Cloning and sequence analysis of the *Blumea balsamifera* DC farnesyl diphosphate synthase gene

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ABSTRACT. *Blumea balsamifera* DC is a member of the Compositae family and is frequently used as traditional Chinese medicine. *Blumea balsamifera* is rich in monoterpenes, which possess a variety of pharmacological activities, such as antioxidant, anti-bacteria, and anti-viral activities. Farnesyl diphosphate synthase (FPS) is a key enzyme in the biosynthetic pathway of terpenes, playing an important regulatory role in plant growth, such as resistance and secondary metabolism. Based on the conserved oligo amino acid residues of published *FPS* genes from other higher plant species, a cDNA sequence, designated *BbFPS*, was isolated from *B. balsamifera* DC using polymerase chain reaction. The clones were an average of 1.6 kb and contained an open reading frame that predicted a polypeptide of 342 amino acids with 89.07% identity to FPS from other plants. The deduced amino acid sequence was dominated by hydrophobic regions and contained 2 highly conserved DDxxD motifs that are essential for proper functioning of FPS. Phylogenetic analysis indicated that *FPS* grouped with other

composite families. Prediction of secondary structure and subcellular localization suggested that alpha helices made up 70% of the amino acids of the sequence.

Key words: *Blumea balsamifera* DC; Farnesyl diphosphate synthase; Phylogenetic analysis; Secondary structure; Subcellular localization

INTRODUCTION

Blumea balsamifera DC is one of the most important medicinal plants in China and is chiefly distributed in southern China. It has long been used as a tea and as a cure for various disorders such as rheumatism and hypertension. The leaves possess various physiological activities, including plasmin-inhibitory, antifungal, and liver-protective effects (Norikura et al., 2008; Saewan et al., 2011). An infusion of the leaves is used as a stomachic, carminative, diaphoretic, expectorant, and emmenagogue. A decoction from fresh leaves alone or in combination with other plants is used as a bath for women after giving birth (Ruangrungsi et al., 1985). Several studies on the chemical constituents of *B. balsamifera* DC have been reported and a number of flavanoids have been isolated from this plant. The essential oil of *B. balsamifera* contains l-borneol, cineole, limonene, and palmitic and myristic acid, sesquiterpene alcohol, dimethyl ether, pyrocatechic tannin, glycoside, and levorotatory camphor (Ruangrungsi et al., 1981; Barua and Sharma, 1992; Fazilatun et al., 2001, 2004; Ali et al., 2005). Additionally, the flavonoids present in *B. balsamifera* DC leaves extracts are potent antioxidants (Fazilatun et al., 2004).

Farnesyl diphosphate synthase (FPS) is a key enzyme in the biosynthetic pathway of terpenes, playing an important regulatory role in plant growth, such as resistance and secondary metabolism. It catalyzes 2 consecutive condensations of isopentenyl diphosphate with allylic diphosphates, dimethylallyl diphosphate, and the resulting geranyl diphosphate into FPP, which is used in the biosynthesis of sterols, dolichols, mitochondrial electron transfer chain components, prenylated proteins, and a wide range of sesquiterpenoids including phytoalexins. Genes encoding FPPS have been cloned from many species, including rat (Clarke et al., 1987), human (Sheares et al., 1989), *Saccharomyces cerevisiae* (Anderson et al., 1989), *Arabidopsis thaliana* (Delourme et al., 1994), and *Lupinus albus* (Attucci et al., 1995).

In this study, based on similarity of cDNA sequences, homologous primers were designed according to the highly conserved region of Farnesyl diphosphate synthase (FPS) genes of other species, and then the full-length cDNA sequence of *FPS* was isolated from *B. balsamifera* DC using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). Sequence analyses were conducted using the DNAMAN, DNAStar, Mega, and BLAST (http://blast.be-md.ncbi.nlm.nih.gov/Blast.cgi) software.

MATERIAL AND METHODS

Plant materials

B. balsamifera DC was grown in a natural environment at the experimental fields of Tropical Crops Genetic Resources Institute. Young leaves were harvested, snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

RNA extraction and RT reactions

Total RNA was isolated from young leaves using the Plant RNA Extraction Kit (Tiangen, Beijing, China). RNA concentration was determined spectrophotometrically, and verified by ethidium bromide staining on an agarose gel. Total RNA was then treated with RNase-free DNaseI (TaKaRa, Shiga, Japan) to remove any contaminating genomic DNA, and approximately 3 µg RNA was used as a template for the first cDNA synthesis using TaKaRa reverse transcription reagents following manufacturer instructions; samples were stored at -20°C.

Isolation of partial cDNA clones

Clones were amplified using the cDNA described above as a template and PF1/PR1 primers designed from the FPS sequences conserved in other plant species. The primer sequences were as follows: PF1: 5'-CTTGGTTGGTGCATTGAATGG-3' and PR1: 5'-TAAAAT GAGT AGTAAGCAGT TTTG-3'. PCR was performed as follows: 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min, then 72°C for 7 min. One DNA fragment with the same expected size was generated, subcloned into the vector pMD19-T (Promega, Madison, WI, USA), and sequenced.

RACE

The 5'-ends of FPS cDNAs were amplified using the SMART and RACE methods with the 5'-RACE System (BD-Clontech, Palo Alto, CA, USA) according to manufacturer instructions with 2 different antisense primers: PR2 (CGGCGATGAATAGACAATG) and PR3 (CCAGAGGCTGTTTGGAACT), which were deduced from the 5'-region specific to each of the clones described above (Alam et al., 2010). Amplification of the 3'-end was performed using the 3'-RACE method as described by Schmidt et al. (2010). The primers used for 3'-RACE were the oligo (dT) 17 adaptor primer (for first-strand cDNA synthesis) and the forward primer PF2 (CCCTGTTGGTTTAGATTACCC) according to manufacturer instructions.

Based on the sequence information of the 5'- and 3'-ends, the sense/antisense primer pairs PF4 (ATGAGCAGCGATCTGAAGT) and PR6 (CTACTTTTGCCTCTTGTATA TCTTTC) were designed to amplify the open reading frame of FPS. PCR fragments were subcloned into the vector pMD19 -T (Promega) and transformed into *Escherichia coli* JM109 cells. The nucleotide sequence was determined by Invitrogen (Carlsbad, CA, USA).

Sequence analyses

The nucleotide sequence from our cDNA clone and the deduced amino acid (aa) sequence were identified using the National Center for Biotechnology Information BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignment was conducted using the DNAMAN software (version 4.0, Lynnon Biosoft, Quebec, Canada). A phylogenetic tree was constructed using the neighbor-joining algorithm, implemented in the MEGA4.0 software (Tamura et al., 2007). The cDNA sequence and deduced aa sequence were designated as BbFPS.

RESULTS AND DISCUSSION

Nucleotide sequence analysis

Using the degenerate primers PF1/PR1, a distinct cDNA fragment was amplified with an expected size of approximately 600 base pairs (bp), containing an open reading frame encoding a 200-aa protein. Alignment of the deduced aa sequences showed a high degree of identity between our cloned fragment and the central coding regions of known plant FPS sequences. The fragment was then used to design gene-specific primers to clone the full-length FPS sequence. A 500-bp fragment of 5' RACE and a 900-bp fragment of 3' RACE were amplified; both sequences were determined. BLAST searches of the deduced aa sequences revealed correspondence with known FPS genes. Sequence comparisons of the 5' and 3' ends with the central region of the gene indicated a perfect match between overlapping regions, suggesting that these sequences represent the missing regions of FPS genes. Based on this sequence data, specific primers were designed and the full-length cDNAs were amplified, cloned, and sequenced, revealing 100% identities to the expected FPS sequence. Our sequence analysis indicated that the full-length cloned cDNA (1597 bp) contained a 1029-bp open reading frame encoding 342 aa residues with an estimated molecular mass of 39.188 kDa (Figure 1).

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ORIGIN

   1    CTCTTACAAA CACACACTCA CACACACATT ACTCGTGTAT CTCGTTTTTC AGCGAAGGAC
  61    AGCTGCATCT TCTCCTTACA AATACCCACA CCCAATTCCT TCCCTCTCAA CACACACATT
 121    AACTGCACAC TATTGACCAA CAGATTTACC AGAATCTGAT ATTTTGCCCA TTTCACAACT
 181    CGCAAAATCA ACAATGAGCA GCGATCTGAA GTCCAGATTC CTCCAAGTTT ACGAGACTCT
 241    TAAATCAGAG TTAATCAACG ATCCGGCCTT CGAGTTCGAT GACGATTCTC GTCAATGGGT
 301    CGCGAAGATG CTTGACTACA ACGTACCTGG AGGAAAGCTG AATCGGGGCC TCTCTGTTGT
 361    TGATAGCTAC CAGTTGCTCA AAGGAGGAAA ATTGACCGAT GATGAAATTT TTCTTGCATC
 421    TGCCCTTGGT TGGTGCATTG AATGGCTTCA AGCATACTTT CTTGTGCTCG ATGATATCAT
 481    GGATGAGTCT CATACACGCA GAGGTCAACC CTGTTGGTTT AGATTACCCA AGGTTGGTAT
 541    GATTGCTGCA AATGATGGAA TAATCCTTCG TAACCATATC CCAAGAATTC TCAAGAAACA
 601    TTTCCGAGGA AAGCCATATT ATGTGGATCT AGTGGACCTG TTCAACGAGG TAGAGTTCCA
 661    AACAGCCTCT GGACAGATGA TTGATTTGAT CACTACTCTT GTCGGAGAGA AAGATCTCTC
 721    AAAATATTCA TTGTCTATTC ATCGCCGAAT TGTTCAGTAC AAAACTGCTT ACTACTCATT
 781    TTACCTTCCT GTTGCGTGTG CACTCCTTAT GTTCGGGGAG GATCTAGACA AGCATGTTGA
 841    AGTGAAGAAC GTACTCGTTG AAATGGGCAC CTATTTTCAA GTTCAGGATG ATTATCTAGA
 901    CTGTTTTGGT GCTCCGGAGG TGATTGGAAA GATAGGAACT GACATTGAAG ACTTTAAGTG
 961    CTCGTGGTTA GTTGTGAAAG CTTTGGAACT TGCCAATGAC GAACAAAAGA AAGTCCTACA
1021    TGAGAACTAT GGGAAAAAAG ATCCCTCCTC CGTTGCAAAA GTGAAGGAGC TATACAACAC
1081    TCTCAATCTT CAGGGTGTCT TCGAAGAGTA TGAGAACACG AGTTACAAGA AGCTGATCAC
1141    GTCCATTGAA GGTCACCCAA GCAAAGCAGT ACAAGCAGTG TTGAAATCTT TCTTAGGAAA
1201    GATATACAAG AGGCAAAAGT AGGTAATTTA TTGTGCCAAT TGATTCTGA TTTTGAGCCC
1261    CGAACGGAAA ATCCTTTGCA TTCTCTTACA AAGGAGGTGA TGTTTGTATC TTATTGTCAT
1321    TGTATTTTTG CATTCTTCCT AAGAATACAT TTAGTTATCT TTTTTAGCAT GTGTAATAAG
1381    ACTTCTACGT TGTTTGTAAT GTATCTCATT TTTTCAGTGT GACGGTGTAA GTGAGTGAAG
1441    GGATACCAAC CTCCTAAAAA CCGTCTTGTA GTTTTTAATT TCTTCCATAT GATGTATCTC
1501    CCTTTTTCCA CATTTGTTAT GATGCATGCA TTAATTTTGA AATTGGAAAC AGCCGAAAGA
1561    TGACTATAAG ATGTGGATAT GTAAAAATGA AATGGAG
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Figure 1. Nucleotide sequence of *BbFPS* gene from *Blumea balsamifera* DC. The open reading frame of the sequence is shaded in gray, and the initiation and termination codons are indicated in boxes.

Amino acid sequence analysis

Using the ProtParam of the ExPASy program (http://www.expasy.ch/), the *B. balsamifera* DC BbFPS protein was analyzed. The results showed that the BbFPS protein contained 342 aa, including 64 negatively charged residues and 41 positively charged residues. The molecular weight was determined to be 39.188 kDa. When the BbFPS aa sequence was compared with those of other plants (*Arabidopsis lyrata*, *Aralia elata*, *Centella asiatica*, *Citrus* x *microcarpa*, *Eleutherococcus senticosus*, *Glycyrrhiza uralensis*, *Helianthus annuus*, *Hevea brasiliensis*, and *Leibnitzia anandria*), no significant differences were found. Alignment of the deduced BbFPS aa sequence with other plant FPS polypeptides showed an identity of approximately 89.07% between the predicted amino acid sequences derived from the *B. balsamifera* DC BbFPS and other plants (*A. lyrata*, *A. elata*, *C. asiatica*, *C.* x *microcarpa*, *E. senticosus*, *G. uralensis*, *H. annuus*, *H. brasiliensis*, and *L. anandria*) (Figure 2).
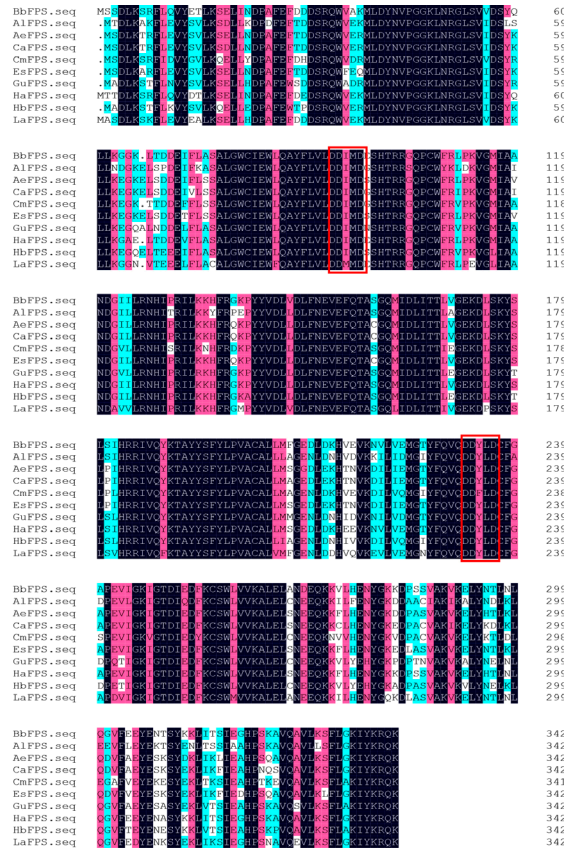

Figure 2. Alignment of predicted amino acid sequences of *Blumea balsamifera* DC *BbTPS* and other plant mono-TPS. Sequences used for the analysis were obtained from GenBank, the Latin name and accession numbers were as follows: AlFPS (*Arabidopsis lyrata* XP 002863368.1), AeFPS (*Aralia elata* ADK12004.1), Ca (*Centella asiatica* AAV58896.1), CmFPS (*Citrus x microcarpa* AAK68152.1), AeFPS (*Eleutherococcus senticosus* AEY77151.1), AeFPS (*Glycyrrhiza uralensis* ADE18770.1), HaFPS (*Helianthus annuus* AFW98437.1), HbFPS (*Hevea brasiliensis* AAM98379.1), and La (*Leibnitzia anandria* AFW98439.1). DDxxD motifs indicated in redbox.

The deduced aa sequence of BbFPS contained 2 common terpene synthase family motifs, including DDxxD (aspartate-rich motifs), which is found in nearly all terpene synthases from bacteria and plants and is a putative substrate binding site (Back and Chappell 1995; Bohlmann et al., 1998; Chen, 2007). The DDxxD motif is thought to be a binding site for the diphosphate moieties of the isopentenyl diphosphate and allylic substrates (Ohnuma et al., 1996). Our results are consistent with those of previous studies.

Evolutionary relationship of *BbFPS* gene

To determine phylogenetic relationships for the *B. balsamifera* DC BbFPS protein, the deduced aa sequence was aligned with those of other plant species and a neighbor-joining tree was constructed. Sequences were selected to cover most of the plant species available and to avoid redundancies. As shown in Figure 3, our results revealed that plant FPS proteins were classified into 3 major branches. The phylogenetic tree showed that plants from the same or similar families were clustered into 1 class, such as *Achillea asiatica*, *L. anandria*, *H. annuus*, *Matricaria chamomilla*, *Leucanthemum vulgare*, and *Chrysanthemum lavandulifolium*, which belong to the Asteraceae family. *B. balsamifera* DC BbFPS is also an Asteraceae plant. This indicated that while phylogenetic analysis of the plant FPS aa did not completely reflect the natural evolution of the relationship between plants, the results might be useful for determining genetic relationships between different plant species (Li and Wang, 2007).

Prediction of protein secondary and 3-D structures

Prediction of secondary structure of the deduced aa of BbFPS was conducted using the Lasergene software (DNASTAR, Inc., Madison, WI, USA) using the formula described by Garnier et al. (1978). Our results showed that the *B. balsamifera* DC BbFPS protein contained alpha helices, beta strands, beta-turns, and coil (Figure 4). Among these, alpha helices made up 70% of the aa sequence.

Analysis of the hydrophilic plot of our aa sequence showed that the hydrophobic areas were mainly in the 3 sections: the N-terminus and C-terminus of the sequence were mainly composed of hydrophilic plots, while in the middle of the BbFPS protein, hydrophilic and hydrophobic aa were alternating. This distribution type of hydrophilic/hydrophobic aa section is closely related to the function of enzyme (Peters et al., 2003; Hyatt et al., 2005, 2007).

To increase the understanding of the 3-D structure of *B. balsamifera* DC *BbFPS* genes, homology-based protein models were constructed using the 3-D crystal structure of the farnesyl diphosphate synthase FDS-5, chloroplastic-farnesyl diphosphate synthase 1 chimera with the Swiss-model online service (Benkert et al., 2011) (http://swissmodel.expasy.org/workspace/index.php). The structure of the enzyme was dominated by 29-helices. The aa of the DDXXD motif (aa indicated with a black dot in Figure 2) was in the area of the enzyme's functional domains, suggesting a functional role for these residues. The QMEAN4 composite score indicated that the prediction model was similar to the actual 3-D structure of BbFPS (Figure 5). The isolation of farnesyl diphosphate synthase gene lays a solid foundation for *Blumea balsamifera* terpenoid biosynthesis metabolism.
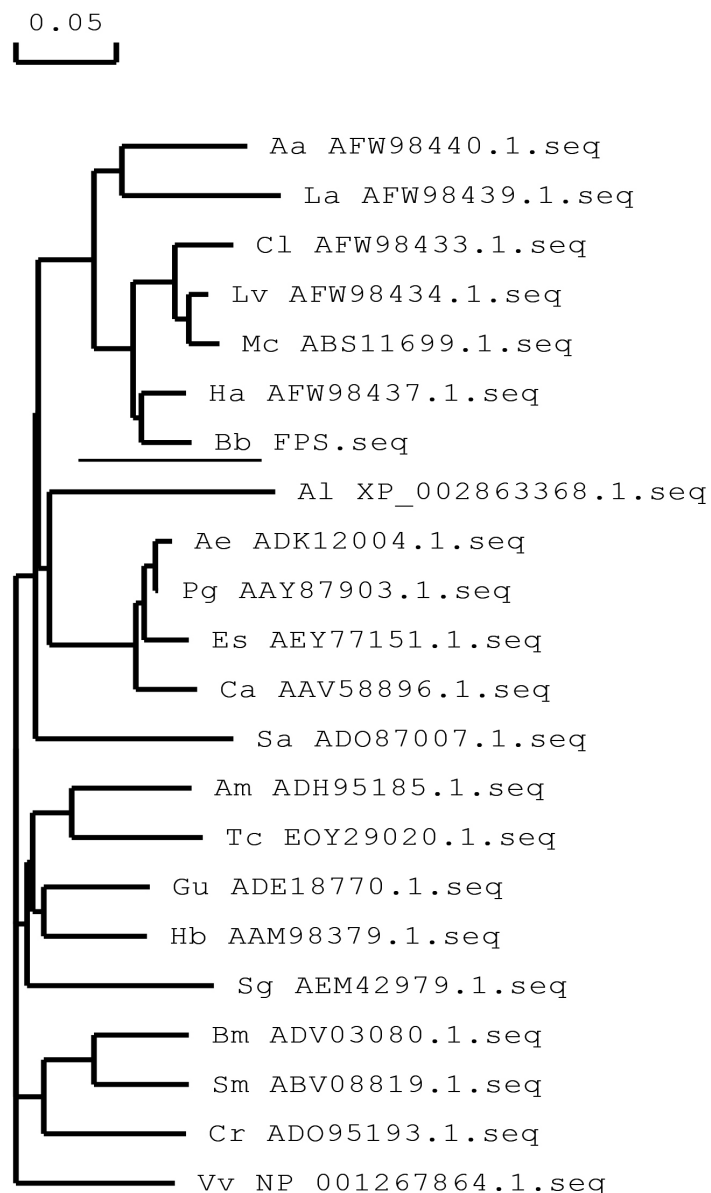
Figure 3. Phylogenetic tree of BbFPS protein from *Blumea balsamifera* DC and other plant species. Sequences used for the analysis were obtained from GenBank. The Latin name and accession number were as follows: *Achillea asiatica* (AFW98440.1), *Aquilaria microcarpa* (ADH95185.1), *Arabidopsis lyrata* (XP 002863368.1), *Aralia elata* (ADK12004.1), *Bacopa monnieri* (ADV03080.1), *Catharanthus roseus* (ADO95193.1), *Centella asiatica* (AAV58896.1), *Chrysanthemum lavandulifolium* (AFW98433.1), *Eleutherococcus senticosus* (AEY77151.1), *Glycyrrhiza uralensis* (ADE18770.1), *Helianthus annuus* (AFW98437.1), *Hevea brasiliensis* (AAM98379.1), *Leibnitzia anandria* (AFW98439.1), *Leucanthemum vulgare* (AFW98434.1), *Panax ginseng* (AAY87903.1), *Matricaria chamomilla* (ABS11699.1), *Salvia miltiorrhiza* (ABV08819.1), *Santalum album* (ADO87007.1), *Siraitia grosvenorii* (AEM42979.1), *Theobroma cacao* (EOY29020.1), and *Vitis vinifera* (NP 001267864.1).
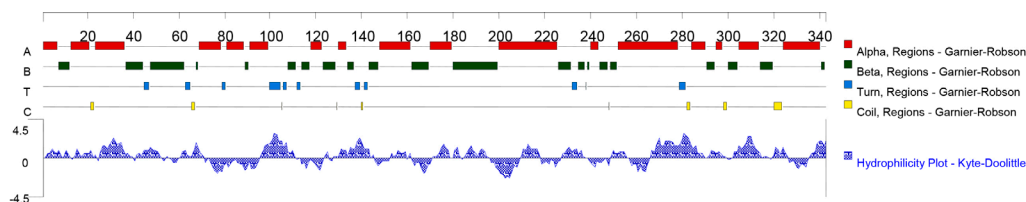
Figure 4. Prediction of secondary structure of *BbFPS* deduced amino acids sequence; barrel structures are noted above the amino acids sequence. A: alpha helix, B: beta strands, C: beta turns, D: coil.
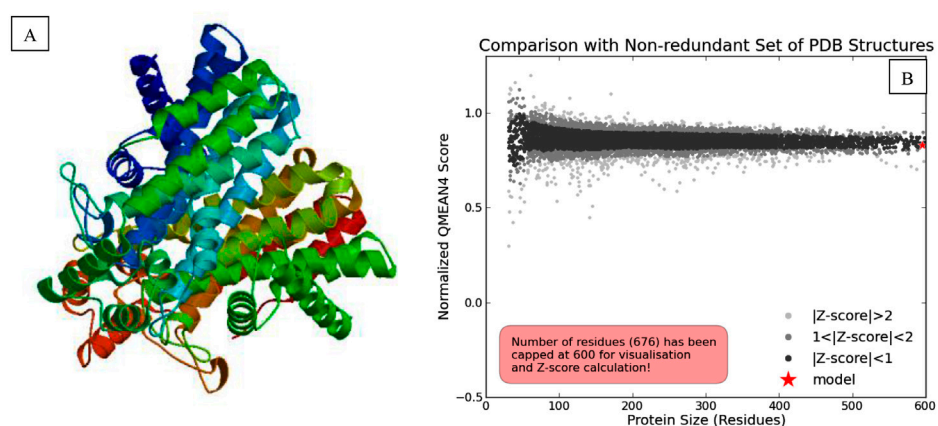

Figure 5. A. 3-D structure of *Blumea balsamifera* DCBbFPS, homology-based on the 3-D crystal structure of the farnesyl diphosphate synthase FDS-5, chloroplastic-farnesyl diphosphate synthase 1 chimera. **B.** QMEAN scores for this model.

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