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Cloning and functional characterization of the β-amyrin synthase gene from *Bupleurum chinense*

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Abstract

Bupleurum chinense DC., a source of the traditional Chinese medicine *Bupleuri Radix*, is rich in triterpenoid saikosaponins with high pharmacological activities. The enzyme β-amyrin synthase (BAS), which converts the precursor 2,3-oxidosqualene to produce the triterpene skeleton, is crucial for the biosynthesis of triterpenoid saponins. In this study, we cloned the full-length sequence of the *BAS* gene from *B. chinense*, conducted a bioinformatics analysis, and expressed it in *Saccharomyces cerevisiae* to investigate its function. The cDNA of β-amyrin synthase (*BcBAS*, GenBank accession number: MN186093) cloned from aseptic seedlings of *B. chinense* was 2 307 bp with a 2 286 bp open reading frame coding for 761 amino acids. Phylogenetic analysis suggests that the BcBAS protein was closely related to the BAS proteins from *Panax ginseng* and *Betula platyphylla* as chromatography mass spectrometry analysis showed that the enzymatic product was indeed β-amyrin, the precursor of oleanane type triterpenes. Overall, our findings lay the foundation for in-depth analysis of the biosynthesis pathway of saikosaponins.

Additional key words: *Saccharomyces cerevisiae,* saikosaponins.

Introduction

Bupleuri Radix, a traditional Chinese herbal medicine, is widely used in China, Japan, South Korea, and countries with the same cultural influence. The most popular source of it, *B. chinense*, is rich in triterpenoid saikosaponins (Wang *et al.* 2018). Modern pharmacological research shows that saikosaponins isolated from *B. chinense* such as saikosaponin *a* (SSa), saikosaponin *c* (SSc), and saikosaponin *d* (SSd) have significant antiinflammatory (Chen *et al.* 2018), antidepressant (Sun *et al*. 2018), antitumor (Feng *et al.* 2017), hepatoprotective (Ashour and Wink 2011) and immunomodulatory (Yen *et al.* 2010) effects, with a broad application foreground and high development potential.

Saikosaponin is an oleanane type triterpene composed of isoprene units. Its biosynthesis pathway is consistent with the general rule of triterpenoid biosynthesis (Haralampidis *et al.* 2002), which is divided into three stages: *1*) initial stage: synthesis of the precursors sopentenyl pyrophosphate and dimethylallyl diphosphate; *2*) skeleton stage: squalene epoxidase (SE) catalyzes the production of 2,3-oxidosqualene promoting the formation of the oleanane type triterpene skeleton; *3*) modification stage: variety of saikosaponins *via* modification by cytochrome P450s and UDP-glycosyltransferases (Lin *et al.* 2013). The enzyme β-amyrin synthase (BAS), which belongs to the oxidosqualene cyclase (OSC) superfamily, plays an important role in the biosynthesis of triterpenoid saponins. It converts the precursor 2,3-oxidosqualene to produce β-amyrin, the basic triterpene skeleton (Yan *et al.* 2015). The characterization of BAS is crucial for the functional analysis of downstream key enzyme genes cytochrome P450s (Jo *et al.* 2017) and UDP-glycosyltransferases (Xu *et al.* 2016).

In our research, we used the comparative transcriptome data of *B. chinense* to obtain the full-length sequence of the *BAS* gene, after which bioinformatics analysis was performed. The gene was subsequently introduced into *Saccharomyces cerevisiae* for heterologous expression, gas chromatography-mass spectrometry (GC-MS) was used to determine the product for functional verification. This research contributes to the analysis of saikosaponin biosynthesis.

Materials and methods

Plants: The aseptic seedlings of *Bupleurum chinense* DC. were collected in Fangshan District, Beijing, China

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Abbreviation: BAS - β-amyrin synthase; CAS - cycloartenol synthase; GC-MS - gas chromatography-mass spectrometry; LUS - lupeol synthase; OSC - oxidosqualene cyclase; SE - squalene epoxidase.
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(E115°50′2″ , N39°42′53″), and identified by our research group. The seedlings were cultivated on Muraschige and Skoog medium at a temperature of 25 °C, a 40 % relative humidity, an 8-h photoperiod, and an irradiance of 60 μmol m^{-2} s^{-1} for 15 d.

Extraction of RNA and reverse transcription: Total RNA was extracted from the aseptic seedlings of *B. chinense* according to the instruction manual of the *Eastep Super* total RNA extraction kit (*Promega*, Madison, WI, USA), and reverse-transcribed using a *SMARSTer™ RACE 5'/3'* kit (*TaKaRa*, Tokyo, Japan).

Cloning the *BAS* gene from *B. chinense*: The full-length unigene annotated as *BAS* was selected from the *B. chinense* transcriptome and *Primer Premier 5.0* software was used to design specific primers (Zhang and Gao 2004). The cDNA served as a template for PCR amplification using 2× *Phusion Master Mix* (*NEB*, Ipswich, MA, USA). The program encompassed denaturation at 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 55 °C for 15 s and 72 °C for 1 min, and a final elongation at 72 °C for 7 min. The PCR product was subcloned into the cloning vector *pEASY-Blunt* (*TransGen Biotech*, Beijing, China), and then transformed into *E. coli* Trans1-T1 competent cells (*TransGen Biotech*). Positive clones were screened by colony PCR and confirmed by sequencing.

Bioinformatic analysis: The full-length sequence of *BcBAS* was analyzed by searching against the *National Center for Biotechnology Information* database (https://www.ncbi.nlm.nih.gov), analyzing the encoded amino acid sequence and homology. *DNAMAN* software was used for amino acid multiple sequence alignment. Phylogenetic analysis was carried out using *MEGA 6.0* software. Physicochemical properties were analyzed using the online tool *ProtParam* (https://web.expasy.org/protparam). *TMHMM* (https://www.cbs.dtu.dk/services/TMHMM-2.0) was used to predict transmembrane regions. Secondary structure prediction was performed using *SPOMA* (https://npsa-prabi.ibcp.fr/cgi-bin). The 3D structure was predicted using *SWISS-MODEL* (https://swissmodel.expasy.org) (Lin *et al.* 2018).

Functional characterization in *Saccharomyces cerevisiae*: The open reading frame of *BcBAS* was ligated into a double-digested (*Bam*HI and *Xho*I restriction enzymes) pYES2 expression vector (*Invitrogen*, Carlsbad, USA) using the *Seamless* cloning kit (*Beyotime*, Beijing, China). The recombinant plasmid was named pYES2-*BcBAS* and transformed into lanosterol synthase deficient *Saccharomyces cerevisiae* by *Frozen-EZ Yeast Transformation II™* kit (*Zymo Research*, Los Angeles, CA, USA). The empty pYES2 plasmid was used as a control. Positive transformants were seeded into 20 cm^3 of *SC-U dropout* medium (*Ura minus* medium 8 g dm^{-3} with 20 g dm^{-3} glucose) and cultivated at 30 °C for 48 h. Then, the cells were collected by centrifugation (3 000 *g*, 4 min), resuspended in 20 cm^3 of SC-U induction medium and grown at 30 °C for 48 h (Lu *et al.* 2018).

Determination of the enzymatic product by GC-MS: The cells were collected by centrifugation (5 000 *g*, 4 min), and heated at 85 °C under reflux with 10 cm^3 of extraction solution containing 20 % (v/v) KOH + 50 % (v/v) ethanol for 5 min. Equal volume of hexane was added and extracted three times. The upper phase was combined and dried by rotary evaporator. Then the residue was re-dissolved in a small volume of hexane. After drying with N_2, the extract was derivatized with N,O-bis (trimethylsilyl) trifluoroacetamide and pyridineat at 80 °C for 1 h, and dried again. Finally, the sample was dissolved in 0.3 cm^3 of hexane and analyzed by the *Agilent 7890B* gas chromatography system equipped with a *DB-5 MS* column (15 m × 250 mm × 0.1 mm). The carrier gas was high purity helium with a flow rate of 1 cm^3 min^{-1}. The inlet temperature was 280 °C and the injection volume was 1 mm^3. The ion source temperature was 250 °C and the ionization voltage was 70 eV. The temperature program was as follows: after keeping the column temperature at 180 °C for 1 min, the temperature was raised to 280 °C at a rate of 20 °C min^{-1}, then to 300 °C at a rate of 2 °C min^{-1} with a 2 min hold (Yin *et al.* 2018).

Results

The cDNA of *BcBAS* was 2 307 bp, with a 2 286 bp (11-2296 bp) open reading frame encoding 761 amino acids. The molecular mass of BcBAS protein was 87.527 kDa and the theoretical pI was 6.05. The total number of negatively charged residues (Asp + Glu) was 89 and positively charged residues (Arg + Lys) 76. According to calculations, the instability index (II) was 44.35, the aliphatic index was 76.69, and grand average of hydropathicity (GRAVY) value of the protein was -0.328.

The amino acid sequence of *BcBAS* showed high similarity with the reported BAS protein sequences from other plants found in *BLAST* search. It had 83.38, 83, 81.6, and 80.5 % identity to the enzymes from *Betula platyphylla* (Q8W3Z1.1), *Bruguiera gymnorhiza* (A8CDT2.1), *Panax ginseng* (O82140.1), and *Glycyrrhiza glabra* (Q9MB42.1), respectively. Multiple sequence alignment showed that BcBAS protein contained conserved regions typical of the OSC gene superfamily, such as the MWCYCR motif, DCTAE motif, and QW (QXXXGXW or QXXGXXXW) motifs (Fig. 1).

The phylogenetic tree was constructed based on known OSC protein sequences from other plants downloaded from the *National Center for Biotechnology Information*. These OSC protein sequences were grouped into three main branches: BAS, lupeol synthase (LUS), and cycloartenol synthase (CAS). BcBAS clustered with other BAS proteins, showing a close relationship, while being far from the LUS and CAS proteins (Fig. 2).

The *SOPMA* analysis showed that the secondary structure of BcBAS protein encompassed 341 residues in α-helices (44.81 %), 100 residues in extended strands (13.14 %), 55 in β-turns (7.23 %), and 265 in random coils (34.82 %). The BcBAS protein had no transmembrane regions according to *TMHMM* analysis. The human

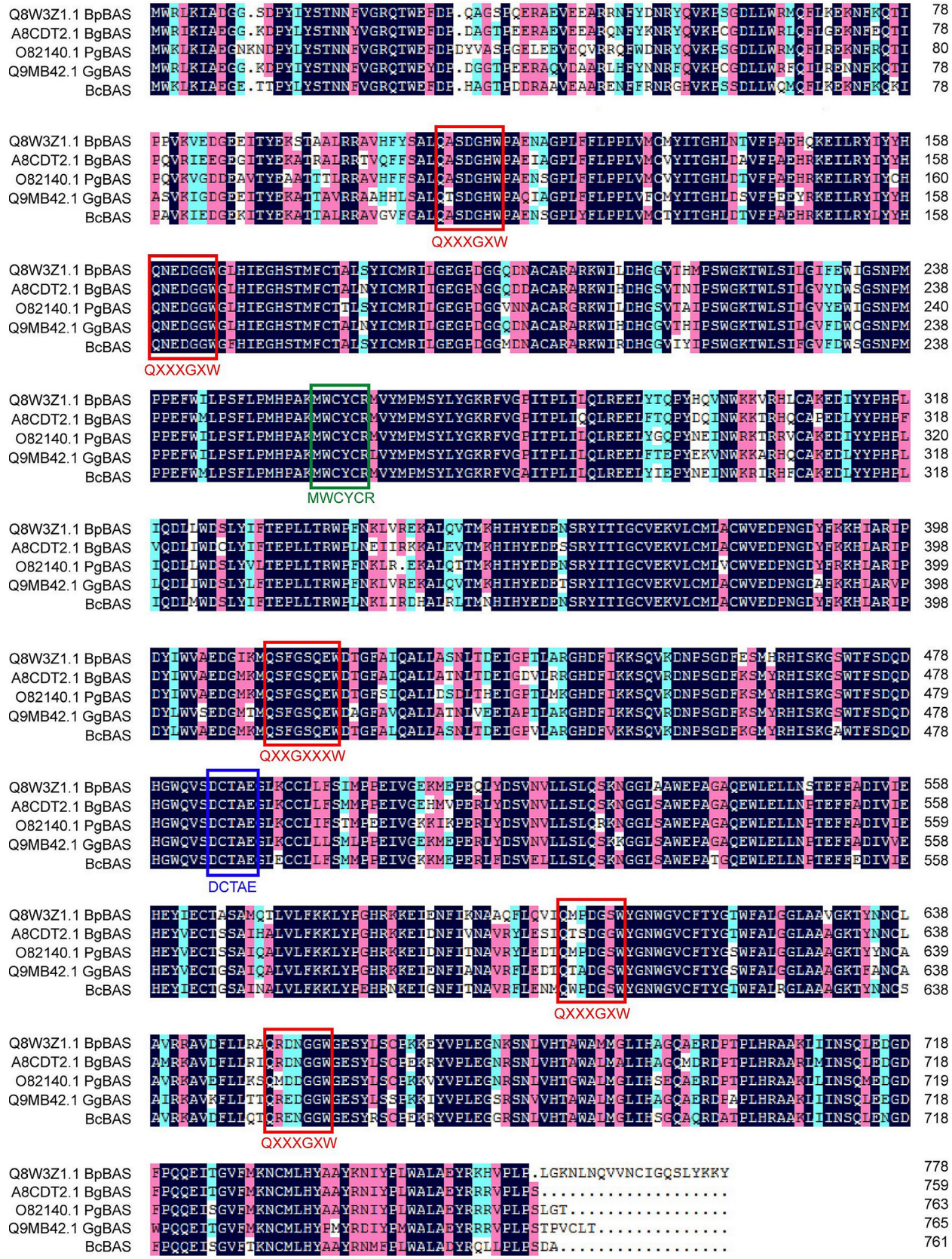

Fig. 1. The multiple sequence alignment of β-amyrin synthase proteins from different plant species. Four QXXXGXW motifs and one QXXGXXXW motif are shown in *red boxes*, one MWCYCR motif is shown in a *green box*, and one DCTAE motif is shown in a *blue box*.

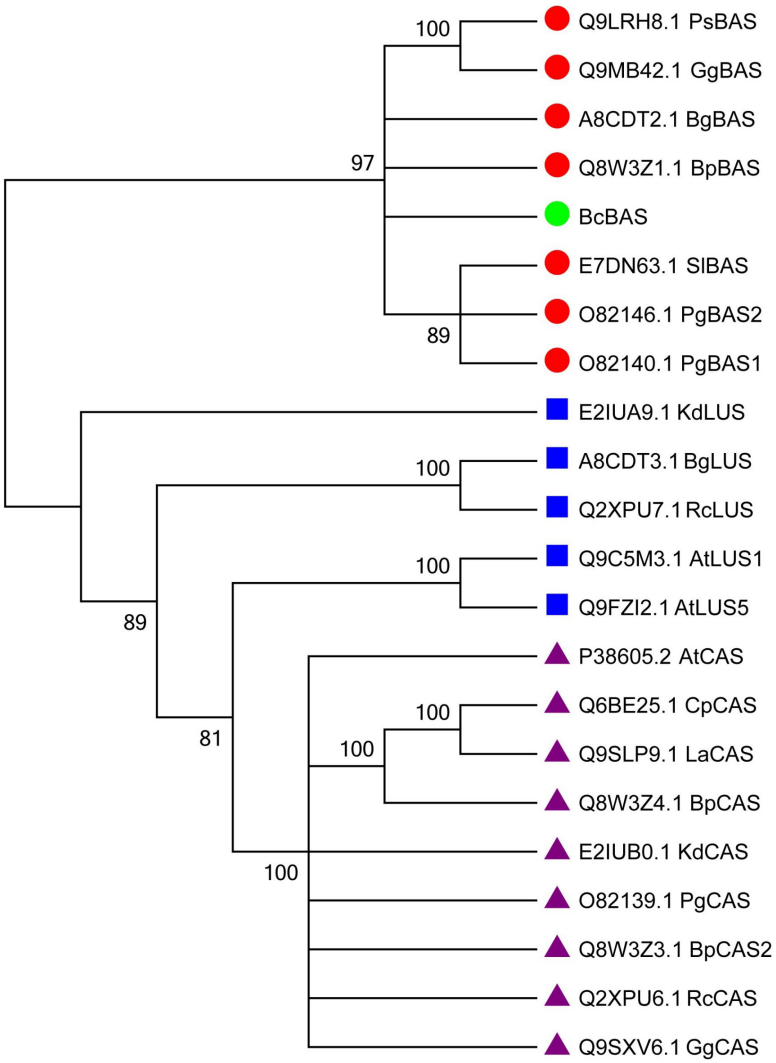

Fig. 2. The phylogenetic tree of *Bupleurum chinense* β-amyrin synthase (BcBAS) and other known oxidosqualene cyclases. An evaluation method of the bootstrap was applied for 1 000 times (bootstrap value ≥ 80). *Green circle* - *Bupleurum chinense* β-amyrin synthase (BcBAS),; *red circles* - BAS from other plants, *blue squares* - lupeol synthase (LUS), *purple triangles* - cycloartenol synthase (CAS). Ps - *Pisum sativum*, Gg - *Glycyrrhiza glabra*, Bg - *Bruguiera gymnorhiza*, Bp - *Betula platyphylla*, Sl - *Solanum lycopersicum*, Pg - *Panax ginseng*, Kd - *Kalanchoe daigremontiana*, Rc - *Ricinus communis*, At - *Arabidopsis thaliana*, Cp - *Cucurbita pepo*, La - *Luffa aegyptiaca*.

CAS protein (1w6j.1.A), which shares 44.16 % sequence similarity with BcBAS, was used as template. The amino acid residues 69-756 were utilized in the modeling process (Fig. 3).

The *BcBAS* coding sequence was ligated into the pYES2 yeast expression vector, namely pYES2-*BcBAS*, and the positive colony was verified (Fig. 4). The plasmid pYES2- *BcBAS* was transformed into *Saccharomyces cerevisiae* and the empty pYES2 plasmid was used as a control. The enzymatic product of the heterologously expressed BcBAS protein was detected by GC-MS. A single new compound was found in the extract of cells expressing BcBAS. It was confirmed as β-amyrin by comparing the retention time and ion fragment information with the authentic standard (Figs 1 and 2 Suppl.). There were three major characteristic ion fragments of β-amyrin at m/z 189.0, 203.0, and 218.0 (Fig. 2 Suppl.).

Discussion

Triterpenoid saponins are a diverse class of plant secondary metabolites with significant pharmacological activities, including anti-tumor, anti-viral, and anti-inflammatory effects. Tetracyclic and pentacyclic triterpenes, whose main representatives are dammarane and oleanane, respectively, are two major categories of triterpenoid saponins. The manufacture of triterpenoid saponins mainly relies on extraction from plants, synthesis through chemical modification of natural raw materials, and biosynthesis (Augustin *et al.* 2011). However, the traditional extraction processes have low yields and waste large amounts of

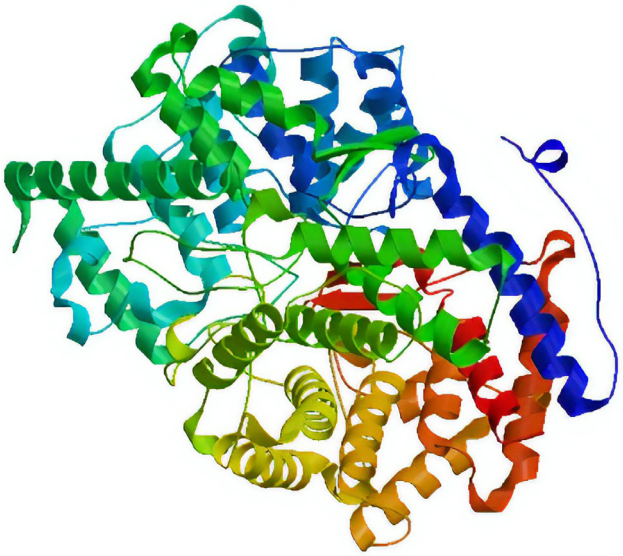

Fig. 3. The homology model of the three-dimensional structure of *Bupleurum chinense* β-amyrin synthase protein.

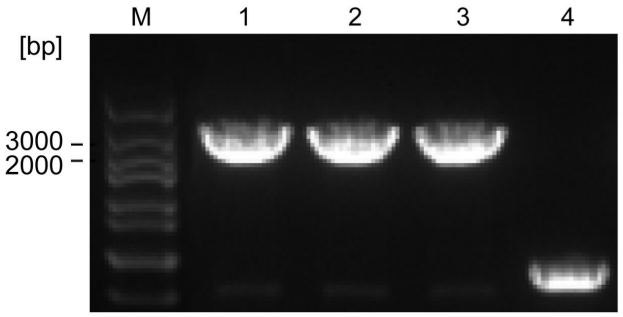

Fig. 4. The ddentification of the positive colony by PCR. M - DNA marker; 1,2,3 - pYES2-*BcBAS* recombinant vector; 4 - empty pYES2 vector.

raw materials. Synthesis through chemical modification faces problems such as complicated processes and many by-products. In recent years, the application of synthetic biology to analyze and regulate the triterpenoid saponin biosynthesis pathway has been studied extensively, with efforts to obtain triterpenoid saponins through genetic engineering. There have been significant advances in the study of triterpenoid saponin biosynthesis in *Panax ginseng* (Yang *et al.* 2018), *P. notoginseng* (Deng *et al.* 2017) and *P. quinquefolius* (Wang *et al.* 2014).

Saikosaponin is an oleanane-type pentacyclic triterpenoid saponin, and BAS, which channels the precursor 2,3-oxidosqualene into saponin synthesis, is a key enzyme in its synthesis pathway. Kushiro *et al.* (1998) successfully cloned the full-length cDNA sequence of *BAS* from ginseng hairy roots in 1998, and observed the production of β-amyrin in mutant yeast lacking lanosterol synthase for the first time. Liu *et al.* (2014) studied the effect of the co-expression of the *BAS* and *SQS* genes on the metabolic pathway of glycyrrhizic acid, discovering that the co-expression contributed to the accumulation of the metabolite β-amyrin. Sun *et al.* (2018) cloned the *BAS* gene from *Hedera helix* for the first time and verified the positive correlation between *HhBAS* expression detected by RT-qPCR and the content of triterpenoid saponin.

In this study, we successfully cloned the *BAS* gene from aseptic seedlings of *Bupleurum chinense*. The bioinformatics analysis confirmed that the gene was a member of the oxidosqualene cyclase family with more than 80 % sequence similarity with other known BAS proteins, containing the conserved DCTAE motif associated with substrate binding, as well as QW motifs that stabilize carbocations in the cyclization reaction (Sikkema *et al.* 1994). Phylogenetic analysis revealed that BcBAS was closely related to *B. gymnorhiza* and *B. platyphylla* BAS. We assumed that BcBAS could convert 2,3-oxidosqualene to produce β-amyrin in *B. chinense*. Therefore, the heterologous yeast expression of this gene was performed to confirm our idea. The enzymatic product was detected and confirmed to be β-amyrin by GC-MS, completing the functional verification of the *BcBAS* gene. What's more, the 3D modeling structure of BcBAS protein could be used in molecular docking to predict enzyme active sites, which laid a foundation for the modification of enzyme activity by site-directed mutation technique in the future. In a nutshell, the characterization of *BcBAS* gene could give a new potential genetic engineering approach to improve the accumulation of saikosaponins by gene over-expression or other methods in engineered yeast and plant systems. This study could greatly promote the analysis and regulation of saikosaponin biosynthesis pathway.

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