



Overexpression of BcbZIP134 negatively regulates the biosynthesis of saikosaponins

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Abstract

Chaihu, the dried roots of several *Bupleurum* species, are widely used as herbal drugs. Triterpene saponins, as saikosaponins (SSs) in Chaihu, exhibit a wide range of pharmacological actions, including significant anti-inflammatory, immunomodulatory, hepatoprotective, antioxidant, anti-tumor and antiviral activities. To explore the regulatory biosynthesis of SS in *Bupleurum chinense* roots, we screened putative transcription factor (TF) genes from transcriptome data. Here, we report one of the bZIP TF genes, named *BcbZIP134*, which belongs to bZIP G subfamily. The expression of *BcbZIP134* was elevated in MeJA-treated adventitious roots of *B. chinense*. No obvious differential expression of *BcbZIP134* was detected in tissues (root, stem, leaf, flower and fruit) of *B. chinense*. Overexpressing *BcbZIP134* in *B. chinense* hairy roots revealed that the content of the main SS monomers a, c and d were decreased compared to control hairy roots. Furthermore, the contents of a few other minor SS monomers were also affected when compared to the controls. The results indicate that *BcbZIP134* may play a negative regulatory role in SS biosynthesis.

Key Message

Chaihu, the dried roots of several *Bupleurum* species, are successfully and widely used as herbal drugs. Triterpene saponins, as saikosaponins (SSs) in Chaihu, exhibit a wide range of pharmacological actions, including significant anti-inflammatory, immunomodulatory, hepatoprotective, antioxidant, anti-tumor and antiviral activities. To explore the regulatory biosynthesis of SS in *B. chinense* roots, we screened putative transcription factor (TF) genes from transcriptome data. Here, we report one of the bZIP TF genes, named *BcbZIP134*, which encodes a predicted 410 amino acid protein belonging to bZIP G subfamily. The expression of *BcbZIP134* was elevated in MeJA-treated adventitious roots of *B. chinense*, and no obvious differential expression of *BcbZIP134* was detected in tissues (root, stem, leaf, flower and fruit) of *B. chinense*. Overexpressing *BcbZIP134* in *B. chinense* hairy roots revealed that the content of the main SS monomers a, c and d were decreased compared to control hairy roots. Furthermore, the contents of a few other minor SS monomers were also affected when compared to the controls. The results indicate that *BcbZIP134* may play a negative regulatory role in SS biosynthesis.

Keywords *Bupleurum chinense* DC. · bZIP transcription factor *BcbZIP134* · Hairy root · Overexpression · Biosynthesis of saikosaponin

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Introduction

Saikosaponins (SSs), which are triterpene saponins, are the main active components in *Radix Bupleuri*, the roots of some species from the genus *Bupleurum*. To date, more than 120 glycosylated oleanane-type and ursane-type saponins have been isolated from *Bupleurum*. The predominant SS monomers in *Bupleurum* are SS a, SS c and SS d, of which structures are shown in Fig. 1. Isolated SS monomers such as SS a, SS c, SS d, SS b1 and SS b2 or an SS mixture displayed pharmacological activity on Hepatitis B replication,

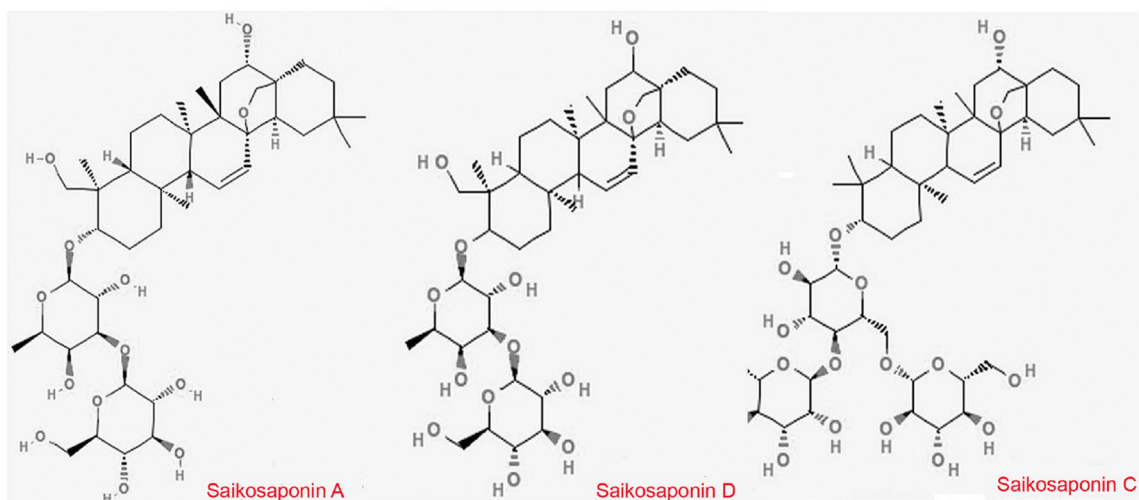


Fig. 1 The structures of SS a, SS d and SS c

acute and chronic inflammation, T cells and B cells proliferation, lipid peroxidation and PGE2 production (Ashour et al. 2011). The putative SS biosynthetic pathway initiates the isoprenoid pathway, mediates the cyclization of oxidosqualene and then undergoes some modifications of oxidation, glycosylation and other secondary transformations (Lin et al. 2013). The genes encoding some of the biosynthetic enzymes were cloned mainly from *B. chinense*, *B. falcatum* and *B. kaoi* (Gao et al. 2015; Moses et al. 2014; Chen et al. 2007; Kim et al. 2006). Previous studies have shown that some endogenous signal molecules, such as methyl jasmonate (MeJA) and abscisic acid (ABA), or some exogenous fungal elicitors could elevate the biosynthesis of SSSs (Chen et al. 2007; Aoyagi et al. 2001; Kim et al. 2011). When potted *B. chinense* seedlings were subjected to progressive drought stress, both SS a and SS d content increased and were accompanied by an enhanced content of active oxygen species (Zhu et al. 2009a, b). However, the molecular regulatory mechanism of the SS biosynthesis remains elusive.

Regarding the regulation of plant terpenoid biosynthesis, a few transcription factors (TFs) belonging to different families have been identified. For example, AabZIP1, AaWRKY1, AaERF1 and AaERF2 from *Artemisia annua* were shown to regulate the biosynthesis of the sesquiterpenoid artemisinin. AabZIP1 activated the expression of the two key genes encoding amorpha-4,11-diene synthase (ADS) and CYP71AV1 and its activation was enhanced by ABA treatment (Zhang et al. 2015a, b). AaWRKY1 bound to the W-box of the promoter of the ADS gene and activated its expression (Ma et al. 2009). AaERF1 and AaERF2 bound to the CRTDREHVCBF2 and RAV1AAT motifs present in both ADS and CYP71AV1 promoters, and the overexpression of either of these TFs resulted in increased accumulation of artemisinin and artemisinic acid (Yu et al.

2012). CrMYC2, ORCA2, CrGBF1 and CrGBF2 were TFs which were found regulating the biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus* (Siberil et al. 2001; Memelink et al. 2007; Zhang et al. 2011a, b). In addition, several TFs regulating the triterpene (saponin) biosynthesis have been identified. In *Medicago truncatula*, two homologous jasmonate-inducible TFs of the basic helix-loop-helix family (bHLH), Triterpene saponin biosynthesis activating regulator1 (TSAR1) and TSAR2, were found directly binding to the N-box in the promoter of the gene encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (HMGR1) to enhance triterpene saponin biosynthesis (Mertens et al. 2016). Another bHLH TF in *glycyrrhiza uralensis*, GubHLH3, was shown to regulate the expression of soyasaponin biosynthetic genes positively (Tamura et al. 2018).

The basic leucine zipper (bZIP) is a large family of TFs in plants and the members of this family contain a conserved DNA-binding basic region and a dimerized leucine zipper region. Diverse roles have been found for bZIP TFs especially in plant stress response and hormone signal transduction (Hurst et al. 1995; Llorca et al. 2014). The bZIP-family in *Arabidopsis thaliana* was sorted into 13 groups comprising 78 members. The members in the same groups tended to have similar structural features and functions (Dröge-Laser et al. 2018). In addition to terpenoids, bZIP TF members were identified by their regulation of the biosynthesis of other metabolites, such as HY5 and HYH from *A. thaliana*, regulating the synthesis of anthocyanins and improving the cold resistance by regulating the key gene DFR of the anthocyanin synthesis pathway (Zhang et al. 2011a, b).

Bupleurum chinense DC. is the dominant source species of Radix Bupleuri and has been domesticated for several decades in China. To explore the biosynthesis and

regulation of SSs in *B. chinense*, we previously analyzed the transcriptome of *B. chinense* intending to search for genes that may participate in the biosynthesis of SSs (Sui et al. 2011). The transcriptomes of the roots of *B. chinense* and another *Bupleurum* species, *B. scorzonerifolium*, were compared to find clues about the regulation of SS biosynthesis because SSs are easily detected in roots of the former while hardly detected at all in roots of the latter (Sui et al. 2014). A number of genes encoding TFs were found to be exceptionally expressed in only one of the two species or were distinctly expressed in both species. In this study, the impact on SS biosynthesis of one of the TFs genes, *BcbZIP134*, was further explored. *BcbZIP134* was overexpressed in the hairy roots of *B. chinense* by *Agrobacterium rhizogenes*-intermediated transformation. The SS compounds were assayed by high performance liquid chromatography quadrupole/time of flight-mass spectrometry/mass spectrometry (HPLC–QTOF–MS/MS).

Materials and methods

Candidate TF genes selection

To identify candidate TF genes that may be involved with SS biosynthesis (The putative biosynthetic pathway presented in our previous report (Sui et al. 2011)), we first analyzed the category of the TFs based on their sequences obtained from the transcriptome data of *B. chinense* and *B. scorzonerifolium*. Those that differentially expressed in the roots of both species and that belonged to the families of AP2, bZIP and bHLH were selected for further screening. A total of 26 TF genes, including 18 AP2 genes, four bZIP genes and four bHLH genes, were analyzed by expression profiling in MeJA-treated *B. chinense* adventitious roots by quantitative real time polymerase chain reaction (qRT-PCR). Next, the contents of SS a, SS c and SS d were assayed by HPLC. Six TF genes, including *BcbZIP134*, exhibited elevated expression accompanied by enhanced levels of SSs. Then, the expression of the six genes in roots, stems, leaves, flowers and fruits of *B. chinense* were tested by qRT-PCR. The experimental protocols of adventitious root culture and qRT-PCR were done according to previous reports (Sui et al. 2014; Kusakari et al. 2000). In addition, the time course of the expression of these genes after MeJA treatment was assayed. MeJA was added to the media at a final concentration of 200 μ M when adventitious roots were transferred to the second step culture media (Aoyagi et al. 2001). The roots were collected at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h and 5 days after MeJA treatment and were immediately frozen in liquid nitrogen then stored at -80 °C until needed. Roots in six bottles were treated as one replicate and three replicates were prepared. In addition, the collection of tissues from *B.*

chinense was done according to our previous report (Dong et al. 2011).

Extraction of SSs and HPLC Analysis

The adventitious roots were dried for 72 h using a freeze-drier (LGJ-18, Beijing Songyuan Huaxing Technology Develop Co. Ltd., China). Powdered dried roots (0.5 g) of each sample were ultrasonically extracted with 25 mL of a 5% ammonia/methanol solution in 100 mL flasks for 30 min. The extract was filtered and then evaporated until dry using 70 °C water baths. The residue was dissolved in chromatographic methanol and adjusted to 5 mL using a volumetric flask. Before injection, the solution was filtered using a 0.45 μ m microfiltration membrane. The reference standards of SS a, SS c and SS d were purchased from the National Institutes for Food and Drug Control, Beijing, China. The HPLC (Waters 1525 Binary HPLC Pump, USA) and an ASB-vensil C18 column (250 mm \times 4.6 mm, 5 μ m) were used. A gradient elution of A (water/0.05% NH_4OH) and B (acetonitrile) was used as follows: 0–15 min, 10–85% B; 15–18 min, 85% B; 18–22 min, 85–10% B; and 22–27 min, 10% B. The detection was set at 210 nm. The injection volume was 20 μ L, the flow rate was 1.5 mL/min and the column temperature was 40 ± 0.15 °C. The total content of SS a, SS c, and SS d was calculated for comparison because these three SSs are the dominant SSs in *B. chinense*.

Full-length cDNA cloning and bioinformatic analysis of *BcbZIP134*

To verify the sequence of *BcbZIP134* from transcriptome data and to obtain the full-length cDNA sequence, rapid amplification of cDNA ends (RACE) PCR was used to amplify the 5' and 3' ends of the sequences using a SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories, CA, USA). Then, the full-length cDNA was amplified using specific primers in one PCR reaction. PCR amplification conditions for the 3' and 5' RACE were as follows: five cycles at 94 °C for 30 s and 72 °C for 3 min; five cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 25 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min; a final extension for 10 min at 72 °C; and then cooled to 4 °C. All primers were designed using Primer Premier 5.0 (Primer Biosoft International, Palo Alto, California, USA) and are listed in the Appendix. Search for similar sequences of *BcbZIP134* was carried out using the blastx suite at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The amino acid sequences of hits with high scores were downloaded and then were used to construct a phylogenetic tree using the MEGA7 software. Alignment of these sequences was conducted using

DNAMAN in order to show the conserved motifs and identity between *BcbZIP134* and another single sequence.

Agrobacterium rhizogenes-mediated transformation of BcbZIP134

Bupleurum chinense seeds were grown on MS solid medium, after surface sterilization in 20% sodium hypochlorite solution for 15 min. The tender leaves of sterile seedlings were used for *A. rhizogenes* infection. A single colony of *A. rhizogenes* ACC10060 strain carrying the pK2GW7 plasmid with the *BcbZIP134* and a spectinomycin resistance gene (*NptII*) was inoculated into 5 mL of lysogeny broth (LB) medium supplemented with spectinomycin (50 mg/L) and rifampin (50 mg/L) and cultured overnight at 28 °C with shaking (180 rpm). Then, the cultures were expanded into a larger volume, and culturing was continued until the OD₆₀₀ was approximately 0.8. The cultures were centrifuged for 10 min at 5000 rpm at 4 °C and were resuspended in equal volume of MS liquid medium containing 200 µM acetosyringone. The explants were dipped into the infection solution for 20 min and were cocultured on Murashige and Skoog (MS) medium at 25 °C in the dark for 3 days after being blotted dry on sterile filter paper. The explants were then transferred to MS medium containing 500 mg/L of cefotaxime. The hairy roots started to develop after about 2 weeks of cultivation. Hairy roots of approximately 2 cm in length were excised from explants and cultured in fresh MS medium containing 500 mg/L cefotaxime in the dark. After approximately 4 weeks, these hairy roots were transferred to MS liquid medium, cultured in the dark with shaking (150 rpm) and maintained as separate independent lines. The roots were transferred to fresh medium once every 4 weeks until the quantity of each independent line was enough for HPLC analysis. The hairy root lines that were induced by nontransformed *A. rhizogenes* were used as controls. The transgenic hairy root lines were verified by PCR amplification with primers for 35 S promoter and 35 S terminator in the vector. The overexpression of *BcbZIP134* was evaluated by real-time qRT-PCR.

SSs profile analysis in BcbZIP134-overexpressed hairy root lines

Profiles of the SSs were analyzed for five independent lines overexpressing *BcbZIP134*. The SS extraction method was identical to the method used to extract SSs from adventitious roots of *B. chinense* described above, except a procedure of solid-phase microextraction (SPME) was added. An SPME column (Bonna-Agela Cleanert S C18, China) was used. First, the column was activated with 5 mL of methanol followed by 5 mL of chromatographic water washing. The materials in 20% methanol were slowly added to the

activated SPME column, and, after the sample was completely loaded into the column, 3 mL 20% aqueous methanol solution was used for washing and then the column was vacuum dried. Then, an 85% aqueous methanol solution was used to elute with a final volume of 2 mL. Finally, the eluted solution was blow-dried with nitrogen and dissolved into 500 µL of methanol for assay by HPLC–QTOF–MS/MS (Agilent APCI–QTOF).

HPLC separation was performed on an Agilent Zorbax Extend-C18 (1.8 µm 2.1 × 100 mm) column using an Agilent 1260 Series system (Agilent, USA). The total flow rate was 0.2 mL/min, and the total elution program was 36 min with an optimized linear gradient elution of A (5 mM ammonium acetate) and B (acetonitrile) as follows: 0–15 min, 15–85% B; 15–20 min, 85% B; 20–25 min, 85–100% B; 25–31 min, 100% B; 31–31.1 min, 100–10% B; and 31.1–36 min, 10% B. The column temperature was maintained at 35 °C. The injection volume was 50 µL.

Mass spectra were acquired with an Agilent Accurate-Mass-Q-TOF MS 6520 system equipped with an atmospheric chemical ionization (APCI) source. All MS experiments were detected in the positive ionization mode. For Q-TOF/MS conditions, fragmentor and capillary voltages were kept at 240 and 3500 V, respectively. The temperature of the drying gas was set at 300 °C. The flow rate of the drying gas and the pressure of the nebulizer were 10 L/min and 25 psi, respectively. Full-scan spectra were acquired over a scan range of *m/z* 80–2500 at 1.03 spectra/s.

The peaks on the representative total ions current chromatograms representing SS a, SS c and SS d were identified by comparison of their chromatographic retention times, accurate molecular weights and characteristic mass fragment ions with those of the references standards. Other peaks were tentatively identified by comparison of their accurate mass data with those reported in the literature. Detailed information related to the illustration of peaks which showed variation between transformed and control samples is shown in Table 1. The quantification of SS a, SS c and SS d was calculated by comparison with reference standards. In addition, the relative quantification of other SS monomers was evaluated by comparison of the peak areas between those in transformed and control samples.

Results

Screening and cloning of BcbZIP134

A total of 26 TF genes were assayed for their expression profiles, and the results were combined with the SSs content assays in MeJA-treated *B. chinense* adventitious roots. Among those TF genes, the expression of *BcbZIP134* was elevated after MeJA treatment (Fig. 2a). The significant

Table 1 Comparison of minor SS monomers that were predicted by references between *BcbZIP134*-overexpressed hairy root lines and controls

Peak	t_R (second)	Identification	Extract molecular weight	Positive APCI-TOF-MS ¹ (m/z)	Positive APCI-TOF-MS ² (m/z)	CK	19	43	54	55	60
1 Zhu et al. 2017; Fang et al. 2017	680	C ₄₈ H ₇₈ O ₁₈ (unknown)	942.5188	925.5285 [M+H-H ₂ O] ⁺	763.3413 [M+H-H ₂ O-Glc] ⁺ 719.4265 [M+H-H ₂ O-C ₂ H ₂ O-Glc] ⁺	100% ± 41.2%	29.7% ± 9%*	28% ± 13%*	53.9% ± 18.4%	17% ± 14%*	43% ± 9%
2 Zhu et al. 2017; Fang et al. 2017	728	C ₄₄ H ₇₀ O ₁₄ (acetyl-SS a or -SS d)	822.4838	805.4859 [M+H-H ₂ O] ⁺	455.3606 [M+H-H ₂ O-C ₂ H ₂ O-FucGlc] ⁺ 437.3505 [M+H-2H ₂ O-C ₂ H ₂ O-FucGlc] ⁺ 419.3344 [M+H-3H ₂ O-C ₂ H ₂ O-FucGlc] ⁺	100% ± 6.7%	37.9% ± 5%***	208% ± 14.8%**	203% ± 64.7%*	105% ± 14%	58.7% ± 23.6%*
3 Zhu et al. 2017	774	C ₄₅ H ₇₀ O ₁₈ (unknown)	899.4635	882.5350 [M+H-H ₂ O] ⁺	865.5102 829.4835	100% ± 11.9%	23.7% ± 35.6%*	86.7 ± 23.2%	117% ± 63.8%	6.8% ± 3.5%*	89% ± 9.7%
4 Zhu et al. 2017; Fang et al. 2017	816	C ₄₄ H ₇₀ O ₁₄ (acetyl-SS a)	822.4838	the same as compound 2	the same as compound 2	100% ± 42.5%	32.3 ± 28.3%	232% ± 222%	278% ± 211%	88.6 ± 66.4%	112% ± 195%
5 Zhu et al. 2017; Fang et al. 2017	864	C ₄₄ H ₇₀ O ₁₄ (acetyl-SS a)	822.4838	the same as compound 2	The same as compound 2	100% ± 41.5%	7.8% ± 3.2%*	21.2% ± 22%	42.6% ± 20.2%*	11.7% ± 15.4%	25% ± 22%
6 Zhu et al. 2017	872	C ₄₂ H ₆₈ O ₁₂ (SS e)	764.4728	747.4827 [M+H-H ₂ O] ⁺	729.4703 [M+H-2H ₂ O] ⁺ 567.4196 [M+H-2H ₂ O-Glc] ⁺ 439.3638 [M+H-H ₂ O-FucGlc] ⁺	100% ± 25.7%	0***	17.2% ± 29.9%	34.1% ± 30.3%	0***	22.06% ± 19.6%*
7 Fang et al. 2017	878	C ₃₇ H ₆₂ O ₇ (unknown)	618.4216	601.4236 [M+H-H ₂ O] ⁺	455.3618 [M+H-164] ⁺	100% ± 40.3%	0***	0***	0***	0***	0***
8 Zhu et al. 2017; Fang et al. 2017	892	C ₄₄ H ₇₀ O ₁₄ (acetyl-SS a)	822.4838	the same as compound 2	the same as compound 2	100% ± 34%	18% ± 29.6%*	23.2% ± 17.4%	69% ± 65%	15.6% ± 14.3%	20.4% ± 30.7%
9 Zhu et al. 2017; Fang et al. 2017	1003	C ₄₄ H ₇₀ O ₁₄ (acetyl-SS d)	822.4838	the same as compound 2	the same as compound 2	100% ± 63.1%	3.02% ± 2.9%	32.7% ± 28.3%	20.3% ± 9.4%	21.2% ± 5.3%	20% ± 29.7%

The content differences were calculated as percentages of the peak area based on controls and the deviation obtained from the samples collected at three time periods during growth. T tests (* $p = 0.05$, and *** $p = 0.01$) were conducted between transgenic lines and controls

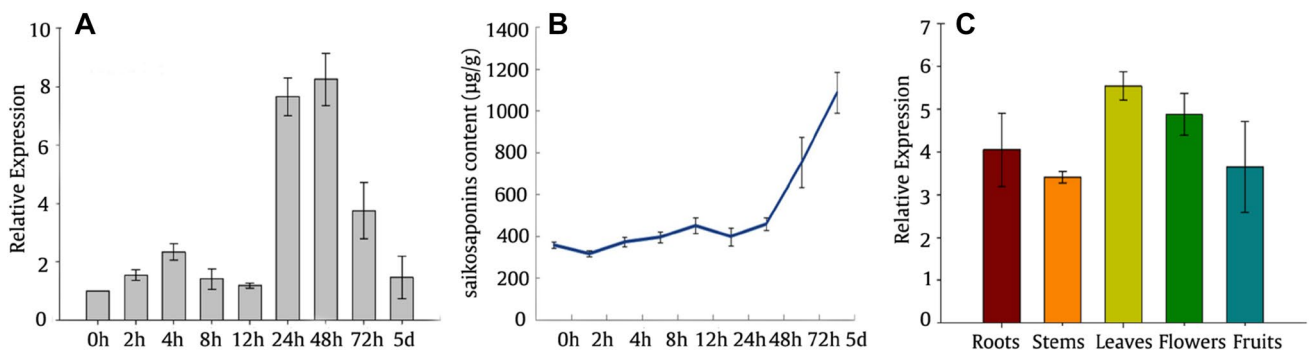


Fig. 2 Expression of *BcbZIP134* in MeJA-treated adventitious roots and different plant tissues. Expression of *BcbZIP134* (a) and the content of SSs (b) at 0, 2, 4, 8, 12, 24, 48, 72 h and 5 days after 200 μ M MeJA added into the medium. The relative expression was calculated

using the $2^{-\Delta\Delta Ct}$ formula with the expression in nontreated roots (0 h) as the calibrator. The content of SSs was calculated as the sum of SS a, SS c and SS d. c Expression of *BcbZIP134* in roots, stems, leaves, flowers and fruits was calculated using the $2^{-\Delta Ct}$ formula

elevation started at 24 h after MeJA treatment but gradually decreased beginning 72 h after treatment. This changing tendency was slightly ahead of the rapid increase in the

accumulation SSs (Fig. 2b). The expression of *BcbZIP134* in the roots, stems, leaves, flowers and fruits were tested and the results did not exhibit any remarkable differences

among tissues (Fig. 2c). Except for *BcbZIP134*, expressions of five other TF genes were enhanced by MeJA treatment. We present here the relevant results of *BcbZIP134*. Further exploration in the function of the five TF genes are underway and will be reported later.

Sequence character of BcbZIP134

The full-length cDNA of *BcbZIP134* was generated using RACE PCR. It included an open reading frame of 1,230 bp, encoding a predicted 410 amino acid protein. Analysis of the deduced amino acid sequence revealed that this protein had one typical bZIP TF DNA binding domain at aa 288–351. The sequence alignment showed that *BcbZIP134* had high sequence identity with bZIP TF 16-like and HBP-1a proteins, which belong to the G group of bZIP TFs from various plant species, such as *Daucus carota* subsp. *Sativus*, *Petroselinum crispum*, *Citrus clementina*, and *Coffea canephora*. Definite functions of these proteins homologous to *BcbZIP134* are still unknown. The phylogenetic tree was shown as Fig. 3 and the alignment was shown as Fig. 4. Similar to other members of the G group of bZIP TF, *BcbZIP134* had characteristic domains 1, 2, 3 which are parts of a proline-rich activation domain, as well as a bZIP domain (labeled in Fig. 4).

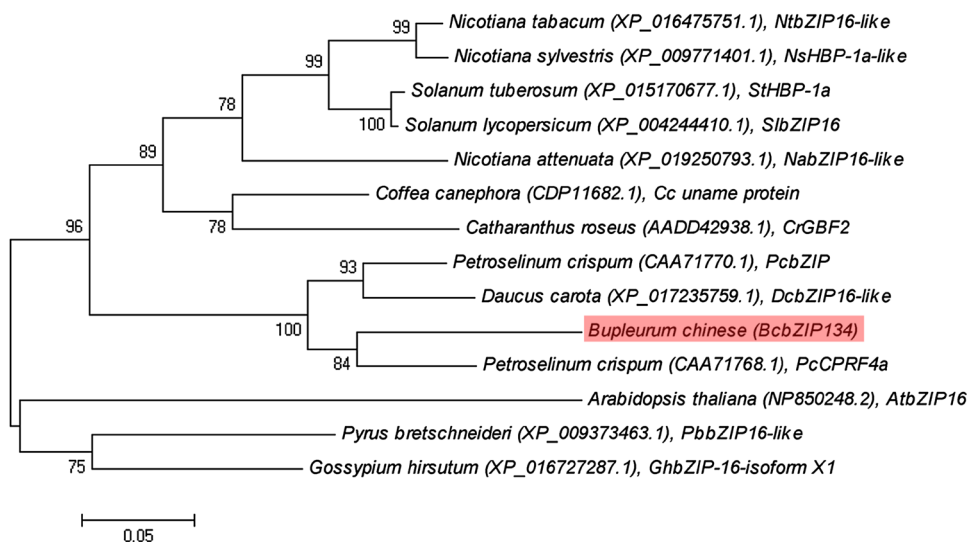
Overexpression of BcbZIP134 in hairy roots of *B. chinense*

The plant overexpression vector pK2GW7 harboring *BcbZIP134* driven by the 35S promoter was transferred into *A. rhizogenes* for hairy roots generation. The overexpressing hairy root lines were screened using real-time qRT-PCR and five independent lines, 19, 43, 54, 55 and 60, with considerably overexpressed *BcbZIP134* transcript

levels selected for the SSs contents assay (Fig. 5a). Compared with control hairy roots, *BcbZIP134*-overexpressing transgenic hairy roots grew slightly faster but stouter, and no difference in color appearance. The content of SS a, SS c and SS d, the main monomers of SS, were calculated and compared between *BcbZIP134*-overexpressed lines and controls. The content of SS a showed significant decrease in all of the overexpressed lines (Fig. 5c). The content of SS c in two lines 19 and 60, and the content of SS d in three lines 19, 55 and 60 significantly decreased (Fig. 5d, e). In all, the total content of SS a, SS c and SS d decreased significantly in all of the overexpressed lines (Fig. 5b).

In addition to the major SS a, SS c and SS d components, other minor SS monomers predicted by previous references were also assayed. One unknown compound (C37H62O7, compound 7 in Table 1) only existed in the controls. Five compounds (C48H78O18 unknown compound 1; C45H70O18 unknown compound 3; C44H70O14 acetyl-SS a compound 5; C42H68O12 SS e compound 6 and C44H70O14 acetyl-SS a compound 8 in Table 1) had significantly lower content in certain *BcbZIP134*-overexpressed lines than in the controls, and due to the large diversity seen between samples within the same line, the high standard deviation for these lines caused the statistical nonsignificance. The content of two compounds (C44H70O14 acetyl-SS a compound four and C44H70O14 acetyl-SS d compound 9) was decreased but was not statistically significant. Only lines 43 and 54 had an increased content of compound 2 (C44H70O14 acetyl-SS a or -SS d), which was the opposite of the others, showing inconsistent variation among individual lines. As a whole, the overexpression of *BcbZIP134* caused a significant decrease of the total SS content in hairy roots, especially SS a, which may play a negative regulatory role in the SS synthesis pathways.

Fig. 3 The phylogenetic tree of *BcbZIP134* and its homologous proteins was constructed by MEGA7. Alignment of all sequences to show the conserved motifs was presented in Fig. 4



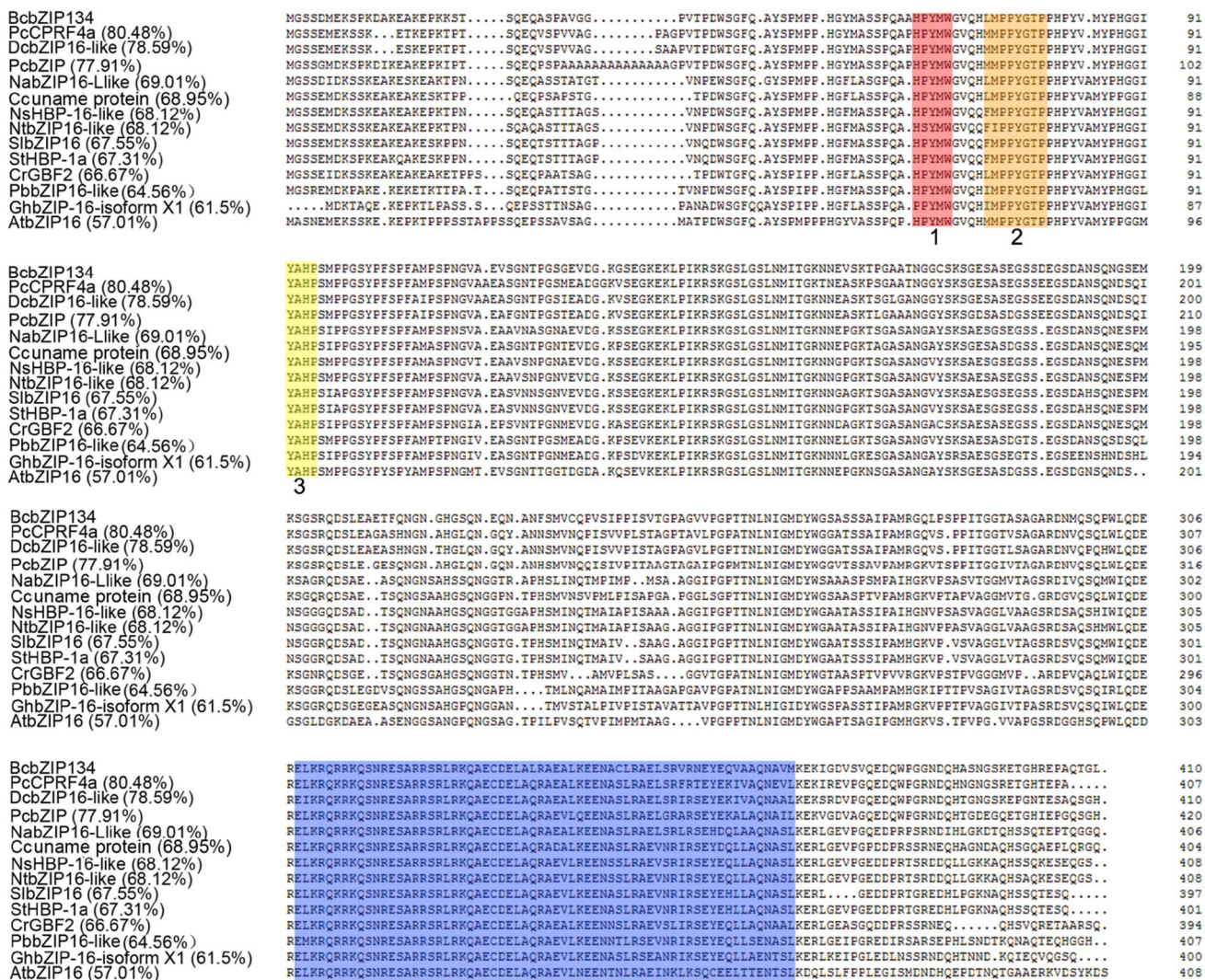


Fig. 4 Alignment of the amino acid sequence of *BcbZIP134* and its homologous proteins from other plants. The conserved motifs are labeled. Motifs one, two and three were identified according to a reference (Jakoby et al. 2002) as part of a proline-rich activation domain

in bZIP group G. The identity of each protein with *BcbZIP134* is given in the brackets after the protein name. The plant source and GenBank accession numbers of all these sequences were shown in Fig. 3

Discussion

In this study, hairy root transgenic systems were used to explore gene function. Unlike many other plants, most medicinal plants lack a transgenic model system, and this has hindered research progress on functional genes. Hairy root transgenic systems have been used as an effective model in a few medicinal plant species and other crops, such as ginseng (Zhang et al. 2015a, b), Danshen (Xu et al. 2017), *Scutellaria baicalensis* (Zhao et al. 2018), and *M. truncatula* (Merten et al. 2016). These systems are practical, considering the long growth period of the roots and since roots are used as the therapeutic parts for most medicinal plant species. In addition, a recent study reported the production

of sapogenins (stigmasterol and hecogenin) using genetically transformed hairy roots of *Chlorophytum borivillanum* (Bathoju et al. 2017). Here, for the first time, we report the use of transgenic hairy roots of *B. chinense* to study gene functions. We believe this system will promote biological and genetic studies on this herb that is used in both traditional and modern medicine. When we overexpressed the cDNA of *BcbZIP134* using the hairy root transgenic system, negative regulation function of this TF on SS biosynthesis was deduced. While in our primary screening with MeJA treated adventitious roots, both expression of *BcbZIP134* and content of SSs were elevated. This suggests a positive regulation function of *BcbZIP134* to the SS biosynthesis. It implied that the increase of SSs may be regulated

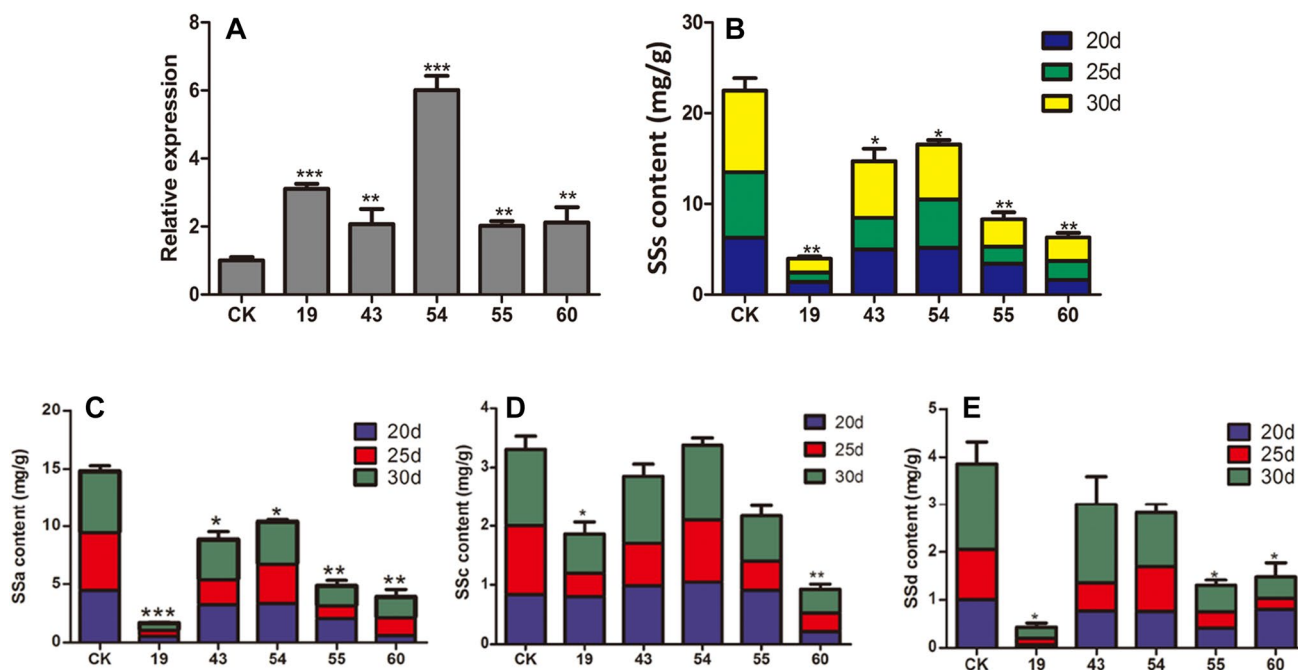


Fig. 5 Transcript abundance and SS content in *B. chinense* *BcbZIP134*-overexpressing hairy roots lines and controls. **a** Transcript abundance showing significantly elevated *BcbZIP134* levels in the overexpressed lines. **b** Comparison of the total content of SS a, SS c and SS d showing significant decreases in the overexpressed lines. **c–e** The individual contents of SS a, SS c and SS d are com-

pared showing altered changes in different overexpressed lines. Hairy root samples collected at three times (20 days, 25 days, and 30 days after changing new culture media) during growth were treated as three replicates and the symbol (*) represents statistical significance by Student's *t* tests

by multiple ways and it is the combined results of different regulations. The relationship among SS biosynthesis and MeJA-responsive genes needs to be explored further.

The biosynthesis of secondary metabolites can be affected by developmental and environmental conditions. Although dozens of different SS monomer compounds have been detected in the roots of *Bupleurum*, little investigation has been conducted on the biosynthesis and regulation of different SS monomers. This is especially true for minor compounds and their mutual transformation in a given *Bupleurum* species (Li et al. 2015). In this study, we compared the content of SS a, SS c and SS d, the three major SS monomers in *Bupleurum*, with reference standard compounds. Other minor compounds were assayed based on the literature reports (Zhu et al. 2017; Fang et al. 2017). Nine minor compounds showed content variation (listed in Table 1). Other SS compounds such as SS f, SS h, SS i and SS b were not detected in either *BcbZIP134*-overexpressed lines or controls. This may indicate that differences exist between hairy roots and other plant roots, or that differences exist in hairy roots cultivated for different lengths of time. We have noticed that the hairy roots cultivated for 2 months after new culture media was changed usually synthesized more SS. Additionally, seven of the nine compounds were significantly decreased in transgenic line 19, combined with the

lowest content of SS a, SS c and SS d in it, which showed the transgenic line 19 was most affected. While the transgenic line 54 showed most abundance of transcripts compared with other transgenic lines. It did not correspondingly possess the lowest content of SSs, although significant decrease in total contents of SS a, SS c and SS d were observed. It indicates that some unknown mechanism may exist to fine tune the accumulation of SSs in *B. chinense* and this mechanism functions when SS biosynthesis is severely threatened.

It has been reported that salt and drought stress can potentially increase pharmaceutically significant secondary compounds in plants (Selmar et al. 2008; Nasrollahi et al. 2014) and that drought stress, fertilizer or altered planting time influences the SS a, SS c and SS d content in *Bupleurum* roots (Zhang et al. 2016; Lee et al. 2002; Zhu et al. 2009a, b). The underlying molecular regulatory mechanisms of these changes have seldom been explored. As for other plant species, a few TFs have been reported to regulate the biosynthesis of terpenoids. Those belonging to the bZIP family include AabZIP1 from *Artemisia annua* (Zhang et al. 2015a, b) and OsTGAP1 and OsbZIP79 from *Oryza sativa* (Okada et al. 2009; Miyamoto et al. 2014). OsTGAP1 exerts an essential role on the expression of the clustered genes for momilactone biosynthesis and functions as a key regulator of the coordinated transcription of genes involved in

inductive diterpenoid phytoalexin production in rice (Okada et al. 2009). OsbZIP79 interacts with OsTGAP1 and functions as a negative regulator of diterpenoid phytoalexin production that is triggered by a chitin oligosaccharide elicitor in rice cells (Miyamoto et al. 2014). It may be speculated that TFs play vital linking roles between stress responses and secondary metabolism in plants and much related to be discovered.

Among the *A. thaliana* bZIP-family, Group G contains five members: AtbZIP16, AtbZIP68, AtbZIP41 (AtGBF1), AtbZIP54 (AtGBF2) and AtbZIP55 (AtGBF3) (Dröge-Laser et al. 2018). The amino acid sequence of BcbZIP134 shares a high level of identity with bZIP TF 16-like proteins. It has been found that AtbZIP16 is a negative regulator in light-mediated inhibition of cell elongation but is a positive regulator in light-regulated seed germination. AtbZIP16 negatively regulates and AtbZIP68 and AtGBF1 positively regulate the expression of light-harvesting chlorophyll a/b-binding protein (LHCb2.4) (Shen et al. 2008; Shaikhali et al. 2012). In addition, GBF1-interacting protein 1 (GIP1) functions to promote hypocotyl elongation during the early stages of *A. thaliana* seedling development by regulating the repression effect by bZIP16 and the activation effect by bZIP68 and GBF1 on LHCb2.4 expression (Shaikhali et al. 2015). The environmentally induced changes in the redox state regulate the activity of members of the G-group of bZIP TFs (Shaikhali et al. 2012). Moreover, GBF1 interacts with the bHLH master regulator MYC2 and the CAM7-TF in *Arabidopsis*. GBF1 physically interacts with MYC2 and inhibits each other through non-DNA binding heterodimers (Maurya et al. 2015), and important functions of GBF1 have been established in promoting lateral root development and natural senescence (Dröge-Laser et al. 2018). GBF from *Catharanthus roseus*, CrGBF1 and CrGBF2 (66.67% identity with BcbZIP134) play negative regulatory roles in the biosynthesis of terpenoid indole alkaloids (TIAs). Both CrGBF1 and CrGBF2 regulate the expression of strictosidine synthase gene (*Str*) via direct interaction with the G-box in the promoter of *Str* (Sibéril et al. 2001). These studies demonstrate the complexity of the regulatory network in functioning bZIP TF G-group members. A recent study showed that repressors CrGBFs dimerize with activator CrMYC2, and CrGBF1 binds to the same cis-elements (T/G-box) as CrMYC2 in the target gene promoters, suggesting that CrGBFs antagonize CrMYC2 transactivation possibly by competitive binding to the T/G-box in the target promoters and/or protein–protein interaction that forms a non-DNA binding complex that prevents CrMYC2 from binding to its target promoters, thus fine-tune the accumulation of TIAs (Sui et al. 2018). Earlier studies have shown that the DNA binding activity of parsley CPRF4a, with which *BcbZIP134* shares the highest sequence identity (80.48%), is modulated in

a phosphorylation-dependent manner and that cytosolic components are involved in the regulation of this process (Wellmer et al. 2001). Therefore, much effort is still needed to explore how *BcbZIP134* performs its regulatory functions in *B. chinense* and whether it functions as repressor of SS biosynthesis through competing the binding site in promoters of enzymatic genes with some activators. In the model reported here, we have determined that *BcbZIP134* overexpression led to the negative regulation of SS biosynthesis.

Conclusions

BcbZIP134, isolated from transcriptome of *B. chinense*, encodes a TF transcription factor belonging to bZIP G subfamily. MeJA can induce the high expression of *BcbZIP134* in adventitious roots of *B. chinense*. Hairy roots of *B. chinense* overexpressing *BcbZIP134* biosynthesize significantly less content of the main SS monomers a, c and d when compared to control hairy roots. The contents of a few other minor SS monomers such as acetyl-SS a, acetyl-SS d and unknown compounds were also decreased on different extent. It is revealed that *BcbZIP134* negatively regulates the biosynthesis of saikosaponin in *B. chinense*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Appendix: Primers used in the present study

Purposes	Names	Sequences (5′–3′)
qRT-PCR for β -Tubulin	Tu-F	ATGTTTCAGGCGCAAG GCTT
	Tu-R	TCTGCAACCGGGTCA TTCAT
qRT-PCR for Actin	At-F	TCACCATTGGAGCTG AGAGATTC
	At-R	CTGCAGCTTCCATTC CAATCA
qRT-PCR for <i>BcbZIP134</i>	bZIP134-F	CCTCCATTAGTGGCT GTTCC
	bZIP134-R	CTTATCCATTTCAGTC CATTTC
3′-RACE for <i>BcbZIP134</i>	GSP3	GCGGTGAAGTGGATG GTAAGGGGTCTGA
	NGSP3	TGGATGGTAAGGGGT CTGAGGGGAAGGA
5′-RACE for <i>BcbZIP134</i>	GSP5	TCCTTCCCCTCAGAC CCCTTACCATCCA
	NGSP5	CCCCTTACCATCCAC TTCACCGCTTCC
Full-length cloning PCR for <i>BcbZIP134</i>	FL134-F	TGTTTCGAGTTGAGTT AGAAGCTACAAT
	FL134-R	GAACACCCATCTAAA TGCATACAAG
Construction of over-expression vectors for <i>BcbZIP134</i>	OE134-F	GGGGACAAGTTTGTGA CAAAAAAGCAGGCTT GATGGGTAGTA GTG ATATGGA AAAATC
	OE134-R	GGGGACCACTTTGTGA CAAGAAAAGCTGGGTG TCAGAGACCAGTTTG TGCGGGCTCTC
Verification for <i>BcbZIP134</i> insertion	P35S	GACGCACAATCCAC TATCC
	T35S	GCTCAACACATGAGC GAAAC
qRT-PCR for <i>HMGR</i>	HMGR-F	TATAACTGGCGATGT GGTGAAG
	HMGR-R	GTGGCTATGAAGATT GCAGAAAC
qRT-PCR for <i>FPS</i>	FPS-F	CTCACACGCAGAG GTCAA
	FPS-R	TGGGGATATGGTTGC GAAG
qRT-PCR for <i>IPPI</i>	IPPI-F	AGGTGACATTCCCTT TGTTG
	IPPI-R	AGAAGCTTCTCTGT GCAGC
qRT-PCR for <i>SQS</i>	SQS-F	AGGTTACCAGGAAGC AATAGAA
	SQS-R	CGACAACCCTAATCC AACAAAG

Purposes	Names	Sequences (5′–3′)
qRT-PCR for <i>SQE</i>	SQE-F	GGTGGAGGAATGACG GTTGC
	SQE-R	TACGGGCTTGCGAAG GGTGT
qRT-PCR for β -AS	AS-F	ACATGGCTTTTCGATA CTCGG
	AS-R	ATTTTCGCTGGATGC ATAGG

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