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ORIGINAL ARTICLE

A cytochrome P450 CYP81AM1 from *Tripterygium wilfordii* catalyses the C-15 hydroxylation of dehydroabietic acid

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Abstract

Main conclusion **A novel cytochrome P450 from *Tripterygium wilfordii*, CYP81AM1, specifically catalyses the C-15 hydroxylation of dehydroabietic acid. This is the first CYP450 to be found in plants with this function.**

Abstract Cytochrome P450 oxygenases (CYPs) play an important role in the post-modification in biosynthesis of plant bioactive terpenoids. Here, we found that CYP81AM1 can catalyze the formation of 15-hydroxydehydroabietic acid by in vitro enzymatic reactions and in vivo yeast feeding assays. This is the first study to show that CYP81 family enzymes are involved in the hydroxylation of abietane diterpenoids. At the same time, we found that CYP81AM1 could not catalyse abietatriene and dehydroabietinol, suggesting that the occurrence of the reaction may be related to the carboxyl group. Through molecular docking and site mutations, it was found that some amino acid sites (F104, K107) near the carboxyl group had an important effect on enzyme activity, also suggesting that the carboxyl group played an important role in the occurrence of the reaction.

Keywords CYP81AM1 · 15-hydroxydehydroabietic acid · *Tripterygium wilfordii* · Molecular docking · Mutagenesis

Introduction

Tripterygium wilfordii (Hook. f.) is widely used as a traditional Chinese herb called Leigongteng, which possesses immunosuppressive, anti-inflammatory and anticancer properties (Wang et al. 2018; Zhou et al. 2019). Much of the remarkable bioactivities can be attributed to bioactive diterpenoids, particularly abietane-type diterpenoids, such as triptolide and its hydroxylated derivative triptolidenol. Triptolide was first identified by Kupchan et al. (1972), and is the most famous active diterpene with immunosuppressive and anti-tumour properties. Triptolidenol is a 15-hydroxylated derivative of triptolide that was first identified by Deng et al. (1985). It has been reported that triptolidenol has stronger anti-inflammatory and immunosuppressive effects than triptolide (Gu and Gao 1994). In addition to triptolidenol, many of the other abietane diterpenoids with C-15 hydroxylation have been isolated from *T. wilfordii* (Ni et al. 2015). The pharmaceutical potential of these abietane-type diterpenoids in *T. wilfordii* has spurred interest in investigating their biosynthetic pathways and

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consequent the heterologous bio-production via synthetic biology strategies.

Many studies have focused on elucidating the biosynthetic pathways of abietane-type diterpenoids in *T. wilfordii*. The universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP) was converted to the abietane-type diterpenoid skeleton miltiradiene through sequential cyclization catalysed by the relevant diterpene synthases (Andersen-Ranberg et al. 2016; Hansen et al. 2017; Inabuy et al. 2017; Su et al. 2016). The intermediate miltiradiene underwent several post-modifications catalyzed by CYPs or some other tailoring enzymes, to form various diterpene structures.

CYP oxidation reactions were first described in 1955 (Klingenberg 1958), and all these CYP enzymes generally couple to the reduction of oxygen. O_2 from air to insert one oxygen atom into a C–H bond of the substrate and the second oxygen atom is released as water (Colthart et al. 2016). Cytochrome P450 reductase (CPR) is the natural redox partner of microsomal CYP450 enzymes that can support the activities of CYPs. There are numerous CYPs in plants that play an important role in generating the structural diversity of terpenoids, as > 97% of terpenoids are oxygenated via the biocatalysis of CYPs (Hamberger and Bak 2013; Guo et al. 2016). Although in the last 10 years, there has been a huge acceleration in plant CYP gene discovery and functional studies, the function of more than 70% CYPs found in the model plant *Arabidopsis* genome is still unknown or poorly understood (Søren Bak et al. 2011). There are still daunting challenges to the identification of CYPs associated with the biosynthesis of particular natural products. Tu identified the first CYP450 CYP728B70 in *T. wilfordii* that can catalyse oxidation of a methyl moiety to the acid moiety of dehydroabietic acid and they demonstrated that dehydroabietic acid may be a precursor compound of triptolide by gene overexpression study (Tu et al. 2020). Until now, no other functional studies of CYPs have been reported in *T. wilfordii*.

Here, we cloned a novel cytochrome P450 from *T. wilfordii*, CYP81AM1, and identified that it can catalyse the formation of 15-hydroxydehydroabietic acid from dehydroabietic acid in vitro enzymatic reactions and in vivo yeast feeding assays. This is the first CYP450 to be found in plants with this function and no function has been previously reported for any member of this CYP81AM subfamily, which will provide experience for the exploration of plant CYP450s related to diterpenoid biosynthesis. Through molecular docking and site mutations, some amino acid sites (F104A, K107A) near the carboxyl group were found to have an important effect on enzyme activity, suggesting that the carboxyl group of the substrate plays an important role in the occurrence of the reaction.

Table 1 GeneBank accession numbers of CYPs with similar sequence

Gene name	Gene bank	Gene name	Gene bank
PsiCYP720B10	ADR78281.1	CfCYP71D381	AMZ03386.1
PsiCYP720B12	ADR78270.1	OsCYP99A2	Q7X7X4.2
PsiCYP720B15	ADR78271.1	OsCYP76M8	Q6YTF1.1
PsiCYP720B16	ADR78272.1	OsCYP76M7	Q69X58.1
PsiCYP720B2	ADR78275.1	NtCYP71D16	AAD47832.1
PsiCYP720B4	ADR78276.1	RoCYP76AH22	AJQ30187.1
PsiCYP720B7	ADR78279.1	RoCYP76AH23	AJQ30188.1
PsiCYP720B8	ADR78280.1	SfCYP76AH24	AJQ30186.1
PsiCYP720B9	ADR78283.1	SmCYP76AH1	AGN04215.1
PbCYP720B2	AIK01725.1	SmCYP76AH3	AMB36496.1
PcCYP720B1	AIK01729.1	CfCYP76AH10	AMZ03390.1
PcCYP720B11	AIK01733.1	CfCYP76AH11	AMZ03393.1
PcCYP720B12	AIK01734.1	CfCYP76AH15	AMZ03402.1
PcCYP720B2	AIK01730.1	CfCYP76AH16	AMZ03403.1
SpCYP71BE52	ALM25794.1	CfCYP76AH17	AMZ03404.1
ElCYP71D445	AMY98418.1	CfCYP76AH8	AMZ03392.1
EpCYP71D365	ANO43023.1	CfCYP76AH9	AMZ03392.1
SmCYP76AK3	AJD25183.1	RoCYP76AK7	AOW42545.1
SpCYP76AK6	ALM25797.1	RoCYP76AK8	AOW42546.1
TwCYP81AM1	XP 038694163.1	SfCYP76AK6	AOW42544.1
CcCYP81D11	XP 006434691.1	SmCYP76AK1	AMB36497.1
SlCYP81D1	XP 025886630.1	SmCYP76AK2	AJD25182.1
MnCYP81E8	XP 024027369.1	CcCYP81E8	XP 006425998.1
RnCYP2A1	P11711	HmCYP71D55	A6YIH8
TvCYP71AJ6	AKJ23347		

Materials and methods

Plant materials

Tripterygium wilfordii suspension cells were initiated from calluses of *T. wilfordii* and cultured in Murashige and Skoog (MS) medium containing 30 g L^{-1} sucrose supplemented with 0.1 mg L^{-1} kinetin (KT), 0.5 mg L^{-1} indole-3-butyric acid (IBA), and 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). These cell suspensions were cultured in the dark at 25 ± 1 °C and 120 rpm with rotary shaking as described previously (Guan et al. 2017).

Bioinformatic analysis

The nucleotide sequence of CYP81AM1 was analysed for its open reading frame and homology to other CYP sequences (https://blast.ncbi.nlm.nih.gov/) and the accession numbers

are provided in Table 1. Phylogenetic analysis was performed using MEGA 7.0 software via maximum likelihood tree analysis (1000 bootstraps) (Sudhir Kumar and Tamura 2016). Multiple sequence alignments were carried out using DNAMAN 8.0 software used to identify substrate recognition sites (SRSs) (Lynnon Biosoft, USA).

RNA isolation, cDNA synthesis, and gene cloning

Total RNA was extracted from the suspension cells using an RNA Extraction Kit (Promega, Shanghai, China), and first-strand cDNA was reverse-transcribed from the total RNA using the Fast Quant RT Kit (with gDNase) (Tian Gen Biotech, Beijing, China). Full-length cDNA was cloned from cDNA isolated from *T. wilfordii* suspension cells using Phusion high-fidelity Master Mix (NEB, USA). All the primers used for cloning are listed in Supplementary Table S1.

Heterologous expression of CYP81AM1 in yeast

To ensure a more efficient reduction of CYP450s, *TwCPR3* was ligated into the yeast epitope-tagging vector pESC-Leu via the *Bam*HI site. Then the ORF region of *CYP81AM1* was ligated into the pESC-leu:: (TwCPR3) construct via the *Not* I site. These constructs were transformed into *E. coli* DH5α cells and then screened in Luria–Bertani medium containing 100 mg L^{-1} ampicillin at 37 °C in the dark. Positive colonies were verified by sequencing. The constructed vectors were transformed into the *Saccharomyces cerevisiae* WAT11U strain using Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA, USA). The construct pESC-leu:: (TwCPR3) was used as a control. The transformants were cultured on solid synthetic complete medium without leucine (SC-Leu) for selection. The positive transformant was then cultured in 100 mL of SD-Leu liquid medium containing 20 g L^{-1} glucose in a shaker at 30 °C and 230 rpm to an OD600 of 1.8–2. Cells were centrifuged and re-suspended in 100 mL of yeast peptone galactose (YPL) induction medium (10 g L^{-1} yeast extract, 20 g L^{-1} bactopeptone, and 20 g L^{-1} galactose) and grown for 12 h at 30 °C and 160 rpm to induce recombinant protein expression.

In vitro enzymatic activity assay

Microsomes were prepared based on the reported method with some modifications (Guo 2013). The induced cells were centrifuged (2000 × *g*, 4 °C, 5 min) and re-suspended in 20 mL of TEK buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1 M KCl for 5 min at room temperature. Cells were centrifuged again (2000 × *g*, 4 °C, 5 min) and re-suspended in 50 mL of TESB buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.6 M sorbitol, and then left on ice for 10 min. The cell suspension was lysed for 7 min at 4 °C and 12,000 psi using a nano-homogenizer machine (AH-1500, ATS Engineering Limited, Canada), and then centrifuged (12,000 × g, 4 °C, 15 min) to collect the supernatant. NaCl (final concentration of 0.15 M) and polyethylene glycol (PEG) – 4000 (final concentration of 0.1 g mL^{-1}) were added to the supernatant, and left on ice for 15 min. The microsomal fractions were collected by centrifugation (12,000 × *g*, 4 °C, 20 min), and re-suspended in TEG buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 20% (v/v) glycerol, which was kept frozen at – 80 °C for months. In vitro hydroxylation assays were conducted in a total volume of 500 µL of 100 mM Tris–HCl (pH 7.5), containing 500 µM NADPH, 5 µM FAD, 5 µM FMN, 5 mM glucose-6-phosphate, 1 unit mL^{-1} glucose-6-phosphate dehydrogenase, 0.5 mg of microsomal protein, and 100 µM substrates. The assays were incubated with shaking for 3 h at 30 °C and 100 rpm, and the reaction system was extracted three times with an equal volume of ethyl acetate. The combined organic phases were dried and methylated with diazomethane (Aladdin Industrial Inc., Shanghai, China) (Su et al. 2016). The methylated samples were re-dried and then dissolved in 100 µL of ethyl acetate. The analysis was performed on an Agilent 7000 gas chromatograph.

Yeast in vivo assays

The induced cells were centrifuged and re-suspended in 30 mL of yeast peptone galactose (YPL) induction medium and grown for 12 h at 30 °C. Then, dehydroabietic acid or other substrates were added to the yeast cultures to final concentrations of 100 µM and fermented for another 48 h. Yeast cells were lysed, and extracted three times with an equal volume of ethyl acetate. The combined organic phases were dried and methylated before GC–MS analysis, as described above.

Substrate recognition site (SRS) analysis of CYP81AM1

SRS regions in CYP81AM1 were speculated by aligning with previously established SRS regions in CYP2A1 from *Rattus norvegicus* (GenBank: P11711), CYP71D55 from *Hyoscyamus muticus* (GenBank: A6YIH8) and CYP71AJ6 from *Tapsia villosa* (GenBank: AKJ23347) (Gotoh 1992; Dueholm et al. 2015; Forman et al. 2018).

Molecular docking and site-directed mutagenesis

The three-dimensional protein structures of the CYP81AM1 model with hemes were generated using Zhanglab (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The substrate dehydroabietic acid was docked with the model structures using Sybyl X-1.3 (El Cerrito, CA, USA). Docking results were visualized using PYMOL. Site-directed mutagenesis

of CYP81AM1 was conducted using the pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech). Amino acids belonging to 6 SRSs within a 4A distance from the substrate molecule were selected and mutated into alanine to investigate the influence of this site on enzyme activity. The mutants were amplified from their templates using specific primers and were verified by sequencing. The positive mutants were transformed into the *S. cerevisiae* WAT11U strain. To make the quantitative results more accurate, these mutants were pre-cultured in a shaker at 30 °C and 230 rpm for 48 h, then transferred into 30 mL fresh SD-Leu medium with an initial OD600 of 0.05 and fermented under the same conditions for 48 h. Cells were centrifuged and re-suspended in 30 mL of yeast peptone galactose (YPL) induction medium and grown for 12 h at 30 °C. Then dehydroabietic acid was added to the yeast cultures to final concentrations of 50 µM and fermented for another 48 h. Yeast cells were lysed, extracted twice with an equal volume of ethyl acetate, and methylated before GC–MS analysis, as described above.

Statistical analyses

The product content measurement data after site-directed mutagenesis are presented as the mean ± standard deviation (S.D.) in GraphPad Prism 7.0. Each mutant had three biological replicates, therefore, $n = 3$. Statistically significant differences between the wild-type group and the mutant

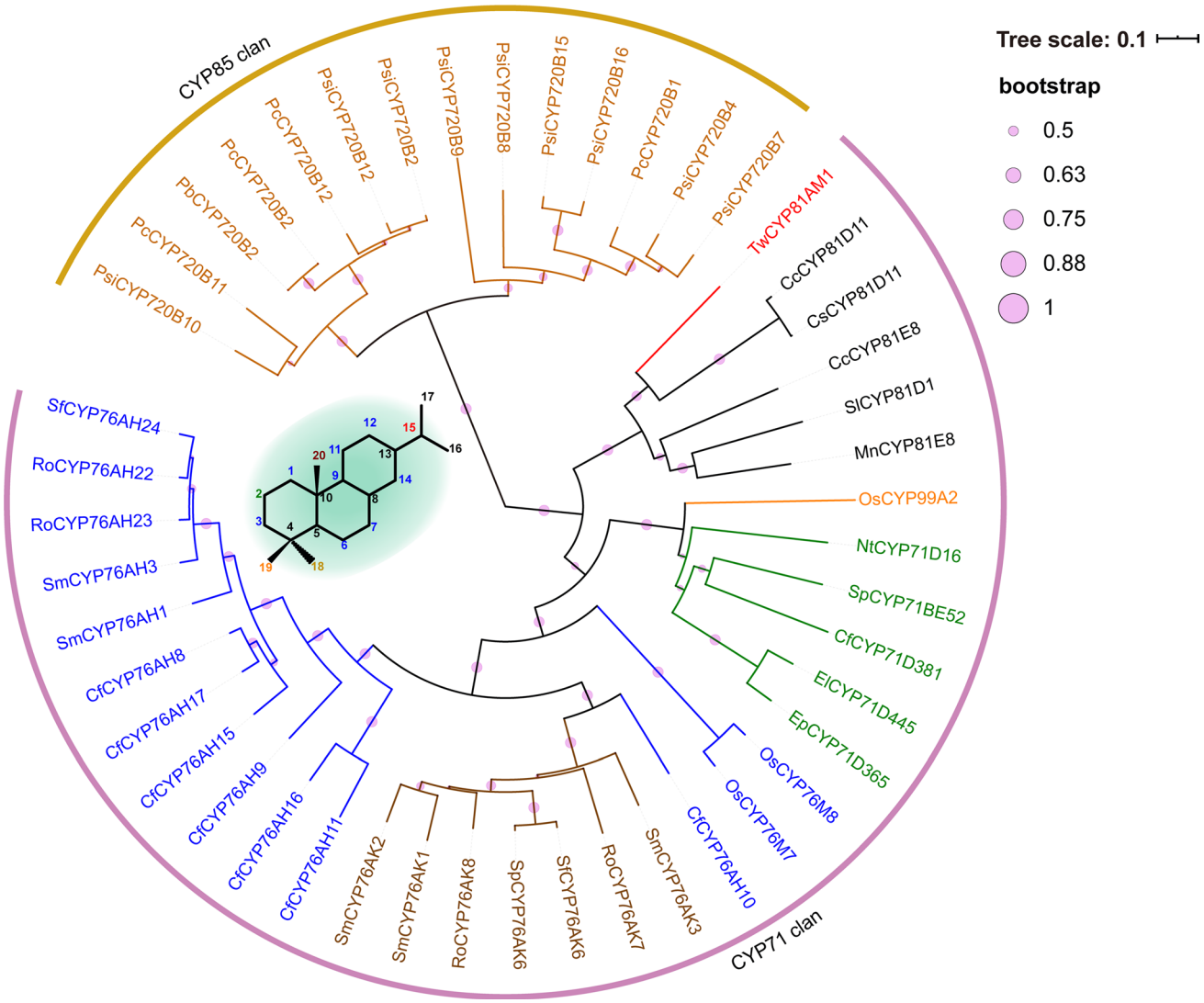

Fig. 1 Position of CYP81AM1 in a phylogenetic tree of the CYP450 superfamily (*Cc Citrus clementina*; *Psi Picea sitchensis*; *Pb Pinus banksiana*; *Pc Pinus contorta*; *Sp Salvia pomifera*; *El Euphorbia lathyrism*; *Ep Euphorbia peplus*; *Tw Tripterygium wilfordii*; *Sl Solanum lycopersicum*; *Mn Morus notabilis*; *Cf Plectranthus barbatus*; *Os Oryza sativa japonica* Group; *Nt Nicotiana tabacum*; *Ro Salvia rosmarinus*; *Sf Salvia fruticose*)

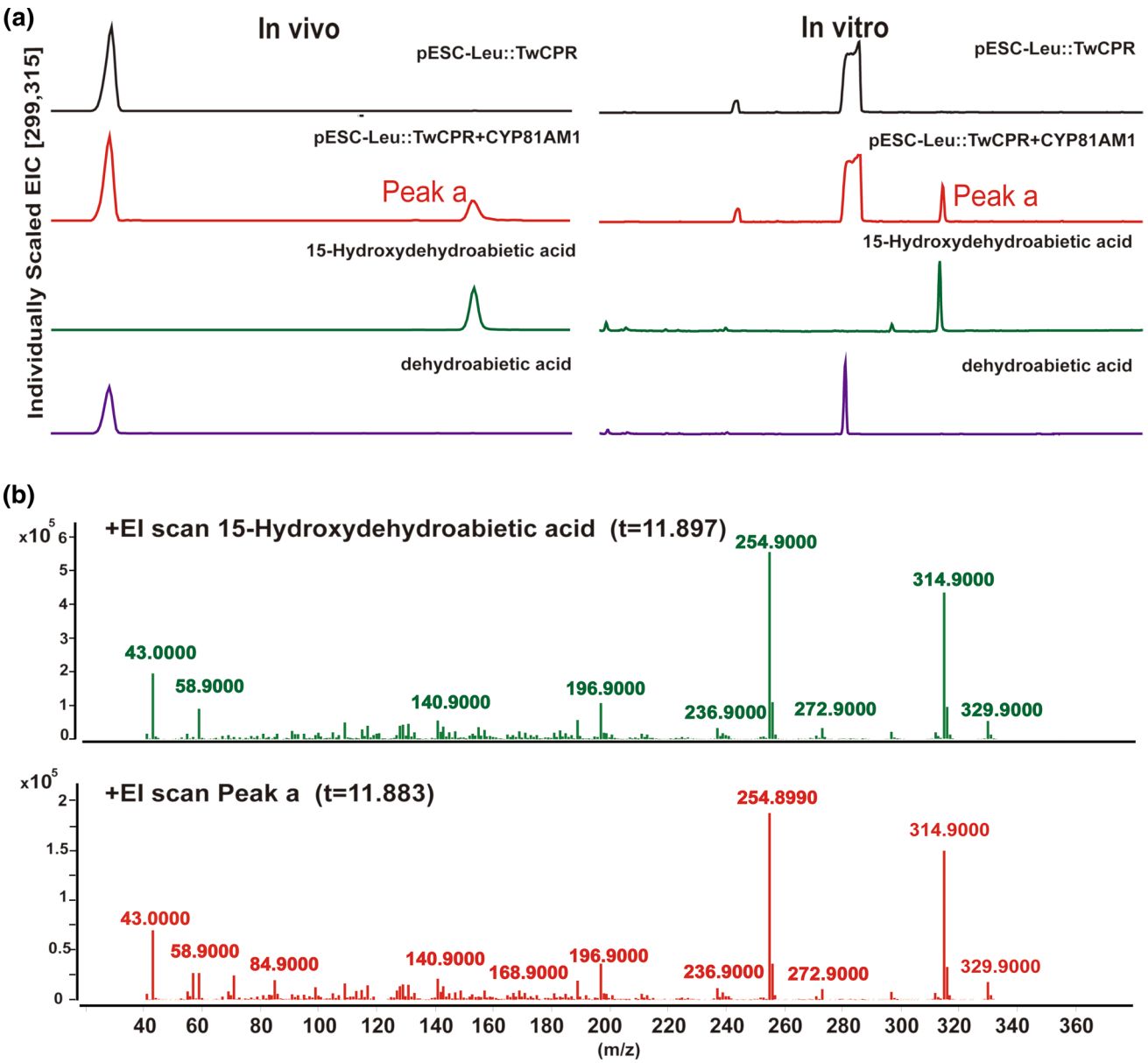

Fig. 2 Oxidation of dehydroabietic acid in vitro and in vivo assays with CYP81AM1. **a** GC–MS chromatogram of extracts from the reaction containing CYP81AM1, extracted ion chromatograms (EICs) at an m/z of 299 (EIC 299) and 315 (EIC 315) were shown. **b** System-extracted ion component spectra of Peak a and the products standards 15-Hydroxydehydroabietic acid at the indicated retention time

group were analysed using the independent samples *t* test (*$P<0.05$, **$P<0.01$) by SPSS Statistics 20.0 software.

Results

Bioinformatic analysis and the full-length sequence of *CYP81AM1*

The amplification guided by specific primers resulted in the full length of CYP81AM1, containing 1500 bp and encoding 499 amino acids. To further explore the evolutionary relationships between CYP81AM1 and other CYPs that may be involved in the oxidative modifications in different carbon atoms of dehydroabietic acid or some other CYPs from the CYP81 family, a phylogenetic tree was constructed based on the comparison of amino acid sequences of these CYPs (Fig. 1). For the oxidative modification of dehydroabietic acid, previously reported CYP450s mostly act on ring carbon atoms, which mostly belong to the CYP76AH subfamily, CYP76AM subfamily and CYP71D subfamily, while the oxidation genes of non-ring carbon atoms mostly belong

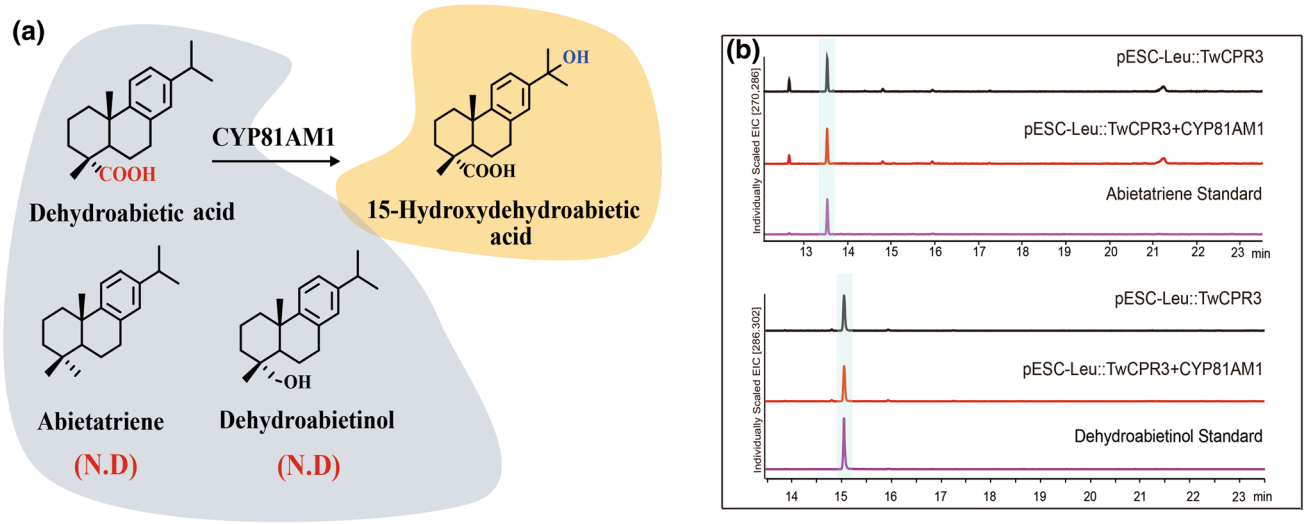

Fig. 3 Oxidation of abietatriene and dehydroabietinol in vivo assays with CYP81AM1. **a** Abietatriene and dehydroabietinol which have similar structures to dehydroabietic acid; **b** GC–MS analysis of extracts from the enzymatic reaction with abietatriene and dehydroabietinol

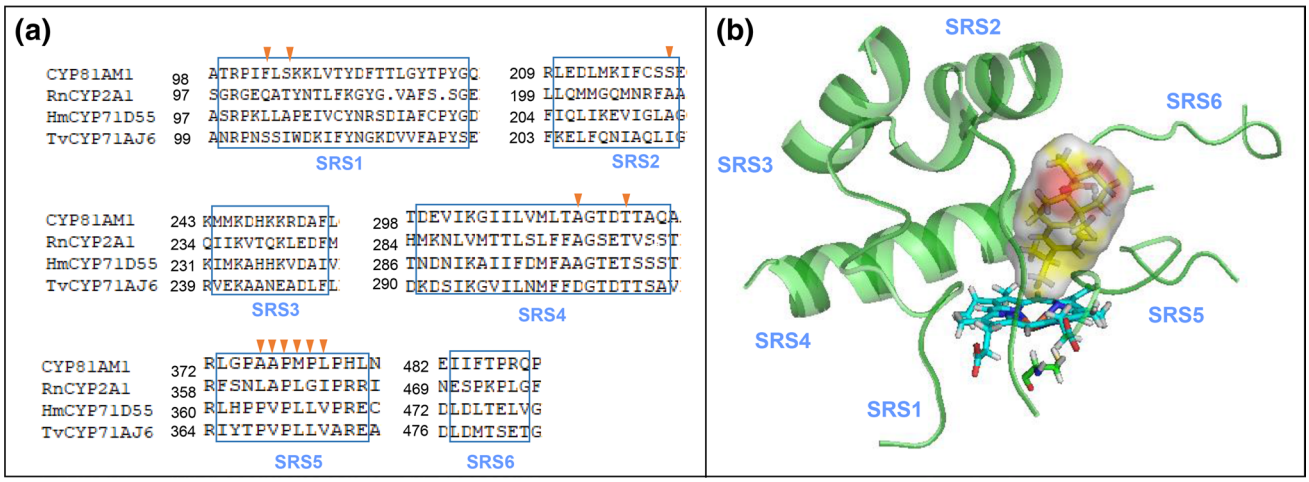

Fig. 4 Potential pre-catalytic SRS regions of CYP81AM1. **a** Alignment of sequences in substrate recognition sites of CYP81AM1 and other representative CYPs; **b** homology model of CYP81AM1 with visualized SRSs

to the CYP720B subfamily and CYP76AK subfamily. CYP81AM1 is more closely related to CYP720B subfamily based on evolutionary relationships.

CYP81AM1 Acts as a 15-Hydroxydehydroabietic acid synthase

The ability of CYP81AM1 to react with dehydroabietic acid was examined by recombinant expression in *S. cerevisiae* WAT11U, followed by in vitro assays using microsomal preparations and in vivo substrate feeding experiments. Notably, dehydroabietic acid could be converted to an oxidized derivative with the catalysis of CYP81AM1, which was determined to be 15-hydroxydehydroabietic acid by comparison with an authentic standard (Fig. 2).

Substrate specificity of CYP81AM1

In vivo yeast feeding assays of CYP81AM1 recombinant yeasts were then assayed with abietatriene and dehydroabietinol which have similar structures to dehydroabietic acid, followed by GC–MS analysis (Fig. 3). However, no corresponding hydroxylation products were detected (N.D.), which indicates that CYP81AM1 has certain substrate specificity.

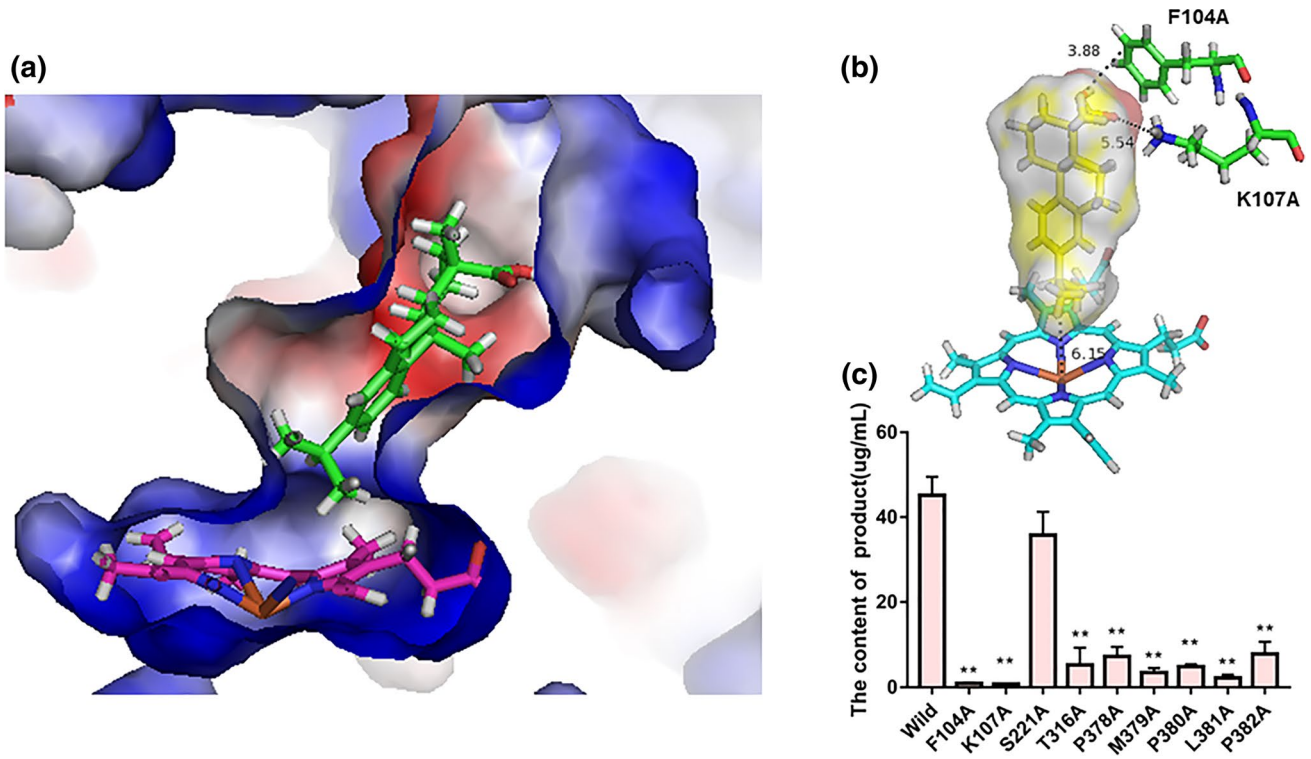

Fig. 5 Key amino acid site mutations of CYP81AM1. **a** Active cavity composed of different surface potentials that have heme (Rose) and substrate (Green). **b** Relative position of residues F104 and K107 to the substrate. **c** Relative yields of the product of candidate active sites near the substrate ($n=3$, $^{*}P<0.05$, $^{**}P<0.01$)

Substrate recognition sites (SRSs) of CYP81AM1

Despite huge variation between the enzymes in the CYP450 superfamily, six substrate recognition sites (SRS1-6) have been identified as essential residues in the active sites (Gotoh 1992). To further understand the interaction relationship between the substrate and CYP81AM1, the substrate recognition sites of CYP81AM1 were speculated based on reports in the existing literature. Six SRSs of CYP81AM1 were visualized using PYMOL (Fig. 4).

Substrate docking and site-directed mutagenesis

To gain deeper insights into the substrate binding of dehydroabietic acid to the active sites of CYP81AM1, docking experiments were performed using SYBYL-X1.3 software. The best docking scores for dehydroabietic acid were obtained with orientation of the isopropyl group of the substrates directly above the heme prosthetic group (Fig. 5a). The active cavity is composed of different surface potentials with heme and substrate. After alanine mutation of the candidate sites, it can be seen that the product content is significantly reduced except for S221A, the mutation of most sites reduced the enzyme activity to a certain extent, especially the mutants F104A and K107A whose spatial position is close to the carboxyl group almost lost its catalytic activity (Fig. 5c). These results suggest that the carboxyl group of dehydroabietic acid plays an important role in the reaction.

Discussion

Cytochrome P450 enzymes are key players in generating tremendous chemical diversity of terpenoid products. These heme-dependent enzymes catalyse a diverse array of reactions, such as oxidation, acylation, methylation and glycosylation (Rudolf et al. 2017). In plant terpene biosynthesis, oxidation of the hydrocarbon backbone produced by terpene synthases is typically carried out by cytochrome P450 oxygenases (Bathe and Tissier 2019). However, functional characterization of eukaryotic CYPs remains challenging, particularly in plants (Renault et al. 2014). Tu et al. (2020) found that CYP728B70 catalysed the formation of dehydroabietic acid and speculated that it may be involved in the biosynthetic pathway of triptolide. In addition to the representative compound triptolide, there are a variety of abietane diterpenoids with C-15 hydroxylation in *T. wilfordii* (Ni et al. 2019; Chen et al. 2018), but the enzyme catalysing C-15 hydroxylation has not been reported (Fig. 6). As a key enzyme for catalyzing dehydroabietic acid to

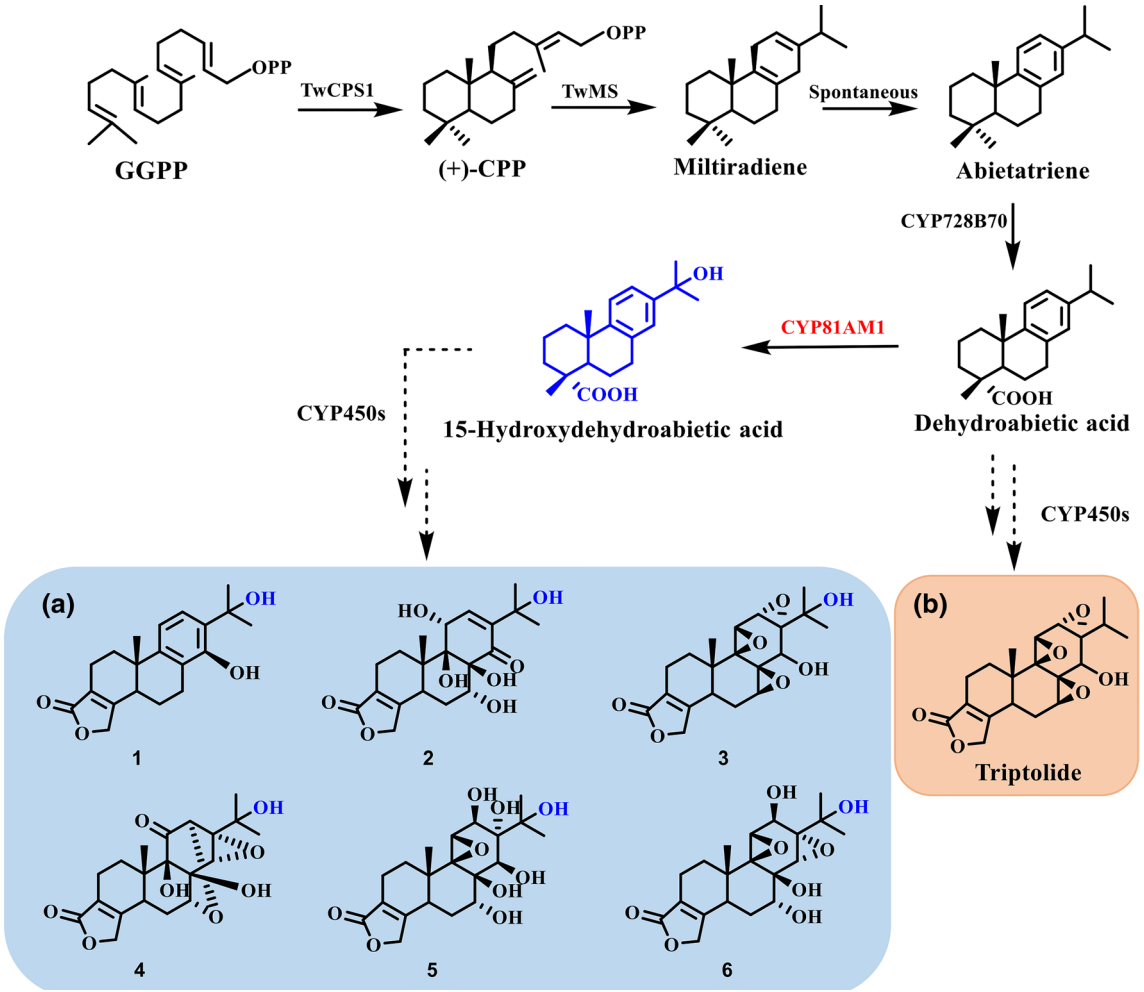

Fig. 6 Possible biosynthetic pathways of active abietane diterpenoids in *Tripterygium wilfordii*. **a** Structure of diterpenoids with 15-hydroxylations, compound 3: triptolidenol; **b** representative active diterpenes: triptolide

15-hydroxydehydroabietic acid, CYP81AM1 was speculated to be involved in the biosynthetic pathways of a series of C-15 hydroxylated derivatives. We plan to investigate the accumulation of these derivatives such as triptolidenol, after the RNA interference of CYP81AM1 in suspension cells for further verification.

The CYP81 family includes multiple members, only a few of which have been subjected to functional analysis. *Medicago truncatula* CYP81Es act as isoflavone C-2' and C-3' hydroxylases (Chang-Jun Liu et al. 2003), while the *Sesamum indicum* SiCYP81Q1 protein catalyses the synthesis of lignan sesamin (Ono et al. 2006). This is the first study to show that CYP81 family enzymes are also involved in the biosynthesis of diterpenoids. CYP81AM1 has certain substrate specificity and can only catalyse the oxidation of dehydroabietic acid but not abietatriene and dehydroabietinol. Through site-directed mutagenesis experiments, it was found that some amino acid sites (F104A, K107A) near the carboxyl group had an important effect on enzyme activity. We speculate that there is a certain interaction between the carboxyl group of the substrate molecule and the enzyme molecule, which makes the orientation of the isopropyl group of the substrate close to the heme center. In conclusion, the functional characterization of CYP81AM1 is of great significance. As the first member of the CYP81AM subfamily, CYP81AM1's catalytic function was also first found in plant CYPs which will lay a foundation for functional exploration of CYP450s in plants.

Author contribution statement JW, WG, and LH designed the experiments. JW, PS, LG, YZ, JW, YL and YY performed the experiments. JW, YZ and LT analyzed the data. JW and WG wrote the manuscript. All authors read and approved the final manuscript.

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Data availability The data supporting the findings of this study are available from the corresponding author Wei Gao, on reasonable request.

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