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ORIGINAL RESEARCH PAPER

Overexpression and RNA interference of *TwDXR* regulate the accumulation of terpenoid active ingredients in *Tripterygium wilfordii*

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Abstract
Objective To examine the putative regulatory role of *TwDXR* in terpenoid biosynthesis and terpenoid biosynthetic pathway-related gene expression, through overexpression and RNA interference with *TwDXR*.
Results We obtained 1410 and 454 bp *TwDXR*-specific fragments to construct overexpression and RNAi vectors. qRT-PCR was used to detect the expression of *TwDXR* and terpenoid biosynthesis pathway-related genes. The overexpression of *TwDXR* led to a 285% upregulation and the *TwDXR* RNAi led to a reduction to 26% of the control (empty vector-transformed cells) levels. However, pathway-related genes displayed different trends. When *TwDXR* was overexpressed, *TwDXS* expression decreased by 31% but increased to 198% when *TwDXR* expression was inhibited. The accumulation of terpenoids was also assayed. In the overexpression group, differences were not significant whereas the contents of triptolide and celastrol in the *TwDXR* RNAi samples were diminished by 27.3 and 24.0%, respectively.
Conclusion The feedback regulation of gene transcription and the accumulation of terpenoids in terpenoid biosynthesis in *Tripterygium wilfordii* were verified by *TwDXR* overexpression and RNAi experiments.

Keywords Celastrol · Gene expression analysis · Overexpression · RNAi · Terpenoid biosynthesis · Triptolide · *TwDXR*

Introduction

Tripterygium wilfordii Hook. f. is a medicinal plant that possesses anticancer, immunosuppressive and

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anti-inflammatory properties (Zheng et al. 2014). Triptolide and celastrol are the main active terpenoids displaying curative effects (Lv et al. 2015). Triptolide inhibits the transcription of RNA polymerase II—mediated through its covalent binding to human Xeroderma Pigmentosum Type B (XPB) and DNA-dependent ATPase inhibition (Titov et al. 2011). Celastrol is also effective in the treatment of Alzheimer's disease and obesity (Liu et al. 2015).

Overexpression and RNAi experiments are widely used because of their direct and effective gene function validation. To seek out potential novel therapeutic targets, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGS1), geranylgeranyl diphosphate synthase 1 (GGPS1), and sterol regulatory element binding transcription factor 2 (SREBP2) in the mevalonic acid (MVA) pathway, were individually knocked down. Pandyra et al. (2015) found that knock-down of SREBP2-specific cancer cells can completely abrogate the upregulation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and HMGS1. This intuitive discovery helped to identify potential new therapeutic targets. After *AtMYB41* was overexpressed, genes in multiple biological processes showed differential expression between transgenic and wild-type plants, suggesting that *AtMYB41* plays a direct or indirect role in the regulation of transcription (Cominelli et al. 2008). Differences in gene expression are frequently accompanied by changes in metabolites. Bulley et al. (2009) overexpressed the GDP-L-galactose guanyltransferase gene in *Arabidopsis*, which resulted in a fourfold increase in L-ascorbic acid.

There is evidence that 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) represents a rate-limiting enzyme in the plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, and thus plays an important role in regulating terpenoid production. *Salvia miltiorrhiza* hairy roots under hyperosmotic stress or after yeast treatment, showed an increased expression level of *DXR* which was correlated with an increased accumulation of diterpenoid tanshinones (Wu et al. 2009). Tong et al. (2015) cloned the *TwDXR* gene of *Tripterygium wilfordii*, and they suggested that *TwDXR* overexpression could promote the rate of terpenoid biosynthesis and thereby increase the concentration of lycopene in a complementary colour experiment. Based on this observation, *TwDXR* expression thus is linked with the biosynthesis rate of terpenoids in *Tripterygium wilfordii*. However, the correlation between *TwDXR* expression and triptolide and celastrol biosynthesis has not yet been studied. In this study, we used gateway technology to construct overexpression and RNAi expression vectors and the gene gun technique to transform vectors into *Tripterygium wilfordii* suspension cells. Contents of triptolide and celastrol were measured, and the expression of related genes was also determined. Hence, we demonstrated correlations of *TwDXR* gene expression with that of other genes in terpenoid biosynthesis. Furthermore, we demonstrated that there are effects on the accumulation of triptolide and celastrol as active components.

Materials and methods

Entry vector construction

Specific primers were designed by Primer Premier 5.0 software according to the available *TwDXR* sequence. The overexpression product corresponds to its full open reading frame, and the RNAi product contained a specific 400–500 bp fragment. Primer sequences are listed in Supplementary Table 1. Target genes were amplified under the guidelines of Phusion HF Master Mix (NEB), with the *TwDXR* plasmid being used as a template. After detection of the correct sequence length, target genes were inserted into a pENTR SD/D-TOPO entry vector (Invitrogen). The recombinant vectors were transformed into *E. coli* Trans5α cells and screened by 50 mg kanamycin l^{-1}. M13 F/R primers were used to verify that cloned sequences were successfully inserted.

Expression vector construction and transformation

According to the Gateway LR Clonase TM II Enzyme Mix (Invitrogen) manufacturer's instructions, overexpression and RNAi target sequences were transferred from entry vectors into pH7WG2D and pK7GWIWG2D, respectively (Cheng et al. 2014). The recombinant plasmids were used to transform *E. coli* Trans5α competent cells, and positive colonies were selected on 100 mg spectinomycin l^{-1}, followed by pH7 and pK7 sequencing. The extraction of plasmids was performed in accordance with the Plasmid Maxi Kit (OMEGA). Gene gun (PDS

100/He, Bio-Rad) bombardment was used to transform recombinant expression vectors into *Tripterygium wilfordii* suspension cells. Corresponding empty vectors pH7WG2D and pK7GWIWG2D were also transformed in parallel. Each plasmid was transformed into five biological replicates (specific bombardment protocol unpublished).

Verification of successful transformation with expression vectors

Total RNA from all samples was extracted using the Total RNA Extraction Kit (Promega, Shanghai, China). In addition the first-stand cDNA was reverse-transcribed from total RNA employing the FastQuant RT kit (with gDNase) (Tiangen Biotech, Beijing, China). There is an Enhanced Green Fluorescent Protein (EGFP) fragment in the pH7WG2D and pK7GWIWG2D vectors. Therefore, EGFP primers (Supplementary Table 1) were utilized to amplify the EGFP fragment to verify the successful transfer of expression vectors into suspension cells.

Transcriptional expression analysis of *TwDXR*

The cDNA obtained above was used as the templates for qRT-PCR. qRT-PCR primers (Supplementary Table 2) and the reagents were added in accordance with the KAPA SYBR FAST qPCR Master Mix Kit (KAPA Biosystems) manufacturer's instructions. We used a LightCycler 480 II (Roche, Switzerland) to assay the expression of *TwDXR*, and three technical replicates were conducted to reduce errors. β-Actin gene expression was used as an endogenous control, and the relative expression of *TwDXR* was analysed by the $2^{-\Delta\Delta Ct}$ method (Rao et al. 2013).

Expression analysis of genes involved in terpenoid biosynthesis pathways

The relative expression was analysed for the MEP pathway genes *TwDXS* and *TwMCT* and for *TwHMGS* and *TwHMGR*, which are involved in the MVA pathway. We analysed *TwGGPS* and *TwFPS*, which are further downstream of the pathways, under the conditions of *TwDXR* overexpression and RNAi as described for *TwDXR* using specific qRT-PCR primers that are listed in Supplementary Table 2.

Extraction and determination of biologically active terpenoids in *Tripterygium wilfordii*

The remaining suspension cells were powdered in liquid N_2 and freeze-dried for 36 h. We precisely weighed freeze-dried samples ($\sim$ 20 mg). Samples were soaked in 1 ml 80% (v/v) methanol overnight at 4 °C, followed by 40 kHz ultrasonication at 25 °C for 30 min. After centrifugation at 12,000×g for 10 min, the supernatant was filtered through a 0.22 μm PTFE microporous membrane. Triptolide and celastrol contents were detected using Acquity UPLC-Xevo TQ MS (Waters), and an Acquity UPLC BEH C18 chromatographic column (2.1 × 100 mm, 1.7 μm, Waters). The mobile phase A was 100% acetonitrile and mobile phase B was 0.1% (v/v) formic acid in water. The elution programme was 50% A at the beginning, 50–95% A at 0–2.5 min, 95% A at 2.5–3 min, 95–50% A at 3–3.5 min, and 50% A at 3.5–5 min. The flow rate was 0.6 ml min^{-1}. The injection volume was 5 μl, and the column was kept at 40 °C. Each biological replicate was run three times.

Results

Generation of *TwDXR* overexpression and RNAi expression vectors

Specific primer amplification resulted in 1410 bp *TwDXR* overexpression and 454 bp *TwDXR* RNAi target fragments. The target band electrophoresis is shown in Fig. 1a. EGFP primer amplification was found in all samples that contained 800 bp EGFP fragments, indicating that the vectors were successfully transformed into the suspension cells. Partial electrophoresis bands are shown in Fig. 1b.

Expression analysis of *TwDXR*

Relative expression levels of *TwDXR* in treatment and control groups were determined by qRT-PCR (Fig. 2a). In the overexpression group, *TwDXR* gene expression was increased to almost four times that of the control. In the RNAi group, its expression was reduced to 0.26 times that of the control (empty vector transformation). The values determined were statistically significant at $P < 0.01$.

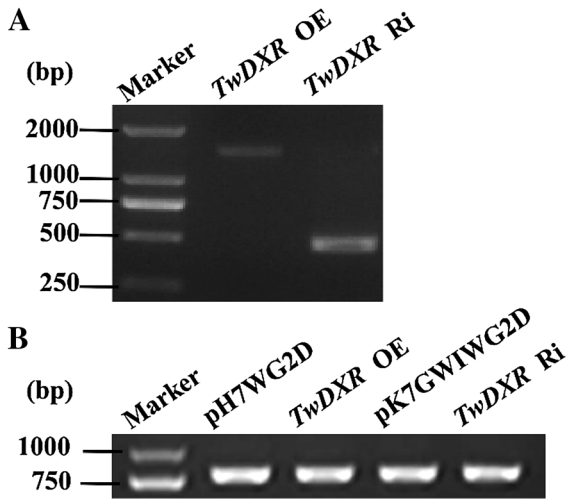

Fig. 1 Agarose gel electrophoresis of target fragments. **a** *TwDXR* overexpression and RNAi fragments. *TwDXR* overexpression fragment of 1410 bp, RNAi fragment of 454 bp. **b** Verification of successful expression vector transformation. The bands show the 800 bp EGFP fragments. Overexpression is the abbreviation for overexpression; Ri is RNAi

Expression analysis of other genes involved in terpenoid biosynthesis

The relative gene expression analysis of *TwDXS*, *TwMCT*, *TwHMGS*, *TwHMGR*, *TwGGPS* and *TwFPS* is displayed in Fig. 2b. Under the *TwDXR* overexpression condition, in contrast to the relative expression of *TwDXS* decreasing by 0.3-fold, the expression of *TwGGPS*, *TwFPS*, *TwHMGS* and *TwHMGR* increased to 1.5-, 1.4-, 1.5- and 1.4-fold, respectively, and *TwMCT* was not significantly affected. The relative expression of *TwDXS* doubled, but *TwMCT*, *TwFPS*, and *TwHMGS* expression decreased to 0.7-, 0.8- and 0.5-fold under condition of *TwDXR* RNAi silencing. However, there was no significant difference in *TwGGPS* or *TwHMGR* expression with that of the empty vector-transformed cells.

Contents of effective terpenoids in *Tripterygium wilfordii*

The contents of triptolide and celastrol were determined in *Tripterygium wilfordii* suspension cells in which the *TwDXR* gene was overexpressed or partially silenced by RNAi. After having assayed five biological replicates, there was no significant difference found in either triptolide or celastrol contents compared to the control when *TwDXR* was overexpressed. In contrast to this, triptolide and celastrol contents showed significant differences under the *TwDXR* RNAi condition ($P < 0.05$). The triptolide content was found to be 36.3 ± 4.2 and 28 ± 2.5 μg g^{-1}, and the celastrol content was 44.3 ± 6 and 34.3 ± 3.3 μg·g^{-1} in the pK7GWIWG2D controls and the *TwDXR* RNAi group, respectively (Fig. 3).

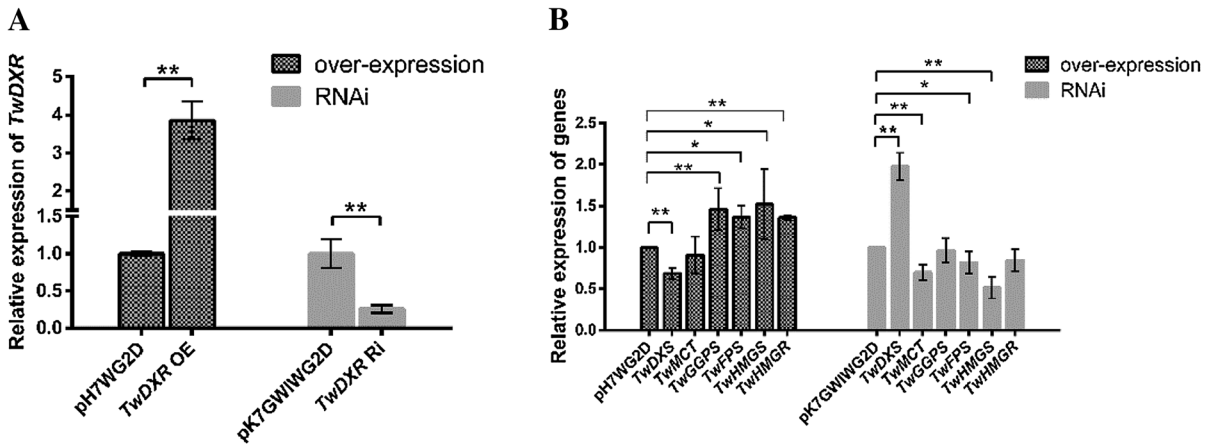

Fig. 2 Relative expression analysis of genes in the terpenoid biosynthetic pathway. **a** Relative expression of *TwDXR*. **b** Relative expression of related genes in the terpenoid biosynthetic pathway. The relative expression of genes were analysed by the $2^{-\Delta\Delta Ct}$ method, and the data represent the average ± standard deviation of five biological replicates and three technical replicates. A double asterisk indicates $P < 0.01$ and a single asterisk indicates $P < 0.05$, n = 5

Discussion

In plants, isoprenoids are synthesized by two independent pathways: the cytosolic MVA pathway and the plastidial MEP pathway (Vranová et al. 2013). Pyruvate and glyceraldehyde 3-phosphate (G3P) in the MEP pathway produce isopentenyl pyrophosphate (IPP) via catalysis by a series of enzymes, and IPP can transfer through the plastid envelope membrane, which among others serve to link the MVA and MEP pathways (Hemmerlin et al. 2012). The universal isoprenoid precursor IPP and its structural isomer dimethylallyl pyrophosphate (DMAPP) produce terpenoids, carotenoids, chlorophylls, sterols, and gibberellins via a series of reactions (Pulido et al. 2012). During their studies on taxadiene biosynthesis in *Taxus chinensis*, Engels et al. (2008) found that when taxadiene synthase was overexpressed alone, the amount of taxadiene did not increase due to the lack of geranylgeranyl diphosphate. Furthermore and to some extent surprisingly, co-expression of taxadiene synthase and geranylgeranyl diphosphate synthase (GGPS) failed to increase taxadiene levels. They attributed this effect to some the negative feedback regulation within the pathway. Based on this finding, they tried to co-express taxadiene synthase, GGPS and yeast HMG-CoA reductase (tHMG1) in yeast, which prevented steroid-based negative feedback in the MVA pathway and led to inhibit steroid production, resulting in a taxadiene increase of 50%. In our study, metabolite contents did not increase under the *TwDXR* overexpression condition, which might be related to the flux of different metabolites and single gene overexpression.

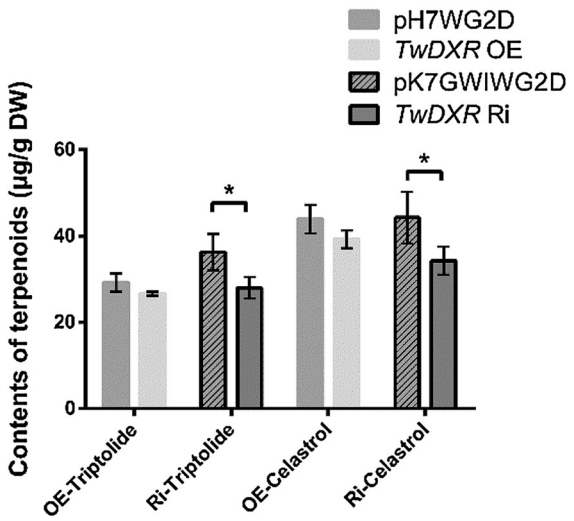

Fig. 3 Contents of triptolide and celastrol in *Tripterygium wilfordii* suspension cells. The data represent the average ± standard deviation of five biological replicates and three technical replicates. Overexpression is the abbreviation for overexpression; Ri is the abbreviation for RNAi. A single asterisk indicates $P < 0.05$, n = 5

The *DXR* gene codes for the first rate-limiting enzyme in the plant MEP pathway (Carretero-Paulet et al. 2002) and has become a new target for antimalarial and antibacterial drugs (Singh et al. 2007). Overexpression of *DXR* in tobacco led to an increase in the content of various isoprenoids such as β-carotene, chlorophyll a, antheraxanthin and lutein (Hasunuma et al. 2008). It can also regulate biosynthesis of terpenoids. *DXR* overexpression in peppermint improved flux to monoterpene production resulting in an about 50% increase in the yield of the essential oil, without any major change in the monoterpene composition (Mahmoud and Croteau 2001). The content of different abietanic diterpenes was 3.3-fold increased compared to the control when *AtDXR* was ectopically overexpressed in *Salvia sclarea* hairy root, and even a 4.6-fold increase in aethiopinone, a abietane-quinone type diterpene (Vaccaro et al. 2014). Similar results were observed in *Artemisia annua*, with a more than two-fold increase in artemisinin production (Xiang et al. 2012). Fosmidomycin, an MEP pathway inhibitor, binds specifically to SmDXR, leading to the inhibition of tanshinone production in *Salvia miltiorrhiza* hairy roots (Yang et al. 2012). Disruption of *DXR* led to some deficiencies in gibberellic acids (GAs), abscisic acid (ABA) and photosynthetic pigments, producing more albino sepals and yellow inflorescences, which could be partially rescued by exogenous application of hormones (Xing et al. 2010). In this study, we not only detected the transcriptional expression of *TwDXR*, but also of crucial genes being involved in terpenoid biosynthesis after *TwDXR* overexpression and interference (Fig. 4). It can be seen that *TwDXS* and *TwDXR* showed some opposite trends, which might be related to the action of negative feedback on the expression of genes in plants (Xi et al. 2010). The residual genes showed the same tendency in expression as did *TwDXR*; this phenomenon can be attributed to gene coregulation and signal transduction in the process of terpenoid biosynthesis (Schnee et al. 2006).

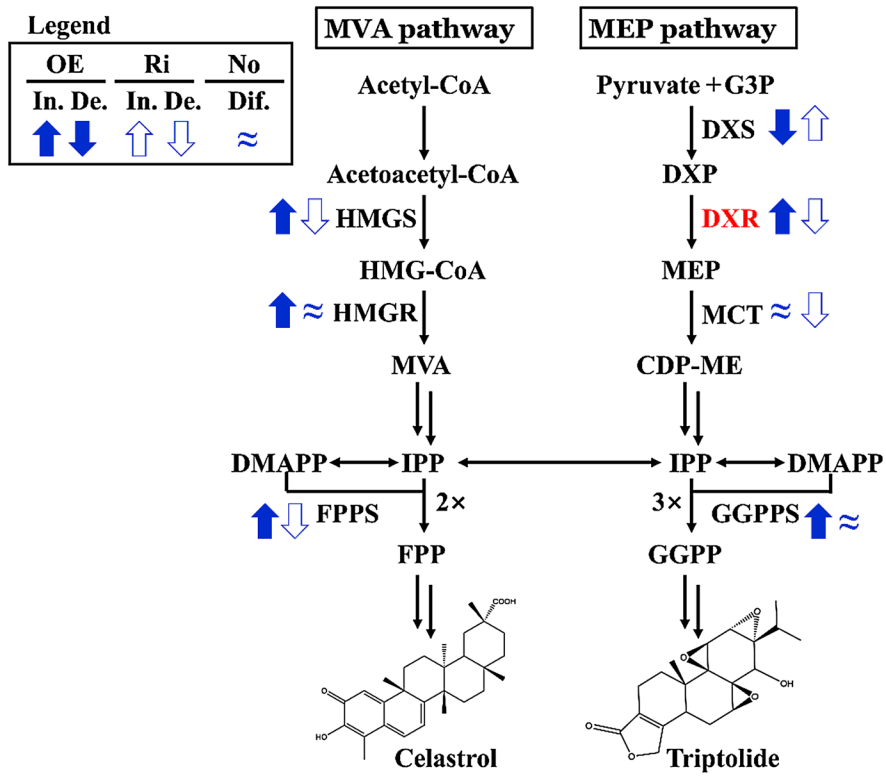

Fig. 4 Overview of the terpenoid biosynthesis pathway and gene expression tendencies. Overexpression indicates *TwDXR* overexpression, and solid blue arrows upward or downward represent corresponding up- or downregulation of gene expression. Ri indicates *TwDXR* RNAi, and the direction of the hollow blue arrows represent the same action as with the overexpression. No Dif. signifies no significant difference between the control and treatment. De., decrease; In., increase. *TwDXR* is marked in red for emphasis. A single arrow indicates a one-step reaction, and a double arrow represents a multiple-step reaction. The purpose of this figure is to summarize the whole study, and the data come from the figures above

Furthermore, the essential function of *TwDXR* in vivo was verified by experiments.

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Supporting information Supplementary Table 1—Primers used for RNAi expression vector construction.

Supplementary Table 2—Primers used for quantitative RT-PCR.

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