	5.76	41.17
TwPMK	KR260990	1789	33–1562	504	5.42	55.25
TwMVD	KR260991	1413	34–1293	377	6.04	46.52

squalene is the precursor of triterpenoids. Squalene was catalyzed by squalene epoxidase and specific oxidosqualene cyclase to form celastrol (Figure 1).

Methyl jasmonate (MJ) is an important regulator in plant development and fertility. It is a fragrant volatile compound and plays the role by MJ signaling [10]. Many researches have demonstrated that the MJ signaling pathway is connected to other pathways, and the network of the pathways has influence on the secondary metabolism [11,12].

It is essential to obtain the gene information about the terpenoids biosynthesis pathway in *T. wilfordii* for improving the celastrol production through synthetic biology strategies. However, the enzymes involved in the biosynthesis of terpenoids in *T. wilfordii* are quite limited. In a previous study, we firstly cloned the full-length gene encoding 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) in *T. wilfordii* [13]. In this recent study, we first cloned the full-length cDNA of the other genes in the MVA pathway in *T. wilfordii*. MJ was used to treat the suspension cells of *T. wilfordii,* and the post-treatment expression levels of the MVA pathway genes were detected systematically. Meanwhile, the accumulation of celastrol after the treatment was determined by HPLC method to investigate the relative relationship between the MVA pathway and the accumulation of triterpenoids in *T. wilfordii*.

2. Results

2.1. Cloning and sequence analyses of the genes

The full-length sequences of the MVA pathway genes in *T. wilfordii* were obtained from this study. The pathway contains 2 AACT genes, 1 HMGS gene, 2 HMGR genes, 1 MVK gene, 1 PMK gene, and 1 MVD gene. According to the National Center for Biotechnology Information (NCBI) database, one of the AACTs (accession number, KR297934) and the HMGS (accession number, KM978213) were reported before. Therefore, the AACT sequence we got in this study was designated as *Tw*AACT2. The other ORFs of the genes obtained in this study were named as *TwHMGR1, TwHMGR2, TwMVK, TwPMK, and TwMVD*. The lengths of cDNA and amino acid residues as well as the ORF sites, the theoretical isoelectric points (pIs), MW, and the accession numbers of the genes are shown in Table 1.

2.2. Multiple alignment analysis of the genes

All the six MVA pathway genes we got in the study presented high identities with the sequences of the same family in GenBank according to the BLAST and the multiple aligned analyses. The results show that the MVA pathway genes are highly conserved in plants. The figures of the multiple alignment analyses can be found in the supplement materials.

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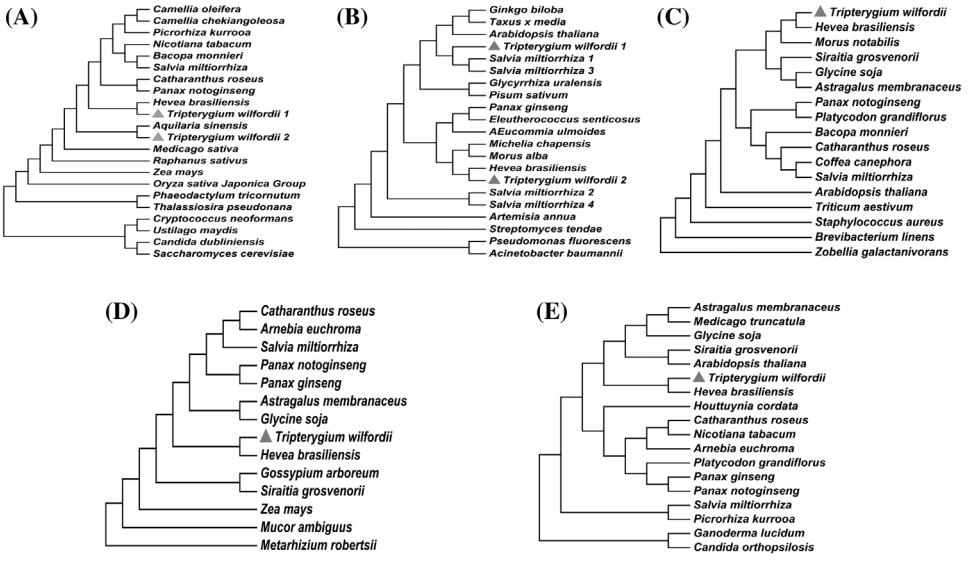

Figure 2. Phylogenetic trees of MVA pathway enzymes. (A) phylogenetic tree of AACTs; (B) phylogenetic tree of HMGRs; (C) phylogenetic tree of MVKs; (D) phylogenetic tree of PMKs; (E) phylogenetic tree of MVDs.

*Tw*AACT2 showed 80.48% identity to *Tw*AACT1 and also had high similarity to AACTs from other plants such as *Hevea brasiliensis* (83%, AAL18924.1), *Aquilaria sinensis* (83%, AHZ96597.1), and *Bacopa monnieri* (83%, ACU87560.2). The thiolase conserved site exists between 346 and 362 amino acids and the thiolase active site exists between 381 and 394 amino acids according to the functional domain analysis.

*Tw*HMGR1 showed 72.45% identity to *Tw*HMGR2. The two *Tw*HMGRs both contain two HMG-CoA binding sites: EMP(V/I)GY(V/I)QIP and TTEGCLVA, two NADP(H) binding sites: DAMGMNM and GTVGGGT. *Tw*HMGR1 and *Tw*HMGR2 both had high similarity to HMGRs from other plants. *Tw*HMGR1 showed 81% identity to *Lc*HMGR (AET72043.1), 78% identity to *Hb*HMGR (BAF98281.1), and 79% identity to *Tm*HMGR (AAY68034.1). *Tw*HMGR2 showed 83% identity to *El*HMGR (AFZ93642.1), 86% identity to *Hb*HMGR (BAF98281.1), and 82% identity to *Bp*HMGR (AHX36946.1).

*Tw*MVK showed 76% identity to *Mn*MVK (XP010105842.1), 77% identity to *Hb*MVK (AAL18925.1), and 74% identity to *Cr*MVK (ADR65111.1).The MVK conserved site exists between 138 and 149 amino acid.

*Tw*PMK showed 77% identity to *Am*PMK (AID51440.1), 75% identity to *Pn*PMK (AIK21784.1) and *Sg*PMK (AEM42973.1), and 78% identity to *Hb*PMK (AAL18926.1).

*Tw*MVD showed 84% identity to *Hb*MVD (AAL18927.1), 82% identity to *Am*MVD (AID51442.1), and 82% identity to *Gs*MVD (KHN11470.1).

2.3. Phylogenetic tree construction

The phylogenetic trees were constructed by neighbor-joining method according to the deduced amino acid sequence of the MVA pathway genes and the same gene families from different hosts (Figure 2). The MVA pathway genes in *T. wilfordii* were all clustered in plants. However, *Tw*AACT2 did not cluster with *Tw*AACT1 and neither did *Tw*HMGR1 clustered

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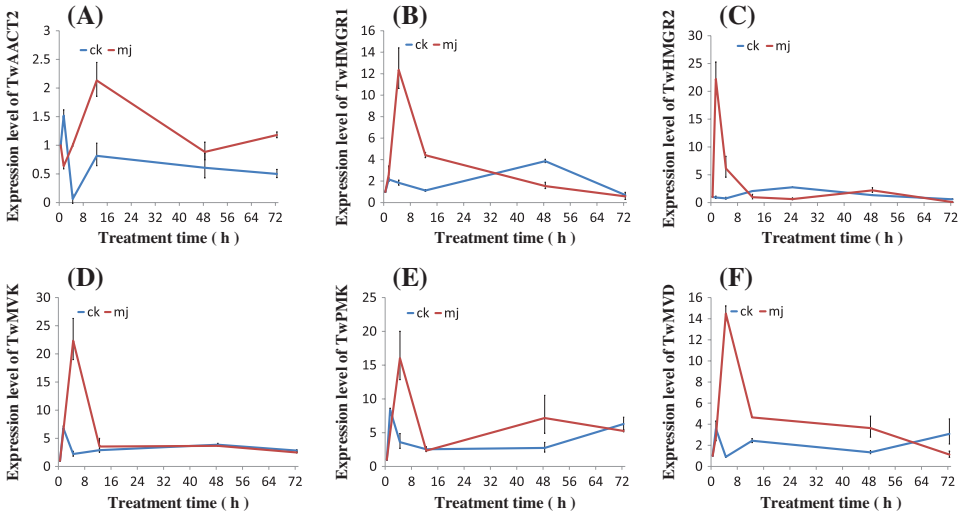

Figure 3. Expression levels of MVA pathway genes after MJ treatment. CK, the control group. MJ, the MJ treatment group. (A) Expression levels of *Tw*AACT2; (B) Expression levels of *Tw*HMGR1; (C) Expression levels of *Tw*HMGR2; (D) Expression levels of *Tw*MVK; (E) Expression levels of *Tw*PMK; (F) Expression levels of *Tw*MVD.

with *Tw*HMGR2. From the phylogenetic trees of different gene families, the sequences we cloned from *T. wilfordii* were close to those in *H. brasiliensis*, which is a plant known to produce isoprenoid polymer.

2.4. Expressions of the MVA pathway genes after MJ treatment

The real-time quantity results showed that the MVA pathway genes expression levels were significantly induced by 50 μM MJ in the suspension cell cultures. The expression levels of the genes reached the highest point at an early time after the induction. As the time gets longer, the expression levels get similar to that in the CK group. However, the expression level of *Tw*AACT was a little different from that of the other genes though it was also induced by MJ. It did not get to a very high expression level and the highest expression level was 2.13 times of that at 0 h. Also, the expression level was increased later than the other MVA pathway genes. TwHMGR1, TwMVK, TwPMK, and TwMVD reached the highest level at 4 h after the treatment, and the expression levels were 12.36, 22.33, 16.02, and 14.49 times of that at 0 h, respectively. TwHMGR2 was the most quickly responded gene in the pathway and got to the highest expression level (22.22 times of that at 0 h) earliest at 1 h after MJ treatment (Figure 3).

2.5. Chemical analysis of celastrol

The product of celastrol in suspension cells of *T. wilfordii* was detected at 0, 4, 48, 72, 120, 288 h after MJ treatment (Figure 4). The original production of celastrol in the suspension cells was quite low (0.0471 mg g^{-1}). The fold change of the accumulation was significant and takes a longer time than the gene expressions. The accumulation reached the highest

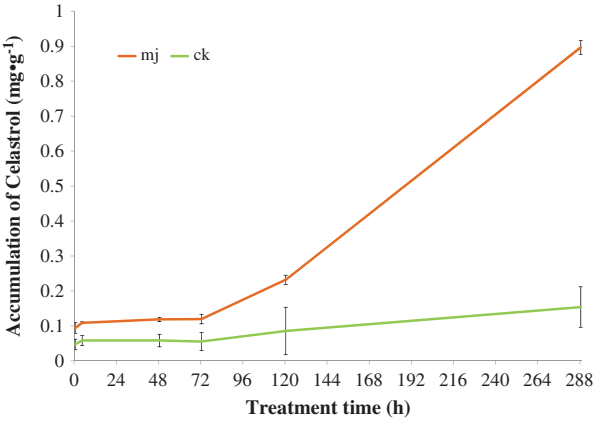

Figure 4. Accumulation of celastrol after MJ treatment.

of 0.742 mg g^{-1} in the MJ group while the content in the CK group was 0.154 mg g^{-1} at 288 h after the treatment.

3. Discussion

The biosynthesis of triterpenoids in higher plants is quite complex with many enzymes involved in. Meanwhile, the special structures of the bioactive products make it hard to derive through traditional chemical synthetic methods. Therefore, analysis of biosynthesis pathways of the bioactive compounds, such as celastrol, is essential and basic in regulating its product by various strategies. Researchers have successfully explored the terpenoids biosynthesis in other medical plants such as *Artemisia annua* [14], *Taxus chinensis* [15], *Panax ginseng* [16] and found new ways to produce the bioactive compounds. The sequences of the genes and proteins involved in the synthesis made it possible for further investigation.

The series reactions of the MVA pathway provide IPP for terpenoids biosynthesis in *T. wilfordii*. According to our results, the pathway contains 2 *AACT* gene, 1*HMGS* gene, 2 *HMGR* genes, 1 *MVK* gene, 1 *PMK* gene, and 1 *MVD* gene in *T. wilfordii*. In this study, we first reported the full-length genes in the MVA pathway including 1 AACT genes, 2 HMGR genes, 1 MVK gene, 1 PMK gene, and 1 MVD gene in *T. wilfordii*. The bioinformation of the sequences was analyzed according to the data reported in NCBI. All the MVA pathway genes in *T. wilfordii* present high similarities with those in other higher plants. The phylogenetic trees of the enzymes showed that the MVA pathway enzymes in *T. wilfordii* have high identities with those in *H. brasiliensis*.

In order to find the relationship between the gene expressions and accumulation of celastrol, we induced the suspension cells with MJ. The MVA pathway genes in *T. wilfordii* were highly induced at an early time and rapidly recovered. The accumulation of celastrol was also highly improved by MJ. The content of celastrol in the MJ group was 4.82 times of that in the CK group and the production of celastrol reached 0.742 mg g^{-1}. Studies have demonstrated that MJ can influence the secondary metabolites including terpenoids, alkaloids, and phenylpropanoids [17,18] by reprogramming of the transcriptome. A recent study on the regulating mechanism of MJ demonstrated that the bHLH transcription factors *Tc*JAMYC1, *Tc*JAMYC2, and *Tc*JAMYC4 negatively regulate the promoters of the terpenoids

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synthase in *Taxus cuspidata* cultured cells [19]. From the results we got in this study and the previous founding that MJ treatment has influence on different kinds of terpenoids [20], MJ may regulate the formation of terpenoids backbone. Meanwhile, it is possible to find a kind of responding transcription factor that regulates the MVA pathway promoter.

The MVA pathway involves the reactions that provide material for the biosynthesis of triterpenoids, which are common in plants. Therefore, the results in this study not only limited to *T. wilfordii* but also provided a clue to explore the mechanism of MJ in regulating the secondary metabolism of plants. However, MJ also has effects on the downstream reactions of triterpenoids biosynthesis, which has demonstrated in other plants [21]. The downstream effects of MJ in *T. wilfordii* are also important factor with regard to the accumulation of celastrol.

In conclusion, we cloned the full-length cDNA of the MVA pathway genes in *T. wilfordii*. The suspension cells of *T. wilfordii* were induced by MJ, and the expression levels of MVA pathway genes were determined. The accumulation of celastrol in the suspension cells was quantified and co-analyzed with the genes expression levels. The sequences in this study enriched the gene information in the widely used traditional Chinese medicinal plant and provided gene blocks for synthetic biology in producing triterpenoids. The sequences can also be used in studies on evolution of the plant of different subspecies [22]. The treatment method we explored is a viable way to improve the production of celastrol in suspension cells of *T. wilfordii* . In addition, the results implied that the regulation of MJ in secondary metabolism of triterpenoids may be related to the MVA pathway. The sequence information of enzymes will provide basic elements for the further investigation of celastrol biosynthesis in *T. wilfordii*.

4. Experimental

4.1. Plant material and cell cultivation

The *T. wilfordii* suspension cells were deduced from the aseptic seedling as previously reported [23]. The MS (Murashige and Skoog) medium contained 30 g L^{-1} sucrose, 8 g L^{-1} agar, and 0.5 mg L^{-1} 2, 4-D (2,4-dichlorophenoxyacetic acid) + 0.1 mg L^{-1} KT (kinetin) + 0.5 mg L^{-1} IBA (indole-3-butyric acid). All suspension cell cultures were maintained at 25 ± 1 °C with shaking at 120 rpm in dark.

The suspension cells were elicited with 50 μM MJ after 10 days of cultivation and DMSO was added in the control group. The cells were harvested at 0, 1, 4, 12, 24, 48, 72, 120, 288 h after MJ treatment and stored at −80 °C for RNA isolation and chemical detection.

4.2. RNA isolation

The *T. wilfordii* suspension cells harvested at 0, 1, 4, 12, 48, 72 h were used for RNA isolation. The total RNA was isolated by CTAB (cetyl-trimethylammonium bromide) method [24].

4.3. Cloning of full-length cDNAs in MVA pathway

Total RNA was reverse-transcripted into first-stand cDNA with SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories INC, CA, USA) according to the manufacturer's instructions. The primers of rapid amplification of cDNA ends (RACE) were designed

Table 2. Primers sequences.

Primer name	Primer sequences (5′–3′)
AACT-F	AGGTCGTAGCAGATGAACAAA
AACT-R	GGAATGAGGAGTCACCAGAA
HMGR1–5′RACE	CGACCTTGGGCTTGACTACGACA
HMGR1-F	ATCCCTTTTCGACTACTTGGCTC
HMGR1-R	TTCGCATCCTCTCAACAACTATCTA
HMGR2–5′RACE	ACTTCAACTCCGCTGCTCTTCTGGC
HMGR2–3′RACE	TCCATCTCCGACCCCGAAAGCATC
HMGR2-F	GCCTACAATCACATTTTCGATCTT
HMGR2-R	GTAGTTCAATCATGGGATCACTTTCT
MVK-F	AGAGGAACCAAATTGGATT
MVK-R	TTGAATGTGGAGCAAGTTAT
PMK-F	GATAGCTCCGTCGTCTTCG
PMK-R	GACCAAACAGAGTTTAACTACCAA
MVD-F	GAGGTGGGTTTCCTGCTATTCGTTT
MVD-R	GGATAGACTACTCTGCCGAGAA
AACT-qF	GGGTTACGTGTAATTGCTAAGATC
AACT-qR	TTGAGAAGCCTCCAGACCAG
HMGR1-qF	GGTTTCTTTGGCGTTGACTT
HMGR1-qR	GACGACCTTGGGCTTGACTA
HMGR2-qF	GCCTTTGCTGCTGGACGACTA
HMGR2-qR	CCGCTGCTCTTCTGGCTGAC
MVK-qF	TGGGTGGTTATCATACGAGGAG
MVK-qR	AGTGATGAGCATTCTGAGTGGG
PMK-qF	GACTGCCTTTGACACCCG
PMK-qR	CCACTGCCAACTTTCCCT
MVD-qF	GAAACAAGTAGCACCTCAGGAAT
MVD-qR	TGCGTAAACGAAGCGAAAT
β-actin-F	AGGAACCACCGATCCAGACA
β-actin-R	GGTGCCCTGAGGTCCTGTT

based on the transcriptome sequencing data of *T. wilfordii* we got prior. The products were cloned into pEASY-T_3 (TRANSGEN BIOTECH, Beijing, China) cloning vector and sequenced to get the full-length sequence. The full-length primers were designed based on the assembled sequences. The products were also cloned into pEASY-T_3 cloning vector and sequenced (Table 2).

4.4. Bioinformatics analysis

The full-length sequences of the MVA pathway genes were blasted against NCBI database. The open reading frame (ORF) and amino acid sequence were deduced by ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The amino acid sequences were firstly aligned with those of *T. wilfordii* that have been reported and then with other plants downloaded from GenBank. The phylogenetic trees were constructed by neighbor-joining method using MEGA5.1. The theoretical isoelectric and the calculated molecular weight were calculated on ExPASy (http://web.expasy.org/). The secondary structures were predicted by PredictProtein (http://www.predictprotein.org/).

4.5. Quantitative real-time PCR

The first strand cDNA was synthesized by total RNA with TIANScriptII RT Kit (TIANGEN BIOTECH(BEIJING) CO., Ltd.) according to the manufacturer's protocol. The relative mRNA quantities were detected using KAPA SYBR® FAST qPCR Kit (KAPA Biosystems,

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Inc, MA, USA) according to the manufacturer's protocol on Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, NY, USA). *β-actin* was chosen as the housekeeper in modifying the expression of the target genes. The expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

4.6. Chemical analysis of celastrol

The *T. Wilfordii* suspension cells materials were harvested for HPLC analysis at 0, 4, 48, 72, 120, 288 h after the treatment. The harvested cell suspensions were freeze-dried for 8 h. The samples were then prepared for detection according to a reported method [25]. Analysis was performed using an Agilent 1260 HPLC system (Agilent, CA, USA) with an Agilent Eclipse XDB-C18 analytical column (5 μm, 4.6 × 250 mm; Agilent, USA). The mobile phase consisted of water (A) and acetonitrile (B) was set at a flow rate of 0.8 ml/min. The samples were then eluted with 38% (B) for 12 min. The detection wavelength was 210 nm. The injection volume was 10 μl.

Disclosure statement

No potential conflict of interest was reported by the authors.

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