CHAPTER 3
RESULTS

3.1 Cloning and DNA sequencing of a full-length cDNA encoding for *dxr* gene

3.1.1 Degenerated primers design

Degenerated *dxr* oligonucleotides were designed from the highly conserved amino acid sequences among the plants species: *Arabidopsis thaliana* (accession no. NP_201085), *Oryza sativa* (accession no. BAF03694), *Lycopersicon esculentum* (accession no. AAK96063), *Catharantus roseus* (accession no. AAF65145), *Stevia rebaudiana* (accession no. CAD22156), and *Artemesia annua* (accession no. AAW28998). By using BLAST and Gene Doc programs, the alignment of deduced amino acids revealed the highly conserved regions as shown in Fig. 3.1. The degenerated primers were designed from the regions that covered the inner part of the Dxr with the omission of the plastid leader sequences (2.2.5). The sense primers were P101S, P157S, P193S and the antisense primers were P274A, P297A, P383A.

3.1.2 Total RNA from *C. stellatopilosus* young leaves

Total RNA from *C. stellatopilosus* young leaves was isolated according to method described in 2.3.1.1. After precipitation the total RNA with 4 M LiCl, the precipitate was washed with ethanol and dried under vacuum. The RNA was re-dissolved in 100 µl of DEPC water. The concentration of the RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The concentration of total RNA was calculated according to the equation (2.3.1). The concentration of the resulting RNA was 2.25 µg/µl. The purity of the total RNA was measured by means of spectrophotometer analysis (A_{260}/A_{280}) and agarose gel electrophoresis analysis. The ratio of the A_{260}/A_{280} of the isolated RNA was 1.678.

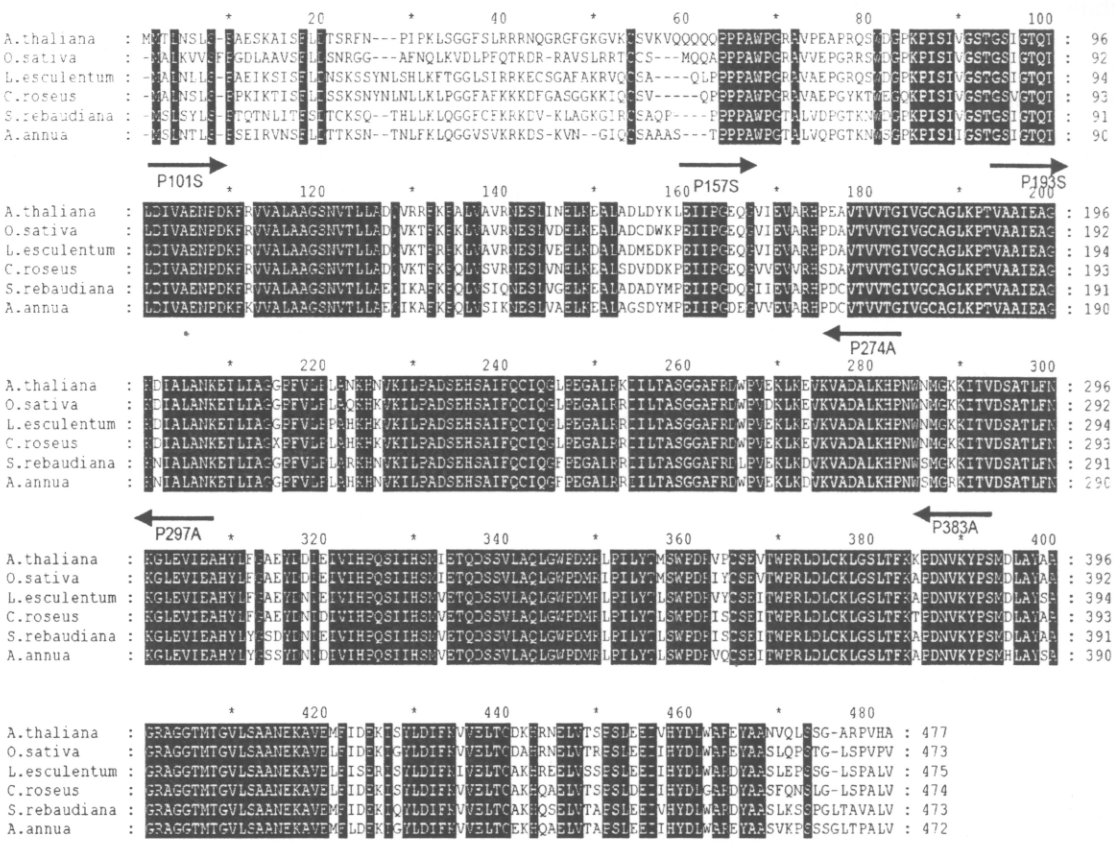

Figure 3.1 Multi-alignment of amino acid sequences of higher plant Dxrs. The identical amino acids were shown in white with black background. The arrows showed the directions of senses and antisense primers.

3.1.3 Cloning of core fragment, 5'- and 3'-ends

For the core fragment amplification (2.3.3.1), the PCR product was successfully obtained from the PCR reaction containing P101S and P297A as primers and cDNA (2.3.2) as template. The resulting PCR product was *ca* 600-bp DNA fragment on agarose gel electrophoresis (Fig. 3.2). The PCR product was purified on agarose gel electrophoresis (2.3.4.1). The purified fragment was ligated with the pDrive vector and transformed into *E. coli* TOP10 competent cells (2.3.4). After selection the white colonies, the recombinant plasmid was isolated and the resulting plasmid was analyzed for restriction site analysis (2.3.5). The double strands DNA were sequenced (2.3.7). The DNA sequence analysis of PCR product revealed the 600-bp DNA fragment.

The 5'- and 3'-ends of *dxr* gene were obtained by RACE method (2.3.3.2). Two gene specific primers for both sides were designed based on the nucleotide

sequence of the core fragment of *dxr* gene (Table 2.1, Scheme 2.2). The 5'- and 3'- ends were amplified using two steps PCR.

For 5'-end amplification, the cDNA template has been modified by adding the Poly-A addition prior using in the PCR reaction. The 1st PCR was performed with RACE32 and 5'P_180A for forward and reverse primers, respectively. The 1st PCR product was purified using the PCR purification kit (Qiagen) and used as template for the 2nd PCR. RACE 17 and 5'P_142A were used for amplification of the 2nd PCR. The PCR product was analyzed by 1.2% agarose gel electrophoresis. As shown in Fig. 3.3 (lane 2), the PCR product of 500-bp was obtained. For 3'-end amplification, the 1st PCR was performed with 3'P_228S and RACE32 for forward and reverse primers, respectively. The 1st PCR product was purified using the PCR purification kit (Qiagen) and used as template for the 2nd PCR. 3'P_374S and RACE17 were used for amplification of the 2nd PCR. The PCR product was analyzed by 1.2% agarose gel electrophoresis. As shown in Fig. 3.3 (lane 3), the PCR product of 1100-bp was obtained.

The PCR products were purified on agarose gel electrophoresis (2.3.4.1). The purified PCR fragments were ligated with the pDrive vector and transformed into *E. coli* TOP10 competent cells (2.3.4). After selection the white colonies, the recombinant plasmid was isolated and the resulting plasmid was analyzed for restriction site analysis (2.3.5). The double strands of DNA fragments of 5'- and 3'- ends were sequenced. The resulting DNA analysis revealed the 500-bp for 5'-end and 1100-bp for 3'-end.

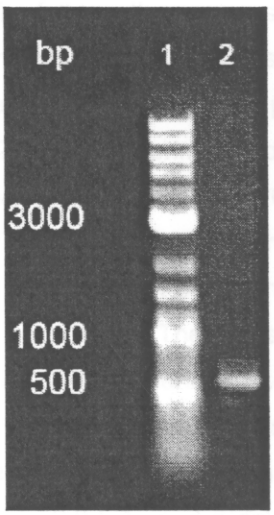

Figure 3.2 PCR product of core fragment was amplified by two steps RT-PCR and analyzed by 1.2% agarose gel electrophoresis

Lane 1: DNA marker

Lane 2: core fragment of *dxr* gene

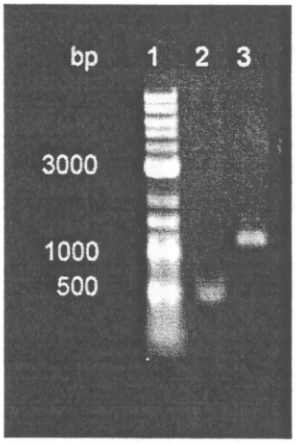

Figure 3.3 1.2% Agarose gel electrophoresis of the PCR products of 5'- and 3'-end fragments.

Lane 1: DNA marker

Lane 2: 5'-end of *dxr* gene

Lane 3: 3'-end of *dxr* gene

3.1.4 Cloning of a full-length cDNA for *dxr* gene (*Csdxr*)

By aligning and assembling the DNA sequences of the core fragment, 5'-end and 3'-end products of *dxr* gene of *C. stellatopilosus*, the two-sets of specific primers for the full-length gene were designed. The full-length gene was amplified using the two-steps PCRs (2.3.3.3). The 1st PCR was performed using PDXR-S1 and PDXR-A1 for forward primer and reverse primers, respectively. The 1st PCR product was purified and used as template for the 2nd PCR. PDXR-S2 and PDXR-A2, a forward primer and a reverse primer, respectively were used for 2nd PCR reaction. Following the 1st and 2nd PCR reactions, the PCR product was obtained as shown in Fig. 3.4. The full- length PCR product was purified, ligated to pDrive and transformed into *E. coli* TOP10 competent cells. The recombinant plasmid was isolated and the double strand DNA was sequenced. The full-length cDNA sequence of *Csdxr* was deduced and subsequently confirmed by sequencing, which was 2055 bp and has an open reading frame of 1404-bp starting with an initiation codon ATG and ending with a termination codon TGA. The protein encoded cDNA of *dxr* gene has 468 amino acid residues (Fig. 3.5) with a predicted molecular mass of 50.6 kDa and a calculated isoelectric point of 5.64. The nucleotide and deduced amino acid sequences of the CsDxr have been submitted to GenBank. The assigned accession numbers are EF451544 and ABO38177, respectively.

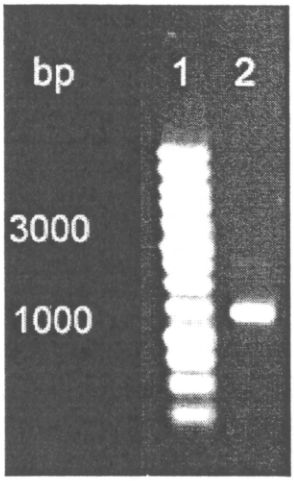

Figure 3.4 1.2% Agarose gel electrophoresis of the PCR products of *Csdxr*
Lane 1 DNA marker
Lane 2 full-length of *dxr* gene

3.2 Comparison of the amino acid sequences of Dxr from higher plants and analysis of conserved residues

The full-length of *dxr* gene from 3.1 was analyzed and translated to the deduced amino acids by using the DNASIS V3.5 software and the CLUSTAL W (1.82). The deduced amino acid sequence of *dxr* from *C. stellatopilosus* (CsDxr) was compared with the amino acid sequence of several representatives Dxr from other plants such as *Hevea brasiliensis, Arabidopsis thaliana, Lycopersicon esculentum, Catharanthus roseus, Stevia rebaudiana and Artemisia annua*. The comparison of sequences was conducted through databases and the alignment was performed using Gene Doc program. The result suggested that CsDxr belongs to the Dxr family as shown the highly conserved to Dxr from other higher plants. The percent identity of the amino acid sequences of CsDxr and Dxr from other plants is ranged from 76-92% (Table 3.2). In contrast, the CsDxr is similar to *Escherichia coli* and *Plasmodium falciparum* with the percent identity of 41 and 34, respectively.

Obviously, all of the sequences at N-terminal region were more different than at the C-terminal region. From the amino acid sequence analyzes among the higher plants, the CsDxr can be divided to three domains. The first domain at N-terminal was predicted by TargetP predictor suggested a chloroplast transit peptide at the N-terminal of 44 amino acids. The second domain contained an extended Pro-rich motif at the N terminus of the CsDxr. The other domain was the highly conserved region of NADPH binding motif (GSTGSIGT) (Fig 3.6).

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gactcgagtcgacatcgatttttttttttttttccattctatccccagctccaggccaca 60
tatttcgtattcctctgagttggaatttttgaattttgattggATGgctcttaatttgc 120
                                                M  A  L  N  L   5
tttctcctgctgaaatcaaatccatttccttcttagactccgccaagtccaaccagcttc 180
L  S  P  A  E  I  K  S  I  S  F  L  D  S  A  K  S  N  Q  L   25
ccaagcttacaggtagtttcagtttgaagagaaaggatttgggcaggaaagtgcaatgtt 240
P  K  L  T  G  S  F  S  L  K  R  K  D  L  G  R  K  V  Q  C   45
ctgttcagtctcctcctccaccagcctggccgggaacagcttttccagaaccaggccgta 300
S  V  Q  S  P  P  P  P  A  W  P  G  T  A  F  P  E  P  G  R   65
agacttgggatggtccaaagcctatttctattgttggatccactggctccattggtactc 360
K  T  W  D  G  P  K  P  I  S  I  V  G  S  T  G  S  I  G  T   85
agacattggatatagtggcagagaatcctgaaaaatttaaagtggtggcacttgcagctg 420
Q  T  L  D  I  V  A  E  N  P  E  K  F  K  V  V  A  L  A  A   105
gttcaaatgtcactcttcttgctgatcaggtgaaaaccttcaaacctcagcttgttgcag 480
G  S  N  V  T  L  L  A  D  Q  V  K  T  F  K  P  Q  L  V  A   125
ttaggaatgagtctttagttgatgaactcaaagaagctttggctgatcttgaagaaaagc 540
V  R  N  E  S  L  V  D  E  L  K  E  A  L  A  D  L  E  E  K   145
ctgagattattcctggggaggaaggagttgttgaggttgcccgccatcctgatgctgcca 600
P  E  I  I  P  G  E  E  G  V  V  E  V  A  R  H  P  D  A  A   165
gtgttgttactggtatagtcggctgtgcaggcttaaggcctacggtggctgcaatagaag 660
S  V  V  T  G  I  V  G  C  A  G  L  R  P  T  V  A  A  I  E   185
ctggaaaagacatatgcttggccaacaaagagactttgattgctggaggtccgtttgtcc 720
A  G  K  D  I  C  L  A  N  K  E  T  L  I  A  G  G  P  F  V   205
ttcctcttgctaacaaatataacgttaaaattcttccagctgattcagaacattctgcta 780
L  P  L  A  N  K  Y  N  V  K  I  L  P  A  D  S  E  H  S  A   225
tatttcagtgtattcaaggcctacctgaaggtgcattgcggcgcattattttaactgctt 840
I  F  Q  C  I  Q  G  L  P  E  G  A  L  R  R  I  I  L  T  A   245
ctggtggggctttcagggattggcctgtagaaaaactgaaagatgttaaggtagctgatg 900
S  G  G  A  F  R  D  W  P  V  E  K  L  K  D  V  K  V  A  D   265
ctttgaagcaccccaactggaatatgggaaaaaagattactgtcgactcagctacccttt 960
A  L  K  H  P  N  W  N  M  G  K  K  I  T  V  D  S  A  T  L   285
tcaacaagggtttagaagttattgaagcccattatctgtttggagctgattatgataata 1020
F  N  K  G  L  E  V  I  E  A  H  Y  L  F  G  A  D  Y  D  N   305
ttgagattgtaattcatccccaatctataatacattcaatggttgaaacacaggattcat 1080
I  E  I  V  I  H  P  Q  S  I  I  H  S  M  V  E  T  Q  D  S   325
ctgttcttgcacaacttgggtggcctgatatgcgcttaccaattctatacaccatgtcat 1140
S  V  L  A  Q  L  G  W  P  D  M  R  L  P  I  L  Y  T  M  S   345
ggcctgacagaatttactgctctgaaataacatggcctcgccttgatctttgcaagcaag 1200
W  P  D  R  I  Y  C  S  E  I  T  W  P  R  L  D  L  C  K  Q   365
gttctctaacatttaaagctcctgataatgtaaaatacccatctatggatcttgcctatg 1260
G  S  L  T  F  K  A  P  D  N  V  K  Y  P  S  M  D  L  A  Y   385
ctgctggaagggctggaggcaccatgacaggagtccttagtgcggctaatgagaaagctg 1320
A  A  G  R  A  G  G  T  M  T  G  V  L  S  A  A  N  E  K  A   405
tggagatgttcatcgatgaaaagataagttatctcgacattttcaagattgtggagctaa 1380
V  E  M  F  I  D  E  K  I  S  Y  L  D  I  F  K  I  V  E  L   425
cctgcaataagcatcgggcggaattggtagccaccccatcgctggaggagattatacact 1440
T  C  N  K  H  R  A  E  L  V  A  T  P  S  L  E  E  I  I  H   445
atgacttgtgggcacgggagtatgctgccagtttgcaaacctctggtcgaagtcctgttc 1500
Y  D  L  W  A  R  E  Y  A  A  S  L  Q  T  S  G  R  S  P  V   465
ttgcaTGAtcgcctatgctgctggaagggctggaggcaccatgacaggagtccttagtgc 1560
L  A  *                                                       468
ggctaatgagaaagctgtggagatgttcatcgatgaaaagataagttatctcgacatttt 1620
caagattgtggagctaacctgcaataagcatcgggcggaattggtagccaccccatcgct 1680
ggaggagattatacactatgacttgtgggcacgggagtatgctgccagtttgcaaacctc 1740
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tttttgaaactggaggataggtgttatactcctgtagaagaagactggcattcttgccgg 1860
ggtttgtggggtcattttgcaaatgtatcatatataaattgtcaatctgacatccgaaac 1920
gtttcccccccatccattttgcagtcattatataattgctgtacttgtgatattcagata 1980
tgaaaattaatttttcaaggtgatacaagggaagaatttatattggaaaaaaaaaaaaaaa 2040
aaaaaaaaaaaaaa                                                 2055
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Figure 3.5 Nucleotide and amino acid sequences of *Csdxr*

ATG = Start codon, **TGA** = Stop codon (*).

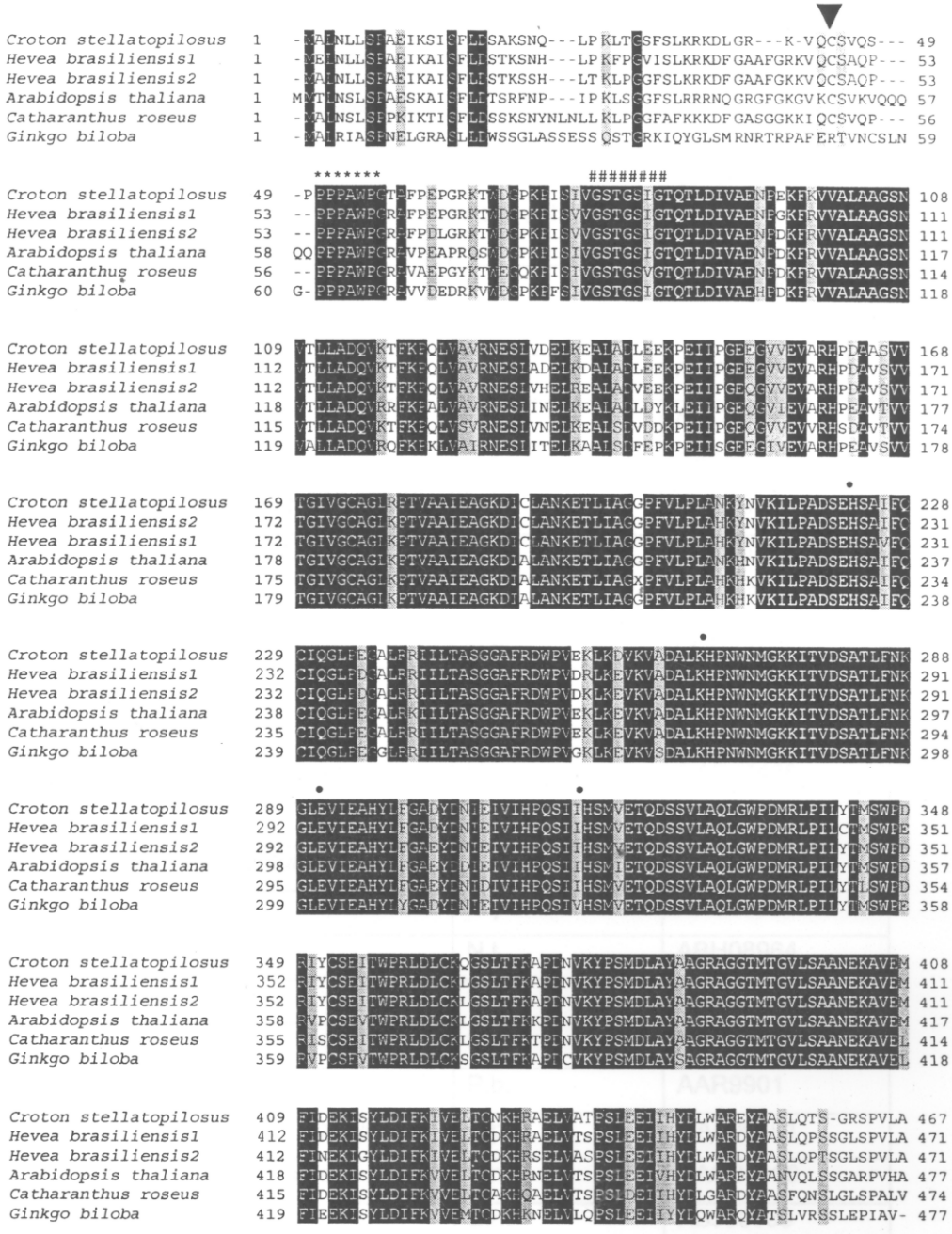

Figure 3.6 Alignment of deduced amino acid sequence of CSDXR and other plant DXRs. Identical residues among six proteins are indicated in inverse contrast. Similarity residues are indicated in shading. Cleavage site of chloroplast transit peptide is indicated by filled triangle. Residues representating a proline-rich motif are indicated by asterisks. Residues representating a putative NADPH-binding site are indicated by #. Putative catalytic residues are indicated by •. GenBank accession numbers as follows: *C. stellatopilosus* (**EF451544**), *H. brasiliensis1* (**DQ437514**), *H. brasiliensis2* (**AY502937**), *A. thaliana* (**NM125674**), *C. roseus* (**AF250235**) and *G. biloba* (**AY443101**).

Table 3.1 List of Dxr from plants, bacteria and malaria parasite from the National Center for Biotechnology Information (NCBI) database

Species	**Abbreviations**	**Accession number**
Antirrhinum majus	A.m.	NP_201085
Arabidopsis thaliana	A.t.	CAB43344
Artemisia annua	A.a.	AAW28998
Camptotheca acuminata	C.a.	ABC86579
Catharanthus roseus	C.r.	AAF65154
Chrysanthemum x morifolium	C.m.	BAE79548
Cistus incanus subsp. creticus	C.i.	AAP56260
Croton stellatopilosus	**C.s.**	**ABO38177**
Ginkgo biloba	G.b.	AAR95700
Hevea brasiliensis 1	H.b.1	AAS94121
Hevea brasiliensis 2	H.b.2	ABD92702
Hordeum vulgare subsp. vulgare	H.v.	CAE47438
Linum usitatissimum	L.u.	CAF22092
Lycopersicon esculentum	L.e.	AAK96063
Mentha x piperita	M.p.	AAD24768
Nicotiana tabacum	N.t.	ABH08964
Oryza sativa	O.s.	BAF03694
Picrorhiza kurrooa	P.k.	ABC74566
Plectranthus barbatus	P.b.	AAR9901
Populus alba x Populus tremula	P.p.	CAE00491
Pueraria montana var. lobata	P.m.	AAQ84168
Salvia miltiorrhiza	S.m	ABJ80680
Stevia rebaudiana	S.r.	CAD22156
Taxus cuspidata	T.c.	AAT47184
Taxus x media	T.m.	AAU87836
Zea mays	Z.m.	CAC03581
Plasmodium falciparum	P.f.	AAD03739
Escherichia coli	E.c.	1Q0Q_B

Table 3.2 Percent identity of amino acid sequences of CsDxr and other plant Dxrs, bacteria and malaria parasite.

Sample	C.s.	A.m.	A.t.	A.a.	C.r.	C.a.	C.m.	C.i.	G.b.	H.b. 1	H.b. 2	H.v.	L.u.	L.e.	M.p.	N.r.	O.s.	P.k.	P.b.	P.p.	P.m.	S.m	S.r.	T.c.	T.m.	Z.m.
C.s.	100																									
A.m.	82	100																								
A.t.	84	84	100																							
A.a.	82	79	79	100																						
C.r.	85	87	83	84	100																					
C.a.	82	80	81	76	81	100																				
C.m.	81	78	78	98	81	76	100																			
C.i.	81	78	78	79	77	75	76	100																		
G.b.	78	76	75	74	73	85	73	72	100																	
H.b.1	91	83	84	80	86	83	79	81	78	100																
H.b.2	92	84	85	80	87	83	80	81	78	94	100															
H.v.	79	78	79	76	78	75	74	71	75	77	78	100														
L.u.	85	82	81	81	83	79	80	77	75	83	83	76	100													
L.e.	86	86	84	81	87	83	81	80	79	87	88	79	82	100												
M.p.	81	85	82	80	84	80	79	76	72	83	84	78	79	84	100											
N.t.	85	87	84	80	87	83	79	78	79	85	87	78	84	93	85	100										
O.s.	82	81	83	76	82	78	74	73	76	83	83	82	79	83	80	83	100									
P.k.	83	90	82	80	84	78	79	77	74	83	84	77	81	85	85	86	80	100								
P.b.	80	84	80	80	84	80	79	76	75	81	83	80	81	85	80	85	80	85	100							
P.p.	86	80	82	79	82	79	78	77	74	85	86	74	81	81	79	81	78	79	78	100						
P.m.	76	73	73	74	73	70	73	71	76	75	76	72	73	74	72	74	71	76	73	74	100					
S.m	83	87	82	80	86	81	78	75	75	83	84	77	78	85	94	86	81	85	90	79	73	100				
S.r.	82	80	80	86	81	77	86	77	73	80	81	75	81	81	81	80	77	81	79	78	72	80	100			
T.c.	79	77	75	77	74	83	76	73	84	77	76	76	77	78	73	79	77	75	74	75	76	77	76	100		
T.m.	78	75	74	74	73	81	73	71	85	76	75	75	76	77	72	79	77	74	74	72	74	75	74	97	100	
Z.m.	80	81	84	77	83	79	77	76	75	83	83	88	79	85	81	85	91	79	90	78	73	81	79	77	75	100
E.c.	41	41	41	41	41	40	41	41	40	41	41	41	41	41	40	41	41	40	41	38	41	40	41	41	40	41
P.f.	34	34	34	34	32	33	33	34	33	33	34	32	34	34	34	35	32	33	34	31	35	34	35	34	33	34

3.3 Phylogenetic analysis

Phylogenetic analysis of the Dxr enzymes from different species: higher plants, gymnosperms, eubacteria and malaria parasite was generated with MEGA version 3.1 as shown in Fig. 3.7. Altogether with the percent identity from Table 3.2, the plants Dxr can be divided into two groups, which are gymnosperm (78-79% identity) and angiosperm (80-90% identity). The amino acid sequence of CsDxr revealed high homology throughout the entire coding regions with: *Hevea braziliensis* (91% identity), *Lycopersicon esculentum* (86% identity), *Populus alba* x *Populus tremula* (86% identity), *Catharanthus roseus* (85% identity), *Nicotiana tabacum* (85% identity), *Linum usitatissimum* (85% identity) for instance. From the phylogenetic tree, the CsDxr obtained from this study belongs to angiosperms group and exhibits closely relationship to *Hevea brasiliensis*. The results are in agreement as those plants are belonging to the family of Euphorbiaceae. All the analysis results strongly suggest that CsDxr is a plant Dxr protein involved in the IPP biosynthesis via the DXP pathway.

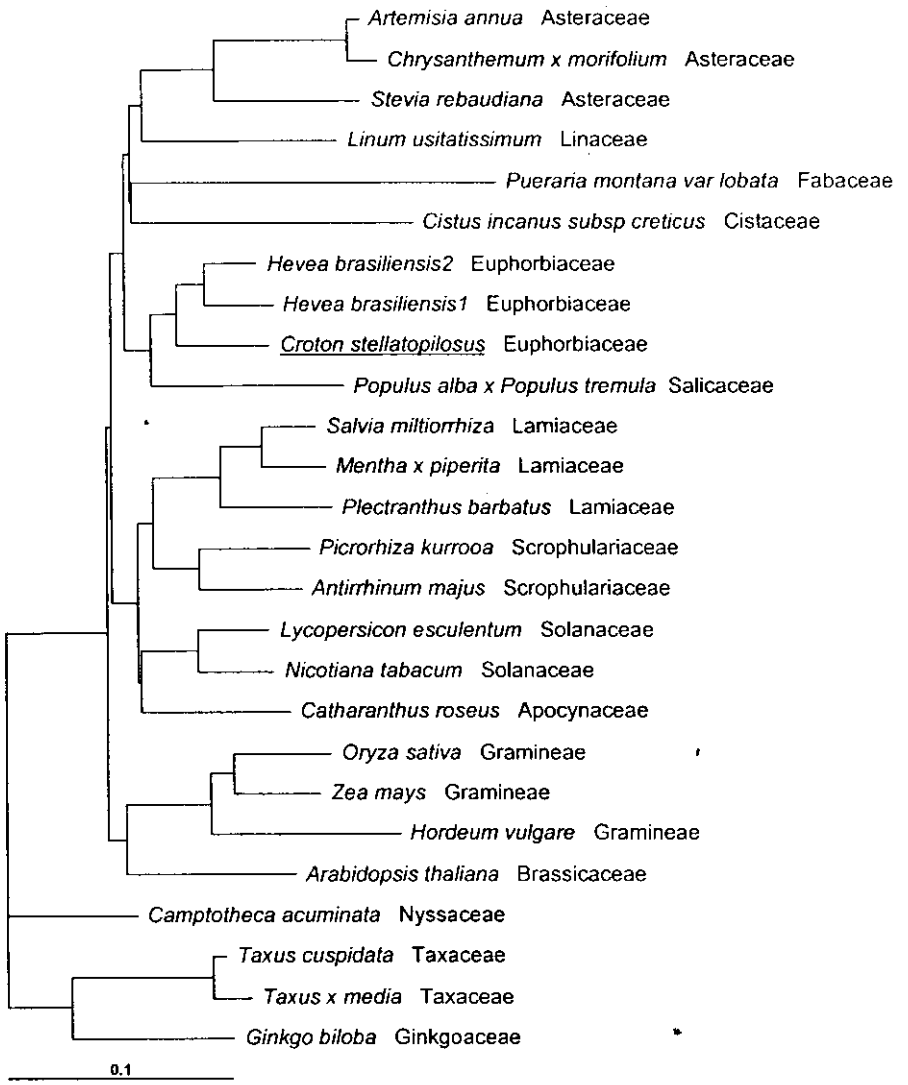

Figure 3.7 A molecular phylogenetic tree of the deduced amino acid sequences of the plant DXRs. The tree was constructed by CLUSTALW-XXL and viewed with Tree View version 1.6.6. The DXRs mentioned in the tree are including: *A. majus* (GenBank accession no. **AY770406**), *A. thaliana* (GenBank accession no. **NM125674**), *A. annua* (GenBank accession no. **AF182287**), *C. acuminata* (GenBank accession no. **DQ355159**), *C. roseus* (GenBank accession no. **AF250235**), *C. morifolium* (GenBank accession no. **AB205045**), *C. incanus*(GenBank accession no. **AY315651**), *C. stellatopilosus* (GenBank accession no. **EF451544**), *G. biloba* (GenBank accession no. **AY443101**), *H. brasiliensis1* (GenBank accession no. **DQ437514**), *H. brasiliensis2* (GenBank accession no. **AY502937**), *H. vulgare* (GenBank accession no. **AJ583446**), *L. usitatissimum* (GenBank accession no. **AJ623266**), *L. esculentum* (GenBank accession no. **AF331705**), *M. piperita* (GenBank accession no. **AF116825**), *N. tabacum* (GenBank accession no. **DQ839130**), *O. sativa* (GenBank accession no. **AK099702**), *P. kurrooa* (GenBank accession no. **DQ347963**), *P. barbatus* (GenBank accession no. **AY515699**), *P. alba x P. tremula* (GenBank accession no. **AJ574852**), *P. montana var. Lobata* (GenBank accession no. **AY315651**), *S. miltiorrhiza* (GenBank accession no. **DQ437514**), *S. rebaudiana* (GenBank accession no. **AY834755**), *T. cuspidata* (GenBank accession no. **AY575140**), *T. media* (GenBank accession no. **AY588482**) *and Z. Mays* (GenBank accession no. **AJ297566**).

3.4 Expression of the *dxr* gene in organs of *C. stellatopilosus*

3.4.1 Determination of cycling parameter for RT-PCR

The protocol of polymerase chain reaction for study on the expression profile was optimized in term of the cycling parameter. It is important to select the appropriate number of cycles so that the amplification product is clearly visible on an agarose gel and can be quantified by gel documentation. The total RNA of the young leave was used as material for the cycling number determination. The PCR reaction was performed using PDXRF-S2 for forward primer and 5'P_142A for reverse primer. The numbers of cycles were varied from 20, 25, 30, 35, 40, 45 and 50 cycles under the same thermal profile. The amplification products were analyzed by 1.2% agarose gel electrophoresis and the band intensity was measured as a relative intensity by densitometric analysis using gel documentation (2.3.8). The relationship between the relative intensity and number of cycles was plotted. The result showed that the intensity of the amplification product was related to the cycling number like sigmoid curve, which composed of initial phase (20-25 cycles), exponential phase (30-40 cycles) and plateau phase (45-50 cycles). The optimal cycling parameter should be the number that is in the exponential phase. Thus, the cycling number of 40 cycles was chosen for the investigation of the *dxr* gene expression profile in *C. stellatopilosus*.

3.4.2 Semiquantitative RT-PCR

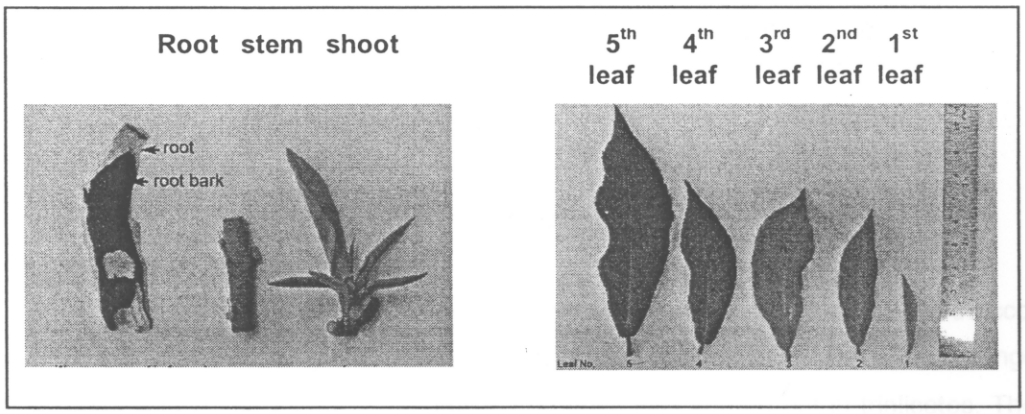

Figure 3.8 Various parts of *C. stellatopilosus*

Semiquantitative RT-PCR was carried out to investigate the expression profiles of *dxr* in various parts of *C. stellatopilosus* including shoot, 1st-5th leaf, stem and root (Fig. 3.8). Aliquots of 500 ng total RNA extracted from each sample was used as templates in two-steps RT-PCR reaction with the forward primer PDXRF-S2 and the reverse primer 5'P_142A specific to the coding sequence of part of full-length of *dxr*. The specific primers are PDXRF-S2 forward primer and 5'P_142A reverse primer were used to amplify 312 base pair fragment of *dxr* cDNA. For the

control gene, a house-keeping gene (18S rRNA) was amplified, 300 base pair fragment using 18s-0.5F for forward primer and 18s-0.5R for reverse primer.

All PCR products containing samples and the house keeping gene were separated by 1.2 % agarose gel electrophoresis and band intensity was measured by densitometric analysis using gel documentation. mRNA expression of *dxr* transcripts at various parts of *C. stellatopilosus* were evaluated by using the intensity ratio of the *dxr* band with 18S rRNA. The expression pattern analysis revealed that *dxr* mRNA were detected only in shoot and leaves with at similar expression levels, not in stem and root as shown in Table 3.3.

Table 3.3 The mRNA expression of *dxr* gene in various parts of *C. stellatopilosus*

sample	Intensity		Relative intensity (*dxr*/18S rRNA)
	dxr	18S rRNA	
1st leaf	10.80	13.20	0.82 ± 0.08
2nd leaf	10.90	13.30	0.82 ± 0.10
3rd leaf	10.60	13.00	0.82 ± 0.06
4th leaf	10.50	12.90	0.81 ± 0.09
5th leaf	11.00	12.90	0.85 ± 0.05
shoot	4.50	13.00	0.35 ± 0.04
stem	0.20	12.80	0.02 ± 0.00
root	0.10	12.80	0.01 ± 0.00

3.5 Quantitative determination of plaunotol

3.5.1 Calibration curve of plaunotol

The calibration curve of the authentic plaunotol was established according to the method 2.3.9. The linearity of the plaunotol calibration curve was observed in the range from 0.01-0.50 μg/μl with R^2 of 0.9984. Each calibration point was carried out in triplicates. The calibration curve is shown in Fig. 3.9. The detection limit was the concentration less than 0.4 μg.

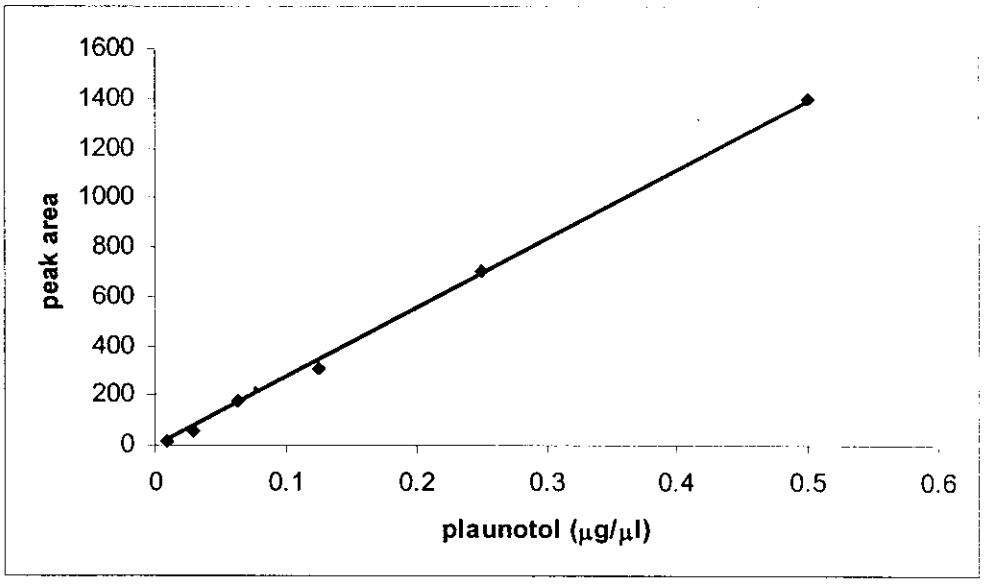

Figure 3.9 Calibration curve of plaunotol

3.5.2 Determination of plaunotol content in organs of *C. stellatopilosus*

The plant materials, which were used for total RNA extraction, were also used for determination of plaunotol content. The extraction of each sample was performed and plaunotol content was determined using gas chromatography (2.3.9). As shown in Table 3.4, the plaunotol was accumulated dominantly in plant leaves, especially the 1st-2nd leaves and declined in the 3rd-5th and stem. However, the plaunotol could not be detected in shoot and root under tested condition.

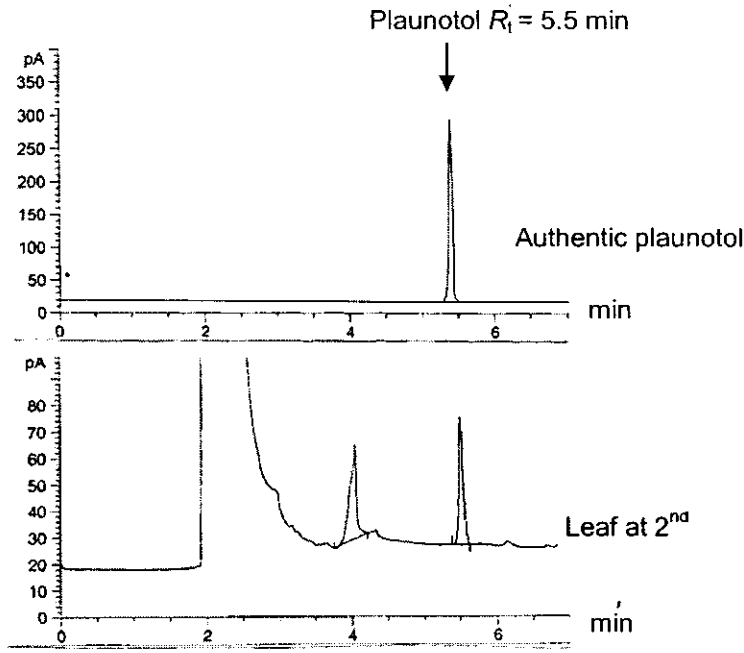

Figure 3.10 Example GC chromatograms of authentic plaunotol and at 2nd position leaf

Table 3.4 Plaunotol content from various parts of *C. stellatopilosus*

Sample	Weight (mg)	Plaunotol content (μg)	Plaunotol content (%w/w) ± SD (n = 3)
1st leaf	0.6	8.92	1.48 ± 0.08
2nd leaf	1.1	18.51	1.68 ± 0.16
3rd leaf	0.9	8.33	0.92 ± 0.17
4th leaf	5.8	4.33	0.07 ± 0.01
5th leaf	0.5	2.56	0.51 ± 0.04
shoot	5.7	0	n.d.
stem	1.7	4.01	0.24 ± 0.04
root	2.7	0	n.d.

n.d. = not determined (≤ 0.4 μg)

3.6 Correlation of *dxr* transcript levels and plaunotol biosynthesis

The correlation between the patterns of *dxr* expressions and plaunotol accumulation suggested the functional of the gene in the plaunotol biosynthesis. As shown in Fig. 3.13, the *dxr* gene transcripts were affected by the various organs in *C. stellatopilosus*. The results showed that *dxr* transcripts were detected in leaves, albeit low amount in shoot. The profile of the plaunotol accumulation was found to increase from 1st leaf to 2nd leaf reach to maximum and then declined gradually from 3rd -5th leaves. From this data, it can be concluded that the *dxr* gene was associated with the plaunotol production in leaves. However, the rarely *dxr* transcripts were found in stem and root, which was correlated to the absence or low amount of plaunotol content.

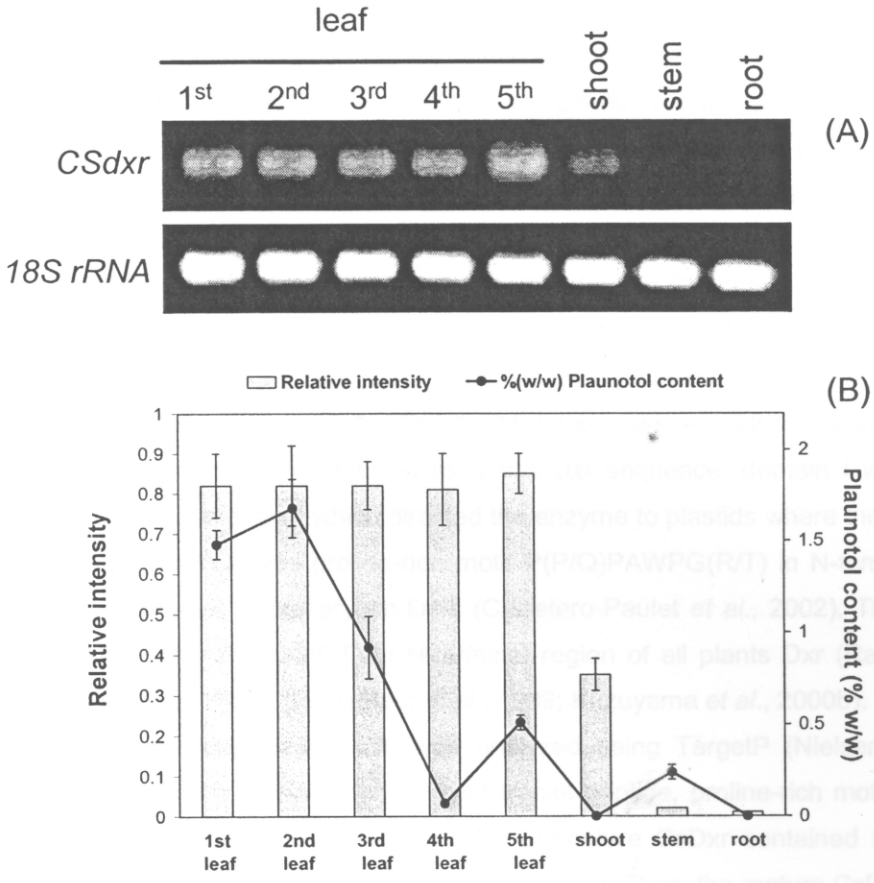

Figure 3.11 Semiquantitative RT-PCR analysis of *CSdxr* expression in various tissues of *Croton stellatopilosus*. (A). Pattern of *CSdxr* transcripts in comparison to *18S rRNA* as a loading control. (B).Correlation of relative intensity of each transcript and plaunotol content.