Sterols, essential eukaryotic constituents, are biosynthesized through either cyclic triterpenes, lanosterol (fungi and animals) or cycloartenol (plants). The cDNA for OSC7 of *Lotus japonicus* was shown to encode lanosterol synthase (LAS) by the complementation of a LAS-deficient mutant yeast and structural identification of the accumulated lanosterol. A double site-directed mutant of OSC7, in which amino acid residues crucial for the reaction specificity were changed to the cycloartenol synthase (CAS) type, produced parkeol and cycloartenol. The multiple amino acid sequence alignment of a conserved region suggests that the LAS of different eukaryotic lineages emerged from the ancestral CAS by convergent evolution.

**Keywords:** Cycloartenol — Lanosterol — *Lotus japonicus* — Oxidosqualene cyclase.

Abbreviations: BAS, β-amyrin synthase; CAS, cycloartenol synthase; EI-MS, electron ionization–mass spectrometry; GC–MS, gas chromatography–mass spectrometry; LAS, lanosterol synthase; LUP, lupeol synthase; NMR, nuclear magnetic resonance; ORF, open reading frame; OSC, oxidosqualene cyclase; TLC, thin-layer chromatography.

The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GenBank database under accession numbers AB244670 (cOSC6) and AB244671 (cOSC7).

Sterols are essential membrane constituents in eukaryotes. A cyclic triterpene monoalcohol lanosterol is an intermediate for ergosterol and cholesterol in fungi and animals, respectively. Plants accumulate specific sterols designated phytosterols in addition to cholesterol, and they are believed to be biosynthesized via another cyclic triterpene, cycloartenol (Grunwald 1980, and references cited therein). During sterol biosynthesis, 2,3-oxidosqualene is converted into the cyclic triterpene monoalcohols by cycloartenol synthase (CAS) or lanosterol synthase (LAS), collectively designated oxidosqualene cyclase (OSC). Both CAS and LAS cyclize oxidosqualene into the protosteryl cation and shift the positive charge of the cation from C-20 towards C-9 or C-8 by a series of 1,2-bond shifts of hydrides or methyl groups. CAS deprotonates H-19 of the C-9 carboxylation giving a C–C bond between C-9 and C-19 and, in some cases, also deprotonates H-11 to produce parkeol as a byproduct (Godzina et al. 2000, Lodeiro et al. 2005). On the other hand, LAS deprotonates H-9 of the lanosterol (C-8) carboxylation, or H-8 of the C-9 cation, to give a double bond between C-8 and C-9 (Fig. 1). In addition to CAS, plants also have many OSCs that form cyclic triterpenes through the dammarenyl cation, and accumulate diverse classes of triterpenes. We identified genes for CAS, lupeol synthase (LUP) and β-amyrin synthase (BAS) from *Lotus japonicus* (Sawai et al. 2006), but the functions of two OSCs (OSC6 and OSC7) remained unknown. The neighbor-joining phylogenetic tree of plant OSCs suggested that eudicot OSCs should be categorized into four groups. Groups II, III and IV are represented by CAS, LUP and BAS, respectively. In this study, we identified the function of OSC7 as LAS, and discuss the evolutionary relationship between CAS and LAS in eukaryotes.

Previous comparison of the OSC gene structures of *L. japonicus* and *Arabidopsis thaliana* showed that the length of most exons is conserved but that the length of exons 4, 7 and 9 is polymorphic (Sawai et al. 2006). The length polymorphism of the three exons was used as a criterion for the cladistic analysis, together with cyclization intermediates and substrate usage (Fig. 2). The outgroups for the seed plant OSCs are the LAS and CAS of other eukaryotes. Group II OSCs (CAS), which use the same cyclization intermediate as the outgroups, were placed at the most basal position, and the other groups were ordered into the most parsimonious tree. The cladogram thus obtained shows the deduced evolutionary polarity from group I and group II to group IV (Fig. 2).

We presumed that some group I OSCs have the same catalytic function as one of the outgroups, LAS. The expression vectors constructed from the cDNAs and pYES2 were used to transform a LAS-deficient (*erg7*) yeast strain GIL77 (Kushiro et al. 1998). *cOSC7* complemented the *erg7* mutation only when its expression was induced by galactose (Fig. 3). To identify the reaction product of OSC7, yeast GIL77 transformed with *cOSC7* was cultured on a large scale with an inhibitor of
Fig. 1  Formation of lanosterol, parkeol and cycloartenol. OSC catalyzes the 2,3-oxidosqualene cyclization, carbocation rearrangement and deprotonation of the cation intermediate. Amounts relative to the most abundant products in native and mutant OSCs are also shown.

Fig. 2  A cladogram of OSCs from *L. japonicus*, *A. thaliana* and other organisms based on the exon length, cyclization intermediates and substrate usage. The outgroups of seed plant OSCs are CAS and LAS of other eukaryotes. Group II OSCs (CAS) are postulated to be the most ancestral among the seed plant OSCs because they produce triterpene alcohol via the protosteryl cation, like the outgroups. The derived character state is indicated at each internode. The exon lengths of *L. japonicus* and *A. thaliana* OSCs (Sawai et al. 2006) are concisely shown.
cytochrome P450, ketoconazol, to prevent the lanosterol produced from oxidative demethylation by yeast CYP51. 1H- and 13C-nuclear magnetic resonance (NMR) spectra (Supplementary data) of the purified triterpenes suggested the presence of lanosterol plus a minor constituent. Lanosterol was further unequivocally identified by direct electron ionization mass spectrometry (EI-MS) and two-dimensional NMR in comparison with the authentic sample. Gas chromatography-mass spectrometry (GC-MS) analysis revealed parkeol as the minor constituent. The amount of parkeol relative to lanosterol was estimated to be 0.01 by integration of the 1H-NMR signal intensities (Fig. 1). On the other hand, cOSC6 did not complement the erg7 mutation (Fig. 3), and no reaction product was detected in the transgenic GIL77 cultured on a large scale.

A previous mutagenic study of Arabidopsis CAS1 showed that a double mutant, His477Asn Ile481Val, produced 99% lanosterol and 1% parkeol (Lodeiro et al. 2005). The corresponding residues of OSC7 are asparagine and valine, and thus the OSC7 double mutant, which has histidine and isoleucine at the loci, was expected to produce cycloartenol. Yeast strain GIL77 was thus transformed with the expression vector to produce the OSC7 Asn478His Val482Ile mutant protein. The triterpene monoalcohol fraction was prepared from the cultured transformant yeast. Signals of the 1H- and 13C-NMR spectra for the major and minor constituents were consistent with those of parkeol (Itoh et al. 1976, Hart et al. 1999) and cycloartenol, respectively. The two constituents were separated further by reversed phase HPLC. The NMR spectra of the parkeol fraction also showed minor signals corresponding to lanosterol, which was further confirmed by GC-MS with trimethylsilyl derivatization. The NMR (Supplementary data) and EI-MS spectra of the cycloartenol fraction were in good agreement with the authentic sample.

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**Fig. 3** Complementation of the yeast erg7 mutation by cOSC7 of L. japonicus. A LAS-deficient yeast strain GIL77 (erg7) was transformed with L. japonicus cOSC6 and cOSC7, together with cOSC1, cOSC3 and cOSC5 encoding BAS, LUP and CAS, respectively. Ten-fold serial dilutions of the cell suspensions were spotted onto the galactose induction test plate (A), the negative control plate added with glucose instead of galactose (B) and the positive control plate supplemented with ergosterol (C), and incubated at 28°C for 4 d.

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**Fig. 4** A multiple amino acid sequence alignment of the conserved region critical for the reaction specificity among representative OSCs from various lineages. The major eukaryotic supergroups proposed by Adl et al. (2005) are indicated. Asterisks denote the amino acid residues whose mutation in Arabidopsis CAS1 (At2g07050) altered the catalytic function to LAS (Lodeiro et al. 2005).
with those obtained with the authentic sample. The amounts of cycloartenol and lanosterol relative to parkeol were estimated to be 0.16 and 0.05, respectively, by the $^1$$^H$-NMR integration (Fig. 1).

The CAS reaction is thought to be terminated by the H-19 deprotonation of the C-9 cation intermediate (Fig. 1). In the OSC7 mutant, the base for deprotonation is likely to be closer to C-11 than C-19, and thus parkeol was preferentially produced. Other amino acid residues in OSC7 possibly have been optimized for efficient lanosterol formation. Lanosterol is distributed to some plants (Itoh et al. 1977, Willuhn et al. 1983, Fernandes-Ferreira et al. 1990). Other group I OSCs, At3g45130 of A. thaliana and PnZ of Panax ginseng, were also shown to be LAS (Suzuki et al. 2006). Because several group I OSCs have been recorded in databases, LAS may be generally distributed to eudicots.

The results of this study may offer clues to the evolutionary relationship between CAS and LAS in eukaryotes. The phylogenetic tree based on the coding sequences by the maximum likelihood method showed the monophyly of the OSCs in groups I, III and IV, suggesting that OSCs in group I, represented by LAS, are more apomorphic (derived) than group II OSCs (CAS) (Supplementary Fig. S1). The plant LAS is thus most likely to have diverged from the ancestral CAS. This idea is supported by the unilateral capability of the functional conversion of CAS and LAS, i.e. the almost complete conversion of Arabidopsis CAS1 to LAS by two amino acid substitutions (Lodeiro et al. 2005) and the incomplete change of Lotus OSC7 by the reverse substitutions (Fig. 1).

OSC7s have been found in several eukaryotes and a few eubacteria. Considering recent systematic studies on eukaryotes (Richards and Cavalier-Smith 2005), homoplastic evolution of CAS and LAS is suggested. The postulated polarity of the molecular evolution can be extended to general evolution of the phylogenetic tree. In CAS, the amino acid residues of this region are highly conserved, and in particular those corresponding to Tyr410, His477 and Ile481 of Arabidopsis CAS1 (At2g07050) are completely identical even in the bacterium Stigmatella aurantiaca. On the other hand, the sequences of this region in LAS are less conserved, and the residues in the positions of Tyr410 and His477 are diverse: tyrosine–histidine in eubacteria, tyrosine–asparagine in Archaeplastida, tyrosine–glutamine in Excavata, threonine–glutamine in fungi and threonine–cysteine in animals. This strongly suggests that LAS emerged from the ancestral CAS in different lineages by convergent evolution, which is consistent with some previously proposed hypotheses (Ourisson 1994, and references cited therein).

Among the lineages so far investigated, only eudicots have both CAS and LAS. Although no findings contradict the idea that cycloartenol is the principal cyclic triterpene intermediate for plant sterol biosynthesis, plant lanosterol may act as an alternative intermediate for the sterols or a precursor of other significant metabolites. Identification of lanosterol-metabolizing enzymes is the key to understanding the physiological roles of plant lanosterol.

**Materials and Methods**

The cDNA template was prepared from the mRNA isolated from 4-week-old whole plants of L. japonicus (Regel) Larsen accession Gifu B-129. To amplify the full open reading frames (ORFs) of cOSC6 and cOSC7, two sets of primers were designed based on the 3′- and 5′-untranslated regions: OSC6/FL (5′-ATAGAAGAGCAGAAATTTACTTCCGTTATG-3′) and OSC6/FU (5′-ATTAAATAGTTCAGAAAGAGAAATTGAGAAG-3′) for cOSC6; and OSC7/FL (5′-AAACTCTCCCAATATTTTTATGGAGATG-3′) and OSC7/FU (5′-AGAAGGAGAAATGAAAAATTTGAGAAG-3′) for cOSC7. The PCR products were cloned into a pT7-Blue T vector (Novagen, San Diego, CA, USA). The full ORF of cOSC6 was subcloned into the KpnI and SpHⅠ sites of a yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA). The full ORF of cOSC7 was transferred to the KpnI and XhoⅠ sites of the pYES2 vector by PCR subcloning with a primer set containing a KpnI or XhoⅠ site (shown in lower case): OSC7/FU_Kpn (5′-TAAAATTAGAAGAGAAATTAGACAGTGTAATGCGAAATG-3′) and OSC7/FU_Xho (5′-CTCCTAATAATTGTCTctcgagGA-TGCAAGTCGTTTTATTTTTGACAC-3′).

Saccharomyces cerevisiae GIL77 (gall2 hem3-6 erg2 ura3-167) (Kushiro et al. 1998) was transformed with cOSC1 to cOSC7 cloned into pYES2. The transformants were cultured in synthetic complete medium without uracil (SC-U) supplemented with 20 µg ml$^{-1}$ ergosterol (E), 5 mg ml$^{-1}$ Tween 80 (T) and 13 µg ml$^{-1}$ hemin (H). After 1 d, these cells were collected and resuspended in water. Ten-fold serial dilutions (5 µl each) were placed on the galactose induction test plate (SC-U+T+H containing 2% galactose), the negative control plate (SC-U+T+E+H containing 2% glucose), and the positive control plate (SC-U+T+E+H containing 2% glucose). The plates were incubated at 28°C for 4 d.

Site-directed mutagenesis was carried out with the GeneEditor in vitro site-directed mutagenesis system (Promega, Madison, WI, USA) using cOSC7 as the template and an N478H-V482I mutagenic primer, 5′-GGATATCGGCTTTATCTCTGTGTC-3′. The PCR product was then subcloned into the pT7-Blue T vector, the transformants were cultured in SC-U+T+H medium containing 2% galactose and 4 mM ketoconazol, and monitored at 205 nm.

ELISA and GC–MS were performed using a direct inlet at the ionization voltage of 70 eV. The samples for GC–MS were trimethylsilylated with N-methyl-N-(trimethylsilyl) trifluoroaceticamide (Sigma-Aldrich, St Louis, MO, USA) at 80°C for 30 min and injected into a GC 6890 (Agilent Technologies, Palo Alto, CA, USA).
CA, USA) equipped with an Rtx-35ms column (30 m, 0.25 mm i.d., 0.10 µm df; Restek, Bellefonte, PA, USA) coupled to a Pegasus III MS system (Leco, St Joseph, MI, USA). The GC–MS conditions were: injector at 280°C, column oven maintained at 80°C for 3 min, then elevated to 280°C (10°C min⁻¹) and splitless injection. NMR spectra were measured with ECA-500 (JEOL) in CDCl₃ with tetramethylsilane as an internal standard. Authentic samples of lanosterol and cycloartenol were purchased from Sigma-Aldrich and Extrasynthése (Genay, France), respectively.

Multiple alignment was created using CLUSTAL W (Thompson et al. 1994). Accession numbers are: *S. aurantiaca*, CAD39196; *Dictyostelium discoideum*, AAF80384; *Abies magnifica*, AAG44096; OSC5 of *L. japonicus*, BAE53431; At2g07050 (CAS1) of *A. thaliana*, AAN64509; *Methyllococcus capsulatus*, YP_115266; At3g45130 of *A. thaliana*, NP_190099; OSCPZ1 of *P. ginseng*, BAA33462; *Leishmania major*, CAJ02110; *Trypanosoma brucei*, AA12587; *S. cerevisiae*, NP_011939; *Schizosaccharomyces pombe*, NP_593702; *Cryptococcus neoformans*, EAL21328; *Homo sapiens*, P48449; and *Mus musculus*, Q8BLN5.

The sequence data for a putative CAS of *Cyanidioschyzon merolae* and a putative LAS of *Gemmata obscuriglobus* are available on the web sites of their genome projects, http://merolae.biol.s.u-tokyo.ac.jp/db/cds_view.cgi?locus=CM1009C (as locus CM1009C) and http://gmn.tigr.org/tdb/mbd/mbdinprogress.html (as contig:4355:g_obscuriglobus), respectively.

**Supplementary material**

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

**Acknowledgments**

The authors are grateful to Dr. Yutaka Ebizuka (*The University of Tokyo*) for yeast GIL77. They also thank Nobuhiko Morishita and Ryouuke Sasaki (*Kazusa DNA Research Institute*) for technical assistance. This work was supported by the Japan Society for the Promotion of Science [Grant-in-Aid for Scientific Research (C) No. 14540603], Ministry of Education, Culture, Sports, Science and Technology of Japan [Grant-in-Aid for COE Research] and the New Energy and Industrial Technology Development Organization (Development of Fundamental Technologies for Controlling the Process of Material Production of Plants).

**References**


(Received February 21, 2006; Accepted March 7, 2006)