Cloning and Functional Analysis of a β-Amyrin Synthase Gene Associated with Oleanolic Acid Biosynthesis in *Gentiana straminea* Maxim.

Yanling Liu,[a,#] Yunfei Cai,[b,#] Zhongjuan Zhao,[a] Junfeng Wang,[a] Jing Li,[c] Wei Xin,[a] Guangmin Xia,[a] and Fengning Xiang*,[a]

[a] *The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Science, Shandong University; Shanda Nanlu No. 27, Jinan 250100, P.R.China:* [b] *Department of Bioscience and Biotechnology, Shandong Institute of Education; Jinan 250013, P.R.China: and* [c] *College of Pharmaceutical Science, Nankai University; Tianjin 300071, P.R.China.* Received December 22, 2008; accepted February 23, 2009; published online February 27, 2009

Phytosterols and triterpenes are synthesized by oxidosqualene cyclases (OSCs) *via* the isoprenoid pathway. Here, *GsAS1*—a full-length β-amyrin synthase cDNA isolated from *Gentiana straminea* Maxim.—was characterized. Its open reading frame consists of 2268 bp, predicted to encode a 756 residue protein containing four QW and one Asp-Cys-Thr-Ala-Glu (DCTAE) motifs, which are both well conserved among known triterpene synthases. The deduced GsAS1 peptide sequence shares 76.2% homology with that of *Panax ginseng* β-amyrin synthase. A phylogenetic analysis showed that GsAS1 is closely related to other plant OSCs, and particularly to the β-amyrin synthases. When the *GsAS1* sequence was heterologously expressed in *Escherichia coli*, an 88 kDa gene product was produced, and this reacted with the appropriate antibody. The sequence was also heterologously expressed in the *Pichia pastoris* yeast. *GsAS1* is expressed in a tissue-specific manner, with its expression in the leaf being *ca.* 4.5-fold than that in the root, and nearly three-fold than that in the stem. *GsAS1* expression was up-regulated by treatment with methyl jasmonate (MeJA) over a period from 6 h to 10 d post treatment. The accumulation oleanolic acid increased after induction by MeJA.

Key words *Gentiana straminea*; β-amyrin synthase; methyl jasmonate; *Pichia pastoris*; triterpenoid

Gentiana straminea Maxim. (Mahuaqinjiao) belongs to the *Gentianaceae* family, and is an important medicinal plant in China. It is found on the Qinghai-Tibetan plateau at altitudes between 2000 and 5000 m asl,[1] and is a rich source of secoiridoid glucosides, including gentiopicrosidel, loganic acid, swertiamarin, oleanolic acid and roburic acid,[2] all of which have been associated with medicinal properties. The triterpenoid oleanolic acid has been used to treat hepatitis and a number of diseases of the immune system.[3,4] Wild populations of *G. straminea* have been threatened by excessive excavation, and its seeds germinate poorly in low altitude environments.[5] Hence there is a need to enhance the production of oleanolic acid by secondary metabolic engineering in the condition of raw *G. straminea* limited.

β-amyrin is thought to act as a precursor for oleanolic acid, and is associated with oleanolic acid production.[6] It is synthesized within the isoprenoid pathway by the cyclization of 2,3-oxidosqualene, catalysed by β-amyrin synthase (an oxidosqualene cyclase, or OSC) (Fig. 1). A number of OSCs have been cloned to date, and their enzyme functions identified by heterologous expression in yeast.[7,8] These enzymes can have either mono- or multifunctional activity in higher plants, and yield various triterpene products.[9—11] Furthermore, some research shows that OSCs are key enzymes in the isoprenoid pathway to form different products.[12—16] Thus β-amyrin synthase in *G. straminea* may represent a key enzyme for oleanolic acid synthesis, and the cloning of its encoding gene is required for any effort to raise the content of a secondary metabolite such as oleanolic acid using metabolic engineering.

Methyl jasmonate (MeJA) is an elicitor which enhances the content of triterpene saponins.[17—20] The indications are that the accumulation of triterpene saponins is related to OSC over-expression, mediated by MeJA. Thus, the relationship between OSC gene expression and oleanolic acid production in the presence of MeJA may be critical to our understanding of the mechanism of secondary product accumulation. Here, we describe the characteristics of *G. straminea* β-amyrin synthase, and show how manipulation of its expression level affects the quantity of oleanolic acid in the presence of MeJA.

MATERIALS AND METHODS

Plant Materials *G. straminea* samples were collected from Yushu Country, Qinghai Province, China. Voucher specimens have been deposited in Qinghai Normal University.

Bacterial and Yeast Strains and Vectors *Escherichia coli* strain BL21(DE3) and the pET28(a) vector provided a prokayotic expression system. Bacterial cells were grown at 37 °C in LB medium containing 0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl and 100 μg/ml kanamycin. *Pichia pastoris* strain X-33 and the pPICZA vector provided an eukaryotic expression system. Yeast cells were grown at 30 °C in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose supplemented with 100 μg/ml Zeocin. Media were solidified with 2% (w/v) agar.

cDNA Preparation Total RNA of *G. straminea* was extracted using the Trizol reagent (Invitrogen, U.S.A.), following the manufacturer's instructions. The quality and concentration of RNA were assessed by ethidium bromide-stained agarose (1% w/v) gel electrophoresis and spectrophotometry. The RNA was converted to cDNA by providing 1 μg total RNA as a template in a 20 μl reverse-transcription reaction using M-MLV reverse transcriptase (TaKaRa, Tokyo, Japan) in the presence of 1 μl 0.5 μg/μl oligo$(dT)_{18}$ (TaKaRa, Tokyo, Japan) and 2 μl 10 mM deoxy-ribonucleoside triphosphate (dNTP) (TaKaRa, Tokyo, Japan).

* To whom correspondence should be addressed. e-mail: xfn0990@sdu.edu.cn

# These authors contributed equally to this work.

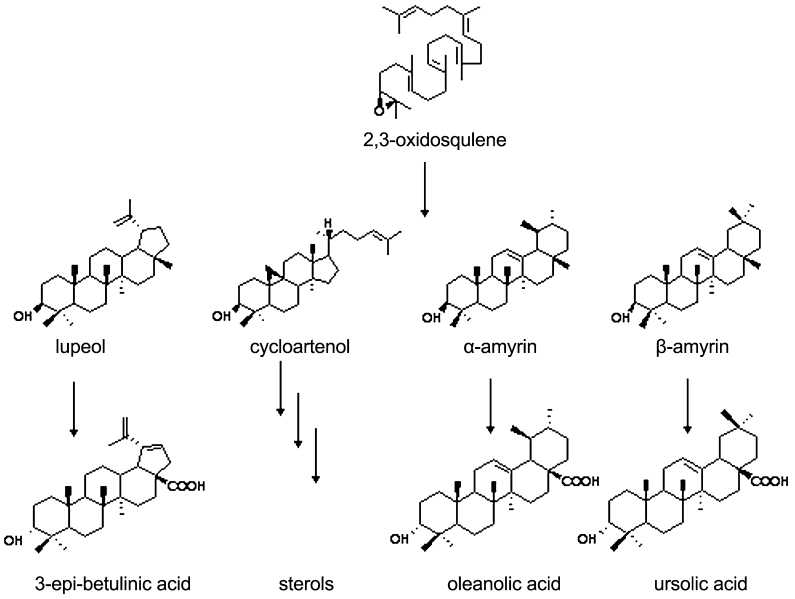

Fig. 1. The Cyclization of 2,3-Oxidosqualene to the Triterpenoids Lupeol, α-Amyrin and β-Amyrin, and the Sterols Cycloartenol and Parkeol, Catalysed by Lupeol Synthase, α-Amyrin Synthase, β-Amyrin Synthase, Cycloartenol Synthase, and Parkeol Synthase

Oleanolic acid is formed from β-amyrin following a series of reactions.

Isolation of the *G. straminea* β-Amyrin Synthase cDNA, and Its Transformation into *E. coli* and *P. pichea* Based on highly conserved regions of OSC sequences deposited in GenBank (specifically, AB014057 and AB009030 from *Panax ginseng*, AB055512 from *Betula platyphylla*, AY836006 from *Aster sedifolius*, AB206469 from *Euphorbia tirucalli*) the degenerate primer pair q3 (5′-TGGCTTTC-GAT(A/T)CTTGGA) and q2 (5′-CCACC(G/A)TTTTT-(G/A)CTCTGTA) were designed. Using *G. straminea* cDNA as a template, a 35 cycle PCR using High Fidelity DNA Polymerase Ex *Taq* (TaKaRa, Tokyo, Japan) was carried out. The cycling parameters were 94 °C/40 s, 49 °C/40 s and 72 °C/60 s, with a 10 min final extension at 72 °C. The PCR amplified product was purified from an agarose gel, cloned into pMD18-T (TaKaRa, Tokyo, Japan) and transformed into *E. coli* strain DH10B. The nucleotide sequence of the insert was determined by reported sequences of other species and used to obtain overlapping 5′ and 3′ clones by Rapid-Amplification of cDNA ends (RACE), based on the 5′ and 3′ Full RACE core sets (TaKaRa, Tokyo, Japan) with primers 5′-CTCAACCCAACAAGCAAGC-3′ and 5′-GTATGCTTGC-TTGTTGGGT-3′. Each of ten independent 5′ and 3′ clones was sequenced, and these sequences were aligned to obtain a consensus sequence. The C-terminal and N-terminal PCR primers used to amplify the full-length cDNA (5′-GAATTCATGTGGAGGCTAAAAATCGG-3′ and 5′-CTC-GAGCGTCTCCTACGGCACTTGCTTGCG-3′) included a restriction site (underlined) for cloning into pET28(a) and pPICZA. This PCR product was cloned into pMD18-T and transformed into *E. coli* strain DH10B for sequencing. The full-length cDNA was designated *GsAS1*.

The *GsAS1* PCR product was sticky-end ligated with pET28(a) and the ligation product was transformed into *E. coli* strain BL21 competent cells, with an empty pET28(a) plasmid representing a negative control. *P. pastoris* strain X-33 was transformed with pPICZA-GsAS1 using the Pichia EasyComp kit (Invitrogen), with an empty pPICZA plasmid representing a negative control.

Heterologous Expression of *GsAS1* *E. coli* carrying the recombinant pET28(a)-GsAS1 plasmid or the negative control pET28(a) were grown in 5 ml LB medium containing 100 μg/ml kanamycin. Cultures were shaken at 200 rpm/min and held at 37 °C before heterologous expression was induced by the addition of 1 mM IPTG. After 4 h, the bacteria were pelleted by centrifugation and re-suspended in 2×sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Then it was boiled for 10 min before spotting. The yeast vector pPICZA-GsAS1 is under the control of the methanol-inducible promoter 5′AOX and a 20 μl sample of each was loaded on a 10% SDS-PAGE gel and electrophoresed. Proteins were visualized by staining in Coomassie Brilliant Blue R-250 (Sigma, U.S.A.). Heterologous proteins were detected by Western blotting, performed essentially using the method of Towbin.[21] After electrophoresis, the proteins were transferred to a nitrocellulose filter (Rockville Center, NY, U.S.A.), which was blocked with 5% (w/v) BSA in TBS-Tween 20 (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% (v/v) Tween 20) at 37 °C for 3 h, incubated for 10 h at room temperature in the same solution containing 0.2 μg/ml of a rabbit polyclonal antibody (His antibody, supplied by Saierbio) recognising HuIFN-beta (PBL Biomedical Laboratories). The filter was then washed three times in TBS-Tween 20, and exposed to the secondary antibody (anti-Mouse IgG, Promega) diluted 1 : 7500 for 2 h at 37 °C. Finally, the filter was rinsed three times in TBS-Tween 20, once in TBS, and incubated for 5 min in 20 mM Tris–HCl, pH 7.5, 40 mg/ml DAB, 80 mg/ml $NiCl_2$, 3% (v/v) H_2O_2. The histochemical reaction was stopped by the addition of water. *P. pastoris* carrying the recombinant pPICZA-GsAS1 plasmid or the negative control pPICZA were grown at 28—30 °C in MGY medium (250—300 rpm) until the culture reaches an OD600=2—6. Then after centrifugation, the resuspended cell pellet grew in MM medium for 4 d and add 100% methanol to a final concentration of 0.5% every 24 h.

The extraction of sample and SDS-PAGE were done using the EasySelect Pichia Expression Kit (Invitrogen, U.S.A.).

Gas Chromatography-Electron Impact Mass Spectrometry (GC-EI-MS) The yeast cells were extracted with methanol–chloroform (2 : 1, v/v) and this extract was analysed by GC-EI-MS, using a Micromass GCT gas chromatograph-mass spectrometer (England) fitted with a DB-5 MS column (0.25 mm×30 m, 0.25 μm film thickness) (JW Scientific, 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 20—600 Da. The column temperature was set at 200 °C for 2 min, then elevated to 300 °C at 15 °C/min, and held at 300 °C for 7 min.

Phylogenetic Analysis of the GsAS1 Peptide Sequence A phylogenetic tree was constructed using CLUSTAL W[22] based on the neighbour-joining algorithm. The comparator sequences (obtained from GenBank) were the β-amyrin synthases AT1G78950 (*Arabidopsis thaliana*), AT1G78955 (CAMS1, *Arabidopsis thaliana*), AY095999 (AMS1, *Glycine max*), AF478453A (MY1, *Medicago truncatula*), EU330197 (BAS, *Artemisia annua*), EU400220 (BAS, *Bupleurum Chinense*), AB289585 (BgbAS, *Bruguiera gymnorrhiza*), DQ915167 (BS, *Vaccaria hispanica*), AB206469 (EtAS, *E. tirucalli*), AB037203 (GgbAS1, *Glycyrrhiza glabra*), AB055512 (OSCBPY, *B. platyphylla*), AB009030 (OSCPNY1, *P. ginseng*), AB014057 (OSCPNY2, *P. ginseng*), AB034802 (OSCPSY, *Pisum sativum*), AB181244 (OSC1, *Lotus japonicus*) and AF478455 (AMY2, *Lotus japonicus*), AY836006 (OXA1, *A. sedifolius*) and EF107623 (PTAS, *Polygala tenuifolia*); the mixed amyrin synthases AB291240 (OEA, *Olea europaea*) and AB034803 (OSCPSM, *P. sativum*); the lupeol synthases At1g78960 (ATLUP2, *A. thaliana*) and At1g78970 (LUP1, *A. thaliana*), AB289586 (BgLUS, *B. gymnorrhiza*), AB116228 (GgLUS1, *G. glabra*), AB025343 (OEW, *O. europaea*), AB181245 (OSC3, *L. japonicus*), AB055511 (OSCBPW, *B. platyphylla*) and AB025345 (TRW, *Taraxacum mongolicum*); the cycloartenol synthases U02555 (cas1, *Dioscorea alata*), AB055510 (CASBPX2, *B. platyphylla*), AY618690 (CC4719, *Avena strigosa*), AB058507 (CSOSC1, *Costus speciosus*), AB181246 (OSC5, *L. japonicus*) and AB009029 (OSCPNX1, *P. ginseng*); the oxidosqualene cyclases AB206470 (EtOSC, *E. tirucalli*) and AB055513 (OSCBPD, *B. platyphylla*); the multifunctional triterpene synthases AB257507 (KcMS, *Kandelia candel*) and AB263203 (RsM1, *Rhizophora stylosa*); and the isomultiflorenol synthase AB058643 (LclMS1, *Luffa cylindrica*).

***GsAS1* Expression in Planta** Roots, stems and leaves were harvested from mature *G. straminea* plants, snap frozen in liquid nitrogen and ground to a powder. RNA extracted from the powder using the Trizol reagent served as the template for a RT-PCR based on the *GsAS1* primer pair 5′-TCGCTGAGGAAAGGGAAGAGGTT-3′ and 5′-TAGTGCTATGACCCTCTATGTGC-3′. RT-PCR products were separated by agarose gel electophoresis. A 760 bp fragment of the actin gene was used to normalize the RT-PCR signal. This amplicon was generated by primers designed from the *P. ginseng* actin sequence, specifically 5′-TGGCATCACACTTTCTAC-3′ and 5′-CCTCCGATCCAGACACT-3′. Quantitative Real-time PCR experiments were carried out in PTC-200 Peltier Thermal Cycler (BioRad, America). Using actin amplicon of 142 bp primed by 5′-TCGTGTTGCCCCAGAAGAG-3′ and 5′-GGAAAGATCGGCTTGAATAGCA-3′ as a positive control and based on 10 times concentration gradients. Fluorogenic quantitative PCR was performed between 2 and 20000 μg/l concentration gradients. A 92 bp *GsAS1* amplicon primed by 5′-CGGCTCTTTGTTCTTTCTTCCT-3′ and 5′-TTTCGTGTGTATGCTCCGTTG-3′ was used as the target gene. The amount of its expression was extrapolated from the standard curve and normalized to actin. Data were analyzed using Rotor-Gene v5.0 (Corbett Research). The PCR used three-step cycling conditions of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min. PCR products were detectable in the presence of SYBR Green. The reaction mixture (25 μl) contained 1 μl cDNA, 1×SYBR Green Real-Time PCR Master Mix (#QPK-201; TOYOBO Co., Ltd., Osaka, Japan), and 6.25 pmol of each primer.

***GsAS1* Expression Elicited by MeJA** *G. straminea* plants of one year old were treated with 0.1 mM MeJA. After 6 h, 12 h, 24 h, 48 h, 4 d, 7 d, 10 d and 14 d after the treatment, the treated materials of the whole plant was harvested, snap frozen in liquid nitrogen and stored at −80 °C.

HPLC-Based Quantification of Oleanolic Acid The whole *G. straminea* plants materials were shade-dried for 7 d and ground to a powder. To each 0.3 g sample, 3 ml methanol was added, the suspension sonicated for 1 h at room temperature, and then passed through a 0.45 μm membrane filter. A 10 μl aliquot of each sample was used for injection into an HPLC device, equipped with an Ultrasphere C18 column (150 mm×4.6 mm i.d., Phenomenex). The mobile phase was methanol : water (90 : 10). The effluent was monitored at 209 nm and the flow-rate was maintained at 0.8 ml min^{-1}. The peak area of oleanolic acid was integrated by a Class-vp5.0 system, using an external standard. A standard calibration curve was assembled from a range of concentrations (20—1000 μg/ml) of oleanolic acid, provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

RESULTS

Isolation of *G. straminea* β-Amyrin Synthase cDNA A 920 bp segment amplified using the degenerate primers designed according to the homologous sequences of β-amyrin synthase in other species was obtained. All 20 independent clones shared the identical nucleotide sequence by sequencing. Analysis of the full length cDNA *GsAS1*, obtained by 5′- and 3′-RACE, showed a single open reading frame of length 2268 bp. The nucleotide sequence of *GsAS1* shares a high level of homology with the β-amyrin synthases of *P. ginseng* (AB014057, 76.2%), *A. sedifolius* (AY836006, 73.1%), *G. glabra* (AB037203, 73.1%), *B. platyphylla* (AB055512, 74.2%) and *E. tirucalli* (AB206469, 74.3%) and so on (data not shown). GsAS1 includes four copies of the QW motif [QXXXGXW] (Fig. 2), which are thought to be involved in the stabilization of carbocationic intermediates formed during the cyclization of OSCs; and one Asp-Cys-Thr-Ala-Glu (DCTAE) motif (Fig. 2), a sequence common to all known OSCs[23—25] including β-amyrin synthase. On this basis, we were able to conclude that *GsAS1* is a β-amyrin synthase gene. The sequence encodes a 756 residue polypepetide of

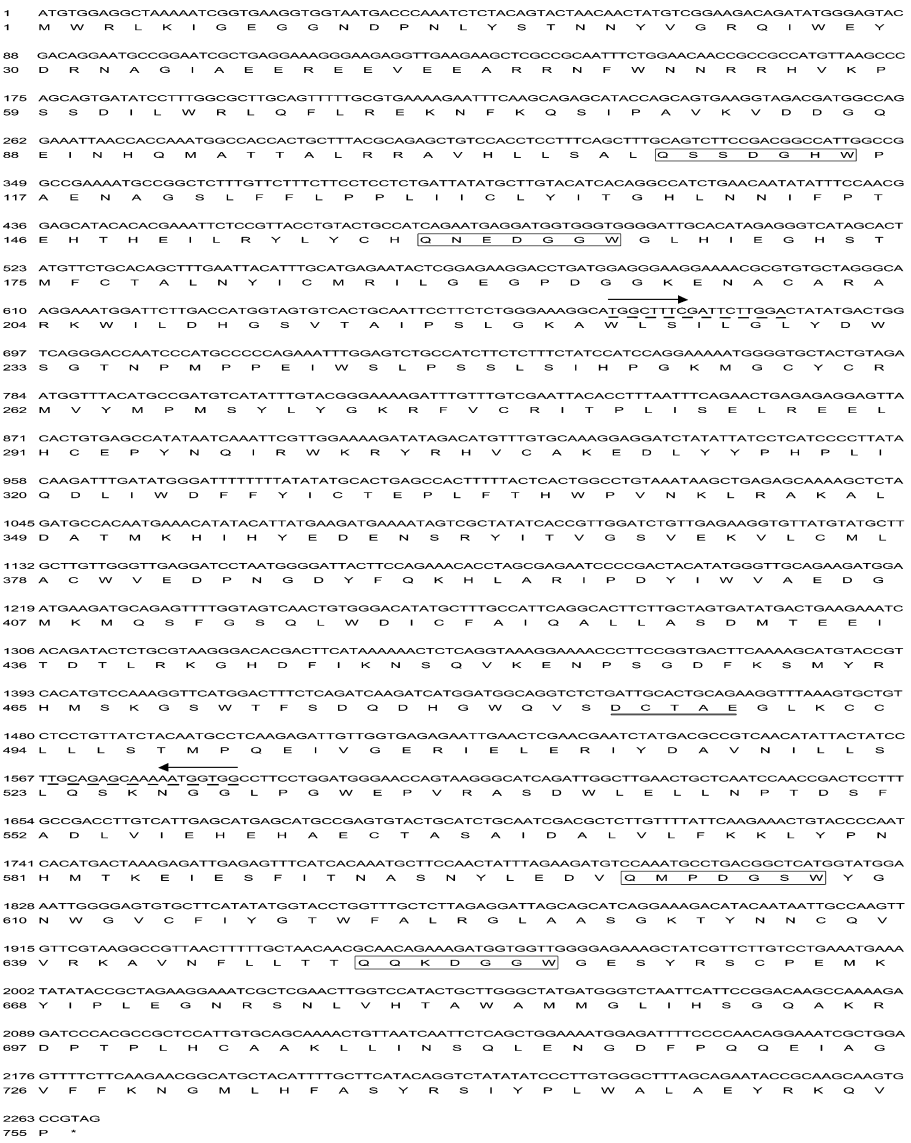

Fig. 2. Alignment of the GsAS1 Sequence with Those of Other β-Amyrin Synthases

The DCTAE motif is underlined with continuous line, and the QW motifs are boxed. The sequence between the arrowhead is the 920 bp segment amplified using the degenerate primers and the primer sequences are underlined with broken line.

predicted molecular weight 88.0 kDa. A phylogenetic analysis to establish the relationship between GsAS1 and other OSC sequences showed that the GsAS1 sequence closely resembles those of a number of other oxidosqualene cyclases, and that the gene belongs to a branch of the dicotyledonenous monofunctional β-amyrin synthases (Fig. 3).

***GsAS1* Expression in Planta** Both semi-quantitative RT-PCR and real-time PCR were used to assess the expression of *GsAS1* in a range of plant organs. The level in the leaves was 4.5 fold that in the roots, and 2.8 times that in the stems (Figs. 4a, b).

Heterologous Expression of *GsAS1* SDS-PAGE analysis identified a protein of molecular mass 88 kDa expressed by the *E. coli* strain BL21 when induced by IPTG (Fig. 5a). Immunoblotting of this separation revealed that this product was a his-tag fusion protein (Fig. 5b), its molecular weight was a little greater than that of the native GsAS1 protein, and suggested that some post-transational modification had occurred. The protein was not expressed in the negative control bacteria carrying an empty pET28(a) plasmid.

Similarly, SDS-PAGE separation of the translation products of the transformed *P. pastoris* X-33 cells identified a recombinant protein of molecular mass 88 kDa (date not shown). The retention time of this product when subjected to GC-EI-MS was 15.20 min, which is identical to the retention time of standard β-amyrin. This product was not present in the negative control carrying an empty vector (Fig. 6A), showing that in *P. pastoris*, GsAS1 could be activated to catalyse the synthesis of β-amyrin. Furthermore, the mass spectrum of the heterologous expression product was the same as that of the β-amyrin standard sample, containing various components of the chemical structure of β-amyrin, including *m*/*z* 218 (C-ring fragment peak), 203 [*m*/*z* 218$-CH_3$] and so on (Fig. 6B).

***GsAS1* Expression and Oleanolic Acid Content Following Elicitation by MeJA** The temporal expression of *GsAS1* following treatment with MeJA was assessed by semi-quantitative RT-PCR. This analysis showed that *GsAS1*

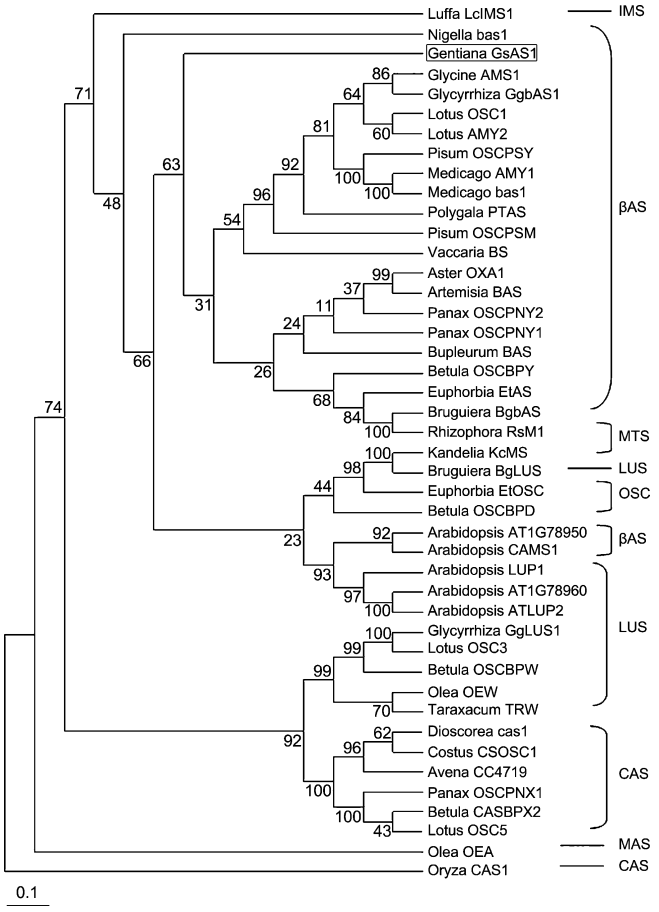

Fig. 3. A Phylogenetic Analysis of the Deduced Peptide Sequences of GsAS1 and Other Plants

G. straminea GsAS1 was used as the outgroup. IMS: isomultiflorenol synthase; βAS: β-amyrin synthase (monofunctional type); MTS: multifunctional triterpene synthase; LUS, lupeol synthase; OSC: putative oxidosqualene cyclase; CAS, cycloartenol synthase; MAS: mixed amyrin synthase.

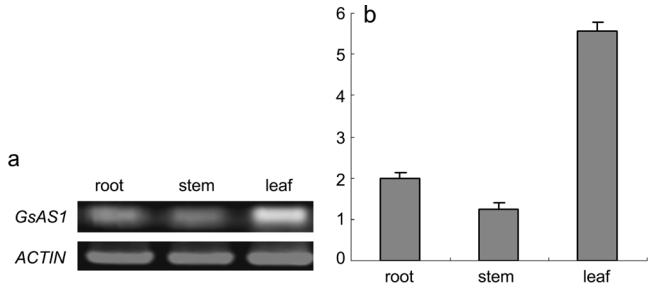

Fig. 4. *GsAS1* Expression in Various Plant Organs, as Assessed by (a) Semi-quantitative RT-PCR and (b) Real-Time PCR

expression could be induced by MeJA during the growth phase, and that the *GsAS1* mRNA level peaked between 24 h and 48 h after treatment, and then decreased to minimum at 14 d (Fig. 7). Non-elicited plants accumulated to a maximum of 0.2—0.3 mg/g oleanolic acid, which is approximately six fold less than that accumulated by MeJA-elicited plants. Oleanolic acid accumulation rose after MeJA treatment was applied, and the rate of increase in this accumulation peaked between 24 h and 48 h. Its maximum content (1.2 mg/g) was reached at 14 d (Fig. 8).

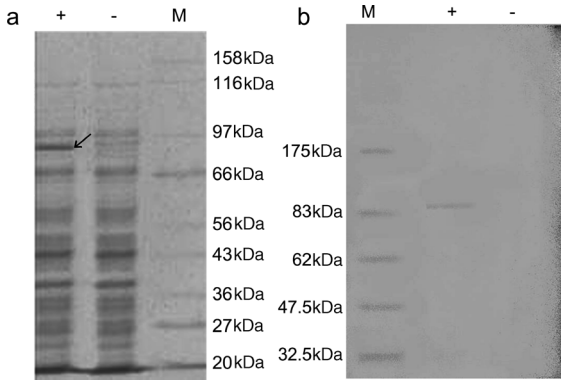

Fig. 5. Heterologous Expression of *GsAS1* in a Prokaryotic Host

(a) SDS-PAGE separation identifying a product of molecular mass 88 kDa (arrowhead). (b) a Western blot identifying a product of molecular mass >90 kDa.

DISCUSSION

We have described the isolation of a full length β-amyrin synthase cDNA from *G. straminea*. The utility of this cDNA sequence will lie as a tool to clarify the role(s) of β-amyrin in *G. straminea*. The GC-EI-MS analysis of the GsAS1 catalysate demonstrated that it is the triterpene β-amyrin. In addition, in Fig. 6A(c), several other peaks near the one of 15.20 min were found, but they were not triterpene products (data not shown). Thus this indicated that β-amyrin was the unique catalysate of GsAS1. This result was consistent with the outcome of the phylogenetic analysis which grouped GsAS1 with other monofunctional β-amyrin synthases. Thus we believe that GsAS1 is a monofunctional β-amyrin synthase, similar to a number of other plant species β-amyrin synthases.[26—28]

According to the relationship of the distance in the phylogenetic tree, GsAS1 showed highly similarity with other β-amyrin synthase of dicotyledonous plants. For all the OSCs mainly including β-amyrin synthase, lupeol synthase and cycloartenol synthase, there had been always two opinions in the evolution. One is that all the OSCs of sterol and triterpene pathways may share a common ancestral origin.[6] The other is that the sequential evolution of lupeol synthase and β-amyrin synthase came from ancestral cycloartenol synthase.[14] From Fig. 4 we could see that a part of lupeol synthase showed highly similarity to cycloartenol synthase and another part of lupeol synthase and β-amyrin synthase belongs to a branch, which coincided with the opinion that they all came from the same ancestral origin.

A large number of studies has shown that MeJA is a key signalling molecule in the elicitation process leading to the accumulation of secondary metabolites.[29] Frequently MeJA has been implicated in stimulating β-amyrin synthase mRNA levels and in accumulating the triterpene saponin.[30—32] However, other data conflict with these results.[33,34] The present data are supportive of the former view, since the *GsAS1* transcript was up-regulated by MeJA treatment over a prolonged time period (6 h to 10 d post treatment) and reached a peak of expression at 48 h; in addition, the accumulation of oleanolic acid was at its most rapid at 48 h. These events are consistent with a scenario whereby *GsAS1* transcript and the accumulation of its downstream product oleanolic acid are simultane-

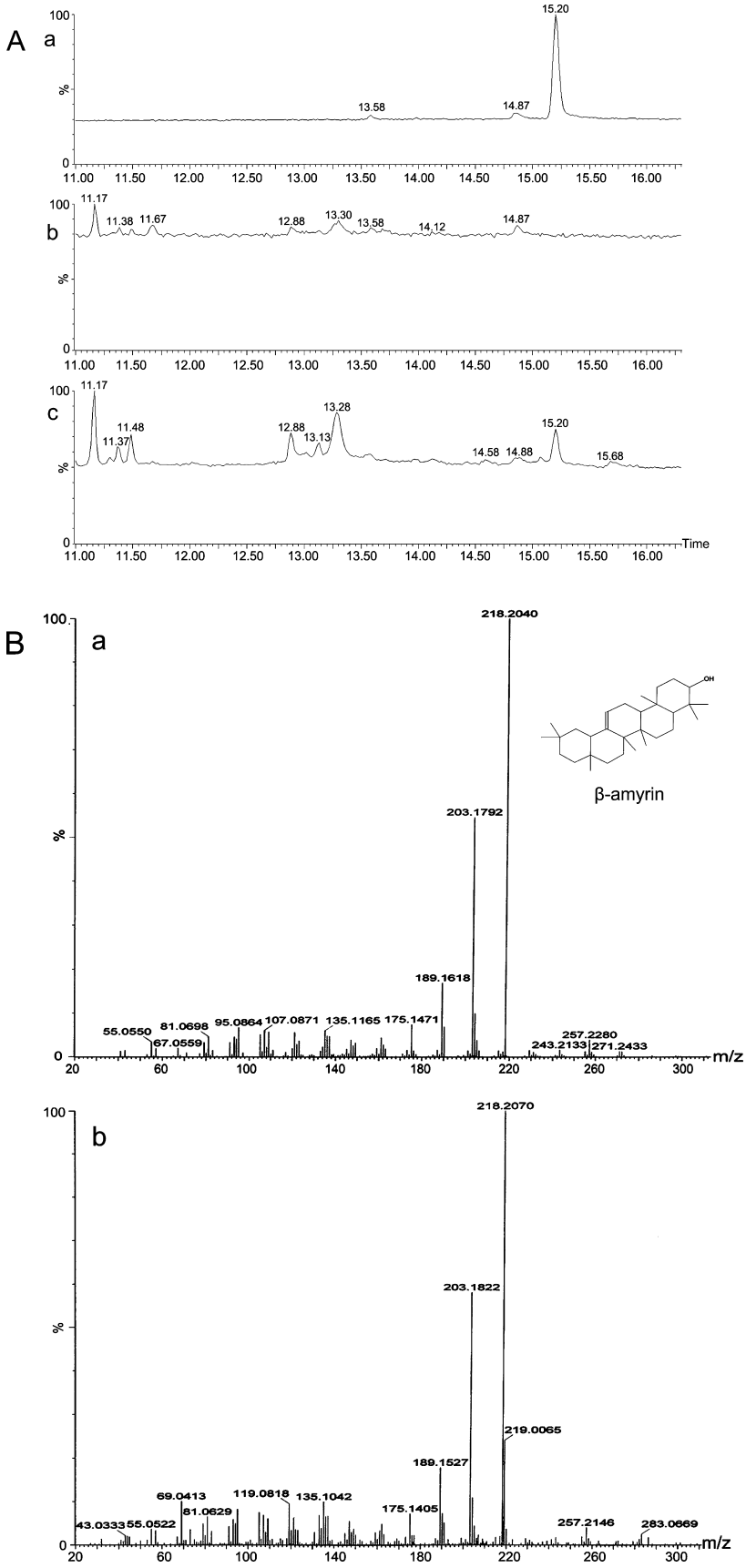

Fig. 6. GC-EI-MS Analysis of the GsAS1 Product

(A) A TIC chromatogram of the cell extract from wild type *P. pichia* including a standard sample of β-amyrin (a), transformed yeast carrying either an empty vector (b) or pPICZA-GsAS1 (c). In (b) and (c), ergosterol is the substrate of the reaction (11.17 min) and β-amyrin product (15.20 min). (B) Mass spectra of (a) a standard sample of β-amyrin, (b) the peak present in the yeast transformed with pPICZA-GsAS1, but absent when transformed with pPICZA.

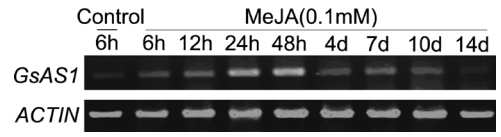

Fig. 7. *GsAS1* Gene Transcripts in *G. straminea* after Treatment with 0.1 mM MeJA

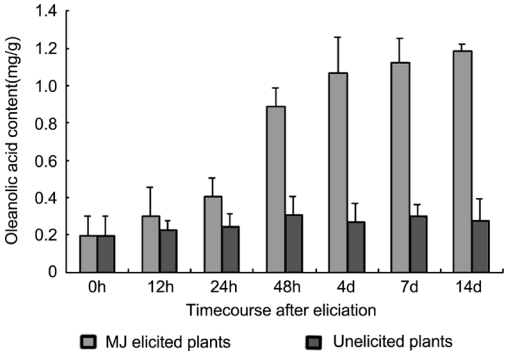

Fig. 8. HPLC Analysis of the Oleanolic Acid Present in *G. straminea* Following Treatment with 0.1 mM MeJA

ously subject to MeJA regulation. The content of oleanolic acid continued up to until 14 d after treatment, indicating that the synthesis of oleanolic acid was enhanced by MeJA treatment over a long time period.

Acknowledgements We thank the National Natural Science Foundation of China (Grant Nos. 30771116 and 30470154), the Chinese Ministry of Education New Century Training Program Foundation (Grant No. NCET-05-0581), the Chinese Natural Education Ministry Doctor Station Foundation Fellowship (Grant No. 20050422015), and Excellent Youth Foundation of Shandong Province of China (Grant No. JQ200810) for financial support. The authors thank Professor Robert Koebner for his help in editing this manuscript.

REFERENCES

1) Ho T. N., Liu S. W., "A Worldwide Monograph of Gentiana," Science Press, Beijing, 2001, p. 176.
2) Chen Q. L., Zhang Y., Tu G. Z., *China Journal of Chinese Materia Medica*, **19**, 1519—1522 (2005).
3) Laus G., *Phytother. Res.*, **18**, 259—274. (2004).
4) Yu F. R., Yu F. H., Li R. D., Wang R., *J. Ethnopharmacol.*, **95**, 77—81 (2004).
5) Tan R. X., Kong L. D., *Phytochemistry*, **46**, 1035—1038 (1997).
6) Haralampidis K., Trojanowska M., Osbourn A. E., *Adv. Biochem. Eng. Biotechnol.*, **75**, 31—49 (2002).
7) Kushiro T., Shibuya M., Masuda K., Ebizuka Y., *Tetrahedron Lett.*, **41**, 7705—7710 (2000).
8) Morita M., Shibuya M., Kushiro T., Masuda K., Ebizuka Y., *Eur. J. Biochem.*, **267**, 3453—3460 (2000).
9) Kushiro T., Shibuya M., Ebizuka Y., *Excerpta Medica International Congress Series*, **1157**, 421—428 (1998b).
10) Hayashi H., Huang P., Kirakosyan A., Inoue K., Hiraoka N., Ikeshiro Y., Kushiro T., Shibuta M., Ebizuka Y., *Biol. Pharm. Bull.*, **24**, 912—916 (2001).
11) Haralampidis K., Bryan G., Qi X., Papadopoulou K., Bakht S., Melton R., Osbourn A., *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 13431—13436 (2001).
12) Suzuki H., Achnine L., Xu R., Matsuda S. P., Dixon R. A., *Plant J.*, **32**, 1033—1048 (2002).
13) Iturbe-Ormaetxe I., Haralampidis K., Papadopoulou K., Osbourn A. E., *Plant Mol. Biol.*, **51**, 731—743 (2003).
14) Zhang H., Shibuya M., Yokota S., Ebizuka Y., *Biol. Pharm. Bull.*, **26**, 642—650 (2003).
15) Kajikawa M., Yamato K. T., Fukuzawa H., Sakai Y., Uchida H., Ohyama K., *Phytochemistry*, **66**, 1759—1766 (2005).
16) Meesapyodsuk D., Balsevich J., Reed D. W., Covello P. S., *Plant Physiol.*, **143**, 959—969 (2007).
17) Wasternack C., Parthier B., *Trends Plant Sci.*, **2**, 302—307 (1997).
18) Creelman R. A., Mullet J. E., *Plant Mol. Biol.*, **48**, 355—381 (1997).
19) Hayashi H., Huang P. Y., Inoue K., *Plant Cell Physiol.*, **44**, 404—411(2003).
20) Kim O. T., Kim M. Y., Hwang S. J., Ahn J. C., Hwang B., *Biotechnology and Bioprocess Engineering*, **10**, 16—22 (2005).
21) Towbin H., Staehelin T., Gordon J., *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350—4354 (1979).
22) Thompson J. D., Higgins D. G., Gibson T. J., *Nucleic Acid Res.*, **22**, 4673—4680, (1994).
23) Poralla K., Hewelt A., Prestwich G. D., Abe I., Reipen I., Sprenger G., *Trends Biochem. Sci.*, **19**, 157—158 (1994).
24) Abe I., Prestwich G. D., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 9274—9278 (1995).
25) Kushiro T., Shibuya M., Masuda K., Ebizuka Y., *Tetrahedron Lett.*, **41**, 7705—7710 (2000).
26) Hayashi H., Huang P., Takada S., Obinata M., Inoue K., Shibuya M., Ebizuka Y., *Biol. Pharm. Bull.*, **27**, 1086—1092 (2004).
27) Kushiro T., Shibuya M., Ebizuka Y., *Eur. J. Biochem.*, **256**, 238—244 (1998).
28) Sawai S., Shindo T., Sato S., Kaneko T., Tabata S., Ayabe S., Aoki T., *Plant Sci.*, **170**, 247—257 (2006).
29) Ebel J., Mithofer A., *Planta*, **206**, 335—348 (1998).
30) Lu M. B., Wong H. L., Teng W. L., *Plant Cell Rep.*, **20**, 647—677(2001).
31) Hayashi H., Huang P. Y., Inoue K., *Plant Cell Physiol.*, **44**, 404—411 (2003).
32) Shibuya M., Adachi S., Ebizuka Y., *Tetrahedron*, **60**, 6995—7003 (2004).
33) Aoyagi H., Kobayashi Y., Yamada K., Yokoyama K., Kusakari K., Tanaka H., *Appl. Microbiol. Biotechnol.*, **57**, 482—488 (2001).
34) Kim O. T., Kim M. Y., Hong M. H., Ahn J. C., Hwang B., *Plant Cell Rep.*, **23**, 339—344 (2004).