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Anthocyanidin 3-glucoside malonyltransferase from *Dahlia variabilis*

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Abstract

Cyanic flowers of *Dahlia variabilis* contained monomalonylated anthocyanins, 3-(6″-malonylglucoside)-5-glucosides of pelargonidin and cyanidin, and dimalonylated anthocyanins, 3,5-di(malonylglucoside)s of pelargonidin and cyanidin, in addition to nonmalonylated 3,5-diglucosides of these anthocyanidins. Enzyme extracts from this plant catalyzed the malonylation of anthocyanidin 3-glucoside to anthocyanidin 3-(6″-malonylglucoside), but not the 3,5-diglucoside to 3-(6″-malonylglucoside)-5-glucoside or 3,5-di(malonylglucoside). The amounts of this enzyme were also examined for cyanic and acyanic cultivars.

Keywords: *Dahlia variabilis*; Compositae; Flower buds; Anthocyanin biosynthesis; Acylated anthocyanins; Malonyltransferase

1. Introduction

Enzymes that acylate anthocyanins with aliphatic acids such as malonic acid (Teusch & Forkmann, 1987; Ino, Nishiyama, & Yamaguchi, 1993; Yamaguchi, Kawanobu, Maki, & Ino, 1996), acetic acid (Ino & Yamaguchi, 1993) and succinic acid (Yamaguchi, Maki, Ohishi, & Ino, 1995) have been characterized using flower or leaf extracts from a variety of plants. All the enzymes investigated so far have been found to catalyze the transfer of an acyl group from a CoA-ester to the 3-glucoside of anthocyanidins. In *Zinnia elegans* (Ino & Yamaguchi, 1993) and *Centaurea cyanus* (Yamaguchi et al., 1995), which contain 3,5-diglucosides of anthocyanidins acylated with acetic acid and succinic acid, respectively, the acylation of 3-glucosides occurs prior to the formation of 3,5-diglucosides, as indicated by analysis of the substrate specificity of the acyltransferases. A comparison of specific activity values for extracts from cyanic and acyanic sources in the same plant species has shown a gene(s) for the enzyme(s) likely to be expressed independently of the genes for anthocyanidin biosynthesis (Ino & Yamaguchi, 1993; Ino et al., 1993; Yamaguchi et al., 1996).

Malonyltransferase has been examined using flowers of *Callistephus chinensis* (Teusch & Forkmann, 1987) and *Dendranthema morifolium* (Ino et al., 1993) or leaves of *Lactuca sativa* (Yamaguchi et al., 1996), which contain the monoglucosyl anthocyanins, 3-glucoside of pelargonidin (Pg 3G) or cyanidin (Cy 3G) acylated with malonic acid. In contrast to the above plants, flowers of *Dahlia variabilis* contain diglucosyl anthocyanins, the 3,5-diglucosides of cyanidin and pelargonidin acylated with malonic acid (Takeda, Harborne, & Self, 1986; Harborne & Grayer, 1988). We were, therefore, prompted to determine the order in which the malonylation of 3-glucosides and the formation of 3,5-diglucosides occurred in the biosynthesis

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Table 1
HPLC and FAB-MS analyses of major anthocyanins contained in purple flowers of *Dahlia variabilis*

Pigment	R_t (min)	$[M]^+$	Identified pigment
1	18.0	611	cyanidin 3,5-diglucoside (Cy 3G5G)[a]
2	21.5	595	pelargonidin 3,5-diglucoside (Pg 3G5G)
3	26.1	697	cyanidin 3-(6″-malonylglucoside)-5-glucoside (Cy 3MG5G)
4	30.1	783	cyanidin 3,5-di(malonylglucoside) (Cy 3MG5MG)
5	30.9	681	pelargonidin 3-(6″-malonylglucoside)-5-glucoside (Pg 3MG5G)
6	35.1	767	pelargonidin 3,5-di(malonylglucoside) (Pg 3MG5MG)

[a] Abbreviations used are shown in parentheses.

of malonylated anthocyanins. In the present study, the properties of malonyltransferase extracted from flowers of *D. variabilis* have been examined and these enzymes from cyanic and acyanic cultivars have been compared.

2. Results and discussion

HPLC analysis of anthocyanins extracted from purple flowers of *D. variabilis* indicated 6 major pigments, **1**–**6**. The FAB-MS spectra of purified pigments **1** to **6** gave $[M]^+$ corresponding to MW for Cy 3G5G, Pg 3G5G, Cy 3MG5G, Cy 3MG5MG, Pg 3MG5G and Pg 3MG5MG, respectively. The results were basically in agreement with those of Takeda et al. (1986) and Harborne and Grayer, (1988) and are summarized in Table 1.

Crude protein extracts from purple flowers were incubated with Pg 3G or Cy 3G as the acyl acceptor and malonyl-CoA as the acyl donor under standard conditions. Each reaction product was identified by HPLC and TLC as pelargonidin or cyanidin 3-(6″-malonylglucoside) (Pg 3MG or Cy 3MG) (Table 2).

Malonyltransferase activity was maximal at pH 7.5, with half maximal activity at pH 6.4 and 9.7. *Ea* and that for denaturation were 64 and 151 kJ mol^{-1}, respectively. The rate of acylation reached a maximum in turn with malonyl-CoA at 300 μM and succinyl-CoA at 400 μM and with 3-glucosides of cyanidin, pelargonidin, peonidin (Pn 3G), delphinidin (Dp 3G), petunidin (Pt 3G) and malvidin (Mv 3G) at 500 μM. Acylation was not significantly affected by any of the various divalent ions tested, except Cu^{2+}, which caused inhibition. No significant inhibitory effect was observed with diethylpyrocarbonate, diethyldithiocarbamate or *N*-ethylmaleimide.

Table 2
TLC and HPLC analyses of anthocyanins produced by the malonyltransferase reaction

Compound	$R_f \times 100$ in				R_t (min)
	AHW	BFW	BuHCl	10F	
Reaction product 1[a]	37	74	58	33	20.4
Reaction product 2[a]	24	65	36	21	16.5
Authentic anthocyanin					
Pg 3G	34	68	39	28	13.8
Cy 3G	18	56	20	18	10.7
Pg 3MG	37	74	58	33	20.4
Cy 3MG	24	64	36	21	16.5

[a] Reaction products 1 and 2 were obtained using Pg 3G and Cy 3G as acyl acceptors, respectively.

Various mono- and diglucosyl anthocyanins were examined for their ability to serve as acyl acceptors (Table 3). As observed previously for other aliphatic acyltransferases (Teusch & Forkmann, 1987; Ino & Yamaguchi, 1993; Ino et al., 1993; Yamaguchi et al., 1995; Yamaguchi et al., 1996), all the monoglucosyl anthocyanins tested functioned equally well as acceptors, while neither Pg 3G5G nor Cy 3G5G, (diglucosyl anthocyanins), served as a substrate. The malonylation of 3-glucoside would thus appear to precede the formation of 3,5-diglucoside, as noted previously in the acetylation (Ino & Yamaguchi, 1993) and succinylation (Yamaguchi et al., 1995) of diglucosyl anthocyanin.

In addition to malonyl-CoA, succinyl-CoA served to a limited degree as a donor (ca. 7% of malonyl-CoA), while acetyl-CoA was ineffective (Table 4). These results are consistent with those for the malonyltransferases from *Callistephus chinensis* (Teusch & Forkmann, 1987) and *Lactuca sativa* (Yamaguchi et al., 1996).

The protein content in enzyme extracts from four cyanic and two acyanic cultivars amounted to ca. 2.1 to 2.5 mg per g fr. wt of flower petals. These enzyme extracts were examined for the specific activity using Pg 3G or Cy 3G as the acyl acceptor (Table 5). Values of the total malonyltransferase activity per g fr. wt of flower petals were closely proportional to those of the specific activity. Regardless of the anthocyanin used, the specific activity was basically the same for all cyanic cultivars irrespective of flower colour or amount of anthocyanin, as previously observed for acyltransferases from *Zinnia* (Ino & Yamaguchi, 1993) and *Centaurea* (Yamaguchi et al., 1995). The specific activity of extracts from acyanic plant sources showed a rather complicated situation, being considerably high in white flowers and extremely low in yellow ones.

Table 3
Substrate specificity of malonyltransferase for various anthocyanins

Anthocyanin	Reaction product[a]	Relative activity (%)[b]
Cy 3G	cyanidin 3-(6″-malonylglucoside)	100
Pg 3G	pelargonidin 3-(6″-malonylglucoside)	92
Pn 3G	peonidin 3-(6″-malonylglucoside)	96
Dp 3G	delphinidin 3-(6″-malonylglucoside)	121
Pt 3G	petunidin 3-(6″-malonylglucoside)	110
Mv 3G	malvidin 3-(6″-malonylglucoside)	123
Cy 3G5G	not detected	0
Pg 3G5G	not detected	0

[a] Malonylated forms of anthocyanins were identified by co-chromatography on HPLC and TLC using authentic anthocyanins purified from *Hibiscus syriacus* (Kim, Nonaka, Fujieda, & Uemoto, 1989).
[b] The malonyltransferase activity obtained with Cy 3G was expressed as 100%.

This could mean that the gene encoding malonyltransferase is expressed independently of genes for anthocyanidin biosynthesis in the white flowers. The genetic basis of the anthocyanin-free phenotype in the yellow flowers, on the other hand, appears to involve suppression of both anthocyanidin genes and the malonyltransferase gene.

3. Experimental

3.1. Plant materials

Flowers and flower buds of *D. variabilis* were collected at Minami-Kyushu University.

3.2. Chemicals

Authentic anthocyanins and pigments 1 to 6 were isolated and purified as in Yamaguchi, Terahara, and Shizukuishi (1990) and Yamaguchi et al. (1996). Aliphatic CoA-esters were from Sigma.

3.3. Enzyme preparation

Two to four flower buds were collected immediately before flowering. Petals (1.0 g) of these flower buds were homogenized in an ice-chilled mortar with 0.8 g quartz, 1.0 g PVP and 3 ml of KP_i buffer (pH 7.0) containing 10 mM L-cysteine. The following steps were performed at 5°C: the homogenate was centrifuged for 5 min at 10000*g* twice, with the resulting crude extract freed from low M_r substances by filtration over a Sephadex G-50 column (1.0 × 10 cm), which had been equilibrated with the same buffer. The enzyme preparation could be stored at −20°C for a few weeks without appreciable loss of enzyme activity.

Table 4
Substrate specificity of acyltransferase for CoA-esters

CoA-ester	Reaction product	Relative activity (%)[a]
Malonyl-CoA	cyanidin 3-(6″-malonylglucoside)	100
Succinyl-CoA[b]	cyanidin 3-succinylglucoside	7
Acetyl-CoA[b]	not detected	0

[a] The acyltransferase activity obtained with malonyl-CoA was expressed as 100%.
[b] The reaction product was analyzed by co-chromatography on HPLC using the acylated anthocyanin produced by succinyltransferase (Yamaguchi et al., 1995) or acetyltransferase (Ino & Yamaguchi, 1993).

3.4. Protein determination and enzyme assay

Protein was determined by the method of Bradford (1976). The standard reaction mixture contained 20 μl enzyme extract, 500 μM Cy 3G, 300 μM malonyl-CoA and 70 μl of 0.1 M KP_i buffer (pH 7.5) containing 10 mM L-cysteine in a total volume of 100 μl. After incubation of the reaction mixture for 20 min at 35°C, the reaction was terminated as in Folch, Lees, and Sloane-Stanley (1957). Anthocyanins in the upper phase were analyzed by HPLC and confirmed in part by TLC.

3.5. Characterization of enzymes

Enzymes in crude extracts from purple flowers were characterized by standard procedures (Ino & Yamaguchi, 1993; Ino et al., 1993), unless otherwise stated. Effects on acylation were tested using 1 mM each divalent ion or enzyme inhibitor. Substrate specificity for acyl acceptor was determined using 500 μM each anthocyanin and 300 μM malonyl-CoA. That for the acyl donor was determined using 500 μM Cy 3G and 300 μM (malonyl- and acetyl-CoA) or 400 μM (succinyl-CoA) CoA-esters.

3.6. Analytical methods

TLC was carried out on microcrystalline cellulose plates (Avicel SF, Funakoshi) in solvents, AHW

Table 5
Content of major anthocyanins and specific activity of malonyltransferase in cyanic and acyanic flowers of *Dahlia variabilis*

Flower colour	Content of major anthocyanin (%)[a]						Specific activity (10^{-1} pkat μg^{-1}) (substrate)	
	Pg 3G5G	Pg 3MG5G	Pg 3MG5MG	Cy 3G5G	Cy 3MG5G	Cy 3MG5MG	Pg 3G	Cy 3G
Purple	3.4	7.6	5.1	3.3	38.1	37.4	4.18	4.52
Red	8.5	5.8	7.5	4.5	23.7	44.5	3.67	4.37
Orange	8.9	1.0	10.2	3.7	19.7	54.1	2.99	3.52
Pink	12.5	1.1	5.6	3.7	14.7	56.7	3.06	4.13
Yellow	ND[b]	ND	ND	ND	ND	ND	0.21	< 0.1
White	ND	ND	ND	ND	ND	ND	2.98	2.62

[a] Content of major anthocyanins extracted from flower petals with 50% HOAc was determined by HPLC.
[b] Not detected.

(HOAc–HCl–H_2O) (15:3:82), BFW (*n*-BuOH–HCO_2H–H_2O) (4:1:2), BuHCl (*n*-BuOH–2 M HCl) (1:1), 10F (HCO_2H–H_2O) (1:9). HPLC (Hitachi LH 6200) was conducted at 520 nm on a Wakosil-II 5C18HG column (4.6 i.d. × 250 mm, 5 μm, Wako) with a linear gradient elution from 15 to 40% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O). FAB-MS spectra were recorded using a JEOL-SX102A in the positive mode with thioglycerol matrix. This experiment was performed only once because of limited amounts of samples, while all of those described above were done at least twice.

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