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METHYLATION OF ANTHOCYANINS BY CELL-FREE EXTRACTS OF FLOWER BUDS OF *PETUNIA HYBRIDA*

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Key Word Index—*Petunia hybrida*; Solanaceae; anthocyanins; *O*-methyltransferase; cinnamic acid.

Abstract—An *O*-methyltransferase activity which catalyses the methylation of anthocyanins was extracted from flowerbuds of *Petunia hybrida*. The methyltransferase uses *S*-adenosyl-L-methionine as methyl donor. Only anthocyanidin 3(*p*-coumaroyl)rutinosido-5-glucoside was methylated. No methylating activity towards anthocyanidins, anthocyanidin 3-glucosides, anthocyanidin 3-rutinosides, caffeic acid or *p*-coumaric acid could be detected.

INTRODUCTION

In flowers of *Petunia hybrida* glycosides of methylated as well as unmethylated anthocyanins are present [1]. Several studies have shown the presence of *O*-methyltransferases in plants which catalyse the methylation of hydroxycinnamic acids or flavonoids [2–5]. According to the early work by Hess [6], anthocyanins are very poor substrates for methylating enzymes in *Petunia hybrida*, whereas cinnamic acids act more efficiently. He suggested, therefore, that during biosynthesis methylated anthocyanins were formed from the incorporation of methylated cinnamic acids. This contradicts the model of biosynthesis of anthocyanins, based on genetic studies in *Petunia hybrida*, more recently postulated by Wiering [1]; he showed that in *Petunia hybrida* two pairs of duplicate genes, Mt1/Mt2 and Mf1/Mf2, are responsible for the methylation at the 3'- and 5'-positions, respectively, of the anthocyanin molecule (Fig. 1). These genes are only expressed if two other genes (Rt and Gf), responsible for glucosylation, rhamnosylation and acylation of the anthocyanin, are both present in the dominant allele. Thus, Wiering's [1] model implies that the methylation of anthocyanins is determined at the C-15-level.

In this paper we report biochemical evidence supporting this model. We show the existence of an enzyme methylating anthocyanins but not cinnamic acids.

RESULTS AND DISCUSSION

HPLC analysis

HPLC analysis proved to be a valuable tool in studying the enzymatic methylation of anthocyanins. The method has the advantage of being rapid and it makes the use of radioactively labelled compounds unnecessary. In Table 1 we present the retention times of various anthocyani(di)ns and cinnamic acid derivatives used in this study.

Methylation of anthocyanidin 3-(p-*coumaroyl*) *rutinosido-5-glucosides*

When a protein extract of the mutant W39 was used in the assay system, cyanidin 3-(*p*-coumaroyl)rutinosido-5-glucoside (cyanidin 3RGac5G) was methylated to the corresponding peonidin derivative. Accordingly, delphinidin 3RGac5G and petunidin 3RGac5G respectively were methylated to the corresponding malvidin derivative. The identity of the products was confirmed by TLC analysis of hydrolysed samples. In the absence of SAM or enzyme, no methylation was observed. The methylating activity was proportional to the amount of protein up to 0.5 mg/ml and was linear in time for at least 15 min. Saturating concentrations of anthocyanins were reached at 60 μM cyanidin 3RGac5G and at 30 μM petunidin 3RGac5G. The optimal concentration of SAM was 0.25 mM. A protein extract of the mutant M30 used under optimal conditions, did not catalyse methylation of the anthocyanins. This means that the methylating activity in this mutant homozygous recessive for the methylating genes, is less than 0.1% of the activity of W39. The relation between methylating activity and pH showed that maximal activity was achieved at pH 7.5. Half-maximal activity was found at pHs 6.8 and 10.0. The curve has a broad shoulder at higher pHs. The curve had the same shape when a mixture of phosphate, Tris and glycine were used, ruling out secondary effects due to different buffer systems. During flower-bud development the ratio between the activity at pHs 7.5 and 9.5 changes; the activity at higher pHs was more pronounced when younger buds were used as source of the enzyme. This might indicate the presence of at least two different methyltransferases. Without Mg^{2+} the activity was only 50% of the activity at standard conditions (4 mM Mg^{2+}). We have not calculated K_m values, because there are four genes controlling methylation of anthocyanins in *Petunia hybrida* and, most likely, more than one methylating enzyme.

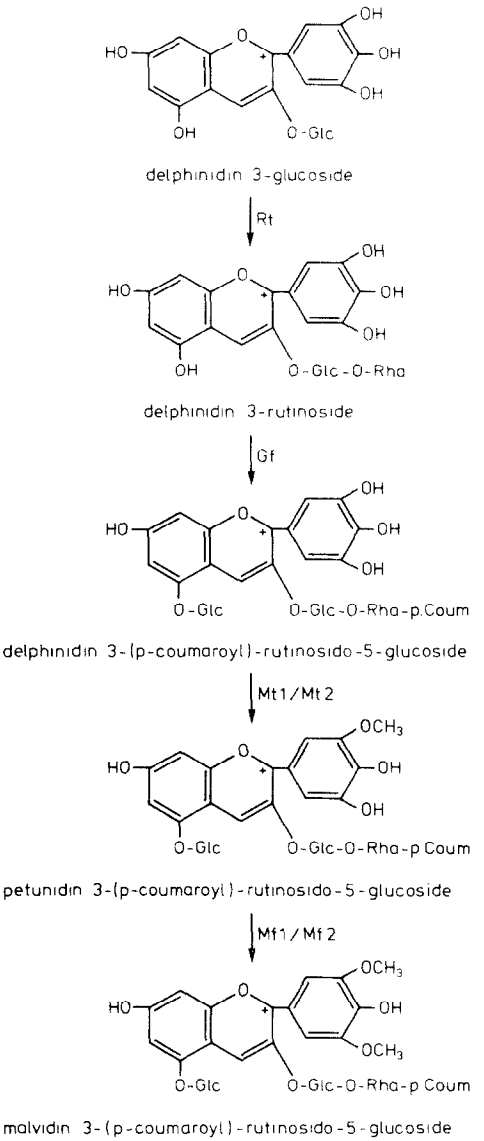

Fig. 1. Genes for glucosylation, rhamnosylation and methylation of anthocyanins in *Petunia hybrida*. Redrawn from ref. [1].

Substrate specificity

When the gene Rt is homozygous recessive, anthocyanidin 3-glucosides accumulate in the flowers. Anthocyanidin 3-rutinosides are found when the gene Gf is homozygous recessive (Fig. 1), even when dominant alleles of the methylation genes are present. When we used cyanidin 3-glucoside, cyanidin 3-rutinoside or cyanidin as substrate at 60 μM, no methylated product could be detected. At substrate concentrations of 1 mM small amounts of methylated products were found (Table 2). The incubations were carried out at three pHs: 7.5, 8.5 and 9.5. The methylation of cyanidin and cyanidin rutinoside at 1 mM only occurred at pH 7.5. *p*-Coumaric acid or caffeic acid used as substrates (60 μM and 1 mM) never gave rise to detectable amounts of methylated product. Only with anthocyanidin 3-(*p*-coumaroyl)rutinosido-5-glucosides was significant activity detected and at all three pHs. In these experiments the substrates were solubilized in EGME (ethylene glycol monomethyl ether). Control experiments with cyanidin 3RGac5G as substrate showed that the amount of EGME used (15 μl in 200 μl) did not inhibit the enzyme. We conclude that the methylating enzyme(s) show a high degree of substrate specificity. In soybean cell cultures [3] and tulip anthers [4] two methylating activities were found, one specific for substituted cinnamic acids and one for flavonoids. *Petunia* flower buds, however, show methylating activity only with anthocyanins as substrate. This is in agreement with the fact that flowers of this mutant do not contain any ferulic acid or sinapic acid, the most common methylated cinnamic acids. Thus methylation of anthocyanins in *Petunia hybrida* is under control of the genes Mt1/Mt2 and Mf1/Mf2 and takes place at the C-15-level. The methylating enzyme(s) responsible for this modification are very specific with regard to their substrate and only methylate(s) anthocyanidin 3-(*p*-coumaroyl)rutinosido-5-glucosides.

EXPERIMENTAL

Plant material. Clones of the mutants W39 and M30 of *Petunia hybrida* were grown in the greenhouse. W39 is homozygous recessive for the gene An3 [7] and dominant for one of the genes Mt1/Mt2 and Mf1/Mf2 [1]. M30 is homozygous recessive for the methylation genes.

Preparation of cell-free extracts. All steps were performed at 4°. Limbs of buds in developmental stage III according to ref. [8] were used. Ten limbs were homogenized in 2 ml 50 mM KPi buffer pH 7.5, containing 1.4 mM β-mercaptoethanol and Dowex 1 × 2 in an amount equal to the wet wt of the limbs. The limbs were homogenized for 2 min in the Ultra-turrax (Janke U. Kunkel, Ika-werk) and thereafter centrifuged (20 min, 38000 *g*) in a MSE 18 centrifuge. The supernatant was used for methyltransferase assay. Protein was determined using the Bio-Rad Protein Assay [9].

Methyltransferase assay. The standard assay consisted of 60 μM anthocyanin substrate in 15 μl 5 mM HCl, 0.5 mM *S*-adenosyl-L-methionine (SAM), 4 mM $MgCl_2$, 75 mM KPi pH 7.5 and 50 μl enzyme-extract in a total volume of 200 μl. For determining the activity at different pH values 75 mM KPi was used at pH 6.5–7.5, 75 mM Tris–HCl at pH 8.0–9.0 and glycine–NaOH at pH 9.5–10.5. The reaction was allowed to proceed for 3–10 min at 37° and was stopped by adding 800 μl of a mixture containing two parts of $CHCl_3$ and one part of MeOH–HCl 1% (v/v). Fifty μl of the upper phase of this Folch partition [10] was subjected to HPLC analysis. Quantitation of the product was done by measuring peak area. Controls contained homogenization buffer instead of enzyme-extract or were run without addition of SAM. Samples were run in duplicate.

Isolation of anthocyanins. Cyanidine 3-(*p*-coumaroyl)-rutinosido-5-glucoside, dephinidin 3-(*p*-coumaroyl)rutinosido-5-glucoside, petunidin 3-(*p*-coumaroyl)rutinosido-5-glucoside, cyanidin 3-rutinoside and cyanidin 3-glucoside were isolated from appropriate geno-types of *Petunia hybrida* [7]. Flower limbs were extracted overnight in MeOH–0.5% HCl at 20°. The extract was evaporated at 35° *in vacuo* and chromatographed on Whatman No. 3 paper in *n*-BuOH–HOAc–H_2O (4:1:5). The anthocyanin was extracted from the paper for 60 min with

Table 1. Retention times of phenolic compounds using HPLC analysis

Compound	Retention time (min) Elution with formic acid–methanol–water	Elution with acetic acid–methanol–water
Cyanidin	3.6	—
Peonidin	5.7	—
Cyanidin 3-glucoside	1.4	—
Cyanidin 3-rutinoside	1.6	—
Cyanidin 3RGac5G*	3.2	—
Peonidin 3RGac5G	4.6	—
Delphinidin 3RGac5G	2.4	—
Petunidin 3RGac5G	3.4	—
Malvidin 3RGac5G	4.6	—
p-Coumaric acid	—	7.4
Caffeic acid	—	3.9
Ferulic acid	—	9.6

See Experimental for a detailed description of the elution systems.
*3RGac5G—3-(*p*-coumaroyl)rutinosido-5-glucoside.

Table 2. Methylation of various phenolic compounds

Substrate	Concn in assay (m/M)	Sp. act. (pKat/mg protein)
Cyanidin	1	10
Cyanidin 3-glucoside	1	< 0.3
Cyanidin 3-rutinoside	1	17
Cyanidin 3RGac5G*	0.06	125
p-Coumaric acid	1	< 0.3
Caffeic acid	1	< 0.3

Incubations were carried out for 10 or 1 min (Cya 3RGac5G). The protein concentration was 0.5 mg/ml.
*3RGac5G—3-(*p*-coumaroyl)rutinosido-5-glucoside.

MeOH–0.5% HCl. This extract was evaporated and re-chromatographed on Whatman No. 3 paper with HOAc–conc. HCl–H_2O (15 : 3, 3 : 81.4). The anthocyanin was again extracted with MeOH–0.5% HCl and kept at −20°.

Identification of anthocyanidins. Anthocyanidins were identified by TLC after hydrolysis for 1 hr in 2 N HCl at 100°. Extracts were chromatographed on cellulose plates (Merck) with HOAc–conc. HCl–H_2O (30 : 3 : 10). Samples were run together with reference anthocyanidins obtained after hydrolysis of extracts from appropriate genotypes of *Petunia hybrida* [7].

HPLC analysis. For HPLC analysis a Perkin–Elmer series 3 HPLC equipped with a Lichrosorb 10RP 18 column (Chrompack, dimensions 24 × 0.4 mm) was used. The Perkin–Elmer LC 75 detector was applied. Anthocyanins were run for 7 min on a gradient between 17.5 and 25% MeOH in 10% HCO_2H in H_2O. The flow rate was 4 ml/min at 45° and extinctions were measured at 530 nm (derivatives of cyanidin) or 540 nm (derivatives of delphinidin). Cinnamic acid derivatives were analysed at isocratic elution with MeOH–HOAc–H_2O (12.5 : 5 : 82.5). The flow rate was 3 ml/min and extinctions were measured at 315 nm.

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