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Identification of a cDNA Encoding Malonyl-Coenzyme A: Anthocyanidin 3-*O*-Glucoside 6"-*O*-Malonyltransferase from Cineraria (*Senecio cruentus*) Flowers

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Abstract

"Cinerarin" is a polyacylated anthocyanin that is responsible for the blue coloration of cineraria (*Senecio cruentus*) flowers. We isolated a full-length cDNA (*Sc3MaT*) encoding a putative anthocyanin acyltransferase from *S. cruentus*. The *Sc3MaT* cDNA was expressed in *Escherichia coli* cells and the expression product was purified to homogeneity and functionally characterized. The Sc3MaT could catalyze the regiospecific malonyl transfer from malonyl-CoA (K_m, 61 μM) to pelargonidin 3-*O*-glucoside (K_m, 11 μM) to produce pelargonidin 3-*O*-6"-*O*-malonylglucoside with a k_{cat} value of 8.8 s^{-1}. The specificities for acyl donors and acceptors were highly restricted to malonyl-CoA and anthocyanidin 3-*O*-glucoside, respectively. Therefore, it may be concluded that Sc3MaT is a malonyl-CoA:anthocyanidin 3-*O*-glucoside-6"-*O*-malonyltransferase. The other enzymatic properties of Sc3MaT were comparable with those of known anthocyanin acyltransferases. Because a reaction product of Sc3MaT, delphinidin 3-*O*-6"-*O*-malonyglucoside, constitutes a part of cinerarin, Sc3MaT is probably involved in the cinerarin biosynthesis in this plant.

Accession number: AY190121.

Key words: anthocyanin, cinerarin, malonyltransferase, *Senecio cruentus*.

Abbreviations

Gt5AT, hydroxycinnamoyl-CoA:anthocyanin 5-*O*-glucoside-6"-*O*-acyltransferase from *Gentiana triflora*; Pf3AT, hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucoside-6"-*O*-acyltransferase from *Perilla frutescens*; Pf5MaT, malonyl-CoA:anthocyanin 5-*O*-glucoside-6"'-*O*-malonyltransferase from *P. frutescens*; Ss5MaT1, malonyl-CoA:anthocyanin 5-*O*-glucoside-6'"-*O*-malonyltransferase from *Salvia splendens*.

Introduction

Orange to blue coloration of flowers in nature is, in most cases, provided by anthocyanins (Brouillard and Dangles, 1994; Strack and Wray, 1994). The structures of anthocyanidins, the chromophore part of anthocyanins, greatly depend on pHs, which, therefore, significantly affect the coloration and stability of the pigments in aqueous systems. At the pHs of vacuoles (pH 4-6, depending on petal cells) in which the pigments generally occur, anthocyanins would not be expected to exist stably in their colored forms, especially in blue, unless there were some mechanism for the maintenance of the color structure, such as, for example, aliphatic and aromatic acylations of pigments, co-pigmentation, and metal complexation (Goto and Kondo, 1991; Brouillard and Dangles, 1994; Yabuya *et al.*, 2000).

In the blue flowers of cineraria (*Senecio cruentus*), the coloration is predominantly provided by polyacylated anthocyanin, called "cinerarin" (**Fig. 1**) (Goto *et al.*, 1984). It is quite interesting that

cinerarin shows a stable blue color in *in vitro* aqueous systems in a wide pH range (pH 3.5-7) without supplementary factors including co-pigments, metal ions, and elevated pHs. It has been proposed that such stable blue coloration of cinerarin occurs as a result of the specific conformation of the pigments in aqueous systems provided by the intramolecular $\pi-\pi$ stacking (Goto and Kondo, 1991) of caffeyl groups. Thus, metabolic engineering of flavonoid biosynthesis leading to the accumulation of cinerarin in flowers may be a simple and effective way to engineer blue flowers (Tanaka *et al.*, 1998). In the biosynthesis of cinerarin, the pathway leading to the delphinidin 3-*O*-glucoside portion of this pigment is well established and conserved among plant species (Heller and Forkmann, 1994). However, the identities of enzymes involved in the subsequent modifications of the "delphinidin 3-*O*-glucoside" portion of the pigment, such as aliphatic and aromatic acyltransferases and glucosyltransferases, have never been identified in this plant species.

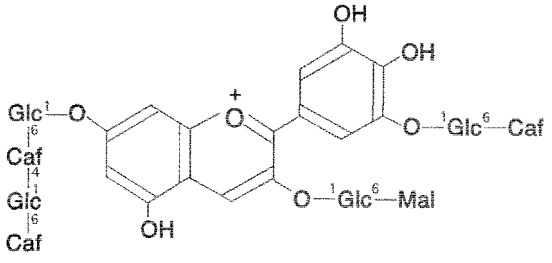


Fig. 1 The structure of cinerarin, the major anthocyanin in blue flowers of *S. cruentus*. Glc, β-D-glucosyl group; Caf, caffeyl group; Mal, malonyl group.

During the course of studies on enzymes involved in the late stage of the cinerarin biosynthesis, there was interest in the published sequence of a partial cDNA with no established biochemical function from *S. cruentus* (GenBank accession number E12757), which had previously been obtained on the basis of sequence similarity to known anthocyanin acyltransferases (Fujiwara *et al.*, 1998). The deduced amino acid sequence of this partial cDNA, called *ScAT48*, contains the motifs His-Xaa_3-Asp (motif 1, **Fig. 2**) and Asp-Phe-Gly-Trp-Gly (motif 3), which are conserved among the members of the versatile plant acyltransferase family, a large family of acyltransferases with diverse biochemical functions in the secondary metabolisms in plants (Suzuki *et al.*, 2001). A signature sequence of anthocyanin acyltransferases, Tyr-Phe-Gly-Asn-Cys (motif 2; Suzuki *et al.*, 2001), is also identified in the deduced amino acid sequence of this partial cDNA. These results suggest that ScAT48 may be an anthocyanin acyltransferase involved in the cinerarin biosynthesis.

Here, the isolation of the full-length *ScAT48* cDNA (termed *Sc3MaT*) is described, in addition to its heterologous expression in *Escherichia coli* cells and functional characterization of the expression product, to establish that Sc3MaT is a malonyl-CoA:anthocyanidin 3-*O*-glucoside 6"-*O*-malonyltransferase. Because this enzyme produces delphinidin 3-*O*-6"-*O*-malonylglucoside from delphinidin 3-*O*-glucoside, it should be involved in the biosynthesis of cinerarin in the flowers of *S. cruentus*.

		Motif 1		Motif 2		Motif 3
Sc3MaT	173	NHHSLGDAS	323	TAYFGNCG	399	DFDFGWGKP
Ss5MaT1	165	NHHCLGDAR	310	ENYFGNCI	388	KADFGWGKA
Pf5MaT	163	NHHCLGDAR	309	ANYFGNCI	387	NADFGWGKA
Pf3AT		NHHTVSDAP		ENYFGNCL		GADFGWGKA
Gt5AT	172	AHHSIADAK	331	PNYFGNCL	409	GVDFGWGKP
BEAT	150	FNHIIGDMF	291	NDVSGNFF	375	EVDFGWGIP
DAT	156	ISHKVADGG	209	QNSVGNLV	378	DVDFGWGKP
TcTAT	162	FHHGVCDGA	296	SGYYGNSI	371	EVDFGWGNA
HCBT	162	QHHHACDGM	307	KGYCGNVV	391	AMDFGWGSP

Fig. 2 Alignment of amino acid sequences of motifs 1, 2, and 3 (boxed) of the plant versatile acyltransferase family enzymes. Enzymes used for alignment are Sc3MaT (this study), Ss5MaT1 (Genbank accession number, AF405707); Pf3AT, hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucoside-6"-*O*-acyltransferase of *P. frutescens* (BAA93475); Gt5AT, hydroxycinnamoyl-CoA:anthocyanin 5-*O*-glucoside-6"-*O*-acyltransferase of *Gentiana triflora* (BAA74428); BEAT, acetyl CoA: benzylalcohol acetyltransferase of *Clarkia breweri* (AAC18062); DAT, deacetylvindoline 4-*O*-acetyltransferase of *Catharanthus roseus* (AAC99311); TcTAT, taxadienol acetyltransferase of *Taxus cuspidata* (AAF34254); HCBT, hydroxycinnamoyl/benzyl-CoA:anthranilate *N*-hydroxycinnamoyl/benzoyltransferase of *Dianthus caryophyllus* (CAB06430).

Materials and Methods

Plant materials and anthocyanins

Blue flowers of *S. cruentus* were purchased from a local market. Petals of the flowers were isolated and stored at −80 ℃ until use. Anthocyanins were isolated and purified as described previously (Suzuki *et al.*, 2001) and used as authentic samples after confirmation of their structures by instrumental analysis.

Cloning of Sc3MaT cDNA

A cDNA library was constructed with poly(A)$^+$ RNA from *S. cruentus* petals using the λZAPII-cDNA synthesis kit (Stratagene; Heidelberg, Germany) according to the manufacturer's instructions. The cDNA library was then used as a template for PCR amplification using two *ScAT48*-specific primers (5'-GGCTCGGGTATATCACTAGGAATG-3' and 5'-ACCAGCAACACCAATCTTCCTAGC-3') synthesized on the basis of the *ScAT48* sequence (GenBank accession number E12757). The PCR amplification was carried out for 30 cycles with one cycle comprising 95 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 1 min. The amplified DNA fragment (690 bp) was cloned into TOPO-pCR2.1 (Invitrogen; Carlsbad, CA, U.S.A.) and confirmed as the partial cDNA of *ScAT48* by sequencing using a Dye-Terminator Cycle Sequence kit (Beckman Coulter, Fullerton, CA, U.S.A.) with a CEQ 2000 DNA analysis system (Beckman Coulter). Using this partial cDNA as a template, a DIG-labeled *ScAT48*-specific probe was synthesized with a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel, Switzerland).

The cDNA library was screened by plaque hybridization with the DIG-labeled *ScAT48*-specific probe. Plaque lifts were taken onto Hybond-N (Amersham Biosciences; Piscataway, NJ, U.S.A.). The hybridization was performed at 37 ℃ for 16 h in 30% (v/v) formamide, 5x SSC, 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine, 2% (w/v) blocking reagent (Roche Diagnostics), and 100 ng ml^{-1} DIG-labeled probe. The membranes were washed twice in 0.1x SSC and 0.1% (w/v) SDS at 55 ℃ for 15 min. DNAs were detected using a DIG DNA Labeling and Detection Kit (Roche Diagnostics). The cDNAs were rescued in pBluescript SK(−) vector following the *in vivo* excision protocol for λZAP and sequenced. We designated the full-length *ScAT48* cDNA as *Sc3MaT* on the basis of its catalytic function clarified in this study and its nucleotide sequence has been submitted to the *Genbank*™*/DDBJ* with accession number AY190121.

Heterologous expression of Sc3MaT

To facilitate cloning into the vector pQE-30 (QIAGEN; Hilden, Germany), two restriction sites were introduced to a full-length *Sc3MaT* cDNA by PCR using a forward primer (5'-GAGCTCatgGATTCCATTCCTTG-3') containing a *Sac*I site (underlined) upstream of the initiation codon (lowercase), a reverse primer (5'-GGTACCttaCAATGAAACTTGAC-3') containing a *Kpn*I site (underlined) downstream of the stop codon (lowercase), and pBluescript containing full-length *Sc3MaT* cDNA as a template. The amplified DNA fragment digested with *Sac*I and *Kpn*I was cloned into *Sac*I and *Kpn*I sites in pQE-30, and the inserted DNA sequence was confirmed to be identical with the ORF sequence of *Sc3MaT* by sequencing. The resulting construct was transformed into *E. coli* JM109 cells. The *E. coli* cells were grown at 30 ℃ in an LB medium supplemented with 50 μg ml^{-1} ampicillin until the absorbance at 600 nm reached about 0.5, and then expression of Sc3MaT was induced by addition of isopropyl β-thiogalactopyranoside to a final concentration of 0.5 mM. The cells were further cultivated for 4 h and collected by centrifugation. The recombinant Sc3MaT was purified to apparent homogeneity, as judged by sodium dodecyl sulfate polyacrylamide gel-electrophoresis analysis (Laemmli *et al.*, 1970), from crude extracts of the transformant cells by a combination of the Ni-NTA agarose (QIAGEN), High-Q (Bio-Rad, CA, U.S.A.), and Phenyl Superose HR 5/5 (Amersham Biosciences) chromatographies, as described previously (Suzuki *et al.*, 2001). The proteins on the gels were visualized by Coomassie Brilliant Blue.

Anthocyanin acyltransferase assay

The standard reaction mixture (final volume, 100 μl) consisted of 20 mM potassium phosphate, pH 7.0, 120 μM anthocyanin substrate, 60 μM malonyl-CoA (final concentration), and enzyme. For routine assays, pelargonidin 3-*O*-glucoside was used as an anthocyanin substrate. The mixture without enzyme was preincubated at 30 ℃, and the reaction was started by the addition of the enzyme. After incubation at 30 ℃ for 20 min, the reaction was stopped by the addition of 200 μl of ice-cold 0.5% (v/v) trifluoroacetic acid. Anthocyanins in the reaction mixture were routinely analyzed by reversed-phase HPLC using a RAININ Dynamax HPLC system (Rainin Instruments Co., Woburn, MA, U.S.A.) equipped with a SHIMADZU SPD-10A VP UV-VIS detector: column, Shodex Asahipak ODP-50 4E (4.6 mm x 250 mm; Shoko, Tokyo); flow rate, 0.7 ml min^{-1}; solvent A, 0.5% v/v trifluoroacetic acid; solvent B, 0.5% (v/v) trifluoroacetic

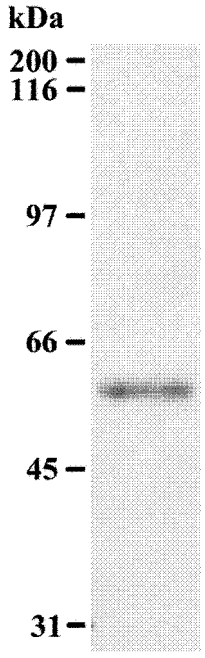
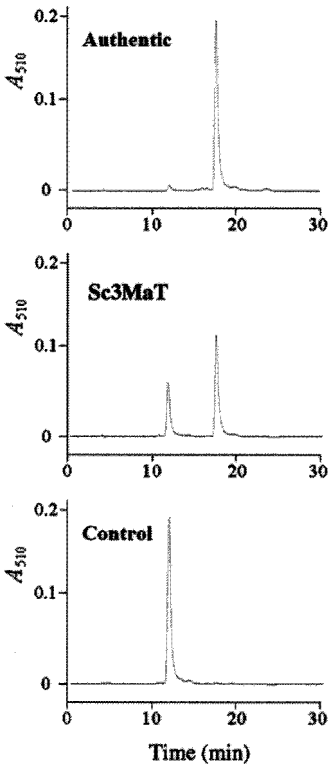


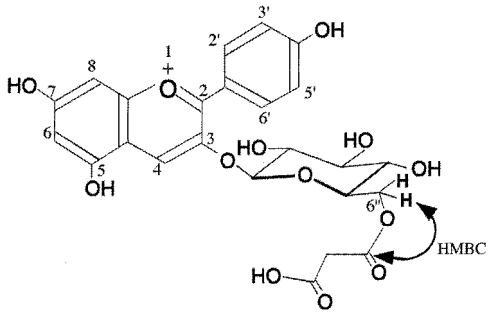
Fig. 3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of Sc3MaT purified from the *E. coli* transformant cells. The scale indicates molecular sizes (in kDa) of marker proteins.

acid in 50% v/v acetonitrile. After 100 μl of the reaction mixture was injected into the column that was equilibrated with 45% B, the column was initially developed isocratically for 3 min, followed by linear gradients from 45% B to 55% B in 15 min and from 55% B to 100% B in 1 min. The column was then washed isocratically with 100% B for 5 min, followed by a linear gradient from 100% B to 45% B in 1 min. The chromatograms were obtained with detection at 510 nm, and anthocyanins were identified by comparing their retention times with those of authentic samples as described previously (Suzuki *et al.*, 2001).

(A)



(B)



Proton		δ (^{1}H)
Aglycone	4	9.00 s
	6	6.92 d (5.0)
	8	6.70 d (1.5)
	2'	8.59 dd (5, 9.4)
	3'	7.06 dd (5.0, 10.0)
	5'	7.06 dd (5.0, 10.0)
	6'	8.59 dd (5.0, 9.4)
3-Glucoside	1"	5.25 d (7.9, 9.7)
	2"	3.74 dd (7.9, 9.2)
	3"	3.59 t (9.1)
	4"	3.48 t (9.3)
	5"	3.80 m
	6"	4.43 dd (7.4, 12.0); 4.55 dd (2, 12)

Fig. 4 Identification of the reaction product of Sc3MaT-catalyzed malonyl transfer to pelargonidin 3-*O*-glucoside. (A) Identification by HPLC. *Top panel*, HPLC profile of the authentic pelargonidin 3-*O*-6"-*O*-malonylglucoside; *middle panel*, HPLC profile of a reaction mixture of enzymatic malonyl transfer containing pelargonidin 3-*O*-glucoside, malonyl-CoA, and recombinant Sc3MaT; *bottom panel*, HPLC profile of a control reaction mixture where the recombinant Sc3MaT was omitted. For details of the reaction mixtures and HPLC conditions, see Materials and Methods. (B) Structure and NMR assignments of the product (pelargonidin 3-*O*-6"-*O*-malonylglucoside). ^{1}H- and ^{13}C-NMR, and HMBC spectra were measured with a Bruker DMX-500 system at 500 MHz in 10% CF_3COOD/CD_3OD at 25 ℃, where D denotes deuterium atom. The residual proton peak of deuterated solvent (δ 3.3) was used as an internal standard. A bent double-headed arrow indicates HMBC cross peaks between C1 of the malonyl group (δ 169) and 6''' hydrogen atoms of 3 glucosyl moiety (δ 4 43 and 4 55)

Results and Discussion

The cDNA library of 20,000 recombinants was stringently screened with the *ScAT48*-specific probe to obtain 7 positive clones. The cDNAs were rescued in pBluescript SK(-) from these clones and sequenced. Two full-length cDNAs encoding *ScAT48* were obtained, and it was revealed that the published *ScAT48* sequence lacked the 5'-terminal portion by 19 bp. The full-length cDNA, which we re-designated *Sc3MaT* on the basis of its specificity (see below), encoded a protein of 461 amino acids and had a calculated molecular mass of 51,320 Da. The identities of the total amino acid sequence of Sc3MaT to those of anthocyanin acyltransferases were 39% to Ss5MaT1 (Suzuki et al, 2001), 35% to Gt5AT (Fujiwara *et al.*, 1998), and 33% to Pf3AT (Yonekura-Sakakibara *et al.*, 2000).

Sc3MaT was expressed under the control of T5 promoter in *E. coli* JM109 cells as an in-frame *N*-terminal fusion with a His_6 tag. It was a soluble, catalytically active protein with an estimated molecular mass of 51 kDa and was purified to homogeneity with an activity yield of 4% (**Fig. 3**). The reaction of the recombinant enzyme with pelargonidin 3-*O*-glucoside and malonyl-CoA yielded a single reaction product, which was predicted to be a monomalonylated form of pelargonidin 3-*O*-glucoside on the basis of HPLC (**Fig. 4A**) and MS analyses (*m/z* 519[M]$^+$). NMR analyses of the product (**Fig. 4B**) showed downfield shifts (by 0.5-0.7 ppm) of resonance of 6"-hydrogens of 3-glucosyl moiety. ^{1}H-detected multiple-bond connectivity (HMBC) cross peaks were also observed between the *C*-1 carbonyl carbon of malonyl group and 6"-hydrogens, indicating that it was pelargonidin 3-*O*-6"-*O*-malonylglucoside. Therefore, the purified enzyme catalyzed the regiospecific transfer of the malonyl group to the 6"-hydroxyl group of the 3-glucosyl moiety of pelargonidin 3-*O*-glucoside (**Fig. 5**). The acyl donor and acceptor specificities of the Sc3MaT-catalyzed acyl transfer (**Table 1**) have been further analyzed. In addition to pelargonidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside could also serve as good malonyl acceptors; however, pelargonidin 3,5-*O*-diglucoside and quercetin 3-*O*-glucoside could not serve as substrates. For acyl donors, the enzyme showed the highest activity with malonyl-CoA. Methylmalonyl-CoA and succinyl-

Table 1 Substrate specificity of Sc3MaT

Substrate	Relative activity (%)[1]
Acyl Acceptor[2]	
Pelargonidin 3-*O*-glucoside	100
Cyanidin 3-*O*-glucoside	110
Delphinidin 3-*O*-glucoside	153
Pelargonidin 3,5-*O*-diglucoside	ND
Quercetin 3-*O*-glucoside	ND
Acyl donor[3]	
Malonyl-CoA	100
Methylmalonyl-CoA	15
Succinyl-CoA	20
Acetyl-CoA	ND
4-Coumaroyl-CoA	ND

[1] For the relative activities of acyl acceptors (final concentration, 120 μM) and donors (60 μM), the specific activity with malonyl-CoA and pelargonidin 3-*O*-glucoside was taken to be 100%. Assay conditions were described under "Materials and Methods".

[2] The reactions were performed with malonyl-CoA as an acyl donor.

[3] The reactions were performed with pelargonidin 3-*O*-glucoside as an acyl acceptor.

ND, Below detection limit (less than 0.5%).

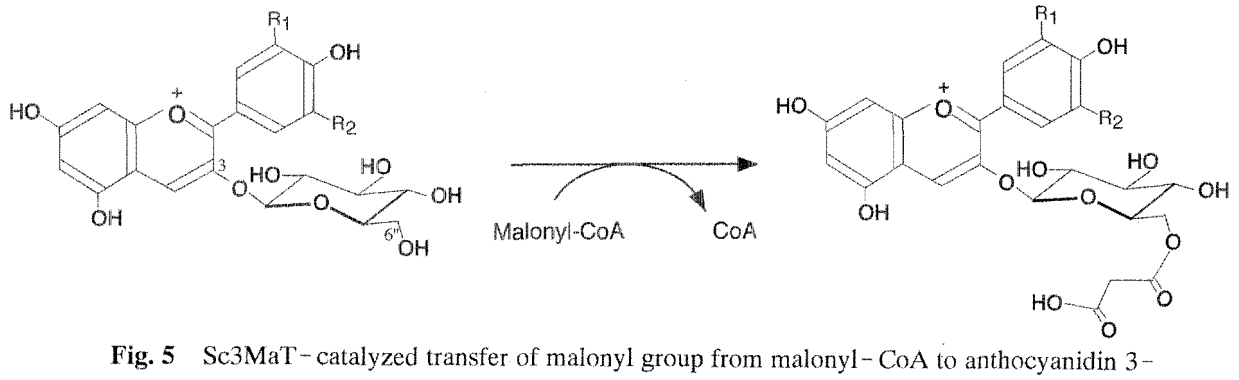


Fig. 5 Sc3MaT-catalyzed transfer of malonyl group from malonyl-CoA to anthocyanidin 3-*O*-glucoside. Malonyl group is specifically transferred to 6"-hydroxyl group of the anthocyanin. Aglycons are pelargonidin (R_1 = H, R_2 = H), cyanidin (R_1 = OH, R_2 = H), and delphinidin (R_1 = OH, R_2 = OH)

CoA could also serve as weak substrates, whereas acetyl-CoA and 4-coumaroyl-CoA were inert as acyl donors. Thus, the acyl donor and acceptor specificities were highly restricted to anthocyanidin 3-*O*-glucosides and malonyl-CoA, respectivly. These results led to the conclusion that Sc3MaT is a malonyl-CoA:anthocyanidin 3-*O*-glucoside-6"-*O*-malonyltransferase (**Fig. 5**). The kinetic parameters for the malonyl transfer to pelargonidin 3-*O*-glucoside under the standard assay conditions were as follows: k_{cat}, 8.8 s^{-1}; K_m for malonyl-CoA, 61 μM; and K_m for pelargonidin 3-*O*-glucoside, 11 μM. These values were comparable with those previously reported for known anthocyanin acyltransferases (Fujiwara *et al.*, 1998; Yonekura-Sakakibara *et al.*, 2000; Suzuki *et al.*, 2001).

The optimum pH for the enzymatic malonyl transfer to pelargonidin 3-*O*-glucoside was 7.5. The enzyme was stable between pHs 5.5-7.5 (at 20 ℃ for 20 h) and below 30 ℃ (at pH 7.0 for 20 min). The enzyme activity was completely abolished after incubation of the enzyme with 5 mM *N*-ethylmaleimide, 0.1 mM $CuCl_2$, or 0.1 mM $HgCl_2$ at 20 ℃ for 20 min and was partially inhibited by 0.1 mM $CdCl_2$ (residual activity, 52%), 0.1 mM $MgCl_2$ (48%), 0.1 mM $ZnCl_2$ (34%), 0.1 mM acetyl-CoA (37%), and 0.1 mM coenzyme A (33%). Other metal ions (Ca^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Sn^{2+}; 0.1 mM as chloride) and EDTA (0.1 mM) had negligible effects on the enzyme activity. These enzymatic properties were also closely related to those of known anthocyanin acyltransferases (Fujiwara *et al.*, 1998; Yonekura-Sakakibara *et al.*, 2000; Suzuki *et al.*, 2001). Because the reaction product, delphinidin 3-*O*-6"-*O*-malonylglucoside, is a part of cinerarin (**Fig. 1**) and should serve as a precursor for cinerarin biosynthesis, it is highly likely that Sc3MaT is involved in the biosynthesis of cinerarin in *S. cruentus*. Sc3MaT may serve as a tool for the metabolic engineering of flavonoid biosynthesis leading to the accumulation of cinerarin in flowers, which results in the coloration of blue flowers.

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