A Letter

Casbene Synthase Gene Cloned from *Euphorbia fischeriana* Steud.

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Abstract Casbene synthase may play an important role in the biosynthesis of tigliane type terpenoid. Casbene synthase gene, named as EfCS (GenBank accession number JN862821), was cloned from the root of *Euphorbia fischeriana* Steud. using RACE-PCR method. The fragment was 1969 bp in length and contained an open reading frame coding a polypeptide of 602 amino acids, with a typical structure of terpenoid synthase gene. With biochemical analysis of the amino acid sequence coding CS gene, the results indicated that the theoretical isoelectric point of CS was 5.36 and the molecular weight was 69,364 kD. By hydrophobic analysis, we found the overall performance of CS was hydrophilic. The lead peptide analysis revealed that the protein tertiary structure prediction showed that EfCS, the casbene synthase obtained in this study, belonged to class I terpene synthase.

Keywords *Euphorbia fischeriana*; Casbene synthase

Background

In Euphorbiaceae plants, there are a wide variety of terpenoids, ranging from monoterpenic, sesquiterpenic, diterpenic to triterpenic. Many of these compounds have been investigated for their toxicity or their potential therapeutic activity (Cox, 1993; Barlow et al., 2005; Cragg and Newman, 2005; Li et al., 2009). Prostratin (12-deoxyphorbal-13-acetate), which belongs to tigliane type diterpenic, is a kind of phorbol esters. It was isolated by American National Cancer Institute (NCI) in 1992, and was confirmed with good resistance to AIDS virus (Gustafson et al., 1992). Therefore, tigliane biosynthesis pathway is a hot research topic in this field in recent year.

Casbene synthase (CS; EC 4.6.1.7), the key enzyme in the pathway of tigliane type diterpene biosynthesis, can produce intermediate product cembrane by catalyzed geranylgeranyl diphosphate (GGPP). Cembrane is then converted into casbene. Casbene is converted into tigliane through a series of electron transfer (Schmidt, 1987). Some CS genes were cloned from plants, such as *Ricinus communis*, *Triadica sebifera*, *Euphorbia esula*, *Euphorbia resinifera* and *Homalanthus nutans* (Kirby et al., 2010). Genome sequencing of *Jatropha curcas* was completed by S. Sato in 2011 and 9 CS genes were found in the *J. curcas* genome, one of the 9 CS genes was regarded as a pseudogene and the amino acid sequences of 6 CS genes were published from species (Sato et al., 2011). As far as we known, Shanghai Institute of Materia Medica had isolated prostratin from the root of *E. fischeriana* Steud (Liu et al., 1996; Ma et al., 1997; Wang et al., 2006), but the CS gene has not been cloned from this species.

In this study, we cloned a casbene synthase gene from *E. fischeriana* with RACE-PCR technique and analyzed this enzyme using bioinformatics methods.

1 Results and Analysis

1.1 CS gene fragment from *E. fischeriana*

A fragment of CS about 591 bp in length was obtained from the *E. fischeriana* transcriptome database.
1.2 Cloning and analysis of the full-length CS gene of E. fischeriana

The total RNA were isolated from the tuberous root of E. fischeriana and synthesized to cDNA. With the cDNA as template and a pair of specific primers RP1 and UPM, we carried out the 3’RACE PCR amplification. A fragment of CS about 1 201 bp in length was obtained (Figure 1). We also used specific primers LP1 and UPM for the first run 5’RACE PCR and then used its PCR product as the template with primers LP2 and NUP for the next run for the nested PCR, a 803 bp in length of PCR product was obtained (Figure 1). According to the sequence of these two fragments, we designed full-length primers (EfCS1–1 and EfCS2–1) for ORF amplification and obtained a 1 809 bp fragment after sequencing.

Figure 1 EfCS gene PCR products
Note: M: DNA Marker DL2000; 1: Amplification product of EfCS 3’ RACE; 2: Amplification product of EfCS 5’ RACE; 3: Amplification product of EfCS ORF

With the sequences of fragments that we got by RT-PCR and RACE-PCR, after combining, the full-length sequence of CS gene 1 969 bp was obtained, named as EfCS (GenBank accession No. JN862821). ORF Finder analysis (NCBI ORF finder website) indicated that the EfCS full-length sequence contained a 1 809 bp open reading frame, a 38 bp 5’ untranslated region and a 122 base pair 3’ untranslated region, encoding a protein with 602 amino acids.

1.3 Bioinformatics analysis

Using ProtParam online tools (Gasteiger et al., 2003), we analyzed the amino acid sequence coding by CS gene. We found the theoretical isoelectric point of CS was 5.36 and the molecular weight was 69.364 kD. ProtScal (Kyce and Doolittle, 1982) was common used to analyze protein hydrophobicity. With setting parameters as default, the ProtScal analysis results showed that the maximum value of hydrophobicity was 2.978 (V347), and the minimum value was -3.067 (N273). The overall performance of EfCS is hydrophobicity. Choosing online tool TargetP 1.1 Server (Emanuelsson et al., 2000) in plant version and with default setting parameters, we analyzed the EfCS amino acid sequence, and the result showed that the protein had chloroplasts guide peptide and the reliable level was II. Predicting the second structure of CS by SOPMA (Geourjon and Deleage, 1995) suggested that the protein was mainly composed by 25 α-helices with 391 amino acids, 13 extended chains with 40 amino acids, 10 β-turns with 40 amino acids and 28 random coils with 192 amino acids, which accounted for 64.95%, 6.64%, 3.16% and 25.25% respectively. Using SMART (Schultz et al., 1998) to analyze the protein, we found that this gene had two function domains Terpene_synth and Terpene_synth_C which were specific to terpene synthase, locating at the 74 to 249 amino acids and the 279 to 547 amino acids respectively. Using an automated homology modeling program of ESyPred3D Web Server 1.0 (Lambert et al., 2002) and setting parameters as neural net and new screening, tertiary structure prediction was performed. As shown in figure 2, we got tertiary structure of CS protein. The result showed that our EfCS belonged to class I terpene synthase (α domain, blue) (Cao et al., 2010), with two metal ion binding regions DDXXD and (N,D)DXX(S,T)XXXE (red and orange, respectively) (Köksal et al., 2011). At the same time, there was a small retrogressive region (β domain, green), that always played a role in class II terpene synthase (Wendt et al., 1997). The purple part was the first α-helix, which worked in class I terpene synthase to cap the active center of enzyme (Köksal et al., 2011).

Figure 2 Predicted three-dimensional structure of EfCS
Note: Blue: α domain; Red: metal-binding motif DDXXD; Orange: metal-binding motif (N, D) DXX (S, T) XXXE; Green: β domain; Purple: the first α helix
2 Discussion

Casbine synthase is a key enzyme in the tigliane biosynthesis pathway in all probability. In this report, CS gene was isolated from E. fischeriana using RACE-PCR. By bioinformatics analysis, we found CS was an acidic protein, with theoretical isoelectric point of 5.36, and the overall performance of CS was hydrophilic. These results would provide useful information for further study of CS. Because of the substrate for CS mainly locate in chloroplastid, the lead peptide for transporting to chloroplastid is a key domain for its appropriate function (Kirby et al., 2010). The prediction showed that CS had chloroplastid lead peptide, supported by the result of ChloroP 1.1 analysis. Secondary structure and tertiary structure prediction showed that EiCS belonged to class 1 terpene synthase, providing evidence for testing its activity and analyzing its function in vitro.

There are many compounds have been isolated from E. fischeriana, a lot of them have insecticide, antiseptic and anticancer activity (Liu et al., 1988; Liu et al., 2000; Zhao et al., 2000; Ma et al., 2005, China Science and Technology Information, 12: 65,89). Prostratin is not enough for its commercial production. Therefore, as a candidate enzyme in the pathway of prostratin biosynthesis, isolation of casbine synthase gene is an important step towards improving the yield of prostratin in E. fischeriana.

3 Materials and Methods

3.1 Plant material

The tuberous root of E. fischeriana was collected from Jiagedaqi in Heilongjiang Province. DH5α cells (Escherichia coli) were bought from TAKARA Inc.

3.2 Extraction of total RNA and synthesis of cDNA

Following the manual instructions, total RNA was extracted by RNeasy Mini Kit (QIAGEN, Germany). Using SMARTer™ RACE cDNA Amplification Kit (Clontech, Dalian, China), we synthesized 5′ and 3′ RACE Ready cDNA according to the protocol provided by Clontech.

3.3 Cloning and sequencing of target gene

According to the EST fragment from the transcriptome database of E. fischeriana, a specific primer RP1 (5′-ACATAACCAATGCCCTGGAACAACCT-3′) was designed. 3′ RACE PCR was performed by using RP1 and UPM (5′-CTAATACGACTCACTATAGGGCAA GCAGGTGTATCAACCGAGTGTG-3′). UPM primer was provided by the SMARTer™ RACE cDNA Amplification Kit. The PCR condition is as follow: 94°C 5 min; 94°C 1 min, 55°C 1 min, 72°C 1 min, 35 cycles; 72°C 10 min. Using specific primer LP1 (5′-CATGAATCCAGGTGCTGACCAGAGG-3′) and UPM, the first round of 5′ RACE PCR was performed. The PCR condition is as follow: 94°C 3 min, 94°C 1 min, 55°C 1 min, 72°C 1 min, 35 cycles; 72°C 10 min. Diluting the first round PCR products for 100 times as template, we performed the second round amplification with specific primers pairs of LP2 (5′-CCATAGTAGGGAAGTTTGCTCCAGGGCA-3′) and NUP (5′-CTAATACGACTCACTATAGGGC-3′). The condition is reported as follow: 94°C 3 minutes; 94°C 1 minute, 55°C 1 minute, 72°C 1 min, 35 cycles; 72°C 10 min. Target fragments were cloned to pMD20-T respectively (TAKARA, Dalian, China) according to the manual of the kit. Gene sequencing was completed by Beijing Genomics Institute. According to the full-length sequence obtained from 3′-RACE and 5′-RACE, we designed two specific primers EFCS1-1 (5′-ATGGCATGCAACCAGCAGC-3′) and EFCS2-1 (5′-TAAAGTAAATAGGATCAAC GAAC-3′) to clone the full-length ORF and the full-length PCR products was ligated to pMD20-T vector for sequencing.

3.4 Sequence analysis of target gene

The target gene sequence was analyzed by online bioinformatics soft wares (Table 1). The analysis of CS open reading frame was worked out by NCBI-ORF Finder. The composition and physicochemical property of amino acids sequence was analyzed by ProtParam online tools. The prediction of lead peptide of protein was completed by TargetP 1.1 and ChloroP 1.1. Second structure and function domain were predicted by SMART and SOPMA, respectively. Protein tertiary structure prediction was completed by ESyPred3D online tool.
Table 1 Websites of the bioinformatics software online tools

| Tool name      | Website                                                                 |
|----------------|-------------------------------------------------------------------------|
| NCBI-ORF Finder | http://www.Ncbi.nlm.nih.gov/gorf/gorf.html                              |
| ProtParam      | http://www.expasy.ch/tools/protparam.html                               |
| TargetP 1.1    | http://www.cbs.dtu.dk/services/TargetP/                                  |
| ChloroP 1.1    | http://www.cbs.dtu.dk/services/ChloroP/                                  |
| ProtScale      | http://www.expasy.ch/tools/protscale.html                               |
| SMART          | http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1            |
| SOPMA          | http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html|
| ESyPred3D      | http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/          |

Authors' Contributions

HWL performed the experiment; HWL and YFY completed the data analysis and drafted the manuscript; QT took part in the performance of RACE; NZ took part in the extraction of RNA; DYQ conceived and took charge the overall study. All authors have read and approved the final manuscript.

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References

Barlow A.J., Compton B.J., Hertwich U., Lorimer S.D., and Weavers R.T., 2005, Sesquiterpenes from the New Zealand liverwort Lepidolaena hodgesonian. Journal of Natural Products, 68(6): 825-831 http://dx.doi.org/10.1021/jp050058x PMid:15974603
Cao R., Zhang Y.H., Mann F.M., Huang C.C., Mukkamala D., Hudock M.P., Mead M.E., Prisc S., Wang K., Lin E.Y., Chang T.K., Peters R.J., and Oldfield E., 2010, Diterpene cyclases and the nature of the isoprene fold, Proteins: Struct. Funct. Bioinf., 78(11): 2417-2432 http://dx.doi.org/10.1002/prot.22751 PMid:20602361
Cox P.A., 1993, Saving the ethnopharmacological heritage of Samoa, Journal of Ethnopharmacology, 38(2-3): 181-188 http://dx.doi.org/10.1016/0378-8741(93)90014-V
Cragg G.M., and Newman D.J., 2005, Biodiversity: A continuing source of novel drug leads, Pure and Applied Chemistry, 77(1): 7-24 http://dx.doi.org/10.1351/pac200577010007
Emanuelsson O., Nielsen H., Brunak S., and Hejne G.V., 2000, Predicting subcellular localization of proteins based on their N-terminal smino acid sequence, Journal of Molecular Biology, 304(4): 1005-1016 http://dx.doi.org/10.1006/jmbi.2000.3903 PMid:10819285
Gasteiger E., Gattiker A., Hoogland C., Ivanyi I., Appel R.D., and Bairoch A., 2003, ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Research, 31(13): 3784-3788 http://dx.doi.org/10.1093/nar/gkq563 PMid:12824418 PMid:168970
Geourjon C., and Deleage G., 1995, SOPMA: significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments, Bioinformatics, 11(6): 681-684 http://dx.doi.org/10.1093/bioinformatics/11.6.681
Gustafsson K.R., Cardellina J.H., McMahon J.B., Gulakowski R.J., Ishtooya J., Szallasi Z., Lewin N.E., Blumberg PM., Weislow O.S., Beutler J.A., Buckheit R.W.J., Cragg GM., Cox P.A., Bader J.P., and Boyd M.R., 1992, Non-promoting phorbol from the Samoan medicinal plant, Homalanthus nutans, inhibits cell killing by HIV-1, Journal of Medicinal Chemistry, 35(11): 1978-1986 http://dx.doi.org/10.1021/jm00089a006 PMid:15978583
Kirby J., Nishimoto M., Park J.G., Withers S.T., Nowroozi F., Behrendt D., Garcia E.J., Fortman J.L., Johnson H.E., Anderson J.V., and Keasing J.D., 2010, Cloning of casbene and neocobrene synthases from Euphorbiaceae plants and expression in Saccharomyces cerevisiae, Phytochemistry, 71(13): 1466-1473 http://dx.doi.org/10.1016/j.phytochem.2010.06.001 PMid:20594566
Koksal M., Jin Y., Coates R.M., Croteau R., and Christianson D.W., 2011, Taxadiene synthase structure and evolution of modular architecture in terpenic biosynthesis, Nature, 469(6): 116-120 http://dx.doi.org/10.1038/nature09628 PMid:21160477 PMid:3059769
Kye J., and Doolittle R.F., 1982, A simple method for displaying the hydropathic character of a protein, Journal of Molecular Biology, 157(1): 105-132 http://dx.doi.org/10.1016/0022-2836(82)90515-0
Lambert C., Leonard N., Bolli X.D., and Depiereux E., 2002, ESyPred3D: Prediction of proteins 3D structures, Bioinformatics, 18(9): 1250-1256 http://dx.doi.org/10.1093/bioinformatics/18.9.1250 PMid:12217917
Li W., Fu H., Bai H., Sasaki T., Kato H., and Koike K., 2009, Triterpenoid saponins from Rhus elliptic var. obcordatus, Journal of Natural Products, 72(10): 1755-1760 http://dx.doi.org/10.1021/np900237a PMid:19795885
Liu G.F., Fui Y.Q., Yang Z.Q., Zhao H.Q., and Fan M.X., 1988, Isolation and identification of antitumor constituents of diterpenoids lectone in Euphorbia fischeriana, Chinese Journal of Chinese Materia Medica, 13(5): 35-36
Liu W.Z., He F.L., Ruan Z.Y., Gu X.F., Wu X.Y., and Qin G.W., 2000, Studies on Euphorbia fischeriana diterpenoid lactones inhibitory effect on human tumor cells in vitro, Journal of Chinese Medicinal Materials, 23(10): 623-625
Liu W.Z., Wu X.Y., Yang G.J., Ma Q.G., Zhou T.X., and Qin G.W., 1996, 12-Deoxyphorbol esters from Euphorbia fischeriana, Chinese Chemical Lett., 7(10): 917-918
Ma Q., Liu W.Z., Wu X.Y., Zhou T.X., and Qin G.W., 1997, Diterpenoids from Euphorbia fischeriana, Phytochemistry, 44(4): 663-666 http://dx.doi.org/10.1016/S0031-133X(00)00605-X
Sato S., Hirakawa H., Isobe S., Fukai E., Watanabe A., Kato M., Kawashima...
K. Minami C., Muraki A., Nakazaki N., Takahashi C., Nakayama S., Kishida Y., Kohara M., Yamada M., Tsuruoka H., Sasamoto S., Tabata S., Aizu A., Toyoda A., Shini T., Minakuchi Y., Kohara Y., Fujiyama A., Tsueimoto S., Kajiyama S., Makigano E., Ohmido N., Shibagaki N., Cartagena J.A., Wada N., Kohinata T., Atefhe A., Yuasa S., Matsuoka S., and Fukui K., 2011, Sequence analysis of the genome of an oil-bearing tree: Jatropha curcas L., DNA Res., 18(1): 65-76 http://dx.doi.org/10.1093/dnares/dsq030 PMid:21149391 PMCid:3041505

Schmidt R.J., 1987, The biosynthesis of tigliane and related diterpenoids: an intriguing problem, Botanical Journal of the Linnean Society, 94(1-2): 221-230 http://dx.doi.org/10.1111/j.1095-8339.1987.tb01047.x

Schultz J., Milpetz F., Bork P., and Ponting C.P., 1998, SMART, a simple modular architecture research tool: identification of signaling domains, Proc. Natl. Acad. Sci., USA, 95(11): 5857-5864 http://dx.doi.org/10.1073/pnas.95.11.5857

Wang Y.B., Huang R., Wang H.B., Jin H.Z., Lou L.G., and Qin G.W., 2006, Diterpenoids from the roots of Euphorbia fischeriana, Journal of Natural Products, 69(6): 967-970 http://dx.doi.org/10.1021/np0600088 PMid:16792421

Wendt K.U., Poralla K., and Schulz G.E., 1997, Structure and function of a squalene cyclase, Science, 277(19): 1811-1815 http://dx.doi.org/10.1126/science.277.5333.1811 PMid:9295270

Zhao K.J., Yang J., and Zhang P.Z., 2000, Comparative observation on the inhibitory effects against tuberculous bacillus of 5 extracts of the roots of Euphorbia fischeriana, Lishizhen Medicine and Materia Medica Research, 11(7): 589-590