Original Paper

Plant Biotechnology, **20** (3), 207–214 (2003)

Expression of the Chalcone Synthase Gene in *Scutellaria baicalensis* Hairy Root Cultures Was Unusually Reduced by Environmental Stresses

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Received 6 December 2002; accepted 15 May 2003

Abstract

A cDNA clone, named as *SbCHS* cDNA, encoding chalcone synthase (CHS, EC 2.3.1.74) was isolated from a cDNA library derived from hairy root cultures of *Scutellaria baicalensis* Georgi by screening with a 1.4 kbp full length *CHS* cDNA of *Phaseolus vulgaris* as the probe. Complete nucleotide sequence of the *SbCHS* cDNA contained 1170-base pair open reading frame encoding 390 amino acid residues. The deduced amino acid sequence of *SbCHS* cDNA exhibited 82.1% identity with CHS of *P. vulgaris*. *SbCHS* mRNA expression in *S. baicalensis* hairy roots was unusually reduced by UV light irradiation, wounding, and yeast extract, as shown by Northern blot analysis. Greater formation of naringenin chalcone (100%) than of pinocembrin chalcone (67.4%) was observed by using thin-layer chromatography (TLC) to assess the enzymatic activity of recombinant SbCHS expressed in *Escherichia coli*.

Accession number: AB008748.

Key words: chalcone synthase, environmental stresses, gene expression, hairy root cultures, *Scutellaria baicalensis*.

Abbreviations

CHS, chalcone synthase; Sb, *Scutellaria baicalensis*.

Introduction

The dried root of *Scutellaria baicalensis* Georgi (Labiatae) is a well-known drug in traditional Chinese medicine used for treatment of arteriosclerosis, bronchitis, diarrhea, hepatitis and tumors (Chiang, 1977; Tang and Eisenbrand, 1992). In a previous paper, we reported that hairy root cultures of *S. baicalensis* were established and sixteen flavonoids, including a new flavone glucoside, were isolated from the *S. baicalensis* hairy root cultures (Zhou *et al.*, 1997). Among these sixteen flavonoids, no one has a hydroxyl group at 4' of the B-ring. Only a few flavonoids lacking a hydroxyl group at 4' of B-ring have been reported from other plant species such as *Cephalocereus senilis* (Liu *et al.*, 1993a, b). Flavonoids are widely distributed group of plant secondary metabolites. Chalcone synthase (CHS, EC 2.3.1.74) is the key enzyme in the biosynthetic pathway of all classes of flavonoids. It catalyzes the formation of the basic structure of flavonoids, naringenin chalcone, through condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA (**Fig. 1** pathway II) (Heller and Forkmann, 1988). As one of the results indicated, there must be a hydroxyl group at the 4' site of the B-ring in typical flavonoids.

Many *CHS* genes have been isolated from gymnosperm plants to angiosperm plants including dicotyledonous and monocotyledonous plants. Only two unusual *CHS* genes isolated from *Pinus sylvestris* (Fliegmann *et al.*, 1992) and barley leaves (Christensen *et al.*, 1998) were reported. Recently, Morita *et al.* (1998) reported that CHS from the *S. baicalensis* plant showed dual specificity for substrates such as 4-coumaroyl-CoA and cinnamoyl-CoA.

Generally, the synthesis of flavonoid compounds in response to environmental stresses has been implicated as a major defense response of higher

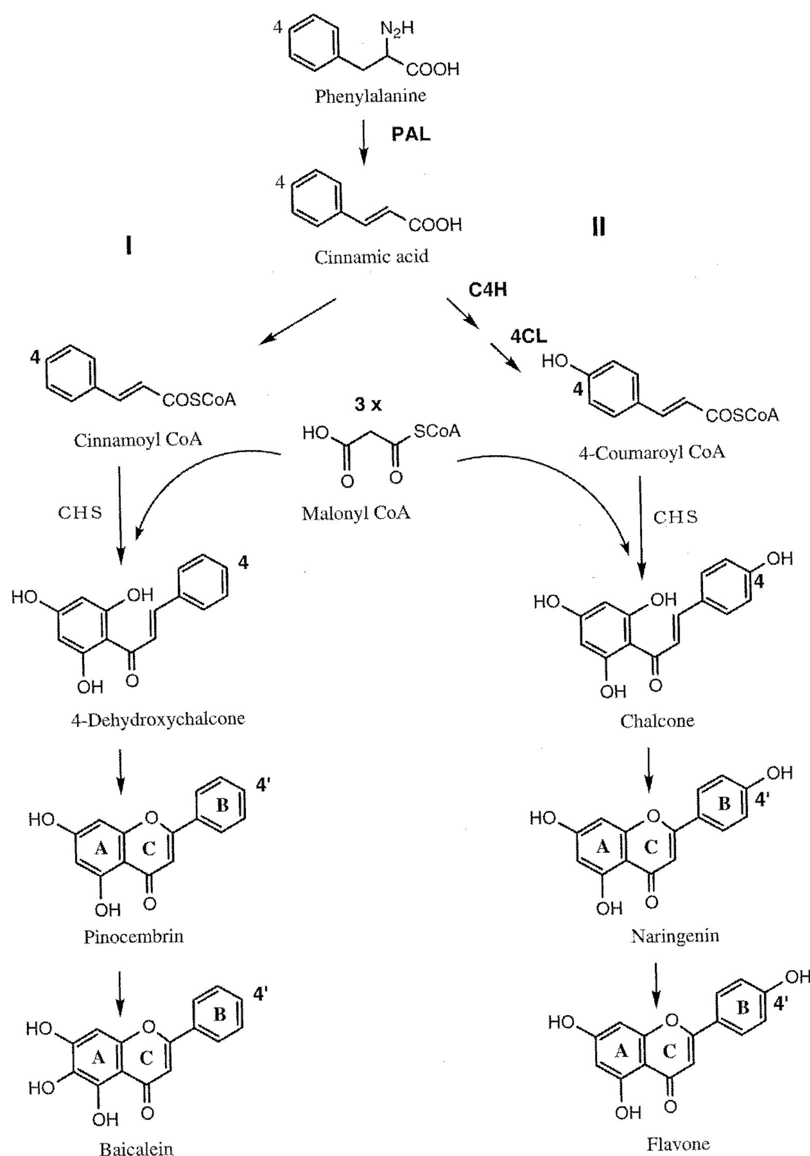

Fig. 1 Possible biosynthetic pathway of flavonoids. I, Possible biosynthetic pathway of unusual flavonoids in hairy roots of *Scutellaria baicalensis*. II, shows biosynthetic pathway of usual flavonoids.
PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoAligase; CHS, chalcone synthase.

plants (Bell, 1981). Moreover, expression of CHS genes were induced by external environmental stresses such as UV light irradiation (Kreuzaler *et al.*, 1983; Chappell and Hahlbrock, 1984; Tunen *et al.*, 1988; Batschauer *et al.*, 1991; Christie and Jenkins, 1996; Fuglevand *et al.*, 1996; Logemann *et al.*, 2000), wounding (Schmelzer *et al.*, 1988; Junghans *et al.*, 1993; Sallaud *et al.*, 1995; Richard *et al.*, 2000), yeast extract elicitor (Ryder *et al.*, 1984; Grab *et al.*, 1985; Hedrick *et al.*, 1988), and variation of temperature (Gong *et al.*, 1997).

In order to clarify the expression of the *CHS* gene in response to environmental stresses, Northern blot analyses were carried out using the *S. baicalensis* hairy root cultures treated with stresses such as UV light irradiation, wounding and yeast extract elicitation. Also, to clarify the catalytic property of the CHS from *S. baicalensis* hairy root cultures, the recombinant CHS expressed in *Escherichia coli* was examined for its ability to utilize both cinnamoyl-CoA and 4-coumaroyl-CoA, like that of the CHS from the *S. baicalensis* plant (Morita *et al.*, 1998) (**Fig. 1** pathway I).

In this paper, we report the cloning and expression in *E. coli* of a cDNA encoding CHS from *S. baicalensis* hairy root that encodes the enzyme which is responsible for catalysing both cinnamoyl-CoA and 4-coumaroyl-CoA, and the negative response of the gene expression to external environmental stresses.

Materials and Methods

Plant material, bacterial strains and vectors

Hairy roots of *Scutellaria baicalensis* Georgi (SbpBI121 strain) were initiated from seedlings and grown in Gamborg's B5 medium (Gamborg *et al.*, 1968) as previously described (Zhou *et al.*, 1997). *Escherichia coli* strains XL1-Blue MRF', SOLR, JM109 and BL21(DE3) were used for the standard molecular biology procedures. pBluescript SKII and expression vector pET21a were purchased from Stratagene and Novagene, respectively.

Construction and screening of a cDNA library

The total RNA from 20 g of 5-weeks-old hairy roots was prepared according to the guanidine thiocyanate and phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Poly $(A)^+$ RNA was prepared from the total RNA using a mRNA Separator Kit (Clontech, CA, USA). The cDNA was prepared using 5 μg of poly $(A)^+$ RNA according to the instructions from the Great Lengths™ cDNA Synthesis Kit (Clontech, CA, USA). The first-strand cDNA was synthesized using MMLV reverse transcriptase. For second-strand synthesis, RNase H, *E. coli* DNA polymerase, and *E. coli* DNA ligase were used; then the ds cDNA was treated with T4 DNA polymerase to create ends. The cDNA was ligated to *Eco*RI-*Not*I-*Sal*I adaptors, and then cloned into the *Eco*RI site of λ ZAP II. Packaging and plating on selective *E. coli* XL1-Blue MRF' cells were carried out according to the instruction from the Predigested Lambda ZAPII/*Eco*RI/CIAP Cloning Kit (Stratagene, USA).

The cDNA library (2.7×10^5 p.f.u.) was screened by plaque hybridization (Benton and Davis, 1977) using ^{32}P-labeled 1.4 kbp full length *CHS* cDNA of *Phaseolus vulgaris* (Ryder *et al.*, 1984) as the probe. Hybridization on Hybond-N^+ membranes (Amersham, Bucks., UK) was carried out at 65 ℃ in 5x SSPE (1x SSPE: 0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.7), 1% SDS, 5x Denhardt's solution (1x Denhardt's solution: 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), and 0.1 mg ml^{-1} salmon sperm DNA. Final washing of the membranes was performed in 2x SSPE, 0.1% SDS at 65 ℃ for 20 min.

CHScDNA sequencing

One positive plaque was isolated. The insert of the positive clone was subcloned into the *Eco*RI site of pBluescript SK II (-) vector (Stratagene) by *in vivo* excision, and the resultant clone was named as pSbCHS. Sequencing was carried out by the dideoxy-chain termination method with Thermo sequenase (Amersham, Bucks., UK) using a DNA sequencer (model DSQ 2000L; Shimadzu, Kyoto, Japan).

Experimental environmental stresses treatments of the hairy roots of S. baicalensis

Various experimental environmental biotic and abiotic stresses were added to the hairy roots of *S. baicalensis* after 5 weeks culture in B5 liquid medium. The biotic stress was carried out by adding yeast extracts to the medium of hairy root cultures to a level of 1 mg ml^{-1} yeast extracts in the medium. The abiotic stresses were carried out by two methods. The first method was UV light irradiation carried out by irradiating the hairy roots with 254 nm UV light located at least 20 cm above the hairy roots. The second method was wounding treatment carried out by aseptically cutting the hairy roots into one-cm-long segments with a sterile knife inside a cell culture hood.

RNA blot analysis

Total RNA was isolated from the hairy roots exposed to various stresses for 0, 0.5, 1, 4, 8, 24 and 48 h by the same method as used for construction of the cDNA library. For Northern hybridization analysis, equivalent amounts of RNA (10 μg) were separated on a 1.2% agarose gel containing formaldehyde under denaturing conditions. Equal loading of RNA was confirmed by ethidium bromide staining of the gel, transferred onto Hybond-N^+ membrane (Amersham), and then fixed by UV irradiation. Hybridization of the membranes was carried out according to the manufacturer's protocol (Amersham). RNA blots were probed with ^{32}P-labeled *SbCHS* cDNA prepared with a Random Primer DNA Labeling Kit version 2 (Takara, Shiga, Japan) for 15 h at 65 ℃. Membranes were washed twice with 2x SSPE, 0.1% SDS at 65 ℃ for 20 minutes. The signals for hybridization were detected using a BAS2000II bioimaging analyzer (Fuji Film, Tokyo, Japan).

Heterologous expression of SbCHS cDNA in E. coli and enzyme extraction

*Nde*I and *Bam*HI sites were created on both sides of the coding region for the *SbCHS* cDNA by the polymerase chain reaction (PCR). The amplified DNA was digested with restriction enzymes, *Nde*I and *Bam*HI, and then ligated into the same sites of an *E. coli* expression vector pET-21a (Novagene). *E. coli* BL21 (DE3) pLysS was transformed with the resulting expression vector pSbCHSEX. The trans-

formed *E. coli* cells were precultured in LB medium (Sambrook *et al.*, 1989) supplemented with carbenicillin (50 mg l^{-1}), 100 μl of the culture was inoculated into 10 ml of the same medium. After incubation at 37 ℃ for 3 hours, recombinant SbCHS was induced with 1 mM of isopropyl 1-thio-β-D-galactoside (IPTG) at 30 ℃ for 3 h. All subsequent operations were conducted at 4 ℃. The cells were collected by centrifugation, washed, and then resuspended in 0.1 M phosphate buffer, pH 7.0, containing 20 mM sodium ascorbate. The solution was sonicated and centrifuged. The supernatant was used as the crude enzyme extract.

SbCHS assay

The enzyme activity of the recombinant SbCHS *in vitro* was determined by thin-layer chromatography (TLC). The reaction mixture containing 400 μl 0.1 M K-Pi buffer, pH 7.0, 10 μl [2-^{14}C] malonyl-CoA (0.74 kBq), 5 μl 4-coumaroyl-CoA (5 nmol) or 5 μl cinnamoyl-CoA (5 nmol), and 100 μl of the crude enzyme extract from transformed *E. coli* in a total volume of 515 μl was incubated at 30 ℃ for one hour. The reaction was terminated by the addition of 20 μl of acetic acid. The resulting solution was partitioned with ethyl acetate (1 ml). The ethyl acetate phase was collected and evaporated under vacuum. The residue was re-dissolved in 10 μl MeOH and subjected to TLC analysis. TLC analysis was performed on silica gel (Merck) plates as adsorbent (stationary phase) and the mixture solvents, toluene : ethyl acetate : methanol : light petroleum ether (6:4:1:3, v/v), as the mobile phase. Equivalent amounts of reaction solutions were spotted. The reaction products were detected using a BAS2000II bioimaging analyzer (Fuji Film, Tokyo, Japan).

Results

Isolation and characterization of full-length cDNA encoding CHS from S. baicalensis hairy roots

In order to isolate the cDNA encoding CHS from *S. baicalensis* hairy roots, plaque hybridization was carried out with a 1.4 kbp full length CHS cDNA of *P. vulgaris* previously isolated by Ryder *et al.* (1984). One positive plaque was isolated from a λ ZAPII cDNA library (2.7x10^5 p.f.u.) constructed from poly (A)$^+$ RNA isolated from hairy roots of *S. baicalensis* cultured in B5 liquid medium for 5 weeks. The positive clone was subcloned into pBluescript SK II (-), its nucleotide sequence was determined. SbCHS cDNA contained 1170-base pair open reading frame encoding a polypeptide of 390 amino acids (**Fig. 2**).

The deduced amino acid sequence of *SbCHS*

```
   1  caaacatcaaccaccacttctcacttctctctcttccaccactccaattacctccttctccggcgacaatggtgaccgttgaagaattcc  90
   1                                                                      M  V  T  V  E  E  F  H   8

  91  accgggcaacaagggcagagggccccgccaccgtcttggccatcggcacggccaatcccccaaactgcgttgagcagtccacttatgctg  180
   9   R  A  T  R  A  E  G  P  A  T  V  L  A  I  G  T  A  N  P  P  N  C  V  E  Q  S  T  Y  A  D  38

 181  attactactttcgcatttgtaaaagtgaacacttgactgatcttaaaaagaagtttgaccgcatgtgcgaaaagtcctgcatcaagaaac  270
  39   Y  Y  F  R  I  C  K  S  E  H  L  T  D  L  K  K  K  F  D  R  M  C  E  K  S  C  I  K  K  R  68

 271  gttacatgcatttaacggaggagtttctgaaggagaacgacaacttcacggcgtacgaggctccttcactggatgcccggcaggacatag  360
  69   Y  M  H  L  T  E  E  F  L  K  E  N  D  N  F  T  A  Y  E  A  P  S  L  D  A  R  Q  D  I  V  98

 361  tggtggtggagatcccgaagctggggaaggaggctgcccagaaggcgatcaaggaatgggggcagcccaagtccaagatcacccacgtca  450
  99   V  V  E  I  P  K  L  G  K  E  A  A  Q  K  A  I  K  E  W  G  Q  P  K  S  K  I  T  H  V  I  128

 451  tcttctgcactaccagcggcgtcgacatgcccggcgcagactaccagatcaccaagctcctcggcctccgcccctccgtcaagcgcttca  540
 129   F  C  T  T  S  G  V  D  M  P  G  A  D  Y  Q  I  T  K  L  L  G  L  R  P  S  V  K  R  F  M  158

 541  tgatgtaccagcagggctgcttcgccggcggcacggtgctccgcatggccaaggacctggctgagaacaacgccggagctagggttctcg  630
 159   M  Y  Q  Q  G  C  F  A  G  G  T  V  L  R  M  A  K  D  L  A  E  N  N  A  G  A  R  V  L  V  188

 631  tcgtctgctccgagatcaccgccatcactttccgggggcccagcgacacccacctcgacagcctggtcggccaggccttgttcggcgacg  720
 189   V  C  S  E  I  T  A  I  T  F  R  G  P  S  D  T  H  L  D  S  L  V  G  Q  A  L  F  G  D  G  218

 721  gcgccgcggcggtcatcgttggctccgaccccatcgtgggggtggagcggccgctcttccagctggtctcggcggcgcagacgattctcc  810
 219   A  A  A  V  I  V  G  S  D  P  I  V  G  V  E  R  P  L  F  Q  L  V  S  A  A  Q  T  I  L  P  248

 811  ctgacagtgagggcgccattgatggccacgtccgcgaggttgggctgaccttccacctcctcaaggacgtccccgggctgatctccaaga  900
 249   D  S  E  G  A  I  D  G  H  V  R  E  V  G  L  T  F  H  L  L  K  D  V  P  G  L  I  S  K  N  278

 901  acatcgagaagagcctgaaggaggccttcgcgccgctgggcatctccgactggaactccctcttctggatcgtgcaccccggaggtcccg  990
 279   I  E  K  S  L  K  E  A  F  A  P  L  G  I  S  D  W  N  S  L  F  W  I  V  H  P  G  G  P  A  308

 991  ccattctggaccaggtggaggagaagctcgggctcaagcccgagatcatggtcccgaccaggcacgtgcttagcgaatacggggaacatgt  1080
 309   I  L  D  Q  V  E  E  K  L  G  L  K  P  E  I  M  V  P  T  R  H  V  L  S  E  Y  G  N  M  S  338

1081  ccagcgcctgcgttctcttcgtgatggatgagatgaggaaggcctccgccaaggacggctgcaccaccaccggagaagggaaggactggg  1170
 339   S  A  C  V  L  F  V  M  D  E  M  R  K  A  S  A  K  D  G  C  T  T  T  G  E  G  K  D  W  G  368

1171  gggttcttttcggcttcggcccgggcctcaccgttgagactgtggttctgcatagtgtgcctctcaattgaggaggagtggtggtgtttg  1260
 369   V  L  F  G  F  G  P  G  L  T  V  E  T  V  V  L  H  S  V  P  L  N  *                       390

1261  aatgagatattggctgaaatgagtcatatttcgtcatattattattatttttaatataatatcttgtaaagtatggaatttcattcatg  1350

1351  ctctacatttatc                                                                              1363
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Fig. 2 Nucleotide and deduced amino acid sequences of a cDNA clone, pSbCHS encoding chalcone synthase (CHS) from hairy root cultures of *Scutellaria baicalensis.*
The initiation codon (ATG) for chalcone synthase open reading frame is underlined. The stop codon is marked with an asterisk. Black shaded sequences indicate PROSITE pattern of the chalcone and stilbene synthases active site (Falquet *et al.*, 2002).

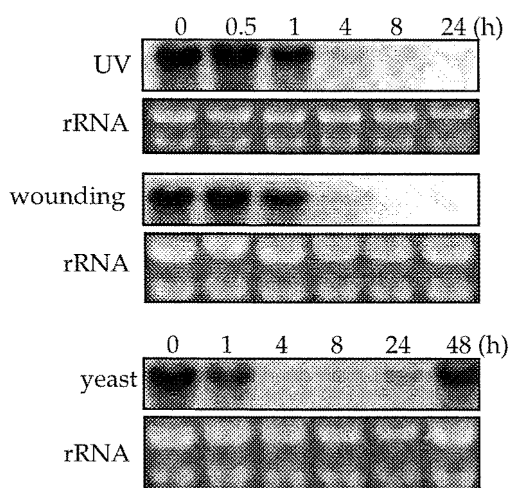

Fig. 3 Northern blot analysis of chalcone synthase mRNA levels in hairy root cultures of *Scutellaria baicalensis* exposed to various environmental stresses.
The hairy roots of *S. baicalensis* were cultured in B5 liquid medium for 5 weeks, then exposed to 254 nm UV light irradiation (UV); wounding treatment by aseptically cutting the hairy roots into one_cm_long segments(wounding); elicitor (1 mg ml^{-1} yeast extracts contained in the medium) (yeast). Total RNA was isolated from the hairy roots harvested at 0, 0.5, 1, 4, 8, 24 and 48 h after exposed to the above various stresses. A 10 μg portion of total RNA was loaded per lane. ^{32}P-labeled SbCHS cDNA was used as the probe. Hybridization of the membranes was carried out as described under "Materials and Methods". Ethidium bromide stained gels were shown beneath each stress treatment to show an equal loading of total RNA.

includes the PROSITE pattern of chalcone and stilbene synthase active site (Falquet *et al.*, 2002) and exhibits 81.0% identity with CHS of *P. vulgaris*, which was used as a probe to isolate SbCHS cDNA, and 81.2% identity with CHS of *P. sylvestris*, which has been reported to use both cinnamoyl-CoA and 4-coumaroyl-CoA (Fliegmann *et al.*, 1992).

Expression of SbCHS gene in response to various environmental stresses

To investigate the regulation of UV light irradiation, wounding and yeast elicitor on *SbCHS* gene expression, the hairy roots of *S. baicalensis* cultured in B5 liquid medium for 5 weeks were exposed to the above various environmental stresses. *SbCHS* mRNA levels were determined at 0, 0.5, 1, 4, 8 and 24 h after UV light irradiation and wounding treatments, and at 0, 1, 4, 8, 24 and 48 h after yeast extract elicitor treatment.

Interestingly, *SbCHS* gene was expressed in an intact hairy roots of *S. baicalensis* (control), however its expression was unusually reduced and inhibited by environmental stresses such as UV light irradiation, wounding and yeast extract elicitor as shown in **Fig. 3**.

Expression of isolated SbCHS cDNA in E. coli, and substrate specificity of the recombinant SbCHS in vitro

To confirm whether the cDNA isolated from the hairy roots of *S. baicalensis* encodes catalytically active CHS, the cDNA was expressed in *E. coli*. The coding region of the *SbCHS* cDNA insert was subcloned into an *E. coli* expression vector pET-21a, resulting in the construction of pSbCHSEX. The crude protein extract of *E. coli* BL21 (DE3) pLysS transformed with pSbCHSEX was assayed for CHS *in vitro*. Overexpressed recombinant SbCHS protein was detectable on CBB stained SDS-PAGE gel (data not shown). As shown in **Fig. 4**, cinnamoyl CoA was converted to pinocembrin in the presence of [2-^{14}C] malonyl-CoA and the crude protein extract from *E. coli* transformed with pSbCHSEX. The identity of pinocembrin formed by the reaction *in vitro* was confirmed by co-chromatography on TLC with authentic pinocembrin, and detected by using a BAS2000II bioimaging analyzer. No radioactive spot was detected when crude protein extract from *E. coli* transformed with an empty vector pET-21a was used as a negative control (data not shown). This result confirmed that the *SbCHS* cDNA from the hairy roots of *S. baicalensis* encodes CHS that catalyzes the formation of pinocembrin chalcone through condensation of one molecule of cinnamoyl-CoA with three molecules of malonyl-CoA. The substrate specificity of recombinant SbCHS *in vitro* was examined. The crude protein extracts from *E. coli* transformed with pSbCHSEX were incubated with radiolabeled [2-^{14}C] malonyl-CoA, and 4-coumaroyl-CoA or cinnamoyl-CoA at 30℃ for one hour. The formation of naringenin chalcone through condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA was demonstrated in the cell-free extract of cultured *Glycyrrhiza echinata* cells treated with yeast extract elicitor (Ayabe *et al.*, 1988). A comparable experiment was carried out with elicited cultured *G. echinata* cells instead of the recombinant SbCHS crude protein extracts from *E. coli*. The reaction products were analyzed by TLC (**Fig. 4**) and quantified by using a BAS2000II bioimaging analyzer (**Table 1**). As shown in **Fig. 4**, the recombinant SbCHS catalyzes the formation of pinocembrin chalcone *in vitro*, through conden-

Table 1 Substrate specificity of the recombinant SbCHS expressed in *E. coli*. Quantitative analysis was carried out by BAS2000II bioimaging analyzer

Enzyme source	Naringenin	pinocembrin
SbCHS crude protein	100%	67.40%
Elicited *Glycyrrhiza echinata cells*	100%	84.60%

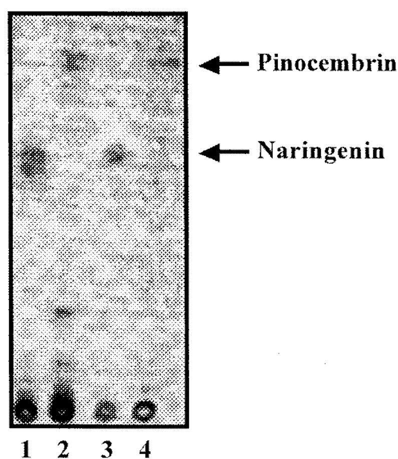

Fig. 4 TLC analysis of the reaction product of the recombinant SbCHS expressed in *E. coli*.
The reaction mixture contained 400 μl 0.1 M K-Pi buffer, pH 7.0, 10 μl [2-^{14}C] malonyl CoA (0.74 kBq), 5 μl 4-coumaroyl CoA (5 nmol) (lane 1) or 5 μl cinnamoyl CoA (5 nmol) (lane 2), and 100 μl of the crude enzyme extract from transformed *E. coli*, and was incubated at 30 ℃ for one hour. See "Materials and Methods" for the SbCHS assay. Equivalent amounts of reaction solutions were spotted on a TLC plate. TLC analysis was carried out with silica gel as the adsorbent (stationary phase) and the solvent mixture, toluene:ethyl acetate:methanol:light petroleum ether (6:4:1:3) as the mobile phase. The reaction products were detected using a BAS2000II bioimaging analyzer. Lanes 3 and 4 were reaction products from elicited cultured *Glycyrrhiza echinata* cells with [2-^{14}C] malonyl CoA (0.74 kBq), 4-coumaroyl CoA (5 nmol) or 5 μl cinnamoyl CoA (5 nmol), respectively.

sation of one molecule of cinnamoyl-CoA with three molecules of malonyl-CoA, and also catalyzes the formation of naringenin chalcone, through condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA. From the results of quantitative analysis, greater formation of naringenin chalcone (100%) than formation of pinocembrin chalcone (67.4%) are shown in **Table 1**. A hundred percent formation of naringenin chalcone and 84.6% formation of pinocembrin chalcone were obtained by using elicited cultured *G. echinata* cells.

Discussion

There have been a great number of reports that *CHS* gene expression in different plants was induced by various environmental stresses. For example, induction of *CHS* gene expression by UV light have been reported in plants, parsley leaves (*Petroselinum crispum*) (Schmelzer *et al.*, 1988), cell suspension cultures of parsley (*P. crispum*) (Logemann *et al.*, 2000), cell suspension cultures of parsley (*P. hortense*) (Kreuzaler *et al.*, 1983; Chappell and Hahlbrock, 1984), mustard (*Sinapis alba*) (Batschauer *et al.*, 1991), *Petunia hybrida* (Tunen *et al.*, 1988), *Arabidopsis* (Christie and Jenkins, 1996; Fuglevand *et al.*, 1996); by wounding in plants, alfalfa (*Medicago sativa*) (Junghans *et al.*, 1993; Sallaud *et al.*, 1995), white spruce (*Picea glauca*) (Richard *et al.*, 2000); by elicitor in plants, *Phaseolus vulgaris* cell cultures (Ryder *et al.*, 1984; Hedrick *et al.*, 1988), alfalfa (*M. sativa*) (Junghans *et al.*, 1993), soybean (*Glycine max*) (Grab *et al.*, 1985); and by temperature-changing in plants, *Perilla frutescens* (Gong *et al.*, 1997).

Because *CHS* gene expression is usually induced by environmental stresses as mentioned above, we repeated three times experiments on the expression of the *SbCHS* gene in response to various environmental stresses; the results remained the same. We also determined the contents of baicalin and baicalein, the major bioactive compounds in the hairy roots of *S. baicalensis* in response to the above environmental stresses. All these environmental stresses, UV light irradiation, wounding, yeast extract elicitor, and temperature-changing treatments, did not affect the synthesis of baicalin and baicalein in the hairy roots of *S. baicalensis* (data not shown).

The hairy roots of *S. baicalensis* were obtained after *Agrobacterium* T-DNA was transferred from *Agrobacterium rhizogenes* to *S. baicalensis* genomic DNA (Zhou *et al.*, 1997). The *Agrobacterium* T-DNA could be considered as an internal stress constantly inducing the *SbCHS* mRNA expression in the hairy roots of *S. baicalensis*. External stresses may not induce and may repress *SbCHS* gene expression that is constantly induced by the internal stress. This hypotheses may help to explain why

those environmental stresses, UV light irradiation, wounding and yeast elicitor, reduced and inhibited the existence of the *SbCHS* gene expression in the hairy roots of *S. baicalensis*.

The previous results of the chemical analysis showed that *S. baicalensis* hairy root can biosynthesize mainly the 4'-dehydroxy compounds, such as baicalin and woogonin, derived from 4'-dehydroxychalcone via pinocembrin, *i.e.* via pathway I in **Fig. 1** (Zhou *et al.*, 1997). Therefore, it was expected that CHS isolated from the hairy root of *S. baicalensis* will show higher specificity for cinnamoyl CoA than for 4-coumaroyl CoA. The result, however, showed almost the same specificity pattern as that of *G. echinata* cells having the CHS activity for 4-coumaroyl CoA (**Table 1**).

Acknowledgments

We would like to take this opportunity to thank Prof. C. J. Lamb (Plant Biology Laboratory, The Salk Institute for Biological Studies, CA, USA), for the kind supply of CHS cDNA of *Phaseolus vulgaris*; Prof. S. Ayabe (Department of Applied Biological Science, College of Biological Resource Sciences, Nihon University, Fujisawa, Japan) for the kind supply of cultured *Glycyrrhiza echinata* cells. We are also indebted to Dr. H. Hayashi (Gifu Pharmaceutical University, Gifu, Japan) for the kind supply of authentic pinocembrin.

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