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Friedelane-type triterpene cyclase in celastrol biosynthesis from *Tripterygium wilfordii* and its application for triterpenes biosynthesis in yeast

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Summary

Cela trol i a promi ing bioactive compound i olated from *Tripterygium wilfordii* and ha been proven to po e many encouraging preclinical application . However, the cela trol bio ynthetic pathway i poorly under tood, e pecially the key oxido qualene cycla e (OSC) re pon ible for cyclization of the main caffold.

Here, we report on the i olation and characterization of three OSC from *T. wilfordii*: *Tw*OSC1, *Tw*OSC2 and *Tw*OSC3. Both *Tw*OSC1 and *Tw*OSC3 were multi-product friedelin yntha e , while *Tw*OSC2 wa a β-amyrin yntha e.

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We further found that *Tw*OSC1 and *Tw*OSC3 were involved in the bio ynthe i of cela trol and that their common product, friedelin, wa a precur or of cela trol. We then recon tituted the bio ynthetic pathway of friedelin in engineered yea t con tructed by the CRISPR/Ca 9 y tem, with protein modification and medium optimization, leading to heterologou production of friedelin at 37.07 mg L^{-1} in a hake fla k culture.

Our tudy wa the fir t to identify the gene re pon ible for bio ynthe i of the main caffold of cela trol and other triterpene in *T. wilfordii*. A friedelin ha been found in many plant , the re ult and approache de cribed here laid a olid foundation for further explaining the bio ynthe i of cela trol and related triterpenoid . Moreover, our re ult provide in ight for metabolic engineering of friedelane-type triterpene .

Key words: bio ynthe i , cela trol, oxido qualene cycla e (OSC), *Tripterygium wilfordii*, triterpene .

Introduction

Tripterygium wilfordii Hook.F i a medicinal plant known in traditional Chine e medicine a 'Lei gong teng' (al o called thunder god vine), the debarked root of which ha a long hi tory of u e in the treatment of rheumati m and inflammation (Jiang, 1994; Tao & Lip ky, 2000; Zhou, ZL *et al.*, 2012). Some li ted drug developed from *T. wilfordii* including Tripterygium glyco ide tablet and Leigongteng tablet , have al o been u ed to treat autoimmune and inflammatory di ea e in China ince the 1970 (Li, 2000; Tao *et al.*, 2001; Yang *et al.*, 2005). A lot of pharmacological tudie have ugge ted that extract of *T. wilfordii* can be u ed to treat tumour (Wong *et al.*, 2012), nephrotic yndrome (Xu *et al.*, 2009), HIV (Duan *et al.*,

Crew , 2007). Cela trol i a friedelane-type triterpene that wa fir t i olated from the root of *T. wilfordii*, and wa found to be effective in the treatment of inflammatory, autoimmune di ea e (A try *et al.*, 2015), Alzheimer' di ea e (Alli on *et al.*, 2001) and cancer (Yang, H *et al.*, 2006; Pang *et al.*, 2010). Intere tingly, re earcher al o found thi compound to be a leptin en itizer and a promi ing agent for the treatment of obe ity (Liu *et al.*, 2015; Ma *et al.*, 2015).

De pite con iderable pharmaceutical intere t, the low cela trol level , low growth rate and re tricted habitat of thi plant have all limited the further tudy and application of cela trol. To addre the above i ue , method have been developed to obtain a u tainable and reliable upply, uch a cell culture y tem and total ynthe i (Su *et al.*, 2014; Camelio *et al.*, 2015). In recent year , metabolic engineering in microorgani m ha been proven to be a promi ing method for the production of high-value natural product (Zhou, YJ *et al.*, 2012; Paddon *et al.*, 2013; Galanie *et al.*, 2015; Lau & Sattely, 2015). However, the bio ynthetic pathway of cela trol remain unknown. The pharmaceutical potential of triptolide ha led many re earcher to inve tigate the bio ynthetic pathway of thi co2.1( );6(und)-10.6(Foharnie)] TJETBT0 Tc

Mo t triterpene caffold are cyclized via the chair-chair-chair (CCC) conformation cataly ed by OSC, while terol are cyclized via chair-boat-chair (CBC) conformation (Fig.2). The cyclization of oxido qualene involved four main tep :(a) binding of the ub trate and predetermination the ring y tem, (b) initiation of the reaction by protonation of the epoxide, (c) cyclization and rearrangement of carbocation pecie , and (d) termination by deprotonation or water capture to yield a final terpene product (Thimmappa *et al.*, 2014). More than 200 different triterpene keleton have been di covered from natural re ource or enzymatic reaction (Xu *et al.*, 2004). Friedelin i one of the mo t highly rearranged triterpene known in plant .

Here, we aimed to identify the OSC re pon ible for the bio ynthe i of cela trol. Three candidate OSC gene were creened from the tran criptome of *T. wilfordii*. *Tw*OSC1 and *Tw*OSC3 are friedelane-type triterpene cycla e re pon ible for the bio ynthe i of cela trol, while *Tw*OSC2 i a β-amyrin yntha e. Both *Tw*OSC1 and *Tw*OSC3 can cyclize 2, 3-oxido qualene to friedelin a the major product, with β-amyrin and α-amyrin a the minor product . We al o proved that friedelin i a precur or of cela trol. In addition, the yield of friedelin in the engineered yea t wa al o improved by u ing ite-directed mutagene i , the CRISPR/Ca 9 y tem and medium optimization. Our finding laid the foundation for exploration of the bio ynthetic pathway of cela trol and other friedelane-type triterpene and provided in ight into the bio ynthetic pathway of friedelane-type, oleanane-type and ur ane-type triterpene in *T. wilfordii* and other plant .

Materials and Methods

Plant materials

T. wilfordii cell u pen ion were initiated from callu e of *T. wilfordii* and cultured in Mura hige and Skoog (MS) medium containing 30 g L^{-1} ucro e with 0.1 mg L^{-1} kinetin (KT), 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} indole-3-butyric acid (IBA). The e cell u pen ion were cultured in the dark at 25 °C with rotary haking at 120 rpm a de cribed previou ly (Guan *et al.*, 2017). Mature *T. wilfordii* plant ti ue were collected from the Yongan national fore t in Fujian Province, China. Sample of root, tem,

MeJA-mediated induction of *T. wilfordii* suspension cells

The *T. wilfordii* u pen ion cell were treated with methyl ja monate (MeJA) at a final concentration of 50 mol L^{-1} after ubculturing. The control group wa treated with the ame volume of carrier olution. After 0, 4, 8, 12, 24, 48, 72, and 360 h, the u pen ion cell were harve ted in liquid nitrogen and then tored at -80 °C before RNA extraction or UPLC analy i . Four biological duplicate were prepared for each time point in each group.

Quantitative real-time PCR

The total RNA of the treated u pen ion cell wa extracted by the modified CTAB method a de cribed above. Fir t- tand cDNA for quantitative real-time PCR (qRT-PCR) wa rever e tran cribed from the total RNA according to manufacturer' in truction for the Fa tQuant RT Kit (Tiangen Biotech, Beijing, China). qRT-PCR were performed with gene pecific primer (Table S1) and the KAPA SYBR® FAST qPCR Kit (KAPA Bio y tem , Ma achu ett , USA) on an Applied Bio y tem QuantStudio 5 real-time PCR Sy tem (Applied Bio y tem , New York, USA). The PCR condition were a follow : an initial incubation at 95 □ for 3 min, followed by 45 cycle of 95 □ for 3 and 60 □ for 30 and then by a melting curve cycle. Elongation factor 1 α (EF1α) wa u ed a a reference gene (Miao *et al.*, 2015). Relative tran cript abundance wa evaluated u ing the $2^{-\ Ct}$ method (Livak & Schmittgen, 2001) with triplicate mea urement from four biological replicate .

UPLC analysis of celastrol content in induced *T. wilfordii* suspension cells and plant tissues

The *T. wilfordii* u pen ion cell and powder of plant ti ue were tored at -80 °C for at lea t 4 h prior to freeze drying for 48 h (Chri t ALPHA 1-2, Germany). Approximately 50 mg of each ample wa u pended in 1 mL of 80% (v/v) methanol overnight at room temperature (25 °C), and then extracted by onication in an ultra onic water bath for 60 min. After centrifugation for 2 min, 10000× g and room temperature, the upernatant wa filtered through a 0.22- m membrane filter (polytetrafluoroethylene) before UPLC analy i .

The analy e were conducted u ing an Agilent 1290 Infinity II Efficient UHPLC y tem quipped with a DAD detector. Chromatographic eparation wa conducted u ing a Water ACQUITY UPLC HSS T3 analytical column (2.1×100 mm, 1.8 m) maintained at 40 °C. The mobile pha e, con i ting of a mixture of 0.1% (v/v) formic acid in water (A) and acetonitrile (C), wa pumped at a flow rate of 0.4 mL/min. The gradient program wa 30% C at 0 min, 35% C at 5-8 min, 70% C at 15 min, and 90% C at 21 min. The detection wavelength wa 425 nm, and UV pectra from 190 to 500 nm were al o recorded. The injection volume wa 5 L.

Functional identification in yeast

The ORF of *Tw*OSC were amplified and ub-cloned into the pYES2 expre ion vector (Invitrogen) eparately. The ORF of *Tw*OSC1 and *Tw*OSC2 were in erted between the *Kpn*I-*Not*I ite of pYES2 via dige tion by the corre ponding re triction endonuclea e . *Tw*OSC3 wa in erted between the *Kpn*I-*Bam*HI ite of pYES2 in the ame manner. All primer are hown in Table S1. The con tructed vector were tran formed into lano terol yntha e-deficient yea t (*Saccharomyces cerevisiae*, purcha ed from ATCC, cell line number: 4021900, -ERG7, -*ura*) u ing the Frozen-EZ Yea t Tran formation II Kit (ZYMO RESEARCH). The pYES2 vector wa tran formed into yea t a a control.

The tran formant were cultured on olid ynthetic complete medium without uracil (SC-Ura) for election. The po itive tran formant wa then cultured in 20 mL of Sc-Ura medium containing 2% gluco e and incubated with haking at 220 rpm and 30 °C for 2 day . Then, the cell were collected and induced in 20 mL of Sc-Ura medium with 2% galacto e in place of gluco e and further cultured at 220 rpm and 30 °C for 12 h. After induction, the yea t cell were collected and re- u pended in 0.1 M pota ium pho phate buffer (pH 7.0) with 2% gluco e, and cultured at 220 rpm and 30 °C for 1 day. Finally, the yea t cell were collected and ly ed with 10 mL of 20% KOH and 50% EtOH by ultra onic extraction for 1 h or refluxed for 5 min with 10 mL of 20% KOH and 50% EtOH. The upernatant wa extracted with 10 mL of hexane three time . All the extract were combined and then evaporated by rotary evaporation.

The extract were di olved in pyridine and derivatized with N-methyl-N-(trimethyl ilyl) trifluoroacetamide at 70 °C for 1 h. Then, the derived extract were evaporated under N_2 and di olved in 1 mL trichloromethane ($CHCl_3$) for analy i by ga chromatography and ma pectrometry (GC-MS). The analy i wa performed on an Agilent 7000 ga chromatograph (plit, 20:1; injector temperature, 250 □) with a DB-5m (15 m×250 μm×0.1 μm) capillary column. One microliter of the concentrated organic pha e wa then injected under a He flow rate of 1 mL/min with a temperature program of 1 min at 50 °C, followed by a gradient from 50 to 260 °C at 50 °C min^{-1} and then, to 272 °C at 1 °C min^{-1} with a 4 min held. The ion trap temperature wa 230 □. The electron energy wa 70 eV. Spectra were recorded in the range of 10-550 m/z.

RNAi study

A pecific 300-500 bp fragment wa elected ba ed on the bla t analy i in *T. wilfordii* tran criptome databa e (Fig.S1 and Fig. S2) and amplified from the ORF of each *Tw*OSC by pecific primer (Table S1) and thenerted the vector pK7GWIWG2D (Invitrogen) by the Gateway cloning y tem (Invitrogen). The vector containing each fragment were tran formed into *E. coli* and then verified by PCR and equencing. The re ulting vector were tran formed into *T. wilfordii* u pen ion cell according to Zhao *et al.* (Zhao *et al.*, 2017). Su pen ion cell in the logarithmic growth pha e were plated on MS olid medium (pH = 5.8) and grown for 7 day before tran formation. The empty vector wa tran formed into *T. wilfordii* u pen ion cell a a control, and all ample had five biological duplicate . The tran formed cell u pen ion were incubated for 7 day on MS medium and then tran ferred o3to election medium (olid MS medium upplemented with 0.5 mg L^{-1} 2, 4-D, 0.1 mg L^{-1} KT, 0.5 mg L^{-1} IBA) upplemented with 100 mg L^{-1} kanmynfor election of tran formed cell u pen ion . Po itive tran formed cell u pen ion were further inoculated in the electionS medium foculture growthith hng. After three generation of creening, the tran formed cell u pen ion were harve ted to mea ure gene expre ion and cela trol level .

Strain construction and fermentation

Specific gRNA targeting *rox1*, *ypl062w* and *yjl064w* were de igned by the open- ource tool at http://yea triction.tnw.tudelft.nl. Single and multiple gRNA expre ion vector for *yjl064w*, *ypl062w* and *rox1* were con tructed a de cribed previou ly (Man *et al.*

Results

Cloning and sequence analysis of the *Tw*OSCs

In our previou tudy, we equenced and reported a tran criptomic library (SRA acce ion number: SRR6001265) of *T. wilfordii* (Su *et al.*, 2017). Tran cript of OSC candidate were creened via homology-ba ed earche of the tran criptome. Three full-length cDNA equence of OSC (*Tw*OSC1, *Tw*OSC2 and *Tw*OSC3) were obtained by polymera e chain reaction and equencing (GenBank acce ion number KY885467, KY885468 and KY885469). Protein domain analy i howed that all the protein have a terpenoid cycla e/protein prenyltran fera e alpha-alpha toroid domain, but only *Tw*OSC2 ha the con erved ite of terpene yntha e (Fig. S4). Ba ed on the multiple equence alignment , all three *Tw*OSC were homologou (average imilarity identified a 76.46%) to OSC in everal other pecie (Fig. S5).The three *Tw*OSC all have the con erved region DCTAE of the OSC family (Abe & Pre twich, 1995), which i a ociated with ub trate binding. The con erved repetitive QW (QXXXXXW) motif alway appear a four to eight repeat in the OSC family (Haralampidi *et al.*, 2002) and appear a four repeat in the *Tw*OSC . In addition, the *Tw*OSC2 and *Tw*OSC3 both have the MWCYCR motif, which i predicted to be a highly con erved motif of β-amyrin yntha e (Tet uo Ku hiro *et al.*, 2000).

Phylogenetic tree construction

The re ult of the phylogenetic analy i howed that *Tw*OSC2 wa different from *Tw*OSC1 and *Tw*OSC3, which clu tered with the previou ly characterized β-amyrin yntha e gene *Ks*bAS, *Hh*bAS and *Ae*bAS (Fig. 3). In contra t, *Tw*OSC1 and *Tw*

*Kd*LUS, re pectively). The e re ult indicated that *Tw*OSC1 and

Functional characterization of *Tw*OSCs in yeast

To identify the function of the putative OSC from *T. wilfordii*, the complete ORF of all the *Tw*OSC were ub-cloned into the vector pYES2 and then tran formed into lano terol yntha e-deficient yea t. In parallel, the empty vector wa tran formed into the yea t mutant to erve a a negative control. After culturing and induction, the metabolite extract were extracted and analy ed by GC-MS. Ba ed on the re ult of GC analy i (Fig. 5), the extract from yea t harboring *Tw*OSC2 contained only one product, which wa confirmed to be *β*-amyrin. Yea t harboring *Tw*OSC1 and *Tw*OSC3 contained friedelin, *β*-amyrin and *α*-amyrin, with friedelin a the major product. The tructure of all the e product were explained by compari on of ma pectral characteri tic with tho e of authentic tandard (Fig. S7, S8 and S9). Thi re ult from GC-MS analy i ugge ted that *Tw*OSC1 and *Tw*OSC3 were multi-product friedelin yntha e and that *Tw*OSC2 wa a *β*-amyrin yntha e.

RNAi identified candidate *Tw*OSCs for biosynthesis of celastrol

To further inve tigate the *Tw*OSC re pon ible for the bio ynthe i of cela trol, *Tw*OSC1, *Tw*OSC2 and *Tw*OSC3 were elected a candidate gene for the RNA interference (RNAi) tudy. In our early work, a table and highly efficient tran formation y tem uitable for *T. wilfordii* cell u pen ion wa developed ba ed on particle bombardment (Zhao *et al.*, 2017). Here, we u ed thi tran formation y tem to tran form the candidate gene . pK7GWIWG2D(II)-*Tw*OSC1, -*Tw*OSC2, -*Tw*OSC3 and the empty vector were eparately tran formed into *T. wilfordii* cell and creened on MS olid medium with kanamycin. *Tw*OSC1 expre ion wa uppre ed by 42.5% and cela trol level decrea ed by 32.0% compared with the control. *Tw*OSC3 expre ion wa uppre ed by 37.3%, and cela trol level decrea ed by 84.2%. In contra t, RNAi uppre ed the tran cription of *Tw*OSC2 by 47% and increa ed cela trol level by 9.5% (Fig. 6a, b, c). RNAi combined with gene expre ion analy i and UPLC analy i of cela trol profile howed that *Tw*OSC1 and *Tw*OSC3 were involved in the bio ynthe i of cela trol and that *Tw*OSC3 may have a tronger effect than *Tw*OSC1 on cela trol bio ynthe i .

Friedelin is a precursor of celastrol

Ba ed on the re ult of the RNAi tudy and the ob erved ti ue expre ion pattern, we determined that *Tw*OSC1 and *Tw*OSC3 were re pon ible for the bio ynthe i of cela trol. The GC-MS analy i howed that both *Tw*OSC1 and *Tw*OSC3 can produce friedelin, which i al o a friedelane-type triterpene, imilar to cela trol. To further inve tigate the potential role of friedelin in cela trol bio ynthe i , we fed friedelin to u pen ion cell culture , which led to a ignificant increa e in cela trol accumulation compared with the control group after 7 day (Fig. 6d). Thi finding indicated that friedelin did erve a a precur or to cela trol.

Site-directed mutagenesis of *Tw*OSC proteins

The reaction cataly ed by OSC i one of the mo t complicated reaction , and can yield a wide array of triterpene keleton derived from the ame imple ub trate 2, 3-oxido qualene. However, the exact mechani m underlying thi product diver ity remain unknown. Enzyme with a ingle product and high yield will be helpful for exploration of the ub equent bio ynthe i pathway and further commercial application. Thu , we wanted to identify the crucial amino acid that are involved in the production of friedelin. Recently, Souza-Moreira *et al.* found that the amino acid two po ition up tream of the DCTAE ite in friedelin yntha e, u ually a Leucine (L) re idue, wa required for friedelin production (Souzamoreira *et al.*, 2016). Multiple equence alignment of the friedelin-producing OSC and molecular docking of *Tw*OSC1 and *Tw*OSC3 howed that the L re idue wa unique to friedelin-producing OSC and wa near the active ite of the e enzyme (Fig. 7a, b).

To further inve tigate thi re idue in friedelin yntha e, we con tructed additional mutant by ite-directed mutagene i . Sub titution of the L re idue with phenylalanine (F), hi tidine (H), proline (P) or arginine (R) in *Tw*OSC1 and ub titution of the L re idue with F, P, R, or alanine (A) in *Tw*OSC3 aboli hed all the product , but ub titution of the L re idue with valine (V) decrea ed the production of friedelin and increa ed the production of β-amyrin. Sub titution of the L re idue with Serine (S) in *Tw*OSC3 al o aboli hed the production of friedelin and increa ed the production of β-amyrin (Fig. S10 and S11).

Moreover, ub titution of the L re idue with i oleucine (I) in *Tw*OSC1 and *Tw*OSC3 did not impact friedelin production much but increa e the production of *β*-amyrin. In addition, we al o con tructed a mutant of *Tw*OSC2 to determine whether ub titution of the V re idue at the ame po ition with L could change the product to friedelin. However, thi ub titution in *Tw*OSC2 did not lead to friedelin production and decrea ed the yield of *β*-amyrin. Fortunately, we found a re idue in *Tw*OSC1 (Threonine (T) 502) that can increa e the production of friedelin. The be t mutant wa T502E (Glutamic acid), which produced 10.63 mg L^{-1} friedelin in a hake fla k culture. The level of all the product of the *Tw*OSC and mutant are hown in Fig. 7c and Table S3.

Improvement of the yield of friedelin in engineered *S. cerevisiae*

To improve the yield of friedelin, two key gene in the triterpene pathway were overexpre ed to enhance the metabolic flux toward (3S)-2, 3-oxido qualene. ERG9 and tHMG1 from *S. cerevisiae* were eparately cloned into the pESC-leu vector then tran formed into the yea t BY4741. In addition to overexpre ing the key gene in the triterpene pathway, three gene were deleted to further improve the production of friedelin (Fig. 8a). The e gene included a tran criptional regulator, *rox1* and two other gene , namely, *ypl062w* and *yjl064w. Rox1* wa reported to repre gene in the MVA pathway and ergo terol bio ynthe i while *ypl062w* and *yjl064w* could improve MVA pathway flux when knocked out and e pecially when combined (Özaydın *et al.*, 2013). When pYES2-*Tw*OSC1^{T502E} wa tran formed into train ZH1, train BY4741 and the lano terol yntha e-deficient train cultured under the ame condition , the highe t yield wa 22.52 mg L^{-1} in train ZH1, which wa higher than yield in the lano terol yntha e-deficient train and almo t two-fold higher than the yield in the parent train BY4741(Fig. 8b). After medium optimization, the highe t yield of train ZH1 could be improved to 37.07 mg L^{-1}. Thi i the fir t tudy on the bio ynthe i of friedelin in engineered yea t with a high yield.

Discussion

T. wilfordii i known to contain a diver ity of triterpene , and cela trol wa one of the valuable compound i olated from *T. wilfordii*. Here, we focu ed on the OSC that were

The mechani tic diver ity of OSC remain intriguing. Although many attempt have been made to tudy the underlying mechani m (Hart *et al.*, 1999; Herrera *et al.*, 2000; Tet uo Ku hiro *et al.*, 2000; Liu *et al.*, 2012; Salmon *et al.*, 2016; Xue *et al.*, 2018), the mechani m of conver ion between caffold remain poorly under tood. Sequence analy i howed that all three *Tw*OSC contained terpenoid cycla e/protein prenyltran fera e alpha-alpha toroid domain , but only *Tw*OSC2 contained con erved ite of terpene yntha e, which i rich in aromatic re idue , in the C-terminal region. Multiple equence alignment howed that all the e protein contained the con erved DCTAE motif and four con erved repetitive QW motif . Moreover, *Tw*OSC2 and *Tw*OSC3 both had the MWCYCR motif, which i important for β-amyrin yntha e. However, we found that *Tw*OSC1 (containing the IWCYCR motif) a well a *Tw*OSC2 and *Tw*OSC3 could produce β-amyrin. Tet uo *et al.* found that the Tyrptophan re idue in the MWCYCR motif wa re pon ible for the formation of pentacyclic triterpene and that ub titution of thi re idue with H re ulted in the production of tetracyclic dammaradienol (Tet uo Ku hiro *et al.*, 2000). Here, our re ult ugge ted that the methionine re idue in the MWCYCR motif wa not nece ary for the production of β-amyrin.

To identify the key re idue involved in functional conver ion between friedelin and β-amyrin, we performed ite-directed mutagene i . Our re ult howed that ub titution of L re iduedn ou21.32.ph5.5 I

re idue with L in *Tw*OSC2 did not lead to friedelin production and decrea ed the yield of *β*-amyrin, which al o confirmed thi inference. Thi re ult trongly indicated that the L re idue wa important for the bio ynthe i of friedelin but not the only factor influencing the production of friedelin. In the other ite of *Tw*OSC1 (T502), we found that ub titution of T

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Fig. S1 cDNA equence of *Tw*OSC1-3 u ed to create RNAi vector .

Fig. S2

Figure Legends

Fig. 1 Propo ed biogenetic pathway for cela trol in *Tripterygium wilfordii.* The red olid arrow indicate a bio ynthetic reaction tep identified in thi work, and each da hed arrow indicate one or multiple propo ed tep reaction . All triterpenoid intermediate in the propo ed pathway have been reported in *T. wilfordii.*

Fig. 2 The propo ed oxido qualene cyclization mechani m . Friedelin i a mo t rearranged friedelane-type triterpene found in many plant including *Tripterygium wilfordii*.

Fig. 3 Phylogenetic tree of *Tw*OSC and the characterized oxido qualene cycla e (OSC) from other pecie . The phylogenetic tree wa created by MEGA 6.0and the neighbour-joining method. The boot trap confidence value were obtained ba ed on 1000 replicate . The *Tw*OSC from *Tripterygium wilfordii* are highlighted with red diamond.

Fig. 4 Analy i of methyl ja monate (MeJA)-induced and ti ue expre ion of *Tw*OSC combined with cela trol di tribution. (a) Relative expre ion of *Tw*OSC in the MeJA induced *Tripterygium wilfordii* u pen ion cell . CK: control group; MJ: MeJA treated group. (b) Cela trol content in MeJA-induced *T. wilfordii* u pen ion cell . CK: control group; MJ: MeJA treated group. (c) Relative expre ion of *Tw*OSC in different *T. wilfordii* ti ue . (d) *T. wilfordii* plant and di tribution of cela trol in *T. wilfordii*. Error bar repre ent SD ba ed on triplicate mea urement of at lea t four biological replicate .

Fig. 5 Ga chromatography-ma pectrom61.96035] TJ(r)3C-MSi.) 61.9635] TJ SD61.96 SD o TJf(r)3. 2(61.965

after 0 and 7day . Student' t-te t wa u ed to te t tati tically ignificant difference of increa ed cela trol level between control group and feeding group. The data repre ent the average ± SD of at lea t four independent line of u pen ion cell .

Fig. 7 Molecular docking and mutagene i a ay of *Tw*OSC from *Tripterygium wilfordii*. (a) Sequence repre entation in the DCTAE motif of *Tw*OSC . Acce ion number and abbreviation are provided in Supporting Information Table S4. (b) Molecular docking of *Tw*OSC1 and *Tw*OSC3 with friedelin. (c) Product analy i of the mutant . The activitie of wild-type and all the mutant are pre ented a the mean ± SD , $n = 5$.

Fig. 8 Strain con truction and friedelin production u ing *Tw*OSC1^{T502E} from *Tripterygium wilfordii*. (a) A trategy to con truct train ZH1. (b) The level of product in the three train containing *Tw*OSC1^{T502E}. BY4741 tand for train BY4741 containing *Tw*OSC1^{T502E}; GIL77 tand for the lano terol yntha e-deficient train containing *Tw*OSC1^{T502E}; ZH1 tand for train ZH1 containing *Tw*OSC1^{T502E}; and ZH1-MO tand for train ZH1 containing *Tw*OSC1^{T502E} in the optimization medium. The data are pre ented a the mean ± SD , n = 3.

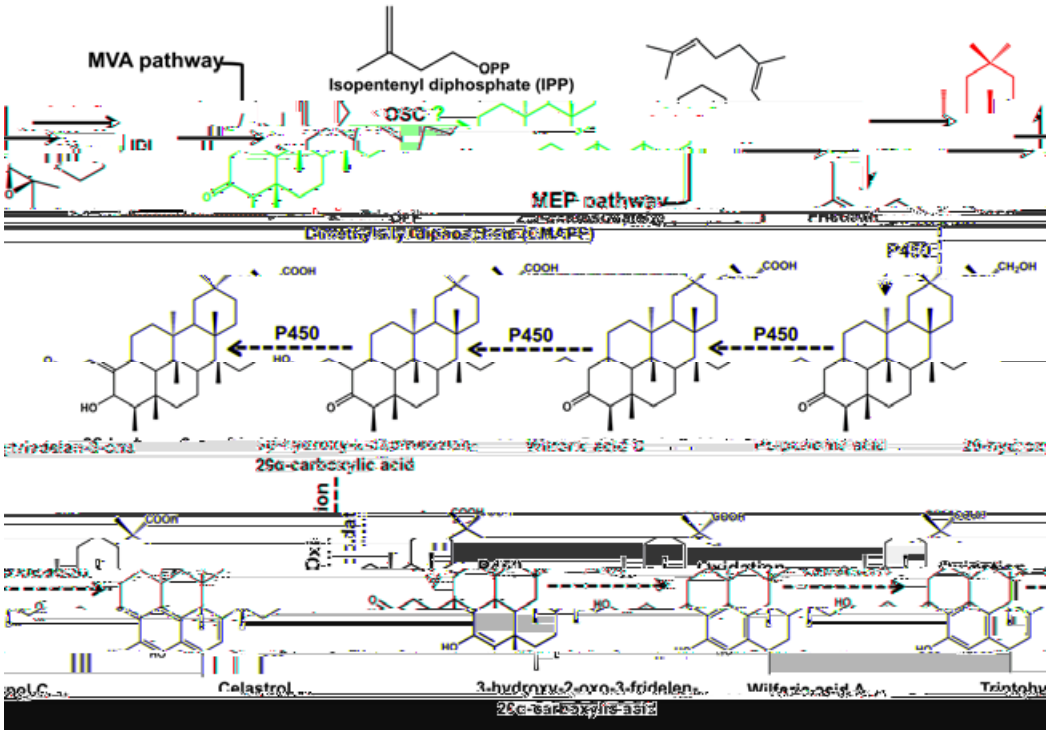


Fig. 1

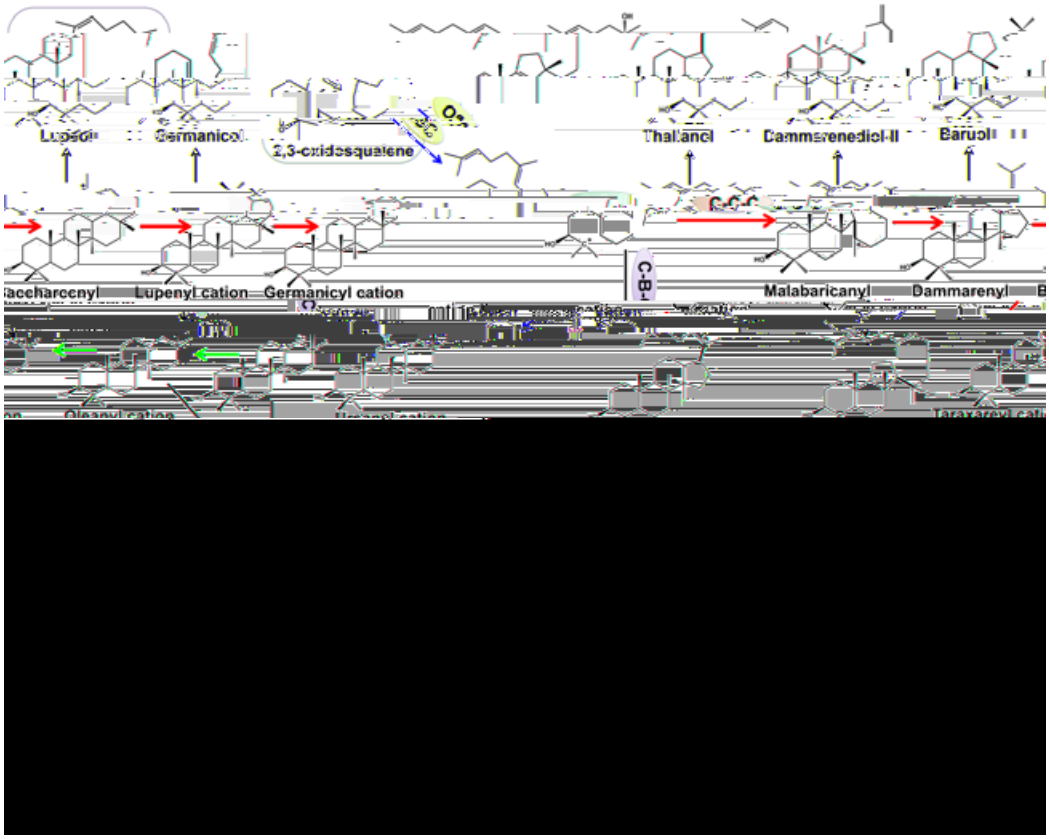


Fig. 2

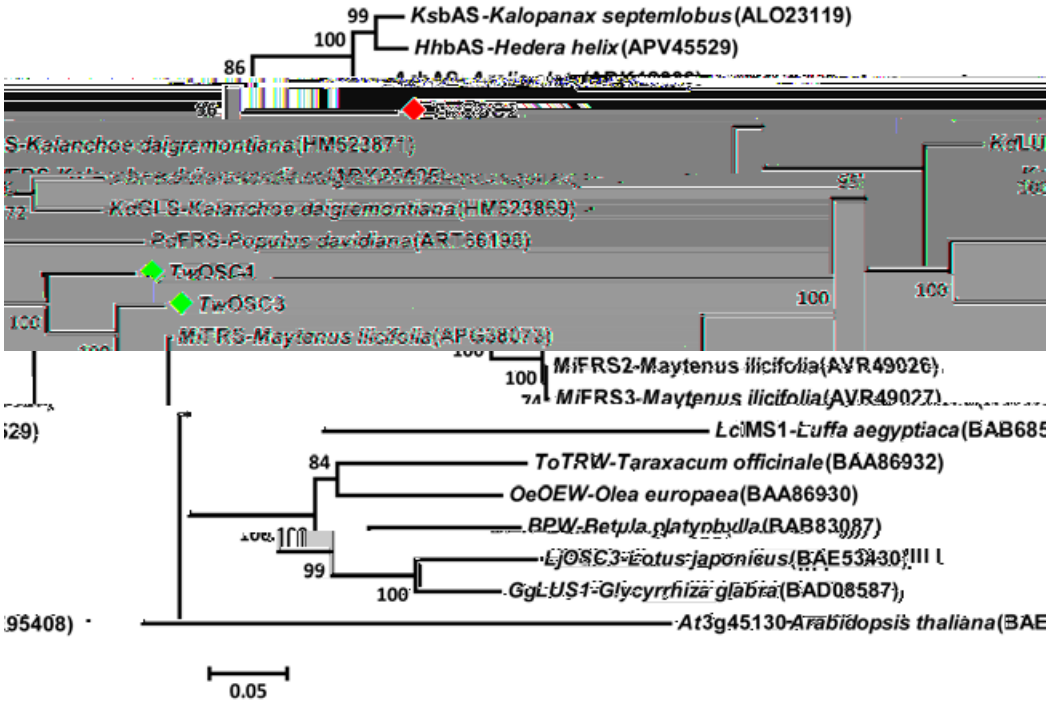
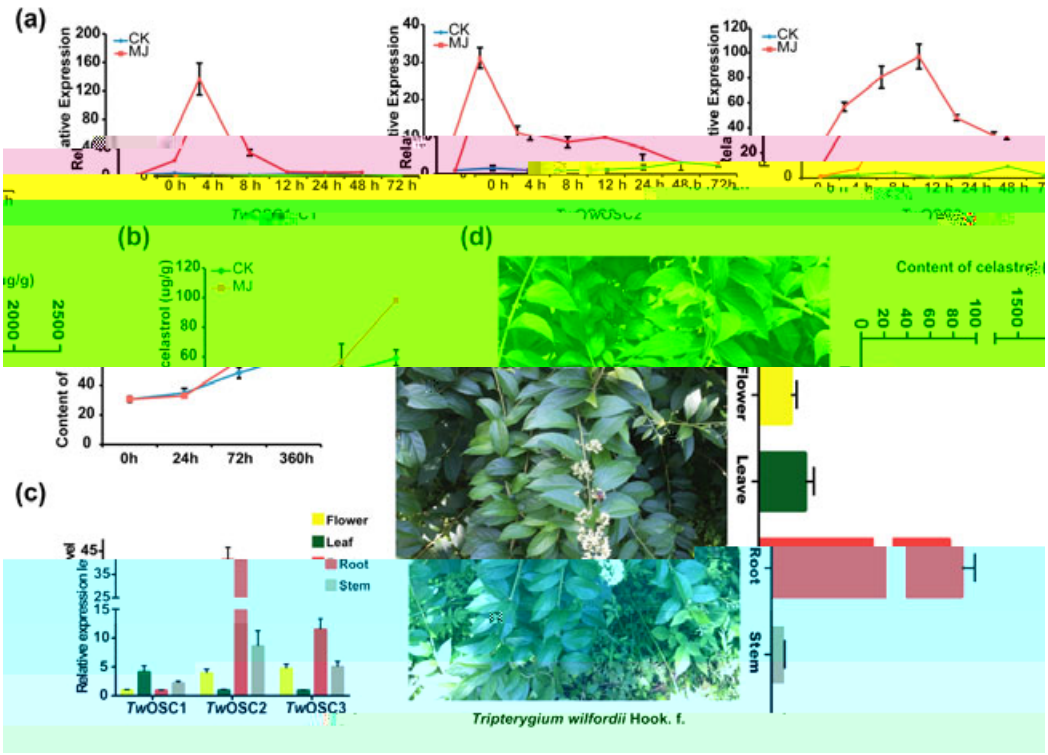


Fig. 3



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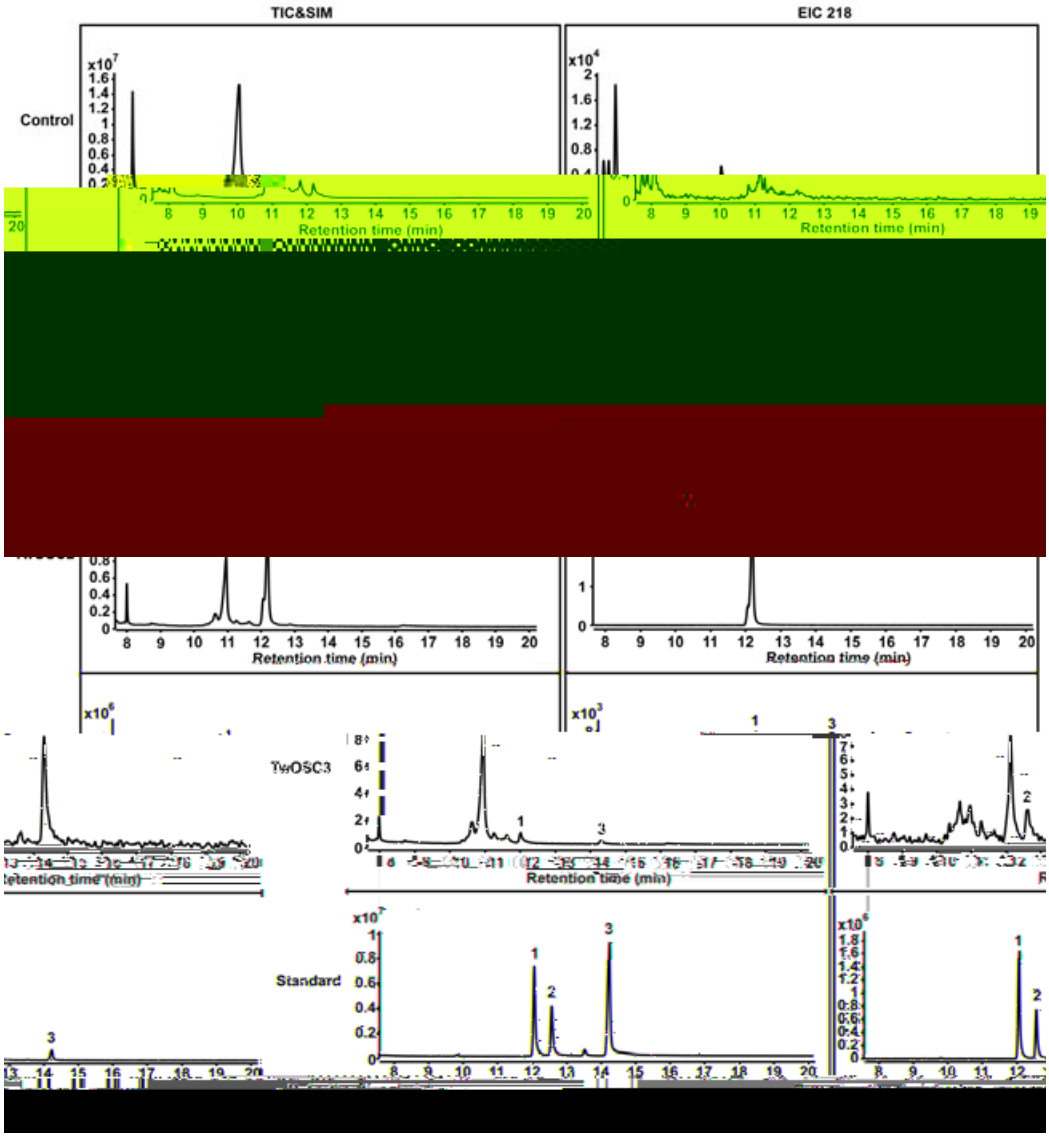

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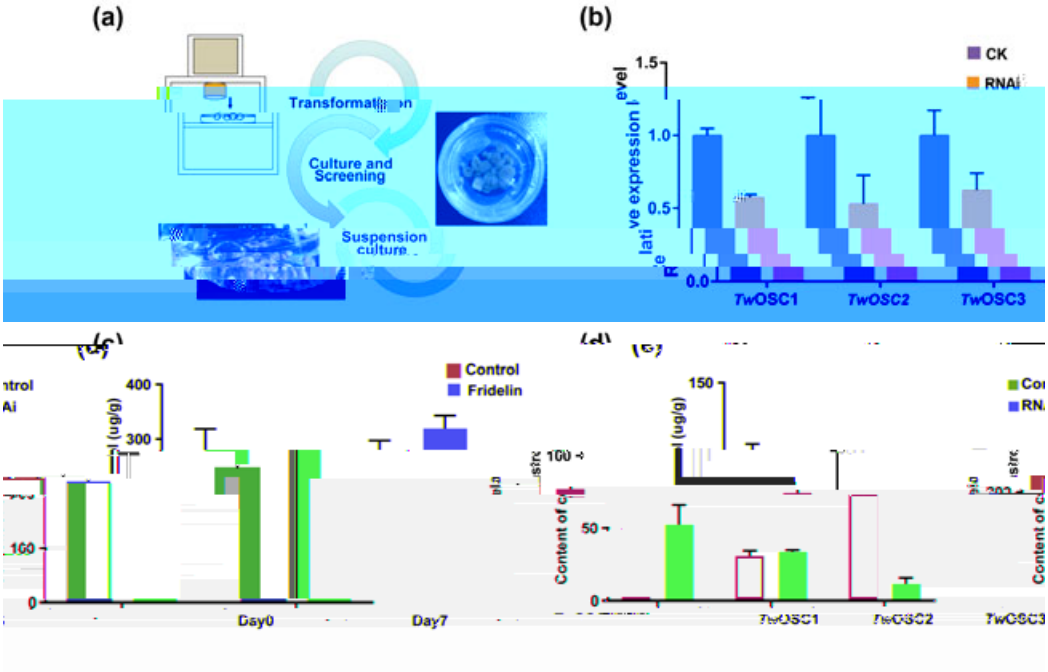

Fig. 6

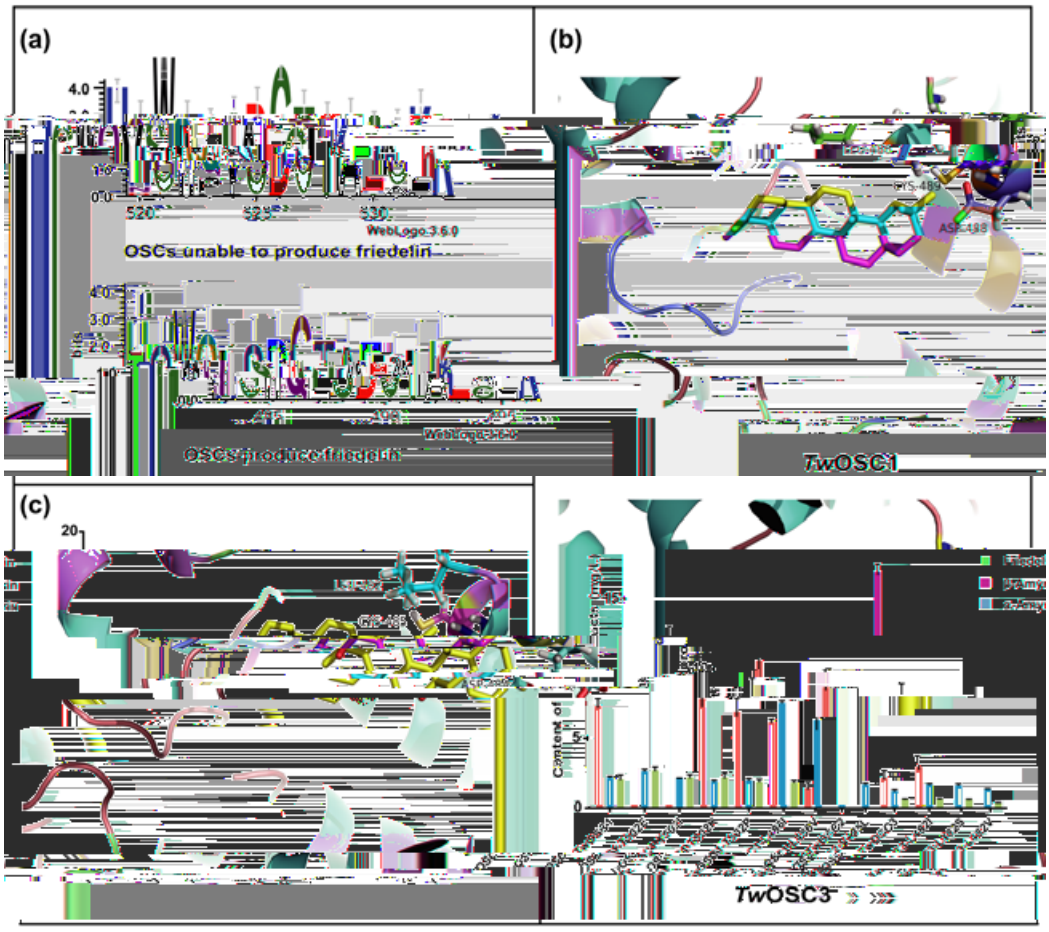

Fig. 7

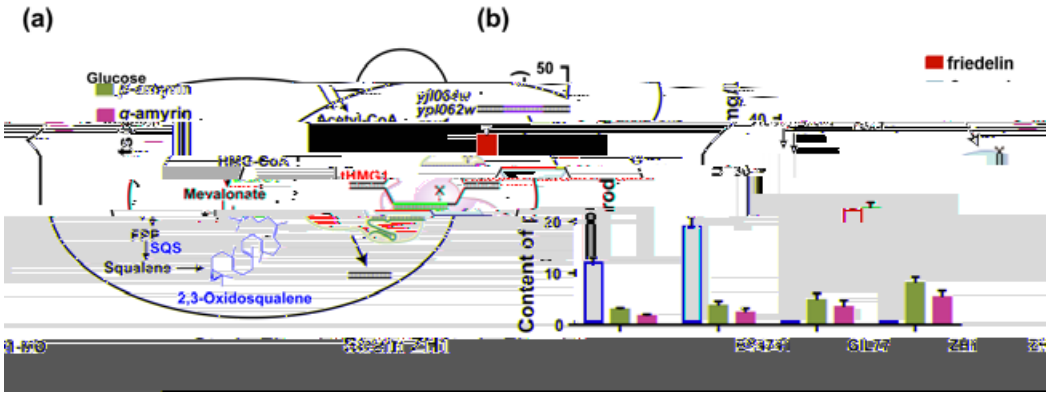

Fig. 8