Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 67 (2006) 2517–2524
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Molecular cloning and functional expression of a multifunctional triterpene synthase cDNA from a mangrove species *Kandelia candel* (L.) Druce ☆

Mohammad Basyuni [a], Hirosuke Oku [b,*], Masashi Inafuku [a], Shigeyuki Baba [c], Hironori Iwasaki [b], Keichiro Oshiro [d], Takafumi Okabe [d], Masaaki Shibuya [e], Yutaka Ebizuka [e]

[a] *United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24, Korimoto, Kagoshima 890-0065, Japan*
[b] *Division of Molecular Biotechnology, Center of Molecular Bioscience, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan*
[c] *Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan*
[d] *Graduate School of Agriculture, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan*
[e] *Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo Bunkyo-ku, Tokyo 113-0033, Japan*

Received 22 June 2006; received in revised form 4 September 2006

Abstract

Homology based PCRs with degenerate primers designed from the conserved sequences among the known oxidosqualene cylases (OSCs) have resulted in cloning of a triterpene synthase (*KcMS*) from the young roots of *Kandelia candel* (L.) Druce (Rhizophoraceae). *KcMS* consists of a 2286 bp open reading frame, which codes for 761 amino acids. The deduced amino acid sequence showed 79% homology to a lupeol synthase from *Ricinus communis* suggesting it to be a lupeol synthase of *K. candel*. *KcMS* was expressed in a lanosterol synthase deficient yeast with the expression vector pYES2 under the control of *GAL1* promoter. GC–MS analysis showed that the transformant accumulated a mixture of lupeol, β-amyrin and α-amyrin in a 2:1:1 ratio, indicating that *KcMS* encodes a multifunctional triterpene synthase, although it showed high sequence homology to a *R. communis* lupeol synthase. This is the first OSC cloning from mangrove tree species.

Keywords: *Kandelia candel* (L.) druce; Rhizophoraceae; Mangrove; Oxidosqualene cyclase; Multifunctional triterpene synthase; Lupeol; β-Amyrin; α-Amyrin

1. Introduction

Mangrove plants are distributed in intertidal tropical and subtropical regions and are rich sources of pentacyclic triterpenoids (Wannigama et al., 1981; Hogg and Gillan, 1984; Ghosh et al., 1985; Koch et al., 2003). Several biological activities of mangrove triterpenoids have been reported. For example, an extract of *Rhizophora apiculata* has been used as a folklore medicine in Thailand and its biologically active compound has been identified as a pentacyclic triterpenoid (Kokpol et al., 1990). Triterpenoids from *Acanthus illicifolius* have also been reported to show antileukemic activity (Kokpol et al., 1986). However, the physiological functions, if any, in mangrove plants have been rarely reported.

Biosynthesis of triterpene branches away from that of the phytosterols at the cyclization step of 2,3-oxidosqualene (**1**), with the latter being catalyzed by oxidosqualene cyclases (OSCs) (Abe et al., 1993). Diverse skeleta of triterpenes such as lupane (lupeol) (**2**), oleanane (β-amyrin) (**3**)

☆ *Note*: The nucleotide sequence reported in this paper appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Databases under the accession number AB257507 (*KcMS*).

* Corresponding author. Tel./fax: +81 98 895 8972.
E-mail address: okuhiros@comb.u-ryukyu.ac.jp (H. Oku).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.09.016

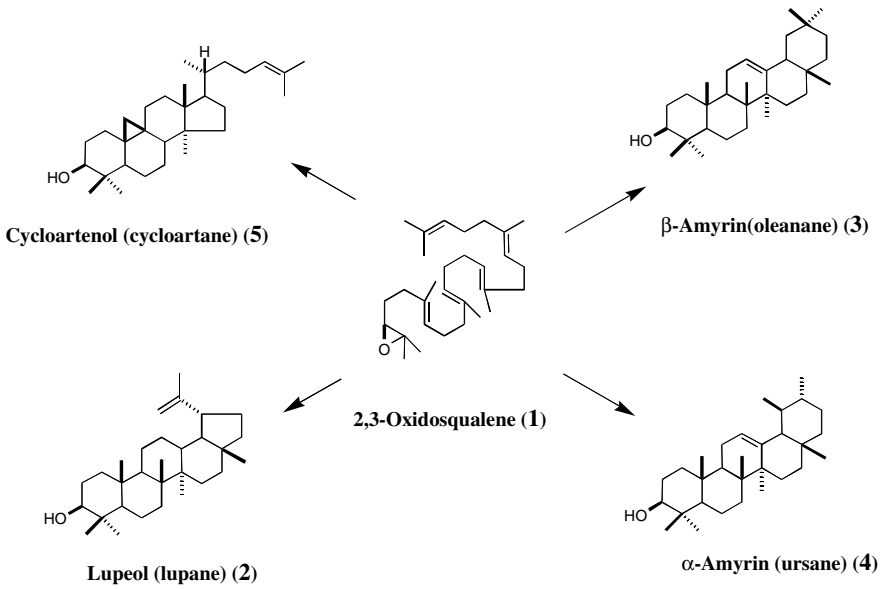

Fig. 1. Cyclization of 2,3-oxidosqualene (**1**) to lupeol (**2**), β-amyrin (**3**), α-amyrin (**4**) and cycloartenol (**5**).

and ursane (α-amyrin) (**4**) are constructed by OSCs (Fig. 1). A number of OSCs have been cloned and their enzyme functions identified by expression in lanosterol synthase deficient yeast (Xiang et al., 2006, and cited therein). These studies revealed the presence of two types of OSCs in higher plants. The first type are monofunctional synthases yielding one specific product, as exemplified by β-amyrin synthases (Hayashi et al., 2004; Iturbe-Ormaetxe et al., 2003; Kajikawa et al., 2005; Kushiro et al., 1998; Morita et al., 2000; Sawai et al., 2006; Zhang et al., 2003), lupeol synthases (Guhling et al., 2006; Hayashi et al., 2004; Shibuya et al., 1999; Zhang et al., 2003), isomultiflorenol synthase (Hayashi et al., 2001), cucurbitadienol synthase (Shibuya et al., 2004), thalianol synthase (Fazio et al., 2004), marneral synthase (Xiong et al., 2006) and arabidiol synthase (Xiang et al., 2006). The second are multifunctional synthases producing more than one product, that include a multifunctional lupeol synthase (LUP1) (Segura et al., 2000) and another multifunctional OSC yielding more than nine products (Kushiro et al., 2000) from *Arabidopsis thaliana*, as well as a mixed amyrin synthase from *Pisum sativum* (Morita et al., 2000), etc. It is noteworthy that more than one hundred triterpene skeleta are elaborated at the cyclization step of **1** by contribution of both mono- and multi-functional triterpene synthases.

Despite the ubiquitous distribution of triterpenes in the plant kingdom, their physiological functions in the producing plants are not well understood, especially for those from mangrove species. Triterpenoids from *R. mangle* may be functioning as chemical defense substances as they show insecticidal activity (Williams, 1999), and our recent study provided a view that mangrove triterpenes may play a role in the defense system against salt stress (Oku et al., 2003). In this context, OSCs are likely to take part in defense mechanisms, as they are the first committed enzymes to triterpene biosynthesis. So far, no information on any OSCs from mangrove species has been reported. In order to obtain more insight into the physiological function of triterpenes in mangrove plants, the present study therefore has undertaken to clone OSC cDNA from *Kandelia candel* (Rhizophoraceae). This species is widely distributed in the Okinawan islands, Japan and produces a large amount of triterpenes (Majumdar and Patra, 1976; Oku et al., 2003). Herein we report the first example of a multifunctional OSC from mangrove tree species.

2. Results and discussion

2.1. Cloning of OSC cDNA from K. candel

In order to clone a *K. candel* triterpene synthase, PCRs were performed using degenerate primers designed from the highly conserved regions of known OSCs as described previously (Kushiro et al., 1998). The amplified DNA fragment (445 bp in length) was cloned into a TOPO 10 vector (Invitrogen). More than three clones were sequenced and all showed an identical sequence. 3′-RACE and 5′-RACE (Frohman et al., 1988) were performed to clone 3′- and 5′-ends of the obtained clone with GeneRacer™ Kit (Invitrogen) and yielded a full-length sequence of the cDNA, which was named *KcMS*.

The ORF of *KcMS* consists of 2286 bp that encodes a 761 amino acid polypeptide, which included five QW motives (Poralla et al., 1994) and a DCTAE motif (Abe and Prestwich, 1994). The deduced amino acid sequence of *KcMS* showed significant homology to known triterpene synthases. Among them, the highest homology of 79% was shown with a *Ricinus communis* lupeol synthase (Guhling et al., 2006). These results suggested that *KcMS* encodes triterpene synthase, most probably lupeol synthase.

2.2. Functional expression of *KcMS* in yeast

In order to investigate the function of the *KcMS* clone, functional expression in yeast was undertaken. *KcMS* cDNA was ligated to a yeast expression vector pYES2 (Invitrogen), and expressed under control of the GAL1 promoter in a lanosterol synthase deficient yeast mutant GIL77 that accumulates oxidosqualene inside cells (Kushiro et al., 1998). Introduction of *KcMS* gene into GIL77 resulted in the production of new compounds with the same mobility as **3** on thin layer chromatography (TLC) (Fig. 2). However, its amount was smaller than that of the known OSCs such as β-amyrin synthase from *Panax ginseng* (Kushiro et al., 1998) (data not shown). Prolonged culturing slightly increased the band intensity but not to a great extent.

In order to identify the chemical structures, products were analyzed by gas chromatography (GC) and by gas chromatography/mass spectrometry (GC–MS) as described in Section 4.

As shown in Fig. 3, three product peaks 1, 2 and 3, in an 1:2:1 intensity were detected by GC and identified as **3**, **2** and **4**, respectively, by comparing their Rts and mass fragmentation patterns using GC–MS with those of authentic standards. These results established that *KcMS* encodes a multifunctional triterpene synthase. Although *KcMS* shares high sequence homology (79%) with *R. communis* lupeol synthase, its product specificity is leaky as it produces significant amounts of **3** and **4** in addition to the major product **2**. So far, six multifunctional triterpene synthases have been identified (LUP1(At1g78970), Herrera et al., 1998; PSM, Morita et al., 2000; At1g78960, Kushiro et al., 2000; LjAMY2, Iturbe-Ormaetxe et al., 2003; At1g66960, Ebizuka et al., 2003; At1g78500, Ebizuka et al., 2003) as mentioned above and some produce **2** as the major product. Their product patterns, however, are not identical and differ from each other. *KcMS* is thus a new type of multifunctional OSCs with unique product specificity.

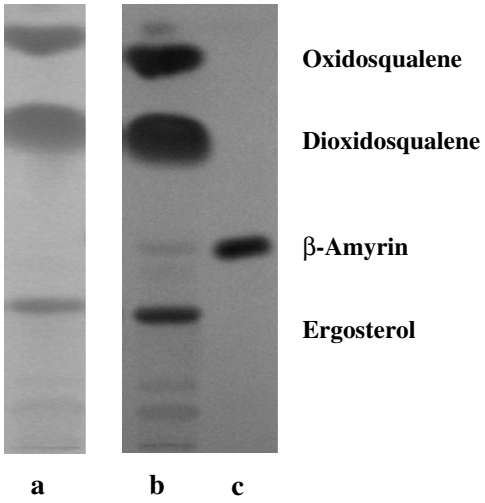

Fig. 2. TLC analysis of hexane extracts from transformed GIL77. TLC was developed with benzene/acetone (19:1). To visualize the chromatograms, the plates were immersed in phosphoric acid/33% acetic acid/sulfuric acid/0.5% copper sulfate (5:5:0.5:90, by vol) for 10 s, and heated at 140 °C for 15 min (Brod et al., 1991). (a) empty vector (pYES2) as control; (b) lipid profile of extract from transformants (pYES2-*KcMS*) incubated 72 h after induction; (c) **3** standard.

2.3. Phylogenetic analysis of *KcMS*

To analyze the phylogenetic relationship of *KcMS* among plant OSCs, a phylogenetic tree was constructed (Fig. 4). Of these, cycloartenol synthases (CAS), lupeol synthases (LUS) and β-amyrin synthases (βAS) clones together. Based on the relationship between the distance in the tree and the reaction mechanism, a sequential evolution of lupeol synthase and β-amyrin synthase from ancestral cycloartenol synthase has been proposed (Shibuya et al., 1999; Wu and Griffin, 2002; Zhang et al., 2003). On the other hand, multifunctional synthases do not form a branch but scatter in the tree and are proposed to be at an evolutionally transient state from one product specific OSC to another OSC. Recently, a new product specific lupeol synthase, which does not join the lupeol synthase branch but is instead located close to the β-amyrin branch has been cloned from *R. communis*. *KcMS* shares highest homology with this clone, and thus might be an evolving clone from this new lupeol synthase to β-amyrin synthase.

2.4. Mangrove triterpenes

Our previous study demonstrated the presence of triterpenes (**2**, **3** and **4**) as fatty acid esters in the roots and leaves of *K. candel* (Oku et al., 2003). The product pattern of *KcMS* in the transformed yeast is almost identical to that of the triterpenes isolated from this plant. However, we cannot exclude the presence of a product specific β-amyrin synthase and lupeol synthase in this plant, since they are widely distributed in higher plants.

Our previous study also showed that the free triterpene concentration was higher in the outer part of the roots than inner part, and the proportion of free triterpenes increased with salinity in both the leaves and roots of *K. candel* (Oku et al., 2003). These results might suggest that mangrove triterpenes play a role in the protection from external invasion by microbes, and/or in maintaining the physiological condition with changes in salt concentration, which is often caused by exposure to marine water. The obtained cDNA will be useful and essential to clarify the possible roles of triterpenes in mangroves.

3. Concluding remarks

It has been argued for a long time whether triterpene synthases yielding different products are distinct proteins, or if they are generated post translationally from one gene product. This is because activities of OSCs are susceptible

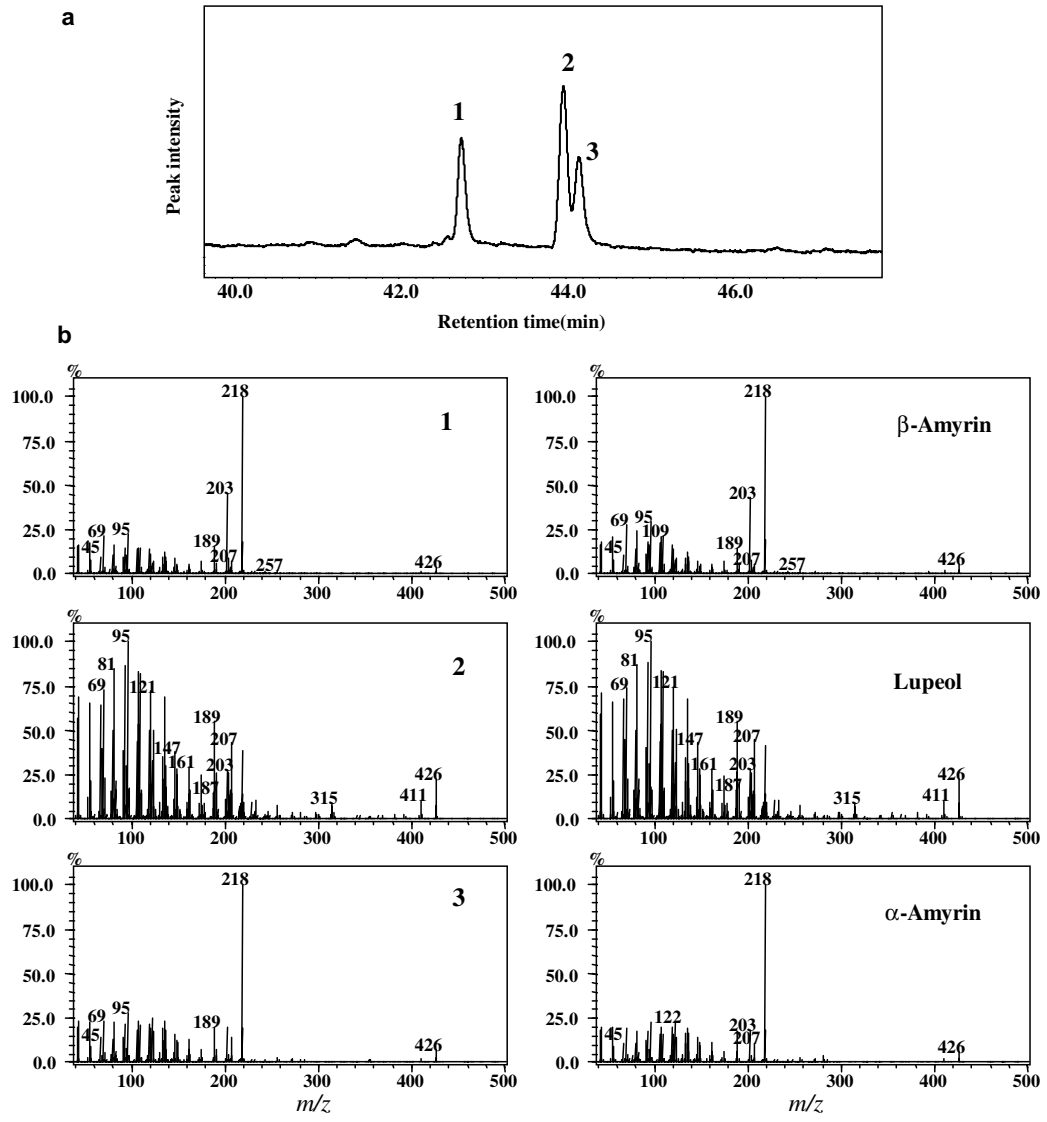

Fig. 3. GC–MS analysis of extracts from transformed GIL 77. Gas-chromatogram was monitored by FID (a). Left column of b (1–3) indicates EI-mass spectra of the products corresponding to peaks 1, 2 and 3. EI-mass spectra of authentic standards are shown in the right column of b.

to changes in pH, detergent and electrolyte concentrations (Baisted, 1971). A number of successful clonings of triterpene synthases has proved the presence of multiple OSCs as distinct proteins even in one plant species. However, this does not exclude the possibility of functional changes by post-translational modifications. Especially in the mangrove trees, such a possibility cannot completely be eliminated, since the amounts and compositions of the triterpenoids in roots of *Bruguiera gymnorrhiza* and *K. candel* vary with salt concentrations (Oku et al., 2003). These changes may be ascribed to changes in catalytic efficiency and/or specificity of *KcMS* by alteration in tertiary protein structure, or in changes in expression of other OSCs with different functions by transcriptional regulation. A detailed biochemical analysis of *KcMS* in vitro and further cloning of other OSCs from mangrove species should provide an answer to these interesting and important questions.

4. Experimental

4.1. Plant and culture conditions

Fresh young roots of *K. candel* were collected at Okukubi River, Okinawa, Japan. These materials were immediately frozen using liquid N_2 and stored at −80 °C for RNA preparation. Yeast strain GIL77 (*gal2 hem3-6 erg7 ura3-167*) was used as the host, and maintained on a YPD medium (1.0% yeast extract, 2.0% peptone, 2.0% dextrose) supplemented with hemin (13 μg/ml), ergosterol (20 μg/ml) and Tween 80 (5 mg/ml). Transformation of the yeast mutant was carried out by the Frozen-EZ Yeast Transformation II™ Kit (ZYMO RESEARCH). The transformant was cultured in synthetic complete medium without uracil (SC-Ura) supplemented with hemin and ergosterol (the same concentration as described above) at 30 °C with shaking (220 rpm).

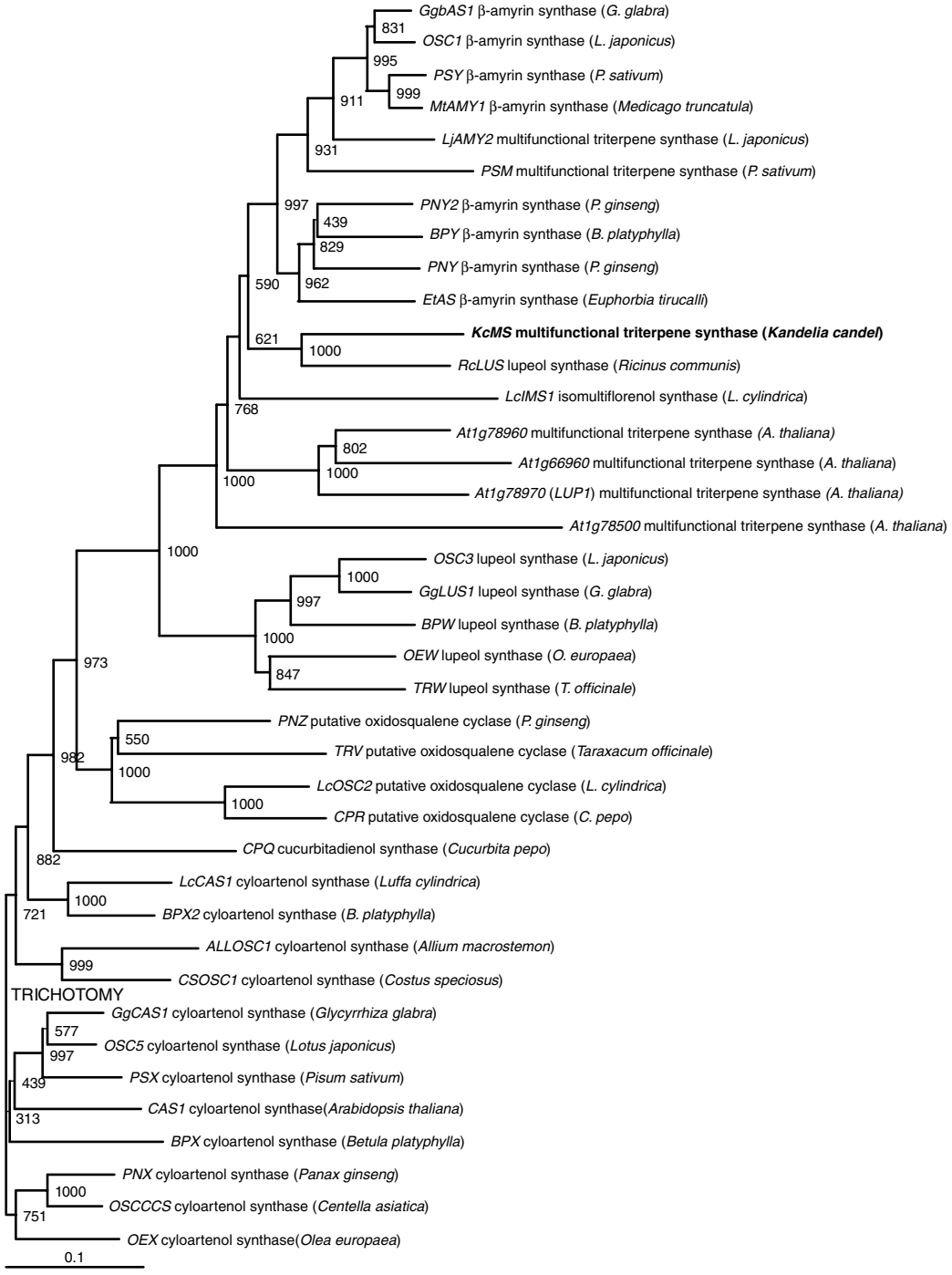

Fig. 4. Phylogenetic tree of plant OSCs including *KcMS* from *K. candel*. The phylogenetic tree was constructed using CLUSTAL W based on neighbor-joining method. The indicated scale represents 0.1 amino acid substitutions per site. Numbers indicate bootstrap values from 1000 replicates. The DDBJ/GenBank/EMBL accession numbers of the sequence used this analysis are indicated in Section 4.

4.2. cDNA preparation

Total RNA was extracted from young root of *K. candel* using CTAB method (Simamoto and Sasaki, 1997) with a few minor modifications to improve the yield. Total RNA (2.42 μg) was reverse-transcribed with 0.5 μg oligo dT primer (RACE 32, 5′-GACTCGAGTCGACATCGA-TTTTTTTTTTTTTT-3′) to produce a cDNA in total volume of 20 μl for 5 min at 65 °C, 1 h at 50 °C, and 5 min at 85 °C, and using cloned AMV First-Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. The resulting cDNA mixture was diluted with 50 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and directly used as a template for the following PCRs.

4.3. Cloning of core fragment

Four degenerate oligonucleotide primers (161S, 463S, 603A and 701A) corresponding to the highly conserved regions of known oxidosqualene cylases (OSCs) enzymes were synthesized. The nucleotide sequences of these primers are as follows: 161S = 5′-GAYGGIGGITGGGIYTICA-3′ (DGGWGLH), 463S = 5′-MGICAYATHWSIAARGG-3′ (RHISKGSW), 603A = 5′-CCCCARTTI- CCRTACCAISWICCRTC-3′ (DGSWYGNWG), 701A = 5′-CKRTAYTCIGCIARIGCCCA-3′ (FPMWALAEY). First PCR with 161S and 711A primers (10 ng) was performed by Ex Taq DNA polymerase (TAKARA BIO INC, Kyoto, Japan) with dNTP (0.2 mM) in a final volume of 100 μl according to the manufacturer's protocol. PCR amplification scheme was for 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 3 min 72 °C, with final extension of 10 min at 72 °C. The first PCR product was applied on Centricon centrifugal filter devices YM-30 (Millipore) to remove the primers, and the volume was adjusted to 100 μl. The second PCR was carried out with 463S and 603A primers (10 ng) and 3 μl of the first PCR product as template under the same conditions as the first PCR. The PCR product (≈445 bp) was separated using 1% agarose gel GTG and purified by Suprec-01 filter (TAKARA BIO INC). The purified fragment was ligated to a plasmid vector of TOPO 10 (Invitrogen) and propagated in *Escherichia coli*, and sequenced by ABI PRISM^{TM} 3100-Avant Genetic Analyzer (Applied Biosystems) with Bigdye^{R} Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). All the PCR products were subcloned and sequenced by the same procedure.

4.4. Cloning of 3′- and 5′- end amplification of cDNA

The "rapid amplification of cDNA ends" (RACE) method (Frohman et al., 1988) was applied for the 3′-end and 5′-end amplification using GeneRacer^{TM} kit (Invitrogen). Based on the sequence of the core fragment, for 3′-RACE amplification, two specific oligonucleotide primers: S1 (5′-TGATGCCCCCTGAACTTG-3′) and S2 (5′-CCTGAGCACAGGAGGAAAG-3′ were synthesized. First PCR was carried out with S1 and GeneRacer 3′ primer (5′-GCTGTCAACGATACGCTACGTAA- CG-3′) using *K. candel* cDNA as template, and the nested PCR with S2 and GeneRacer 3′ nested (5′-CGCTACGTAACGGCATGACAGTG-3′) primer was performed with the first PCR product as template, as described for the core fragment amplification except for that annealing temperature was 55 °C.

For 5′-RACE amplification, two specific primers were synthesized i.e. A1 (5′-GAAACTCCACTGGATTAAGCCA-3′) and A2 (5′-GAGGTTCCATCTTCCTACCA-3′). 5′-RACE was carried out as described above using A1 and GeneRacer 5′ primer (5′-CGACTGGAGCACGAGGACACTGA-3′) for first PCR with cDNA template prepared by superscript III RT GeneRacer (Invitrogen). The second PCR was conducted with A2 and GeneRace 5′ nested primer (5′-GGACACTGACATGGACTGAAGGAGTA- 3′) using the first PCR product as template DNA and with an annealing temperature of 57 °C.

4.5. Cloning of the full-length cDNA

The full-length cDNA for *KcMS* was obtained using N-terminal and C-terminal primers, with introduction of a *Kpn*I site and *Xho*I site immediately upstream of the ATG codon and downstream of the TGA codon. The sequences were as follows: Kpn-KC-N1 (5′-GACT**GGTACC**ATGTGGAGGCT- TAAGATTGCA-3′; *Kpn*I site in bold face), Xho-KC-C2 (5′-GGC**CTCGAG**ATTTTTGGCCTATTT- CAATCA -3′; *Xho*I site in bold face). PCR was performed with each set of primers with *K. candel* cDNA template and annealing temperature of 55 °C. The obtained full-length cDNA was sequenced in both strands.

4.6. Expression in yeast

The 2.3-kb PCR product was digested with *Kpn*I and *Xho*I and ligated into *Kpn*I and *Xho*I sites of pYES2 (Invitrogen) to construct the plasmid *KcMS*. The identity of the inserted DNA was verified by sequencing. The mutant GIL77 lacking lanosterol synthase activity was transformed with the plasmid using Frozen-EZ Yeast Transformation II^{TM} kit (ZYMO RESEARCH). The transformant of OSC-*KcMS* was inoculated in 25 ml synthetic complete medium without uracil (SC-Ura), containing hemin (13 μg/ml), ergosterol (20 μg/ml) and Tween-80 (5 mg/ml), and incubated at 30 °C for 2 days. Then, the medium was changed to SC-Ura supplemented with 2% galactose for glucose. Cells were incubated at 30 °C for 10 h, harvested by centrifugation at 3000 rpm for 5 min, resuspended in 20 ml of 0.1 M potassium phosphate buffer pH 7.0 and 3% glucose and hemin, and incubated another 72 h. Cell pellets were collected and refluxed with 2 ml 20% KOH/50% ethanol at 90 °C for 10 min. After extraction with the same volume of hexane, the extract was concentrated and applied to a TLC plate (Merck), which was developed with benzene/acetone (19:1, v/v). The fraction corresponding to the triterpene mono-alcohol was excised, extracted with $CHCl_3$–MeOH (2:1) and used for either GC or GC–MS analyses.

4.7. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC–MS) of triterpenoids

The reaction products of OSC in the extract were directly analyzed using a gas chromatograph equipped with a flame ionization detector (Shimadzu, Kyoto, Japan). The column used was CBPI-M50-025 (0.25 mm ID × 50 m, Shimadzu), with the column temperature program set at 50 °C for 1 min, then raised to 300 °C with a rate of 10 °C/min, and held at 300 °C for 26 min. The carrier gas was He with

a flow rate of 20 cm/s, and the temperatures for injector and detector were 250 °C and 300 °C, respectively. The mass spectrometer used was a GC–MS QP-2010 (Shimadzu), with the column and GC conditions as described above. Ionization of sample was by electron impact (EI) at 70 eV to estimate the chemical structure, or by chemical ionization with methane as a reaction gas to determine the molecular weight. Similarity searches of the spectra were carried out using the mass-spectrum library (Nist 107). Authentic standards of **2**, **3** and **4** were purchased from Extrasynthese, Genay, France.

4.8. Phylogenetic analysis of amino acid sequence

Sequence alignments of deduced amino acid *KcMS* were performed using the CLUSTAL W program (Thompson et al., 1994). Phylogenetic analysis of deduced amino acid alignment was conducted with CLUSTAL W program followed by drawing with TreeView software (Page, 1996) based on a neighbor-joining method. Bootstrap analysis with 1000 replications was used to assess the strength of the nodes in the tree (Felsenstein, 1985). The DDBJ/GenBank/EMBL accession numbers of the sequence used this analysis are as follows: AB025344 (*OEX*), AY520819 (*OSCCCS*), AB009029 (*PNX*), AB055509 (*BPX*),AB055510 (*BPX2*), U02555 (*CAS1*), AB025968 (*GgCAS1*), D89619 (*PSX*), AB181246 (*OSC5*),AB058507 (*CSOSC1*), AB025353 (*ALLOSC1*), AB033334 (*LcCAS1*), AB116238 (*CPQ*), AB116239 (*CPR*),AB033335 (*LcOSC2*), AB025346 (*TRV*), AB009031 (*PNZ*), AB025345 (*TRW*), AB025343 (*OEW*),AB055511 (*BPW*), AB116228 (*GgLUS1*), AB181245 (*OSC3*), AC007260 (*At1g78500*(*T30F21.16*)), U49919 (*At1g78970*(*LUP1*)), AC007152 (*At1g66960*-(*F*1019.4)), AC002986 (*At1g78960*(*YUP8H12R.43*)), AB058643 (*LcIMS1*), DQ268869 (*RcLUS*), AB257507 (*KcMS*), AB206469 (*EtAS*), AB009030 (*PNY*), AB014057 (*PNY2*), AB055512 (*BPY*),AB034803 (*PSM*), AF478455 (*LjAMY2*), AB034802 (*PSY*), AF478453 (*MtAMY1*), AB181244 (*OSC1*),AB037203 (GgbAS1).

Recently, enzyme function of *PNZ* was identified as lanosterol synthase (Suzuki et al., 2006).

Acknowledgements

One of the authors, Mohammad Basyuni, thanks the Ministry of Education, Culture, Sports, Science and Technology of Japan (Monbukagakusho), for a scholarship to study in Japan. A part of this study was supported by a Grant-in Aid for Scientific Research (C 15580131) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Abe, I., Prestwich, G.D., 1994. Active site mapping of affinity-labeled rat oxidosqualene cyclase. J. Biol. Chem. 269, 802–804.

Abe, I., Rohmer, M., Prestwich, G.D., 1993. Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. Chem. Rev. 93, 2189–2208.

Baisted, D.J., 1971. Sterol and triterpene synthesis in the developing and germinating pea seed. Biochem. J. 124, 375–383.

Brod, J., Bavelier, E., Justine, P., Weerheim, A., Ponec, M., 1991. Acylceramides and lanosterol-lipid markers of terminal differentiation in cultured human keratinocytes: modulating effect of retinoic acid. In Vitro Cell. Dev. Biol. 27A, 163–168.

Ebizuka, Y., Katsube, Y., Tsutsumi, T., Kushiro, T., Shibuya, S., 2003. Functional genomics approach to the study of triterpene biosynthesis. Pure Appl. Chem. 75, 361–366.

Fazio, G.C., Xu, R., Matsuda, S.P.T., 2004. Genome mining to identify new plant triterpenoids. J. Am. Chem. Soc. 126, 5678–5679.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Frohman, M.A., Dush, M.K., Martin, G.R., 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85, 8998–9002.

Ghosh, A., Misra, S., Dutta, A.K., Choudhury, A., 1985. Pentacyclic triterpenoids and sterols from seven species of mangrove. Phytochemistry 24, 1725–1727.

Guhling, O., Hobl, B., Yeats, T., Jetter, R., 2006. Cloning and characterization of a lupeol synthase involved in the synthesis of epicuticular wax crystals on stem and hypocotyls surfaces of *Ricinus communis*. Arch. Biochem. Biophys. 448, 60–72.

Hayashi, H., Huang, P., Inoue, K., Hiraoka, N., Ikeshiro, Y., Yazaki, K., Tanaka, S., Kushiro, T., Shibuya, M., Ebizuka, Y., 2001. Molecular cloning and characterization of isomultiflorenol synthase, a new triterpene synthase from *Luffa cylindrica*, involved in biosynthesis of bryonolic acid. Eur. J. Biochem. 268, 6311–6317.

Hayashi, H., Huang, P., Takada, S., Obinata, M., Inoue, K., Shibuya, M., Ebizuka, Y., 2004. Differential expression of three oxidosqualene cyclase mRNAs in *Glycyrrhiza glabra*. Biol. Pharm. Bull 27, 1086–1092.

Herrera, J.B.R., Bartel, B., Wilson, W.K., Matsuda, S.P.T., 1998. Cloning and characterization of the *Arabidopsis thaliana* lupeol synthase gene. Phytochemistry 49, 1905–1911.

Hogg, R.W., Gillan, F.T., 1984. Fatty acids, sterols and hydrocarbons in the leaves from eleven species of mangrove. Phytochemistry 23, 93–97.

Iturbe-Ormaetxe, I., Haralampidis, K., Papadopoulou, K., Osbourn, A.E., 2003. Molecular cloning and characterization of triterpene synthases from *Medicago truncatula* and *Lotus japonicus*. Plant Mol. Biol. 51, 731–743.

Kajikawa, M., Yamato, K.T., Fukuzawa, H., Sakai, Y., Uchida, H., Ohyama, K., 2005. Cloning and characterization of a cDNA encoding β-amyrin synthase from petroleum plant *Euphorbia tirucalli* L. Phytochemistry 66, 1759–1766.

Koch, B.P., Rullkotter, J., Lara, R.J., 2003. Evaluation of triterpenoids and sterols as organic matter biomarkers in a mangrove ecosystem in Northen Brazil. Wetl. Ecol. Manag. 11, 257–263.

Kokpol, U., Chavasiri, W., Chittawong, V., Miles, D.H., 1990. Taraxeryl *cis-p*-hydroxycinnamate, a novel taraxeryl from *Rhizophora mucronata*. J. Nat. Prod. 53, 953–955.

Kokpol, U., Chittawong, V., Miles, D.H., 1986. Chemical constituents of the roots of *Acanthus illicifolius*. J. Nat. Prod. 49, 355–356.

Kushiro, T., Shibuya, M., Ebizuka, Y., 1998. β-amyrin synthase: cloning of oxidosqualene cyclase that catalyzes the formation of the most popular triterpene among higher plants. Eur. J. Biochem. 256, 238–244.

Kushiro, T., Shibuya, M., Ebizuka, Y., 2000. A novel multifunctional triterpene synthase from *Arabidopsis thaliana*. Tetrahedron Lett. 41, 7705–7710.

Majumdar, S.G., Patra, G., 1976. Chemical investigation of some mangrove species. Part III. Rhizophoracea, a mangrove family. J. Indian Chem. Soc. 53, 1241–1242.

Morita, M., Shibuya, M., Kushiro, T., Masuda, K., Ebizuka, Y., 2000. Molecular cloning and functional expression of triterpene synthases from pea (*Pisum sativum*): new α-amyrin producing enzyme is a multifunctional triterpene synthase. Eur. J. Biochem. 267, 3453–3460.

Oku, H., Baba, S., Koga, H., Takara, K., Iwasaki, H., 2003. Lipid composition of mangroves and its relevance to salt tolerance. J. Plant Res. 116, 37–45.

Page, R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Poralla, K., Hewelt, A., Prestwich, G.D., Abe, I., Reipen, I., Sprenger, G., 1994. A specific amino acid repeat in squalene and oxidosqualene cyclases. Trend Biochem. Sci. 19, 157–158.

Sawai, S., Shindo, T., Sato, S., Kaneko, T., Tabata, S., Ayabe, S., Aoki, T., 2006. Functional and structural analysis of genes encoding oxidosqualene cyclases of *Lotus japonicus*. Plant Sci. 170, 247–257.

Segura, M.J.R., Meyer, M.M., Matsuda, S.P.T., 2000. *Arabidopsis thaliana* LUP1 converts oxidosqualene to multiple triterpene alcohols and a triterpene diol. Org. Lett. 2, 2257–2259.

Shibuya, M., Zhang, H., Endo, A., Shishikura, K., Kushiro, T., Ebizuka, Y., 1999. Two branches of the lupeol synthase gene in the molecular evolution of plant oxidosqualene cyclases. Eur. J. Biochem. 266, 302–307.

Shibuya, M., Adachi, S., Ebizuka, Y., 2004. Cucurbitadienol synthase, the first committed enzyme for cucurbitacin b iosynthesis, is a distinct enzyme from cycloartenol synthase for phytosterol biosynthesis. Tetrahedron 60, 6995–7003.

Simamoto, K., Sasaki, T., 1997. Sinpan Shokubutsu no PCR Jikken Protocol. Shujunsa, pp. 60–61. (Japanese).

Suzuki, M., Xiang, T., Ohyama, K., Seki, H., Saito, K., Muranaka, T., Hayashi, H., Katsube, Y., Kushiro, T., Shibuya, M., Ebizuka, Y., 2006. Lanosterol synthase in dicotyledonous plants. Plant Cell Physiol. 47, 565–571.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res. 22, 4673–4680.

Wannigama, G.P., Volkman, J.K., Gillan, F.T., Nichols, P.D., Johns, R.B., 1981. A comparison of lipid components of the fresh and dead leaves and pneumatophores of the mangrove *Avicennia marina*. Phytochemistry 20, 659–666.

Williams, L.A.D., 1999. *Rhizophora mangle* (Rhizophoraceae) triterpenoids with insectidal activity. Naturwissenschaften 86, 450–452.

Wu, T.K., Griffin, J.H., 2002. Conversion of a plant oxidosqualene-cycloartenol synthase to an oxidosqualene-lanosterol cyclase by random mutagenesis. Biochemistry 41, 8238–8244.

Xiang, T., Shibuya, M., Katsube, Y., Tsutsumi, T., Otsuka, M., Zhang, H., Masuda, K., Ebizuka, Y., 2006. A new triterpene synthase from *Arabidopsis thaliana* produces a tricyclic triterpene with two hydroxyl groups. Org. Lett. 8, 2835–2838.

Xiong, Q., Wilson, W.K., Matsuda, S.P.T., 2006. An Arabidopsis oxidosqualenecyclase catalyzes iridal skeleton formation via Grob fragmentation. Angew. Chem. Int. Ed. 45, 1285–1288.

Zhang, H., Shibuya, M., Yokota, S., Ebizuka, Y., 2003. Oxidosqualene cyclases from cell suspension cultures of *Betula platyphylla* var. *japonica*: molecular evolution of oxidosqualene cyclases in higher plants. Biol. Pharm. Bull. 26, 642–650.