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FLAVANONES AND DIHYDROFLAVONOLS AS BIOSYNTHETIC INTERMEDIATES IN *MATTHIOLA INCANA*

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Key Word Index—*Matthiola incana*; Cruciferae; biosynthesis; flavanones; dihydroflavonols; precursors.

Abstract—In anthocyanin-producing flowers of *Matthiola incana*, the presence of naringenin, naringenin 7-glucoside, dihydrokaempferol and dihydrokaempferol 7-glucoside could be demonstrated. The four isolated compounds initiated anthocyanin synthesis after administration to acyanic flowers of genetically defined lines of *Matthiola incana* and *Antirrhinum majus*. Therefore, these compounds cannot be regarded as end-products but rather as intermediates in anthocyanin biosynthesis. Furthermore, naringenin 7-glucoside and dihydrokaempferol 7-glucoside most probably act as a pool for their aglycones, which serve as the actual substrates.

INTRODUCTION

Much chemical and genetic information concerning the biosynthesis or modification of flavonoids is available in *Matthiola incana*. Chemogenetic investigations revealed three complementary basic factors (e, f and g) for anthocyanin formation [1] and four genes (b, l, u and v) for anthocyanin modification [2]. Furthermore, a number of genes influencing the rate of anthocyanin synthesis are known [3, 4]. In spite of this appreciable chemogenetic information, the nature and sequence of the individual biosynthetic steps resulting in flavonol and anthocyanin production are still unknown for *Matthiola incana*. The demonstration of biosynthetic intermediates should contribute to a more detailed knowledge of the pathway of flavonoid synthesis. In this paper, the identification of flavanones and dihydroflavonols in flowers of an anthocyanin-producing line of *Matthiola incana* is described and their biosynthetic function is discussed.

RESULTS AND DISCUSSION

After extraction of the flowers with ethyl acetate, spectral measurements on the extract revealed a distinct peak at *ca* 287 nm (MeOH) probably corresponding to flavanones and/or dihydroflavonols. From many acyanic and cyanic lines tested for the presence and intensity of this peak, line 09, which is known to accumulate pelargonidin derivatives in very high concentration in flowers [5], was found to have the highest absorbance in this region.

Extracts of flowers of line 09 were separated on paper using 15% HOAc. The borohydride–HCl test for flavanones and dihydroflavonols [6] revealed four compounds. Compounds 1 and 3 (R_fs 0.43 and 0.67) gave intense red colour reactions, whereas compounds 2 and 4 (R_fs 0.56 and 0.80) gave only a weak light brown reaction. However, after zinc–HCl reduction, 2 and 4 produced a brilliant red colour on chromatograms which is characteristic for dihydroflavonols [7]. The four compounds were further purified chromatographically and identified by TLC in four solvents and by spectral analysis [8] in comparison with authentic samples. Compound 1 corresponded to naringenin and 2 to dihydrokaempferol; furthermore, bisulfite oxidation [9] of 2 yielded kaempferol. Acid hydrolysis and identification of the products by TLC proved that 3 and 4 were glucosides of 1 and 2, respectively. Treatment with β-glucosidase overnight also released the aglycones indicating that the sugar is β-linked. The spectrum of 3 (λ_{max}283 nm, MeOH) and 4 (λ_{max}287 nm, MeOH) did not show a shift after addition of sodium acetate indicating that the glucose residue in both compounds is attached at position 7 [8]. From these results, 3 is naringenin 7-β-glucoside and 4 is dihydrokaempferol 7-β-glucoside.

In all developmental stages of the flowers, dihydrokaempferol 7-glucoside accumulated in greater amounts than naringenin 7-glucoside. The concentration of the

Table 1. Anthocyanins formed from the compounds isolated and from authentic flavonoids after administration to acyanic flowers of genetically defined lines of *Matthiola incana* and *Antirrhinum majus*

Testlines	Compounds administered 1	2	3	4	NAR*	N7G*	DHK*	DHQ*
M. incana (line 18b)	Pg*	Pg	Pg	Pg	Pg	Pg	Pg	Cy*
A. majus (*inc eos* mutant)	—	Pg	—	Pg	—	—	Pg	Cy

* NAR = Naringenin; N7G = naringenin 7-glucoside; DHK = dihydrokaempferol; DHQ = dihydroquercetin; Pg = pelargonidin derivatives; Cy = cyanidin derivatives.

aglycones was always lower, but they were also found in ether extracts of fresh flowers prepared under liquid nitrogen and are therefore not formed from their glucosides during the extraction procedure.

The question of whether the four flavonoids isolated are real intermediates in anthocyanin biosynthesis, or possibly end-products, was investigated by administering them as precursors for anthocyanin formation to flowers of genetically defined acyanic lines, which are known to produce anthocyanins from flavanones and/or dihydroflavonols [10, 11] (Table 1). The ready conversion into anthocyanins proved that both the aglycones and their 7-glucosides are real intermediates. Furthermore, the formation of pelargonidin in flowers of the line 18b confirmed that in all four compounds the B ring is only hydroxylated in the 4'-position, and by using the *inc eos* mutant of *Antirrhinum majus*, which can only produce anthocyanins from dihydroflavonols [10], a clear distinction between 1 and 3 as flavanones and 2 and 4 as dihydroflavonols could be made (Table 1).

The possible biosynthetic interrelations of the four flavonoids and their conversion into anthocyanins are outlined in Fig. 1. Two different roles could be assigned to the 7-glucosides. In the first, they only act as a pool for their aglycones which are the real substrates; glucosylation of the aglycones to the 7-glucosides and hydrolysis of the glucosides to the aglycones would therefore occur in the flowers. In the second, the 7-glucosides might be directly involved in the anthocyanin pathway, namely in the sequence naringenin → naringenin 7-glucoside → dihydrokaempferol 7-glucoside → dihydrokaempferol. In this case, no reverse reaction from naringenin 7-glucoside to naringenin and no glucosylation of dihydrokaempferol to dihydrokaempferol 7-glucoside would be expected *in vivo*.

The biosynthetic interrelations of naringenin, naringenin 7-glucoside, dihydrokaempferol and dihydrokaempferol 7-glucoside were studied by administering them to acyanic flowers of line 20 (genotype: ee ff g^+g^+ bb), in which the anthocyanin pathway is blocked before flavanone synthesis (recessive f) and additionally after dihydroflavonol formation (recessive e). However, the conversion of flavanones to dihydroflavonols is not impaired in this line. Because both aglycones are readily glucosylated in the 7-position and both 7-glucosides can again be converted to the corresponding aglycones *in vivo* (Table 2), the 7-glucosides most probably act as a pool for their aglycones which serve as the immediate substrates for the anthocyanins. A similar interpretation has been proposed for the occurrence of dihydroflavonol glucosides in *Prunus mahaleb* [12] and *Petunia hybrida* [13]. Possibly the glucosides are more stable in the flowers than the free phenols or they are involved in transport.

Table 2. Presence and concentration of flavanones and dihydroflavonols in acyanic flowers of line 20 after administration of the four compounds isolated from flowers of line 09

Compounds administered	NAR*	N7G*	DHK*	DHK7G*
None	–	–	–	–
1 (NAR)	+	+++	++	++
2 (N7G)	+	++	++	+++
3 (DHK)	–	–	++	+++
4 (DHK7G)	–	–	++	++

Concentrations: – not detectable, + low, ++ medium, +++ high.

*NAR = naringenin; N7G = naringenin 7-glucoside; DHK = dihydrokaempferol; DHK7G = dihydrokaempferol 7-glucoside.

EXPERIMENTAL

Plant material. The line 09 (genotype: $e^+e^+f^+f^+g^+g^+$ bb) producing pelargonidin derivatives in the flowers was used for the isolation of flavanones and dihydroflavonols. The supplementation experiments were performed on acyanic flowers of the *inc eos* mutant of *Antirrhinum majus*, where the anthocyanin pathway is blocked between flavanones and dihydroflavonols [10], and on acyanic flowers of line 18b of *Matthiola incana* (genotype: e^+e^+ ff g^+g^+ bb). In flowers of line 18b, pelargonidin is produced after imbibition of naringenin or dihydrokaempferol and the synthesis of cyanidin can only be initiated by a suitable substituted precursor like dihydroquercetin. Line 20 of *Matthiola incana* (genotype: ee ff g^+g^+ bb) was used for studying the interrelations of the four isolated flavonoids. Because of two blocks in the anthocyanin pathway (recessive e and f), the flowers of line 20 are not only completely free of flavanones and dihydroflavonols, but also unable to use these intermediates for anthocyanin formation. The plant material was cultivated and harvested during the summer months of 1978 in the experimental garden of this institute.

Flavonoids. Naringenin, naringin, dihydroquercetin and kaempferol were obtained from Roth, Germany. Dihydrokaempferol was synthesized according to ref. [14]. Naringenin 7-glucoside was prepared by partial hydrolysis of naringin.

Chromatography. The following solvents were used: BAW, *n*-BuOH–HOAc–H_2O (4:1:5); CAW, $CHCl_3$–HOAc–H_2O (10:9:1); 15% HOAc and H_2O for flavonoids and BAW (6:2:2), and BEW, *n*-BuOH–EtOH–H_2O (4:1:2.2) for sugars.

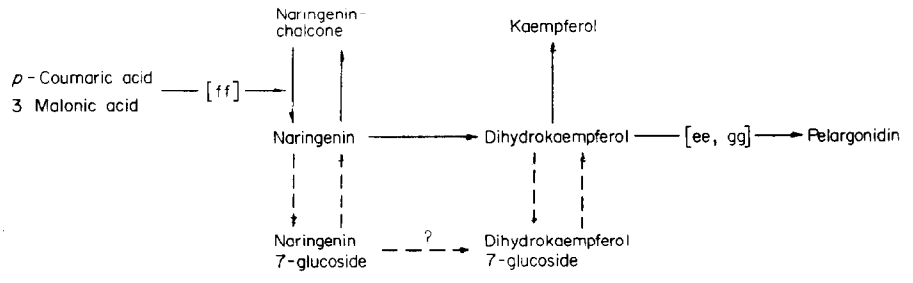

Fig. 1. The possible biosynthetic interrelations of the flavanones and dihydroflavonols isolated from flowers of line 09 and the position of the genetic blocks in the pathway of anthocyanin biosynthesis in *Mathiola incana*.

For the determination of R_f values, purified compounds and authentic samples were chromatographed on 0.1 mm cellulose TLC plates (Schleicher and Schüll). For sugars, 0.5 mm cellulose TLC plates were used.

Isolation and purification of compounds. The anthocyanin-containing parts of flowers and buds of line 09 were extracted with EtOAc (2 ×) within 48 hr at 4°. By using EtOAc as solvent, anthocyanins remained largely in the flowers while other flavonoids were readily extracted. The combined EtOAc extracts were concd and separated on Whatman 3MM paper with 15% HOAc. After colour reactions on small strips of the chromatogram, the parts containing flavanones or dihydroflavonols were eluted with MeOH. Further purification was achieved by successive chromatography on paper 2043b (Schleicher and Schüll) using BAW and H_2O.

Spectral analysis. The identification of the compounds by spectral measurements was performed according to ref. [8].

Hydrolysis of compounds. Methanolic soln (1 ml) containing a few mg of the isolated compound was hydrolysed by heating with 1 ml 10% HCl at 90° for 1 hr under N_2. Ether extraction of the hydrolysate yielded the aglycone which was identified by spectral analysis and co-chromatography with authentic samples. The extracted H_2O phase was concd and the sugar identified by co-chromatography with appropriate standards. Enzymic hydrolysis was performed according to ref. [8] with β-glucosidase (Serva).

Oxidation of dihydroflavonol to flavonol. The method described by Kho *et al.* [9] was used. The flavonol formed was identified by spectral analysis and co-chromatography with authentic flavonols.

Precursor experiments. Method 2 [11] was used for the administration of the compounds to acyanic flowers. After 24 hr incubation, the petals were washed with distilled H_2O in order to remove non-utilized precursors from the surface of the petals. After drying the petal surface, the products formed in the petals were extracted with 1% MeOH–HCl (in case of anthocyanins) or EtOAc (in case of flavanones and dihydroflavonols). Standard procedures were used for the identification of anthocyanidins [15]. Flavanones and dihydroflavonols were identified by co-chromatography with appropriate markers.

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