

cDNA cloning and characterization of chalcone isomerase-fold proteins from snapdragon (*Antirrhinum majus* L.) flowers

Naoto Fujino^{1,†}, Tatsuya Yamazaki^{1,†}, Yanbing Li¹, Kota Kera¹, Erika Furuhashi¹, Satoshi Yamashita¹, Yasumasa Morita^{2,a}, Masayoshi Nakayama², Seiji Takahashi¹, Toru Nakayama^{1,*}

¹Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Sendai, Miyagi 980-8579, Japan; ²NARO Institute of Floricultural Science, Tsukuba, Ibaraki 305-8666, Japan

*E-mail: nakayama@seika.che.tohoku.ac.jp Tel & Fax: +81-22-795-7270

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Abstract cDNAs coding for the chalcone isomerase-fold proteins (CIFP) of the snapdragon (*Antirrhinum majus* L.) were cloned and characterized. One of these CIFPs was a Cluster-1 member of the CIFP family, which was a catalytically active chalcone isomerase and thus termed AmCHI1. The recombinant AmCHI1 could act on 2',4,4',6'-tetrahydroxychalcone (THC) and 2',3,4,4',6'-pentahydroxychalcone (PHC) to produce naringenin and eriodictyol with k_{cat}/K_m values of $0.25\text{ s}^{-1}\mu\text{M}^{-1}$ and $0.071\text{ s}^{-1}\mu\text{M}^{-1}$, respectively, at pH 7.5 and 4°C. The enzyme could not act on 4'-O-glucosides of THC and PHC. In the yellow snapdragon petals (cv. Yellow Butterfly), the temporal expression patterns of *AmCHI1* were consistent with the observed temporal accumulation patterns of flavones. Thus, regulation of the *AmCHI1* transcription and substrate specificity of the expressed AmCHI1 should serve as the key mechanisms that allows for partitioning of the flavonoid biosynthetic pathways into the aurones and the non-aurone flavonoids in snapdragon petal cells. The other CIFP cDNA, *AmCIFP4*, was a Cluster-4 member of the family and was similar in its primary structure to enhancers of the flavonoid production of torenia (*Torenia* × *hybrida*) and petunia (*Petunia* × *hybrida*). *AmCIFP4* was more abundantly expressed than *AmCHI1* irrespective of flower color.

Key words: *Antirrhinum majus* L., aurone, chalcone isomerase, enhancer of flavonoid production, snapdragon.

The snapdragon (*Antirrhinum majus* L.) is an important ornamental plant with a wide variety of flower colors (Schwarz-Sommer et al. 2003), which arise exclusively from flavonoids. The yellow color of this flower is mainly provided by the 6-O-glucosides of aurones (Sato et al. 2001), and the reddish color derives from anthocyanins (Figure 1). Small amounts of 4'-O-glucosides of chalcones [2',4,4',6'-tetrahydroxychalcone (THC) and 2',3,4,4',6'-pentahydroxychalcone (PHC)] accumulate in the petals and are partly responsible for the yellow color (Figure 1) (Gilbert 1973; Sato et al. 2001). Glycosides of flavones and flavonols also occur in petals of different colors and may play a role in the modulation of flower

color (Asen et al. 1972; Harborne 1963).

The biosynthetic pathways of flavones, flavonols and anthocyanins are conserved among seed plants, and chalcone isomerase (CHI, EC 5.5.1.6) serves as the first committed enzyme for the biosyntheses of these flavonoids, catalyzing the stereo-specific isomerization of chalcones to produce (2S)-flavanone (Figure 1). The distribution of aurones, however, is sporadic in plants and their biosynthetic pathways may vary with the plant species (Farang et al. 2009; Nakayama et al. 2000; Nakayama et al. 2001). In snapdragon petal cells, 6-O-glucosides of aurones [aureusidin 6-O-glucoside (Aur-G) and bracteatin 6-O-glucoside (Brc-G), see

Abbreviations: AmAS1, aureusidin synthase of *Antirrhinum majus*; AmCHI1, chalcone isomerase of *A. majus*; AmC4'GT, UDP-glucose:chalcone 4'-O-glucosyltransferase of *A. majus*; AmCHS1, chalcone synthase of *A. majus*; AmFNS1, flavone synthase (type II) of *A. majus*; Aur, aureusidin; Aur-G, Aur 6-O-glucoside; Brc, bracteatin; Brc-G, Brc 6-O-glucoside; CHI, chalcone isomerase; CHS, chalcone synthase; CIFP, chalcone isomerase-fold protein; EFP, enhancer of flavonoid production; FAP, fatty-acid binding protein; EST, expressed sequence tag; F3'H, flavonoid 3'-hydroxylase; rAmCHI1, recombinant AmCHI1; PHC, 2',3,4,4',6'-pentahydroxychalcone; PHC-G, PHC 4'-O-glucoside; RT-PCR, reverse transcription polymerase chain reaction; THC, 2',4,4',6'-tetrahydroxychalcone; THC-G, THC 4'-O-glucoside.

[†]These authors contributed equally to this work.

^aPresent address: Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0795, Japan.

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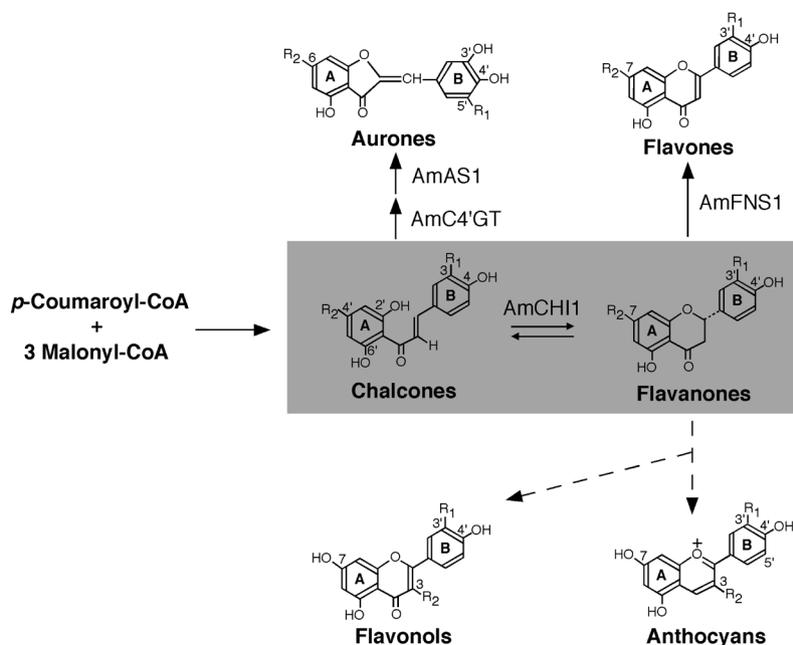


Figure 1. Structures of flavonoids (chalcones, aurones, flavanones, flavones, flavonols, and anthocyanins) that are typically found in snapdragon petal cells and their metabolic relationships. Key positional numberings are labeled on the structures. Note that the positional numbering in the chalcone structure is different from those of the other flavonoids. A and B indicate A- and B-rings in the flavonoid structures. The CHI-catalyzed reaction is shown with a gray background. Names of flavonoids are as follows: aurones [R₁=H, R₂=OH: aureusidin (Aur); R₁=H, R₂=OGlc: Aur 6-*O*-glucoside (Aur-G); R₁=OH, R₂=OH: bracteatin (Brc); R₁=OH, R₂=OGlc: Brc 6-*O*-glucoside (Brc-G)], chalcones [R₁=H, R₂=OH: 2',4,4',6'-tetrahydroxychalcone (THC); R₁=H, R₂=OGlc: THC 4'-*O*-glucoside (THC-G); R₁=OH, R₂=OH: 2',3,4,4',6'-pentahydroxychalcone (PHC); R₁=OH, R₂=OGlc: PHC 4'-*O*-glucoside (PHC-G)], flavanones [R₁=H, R₂=OH: naringenin; R₁=OH, R₂=OH: eriodictyol]; flavones [R₁=H, R₂=OH: apigenin; R₁=H, R₂=*O*-glucuronide: apigenin 7-*O*-glucuronide; R₁=OH, R₂=OH: luteolin; R₁=OH, R₂=*O*-glucuronide: luteolin 7-*O*-glucuronide; R₁=OCH₃, R₂=OH: chrysoeriol; R₁=OCH₃, R₂=*O*-glucuronide: chrysoeriol 7-*O*-glucuronide], anthocyanins [R₁=H, R₂=OH: pelargonidin; R₁=H, R₂=*O*-rutinoside: pelargonidin 3-*O*-rutinoside; R₁=OH, R₂=OH: cyanidin; R₁=OH, R₂=*O*-rutinoside: cyanidin 3-*O*-rutinoside; R₁=OCH₃, R₂=OH: peonidin; R₁=OCH₃, R₂=*O*-rutinoside: peonidin 3-*O*-rutinoside], flavonols [R₁=H, R₂=OH: kaempferol; R₁=H, R₂=*O*-Glc: kaempferol 3-*O*-glucoside; R₁=OH, R₂=OH: quercetin; R₁=OH, R₂=*O*-Glc: quercetin 3-*O*-glucoside].

Figure 1] are oxidatively produced from THC 4'-*O*-glucoside (THC-G) and PHC 4'-*O*-glucoside (PHC-G) in vacuoles by the action of aureusidin synthase (AmAS1, EC1.21.3.6), a chalcone-specific homolog of plant polyphenol oxidase (Nakayama et al. 2000; Nakayama et al. 2001). THC-G and PHC-G are produced from THC and PHC in the cytoplasm by the action of chalcone 4'-*O*-glucosyltransferase (AmC4'GT), and are subsequently transported to vacuoles (Ono et al. 2006a; Ono et al. 2006b). Thus, in the snapdragon, chalcones serve as a bifurcation in the flavonoid biosynthetic pathway (Figure 1), where the CHI-catalyzed production of (2S)-flavanone entails the formation of “non-aurone” flavonoids (i.e., flavones, flavonols, and anthocyanins) while the AmC4'GT-catalyzed glucosylation entails the formation of aurones. An elucidation of specificity, biochemistry, and expression patterns of snapdragon CHI should provide an important clue toward a better understanding of the regulation of the production of the aurones and the non-aurone flavonoids in snapdragon petal cells.

Recent phylogenetic and structural analyses of CHIs and CHI-related proteins have revealed the occurrence of four distinct phylogenetic clusters of

chalcone isomerase-fold proteins (CIFP) (Figure 2) (Ngaki et al. 2012; Ralston et al. 2005; Shimada et al. 2003). CIFPs of Cluster 1 represent the type-I CHIs that generally occur in vascular plants and act exclusively on 6'-hydroxychalcones while CIFPs of Cluster 2 are the type-II CHIs that specifically occur in leguminous plants and display activities toward both 6'-deoxy- and 6'-hydroxychalcones (Ralston et al. 2005; Shimada et al. 2003). Unlike CIFPs of these two clusters, the CIFPs of Clusters 3 and 4 are at least partly devoid of the machinery that is required for the catalytic functioning of CHI—the Cluster-3 CIFPs represent fatty acid-binding proteins (Ngaki et al. 2012), and the Cluster-4 CIFPs may serve as enhancers of the flavonoid production (termed EFPs) (Morita et al. 2014) that is involved in the regulatory mechanism of flower coloration in several plant species.

Because the snapdragon flower shows a wide variety of colors, it is of interest to examine the occurrence and genetic as well as biochemical aspects of CIFPs that are related to flavonoid biosynthesis in this flower. In the present study, we identified two distinct CIFPs that were commonly expressed in different commercial varieties of snapdragon petals (i.e., different colors). One of these

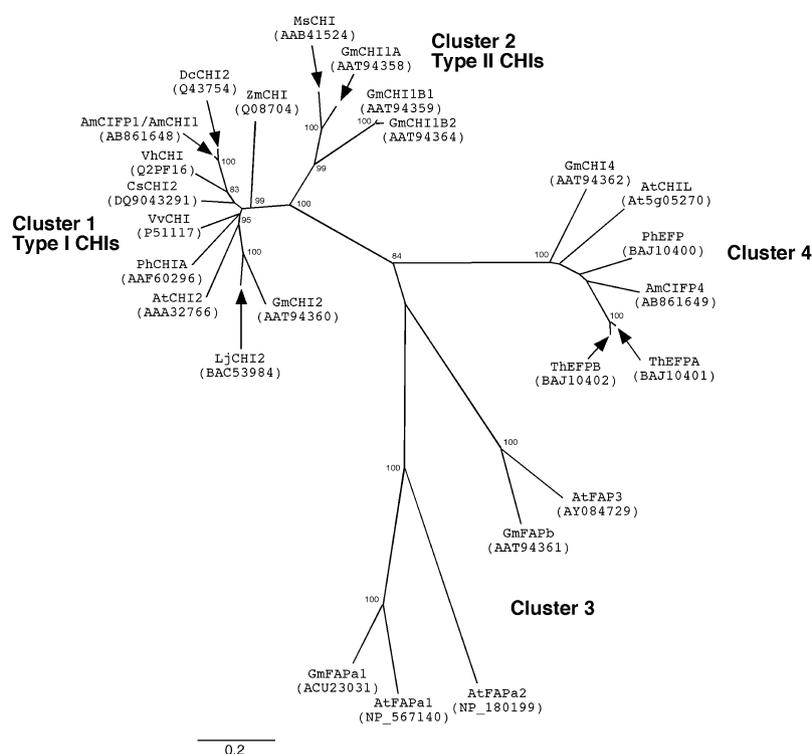


Figure 2. (A) A neighbor-joining phylogenetic tree of CIFPs based on deduced amino acid sequences. Amino acid sequences were analyzed using the CLUSTALW program. Numbers next to the nodes are bootstrap values greater than 80 from 100 replications. The bar indicates a genetic distance of 0.2. The deduced amino acid sequences of CIFPs were retrieved from the DDBJ/EMBL/GenBank databases. Accession numbers of CIFPs are shown with parentheses. Abbreviations of protein names are as follows: CHI, chalcone isomerase; CHIL, chalcone isomerase-like; EFP, enhancer of flavonoid production; FAP, fatty-acid binding protein. Abbreviations of plant species names are as follows: Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; Cs, *Camellia sinensis*; Dc, *Dianthus caryophyllus*; Lj, *Lotus japonicus*; Ms, *Medicago sativa*; Ph, *Petunia*×*hybrida*; Vh, *Verbena*×*hybrida*; Vv, *Vitis vinifera*; Zm, *Zea mays*.

CIFPs was a Cluster-1 protein (AmCIFP1; also termed AmCHI1), whereas the other was a CIFP of Cluster 4 (termed AmCIFP4). Comparative transcription analyses of these CIFPs were carried out and the biochemistry of AmCIFP1/AmCHI1 has been established. The controlled expression and substrate specificity of AmCIFP1/AmCHI1 should serve as the key mechanisms that allows for a partitioning of the flavonoid biosynthetic pathways into the aurones and the non-aurone flavonoids found in snapdragon petal cells.

Materials and methods

Plant materials and flavonoids

Snapdragon flowers of different colors [i.e., cv. Yellow Butterfly (yellow); cv. Floral Showers (yellow, red, and white)] were purchased from a local market in Sendai, Japan. The seeds of cv. Athlete Purple (purple) were from the Sakata Seed Co. (Yokohama, Japan). The buds and flowers of the snapdragons were stored at -80°C until use. 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). Naringenin

and eriodictyol were purchased from Extrasynthèse, Genay, France, and THC and PHC were synthesized from naringenin and eriodictyol, respectively, as described previously (Moustafa and Wong 1967). THC-G, THC 2'-O-glucoside, and PHC-G were obtained as described previously (Sato et al. 2001).

Cloning of AmCIFP cDNAs

Total RNA was prepared from the petals (stages 2–5) of different cultivars of snapdragons by using an RNeasy Plant Mini Kit (Qiagen, Tokyo Japan). For the cloning of *AmCIFP1* cDNA, total RNA was used for the synthesis of dsDNAs using the 3'-Full RACE Core Set (Takara Bio, Shiga, Japan). A forward primer, 5'-ATGGCTGAGATCACCAATT C-3' (italics indicates the translation initiation codon), was designed based on the registered nucleotide sequence of a snapdragon CHI (GenBank accession number, M68326) (Martin et al. 1991); a reverse primer that was used, 5'-CTGATCTAGAGG TACCGGATCC-3', was included in the kit. The amplified fragment, *AmCIFP1*, was approximately 0.9 kbp in length, was gel-purified, was recovered by using the UltraClean 15 DNA Purification kit (MO BIO Laboratories, Carlsbad, CA, USA), and was cloned into a pTA2 vector (Takara Bio, Shiga, Japan) using the TArget Clone -Plus- kit (Toyobo, Osaka, Japan). A recombinant plasmid, pTA2-AmCIFP1, was used to transform *Escherichia coli* DH5 α cells, which were then plated on Luria-

Bertani (LB) agar medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin, 0.5 mM isopropyl β -thiogalactoside, and 0.004% X-gal followed by incubation at 37°C overnight. White colonies grown on the plates were randomly selected. The transformant cells were grown at 37°C overnight in an LB medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin. The recombinant plasmids were recovered from the cells by using a GenEute Plasmid Mini-prep kit (Sigma-Aldrich, St. Louis, MO, USA), and were subjected to sequencing using a Dye-Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA, USA) with a CEQ 2000 DNA analysis system (Beckman Coulter).

For the cloning of *AmCIFP4* cDNA, the total RNA (2 μg , see above) that had been prepared from the buds of the cv. Athlete Purple was used for first-strand cDNA synthesis by using Invitrogen SuperScript III reverse transcriptase (Life Technologies Japan; Tokyo) with the GeneRacer Oligo dT primer (Life Technologies Japan). The 3' ends of the *AmCIFP4* cDNA were then amplified by PCR by using PrimeSTAR GXL polymerase (Takara Bio) with the ThCHI4-F1 primer (5'-GGA ATC ACA GAC ATT GAG ATA CA-3') (Morita et al. 2014) and the GeneRacer Oligo dT primer. The total RNA (5 μg) was also used to obtain the 5' ends of the *AmCIFP4* cDNA by using an Invitrogen GeneRacer Kit (Life Technologies Japan). Briefly, the GeneRacer RNA Oligo that was included in the kit was ligated to the 5' end of the 5' cap-removed mRNAs using T4 RNA ligase, followed by first-strand cDNA synthesis by using Invitrogen SuperScript III reverse transcriptase with a GeneRacer Oligo dT primer. The 5' ends of the *AmCIFP4* cDNA were amplified using PrimeSTAR GXL polymerase with a reverse gene-specific primer AmCHI4-R1 (5'-CACAACTT GTCCACCGG TGCTG-3') and the GeneRacer 5' Primer that was included in the kit. The nucleotide sequence of the full-length *AmCIFP4* cDNA was confirmed essentially as described above.

Heterologous expression of *AmCIFP1* cDNA in *E. coli* cells

The *AmCIFP1* cDNA was amplified by PCR with a forward primer 5'-CACCATGGCTGA GATCACCC-3' and a reverse primer 5'-TCA TTC CTT TTT GGG ATT CTC ATG TTG-3' using the pTA2-*AmCIFP1* plasmid (see above) as the template. The amplified DNA was gel-purified and cloned into the pENTR/TEV/D-TOPO vector (Life Technologies Japan). The *AmCIFP1* cDNA was introduced into pDEST17 by means of LR recombination reaction using a kit (*E. coli* Expression System with Gateway Technology) according to the manufacturer's guidelines. The resultant plasmid, pDEST-*AmCIFP1*, was used to transform the cells of *E. coli* BL21(DE3). Transformant cells were grown in an LB broth containing 50 $\mu\text{g}/\text{ml}$ ampicillin with shaking at 37°C overnight. Ten milliliters of the culture were inoculated into 1,000 ml of LB broth, and the cells were grown with shaking at 37°C. When optical density at 600 nm of the culture reached 0.4, isopropyl 1- β -D-thiogalactoside was added to the culture at a final concentration of 1.0 mM, followed by cultivation at 18°C for an additional 12 h.

All subsequent methods were performed at 0–4°C. The cells were harvested by centrifugation at 4,000 $\times g$ for 20 min and resuspended in 50 mM Hepes-NaOH buffer, pH 8.0, containing 2 mM 2-mercaptoethanol, 20 mM imidazole, 0.5 M NaCl, 10% glycerol, and 1% Tween 20. The cell suspension was subjected to ultrasonication, (20 Hz, 1 min, ten times) and the resultant debris was removed by centrifugation at 8,000 $\times g$ for 25 min. The supernatant was applied to a HisTrap HP column (1 ml, GE Healthcare Life Sciences, Piscataway, NJ, USA) and equilibrated with buffer A (50 mM Hepes-NaOH buffer, pH 7.5) containing 20 mM imidazole. The column was washed with 10 ml of the equilibration buffer, and the enzyme was eluted with buffer A containing 150 mM imidazole. The fraction eluted with 150 mM imidazole was concentrated and dialyzed against buffer A. The enzyme solution was concentrated using an Amicon Ultra-15 Centrifugal Filter Device (10,000 MWCO; Millipore, Billerica, MA, USA). SDS-PAGE of the purified enzymes was carried out using a 10% gel according to a method established by Laemmli (Laemmli, 1970).

Enzyme assays

Method I: The standard assay system for rAmCHI1 consisted of 50 mM potassium phosphate, pH 7.5, 25 μM THC, 5% ethanol, and enzyme in a final volume of 400 μl . The reaction mixture without the substrate was incubated at 4°C, and the reaction was started by the addition of the substrate in ethanol. Time-dependent decreases in absorbance at 368 nm (for THC) or 378 nm (for PHC) were monitored at 4°C with a U-2000 Hitachi double-beam spectrophotometer. Initial velocity measurements were conducted in the standard assay system with varied concentrations (5–35 μM) of either THC or PHC. All assays were corrected for non-enzymatic cyclization reactions, which were determined under the same conditions except that the enzyme was previously inactivated by incubation at 98°C for 15 min. Initial velocities at different substrate concentrations were obtained from three independent determinations and fitted to the Michaelis-Menten equation by non-linear regression using a computer program. The effects of pH on the enzyme activity were examined under the standard assay conditions in the presence or absence of the enzyme using the following buffers (50 mM): sodium acetate (pH 4.0–5.5), potassium phosphate (pH 5.5–7.5), Hepes-NaOH (pH 7.5–8.5), and glycine-NaOH (pH 8.5–10.0).

Method II: The standard reaction mixture (100 μl) consisted of 1 mM THC, 50 mM Hepes-NaOH, pH 7.5, 5% (v/v) ethanol, and enzyme. The mixture without THC was incubated at 25°C for 5 min, and the reaction was started by the addition of THC. After incubation at 25°C for 1 min, the reaction was stopped by adding 100 μl of ice-cold 2.5% (v/v) trifluoroacetic acid in ethanol. Flavonoids in the resultant mixture were analyzed by reversed-phase HPLC using a Shimadzu Prominence system (Shimadzu, Kyoto, Japan) under the following analytical conditions: column, J'sphere ODS M80 (4.6 \times 150 mm; YMC, Kyoto, Japan); flow rate, 0.7 ml \cdot min⁻¹; solvent A, 0.1% (v/v) trifluoroacetic acid in water; solvent B, 0.1% (v/v)

trifluoroacetic acid in a 9:1 (v/v) mixture of acetonitrile and water. The column was previously equilibrated with 10% B. After injection (50 μ l), the column was initially developed isocratically with 10% B for 5 min, followed by a linear gradient from 10% B to 100% B in 30 min. The column was then washed isocratically with 100% B for 5 min, followed by a linear gradient from 100% B to 10% B in 1 min. The chromatograms were obtained with detection at 290 nm (for flavanones) and 360 nm (for chalcones).

Quantitative real-time RT-PCR

Total RNAs were prepared from petals (stages 1–6) of various commercial varieties of snapdragon (see above) using a hot phenol method (Shirzadegan et al. 1991) and subsequently were treated with DNase I (Takara Bio). The transcripts of *AmCIFPs*, *AmCHS1* (Hatayama et al. 2006), *AmFNS1* (Akashi et al. 1999), *AmC4'GT* (Ono et al. 2006a), *AmAS1* (Nakayama et al. 2000), and *Ubiquitin* (GenBank accession number, X67957) in the total RNA samples were quantified by quantitative real-time RT-PCR on a LightCycler Quick System model 330 (Roche Diagnostics), or illumina Eco Real-time PCR system (illumina, San Diego, CA, USA), using a kit SYBR Select Master Mix (Life technologies Japan). The specific primers used for quantification were designed using Primer3 (Rozen and Skaletsky 2000), and are shown in Supplementary Table 1S. The standard thermal profile for PCRs was as follows: 50°C for 2 min, 95°C for 2 min, and 40 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 1 min. Each expression value was quantified in comparison with standard molecular amounts and normalized by that for *Ubiquitin*.

Flavonoid analyses

Weighed quantities of frozen buds or petals (1–2 g for each stage; calyces were removed) were pulverized in liquid nitrogen in a mortar. The flavonoids were extracted with 8–16 ml of 0.1% trifluoroacetic acid (by vol.) in a 1:1 mixture of acetonitrile and water. After centrifugation at 10,000 $\times g$ for 10 min, the supernatant was analyzed for flavonoids by reversed-phase HPLC using a Shimadzu Prominence system (Shimadzu, Kyoto, Japan) under the following conditions: column, *J'sphere* ODS M80 (4.6 \times 150 mm; YMC, Kyoto, Japan); flow rate, 0.7 ml \cdot min⁻¹; solvent A, 0.1% (v/v) trifluoroacetic acid in water; and, solvent B, 0.1% (v/v) trifluoroacetic acid in a 9:1 mixture of acetonitrile and water. The column was previously equilibrated with 15% B. After injection (50 μ l), the column was isocratically developed with 15% B, followed by five successive linear gradients of solvent B: 15% B to 20% B in 5 min, 20% B to 23% B in 5 min, 23% B to 37% B in 20 min, and 37% B to 60% B in 16 min. The column was then developed with a linear gradient from 60% B to 100% B in 1 min and subsequently washed isocratically with 100% B for 5 min, followed by a linear gradient from 100% B to 15% B in 1 min. It must be mentioned that total flavones were determined after conversion of their glucuronides to aglycons. Briefly, the extracts (see above; 20 μ l) were neutralized with NaOH, mixed with 180 μ l

of water containing 14 units of β -glucuronidase (Roche Applied Science; Mannheim, Germany), and incubated at 37°C for 20 min, and subsequently the mixture was subjected to HPLC analysis as described above. The chromatograms were obtained with detection at 290 nm (for flavanones and other flavonoids), 371 nm (for flavones and chalcones), and 405 nm (for aurones). The standard curves of each flavonoid were obtained by HPLC analysis of the flavonoid standards after appropriate dilutions of their stock solutions.

Results

Isolation of *AmCIFP* cDNAs expressed in snapdragon buds

Total RNA was prepared from the buds of a yellow snapdragon (cv. Yellow Butterfly). A 0.9-kbp DNA fragment was amplified by RT-PCR with a primer set designed on the basis of the reported nucleotide sequence of the M68326 *CHI* (see Materials and methods) using the total RNA as a template. The cDNA that was exclusively amplified encoded a protein consisting of 221 amino acids with a calculated molecular mass of 24.1 kDa, and were tentatively termed *AmCIFP1* (DDBJ accession number, AB861648). The deduced amino acid sequence of *AmCIFP1* was identical to that of the clone 018_4_05_n17 of *A. majus* EST (EMBL Accession code, AJ798794.1) and showed a high similarity to those of the type-I CHIs of carnations (DcCHI2 of *Dianthus caryophyllus*; GenBank accession number, Z67989.1; 96% identity) and tea leaves (CsCHI2 of *Camellia sinensis*; GenBank accession number, DQ904329.1; 80% identity). Phylogenetic analysis clearly showed that *AmCIFP1* is a Cluster-1 member of the *CIFP* family (Ngaki et al. 2012; Ralston et al. 2005; Shimada et al. 2003) (Figure 2).

It has recently been shown that the Cluster-4 *CIFPs* may serve as EFPs that is involved in the regulatory mechanism of flower coloration in several plant species (Morita et al. 2014). Thus, to examine the occurrence of the expressed *CIFP4* gene in snapdragon flowers, we attempted to amplify *CIFP4* gene using the total RNA prepared from the buds of cv. Athlete Purple by RT-PCR with a primer set designed on the basis of the nucleotide sequences of the Cluster-4 *CIFPs* of torenia (i.e., TheFPA and TheFPB; Figure 2) (Morita et al. 2014). As a result, a cDNA encoding a protein consisting of 212 amino acids with a calculated molecular mass of 23.5 kDa was amplified, which was a Cluster-4 member of the *CIFP* family (Morita et al. 2014; Ngaki et al. 2012), as expected, and thus was termed *AmCIFP4* (Figure 2). The deduced amino acid sequence of *AmCIFP4* (DDBJ accession number, AB861649) was 27% identical to *AmCIFP1* and showed a high similarity to the EFPs of petunias (PhEFP, 77%) and torenias (TheFPA, 73%; TheFPB, 71%) (Morita et al. 2014).

CHIs have been shown to be cytoplasmic proteins that can also be localized on the cytoplasmic surface of ER and tonoplast and within nuclei (Saslowky and Winkel-Shirley 2001; Saslowky et al. 2005). When the primary structures of AmCIFP1/AmCHI1 and AmCIFP4 were analyzed by using TargetP (<http://www.cbs.dtu.dk/services/TargetP/>), WoLF PSORT (<http://wolfsort.seq.cbrc.jp/>), and iPSORT (<http://hc.ims.u-tokyo.ac.jp/iPSORT/>) programs, neither the signal sequence for secretion nor the translocation to any organelles could be identified. This implied that both homologs might potentially be cytoplasmic proteins. Subcellular localization of these CIFPs should be analyzed by experimentation in future studies.

Biochemical properties

The *AmCIFP1* cDNA were heterologously expressed at 18°C under the T7 promoter in *E. coli* BL21(DE3) cells as an N-terminal fusion with a His₆ tag. However, despite our attempts, the *AmCIFP4* cDNA could not be successfully expressed with our heterologous *E. coli* systems. Although a large fraction of the expressed AmCIFP1 was obtained as an insoluble inclusion body, a small fraction occurred as a soluble, catalytically active protein with an estimated molecular mass of 28.7 kDa. Thus, the soluble fraction of the expressed AmCIFP1 was purified to homogeneity by means of nickel affinity chromatography (Figure 3A), and the biochemistry of AmCIFP1 (hereafter termed AmCHI1) was studied in detail.

The recombinant AmCHI1 (rAmCHI1) efficiently catalyzed the isomerization of THC to produce naringenin (Figure 3B, C). Although THC can also be isomerized to naringenin in a non-enzymatic manner (Mol et al. 1985), this non-enzymatic reaction was negligible and took place very slowly at 4°C under the assay conditions employed in the present study (Figure 3C). Thus, the initial velocity assays of the rAmCHI1-catalyzed reaction could be conveniently carried out at 4°C. Substrate specificity of rAmCHI1 was then analyzed using the related chalcones and their glucosides [i.e., PHC, THC-G, PHC-G, and THC-2'-*O*-glucoside]. The results showed that rAmCHI1 could also utilize PHC as a substrate to produce eriodictyol (relative activity, 9% with the activity for THC taken to be 100%). All chalcone glucosides examined were inert as substrates. The kinetic parameters for the rAmCHI1 of THC and PHC are summarized in Table 1. The k_{cat} value for THC was 10-fold higher than the value for PHC, while the K_m for THC was 3-fold larger than the value for PHC; thus, the catalytic efficiency (k_{cat}/K_m) for the rAmCHI1 of THC was 3.5-fold higher than that of PHC. The optimum pH for the rAmCHI1-catalyzed isomerization of THC ranged from 7.5 to 8.5.

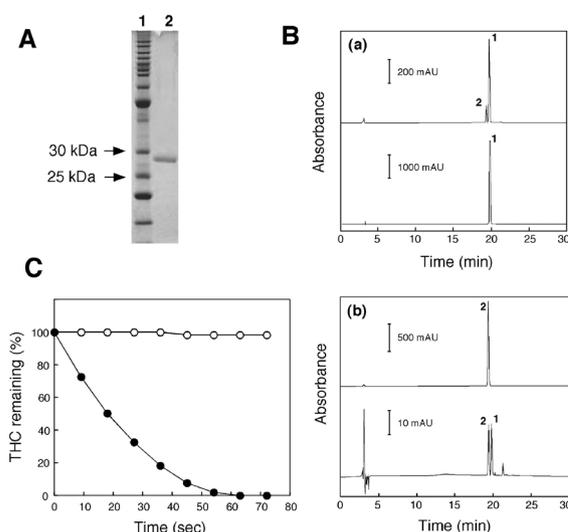


Figure 3. Analysis of the reaction as catalyzed by purified rAmCHI1. (A) SDS-PAGE of purified rAmCHI1. The purified enzyme (lane 2) was simultaneously subjected to electrophoresis with standard proteins (lane 1). Molecular sizes are indicated by arrows. (B) Reversed-phase HPLC analysis of the isomerization of THC (peak 1) to naringenin (peak 2). Panel (a), control reaction mixture with heat-inactivated enzyme. Naringenin was also found in the control reaction because of a spontaneous cyclization of the substrate. Panel (b), reaction mixture with rAmCHI1. Upper and lower chromatograms in each panel are those obtained with detection at 290 nm and 370 nm, respectively. (C) Course of isomerization of THC to naringenin. The reaction was monitored spectrophotometrically at an absorbance of 360 nm at 4°C. Closed circles, progress curve with rAmCHI1; open circles, control progress curve with heat-inactivated enzyme. For experimental details, see Materials and methods.

Table 1. Kinetic parameters of rAmCHI1.

Substrate	k_{cat} [s ⁻¹]	K_m [μM]	k_{cat}/K_m [s ⁻¹ ·μM ⁻¹]
THC	1.7	7.0	0.25
PHC	0.16	2.3	0.071

Kinetic parameters for the isomerization of chalcones to naringenins, as catalyzed by rAmCHI1, were determined at pH 7.5 and 4°C by Method I as described under Materials and methods. Values are expressed as the mean for three measurements.

Transcription and flavonoid analyses

The temporal expressions of *AmCHI1* and *AmCIFP4* in the snapdragon petals of cv. Floral Showers of different colors (yellow, red, and white) were analyzed by means of quantitative real-time RT-PCR (Figure 4). The results showed that the expression levels of *AmCHI1* were highest in the red petals, followed by yellow and white petals. In red petals, an appreciable level of the *AmCHI1* transcript was found from the beginning of flower development (stage 1), showing the highest level at stage 2, followed by a gradual diminution from stages 4 through 6 (Figure 4, left panel (b)). In the yellow and white petals, the *AmCHI1* transcript was also found from stage 1, the most abundant at stage 3, and then diminished to a very low level at stage 6 (Figure 4, left panels (a, c)). The expression levels of *AmCIFP4* in the red, yellow, and white petals were approximately 10-fold

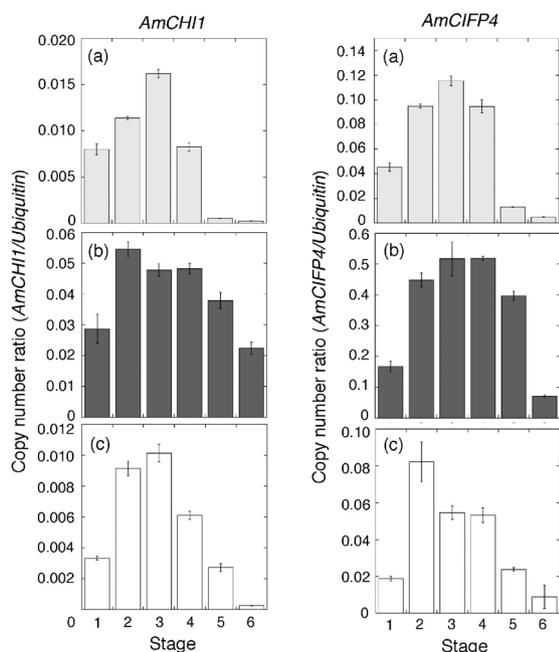


Figure 4. Changes of expression levels of *AmCHI1* (left panels) and *AmCIFP4* (right panels) in petals of different colors during the flower development. Panels (a), cv. Floral Shower Yellow; panels (b), cv. Floral Shower Red; panels (c), Floral Shower White. Transcription levels of *AmCIFPs* and *ubiquitin* genes in snapdragon petals were determined by means of quantitative real time PCR. The ratios of copy numbers of *AmCIFPs* to copy numbers of *ubiquitin* were monitored during the development of flowers of different colors.

higher than the *AmCHI1* expression levels in the petals of corresponding colors, and showed the highest levels at stages 2–4 (Figure 4, right panels).

We also analyzed the temporal expressions of the following flavonoid biosynthetic genes in yellow petals (cv. Yellow Butterfly) (Figure 5B) and made comparisons with those of *AmCHI1* and *AmCIFP4* in the same petals (Figure 5A): chalcone synthase (*AmCHS1*, also termed *Nivea* (Hatayama et al. 2006)), type-II flavone synthase (*AmFNS1* (Akashi et al. 1999)), aurone biosynthetic enzymes (*AmC4'GT* (Ono et al. 2006a), and *AmAS1* (Nakayama et al. 2000)). Temporal expression patterns of *AmCHI1* and *AmCIFP4* in this commercial variety were similar to those observed with cv. Floral Shower Yellow (Figure 4) although copy number ratios (*GOI/ubiquitin*) were lower. The expression levels of *AmCHS1* were greater than those of *AmCHI1* and *AmCIFP4*, showing the highest levels in stage 5 (Figure 5B, left upper panel). *AmFNS1* was expressed from the beginning of flower development (stage 1), showing the highest level in stage 4 (Figure 5B, left lower panel). The temporal expression patterns of the aurone biosynthetic genes (*AmC4'GT* and *AmAS1*) were different from those of *AmCHS1*, *AmCHI1*, and *AmFNS1*. Namely, the transcript of *AmC4'GT* was essentially absent in stages 1–2 and remarkable increases in the transcription levels of both of the *AmC4'GT* and *AmAS1* genes were observed after stage 5 (Figure 5B,

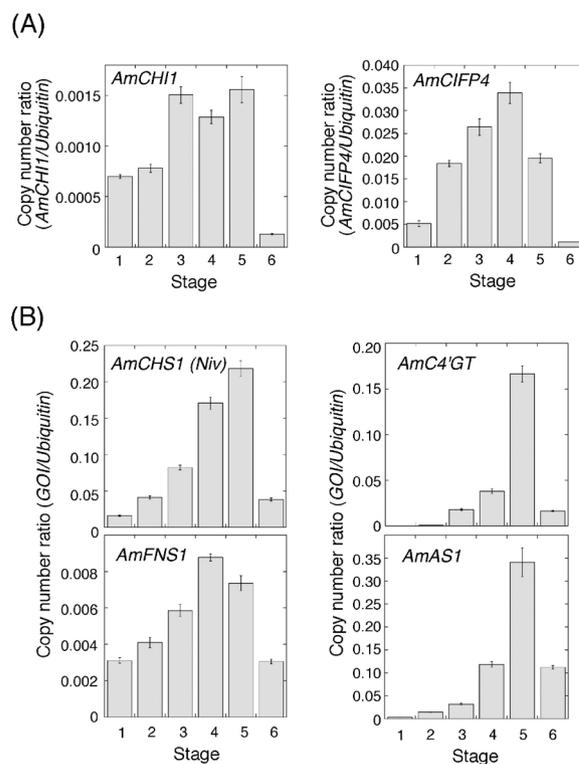


Figure 5. Changes of transcription levels of (A) *AmCIFP1/AmCHI1* (left panel), *AmCIFP4* (right panel) and (B) other flavonoid biosynthetic genes (*AmCHS1*, *AmFNS1*, *AmC4'GT*, *AmAS1*) during the development of yellow snapdragon petals (cv. Yellow Butterfly). Genes of interest (*GOI*) and *ubiquitin* genes in yellow petals were determined by means of quantitative real time RT-PCR. The ratios, copy numbers of *GOI* to copy numbers of *ubiquitin*, were monitored during the development of flowers. The average values of three or four independent determinations of the ratios are presented with error bars indicating \pm S.D.s. For a definition of the developmental stages of snapdragon flowers, see Materials and methods.

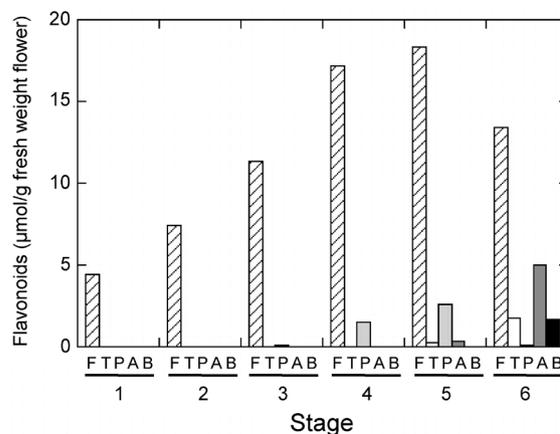


Figure 6. Changes in the accumulation levels of flavonoids in yellow snapdragon petals (cv. Yellow Butterfly) during flower development. Flavonoids are: apigenin glucuronides (F), hatching bars; THC-G (T), white bars; PHC-G (P), light gray bars; Aur-G (A), dark gray bars; and Brc-G (B), black bars.

right panels).

Temporal changes in the flavonoid contents in the yellow petals (cv. Yellow Butterfly) were then analyzed to examine their consistency with the observed expression patterns of these flavonoid biosynthetic genes (Figure 6). The results showed that glycosides (mainly glucuronides) of apigenin abundantly occurred from the beginning of flower development (i.e. stage 1) and the highest level of its accumulation was found in stages 4–5. By contrast, Aur-G, Brc-G, and THC-G were absent in stages 1–3, and the amounts of these flavonoids reached the highest levels in stage 6. Overall, the temporal accumulation patterns of flavones were correlated with the temporal expression patterns of *AmCHI1* and *AmFNS1*. The expression of aurone biosynthetic genes (*AmC4'GT* and *AmAS1*) preceded the accumulation of Aur-G and Brc-G.

Discussion

AmC1FP1/AmCHI1

The deduced amino acid sequence of *AmCHI1* displayed a 56% identity to that of *MsCHI*, a type-II CHI, which has been subjected to intense structural and mechanistic studies (Hur and Bruice 2003; Hur et al. 2004; Jez et al. 2000; Jez et al. 2002; Jez and Noel 2002; Ruiz-Pernia et al. 2008). Those studies revealed the importance of hydrogen bonds in the active site of *MsCHI* for catalysis, which involved Thr48, Tyr106, Asn113 and Thr190 as key residues (Figure 7). These key residues were also conserved in the primary structure of *AmCHI1* (Figure 7), although the position corresponding to the Thr190 of *MsCHI* was occupied by serine residue in the primary structures of *AmCHI* and other type-I enzymes. These observations implied a common reaction mechanism for flavanone formation as catalyzed by type-I and type-II CHI enzymes.

In the present study, we showed that the cDNAs of *AmC1FP1/AmCHI1* were commonly expressed

irrespective of flower color, but with temporal expression patterns varying somewhat (Figure 4). Moreover, a comparison among the temporal expression patterns of *AmCHI1* and other flavonoid biosynthetic genes as well as the temporal accumulation patterns of flavonoids (flavones, aurones, and chalcones) in yellow petals (cv. Yellow Butterfly; Figures 5 and 6) provided important information relative to the functional significance of *AmCHI1* in the regulation of flavonoid biosyntheses in the flower (Figure 1), as follows. Appreciable levels of transcripts of *AmCHI1* and *AmFNS1* occurred in the yellow petals from the beginning of flower development (stage 1), consistent with the temporal accumulation patterns of flavones (apigenin glucuronides, Figure 6), which already occurred in a large amount in stage 1. By contrast, in this yellow variety, the transcription levels of aurone biosynthetic genes (*AmC4'GT* and *AmAS1*) were very low in early stages (i.e., stages 1 and 2) and the highest in stage 5 while the *AmCHI1* transcription level was the lowest in stage 6. Aurones were completely absent during stages 1–4 and begin to accumulate only after stage 5 (Figure 6). These observations suggested that *AmCHI1* should be coordinately expressed with flavone biosynthesis during early stages of flower development and diminution of its expression levels in late stages should at least partly play a role in switching the flavonoid pathways (Figure 1) from flavone biosynthesis (stages 1–4) to aurone biosynthesis (stages 5–6). It would be also noteworthy here that a small amount of PHC-G accumulates in stages 4–5 and subsequently diminished to a negligible level with concomitant formation of Aur-G and Brc-G in stage 6 (Figure 6). Because *AmC4'GT* and *AmAS1* were expressed in stages 5 and 6 (see Figure 5), these results implied that some factors other than these biosynthetic enzymes might also critically govern the production of aurones in vacuoles.

The results of previous chemogenetic analyses of the snapdragon flavonoids would also be worth

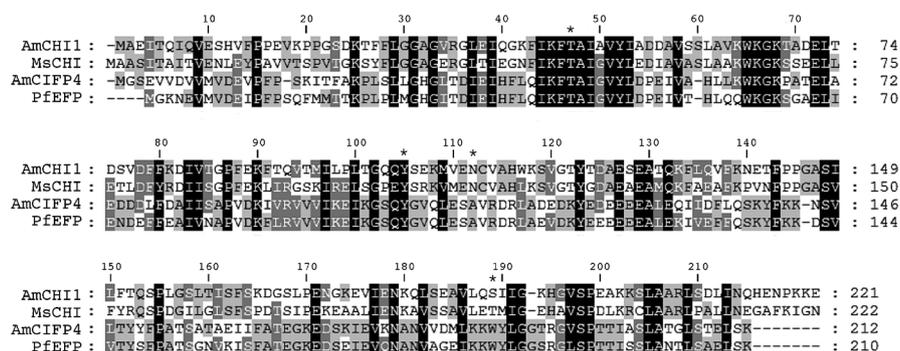


Figure 7. Alignment of the deduced amino acid sequences of *AmCHI1*, *MsCHI*, *AmC1FP4*, and *PhEFP*. Amino acid numbering of the *AmCHI1* sequence is shown above the alignment. Similar amino acid residues among 4 and 3 sequences are shown with black and dark gray backgrounds, respectively, and those between 2 sequences are shown with a light gray background. The locations of the amino acid residues that are involved in the active-site hydrogen-bond network in the crystal structure of *MsCHI* complexed with naringenin (Jez et al. 2000; Jez et al. 2002; Jez Noel 2002) are indicated by asterisks above the alignment.

discussing in conjunction with substrate specificity of AmCHI1. The glycosides of flavones, flavonols, and anthocyanidins with the 3',4'-hydroxy B ring (i.e., the glycosides of luteolin, quercetin, and cyanidin; Figure 1) accumulated only in the presence of the dominant allele *Eosinea* (*Eos*) encoding flavonoid 3'-hydroxylase (F3'H, EC 1.14.13.21), while the glycosides of the 4'-hydroxy flavonoids (i.e., apigenin, kaempferol, and pelargonidin; Figure 1) accumulated in the petals with the *eos eos* genotype (Forkmann and Stotz 1981). PHC-G occurred in the petals of snapdragons with the *eos eos* genotype containing apigenin glycosides (Gilbert 1973; Sato et al. 2001; see also Figure 6 for other example); PHC did not serve as a precursor of the flavonoids with a 3',4'-dihydroxy B ring. Specificity analysis of rAmCHI1, however, showed that the enzyme was capable of acting on PHC to produce eriodictyol, which can potentially be metabolized to produce flavonoids with a 3',4'-dihydroxy B ring. Thus, in snapdragon petal cells, PHC has to be functionally separated from AmCHI1 to escape the action of the enzyme. In this context, it is very important to note that THC-G and PHC-G were inert as substrates for rAmCHI1. The inability of CHI to act on these chalcone glucosides could be structurally rationalized by the crystal structure of MsCHI complexed with naringenin, which shows that the 7-hydroxy group of the bound substrate (corresponding to 4'-hydroxyl group of chalcones) is hydrogen bonded with side chains of Thr190 and Asn113 in the substrate pocket of MsCHI (Jez et al. 2000)—the 4'-*O*-glucosyl group of THC-G and PHC-G should hamper the binding of these substrates to rAmCHI1. Thus, the inability of this enzyme to act on chalcone 4'-*O*-glucosides should provide one of the key mechanisms that allow chalcones to escape from the action of AmCHI1. Namely, once PHC is produced in the cytoplasm, it is immediately glucosylated by the action of AmC4'GT, which is a cytoplasmic enzyme (Ono et al. 2006a; Ono et al. 2006b). AmCHI1 cannot act on the resultant PHC-G, which is removed from the cytoplasmic environment by translocation to the vacuole lumen, allowing its exclusive utilization as a substrate for aureusidin synthase (Nakayama et al. 2000; Ono et al. 2006a; Ono et al. 2006b).

AmC1FP4

Sequence comparison showed that two catalytic residues of MsCHI (Asn113 and Thr190) are not conserved in the primary structures of AmC1FP4 and other type-4 CHI proteins and are replaced by alanine and tryptophan, respectively (Figure 7). Previous mutational studies of MsCHI showed that replacement of Asn113 and Thr190 by alanine resulted in 6- to 7-fold reductions in k_{cat} , 2- to 3-fold increases in K_{m} , and 14- to 20-fold reductions in the $k_{\text{cat}}/K_{\text{m}}$ values of the enzyme, as assayed using

4,2',4'-trihydroxychalcone as a substrate. Although we could not examine the ability of AmC1FP4 to catalyze the CHI reaction in the present work, these observations suggest that AmC1FP4 might show only a very low CHI activity, if any, hence any catalytic role of this protein as a CHI in flavonoid biosynthesis in snapdragons would be unlikely. Consistently, it was shown that the CHI mutants of *Ipomoea nil* were devoid of anthocyanins although these mutants likely expressed *C1FP4* (Iida et al. 2004).

In a recent study, the red color of a morning glory flower (i.e., production of anthocyanins) was reduced by the suppression of a gene coding for a homolog of cluster-4 C1FP (termed here C1FP4) (Morita et al. 2014). This observation was further confirmed with other flowers such as petunias (PhEFP; see Figure 2) and torenias (ThEFPa and ThEFPb) (Morita et al. 2014), suggesting that C1FP4 proteins may generally play a role in enhancing anthocyanin (or flavonoid) production, although mechanistic aspects of these observations remain to be clarified. Thus, these C1FP4 proteins have been referred to as the “enhancers of flavonoid production (EFPs).” In snapdragon flowers, *AmC1FP4* was more abundantly expressed than *AmCHI1* irrespective of flower color (Figure 4 and Figure 5A). It would be tempting to speculate that AmC1FP4 might also serve as an EFP in the flower coloration of snapdragons, and this possibility should be examined in future studies.

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