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Journal of Molecular Catalysis B: Enzymatic 23 (2003) 117–132
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Review

Anthocyanin acyltransferases: specificities, mechanism, phylogenetics, and applications

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Received 6 February 2003; accepted 19 March 2003

Dedicated to Professor Kenji Soda in the honor of his 70th birthday

Abstract

Anthocyanins are responsible for the orange to blue coloration of flowers, fruits, and leaves. They are beneficial to human health and widely used as food colorants. Anthocyanin acyltransferases (AATs) are the plant enzymes that catalyze the regiospecific acyl transfer from acyl-CoA to the sugar moiety of anthocyanins. AATs are classified on the basis of their acyl-donor specificity into two categories; i.e. aliphatic and aromatic acyltransferases. However, the acyl-acceptor specificity of AAT differs greatly with the enzyme. Primary structural analyses of several AATs revealed that AATs form a subfamily within the versatile acyltransferase family and share highly conserved sequences such as motif 1 (-His-Xaa$_3$-Asp-) and motif 3 (-Asp-Phe-Gly-Trp-Gly-) with each other. It is proposed that AAT-catalyzed acyl transfer proceeds with a general acid/base mechanism, where the enzyme and both acyl donor and acyl acceptor form a ternary complex before catalysis can occur. The histidine and aspartic acid residues located at motifs 1 and 3, respectively, appear to play very important roles during the proposed general acid/base catalysis. AAT cDNAs have been expressed in heterologous systems, providing a basis for applications of AATs in biotechnology, such as flower color modification and food colorant production by metabolic engineering of anthocyanin biosynthesis in plants.

Keywords: Anthocyanin; Acyltransferase; Malonyl-CoA; Hydroxycinnmanoyl-CoA; Versatile plant acyltransferase family; Plant secondary metabolism; Flower color

1. Introduction

Anthocyanins, the largest subclass of plant flavonoids, are in most cases responsible for the orange to blue colors of flowers and other organs of plants [1] and play important roles in attracting pollinators and protecting plants from various stresses [2]. The general structures, nomenclatures, and positional numbering of anthocyanins are shown in Fig. 1. Anthocyanins are associated, in many ways, with the sensory and nutritional quality of foods of plant origin [2,3]. Some anthocyanins, such as those extracted from perilla (*Perilla frutescens*), red cabbage (*Brassica oleracea*), and sweet potato (*Ipomea batatas*), have been used as natural food colorants with high degree of safety to human health. Moreover, it has recently been shown that anthocyanins have a variety of bioactivities of medical interest, such as antioxidant activity, anti-inflammatory activity, anti-mutagenic activity, and visual function-improving effects [2,3].

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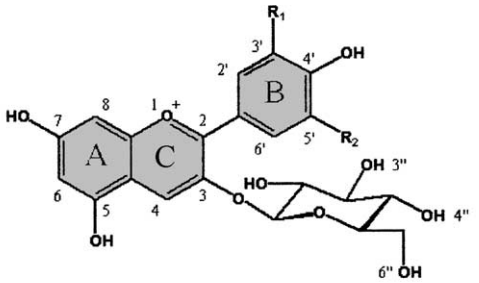
doi:10.1016/S1381-1177(03)00078-X



1a, $R_1 = R_2 = H$, Callistephin (pelargonidin 3-*O*-glucoside)
1b, $R_1 = H$, $R_2 = OH$, Chrysanthemin (cyanidin 3-*O*-glucoside)
1c, $R_1 = R_2 = OH$, Myrtillin (delphinidin 3-*O*-glucoside)

Fig. 1. General structure of anthocyanins. Anthocyanins are the glycosides of anthocyanidins, which are the chromophore aglycons of the pigments. Anthocyanidins (shadowed) have a common 2-(4′-hydroxyphenyl)-3,5,7-trihydroxybenzopyrylium (or 3,4′,5,7-tetrahydroxyflavylium) structure (at acidic pHs; see below). A, B, and C indicate A-, B-, and C-rings in the anthocyanidin structures, respectively. For positional numbering of the anthocyanidin structure, prime numbers are used for the positions on the B-ring (which is always located on the right of the structure), whereas the positions of the A- and C-rings are identified by numbers without a prime. Anthocyanidins with 4′-hydroxy; 3′,4′-dihydroxy; and 3′,4′,5′-trihydroxy B-rings are called pelargonidin, cyanidin, and delphinidin, respectively. Glycosyl group(s), such as glucosyl group(s), is linked in its β-anomeric configuration to 3-, 5-, 7-, and/or 3′-hydroxy groups of anthocyanidin to form anthocyanins (see also Fig. 2). Positional numbering of hydroxyls of 3-*O*-glycosyl and 5-*O*-glycosyl moieties is indicated with double and triple primes, respectively. It should be mentioned that, in in vitro aqueous systems, anthocyanin is proposed to exist as several distinct forms, depending on pH, as follows [43]. At acidic pHs (pH < 2), anthocyanin is present in its cationic flavylium form (red in color in aqueous solution), as shown in this figure. At physiological pHs of 4–7, however, anthocyanin is spontaneously tautomerized to 7-quinonoidal and 4-quinonoidal base isomers (bluish-purple to blue in color; structures not shown). At pHs higher than 5, the anthocyanins also undergo hydration to produce a colorless carbinol pseudobase species (structures not shown), and this hydration process is closely related to the instability of the anthocyanin coloration at these pHs (see Sections 1 and 7.2).

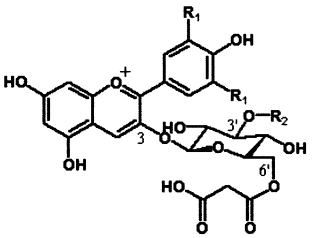
Anthocyanins exist as their acylated forms in many plant species [1,4,5]. The structures of such acylated anthocyanins are exemplified by structures **2**–**7** in Fig. 2. Two major types of acyl substituents of anthocyanins exist, i.e. aromatic and aliphatic acyl groups, both of which are commonly linked to a hydroxy group of a glycosyl moiety of anthocyanins. It has been proposed that aromatic acylation makes anthocyanins more stable and bluer by intramolecular stacking of the anthocyanins with polyphenols [6] and that the aliphatic acylation of anthocyanin, such as malonylation, is important for enhancing the pigment solubility in water [7], protecting glycosides from enzymatic degradation [8], stabilizing anthocyanin structures [8,9], or uptaking anthocyanins into vacuoles [10]. Furthermore, anthocyanin acylation should be of nutritional and biomedical importance because some of the bioactivities of anthocyanins have been shown to be strongly modulated by acylation [11].

During anthocyanin biosynthesis, acylation generally takes place on the glycosyl moieties of anthocyanins after the formation of anthocyanidin 3-*O*-glucosides [7,12], a core part of the pigment, and is catalyzed by anthocyanin acyltransferases (AATs), a group of anthocyanin-specific, acyl-CoA-dependent plant enzymes.[1] Although the pathway leading to anthocyanidin 3-*O*-glucosides were well studied, AATs were only recently characterized in terms of biochemistry and molecular biology. Recent advances in genetic engineering to modify flower color and the elucidation of a variety of bioactivities of anthocyanins have attracted increasing attention to the anthocyanin acylation by AATs and their applications in biotechnology [12]. In this review, we will describe the current knowledge on the enzymology of AATs and focus on their specificities, reaction mechanism, phylogenetic relationship with other acyltransferases, and potential applications in biotechnology.

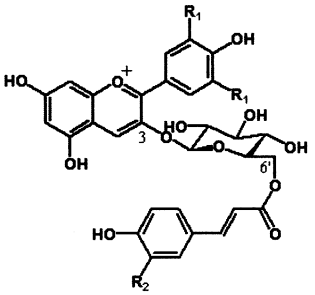
2. Occurrence of AATs in plants

AAT activities have been shown to occur in the cell cultures, flowers, and other plant organs that accumulate acylated anthocyanins. Some AAT activities have also been identified in acyanic ones ([13]; Suzuki et al., unpublished results). An AAT activity that catalyzes the acylation of the 3-*O*-rhamnosyl group of anthocyanidin 3-*O*-rutinoside was first demonstrated in the flowers of *Silene dioica* in 1980 [14]. Since then, many AATs with different acyl-donor specificity, acyl-acceptor specificity, and regiospecificity of acyl

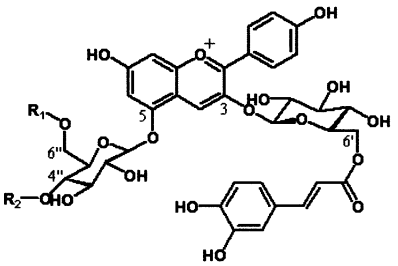
[1] An activity of 1-*O*-acylglycoside-dependent AAT has also been identified in cell cultures of *Daucus carota*. This enzyme catalyzes the acyl transfer from hydroxycinnamoyl-1-*O*-glucosides to position 6 of the glucose moiety of cyanidin 3-*O*-(6″-*O*-glucosido-2″-*O*-xylosido)galactoside [46].



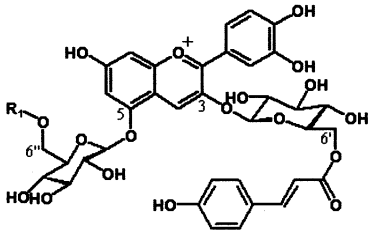
2a, R_1 = H or OH, R_2 = H
2b, R_1 = H or OH, R_2 = malonyl-



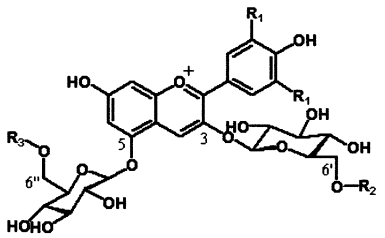
3a, R_1 = H or OH, R_2 = H
3b, R_1 = H or OH, R_2 = OH



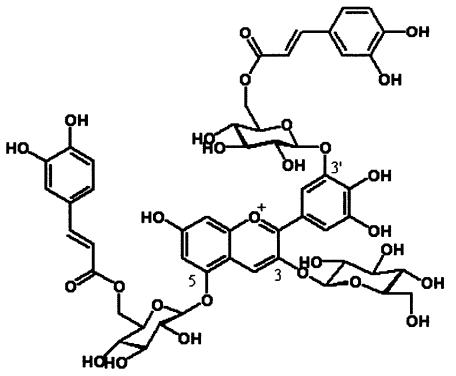
4a, R_1 = R_2 = H, Bisdemalonylsalvianin
4b, R_1 = malonyl-, R_2 = H, Monodemalonylsalvianin
4c, R_1 = R_2 = malonyl-, Salvianin



5a, R_1 = H, Shisonin
5b, R_1 = malonyl-, Malonylshisonin



6a, R_1 = H or OH, R_2 = R_3 = H
6b, R_1 = H or OH, R_2 = malonyl-, R_3 = H
6c, R_1 = H or OH, R_2= acetyl, R_3 = H
6d, R_1= H or OH, R_2 = R_3 = malonyl-
6e, R_1= H or OH, R_2= R_3 = acetyl-
6f, R_1= H or OH, R_2=H, R_3 = *p*-coumaryl- or caffeyl-



7, Gentiodelphin

Fig. 2. Example of acylated anthocyanins. Hundreds of acylated forms of anthocyanins have been found in plants.

Table 1
Identification of acyl-CoA-dependent AATases and related activities in plants[a]

Source	Enzyme	Abbreviation	References
Aliphatic acyltransferases			
Cicer arietinum roots	Malonyl-CoA:isoflavone 7-*O*-glucoside 6″-*O*-malonyltransferase[b]		[47]
Callistephus chinensis flowers	Malonyl-CoA:anthocyanidin 3-*O*-glucoside malonyltransferase		[26]
Zinnia elegans flowers	Acetyl-CoA:anthocyanidin 3-*O*-glucoside acetyltransferase		[28]
Centaurea cyanus flowers	Succinyl-CoA:anthocyanidin 3-*O*-glucoside succinyltransferase		[27]
Dendranthema morifolium flower buds	Malonyl-CoA:anthocyanidin 3-*O*-glucoside malonyltransferase		[48]
Lactuca sativa leaves	Malonyl-CoA:anthocyanidin 3-*O*-glucoside malonyltransferase		[49]
Petroselium hortense cell cultures	Malonyl-CoA:flavone/flanvonol 7-*O*-glucoside malonyltransferase[b]		[50]
P. hortense cell cultures	Malonyl-CoA:flanvonol 3-*O*-glucoside malonyltransferase[b]		[50]
Ajuga reptans cell cultures	Malonyl-CoA:anthocyanin malonyltransferase		[13]
Salvia splendens flowers	Malonyl-CoA:anthocyanin 5-*O*-glucoside 6‴-*O*-malonyltransferase	Ss5MaT1	[22]
S. splendens flowers	Malonyl-CoA:anthocyanin 5-*O*-glucoside 4‴-*O*-malonyltransferase	Ss5MaT2	[22]
Dahlia variabilis flowers	Malonyl-CoA:anthocyanidin 3-O-glucoside 6‴-*O*-malonyltransferase	Dv3MaT	[8,51]
Senecio cruentus flowers	Malonyl-CoA:anthocyanidin 3-*O*-glucoside 6″-*O*-malonyltransferase	Sc3MaT	[24]
Perilla frutescens leaves	Malonyl-CoA:anthocyanin 5-*O*-glucoside 6‴-*O*-malonyltransferase	Pf5MaT	[22,52]
Aromatic acyltransferases			
Silene dioica flowers	Hydroxycinnamoly-CoA:anthocyanidin 3-*O*-rhamnosyl(1 → 6) glucoside 4‴-*O*-hydroxycinnamoyltransferase		[14]
Matthiola incana flowers	Hydroxycinnamoly-CoA:anthocyanidin 3-*O*-glucoside hydroxycinnamoyltransferase		[53]
A. reptans cell cultures	Hydroxycinnamoly-CoA:anthocyanin hydroxycinnamoyltransferase		[13]
Gentiana triflora flowers	Hydroxycinnamoly-CoA:anthocyanidin 3,5-*O*-diglucoside 6‴-*O*-hydroxycinnamoyltransferase	Gt5AT	[16,17]
P. frutescens leaves	Hydroxycinnamoly-CoA:anthocyanin 3-*O*-glucoside 6‴-*O*-hydroxycinnamoyltransferase	Pf3AT	[19,20]
Pisum sativum seedlings	Hydroxycinnamoly-CoA:flavonol 3-triglucoside hydroxycinnamoyltransferase[b]		[54]

[a] Several nomenclatures have been used for AATs. These enzymes can be best specified in terms of their acyl-donor specificity, acyl-acceptor specificity, and regiospecificity of acyl transfer (see Section 3 for details) using the systematic nomenclature proposed by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, http://www.chem.qmul.ac.uk/iubmb/enzyme/). Thus, for example, Ss5MaT1, which catalyzes the regiospecific transfer of malonyl group from malonyl-CoA to the 6‴-hydroxyl group of anthocyanin 5-*O*-glucosides, is designated as "malonyl-CoA:anthocyanin 5-*O*-glucoside 6‴-*O*-malonyltransferase", where the underlining indicates the site of acyl transfer. However, AATs may more simply be designated as, for example, anthocyanin malonyltransferases and anthocyanin hydroxycinnamoyltransferases. These names have frequently been associated with the specification of the glycosyl moiety (e.g. 3-*O*-glucosyl, 5-*O*-glucosyl, 7-*O*-glucosyl, or 3′-*O*-glucosyl) to be acylated, with emphasis on its significance in biosynthesis. Thus, Ss5MaT1 and Ss5MaT2 may also be called anthocyanin 5-malonyltransferases.

[b] These enzymes are flavonoid glucoside acyltransferases.

transfer have been identified (Table 1). The first purification to homogeneity and cDNA cloning of AAT were achieved with hydroxycinnamoyl-CoA:anthocyanin 3,5-*O*-diglucoside 6‴-*O*-hydroxycinnamoyltransferase, Gt5AT, which is involved in the biosynthesis of gentiodelphin (**7**) [15] in the blue flowers of gentian (*Gentiana triflora*) [16,17]. To date, the following AATs have also been cloned, sequenced, and biochemically characterized in their native and/or recombinant forms (Fig. 3): Pf3AT, hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucoside 6″-*O*-hydroxycinnamoyltransferase, which is involved in the biosynthesis of malonylshisonin (**5b**) [18] in the red forma leaves of *P. frutescens* [19,20]; Ss5MaT1, malonyl-CoA:anthocyanin 5-*O*-glucoside 6‴-*O*-malonyltransferase, which is involved in the biosynthesis of salvianin (**4c**) [21] in the scarlet sage (*Salvia splendens*) flowers [22]; Dv3MaT,

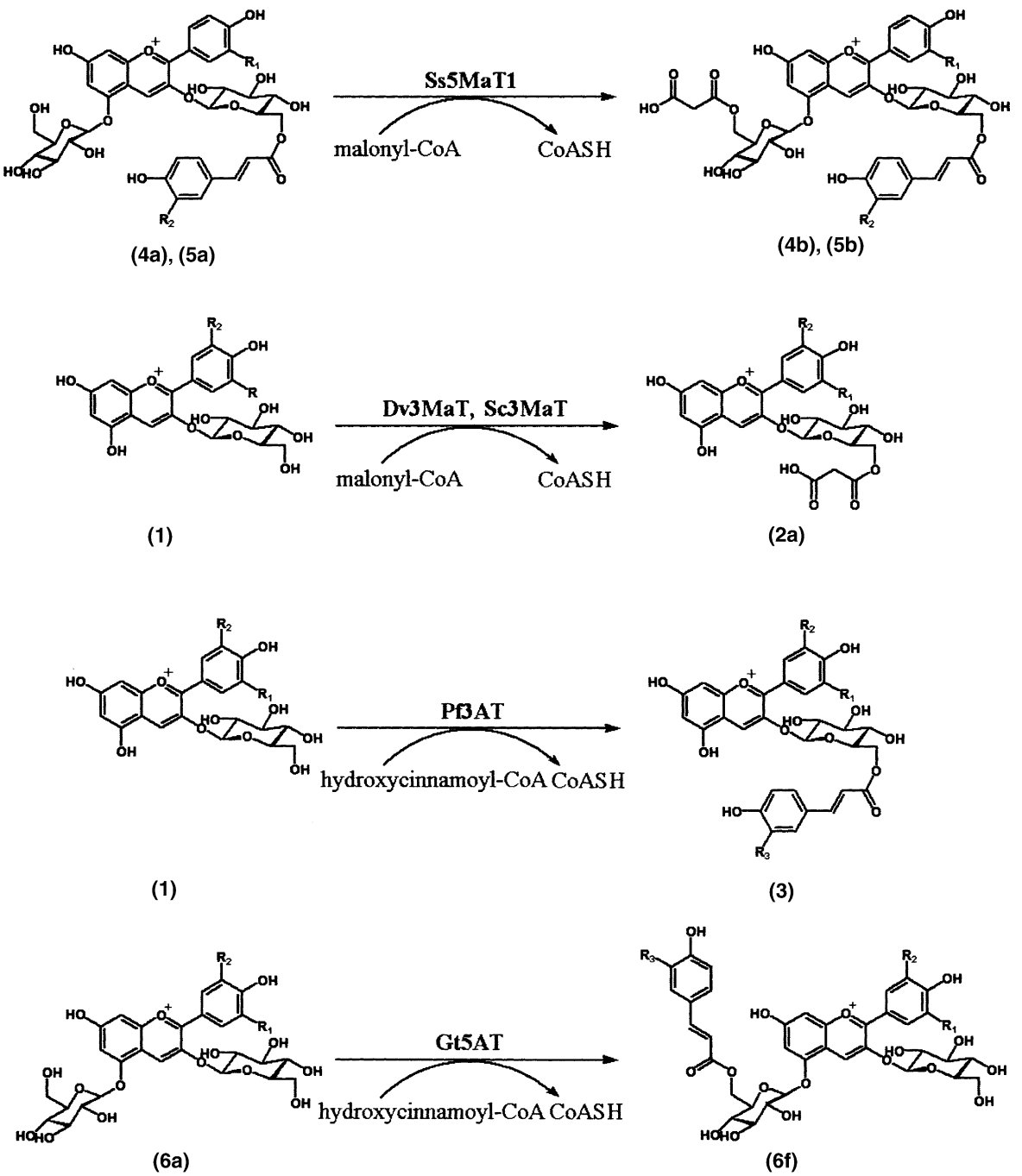


Fig. 3. Regiospecific acyl transfer reactions catalyzed by some AATs. Enzymes are: Ss5MaT1, malonyl-CoA:anthocyanin 5-*O*-glucoside 6‴-*O*-malonyltransferase of *S. splendens*; Dv3MaT, malonyl-CoA:anthocyanin 3-*O*-glucoside 6″-*O*-malonyltransferase of *D. variabilis*; Sc3MaT, malonyl-CoA:anthocyanin 3-*O*-glucoside 6″-*O*-malonyltransferase of *Senecio cruentus*; Pf3AT, hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucoside 6″-*O*-hydroxycinnamoyltransferase of *P. frutescens*; Gt5AT, hydroxycinnamoyl-CoA:anthocyanin 3,5-*O*-diglucoside 6‴-*O*-hydroxycinnamoyltransferase of *G. triflora*.

malonyl-CoA:anthocyanidin 3-*O*-glucoside 6″-*O*-malonyltransferase, which is involved in the biosynthesis of anthocyanidin 3-*O*-glucoside-6″-*O*-malonylglucoside (**2a**) in the red dahlia (*Dahlia variabilis*) flowers [8]; and Sc3MaT, malonyl-CoA:anthocyanidin 3-*O*-glucoside 6″-*O*-malonyltransferase, which is involved in the biosynthesis of cinerarin [23] in the blue cineraria (*Senecio cruentus*) flowers [24]. Because these enzymes are currently the best characterized AATs in terms of their primary structures, specificities, and molecular properties, this review will mainly deal with these five AATs to survey their primary structure/function as well as the phylogenetic relationships of AATs.

3. Specificities of AATs

3.1. Acyl-donor specificity

AATs have been shown to be clearly classified into two distinct categories on the basis of their acyl-donor specificity, i.e. aliphatic acyltransferases and aromatic acyltransferases (Table 1). Aliphatic acyltransferases do not act on aromatic acyl-CoAs, such as *p*-coumaroyl-CoA and caffeoyl-CoA, and vice versa. This is illustrated by the data presented in Table 2, which shows the acyl-donor specificities of some AATs evaluated with their homogeneous enzyme preparations.

The malonyltransferases presented in Table 2, i.e. Ss5MaT1, Dv3MaT, and Sc3MaT, also utilize methylmalonyl-CoA and succinyl-CoA as weak acyl donors. The relatively broad specificity of malonyltransferases for aliphatic acyl-CoAs was also observed with enzymes from the roots of *Cicer arientinum* [25] and the flowers of *Callistephus chinensis* [26], for which succinyl-, glutaryl-, methylmalonyl-, and acetyl-CoAs could serve as weak substrates. Succinyltransferase of *Centaurea cyanus* flowers appears to exhibit a comparable level of malonyltransferase activity [27]. This enzyme has been designated as "succinyltransferase" because the major anthocyanins in the flowers exist in their succinylated forms. The

Table 2
The acyl-donor specificity of purified AATs

Acyl donor	AAT				
	Ss5MaT1[a]	Dv3MaT[b]	Sc3MaT[c]	Pf3AT	Gt5At[f]
Aliphatic acyl-CoA					
Malonyl-CoA	100 (57)	100 (18.8)	100 (61)	–	–
Acetyl-CoA	2	–	–		
Methylmalonyl-CoA	3	11	15		
Succinyl-CoA	49	16	20		
Aromatic acyl-CoA					
p-Coumaroyl-CoA				61[d] (24)[e]	nd (190)
Caffeoyl-CoA	–[g]	–		100[d] (45)[e]	nd (65)

Relative percentage activities of acyl donors are shown with their K_m values (in parenthesis; in μM). For the acyl acceptors and their concentrations used for evaluation, see footnotes to this table. nd: not determined.

[a] Values are quoted from [22]. Relative activities were determined at acyl-CoA and bisdemalonylsalvianin (**4a**) concentrations of 60 and 120 μM, respectively. The enzyme activity with malonyl-CoA is taken to be 100%. For the determination of the K_m value, the concentration of **4a** was set at saturating levels.

[b] Values are quoted from [8]. Relative activities were determined at acyl-CoA and pelargonidin 3-*O*-glucoside concentrations of 60 and 120 μM, respectively. The enzyme activity with malonyl-CoA is taken to be 100%. The K_m value was determined at the saturating concentration of pelargonidin 3-*O*-glucoside.

[c] Values are quoted from [24]. Assay conditions are same as in the previous footnote of this table.

[d] Values are quoted from [20]. Relative activities were determined at acyl-CoA and pelargonidin 3,5-*O*-diglucoside concentrations of 220 and 200 μM, respectively. The enzyme activity with caffeoyl-CoA is taken to be 100%.

[e] Values are quoted from [19].

[f] Values are quoted from [16,17]. K_m values were determined using 480 μM delphinidin 3,5-*O*-diglucoside as a counter substrate. The k_{cat} values are not reported for Gt5AT.

[g] (–) Relative activity is less than 0.1%.

extracts of the *Zinnia elegans* flowers have been shown to contain an anthocyanin acetyltransferase activity that is responsible for the production of the acetylated anthocyanins in the flower [28]; the specificity of this enzyme toward aliphatic acyl-CoAs has not been clarified.

Homogeneous preparations of Gt5AT and Pf3AT exclusively utilize hydroxycinnamoyl-CoAs as acyl donors but do not utilize malonyl-CoA (Table 2). Strict specificity for aromatic acyl-CoAs has also been observed so far for many other anthocyanin hydroxycinnamoyltransferases (Table 1).

3.2. Regiospecificity of acyl transfer

Many AATs have been shown to have strict specificities for the position of the acylation of anthocyanins. For example, Dv3MaT, Sc3MaT, and Pf3AT specifically acylate the 6″-hydroxyl group of the 3-*O*-glucoside moiety of anthocyanins (Fig. 3). Three AATs with distinct regiospecificity are involved in the biosynthesis of the salvianin (**4c**), which carries two malonyl groups at its 4‴- and 6‴-positions, along with a caffeyl group at its 6″-position. Specificity analyses suggested that, during salvianin biosynthesis, 6″-*O*-caffeylation takes place first, followed by 6‴-*O*-malonylation and 4‴-*O*-malonylation, in this order. The 6″-*O*-caffeylation should be catalyzed by a 3-aromatic acyltransferase, and the 6‴-*O*-malonylation (**4a**–**4b**, see also Fig. 3) and 4‴-*O*-malonylation (**4b**–**4c**) are shown to be catalyzed by two distinct malonyltransferases called Ss5MaT1 and Ss5MaT2 (Table 1), respectively [22].

3.3. Acyl-acceptor specificity

It has been generally observed that the number of B-ring hydroxyls of substrate anthocyanin (Fig. 1) does not affect their reactivity to AATs, which, therefore, can equally act on any anthocyanidin types—the pelargonidin, cyanidin, and delphinidin (Table 3). Moreover, some AATs have even been shown to act on flavonoid glycosides, such as quercetin 3-*O*-glucoside, though weakly (Table 3). Depending on the enzymes, the glycosylation and acylation of substrate anthocyanin may affect their reactivity to AATs, as illustrated by the following observations. (i) Ss5MaT1 acts on anthocyanidin 3-*O*-(6-*O*″-hydroxycinnamylglucoside)-5-*O*-glucosides (**4a** and **5a**) but not on either anthocyanidin 3,5-*O*-diglucosides (**6a**) or anthocyanidin 3-*O*-(6-*O*″-malonylglucoside)-5-*O*-glucosides (**6b**) [22]. In contrast, however, Gt5AT acts on **6a**, **6b**, and **6c** but not on either **4a** or **5a** [16,17] (Table 3). It should be mentioned that *p*-coumaric acid, which mimics the 6-*O*″-hydroxycinnamyl moiety of substrates **4a** and **5a**, is a dead-end competitive inhibitor of Ss5MaT1 with respect to substrate anthocyanins [22]. These results suggest the absolute requirement of the 6″-*O*-hydroxycinnamyl group at the 3-*O*-glucosyl moiety of the substrate for its efficient binding to Ss5MaT1. (ii) Anthocyanidin 3-*O*-glucosides (**1**, Fig. 1) are very good acyl acceptors for both Dv3MaT and Pf3AT. Pf3AT acts on anthocyanidin 3,5-*O*-diglucoside (**6a**) as well, whereas Dv3MaT does not (Table 3). Such broad substrate specificity toward anthocyanins contributes to forming “metabolic grid” in the biosynthetic pathway. (iii) The reaction products of the AAT-catalyzed acylation of anthocyanins no longer serve as acyl acceptors. Thus, for example, the following acylated anthocyanins are inert as substrates for respective enzymes: anthocyanins **4b** and **5b** for Ss5MaT1; anthocyanin **2a** for Dv3MaT; and anthocyanins **3**, **4a**, and **5a** for Pf3AT. In contrast to these observations, AAT activities of the *Ajuga reptans* cell cultures appear to have relatively broad acyl-acceptor specificities with respect to the modifications (glycosylation and acylation) of the substrates [13]. One of the hydroxycinnamoyltransferase activities of *A. reptans* showed the ability of *p*-coumaroyl transfer to anthocyanidin 3-*O*-sophorosides, 3,5-*O*-diglucosides, and 3-*O*-sophoroside-5-*O*-glucosides. An anthocyanin malonyltransferase activity of the same plant species is also capable of catalyzing malonyl transfer to anthocyanidin 3-*O*-glucosides, 3,5-*O*-diglucosides, 3-*O*-sophoroside-5-*O*-glucosides, and 3-*O*-6″-*O*-*p*-coumarylglucoside-5-*O*-glucosides.

It is important to note that a wide variety of polyacylated anthocyanins, such as Heavenly Blue anthocyanins in morning glory, are known to exist in nature [5], and the observed strict acyl-donor specificities, acyl-acceptor specificities, and regiospecificities of several AATs suggest that the polyacylation of anthocyanins may be, in many cases, completed by the actions of a series of distinct AATs, each of which is responsible for one specific acylation of substrate

Table 3
The acyl-acceptor specificity of purified AATs

Acyl acceptor	Ss5MaT1[a]	Dv3MaT[b]	Sc3MaT[c]	Pf3AT	Gt5AT[f]
Anthocyanidin 3-*O*-glucosides					
1a	–[h]	100 (47)	100 (11)	100[d] (6)[e]	–
1b		111 (40)	110 (nd)	94[d] (11)[e]	
1c		117 (46)	153 (nd)	77[d] (19)[e]	
2a		–			
3a				–	
Quercetin 3-*O*-glucoside[g]		15 (81)	–		
Anthocyanidin 3,5-*O*-diglucosides					
4a	100 (101)	–		–	–
4b	–				
5a	55 (36)	–		–	–
5b	–				
6a ($R_1 = R_2 = H$)	–	–	–	177[d] (138)[e]	nd (172)
6a ($R_1 = OH$, $R_2 = H$)				77[d] (87)[e]	nd (113)
6a ($R_1 = R_2 = OH$)				48[d] (227)[e]	nd (180)
6b	–	–			nd (461)
6c					nd (777)
6f					–

Relative percentage activities of acyl acceptors are shown with their K_m values (in parentheses; in μM). For the acyl donors and their concentrations used for evaluation, see footnotes to this table. nd: not determined.

[a] Values are obtained from [22]. The k_{cat} value for bisdemalonylsalvianin (**4a**) is taken to be 100%. K_m values were determined at the saturating concentration of malonyl-CoA.

[b] Values are quoted from [8]. Relative activities were determined at malonyl-CoA and acyl-acceptor concentrations of 60 and 120 μM, respectively. The enzyme activity with pelargonidin 3-*O*-glucoside is taken to be 100%. K_m values were determined at the saturating concentration of malonyl-CoA.

[c] Values are quoted from [24]. Relative activities were determined at malonyl-CoA and acyl-acceptor concentrations of 60 and 120 μM, respectively. The enzyme activity with pelargonidin 3-*O*-glucoside is taken to be 100%. The K_m value was determined at the saturating concentration of malonyl-CoA.

[d] Values are calculated from those presented in [20]. Relative activities were determined at caffeoyl-CoA and acyl-acceptor concentrations of 220 and 200 μM, respectively. The value with pelargonidin 3-*O*-glucoside is taken to be 100%.

[e] Values are quoted from [19].

[f] Values are quoted from [16,17]. K_m values were determined using 430 μM caffeoyl-CoA as a counter substrate. The k_{cat} values are not reported for Gt5AT.

[g] This is a flavonol glucoside.

[h] (–) Relative activity is less than 0.1%.

anthocyanin to provide the necessary precursor for subsequent modifications. Corresponding to a variety of acylation types of anthocyanins that exist in nature, there must be a wide variety of AATs with different specificities, and, currently, most of these enzymes are yet to be isolated.

4. Other enzymatic profiles

Ss5MaT1, Dv3MaT, Sc3MaT, Gt5AT, and Pf3AT are monomeric proteins of 446–469 amino acids with an approximate molecular mass of 50 kDa. The p*I* value of native Gt5AT was determined by isoelectric focusing to be 4.6; predicted p*I* values of other AATs (Ss5MaT1, Dv3MaT, Sc3MaT, and Pf3AT) range from acidic to neutral regions. The optimum pHs for the catalytic activities of these AATs are at neutral to slightly alkaline pHs (7.0–8.5), as was observed for many other AATs listed in Table 1. The k_{cat} values (at pH 7.0 and 30 °C) are reported for the following malonyltransferases: Ss5MaT1, 7.8 s^{-1} [acceptor, bisdemalonylsalvianin (**4a**); [22]]; Dv3MaT, 7.3 s^{-1} [pelargonidin 3-*O*-glucoside (**1**); [8]]; Sc3MaT, 8.8 s^{-1} [pelargoni-

din 3-*O*-glucoside (**1**); [24]]. The sensitivity of AATs to metal ions varies with the source of enzyme. Gt5AT activity is enhanced 1.2-fold by 1 mM Zn^{2+} [16], which is highly inhibitory to Pf3AT [19] and shows a negligible effect on many other AATs. Some AATs are strongly inhibited by 0.1–1 mM Cu^{2+} (e.g. Ss5MaT [22], Dv3MaT [8], Sc3MaT [24], and *C. chinensis* AAT [26]) and 0.1 mM Hg^{2+} (e.g. Ss5MaT, Dv3MaT, and ScMaT). Ethylenediaminetetraacetic acid generally shows no or only small inhibitory effects on AATs, suggesting that divalent metal ions are not essential for AAT activity. Many AATs are strongly inhibited by *p*-chloromercuribenzoic acid, an SH reagent. These AATs are also highly (e.g. Ss5MaT [22], Dv3MaT [8], and Sc3MaT [24]) or partially (e.g. Gt5AT and Pf3AT [20]) inactivated by 1 mM *N*-ethylmaleimide. The highly sensitive nature of Ss5MaT1 to the cysteine modifiers (Cu^{2+}, Hg^{2+}, and SH reagents) is consistent with the observed instability of this enzyme to oxidation during purification, for which the addition of a reducing agent such as 2-mercaptoethanol was essential to attain the maximum yield of enzyme activity [22]. Some AATs (e.g. Pf3AT and Ss5MaT1) are shown to be inactivated by 1 mM diethylpyrocarbonate, a histidine modifier, which is also known to inactivate other acyltransferases of plants [29] (see below).

5. Phylogenetics and primary structural characteristics of AATs

The analyses of the deduced amino acid sequences of Ss5MaT1, Dv3MaT, Sc3MaT, Gt5AT, and Pf3AT revealed that these AATs have consensus sequences (-His-Xaa$_3$-Asp- (motif 1, Fig. 4) and -Asp-Phe-Gly-Trp-Gly- (motif 3)) that have been specifically identified in the members of the versatile acyltransferase (VAT) family (also called BAHD superfamily [29,30] or VPAT family [8,22]), indicating the close phylogenetic relationship of these AATs with this family. The VAT family has been identified as a protein family consisting of acyltransferases involved in the secondary metabolisms in plants and fungi (Fig. 5), such as the biosyntheses of phytoalexins [31], vindoline [29], benzylacetate [32], taxol [33–35], and morphine [36]. These biochemically characterized VATs show only low sequence similarities to each other (20–40% identity) and should yet represent only a small fraction of the total membership of the family because *Arabidopsis* was recently estimated to contain about 70 related genes of the family [37], most of whose biochemical roles have yet to be determined. In addition to the suggested widespread occurrence of members of this family in plants, a limited number of the family members have also been identified in fungal species [30]. These observations suggest that this new acyltransferase family covers highly divergent members in terms of primary structure and biochemical functions. In addition, despite their similarity in the biochemical role, Ss5MaT1, Dv3MaT, Sc3MaT, Gt5AT, and Pf3AT show only 30–60% sequence identity with each other. The molecular phylogenetic tree of the amino acid sequences of the VAT family members constructed by the neighbor-joining method suggests that these AATs may be categorized into a subfamily that is

		Motif 1		Motif 2		Motif 3
Ss5MaT1*	165	NHHCLGDAR	310	ENYFGNCI	388	KADFGWGKA
Dv3MaT*	171	NHHSLSDAN	322	ENYFGNCS	398	DIDFGWGKA
Sc3MaT*	173	NHHSLGDAS	323	TAYFGNCG	399	DFDFGWGKP
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. 4. Alignment of amino acid sequences of motifs 1, 2, and 3 (boxed) of AATs (indicated by an ✱) and other members of the VAT family. GenBank™ accession numbers for amino acid sequences are as follows: Ss5MaT1, AAL50566; Dv3MaT, AF489108; Sc3MaT, AY190121; Pf3AT, BAA93475; and Gt5AT, BAA74428. Explanation of enzyme names of other VATs and accession numbers for their amino acid sequences are described in the legend to Fig. 6.

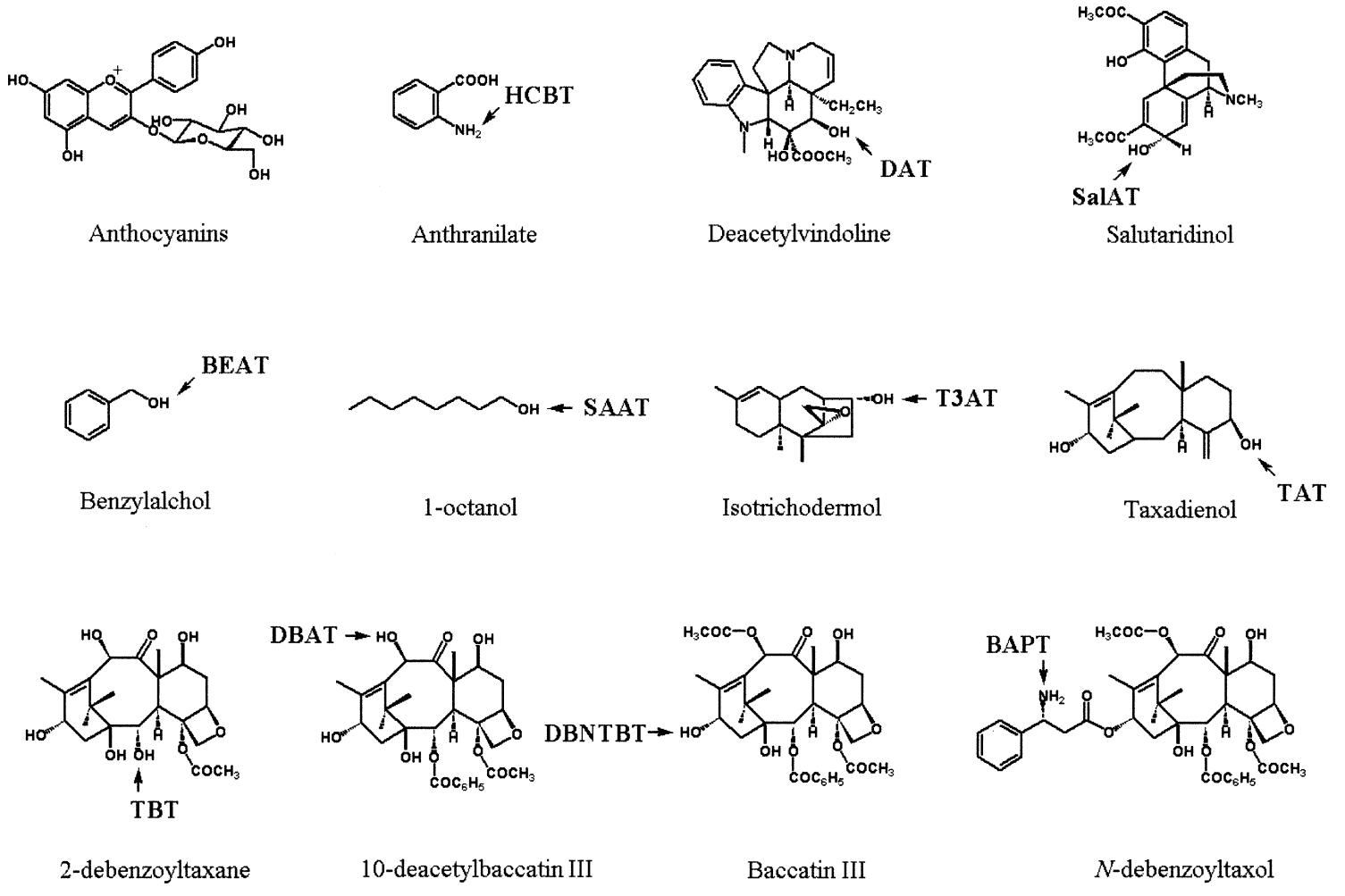


Fig. 5. Acyl acceptors of VATs. The site of acylation of each substrate is indicated by an arrow with an abbreviated enzyme name (see legend of Fig. 6). These enzymes are involved in the production of the following secondary metabolites: HCBT, methoxydianthramide B (phytoalexin); DAT, vindolin; SalAT, morphine; BEAT, benzylacetate (floral scent); SAAT, alcohol acetate (fruit flavor); T3AT, trichothecene mycotoxins; TAT, TBT, DBAT, DBNTBT, and BAPT, taxol (anti-cancer drug).

phylogenetically separate from other VATs (Fig. 6). It must also be mentioned that Ss5MaT1, Dv3MaT, Sc3MaT, Gt5AT, and Pf3AT also share the sequence -Tyr-Phe-Gly-Asn-Cys- (motif 2) with each other [8,22]. This motif may serve as a signature sequence of AATs; indeed, the Dv3MaT cDNA had been specifically isolated by a homology-based strategy taking full advantage of the specific conservation of motif 2 in AATs [8].

Because the three-dimensional structures of AATs and other VATs are not yet known, intensive sequence comparison studies using these enzymes have been carried out to explore any primary structural characteristics that may be responsible for catalytic activity, acyl-donor specificity, acyl-acceptor specificity, and the regiospecificity of the acyl transfer of enzymes [8]. It has been pointed out that a motif 1-like sequence also occurs in the sequence of chloramphenicol

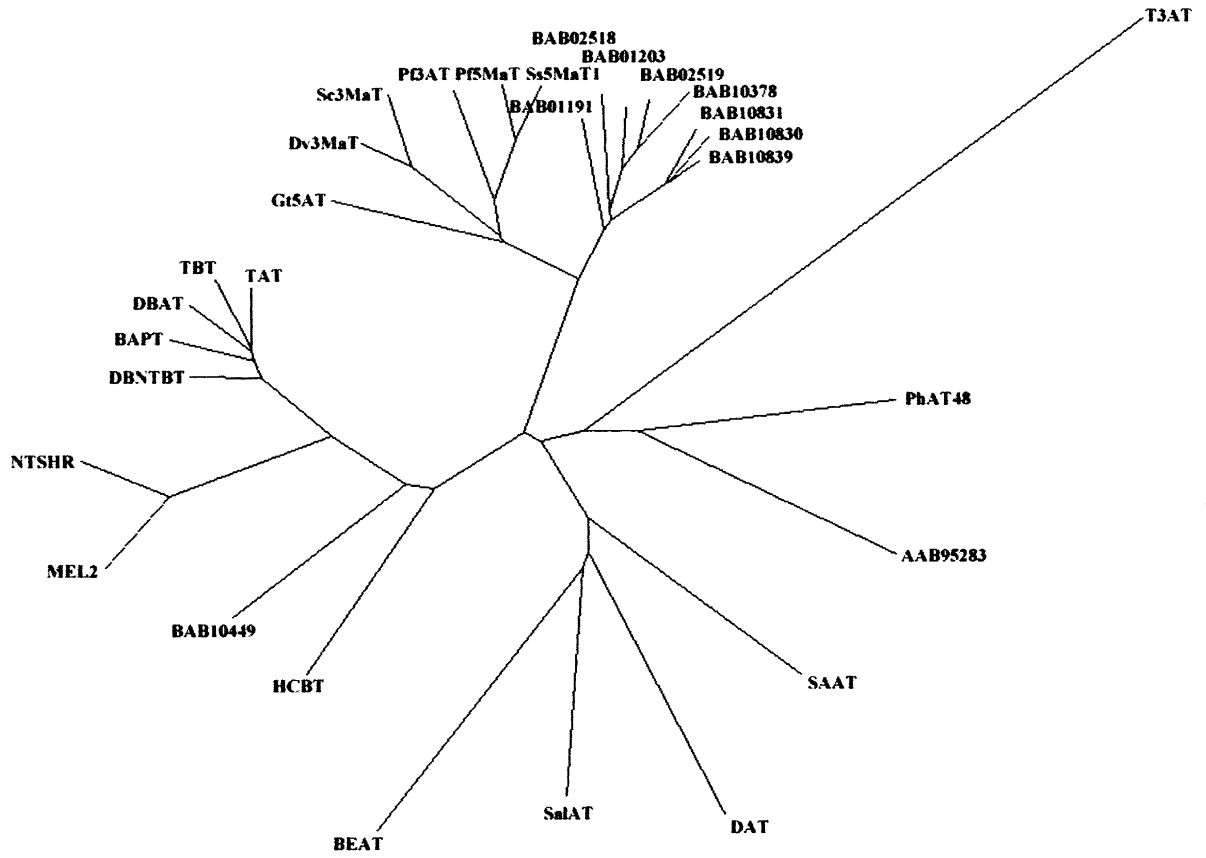


Fig. 6. A non-rooted molecular phylogenetic tree of the VAT family. The tree was constructed by the neighbor-joining method. Enzymes used for alignment are as follows: Pf5MaT, malonyl-CoA:anthocyanin 5-*O*-glucoside 6‴-*O*-malonyltransferase of *P. frutescens* (AF405204); HCBT, hydroxycinnamoyl/benzoyl-CoA:anthranilate *N*-hydroxycinnamoyl/benzoyltransferase of *Dianthus caryophyllus* (CAB06430); DAT, deacetylvindoline 4-*O*-acetyltransferase of *Catharanthus roseus* (AAC99311); SalAT, salutaridinol 7-*O*-acetyltransferase of *Papaver somniferum* (AAK73661); BEAT, benzylalcohol acetyltransferase of *Clarkia breweri* (AAC18062); SAAT, alcohol acetyltransferase of *Fragaria ananassa* (AAG13130); T3AT, trichothecene 3-*O*-acetyltransferase of *Fusarium graminearum* (BAA24430); TAT, taxadienol acetyltransferase of *Taxus cuspidata* (AAF34254); TBT, taxane benozoyltransferase of *T. cuspidata* (AAG38049); DBAT, 10-deacetylbaccatin III-10-*O*-acetyltransferase of *T. cuspidata* (AAF27621); DBNTBT, baccatin III (3-amino-3-phenylpropanoyl)transferase of *T. cuspidata* (AAM75818); BAPT, *N*-debenzoyltaxol *N*-benzoyltransferase of *T. cuspidata* (AAL92459); and NTSHR, Hsr201 protein of *Nicotiana tabacum* (T03274). The following are putative acyltransferases whose biochemical function is not clarified: *Arabidopsis thaliana* (BAB01191, BAB02518, BAB02519, BAB10378, BAB10831, BAB10830, BAB10829, BAB10449, and AAB95283), MEL2 of *Cucumis melo* (CAA94432), PfAT48 of *Petunia hybrida* (BAA93453), and GAT106 of *G. triflora* (BAA93452).

O-acetyltransferase (CAT) [29], in which the histidine residue located in the sequence is proposed to act as a general acid/base catalyst during catalysis [38]. These observations, as well as the results of chemical modification studies of VATs [20,29], have suggested that the invariant histidine of motif 1 of VATs may be important for catalytic activity [29]. Moreover, a comparison of 12 VAT sequences as well as the mutagenesis studies of Ss5MaT1 has revealed that the aspartic acid residue in motif 3 is also very important for the catalytic activity [39] (see Section 6 for details). Although motif 2 has been specifically identified with AATs, the importance of motif 2 for the specificity of anthocyanin binding remains to be demonstrated. Mutagenesis studies of Ss5MaT1 revealed that the replacement of the asparagine residue in this motif by alanine resulted in 100-fold diminution in the k_{cat} value of this enzyme but did not significantly alter the K_m values for malonyl-CoA and anthocyanins [39], suggesting that, at least, the asparagine residue in this motif should not be responsible for the affinity of this enzyme to anthocyanins.

The alignment of AAT sequences further led to identify several conservations of amino acid residues within each set of sequences of enzymes exhibiting the same acyl-donor specificity [8]. For example, Arg^{120} of Ss5MaT1 is found to be invariant in the sequences of all malonyltransferases compared (Fig. 2; corresponding to Arg^{126} and Arg^{128} of Dv3MaT1 and ScMaT, respectively). Furthermore, basic amino acid residues (Lys and Arg) are conserved at a position corresponding to position 353 of Ss5MaT1. It is likely that one or both of these cationic residues are involved in the specific binding of malonyl-CoA via electrostatic interactions in these malonyltransferases. Similarly, some aromatic amino acid residues are found to be conserved in the sequences of Gt5AT and Pf3AT (e.g. Tyr^{98}, Phe^{182}, Tyr^{314}, and Phe^{396} of Gt5AT), one of which may be responsible for possible aromatic interactions in the specific binding of aromatic acyl-CoA by these aromatic acyltransferases. These observations warrant future site-directed mutagenesis and X-ray crystallographic studies to probe their role in acyl-CoA binding. At present, it is difficult to find any relevance of the characteristics of the amino acid sequence or residue(s) to the regiospecificity of acyl transfer.

The PSORT analysis of the amino acid sequence of Gt5AT [17] as well as those of other AATs (Ss5MaT1, Dv3MaT, Sc3MaT, and Pf3AT; unpublished results) revealed that these proteins have no signal for intracellular translocation and no transmembrane domain in their sequences. The immunochemical analysis of the subcellular localization of Gt5AT showed that this enzyme is localized in the cytosol of the outer epidermal cells of gentian petals [17], although there was a criticism to this conclusion (Tanaka, personal communication). The observed optimum pHs for the catalytic activity of AATs (see Section 4) are consistent with their subcellular localization in the cytosol.

6. Proposed catalytic mechanism

6.1. Kinetic mechanism

The kinetic and catalytic mechanisms of AATs have been the most intensively studied with Ss5MaT1 [39]. The steady-state kinetic studies of inhibitions of Ss5MaT1-catalyzed acyl transfer have been carried out using product inhibitors [CoA-SH and malonylshisonin (**5b**), a malonylated anthocyanin product] [39] as well as the dead-end inhibitor, *p*-coumaric acid, which mimics an aromatic acyl group linked to the 3-glucosidic moiety of an anthocyanin substrate [22] (see Section 3.3). The results of these inhibition studies predicted that Ss5MaT1-catalyzed acyl transfer should proceed with the compulsory ordered Bi Bi mechanism, where malonyl-CoA is the first substrate to bind to the enzyme and CoA-SH is the last product to leave (Fig. 7A). During the catalysis, a ternary complex consisting of acyl-CoA, an acyl acceptor, and an enzyme should be formed prior to chemical catalysis.

6.2. General acid/base mechanism of Ss5MaT1-catalyzed acyl transfer

The formation of such ternary complex has also been identified in the kinetic mechanisms of other acyl-CoA-dependent acyltransferases, such as CAT [38] and histone *N*-acetyltransferase (HAT) [40], which are phylogenetically distant from AATs and other VATs. The catalytic mechanisms of CAT and HAT have been established in relation to their three-dimensional structures and shown to share common mechanistic features of acyl transfer: in the ternary complex, a general base activates the nucleophilic

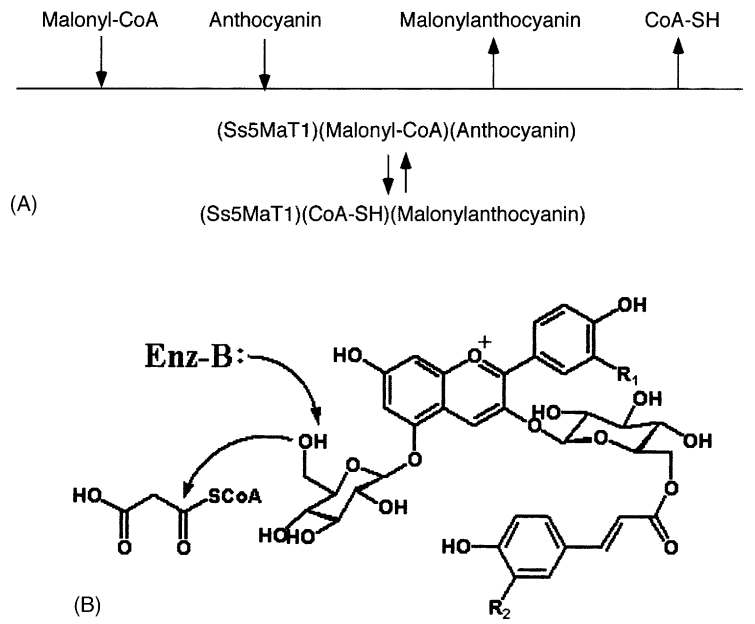


Fig. 7. The proposed kinetic mechanism (A) and catalytic mechanism (B) of AAT. The most likely candidates for the general base (Enz-B:) involved in the Ss5MaT1 catalysis are His^{167} and/or Asp^{390}.

group of the acyl acceptor by deprotonation, promoting its nucleophilic attack on the carbonyl carbon of the acyl-CoA. It is proposed that the residues to fulfill the role of a general base during catalysis are His^{195} and Glu^{173} in CAT [38] and HAT [41], respectively. Because the kinetic mechanism of Ss5MaT1-catalyzed malonyl transfer also agrees with the ternary-complex mechanism, the Ss5MaT1 catalytic mechanism likely shares such common features with the CAT and HAT mechanisms. Thus, in the ternary [malonyl-CoA-anthocyanin-Ss5MaT1] complex, a general base would deprotonate the 6‴-hydroxyl of the acyl acceptor to facilitate its nucleophilic attack on the carbonyl carbon of acyl-CoA (Fig. 7B). Phylogenetically distant groups of proteins could be evolved to enzymes having such the common mechanism of acyl transfer, and this will be an interesting issue to be addressed in future studies.

6.3. *Importance of His^{167} and Asp^{390} during Ss5MaT1 catalysis*

To probe the functional amino acid residues of Ss5MaT1 catalysis on the basis of general acid/base mechanism presented above, alanine-scanning mutagenesis studies have been carried out [39]. Because the three-dimensional structure of Ss5MaT1 is not yet known, intensive sequence comparison of Ss5MaT1 with other 11 VATs has been performed to select targets for mutagenesis. Eight polar or ionizable amino acid residues that are invariant among 12 VATs were identified and replaced by alanine, and the mutant enzymes were kinetically characterized. A significant diminution of the k_{cat} value was observed with the substitution of His^{167} (relative k_{cat}, 0.02%) and Asp^{390} (less than 0.01%), strongly suggesting that these residues are very important for the proposed general acid/base mechanism of Ss5MaT1 catalysis. The log k_{cat} versus pH plots of the Ss5MaT1-catalyzed malonyl transfer suggested that a deprotonated active-site group of a $pK_a = 7.0 \pm 0.1$ may be involved in the catalytic steps of the "substrate to product" conversion in the ternary enzyme–substrate complex. Although the pK_a value of 7.0 ± 0.1 likely reflects the ionization of the histidine residue [42], to which His^{167} could be assigned, it is also possible that this value represents the ionization of a perturbed carboxylic acid residue (i.e. aspartic acid or glutamic acid residue; e.g. Asp^{390}) or that of some other residue within the specialized environment of

the complex [42]. It should be pointed out that there are many examples of enzyme mechanisms, such as those of serine proteinases, α/β hydrolases, and CAT (see above), which utilize a histidine residue as a general base to deprotonate the primary-alcoholic hydroxyl group to enhance the nucleophilicity of the hydroxyl oxygen. Thus, His^{167} itself might serve as the general base involved in the Ss5MaT1 mechanism. It is tempting to speculate that, in the Ss5MaT1 mechanism, there may be a "carboxylate-imidazole (His^{167})-hydroxyl (substrate)" relay system, which is analogous to the catalytic triads of serine proteinases and α/β hydrolases, as the machinery to enhance the nucleophilicity of the hydroxyl group of substrate anthocyanin: Asp^{390} could be a carboxylate member of such a system. Three-dimensional structural information on AATs is necessary to address these issues.

7. Potential applications of AATs

7.1. Heterologous expressions

The AAT cDNAs have been expressed as a soluble, catalytically active form of protein in prokaryotic and eukaryotic heterologous systems using *Escherichia coli* [8,17,20,22,24] and yeasts [17,20], respectively, as host strains. Moreover, Dv3MaT cDNA has been introduced into petunia (*Petunia hybrida*), whose red flower color is provided by cyanidin 3-*O*-glucoside and 3,5-*O*-diglucoside, and functionally expressed in the cells of the transgenic plant, resulting in the accumulation of cyanidin 3-*O*-6″-*O*-malonylglucoside and cyanidin 3-*O*-6″-*O*-malonylglucoside 5-glucoside in the flowers [8]. Successful expressions of AAT cDNAs in heterologous systems should provide the basis for applications of AATs in biotechnology, some of which are exemplified below.

7.2. Metabolic engineering to modify flower color

To date, many attempts have been made to engineer the flavonoid pathway in pursuit of flower color novelty by genetic engineering approaches [3,12]. Metabolic engineering of the anthocyanin biosynthesis to create blue flowers such as "blue rose"—an unknown blue cultivar of *Rosa hybrida*—is one of such examples. As mentioned in Section 1, aromatic acylation is known to stabilize the coloration of anthocyanins at pHs of intracellular milieus of flowers (pH 4–7; see also legend to Fig. 1) and to intensify the blue color as a result of a bathochromic effect; these effects are enhanced as the number of aromatic groups increases [43]. Thus, the accumulation of polyacylated anthocyanins carrying multiple aromatic acyl functions in the flowers of transgenic plants may serve as one of strategies for the creation of blue flowers, and genes coding for anthocyanin aromatic acyltransferases with different specificities will be important tools for such flower color modification [12,17,20,24].

Malonylation (or aliphatic acylation) is also an important modification of anthocyanins to control flower color. Although malonylation does not intrinsically alter the absorption spectra of anthocyanins, it enhances the stability of anthocyanin coloration and prevents the pigments from the attack of β-glycosidase [8]. Indeed, many anthocyanins exist to be malonylated as their ultimate stable forms in many plant species; therefore, malonylation should also serve as a strategy for pigment stabilization in the flowers. Moreover, multiple modifications of anthocyanins may proceed in a sequential manner (see Section 3.3), and the malonylation of anthocyanins should provide the necessary intermediates for subsequent modifications. Overexpression of malonyltransferase genes in the heterologous plants may overcome endogeneous anthocyanin modification and result in color-modified plants.

7.3. Modification of the functional properties of anthocyanins by acylation

Acylation is a powerful means for the structural and functional modification of medicines and many other bioactive compounds. Acylation may also modify the bioactivities of anthocyanins because the antimutagenic activities of anthocyanins purified from sweet potato with purple-colored flesh are shown to be significantly higher than those of their deacylated forms [11]. Moreover, the acylation of anthocyanins may improve the solubilities, physicochemical stabilities, biological half-lives, membrane permeabilities, and intestinal absorption of the pigments. Thus, there has been a growing interest in the modification of the functional properties of anthocyanins by means of specific acylations. Until recently, the use of specific AATs for this purpose had been hampered by

the restricted availability of enzymes. An alternative may be to use transacylation catalyzed by lipases and esterases. In this case, however, there exists the problem of regiocontrol with respect to the acyl acceptor that carries multiple hydroxyl functions. Recently, the regioselective acylation of cyanidin 3-*O*-glucoside with aromatic acids has been attained by means of cultured cells of *Ipomoea batatas* that probably contain an aromatic AAT activity [44]. This enzymatic method has been proved to be useful for the synthesis of acylated anthocyanins, whose productivity exceeds that of lipase-catalyzed transesterification. During the past several years, it has become possible to obtain large amounts of AATs in heterologous systems as described above, and this would allow the efficient and specific acylation of anthocyanins to enhance their health-giving functions.

Finally, the importance in biotechnology may be extended to other VATs because they are involved in the syntheses of a wide variety of metabolites of pharmaceutical and agricultural importance, such as taxol (an anti-cancer drug) and morphine (an anodyne). Commercially feasible total chemical syntheses have not yet been achieved for some of these industrially important compounds [36], and VATs should serve as useful molecular catalysts for regiospecific acylation for the production of compounds of such importance. Moreover, VATs will be an important target in efforts to specifically accumulate such compounds in large amounts in plants or other alternative biotechnological sources through metabolic engineering approaches. Further studies will be needed to overexpress VAT genes in transgenic plants and cells to establish the efficient productions [45] of these useful compounds.

Acknowledgements

We would like to express our deep gratitude to Dr. Yoshikazu Tanaka, Suntory Ltd., for critical reading of the manuscript.

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