

BIOSYNTHESIS OF PROANTHOCYANIDINS IN BARLEY: GENETIC CONTROL OF THE CONVERSION OF DIHYDROQUERCETIN TO CATECHIN AND PROCYANIDINS

by

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The conversion of dihydroquercetin to catechin and procyanidin was studied in maturing wild type barley (*Hordeum vulgare* L., cv. Nordal) seeds and proanthocyanidin free mutants blocked in four different genes, *ant 13*, *ant 17*, *ant 18* and *ant 19*. In the wild type barley grown under controlled conditions, maximal rate of synthesis of catechin, procyanidin B3 and procyanidin C2 occurred 8-16 days after flowering. Dihydroquercetin was radioactively labelled by feeding (¹⁴C)-acetate and (2-¹⁴C)-acetate to flowerbuds of a petunia mutant accumulating this flavonoid. When fed to pericarp-testa tissue of wild type barley labelled catechin, procyanidin B3 and procyanidin C2 were synthesized establishing dihydroquercetin as a precursor of these compounds. In addition labelled 2,3-*trans*-3,4-*cis*-leucocyanidin was synthesized indicating that this compound is an intermediate. The leucocyanidin was identified by co-chromatography with an authentic standard prepared chemically by reduction of dihydroquercetin with NaBH₄. The major product of this reduction, however, was the 2,3-*trans*-3,4-*trans*-leucocyanidin. Only mutant *ant 18*-102 accumulated dihydroquercetin in the seeds. Feeding (¹⁴C)-dihydroquercetin to pericarp-testa tissue from the mutants revealed that *ant 17*-139 was capable of synthesizing significant amounts of labelled catechin and procyanidin, whereas *ant 13*-101, *ant 13*-152, *ant 18*-102 and *ant 19*-109 synthesized none or only very small amounts of these compounds. It is concluded that (i) *ant 18* controls the reduction of dihydroquercetin to 2,3-*trans*-3,4-*cis*-leucocyanidin, (ii) *ant 19* controls the reduction of the leucocyanidin to catechin, and (iii) *ant 13* and *ant 17* control unidentified steps prior to dihydroquercetin.

Abbreviations: BAW = n-butanol-acetic acid-water; BW = n-butanol-water; CAW = chloroform-acetic acid-water; ¹H NMR = proton nuclear magnetic resonance; HOAc = acetic acid; HPLC = high pressure liquid chromatography; MS = mass spectroscopy; sBAWC = s-butanol-acetic acid-water-chloroform; 3,4-*cis*-diol = (2R,3S,4S)-3,4,5,7,3',4'-hexahydroxyflavan; 3,4-*trans*-diol = (2R,3S,4R)-3,4,5,7,3',4'-hexahydroxyflavan; TLC = thin layer chromatography; UV = ultra violet; WsB = water saturated s-butanol.

1. INTRODUCTION

Proanthocyanidins (condensed tannins) are widely distributed in the plant kingdom (21, 23). Those of barley are located in the seed, and are based exclusively on two flavan-3-ol units, namely (+)-catechin and (+)-gallocatechin, which give rise to procyanidins and prodelphinidins (29, 33, 34). Their synthesis and that of the anthocyanins in barley are controlled by the *ant* genes (27, 28, 29). Nineteen complementing genes, *ant 1* to *ant 19*, have been identified by JENDE-STRID (28). Mutants in *ant 13*, *ant 17* and *ant 18* are blocked in both proanthocyanidin and anthocyanin syntheses, mutants in *ant 19* are blocked only in the proanthocyanidin syntheses, whereas mutants in *ant 1* to *ant 12* and *ant 14* to *ant 16* are blocked only in anthocyanin synthesis (28).

The enzymology of flavonoid biosynthesis is well established from the level of phenylalanine to dihydroquercetin (10). Though these studies have dealt with the biosynthesis of flavonoids other than proanthocyanidins, tracer experiments using phenylalanine (40), cinnamic acid (26), chalcone (35), and dihydrokaempferol (44) have demonstrated that procyanidins are synthesized according to the general scheme for flavonoid formation. Dihydroquercetin is a known precursor of cyanidin (13) and is also believed to be a precursor of catechin and procyanidins (18, 22, 23, 26, 37, 39), but this has not been confirmed by tracer experiments. Little information is available concerning the biosynthetic steps leading from the presumed dihydroquercetin precursor to catechin and procyanidins. The latter compounds are, however, easily synthesized nonenzymatically by reduction of dihydroquercetin and condensation of the resulting flavan-3,4-diol with catechin in acid (4, 8, 9, 11). Enzymatic reduction of dihydroquercetin to a flavan-3,4-diol has been reported by STAFFORD and LESTER (41).

As a first step toward preparing cell free systems containing the enzymes involved in catechin and procyanidin synthesis, the fate of (¹⁴C)-dihydroquercetin fed to seeds of wild type was studied. The results presented establish dihydroquercetin as a precursor of catechin and procyanidins. Furthermore, the sites of action of *ant 13*, *ant 17*, *ant 18* and *ant 19* gene products

in the biosynthetic pathway were established relative to dihydroquercetin on the basis of dihydroquercetin contents in the mutant seeds and the capabilities of the latter to convert (¹⁴C)-dihydroquercetin into catechin and procyanidins.

2. MATERIALS AND METHODS

2.1. Plant materials

2.1.1. Barley

Wild type barley (*Hordeum vulgare* L., cv. Nordal) and the proanthocyanidin deficient mutants *ant 13*-101, *ant 17*-139, *ant 18*-102 (all induced in Nordal), *ant 19*-109 (induced in Alf) and *ant 13*-152 (induced in Triumph) were used in the present study. The barley plants were grown in a Weiss Model 20 RB/5-JU-P growth chamber in continuous light (24,000 lux) supplied by Osram HQI-E, 400 W/D lamps under a thermoperiod of 16 hours at 17 °C and 8 hours at 12 °C.

2.1.2. *Petunia mutant W78*

The white flowering petunia mutant W78 (*Petunia hybrida* Hort.) is blocked in the biosynthesis of anthocyanins in the petals. The genotype of W78 is *an1an1Ht1Ht1hf1hf1ffl* which results in accumulation of dihydroquercetin-7-glucosides and dihydroquercetin-4'-glucosides (14, SCHRAM pers. comm.). Seeds of W78 were a kind gift from Dr. A.W. SCHRAM, University of Amsterdam, The Netherlands. The petunia plants were cultivated in a greenhouse, maintained at 20 °C with supplemental light during the winter months from Osram HQI-E, 400 W/D lamps.

2.2. Chemicals

(+)-Dihydroquercetin was obtained from Serva (Heidelberg, West Germany). (+)-Catechin and NaBH₄ were purchased from Fluka AG (Buchs, Switzerland). β-Glucosidase isolated from almonds was from Sigma (St. Louis, USA). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). Sodium (1-¹⁴C)-acetate (57.0 mCi×mmol⁻¹) and sodium (2-¹⁴C)-acetate (58.3

mCi · mmol⁻¹) were purchased from the Radiochemical Centre (Amersham, England). Standards of procyanidin B3 (catechin-(4 α → 8)-catechin, nomenclature as suggested in ref. 24), procyanidin C2 (catechin-(4 α → 8)-catechin-(4 α → 8)-catechin), prodelphinidin B3 (gallo catechin-(4 α → 8)-catechin), trimer-1 (gallo catechin-(4 α → 8)-gallo catechin(4 α → 8)-catechin) and trimer-2 (gallo catechin-(4 α → 8)-catechin-(4 α → 8)-catechin), all isolated from barley (33, 34), and dihydromyricetin were a kind gift from cand. polyt. H. OUTTRUP, Department of Brewing Chemistry, Carlsberg Research Laboratory.

2.3. Chromatography

2.3.1. High Pressure Liquid Chromatography (HPLC)

Analyses were performed using a Waters Assoc. (Milford, Mass., USA) instrument composed of two Model 6000A solvent delivery systems, a WISP Model 710B sample injector, a Model 450 wavelength detector, a data module and a Model 720 system controller. Four HPLC systems were used for separation of flavonoids:

I. A μ Bondapak phenyl column, 30 cm \times 3.9 mm (Waters Assoc.), eluted with a linear gradient from 2% to 10% acetic acid in 30 min and maintained at 10% acetic acid for 25 min. The flowrate was 1 ml · min⁻¹ (modified from ref. 29).

II. A μ Bondapak C₁₈ column, 30 cm \times 7.9 mm (Waters Assoc.), eluted isocratically for 10 min with methanol:acetic acid:water (20:5:75, v/v/v) and then isocratically for 20 min with methanol:acetic acid:water (40:5:55, v/v/v). The flowrate was 2 ml · min⁻¹.

III. A μ Bondapak C₁₈ column, 30 cm \times 7.9 mm (Waters Assoc.), eluted with a linear gradient from methanol:acetic acid:water (20:5:75, v/v/v) to methanol:acetic acid:water (40:5:55, v/v/v) in 20 min and maintained at the latter for 10 min. The flowrate was 2 ml · min⁻¹.

IV. A μ Bondapak C₁₈ column, 30 cm \times 3.9 mm (Waters Assoc.), eluted with a linear gradient from 2% to 10% acetic acid in 30 min. The flowrate was 2 ml · min⁻¹.

The chromatography was carried out at 20 °C, and elution was monitored spectrophotometri-

cally at 280 nm in all systems. Concentrations of flavonoids were calculated from the peak areas. Table I shows elution volumes of proanthocyanidins and suspected intermediates of proanthocyanidin biosynthesis in the appropriate systems.

2.3.2. Thin layer chromatography (TLC)

Analytical separations were performed on Merck (Darmstadt, West Germany) precoated cellulose F₂₅₄ plates (0.1 mm) unless otherwise stated. Preparative plates (0.5 mm) were prepared from a slurry of Avicel-cellulose (Merck). The following solvent systems were used for separation of proanthocyanidins and suspected intermediates of proanthocyanidin biosynthesis: (i) CAW, CHCl₃:acetic acid:H₂O (10:9:1, v/v/v), (ii) BAW, n-butanol:acetic acid:H₂O (4:1:5, v/v/v, upper phase), (iii) 15% acetic acid (HOAc) (v/v), (iv) sBAWC, s-butanol:acetic acid:H₂O:CHCl₃ (7:1:2:1 v/v/v/v), (v) 6% acetic acid (HOAc) (v/v), (vi) BW, n-butanol:H₂O (7:2, v/v), (vii) WsB, H₂O-saturated s-butanol. Pertinent R_f values are given in Table I.

Flavonoids were visualized under UV-light or by spraying with a mixture of 1% (w/v) FeCl₃·6H₂O and 1% (w/v) K₃Fe(CN)₆ (1:1, v/v) which produces a blue color with most phenolics (38). Catechin and proanthocyanidins were also detected by spraying with a mixture of 5% (w/v) vanillin in ethanol and concentrated HCl (4:1, v/v) which produces a red color with these compounds (32).

2.4. Determination of catechin and proanthocyanidin content in maturing wild type barley seeds

Spikes were collected at regular intervals after flowering, the awns were cut off, and the spikes were frozen in liquid nitrogen and stored at -20 °C until used for extraction similar to that in ref. 29. 50 seeds (0.57-5.0 g) from each developmental stage were homogenized with a Polytron Type PT 10-35 homogenizer in 25 ml 75% (v/v) aqueous acetone. The slurry was centrifuged for 10 min at 25,000 \times g and the pellet reextracted twice with 25 ml 75% acetone. The combined extracts were filtered and delipidated

Table I.

Elution volumes on HPLC and R_f values on TLC of proanthocyanidins and suspected intermediates of proanthocyanidin biosynthesis. The chromatographic systems are detailed in section 2.3.

Flavonoid	Elution volume (ml)				R_f value						
	I	II	III	IV	CAW	BAW	15% HOAc	6% sBAWC	HOAc	BW	WsB
Catechin	25.2	-	-	24.4	-	0.71	-	0.66	-	-	-
Procyanidin B3	23.4	-	-	19.9	-	-	-	0.44	-	-	-
Procyanidin C2	29.3	-	-	21.3	-	-	-	0.33	-	-	-
2,3-trans-3,4-trans- Leucocyanidin	-	-	-	21.6	-	0.69	-	0.61	0.46	0.59	0.71
2,3-trans-3,4-cis- Leucocyanidin	-	-	-	14.0	-	0.69	-	0.48	0.45	0.48	0.58
Dihydroquercetin	-	40.2	29.7	49.9	0.34	0.84	0.53	0.86	-	-	-
Dihydromyricetin	-	-	-	29.4	-	-	-	0.75	-	-	-
Prodelphinidin B3	-	-	-	12.6	-	-	-	0.32	-	-	-
Trimer-1	-	-	-	11.3	-	-	-	0.14	-	-	-
Trimer-2	-	-	-	15.5	-	-	-	0.24	-	-	-

with 3×25 ml petroleum ether (bp. 40-60 °C). The acetone was removed from the extract in vacuo at 30 °C. Five ml 96% ethanol was added to prevent precipitation and the volume was adjusted to 25 ml with water. 100 µl was subjected to HPLC analysis in system I (section 2.3.1) and the concentrations of catechin, procyanidin B3 and procyanidin C2 were calculated from peak areas. The identities of peaks were confirmed by the vanillin test (section 2.10) and by the conversion of procyanidins to cyanidin upon hydrolysis (section 2.10).

2.5. Isolation of dihydroquercetin from barley seeds

Wild type, *ant 13-101*, *ant 17-139*, *ant 18-102* and *ant 19-109* seeds (28 days after flowering) were assayed for the presence of dihydroquercetin. 200 seeds were homogenized with the Polytron in 100 ml acetone. The slurry was centrifuged for 10 min at 20,000 × g and the pellet was reextracted twice with 50 ml acetone. The combined extracts were filtered and evaporated to dryness in vacuo at 30 °C. The residue was dissolved in 10 ml methanol and hydrolyzed with 10 ml 2 M-HCl for 15 min at 100 °C to produce the aglucone. The hydrolysate was extracted with ether (4×20 ml). The ether extracts

were dried over Na_2SO_4 and evaporated to dryness in vacuo at 30 °C. The residue was dissolved in methanol and applied to a preparative TLC cellulose plate (0.5 mm) which was developed in CAW. The zone corresponding to dihydroquercetin was scraped off and the gel eluted with methanol (3×5 ml). After concentration to 400 µl an aliquot was subjected to HPLC analysis using system II (section 2.3.1).

2.6. Synthesis and isolation of (^{14}C)-dihydroquercetin

(^{14}C)-Dihydroquercetin was prepared by feeding ^{14}C -labelled acetate to excised flowerbuds of the dihydroquercetin accumulating petunia mutant W78. 25 buds were surface sterilized and dissected as described by KHO et al. (31). The corollas were incubated in a petri dish (90×15 mm) with 10 ml petunia culture medium (31) containing 1 mCi sodium ($1\text{-}^{14}\text{C}$)-acetate and 1 mCi sodium ($2\text{-}^{14}\text{C}$)-acetate. The incubation was carried out for 24 hours at 24 °C and 2,500 lux supplied by an Osram HQI-E, 400 W/D lamp.

After incubation the buds were washed with water and homogenized in a mortar. The homogenate was extracted with 75% acetone (6×25 ml) and the combined acetone extracts were delipidated with 3×50 ml petroleum ether. The

extract was concentrated to a 10 ml water phase in vacuo at 30 °C and hydrolyzed overnight at 20 °C with 3 mg (18.9 units) of β -glucosidase to produce the aglucone of dihydroquercetin. Dihydroquercetin was extracted into ether (6 \times 10 ml). The ether extracts were dried over Na₂SO₄ and evaporated to dryness in vacuo at 30 °C. The residue was dissolved in 1 ml methanol and the labelled dihydroquercetin was finally purified by semi-preparative HPLC using system III (section 2.3.1). After removal of methanol in vacuo, the combined collected eluates were freeze-dried. The purified (¹⁴C)-dihydroquercetin was dissolved in methanol and stored at -20 °C.

2.7. Chemical synthesis of leucocyanidins

2.7.1. Synthesis and isolation of two isomeric flavan-3,4-diols

Leucocyanidins (3,4,5,7,3',4'-hexahydroxyflavans) were synthesized by reduction of dihydroquercetin (0.5 g) with NaBH₄ (0.25 g), and purified by chromatography on a Sephadex LH-20 column using ethanol, as described in detail by PORTER and FOO (36). Elution was followed by subjecting aliquots of the fractions to TLC analysis in 6% HOAc and HPLC analysis using system IV (section 2.3.1). The major 2,3-*trans*-3,4-*trans*-leucocyanidin isomer ((2R,3S,4R)-3,4,5,7,3',4'-hexahydroxyflavan = 3,4-*trans*-diol), also isolated by PORTER and FOO, eluted in fractions 22-39. After concentration of the pooled fractions in vacuo at 30 °C and rechromatography, a pure preparation of the 3,4-*trans*-diol was obtained, as judged from HPLC and TLC analyses.

A minor 2,3-*trans*-3,4-*cis*-leucocyanidin isomer ((2R,3S,4S)-3,4,5,7,3',4'-hexahydroxyflavan = 3,4-*cis*-diol), not isolated by PORTER and FOO, eluted in fractions 15-22. Fractions 15-19 were pooled and concentrated in vacuo at 30 °C. HPLC using system IV revealed only a few minor contaminating peaks. TLC analysis, however, showed the presence of many prominent components having greater R_f values than the 3,4-*cis*-diol and giving a positive reaction with the vanillin reagent. The 3,4-*cis*-diol was further purified by HPLC using system IV and immediately freeze-drying the eluate. Recoveries of 70-90% were obtained in this way. The final

preparation of the 3,4-*cis*-diol contained only a few minor, vanillin positive contaminants that migrated more slowly than the 3,4-*cis*-diol on TLC plates developed in BW and WsB (section 2.3.2). Presumably they are self-condensation products of the 3,4-*cis*-diol.

2.7.2. Methylation of leucocyanidins

To obtain 5,7,3',4'-tetramethyl ethers of the leucocyanidins needed for mass spectroscopy (MS), the two 3,4-diols were separately dissolved in methanol and methylated with an excess of diazomethane in ether for 48 hours at -15 °C. The reaction mixture was then evaporated to dryness under a stream of N₂. The residue was dissolved in acetone and applied to a Merck precoated kieselgel 60F₂₅₄ plate (0.25 mm) which was developed in benzene:acetone (4:1, v/v) (36). The methylation resulted in one major UV-absorbing band for each 3,4-diol. R_f values of 0.17 and 0.22 were obtained for the tetramethyl ether of the 3,4-*trans*- and 3,4-*cis*-diols, respectively. The tetramethyl ethers were eluted from TLC plates with acetone.

2.7.3. Condensation of the 3,4-*cis*-diol with catechin

To 124 nmol (¹⁴C)-catechin (5,300 cpm) in 1 ml water was added 37.5 nmol 2,3-*trans*-3,4-*cis*-leucocyanidin in five μ l methanol. The mixture was acidified to pH 1.2 with 10 μ l 8 M-HCl and incubated for 1 hour at room temperature. The flavonoids were extracted into ethyl acetate (5 \times 1 ml). The combined extracts were dried over Na₂SO₄ and evaporated to dryness under a stream of N₂. The residue was dissolved in methanol and applied to a TLC plate which was developed in sBAWC (section 2.3.2). The plate was scanned for radioactivity and the radioactive products formed were identified as described in section 2.8. A control lacking the 3,4-*cis*-diol was included.

2.8. Administration of (¹⁴C)-dihydroquercetin to barley seeds and isolation of labelled products

Spikes were harvested 12 days after flowering

and 25 seeds were picked at random. The tissue consisting of pericarp and testa was isolated manually by removing the husk and squeezing out the endosperm and the adhering aleurone layer. The pericarp-testa tissue (0.46-0.52 g) was incubated in a 25 ml Erlenmeyer flask in 2 ml 1 mM-chloramphenicol solution containing 1 μCi (^{14}C)-dihydroquercetin (2.6 $\text{mCi} \cdot \text{mmol}^{-1}$). The incubation lasted 24 hours at 20 °C during which time 1,700 lux were supplied by an Osram HQI-E, 400 W/D lamp.

After incubation the tissue was washed with water and homogenized with the Polytron in 10 ml 75% acetone. 100 μg catechin, 100 μg procyanidin B3, 190 μg 3,4-*trans*-diol and 120 μg 3,4-*cis*-diol in 220 μl methanol were added as carriers. The slurry was centrifuged and the pellet extracted with 75% acetone (2 \times 5 ml). The combined extracts were delipidated with 3 \times 5 ml petroleum ether and concentrated to a 5 ml water phase in vacuo at 30 °C. The flavonoids were extracted into ethyl acetate (4 \times 5 ml). The combined ethyl acetate extracts were dried over Na_2SO_4 and evaporated to dryness in vacuo at 30 °C. The residue was dissolved in 100 μl methanol. Identification of labelled catechin and procyanidins, and quantitation of radioactivity in the products were carried out using two different methods:

(i) 25 μl of the final extract was applied to a TLC plate which was developed in sBAWC. The plate was scanned for radioactivity, and the radioactive zones corresponding to catechin, procyanidin B3 and procyanidin C2 were scraped off and the gel eluted with water (3 \times 2 ml). The samples were freeze-dried and redissolved in methanol. An aliquot was subjected to HPLC using system IV (section 2.3.1). The peaks were collected and counted.

(ii) 25 μl of the final extract was subjected to HPLC using system IV. One ml fractions were collected and counted to localize the radioactivity. Another 25 μl was then injected, and the appropriate fractions containing the labelled catechin, procyanidin and leucocyanidin peaks were collected. The samples were freeze-dried, dissolved in methanol and identified by TLC using sBAWC as the developing system.

2.9. Determination of radioactivity

TLC plates (5 \times 20 cm) were scanned for radioactivity using a Packard Model 7201 radiochromatogram scanner (Radio-TLC). Samples were counted in 10 ml of a scintillation liquid composed of 7.5 g 2,5'-diphenyloxazole (PPO), 0.15 g 2,2'-p-phenylen-bis-(5-phenyloxazole) (POPOP), 0.51 Triton X-100 per l of xylen using a Beckman LS 3155T liquid scintillation spectrometer. The counting efficiency of ^{14}C was 95.3%.

2.10. Other analytical methods

A vanillin reagent consisting of 1% (w/v) vanillin in concentrated HCl was used in a test tube assay to detect catechin and procyanidins (43). Procyanidins were also detected by the production of cyanidin upon hydrolysis with 5 M-HCl/ethanol for 20 min at 80 °C (29). MS was performed on a VG 7070 F instrument equipped with a VG 2035 Data System. The samples were inserted directly (200 °C, 70 eV).

3. RESULTS

3.1. Time course of catechin and procyanidin biosynthesis in maturing wild type barley seeds

To determine the developmental stage during barley seed maturation where maximal catechin and procyanidin synthesis occur, wild type seeds were collected at four day intervals from the time of flowering to 28 days after flowering when the seeds were physiologically mature. Acetone extracts of the seeds were analysed by HPLC and the concentrations of catechin, procyanidin B3 and procyanidin C2 were calculated from the peak areas. Figure 1 shows high pressure liquid chromatograms of acetone extracts of seeds collected 8, 16 and 28 days after flowering. The prodelphinidins known to be present in barley (29, 33, 34) were eluted within the first 22 ml. Since they were not satisfactorily separated from other UV-absorbing compounds, they are not included in the time course study.

Figure 2 shows the time course of catechin, procyanidin B3 and procyanidin C2 synthesis during seed maturation. Catechin appears in the seeds four days after flowering, increases rapidly

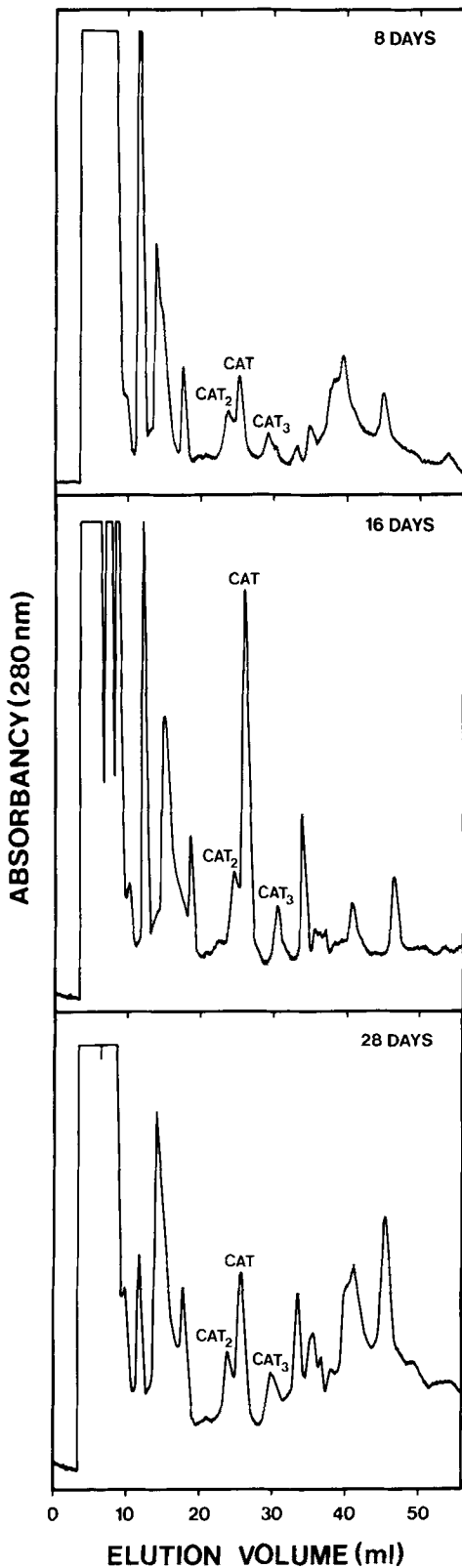


Figure 1. High pressure liquid chromatograms of acetone extracts of wild type barley seeds harvested 8, 16 and 28 days after flowering. Separation was carried out using HPLC system I (section 2.3.1). CAT = catechin, CAT₂ = procyanidin B3, CAT₃ = procyanidin C2.

and reaches a maximum of approximately 97 nmoles·seed⁻¹ 18 days after flowering. Then the content of catechin decreases, reaching a constant level of approximately 40 nmoles·seed⁻¹. The procyanidins B3 and C2 appear in the seeds 4-8 days after flowering and are synthesized until approximately 20 days after flowering. Then the contents of procyanidin B3 and procyanidin C2 remain constant at 9.7 and 4.3 nmoles·seed⁻¹, respectively. The biosyntheses of both catechin, procyanidin B3 and procyanidin C2 are in an almost linear phase 8-16 days after flowering and the rates of synthesis are 10, 0.9 and 0.5 nmoles·seed⁻¹·day⁻¹, respectively. Anthocyanins were visually detected in the husk from the eighth day after flowering.

That catechin appears first in the seed followed by procyanidin B3 and then procyanidin

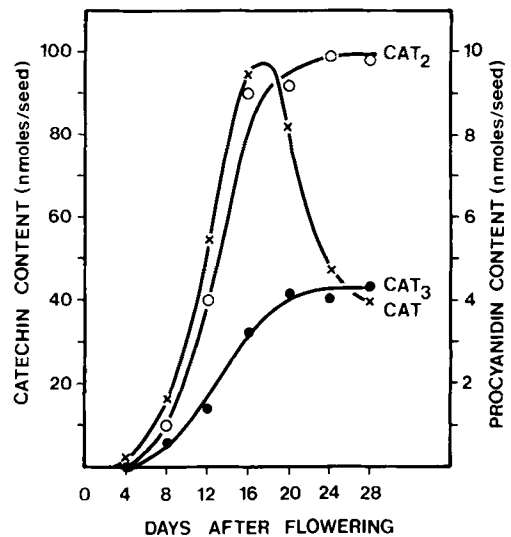


Figure 2. Time course of catechin and procyanidin biosynthesis in maturing wild type seeds. CAT = catechin, CAT₂ = procyanidin B3, CAT₃ = procyanidin C2.

C2 is in agreement with the idea that procyanidins in general are synthesized sequentially from monomer through dimer and trimer to higher polymers (19, 23).

The observed decline in catechin content after reaching a maximum is in agreement with other time course studies in barley and sorghum. BRANDON et al. (5) investigating field grown barley found that the most significant difference between a sample of seeds where chlorophyll was still present and a mature sample was the relatively high concentration of catechin in the former. In sorghum the decline in catechin content was found to be associated with the synthesis of procyanidins of higher molecular weights (15, 16, 19). The decrease in catechin concentration observed in the present study, however, was not associated with synthesis of procyanidin oligomers, since the contents of procyanidin B3 and procyanidin C2 remained constant. No procyanidins consisting of more than three catechin units have been identified in the Nordal barley used (29, 33, 34). Marked changes in the prodelfinidin content were not detectable during the decline in catechin content. The reason for the decrease in catechin concentration is unknown. Possibly catechin in contrast to the procyanidins is degraded by the plant when the synthesis of procyanidins has stopped. Some evidence exists that flavan-3-ols such as catechin are catabolized in vivo (3). Furthermore, STAFFORD et al. (40) have observed production of $^{14}\text{CO}_2$ upon feeding (^{14}C)-catechin to cell cultures of Douglas fir. Another explanation for the apparent decline in catechin content would be that catechin in barley is modified into yet unidentified forms that escape detection with the isolation procedure used, e.g. glucosylated (30) or esterified to gallic acid (42).

In the mature barley seeds, the content of catechin is much greater than that of procyanidin B3 which in turn is twice that of procyanidin C2. The presented data provide only very weak if any support for the proposal (17) that an increase in molecular weight of proanthocyanidins occurs upon tissue maturation. This is in marked contrast to the situation in sorghum where catechin and dimer (epicatechin-(4 β →8)-catechin) are only present in trace amounts and

polymeric procyanidins dominate in mature seeds (15, 16, 19). This time course study is, however, in agreement with the hypothesis (22) that a steady state concentration of procyanidins is maintained in the vegetative tissues of the plant after an initial burst of procyanidin synthesis.

3.2. Dihydroquercetin content in wild type and proanthocyanidin free barley mutants

As a first approach to localize the sites of action of *ant 13*, *ant 17*, *ant 18* and *ant 19* in the biosynthetic pathway to catechin and procyanidins in barley seeds, the genetic blocks were determined relative to the postulated dihydroquercetin intermediate.

Initially, seeds of *ant 13-101*, *ant 17-139*, *ant 18-102*, *ant 19-109* and wild type were assayed for the presence of dihydroquercetin as described in section 2.5. HPLC analysis of the extracts (Figure 3) revealed that *ant 18-102* was the only mutant accumulating dihydroquercetin and that trace amounts might exist in wild type. The identity of the major HPLC peak in the *ant 18-102* sample as dihydroquercetin was confirmed by co-TLC with an authentic standard in CAW, BAW and 15% HOAc. The content of dihydroquercetin in the *ant 18-102* mutant was 2.0 nmoles \cdot seed $^{-1}$. All was present as glucosides as judged from unhydrolyzed and hydrolyzed samples. The extent of dihydroquercetin accumulation in *ant 18-102* is much smaller than expected when compared to the contents of catechin, procyanidin B3 and procyanidin C2 in wild type seeds of the same developmental stage. Based on catechin units incorporated this content is 72 nmoles \cdot seed $^{-1}$. Whether the dihydroquercetin synthesized in *ant 18-102* seeds is (i) converted into other flavonoid products, e.g. quercetin glucosides, (ii) catabolized, as might be the case for catechin, or (iii) inhibited by an unknown regulatory mechanism which prevents an extensive accumulation of dihydroquercetin is unknown. The small but significant accumulation of dihydroquercetin in a mutant blocked in the biosynthesis of catechin and proanthocyanidins strongly supports the idea that dihydroquercetin

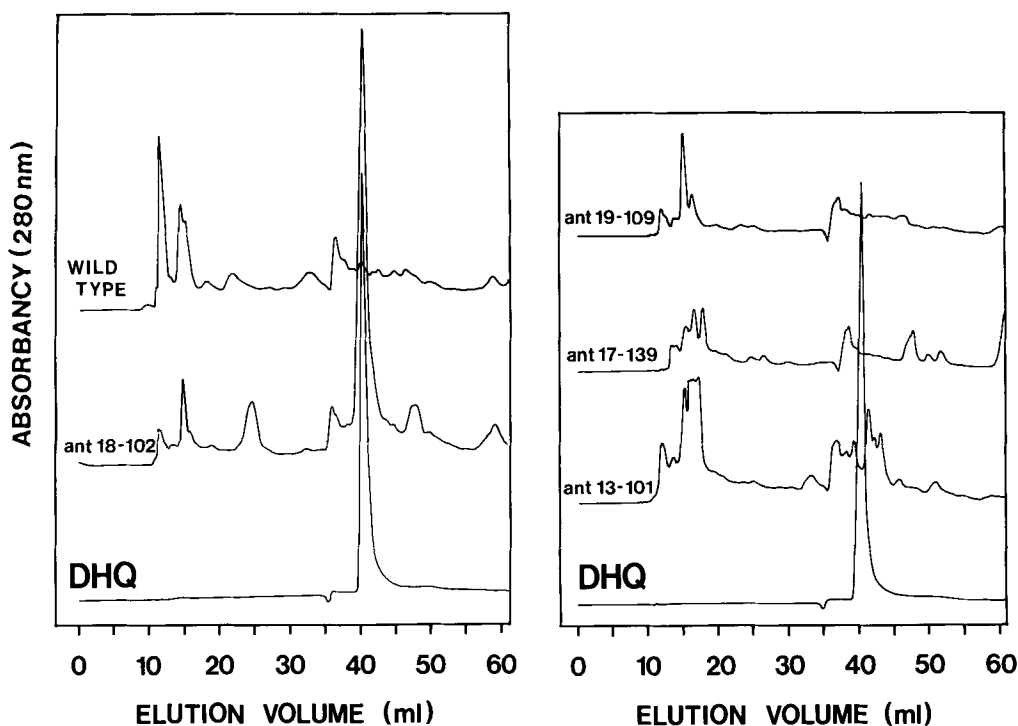


Figure 3. High pressure liquid chromatograms of extracts of wild type, *ant 18-102*, *ant 19-109*, *ant 17-139* and *ant 13-101* seeds harvested 28 days after flowering. Separation was carried out using HPLC system II (section 2.3.1) and injecting 100 μ l. DHQ = dihydroquercetin standard.

is an intermediate in the biosynthetic pathway leading to these compounds.

On the basis of the dihydroquercetin measurements, no conclusions can be drawn regarding the sites of action of *ant 13-101*, *ant 17-139* and *ant 19-109*.

3.3. Synthesis and identification of (14 C)-dihydroquercetin

Another way to localize the genetic blockages in the proanthocyanidin free mutants is to establish if any of the mutants are able to convert dihydroquercetin into catechin and procyanidins. For this purpose, and to establish dihydroquercetin as a precursor, (14 C)-dihydroquercetin was synthesized.

Ring A of the flavonoid skeleton is formed by a head-to-tail condensation of three molecules of malonyl-CoA which is synthesized from acetate, coenzyme A and CO_2 (10, 18, 20). (14 C)-Dihydroquercetin, uniformly labelled in

the A-ring (Figure 4), was thus prepared by feeding (14 C)-acetate and (14 C)-acetate to flowerbuds of the dihydroquercetin accumulating petunia mutant W78. The buds used were 25-35 mm in length. At this developmental stage the synthesis of dihydroquercetin has just entered the linear phase and only a small amount of dihydroquercetin is present. Time course studies on the incorporation of (14 C)-acetate into

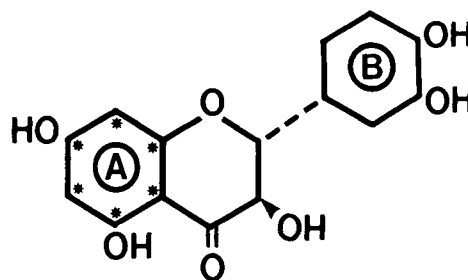


Figure 4. (14 C)-dihydroquercetin, uniformly labelled in the A-ring.

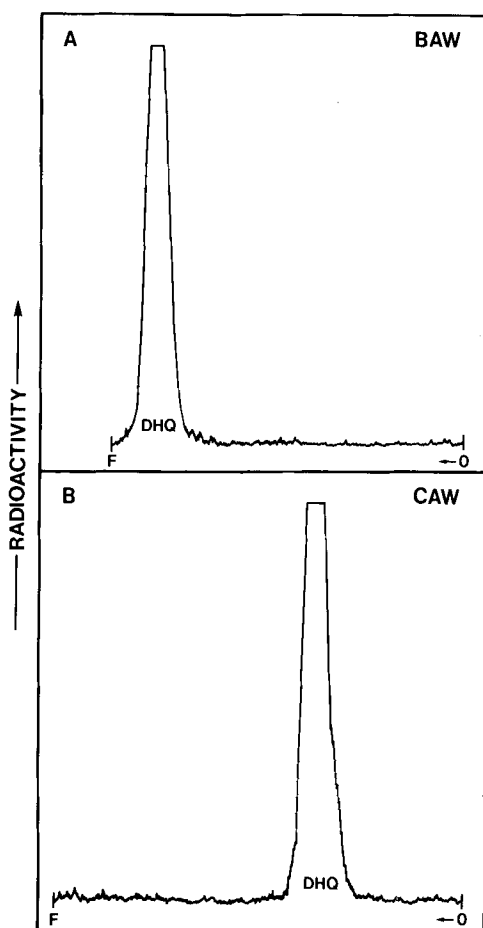


Figure 5. Radio-thin layer chromatograms of the purified (^{14}C)-dihydroquercetin, synthesized by feeding ($1\text{-}^{14}\text{C}$)-acetate and ($2\text{-}^{14}\text{C}$)-acetate to buds of petunia mutant W78. Plates spotted with ca. 200,000 cpm were developed in (A) BAW or (B) CAW (section 2.3.2). DHQ = dihydroquercetin, O = origin, F = solvent front.

dihydroquercetin revealed that an incubation period of 24 hours offered a reasonable compromise between the specific activity of dihydroquercetin, which reached a maximum after approximately 12 hours and then declined, and the total amount of radioactivity incorporated into dihydroquercetin.

25 buds took up 90% of the radioactivity when incubated for 24 hours with 2 mCi (^{14}C)-acetate. Approximately 40% of the radioactivity was found in the acetone extracts (see section 2.6), but the majority of this radioactivity was incor-

porated into lipids and was removed by extracting with petroleum ether. The yield of purified (^{14}C)-dihydroquercetin ranged from 44 to 52 μCi in three different experiments and specific activities of 2.6, 3.1 and 4.2 $\text{mCi} \cdot \text{mmol}^{-1}$ were obtained. The purity of the final (^{14}C)-dihydroquercetin preparation was checked by TLC (Figure 5) and by HPLC (Figure 6). Dihydroquercetin was found to be the only radioactively labelled compound in the preparation, but some minor UV-absorbing compounds were also present having slightly larger elution volumes than dihydroquercetin on HPLC. The labelled compound was simultaneously, conclusively identified as dihydroquercetin by co-chromatography with an authentic standard.

This *in vivo* synthesis of (^{14}C)-dihydroquercetin seems to be a satisfactory alternative to the *in vitro* synthesis of labelled flavonoids from ($2\text{-}^{14}\text{C}$)-malonyl-CoA and 4-coumaroyl-CoA (or caffeoyl-CoA), requiring chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase and, if using 4-coumaroyl-CoA, a flavonoid-3'-hydroxylase in addition (6, 12).

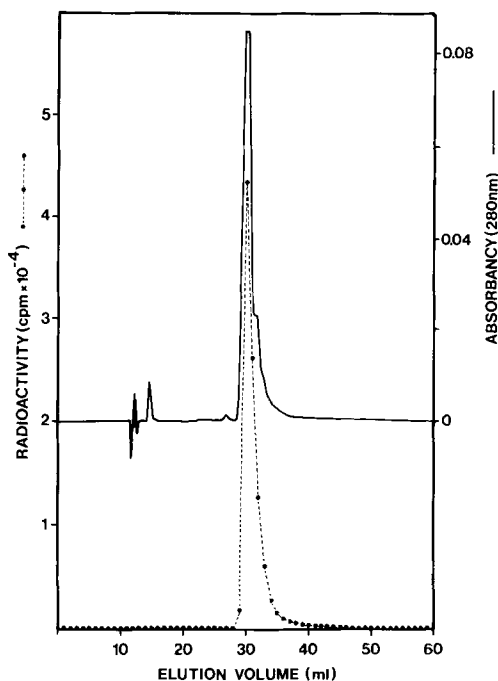


Figure 6. High pressure liquid chromatogram of the purified (^{14}C)-dihydroquercetin. HPLC system III (section 2.3.1) was used.

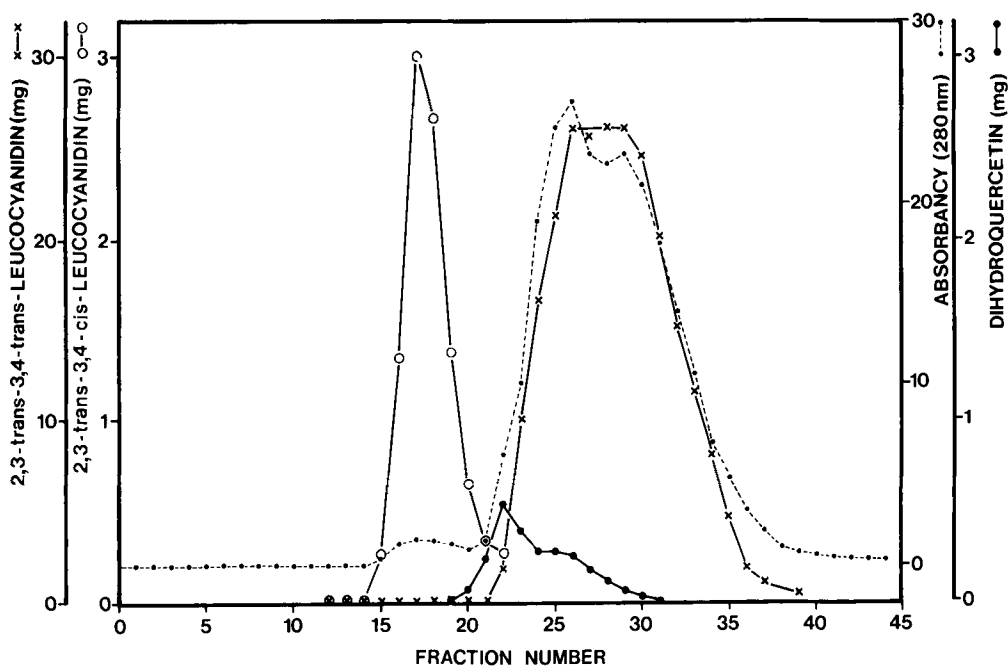


Figure 7. Elution profiles of 2,3-*trans*-3,4-*trans*-leucocyanidin, 2,3-*trans*-3,4-*cis*-leucocyanidin and dihydroquercetin which have been separated by a Sephadex LH-20 column (2.5×35 cm) eluted with ethanol. The flowrate was 16 ml · h⁻¹ and 16 ml fractions were collected. An aliquot of each fraction was analysed by HPLC using system IV (section 2.3.1) to determine the concentrations of the flavonoids.

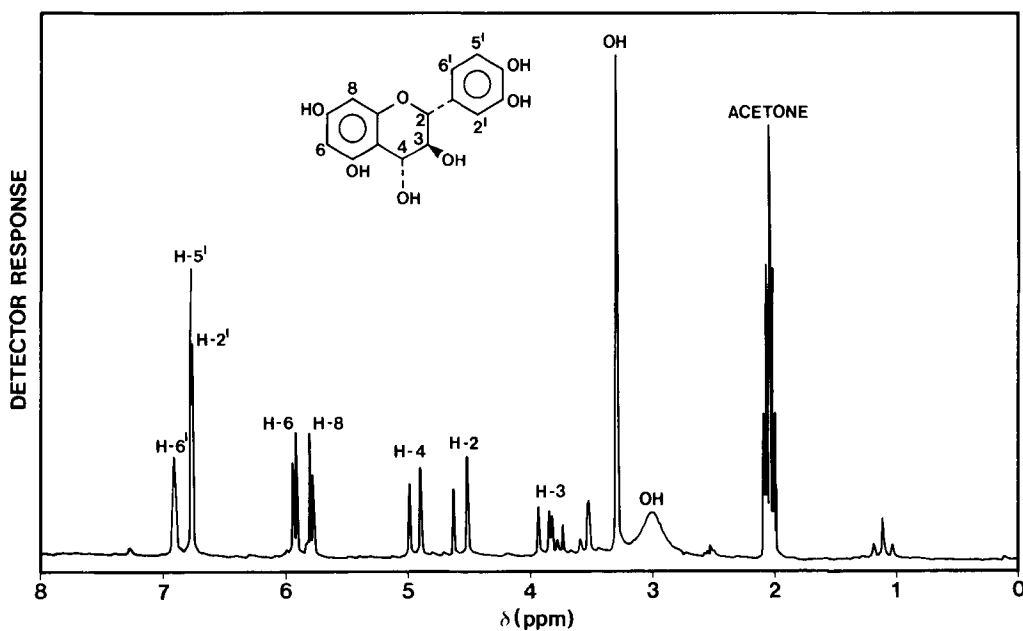


Figure 8. 90-MHz ¹H NMR spectrum of (2R,3S,4R)-3,4,5,7,3',4'-hexahydroxyflavan (2,3-*trans*-3,4-*trans*-leucocyanidin) in d₆-acetone at 20 °C. Acetone was used as internal standard.

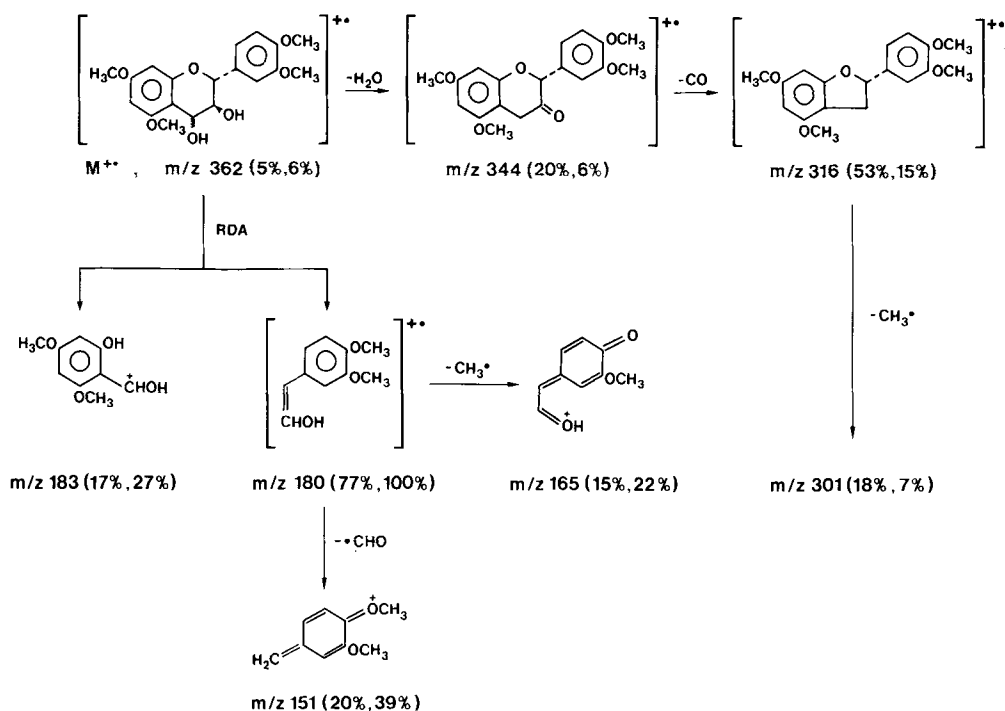


Figure 9. Origin of the important mass ion fragments of (2R,3S,4R and S)-5,7,3',4'-tetramethoxyflavan-3,4-diol. The relative intensities obtained are given in parentheses (that for the 3,4-*trans*-diol to the left, and that for the 3,4-*cis*-diol to the right). RDA = retro Diels-Alder. Modified from ref. 7.

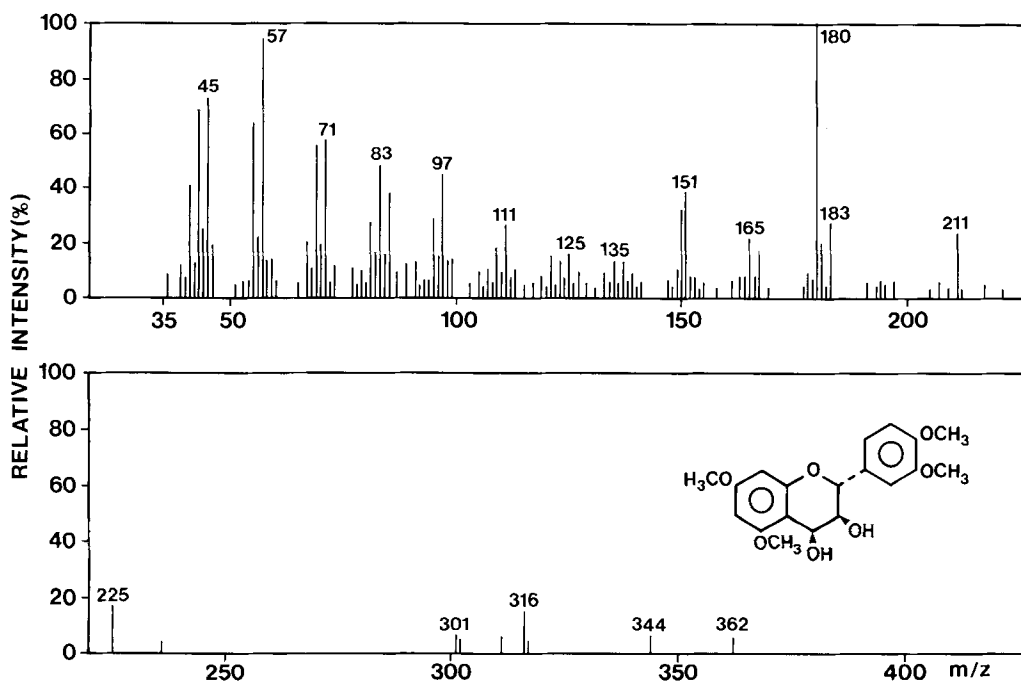


Figure 10. Mass spectrum of the tetramethyl ether of the minor leucocyanidin isomer ((2R,3S,4S)-5,7,3',4'-tetramethoxyflavan-3,4-diol).

3.4. Synthesis and characterization of two isomeric leucocyanidins

Leucocyanidin (a flavan-3,4-diol) is a suspected intermediate in the biosynthesis of catechin and procyanidins (4, 22, 23, 26, 37, 41). Two isomeric leucocyanidins (the 3,4-*trans* and 3,4-*cis* isomers) were therefore synthesized chemically to allow determination of their chromatographic properties. This information would enable the detection and identification of a radioactively labelled leucocyanidin isomer if synthesized by barley seeds fed (^{14}C)-dihydroquercetin.

The two isomeric leucocyanidins formed by reduction of (+)-dihydroquercetin with NaBH_4 were separated by column chromatography (Figure 7). The yields of the major and minor isomer were 50.0% and 2.3%, respectively. Only 0.5% of the starting dihydroquercetin was recovered as such. The rest was probably converted to self-condensation products of the leucocyanidins.

The major isomer was conclusively identified as (2R,3S,4R)-3,4,5,7,3',4'-hexahydroxyflavan (3,4-*trans*-diol) by ^1H NMR and MS. The ^1H NMR spectrum (Figure 8) showed the following characteristic chemical shifts: δ 3.83 (m, H-3), 4.58 (d, $J=10.0$ Hz, H-2), 4.94 (d, $J=7.8$ Hz, H-4), 5.80 (d, $J=2.3$ Hz, H-8), 5.93 (d, $J=2.3$ Hz, H-6), 6.77 (s, H-2'), 6.78 (s, H-5'), 6.91 (s, H-6'). This spectrum is identical to that obtained by PORTER and FOO (36), including the typical large coupling constants ($J_{2,3}=10.0$ Hz, $J_{3,4}=7.8$ Hz) associated with the 2,3-*trans*-3,4-*trans* stereochemistry (2). The mass spectrum of the 5,7,3',4'-tetramethyl ether showed the expected fragmentation pattern (7, 36). The important fragments and their relative intensities are given in Figure 9. A relatively small molecular ion peak (M^+) is seen. The most prominent peak at m/z 180 arises from a retro Diels-Alder fission of the heterocyclic ring.

The minor isomer was also identified as a leucocyanidin by MS of the tetramethyl ether (Figures 9 and 10). Since the 2,3-*trans* stereochemistry of (+)-dihydroquercetin is maintained during the reduction of dihydroquercetin with NaBH_4 (4), the minor leucocyanidin is probably (2R,3S,4S)-3,4,5,7,3',4'-hexahydroxyflavan (3,4-*cis*-diol). The R_f value (0.22) ob-

tained for the tetramethyl ether of the minor isomer by TLC on a kieselgel plate developed in benzene:acetone (4:1, v/v) is in agreement with that reported by PORTER and FOO (36) for (2R,3S,4S)-5,7,3',4'-tetramethoxyflavan-3,4-diol (R_f 0.24). The latter was synthesized by reduction of dihydroquercetin tetramethyl ether with NaBH_4 . Furthermore, the minor leucocyanidin isomer condensed in acid with added (^{14}C)-catechin to form the expected all-*trans* dimer and trimer of catechin (Figure 11). 55% of the diol was recovered in procyanidin B3 and 17% in procyanidin C2.

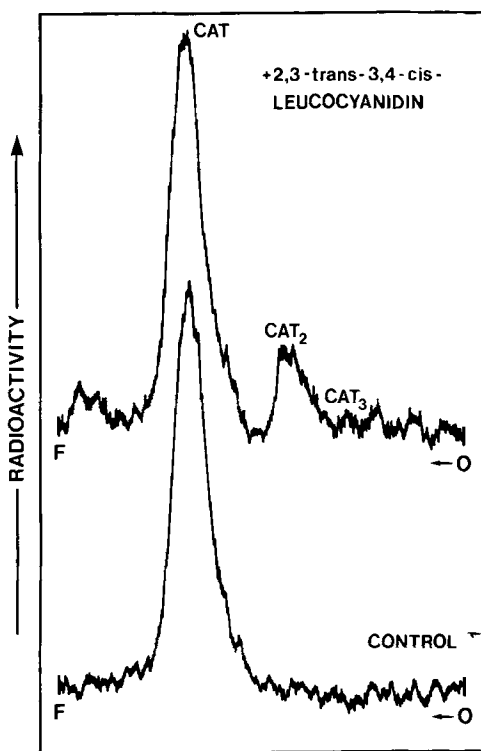


Figure 11. Radio-thin layer chromatograms of products formed by addition of the minor leucocyanidin isomer (2,3-*trans*-3,4-*cis*-leucocyanidin) to an acidic solution of (^{14}C)-catechin (upper) and a control where leucocyanidin was omitted (lower). Strong vanillin positive bands corresponding to procyanidins B3 and C2 were seen when spraying a narrow vertical strip of the upper plate with the vanillin reagent. Separation was carried out using TLC plates, developed in sBAWC (section 2.3.2). CAT = catechin, CAT_2 = procyanidin B3, CAT_3 = procyanidin C2, O = origin, F = solvent front.

Table II.

Incorporation of 1 μCi (^{14}C)-dihydroquercetin into catechin and procyanidin B3 by various tissue preparations of wild type barley seeds (cpm).

Plant tissue ^a	Catechin	Procyanidin B3
Excised spike	0	0
25 seeds minus awns	9,900	2,100
25 seeds minus awns and husks	12,300	3,200
25 seeds minus awns, endosperms and aleurone layers	41,800	9,900
25 seeds minus awns, husks, endosperms and aleurone layers	78,700	11,800

^a Incubation procedure for the excised spike is described in section 3.5.1, and for the other preparations in section 2.8.

Each of the flavan-3,4-diols synthesized showed the characteristics of leucocyanidins (8, 21), i.e., conversion to cyanidin upon hydrolysis in acid, a positive reaction with the vanillin reagent (section 2.10) and a R_f value similar to that of catechin in the BAW solvent system. The chromatographic properties of the 3,4-diols are given in Table I. That the 3,4-*cis*-diol is more water soluble than the 3,4-*trans*-diol is shown by the lower R_f values in three of the five TLC systems used and by the smaller elution volume on the reverse phase HPLC column.

The 3,4-*trans*-diol was highly unstable in acid and epimerized at C-4 to give the 3,4-*cis*-diol. When the 3,4-*trans* isomer was collected from the HPLC (system IV), left in the 5% acetic acid eluate for 10 minutes and reinjected into the HPLC, a 1:1 ratio of 3,4-*trans*-diol and 3,4-*cis*-diol was obtained. The 3,4-*cis* diol did not give rise to other products when kept in the eluate for 10 minutes.

STAFFORD and LESTER (41) have also isolated two 2,3-*trans*-3,4-diol isomers after reduction of dihydroquercetin with NaBH_4 . The less water soluble isomer was the major product isolated as in the present study. The chromatographic properties and the stability to acid reported for these two isomers are very similar to those described in the present study for the 3,4-*trans*-diol and 3,4-*cis*-diol. STAFFORD and LESTER obtained, for example, elution volumes of 13.0 and 22.5 ml, respectively, for the two diols using a C_{18} -Partisil ODS3 HPLC column eluted isocratically with 5% acetic acid. These values are comparable with the elution volumes of 13.05 and 21.60 ml obtained for the 3,4-*cis*-diol and

3,4-*trans*-diol, respectively, using HPLC system IV (section 2.3.1). STAFFORD and LESTER, however, hypothesized that the more water soluble isomer was the 3,4-*trans*-diol and the less water soluble isomer the 3,4-*cis*-diol which is in contrast to the conclusions drawn here on the basis of ^1H NMR and MS.

3.5. Incorporation of (^{14}C)-dihydroquercetin into catechin and procyanidins in maturing barley seeds

3.5.1. Incorporation of label by wild type seeds

For the administration of (^{14}C)-dihydroquercetin to barley seeds, spikes were harvested 12 days after flowering at which developmental stage the biosynthesis of catechin, procyanidin B3 and procyanidin C2 is in a linear phase in wild type seeds (Figure 2). Several techniques for administering 1 μCi (^{14}C)-dihydroquercetin were tested (Table II). No detectable incorporation of label into catechin and procyanidin B3 occurred in intact spikes. The latter had been cut off 1-2 cm below the lowermost glume, stood in 200 μl aqueous solution of (^{14}C)-dihydroquercetin and were left for 24 hours in the Weiss growth chamber under the conditions detailed in section 2.1. When 25 seeds from which the awns were cut off were incubated in an Erlenmeyer flask with 2 ml aqueous precursor solution labelled catechin and procyanidin B3 were formed. A similar extent of incorporation was observed when both husks and awns were removed from the seeds. If, instead of removing the husks, the endosperm and adhering aleurone layers were squeezed out from awnless seeds,

a 2-fold increase of label incorporated into catechin and procyanidin B3 resulted. Even more label was incorporated when the husk, in addition to awn, endosperm and aleurone layer, was removed from the seed and the remaining pericarp-testa tissue incubated.

Labelled dihydroquercetin was not detected in extracts of seeds devoid of the husk, but constituted the majority of label in extracts of seeds containing the husk. In the latter case the labelled dihydroquercetin present in the extracts was apparently not taken up by the pericarp-testa tissue but stuck to the surface of the waxy husk. This may account for the higher incorporation of label into catechin and procyanidin B3 in seeds without husk compared to seeds with husk, since more substrate is available for synthesis when the husk is removed.

The marked increase in label incorporated into catechin and procyanidin B3 when removing the endosperm and the adhering aleurone layer from the seed indicates that the testa layer is the site of catechin and procyanidin biosynthesis. By removing the aleurone layer and endosperm, the testa becomes directly exposed to the incubation medium, facilitating the uptake of substrate by the tissue. Combining this observation with a recent report (1) that catechin and proanthocyanidins are concentrated in the testa of the mature seed leads to the conclusion that these compounds are synthesized and deposited in the same tissue.

The few cell layers constituting the testa were not isolable from the pericarp and therefore the combined pericarp-testa tissue was used in the feeding experiments. A radio-TLC scan of the ethyl acetate extractable products formed by wild type seeds incubated with (^{14}C)-dihydroquercetin is shown in Figure 12 A. Radioactively labelled catechin, procyanidin B3 and procyanidin C2 were synthesized, indicating that dihydroquercetin is an *in vivo* precursor of these compounds. The radioactive band at R_f 0.95 was not identified. It was a yellow compound that could be eluted from the cellulose plate with methanol. TLC in BAW, CAW and 15% HOAc gave R_f values of 0.94, 0.82 and 0.76, respectively. Thus the unknown is not quercetin which is a potential product synthesized by the seeds when feeding (^{14}C)-dihydroquercetin, but

is believed to be a degradation product of (^{14}C)-dihydroquercetin.

The flavonoids of interest were satisfactorily separated by HPLC using system IV (Table I, Figure 13 B), except procyanidin C2, which eluted almost simultaneously with 3,4-*trans*-diol. Subjecting the extract to HPLC analysis revealed several minor radioactive peaks in addition to the major peaks corresponding to catechin and procyanidin B3 (Figure 13 A). The

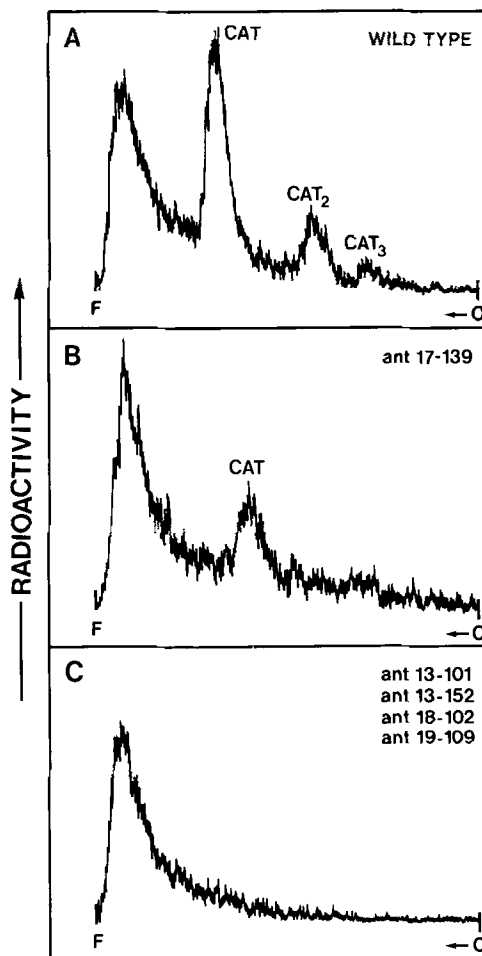


Figure 12. Radio-thin layer chromatograms of ethyl acetate soluble products formed by feeding (^{14}C)-dihydroquercetin to pericarp-testa tissue of (A) wild type, (B) *ant* 17-139, and (C) *ant* 13-101, *ant* 13-152, *ant* 18-102 and *ant* 19-109. From 21,900 to 62,400 cpm were applied to each plate which was developed using sBAWC (section 2.3.2). CAT = catechin, CAT₂ = procyanidin B3, CAT₃ = procyanidin C2, O = origin, F = solvent front.

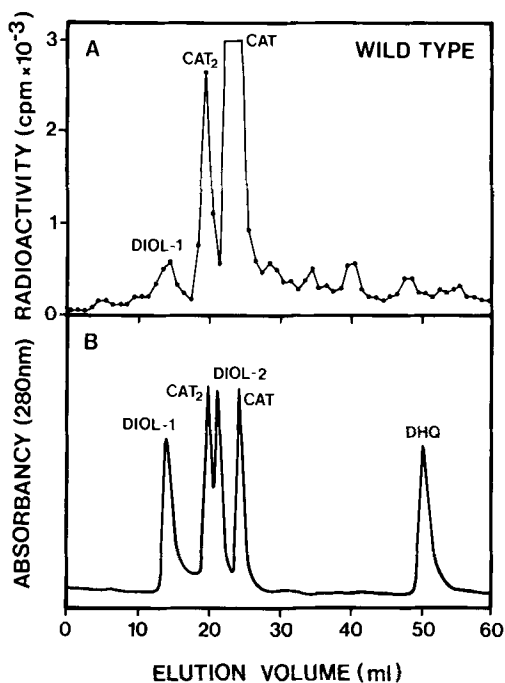


Figure 13. High pressure liquid chromatograms of (A) ethyl acetate soluble products formed by feeding (^{14}C)-dihydroquercetin to pericarp-testa tissue of wild type (25 μl , corresponding to pericarp-testa tissue from 6.25 seeds, was injected), and (B) a mixture of flavonoid standards. Separations were carried out using HPLC system IV (section 2.3.1). DIOL-1 = 2,3-*trans*-3,4-*cis*-leucocyanidin, CAT_2 = procyanidin B3, DIOL-2 = 2,3-*trans*-3,4-*trans*-leucocyanidin, CAT = catechin, DHQ = dihydroquercetin.

compound migrating near the front on the TLC plate was not eluted from the HPLC column with the acetic acid system used. Virtually no labelled dihydroquercetin aglucone was recovered in the plant extract. Probably the substrate, taken up by the pericarp-testa tissue, is immediately glucosylated and thus not detected, since only ethyl acetate soluble products were analysed.

No radioactive peak corresponding to 3,4-*trans*-diol was detected. Formation of the labelled 3,4-*cis*-diol from (^{14}C)-dihydroquercetin indicates that the leucocyanidin with the 3,4-*cis* stereochemistry is an intermediate in catechin and procyanidin biosynthesis. The other minor labelled peaks seen in Figure 13 A were not identified. No label was incorporated into dihydromyricetin and prodelfinidins, i.e., flavonoids having a 3',4',5'-hydroxylation pattern.

The amounts of label incorporated into catechin and procyanidins are shown in Table III. 4.7% of the radioactivity fed was recovered in these flavonoids. Using the rates of synthesis given in section 3.1, the expected ratio can be calculated among label incorporated into catechin, procyanidin B3 and procyanidin C2 as 10:0.9:0.5, assuming that just one catechin unit in the procyanidin molecules is labelled. The observed ratio was 10:1.5:0.44, revealing that 67% more label than expected was incorporated into procyanidin B3. This effect was also obser-

Table III.

Synthesis of catechin and procyanidins by pericarp-testa tissue prepared from 25 seeds of wild type barley and proanthocyanidin free mutants fed 1 μCi (^{14}C)-dihydroquercetin.

Genotype	Catechin		Procyanidin B3		Procyanidin C2		3,4- <i>cis</i> -diol		3,4- <i>trans</i> -diol
	cpm	nmol	cpm	nmol	cpm	nmol	cpm	nmol	cpm
Wild type	78,700	14.3	11,800	2.15 ^a	3,500	0.64 ^a	6,000	1.09	-
<i>ant 17-139</i>	16,500	3.00	4,400	0.40 ^b	-	-	-	-	-
<i>ant 18-102</i>	1,900	0.35	500	0.05 ^b	-	-	-	-	-
<i>ant 19-109</i>	1,700	0.31	2,600	0.24 ^b	-	-	-	-	-
<i>ant 13-101</i>	-	-	-	-	-	-	-	-	-
<i>ant 13-152</i>	-	-	-	-	-	-	-	-	-

^a Calculated on the assumption that only one catechin unit in the procyanidin molecules is labelled (1 nmol = 5,500 cpm).

^b Calculated on the assumption that both catechin units are labelled (1 nmol = 11,000 cpm).

- = less than 100 cpm or 0.02 nmol.

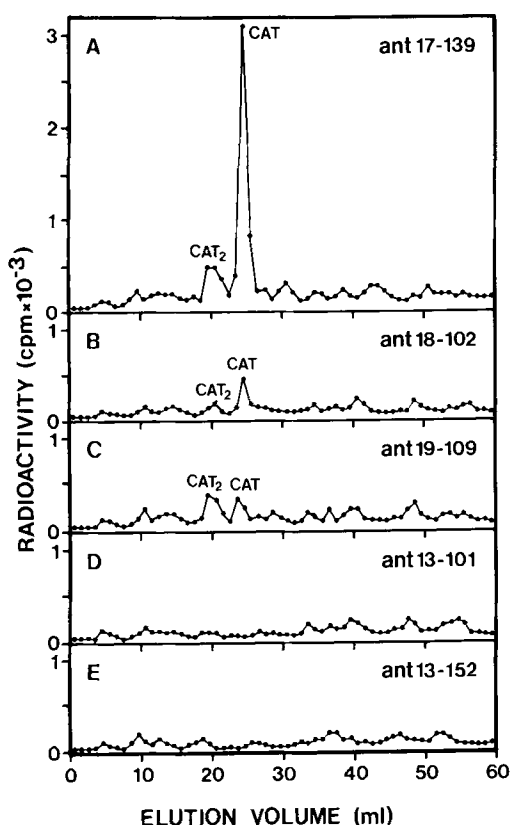


Figure 14. High pressure liquid chromatograms of ethyl acetate soluble products formed by feeding (^{14}C)-dihydroquercetin to pericarp-testa tissue of (A) *ant 17-139*, (B) *ant 18-102*, (C) *ant 19-109*, (D) *ant 13-101*, and (E) *ant 13-152*. See footnote for Figure 13.

ved in the incorporation experiments in Table II, to an even higher extent. Control experiments showed that this effect is not due to a spontaneous condensation during the purification procedure between labelled catechin formed by the seeds and the added leucocyanidin carrier, since the effect was also observed when no leucocyanidin was added. A selective stimulation of the procyanidin B3 synthesis, however, may be occurring *in vitro* but not *in vivo*.

3.5.2. Incorporation of label by seeds of proanthocyanidin free mutants

The results of feeding (^{14}C)-dihydroquercetin to pericarp-testa tissue prepared from proanthocyanidin free mutants belonging to different loci

are presented in Figures 12 B, C, and 14 plus Table III. The mutant *ant 17-139* was capable of synthesizing catechin and procyanidin B3 from dihydroquercetin at a rate of synthesis approximately 20% of that for wild type. The ratio between nmol of catechin and procyanidin B3 formed by *ant 17-139* is 7.5:1, assuming that the specific activity of procyanidin B3 is twice that of catechin and recalling that no pool of unlabelled catechin exists in this proanthocyanidin free mutant. This ratio is in good agreement with that of 6.8:1 obtained for wild type. The mutants *ant 18-102* and *ant 19-109* synthesized such small amounts of catechin and procyanidin B3 that they were not detectable by the radio-TLC analysis (Figure 12 C) but only by HPLC analysis (Figure 14B, C). The rates of catechin and procyanidin B3 synthesis observed for *ant 18-102* were 2.4% and 2.1%, respectively, of those for wild type. The corresponding figures for *ant 19-109* were 2.2% and 11%, respectively. Two mutants in the gene *ant 13* were tested, and both were totally inactive with respect to catechin and procyanidin synthesis.

4. DISCUSSION

4.1. Biosynthetic pathway for the conversion of dihydroquercetin into catechin and procyanidins

A biosynthetic pathway for the conversion of dihydroquercetin into catechin and procyanidins based on the above results is shown in Figure 15.

That dihydroquercetin is a precursor of catechin and procyanidins, as proposed earlier (18, 22, 23, 26, 37, 39), was established by feeding (^{14}C)-dihydroquercetin to pericarp-testa tissue prepared from wild type and recovering the label in the appropriate products. As an alternative to a pathway via dihydroquercetin, HASLAM suggested a pathway via 2-hydroxychalcone (22, 23, 26). This hypothesis is no longer tenable. The simultaneous synthesis of radioactively labelled 2,3-*trans*-3,4-*cis*-leucocyanidin, catechin, procyanidin B3 and procyanidin C2 observed when feeding (^{14}C)-dihydroquercetin to wild type barley seeds strongly suggests that the 3,4-*cis*-diol is an intermediate in catechin and procyanidin synthesis. This is in agreement with

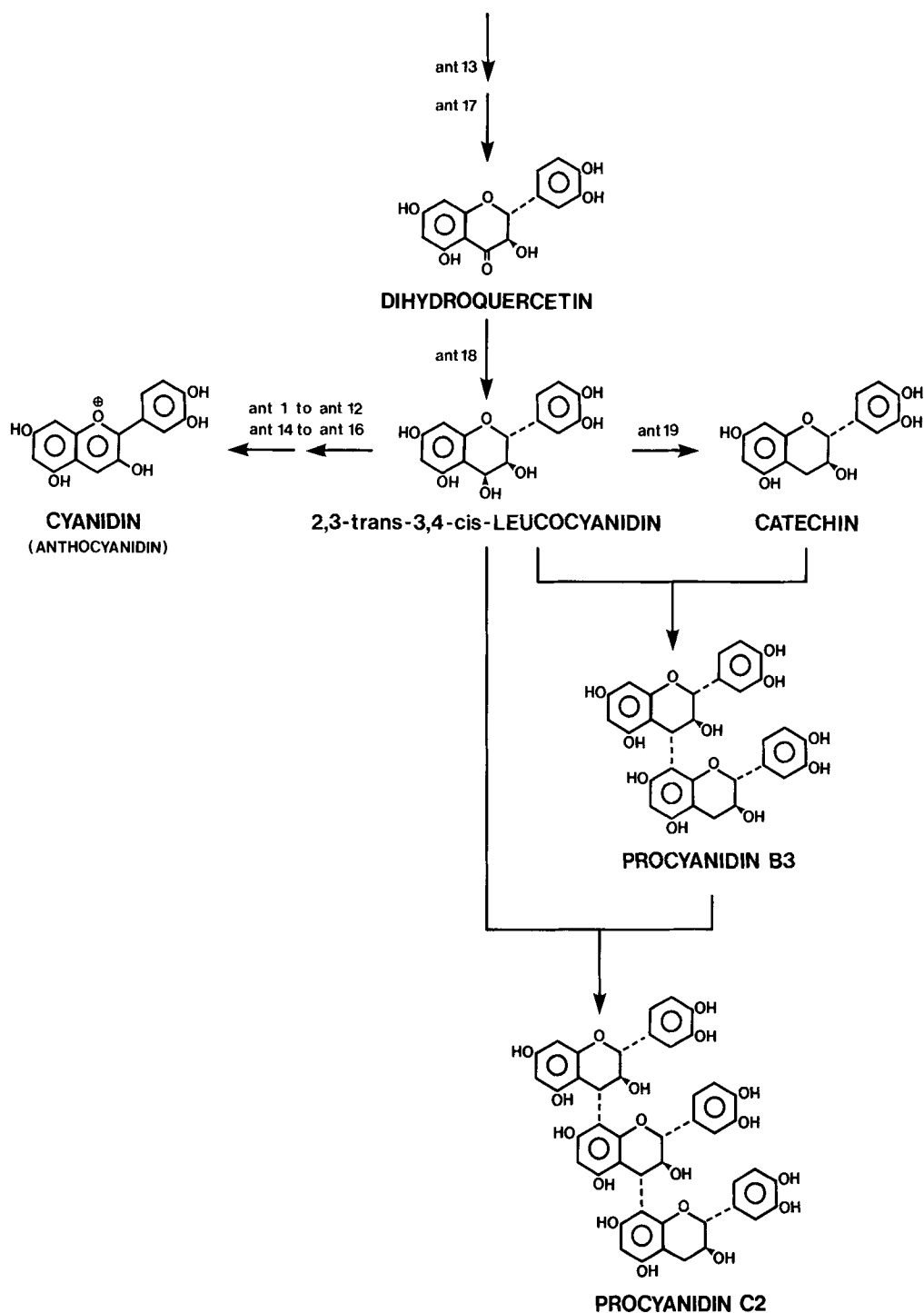


Figure 15. Proposed biosynthetic pathway leading from dihydroquercetin to catechin, procyanidins and cyanidin showing the likely sites of action of 19 *ant* genes.

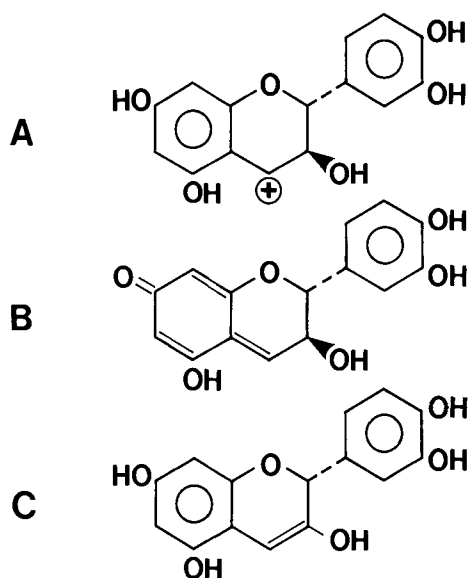


Figure 16. Structures of postulated intermediates in catechin and procyanidin biosynthesis. (A) Flavanyl-4-carbocation, (B) quinone methide, and (C) flav-3-en-3-ol.

the idea that a leucocyanidin is involved in the biosynthesis of these compounds (4, 22, 23, 26, 37, 41). STAFFORD and LESTER (41) have demonstrated a NADPH-dependent reductase activity, capable of converting dihydroquercetin to a flavan-3,4-diol, in crude soluble protein extracts from cell suspension cultures of Douglas fir. The enzymatically produced 3,4-diol was deduced to be the 2,3-*trans*-3,4-*trans* isomer when it produced cyanidin upon acid hydrolysis and it condensed nonenzymatically with catechin in acidic solution to form procyanidin B3 (37, 41). These two criteria, however, can not be used to distinguish the 3,4-*trans*-diol from the 3,4-*cis*-diol, since the same reactions are expected for the 3,4-*cis*-diol. The 3,4-diol formed by the protein extracts of Douglas fir was similar to the minor more water soluble of the two isomers formed by reduction of dihydroquercetin with NaBH₄. In the present study the more water soluble isomer was demonstrated to be the 2,3-*trans*-3,4-*cis* isomer. Thus, both cell suspension cultures of Douglas fir and pericarp-testa tissue of barley most likely synthesize the 2,3-*trans*-3,4-*cis* isomer of leucocyanidin.

(Preliminary experiments using crude protein extracts prepared from pericarp-testa tissue of wild type barley revealed a NADPH-dependent reduction of dihydroquercetin to the 3,4-*cis*-diol).

It has been postulated that oligomeric procyanidins in general are synthesized by the addition of a stereospecific flavanyl-4-carbocation (Figure 16 A) to a flavan-3-ol (catechin or epicatechin) or to an already existing procyanidin chain (4, 22, 23, 26, 37). Support for a condensation of units from separate metabolic pools came from tracer experiments (26, 40), showing that more label was incorporated into the upper unit of a procyanidin dimer than into the lower unit. The status of flavanyl-4-carbocation as an intermediate in the procyanidin biosynthesis is due, however, solely to *in vitro* reactions. The *in vitro* reduction product of dihydroquercetin, mainly 2,3-*trans*-3,4-*trans*-leucocyanidin, is easily condensed with catechin in acid to give all-*trans* procyanidins (4, 8, 9, 11, 25, 41). Since the stereochemistry at C-4 is preserved, the *in vitro* condensation must proceed through a S_N1 mechanism, i.e., through the flavanyl-4-carbocation, as inferred for the *in vivo* synthesis. HEMINGWAY and FOO (25), however, found that the base-catalyzed condensation of 2,3-*trans*-3,4-*trans*-leucocyanidin with catechin through a quinone methide (Figure 16 B) proceeded more rapidly than the acid-catalyzed through the flavanyl-4-carbocation. They concluded that the quinone methide is more likely to be involved in the biosynthesis of procyanidins than the flavan-4-carbocation intermediate.

Whether the flavanyl-4-carbocation or the quinone methide is the actual electrophile condensing with catechin *in vivo*, the highly reactive electrophilic species most probably only exists at the transition state in the active site of an enzyme. That the enzymatic reduction of dihydroquercetin results in the 3,4-*cis*-diol permits one to suggest a third mechanism for the *in vivo* condensation, namely through an S_N2 reaction between the 3,4-*cis*-diol and catechin not involving one of the highly reactive electrophilic species. The inversion of configuration at C-4 associated with this S_N2 mechanism results in formation of the all-*trans* procyanidin dimer

(Figure 15). Thus whatever the mechanism of enzyme catalysis, the 3,4-*cis*-diol can be condensed with catechin to give procyanidin B3 in one enzymatic step.

In the speculative pathway for catechin and proanthocyanidin biosynthesis proposed by HASLAM (22, 23, 26), the reduction of dihydroquercetin to a leucocyanidin was followed by a dehydration of leucocyanidin to give a flav-3-en-3-ol (Figure 16 C). This was thought to be the key intermediate that by protonation gave rise to the postulated flavanyl-4-carbocation. In the biosynthetic pathway presented in Figure 15, the flav-3-en-3-ol intermediate is omitted (37) and the 3,4-*cis*-diol is believed to be the key intermediate. HASLAM further suggested that procyanidins are synthesized when the supply of biological reductant is rate-limiting so that the postulated flavanyl-4-carbocation might escape from the active site of a reductase and react with an already formed reduced product (catechin or epicatechin) to produce dimers, trimers etc. This hypothesis seems unlikely, since the synthesis of catechin, procyanidin B3 and procyanidin C2 occurred simultaneously in barley 8-16 days after flowering (Figure 2) when chlorophyll is still present and biological reductant will probably not be rate-limiting. More likely specific enzymes are involved in the biosynthesis of catechin and procyanidins.

Thus, I propose that at least four enzymes are involved in the conversion of dihydroquercetin to catechin and procyanidins: (i) a reductase, converting dihydroquercetin to its 3,4-*cis*-diol, (ii) a second reductase, converting 3,4-*cis*-diol to catechin, (iii) a condensing enzyme, adding 3,4-*cis*-diol to catechin to form the all-*trans* dimeric procyanidin, (iv) a second condensing enzyme, adding 3,4-*cis*-diol to the procyanidin dimer to form the all-*trans* trimer.

4.2. Site of action of *ant* genes

The most likely sites of action of *ant 13*, *ant 17*, *ant 18* and *ant 19* in the biosynthetic pathway to catechin and procyanidins are indicated in Figure 15, together with the sites of the other *ant* genes (*ant 1* – *ant 12*, *ant 14* – *ant 16*) known to participate in anthocyanin but not proanthocyanidin biosynthesis (27, 28).

The gene *ant 18* appears to control the reduction of dihydroquercetin, since this intermediate accumulated in seeds of *ant 18-102*. The capability of *ant 18-102* seeds to synthesize small amounts of catechin and procyanidin B3 when fed (¹⁴C)-dihydroquercetin is probably due to an incomplete genetic block. The gene *ant 19* is believed to function in the postulated reduction of 2,3-*trans*-3,4-*cis*-leucocyanidin to catechin, since seeds of *ant 19-109* are proanthocyanidin free but capable of synthesizing anthocyanins (28). This mutant is also considered slightly leaky due to a minor synthesis of catechin and procyanidin B3 from (¹⁴C)-dihydroquercetin. No labelled 3,4-*cis*-diol accumulated during the feeding experiments as might be expected. This result can not be attributed to a further conversion of the 3,4-*cis*-diol to anthocyanins, because the latter are synthesized in the husk (27) and not in the pericarp-testa tissue used for the feeding experiments. On the other hand a regulatory mechanism (a feedback inhibition) may prevent an extensive accumulation of the 3,4-*cis*-diol by inhibiting the reduction of dihydroquercetin when the 3,4-*cis*-diol is not metabolized further to catechin and procyanidins.

The leucocyanidin (Figure 15) rather than dihydroquercetin apparently serves as the last common intermediate in the synthesis of anthocyanins and proanthocyanidins, since seeds of *ant 18-102*, though accumulating dihydroquercetin, are not capable of synthesizing anthocyanins (28) in contrast to seeds of *ant 19-109*. The genes *ant 1* to *ant 12* and *ant 14* to *ant 16* must therefore control steps between 3,4-*cis*-diol and anthocyanins.

The gene *ant 17* determines a step prior to dihydroquercetin, since *ant 17-139*, deficient in both proanthocyanidins and anthocyanins (28), was capable of converting (¹⁴C)-dihydroquercetin into catechin and procyanidin. Similarly, the gene *ant 13* functions prior to dihydroquercetin, since mutants in this gene lack proanthocyanidin and anthocyanin (28) as well as dihydroquercetin. Unexpectedly, however, seeds of *ant 13-101* and *ant 13-152* unlike those of *ant 17-139* were not capable of synthesizing catechin and procyanidins from (¹⁴C)-dihydroquercetin. Why the enzymes needed for the

conversion of dihydroquercetin into catechin and procyanidins are present in *ant 17-139* but absent in the *ant 13* mutants is not known. Assuming that *ant 13* blocks a step earlier in the pathway than does *ant 17*, it can be speculated that a given intermediate, responsible for induction of these enzymes, is synthesized in *ant 17-139* but not in the *ant 13* mutants.

In future work of isolating and characterizing the enzymes involved in the conversion of dihydroquercetin into catechin and procyanidin the barley mutants blocked after as well as before dihydroquercetin will provide helpful tools especially in regard to the biosynthesis of these enzymes.

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