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Original Paper

Anthocyanin acyltransferase engineered for the synthesis of a novel polyacylated anthocyanin

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Abstract Some polyacylated anthocyanins, anthocyanins containing two or more aromatic acyl groups, are blue in color within a wide range of pH values, including acidic and neutral conditions even in the absence of any co-pigments or metal ions. We engineered a mutant of a malonyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase of *Salvia splendens* (Ss5MaT1), Ss5AT306, which has an acquired specificity for hydroxycinnamoyl-CoA and is able to produce novel polyacylated anthocyanins. Ss5AT306 showed 6‴-*O*-hydroxycinnamoyltransferase activity toward some anthocyanins in addition to the 6‴-*O*-malonyltransferase activity retained (relative activities for acyl transfer to shisonin: malonyl-CoA, 100%; *p*-coumaroyl-CoA, 132%; caffeoyl-CoA, 103%). This alteration of acyl-donor specificity was achieved by the substitutions of only three contiguous amino acid residues, Val39-Arg40-Arg41, to the corresponding residues of anthocyanin aromatic acyltransferases, Met-Leu-Gln, suggesting that these amino acid residues are key residues governing the malonyl-CoA specificity of Ss5MaT1. Through the use of Ss5AT306, a novel polyacylated anthocyanin, *p*-coumaroylshisonin, with one aromatic acyl group in each of the 3-*O*-glucosyl and 5-*O*-glucosyl moieties of its structure, was produced and characterized *in vitro*. *p*-Coumaroylshisonin was bluer in color and displayed stronger color intensity than did shisonin, implying that accumulation of polyacylated anthocyanins such as *p*-coumaroylshisonin cause the modulation of flower colors.

Key words: Acyltransferase, malonyltransferase, polyacylated anthocyanin, *Salvia splendens*.

Some polyacylated anthocyanins, anthocyanins containing two or more aromatic acyl moieties in their structures, are bluer in color and more stable in a wide variety of physiological conditions than are non-polyacylated anthocyanins. This paper describes an anthocyanin acyltransferase engineered for the production of novel polyacylated anthocyanins and spectral properties of the produced anthocyanin.

Anthocyanins make up a colored class of flavonoids conferring intense orange to blue colors on flower petals. Genetic engineering of the anthocyanin biosynthetic pathway for the modulation of flower colors has attracted much attention during the past decade (Forkmann and Martens 2001; Fukusaki et al. 2004; Ono et al. 2006). Although anthocyanin colors depend on various factors, including pigment structure, environmental pH, co-pigmentation, and/or complexation with metal ions, the aglycon part of an anthocyanin is essentially orange to reddish-purple under acidic conditions of the vacuoles, in which anthocyanins accumulate (Heller and Forkmann 1994). The most common shift of the pigment color to blue under acidic conditions is ascribed to co-pigmentation with colorless phenolics such as flavones and flavonols (Harborne and Williams 2000). However, some polyacylated anthocyanins having two or more aromatic acyl substituents are more stable and bluer in color within a wide range of pH values, including acidic and neutral conditions, even in the absence of co-pigments or metal ions. Examples are gentiodelphin from the gentian (*Gentiana makinoi*), ternatins from the butterfly pea (*Clitoria ternatea*), heavenly blue anthocyanin from the morning glory (*Ipomoea tricolor*), and cinerarin from the cineraria (*Senecio cruentus*) (Goto and Kondo 1991). Their stable blue coloration has

Abbreviations: 3MaT, malonyl-CoA:anthocyanin 3-*O*-glucoside-6″-*O*-malonyltransferase; *AOX1*, alcohol oxidase 1 gene of *Pichia pastoris*; BAHD, benzylalcohol acetyl-, anthocyanin-*O*-hydroxycinnamoyl-, anthranilate-N-hydroxycinnamoyl/benzoyl-, and deacetylvindoline acetyltransferase; Dm3MaT3, malonyl-CoA:anthocyanin 3-*O*-glucoside-6″-*O*-malonyltransferase 3 of *Dendranthema* x *morifolium*; FAB-MS, fast atom bombardment-mass spectrum; Gt5AT, hydroxycinnamoyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-acyltransferase of *G. triflora*; *his*4, histidinol dehydrogenase 4 gene of *Pichia pastoris*; HMBC, ^{1}H-detected multiple-bond connectivity; HPLC, high-performance liquid chromatography; Pf3AT, hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucoside-6″-*O*-acyltransferase of *Perilla frutescens*; Ss5MaT1, malonyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase of *Salvia splendens*; TFA, trifluoroacetic acid; UV-VIS, UV-visible absorption.
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been attributed to intramolecular stacking between the anthocyanin chromophore and the aromatic acyl moiety(s) (Harborne and Williams 2000). Heterologous production of these polyacylated anthocyanins by genetic engineering has the potential to generate transgenic flowers showing novel colors, particularly bluer colors (Tanaka et al. 1998).

The aromatic acyl substituent is generally attached to a hydroxyl group of a glycosyl moiety of an anthocyanin with an ester bond formed by the action of anthocyanin aromatic acyltransferases. Although the biosynthesis of some polyacylated anthocyanins has been well characterized (Fukuchi-Mizutani et al. 2005; Kogawa et al. 2007; Morita et al. 2005; Suzuki et al. 2003), there have been only a few reports regarding the genes encoding anthocyanin aromatic acyltransferases (Fujiwara et al. 1998; Yonekura-Sakakibara et al. 2000; Yoshihara et al. 2006). Hydroxycinnamoyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-acyltransferase of a gentian (*Gentiana triflora*), Gt5AT, the first anthocyanin aromatic acyltransferase to be identified (Fujiwara et al. 1998), is an enzyme that is able to catalyze 6‴-*O*-acylation of anthocyanin **1** (Figure 1). Hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucoside-6″-*O*-acyltransferase of perilla red form (*Perilla frutescens*), Pf3AT, catalyzes 6″-*O*-acylation of anthocyanin **1** (Figure 1) (Yonekura-Sakakibara et al. 2000). Note that Gt5AT is unable to catalyze 6‴-*O*-acylation of anthocyanin **2** (Fujiwara et al. 1998), which are the reaction products derived from anthocyanin **1** by Pf3AT. Basically, polyacylated anthocyanin **5** is not formed by the cooperative actions of Gt5AT and Pf3AT.

The aliphatic acyl group, in addition to the aromatic acyl group, is one of the major acyl substituents in anthocyanins. We have studied plant acyltransferases responsible for the malonylation of flavonoid glucosides, including anthocyanins (Nakayama et al. 2003), and have established that the malonyltransferases, along with Gt5AT and Pf3AT, are members of the BAHD superfamily (St-Pierre and De Luca 2000). The BAHD family members are acyl-CoA-dependent acyltransferases sharing two highly conserved motifs, HXXXD and DFGWG, and are involved in the secondary metabolism of plants and fungi (Nakayama et al. 2003; D'Auria 2006). Malonyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase of scarlet sage (*Salvia splendens*), Ss5MaT1, one of the best-characterized anthocyanin malonyltransferases (Suzuki et al. 2001; Suzuki et al. 2003), catalyzes the specific 6‴-*O*-malonylation of anthocyanin **2** to produce anthocyanin **3** (Figure 1). Neither hydroxycinnamoyl-CoA nor anthocyanin **1** serves as a substrate, in contrast to Gt5AT. In this study, Ss5MaT1 was engineered to an enzyme that can catalyze the 6‴-*O*-hydroxycinnamoylation of anthocyanin **2** by the alteration of acyl-donor specificity from malonyl-CoA specificity to hydroxycinnamoyl-CoA specificity. This enzyme, termed Ss5AT306, was capable of producing a novel polyacylated anthocyanin which was bluer in color and displayed stronger color intensity than did the substrate anthocyanin.

Materials and methods

Chemicals, strains, plasmids, and media

Anthocyanins were kindly provided by Prof. Masa-atsu Yamaguchi (Minami-Kyusyu University, Miyazaki, Japan). *p*-Coumaroyl-CoA and caffeoyl-CoA were kindly provided by Dr. Yoshikazu Tanaka (Suntory Research Center, Osaka, Japan). Other commercially available chemicals were of analytical grade. *Escherichia coli* strains XL1-Blue (Stratagene) and TOP10 (Invitrogen) and plasmids pCR4Blunt-TOPO (Invitrogen), pHIL-D2 (Invitrogen), and pPIC3.5K (Invitrogen) were used for DNA manipulation. *E. coli* JM109, pQE-30 (Qiagen), and *Pichia pastoris* GS115 (Invitrogen) were used for protein production. *P. pastoris* strains were grown at 30°C in MGY medium (1.34% yeast nitrogen base, 0.00004% biotin, and 1% glycerol) or MM medium (1.34% yeast nitrogen base, 0.00004% biotin, and 0.5% methanol).

Construction of plasmids

The plasmid pSs5MaT1 that contains the sequence encoding MRGSHHHHHHGSIEGR-Ss5MaT1 downstream of the *T5* promoter on pQE-30 was constructed in the previous study (Suzuki et al. 2001). For preparation of the Ss5AT306 protein, a derivative of Ss5MaT1 with triple substitutions, Val39Met-Arg40Leu-Arg41Gln, the plasmid pSs5AT306 containing the sequence encoding MRGSHHHHHHGSIEGR-Ss5AT306 downstream of the T5 promoter on pQE-30, was generated from pSs5MaT1 by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the following primer: 5′-CCACTACCACCCC*ATGCTCCAG*CTCCTCTTCTACC-3′ (with the mutation codons shown in italics).

The nucleotide sequence encoding MRGSHHHHHH-GSIEGR-Ss5AT306 on pSs5AT306 was amplified by PCR using the following primers: 5′-GAATTCACT*ATG*AGAGGA-TCG-3′ (with the start codon shown in italics and an EcoRI site shown by underlining) and 5′-GGATCC*TTA*CAATGGTTC-GACGAGCGCCGGAGA-3′ (with the stop codon shown in italics). The amplified fragment was cloned into pCR4Blunt-TOPO and sequenced to confirm the absence of PCR-generated errors. From the plasmid, the sequence containing *Ss5AT306* was excised with EcoRI and cloned in EcoRI sites of pHIL-D2 and pPIC3.5K in the correct orientation, resulting in pHIL306 and pPIK306, respectively. These plasmids contain an expression cassette sequence consisting of the 5′-region of an alcohol oxidase gene of *P. pastoris* (*AOX1*), an *AOX1* promoter that is inducible by methanol, a gene of histidine-tagged Ss5AT306, and a histidinol dehydrogenase gene (*his*4) as a selection marker, followed by the 3′-region of *AOX1*.

Enzyme preparation

Ss5AT306 proteins were produced in cells of *E. coli* JM109 harboring p*Ss5AT306*, extracted from the cells, and purified to apparent homogeneity by column chromatographies using

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Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) and High-Q (Bio-Rad), followed by Phenyl-Sepharose (Amersham), as described previously (Suzuki et al. 2001).

Ss5AT306 proteins were also prepared from *P. pastoris*. A 6.8-kb NotI fragment containing the expression cassette derived from pHIL306 was introduced into *P. pastoris* GS115, which had the histidine auxotrophic genotype (*his4**) by electroporation. A transformant was isolated growing on histidine-deficient medium as a result of double crossover between the expression cassette and the chromosomal *AOX1* gene. pPIK306 linearized by SalI digestion was introduced into the resulting transformant by electroporation. This fragment would be integrated into the chromosomal *his4* and/or *his4** loci by a single crossover(s). The plasmid pPIK306 carries a kanamycin resistance gene conferring G418 resistance to *Pichia* cells. A G418 resistance transformant was isolated. The level of G418 resistance roughly depends on the number of kanamycin resistance genes integrated. The transformant grew on a medium containing 4 mg ml^{-1} G418, implying that it has 7 to 12 copies of kanamycin resistance genes per cell, together with 8 to 13 copies of the expression cassette, one of which is in the *AOX1* locus.

The transformant cells of *P. pastoris* were cultured aerobically in 12 l of MGY medium until OD_{600} reached 2–5. Then they were centrifuged, resuspended in 2 l of MM medium, and further cultured for 60 h according to the manufacturer's guidelines. Ss5AT306 proteins were extracted from the harvested cells by vigorous shaking with glass beads using a Multi-Beads Shocker apparatus, model MBS200 (Yasui Kikai Co., Osaka), and purified by essentially the same method as that described for Ss5AT306 preparation from *E. coli* cells.

Enzyme assay

The standard reaction mixture (100 μl) consisted of 20 mM potassium phosphate (pH 7.0), 60 μM acyl-CoA, 120 μM anthocyanin, and enzyme. After incubation at 30°C for 20 min, the reaction was stopped by adding 200 μl of ice-cold 0.5% trifluoroacetic acid (TFA) in water. The reaction products were analyzed by reversed-phase high-performance liquid chromatography (HPLC) using the Dynamax system (Rainin Instruments) equipped with a UV-visible absorption (UV-VIS) detector model SPD-10A *VP* (Shimadzu). HPLC conditions for the analysis of malonylshisonin and bisdemalonylsalvianin (see Figure 1 for their structures) were as described previously (Suzuki et al. 2001). HPLC conditions for the analysis of anthocyanins having two hydroxycinnamoyl substituents were as follows: column, *J'*sphere ODS-M80 (4.6×150 mm, YMC); column temperature, 20°C; detection, 520 nm; flow rate, 0.7 ml min^{-1}. After 50 μl of the reaction mixture had been injected into a column equilibrated with 18% acetonitrile in water containing 0.1% TFA, the column was initially developed isocratically for 3 min, followed by development by a linear gradient from 18% to 90% acetonitrile in water containing 0.1% TFA for 15 min.

Preparation of a novel polyacylated anthocyanin

The reaction mixture (55 ml) consisted of 20 mM potassium phosphate (pH 7.0), 23 mg *p*-coumaroyl-CoA, 23 mg shisonin (see Figure 1 for its structure), and 3 mg Ss5AT306. After incubation at 30°C for 24 h, the reaction was stopped by the addition of TFA to a final concentration of 0.5%. The reaction product was purified by a preparative HPLC and dried. The HPLC conditions were as follows: column, YMC-Pack ODS-A (10×250 mm, YMC); column temperature, 20°C; detection, 520 nm; flow rate, 3.0 ml min^{-1}. After 1 ml of the reaction mixture had been injected into a column equilibrated with 27% acetonitrile in water containing 0.1% TFA, the column was initially developed isocratically for 3 min, followed by development by a linear gradient from 27% to 45% acetonitrile in water containing 0.1% TFA for 5 min. The anthocyanin was dissolved in methanol containing 0.01% TFA.

NMR and MS analyses

^{1}H-, ^{13}C-NMR, and ^{1}H-detected multiple-bond connectivity (HMBC) spectra were obtained in 10% (v/v) TFA_d-CD_3OD on a DMX 750 spectrometer (Bruker BioSpin, Tsukuba, Japan). Fast atom bombardment-mass spectrum (FAB-MS) was measured on a JMS-700 MStation (JEOL, Tokyo, Japan) in the positive-ion mode.

Results and discussion

Functional alteration of Ss5MaT1

We previously reported the acyl-donor specificity of an anthocyanin malonyltransferase of *S. splendens*, Ss5MaT1 (Suzuki et al. 2001), an enzyme which catalyzes the malonyl transfer reaction from malonyl-CoA to shisonin, yielding malonylshisonin (Figure 1). Acetyl-CoA is an extremely poor acyl donor, as are many other flavonoid glucoside-specific malonyltransferases (Figure 2; see the Figure 2 legend for details). Because the acetyl group is smaller than the malonyl group due to the absence of the terminal carboxylic group, it is unlikely that the inability of acetyl-CoA to serve as a substrate for these malonyltransferases is caused by a steric hindrance between the enzyme molecule and acetyl-CoA. Consequently, we assumed that the malonyl transfer catalysis involved an electrostatic interaction(s) between the negatively charged carboxylate group of a malonyl group of malonyl-CoA and a positively charged side chain of a basic amino acid residue(s) comprising the acyl-donor binding pocket of the enzyme. This idea led to the hypothesis that the substitution of the basic amino acid residue(s) of Ss5MaT1 to the corresponding amino acid residue(s) of aromatic acyltransferases causes an alteration of malonyl-CoA specificity to hydroxycinnamoyl-CoA specificity in Ss5MaT1.

In this study, we focused on Arg40 and Arg41 as the probable candidates for the basic amino acid residue of Ss5MaT1 based on a sequence comparison between flavonoid glucoside-specific malonyltransferases and anthocyanin aromatic acyltransferases (Figure 2, left). One or two arginine residue(s) were found in the region corresponding to Arg40-Arg41 of Ss5MaT1

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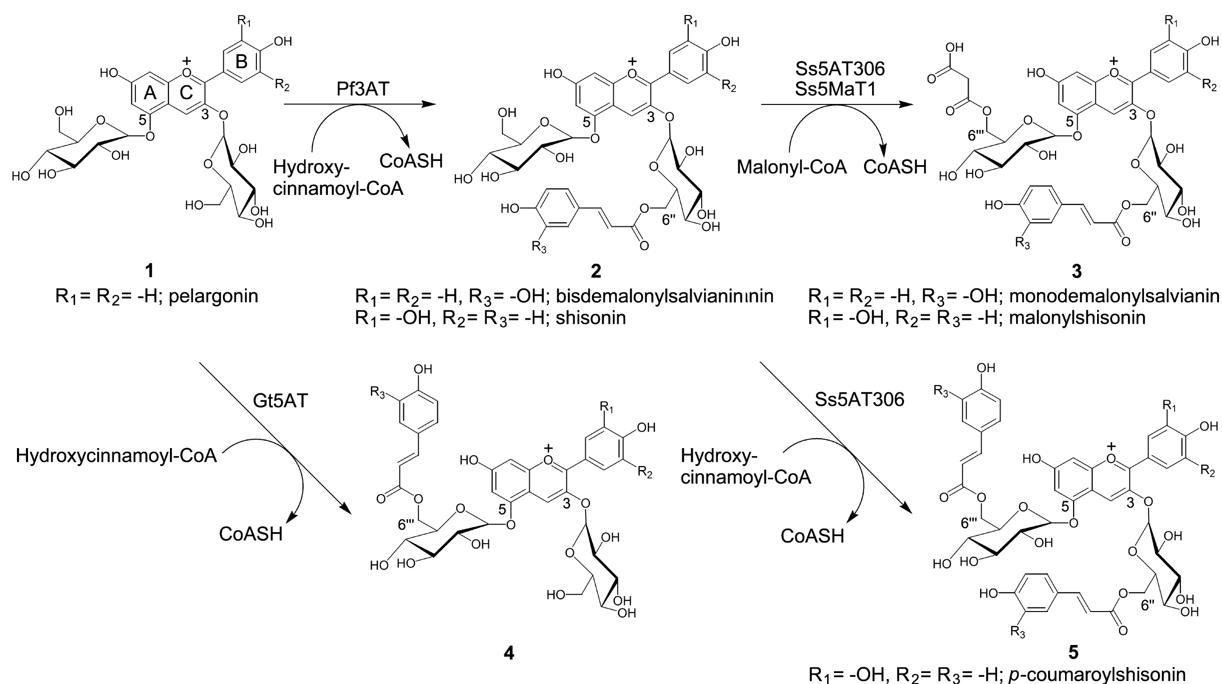

Figure 1. Reactions catalyzed by anthocyanin acyltransferases. Ss5AT306 was generated in this study from an anthocyanin malonyltransferase, Ss5MaT1 (Suzuki et al. 2001). Pf3AT (Yonekura-Sakakibara et al. 2000) and Gt5AT (Fujiwara et al. 1998) are anthocyanin aromatic acyltransferases. Anthocyanins used in this study are represented by their names. Gt5AT is able to catalyze acylations of anthocyanin **1** but not **2**, while Ss5MaT1 and *Ss5AT306* catalyze acylations of anthocyanin **2** but not **1**. The structures of anthocyanins are shown as their flavylium forms. A, B, and C in anthocyanin **1** indicate A-, B-, and C-rings in anthocaynin structures, respectively.

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                                  **                 *
Ss5MaT1(AF405707)     36: YHPVRRLLFY    171: LGDARSIVGFI
Pf5MaT(AF45707)       33: FHPLRRLLFY    169: LGDARSVVGFV
Dv3MaT(AF4892028)     39: FPPVHHLYFY    175: LSDANTRFGFL
Sc3MaT(AY190121)      39: FPPVHHLFFY    177: LGDASTRFNFL
Dm3MaT1(AY298809)     39: FPPVHHVFFY    177: LGDASTRLGFL
Dm3MaT2(AY298810)     39: FPPVHHVFFY    178: VADASTRLGFL
Dm3MaT3(AB290338)     39: SPPINNLFFY    172: LGDASTRFCFL
Lp3MaT1(AY500352)     42: FHPIKRLLFY    178: AGDASSIVGFI
Vh3MaT1(AY500350)     42: FHPIQRLLFY    178: VGDASSIVGFI
Vh3MaT2(AY500351)     46: FHPTQRLLFY    181: VGDASSVVGFI
GmIF7MaT(AB291058)    38: LPPVERLFFY    168: ALDGKSSTLFM
Gt5AT(AB010708)       35: LNKMQSLLFY    171: IADAKSFVMFI
Pf3AT(AB029340)       31: FHPMLQLLFY    164: VSDAPSFLAFI
Ss3AT(AY395719)       35: FHPMLQLIFY    168: VSDAPSFLSFL
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Figure 2. The alignment of amino acid sequences of flavonoid glucoside-specific acyltransferases focusing on Arg40 and Arg41 of Ss5MaT1 (left, *) and that focusing on Arg178 of Dm3MaT3 (right, *). The enzymes used for the alignment are as follows: anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferases, Ss5MaT1 (Suzuki et al. 2001) and Pf5MaT1; anthocyanin 3-*O*-glucoside-6″-*O*-malonyltransferases, Dv3MaT (Suzuki et al. 2002), Sc3MaT (Suzuki et al. 2003), Dm3MaT1 (Suzuki et al. 2004b), Dm3MaT2 (Suzuki et al. 2004b), and Dm3MaT3 (Unno et al. 2007); flavonol 3-*O*-glucoside-6″-*O*-malonyltransferases, Lp3MaT1 (Suzuki et al. 2004a) and Vh3MaT1 (Suzuki et al. 2004a); phenolic glucoside malonyltransferases, Vh3MaT2; isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase, GmIF7MaT (Suzuki et al. 2007); anthocyanin aromatic acyltransferases, Gt5AT (Fujiwara et al. 1998), Pf3AT (Yonekura-Sakakibara et al. 2000), and Ss3AT. The DDBJ/GenBank accession numbers of these enzymes are indicated in parentheses.

among some malonyltransferases but not among aromatic acyltransferases. Malonyl-CoA:anthocyanin 3-*O*-glucoside-6″-*O*-malonyltransferases (3MaTs; i.e., Dv3MaT, Sc3MaT, Dm3MaT1, and Dm3MaT2 in Figure 2) share two histidine residues in the corresponding region. Dm3MaT3 also displays 3MaT activity, the level of which, however, is much lower than those of other malonyltransferases (about 100-fold diminution in k_{cat} values) (Unno et al. 2007). The corresponding region of Dm3MaT3 consists of two asparagines, which are neutral amino acids. The amino acid sequence flanking Val39-Arg40-Arg41 of Ss5MaT1, -HPXXXLLFY-, was identical to that of Pf3AT but not of Gt5AT (Figure 2, left), suggesting that simple substitutions in the sequence of the three contiguous amino acid residues, Val39-Arg40-Arg41, potentially alter the acyl-donor specificity of Ss5MaT1. Thus, Val39-Arg40-Arg41 of Ss5MaT1 were substituted to the amino acid residues, Met-Leu-Gln, corresponding to those of Pf3AT. The resulting mutant is here termed Ss5AT306.

Ss5AT306 was prepared as a histidine-tagged protein from *E. coli* JM109 harboring pSs5AT306. The purified protein was incubated at 30°C for 20 min with *p*-coumaroyl-CoA and shisonin. The reaction products were analyzed by HPLC. As expected, Ss5AT306 showed 6‴-*O*-*p*-coumaroyl transfer activity to shisonin, yielding the polyacylated anthocyanin tentatively named *p*-coumaroylshisonin (see below), in addition to the retained 6‴-*O*-malonyl transfer activity (relative activities: malonyl-CoA, 100%; *p*-coumaroyl-CoA, 132%). Caffeoyl-CoA also served as an acyl donor (relative activity, 103%). These aromatic acyl transfer

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Table 1. Kinetic parameters of acyl transfer activities to shisonin by Ss5AT306 and Ss5MaT1

	k_{cat} ($s^{-1}\times10^{3}$)	K_m for acyl donor (μM)	K_m for acyl acceptor (μM)
Ss5AT306[a]			
Malonyl-CoA	110±11	7.6±1.1	22±4
p-Coumaroyl-CoA	82±11	19±3	37±7
Caffeoyl-CoA	38±7	14±4	37±7
Ss5MaT1[b]			
Malonyl-CoA	870±50	22±3	36±5
p-Coumaroyl-CoA	<1	N. D.[c]	N. D.
Caffeoyl-CoA	<1	N. D.	N. D.

[a] The reactions were carried out at 30°C in a mixture of 20 mM potassium phosphate (pH 7.0), 5–15 μM of acyl-CoA, 10–30 μM of shisonin, and 0.28 μg (59 nM) of enzyme. The kinetic parameters were determined by non-linear least squares of the initial reaction velocities fitting on the equation described previously (Unno et al. 2007; Segel 1975).

[b] These parameters are cited from the previous report (Suzuki et al. 2001).

[c] N. D., not determined.

activities have never been observed in Ss5MaT1 (Table 1). The aromatic and aliphatic acyl donors served as substrates even when bisdemalonylsalvianin (see Figure 1 for its structure) was used as an acyl acceptor (relative activities: malonyl-CoA, 40%; *p*-coumaroyl-CoA, 30%; caffeoyl-CoA, 15%). Ss5AT306 could not act on pelargonin (see Figure 1 for its structure), as in the case of Ss5MaT1. Thus, Ss5MaT1 was successfully engineered to Ss5AT306 with an acquired specificity for hydroxycinnamoyl-CoA. This specificity makes it possible to produce novel anthocyanin **5** from anthocyanin **2**, which can be produced by hydroxycinnamoylation of anthocyanin **1** with Pf3AT (see Figure 1). Even *in planta*, anthocyanin **5** should be produced from anthocyanin **2** by Ss5AT306, in addition to anthocyanin **3**, when hydroxycinnamoyl-CoA exists abundantly as malonyl-CoA in the cells because the k_{cat}/K_m values for hydroxycinnamoyl-CoA of Ss5AT306 (4,300 $s^{-1} M^{-1}$ for *p*-coumaroyl-CoA; 2,700 $s^{-1} M^{-1}$ for caffeoyl-CoA) are comparable to that for malonyl-CoA (14,000 $s^{-1} M^{-1}$).

Mutated sites that affect acyl-donor specificity—A structural consideration

Are the Arg40-Arg41 residues of Ss5MaT1 involved in direct binding with malonyl-CoA? We recently determined the crystal structure of Dm3MaT3 complexed with malonyl-CoA, the first structure of anthocyanin acyltransferases (Unno et al. 2007). This structure revealed an electrostatic interaction between a negatively charged carboxylate group in malonyl-CoA and a positively charged side chain of Arg178 residue of Dm3MaT3. No residue other than Arg178 appeared to be involved in the interaction between the malonyl group and Dm3MaT3. The three contiguous residues of Dm3MaT3, Ile42-Asn43-Asn44, which correspond to Val39-Arg40-Arg41 of Ss5MaT1, constituted a portion of the binding pocket of the acyl acceptor rather than that of the acyl donor, indicating that these three residues of 3MaTs are not involved in malonyl-CoA binding. This result implies that the alteration of the acyl-donor specificity of Ss5MaT1 might arise from conformational changes around the acyl-donor binding site by substitutions of Val39-Arg40-Arg41; however, the possibility of a direct (or indirect) involvement of Arg40-Arg41 in the binding of malonyl-CoA cannot be disregarded because Arg178 is conserved exclusively in 3MaTs but not in other malonyltransferases, including Ss5MaT1 (Figure 2, right), and because the BAHD family of enzymes has a great diversity of substrate binding sites (Unno et al. 2007). For clarification, structural information on Ss5MaT1 and/or Ss5AT306 is needed. Incidentally, anthocyanin aromatic acyltransferases shared phenylalanine at the residue corresponding to Arg178 of Dm3MaT3 (Figure 2, right), suggesting that phenylalanine is a key residue that governs the hydroxycinnamoyl-CoA preference of anthocyanin aromatic acyltransferases (Unno et al. 2007). Provided that the region around Ile175 of Ss5MaT1, which corresponds to the Arg178 residue of Dm3MaT3, constitutes the binding pocket for the acyl donor in Ss5MaT1, substitutions of this region of Ss5AT306 to that of anthocyanin aromatic acyltransferases may cause further alterations of acyl-donor specificity toward hydroxycinnamoyl-CoA preference.

p-Coumaroylshisonin as a novel polyacylated anthocyanin

About 5 mg of *p*-coumaroylshisonin was produced by using Ss5MaT306 prepared from *P. pastoris* transformant cells. ^{1}H NMR analysis of the product showed downfield shifts by 0.45–0.46 ppm of resonance of the 6‴-hydrogens of shisonin. HMBC cross peaks were also observed between the *C*-1 carbonyl carbon of the *p*-coumaroyl group and 6‴-hydrogens. FAB-MS analysis showed molecular ions at *m*/*z* 903.12 $[M]^+$ consistent with those of shisonin esterified with one *p*-coumaric acid ($C_{45}H_{43}O_{20}$). We therefore concluded that the product, *p*-coumaroylshisonin, is cyanidin 3-*O*-(6″-*O*-*p*-coumaroylglucoside)-5-*O*-(6‴-*O*-*p*-coumaroyl-glucoside) (see Figure 1 for its structure). To our knowledge, anthocyanins containing aromatic acyl groups in both of the 3-*O*-glucosyl and 5-*O*-glucosyl moieties, including *p*-coumaroylshisonin, have never been found in nature.

Spectral properties of p-coumaroylshisonin

We compared the spectral properties of *p*-coumaroylshisonin with those of shisonin. The UV-VIS

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spectra of *p*-coumaroylshisonin in water containing 0.5% TFA are shown in Figure 3A, upper panel. The absorption maximum, λ_{max}, in the visible region was at 522 nm, which was 4 nm red-shifted from that of shisonin. A similar red shift of λ_{max} was observed in 6″-*O*-caffeoylation of delphinidin 3,5-*O*-diglucoside (Yonekura-Sakakibara et al. 2000). The molar absorption coefficient at 522 nm was comparable to that at 518 nm of shisonin (27,000 $M^{-1} cm^{-1}$), while the absorption between 250 and 350 nm, including absorption by the *p*-coumaroyl group, was twice as high as that of shisonin. We also determined UV-VIS spectra in buffers (0.1 M sodium phosphate and 0.1 M sodium citrate) adjusted to pH 4.0–9.0. The UV-VIS spectra at pH 5.0 (Figure 3A, middle panel) and pH 7.0 (Figure 3A, lower panel) were shown in Figure 3A. Unfortunately, the UV-VIS spectra showed a single absorption peak in the visible region, with no other absorption peak in the visible and near-infrared regions, unlike the case of gentiodelphin (Yoshida et al. 2000). Yoshida and coworkers (Yoshida et al. 2000) suggested that the caffeoyl group on the glucose attached to the B-ring should make a more important contribution than the 6‴-*O*-caffeoyl group to establish the stable blue color of gentiodelphin by the formation of face-to-face intramolecular stacking. However, the λ_{max} values of *p*-coumaroylshisonin were 3–13 nm (mean, 5.0 nm) longer than those of shisonin, depending on the pH values (4.0–9.0), while the pK_a values estimated from plots of λ_{max} versus pH (data not shown) were almost the same in *p*-coumaroylshisonin (7.56±0.03) and shisonin (7.54±0.04). Moreover, *p*-coumaroylshisonin exhibited a much higher absorption coefficient at λ_{max} than did shisonin in a wide pH range; the differences in the absorption coefficients in both anthocyanins increased as the buffer pH was increased (Figure 3B). Anthocyanins are generally a 7-quinonoidal or 4-quinonoidal base form having a hydrophobic chromophore at neutral pH and are a cationic flavylium form in acidic conditions (Goto and Kondo 1991). The enhancement of the absorption coefficient by increasing the pH value and the higher absorption coefficient of *p*-coumaroylshisonin can be explained by the intermolecular stacking of anthocyanins by hydrophobic interaction, which should take place more efficiently among *p*-coumaroylshisonin than among shisonin. These results imply that the accumulation of polyacylated anthocyanins such as *p*-coumaroylshisonin in large amounts causes the modulation of flower colors with or without co-pigmentation.

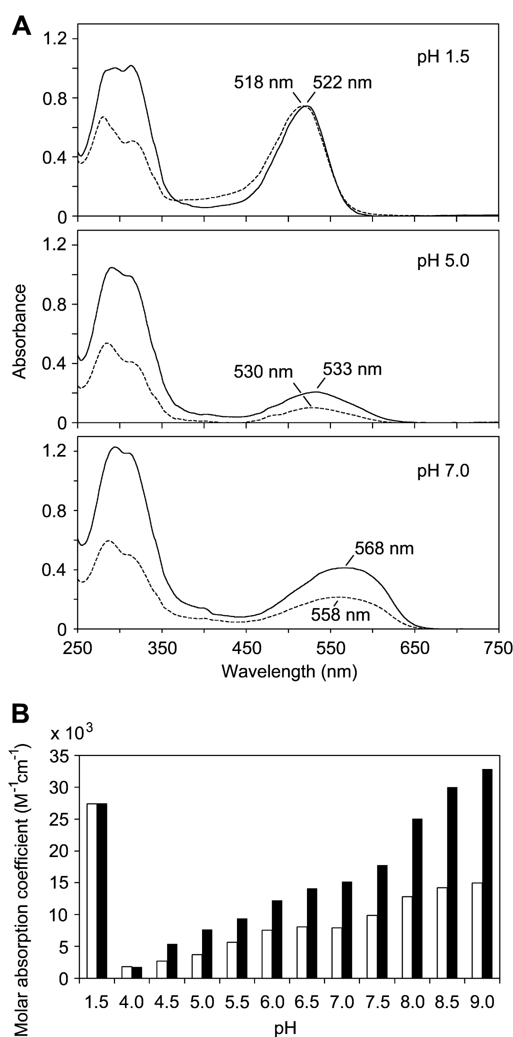

Figure 3. Comparison of spectral characteristics of *p*-coumaroylshisonin and shisonin. (A) UV-VIS spectra in water containing 0.5% TFA at pH 1.5 (upper panel) and in buffers at pH 5.0 (middle panel) and pH 7.0 (lower panel). The buffers consisted of 0.1 M sodium phosphate and 0.1 M sodium citrate. Anthocyanins, 26 μM; solid line, *p*-coumaroylshisonin; dashed line, shisonin. (B) The pH-dependent changes of molar absorption coefficients at the absorption maximum in the visible region. Black bars, *p*-coumaroylshisonin; white bars, shisonin.

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