ISOLATION, SEQUENCE AND BACTERIAL EXPRESSION OF A cDNA FOR CHALCONE SYNTHASE FROM THE CULTURED CELLS OF Pueraria lobata

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cDNA clones for chalcone synthase (CHS) of Pueraria lobata cultured cells were isolated by screening the cDNA library using CHS cDNA of Phaseolus vulgaris as a probe. Analysis of nucleotide sequences of the cloned cDNA revealed a 1170-bp open reading frame that encoded a 390-amino acid polypeptide with an Mr of 43,000. The full-length cDNA was cloned into the expression vector pT7-7. CHS activity was found in the crude extracts of transformed E. coli after induction and two protein bands of ca. 43 and 34 kd were hybridized with anti-persley CHS antiserum.

KEYWORDS biosynthesis; secondary metabolism; chalcone synthase; elicitor; cDNA cloning; DNA sequence analysis; gene expression

The biosynthesis of flavonoids has been extensively studied at the enzyme level in many species of plants, since flavonoids are ubiquitous in higher plants and have a wide variety of physiological functions in the plants. A typical example is the synthesis of phytoalexins in legumes against stress or microbial infection. In the last decade great attention has been paid to the study of the regulatory mechanism of flavonoid biosynthesis, since the recent progress of molecular biology made it possible to study flavonoid biosynthesis at the enzyme and gene levels. The first pathway-specific enzyme in flavonoid biosynthesis is chalcone synthase (CHS), which catalyzes the formation of chalcone from p-coumaroyl and malonyl CoAs. CHS cDNAs were cloned in several species of plants. Studies of genomic CHS DNA were also reported. We have been studying enzymology and the reaction mechanisms of isoflavonoid biosynthesis using cell suspension cultures of Pueraria lobata. This paper reports the cloning, sequencing and bacterial expression of a cDNA for chalcone synthase (CHS) from the cultured cells of P. lobata.

In our previous studies on the enzymology of isoflavonoid biosynthesis we used an endogenous elicitor to induce CHS. Later, yeast extract was found to be a better elicitor to induce the CHS activity in the cultured cells of P. lobata and was used as the elicitor to obtain mRNA. A northern blot analysis (Fig. 1) demonstrated that yeast extract induces CHS synthesis which is regulated at the transcriptional level, since the amount of mRNA in untreated cells was below the detectable level. The size of CHS mRNA, 1.5 kb which is sufficient to encode a protein of 43 kDa, was determined by the SDS-PAGE of purified CHS from the cultured cells of P. lobata. A cDNA library was constructed in λ gt10 and 2.0 x 10⁵ recombinants were screened with 32P labelled CHS cDNA of Phaseolus vulgaris as a probe. Four hybridization-positive clones (designated as PICHSI, PICHSII, PICHSIII and PICHSIV) were obtained and sequenced by a DNA sequencer (Hitachi SQ-3000) after subcloning in pBluescript. The longest cDNA clone, pPICHSI is a full-length clone comprising a single open reading frame (ORF) of 1170 bp that encoded a 390-amino acid polypeptide with an Mr of 43,000, 97 bp of 5' leader sequence and 188 bp of 3' untranslated sequence. A part of the cDNA and deduced amino acid sequences are shown in Fig. 2. The other clones were not full-length cDNAs, but were 625 bp, 838 bp and 474 bp long, respectively. Their sequences of coding regions were the same as that of PICHSI, but

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Fig. 1. RNA Blot Hybridization Analysis of P. lobata CHS mRNA
The total P. lobata RNA (2 μg) was separated on a 1.2% agarose gel, blotted and hybridized with radioactive probes (1.4-kbp EcoRI fragment of pCH15). The mobilities of the 25S (3.4 kb) and 17S (1.8 kb) rRNA are indicated by lines. The size of P. lobata CHS mRNA was estimated to be 1.5 kb. (+): elicitor-challenged with yeast ext, (-): no elicitor-challenged.
the lack a part of the sequence in the 3’ untranslated region, just before poly(A), by 18 bp, 15 bp and 8 bp, respectively. It is not clear that these deletions are due to different origins of the clones or to artifacts during the cloning procedure. Sequence comparison between the ORFs of P. lobata CHSI and Phaseolus vulgaris CHS cDNA showed their high homology, 90.8% at nucleotide level and 95.9% at amino acid level as is expected from their close taxonomical relation.

The isolated cDNA of CHSI has several stop codons in the 5’ leader sequence and a sequence, AAGGA, which is identical to one of the ribosome binding sites (rbs) of E. coli, at 8-bp upstream from the initiation codon ATG. Since this AAGGA sequence was expected to function as rbs in transformed E. coli, we tried to express the isolated cDNA in E. coli. The BamHI fragment of cPICHSI was ligated to the BamHI site of an expression vector pT7-7 (T7 RNA polymerase/T7 promoter expression system)\(^\text{8a}\) without adjusting a frame to the translating frame of the expression vector. The construct was designated as pT7CHS. Induction was effected by raising the temperature, as described by S. Tabor.\(^\text{8b}\) Though no distinct band of expressed CHS appeared at the expected molecular weight of CHS in silver stained SDS-PAGE, more than one distinct band were detected by immunoblotting analysis (western blotting) using anti-parley CHS antisemur (Fig.3).\(^\text{9a}\) The largest band blotted at 42-43 kd (lane 3) coincides with that of purified CHS from P. lobata cell cultures (lane 4).\(^\text{9b}\) and the deduced Mr of a protein coded in the ORF of the cDNA. The other band corresponds to a protein of ca. 34 kd. In addition to the initiation ATG which is accompanied by potential rbs, there are several ATG codons accompanied by potential rbs in the ORF of the cDNA. It is highly probable that translation started from the ATG codon at 208 and/or 247 to yield the protein(s) of ca. 34 k. An analogous case of translation initiated from the middle of ORF occurred in the expression of cDNA for ferredoxin NADP\(^+\) reductase from spinach.\(^\text{10}\)

The protein of low molecular weight of the two expressed proteins of ferredoxin NADP\(^+\) reductase is a product whose translation started from the GTG codon at 84-bp downstream from the initiation codon of ORF. This was caused by the potential rbs, AGGA, at 10 bp upstream from the GTG codon.\(^\text{10}\)

To further determine the expression of the CHS cDNA, the CHS activity was measured using \([^{14}\text{C}]\)-malonyl CoA as a substrate. As shown in Table 1, the CHS activity was found exclusively in the crude extract of E. coli transformed with pT7CHS. The radioactive naringenin, formed by chemical cyclization of naringenin chalcone, a product of the CHS reaction,
was determined by isotopic dilution analysis. A radioactive fraction obtained by HPLC separation was diluted with non-labelled naringenin and recrystallized three times. Each sample showed constant specific activity (first: 257 dpm/mg; second: 246 dpm/mg, and third: 267 dpm/mg). The results rigorously demonstrated that the introduced cDNA was expressed in E. coli to yield CHS with catalytic activity. The CHS activity also appeared in an experiment using pBluescript as the expression vector (data not shown). The results so far obtained are unique, since full length cDNA is expressed by using the potential rbs site present in the cDNA. Successful expression of CHS in E. coli will help in studying the association and cooperative action of the enzymes involved in 5'-deoxychalcone synthesis, and to investigate the stereochemistry of hydrogen loss in the process of aromatization subsequent to the condensation of p-coumaroyl CoA and the three molecules of malonyl CoA.

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REFERENCES AND NOTES

7) The nucleotide sequence of PICHIS I will appear in the DDBJ, EMBL and Genbank Nucleotide Sequence Databases under the accession number D01023.
8) a) S. Tabor, and C.C. Richardson, Proc. Natl. Acad. Sci. USA, 82, 1074 (1985); b) The E. coli carrying pT7CHS or pT7-7 was cultured at 30°C before induction. To induce T7 RNA polymerase gene under the λ P2 promoter, the temperature was raised to 42°C for 30 min., and then lowered to 37°C. The cultivation was continued for 3 h.
9) a) M. Lawton, R.A. Dixon, K. Hahlbrock and C.J. Lamb, Eur. J. Biochem., 129, 593 (1983); b) Sequence comparison between P. lobata CHS and parsley CHS (Genbank, PETCHIS) revealed 70.7% homology at the nucleotide level. As expected from such homology, anti-parisley CHS antiserum reacted to the purified CHS from P. lobata.
11) Naringenin chalcone is reversibly converted to naringenin in a non-enzymatic manner. Its equilibrium lies very far to naringenin.
12) During the course of this study Schröder and his group also succeeded in the expression of CHS cDNA as a fusion protein having catalytic activity in E. coli. The authors thank Prof. Schröder for kindly supplying information.

Table I. CHS Activity in Crude Extract of Transformed E. coli.

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<th>Plasmid</th>
<th>Induction</th>
<th>Naringenin chalcone and naringenin (dpm)</th>
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<tr>
<td></td>
<td>pT7CHS</td>
<td>0 h</td>
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<tr>
<td></td>
<td>pT7-7</td>
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<td>3 h</td>
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After lysis of the cells by lysozyme, the extract was incubated with 14C-Malonyl CoA (1.1x10^5 dpm, 1.05 nmol) and p-coumaroyl CoA (2.5 nmol) at 30°C for 20 min. The product was extracted with ethyl acetate, mixed with non-labeled standard sample and applied to reverse phase HPLC. The fraction of naringenin chalcone and naringenin were combined and radioactivity was measured.

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