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Isolation and characterization of a novel (*S*)-canadine synthase gene from *Coptis chinensis*

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ABSTRACT

Background: Berberine acts primarily as an important active ingredient in *Coptis chinensis* and has been traditionally applied in clinical treatment. Nevertheless, little information has been released about *C. chinensis*, as far as functional genes and biosynthetic pathway of berberine are concerned. Here, we isolated a novel (*S*)-canadine synthase gene (designated as *CAS-1*) from *C. chinensis* by using RT-PCR and RACE techniques.
Results: Bioinformatics analysis showed that the cDNA is 1942 bp in length with a complete open reading frame (ORF) of 1476 bp, and the ORF encodes a polypeptide of 491 amino acids with a calculated molecular mass of 55.29 kDa and a pI value of 8.92. Real-time quantitative PCR analysis showed that *CcCAS-1* was constitutively expressed in leaf, petiole and rhizome tissues, especially in the leaves of *C. chinensis*. However, the results of berberine content in different tissues by high-performance liquid chromatography with photodiode array detection (HPLC-DAD) method showed that the leaves and the petiole tissues have similar content of berberine.
Conclusions: We found that the berberine content in the rhizome was seven times (more or less) than that in the leaves and the petioles. In addition, the full length coding sequence of *CcCAS-1* was inserted into pET-32a and was successfully expressed in *Escherichia coli*, laying a solid foundation for protein purification, activity assay and multi-clonal antibody preparation. Together, our data suggest that *CcCAS-1* is a novel heme-thiolate enzyme essential for berberine biosynthesis in *C. chinensis*.

1. Introduction

Coptis chinensis, belonging to the family Ranunculaceae, has been effectively used as one of the most popular herbal medicines for preventing diarrhea, alleviating the stomach cramps, treating cancer, reducing fever, and the risk of heart disease and stroke [1,2,3,4]. Its main product areas have been distributed in Southwest China, the most famous-region product area is Shizhu Tujia Nationality Autonomous County, and it has been generally accepted as one of frequently used ethnic medicines for a long time in Tujia Nationality [5,6,7]. So far as recent studies are concerned, the main active ingredient in *C. chinensis* is benzylisoquinoline alkaloid such as berberine [8], which is rather difficult to be synthesized chemically and extracted in large amounts [9]. Recently, berberine has drawn increased attention for its high production to satisfy the magnifying demand, so it is very important to study metabolic engineering about berberine in *C. chinensis*. Moreover, berberine had been usually used as a marker to monitor the drug quality in China [10,11].

Up to date, the biosynthetic pathway of berberine has been investigated and known in several species, such as *Coptis japonica* [12], *Thalictrum flavum* [13], and *Eschscholzia californica* [14]. However, in *C. chinensis*, no correlative functional gene has been reported. In the pathway, many genes such as (*S*)-tetrahydroberberine oxidase ((*S*)-*THBO*), (*S*)-norcoclaurine synthase ((*S*)-*NCS*), and (*S*)-canadine synthase (*CAS*), are involved in berberine biosynthesis [9,12,13]. *CAS*, belonging to the P450 family, catalyzes the (*S*)-tetrahydrocolumbamine ((*S*)-THC) to produce (*S*)-tetrahydroberberine ((*S*)-THB) and has been studied in *C. japonica* [12] and *T. flavum* [15]. *CAS* is the downstream gene in the biosynthesis pathway of berberine, and its main function is to form the special methylenedioxy bridge structure.

In this study, we report the molecular cloning and characteristics of a *CAS* gene (*i.e. CcCAS-1*) from *C. chinensis*. We isolated a full-length cDNA based on the sequence homology, and the open reading fragment (ORF) of *CcCAS-1* was successfully expressed in prokaryotic expression vector pET32a(+). This study provides fundamental information for further understanding the mechanism of berberine biosynthesis and future application in the metabolic engineering of *C. chinensis*.

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2. Materials and methods

2.1. Plant materials and RNA isolation

All tissue materials were collected from two-year-old seedlings of *C. chinensis* in the Shizhu Tujia Nationality Autonomous County, Chongqing City, China, and instantly frozen in liquid nitrogen and stored at -80°C for RNA extraction.

Total RNA was extracted from the leaves, petiole and rhizome of *C. chinensis* exposed to 300 mM NaCl by using plant RNA isolation reagent (Tiangen Biotech, Beijing) following the manufacturer's instructions. Total RNA samples of different tissues (leaf, petiole and rhizome) were reversely transcribed by using AMV reserve transcriptase (Takara, Japan) to generate cDNA.

2.2. Cloning the full-length cDNA of CcCAS-1

Based on highly conserved sequences of *CAS* genes, we designed gene specific primers (*CcCAS-1*-Fwd: 5′-ACTAACTCGCCTTATTTT CGG-3′; *CcCAS-1*-Rev: 5′-GCAAAAGCAAAC TGAAGCT-3′) for obtaining the core sequence of *CcCAS-1*. The nucleotide sequences of the 3′ and 5′ ends of *CcCAS-1* were amplified by the RACE method [16], using specific primers (*CcCAS-1* 3-1: 5′-CGTGAACAAGGAGCACAAGA-3′; *CcCAS-1* 3-2: 5′-GGGACTAAGGTAG CCAAGGG-3′; *CcCAS-1* 5-1: 5′-CATAGCCACTAATGCGAATA-3; *CcCAS-1* 5-2: 5′-TTTTTGT GACTGG GGAGGTA-3′). The full-length cDNA was then amplified with *CcCAS-1*-ORF primers (*CcCAS-1*-ORF-Fwd: 5′-CGCGGATCCATGGAGATG AGTCCAC TGC-3′; *CcCAS-1*-ORF- Rev: 5′-CGAGCTCTCAAACATTTCTACC AGCG-3′). *CcCAS-1*-ORF was used to amplify the ORF of *CcCAS-1* gene. All resulting products were sub-cloned into pMD18-T vectors (Takara, Dalian, China) and sequenced (Invitrogen, Shanghai, China), respectively. The specific primers were designed with Primer Primer 5.0 software (Premier Biosoft International, USA). Alignments of amino acid sequences were carried out with the sequence analysis software DNAMAN (Lynnon Biosoft, USA).

2.3. Expression pattern of CcCAS-1 in different tissues

Real-time quantitative PCR (qPCR) was used to evaluate expression levels of *CcCAS-1* in different tissues. The qPCR was performed according to the manufacturer's instruction (TaKaRa, Japan). Each sample was amplified by the gene specific primer (*CcCAS-1*-real time-Fwd: 5′-GGGACTAAGGTAGCCAAGGG-3; *CcCAS-1*-real time-Rev: 5′-GCACTG AAGGGAAGAA ACGA-3) and *18S rRNA* primer (GenBank accession No. DQ406855; internal standard, *Cc*18S-real time-Fwd: 5′-ACCATAAACG ATGCCGACCAG-3′; *Cc*18S-real time-Rev: 5′-CAGC CTTGCGACCATAC TCCC-3′). PCR reactions were carried out under the following conditions: denaturation at 95°C for 10 s, 35 cycles at 95°C for 10 s, 60°C for 20 s and 72°C for 20 s, and then 72°C for 3 min. Quantification of the gene expression was done with comparative CT method. Experiments were performed in triplicate, and the results were represented by their mean ± standard error.

2.4. Construction of recombinant prokaryotic expression vector and expression of CcCAS-1 in Escherichia coli (E. coli)

Using cDNA as the template, the full-length coding region of *CcCAS-1* gene was amplified by PCR using the forward primer *CcCAS-1*-ORF-Fwd containing the *Bam*H I site and the reverse primer *CcCAS-1*-ORF-Rev containing the *Sac* I site. The fragment was digested with *Bam*H I and *Sac* I and gel-purified with the Gel Extraction Kit (Axygen scientific Inc., USA), then integrated into a purified pET32a(+) vector digested with the same enzymes, forming a recombinant plasmid pET32a-*CcCAS-1*. The recombinant plasmid pET32a-*CcCAS-1* and empty vector pET32a(+) were transformed into *E. coli* BL21. The transformed cells were cultured at 37°C in Luria-Bertani (LB) medium until OD_{600} reached 0.6–0.8, isopropyl-b-D-thiogalactoside (IPTG, Merck, Germany) was then added to a final concentration of 1 mmoL L^{-1}, and further incubated at 37°C for 5 h. The cells of an overnight culture (approximately 1 mL) were harvested by centrifuging at 5000 × g for 10 min at 4°C, dissolved in 100 μL 1 × SDS sample buffer and boiled at 100°C for 10 min. The total bacterial protein was analyzed by 12% SDS-PAGE.

2.5. Detection of alkaloid content in different tissues

The clean leaves, petiole and rhizome of *C. chinensis* were crushed into powder, respectively. Approximately 0.1 g of the powder was accurately weighed and treated with 50 mL MeOH–HCl (100:1, v/v) in conical flask with tampon, then handled with ultrasound for 30 min and cooled at room temperature. The weight loss in the ultrasound procedure was compensated using MeOH–HCl. After agitation and filtration, the samples were filtered through 0.45 μm microporous film to yield the sample solution and subsequently subjected to HPLC (Agilent 1100 Series LC system) with DAD detector.

The analytical column was an Xtimate C18 HPLC column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of 30 mmoL/L ammonium bicarbonate buffer containing 0.1% (v/v) triethylamine and 0.7% (v/v) ammonia water (A) and acetonitrile (B). Separation was performed at 30°C with a flow rate of 1.0 mL/min, and the detection wavelength was set at 270 nm. Sample solutions (10 μL) were injected and separated using the following gradient elution program: 10–25% B at 0–15 min, 25–30% B at 15–25 min, and 30–45% B at 25–40 min. The concentrations of active alkaloids in each sample were determined by using the established calibration curves made from the standard compounds.

2.6. Statistical analysis

All the experiments were laid out in completely randomized design with three repetitions. The contents of berberine were presented as mean ± standard error. The statistical difference between the means was estimated at the 5% significance level by the *t* test.

3. Results and discussion

3.1. Cloning of the full-length cDNA of CcCAS-1

Based on the highly conserved sequence of plant *CAS* genes [12], a cDNA fragment of 774 bp was initially isolated with specific primers by the RT-PCR method. 3′- and 5′-RACE were accomplished using the specific primers, fragments of 362 bp and 806 bp were amplified by 3′- and 5′-RACE, respectively. After cutting away the adapter sequences, the 1942 bp cDNA of *CcCAS-1* (GenBank accession No. KC577598) includes a 1476-bp open reading frame (ORF) from 241 bp to 1716 bp of the sequence besides a 240-bp 5′ UTR and a 226-bp 3′ UTR including a PolyA tail. The ORF encodes a polypeptide of 491 amino acids, with a calculated molecular mass of 55.29 kDa and a pI value of 8.92 (http://cn.expasy.org/tools/protparam.html).

3.2. Sequence analysis of putative CcCAS-1 protein

Based on the NCBI database BlastP program analysis, the putative amino acid sequence of CcCAS-1 showed high identities with other organisms such as *C. japonica* (GenBank accession no. AB026122), *Argemone mexicana* (GenBank accession no. EF451151), *Papaver somniferum* (GenBank accession no. GU325750), and *E. californica* (GenBank accession no. EU882969). *CcCAS-1* had three typical conserved regions: a helix K region, an aromatic region, and a heme-binding region at the C-terminal end (Fig. 1) [14]. In addition, *CcCAS-1* had a conserved serine (Ser-296), which played a significant role in oxygen-activation [12,17].

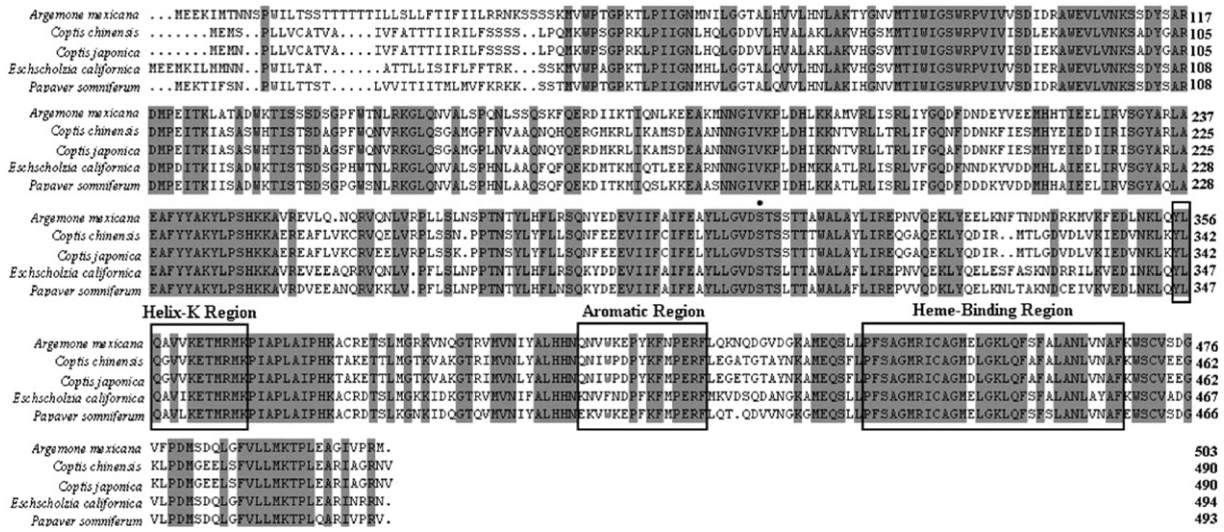

Fig. 1. Alignment and sequence analysis of deduced amino acid sequences of *CcCAS* proteins in *C. chinensis* and other species. Boxes indicate the conserved regions (*i.e.* helix-K region, aromatic region, and heme-binding region). Black dot indicates the position of Ser-296.

3.3. Expression levels of CcCAS-1 in different tissues

To investigate the expression pattern of *CcCAS-1* gene in different tissues of *C. chinensis*, total RNA was isolated from the leaves, petiole and rhizome, respectively; then subjected to real-time quantitative PCR using *18S rRNA* as the internal control. The real-time PCR results indicated that *CcCAS-1* was a constructive expression gene but at different levels. The expression levels from high to low were in the leaves, petiole and rhizome (Fig. 2). From the expression pattern in different tissues, we could infer that in the pathway of berberine, the synthesis of (*S*)-canadine synthase was mainly located in leaves, moreover, the highest content of berberine was in rhizome, which maybe confirm that the process of the reaction of (*S*)-canadine synthase completed principally in leaves, but the accumulation of berberine completed principally in rhizome. At the same time, we speculated that it had specific functions in the expressing tissues [14].

3.4. Prokaryotic expression of CcCAS-1 in E. coli

Recombinant pET32a(+) vector containing *CcCAS-1* was expressed in *E. coli* BL21 and analyzed by SDS–PAGE. As shown in Fig. 3, the recombinant protein pET32a-*CcCAS-1*, with a molecular weight of 73 kD, was observed in *E. coli* BL21 (Fig. 3, Line 2). Due to His-tag proteins with an expected molecular weight of 18 kD (Fig. 3, Line 1), the molecular weight of the *CcCAS-1* protein was about 55 kDa, which was similar to the predicted molecular mass. After induction by IPTG for 5 h, the protein expression showed high level. The results showed that the *CcCAS-1* gene could be highly expressed in *E. coli* cells and the successful expression of recombinant *CcCAS-1* in prokaryote confirmed that the anticipated protein was encoded by *CcCAS-1* gene. Prokaryotic expression of *CcCAS-1* in *E. coli* would lay a solid foundation for protein purification, multi-clonal antibody preparation and the activity assay of canadine synthase.

3.5. Chemical analysis in different tissues

The proposed HPLC-DAD method was subsequently applied to the determination of main alkaloids in different tissues of *C. chinensis* (Fig. 4). In Fig. 4, the peak of berberine was evaluated by comparison with authentic standards. In different tissues (leaf, petiole, and rhizome), the berberine was found in approximately 35 min (retention time), but the content of berberine existed disparity. Similar content of berberine was shown in the leaves (14.95 mg g^{-1}) and the petiole

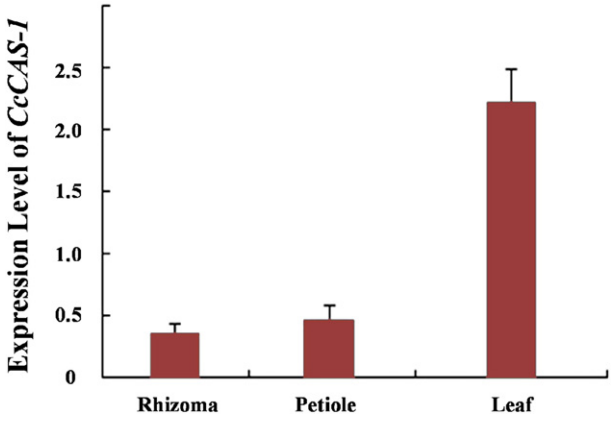

Fig. 2. Expression level of *CcCAS-1* in different tissues of *C. chinensis*. Data represent the mean values ± SE of three replicates.

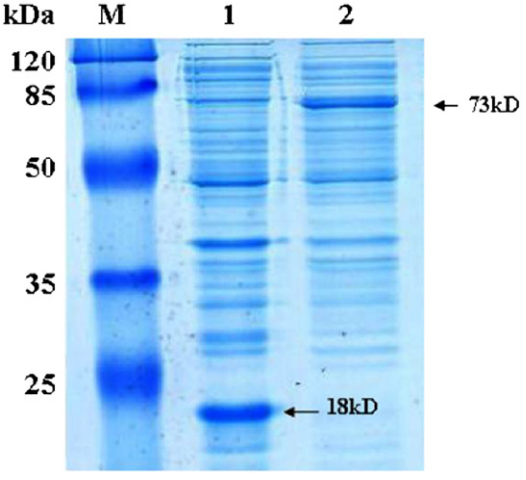

Fig. 3. SDS–PAGE analysis of *CcCAS-1* protein expression in *E. coli* (Coomassie blue staining). Lane M, Protein marker; Lane 1, whole cell lysate of BL21 *E. coli* cells containing the empty vector pET32a(+); Lane 2, whole cell lysate of BL21 *E. coli* cells containing the pET32a-*CcCAS-1*.

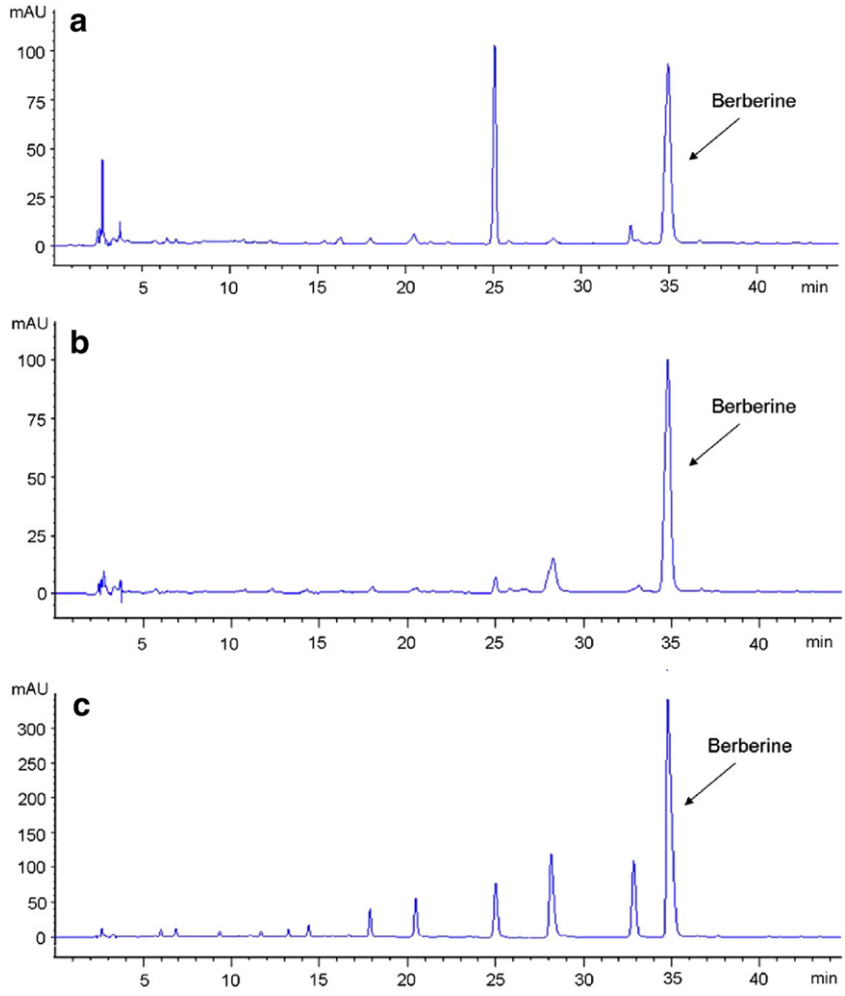

Fig. 4. HPLC-DAD chromatograms in different tissues of *C. chinensis*. (a) leaf; (b) petiole; (c) rhizome. The peak (the retention time were approximately 35 min), which arrows indicated, were berberine in different tissues of *C. chinensis*.

(16.10 mg g^{-1}) tissues, nevertheless, the berberine content was observed approximately seven times(more or less) in the rhizome (110.36 mg g^{-1}) than in the leaves and the petiole [18]. While the other main alkaloids were also detected, there was a significant disparity in the berberine content and the types of alkaloids [19].

3.6. Correlation analyses between berberine content and expression level of CcCAS-1

In recent years, the accumulation of berberine might be mainly affected by the expression level of *CcCAS-1* [12,15]. In our study, the correlation analyses between berberine content and *CcCAS-1* expression level in different tissues were investigated. From the peak and the content of berberine in different tissues (Fig. 4), the rhizome showed the highest content, however, the lowest expression level of *CcCAS-1* was existed in the rhizome. On the contrary, the leaves had the lowest berberine content and the highest expression level of *CcCAS-1*. In petiole, there are middle berberine content and intermediate expression level of *CcCAS-1*. This raises the question of how the expression level of *CcCAS-1* would affect the homeostasis of main alkaloids and regulate the berberine content in *C. chinensis*. From the results, we could not draw a conclusion about the relationship between the content of berberine and the expression level of *CcCAS-1* in different tissues. This was mainly because that there were many functional genes involved in the pathway of berberine biosynthesis, and each gene's expression level might be affected by the internal phytohormones and external environmental factors [20,21,22].

4. Conclusions and future prospect

In this work, we isolated and characterized a novel gene encoding a (*S*)-canadine synthase from *C. chinensis*, which we designated *CcCAS-1*. To our knowledge, this is the first report on the isolation and characterization of the gene from *C. chinensis*. This work would lay a solid foundation and play an important role for further study on the functions of *CcCAS-1* in *C. chinensis*. In considering future work, we will transform or transfer this gene into model plants such as Arabidopsis and tobacco to discover unknown roles.

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Conflict of interest

The authors declare that there is no conflict of interest.

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