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*Full Length Research Paper*

# Molecular cloning and characterization of the gene encoding pinoresinol synthase from Tibet Dysosma

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**Two molecules of coniferyl alcohols are dimerized to form a molecule of pinoresinol , a precursor of podophyllotoxin – antiviral and antitumor agent. The process is catalyzed by pinoresinol synthase (PS). In the present study, the full-length cDNA encoding PS (designated as *DtPS*) was isolated and characterized from Tibet Dysosma, *Dysosma tsayuensis* Ying. The full-length *DtPS* cDNA was 798 bps, and contains a 582-bp open reading frame encoding a 193-amino-acid polypeptide, with a calculated molecular mass of 21.5 kDa and an isoelectric point of 7.24. Comparative analysis indicated that *DtPS* was similar to other plant PSs in terms of base sequence. The gene tissue- expressing pattern analysis indicated that the expression of *DtPS* could be detected in all the detected organs including roots, rhizomes, leaves, petioles, flowers and fruits but at different levels. The highest expression level was found in petiole and in fruit, followed by the roots, and lastly by the rhizome, leaf and flower. Cloning and characterization of the PS gene from Tibet Dysosma will facilitate mapping biosynthesis of podophyllotoxin at the molecular level.**

**Key words:** *Dysosma tsayuensis* Ying, pinoresinol synthase, cloning, expression profile, podophyllotoxin.

## INTRODUCTION

Tibet Dysosma (*Dysosma tsayuensis* Ying) is an endangered plant species that belongs to Berberidaceae. The rhizome is widely used to produce podophyllotoxin - an antiviral and anticancer agent, (Liao et al., 2002). The resource of Tibet Dysosma is very limited because this rare plant only grows in the forests of Southeast Tibet of China, which is about 2500-3500 m above sea level (Lan et al., 2010). Furthermore, the content of podophyllotoxin in the rhizome is low and the rate of accumulation of slow. The limitedness of this resource coupled with the huge demands for the plant makes it an endangered species. The chemical synthesis of podophyllotoxin is successful only at the academic level (Wu et al., 2007). However, the cost of chemical synthesis of podophyllotoxin is too high to meet the commercial standards. Plant cell and hairy root cultures are usually employed to produce high-value natural products (Hu and Du, 2006). Li et al. (2009) established the hairy root culture of *Podophyllum hexandrum* can produce podophyllotoxin. Similarly, Chattopadhyay et al. (2002) reported the production of podophyllotoxin through *P. hexandrum* cell cultures in bioreactor. Yet the rate of production of podophyllotoxin could not meet commercial demand. This is mainly caused by weak biosynthesis due to the rate-limiting enzymatic reactions involved in the biosynthetic pathway of podophyllotoxin. Metabolic engineering has been successfully used to genetically modify the biosynthetic pathway of natural products of interest. For example, the world-famous 'golden rice' is the milestone of plant metabolic engineering (Ye et al., 2000). So, metabolic engineering might be the best way to enhance biosynthesis of podophyllotoxin, which is based on the knowledge of molecular biology and biochemistry of the biosynthetic pathway of podophyllotoxin (Figure 1). In the present study, the gene encoding pinoresinol synthase was firstly cloned and

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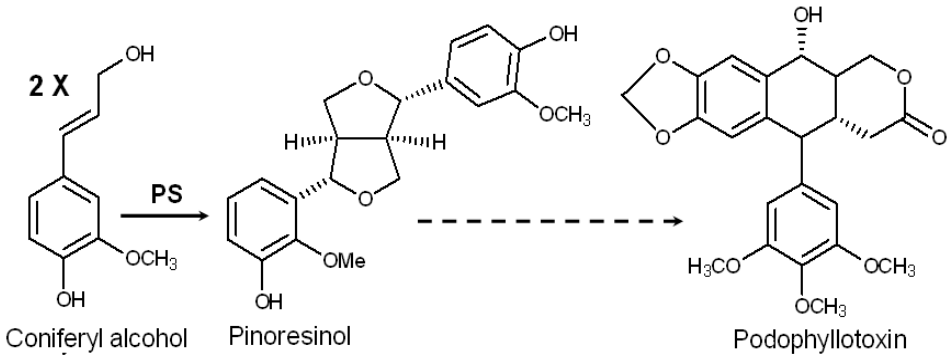


**Figure 1.** The biosynthetic pathway of podophyllotoxin. PS: pinoresinol synthase.

characterized from Tibet Dysosma.

## MATERIALS AND METHODS

The different tissues of Tibet Dysosma were collected from the forest of Nyingchi District (Tibet, China). RNA isolation reagent and reverse transcriptase were produced respectively by Tiangen (Beijing, China) and Clontech (CA, USA). RACE Kit was produced from Clontech (CA, USA). The pGEM® T-easy vector was the product of Promega (WI, USA). The Taq polymerase was made by TaKaRa (Dalian, China). Other chemicals were supplied by Sigma (USA). Primers used in the present study were synthesized by Invitorgen (Shanghai, China). The authoritative sample of podophyllotoxin was extracted and identified by Sigma.

### Isolation of the total RNAs

The method of isolation of the total RNAs from Tibet Dysosma was according to the manufacturer's instruction and the authors' previous reports (Lan et al., 2010).

### Cloning of the full-length cDNA of *DtPS*

Single-stranded cDNAs were synthesized from 5 µg of total RNAs with an oligo (dT)17 primer that were reversely transcribed according to the manufacturer's protocol (PowerScript™, Clontech, USA). After RNaseH treatment, the single-stranded cDNA mixture was used as templates for polymerase chain reaction (PCR) amplification of the fragment of DtPS. A pair of primers (fdtps: 5'-ATGGGAGGAGAAAAAGCTTTCAG -3' and rdtps: 5'-TTACCAACACTCATACAACTTGATATC -3') were used for amplification. The PCR reaction was carried out by denaturing the cDNA at 94°C for 3 min followed by 30 cycles of amplification (94°C for 45 s, 55°C for 45 s and 72°C for 1 min) and by extension at 72°C for 8 min. The fragment of *DtPS* was amplified and subcloned into pGEM T-easy vector followed by sequencing, which was confirmed to be similar to other plant PS genes by blast-n search. The fragment was subsequently used to design the gene-specific primers for the cloning of full-length cDNA of *DtPS* by the technology of rapid amplification of cDNA ends (RACE).

SMART™ RACE cDNA Amplification Kit (Clontech, USA) was used to isolate *DtSD* cDNA 3' end and 5' end. Firstly, the first-Stranded 3'-RACE-ready and 5'-RACE-ready cDNA samples from Tibet Dysosma were prepared according to the manufacturer's protocol (SMART™ RACE cDNA Amplification Kit, User Manual, Clontech). The 3'-RACE-ready cDNA and 5'-RACE-ready cDNA were used as templates for 3'-RACE and 5'-RACE respectively. *DtPS* cDNA's 3' end was amplified using 3'-gene-specific primers and the universal primers provided by the kit. For the first PCR amplification of 3'-RACE, DtPS3-1 (5'-TTCGACGACCCAATTACTCTAG -3') and UPM (Universal Primer Mix, provided by Clontech) were used as the first PCR primers (3'-RACE), and 3'-RACE-ready cDNAs were used as templates. For the nested PCR amplification of 3'-RACE, DtPS3-2 (5'-AGACACATTCCACTCATGGC -3') and NUP (Nested Universal Primer, provided by Clontech) were used as the nested PCR primers (3'-RACE), and the products of the first PCR amplification were used as templates. DtPS cDNA 5' end was amplified using 5'-gene-specific primers and the universal primers provided by the kit. For the first PCR amplification of 5'-RACE, DtPS5-1 (5'-GTCTGCGCCTTGTGGTGCACC -3') and UPM were used as the first PCR primers (5'-RACE), and 5'-RACE-ready cDNAs were used as templates. For the nested PCR amplification of 5'-RACE, DtPS5-2 (5'- GGGGTGAGCTGAAGAACCAGAG -3') and NUP were used as the nested PCR primers (5'-RACE) and the products of the first PCR amplification were used as templates. For the first and nested PCR amplification of *DtPS* cDNA 3' and 5' end, Advantage™ 2 PCR Kit (Clontech, USA) was used.

The first and nested PCR procedures were carried out at the same conditions described in the protocol (SMART™ RACE cDNA Amplification Kit, User Manual, Clontech): 25 cycles (30 s at 94°C, 30 s at 68°C, 3 min at 72°C). By 3'-RACE and 5'-RACE, both ends of *DtPS* were respectively obtained. The products were subcloned into pGEM T-easy vector followed by sequencing. By assembling the sequences of 3'-RACE, 5'-RACE and the core fragment on ContigExpress (Vector NTI Suite 8.0), the full-length cDNA sequence of *DtPS* was obtained. The open reading frame (ORF) of *DtPS* was predicted by ORF Finder on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The full-length cDNA of *DtPS* was isolated by PCR amplification with a pair of primers: DTSDFL (5'- GAAGAGTAGTCATCAGTCCC -3') and DTSDRL (5'-ACAAAACAAATGAGGCTCTTTCAAAG -3'). The PCR procedure was conducted under the following conditions: 3 min at 94°C, 29 cycles (50 s at 94°C, 50 s at 55°C, 2min at 72°C) and 10 min at 72°C. The amplified PCR product was purified and cloned into pGEM T-easy vector and then sequenced.

### Bioinformatic analysis

BLAST was employed to analyze the similarity of sequences (Altschul et al., 1997). CLUXTALX was used for multiple alignment analysis of the full-length PS amino acid sequences (Thompson et

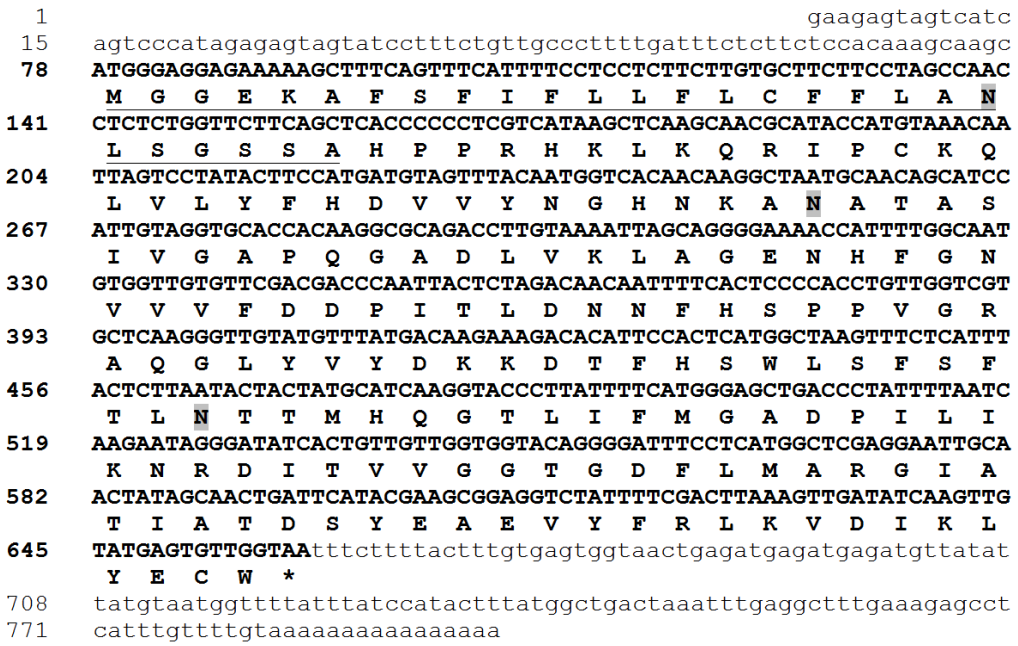


**Figure 2.** The full-length cDNA of DtPS and its deduced amino acids. The coding sequence was shown in bold capital letters, the stop codon marked with an asterisk. The signal peptide was marked with underline and three conservative N-glycosylation was marked in gray background color.

al., 1997). SignalP was used to analyze the signal peptide (Bendtsen et al., 2004).

#### The tissue expression pattern of *DtSD*

The tissue expression pattern of *DtPS* was investigated with semi-quantitative RT-PCR. Aliquots of 0.5 µg total RNA extracted from each sample were used as templates in the one-step RT-PCR reaction with the forward primer fdtps and rdtps specific to the coding sequence of *DtPS* using one-step RT-PCR kit (TaKaRa, Japan). Amplifications were performed in a volume of 25 µl under the following conditions: 50°C for 30 min, 94°C for 2 min followed by 25 cycles of amplification (94°C for 50 s, 55°C for 55 s, 72°C for 1 min). Meanwhile, the 18 S rRNA gene was used to estimate whether the equal amounts of total RNA among samples were used in RT-PCR reaction (Lan et al., 2010).

## RESULTS

### Cloning of the full-length cDNA of DtPS

The core fragment of *DtPS* was specifically amplified with a pair of primers fdtps and rdtps, which was 582 bps in length. The BlastP analysis showed that the fragment of DtPS was similar with other PS genes of plant, such as *Sinopodophyllum hexandrum* (97% similarity) and *Forsythia* x *intermedia* (58% similarity). The comparative analysis strongly suggested that the amplicon was the fragment of *DtPS*. Based on the amplified fragment; the 377-bp 3'-end and 164-bp 5'-ends of *DtPS* were respectively obtained through RACE. By assembling the core fragment with the 3'-end and 5'-end, the putative full-length cDNA of *DtPS* was produced, that was 798 bps. Finally, the physical full-length cDNA of *DtPS* was obtained that was consistent with the assembled sequence. The full-length cDNA of *DtPS* had the 77-bp 5' untranslated region (UTR), the 582-bp coding sequence and the 139-bp 3' UTR harboring 16-bp poly-A tail (Figure 2, which was designated as DtPS with the GenBank accession number, ABD78858.1.

### Bioinformatic analysis

The ORF analysis showed that DtPS encoded a 193-amino-acid polypeptide with a calculated molecular mass of 21.5 kDa and an isoelectric point of 7.24. The BlastP analysis showed that *DtPS* belonged to the dirigent superfamily and the amino acid sequence of *DtPS* was similar with that from Sinopodophyllum hexandrum (97% similarity), *P. peltatum* (97% similarity) and Forsythia x intermedia (58% similarity).

The comparative analysis showed the DtPS was very similar with the reported pinoresinol synthase gene from *P. peltatum* (Xia et al., 2000). The signal peptide analysis resulted that *DtPS* has a 27-amino-acid signal peptide at its N-terminus that was cut in the mature protein of *DtPS*, which was like the PS of *Forsythia* x *intermedia* (Davin and Lewis, 2000). The multiple alignment of PSs of plant origin showed that the similarity of sequence was higher in the catalytic domains than that in the signal peptide

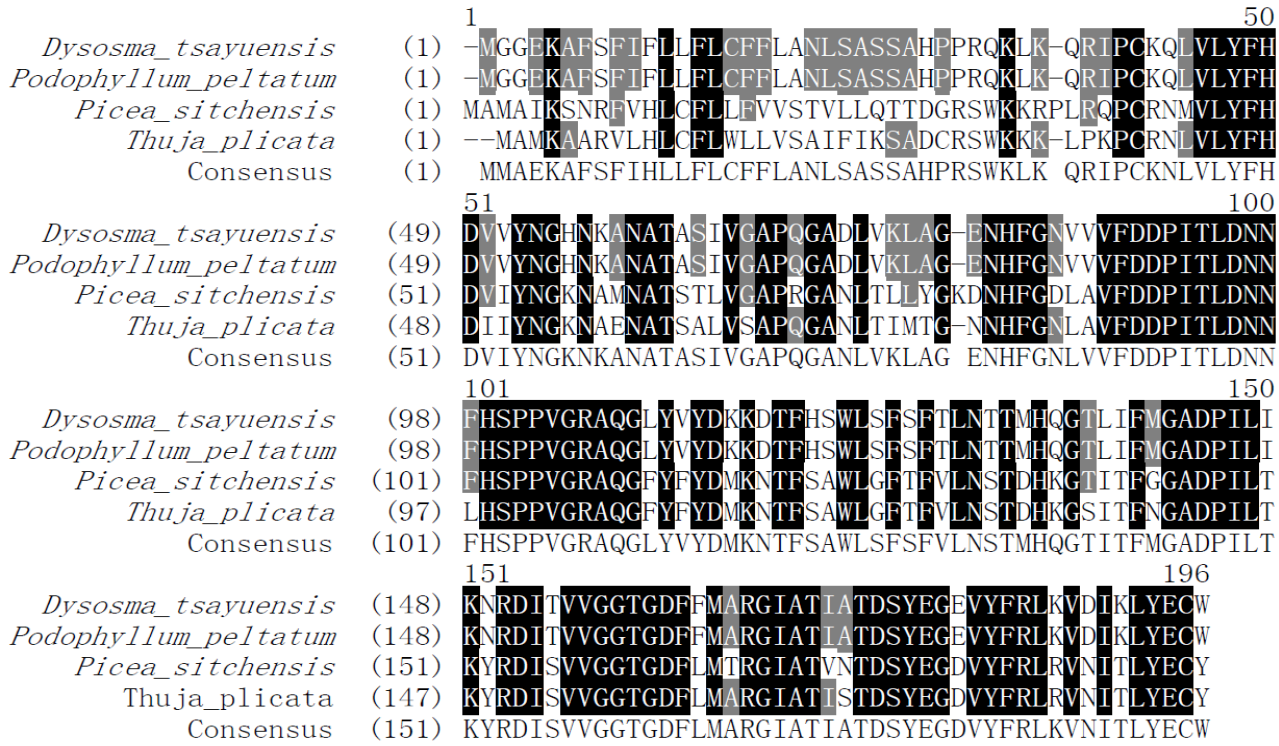


**Figure 3.** The multiple alignments of plant PS enzymes. The identical amino acids were showed in white with black background; the conservative amino acids were showed in white with gray background.

domains Figure 3. According the reported PS from *P. peltatum*, there existed three conservative N-glycosylation sites (Xia et al., 2000). The three N-glycosylation sites were respectively localized at $N^{21}$, $N^{59}$ and $N^{129}$ of DtPS.

## Tissue expression profile of *DtPS*

The semi-quantitative one-step RT-PCR analysis of *DtPS* demonstrated that the *DtPS* gene expressed in all the six detected tissue including root, rhizome, leaf, petiole, flower and fruit but at different levels and at the same time the internal reference gene (*18S rRNA*) expressed in all the six detected tissue but without significant difference. This suggested that difference of *DtPS* expression was caused by difference of tissues. The highest expression level of *DtPS* was found in petioles; the expression level of *DtPS* was higher in fruits than that in roots, rhizomes, leaves and flowers Figure 4. According the authors' previous report, both *DtPS* and *DtSD* showed highest expression levels in petioles (Lan et al., 2010).

## DISCUSSION

Pinoresinol synthase condensed two molecules of coniferyl alcohols to form pinoresinol – a decisive step in the biosynthetic pathway of podophyllotoxin (Federolf et al., 2007). The gene encoding PS was first cloned from podophyllotoxin-producing Tibet Dysosma (Lan et al., 2010). The comparative analysis showed that the sequence of *DtPS* was similar with the reported PS gene from *P. peltatum* (Xia et al., 2000), and the conservative motif of *DtPS* such as the three N-glycosylation sites was similar with the PS of *P. peltatum* (Xia et al., 2000). The tissue expression profile demonstrated that *DtPS* was expressed in all the six tissues examined, but at different levels of expression. This was reasonable because expression of most genes involved in biosynthesis of natural products were related with the status of plant development. For example, the gene encoding H6H (hyoscyamine 6 beta-hydroxylase) - a key gene involved in scopolamine biosynthesis, was expressed in cultured root, native root and anther, but not in the stem, leaf, pistil, petal, and sepal tissues (Suzuki et al., 1999).

The highest level of expression of *DtPS* was found in petioles, just like in the case of *DtSD* in the biosynthesis of podophyllotoxin. Both of *DtPS* and *DtSD* expressed at the much higher levels only in petioles than those in others organs might suggest that the petioles were the main biosynthesizing organ for the precursors of podophyllotoxin. We had previously reported the levels of podophyllotoxin in different tissues of Tibet Dysosma, including roots, rhizomes, leaves, petioles, flowers and

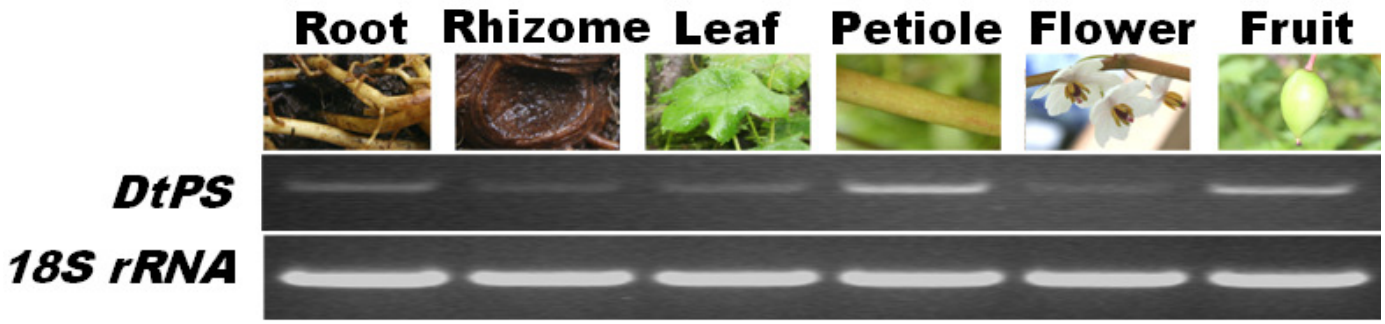


**Figure 4.** Tissue expression profile of *DtPS*.

fruits (Lan et al., 2010). The contents of podophyllotoxin in different tissues were not consistent with the expression of *DtPS* and *DtSD*. The highest content of podophyllotoxin was found in rhizomes (180.5±2.74 µg/g), and then followed by roots (58.8±1.59 µg/g), flowers (40.3±1.24 µg/g), leaves (36.9±1.09 µg/g), petioles (29.9±0.45 µg/g) and fruits (6.03±0.12 µg/g). Even both *DtPS* and *DtSD* expressed at highest levels in petioles, the highest content of podophyllotoxin was not found in petioles but in rhizomes. This strongly suggested that the rhizomes were the storage organs of podophyllotoxin. In summary, cloning and characterization of the gene encoding pinoresinol synthase from Tibet Dysosma will facilitate mapping the biosynthetic pathway of podophyllotoxin at the molecular level and provide a gene of interest that can be employed to genetically modify the biosynthetic pathway of podophyllotoxin.

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