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The Functional Expression of the *CHS-D* and *CHS-E* Genes of the Common Morning Glory (*Ipomoea purpurea*) in *Escherichia coli* and Characterization of Their Gene Products.

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Abstract

The genes encoding chalcone synthase (CHS) in the common morning glory (*Ipomoea purpurea*) comprise a multigene family, and they are divided into two subfamilies. The genes in a subfamily including the *CHS-A*, *CHS-B* and *CHS-C* genes are distantly related to the other known *CHS* sequences in a phylogenetic tree, whereas the *CHS-D* and *CHS-E* genes in another subfamily are more closely related to the well-characterized *CHS* genes.

As an initial step to elucidate biological function of these *CHS* genes in *I. purpurea*, the *CHS-D* and *CHS-E* cDNAs were expressed in *Escherichia coli* with different expression systems. The recombinant CHS-D and CHS-E proteins both showed CHS activity to produce naringenin chalcone.These results are discussed with regard to the biological roles of the *CHS-D* and *CHS-E* genes in flower pigmentation in *I. purpurea*. We have also discussed these CHS-D and CHS-E enzyme as members of plant specific polyketide synthases.

Key words chalcone synthase, *Ipomoea purpurea*, *p*-coumaroylacetoacetic acid synthase, bisnoryangonin

Introduction

Flavonoids play important roles in flower pigmentation and in many other biological functions, including protection against UV light, plant-microbe interactions, and male fertility (Harborne, 1994; Shirley, 1996). Chalcone synthase (CHS) catalyzes a successive condensation of three molecules of malonyl-CoA and one molecule of *p*-coumaroyl-CoA to form naringenin chalcone. The CHS enzyme has been regarded to be a key enzyme for flavonoid biosynthesis and is now considered to be a members of plant-specific polyketides synthases (PKSs) (Schröder, 1997), which catalyzes the linking of acyl-CoA subunits by repetitive decarboxylative condensations (Hopwood, 1997). The *CHS* genes comprise a multigene family in many species, although a few plants such as *Antirrhinum* and *Arabidopsis* are known to carry a single copy of the gene (Harborne, 1994). Durbin *et al.* (1995) have isolated four *CHS* genes, *CHS-A*, *CHS-B* and *CHS-C* and a pseudogene, *CHS-PS*, from *Ipomoea purpurea* and found that they are members of the *Ipomoea CHS* gene family, which is comprised of 13 *CHS* genes from seven *Ipomoea* species. These *Ipomoea CHS* genes are distantly related to the other known *CHS* sequences in a phylogenetic tree.

Another subfamily of the *CHS* genes, *CHS-D* and *CHS-E*, were later isolated from pigmented flower buds of *I. purpurea* (Fukada-Tanaka *et al.*, 1997; Habu *et al.*, 1997). The nucleotide and deduced amino acid sequence of the *CHS-D* showed 79.9 and 85.9% identities to those of *CHS-E*, respectively. The *CHS-D* and *CHS-E* genes do not belong to the previously characterized *Ipomoea* CHS gene subfamily and are more closely related to the *CHS* sequences found in other plant species.

These *CHS* genes in the newly identified *CHS* gene subfamily are expressed predominantly in the pigmented buds, and the *CHS-D* gene is responsible for pigmentation in flower limbs and stems while the *CHS-E* gene is expressed mainly in faintly pigmented flower tubes (Fukada-Tanaka, *et al.*, 1997; Johzuka-Hisatomi *et al.*, 1999; Durbin *et al.*, 2000). Indeed, the mutable *flaked* plant having the transposable element *Tip100* inserted into the *CHS-D* gene produces white flowers with colored spots and sectors as well as green stems with red sectors (Hisatomi *et al.*, 1997; Habu *et al.*, 1998). These variegated phenotypes in flowers and stems are caused by recurrent somatic mutations due to excision of Tip100 from the *CHS-D* genes. Although these lines of circumstantial evidence were consistent with the notion that the *CHS-D* gene indeed encodes the CHS enzyme in anthocyanin biosynthesis pathway which forms naringenin chalcone, no substantial biochemical data concerning enzyme activities of these *CHS* gene products are available. It would be interesting to ask whether all the *CHS* genes belonging to the different subfamily of *I. purpurea* equally retain the enzyme activity of the CHS.

As an initial step to elucidate biological function of these *CHS* genes in *I. purpurea*, we have chosen to characterize the *CHS-D* and *CHS-E* genes, since not only the mode of expression of there genes but also their genomic structures are well documented (Johzuka-Hisatomi *et al.*, 1999; Durbin *et al.*, 2000). We have attempted to clarify the function of them by characterizing the products of the enzymatic reactions that are catalyzed by the recombinant proteins yielded by the functional expression of their cDNAs in *E. coli* K12 derivatives. In this study, we showed that both CHS-D and CHS-E gene products indeed carry the CHS activity to produce naringenin chalcone.

2. Materials and Methods

2.1 Bacterial expression systems used

The full length of cDNAs of the *CHS-D* and *CHS-E* genes were described previously (Fukada-Tanaka *et al.*, 1997; Habu *et al.*, 1997). For the preparation of the recombinant chalcone synthases, we used the bacterial expression systems; the pET system (pET22b(+) in *E. coli* (BL21(DE3)pLysS; Novagen; pRSET C in *E. coli* (BL21(DE3)pLysS; Invitrogen) and the IMPACT system (pCYB3 and pTYB3 in *E. coli* ER2267 and ER2566; New England Biolabs). In the pET system, six histidine residues were added to the C- or N-termini of CHS-D. IMPACT systems are exploited for obtaining intact recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element (Chong *et al.*, 1997).

(1) *Construction of plasmid pSIO11 producing intact-CHS-D proteins*

To introduce *Nco* I and *Sap* I sites at the N- and C-termini of the *CHS-D* ORF, respectively, the primers (Primer 1) 5'-CGAAGCCATGGTGACCGTCG-3' and (Primer 2) 5'-TTTGGCTCTTCCGCATGCTGGGACGCTATG-3' were used for the PCR amplification. **(Fig. 1A)** The PCR reaction with 10 pmol each of these primers with 1 ng of *CHS-D cDNA* was carried out for 30 cycles consisting of denaturation at 93 ℃ for 40 s, annealing at 42 ℃ for 40 s and extension at 72 ℃ for 60 s with 2.5 U of ExTaq DNA polymerase (Takara). The obtained PCR products were digested with *Nco* I and *Sap* I and ligated into *Nco* I and *Sap* I sites of pCYB3 to yield pSIO3. To express the CHS-D gene driven by T-7 promoter, the *Nco* I and *Kpn* I fragment containing the *CHS-D* ORF of pSIO3 was then cloned into the *Nco* I and *Kpn* I sites of pTYB3 to produce pSIO11. The expression plasmid pSIO11 was introduced into *E. coli* ER2566.

(2) *Construction of plasmid pSIO33 for CHS-D-His protein and pSIO57 for His-CHS-D protein*

To construct pSIO33 which can produce the CHS-D derivative with tagged six histidine-residues at its C-terminus (CHS-D-His), the primer (Primer 3) 5'-TAATACGACTCACTATAGGG-3' containing the T7 promoter sequence and the primer (Primer 4) 5'-TGGCGGCCGCTGCTGGGACGCTATG-3' with a *Not* I site were used for amplification of the DNA fragment carrying the *CHS-D* ORF. **(Fig. 1B)** The PCR amplification with 10 pmol each of these primers together with 1 ng of pSIO11 containing the *CHS-D* cDNA sequence was carried out for 30 cycles consisting of denaturation at 93 ℃ for 40 s, annealing at 52 ℃ for 40 s and extension at 72 ℃ for 60 s with 2.5 U of ExTaq DNA polymerase. The obtained PCR products were cleaved with *Xba* I and *Not* I and cloned into the *Xba* I and *Not* I sites of pET22b(+) to yield the plasmid pSIO33.

To obtain pSIO57 producing the CHS-D derivative with tagged six histidine-residues at its N-terminus (His-CHS-D), the primers (Primer 5) 5'-GCTAGCATGGTGACCGTCGAGGAGGTCA-3' with an *Nhe* I site (Primer 6) 5'-CCATGGTTATGCTGGGACGCTATGGAG-3' with an *Nco* I site were employed, and the PCR amplification with 10 pmol each of these primers together with 1 ng of pSIO11 was performed for 30 cycles consisting of denaturation at 93 ℃ for 40 s, annealing at 56 ℃ for 40 s and extension at 72 ℃ for with 2.5 U of ExTaq DNA polymerase. **(Fig. 1C)** The resultant PCR

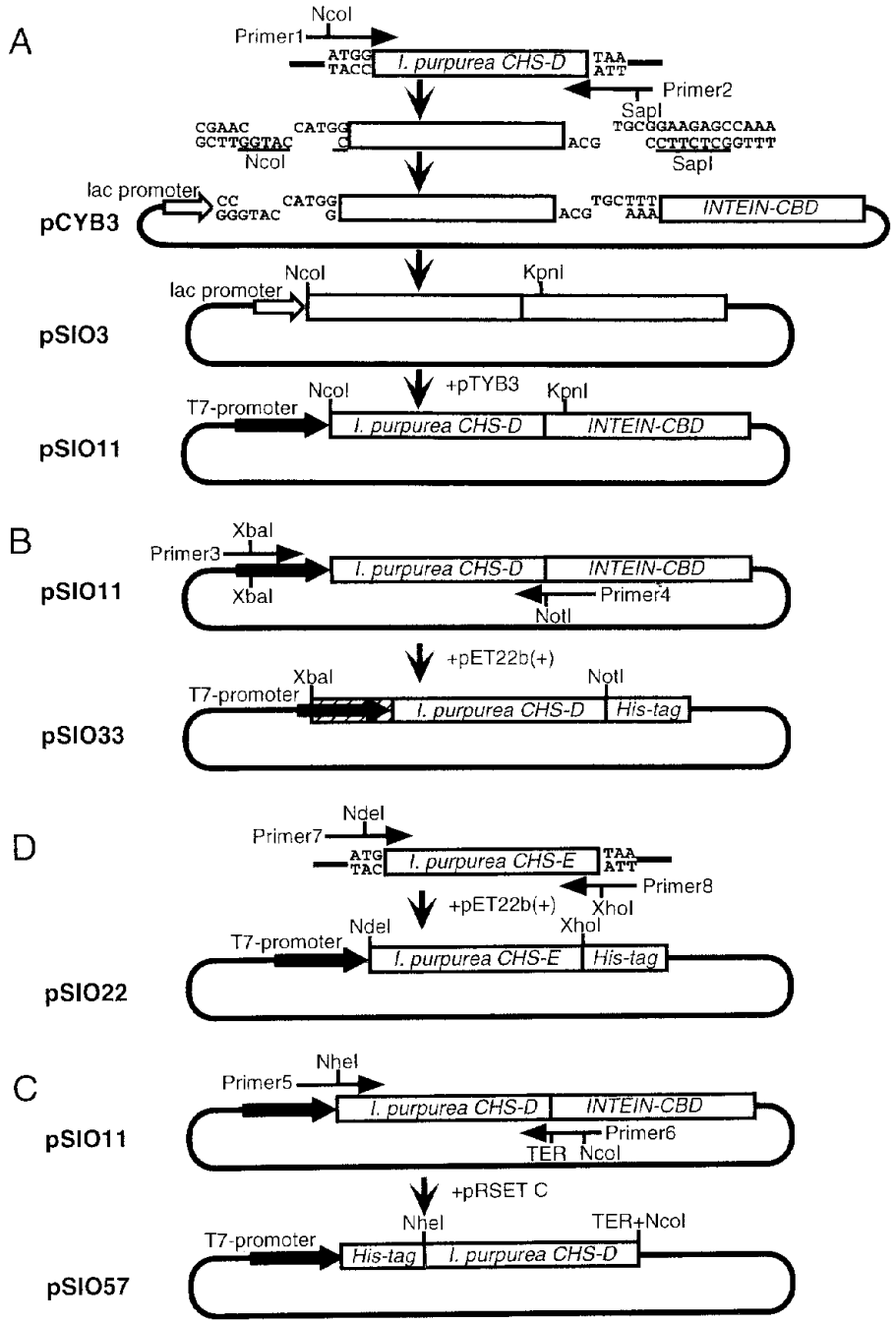

Fig. 1A-D. Prasmid constructs for the heterologous expression of chalcone synthases in *E. coli.* Only the relevalent restriction sites are indicated.TER; stop codon. See Materials and Methods.

products were digested with *Nhe* I and *Nco* I and ligated into the *Nhe* I and *Nco* I sites of pRSET C to yield the plasmid pSIO57. These pSIO33 and pSIO57 plasmids were introduced into *E. coli* BL21(DE3)pLysS for production of recombinant CHS-D-His and His-CHS-D proteins, respectively.

(3) *Construction of plasmid pSIO22 producing CHS-E-His proteins*

For obtaining the CHS-E derivative with tagged six histidine-residues at its C-terminus (CHS-E-His), the primers (Primer 7) 5'-CAGCTACCATATG GTGACCG-3' with a *Nde* I site and (Primer 8) 5'-TTGGCCTCGAGAGCTGAAACACTATG-3' with a *Xho* I site were used to amplify the fragment containing the *CHS-E* ORF. (**Fig. 1D**) The PCR amplification with 10 pmol each of these primer together with 1 ng of the *CHS-E* cDNA was carried out for 30 cycles consisting of denaturation at 93 ℃ for 40s, annealing at 56 ℃ for 40 s and extension at 72 ℃ for 60 s with 2.5 U of ExTaq DNA polymerase. The resultant PCR products were cleaved with *Nde* I and *Xho* I and ligated into the *Nde* I and *Xho* I sites of pET22b(+) to construct the plasmid pSIO22, which was introduced into *E. coli* BL21 (DE3)pLysS for production of recombinant CHS-E

-His proteins.

2.2 Purification of the expression proteins

The following buffers were used for purification of the expression proteins: Buffer I (4 mM imidazole, 40 mM K_2HPO_4, 0.1M NaCl, 0.002% RNase, 0.001% DNase, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.5% polyoxyethylene (10) octylphenyl ether (Triton X-100), pH 7.9), Buffer II (40 mM imidazole, 20 mM K_2HPO_4, 0.5M NaCl, pH 7.9), Buffer III (0.5M imidazole, 15 mM K_2HPO_4, 10% glycerol, pH 7.5), Buffer IV (20 mM K_2HPO_4, 0.5M NaCl, 0.1 mM EDTA, 0.002% RNase, 0.001% DNase, 0.2 mM PMSF, 0.5% Triton X-100, pH 8.0), Buffer V (50 mM β-mercaptoethanol (2-ME), 20 mM K_2HPO_4, 50 mM NaCl, 0.1 mM EDTA, pH 8.0), Buffer VI (20 mM K_2HPO_4, 50 mM NaCl, and 0.1 mM EDTA, pH 8.0), Buffer VII (100 mM K_2HPO_4, 1 mM EDTA, 10% glycerol, pH 8.0).

We purified hexahistidyl-tagged proteins, CHS-D-His, His-CHS-D and CHS-E-His, from *E. coli* BL21(DE3)pLysS containing the pET vector plasmids, pSIO33, pSIO57 and pSIO22, respectively. These *E. coli* strains were grown in 10 m*l* of LB medium containing ampicillin (100 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}) at 37 ℃ to the saturation. The cells were then transferred to one liter of LB medium containing ampicillin (100 μg ml^{-1}) at 37℃ for 3 hours. After cultures were cooled down to 25 ℃, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 1 mM to induce for protein expression, and cultures were further incubated at 25 ℃ for 3 hours. The cells were collected by centrifugation, resuspended in 10 m*l* Buffer I, and lysed by intermittently modulated sonication for 20 min. on ice. The lysate was centrifuged at 10000 x g for 30 min. at 4 ℃ , and then, the supernatant was loaded on to 2 m*l* PROBOND Resin (Invitrogen). The resin was washed with 500 m*l* Buffer II. The bound protein was eluted with 10 m*l* Buffer III.

The *E. coli* strain ER2566 harboring pSIO11 was cultured in 10 m*l* of LB medium containing ampicillin (100 μg ml^{-1}) and tetracycline (25 μg ml^{-1}) and incubated at 37 ℃ until full-growth culture to obtain INTEIN-CBD tagged protein. The cultures were transferred in one liter of LB medium containing ampicillin (100 μg ml^{-1}) and incubated at 37 ℃ for 3 hours. After cultures were cooled down to 25 ℃ , 1 mM IPTG was added to induce for protein expression, and cultures were further incubated at 25 ℃ for 5 hours. The cells were harvested and resuspended in 10 m*l* Buffer IV, and lysed by intermittently modulated sonication for 20 min on ice. The lysate was centrifuged at 10000 x g for 30 min. at 4 ℃, and the supernatant was loaded on to 2 ml Chitin Beads (NEB). The Chitin Beads column washed with one liter of Buffer IV without RNase, DNase and phenylmethylsulfonylfluoride (PMSF). For on-column cleavage reaction (Chong *et al.*, 1997), we were flushed the column with 15 m*l* Buffer V to uniformly distribute 2-ME throughout the column. The column was left at 4 ℃ overnight to release intact-CHS D-His protein and the resultant protein was eluted using 10 m*l* Buffer VI.

The fractions from the affinity columns that contain CHS-D-His, His-CHS-D and CHS-E-His proteins in Buffer III and the intact-CHS-D protein in Buffer IV were concentrated to about 500 μl using CENTRIPREP10 (Amicon) at 4000 x g at 4 ℃. For concomitant desalting, replacement and concentration, 10 m*l* Buffer VII were added to the protein solutions and concentrated using CENTRIPREP10 at 4000 x g at 4 ℃ twice. These manipulations restored the enzyme activity. The protein concentrations were measured by Bio-Rad Protein Assay (Bio-Rad Laboratories)

2.3 Assays of the CHS activities

The standard incubation (300 μl) mixture contains 50 nmol *p*-coumaroyl-CoA (starter CoA-ester), 150 nmol malonyl-CoA and 0.2 nmol protein in the Buffer VII . The reaction was stopped after 2 hours at 30 ℃ by adding 20% HCl (30 μl) to convert the yielded chalcone into isomeric flavanone. The products were extracted with ethyl acetate (EtOAc) (330 μl x 2), and EtOAc layer was washed with distilled water (500 μl) and concentrated by air blowing. The residue was diluted with 20 μl of MeOH (0.1% TFA), and subjected to HPLC (JASCO 880, JASCO) using TSK-gel ODS-80Ts (4.6 x 15 cm, Tosoh) monitoring by a multichannel UV detector (MULTI 340, JASCO) at 290 nm and 360 nm with a flow rate of 0.8 m*l* min^{-1}. A UV spectrum (198-400 nm) was recorded every 0.4 s. The solvents used for gradient elution were solvent A (MeOH, 0.1% TFA) and solvent B (Water, 0.1% TFA). The gradient conditions are as follows: 0-5 min, 30% solvent A in solvent B; 5-17 min, liner gradient to 60% solvent A in solvent B; 17-30 min, 60% solvent A in solvent B. Authentic naringenin gave a peak at retention time of 23.0 min under these conditions. The standard calibration curves were made from solutions of a known concentration of authentic sample. The pET22b (+) expression vector without the CHS sequence was used as a negative control.

2.4 On-line HPLC-ESI MS analysis

A Hewlett-Packard HPLC 1100 series (Wil-

mington, DE) coupled to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA) fitted with an ESI source was used. The separation was carried out on a TSK-gel ODS-80Ts (4.6 x 15 cm, Tosoh) with 45% MeOH in water containing 0.1% TFA as mobile phase. The flow rate of the mobile phase was 0.8 $ml\,min^{-1}$. The ESI capillary temperature and capillary voltage were 275 ℃ and 3.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in the positive mode; over a mass range of *m/z* 150-500, at a range of one scan every 2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV).

3. Results

3.1 The functional expression of CHS-D and CHS-E genes and purification of their gene products from E. coli.

The plasmid pSIO11 to express INTEIN-CBD tagged CHS-D protein was constructed and was expressed in the *E. coli* strain ER2566. The cell free extract that was harvested after induction of IPTG for 5 hours was used to obtain the natural CHS-D enzyme (intact-CHS-D) in sequence by on-column cleavage reaction. The recovery of active enzyme after the cleavage reaction by β-mercaptoethanol (2-ME) was about only 70 μg per one liter culture. However, the enzyme activity as chalcone synthase was retained after the on column reaction using thiol reagent. The low yield of the expressed recombinant protein using the above IMPACT system tempted us to construct an alternate expression system. We chose to synthesize these enzymes with hexahistidyl-terminal extensions to facilitate their purification. For that purpose, we constructed pSIO33 producing CHS-D-His (six histidine residue at the C-terminus) and pSIO57 producing His-CHS-D (six histidine residue at the N-terminus) to express them with the pET system. Both His-tagged constructs produced the recombinant proteins that are readily bound to the Ni^{2+} affinity column. The yields of the purified enzymes were about 2-3 mg per one liter culture. The CHS-E-His (C-terminal His-tagged) protein was expressed in the pET system and the yield of the purified enzyme after the Ni^{2+} affinity column (PROBOND Resin) purification step was almost the same as those of CHS-D-His and His-CHS-D.

3.2 Enzyme activities of the CHS-D and CHS-E proteins

The enzyme activities of three different recombinant CHS-D protein derivatives, intact-CHS-D, CHS-D-His (C-terminal His-tagged) and His-CHS-D (N-terminal His-tagged) , which were expressed in *E. coli*, were examined. The results of the HPLC analyses of the products are shown in the **Fig. 2**. Reaction product profiles of all three enzymes were almost identical. The first major peak at Rt=17.9 was identified as *p*-coumaroyltriacetic acid lactone (CTAL), because it gave a molecular ion at *m/z* 273 $[M+H]^+$ (LC-MS) and a fragment at *m/z* 227(MS/MS). The peak afforded the UV spectrum showing an absorption maximum at 332 nm that is in accord with this structure. The second major peak at Rt=23.0 was identified as naringenin, because it gave also a molecular ion at *m/z* 273 $[M+H]^+$ (LC-MS) as well as the typical fragment of flavanone at *m/z* 153(MS/MS). The structure was further confirmed by the UV spectrum with an absorption maximum at 290 nm. Two small peaks between these two major peaks are inferred to be *trans*- and *cis*-isomers of bisnoryangonin, judging from these mass numbers, $[M+H]^+$ at *m/z* 231 and the characteristic UV absorption at 365 nm (Akiyama, 1998). The above results reveal that all those proteins (intact-CHS-D, CHS-D-His, His-CHS-D) catalyzed naringenin chalcone formation with malonyl-CoA and *p*-coumaroyl-CoA as substrates at the specific activity of 68, 56, 49 $\mu Kat\,kg^{-1}$, respectively. The intact-CHS-D protein that is obtained by cleavage reaction using 2-ME; one of the typical thiol reagents showed the same or somewhat better specific activity for naringenin chalcone than those of His-tagged recombinant protein. The on column cleavage reaction with 30-50 mM of dithiothreitol or 100 mM of hydoxylamine gave the same results (data not shown). It is well known that the chalcone synthase releases bisnoryangonin in much higher relative rate to naringenin when the reaction mixture contains various thiol reagents (Kreuzaler *et al.*, 1975). Our results show that the enzyme recovers its genuine activity when it is assayed free from the thiol reagents. Though the enzyme has once contacted with the thiol reagents. Thus, we can conclude that all those recombinant CHS-D proteins showed the almost same enzyme activities regardless of the intact form or the His-tagged forms.

For the *CHS-E* gene product, we have examined only the recombinant CHS-E-His derivative produced in *E. coli*. As is the case for the CHS-D enzymes, two major products, CTAL and naringenin were detected under the same assay condition (**Fig. 1**). Interestingly, the specific activity of the CHS-E-His proteins was found to be 158 $\mu Kat\,kg^{-1}$, which is about three times higher than that of the corresponding CHS-D-His proteins. It is clear that the *CHS-E* gene also encodes chalcone synthase.

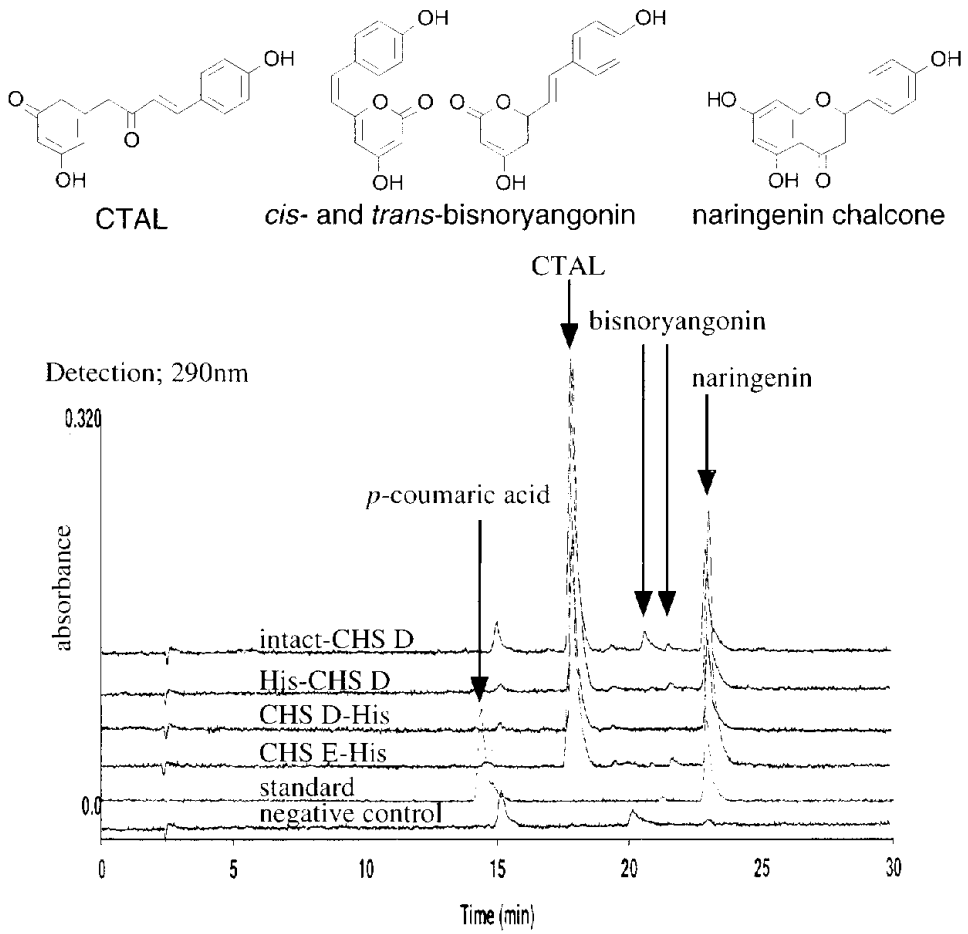

Fig. 2. HPLC elution profiles obtained for assays of heterologously expressed chalcone synthase activities. For assay and HPLC conditions, see Materials and Methods.

4. Discussion

4.1 The CHS-D and CHS-E genes and flower pigmentation

The *CHS* genes in the common morning glory as well as the Japanese morning glory (*Ipomoea nil*) comprise a multigene family (Durbin *et al.*, 1995; Fukada-Tanaka *et al.*, 1997). Among these *CHS* genes, the *CHS-D* gene is the most abundantly expressed in the pigmented flower buds and majorities of the remaining CHS mRNAs are produced from the *CHS-E* gene. The *CHS-D* and *CHS-E* genes are expressed predominantly in flower limbs and tubes, respectively (Fukada-Tanaka *et al.*, 1997; Johzuka-Hisatomi *et al.*, 1999; Durbin *et al.*, 2000). Moreover, the mutable *flaked* plants of *I. purpurea* having the transposable element *Tip100* inserted into the *CHS-D* gene produces white flower limbs with colored spots, and the *CHS-D* gene is scarcely expressed in the young flower buds of the mutable lines (Habu *et al.*, 1997; 1998). However, the flower tubes of these mutants are often lightly pigmented (Habu *et al.*, 1998; Johzuka-Hisatomi *et al.*, 1999). The observation presented here that both *CHS-D* and *CHS-E* genes encode CHS enzymes further confirmed the previous notion that the function of the *CHS-D* and *CHS-E* gene products of *I. purpurea* is the chalcone synthase which is a key enzyme for anthocyanin biosynthesis. Although the specific activity of the CHS-E enzyme seems to be higher than that of the CHS-D protein, the pigmentation in flower tubes is much weaker than that in flower limbs. Probably, this apparent contradiction can be partially explained by the fact that the expression of the *CHS-D* gene is much more abundant than that of the *CHS-E* gene (Fukada-Tanaka *et al.*, 1997; Johzuka-Hisatomi *et al.*, 1999; Durbin *et al.*, 2000). Alternatively, one of the other structural genes in the anthocyanin biosynthesis pathway may be much scarcely expressed in flower tubes than in flower limbs.

Since the genomic as well as the cDNA sequences of the corresponding *CHS* genes between *I. purpurea* and *I. nil* are highly homologous each other (Johzuka-Hisatomi *et al.*, 1999), it is highly likely that both *CHS-D* and *CHS-E* genes of *I. nil* also encode CHS enzymes in anthocyanin biosynthesis pathway. Indeed, we are able to identify that a mutation conferring white flowers in *I. nil* is caused

by insertion of a transposable element into the *CHS-D* gene (A. Hoshino and S. Iida, unpublished results).

4.2 The CHS-D and CHS-E gene products and polyketide synthases

As **Fig. 2** shows, the CHS-D and CHS-E proteins produced CTAL, a well-known by-product of CHS, which was released from the enzyme prematurely in non-physiologically condition. Recently, a new type of the *CHS* gene, termed *HmS* for *p*-coumaroyltriacetic acid synthase, was isolated from *Hydrangea macrophylla* var. *thunbergii*, and its genuine product was found to be *p*-coumaroyltriacetic acid (Akiyama *et al.*, 1999). The sequences of the *CHS-D* and *CHS-E* genes showed higher similarity to that of the *Hms* gene than those of the *CHS-A* and *CHS-B* genes among the two subfamilies of the *CHS* genes, although *Ipomoea* and *Hydrangea* are distantly classified species.

Several plant polyketide synthases that utilized the compound other than phenylpropanoids were reported recently. For example, pyrone synthase, GCHS2, from *Gerbera hybrida* (Asteraceae) (Eckermann *et al.*, 1998; Zuurbier *et al.*, 1998) utilizes acetyl-CoA as its starter substrate, and bibenzyl synthase (BBS) cloned from *Phalaenopsis* sp. utilizes *m*-hydroxyphenyl-CoA and propionyl-CoA as the starters (Preisig-Müller *et al.*, 1995). Acridone synthase (ACS) cloned from *Ruta graveolens* was shown to utilize *N*-methylanthraniloyl-CoA as the starter substances (Junghanns *et al.*, 1995). As mentioned above, the *HmS* gene product from *Hydrangea macrophylla* has been identified to be a linear tetraketide synthase for the first time (Akiyama *et al.*, 1999). In every case, the constituents that are regarded to be derived from the products catalyzed by these polyketide synthases have been isolated from their mother plants, e.g. pyrone glycosides from *Gerbera hybrida* and secologanin derivatives from *Hydrangea macrophylla*.

Both Japanese and common morning glories carry at least five *CHS* genes which can be divided into two subfamilies (Durbin *et al.*, 1995; Fukada-Tanaka *et al.*, 1997). The first subfamily, represented by the *CHS-A* and *CHS-B* genes, are distantly related to the other known *CHS* sequences in a phylogenetic tree, whereas the other subfamily consisting of the *CHS-D* and *CHS-E* genes are more closely related to the well-characterized *CHS* genes. Although these *CHS* genes comprise two exons with an identical intron position, the introns of the *CHS-D* and *CHS-E* genes expressed abundantly in the pigmented buds are much longer than those of other *CHS* genes (Durbin *et al.*, 1995; Johzuka-Hisatomi *et al.*, 1999). We have shown here that the *CHS-D* and *CHS-E* genes in *I. purpurea* indeed encode the chalcone synthases producing naringenin chalcone. It would be extremely interesting to ask whether the *CHS-A* and *CHS-B* gene products bear new polyketide synthase activities or they are also the ordinary CHS enzymes. We are currently characterizing the activities of the CHS-A and CHS-B proteins.

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