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# Cloning and characterization of a cDNA encoding β-amyrin synthase from petroleum plant *Euphorbia tirucalli* L. ☆

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**Abstract**

*Euphorbia tirucalli* L., known as the petroleum plant, produces a large amount of triterpenes, such as β-amyrin. Degenerate RT-PCR based on the sequences conserved among known β-amyrin synthases led to cloning of a putative triterpene synthase cDNA, *EtAS*, from leaves of *E. tirucalli*. The deduced amino acid sequence of the *EtAS* cDNA showed the highest identity of 82% to the *Panax ginseng* β-amyrin synthase. Heterologous expression of the *EtAS* ORF in the methylotrophic yeast, *Pichia pastoris*, resulted in production of β-amyrin, revealing that the *EtAS* cDNA codes for a β-amyrin synthase. This is the first report of a gene involved in the triterpene synthetic pathway from *Euphorbiaceae* plants.


*Keywords:* *Euphorbia tirucalli* L.; *Euphorbiaceae*; Petroleum plant; Heterologous expression; *Pichia pastoris*; Oxidosqualene cyclase; Triterpenoid; β-Amyrin

## 1. Introduction

Sterols are structural components of the membrane system and serve as precursors for various hormones in eukaryotes. Higher plants produce not only sterols but also various skeletal types of triterpenes. Some triterpene saponins, which are synthesized by glycosylation of triterpenes, are thought to be involved in plant defense systems against microbial pathogens or insects (Osbourn, 1996; Papadopoulou et al., 1999; Agrell et al., 2003), however, the functions of most triterpenes are still unclear. All sterols and triterpenes are synthesized from 2,3-oxidosqualene (**1**) (Abe et al., 1993). The cyclizations of **1** into tetracyclic or pentacyclic carbon skeletons of sterols or triterpenes are remarkable as one of the most complicated biosynthetic reactions catalyzed by a single enzyme. The enzymes which perform these reactions belong to the oxidosqualene cyclase (OSC) family. In higher plants, OSC family member cycloartenol synthase is responsible for sterol biosynthesis, and other OSCs for triterpene synthesis.

β-Amyrin (**2**) is one of the most common triterpenes in plants. Its pentacyclic carbon skeleton, which is folded in pre-chair-chair-chair conformation, is derived from the precursor **1** after a series of processes (Fig. 1) mediated by OSC β-amyrin synthase (Kushiro et al., 1998a). Up to now, β-amyrin synthase genes have been isolated from a number of dicots, *Panax ginseng* (*PNY*; Kushiro et al., 1998a, *PNY2*; Kushiro et al., 1998b), *Pisum sativum* (PSY; Morita et al., 2000), *Glycyrrhiza*

☆ The nucleotide sequence data reported in this paper appear in the GenBank/EMBL/DDBJ nucleotide sequence databases with the accession numbers, AB206469 (*EtAS*) and AB206470 (EtOSC).

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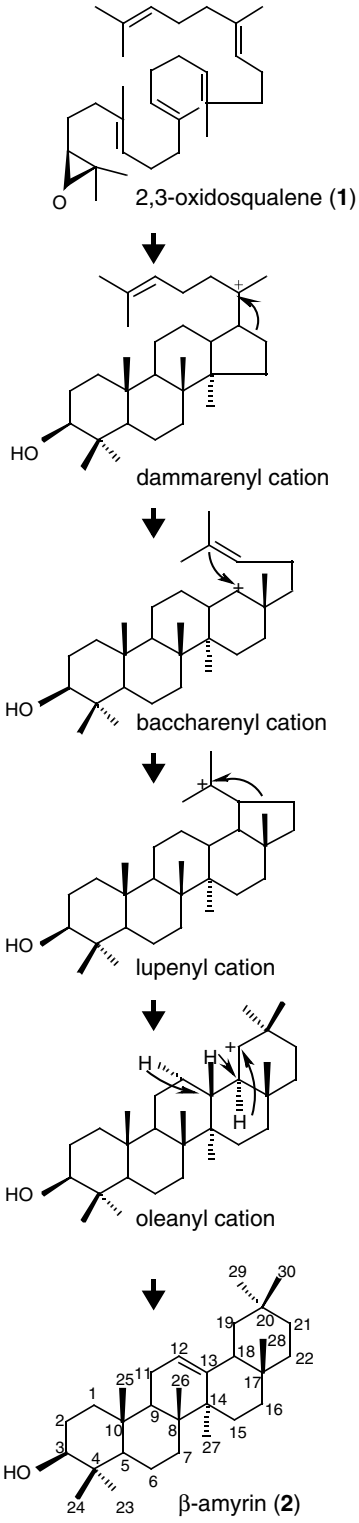


Fig. 1. Cyclization of 2,3-oxidosqualene (**1**) to triterpene β-amyrin (**2**) mediated by β-amyrin synthase.

*glabra* (*GgbAS1*; Hayashi et al., 2001), *Medicago truncatula* (Suzuki et al., 2002) and *Betula platyphylla* (Zhang et al., 2003), and a monocot, *Avena strigosa* (Haralampidis et al., 2001). Another group of OSC genes were isolated from dicot species, *Arabidopsis thaliana* ATLUP1 (Herrera et al., 1998; Segura et al., 2000), *A. thaliana* ATLUP2 (Kushiro et al., 2000; Husselstein-Muller et al., 2001), *Lotus japonicus* LjAMY2 (Iturbe-Ormaetxe et al., 2003) and *P. sativum* PSM (Morita et al., 2000), and a monocot, *Costus speciosus* (Kawano et al., 2002). Enzymes encoded by the latter group of genes catalyze reactions which convert oxidosqualene into not only **2** but also other triterpenes, and thus are "multifunctional", which is in contrast to "monofunctional" β-amyrin synthase.

*Euphorbia tirucalli* produces a large amount of triterpenes and sterols (Ohyama et al., 1984), which makes this plant not only a source of genes encoding enzymes with high activity in the triterpene and sterol biosynthetic pathways, but also a candidate organism for production of useful triterpenes and sterols by transgenic technology. An efficient regeneration system (Uchida et al., 2004) and an EST database (Kajikawa et al., 2004) of *E. tirucalli* have been recently established. Here, we report on the first cloning and characterization of a β-amyrin synthase gene from *E. tirucalli*.

## 2. Results and discussion

### 2.1. Cloning of putative β-amyrin synthase cDNA from E. tirucalli

In order to isolate a *E. tirucalli* β-amyrin synthase gene, degenerate primers were designed from the highly conserved regions of the other plant β-amyrin synthases (Fig. 2). Degenerate PCR was performed using cDNA from leaves including young stems of *E. tirucalli* plants as a template, since this tissue contains **2** (Kajikawa et al., unpublished results). The product amplified was approximately 550-bp in length (data not shown), corresponding to the distance between the degenerate primers in the sequences of β-amyrin synthase genes of the other plants. The DNA fragment was cloned into a T-vector. DNA sequences of the 22 independent clones were all identical. The full length sequence of the cDNA was obtained by 5′- and 3′-RACE and named as *EtAS*. The *EtAS* cDNA contained an ORF of 2289 nt, which codes for 762 amino acid residues. The deduced amino acid sequence of the cDNA showed the highest identity of 82% to the *P. ginseng* β-amyrin synthase PNY (Morita et al., 2000) (Fig. 2). Six copies of QW-motif: [(K/R)(G/A)XX(F/Y/W)(L/I/V)XXXQXXXGXW] (Poralla et al., 1994; Abe and Prestwich, 1995), which occurs repeatedly in the sequences of all known OSCs including β-amyrin synthase, are present in the EtAS sequence, as well as a DCTAE motif, which has been claimed to be in the active site of the rat OSCs, lanosterol synthases (Abe and Prestwich, 1994, 1995). From the high level of

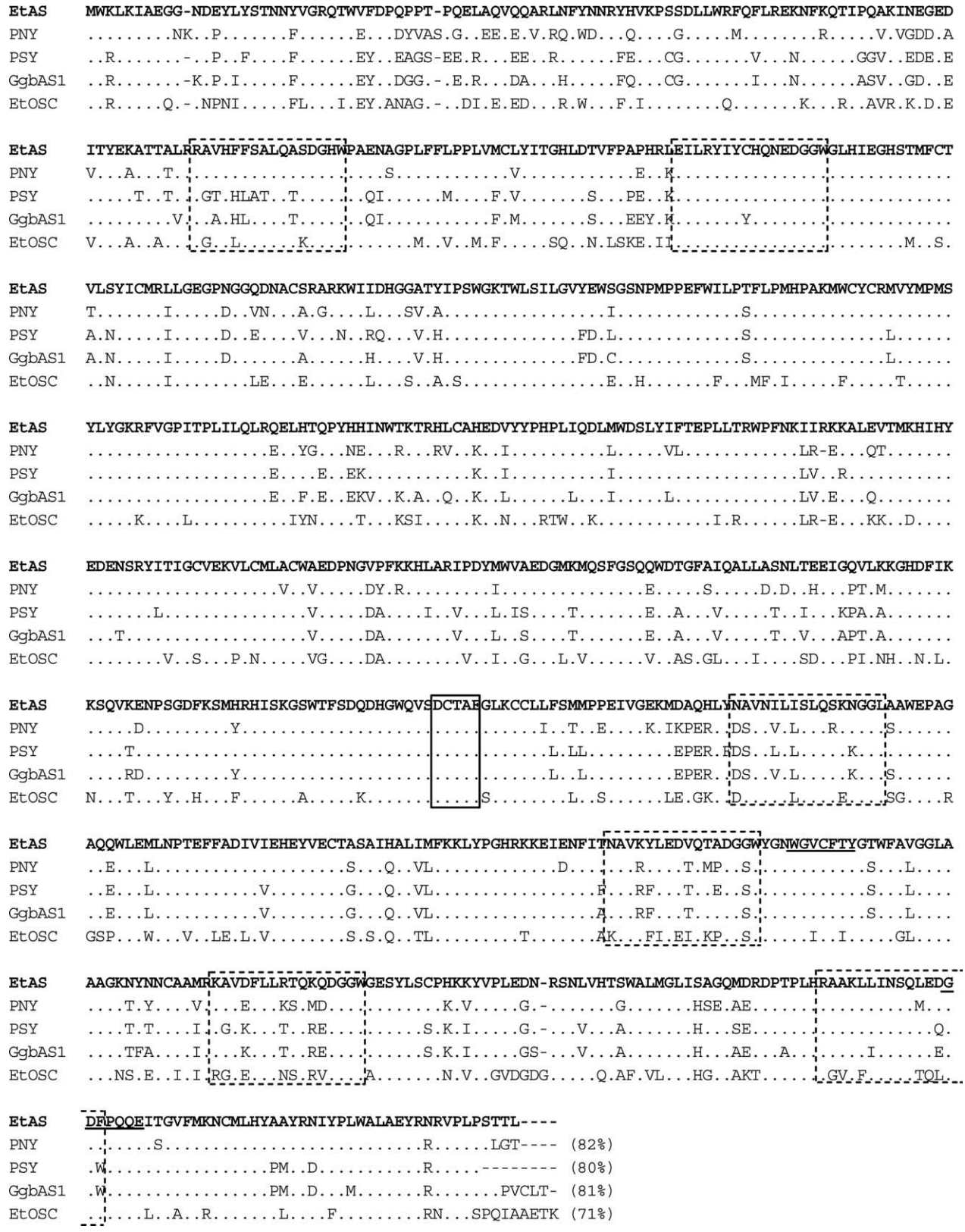


Fig. 2. Alignment of the deduced amino acid sequences of *E. tirucalli* and other β-amyrin synthases. The DCTAE motif is boxed, and the QW motifs are boxed by broken line. Dots indicate residues that are identical to those in the EtAS sequence, and dashes indicate alignment gaps. The portions that the degenerate primers were designed for are indicated by underlines. The sequence identities between the EtAS sequence and the other sequences are indicated in parentheses at the end of each sequence. The DDBJ/GenBank/EMBL accession numbers of the sequences are AB206469 (EtAS), AB009030 (PNY from *Panax ginseng*), AB037203 (GgbAS1 from *G. glabra*), AB034802 (PSY from *Pisum sativum*) and AB206470 (EtOSC).

sequence identity with β-amyrin synthases, the *EtAS* cDNA may code for β-amyrin synthase.

### 2.2. Functional analysis of the EtAS cDNA in Pichia pastoris

In order to elucidate the enzymatic activity of the *EtAS* gene product, the triterpene composition of the methylotrophic yeast *P. pastoris* expressing the *EtAS* gene was analyzed by GC-EIMS. *P. pastoris* cells contain ergosterol as a major sterol component (Sakaki et al., 2001; a peak at 19.1 min in Fig. 3), which is synthesized from oxidosqualene via lanosterol, but not triterpenes such as **2** (see Fig. 3(b)). Therefore, the function of a triterpene synthase gene can be examined in *P. pastoris* without feeding a substrate.

The *EtAS* 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) (pPICZA-EtAS), and

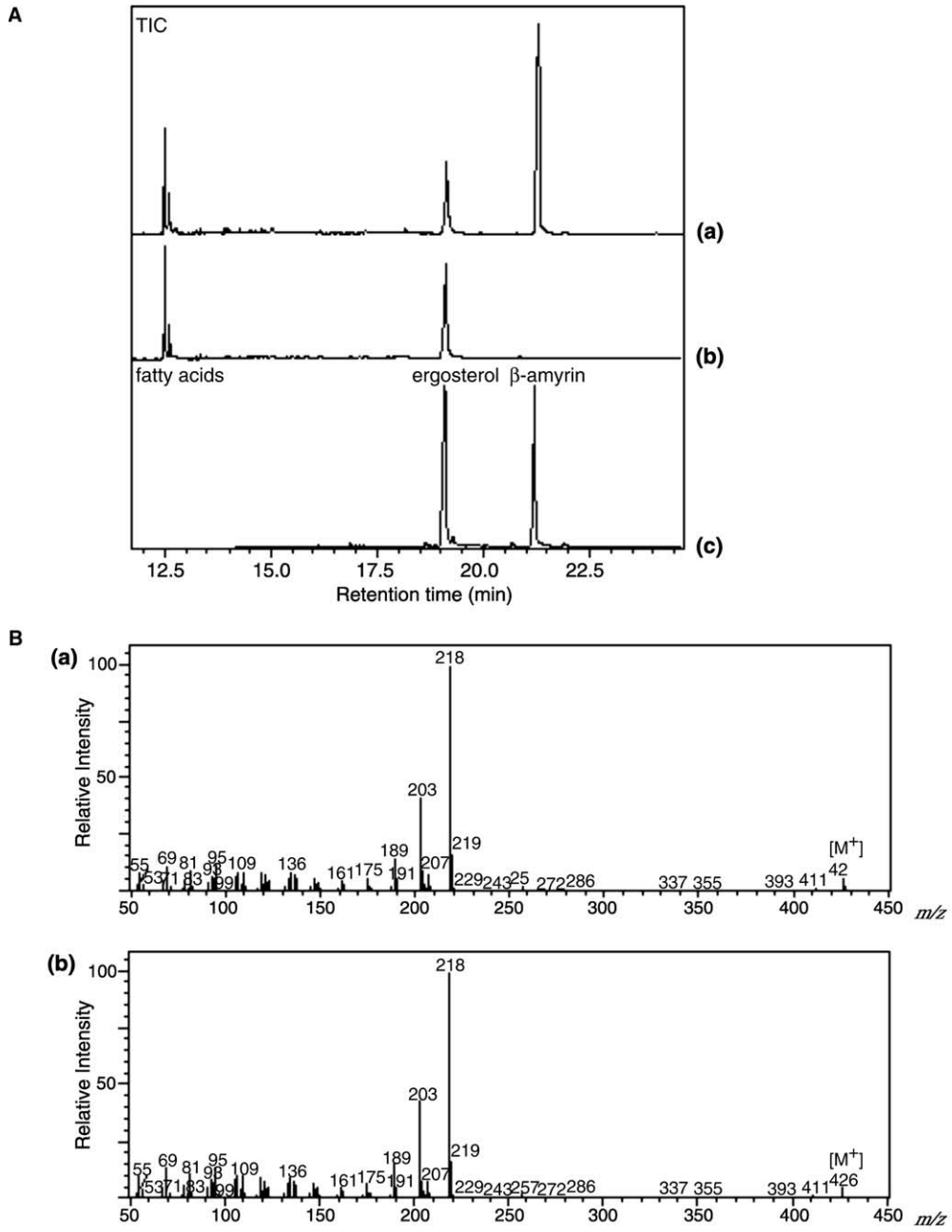


Fig. 3. GC-EIMS analysis of EtAS product. A, TIC chromatogram of the cell extract from the yeast with pPICZA-EtAS (a), with empty vector as a control (b), and mixture of authentic ergosterol (19.1 min) and **2** (21.2 min) (c). B, Mass spectra of authentic **2** (a) and a peak newly detected in the yeast with pPICZA-EtAS (b). *m/z* 426 [$M^+$]; 411 [$M^+$–$CH_3$]; 393 [$M^+$–$H_2O$–$CH_3$]; 218 (C-ring fragment peak, Budzikiewicz et al., 1963); 203 [*m/z* 218-$CH_3$].

expressed in *P. pastoris* cells. Unlike a comparable report by Ruf et al. (2004), where an OSC gene was fused to the HIS tag of pPICZA for subsequent purification and in vitro enzymatic assay, only the *EtAS* ORF was expressed, and methanol–chloroform extracts of the cells were directly subjected to GC-EIMS analysis. As shown in Fig. 3, a novel peak was detected in the yeast expressing *EtAS* at retention time 21.2 min, but not in the control cells carrying empty vector. The retention time and MS fragmentation pattern of the peak were indistinguishable from those of authentic **2**. To further confirm the identity of the product as **2**, preparative-scale culture (4 L) was carried out. The product was extracted and purified by silica-gel column chromatography to give 4 mg sample. $^{1}$H- and $^{13}$C-NMR spectra were identical with the authentic **2**. These results suggest

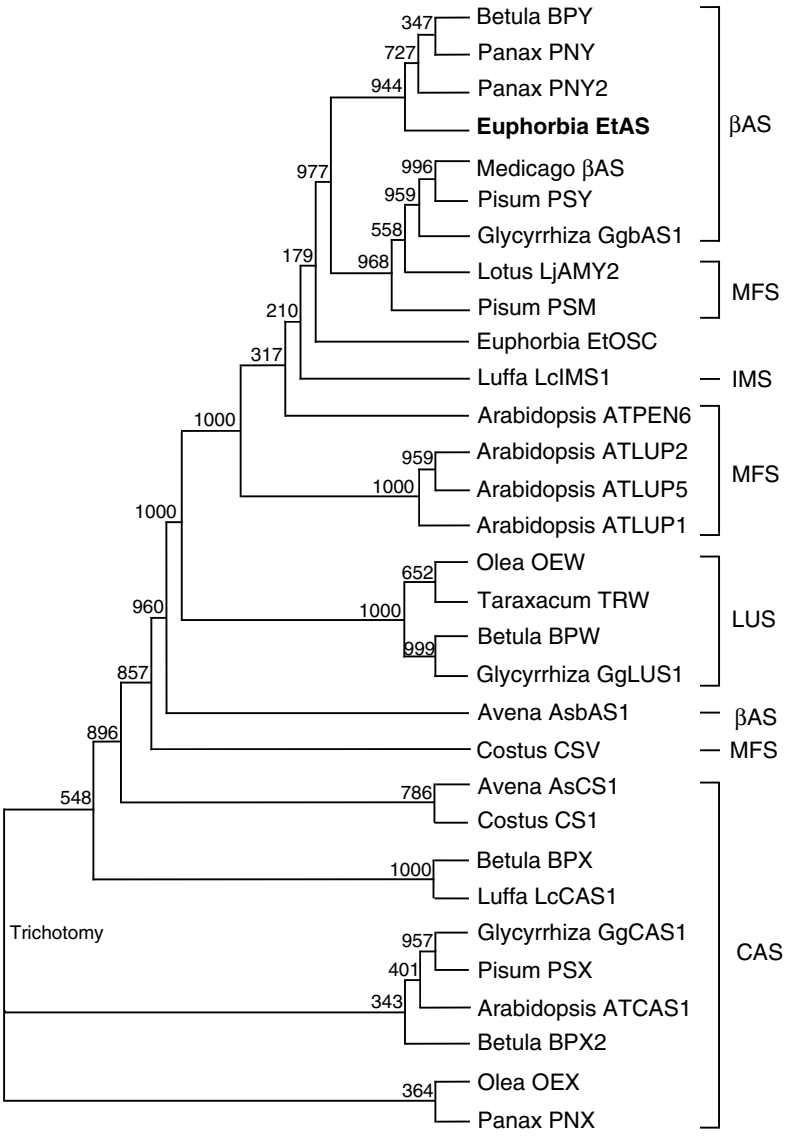


Fig. 4. Phylogenetic tree of the deduced amino acid sequences of EtAS and other plant OSCs. The phylogenetic tree was constructed using the sequences of EtAS and plant OSCs (based on Hayashi et al., 2004). Numbers indicate bootstrap values from 1,000 replicates. The DDBJ/GenBank/EMBL accession numbers of the sequences used in this analysis are as follows: AB055512 (Betula BPY), AB014057 (Panax PNY2), AJ430607 (Medicago βAS), AF478455 (Lotus LjAMY2), AB034803 (Pisum PSM), AB058643 (Luffa LcIMS1), AC007260 (Arabidopsis ATPEN6), AC002986 (Arabidopsis ATLUP2), AC007152 (Arabidopsis ATLUP5), U49919 (Arabidopsis ATLUP1), AB025343 (Olea OEW), AB025345 (Taraxacum TRW), AB055511 (Betula BPW), AB116228 (Glycyrrhiza GgLUS1), AJ311789 (Avena AsbAS1), AB058508 (Costus CSV), AJ311790 (Avena AsCS1), AB058507 (Costus CS1), AB055509 (Betula BPX), AB033334 (Luffa LcCAS1), AB025968 (Glycyrrhiza GgCAS1), D89619 (Pisum PSX), U02555 (Arabidopsis ATCAS1), AB055510 (Betula BPX2), AB025344 (Olea OEX) and AB009029 (Panax PNX), βAS, β-amyrin synthase (monofunctional type); CAS, cycloartenol synthase; IMS, isomultiflorenol synthase; LUS, lupeol synthase; MFS, multifunctional triterpene synthase.

that *EtAS* codes for β-amyrin synthase, and also that *P. pastoris* is a convenient system for detecting enzymatic activities of OSCs.

A phylogenetic tree constructed from the deduced amino acid sequences of plant OSCs is shown in Fig. 4. In the tree, EtAS belongs to a group of the dicot monofunctional β-amyrin synthases. This is consistent with our result of the heterologous expression experiment described above. Several plant species, such as *A. thaliana*, *P. ginseng*, and *P. sativum*, have more than one triterpene synthase genes (Fig. 4). A homologue of the β-amyrin synthase gene, termed *EtOSC*, was also found in the *E. tirucalli* ESTs (Kajikawa et al., 2004; ETC010D09) (Figs. 2 and 4), however, it did not exhibit triterpene synthase activity in the *P. pastoris* expression system under the condition described below (data not shown).

*E. tirucalli* contains many kinds of characteristic triterpenes and their derivatives, such as euphol (McDonald et al., 1949), taraxasterol (Haines and Warren, 1949), tirucallol (Haines and Warren, 1949), euphorbinol (Afza et al., 1979a), cycloeuphornol (Afza et al., 1979b), cycloart-23-ene-3β,25-diol (Khan et al., 1987), glut-5-en-3β-ol (Khan et al., 1987), cycloeuphordenol (Khan et al., 1988a), cyclotirucanenol (Khan et al., 1988b), euphorginol (Rasool et al., 1989) and euphorcinol (Khan et al., 1989), in addition to **2**. Further screening for triterpene synthase genes is necessary to clarify the triterpene biosynthetic pathways in *E. tirucalli*.

## 3. Experimental

### 3.1. Plant and yeast materials

Plants of *E. tirucalli* were cultivated in pots at 30 °C under natural light. Their leaves including young stems were harvested, immediately frozen in liquid nitrogen and stored at −80 °C for RNA preparation. The wild-type *P. pastoris* strain PPY1 (NRRL Y-11430) (Gould et al., 1992) was used for transformation and cultured in a rich YPD medium (1.0% yeast extract, 2.0% peptone, 2.0% glucose) supplemented with 100 μg/ml Zeocin™ (Invitrogen). Recombinant *P. pastoris* cells were cultured in a minimal medium composed of 1.34% yeast nitrogen base with ammonium sulfate without amino acids, $4 \times 10^{-5}$% biotin and a carbon source (1.0% glycerol or 0.5% methanol for gene expression).

### 3.2. E. tirucalli cDNA preparation

Poly(A)$^+$ RNA was isolated from 10 g of the frozen tissues of *E. tirucalli* using Concert Plant RNA Reagent (Invitrogen), and PolyATract System 1000 (Promega, Madison, WI) according to the manufacturers' instructions. One μg of the poly(A)$^+$ RNA was reverse-transcribed using the Ready-To-Go T-primed First Strand kit (Amersham Biosciences, Pollards Wood, UK) with oligo(dT) primer (5′-AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTT-TTTT-3′) in a total volume of 20 μl for 1 h at 42 °C according to the manufacturers' instructions. To remove RNA complementary to the cDNA, *Escherichia coli* RNaseH (Takara Bio, Shiga, Japan) was added to the reaction mix and incubated for 20 min at 37 °C. The resulting cDNA mixture was directly used as a template for PCR.

### 3.3. Cloning of a fragment of β-amyrin synthase

Based on highly conserved sequences, two degenerate primers were synthesized for degenerate PCR: forward primer dOSC-01F: 5′-TGGGSIGTITGYTTYAYICA-3′ (degenerate for the conserved amino acid sequence W(G/A)VCF(T/I)Y) and reverse primer dOSC-02R: 5′-TCYTGYTGIGGRAARTCICC-3′ (degenerate for the conserved amino acid sequence GDFPQQE), where R, Y, S and I are mixtures of nucleotides A/G, C/T, G/C and inosine, respectively. Using the *E. tirucalli* cDNA as a template, degenerate PCR was carried out under the following condition: 35 cycles of 94 °C for 1 min, 45 °C for 1.5 min and 72 °C for 2 min. After separation in 1% (w/v) agarose gel, DNA fragments of the expected size (500–600 bp) were recovered from the gel by Prep-A gene (Promega), ligated into pT7Blue Tvector (EMD/Merck Biosciences, Darmstadt, Germany) and transformed into the *E. coli* ElectroMax™ DH10B cells (Invitrogen). *E. coli* cells were cultured in a low salt Luria-Bertani medium (0.5% yeast extract, 1.0% peptone, 0.5% NaCl) supplemented with 40 μg/ml Zeocin™.

### 3.4. 5′- and 3′-end amplification of the EtAS cDNA

The sequence of mRNA was determined by RACE, using the 5′-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) and the Ready-To-Go T-primed First Strand kit (Amersham Biosciences) with primers, EtAS-08R: 5′-ATGACAGTAGATGTAACGAAGG-3′ and EtAS-01F: 5′-GGAGGTTGGGGAGAAAGCTACC-3′ for 5′- and 3′-RACE of *EtAS*, respectively. One μg of poly(A)$^+$ RNA was reverse-transcribed according to the manufacturers' instructions. Each PCR product was cloned and sequenced.

### 3.5. Sequence and phylogenetic analyses

Multiple sequence alignments were generated using the CLUSTAL W program (Thompson et al., 1994). Phylogenetic analysis of deduced amino acid alignments was performed using the neighbor-joining method

with the program and TreeView software (Page, 1996). Bootstrap analysis with 1000 replicates was used to assess the strength of nodes in the tree (Felsenstein, 1985).

### 3.6. Plasmid construction and yeast transformation

Two primers, EtAS-15F: 5′-GTTGAATTCATCATGTGGAAGCTGAAGATA-3′ and EtAS-13R: 5′-AAATTAAAGAGTAGTGGAAGGC-3′, were used to amplify a DNA fragment of the *EtAS* ORF. PCR was performed under the following condition: 25 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, using *Pyrobest* DNA polymerase (Takara Bio) and the cDNA mixture reverse-transcribed by oligo(dT) primer described above as a template. The amplified cDNA was phosphorylated by T4 DNA kinase (Takara Bio) and subcloned into the *Pml*I site of the *P. pastoris* expression vector pPICZA (Invitrogen) to place the *EtAS* ORF under the control of the methanol-inducible promoter, 5′ *AOX1* (pPICZAEtAS). *P. pastoris* wild-type strain PPY1 was transformed with pPICZA-EtAS and pPICZA using the *Pichia* EasyComp kit (Invitrogen).

### 3.7. Gas chromatography-electron impact mass spectrometry (GC-EIMS) analysis of triterpenoids

Methanol–chloroform (2:1, v/v) extracts of yeast cells were directly subjected to GCEIMS analysis. GC-EIMS spectrometry was conducted on a Shimadzu GCMS-QP2010 (Shimadzu, Kyoto, Japan) fitted with a DB-5 ms column ($\phi$ 0.25 mm × 30 m, 0.25 μm film thickness) (J W Scientific, Folsom, CA) with helium carrier (flow rate 1 ml/min), operating at an ionization voltage of 70 eV with a scan range of 50–550 Da. The column temperature was maintained at 40 °C for 1 min, elevated to 320 °C at (20 °C/min) and then held at 300 °C for 15 min. Authentic ergosterol and **2** (Extrasynthese, Genay, France), were directly subjected to GC-EIMS under the same condition.

### 3.8. $^{1}H$- and $^{13}C$-NMR

For NMR analysis, the yeast transformant with pPICZA-EtAS was cultured for 48 h in 4.0 L-scale of minimal methanol medium for *EtAS* gene expression. Collected cells were refluxed with 200 ml 20% KOH/50% EtOH for 1 h as described by Morita et al. (2000).

The material was extracted with 200 ml hexane two times and concentrated. The extract was purified by a silica gel column chromatography (EMD/Merck Biosciences) with hexane/ethyl acetate (9:1, by vol.). The fractions corresponding to triterpene were collected and evaporated. The purified extract was directly used for NMR measurements on a Bruker ARX500. $^{1}H$- and $^{13}C$-NMR were measured in $CDCl_3$ with trimethylsilate as an internal standard.

$^{1}H$-NMR (500 MHz, $CDCl_3$): $\delta$ (3H, s, C-24), 0.83 (3H, s, C-28), 0.87 (6H, s, C-29, C-30), 0.94 (3H, s, C-25), 0.97 (3H, s, C-26), 1.00 (3H, s, C-23), 1.14 (3H, s, C-27), 3.22 ($^{1}H$, dd, $J$ = 10.9, 4.0 Hz, C-3), 5.18 ($^{1}H$, t, $J$ = 3.5 Hz, C-12).

$^{13}C$-NMR (125 MHz, CDCl3); $\delta$ 15.5 (C-25), 15.6 (C-24), 16.8 (C-26), 18.4 (C-6), 23.5 (C-11), 23.7 (C-30), 26.0 (C-27), 26.1 (C-15), 26.9 (C-16), 27.2 (C-2), 28.1 (C-23), 28.4 (C- 28), 31.1 (C-20), 32.5 (C-17), 32.6 (C-7), 33.3 (C-29), 34.7 (C-21), 36.9 (C-10), 37.1 (C-22), 38.6 (C-1), 38.8 (C-4), 39.8 (C-8), 41.7 (C-14), 46.8 (C-19), 47.2 (C-18), 47.6 (C-9), 55.1 (C-5), 79.0 (C-3), 121.7 (C-12), 145.2 (C-13).

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