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Research paper

Overexpression and RNAi-mediated downregulation of *TwIDI* regulates triptolide and celastrol accumulation in *Tripterygium wilfordii*

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ABSTRACT

The aim of this study was to verify the effects of *TwIDI* (GenBank: KT279355.1) on triptolide and celastrol accumulation in the biosynthesis of terpenoids in *Tripterygium wilfordii* and the regulation of the expression of related genes in the triptolide and celastrol biosynthesis pathway. After bioinformatics analysis of *TwIDI*, we cloned the full-length CDS and a specific 398 bp fragment to construct overexpression and RNAi vectors, respectively. The specific amplification of hygromycin and kanamycin resistance gene fragments confirmed that the expression vectors had been successfully delivered into *Tripterygium wilfordii* suspension cells. qRT-PCR was used to detect the expression of *TwIDI* and related genes in the triptolide and celastrol biosynthesis pathway. The expression of *TwIDI* was increased to 157% of the control group (empty vector) in the overexpression group, and was reduced to 71% of the control group in the RNAi group. Notably, the expression of other genes in the triptolide and celastrol biosynthesis pathway also showed differences. For example, *TwMCS* was reduced to 62% of the control when *TwIDI* was overexpressed and increased to 188% in the RNAi group. The expression of *TwDXS* did not change significantly both during *TwIDI* overexpression and RNAi group. The accumulation of triptolide and celastrol in the suspension cells of *Tripterygium wilfordii* was detected by UPLC, revealing that the contents of triptolide and celastrol were increased 1.36- and 1.20-fold over the control group in the overexpression group, and decreased to 0.16 and 0.36 of the control group in the RNAi group. Based on these findings, the effect on the accumulation of active terpenoids in *Tripterygium wilfordii* and the feedback regulation of genes in the triptolide and celastrol biosynthesis pathway was verified through *TwIDI* overexpression and RNAi experiments.

1. Introduction

Tripterygium wilfordii (Hook. f.) is a woody vine belonging to the Celastraceae family, which is native to China (south of the Yangtze River), Korea, and Japan (Hümbelin et al., 2002; Zeng et al., 2016). It is widely used as a traditional Chinese herb called Leigongteng, which possesses immunosuppressive, anti-inflammatory and anticancer properties (Zheng et al., 2014). Modern research shows that it contains a large variety of active terpenoid compounds (Zetina-Rocha, 1998). Triptolide and celastrol are the two main terpenoid compounds in *Tripterygium wilfordii*, exhibiting a broad range of biological activities, especially anticancer activity (Jiang et al., 2015). Mechanistically, triptolide inhibits DNA-dependent ATPase activity, which leads to the inhibition of RNA polymerase II–mediated transcription and some nucleotide excision repair (Titov et al., 2011). Celastrol has been identified as a novel inhibitor of HSP90 which displays anticancer activity (Zhang et al., 2008). Furthermore, studies have shown that celastrol is a leptin sensitizer and a promising agent for the pharmacological treatment of obesity (Liu et al., 2015).

IPP and DMAPP are the precursor substances common to all terpenoids. They are synthesized by two independent pathways – the mevalonate (MVA) pathway in the cytoplasm and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids. Until 2000, several *IDI* genes, encoding IPP isomerase, were known from eukaryotic organisms (Hümbelin et al., 2002). Isopentenyl diphosphate isomerase (*IDI*) is a particularly important enzyme involved in the biosynthesis of isoprenoids, which catalyzes the transition between DMAPP and IPP involving a protonation/de-protonation reaction (Wouters et al., 2003).

Abbreviations: Tw, *Tripterygium wilfordii*; OE, overexpression; RNAi or Ri, RNA interference; *IDI*, isopentenyl diphosphate isomerase; CDS, coding sequence

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Therefore, IPP and DMAPP are the starting materials for the construction of the terpenoid carbon skeleton and play a vital role in the biosynthesis of terpenoids (Zhou et al., 2013). Tong et al. cloned the full-length *TwIDI* gene and confirmed that it plays a significant role in promoting the flux in the lycopene pathway using a functional color assay (Tong et al., 2015). Overexpression of the *IDI* gene in *Eucommia ulmoides* Oliv. increased the total content of trans-polyisoprenes three-fold to fourfold compared with the wild-type organism (Chen et al., 2012). However, native function verification of *TwIDI* has not been reported yet.

Overexpression and RNAi has been widely used in basic and applied research. Naora et al. found that the most effective double-stranded RNA components are hairpin RNAs which can be transcribed (Naora, 1979). Strong promoters can be used to enhance the expression of exogenous genes. Most dicotyledonous plant transformation vectors use the 35S promoter from *Cauliflower mosaic virus* to overexpress heterologous genes (Benfey and Chua, 1990). Accordingly, we used pH7WG2D (with the 35S promoter), and pK7GWIWG2D (with a hairpin structure) to construct overexpression and RNAi vectors, respectively, using the Gateway cloning system.

The results confirm the effects of *TwIDI* on the accumulation of triptolide and celastrol, which provides a new way to increase the activity of the corresponding terpenoid synthesis pathways.

2. Materials and methods

2.1. Bioinformatic analysis of TwIDI

The nucleotide sequence of *TwIDI* was analyzed for its open reading frame and homology to other *IDI* sequences (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were carried out using DNAMAN 8.0 software (Lynnon Biosoft, USA). Phylogenetic analysis was performed using MEGA 7.0 software (Kumar et al., 2016).

2.2. Entry vector construction

According to the obtained *TwIDI* sequence, specific primers were designed using Primer Premier 5.0 software (PREMIER Biosoft, Canada). The overexpression fragment contained the open reading frame (ORF) of *TwIDI*, and the RNAi fragment was a specific segment of about 400 bp within the ORF. Primer sequences are listed in Supplementary Table 1. The target gene fragments were amplified using Phusion high-fidelity Master Mix (NEB, USA), according to the manufacturer's protocol, with the *TwIDI* plasmid (saved by our laboratory) as the template. The fragments were ligated into the pENTR SD/D-Topo® entry vector (Invitrogen, USA) after gel electrophoresis validation of the BP reaction. The recombinant vectors were transferred into *E. coli* Trans5α competent cells and screened with kanamycin (50 mg/L). M13F/R primers were used to verify the size of the cloned fragment by PCR, and the recombinant plasmids were extracted using the Plasmid Mini Kit (OMEGA, China) after verification of correct sequences.

2.3. Expression vector construction

According to the Gateway procedure, the verified fragments were transferred from the entry vector into the expression vector by the LR reaction: the overexpression fragment was transferred into the pH7WG2D vector, and the RNAi fragment was transferred into the pK7GWIWG2D vector (Zhang et al., 2018). The recombinant vectors were transferred into *E. coli* Trans5α competent cells and screened with spectinomycin (50 mg/L). pH7-F, pK7-F and specific primers-F/R (Supplementary Table 1) of target fragments were used to verify the size of the cloned fragments by PCR, and the recombinant plasmids were extracted using the Plasmid Maxi Kit (OMEGA, China) after verification of correct sequences.

2.4. Delivery of recombinant DNA into Tripterygium wilfordii suspension cells using a gene gun

The suspension cells (maintained by our laboratory) were cultured in MS solid medium for 6–9 days until the logarithmic phase. The recombinant expression vectors were mixed with 1 μm Au microparticles (Bio-Rad, USA) which were used as bullets and bombarded into the suspension cells under high pressure helium using a PDS, 100/He gene gun (Bio-Rad, USA). The empty vectors were also assayed as a parallel control. After the bombardment by the gene gun, the suspension cells were cultured in the original medium at 25 °C in dark for a week. Each plasmid was tested in 5 biological replicates.

2.5. Verification of successful transformation with the expression vector

Total RNA of all samples was extracted using an RNA Extraction Kit (Promega, Shanghai, China), and the first-strand cDNA was reverse-transcribed from the total RNA using the FastQuant RT Kit (with gDNase) (Tiangen Biotech, Beijing, China). The pH7WG2D vector contains a hygromycin resistance cassette (Hyg) and the pK7GWIWG2D vector contains a kanamycin resistance gene (Kan). Therefore, we used Hyg and Kan primers (Supplementary Table 1) to amplify specific fragments, and gel electrophoresis showed the fragment size to verify successful transformation of the suspension cells with the expression vectors.

2.6. Transcription expression analysis of TwIDI

The cDNA obtained through reverse transcription was used as a template for qRT-PCR using the primers listed in Supplementary Table 2. The reaction system was prepared according to the KAPA SYBR FAST qPCR Master Mix Kit (KAPA Biosystems, USA) manufacturer's instructions. A LightCycler 480II (Roche, Switzerland) was used to assay the expression of *TwIDI*. We chose β-actin as the internal control gene and set three technical replicates to reduce errors. The relative expression levels of *TwIDI* were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Expression analysis of genes in the triptolide and celastrol pathway

The expression of related genes in terpenoid biosynthesis pathways was also analyzed in the case of *TwIDI*-overexpression and *TwIDI*-RNAi. We chose *TwHMGS* in the MVA pathway, *TwMCS* in the MEP pathway as well as *TwGGPPS* and *TwSQS* further downstream of the pathways. The corresponding qRT-PCR primers are listed in Supplementary Table 2. We chose β-actin as the internal control gene and set three technical replicates to reduce errors. The relative expression levels of related genes were analyzed using the $2^{-\Delta\Delta CT}$ method.

2.8. Extraction and detection of active terpenoids in Tripterygium wilfordii

The remaining suspension cells were crushed in liquid nitrogen and freeze-dried for 36 h. An aliquot comprising 20 mg of the resulting cell powder was weighed accurately and soaked in 1 mL 80% (v/v) methanol in water overnight at 4 °C, followed by 100 kHZ ultrasonication for 30 min at 25 °C. After centrifugation at 12000 × *g* for 10 min the supernatant was filtered through a 0.22 μm PTFE microporous membrane (DIKMA, China). The contents of triptolide and celastrol in each sample was determined by UPLC (1290 Infinity II; Agilent Technologies, USA) using an Acquity UPLC BEH C18 chromatographic column (2.1 × 100 mm, 1.7 μm, Waters, USA). A series of standard solutions of triptolide and celastrol were prepared with 80% (v/v) methanol in water as solvent (1, 2.5, 3.5, 10, 25 μg/L). The UPLC conditions were as follows: The mobile phase A was acetonitrile, mobile phase B was 0.1% (v/v) formic acid in water. The elution program encompassed 70% A at 0 min, 65% A at 5 min, 65% A at 8 min, 30% A