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Full Length Research Paper

Molecular cloning and characterization of the secoisolariciresinol dehydrogenase gene involved in podophyllotoxin biosynthetic pathway from Tibet Dysosma

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Secoisolariciresinol dehydrogenase catalyzes the conversion of secoisolariciresinol into matairesinol that is a central precursor of antiviral and anti-tumor podophyllotoxin. The full - length cDNA encoding secoisolariciresinol dehydrogenase (designated as DtSD) was cloned and characterized from Tibet Dysosma, *Dysosma tsayuensis* Ying. The full-length DtSD cDNA was 994 bps containing an 837-bp open reading frame encoding a 278-amino-acid polypeptide with a calculated molecular mass of 29.2 kDa and an isoelectric point of 6.32. Comparative analysis indicated that DtSD was similar with other plant SDs at the level of sequence with the highly conserved catalytic motif, Ser-X13-Tyr-X2-Lys. The homology - based structural modeling showed that DtSD was similar to the SD enzyme from *Podophyllum peltatum*. Tissue expression pattern analysis indicated that DtSD expressed in root, rhizome, petiole and fruit but at different levels. The highest expression level was found in petiole, and the lowest in fruit. The expression of DtSD could not be detected in leaf and flower. Finally, the podophyllotoxin were detected in all the six tested tissues including root, rhizome, leaf, petiole, flower and fruit at different levels. The highest content of podophyllotoxin (180.5±2.74 µg/g) was found in rhizome, and then followed by root, flower, leaf and petiole. The lowest content of podophyllotoxin (6.03±0.12 µg/g) was found in fruit. The expression levels of DtSD were not consistent with the content of podophyllotoxin in the six tested tissues. So, this might mean that the biosynthetic tissues of podophyllotoxin precursors were not the storage tissues.

Key words: *Dysosma tsayuensis* Ying, secoisolariciresinol dehydrogenase, cloning, expression profile, podophyllotoxin, content.

INTRODUCTION

Dysosma tsayuensis Ying (also call Tibet Dysosma) is an endangered plant species that only grow in the forests with the altitude of 2500 to 3500 m in Southeast Tibet of China (Ying, 1979). *Dysosma tsayuensis* Ying is widely used to extract the antiviral and anticancer agent, podophyllotoxin, from rhizome (Liao et al., 2002). The limited resource with huge demands for *D. tsayuensis* Ying makes it an endangered plant species. So, it is urgent to find alternative ways to produce podophyllotoxin. The chemical synthesis of podophyllotoxin was academically successful (Macdonald et al., 1988). However, the me-

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thod is so complicated that its price is unbelievable high. Some scientists tried to produce podophyllotoxin through plant cell cultures (Kadkadea, 1982), but the productivity of podophyllotoxin was too low to meet the commercial standards. This is mainly due to existence of the rate-limiting enzymatic steps in the podophyllotoxin biosynthetic pathway. Currently, metabolic engineering has been successfully used to genetically modify the plant secondary metabolite biosynthetic pathways and might be the best strategy to produce the high-value plant natural products including podophyllotoxin in plant. Metabolic engineering of podophyllotoxin is based on mapping its biosynthetic pathway at the levels of molecular biology and biochemistry.

In recent years, the researches on molecular biology and biochemistry of podophyllotoxin biosynthetic pathway are fruitful. A few functional genes involved in the pathway have been isolated and functionally identified, including secoisolariciresinol dehydrogenase (SD) and dirigent protein oxidase (DPO). The SD protein catalyzes the enantiospecific conversion of secoisolariciresinol into matairesinol, a central precursor in planta in the biosynthesis of numerous lignans, including that of the important antiviral and anticancer agent, podophyllotoxin. Based on the sequence of the SD protein from Podophyllum peltatum, the cDNA encoding SD was isolated (Xia et al., 2001). In the present study, we cloned and characterized the secoisolariciresinol dehydrogenase gene involved in the biosynthetic pathway of podophyllotoxin from *Dysosma tsayuensis* Ying for the first time, which can be used to metabolic engineer the podophyllotoxin biosynthetic pathway in Tibet Dysosma.

MATERIALS AND METHODS

Materials

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

Isolation of the total RNAs

All fresh organs including root, rhizome, leaf, petiole, flower and fruit were excised from Tibet Dysosma plants and immediately immersed separately in liquid nitrogen for RNA extraction. Total RNAs were isolated from the plant materials using RNA Plant isolation system (Tiangen, China). After isolation, total RNAs were stored in -85°C for the future usage.

Cloning of the full-length cDNA of *DtSD*

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 *DtSD*. A pair of primers (fdtsd: 5'-ATGGGATCCACTTCTACACCAG -3' and rdtsd: 5'-CAAGCCAATCCATGTTTCAATGCG -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, 57.5°C for 45 s and 72°C for 1 min) and by extension at 72°C for 6 min. The fragment of *DtSD* was amplified and subcloned into pGEM T-easy vector followed by sequencing, which was confirmed to be similar to other plant SD genes by blast-n search. The fragment was subsequently used to design the gene-specific primers for the cloning of full-length cDNA of *DtSD* 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. *DtSD* 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, DtSD3-1 (5'-ACTTGACATAATGTTCGGCAAC-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, DtSD3-2 (5'-GACATTAACGTGTATGGTGC-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. DtSD 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, DtSD5-1 (5'-CCACTAGGTTCCGGACATCCTC-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, DtSD5-2 (5'-GGCCGTGGTCATCTGAGATGTC-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 *DtSD* 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 sec at 94°C, 30 s at 68°C, 3 min at 72°C). By 3'-RACE and 5'-RACE, both ends of *DtSD* 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 6.0), the full-length cDNA sequence of DtSD was obtained. The open reading frame (ORF) of DtSD was predicted by ORF Finder on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The full-length cDNA of *DtSD* was isolated by PCR amplification with a pair of primers: DTSDFL (5'- AAGACATCTTAACAATCTGAATTATTT-3') and DTSDRL (5'-AGAACATATCAAGACAACTTAACAAT-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 50°C, 2 min 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

CLUXTALX was used for multiple alignment analysis of the full-length SD amino acid sequences (Thompson et al., 1997). The sequence's homology-based structural modeling was performed by

Swiss-Model (Arnold et al., 2006) and WebLab ViewerLite was used for 3-D structure displaying.

The tissue expression pattern of DtSD

The tissue expression pattern of DtSD 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 fdtsd and rdtsd specific to the coding sequence of *DtSD* 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 sec, 57.5°C for 45 sec, 72°C for 1min). Meanwhile, the RT-PCR reaction for the house-keeping gene (18S rRNA gene), using specific primers 18sf (5`-GTGACAATGGAACTGGAATGG-3`) and 18sr (5`-AGACGGAGGATAGCGTGAGG-3`), was used to estimate whether the equal amounts of total RNA among samples were used in RT-PCR reaction.

HPLC analysis of Podophyllotoxin

The extraction and detection of podophyllotoxin was performed according to the method established by Cushman et al (Cushman et al., 2005).

RESULTS

Cloning of the full-length cDNA of DtSD

A specially amplified PCR product of 800 bps was obtained at 57℃ using a pair of primers (fdtsd and rdtsd), which was sub-cloned and sequenced to generate an 837-bp nucleotide sequence that was confirmed by sequencing. The BLAST searching results demonstrated that the 837-bp cDNA fragment showed homologous with SD genes from other plant species, such as *Podophyllum peltatum* and *Forsythia* x *intermedia*. These strongly suggested that the core fragment of *DtSD* had been obtained, which provided necessary and enough sequence information for isolating the cDNA ends of *DtSD* by RACE. The 596-bp 3'-end and 192-bp 5'-end of DtSD was respectively obtained by 3'RACE and 5'RACE. By assembling the sequences of 3'-end, 5'-end and the core fragment on ContigExpress, the full-length cDNA sequence of *DtSD* was generated which was 994 bp. Then, the physical full-length *DtSD* cDNA was amplified and confirmed by sequencing. *DtSD* had the 32-bp 5' untranslated region (UTR), the 837-bp coding sequence and the 125-bp 3' UTR including the 14-bp poly-A tail (Figure 1). The ORF finding analysis showed that *DtSD* contained an 837-bp coding sequence encoding a 278-amino-acid polypeptide with a calculated molecular mass of 29.2 kDa and an isoelectric point of 6.32 that were similar with the reported plant SDs such as *Podophyllum peltatum* SD (Xia et al., 2001). Then, DtSD was submitted to GenBank and assigned an accession number: ABD78859.1.

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   1                                          AAGACATCTTAACAATCTGAATTATTTGGACA
  33 ATGGGATTCACTTCTACACCAGCTTCGTCTACCAATAGGTTACAAGATAAAGTAGCC
       M  G  F  T  S  T  P  A  S  S  T  N  R  L  Q  D  K  V  A
  90 ATCATAACAGGGGGAGCAGGTGGGATTGGTGAAACCACAGCAAAATTATTCGTCCGC
       I  I  T  G  G  A  G  G  I  G  E  T  T  A  K  L  F  V  R
 147 TACGGTGCTAAAGTTGTGATAGCAGACATCTCAGATGACCACGGCCAAAAAGTTTGT
       Y  G  A  K  V  V  I  A  D  I  S  D  D  H  G  Q  K  V  C
 204 AAAAACATTGGCTCACCGGACGTGATCTCTTTCGTTCATTGTGATGTGACCAAAGAT
       K  N  I  G  S  P  D  V  I  S  F  V  H  C  D  V  T  K  D
 261 GAGGATGTCCGGAACCTAGTGGATACCACCATAGCCAAGCATGGAAAACTTGACATA
       E  D  V  R  N  L  V  D  T  T  I  A  K  H  G  K  L  D  I
 318 ATGTTCGGCAACGTTGGTGTTCTGAGCACCACTCCTTACAGCATACTGGAAGCTGGA
       M  F  G  N  V  G  V  L  S  T  T  P  Y  S  I  L  E  A  G
 375 AATGAAGATTTCAAGAGAGTTATGGACATTAACGTGTATGGTGCATTTTTAGTAGCC
       N  E  D  F  K  R  V  M  D  I  N  V  Y  G  A  F  L  V  A
 432 AAACACGCAGCCAGAGTCATGATCCCAGCCAAGAAAGGTAGTATAGTATTCACTGCA
       K  H  A  A  R  V  M  I  P  A  K  K  G  S  I  V  F  T  A
 489 AGTATTTCTTCCTTCACAGCAGGAGAAGGTGTGTCGCATGCTTACACCGCAACCAAG
       S  I  S  S  F  T  A  G  E  G  V  S  H  A  Y  T  A  T  K
 546 CATGCTGTCCTTGGATTGACAACCAGCTTATGTACTGAGCTAGGACAGCATGGGATC
       H  A  V  L  G  L  T  T  S  L  C  T  E  L  G  Q  H  G  I
 603 CGAGTGAACTGTGTATCTCCCTATGTGGTTGCATCCCCATTGTTGACAGATGTGTTT
       R  V  N  C  V  S  P  Y  V  V  A  S  P  L  L  T  D  V  F
 660 GGGGTGGATTCTAGTAGGGTTGAGGAATTGGCACATCAAGCTGCAAACCTCAAAGGG
       G  V  D  S  S  R  V  E  E  L  A  H  Q  A  A  N  L  K  G
 717 ATTTTGCTCAGGGCTGAGGATGTGGCCGATGCAGTCGCGTATTTGGCAGGGGATGAG
       I  L  L  R  A  E  D  V  A  D  A  V  A  Y  L  A  G  D  E
 774 TCCAAGTATGTGAGCGGCCTGAACCTTGTTATCGATGGGGGCTACACCAGAACCAAT
       S  K  Y  V  S  G  L  N  L  V  I  D  G  G  Y  T  R  T  N
 831 CCGGCTTTCCCAACTGCATTGAAACATGGATTAGCTTGATTACGTTGCTGCTTGTGT
       P  A  F  P  T  A  L  K  H  G  L  A  *
 888 TTGTGTTTCTATGTCAAAGATGCAGTTTGTCCTAGTTCTTGCTTATCCTTTCATATG
 945 ATGAATAAGAATTGTTAAGTTGTCTTGATATGTTCTAAAAAAAAAAAAAA
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Figure 1. The full-length cDNA sequence and the deduced amino acid sequence of DtSD. The coding sequence was typed in bold; the stop codon (TGA) was marked with an aster; the 5`- and 3`-untranslated regions were shown in normal letters.

Bioinformatic analysis

The BlastP analysis of DtSD amino acid sequence on NCBI showed that DtSD belonged to the protein superfamily of dehydroxylase, and revealed that DtSD was similar with other plant SD proteins such as *Sinopodophyllum hexandrum* SD (98 % similarity), *Podophyllum peltatum* SD (96% similarity) and *Forsythia* x *intermedia* SD (53% similarity). The multiple alignments of SDs from *D*. *tsayuensis*, *P*. *peltatum* and *F*. x *intermedia* demonstrated that all the three SDs had the conserved catalytic motif characterized by the amino acid patter of Ser-X13-Tyr-X2-Lys. In the SD of D. tsayuensis, the motif was Ser164-X13-Tyr178-X2-Lys181, which is the binding site of NAD^+ and substrate (Figure 2). The homology-based structural modeling results showed that the overall structure of DtSD contained 5 β-sheets that were folded to form the active center in which the catalytic motif of Ser164-X13-Tyr178-X2-Lys181 was localized, and 8 α-helices surrounding the active center (Figure 3). It was highly similar with the known structure of the SD protein of *P*. *peltanum*, which had 7β-sheets to form the active center and 8 α-helices surrounding the active center (Buhyun et al., 2005).

The tissue expression pattern of *DtSD*

The semi-quantitative one-step RT-PCR analysis was

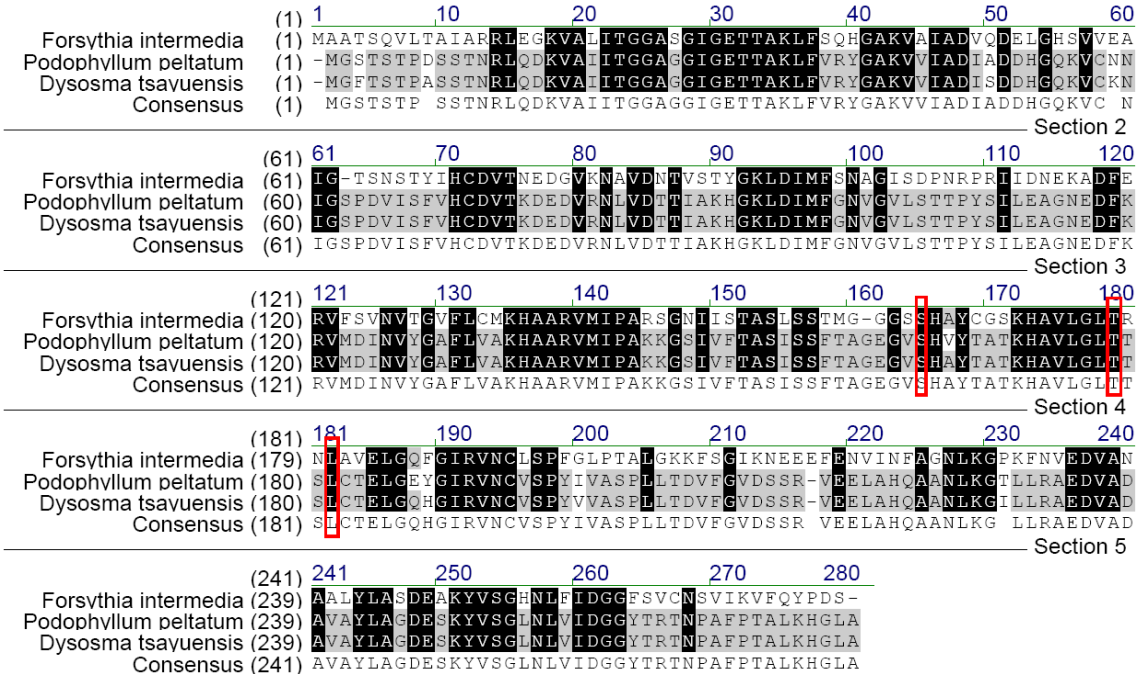

Figure 2. The multiple alignments of amino acid sequences of SDs from plant. The identical amino acids were showed in white with black background and the conserved amino acids were showed in black with gray background. Numbers indicate the position of amino acid residues. The catalytic motif of Ser-X13-Tyr-X2-Lys was boxed.

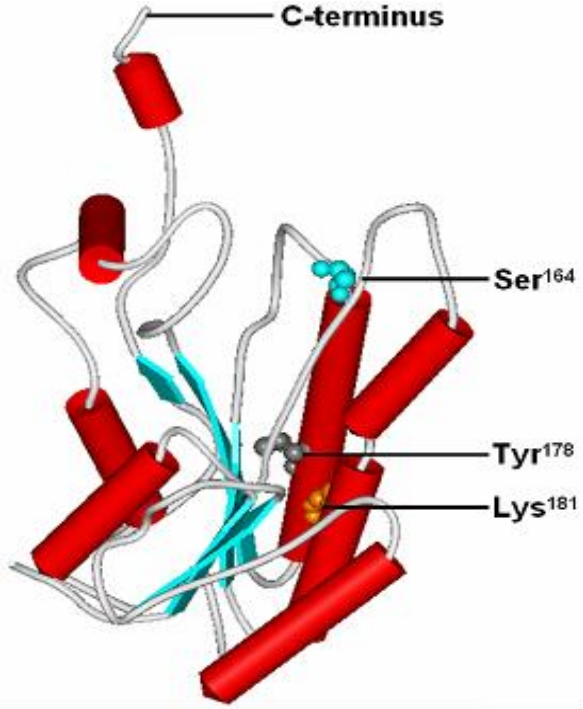

Figure 3. The three-dimensional structure of DtSD monomer established by homology-based modeling. The α-helix, β-sheet and random coil are indicated by pod, arrowed-plate and rope-shape, respectively. The catalytic motif of Ser-X13-Tyr-X2-Lys was indicated by balls.

applied to unveil the tissue expression profile of DtSD in different tissues of Tibet Dysosma including root, rhizome, leaf, petiole, flower and fruit. The results showed that DtSD expressed in root, rhizome, petiole and fruit but at different levels, but the expression of DtSD was not found in leaf and flower. The constitutive 18S rRNA gene had the nearly same expression level in all the tissues. The lowest-level expression of DtSD was found in fruits; the roots and the rhizomes had the middle-level expression of DtSD at the similar levels; the highest-level expression of DtSD was found in petioles (Figure 4). The results of the tissue expression profile of DtSD demonstrated that DtSD was not a constitutive gene in Tibet Dysosma, but a developmentally regulated gene just like other genes involved in secondary metabolic pathways such as hyoscyamine 6β-hydroxylase (H6H) gene from *Atropa benlladonna* (Suzuki et al., 1999) and S-adenosyl-L-methionine: loganic acid methyl transferase (LAMT) gene in *Catharanthus roseus* (Murata et al., 2008).

HPLC analysis of podophyllotoxin

The HPLC method was employed to detect the content of podophyllotoxin in different tissues of Tibet Dysosma. The podophyllotoxin could be detected in root, rhizome, leaf, petiole, flower and fruit of Tibet Dysosma but at different levels. This was the first time to find that the flowers and fruits of Tibet Dysosma contained the podophyllotoxin.

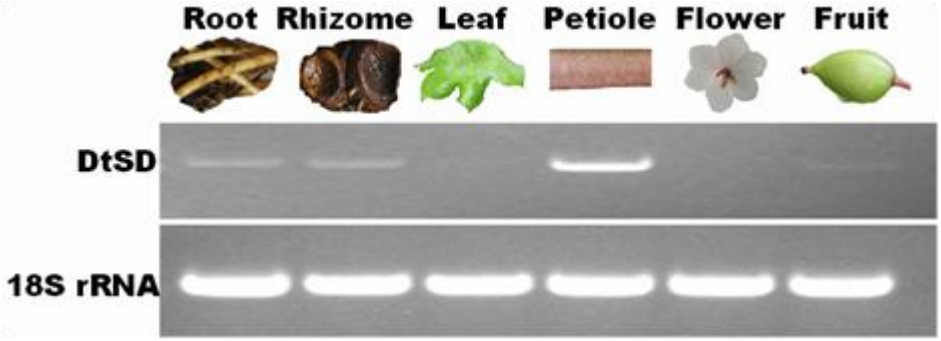

Figure 4. Tissue expression of DtSD in various tissues of Tibet Dysosma.

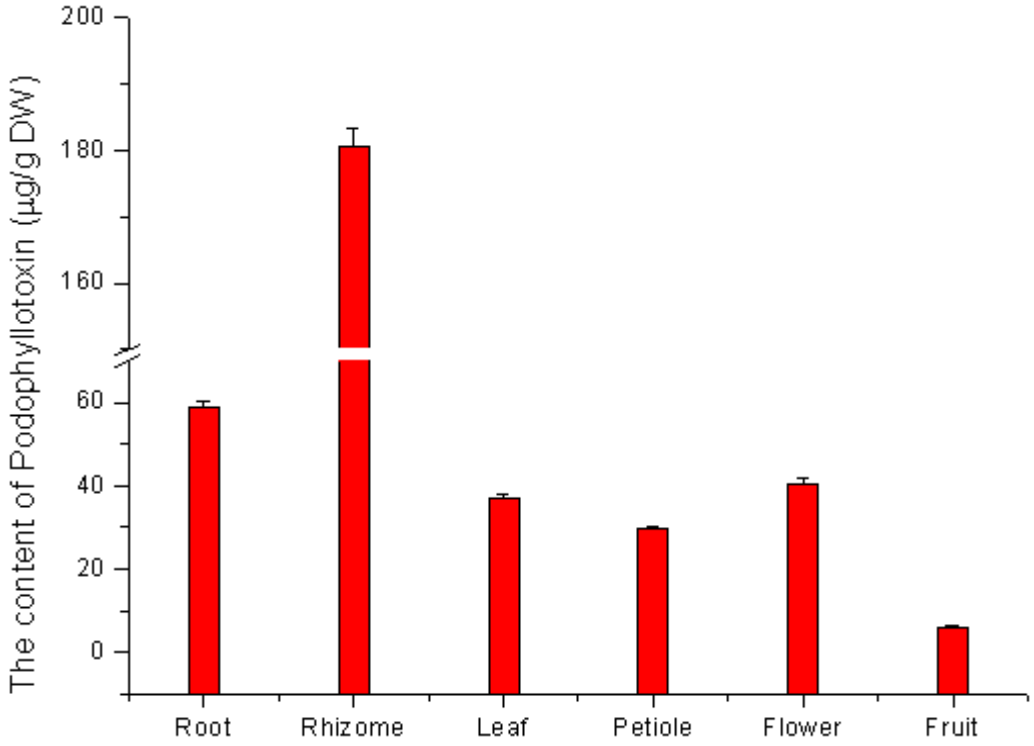

Figure 5. The podophyllotoxin content in various tissues of Tibet Dysosma.

The highest content of podophyllotoxin was found in rhizome (180.5±2.74 μg/g), and then followed by root, flower, leaf and petiole. The lowest content of podophyllotoxin was found in fruit (6.03±0.12 μg/g). The content of podophyllotoxin in rhizomes of Tibet Dysosma was 30 times higher than that in fruits. This suggests that rhizomes are the storage organs of podophyllotoxin of Tibet Dysoma (Figure 5).

DISCUSSION

The secoisolariciresinol dehydrogenase is a key enzyme involved in the biosynthetic pathway of podophyllotoxin (Xia et al., 2001), which might be a necessary gene of interest used in metabolic engineering the biosynthetic pathway of podophyllotoxin. In the present study, we cloned and characterized the full-length cDNA encoding SD from a kind of native herbal plant, *Tibet Dysosma*, for the first time. The protein of DtSD is highly similar with the reported SD proteins from other podophyllotoxin-producing plants including *P. peltanum* and *F.* x *intermedi*a, not only at the level of sequence but also at the level of 3-D structure.

The previous researches always focused on gene cloning and biochemistry of the SD protein, but ignored the gene expression features. We established the tissue expression of DtSD in the present study, and concluded

that DtSD is a developmentally regulated gene because it expressed not in all the tested tissues but only in root, rhizome, petiole and fruit. This is reasonable like other functional genes involved in other secondary metabolic pathways. For example, the *H6H* gene encoding the committed-step enzyme of scopolamine biosynthesis specially expressed in the roots of *Atropa belladonna* (Suzuki et al., 1999); the ADS gene encoding amorpha-4,11-diene synthase involved in artemisinin biosynthesis exclusively expressed in the glandular secretory trichomes of *Artemisia annua* (Olsson et al., 2009). So, the functional genes of the secondary metabolic pathways usually express according to the developmental stages and the living environments. The secondary metabolites may not distribute equally in different tissues, such as the famous anti-tumor Taxol much more abundant in the older bulks than that in any other tissue of yews (Wani et al., 1971), ginkgolides richer in roots than that in other tissues of Ginkgo (Carrier et al., 1998). It is the same case for podophyllotoxin in Tibet Dysosma.

The highest content of podophyllotoxin was found in rhizomes of *Tibet Dysosma* because rhizomes are the storage organs of podophyllotoxin, and this is consistent with the previous report (Liu et al., 2004). The lowest content of podophyllotoxin was found in fruits, which was 30 times lower than that in rhizomes. When comparing the DtSD gene tissue expression profile and the podophyllotoxin tissue content profile, the gene expression did not correlate with the content of podophyllotoxin. That is reasonable because the transportation mechanism of secondary metabolites widely exists in plants. As a result, the biosynthetic organs of secondary metabolites can not be consistent with the storage organs. In summary, cloning and characterization of the SD gene from *Tibet Dysosma* facilitates to understand the molecular mechanisms of podophyllotoxin biosynthesis and provides an important gene of interest to metabolic engineering the podophyllotoxin biosynthetic pathway in Tibet Dysosma.

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REFERENCES

Arnold K, Bordoli L, Kopp J, Schwede T (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22: 195-201.

Buhyun Y, Syed GAM, Laurence BD, Norman GL, ChulHee K (2005). Crystal Structures of Apo-form and Binary/Ternary Complexes of Podophyllum Secoisolariciresinol Dehydrogenase, an Enzyme Involved in Formation of Health-protecting and Plant Defense Lignans. J. Biol. Chem. 280: 12917-12926.

Carrier DJ, van Beek TA, van der Heijden R, Verpoort R (1998). Distribution of ginkgolides and terpenoid biosynthetic activity in G. biloba. Phytochemistry 48: 89-92.

Cushman KE, Maqbool M, Lata H, Bedir E, Khan KA, Moraes RM (2005). Podophyllotoxin content and yield of American mayapple leaves in sun and shade. Hortscience 40: 60-63.

Kadkadea PG (1982). Growth and podophyllotoxin production in callus tissues of Podophyllum peltatum. Plant Sci. Lett. 25: 107-115.

Liao MC, Wang YW, Tu ZB, Xiao PG (2002). Study on consitituents of *Dysosoma tsayunesis* Ying. J. Wuhan Bot. Res. 20: 71-74.

Liu HJ, Xu Y, Su GQ, Li CY, Wang L, Liu YJ (2004). Research Progress on Production of podophyllotoxin from Sinopodophyllum hexandrum. Forestry Studies in China 6: 48-53.

Macdonald I, Durst TJ (1988). A highly stereoselective synthesis of podophyllotoxin and analogues based on an intramolecular Diels-Alder reaction. Organic Chem. 53: 3663–3669.

Murata J, Roepke J, Gordon H, De Luca V (2008). The Leaf Epidermome of Catharanthus roseus Reveals Its Biochemical Specialization. The Plant Cell 20: 524-542.

Olsson ME, Olofsson LM, Lindah AL, Lundgren A, Brodelius M, Brodelius PE (2009). Localization of enzymes of artemisinin biosynthesis to the apical cells of glandular secretory trichomes of Artemisia annua L. Phytochemistry 70: 1123-1128.

Suzuki KI, Yun DJ, Chen XY, Yamada Y, Hashimoto T (1999). An Atropa belladonna hyoscyamine 6 beta-hydroxylase gene is differentially expressed in the root pericycle and anthers. Plant Mol. Biol. 40: 141-152.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876-4882.

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971). Plant antitumor agents. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J. Am. Chem. Soc. 93: 2325-2327.

Xia ZQ, Michael AC, Hélène CP, Laurence BD, Norman GL (2001). Secoisolariciresinol Dehydrogenase Purification, Cloning, and Functional Expression. J. Biol. Chem. 276: 12614-12623.

Ying JS (1979). Study on new genus of Dysosma and Sinopodophyllum in the family of Berberidaceae. Acta Phytotaxonomica Sinica 17: 13-15.