Modification of the B-ring during flavonoid synthesis in *Petunia hybrida*:
Effect of the hydroxylation gene Hfl on dihydroflavonol intermediates

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**Abstract.** The white flowering mutant W48 of *Petunia hybrida* is dominant for the hydroxylation gene Hfl and homozygous recessive for the hydroxylation gene Htl and the anthocyanin gene Anl. Flower buds of this mutant accumulate dihydrokaempferol glucosides. Thus the effect of Hfl being dominant is not the hydroxylation of the C15 skeleton, as is the case in mutants that are able to synthesize anthocyanins. This can be explained either by a feed-back inhibition of the hydroxylation by small amounts of dihydromyricetin (glucosides), or by a controlling effect of the gene Anl on the expression of Hfl. However, the white flowering mutant W58, which is homozygous recessive for the gene Anl and dominant for Hfl, accumulates dihydromyricetin (glucosides). This excludes a possible feed-back inhibition by dihydromyricetin and we conclude that Anl controls the expression of Hfl. Feeding of radioactive malonic acid to isolated flower limbs of mutants able to synthesize anthocyanins, leads to the incorporation of radioactivity into dihydrokaempferol (glucosides) and dihydroquercetin (glucosides). These results show that glucosylation of dihydroflavonols is a normal event in anthocyanin biosynthesis and is not induced by an inhibition of anthocyanin synthesis.

**Key words:** Anthocyanin – Flavonoid – Hydroxylation (flavonoids) – *Petunia*.

**Introduction**

A number of genes involved in anthocyanin synthesis in flowers of *Petunia hybrida* have been described (Wiering et al. 1974; Wiering et al. 1979). They are either involved in the biosynthesis of the C15 skeleton or determine the substitution pattern of the skeleton. The hydroxylation genes Htl and Hfl are responsible for the occurrence of anthocyanins derived from cyanidin or delphinidin resp.

In an earlier paper (Tabak et al. 1978), we demonstrated that the dominant gene Htl controls the hydroxylation of dihydrokaempferol (3,5,7,3' tetrahydroxylavone) to dihydroquercetin (3,5,7,3',4' pentahydroxyflavonane). If anthocyanin synthesis is blocked, as in mutants homozygous recessive for the gene Anl, the effect of Htl is the accumulation of dihydroquercetin (Kho et al. 1975, 1977).

In mutants with Anl homozygous recessive and Hfl dominant one could expect the accumulation of dihydroflavonols with three hydroxyl groups in the B-ring (dihydromyricetin). To test this hypothesis we isolated and characterized dihydroflavonols from flower buds of white flowering mutants homozygous recessive for the genes Anl or An6. The gene An6, as the gene Anl, controls the conversion of dihydroflavonols to anthocyanins (Gerats and Schram, manuscript in preparation).

**Material and methods**

Clones of mutants of *Petunia hybrida* were cultivated in the greenhouse. Mutant W58 was obtained from the "Station d'amélioration des plantes", Dijon, France. This mutant is homozygous recessive for the gene An6 (see Table 1).

For complementation experiments the technique developed by Kho et al. (1975) was used. Limbs of flower buds were cultured for 48 h in petri dishes with 3 ml sterile B2 medium in the dark at 24°C, during which time the substrates were dissolved in the B2 medium. The solutions were sterilized by membrane filtration (Sartorius SM 11307, pore size 0.2 µm). After 48 h of incubation the limbs were washed in distilled water and extracted with 3 ml methanol-HCl (1%), for 1 h at room temperature. The anthocyanin content was determined spectrophotometrically at 530-540 nm.

After determination of the optical densities, the extracts were concentrated to a volume of 1-2 ml. An equal volume of 2 M HCl was added and the mixture was heated for 40 min at 100°C to hydrolyze the anthocyanins. The anthocyanidins were extracted with a few drops of isooamyl alcohol and the extract chromatographed on thin layers of cellulose using as solvent systems propanol-2: 2 M HCl (1:1, v/v) and acetic acid:water:HCl (30:10:3, by
vol.). Relative amounts of the anthocyanidins were measured by scanning the thin layers with a scanning photometer (Zeiss KM 3) at 535 nm.

Dihydroflavonols were purified by fractionating the methanolic extracts on a Sephadex LH20 column (40-4.5 cm) with methanol. Fractions of 4 ml were collected and analyzed on cellulose TLC plates (Merck, FRG) using butanol: acetic acid: water (4:1:5, by vol., upper phase) as the eluant. Dihydroflavonols were further chromatographed on Whatman 3 paper with propanol-2: water (1:1, v/v) as the solvent. Methanol eluates (10 ml) of 2-cm-wide strips of the paper were evaporated and used in complementation experiments. The eluates were finally purified on a Sephadex LH20 column and chromatographically pure compounds were obtained, which could be identified by co-chromatography with reference substances on thin layers of cellulose (E. Merck, FRG). The solvent systems used were butanol: acetic acid: water (4:1:5, by vol., upper phase), chloroform: acetic acid: water (50:45:5, by vol.) and 15% acetic acid (15% HAc). The colorless spots were made visible by spraying with a freshly prepared 0.5% aqueous solution of Echtblausalz B (Merck, FRG).

To obtain information on the ratio between quantities of 7- and 4'-glucosides in flowerbuds, methanolic extracts of flowerbuds were chromatographed on Sephadex LH20. Ratio’s between 7- and 4'-glucosides were obtained by scanning thin-layer chromatograms (cellolose, 15% HAc as eluant) of the individual column fractions with a scanning Photometer (Zeiss KM 3) at 290 nm.

Acid hydrolysis of the dihydroflavonol-glycosides was carried out in a mixture of methanol and 2 M HCl (1:1, v/v) in a fused tube kept in a boiling water bath for 20 min. After ether extraction, the aglycone was identified by thin-layer chromatography with appropriate standards.

The extracted water phase was neutralized by shaking with a solution of 20% (v/v) di-n-octylmethylamine in chloroform. The sugars could be identified by co-chromatography with appropriate standards on thin layers of cellulose. The solvent systems were butanol - benzene - pyridine - water (60:10:30:24, by vol., and butanol - ethanol - water (40:10:22, by vol.).

Feeding experiments with radioactive malonic acid were performed as follows: The limbs of 60 flower buds (25 mm) were rinsed in sterile distilled water, dried on filter paper, and then placed side by side in petri dishes of 60-15 mm. After the addition of 3.77 × 10^6 Bq [C^14]malonic acid in 1 ml sterile distilled water, the petri dishes were placed in a closed glass box of 110.60 mm. During incubation for 3 h at 24 °C in the light, all the water in the petri dishes was absorbed by the flower limbs. After thoroughly washing with distilled water and drying with filter paper the limbs were extracted with acetone. To these extracts purified dihydroflavonols or dihydroflavonol-glucosides were added as a carrier. From these extracts, dihydroflavonols were isolated as described above.

Samples were counted in 10 ml toluene, containing 2,5-di-phenyl oxazole (2 g l^{-1}) and 1,4-bis-(4-methyl-5-phenyl-oxasole-2-yl)-benzene (25 mg l^{-1}) in a Packard liquid scintillation counter.

Chemicals. (+) dihydroquercetin was obtained from Carl Roth A.G., Karlsruhe, FRG. Dihydrokaempferol-7-glucoside and dihydrokaempferol-4'-glucoside were isolated from appropriate genotypes of Petunia hybrida. Dihydrokaempferol was synthesized as described before (Tabak et al. 1978). Radioactive malonic acid was purchased from Amersham (U.K.).

Table 1. Genotypes of mutants of Petunia hybrida

<table>
<thead>
<tr>
<th>Mutant</th>
<th>An1</th>
<th>An3</th>
<th>An6</th>
<th>Ht1</th>
<th>Hf1</th>
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<tr>
<td>W48</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0</td>
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<tr>
<td>W37</td>
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+ = dominant, - = homozygous recessive

Results and discussion

The mutant W48 is homozygous recessive for Anl and dominant for Hf1. This mutant was expected to accumulate dihydroflavonol with hydroxyflavonoids in position 3', 4', and 5' in the B-ring. To confirm this hypothesis we extracted 750 corollas of this mutant with methanol and fractionated this extract on Sephadex LH20. The elution profile (Fig. 1) showed two peaks when monitored at 290 nm. The separate fractions were examined for the presence of substrates (dihydroflavonols) for anthocyanin synthesis. Only the second peak contained precursor material. Further purification of the pooled fractions of the second peak using paper chromatography indicated the presence of two compounds which differed slightly in their absorption maxima, viz. 286 nm and 292 nm. These two compounds were finally purified on a Sephadex LH20 column. (dimensions 40-4.5 cm.) with methanol as the solvent. Acid hydrolysis of these purified intermediates showed that in both cases the agly-