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Original Paper

Biochemical characterization and mutational studies of a chalcone synthase from yellow snapdragon (*Antirrhinum majus*) flowers

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Abstract The 4′-*O*-glucosides of 2′,4,4′,6′-tetrahydroxychalcone (THC) and 2′,3,4,4′,6′-pentahydroxychalcone (PHC) are present in the yellow snapdragon (*Antirrhinum majus*) flowers and serve as direct precursors for the synthesis of aurones, the yellow pigments of the flowers. Despite the metabolic significance of these chalcones in aurone biosynthesis, the biochemical properties of chalcone synthase (CHS), the enzyme responsible for their synthesis, remains to be clarified. In this study, one known CHS cDNA (*niv*, also termed *AmCHS1*) and one CHS-related cDNA, *AmCHS2*, were isolated from yellow snapdragon buds. *AmCHS1* mRNA specifically accumulated in the petals in a coloration-dependent manner, whereas *AmCHS2* mRNA accumulated only negligibly in petals and other organs, corroborating the importance of AmCHS1 in chalcone synthesis in the yellow flower. Recombinant AmCHS1 that was heterologously expressed in *Escherichia coli* cells efficiently utilized both *p*-coumaroyl-CoA and caffeoyl-CoA (relative activity, 50% of the activity for *p*-coumaroyl-CoA) as a starting ester to produce THC and PHC, respectively. Previous studies predicted that a single Thr197Ala or the double Val196Met/Thr197Ala substitution in AmCHS1 may provide a wider space for hydroxycinnamoyl-group binding and might enhance the ability of this enzyme to produce PHC [Austin MB, Noel JP (2003) *Nat Prod Rep* 20: 79–110]. However, these substitutions led to a decreased PHC-producing activity, implying that other factors may also be important for the efficient utilization of caffeoyl-CoA.

Key words: *Antirrhinum majus*, chalcone synthase, snapdragon, 2′,3,4,4′,6′-pentahydroxychalcone, 2′,4,4′,6′-tetrahydroxychalcone.

Chalcone synthase (CHS) catalyzes the sequential, decarboxylative addition of three acetate units from malonyl-CoA to a hydroxycinnamoyl-CoA as a starting precursor, followed by cyclization, to produce a chalcone (Jez and Noel 2000) (Figure 1). In most plant species, *p*-coumaroyl-CoA serves as the precursor of 2′,4,4′,6′-tetrahydroxychalcone (THC), a pivotal precursor of a diverse group of flavonoids.

Small amounts of 4′-*O*-glucosides of THC and 2′,3,4,4′,6′-pentahydroxychalcone (PHC) have been identified in yellow flowers of snapdragon (*Antirrhinum majus*) (Sato et al. 2001). Recent studies suggest that these chalcone glucosides serve as direct precursors of aurones, which are mainly responsible for the yellow color of the flowers (Nakayama et al. 2000; Nakayama et al. 2001; Sato et al. 2001; Ono et al. 2006). Genetic and chemicogenetic evidence to date suggests that *Antirrhinum* should express only a single CHS, referred

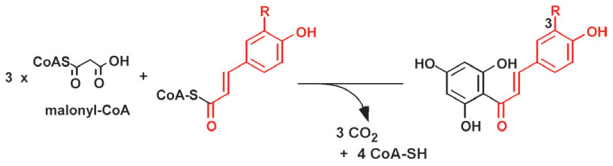

Figure 1. Chalcone synthase-catalyzed reaction. Starting esters are *p*-coumaroyl-CoA (R=H) and caffeoyl-CoA (R=OH), and product chalcones are THC (R=H) and PHC (R=OH). Portions derived from phenylpropanoids in the structures of the substrate and product are shown in red.

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Accession number: *AmCHS2*, AB219808.
Abbreviations: AmCHS1, *Antirrhinum majus* chalcone synthase 1; CHS, Chalcone synthase; HvCHS2, *Hordeum vulgare* chalcone synthase 2; MsCHS2, *Medicago sativa* chalcone synthase 2; PHC, 2′,3,4,4′,6′-pentahydroxychalcone; rAmCHS1, recombinant AmCHS1; RgCHS1, *Ruta graveolens* chalcone synthase 1; THC, 2′,4,4′,6′-tetrahydroxychalcone.
This article can be found at http://www.jspcmb.jp/

to as the *niv* gene, in flavonoid biosynthesis (Spribille and Forkmann 1982; Sommer and Saedler 1986). For example, the interruption of anthocyanin biosynthesis by recessive alleles (*niv*/*niv*) results in the chalk-white "nivea" type of the flower, which lacks chalcone synthase activity (Spribille and Forkmann 1982). Moreover, the integration and excision of transposons (Tam) at the *nivea* locus have been shown to cause variegated phenotypes of flower colors (Bonas et al. 1984). These results corroborate a general role of *niv* in the biosynthesis of flavonoids. However, the biochemical properties of *niv* CHS remain to be clearly established. CHS activity giving rise to THC was previously identified in crude extracts of pink flowers of the snapdragon (Spribille and Forkmann 1982). However, this CHS activity did not lead to the production of PHC.

To establish the enzymology of the snapdragon CHS and enhance our understanding of chalcone biosynthesis in the yellow flower, we undertook a study of CHS activity in crude extracts of yellow snapdragon flowers. However, the activity was marginally low and unstable, and this hampered the biochemical characterization of the enzyme. We therefore isolated *niv* cDNA (also termed here as *AmCHS1*), which is expressed in the petals of yellow snapdragon flowers. During the course of the study, we also isolated another CHS-related cDNA, termed *AmCHS2*, which encoded a novel member of the type III plant polyketide synthase family. The *AmCHS1* appeared to be expressed in a spatially- and temporally-regulated manner in the snapdragon plant, whereas *AmCHS2* was only negligibly expressed in the plant. We overexpressed *AmCHS1* in *Escherichia coli* cells as a catalytically active protein (termed rAmCHS1) in order to biochemically characterize the molecule. In addition, mutational studies were carried out in an attempt to evaluate the molecular basis of the specificity of CHS for the starting ester.

Materials and methods

Plant materials and flavonoids

Yellow snapdragon flowers were purchased from a local market in Sendai, Japan, and their buds and flowers were stored at −80°C until used. The developmental stages of snapdragon flowers are defined as follows: stage 1, closed buds (<10 mm in length); stage 2, buds (10–15 mm in length) with petals visible; stage 3, buds (15–20 mm in length); stage 4, buds (20–25 mm in length); stage 5, buds (30 mm< in length); and stage 6, recently opened flowers (Sato et al. 2001).

Eriodictyol and naringenin were purchased from Extrasynthése, Genay, France, and malonyl-CoA was from Sigma, St Louis, USA. [^{14}C]malonyl-CoA was from Moravek (Brea, CA, USA). *p*-Coumaroyl-CoA and caffeoyl-CoA were obtained as described previously (Suzuki et al. 2004). THC and PHC were synthesized from naringenin and eriodictyol, respectively, as described previously (Moustafa and Wong 1967).

Construction of cDNA library and sequence analysis

Poly(A)+ RNA was prepared from the buds (stages 3–5) of yellow snapdragon and was used for the construction of a cDNA library using the λZAPII-cDNA synthesis kit (Stratagene, La Jolla, CA, USA). A forward primer, 5′-AT(T/C)TA(T/C)TT(T/C)GGIAA(T/C)TG-3′, and a reverse primer, 5′-GATTGGAACAGCTACTCCTG-3′, were designed based on the nucleotide sequence encoding the conserved region of the CHS family of enzymes. The cDNA library was then used as a template for the PCR amplification of a partial CHS cDNA using the above-mentioned PCR primers. The amplified fragment, *CHS01*, which was ~0.36 kbp in length, was cloned into TOPO-pCR2.1 (Invitrogen, Groningen, Netherlands) and subjected to sequencing using a Dye-Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA, USA) with a CEQ 2000 DNA analysis system (Beckman Coulter). The *CHS01* fragment was DIG-labeled using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel, Switzerland). The cDNA library was screened by plaque hybridization with the amplified cDNA fragment as a probe using the PCR DIG Probe Synthesis Kit and the DIG-DNA Labeling and Detection Kit (Roche Diagnostics). The hybridization was performed at 37°C in 5×SSC containing 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine, 2% (w/v) blocking reagent, and 30% (v/v) formamide (Roche Diagnostics). The filters were washed two times in 0.1×SSC and 0.1% (w/v) SDS at 55°C for 15 min.

Heterologous expression of AmCHS cDNAs in E. coli cells

To introduce a *Bgl*II site and an *Ase*I site, PCR was performed using a full-length *AmCHS* cDNAs (cloned into the plasmid pBluescript SK) as a template and a set of specific primers: 5′-AATCTAGATTAATATGGTGACTGTTGAGGAG-3′ and 5′-AATCTAGAGATCTTTAATTAAGCGGCACAC-3′ (for *AmCHS1*) or 5′-GAGCCCTATCTGGTCTGTTAAAA-3′ and 5′-AACATATGCCTCGTGGTTCGA-3′ (for *AmCHS2*). The amplified fragments were digested with *Bgl*II and *Ase*I and then ligated in the site of *Bam*HI-*Nde*I site of a pET15b vector (Novagen, Madison, WI, USA), yielding pHisAmCHS1 and pHisAmCHS2, which were subsequently used to transform *E. coli* BL21(DE3) cells. The recombinant enzymes were expressed in the transformed cells and purified to apparent homogeneity, as described previously (Suh et al. 2000).

Enzyme assays

CHS activity was routinely assayed by high performance liquid chromatography (HPLC) by determining the chalcone and its isomeric flavanone that are produced. The standard assay mixture consisted of 100 mM HEPES buffer (pH 7.5), 30 μM *p*-coumaroyl-CoA or caffeoyl-CoA, 60 μM malonyl-CoA, and enzyme in a final volume of 200 μl. After incubation at 30°C for 10 min, the reaction was stopped by the addition of 200 μl of a 2 : 3 mixture of acetonitrile: H_2O containing 0.6% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid and 4% (by volume) trifluoroacetic acid. Analytical HPLC was performed using a Gilson 305 system, equipped on-line with a Rainin auto-sample injector (model AI-3): column,

YMC *J*'sphere ODS M80 (4.6×150 mm); flow rate, 0.7 ml/min; solvent A, 0.1% (by volume) trifluoroacetic acid in H_2O; solvent B, 0.1% trifluoroacetic acid in a 9 : 1 (by volume) mixture of acetonitrile and H_2O. Compounds in the eluent were detected at both 290 nm and 370 nm. Peak identification of each component was confirmed post-run by photodiode-array spectroscopic analysis from 200 to 600 nm using a Shimadzu SPD M6A system. The amounts of the flavonoids were determined from the peak integrals using authentic samples, which were used for calibration. Protein was quantified by the method of Bradford (1976) with bovine serum albumin as the standard. The radio-assay for CHS reaction was performed essentially as described above, except that [^{14}C]malonyl-CoA (53 mCi/mmol; final concentration, 10 μM), *p*-coumaryol-CoA or caffeoyl-CoA (20 μM), and phosphate buffered saline, pH 7.0, were used. The reaction products were detected by UV absorption at 290 nm and by measurement of radioactivity with a Ramona star radio-HPLC analyzer (Raytest, Tokyo, Japan).

Quantitative real-time RT-PCR

Total RNA was prepared from the individual organs of snapdragon plants using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The *AmCHS1* and *AmCHS2* transcripts were quantified by quantitative real-time PCR on the LightCycler Quick System model 330 (Roche Diagnostics) using the total RNA as a template along with the QuantiTect SYBR Green RT-PCR Kit (Qiagen). Specific primers used for the quantification were 5′-CTACGCCCTTCCGTCAAACGT-3′ and 5′-CACTTCCCTCAAATGCCCGTCA-3′ for *AmCHS1*, and 5′-GAGCCCTATCTGGTCTGTTAAAA-3′ and 5′-AACATATGCCTCGTGGTTCGA-3′ for *AmCHS2*. Thermal cycling conditions were 50°C for 20 min and then 95°C for 15 min, followed by 42 cycles of 95°C for 15 sec, 55°C for 20 sec, and 72°C for 10 sec. The results are presented as the average of five independent determinations with standard errors.

Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli (1970). For Western blotting analyses, proteins in the SDS-PAGE gel were transferred to an Immobilon-P membrane (Millipore, Billerica, MA), which was then blocked with the recommended dilution of a blocking reagent (Amersham Bioscience) at room temperature for 1 h. The blots were probed with 1 : 5000 dilution of the primary anti-rAmCHS1 IgG (rabbit) and 1 : 25000 dilution of the secondary donkey anti-rabbit IgG-horseradish peroxidase conjugate. The immune complexes were visualized by a peroxidase-catalyzed chemiluminescence reaction using an ECL Western blotting kit (Amersham Bioscience) following the manufacturer's recommendations.

Site-directed mutagenesis

Introduction of the single Thr197Ala and the double Val196Met/Thr197Ala substitutions into AmCHS1 was performed on plasmid pHisAmCHS1 using the Quik Change Mutagenesis kit (Stratagene). Individual mutations were verified by DNA sequencing on both orientations. The construct of each mutant was used to transform *E. coli* BL21(DE3) cells. The expressed products were analyzed for their substrate specificity as described above.

Results and discussion

Isolation of CHS-related cDNAs expressed in yellow snapdragon flowers

A cDNA library was prepared from poly(A)+RNA isolated from the buds of the yellow snapdragon. The 0.36-kbp cDNA fragment was amplified by RT-PCR with a primer set designed from the conserved sequence in the CHS family using total RNA as a template. Using the fragment as a probe, the cDNA library, consisting of 150,000 clones, was screened under high stringency conditions and fifteen positive clones were obtained. One of the full-length cDNA, termed *AmCHS1*, encoded a protein consisting of 390 amino acids with a calculated molecular mass of 42,635 Da. The nucleotide sequence of *AmCHS1* cDNA was identical to that of the *niv* gene of the same plant species (TTK line) (Sommer and Saedler 1986; Genbank accession number, X03710). The deduced amino acid sequence of AmCHS1 had an 88% identity to that of RgCHS1 of the common rue (*Ruta graveolens*) (Springob et al. 2000), 84% to the HvCHS2 of barley (*Hordeum vulgare*) (Christensen et al. 1998), and 75% to the MsCHS2 of alfalfa (*Medicago sativa*) (Junghans et al. 1993). Another cDNA, *AmCHS2*, which has not thus far been identified in *A. majus*, encoded a protein (42,958 Da) consisting of 390 amino acids. AmCHS2 was predicted to be a member of the type III polyketide synthase family, judging from its high sequence similarity to known CHSs (64–71% identity) and the conservation of the catalytic residues of CHS (Cys164, His303, and Asn336) (Jez and Noel 2000). The deduced amino acid sequence of AmCHS2 was 72% identical to that of AmCHS1.

The *AmCHS1* and *AmCHS2* were then expressed under the T7 promoter in *Escherichia coli* BL21(DE3) cells as an in-frame N-terminal fusion with a His_6 tag. The heterologous expression of *AmCHS1* yielded a soluble, catalytically active protein with an estimated molecular mass of 45 kDa (14 mg from 1 L of the culture), while that of *AmCHS2* yielded an inclusion body of the expressed protein with no catalytic activity. The recombinant AmCHS1 (rAmCHS1) was purified to homogeneity and its substrate specificity was examined. It catalyzed the consecutive condensation of three acetyl units from malonyl-CoA with a *p*-coumaroyl-CoA to produce THC, which underwent a rapid spontaneous isomerization to naringenin (Mol et al. 1985). It could also utilize caffeoyl-CoA as a starting ester to produce PHC, most of which was identified as its isomeric product eriodictyol. Kinetic parameters for rAmCHS1 are summarized in Table 1. The catalytic efficiency (V_{max}/K_m) for THC-producing activity of rAmCHS1 was 1.6-fold higher than that for its PHC-producing activity.

Table 1. Kinetic parameters of rAmCHS1

Substrate	K_m (μM)	V_{max} (pkat mg^{-1})[a]	V_{max}/K_m (pkat mg^{-1} μM^{-1})
p-Coumaroyl-CoA[b]	18±5	51±6	2.8
Caffeoyl-CoA[b]	15±5	27±4	1.8
Malonyl-CoA[c]	27±9	20±2	0.74

Values are expressed as a mean±SE.

[a] One katal unit of enzyme is defined as the amount of enzyme required to catalyze the formation of 1 mole of chalcone per sec.

[b] Kinetic parameters were determined at a malonyl-CoA concentration of 60 μM.

[c] Kinetic parameters were determined at a caffeoyl-CoA concentration of 20 μM.

There is no significant difference between the K_m values for these hydroxycinnamoyl-CoAs. Optimum pH for the reaction was 7.0 for caffeoyl-CoA and 7.5 for *p*-coumaroyl-CoA. Acetyl-CoA, succinyl-CoA, and methylmalonyl-CoA were all inert as substrates for rAmCHS1.

To evaluate the physiological significance of *AmCHS1* and *AmCHS2* in chalcone synthesis in yellow snapdragon flowers, the spatial and temporal expressions of these cDNAs were analyzed by quantitative real-time RT-PCR (Figure 2A). The *AmCHS1* transcripts were mainly located in the flower buds and the expression was increased with flower development. It should be noted that a previous study reported that the steady-state level of the CHS transcript is maximal at stage 3–5 and then diminishes at stage 6 (Jackson et al. 1992). However, this appears to not always be the case; our finding indicates that the steady-state level of the CHS transcript in the yellow flower appears to be maintained at a high level even in recently opened flowers (*i.e.*, stage 6), and this might depend on the variety or the growth conditions used. *AmCHS2* was expressed only slightly in the organs tested here. Moreover, the expression pattern of *AmCHS1* was correlated with accumulation of chalcones and aurones in the petal during flower development (Sato et al. 2001). The specific expression of AmCHS1 in the petals of aurone-containing flowers (*i.e.*, yellow and orange flowers; Toki 1988) was also confirmed by a Western blotting analysis using anti-rAmCHS1 IgG (rabbit) (Figure 2B). The above results are all consistent with the role of AmCHS1 in chalcone synthesis in yellow snapdragon flowers. It should be noted, however, that some of the previous biochemical and chemicogenetic observations appear to argue against the possible involvement of the PHC-producing activity of AmCHS1 in PHC biosynthesis. CHS that was previously identified in crude extracts of pink snapdragon flowers was unable to utilize caffeoyl-CoA (Spribille and Forkmann 1982). Moreover, 3′4′-hydroxylated flavonoids (luteolin, quercetin, and cyanidin) of snapdragon are only formed in the presence of the dominant allele *Eos* encoding flavonoid 3′-hydroxylase

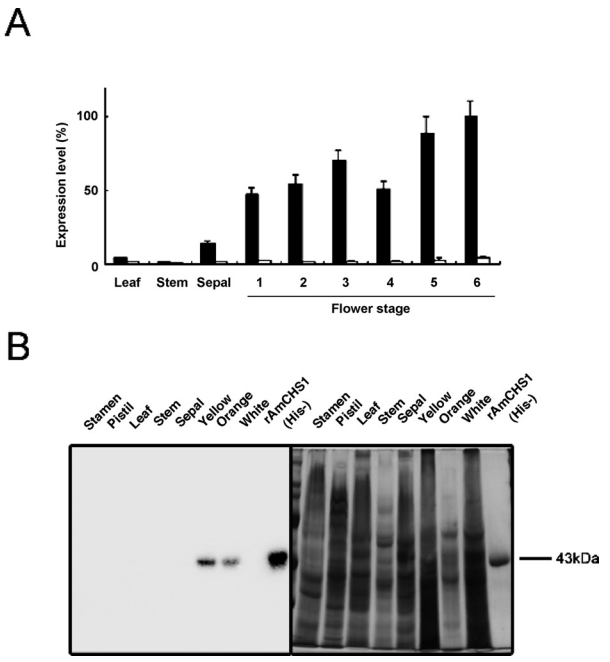

Figure 2. Expression of AmCHS1 in the snapdragon plant. (A) The relative levels of transcription of *AmCHS1* (*black bars*) and *AmCHS2* (*white bars*). Developmental stages of the flowers are defined under Experimental Procedures. Average values of five independent determinations of transcription levels are presented with error bars indicating ±SEs. The transcription level of *AmCHS1* at stage 6 was taken to be 100%. (B) Western blotting analysis and SDS-PAGE analysis of crude extracts prepared from the snapdragon plant. One hundred nanograms of the purified rAmCHS1 without a His_6 tag and 4 μg of proteins extracted from the stamens, pistils, leaves, stems, sepals, and petals of yellow snapdragon flowers (stages 2–6) as well as the petals of orange and white snapdragon flowers (stages 2–6) were electrophoresed and subjected to Western blotting probed with anti-rAmCHS1 IgG (rabbit), as described in Materials and Methods (*left panel*). Another set of the electrophoresed proteins was visualized by silver staining (*right panel*). The rAmCHS1 without a His_6 tag was prepared by treating rAmCHS1 with thrombin followed by passing through a column of HisTrap (1 ml, Amersham Biosciences). The molecular mass of the recombinant protein [rAmCHS1 (His−)] was calculated to be 43 kDa from the deduced amino acid sequence. Flower colors are spectrally defined as described previously (Toki 1988). It should be noted that the absence of expressed AmCHS1 in the petals of the white snapdragon (albino type) is consistent with the *niv*/*niv* genotype of the flower (Toki 1988).

(Spribille and Forkmann 1982). In addition, pink flowers of the genotype *eos*/*eos* snapdragon do not contain cyanidin (Spribille and Forkmann 1982). Thus, we examined the issue of whether the observed PHC-producing activity of rAmCHS1 represented an artifact due to the *in vitro* assay conditions used in our study. When the N-terminal His_6 tag of the recombinant enzyme was removed by proteolytic cleavage with thrombin, the resulting enzyme was still able to utilize caffeoyl-CoA to produce PHC. Moreover, when the CHS reaction with the recombinant enzyme (without His_6 tag) was examined using a crude extract of yellow snapdragon flowers, the enzyme was able to produce both THC and PHC, similar to the CHS reactions that

were run in the buffer without added snapdragon proteins. Therefore, the ability of rAmCHS1 to utilize caffeoyl-CoA does not appear to be an artifact that arose from the presence of the His_6 tag in the recombinant enzyme molecule or the assay conditions. The possibility, however, that substrate specificity of the heterologously-expressed enzyme was inherently altered from that of the native enzyme, cannot be excluded. The 3-hydroxylation, by flavonoid 3′-hydroxylase (Eos), of THC that is produced by the action of AmCHS1 may be a possible alternate route to PHC biosynthesis; but, it is believed that Eos is not involved in the hydroxylation of the chalcone B-ring in the snapdragon (Forkmann and Heller 1999). Further studies will be needed to clarify the biosynthetic route of PHC in the yellow snapdragon flowers.

Mutational studies to study factors that govern the starting-ester specificity of CHS

Although the physiological significance of the observed PHC-producing activity of rAmCHS1 remains to be established, the fact that rAmCHS1 efficiently utilized caffeoyl-CoA makes this enzyme an interesting model for studies of the molecular basis of the starting-ester specificity of CHS. A CHS of barley (*H. vulgare*), HvCHS2, is known to show an unusually high preference for caffeoyl and feruloyl-CoAs, with more than 1.8-times higher activities than that of *p*-coumaroyl-CoA (Christensen et al. 1998). It is noteworthy that CHS of the common rue (*R. graveolans*), RgCHS1, preferentially utilizes *p*-coumaroyl-CoA, but can also utilize cinnamoyl-CoA and caffeoyl-CoA at significant rates (76% and 30% of the activity of *p*-coumaroyl-CoA, respectively) (Springob et al. 2000). It has been suggested that such a preference might be related to the large volume of the hydroxycinnamoyl-group binding pocket of the enzyme (Austin and Noel 2003). The crystal structure of an alfalfa CHS (MsCHS2) that preferentially utilizes *p*-coumaroyl-CoA shows that Thr197* of MsCHS2 is located at the pocket (Ferrer et al. 1999) (The amino acid residues are numbered so as to correspond to the numbering of AmCHS1 on the basis of the alignment shown in Figure 3A, and positional numbering, according to this notation, is indicated by an asterisk). The amino acid residue corresponding to Thr197* of MsCHS2 is alanine in HvCHS2. Thus, it has been suggested that this alanine residue provides a wider space for the binding of the hydroxycinnamoyl group in HvCHS2, making it possible to accommodate caffeoyl-CoA, which is bulkier than *p*-coumaroyl-CoA, in the pocket (Austin and Noel 2003). A comparison of the deduced amino acid sequence of AmCHS1 with those of HvCHS2, MsCHS2, and RgCHS1 revealed that the residue, which corresponds to Thr197* of MsCHS2 is also threonine in AmCHS1 and RgCHS1. This

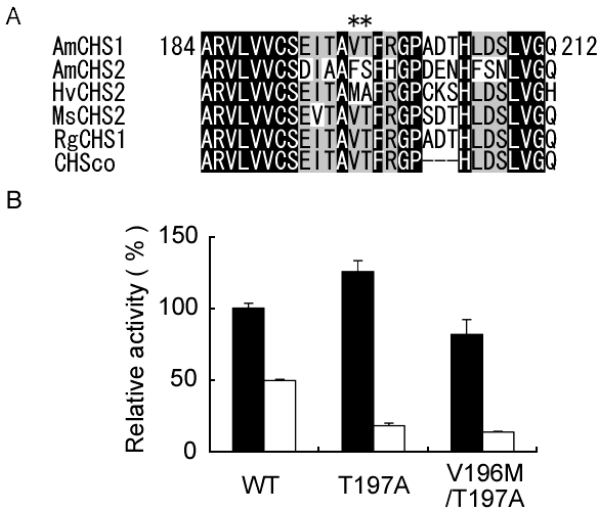

Figure 3. Mutagenic analysis of AmCHS1 mutants. (A) A comparison of amino acid sequences around the putative starting-ester binding pocket of AmCHS1 and AmCHS2 with those of HvCHS2 from barley (*H. vulgare*), MsCHS2 from alfalfa (*M. sativa*), and RgCHS1 from the common rue (*R. graveolens*). CHSco shows the CHS consensus sequence. The numbers refer to the positions in the AmCHS1 sequences from the N-terminus. Mutational sites in this study are marked with *asterisks*. (B) Substrate specifities of AmCHS1 mutants. *black bars*, % THC-producing activity; *white bars*, % PHC-producing activity. Specific THC-producing activity of the wild type is taken as 100%. Average values of four independent determinations of enzyme activity are presented with error bars indicating ±SEs.

comparison also revealed that Val196*, which is conserved in MsCHS2, RgCHS1 and AmCHS1, is specifically replaced by a methionine residue in HvCHS2 (Figure 3A). These observations suggest that the single Thr197Ala or the double Val196Met/Thr197Ala substitution in AmCHS1 may further enhance the ability of this enzyme to produce PHC. To examine the validity of this prediction, the mutants Thr197Ala and Val196Met/Thr197Ala were created, purified to apparent homogeneity, and their specificity was determined. The results showed that the relative PHC-producing activities of these mutants were somewhat decreased compared with that of wild-type CHS (Figure 3B). Thus, it was not possible to enhance the PHC-producing activity of AmCHS1 using point mutations on the basis of the predictions described above. Kinetic studies of wild-type AmCHS1 (Table 1) showed that the difference between the reactivity of *p*-coumaroyl-CoA and caffeoyl-CoA mainly arose from k_{cat} values, but not from K_m values. This suggests that other factors, such as the structural flexibility of the enzyme required for the binding of caffeoyl-CoA and subsequent catalysis, may also be important for attaining a higher ability to utilize caffeoyl-CoA. Finally, it should be pointed out that the double mutant unexpectedly produced a significant amount of a by-product in addition to THC when the starting ester was *p*-coumaroyl-CoA. The identification of the byproduct and mechanistic consideration for the byproduct formation

will be reported elsewhere.

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