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Gene-enzyme relations in the pathway of flavonoid biosynthesis in barley

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Summary. Mutations in genes associated with the pathway of flavonoid biosynthesis can be easily induced in barley, and more than 600 mutants have been isolated in which the biosynthesis of proanthocyanidins is genetically blocked. Diallelic crosses have localized a majority of the proanthocyanidin-free mutants to nine different *ant* genes. In order to characterize the *ant* gene assays for flavanone 3-hydroxylase, flavonoid 3′-hydroxylase and dihydroflavonol reductase activities in protein extracts from developing grains of proanthocyanidin-free mutants in six *ant* genes and wild-type barley were performed. The presence of chalcone synthase enzyme protein in wild-type barley and the mutant *ant* 13–152 was investigated by SDS-PAGE, protein blotting, and reaction with an antiserum against parsley chalcone synthase. The results of these experiments combined with earlier studies of flavonoid content in barley grains of different genotypes characterize the functions of six *ant* genes as follows. The gene *ant* 13 is a regulatory gene affecting the transcription of structural genes in the flavonoid pathway. The genes *ant* 17 and *ant* 22 are probably coding for different components necessary for hydroxylation of flavanones in 3-position. The gene *ant* 18 is the structural gene coding for dihydroflavonol reductase, and *ant* 19 is most likely the structural gene coding for leucoanthocyanidin reductase. *Ant* 21 is probably a regulatory gene affecting the biosynthesis of proanthocyanidins and anthocyanins.

Key words: Proanthocyanidin-free barley mutants – *ant* Genes – Flavonoid biosynthesis – Flavonoid enzymes

Abbreviations: DHK = dihydrokæmpferol; DHQ = dihydroquercetin; CHS = chalcone synthase; CHI = chalcone isomerase; DFR = dihydroflavonol reductase; UFGT = UDP glucosyl:flavonoid 3-O-glucosyltransferase; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC = thin layer chromatography; HPLC = high performance liquid chromatography

Introduction

Barley and sorghum are the only cereals containing proanthocyanidins. The barley proanthocyanidins are located in the testa layer of the grains (Aastrup et al. 1984) and consist of dimers and trimers of (+)-catechin and (+)-gallocatechin, which give rise to procyanidins and prodelphinidins. These flavanoids are able to precipitate proteins in beer, which results in formation of colloidal haze. Beer haze formation can be avoided by use of barley varieties for brewing in which the biosynthesis of proanthocyanidins is genetically blocked (von Wettstein et al. 1977, 1985). Proanthocyanidin-free barley mutants can be induced by chemical mutagens or irradiation and more than 600 such mutants have been isolated. Mutants that are good from an agronomical point of view are used for breeding of high-yielding, proanthocyanidin-free malting barley varieties, and proanthocyanidin-free mutants are also used for studies of the pathway of flavonoid biosynthesis in barley and for characterization of genes associated with this pathway. By diallelic crosses, 474 proanthocyanidin-free mutants have been localized to nine different genes which, upon mutation, block the biosynthesis of proanthocyanidins in the barley grains (Jende-Strid 1990).

Anthocyanin and proanthocyanidin biosynthesis share several common steps (Fig. 1), and mutants in five genes, namely, *ant* 13, *ant* 17, *ant* 18, *ant* 21, and *ant* 22, are anthocyanin-free, whereas mutants in the genes *ant* 19, *ant* 25, *ant* 26, and *ant* 27 synthesize anthocyanin in various organs of the plant. High performance liquid chromatography (HPLC) analysis of mature barley grains has revealed that (+)-catechin, procyanidins B-3 and C-2, prodelphinidin B-3, and three prodelphinidin trimers occur in wild-type barley, but proanthocyanidin-free mutants in the genes *ant* 13, *ant* 17, *ant* 18, *ant* 19, *ant* 21, and *ant* 22 lack these flavanoids (Jende-Strid and Møller 1981). Thin layer chromatography (TLC) analysis of developing grains (around 14 days after flowering) has shown that a small amount of (+)-cat-

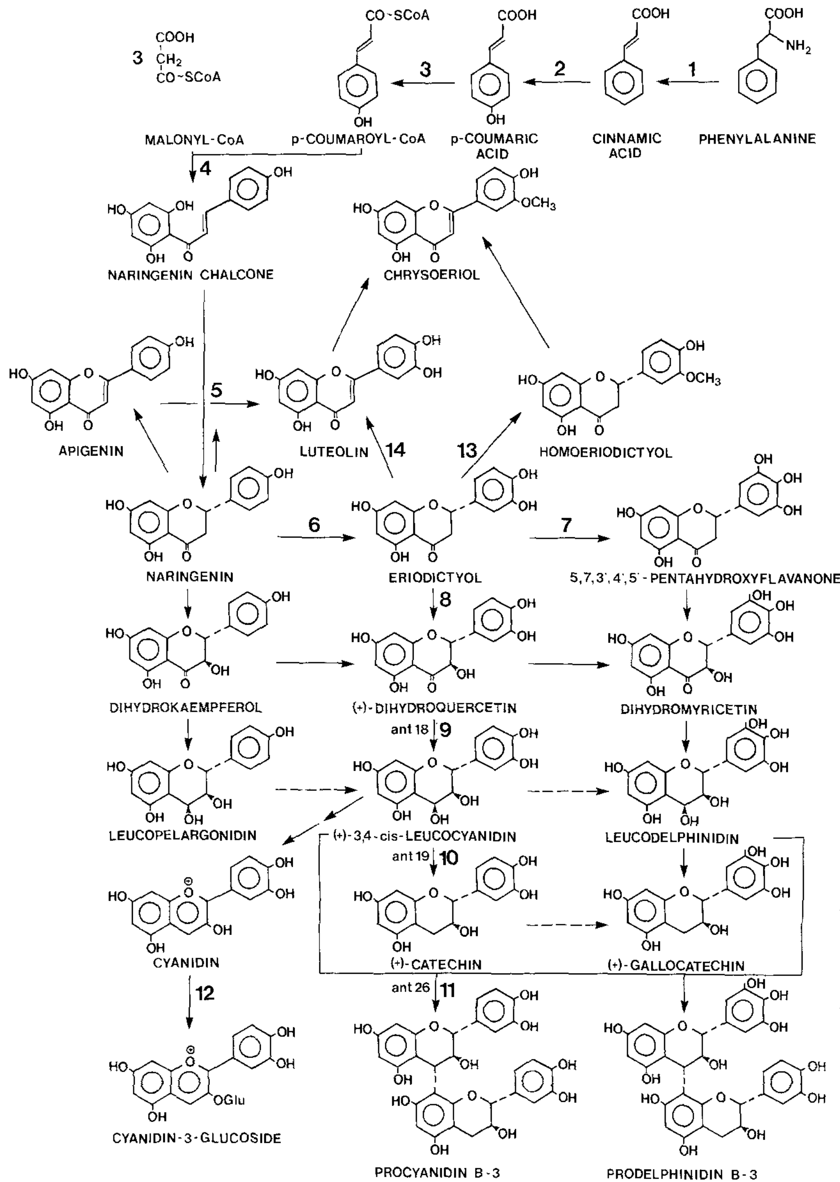

Fig. 1. Biosynthesis of flavonoids in barley. The number refer to enzymes in the flavonoid pathway. *1* phenylalanine ammonia-lyase (PAL), *2* cinnamate 4-hydroxylase, *3* 4-coumarate: CoA ligase (4CL), *4* chalcone synthase (CHS), *5* chalcone isomerase (CHI), *6* flavonoid 3'-hydroxylase, *7* flavonoid 5'-hydroxylase, *8* flavanone 3-hydroxylase, *9* dihydroflavonol reductase (DFR), *10* leucoanthocyanidin reductase, *11* putative flavanol condensing enzyme, *12* UDP glucose:anthocyanin 3-O-glucosyltransferase, *13* flavonoid 3'-O-methyltransferase, *14* flavone synthase. The action sites of some structural *and* genes are indicated

echin and proanthocyanidins (around 5% of wild-type amount) is present in the mutant *ant* 19–109 at that stage of development (Jende-Strid 1990). No flavonoid precursors of (+)-catechin and proanthocyanidins have been detected in grains of wild-type barley or proanthocyanidin-free mutants in the genes *ant* 13, *ant* 19, and *ant* 21. Grains of mutants in the gene *ant* 18 accumulate small amounts of dihydroquercetin (DHQ), which is the precursor of leucocyanidin and (+)-catechin, and mutants in the genes *ant* 17 and *ant* 22 accumulate homoeriodictyol and chrysoeriol (Jende-Strid and Kristiansen 1987; Jende-Strid 1990). These compounds are not precursors of proanthocyanidins.

Proanthocyanidins and anthocyanins are synthesized in a multistep pathway (Fig. 1), and most of the enzymes catalyzing the individual steps have been purified or at least characterized (Forkmann 1989). Chalcone synthase (CHS) is involved in the formation of the first intermediate in the flavonoid pathway, naringenin chalcone, and chalcone isomerase (CHI) converts the naringenin chalcone into naringenin. Flavanone 3-hydroxylase catalyzes the hydroxylation of flavanones in 3-position to yield dihydroflavonols, and the hydroxylation of flavanones and dihydroflavonols in 3'-position is mediated by another enzyme, flavonoid 3'-hydroxylase. Dihydroflavonol reductase (DFR) catalyzes the stereospecific reduction of (+)-dihydroflavonols to flavan 3,4-*cis*-diols (leucoanthocyanidins). Genes coding for chalcone synthase, chalcone isomerase, dihydroflavonol reductase and UDP glucosyl : flavonoid 3-O-glucosyltransferase (UFGT) have been cloned from a number of plant species (Mol et al. 1988).

In order to further characterize the different *ant* genes in barley, studies of the presence and activity of some of the enzymes in the flavonoid pathway have been made. An investigation on DHQ reductase activity in protein extracts of developing grains of wild-type barley and mutants in the genes *ant* 13, *ant* 17, *ant* 18, *ant* 19, *ant* 21, and *ant* 22 was published in 1987 (Jende-Strid and Kris-

tiansen). In this paper results are reported of immunoassays for chalcone synthase protein in wild-type barley and the mutant *ant* 13-152 and studies of flavanone 3-hydroxylase, flavonoid 3′-hydroxylase, and dihydroflavonol reductase activities in wild-type barley and proanthocyanidin-free mutants in the genes *ant* 13, *ant* 17, *ant* 18, *ant* 19, *ant* 21, and *ant* 22.

Materials and methods

Plant material

Barley. The following barley genotypes were studied: two commercial malting barley varieties, Triumph and Nordal, and the proanthocyanidin-free mutants *ant* 13-152 (induced in Triumph), *ant* 17-139 and *ant* 18-102 (induced in Nordal), *ant* 19-109 (induced in Alf), *ant* 21-194 (induced in Georgie), and *ant* 22-1508 (induced in Haruna-Nijo).

Petunia hybrida. Two petunia mutants, W75 and W39, which are blocked in the biosynthesis of anthocyanins in the flower petals, were used for preparation of standards and radioactive substrates for the enzyme assays. The mutant W75 is blocked between the dihydroflavonol and leucoanthocyanidin intermediates, and it accumulates dihydrokaempferol (DHK) glucosides. The mutant W39 is blocked between the flavanone and dihydroflavonol intermediates, and it accumulates mainly eriodictyol glucoside together with small amounts of naringenin glucoside (Doodeman et al. 1982). Seeds of the two mutants were a gift from Dr. A. Gerats, Amsterdam.

Chemicals

Unlabelled (+)-dihydroquercetin (DHQ) was obtained from Serva (Heidelberg, Germany). Naringenin and β-glucosidase were bought from Sigma (St Louis, USA), sodium (1-^{14}C)-acetate and sodium (2-^{14}C)-acetate were from Amersham (England), and Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). Dowex 1 × 2 and Bio-Rad protein assay reagent were supplied by Bio-Rad (Richmond, USA). Rabbit antiserum against parsley chalcone synthase was a gift from Prof. K. Hahlbrock, Cologne, Germany, and POD-coupled swine antirabbit antibodies were bought from Dakopatts A/S (Copenhagen, Denmark).

Chromatography

High Performance Liquid Chromatography (HPLC). Analyses were performed on a Waters Assn. (Millford, USA) instrument. Two systems were used for separation of flavonoids. (I) A μ-Bondapak C_{18} semipreparative column, 30 cm · 7.9 mm (Waters Assn.), eluted isocratically with methanol : acetic acid : water (40 : 5 : 55, v/v/v). Flow rate 2 ml/min. (II) A Nova-Pak C_{18} column, 15 cm × 3.9 mm (Waters Assn.), eluted isocratically with 10% acetic acid. Flow rate 1 ml/min. Elution was monitored spectrophotometrically at 280 nm.

^{14}C-labelled compounds were detected and quantified using a Berthold (Wildbad, Germany) HPLC radioactivity monitor LB 505 equipped with a GT 400 measuring cell (400 μl glass scintillator), connected to Berthold radiochromatography data system LB 500 M (Radio-HPLC).

Thin Layer Chromatography (TLC). Separations were performed on Merck (Darmstadt, Germany) precoated cellulose F_{254} plates (0.1 mm) in five different solvent systems:

(I) CAW (chloroform : acetic acid : water, 10 : 9 : 1, v/v/v).
(II) BAW (butanol : acetic acid : water, 4 : 1 : 5, v/v/v).
(III) BAW (butanol : acetic acid : water, 6 : 1 : 2, v/v/v).
(IV) 6% acetic acid (v/v).
(V) 15% acetic acid (v/v).

Flavonoids were visualized under UV light and flavanols were detected by spraying with a mixture of 5% vanillin in ethanol and concentrated hydrochloric acid (4 : 1, v/v).

Extraction of flavonoids from petunia petals

Twenty fully expanded flowers of petunia mutants W75 or W39 were frozen in liquid nitrogen and homogenized. The resulting powder was extracted with 6 × 20 ml 75% acetone, and the combined extracts were delipidated with 3 × 40 ml petroleum ether. The volume was reduced to 20 ml in vacuo, and the remaining water phase hydrolyzed overnight at room temperature with 6 mg (37 units) of β-glucosidase to remove the sugar moieties from the flavonoid aglucones. Flavonoids were extracted with 6 × 10 ml diethylether and the ether extracts were dried over Na_2SO_4, evaporated to dryness, and dissolved in 2 ml methanol. Dihydrokaempferol, eriodictyol, and naringenin were purified by semipreparative HPLC (system I). The eluates were freeze-dried and the purified compounds were dissolved in methanol.

Synthesis of ^{14}C-labelled flavonoids

(^{14}C)-dihydrokaempferol, (^{14}C)-eriodictyol, and (^{14}C)-naringenin were prepared by feeding sodium (1-^{14}C)-acetate and sodium (2-^{14}C)-acetate to excised flower buds of petunia mutants W75 and W39, according to procedures described by Kho and Bennink (1975) and Kristiansen (1984). Radioactive compounds were extracted and purified using the same methods as described above.

Preparation of enzyme extracts

Protein extracts for flavanone 3-hydroxylase and flavonoid 3′-hydroxylase assays. Awnless barley grains (1.25 g, 8–16 days after flowering) were frozen in liquid nitrogen, homogenized together with 1.25 g of glass beads (250–300 μm), and suspended in 2.5 ml TRIS-HCl buffer (pH 7.5) containing 10% glycerol (v/v) and 20 m*M* Na-ascorbate. The slurry was squeezed through nylon cloth and centrifuged for 20 min at 20,000 × *g*. The supernatant was stirred for 30 min with 10% (w/v) Dowex 1 × 2 (equilibrated with buffer), filtered through nylon cloth, and centrifuged for 20 min at 20,000 × *g*. The supernatant was desalted on a Sephadex G-25 column. A 10% solution of polyethyleneimine (adjusted to pH 7.5 with HCl) was added to the supernatant by stirring until the final concentration of polyethyleneimine was 0.25%. After 20 min stirring, the solution was centrifuged at 20,000 × *g* for 20 min and the supernatant was brought to 40% saturation with $(NH_4)_2SO_4$. After 15 min the precipitate was spun down as described above and the supernatant was brought to 80% saturation with $(NH_4)_2SO_4$ and centrifuged as above. The pellet was dissolved in a small amount of buffer and this solution served as the enzyme source. All steps were carried out at 0 to +4 °C.

Crude protein extracts for enzyme assays and SDS-PAGE gel electrophoresis. Crude protein extracts were prepared from developing barley grains as described by Kristiansen (1986), except that 1,4-dithiothreitol (2 m*M*) in the TRIS-HCl suspension buffer was substituted with sodium ascorbate (5 m*M*) in some of the preparations.

Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad protein reagent and bovine serum albumin as protein standard.

Table 1. Average elution volumes with HPLC and R_f values with TLC of intermediates in the flavonoid pathway. Details of the chromatographic systems are given in the 'Materials and methods' section

Compound	Average retension volumes with HPLC in ml		Average R_f values with TLC				
	System I	System II	CAW	BAW, 4:5:1	BAW 6:1:2	6% Acetic acid	15% Acetic acid
Naringenin	72.0	29.2	0.79	0.94	–	–	0.35
Eriodictyol	46.0	15.2	0.61	0.92	–	–	0.33
Dihydrokæmpferol	33.0	7.9	0.56	0.91	0.93	0.36	0.52
Dihydroquercetin	25.0	4.7	0.35	0.85	0.87	0.36	0.51
Leucopelargonidin	15.0	3.0	0.21	–	0.76	0.49	–

Enzyme assays

Assay for flavanone 3-hydroxylase activity. Assays for flavanone 3-hydroxylase activity were carried out according to Standard assay A developed by Britsch and Grisebach (1986). The assay system contained in a total volume of 200 µl: 2 nmol (^{14}C)-naringenin (25,000 dpm) or 10 nmol (^{14}C)-eriodictyol (25,000 dpm), 20 µmol TRIS-HCl buffer (pH 7.5), 20 µl glycerol, 400 µg catalase, 4 µmol Na-ascorbate, 20 nmol Fe^{2+}, 50 nmol 2-oxoglutarate, and 100–200 µg of protein. The mixture was shaken and incubated for 60 min at +30 °C. Five micrograms of unlabelled DHQ or DHK was added as a carrier and the flavonoids were immediately extracted with 3 × 500 µl ethylacetate. The combined extracts were evaporated to dryness under a stream of nitrogen and the residue was dissolved in 100 µl of water. The radioactive products were separated and quantified by Radio-HPLC (solvent system I). DHK and DHQ were identified by co-chromatography with standard compounds (Table 1).

Assay for flavonoid 3′-hydroxylase activity. Assays for flavonoid 3′-hydroxylase activity were carried out in the same assay system as described above, except that 400 nmol NADPH was added and 5 µg of unlabelled eriodictyol or DHQ was used as a carrier. In addition to (^{14}C)-naringenin (^{14}C)-DHK (11 nmol, 50,000 dpm) was tried as a substrate. Eriodictyol was identified by co-chromatography with standard eriodictyol (Table 1).

Assay for dihydroflavonol reductase activity. Assays for dihydroflavonol reductase activity were made according to Kristiansen (1986). (^{14}C)-DHK (11 nmol, 50,000 dpm) was used as substrate and the amount of protein varied between 100 and 200 µg in the assays. The radioactive products were separated and quantified by Radio-HPLC (Solvent system II).

SDS-PAGE gel electrophoresis and immunoblotting assay. Polypeptides from crude protein extracts of developing barley grains (5–50 µg protein per slot) were separated by SDS-PAGE according to Fling and Gregerson (1986) and immediately transferred to nitrocellulose filters. The electrophoretic blotting procedure and the immunoblotting assay were performed as described by Høyer-Hansen et al. (1985), except that polyclonal rabbit antibodies against parsley chalcone synthase (CHS) were used.

Results

Chalcone synthase (CHS)

The presence of CHS enzyme protein was investigated in developing grains of *ant* 13-152 and Triumph. The CHS antibodies reacted with the CHS protein from the wild-type barley, whereas no CHS protein could be detected in the *ant* 13-152 mutant (Fig. 2). By comparison with standard molecular mass markers it was found that the subunits of the barley CHS enzyme have a molecular mass of ca. 41 kDa, which is in agreement with data from other plant CHS proteins (Kreuzaler et al. 1979, Welle and Grisebach 1987).

Flavanone 3-hydroxylase activity

Enzyme assays with crude protein extracts using (^{14}C)-eriodictyol as a substrate gave poor results. Activity could only be demonstrated in wild-type barley extracts. A maximum amount of 10% of the labelled eriodictyol was converted to DHQ. Partial purification of the protein extracts by ammonium sulphate precipitation improved the enzyme activity. If (^{14}C)-naringenin was used as a substrate, 20% of the labelled naringenin was converted to DHK. All genotypes except *ant* 13-152 showed flavanone 3-hydroxylase activity (Table 2). No flavanone 3-hydroxylase activity was detected in any of the genotypes when (^{14}C)-eriodictyol was used as a substrate for the concentrated proten extracts. Instead, the substrate seemed to disappear from the assay mixture, even if (^{14}C)-eriodictyol was quite stable in control experiments without protein. Eriodictyol might serve as substrate for other more active enzymes in the protein extracts, but the most likely products – homoeriodictyol, chrysoerol, and luteolin – could not be detected either.

Flavonoid 3′-hydroxylase activity

Flavonoid 3′-hydroxylase activity was observed in the assays for flavanone 3-hydroxylase activity in the ammonium-sulphate-precipitated protein extracts, provided that NADPH was added to the assay mixture. Five to ten percent of the labelled naringenin was converted to eriodictyol. Protein extracts from Nordal and the proanthocyanidin-free mutants *ant* 17-139, *ant* 18-102, and *ant* 19-109 all showed approximately the same degree of flavonoid 3′-hydroxylase activity in addition to fla-

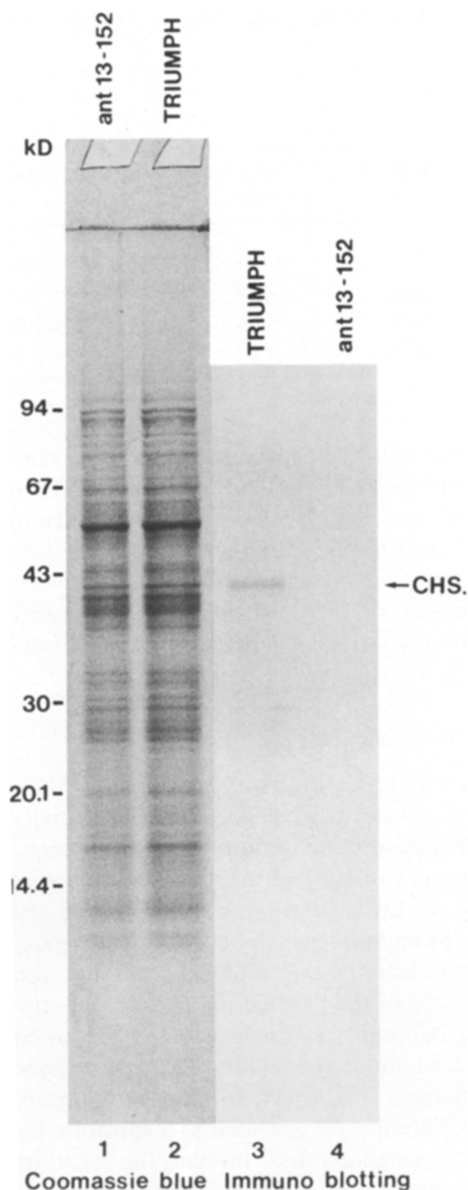

Fig. 2. Polypeptide patterns of barley testa-pericarp tissue from Triumph and *ant* 13-152. The polypeptides (25 μg) were separated on 8–25% SDS-PAGE, transferred to nitrocellulose filters, and probed with chalcone synthase antibodies

vanone 3-hydroxylase activity, whereas extracts from *ant* 13-152 were totally inactive with regard to both enzyme activities (Table 2). If (^{14}C)-DHK was used as a substrate under the same assay conditions, no flavonoid 3′-hydroxylase activity could be demonstrated by any genotype. DHK was not converted to DHQ.

Dihydroflavonol reductase activity

Dihydroflavonol reductase activity in developing barley grains of Nordal barley and proanthocyanidin-free mutants in different genes was studied with ^{14}C-labelled DHK as a substrate. The enzyme activity converted (^{14}C)-DHK to a radioactive compound, which was tentatively identified as leucopelargonidin. As no leucoperlargonidin standard was available, the identification was based on TLC chromatography in three different solvent systems (Table 1) and comparison of the obtained R_f values with published R_f vaues for leucopelargonidin (Heller et al. 1985). As expected, the leucopelargonidin produced a red-colored complex with vanillin in acidic solution. Crude protein extracts from Nordal barley showed the highest enzyme activity followed by extracts from *ant* 17-139, *ant* 22-1508, and *ant* 19-109. *Ant* 21-194

Table 2. Relative flavanone 3-hydroxylase and flavonoid 3′-hydroxylase activities in wild-type barley and proanthocyanidin-free mutants

Genotype	Flavanone-3-hydroxylase		Flavonoid 3′-hydroxylase	
	μg protein	activity, % of wild-type [a]	μg protein	activity, % of wild-type [b]
Wild-type (Nordal)	136	100	128	100
ant 13-152 (Triumph)	184	0	162	0
ant 17-139 (Nordal)	122	82	165	79
ant 18-102 (Nordal)	159	71	150	95
ant 19-109 (Alf)	170	74	148	81
ant 21-194 (Georgie)	179	13	–	ND
ant 22-1508 (Haruna-Nijo)	150	104	–	ND

[a] 100% corresponds to incorporation of 5,200 dpm into DHK
[b] 100% corresponds to incorporation of 2,300 dpm into eriodictyol
ND = not determined

Table 3. Relative dihydroflavonol reductase activity in wild-type barley and proanthocyanidin-free mutants

Genotype	DHK used as substrate		DHQ used as substrate	
	μg protein	activity, % of wild-type [a]	μg protein	activity, % of wild-type [b]
Wild-type (Nordal)	170	100	200	100
ant 13-152 (Triumph)	–	ND	265	2
ant 17-139 (Nordal)	185	95	250	99
ant 18-102 (Nordal)	168	6	272	3
ant 19-109 (Alf)	180	79	250	88
ant 21-194 (Georgie)	160	14	284	20
ant 22-1508 (Haruna-Nijo)	199	92	223	94

[a] 100% corresponds to incorporation of 14,500 dpm into leucopelargonidin
[b] 100% corresponds to incorporation of 31,000 dpm into leucocyanidin
ND = not determined

had decreased enzyme activity and *ant* 18-102 showed almost no activity (Table 3). The results are in agreement with previously published results on dihydroflavonol reductase activity in wild-type barley and proanthocyanidin-free mutants using DHQ as a substrate (Jende-Strid and Kristiansen 1987 and Table 3).

Discussion

Earlier investigations on flavonoid content in barley grains have revealed that only mutants in the *ant* 18 gene accumulate a flavonoid precursor of (+)-catechin and proanthocyanidins, namely, the dihydroflavonol DHQ. Assays for dihydroflavonol reductase activity in crude protein extracts from *ant* 18-102 with two different dihydroflavonols, DHK and DHQ, as substrates showed that the *ant* 18 mutant almost lacked dihydroflavonol reductase activity. *Ant* 18-102 has approximately normal activity of the two enzymes flavanone 3-hydroxylase and flavonoid 3′-hydroxylase. It has been shown by cloning, *in vitro* expression studies, and molecular analyses of *ant* 18 mutants that *ant* 18 is the structural gene for dihydroflavonol reductase in barley and that its nucleotide sequence is homologous to the *A1* gene in maize (K. Kristiansen, unpublished results).

Grains of mutants in the two genes *ant* 17 and *ant* 22 accumulate homoeriodictyol and chrysoeriol. Homoeriodictyol (5,7,4′-trihydroxy-3′-methoxyflavanone) is derived by methylation of eriodictyol, which is the precursor of dihydroquercetin, and chrysoeriol (5,7,4′-trihydroxy-3′-methoxyflavone) is derived either by methylation of the flavone luteolin or by oxidation of homoeriodictyol. The two genes, *ant* 17 and *ant* 22, have been localized to different chromosomes: *ant* 17 is located on chromosome 3 and *ant* 22 on chromosome 1 (Boyd and Falk 1990). In precursor feeding experiments with mutants in the genes *ant* 13, *ant* 17, *ant* 18, and *ant* 19, *ant* 17-139 was the only mutant that could convert significant amounts of ^{14}C-labelled DHQ into (+)-catechin and procyanidin B-3 (Kristiansen 1984). The efficiency of the conversion was 20% of that of the wild-type. It seemed likely that either *ant* 17 or *ant* 22 was the structural gene for flavanone 3-hydroxylase, but in assays for flavanone 3-hydroxylase activity with (^{14}C)-naringenin as a substrate, both *ant* 17-139 and *ant* 22-1508 showed wild-type activity (Table 2). The two mutants had normal activity of dihydroflavonol reductase.

In activity studies with petunia flavanone 3-hydroxylase, only 50% of the enzyme activity was observed when eriodictyol was used as a substrate instead of naringenin (Froemel et al. 1985). It is not known whether the barley flavanone 3-hydroxylase has the same substrate preference. Unfortunately, it has not been possible to demonstrate flavanone 3-hydroxylase activity in protein extracts of the *ant* mutants with (^{14}C)-eriodictyol as a substrate. In *in vitro* experiments the enzyme activity could convert naringenin into DHK, but it is doubtful if this conversion normally occurs *in vivo*. DHK has not so far been detected in barley grains, and the catechin and proanthocyanidins with the same hydroxylation pattern of the B ring as naringenin and DHK (4′-hydroxylation only) have not been detectable in barley. Flavonoid 3′-hydroxylase activity has been demonstrated in wild-type barley and mutants in the genes *ant* 17, *ant* 18, and *ant* 19 with naringenin as a substrate, but DHK could not be converted into DHQ by the enzyme activity under the same assay conditions. Therefore, it is suggested that the main biosynthetic route to proanthocyanidins in barley proceeds via naringenin chalcone, naringenin, eriodictyol, dihydroquercetin, and leucocyanidin. This is in agreement with observations on accumulation of precursors of proanthocyanidins in Douglas fir needles (Stafford 1989).

The ways the genes *ant* 17 and *ant* 22 act are still unclear. Even though flavanone 3-hydroxylase activity was observed with naringenin as a substrate instead of eriodictyol, it might be incorrect to conclude that mutations in two genes affect the activity of the flavanone 3-hydroxylase enzyme protein. Instead, the two genes might code for components required to produce the Fe^{2+}, ascorbate, or 2-oxoglutarate cofactor in the compartment of the cells where it is to be used. Alternatively, it might be possible that mutations in the two genes are associated with overproduction of another enzyme, flavonoid 3′-O-methyltransferase, which is the enzyme responsible for methylation of eriodictyol and luteolin to yield homoeriodictyol and chrysoeriol. In this way, the eriodictyol intermediate could be removed from the flavonoid pathway and the biosynthesis of proanthocyanidins blocked between the eriodictyol and DHQ intermediates.

The proanthocyanidin-free mutant *ant* 19–109 showed approximately normal activity of the three investigated enzymes and it synthesizes wild-type amounts of anthocyanin in the plants. TLC analysis of developing grains revealed that only very small amounts of (+)-catechin was present. Therefore, it is suggested that *ant* 19 is the structural gene for leucoanthocyanidin reductase, the enzyme catalyzing the reduction of (+)-leucocyanidin to (+)-catechin.

No proanthocyanidins or precursors of proanthocyanidins have been detected in the *ant* 13 mutants and *ant* 13-152 had no or almost no flavanone 3-hydroxylase, flavonoid 3′-hydroxylase, or dihydroflavonol reductase activity. The experiments with SDS-PAGE and immunoblotting assays of protein extracts of wild-type barley and *ant* 13-152 showed that no chalcone synthase protein was present in the *ant* 13 mutant. These results indicate that the *ant* 13 gene is working on a regulatory

level. Northern analyses of *ant* 13 mutants with probes from barley cDNA clones coding for chalcone synthase and dihydroflavonol reductase revealed that, at best, 5% of the wild-type amount of chalcone synthase mRNA is produced in the mutants and no mRNA for dihydroflavonol reductase is detected (M. Medlgaard, personal communication). It is inferred that *ant* 13 is a regulatory gene affecting the transcription of at least two structural genes in the flavonoid pathway, namely the genes coding for chalcone synthase and dihydroflavonol reductase (*ant* 18). The *ant* 13 gene might be homologous to the *C1* gene in maize.

The mutant *ant* 21–194 showed reduced activity of the two enzymes flavanone 3-hydroxylase and dihydroflavonol reductase. No precursors of proanthocyanidins have been detected in the grains of mutants in this gene. The *ant* 21 mutants have not been further characterized, but it is possible that *ant* 21 is a regulatory gene affecting the synthesis of anthocyanins and proanthocyanidins.

Conclusions

Ant 13 is regulatory gene acting on the transcriptional level. *Ant* 17 and *ant* 22 might code for different components participating in the hydroxylation of flavanones in 3-position or in the overproduction of the methyltransferase. *Ant* 18 is the structural gene coding for dihydroflavonol reductase. *Ant* 19 is the structural gene coding for leucoanthocyanidin reductase. *Ant* 21 might be a regulatory gene.

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