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Full Length Research Paper

Molecular cloning and characterization of a cDNA encoding cycloartenol synthase from *Fritillaria thunbergii* Miq.

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***Fritillaria thunbergii* Miq., known as the bulbous plants of the genus *fritillaria*, produces a large amount of sterols. Homology based polymerase chain reactions (PCRs) with degenerate primers designed from the conserved sequences among the known cycloartenol synthase (CAS) resulted in cloning of a CAS from the young leaves of *F. thunbergii* Miq. A putative cycloartenol synthase cDNA (*FtCAS*) consists of a 2271 bp open reading frame, which encodes for 756 amino acids. The deduced amino acid sequence shows 82% homology to cycloartenol synthases from *Dioscorea zingiberensis*. Heterologous expression of the *FtCAS* in the methylotrophic yeast, *Pichia pastoris*, resulted in production of cycloartenol. Our results indicate that *FtCA*S encode cycloartenol synthase.**

Key words: *Fritillaria thunbergii* Miq., cycloartenol synthase, cDNA cloning, 2,3-oxidosqualene cyclase.

INTRODUCTION

Higher plants usually produce phytosterols and various triterpenoids. Phytosterols play a role as structural components of the membrane system, and triterpenoids are often involved in plant defense systems against microbial pathogens or insects (Papadopoulou et al., 1999; Agrell et al., 2003). However, the functions of most phytosterols are still unclear. Biosynthesis of phytosterols branches away from that of triterpenes at the cyclization step of 2,3-oxidosqualene, which is catalyzed by oxidosqualene cyclases (OSCs) (Abe et al., 1993). In the OSC family, cycloartenol synthase is responsible for sterol biosynthesis, and other OSCs for triterpene synthesis (Figure 1).

A number of plant OSCs has been cloned and their enzymes functions have been confirmed by expression in lanosterol synthase deficient yeast (Xiang et al., 2006). The cDNAs of monofunctional synthases such as β-amyrin synthase and lupeol synthase have been cloned from different plant species (Kajikawa et al., 2005; Hayashi et al., 2001; Herrera et al., 1998). In addition, the cDNAs of multifunctional triterpene synthase from *Kandelia candel* and *Pisum sativum* have been cloned and functional expressed (Basyuni et al., 2006).

Cycloartenol is an important membrane constituent that can serve as precursors to steroid hormones. As shown in Figure 1, it is formed from (S)-squalene-2, 3-epoxide by a cyclization reaction catalysed by cycloartenol synthase (EC 5.4.99.8.). Up till now, the cDNAs of cycloartenol synthase have been cloned from different plant species (Morita et al., 1997; Hayashi et al., 2000; Basyuni et al., 2007). *F. thunbergii* Miq. (Chinese name, Zhebeimu) is a well-known ingredient in traditional Chinese medicine. Several biological activities of sterols from this plant have been reported. For example, an extract of peimine has been used in clinic for reducing fever and eliminating phlegm (Zhang et al., 2009). Although phytosterols are ubiquitous and indispensable components of cell membrane, their physiological functions in *F. thunbergii* Miq. are still unclear.

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Abbrerviations: CAS, Cycloartenol synthase; **LUS,** lupeol synthase; **bAS,** beta-amyrin synthase; **MFS,** multifunctional triterpene synthase

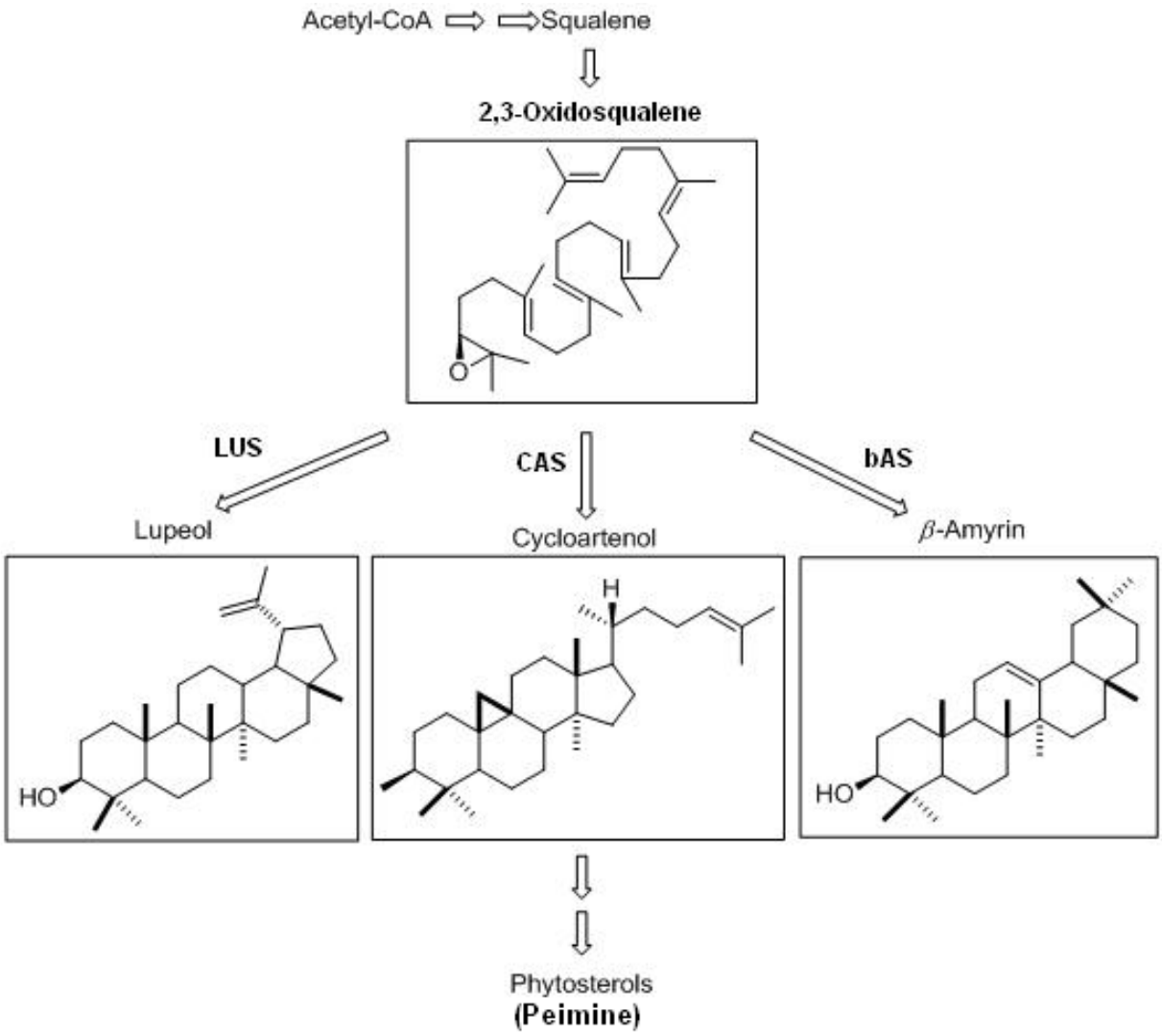

Figure 1. Cyclization reactions in higher plants leading from 2,3-oxidosqualene to phytosterols (cycloartenol) and triterpenoids (lupeol and β-amyrin). CAS, Cycloartenol synthase; LUS, lupeol synthase; bAS, β-amyrin synthase.

Additionally, no OSCs from *F. thunbergii* Miq. species has been reported. In order to obtain insight into the physiological function of sterols in *F. thunbergii* Miq.. This present study therefore cloned cycloartenol synthase cDNA (*FtCAS*) from *F. thunbergii* Miq. and functionally expressed the gene in yeast.

MATERIALS AND METHODS

Plant and materials

Plants of *F. thunbergii* Miq. were cultivated in pots at room temperature under natural light. Their young leaves were harvested, immediately frozen using liquid N_2 and stored at -80°C for RNA preparation. RNA extraction and reverse transcription reagents were obtained as follows: TRIzol® from Invitrogen (San Diego, CA, U.S.A.); Advantage® two polymerase chain reaction (PCR) kit and SMARTer™ rapid amplification of cDNA ends (RACE) cDNA Amplification kit from Clontech (Mountain View, CA, U.S.A.). Yeast strain X-33 (Invitrogen) was used as the host, and maintained on yeast extract peptone dextrose (YPD) medium (1.0% yeast extract, 2.0% peptone, 2.0% dextrose) supplemented with 100μg/ml Zeocin™ (Invitrogen). Plasmid vectors used were as follows: pPICZA from Invitrogen (San Diego, CA, U.S.A.); pTG19-T vector from Generay (Shanghai, China). Recombinant *Pichia pastoris* cells were cultured in a minimal medium composed of 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin and a carbon source (1.0% glycerol or 0.5% methanol for gene expression).

RNA and cDNA preparation

The freezing young leaves of *F. thunbergii* Miq. were grounded into powder by using TRIzol® method (Hayashi et al., 2000), total RNA (2 μg) was reverse-transcribed with 1.0 μl oligo$(dT)_{18}$ primer to produce a cDNA in total volume of 20 μl for 5 min at 65°C, 1 h at

42°C, and 5 min at 70°C, by using RevertAid™ first strand cDNA synthesis kit (Fermentas, #k1621) according to the manufacturer`s protocol. The resulting cDNA mixture was directly used as a template for the amplification of core fragment of *FtCAS* gene. 5` and 3`-RACE Ready cDNA were synthesized by using SMARTer™ RACE cDNA amplification kit (Clontech, Cat.634923) according to the manufacturer`s protocol. The resulting cDNA mixture was diluted with 50 µl Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and used as a template for the following 5`- and 3`-end amplification.

Cloning of core fragment of cycloartenol synthase

Four degenerate oligonucleotide primers (161S, 314S, 314A and 624A) corresponding to the highly conserved regions of known CAS were synthesized. Two PCR reactions with two primer pairs (161S and 314A; 314S and 624A) were performed by *Taq* DNA polymerase (Fermentas, Canada). PCR program was carried out under the following conditions: an initial preheating step of 3 min at 94°C was used to melt double-stranded DNA. Then, a touch-down procedure followed, consisting of 60 s at 94°C, annealing for 50 s at temperatures decreasing from 58 to 53°C during the first six cycles (with 1°C decremental steps in cycles 1 to 6), 52°C during the last 30 cycles, and ending with an extension step at 72°C for 50 s. A total of 36 cycles were performed. The PCR products (about 460 and 930 bp) were separated using 1% agarose gel and purified by suprec-01 filter (TAKARA BIO INC). The purified fragment was ligated to a pTG19-T vector (Generay, China) and transformed to TOP10 (Invitrogen). DNA sequencing was carried out by ABI PRISMTM 3100-Avant Genetic Analyzer (Applied Biosystems) in Genscript (Nanjing) co., Ltd. The nucleotide sequences of these primers are as follows:

161S: 5`-GATGTGCCGGTACCTCTACAAYCAYCARAA-3` (RYLYNHQN),
314S: 5`-GGAACCAGTGCGCCAARGARGAYYT-3` (CAKEDL),
314A: 5`-TGCGGGTAGTACAGGTCCTCYTTNGCRCA-3` (CAKEDL),
624A: 3`-CCGAACCAGGTGCCGTANGTRAARCA-3` (VCFTYG).

5`- and 3`-end amplification of cDNA

RACE method (Frohman et al., 1988) was applied for the 3`-end and 5`-end amplification by using SMARTer™ RACE cDNA Amplification kit (Clontech,USA). Based on the sequence of the core fragments, two specific oligonucleotide primers: 190S and 628S were synthesized for 3`-RACE amplification. First PCR was carried out with 190S and universal primer A mix (UPM) using 3`-RACE-Ready cDNA template prepared by SMARTer™ RACE kit (Clontech,USA), and the nested PCR with 628S and nested universal primer A (NUP,10µM) was performed with the first PCR product as template with an annealing temperature of 68°C.

For 5`-RACE amplification, two specific primers (130A and 29A) were synthesized, 5`-RACE was carried out as described above using 130A and UPM for first PCR with 5`-RACE-Ready cDNA template prepared by SMARTer™ RACE kit (Clontech). The nested PCR was conducted with 29A and NUP using the first PCR product as template DNA. Each above PCR product was cloned and sequenced.
The nucleotide sequences of these primers are as follows:

190S: 5`-TATGCTTTGCTGCTGGGTGGAA-3`
628S: 5`- CCTCTCCTTAATGAACAGCGATGGTGG-3`
Universal Primer A Mix (UPM): (long primer (0.4 µM), short primer (2 µM):
long: primer
5`CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAG AGT-3`
Short primer
5`-CTAATACGACTCACTATAGGGC-3`
Nested Universal Primer A (NUP):
5`-AAGCAGTGGTATCAACGCAGAGT-3`
130A: 5`-CCTCCCTTTCTGCATTGCCCCAT-3`
29A: 5`-CAATGTGCAAGCCCCAACCTCCA-3`

Cloning of the full-length cDNA

The full-length cDNA for *FtCAS* was obtained using N-terminal and C-terminal primers (*FtCAS*-P1, *FtCAS*-P2), with introduction of a *Eco*RI site and *Sac*II site immediately upstream of the ATG codon and downstream of the TGA codon. PCR was performed by KOD-Plus DNA polymerase (TAKARA BIO INC, Kyoto, Japan) with *F. thunbergii* Miq. cDNA template. PCR program was 2 min at 94°C, followed by 30 cycles of 20 s at 94°C and 2 min at 68°C, with final extension of 10 min at 72°C. The obtained full-length cDNA was sequenced in both strands. The primer sequences were designed as follows:

FtCAS-P1 5`-G**GAATTC**ATGTGGCAGCTCAAGATCGCCGAG-3`;(*Eco*RI site in bold face),
FtCAS-P2 5`-TCC**CCGCGG**TTAACAGAGTAGAACACGGG-AGCGA-3`; (*Sac*II site in bold face)

Sequence and phylogenetic analyses

Sequencing was performed on an automated DNA sequencer model 4000L and 4200L (LICOR Inc., Neb) with the Thermo Sequenase™ cycle sequencing kit (Amersham Pharmacia Biotech). Phylogenetic analysis was carried out with a Clustal X (version 2.1) program based on a neighbor-joining method. A phylogenic tree was created with the TreeView program (version 1.6.6). Bootstrap analysis with 1000 replications was used to assess the strength of the nodes in the tree (Felsenstein, 1985). The nucleotide sequence data reported in this paper have been deposited in the GeneBank nucleotide sequence databases under the accession numbers JN596102.

Functional expression in yeast

The 2271 bp PCR product was digested with *Eco*RI and *Sac*II, then ligated with pPICZA (Invitrogen) to place the *FtCAS* open reading frame (ORF) under the control of the methanol-inducible promoter, 5` AOX1 (pPICZA-*FtCAS*). *P.pastoris* wild type strain X-33 was transformed with pPICZA-*FtCAS* and pPICZA using the Pichia EasyComp kit (Invitrogen). *P. pastoris* carrying the recombinant pPICZA-*FtCAS* plasmid or the negative control pPICZA were grown at 28 to 30°C in buffered glycerol-complex medium (BMGY) (250 to 300 rpm) until the OD_{600} of the culture reaches two to six. The cells were collected by centrifugation, resuspended, and inoculated into BMMY medium for four days. Methanol was added into the culture to a final concentration of 0.5% every 24 h. The extraction of sample and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were done using the EasySelect Pichia Expression Kit (Invitrogen, U.S.A.).

Gas chromatography-electron impact mass spectrometry (GC-EIMS) analysis

The yeast cells were extracted with methanol–chloroform (2: 1, v/v)

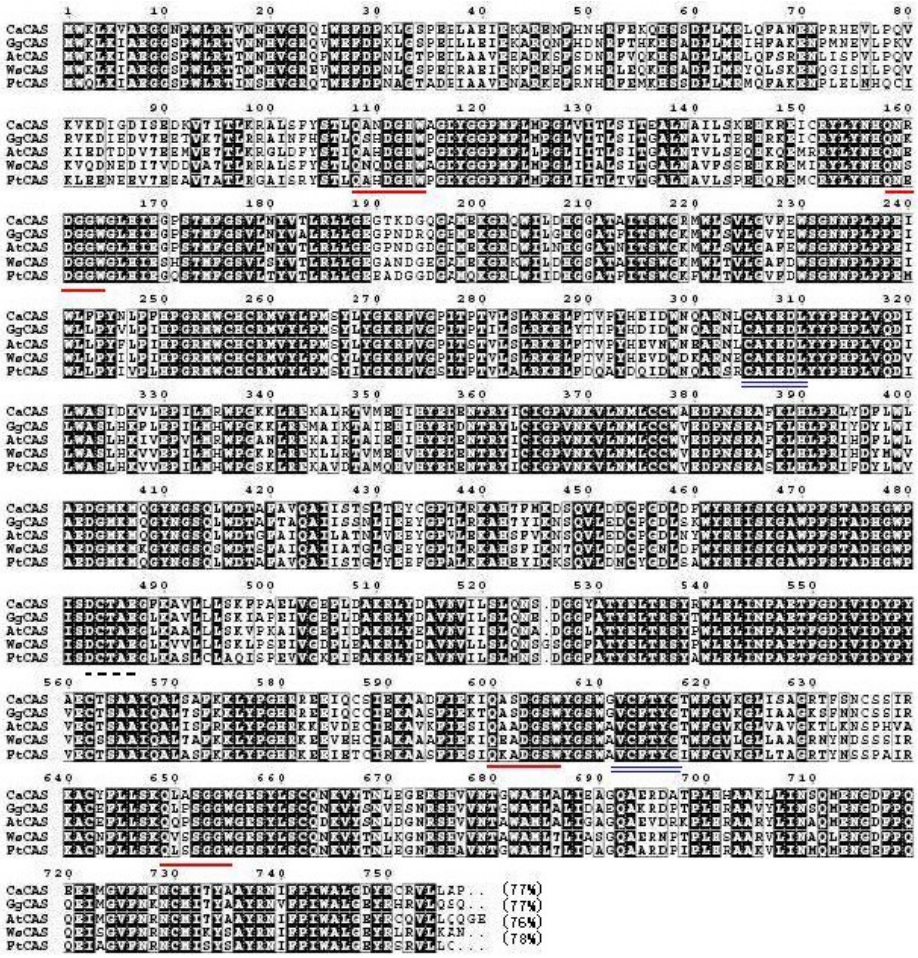

Figure 2. Multiple alignment of deduced amino acid sequences of *F. thunbergii* Miq. and other cycloartenol synthases. The amino acid residues identical in five protein sequences are boxed. The DCTAE motif is marked with a black dotted line, and the QW motifs are marked with red underlines. The highly conserved amino acids which were used to design degenerate primers are marked with blue double underlines. *CaCAS, Centella asiatica* cycloartenol synthase; *GgCAS, Glycyrrhiza glabra* cycloartenol synthase; *AtCAS, Arabidopsis thaliana* cycloartenol synthase; *WsCAS, Withania somnifera* cycloartenol synthase.

and these extracts were directly subjected to GC-EIMS analysis. GC-EIMS spectrometry was conducted on a Micromass GCT gas chromatograph-mass spectrometer (England) fitted with a DB-5MS column (0.25 mm × 30 m, 0.25 µm film thickness) (JWScientific, Folsom, CA, U.S.A.), with a helium flow rate of 1 ml/min, and operating at 70 eV ionization voltage with a scan range of 35 to 600 Da. The column temperature was set at 50°C for 2 min, then elevated to 300°C at 12°C/min, and held at 300°C for 15 min. The injection volume was 1 µl. Authentic cycloartenol (Sigma-Aldrich, Germany,

RESULTS

Cloning of putative cycloartenol synthase cDNA from *F. thunbergii* Miq.

In order to isolate the *CAS* gene of *F. thunbergii* Miq., degenerate primers were designed from the highly conserved regions of the other plant CAS (Figure 2).

PCRs were performed using the degenerate primers as described previously. The amplified core DNA fragments (approximately 460 and 930 bp in length) were cloned into pTG19-T vector (Generay, China). DNA sequencing of more than four clones of two core fragments showed that all clones have an identical sequence. The full length sequence of the cDNA was obtained by 5`-and 3`-RACE and named as *FtCAS*. The *FtCAS* cDNA contains an ORF of 2271 bp that encodes a 756 amino acid polypeptide. The deduced amino acid sequence of the cDNA shows the highest identities of 82, 78, 77, 77, 76, 76, 74 and 59% to the cycloartenol synthases of *Dioscorea zingiberensis, Withania somnifera, Centella asiatica, Glycyrrhiza glabra, Arabidopsis thaliana, P.*

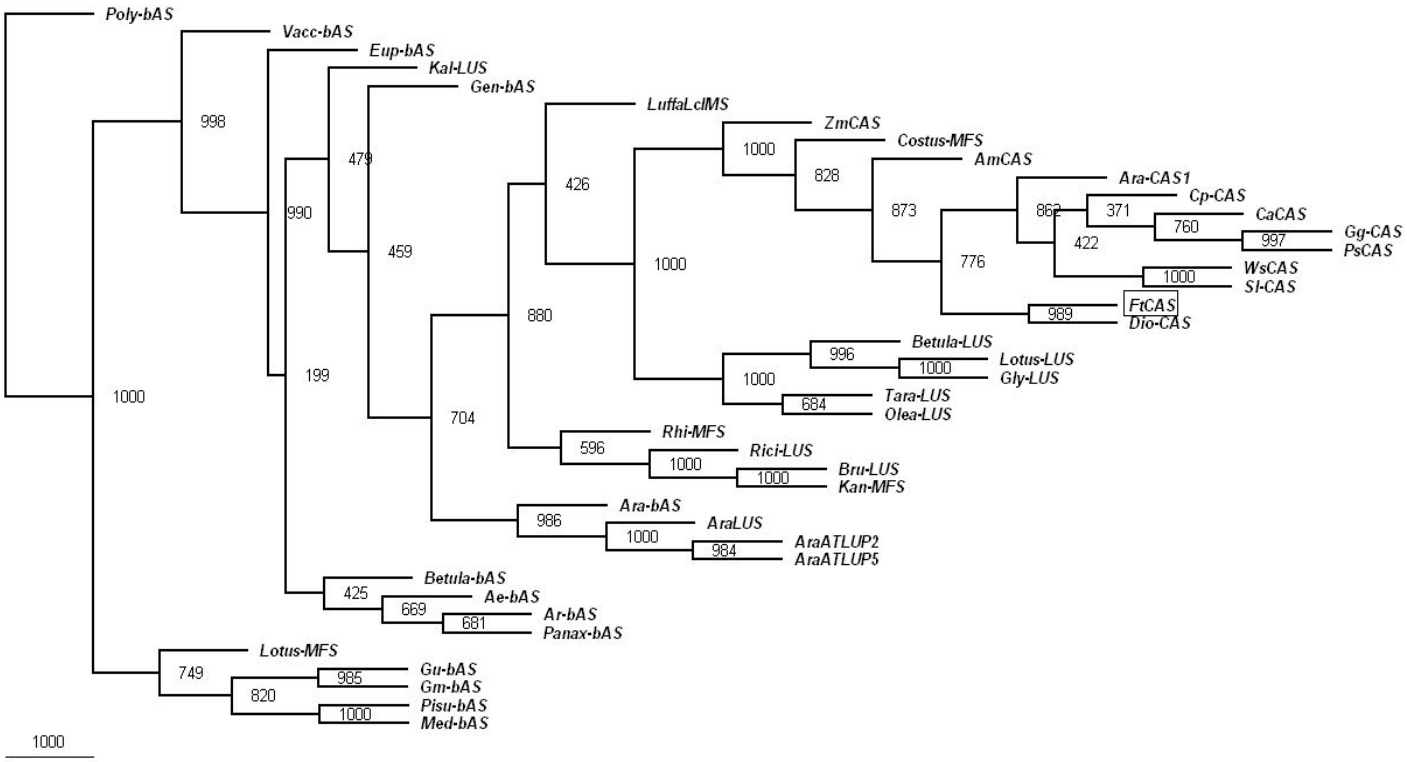

Figure 3. Phylogenetic tree of the deduced amino acid sequences of *FtCAS* and other plant OSCs (*FtCAS* is boxed). Numbers indicate bootstrap values from 1000 replicates. The DDBJ/GenBank/EMBL accession numbers of the sequences used in this analysis are as follows: U49919 (AraLus), DQ268869 (Rici-Lus), AB025345 (Tara-Lus), AB025343 (Olea-Lus), HM623871 (Kal-Lus), AB055511 (Betula-Lus), AB289586 (Bru-Lus), AB181245 (Lotus-Lus), U02555 (Ara-CAS1), AB116237 (Cp-CAS), AY520819 (CaCAS), NM-001158534 (ZmCAS), FN547822 (Dio-CAS), AB025968 (GgCAS), D89619 (PsCAS), EU449280 (Sl-CAS), HM037907 (WsCAS), AB374428 (Ara-bAS), AC024697 (Gen-bAS), FJ627179 (Gu-bAS), HM219225 (Ae-bAS), AY095999 (Gm-bAS), DQ915167 (Vacc-bAS), EF107623 (Poly-bAS), AB014057 (Panax-bAS), AB206469 (Eup-bAS), AY836006 (Ar-bAS), AJ430607 (Med-bAS), AB055512 (Betula-bAS), AB034802 (Pisu-bAS), AB257507 (Kan-MFS), AB263204 (Rhi-MFS), AB058508 (Costus-MFS), AF478455 (Lotus-MFS), AC002986 (AraATLUP2), AC007152 (AraATLUP5).

sativum, Cucurbita pepo, and *Zea mays* respectively (Figure 2). Four copies of QW-motif: [(K/R)(G/A)X X(F/Y/W)(L/I/V)XXXQXXXGXW] (Poralla et al., 1994; Kajikawa et al., 2005), which occurs repeatedly in the sequences of all known OSCs and may be involved in the binding and/or catalysis of squalene and oxidosqualene during the polycyclization reaction, are present in the *FtCAS* sequence. In addition, the DCTAE motif, which is claimed to be the active site of the rat OSCs (Abe, 1994), is observed in the deduced *FtCAS* sequence (Figure 2). These results suggest that *FtCAS* most probably was a cycloartenol synthase.

Phylogenetic analysis of FtCAS

To analyze the phylogenetic relationship of *FtCAS* among plant OSCs, a phylogenetic tree was constructed (Figure 3). Of these, lupeol synthase (LUS), CAS, beta-amyrin synthase (bAS), and multifunctional triterpene synthase (MFS) cluster together. In the tree, *FtCAS* belongs to a group of cycloartenol synthases (Figure 3). This is consistent with our results of the heterologous expression experiment described below.

Functional expression of the *FtCAS* cDNA in the *Pichia pastoris*

In order to elucidate the enzymatic activity of the *FtCAS* gene product, the cycloartenol composition of the methylotrophic yeast *P. pastoris* expressing the *FtCAS* gene was analyzed by GC-EIMS. *P. pastoris* cells contain ergosterol as a major sterol component but not cycloartenol (Sakaki et al., 2001). Therefore, the function of a cycloartenol synthase gene can be examined in *P. pastoris* without feeding a substrate (Kajikawa et al., 2005). The *FtCAS* ORF with its original stop codon was placed under the control of the methanol-inducible AOX1 promoter in the pPICZA expression vector (Invitrogen, Carlsbad, CA), and expressed in *P. pastoris* cells. The methanol–chloroform extracts of the cells were directly subjected to GC-EIMS analysis. As shown in Figure 4A, a novel peak was detected in the yeast expressing *FtCAS*

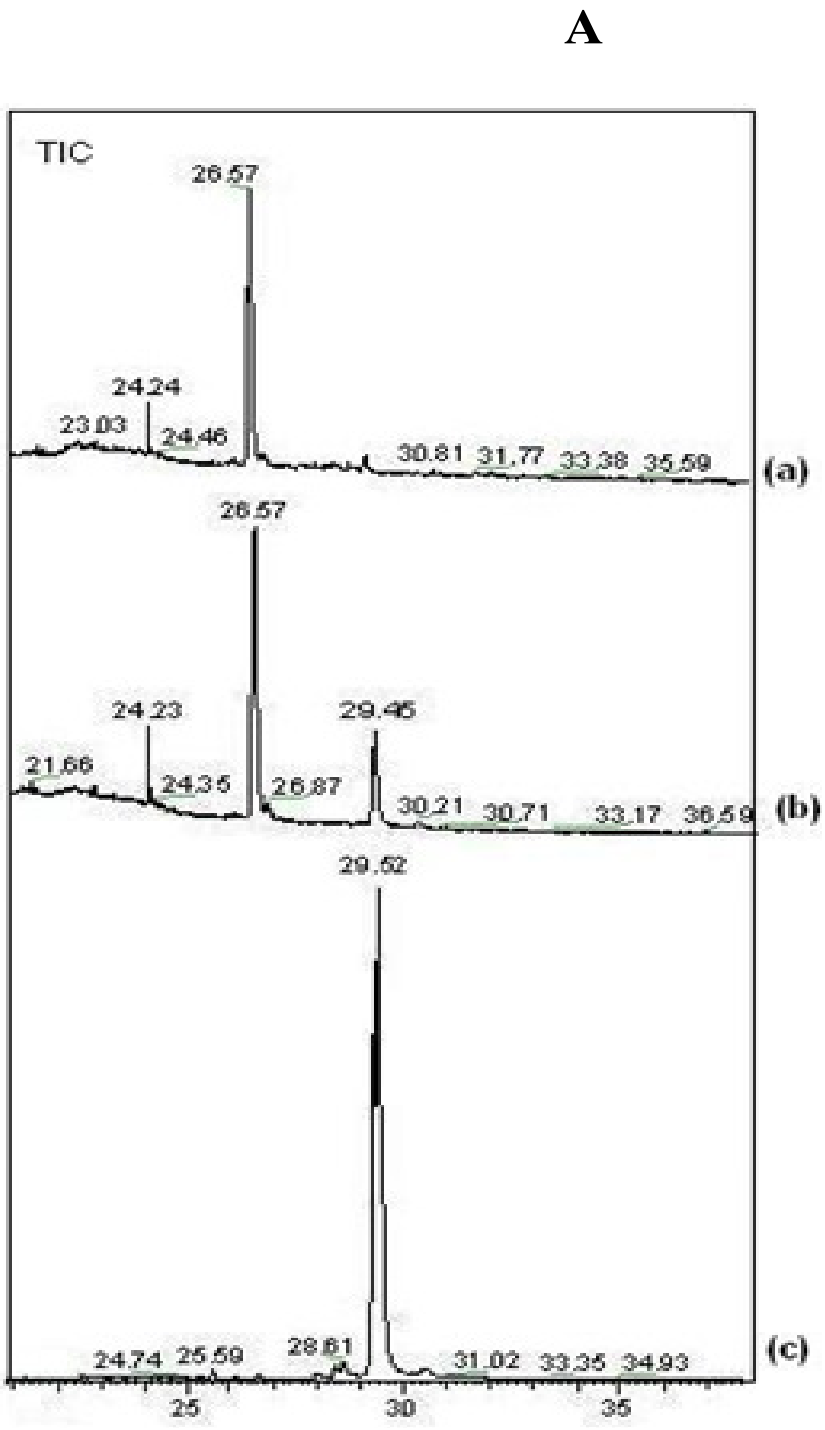

Figure 4. GC-EIMS analysis of *FtCAS* product. (A) TIC chromatogram of the cell extract from the yeast with pPICZA empty vector (a), pPICZA-*FtCAS* (b), and authentic cycloartenol standard (29.52 min) (c). Authentic cycloartenol was purchased from Sigma-Aldrich Co.LLC. Injection volume was 1 µl. (B) EI-mass spectra authentic peak (a) and a peak newly detected in the yeast with pPICZA-*FtCAS* (b). GC-EIMS, Gas chromatography-electron impact mass spectrometry; TIC, total ion current.

at retention time 29.45 min, but not in the control cells carrying empty vector. The retention time and MS fragmentation pattern of the peak were indistinguishable from authentic cycloartenol standard (Figure 4B).

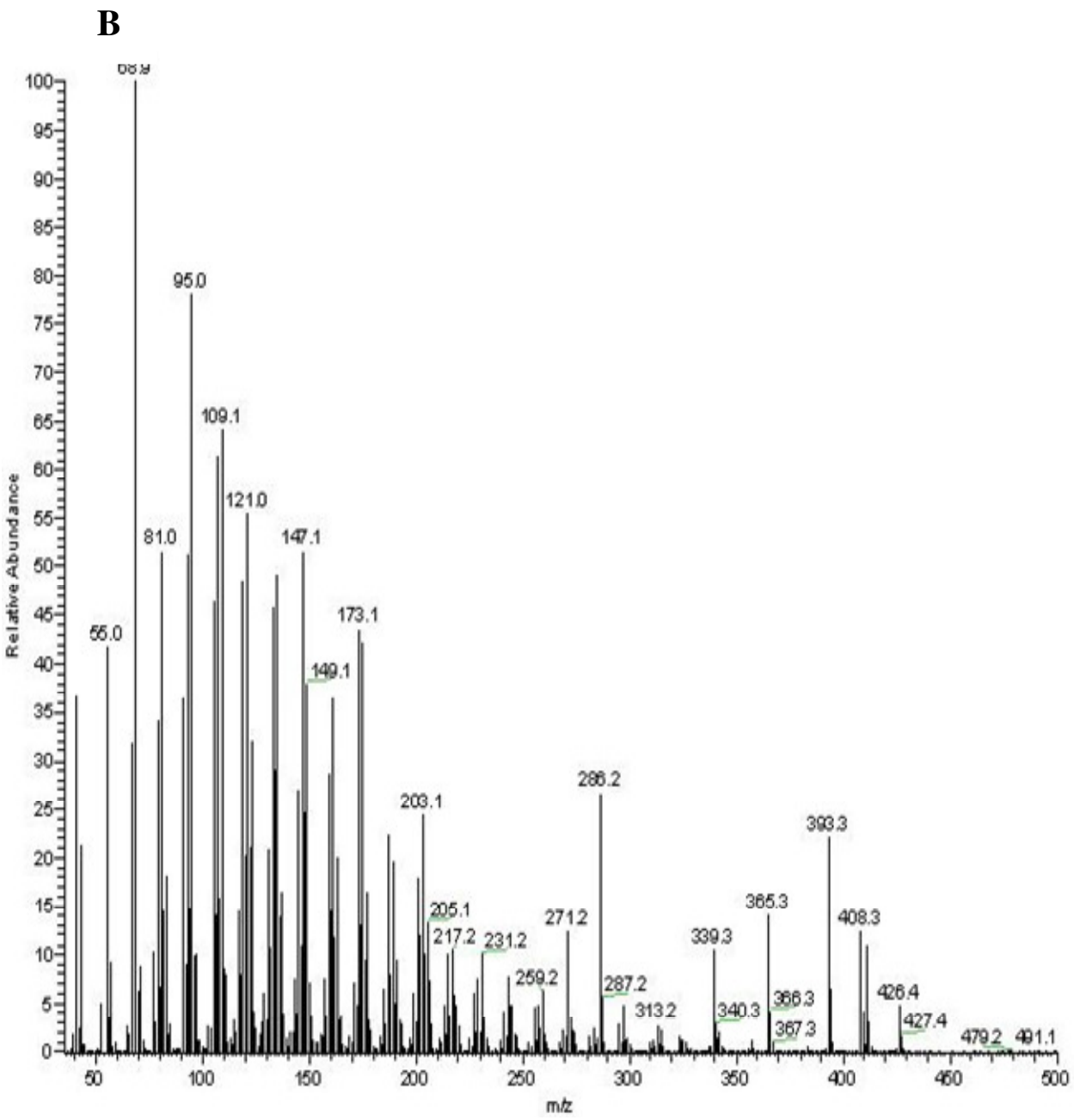

Figure 4. Contd.

DISCUSSION

Higher plants, especially Chinese traditional herbs, produce lots of secondary metabolites such as phytosterols and triterpenoids. OSCs catalyze the cyclization of 2,3-oxidosqualene, a common biosynthetic intermediate of both triterpenoids and phytosterols. This step is situated at the critical branching point for phytosterol and triterpenoid biosyntheses. *F. thunbergii* Miq. is a rich source of steroidal and alkaloid metabolites such as peimine, Peiminine, Propeimine and so on. However, the biosynthetic way of phytosterols is unclear. To well understand these aspects, relevant genes involved in the formation of secondary metabolites have to be cloned and characterized. In this study, we have succeeded in the molecular cloning of cDNA encoding CAS (*FtCAS*) from *F. thunbergii* Miq. Phylogenetic analyses revealed that *FtCAS* is closely related to other plant cycloartenol synthases, showing up to high sequence identity of the deduced amino acid sequences with homologues from other species. As functional characterization of *FtCAS* in the yeast strain X-33 yielded cycloartenol as the major

product, we concluded that *FtCAS* encodes a *F. thunbergii* cycloartenol synthase.

Previous mutagenesis studies on the role of amino acid residues (Tyr410, His477, and Ile481) in CAS have revealed the significant catalytical function (Herrera, et al., 2000; Meyer, et al., 2002). These catalytically important amino acid residues are usually conserved and also found in *FtCAS* (Figure 2). Molecular cloning of CAS in *F. thunbergii* Miq. provides a useful tool to elucidate the peimine biosynthesis of *F. thunbergii.* Further studies on gene expression and regulation of peimine biosynthesis in the roots and bulbus are in progress. It might give more insight into the physiological roles of sterols in fritillaria plant species.

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