Overexpression of *TwSQS*, *TwSE* and *TwOSC* Regulates Celastrol Accumulation in Cambial Meristematic Cells and Dedifferentiated Cells

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Research Article

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Abstract

Squalene synthase (SQS), squalene epoxidase (SE) and oxidosqualene cyclase (OSC) are encoding enzymes in downstream biosynthetic pathway of triterpenoid in plants, but the relationship between the three genes and celastrol accumulation in *Tripterygium wilfordii* (*T. wilfordii*) still remains unknown. Gene transformation platform in plant can be used for fast studying gene function. However, there is no report on the application of cambial meristematic cells (CMCs) and dedifferentiated cells (DDCs) in genetic transformation platforms. Our aim was to study the effects of overexpression of *TwSQS*, *TwSE* and *TwOSC* on celastrol accumulation and terpenoid biosynthetic pathway-related gene expression through CMCs and DDCs platforms. Overexpression vectors of *TwSQS*, *TwSE* and *TwOSC* were constructed by Gateway technology and transferred into *T. wilfordii* CMCs and DDCs by gene gun. The content of celastrol was assayed and it significantly increased in CMCs compared with the control group after overexpression. However, there was no significant increment in celastrol was detected in DDCs. Meanwhile, the relative expression levels of *TwSQS*, *TwSE*, *TwOSC* and terpenoid biosynthetic pathway-related genes were detected. The relative expression levels of *TwSQS*, *TwSE* and *TwOSC* were all increased compared with the control group in both CMCs and DDCs. While the pathway-related genes displayed different expression trends. Therefore, it was verified *in T. wilfordii* CMCs that overexpression of *TwSQS*, *TwSE* and *TwOSC* increased celastrol accumulation and had different effects on the expression of related genes in terpenoid biosynthetic pathway, laying a foundation for further elucidating the downstream biosynthetic pathway of celastrol through *T. wilfordii* CMCs platform.

Key Message

It was verified *in vivo* that overexpression of *Tripterygium wilfordii* squalene synthase, squalene epoxidase and oxidosqualene cyclase promoted celastrol accumulation in cambial meristematic cells.

Introduction

Tripterygium wilfordii Hook. f. (*T. wilfordii*) is a woody medicinal plant and its dried root is a well-known traditional Chinese medicine widely used to treat immune regulation (Li et al. 2005), inflammation (Xu et al. 1985; Zhang et al. 2017b) and tumor (Wang et al. 2016). The pharmacologically active constituents of *T. wilfordii* are terpenoids, mainly included diterpenoid and triterpenoid (Liu et al. 2011). Celastrol is a triterpenoid compound and considered to be one of the most likely natural products to develop into modern medicines (Corson and Crews 2007), which can efficiently treat inflammatory diseases (Guo et al. 2017), tumor (Liu et al. 2019), obesity and metabolic dysfunction (Ma et al. 2015).

In plants, terpenoids are biosynthesized by the mevalonate (MVA) pathway located in the cytoplasm and the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway located in the plastid (Vranova et al. 2013). The isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) which is the common precursor of terpenoids can transfer through the plastid envelope to link the MVA and MEP pathways (Rodriguez-Concepcion and Boronat 2002). Farnesyl diphosphate synthase (FPS) catalyzes

one molecule of DMAPP and two molecules of IPP to form farnesyl pyrophosphate (FPP), and two molecules of FPP can be catalyzed to squalene by squalene synthase (SQS) which is the first enzyme to catalyze the MVA pathway in the biosynthesis steps of the sterols and triterpenoids (ABE et al. 1994). Then, the rate-limiting enzyme squalene epoxidase (SE) (Han et al. 2010) converts squalene to 2,3-oxidosqualene (Zhou et al. 2018). 2,3-oxidosqualene can be subsequently catalyzed by oxidosqualene cyclase (OSC) to friedelin (Zhou et al. 2019) which will be finally transformed to celastrol through a series of biosynthetic reactions. So far, the downstream biosynthetic pathway of celastrol has not been clearly elucidated.

Recently, a stable gene transformation platform of *T. wilfordii* suspension cells for studying gene function has been developed, which is fast, convenient and efficient (Zhao et al. 2017). Cambial meristematic cells (CMCs) are innately undifferentiated cells derived from cambium in plant and they possess characteristics of plant stem cells (Ochoa-Villarreal et al. 2015). CMCs were commonly used as cell culture systems of medical plant, such as *Taxus cuspidata* (Lee et al. 2010), *Catharanthus roseus* (Moon et al. 2015), *Camptotheca acuminata* (Zhang et al. 2017c) and *Tripterygium wilfordii* (Song et al. 2019), from which, CMCs could provide a cost-effective platform for producing higher amounts of important natural products than typical dedifferentiated cells (DDCs). However, there is no report on the application of CMCs and DDCs in genetic transformation platform.

Overexpression of genes that encode enzymes in the biosynthetic pathway can directly affect gene expression levels and alter the yield of direct and end products associated with the encoding enzyme, which was extensively researched in plants. In adventitious roots of *Panax ginseng*, overexpressing *SQS* produced approximately 2 times more phytosterol than the wild type (Lee et al. 2004). After *SE* overexpression, the content of ganoderic acid in *Ganoderma lingzhi* was 2 times higher than that of the wild type (Zhang et al. 2017a). In *Populus davidiana*, overexpression of *OSC* increased friedelin accumulation by 233–445% compared with the non-transgenic plants (Han et al. 2019). However, the effects of overexpression of *TwSQS*, *TwSE* and *TwOSC* on celastrol biosynthesis in *T. wilfordii* CMCs and DDCs still remain unknown.

In this work, we aimed to study the effects of overexpression of *TwSQS*, *TwSE* and *TwOSC* on celastrol accumulation and terpenoid biosynthetic pathway-related gene expression through *T. wilfordii* CMCs and DDCs, laying a foundation for further research on downstream biosynthetic pathway of celastrol.

Materials And Methods

Plant cell suspension cultures of *T. wilfordii* CMCs and DDCs

The plant *Tripterygium wilfordii* Hook. f. was obtained from Yongnan national forest in Fujian Province, China. CMCs and DDCs were isolated from the stem of *T. wilfordii* plant and cultured in Murashige & Skoog (MS) medium (PhytoTechnology Laboratories, Lenexa, KS, USA) as described previously (Song et al. 2019). Briefly, under the sterile condition, cambium, phloem, cortex and epidermal tissue of the *T. wilfordii* stem were peeled off from the xylem. The epidermal tissue side was laid on MS solid medium

that contained 2 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg L⁻¹ naphthalene-acetic acid (NAA), 30 g L⁻¹ sucrose and 8 g L⁻¹ agar. The pH of medium was 5.8-6.0. During the culture process, CMCs was formed from the cambium, and DDCs was formed from the phloem, cortex and epidermis. The characteristic features of CMCs and DDCs were previously identified by our team (Song et al. 2019). 2.5 g (fresh cell weight) cells from each cell line were cultured in 100 mL Erlenmeyer flasks including 40 mL of MS liquid medium. The composition of the liquid medium is the same as the solid medium except for agar. The flasks were placed at 25°C in dark and agitated at 120 rpm. Subculturing was undertaken at 20-day intervals.

Construction of entry vectors

The full-length cDNA of *TwSQS* (Liu et al. 2016), *TwSE* (Zhou et al. 2018) and *TwOSC* (Zhou et al. 2019) (GenBank accession numbers: *TwSQS* KR401220, *TwSE* MG717396 and *TwOSC* KY885467) were previously cloned. The full open reading frame (ORF) of *TwSQS*, *TwSE* and *TwOSC* were amplified by PCR using specific overexpression (OE) primers (**Table S1**) and Phusion High-Fidelity Master Mix (New England Biolabs). Their plasmids were used as templates. After detecting that the sequence lengths of the amplified products were the same as their full ORF, the products were purified and then inserted into pENTR^{TM}/D-TOPO entry vectors using the Cloning Kit (Thermo Fisher Scientific). The recombinant vectors were transformed into *Escherichia coli* (*E. coli*) Trans5α cells (TransGen Biotech, Beijing, China) and then cultured on Luria-Bertani (LB) solid medium with 50 mg L⁻¹ kanamycin for selection of entry vectors. M13 F/R primers (**Table S1**) were used to verify that entry vectors were successfully constructed.

Construction of overexpression vectors

The overexpression target fragments of *TwSQS*, *TwSE* and *TwOSC* were transferred from entry vectors into pH7WG2D vectors using the Gateway LR Clonase^{TM} II Enzyme Mix (Thermo Fisher Scientific), respectively. The promoter of the vector was p35S and the terminator was T35S. The recombinant vectors containing each fragment were transformed into *E. coli* Trans5α cells and selected on LB solid medium with 50 mg L⁻¹ spectinomycin, followed by pH7 (**Table S1**) sequencing. OE primers (**Table S1**) were used to verify that overexpression vectors were successfully constructed. The plasmids of *TwSQS*, *TwSE* and *TwOSC* overexpression vectors were extracted using EZNA® Plasmid Maxi Kit (OMEGA, Norcross, Georgia, USA).

Transfer of overexpression vectors into *T. wilfordii* CMCs and DDCs

At the time of subculturing, CMCs and DDCs were cultured on MS solid medium containing 2 mg L⁻¹ 2,4-D, 2 mg L⁻¹ NAA, 30 g L⁻¹ sucrose and 8 g L⁻¹ agar. The pH of medium was 5.8-6.0. 200 mg (fresh cell weight) cells corresponded to 3 mL medium. Cells grew in dark at 25°C for 7 d before transfer. The plasmids of overexpression vectors were separately transferred into *T. wilfordii* CMCs and DDCs using Gene gun instrument (PDS 100/He, Bio-Rad) and the Gene gun protocol has been described previously in

detail (Zhao et al. 2017). Briefly, the plasmids were mixed with 1 μm gold microparticles (Bio-Rad) and the mixture was then bombarded into the cells using Gene gun under high pressure helium. The empty vector pH7WG2D were transferred into CMCs and DDCs as a control check. After gene transfer, cells were cultured in the original medium in dark at 25°C for 7 d. After 7 d, the cells were separated from the culture medium, and the cells were stored at -80°C for later use. All samples had five biological replicates.

Verification of successful transfer of overexpression vectors in cells

The cell samples were divided into two parts, one for terpenoids content measurement and the other for gene expression determination. Total RNA from all cell samples were extracted using the Total RNA Extraction Kit (Promega, Shanghai, China). Then all RNAs were reverse-transcribed to first-strand cDNAs using the FastQuant RT kit (with gDNase) (Tiangen Biotech, Beijing, China). The hygromycin (Hyg) fragments were amplified in the cDNAs using Hyg F/R primers (**Table S1**) to verify the successful transfer of overexpression vectors in *T. wilfordii* CMCs and DDCs.

Extraction and determination of terpenoids in *T. wilfordii* CMCs and DDCs

The remaining cell samples were freeze-dried *in vacuo* for 48 hours. Then 5 mg dried cells were precisely weighed, soaked in 250 μL n-hexane and overnight at 4°C. Another 5 mg dried cells were also precisely weighed, soaked in 250 μL 80% (v/v) methanol and overnight at 4°C. All cells were ultrasonicated at 25°C and 40 kHz for 1 h the next day. After centrifugation at 10,000 $\times g$ for 10 min, the supernatants were filtered through 0.22 μm PTFE microporous membrane. For determining the content of squalene (direct product of TwSQS enzyme), 2,3-oxidosqualene (direct product of TwSE enzyme) and friedelin (direct product of TwOSC enzyme), the n-hexane extracts were detected using GC-MS (Agilent 7000, Agilent) with a DB-5ms capillary column (15 m $\times$ 250 μm $\times$ 0.1 μm) as described previously (Zhou et al. 2019). The gas chromatography detection conditions were set as follows: the initial column temperature was 50°C, hold for 1 min, then it increased to 260°C at a rate of 50 $^{\circ}\text{C min}^{-1}$, and then increased to 272°C at a rate of 1 $^{\circ}\text{C min}^{-1}$, hold for 4 min. The injection temperature was 250°C and the split ratio was 20: 1. The carrier gas was helium and flow rate was 1 mL min^{-1} . The mass spectrometric detector was operated with ionization energy of 70 eV by electron impact. The ion trap temperature was 230°C and spectra were recorded in the range of 10-550 m/z. The sample injection volume was 1 μL .

For determining the content of celastrol (the end product of MVA pathway containing the three enzymes), the methanol extracts were detected using UPLC (1290 Infinity II, Agilent) with an ACQUITY UPLC® HSS T3 chromatographic column (1.8 μm , 2.1 mm $\times$ 100 mm, Waters). Column temperature was 40°C and the flow rate was 0.4 mL min^{-1} . Gradient elution with 100% acetonitrile (mobile phase A) and 0.1% (v/v) formic acid in water (mobile phase B) was used: 0 min: 70% B, 5 min: 65% B, 15 min: 30% B, 21 min: 10% B. The UV detector monitored at 426.0 nm and the sample injection volume was 5 μL .

Relative expression analysis of *TwSQS*, *TwSE* and *TwOSC*

The determination of gene expression levels was carried out by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). KAPA SYBR® FAST qPCR Master Mix Kit (KAPA Biosystems, Wilmington, Massachusetts, USA) was used to perform qRT-PCR experiment on the QuantStudio 5 real-time PCR system (Thermo Fisher Scientific). The cDNA obtained above was used as the templates for qRT-PCR. The qRT-PCR mixture contained 10 µL of 2 × qPCR Master Mix, 1 µL of cDNA (10 ng), 0.4 µL of 50 × ROX reference Dye low, 0.4 µL of primers (10 µM) and 8.2 µL of double distilled H₂O. The qRT-PCR experiment was performed under the following conditions: at 95°C for 3 min, followed by 40 cycles consisted of 95°C for 3 s and 60°C for 30 s, and then a melting curve cycle: 95°C for 1 s and 60°C for 20 s. *Elongation factor 1α (Ef1α)* was used as the reference gene and the relative expression was analyzed by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) with five biological replicates and three technical replicates. The primer sequences employed were listed in **Table S1**.

Relative expression analysis of genes involved in terpenoid biosynthetic pathway

We also analyzed the relative expression levels of *T. wilfordii* 1-deoxy-D-xylulose-5-phosphate synthase (*TwDXS*) and geranylgeranyl diphosphate synthase (*TwGGPPS*) genes involved in MEP pathway and of hydroxymethylglutaryl-CoA synthase (*TwHMGS*), hydroxymethylglutaryl-CoA reductase (*TwHMGR*), isopentenyl diphosphate isomerase (*TwIDI*), *TwFPS*, *TwSQS*, *TwSE* and *TwOSC* genes involved in MVA pathway of terpenoids in *T. wilfordii* under the conditions of *TwSQS*, *TwSE* and *TwOSC* overexpression. The specific qRT-PCR primers used were listed in **Table S1**. The methods of relative gene expression analysis were same as described in relative expression analysis of *TwSQS*, *TwSE* and *TwOSC*.

Results

Confirmation of *TwSQS*, *TwSE* and *TwOSC* overexpression vectors

The *TwSQS* overexpression fragment of 1241 bp, the *TwSE* overexpression fragment of 1584 bp and the *TwOSC* overexpression fragment of 2298 bp were obtained. The sequencing results were consistent with the ORF sequence of *TwSQS*, *TwSE* and *TwOSC*, confirming that overexpression vectors of the three genes were successfully constructed. Agarose gel electrophoresis showed the length of the target fragments in Fig. 1a. The cDNA of CMCs and DDCs after transfer were amplified by specific primers and the Hyg fragments with the length of 1787 bp were found in all cell samples (Fig. 1b), indicating that the empty vector pH7WG2D and overexpression vectors were successfully transferred into CMCs and DDCs. A schematic diagram of overexpression vector was shown in Fig. 1c and the morphology of CMCs and DDCs was shown in Fig. 2.

Accumulation of squalene, 2,3-oxidosqualene and friedelin in *T. wilfordii* CMCs and DDCs

In order to explore the ability of *TwSQS*, *TwSE* and *TwOSC* enzymes to biosynthesize related terpenoids after overexpression, we detected the content of direct products of the three enzymes in *T. wilfordii* CMCs and DDCs (Fig. 3a, **Fig.S1-S3**). The content of squalene and friedelin were detected in CMCs, but not in

DDCs. In the pH7WG2D control group, *TwSQS* overexpression group and *TwOSC* overexpression group, the content of squalene was 229.5 ± 42.4 , 283.5 ± 5.6 and $369.8 \pm 108.7 \mu\text{g g}^{-1}$, respectively. The content of friedelin was 180.8 ± 4.7 , 226.2 ± 20.2 , 251.6 ± 43.7 and $225.9 \pm 19.4 \mu\text{g g}^{-1}$ in the pH7WG2D control group, *TwSQS* overexpression group, *TwSE* overexpression group and *TwOSC* overexpression group, respectively. Only the content of squalene detected in the *TwSE* overexpression group had no significant differences compared with the control group. However, we did not detect any 2,3-oxidosqualene in both CMCs and DDCs.

Accumulation of celastrol in *T. wilfordii* CMCs and DDCs

The content of end product celastrol was then detected in *T. wilfordii* CMCs and DDCs after overexpression (Fig. 3b, **Fig.S4**). Compared with the control group, the contents of celastrol in CMCs overexpression group showed significant increase. In the pH7WG2D control group, *TwSQS* overexpression group, *TwSE* overexpression group and *TwOSC* overexpression group, the content of celastrol in CMCs was 937.6 ± 106.9 , 1209.8 ± 111.3 , 1217.6 ± 95.0 and $1125.2 \pm 95.7 \mu\text{g g}^{-1}$, respectively. In contrast to this, there was no significant difference in the content of celastrol between DDCs overexpression group and the control group.

Relative expression analysis of *TwSQS*, *TwSE* and *TwOSC* in *T. wilfordii* CMCs and DDCs

To further investigate the transcription levels of overexpressed genes, the relative expression levels of *TwSQS*, *TwSE* and *TwOSC* were determined by qRT-PCR (Fig. 4a). In CMCs, relative expression level of *TwSQS*, *TwSE* and *TwOSC* increased by 2.5, 1.8 and 15.8 times compared with the pH7WG2D control group, respectively. As for DDCs, their relative expression level exhibited 1.3-, 5.1- and 2.9-fold increase compared with the control group, respectively. In both cell lines, the relative expression levels of the three genes showed statistical significance compared with the control group.

Relative expression analysis of other important genes involved in terpenoid biosynthetic pathway

We next analyzed the relative expression levels of other genes encoding important enzymes involved in terpenoid biosynthetic pathway after overexpression of *TwSQS*, *TwSE* and *TwOSC*.

Under the *TwSQS* overexpression condition (Fig. 4b), in terms of CMCs group, the relative expression level of *TwSE*, *TwOSC*, *TwHMGR*, *TwIDI*, *TwDXS* and *TwGGPPS* increased by 1.5, 7.7, 2.8, 3.1, 2.0 and 5.0 times compared with the pH7WG2D control groups. The relative expression levels of *TwFPS* and *TwHMGS* were not statistically significant compared with the control group. As for DDCs group, in contrast to the relative expression level of *TwOSC*, *TwIDI* and *TwGGPPS* decreasing to 0.3-, 0.6- and 0.3-fold, respectively, the relative expression level of *TwSE*, *TwFPS*, *TwHMGS* and *TwDXS* increased by 1.5, 2.7, 1.7 and 1.5 times compared with the control group. Only the relative expression level of *TwHMGR* was not statistically significant.

Under the *TwSE* overexpression condition (Fig. 4c), in terms of CMCs group, the relative expression level of *TwOSC*, *TwHMGS*, *TwHMGR*, *TwIDI* and *TwDXS* increased by 2.1, 2.0, 1.7, 1.7 and 3.3 times compared with the control group, respectively. The relative expression levels of *TwSQS*, *TwFPS* and *TwGGPPS* were not statistically significant. As for DDCs group, only the relative expression level of *TwFPS* and *TwIDI* were statistically significant, increasing by 2.6 and 1.5 times compared with the control group, respectively.

Under the *TwOSC* overexpression condition (Fig. 4d), in terms of CMCs group, apart from relative expression level of *TwDXS* increased by 1.61 times, the relative expression level of *TwSQS*, *TwSE*, *TwFPS*, *TwHMGS*, *TwHMGR*, *TwIDI* and *TwGGPPS* reduced by 0.4-, 0.3-, 0.2-, 0.5-, 0.4-, 0.6- and 0.7-fold compared with the control group, respectively. The relative expression levels of all genes determined were statistically significant. As for DDCs group, compared with the control group, the relative expression level of *TwSQS*, *TwSE* and *TwGGPPS* increased by 3.2, 3.3 and 1.5 times, respectively, but the relative expression level of *TwDXS* decreased to 0.4-fold. The expression levels of *TwFPS*, *TwHMGS*, *TwHMGR* and *TwIDI* were not statistically significant.

Discussion

In our work, we obtained the overexpression vectors of *TwSQS*, *TwSE* and *TwOSC* and they were transferred into *T. wilfordii* CMCs and DDCs. After overexpression, the contents of end product celastrol increased in *T. wilfordii* CMCs, but not in DDCs. Meanwhile, the relative expression level of *TwSQS*, *TwSE* and *TwOSC* were all enhanced in CMCs. These results indicate that *TwSQS*, *TwSE* and *TwOSC* were involved in biosynthesis of celastrol, and that only in *T. wilfordii* CMCs, overexpression of *TwSQS*, *TwSE* and *TwOSC* could increase celastrol accumulation.

The direct product 2,3-oxidosqualene of *TwSE* enzyme was not detected in both *T. wilfordii* CMCs and DDCs in our work. The reason is probably that 2,3-oxidosqualene contains an epoxy group in the molecule, which is usually unstable and may be easily degraded or converted into other compounds (Araki et al. 2016). Another possibility could be that in other biosynthetic pathways, such as sterol and nonsteroidal triterpene biosynthetic pathways, the immediate utilization of the substrate 2,3-oxidosqualene by other downstream enzymes is highly efficient, such as lanosterol synthase and cycloartenol synthase (Babiychuk et al. 2008). Moreover, in our work, squalene and friedelin were detected in CMCs, but not in DDCs. The reason for this phenomenon might be that intermediate products in DDCs were likely to transform into other compounds (such as sterol) soon before detection. Furthermore, overexpression of a gene involved in terpenoid biosynthetic pathway might lead to increase the flux towards the steroid biosynthesis pathway (Engels et al. 2008), which could be the reason why no significant increase in celastrol was detected in DDCs. The biosynthetic pathways of *T. wilfordii* terpenoids and steroids are so complex that further research is required to fully elucidate them.

Vacuoles in plant cells can dynamically store secondary metabolites, including terpenoids, alkaloids, and flavonoids, etc. (De Brito Francisco and Martinoia 2018). Previously, we found that the morphology of

CMCs and DDCs were quite different. A single CMC had a number of vacuoles while a single DDC only had one vacuole (Song et al. 2019). A lot of vacuoles allow celastrol to store in CMCs before detection, which may be another reason for the no significant increment of celastrol in DDCs after gene overexpression.

Overexpression of terpenoid biosynthetic enzyme genes may affect the expression of other genes in the pathway (Tong et al. 2019; Zhang et al. 2018). In our work, relative expression levels of terpenoid biosynthetic pathway-related genes showed different trends after overexpression of *TwSQS*, *TwSE* and *TwOSC* in *T. wilfordii* CMCs and DDCs (Fig. 5). In addition, after overexpressing *TwSQS*, *TwSE* and *TwOSC* in CMCs in our work, the relative expression of *TwDXS* involved in MEP pathway were all increased, leading to an increase in the content of celastrol biosynthesized through MVA pathway, which indicates that *TwDXS*, *TwSQS*, *TwSE* and *TwOSC* seem to have synergistic effects during the biosynthesis of celastrol. Moreover, after *TwOSC* overexpression, though the relative expression levels of terpenoid biosynthetic pathway-related genes detected in CMCs were all decreased except *TwDXS*, the content of celastrol was still increased, which could be attributed to the feedback regulation on the expression of genes in the pathway (Zhang et al. 2018).

In our work, the content of celastrol produced in CMCs was 57.9-, 37.5- and 53.3-fold higher than that of DDCs after *TwSQS*, *TwSE* and *TwOSC* overexpression, respectively. Moreover, the relative expression levels of *TwSQS* and *TwOSC* in CMCs were higher than that of DDCs after overexpression. Higher gene expression might contribute to higher celastrol accumulation in CMCs. Our results demonstrate that CMCs could be used as a better platform than DDCs to study the function of genes involved in the biosynthetic pathway of celastrol.

In conclusion, it was verified in *T. wilfordii* CMCs that overexpression of *TwSQS*, *TwSE* and *TwOSC* increased celastrol accumulation and had different effects on the expression of related genes in terpenoid biosynthetic pathway, which laid a foundation for further elucidating the downstream biosynthetic pathway of celastrol through *T. wilfordii* CMCs platform.

Abbreviations

Tripterygium wilfordii Hook. f.: *T. wilfordii*

Cambial meristematic cells: CMCs

Dedifferentiated cells: DDCs

Declarations

Author contribution statement

YS, XW and WG conceived and designed the research. YS conducted experiments. JZ assisted vector construction. Yf Z and Yj Z assisted gene gun technology. TH, YT and LH assisted data analysis. YS wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Supplementary material

The spectrum of squalene, friedelin and celastrol detected by GC-MS and UPLC (PDF).

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Figures

Figure 1

Construction and transfer verification of TwSQS, TwSE and TwOSC overexpression vectors a TwSQS, TwSE and TwOSC overexpression fragments. TwSQS overexpression fragment of 1241 bp, TwSE overexpression fragment of 1584 bp and TwOSC overexpression fragment of 2298 bp b Verification of successful transfer of overexpression vectors in *T. wilfordii* CMCs and DDCs. Agarose gel electrophoresis showed the hygromycin fragment (1787 bp) c Schematic diagram of overexpression vectors. OE was the abbreviation for overexpression, Hyg was the abbreviation for hygromycin

Figure 2

The morphology of *T. wilfordii* CMCs and DDCs a CMCs were mostly aggregated by small cell groups. Scale bar, 2 mm b DDCs were gathered with large cell groups. Scale bar, 2 mm

Figure 3

Accumulation of squalene, friedelin and celastrol in *T. wilfordii* CMCs and DDCs after overexpressing TwSQS, TwSE and TwOSC a Accumulation of squalene and friedelin in CMCs b Accumulation of celastrol in CMCs and DDCs. OE was the abbreviation for overexpression. DW was the abbreviation for dry weight. The data are summarized as means ± standard deviation. *P<0.05 and **P<0.01, n=5. Student's t test

Figure 4

Relative expression analysis of related genes involved in terpenoid biosynthetic pathway in *T. wilfordii* CMCs and DDCs after overexpression of TwSQS, TwSE and TwOSC a Relative expression levels of TwSQS, TwSE and TwOSC b Relative expression levels of related genes in terpenoid biosynthetic pathway after TwSQS overexpression c Relative expression levels of related genes in terpenoid biosynthetic pathway after TwSE overexpression d Relative expression levels of related genes in terpenoid biosynthetic pathway after TwOSC overexpression. OE was the abbreviation for overexpression. The data are summarized as means $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, $n = 5$. Student's *t* test

Figure 5

The expression trends of genes involved in terpenoid biosynthetic pathway after overexpression of TwSQS, TwSE and TwOSC based on Fig.4. A black single arrow represented one-step reaction, a black double arrow represented multi-step reaction and a black dotted arrow represented unknown steps. The colored upward or downward arrows indicated corresponding increase and decrease in gene expression. Colored horizontal line represented no statistical significance between the overexpression group and the pH7WG2D control group. The SQS, SE and OSC enzymes studied in this work were highlighted in red. OE was the abbreviation for overexpression

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