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Expression of chalcone synthase, dihydroflavonol reductase, and flavanone-3-hydroxylase in mutants of barley deficient in anthocyanin and proanthocyanidin biosynthesis

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Summary. A barley (cv *Triumph*) cDNA library was screened with a cDNA probe encoding flavanone-3-hydroxylase of *Antirrhinum majus*. A full-length clone coding for a protein of 377 amino acids (42 kDa), with an overall homology of 71% and a central domain homology of 85% to the *Antirrhinum* protein, was isolated. This novel barley cDNA and two previously isolated cDNAs encoding chalcone synthase and dihydroquercetin reductase, respectively, were used to study the transcription of the corresponding genes in testa pericarp tissue from *ant 13* mutants of barley. No or very low levels of transcripts are found in mutants *ant 13-152, ant 13-351,* and *ant 13-353*. It is concluded that the gene *Ant 13* encodes a transcription factor operating in the flavonoid biosynthesis of barley. Transcription of the gene for the flavanone-3-hydroxylase (subunit) was also studied in an *ant 17* mutant of barley. Mutant *ant 17-352* transcribes the gene at normal or elevated levels. The mutant is blocked in the synthesis of dihydroquercetin and accumulates derivatives of eriodictyol, the precursor of dihydroquercetin. The combined observations suggest that *Ant 17* is the structural gene for a barley flavanone-3-hydroxylase subunit, and that the mutant allele is a mutation in the structural domain of the gene.

Key words: Barley genes *Ant 13, Ant 17, Ant 18, Ant 22* – Flavonoids – Regulatory gene – Transcription factor

Introduction

Anthocyanins and proanthocyanidins are secondary plant metabolites. They are synthesized via the flavonoid biosynthesis pathway (Fig. 1) and serve a multitude of functions. Flower color stimulated the determination of the chemical structure of flavonoids, many of which are pigments, and this forged early links betweent biochemistry and genetics (Scott-Moncrieff 1939). Flavonoids play central roles in plant defense against biological and environmental stresses such as fungal infection, wounding, and UV irradiation. Under optimal growth conditions, flavonoid biosynthesis is tissue-specific, developmentally controlled, and light-dependent. Stress frequently induces flavonoid biosynthesis in additional tissues of the plant. In response to various stimuli, a coordinate induction or repression of the entire pathway or part of it takes place (Schröder et al. 1979; Lawton et al. 1983; Mehdy and Lamb 1987; van Tunen et al. 1988, 1989).

In the present paper the expression of three of the flavonoid biosynthesis enzymes, chalcone synthase (CHS), dihydroflavonol reductase (DFR), and flavanone-3-hydroxylase (F3H), is analyzed in wild-type and flavonoid mutant lines of barley. The enzymology and gene-enzyme relationships for these three steps in the pathway have been reviewed by Stafford (1989) and Forkmann (1989). CHS synthesizes naringenin chalcone from three molecules of malonyl-CoA and one molecule of coumaroyl-CoA. A compilation of the structural genes known in various plant species is presented in Table 1.

DFR are enzymes reducing dihydroflavonols such as dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydromyricetin (DHM) to the corresponding flavan-3,4-diols (Fig. 1). As cofactor for the reduction of the keto-group at position 4, NADPH is required. The biosynthesis of anthocyanins and proanthocyanidins follows separate routes after the DFR-catalyzed step of the pathway. DFRs differ in their substrate specificity (Forkmann and Ruhnau 1987; Stafford 1989; Forkmann 1989). DFR of *Petunia hybrida* has the highest activity with DHM as substrate and does not reduce DHK. *Zea*

mays DFR cannot reduce DHM. DFR of barley reduces DHK as well as DHQ in vitro (Kristiansen 1986; Jende-Strid 1991). Since leucopelargonidins, the products of DHK reduction, are not detected in barley, and since five of the seven known proanthocyanidins of barley are prodelphinidins, barley DFR seems in vivo to prefer DHQ and DHM as substrates. The plant genes known to encode DFR are listed in Table 1.

F3H catalyzes the hydroxylation in 3-position of flavanones such as naringenin, eriodictyol, and 5,7,3′,4′,5′-pentahydroxy flavanone to yield dihydroflavonols. F3H is a 2-oxoglutarate-dependent dioxygenase requiring Fe^{++}, oxygen, and ascorbate as cofactors (Forkmann et al. 1980; Forkmann and Stotz 1981; Britsch et al. 1981). The enzyme has been purified from *Petunia hybrida* (Britsch and Grisebach 1986; Britsch 1990) and in this species is a dimer composed of two identical subunits of 37 kilo Dalton. As in the case of DFR, different substrate specificities can be expected in different plants or tissues. Partially purified F3H from *Petunia hybrida* readily 3-hydroxylated naringenin and eriodictyol, but failed to catalyze the 3-hydroxylation of the pentahydroxy flavanone. Structural plant genes thus far implicated for F3H are listed in Table 1.

Barley and sorghum are unique among cereals in producing proanthocyanidins during development of the caryopses. In barley the proanthocyanidins are localized in the testa tissue (Aastrup et al. 1984), while in sorghum they are found in both the testa and aleurone tissues. For the plant breeder the content of proanthocyanidins in the grain is of interest, since these compounds survive the malting, mashing, and fermentation operations in the brewing of beer. Permanent haze and chill haze in the beer is caused through precipitation of proteins by proanthocyanidins, and colloidal stability and long-lived brilliance of beer is achieved by stabilizing treatments, which remove protein or proanthocyanidin or both. An alternative approach is to breed proanthocyanidin-free malting-barley varieties (von Wettstein et al. 1977, 1985). Towards this end, more than 600 single gene mutants that are blocked in proanthocyanidin biosynthesis have been induced by chemical mutagenesis or radiation. By diallelic crosses, 474 of the mutants have been assigned to nine different gene loci (Jende-Strid 1988, 1991). Biochemical analysis of these mutants have shown that loci *Ant13, Ant17, Ant18, Ant21,* and *Ant22* control both anthocyanin pigmentation in the vegetative organs of the plant and proanthocyanidin formation in the testa tissue. This implies that these genes determine steps in the synthesis of 2,3-*trans*-3,4-*cis*-leucocyanidin. Mutants in the loci *Ant19, Ant25, Ant26,* and *Ant27* synthesize anthocyanin in the vegetative organs of the plant, but are blocked in the formation of proanthocyanidins in the testa tissue. These genes thus control steps in the branch converting leucocyanidin into proanthocyanidins.

Genetic and biochemical studies of the *Ant* loci of barley address gene-enzyme relations and the identification of structural and regulatory *Ant* genes (Jende-Strid 1991). In this paper a barley cDNA clone with high homology to the F3H gene of *Antirrhinum majus* is presented. Transcription of CHS, DFR, and F3H is analyzed in barley mutant genes *ant13* and *ant17*. The anthocyanin- and proanthocyanidin-free *ant13* mutants analyzed are deficient in the transcription of the structural genes encoding CHS, DFR, and F3H. The *ant17-352* mutation permits transcription of the gene, but does not contain an active enzyme. It is proposed that *Ant17* is the structural gene for a F3H subunit and that the *ant17-352* mutation has occurred in the coding region.

Materials and methods

Plant material

Wild-type barley (*Hordeum vulgare* L., cv *Triumph*) and the proanthocyanidin-deficient mutants *ant13-152, ant13-353, ant13-351,* and *ant17-352* (all induced by sodium azide in *Triumph*) were used in the present study (Jende-Strid 1988). Also analyzed was mutant *ant22-1508* induced in the Japanese cultivar *Haruna-Nijo*. The plants were grown under greenhouse conditions during the summer. Testa pericarp tissue was harvested (unless otherwise stated) 9–10 days after anthesis and used for the preparation of RNA.

Poly (A)$^+$ RNA extraction

For total RNA isolation a modified version of the procedure of Han et al. (1987) was used: 4 g of plant material was soaked in a solution of 5 *M* guanidinium thiocyanate, 8% (v/v) β-mercaptoethanol, 0.1% (w/v) N-lauroyl-sarcosine, 50 m*M* Tris-(hydroxymethyl)-aminomethane (TRIS) (pH 7.5), and 10 m*M* ethylene diamine tetraacetic acid (EDTA). Cells were disrupted in this chaotropic solution on ice using a Polytron (Kinematica GmbH, Lucerne, Switzerland). Insoluble material was spun down and nucleic acids precipitated from the supernatant by addition of an equal volume of ethanol, followed by centrifugation for 20 min at 10,000 × *g* and 0 °C. The pellet was dissolved in 5 ml of the above solution and adjusted to 2.5 *M* LiCl, 0.06% (w/v) N-lauroylsarcosine in a total volume of 20 ml. Incubation for 40 h at 4 °C followed by centrifugation at 15,000 × *g* and 4 °C for 90 min selectively precipitated the RNA fraction of the nucleic acid content of the solution. The pellet was resuspended in 20 ml of a solution of 2.5 *M* LiCl, 2.5 *M* urea and centrifuged as before. The pellet was dissolved in TE buffer [10 m*M* TRIS (pH 7.5), 1 m*M* EDTA, and 0.1% (w/v) N-lauroylsarcosine] and successively extracted with phenol, phenol:chloroform:isoamylalcohol (25:24:1), and chloroform. After each extraction the organic phase was re-extracted with TE buffer. Re-extractions increased yields by 200–400%. RNA was recovered from the pool aqueous phases by standard ehtanol precipitation, the pellet was dissolved in RNase-free water (0.5–1.0 ml) and yields were determined spectrophotometrically by taking a 2 µl aliquot into 1 ml water and measuring at 260 nm (1 OD_{260} unit is 40 µg/ml). Before proceeding to poly (A)$^+$ RNA isolation, the quality of the RNA was estimated by gel electrophoresis.

Poly(A)$^+$ RNA extraction was done by oligo(dT)-cellulose-chromatography in a gravity-driven column using standard procedures (Maniatis et al. 1982), except that 0.1% (w/v) N-lauroyl-

sarcosine was included in all solutions to inhibit RNase activity and increase flow rate in the column. The chromatography was repeated once in order to obtain very pure poly $(A)^+$ RNA.

RNA gel electrophoresis

Minigels were prepared with 1.5% (w/v) agarose (Seakem GTG, FMC) in 1 × MOPS buffer [20 m*M* MOPS (pH 6.8), 5 m*M* Na-acetate, 1 m*M* EDTA], and 4% (w/v) formaldehyde was just prior to pouring the gel. RNA to be analyzed (0.2–2.0 µg) was denatured by heating at 96 °C for 3 min in loading buffer containing 50% (v/v) formamide, 1 × MOPS buffer, and dye (bromophenol blue). Electrophoresis was for 2–3 h at 40 V with occasional stirring to mix the buffer content of the two separate reservoirs of the minigel electrophoresis unit. For visualization of the RNA on an UV transilluminator, ethidium bromide (0.5 µg/ml gel) was included in the agarose gel or, alternatively, in the second wash with RNase-free water prior to RNA blotting.

Probes for analysis of transcription and for colony hybridization

Plasmid DNA was prepared by alkaline lysis according to (Maniatis et al. 1982).

CHS plasmid pcCHS11 (pUC9 containing a full-length cDNA insert of the barley chalcone synthase gene) was obtained from Prof. W. Rohde, Max-Planck-Institute for Plant Breeding, Cologne (Rohde et al. 1987).
DFR plasmid (pSP65 containing a 1,250-bp cDNA fragment of the barley dihydroquercetin reductase gene) was obtained from K. N. Kristiansen, Carlsberg Laboratory.
F3H plasmid pJAM239 (pUC18 containing a 1,357-bp cDNA fragment of the *Antirrhinum majus* mRNA encoding the flavonone-3-hydroxylase) was kindly provided by Dr. Cathie Martin, John Innes Institute, Norwich.
F3H plasmid pc3H5 (pUC12 containing a 1,363 bp barley cDNA fragment encoding flavanone-3-hydroxylase) was isolated in the present experiment.

Template DNA for the synthesis of labeled probes was prepared by releasing the cDNA insert from their vectors with the restriction enzyme *Eco*RI or, in the case pc3H5, with *Eco*RI and *Bam*HI. The inserts were electrophoretically purified twice in a 1.2% (w/v) agarose gel and isolated from the gel with a GeneClean Kit (Bio 101, La Jolla Ca).

Synthesis of radioactively labeled probe DNA was done by the random prime method [Feinberg and Vogelstein 1983; Prime It Kit, Stratagene, La Jolla, Ca (1989)] using α-^{32}P-dCTP (> 3,000 Ci/mmol).

Northern blot and probing

For analysis of transcripts, wild-type (*Triumph*) and *ant* mutant poly $(a)^+$ RNAs were separated in a formaldehyde-containing agarose gel by electrophoresis. The gel was then washed twice in RNase-free water to remove formaldehyde and subsequently equilibrated in 10 × SSC (1.5 *M* NaCl, 0.15 *M* sodium citrate). The RNA was blotted over to a nylon filter (GeneScreen Plus, Du Pont) by capillary action for 16 h. The filter was then exposed to UV light for 2 min, dried, and baked 1 h at 80 °C in a vacuum oven. Hybridizations were performed according to the protocol of the manufacturer, Du Pont, except that the stringency during hybridization was higher (0.3 *M* NaCl and 68 °C), and post-hybridization wash was at 68 °C in 1 × SSC (high stringency).

cDNA synthesis

Complementary DNA was synthesized utilizing the RNaseH approach (Gubler and Hoffmann 1983) and $(dT)_{15}$ oligodeoxynucleotides as primers. Five micrograms *Triumph* poly$(A)^+$ RNA (mRNA) was reverse transcribed using M-MuLV (Moloney Murine Leukemia Virus) reverse transcriptase into first-strand cDNA in a reaction volume of 33 µl. Subsequently, the second strand was synthesized using RNaseH and DNA polymeraseI in a reaction volume of 100 µl. The cDNA ends were trimmed using Klenow enzyme and the cDNA was size-fractionated by agarose gel electrophoresis in a 1.2% agarose gel [electrophoresis buffer: 1 × TAE (Maniatis et al. 1982)]. Double-stranded cDNA ranging in size from 0.6 to 5.0 kbp was isolated from the gel using a GeneClean Kit (Bio 101, La Jolla/Ca).

cDNA cloning

Cloning of the size-selected cDNA was done in the plasmid vector, pUC12, linearized with blunt ends by restriction enzyme digestion with *Sma*I. The single site for *Sma*I is located in the multiple cloning site region of pUC12, permitting a blue/white screening of non recombinant/recombinant clones of the cDNA library. Following linearization and purification, the plasmid was dephosphorylated as follows: 10 µg plasmid was mixed in a buffer of 50 m*M* TRIS (pH 8.0 at 22 °C), 10 m*M* Mg chloride, 0.5 m*M* spermidine HCl, 0.1 m*M* $ZnSO_4$, 0.01 m*M* EDTA. 0.5 units calf intestinal phosphatase (CIP, Boehringer-Mannheim) was added and the reaction was incubated for 15 min at 37 °C followed by 10 min at 56 °C. This procedure was repeated twice. The reaction mixture was heated for 15 min at 62 °C in the presence of 15 m*M* EGTA (complexes Zn^{++} selectively) and the vector was purified by phenol extraction and ethanol precipitation. The suitability of the vector for cloning of cDNA was tested by transforming *E. coli* after ligation with and without a blunt-ended 1-kbp DNA fragment, and by comparing the frequency of transformation to that of the linearized, non ligated pUC12. When that of the vector self-ligation did not exceed that of linear pUC12 and the cloning of the 1-kbp DNA fragment was efficient, giving a low background of blue (non recombinant) clones, the vector was found suitable for cDNA cloning.

The blunt-ended cDNA was ligated in the cloning vector as follows: 200 ng cDNA and 270 ng vector DNA was mixed to give an approximate mol ratio of 2:1 (cDNA to vector). The volume was adjusted to 170 µl to give 66 m*M* TRIS (pH 7.6 at 14 °C), 10 m*M* $MgCl_2$, 10 m*M* DTT, 0.3 m*M* ATP, 1 m*M* spermidine HCl, 1 m*M* hexamine cobalt chloride, and 0.2 mg/ml BSA. Seventeen units T4 DNA ligase (Boehringer-Mannheim) were added and the reaction was incubated for 20 h at 14 °C followed by 6 h at 22 °C.

For screening of this cDNA library, aliquots of 4.2 µl were used in the transformation of *E. coli* strain DH5α (Bethesda Research Laboratory/Life Technologies Inc., Maryland). The protocol followed in transformation was that of D. Hanahan for frozen, competent cells (Hanahan 1983; 1985).

Colony hybridization; cDNA library screening with heterologous cDNA encoding F3H

Following transformation of DH5α, approximately 5,000 transformed cells were planted onto 10 LB agar plates containing 0.1 mg/ml ampicillin, 40 µg/ml X-gal (for the blue staining of non recombinant clones on the plates), 0.2 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) and with an 82 mm nylon filter (GeneScreen Plus, NEF978, NEN Research Products, Du Pont)

on the agar surface. These transformation plates were incubated for 13 h at 37 °C, cooled to 4 °C, and used to prepare replicas by lifting the colony-carrying master filter off the agar plate and placing it on sterile Kleenex tissue, colony-side up. The nylon filter to be used for replica was wetted on a LB agar plate containing ampicillin and laid onto the master filter applying gentle pressure. The replica was subsequently incubated for 3 h at 37 °C on the plate used for wetting. Lysis, fixation, and hybridization were performed as described in the manufacturer's protocol for GeneScreen Plus, NEF 978, with the following changes: hybridization solution included poly (dA) (1 μg/ml) and salmon sperm DNA (30 μg/ml), during prehybridization as well as during hybridization.

Prehybridization was for 1.5 h and hybridization was for 24 h, both at 65 °C. An activity about 1.5×10^5 cpm/ml of the random-primed probe synthesized from the *Antirrhinum majus* F3H-cDNA was used for hybridization.

In the primary screening for hybridizing clones of the barley cDNA library, stringency of washing was 2 × SSC, 50 °C. In the second round of hybridization the temperature of the washing was raised to 60 °C.

As a control for hybridization stringency, DH5α, transformed to ampicillin resistance using non recombinant vector, pUC12, was streaked on a small border area of the replica filter prior to incubation at 37 °C for 3 h. No hybridization signal was expected from this under sufficiently stringent conditions.

Southern blot and probing

To unambiguously establish the plasmid-inserted barley cDNA as the hybridizing entity of the colony, cDNA was excised from the vector by restriction enzyme digestion with *Eco*RI and *Bam*HI of the purified recombinant plasmid. Subsequently, the digest mix was electrophoresed in a 1% GTG agarose minigel and capillary blotted to a piece of nylon filter (GeneScreen Plus, Du Pont). Hybridization with the *Antirrhinum majus* F3H cDNA probe was performed as described for the second round of colony hybridization (60 °C in the 2 × SSC wash).

Sequencing of barley F3H cDNA

Plasmid sequencing was done using a two-step chain termination method and T7 DNA polymerase (Sequenase 2.0, United States Biochemical Corp., Cleveland OH). Sequencing artifacts due to secondary structures in the cDNA were resolved, either by automated fluorescent DNA sequencing using the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems 373A DNA Sequencer, or by substituting dITP for dGTP in the sequencing DNA synthesis reactions. Oligodeoxynucleotides (17-mers) for priming were synthesized on an applied Biosystems 380 A DNA Synthesizer.

The sequence reported in this publication will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number X58138 *H. vulgare* Flavanone-3-hydroxylase cDNA.

Results

Cloning of a full-length cDNA encoding barley flavanone-3-hydroxylase (F3H)

From barley, only two genes relating to the flavonoid biosynthesis were available for the molecular study of this pathway. These were cDNAs for chalcone synthase and dihydroflavonol reductase, respectively. Here, a third cDNA relating to the flavonoid biosynthesis is presented. This encodes flavanone-3-hydroxylase (F3H) (Table 1, Fig. 1).

A wild-type (cv *Triumph)* cDNA library prepared in pUC12 was screened with a nearly full-length F3H cDNA isolated from *Antirrhinum majus* and kindly provided by Dr. Cathie Martin, John Innes Institute, Norwich (Martin et al. 1991). In the first round of colony screening, 15 clones were isolated from a total of 5,000 colonies. In the second round of screening, the temperature of the post-hybridization wash was raised (from 50 °C to 60 °C) and only seven clones showed strong hybridization. From six recombinant clones, plasmids were extracted and analyzed by Southern blot and hybridization with the *A. majus* F3H cDNA. The six insert cDNAs all showed strong hybridization to the probe, and the two largest, c3H2 (1.2 kbp) and c3H5 (1.3 kbp), were chosen for sequence analysis.

It was found that the nucleotide sequences of the two cDNAs are identical, except that c3H5 is 157 nucleotides longer 5′ and c3H2 is 31 nucleotides longer 3′. In combination they provide 1,363 nucleotides transcript-sequence (Fig. 2). c3H5 is a full-length cDNA for barley F3H, as it includes a putative start codon, ATG at position 41-43 (overlined in Fig. 3). This ATG is preceded and followed by the same sequences as the start codon of the barley CHS gene (GATCG-ATG-GCG) (Rohde et al. 1988). An open reading frame, starting with this methionine codon, extends for 1,131 nucleotides, encoding a protein of 377 amino acid residues (42 kDa) (Fig. 3). Comparison between this amino acid sequence and the sequence deduced from the *A. majus* F3H cDNA reveals an overall homology of 71%. It also shows that the F3H sequence extends for 22 additional residues at the C-terminal end when compared to the c3H5-derived sequence. A central stretch of 110 amino acid residues exhibits a homology of 85% to the *A. majus* sequence (residues 174 through 283, shaded sequence in Fig. 3). This protein domain is expected to be indispensable for F3H activity due to the high degree of conservation. The amino terminal 100 residues show 54% homology and the carboxy terminal 33 residues of the barley sequence show less than 25% homology to the *A. majus* F3H amino acid sequence.

Expression of chalcone synthase, dihydroflavonol reductase, and flavanone-3-hydroxylase in proanthocyanidin-free barley mutants

Proanthocyanidins are synthesized in the testa tissue during development of the barley kernel. Kristiansen (1984) determined the time course of catechin and proanthocyanidin accumulation during grain maturation. Catechin, procyanidin B3, and procyanidin C2 increased linearly between 8 and 16 days after anthesis (flowering), after which synthesis declined. The time course of tran-

Table 1. Survey of structural genes encoding chalcone synthase (CHS), dihydroflavonol reductase (DFR), and flavanone-3-hydroxylase (F3H)

Species	Gene	cDNA	Gene clone	Transcriptional regulation	References
CHS					
Antirrhinum majus	*Nivea*	+	+	UV induction	Wienand et al. 1982 Sommer and Saedler 1986 Staiger et al. 1990
Arabidopsis thaliana			+	Light	Feinbaum and Ausubel 1988
Glycine max	Multigene family (>6)		+		Akade et al. 1990
Hordeum vulgare	*Chs*	+	+		Rohde et al. 1987, 1988
Magnolia liliiflora		+			Niesbach-Klösgen et al. 1987
Matthiola incana		+			Epping et al. 1990
Petroselium hortense		+		UV induction	Kreuzaler et al. 1983 Reimold et al. 1983
Petunia hybrida	Multigene family (8)	+	+	2 genes expressed in flower, light/UV induces 4 genes	Reif et al. 1985 Koes et al. 1986, 1987, 1989 and 1990
Phaseolus vulgaris	Multigene family (6–8)	+		Differential induction by pathogens, elicitors wounding, light	Ryder et al. 1984, 1987
Pisum sativum	Multigene family	+		2 genes expressed in petals and root, 3 genes by $CuCl_2$, 2 regulator genes	Harker et al. 1990
Ranunculus acer		+			Niesbach-Klösgen et al. 1987
Sinapis alba		+			Ehmann and Schäfer 1988
Zea mays	*C2*	+	+		Wienand et al. 1986
DFR					
Antirrhinum majus	*Pallida*	+			Martin et al. 1985 Beld et al. 1989
Hordeum vulgare	*Ant18*	+	+	*Ant13* gene product	Rohde et al. 1988 Jende-Strid 1991 K. N. Kristiansen, in preparation
Petunia hybrida	*An6*	+		Several regulatory genes	Beld et al. 1989
Zea mays	A1	+	+	Several regulatory genes	Fedoroff 1983 O'Reilly et al. 1985 Reddy et al. 1987
F3H					
Antirrhinum majus	*Inc*	+	+		Martin et al. 1991 Prescott et al. 1990
Hordeum vulgare	*Ant17*	+			This paper
Petunia hybrida	*An3*	+			Froemel et al. 1985 Martin et al. 1991

script levels was determined for CHS and for DFR in order to see whether or not transcription of the genes encoding the enyzmes parallels the accumulation of the products of the pathway, and to evaluate appropriate sampling times for transcription analysis. Testa pericarp tissue from wild-type barley (cv *Triumph*) was harvested at about 2, 10, and 16 days after anthesis, poly $(A)^+$ RNA was extracted and approximately equal amounts were loaded into six slots of an agarose gel. After electrophoretic separation, the RNA was blotted onto two nylon filters and hybridized with barley CHS and barley DFR probes, respectively. The results of the hybridization with the CHS probe in Fig. 4 indicate the highest steady-state of CHS mRNA to be present at about 10 days after anthesis, in agreement with the time course for product accumulation mentioned above. Hybridization with the DFR probe gave an analogous result; this indicates a coordinated expression of these two genes.

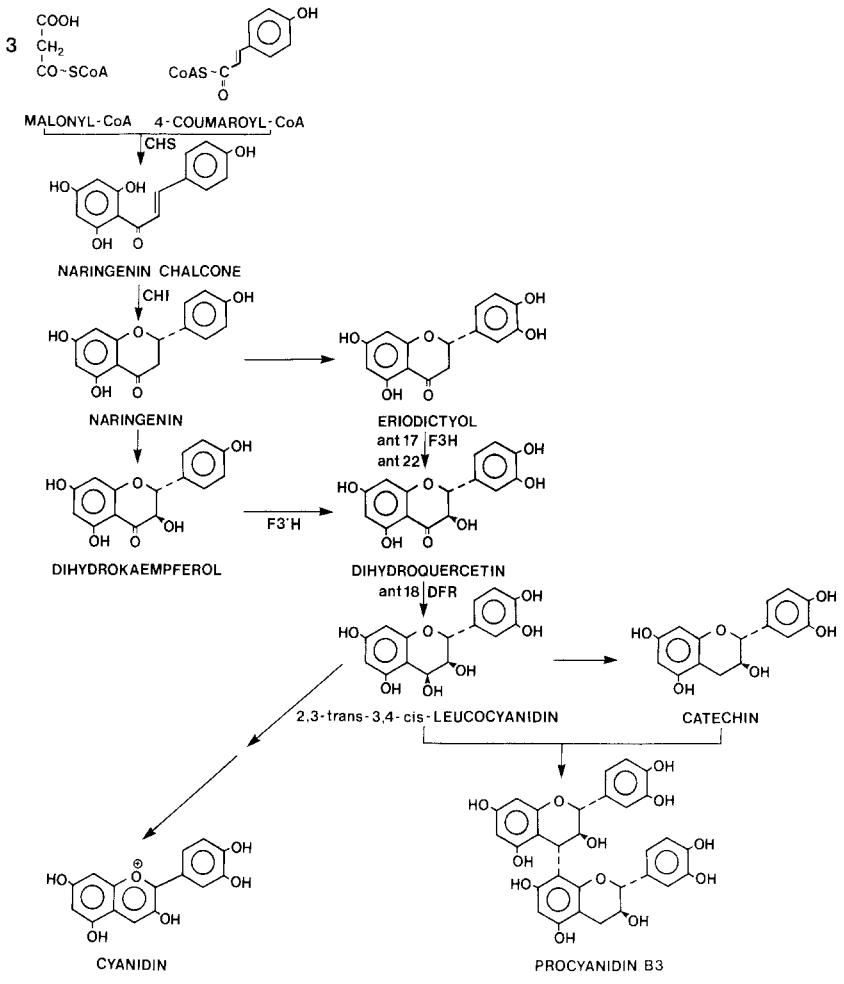

Fig. 1. Biosynthesis of flavonoids in barley (simplified scheme). CHI = chalcone isomerase; CHS = chalcone synthase; F3'H = flavanone-3'-hydroxylase; F3H = flavanone-3-hydroxylase; DFR = dihydroflavonol reductase

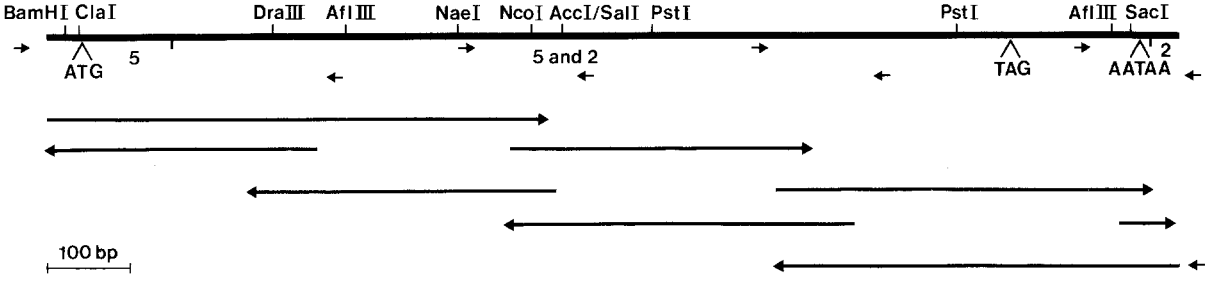

Fig. 2. Sequencing strategy and restriction endonuclease site map of the full-length barley F3H cDNA (1,363 bases). No. 5 indicates the 157 base pairs unique to clone c3H5, no. 2 indicates the 31 bp unique to clone c3H2. Primers (17-mers) used for sequencing with the dideoxy-nulceotide chain termination method are shown as small arrows. ATG is the likely start codon and TAG the corresponding stop codon. A putative poly A-signal (AATAA) is found in the 3'end

Mutant *ant 13-152* (induced in cv *Triumph*) lacks DFR activity, F3H activity, and flavanone-3'-hydroxylase activity (F3'H activity) in the testa pericarp tissue, and no CHS protein could be detected with an antiserum, which readily detects this protein in wild-type barley (Jende-Strid and Kristiansen 1987; Jende-Strid 1991). *Ant 13* is therefore considered to be a regulatory gene. Its product is necessary for the synthesis of several, if not all, enzymes in the flavonoid pathway. Northern blots and hybridization with labeled barley CHS cDNA demonstrates that *ant 13-152* also lacks transcripts for CHS (Fig. 5), while *ant 13-353* synthesizes a small amount of CHS homologous messages, one species with the size of the wild-type transcript (1.6 kb) and a second species,

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1 GGCGACACAAGCGCGCGTAGGATCCCCGGCGTGCGGATCGATGGCGCCGGTGAGCAACGAGACGTTCCTCCCGACGGAGGCGTGGGGGGAGGCCACGCTGCGCCCGTCCTTCGTGCGGGAC
                                  MetAlaProValSerAsnGluThrPheLeuProThrGluAlaTrpGlyGluAlaThrLeuArgProSerPheValArgAsp  2

122 GAGGACGAGAGGCCCAAGGTGGCGCACGACCGCTTCAGCGACGCGGTGCCGCTGATCTCGCTCCACGGCATCGACGGCGCCGCGCCGGGCCCAGATCCGGGACCGCGTGGCCGCCGCCTGC
    GluAspGluArgProLysValAlaHisAspArgPheSerAspAlaValProLeuIleSerLeuHisGlyIleAspGlyAlaArgArgAlaGlnIleArgAspArgValAlaAlaAlaCys  6

242 GAGGACTGGGGCATCTTCCAGGTGATCGACCACGGCGCTGGATGCGGACCTCATCGCCGACATGACGCGCCTGGCTCGCGAGTTCTTCGCGCTGCCCGCCGAGGACAAGCTCCGGTACGAC
    GluAspTrpGlyIlePheGlnValIleAspHisGlyValAspAlaAspLeuIleAlaAspMetThrArgLeuAlaArgGluPhePheAlaLeuProAlaGluAspLysLeuArgTyrAsp 10

362 ATGTCCGGCGGCAAGAAGGGCGGCTTCATCGTCTCCAGCCACCTACAGGGTGAGGCGGTGCAGGACTGGAGGGAGATAGTGACCTACTTCTCGTACCCGGTGAAGGCGCGGGACTACGGG
    MetSerGlyGlyLysLysGlyGlyPheIleValSerSerHisLeuGlnGlyGluAlaValGlnAspTrpArgGluIleValThrTyrPheSerTyrProValLysAlaArgAspTyrGly 14

482 CGGTGGCCGGAGAAGCCGGCGGGGTGGTGCGCGGTGGTGGAGCGGTACAGCGAGCGGCTCATGGGGCTGTCGTGCAATCTGATGGGCGTGCTGTCGGAGGCCATGGGCCTGGAGACGGAG
    ArgTrpProGluLysProAlaGlyTrpCysAlaValValGluArgTyrSerGluArgLeuMetGlyLeuSerCysAsnLeuMetGlyValLeuSerGluAlaMetGlyLeuGluThrGlu 18

602 GCGCTGGCCAAGGCGTGGCGTCGACATGGACCAGAAAGTGGTGGTCAACTTCTACCCGCGGTGCCCGCAGCCCGACCTCACCCTGGGCCTCAAGCGCCACACCGACCCCGGCACCATAACG
    AlaLeuAlaLysAlaCysValAspMetAspGlnLysValValValAsnPheTyrProArgCysProGlnProAspLeuThrLeuGlyLeuLysArgHisThrAspProGlyThrIleThr 22

722 CTCCTCCTGCAGGACCTCGTCGGCGGCCTCCAGGCCACCCGCGACGGCGGCAAGAACTGGATCACCGTCCAGCCCATCTCCGGCGCATTCGTCGTCAACGCTCGGCGACCACGGCCACTTC
    LeuLeuLeuGlnAspLeuValGlyGlyLeuGlnAlaThrArgAspGlyGlyLysAsnTrpIleThrValGlnProIleSerGlyAlaPheValValAsnLeuGlyAspHisGlyHisPhe 26

842 ATGAGCAACGGCAGGTTCAAGAACGCCGACCACCAGGCGGTGGTGAACGGGGAGAGCAGCAGGCTGTCGATCGCGACGTTCCAGAACCCGGCGCCGGACGCGAGGGTGTGGCCGCTGGCG
    MetSerAsnGlyArgPheLysAsnAlaAspHisGlnAlaValValAsnGlyGluSerSerArgLeuSerIleAlaThrPheGlnAsnProAlaProAspAlaArgValTrpProLeuAla 30

962 GTGAGGGAGGGGGAGGAGCCCATACTGGAGGAGCCCATCACCTTCACCGAGATGTACCGCCGCAAGATGGAGCGCGACCTCGACCTCGCCAAGCGCAAGAAGCAGGCCAAGGACCAGCTG
    ValArgGluGlyGluGluProIleLeuGluGluProIleThrPheThrGluMetTyrArgArgLysMetGluArgAspLeuAspLeuAlaLysArgLysLysGlnAlaLysAspGlnLeu 34

1082 ATGCAGCAGCAGCTGCAGCTCCAGCAGCAGCAGGCGGTCGCCGCGCGCGCCCATGCCCACCGCCACCAAGCCCCTCAACGAAATTCTTGCCTAGATCCTTCCGGCGCGCGGGCGGCGATTAA
    MetGlnGlnGlnLeuGlnLeuGlnGlnGlnGlnAlaValAlaAlaAlaProMetProThrAlaThrLysProLeuAsnGluIleLeuAla                              37

1202 ATACTTCAAATTGATGGATGCGTGGGATTGATTCTCCTAAGTACTAGTACGATATAAATTATTGCATGCATATATCCGTACGTGTGTAGCAGGGAGGAGCTCGGCCTGTAATAACGTCGT

1322 GCGTGGAACTGAACCGAGCTAAGCAAGTTCATGAGTTTAGCG
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Fig. 3. Nucleotide and deduced amino acid sequence of the barley F3H cDNA. The start and stop codons as well as the putative poly A-signal are overlined. The central domain, which shows 85% amino acid sequence homology to the *A. majus* sequence, is indicated by shading

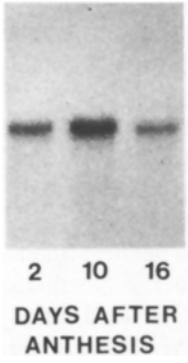

Fig. 4. Northern blot of poly(A)$^+$ RNA from testa pericarp tissue of *Triumph* barley 2, 10, and 16 days after anthesis. The blot was probed with barley chalcone synthase cDNA

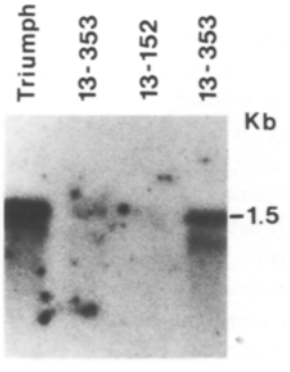

Fig. 5. Northern blot of poly (A)$^+$ RNA from testa pericarp tissue of *Triumph* barley and proanthocyanidin-free mutant *ant* 13-353 (two separate preparations, 10 days after anthesis) and *ant* 13-152 (14 days after anthesis). The blot was probed with barley chalcone synthase cDNA. Exposed for 96 h with an itensifying screen at −75 °C

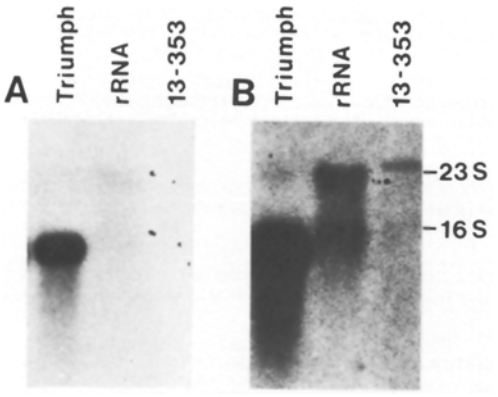

Fig. 6 A, B. Northern blot of poly(A)$^+$ RNA from testa pericarp tissue (9–10 days after anthesis) of *Triumph* barley and the proanthocyanidin-free mutant *ant* 13-353. The blot was probed with barley dihydroflavonol reductase cDNA. **A** 46-h exposure at room-temperature; **B** 2-week exposure with an itensifying screen at −75 °C

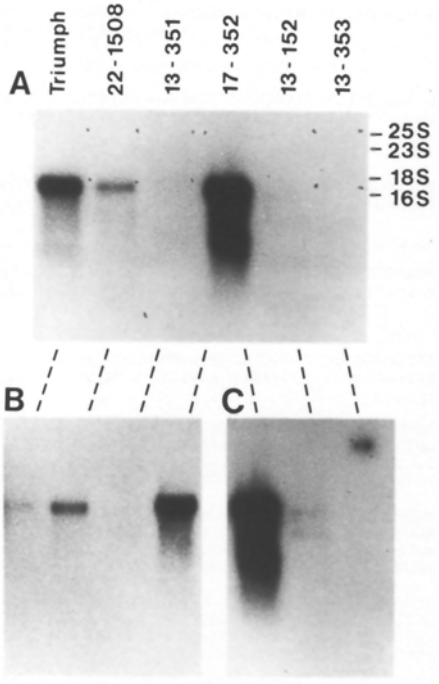

Fig. 7 A–C. Northern blot of poly(A)$^+$ RNA from testa pericarp tissue of *Triumph* barley and the proanthocyanidin-free mutants *ant* 22-1508, *ant* 13-351, *ant* 17-352, *ant* 13-353 (all 9–10 days after anthesis) and *ant* 13-152 (14 days after anthesis). The blot was probed with barley flavanone-3-hydroxylase cDNA (C3H5). **A** 48-h exposure at room-temperature; **B** result from a blot similar to the left *let-hand* part of panel **A. C** 1-week exposure with an intensifying screen at −75 °C of the *right-hand* part of the blot from panel *A*

which is 200–300 nucleotides smaller. When mutant *ant 13-152* is probed with DFR cDNA, no transcript can be detected (data not shown), and the same result is obtained with *ant 13-353* (Fig. 6 A, B). The size markers included in the gel (16S *E. coli* rRNA = 1,540 nucleotides, 23S *E. coli* rRNA = 2.9 kb) permit a size estimate for the wild-type DFR transcript of 1.4 kb. No or very little messenger RNA (mRNA) for F3H is found in mutant *ant 13-152, ant 13-351* and *ant 13-353* (Fig. 7 A). The two weak bands visible after prolonged exposure of the filter (Fig. 7 C) from *ant 13-152* represent two transcripts shorter than the single transcript of the wild type. This could signify two weakly expressed homologous genes.

It can be concluded that the absence of the flavonoid synthesizing enzymes CHS, DFR, and F3H in barley carrying the mutant alleles *ant 13-152, ant 13-351* or *ant 13-353* is due to the absence or insufficient amounts of a transacting factor necessary for the transcription of the genes encoding these three enzymes. It is likely that this transacting factor is the gene product of the *Ant 13* gene, possibly a DNA-binding protein. Very likely this gene product is required also for the transcription of other genes in the flavonoid pathway of barley, such as the gene for flavanone-3'-hydroxylase (F3'H).

Barley mutants in the *ant 17* gene (*ant* 17-139, *ant 17-148, ant 17-151,* and *ant 17-192*) accumulate derivatives of eriodictyol (homoeriodictyol = 5,7,4'-trihydroxy-3'-methoxyflavanone and chrysoeriol = 5,7,4'-trihydroxy-3'-methoxyflavone). Eriodictyol is a substrate for F3H and the precursor of dihydroquercetin, the major dihydroflavonol in barley testa pericarp (Jende-Strid 1985; Jende-Strid and Kristiansen 1987; Jende-Strid 1991). The enzymatic capacity of *ant 17-139* has been investigated and it was found that this mutant contains wild-type amounts of activity of the three enzymes for which it was tested (DFR, F3'H, and F3H). Mutant *ant 22-1508* was found to accumulate the same two flavonoids and to contain the same level of the three enzyme activities (Jende-Strid 1985, 1991); *ant 22-1508* complements *ant 17-105* in anthocyanin and proanthocyanidin biosynthesis (Table 2).

Poly (A)$^+$ RNA was isolated from test pericarp tissue of wild-type barley (cv *Triumph*) and of mutants, *ant 17-352,* and *ant 22-1508*. Probing of Northern blots with c3H5 (encoding the barley F3H) revealed that *ant 17-352* has steady-state levels of the F3H transcript of at least the same order of magnitude as the wild type (Fig. 7 A, B). The mutant *ant 22-1508* seems to transcribe the F3H gene to the same extent as *Triumph* barley. A comparison of Fig. 7 B with 7 A demonstrates that no detailed quantitative conclusions can be drawn from the Northern blots, since the amounts of poly(A)$^+$ RNA loaded varied with the degree of enrichment for poly (A)$^+$ RNA in the RNA preparations loaded.

The complementation analysis in F_1 plants of crosses with the *ant 17* mutant is critical for the evaluation of the results. The complementation tests in Table 2 establish that mutant *ant 17-352* does not complement *ant 17-151*

Table 2. Complementation tests for presence (+) or absence (−) of anthocyanin and proanthocyanidins in crosses between proanthocyanidin-free barley mutants (courtesy, of B. Jende-Strid)

Combination	No. of F_1 plants	Presence (+) or absence (−) of anthocyanin and proanthocyanidin
ant17-352 × *ant17-151*	12	−
ant17-352 × *ant17-151*	16	−
ant13-351 × *ant13-353*	5	−
ant13-353 × *ant13-152*	14	−
ant13-351 × *ant18-148*	6	+
ant13-351 × *ant17-352*	8	+
ant17-352 × *ant13-13*	4	+
ant13-353 × *ant17-151*	4	+
ant13-353 × *ant17-151*	4	+
ant13-353 × *ant17-352*	8	+
ant13-351 × *ant18-164*	5	+
ant13-353 × *ant18-102*	5	+
ant13-353 × *ant18-164*	2	+
ant13-353 × *ant18-164*	13	+
ant17-352 × *ant18-102*	10	+
ant22-1508 × *ant13-13*	7	+
ant22-1508 × *ant17-105*	5	+
ant22-1508 × *ant18-102*	8	+

in proanthocyanidin synthesis. Mutants in *ant17* on the other hand, complement the *ant13*, *ant*18, and *ant22* mutants. It can therefore be inferred that the transcript of mutant allele *ant17-352* encodes an inactive protein, e.g., due to a missense of reading frame shift mutation.

With the exception of the observation that crude protein extracts of mutant *ant17-139* (with the addition of oxoglutarate, Fe^{++}, and ascorbate) in vitro can catalyze hydroxylation of flavanone, the data suggest that *Ant17* is a structural gene for barley F3H.

Discussion

The ant13 gene encodes a transcription factor operating in flavonoid biosynthesis

Mutants in the *Ant13* gene lack anthocyanin pigments in all plant organs, as well as catechin and proanthocyanidins in the testa tissue of the kernels. No precursor flavonoids or side products were accumulated in the *ant13* mutants investigated (Kristiansen 1984; Jende-Strid 1985). When developing testa pericarp tissue of mutants *ant13-101* or *ant13-152* is fed ^{14}C-labeled dihydroquercetin, no catechin or proanthocyanidin is formed, while mutant *ant17-139* converts this precursor readily into catechin and proanthocyanidin, as do mutants *ant18-102* and *ant*19-109 to a limited extent (Kristiansen 1984). This indicates that at least *ant17* mutants, which are blocked in the synthesis of dihydroquercetin, will produce the enzymes operating in the later steps of the pathway. Mutants in the *Ant13* gene have been shown to lack activity of DFR, F3H, and flavanone-3'-hydroxylase (F3'H) and the CHS protein (Jende-Strid and Kristiansen 1987; Jende-Strid 1991). In this article it is shown that two *ant13* mutants transcribe minute amounts of mRNA or none at all from the genes encoding CHS, DFR, and F3H. This is strong evidence that *Ant13* encodes a transcription factor required for the expression of the mentioned genes. In this report *Ant13* resembles the *C1* gene of maize, which regulates the expression of the genes encoding CHS (*C2*), DFR (*A1*), and UDP-glucose: flavonol 3-O-glucosyl transferase (*Bz1*) (Dooner 1983; Dooner and Nelson 1977, 1979; Wienand et al. 1986; Rohde et al. 1987; Schwarz-Sommer et al. 1987; Reddy et al. 1987; Niesbach-Klösgen et al. 1987). Cloning and sequencing of the *C1* gene (Cone et al. 1986; Paz-Ares et al. 1986, 1987) revealed it to be the structural gene for a 29-kDa DNA-binding protein. The NH_2-terminal 120 amino acid sequence of this protein is homologous to the DNA-binding domain of the *myb* proto-oncogene products, and the acidic domain at the carboxyl-terminal end is homologous to the activating regions of the GCN4 and GAL4 proteins of yeast. Franken et al. (1991) has shown that these two domains are indeed of importance in regulating the amount of anthocyanin formed in the aleurone cells of developing and germinating maize kernels. Thus, a reversion of a transposon insertion, which leads to a deletion of a single amino acid in the DNA-binding domain, causes a reduction in pigment formation compared to the wild-type allele. Another reversion caused a reading-frame shift in the acidic domain and a correlated reduction in anthocyanin pigmentation.

Using the maize *C1* cDNA as a probe, three different cDNA clones were isolated from barley kernel, leaf, and apex cDNA libraries (Rhode et al. 1988; Marocco et al. 1989). Very good homology of the deduced amino acid sequence is displayed to the NH_2-terminal 120 amino acids (very likely a DNA-binding domain) of the C_1 protein from maize. The C-terminal parts of the sequences are highly diverse, but all three are relatively acidic. It will be of interest to see whether or not one of these three cDNA clones represents a transcript of the *Ant13* gene.

In *Antirrhinum majus,* mutations in the *Delila* gene influence the expression of genes for F3H (*Incolorata*), DFR (*Pallida*), and UDP-glucose: flavonol 3-O-glucosyl transferase, but not those for CHS or chalcone isomerase (CHI) (Beld et al. 1989).

These three example [from barley, maize, and snapdragon (*A. majus*)] show that plant species differ by the section of the flavonoid biosynthesis pathway, wich is coordinately expressed in a given tissue or in the entire plant.

Molecular analysis of F3H in barley

The identification of the clones c3H2 and c3H5 from barley, as cDNA encoding F3H, rests on the 71% overall amino acid homology and the 85% central domain amino acid homology to the F3H sequence from *Antirrhinum majus*. F3H of *A. majus* is transcribed from the *Inc* (*Incolorata*) gene (Martin et al. 1991). Forkmann and Stotz (1981) correlated the presence of the *Inc* gene with the presence of the F3H, while this enzyme was absent in all genotypes containing a recessive mutant allele of this gene. By transposon tagging, several mutant forms of the *Inc* gene have been cloned and sequenced. Two insertion mutants lacked the F3H transcripts and four others had insertions in the *Inc* gene (Martin et al. 1991). In *Petunia hybrida* the presence of the dominant *An*3 allele was correlated with the presence of flavanone-3-hydroxylase activity, while homozygous recessive plants (*an3*/*an3*) had no or very small amounts of this activity (Froemel et al. 1985). A leaky allele *an3-1* was discovered, which had about 10% of the hydroxylase activity. This was sufficient to support anthocyanin formation, but insufficient to permit flavonol accumulation in the flowers.

Using a cDNA clone of the *Antirrhinum majus* F3H as a probe, cDNA of the *An3* gene has been obtained. An amino acid homology of 87% was found (Martin et al. 1991). In both *Antirrhinum* and *Petunia*, a second gene (*Inc3* and *Ant13*, respectively) has been identified. This codes for a F3H-related protein with 53% amino acid homology (Martin et al. 1991).

Flavanone-3-hydroxylase has been purified from *Petunia hybrida* and characterized (Britsch and Grisebach 1986; Britsch 1990). It requires 2-oxoglutarate, Fe^{++}, and ascorbate as cofactors and has an apparent molecular weight of 37 kDA (as compared to 42 kDa and 45 kDa for the cDNA-deduced molecular weights of *H. vulgare* and *A. majus*). An antibody raised to the purified F3H detected a native protein of 74 kDa, which would mean that the F3H is a homodimer. No F3H could be detected in a white-flowered mutant with the genotype *an3*/*an3*.

F3H is specific for the 3-hydroxylation of flavanones, thus converting naringenin to DHK and eriodictyol to DHQ. F3H from barley and from petunia seem to have different preferences to naringenin and eriodictyol as substrates in vivo (Jende-Strid 1991). In barley, crude protein extracts from developing kernels of wild-type and mutant *ant17-139* hydroxylate naringenin into dihydrokaempferol, but fail to hydroxylate eriodictyol into dihydroquercetin, in spite of the fact that in vivo the latter reaction must be the one occurring in testa tissue, since dihydrokaempferol and catechin and proanthocyanidins with the same hydroxylation pattern of the B-ring as DHK (4'-hydroxylated exclusive) have not been found in barley.

Mutations in the genes *Ant17* and *Ant22* block singly and equally effectively the biosynthesis of anthocyanin and proanthocyanidins. The block is located in the conversion of eriodictyol to DHQ, since feeding of DHQ to mutant tissue yields catechin and proanthocyanidins, and derivatives of eriodictyol are accumulated.

Mutant *ant17-352* transcribes the gene corresponding to the cDNA clone encoding the barley F3H (c3H5) Fig. 7A and B may indicate an overexpression at the transcriptional level. Since F3H functions as a dimer, it is conceivable that this enzyme in barley is a heterodimer of polypeptides encoded by the *Ant17* and the *Ant22* genes, which are located on chromosome 3 and chromosome 1, respectively (Boyd and Falk 1990). In support of such a possibility is the situation recently encountered for the cerulenin binding β-ketoacyl-(acylcarrier protein) synthase from barley chloroplasts (Siggaard-Andersen et al. 1991). While spinach contains a single homo dimeric enzyme, barley houses three dimeric enzymes ($\alpha\alpha$, $\alpha\beta$, $\beta\beta$). The in vitro hydroxylase activities in *ant17* and *ant22* mutants could be spurious and result from activity of the homodimers.

An alternative possibility is that the *Ant22* gene codes for a protein required to provide the cofactors such as Fe^{++}, ascorbate, or 2-oxoglutarate in the compartment of the plant cells, where it is to be used (cf. Jende-Strid 1991).

With the isolation of the F3H cDNA reported in this article, it will be possible to analyze the *Ant17* and *Ant22* genes. In vitro transcription and translation of the cDNA and testing of the polypeptide in the enzymatic assay will reveal whether or not this protein catalyzes the hydroxylation of eriodictyol in the 3-position. Introduction of the F3H cDNA into anthocyanin-free *ant17* seedlings by particle bombardment will give red sectors and determine whether or not *Ant17* encodes a F3H subunit.

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References

Aastrup S, Outtrup H, Erdal K (1984) Location of the proanthocyanidins in the barley grain. Carlsberg Res Commun 49:105–109

Akade S, Kung SD, Dube SK (1990) Nucleotide sequence of one member of soybean chalcone synthase multi-gene family. Nucleic Acids Res 18:3398

Beld M, Martin C, Huits H, Stuitje AR, Gerats AGM (1989) Flavonoid synthesis in *Petunia hybrida:* partial characterization of dihydroflavonol-4-reductase genes. Plant Mol Biol 13:491–502

Boyd PW, Falk DE (1990) Use of pseudolinkage and xenia to locate genes on the barley chromosome map. Barley News 33:106

Britsch L (1990) Purification of flavanone 3β-hydroxylase from *Petunia hybrida*: antibody preparation and characterization of a chemogenetically defined mutant. Arch Biochem Biophys 276:348–354

Britsch L, Grisebach H (1986) Purification and characterization of (2S)-flavanone-3-hydroxylase from *Petunia hybrida*. Eur J Biochem 156:569–577

Britsch L, Heller W, Grisebach H (1981) Conversion of flavanone to flavone, dihydroflavonol and flavonol with an enzyme system from cell cultures of parsley. Z Naturforsch 36:742–750

Cone KC, Burr FA, Burr B (1986) Molecular analysis of the maize anthocyanin regulatory locus *C1*. Proc Natl Acid Sci USA 83:9631–9635

Dooner HK (1983) Coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Mol Gen Genet 189:136–141

Dooner HK, Nelson OE (1977) Genetic control of UDP glucose-flavonol 3-O-glucosyl transferase in the endosperm of maize. Biochem Genet 15:509–515

Dooner HK, Nelson OE (1979) Interaction among *C*, *R* and V_p in the control of the *Bz* glucosyltransferase during endosperm development in maize. Genetics 91:309–315

Ehmann B, Schäfer E (1988) Nucleotide sequences encoding two different chalcone synthases expressed in cotyledons of SAN 9789 treated mustard (*Sinapis alba* L.) Plant Mol Biol 11:869–870

Epping B, Kittel M, Ruhnau B, Hemleben V (1990) Isolation and sequence analysis of a chalcone synthase cDNA of *Matthiola incana* R. Br. (Brassicaceae). Plant Mol Biol 14:1061–1063

Fedoroff N (1983) Controlling elements in maize. In: Shapiro J (ed) Mobile genetic elements. Academic Press, NY, pp 1–63

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol Cell Biol 8:1985–1992

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Forkmann G (1989) Gene-enzyme relations and genetic manipulation of anthocyanin biosynthesis in flowering plants. In: Styles DE, Gavazzi GA, Racchi ML (eds) The genetics of flavonoids. Edizioni unicopli, Milan pp 49–60

Forkmann G, Ruhnau B (1987) Distinct substrate specific of dihydroflavonol-4-reductase from flowers of *Petunia hybrida*. Z Naturforsch 42c:1146–1148

Forkmann G, Stotz G (1981) Genetic control of flavanone-3-hydroxylase activity and flavonoid-3'-hydroxylase activity in *Antirrhinum majus* (Snapdragon). Z Naturforsch 36c:411–416

Forkmann G, Heller W, Grisebach H (1980) Anthocyanin biosynthesis in flowers of *Matthiola incana*. Flavanone-3- and flavonoid-3'-hydroxylases. Z Naturforsch 35c:691–695

Franken P, Niesbach-Klösgen U, Maréchal-Drouard L, Saedler H, Wienand U (1991) The duplicated chalcone synthase genes C2 and *Whp* (white pollen) of *Zea mays* are independently regulated; evidence for transitional control *Whp* expression by the anthocyanin intensifying gene. EMBO J 10:2605–2612

Froemel P, Vlaming P de, Stotz G, Wiering H, Forkman G, Schramm AW (1985) Genetic and biochemical studies on the conversion of flavanones to dihydroflavonols in flowers of *Petunia hybrida*. Theor Appl Genet 70:561–568

Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene 25:263–269

Han JH, Stratowa C, Rutter WJ (1987) Isolation of full-length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and cDNA-cloning. Biochemistry 26:1617–1625

Hanahan D (1983) Studies on transformation of *E. coli* with plasmids. J Mol Biol 166:557–580

Hanahan D (1983) Techniques for transformation of *E. coli*. In: Glover DM (ed) DNA cloning, vol I. IRL Press, Oxford, pp 109–135

Harker CL, Ellis THN, Coen ES (1990) Identification and genetic regulation of the chalcone synthase multigene family in pea. Plant Cell 2:185–194

Jende-Strid B (1985) Phenolic acids in grains of wild-type barley and proanthocyanidin-free mutants. Carlsberg Res Commun 50:1–14

Jende-Strid B (1988) Co-ordinators report: anthocyanin genes. Stock list of *ant* mutants kept at the Carlsberg Laboratory. Barley Genet News 18:74–79

Jende-Strid B (1991) Gene-enzyme relations in the pathway of flavonoid biosynthesis in barley. Theor Appl Genet 81:668–674

Jende-Strid B, Kristiansen KN (1987) Genetics of flavonoid biosynthesis in barley. In: Barley genetics V. Proc 5th Barley Genet. Symp., Okayama, Japan, 1986, pp 445–453

Koes RE, Spelt CE, Reif HJ, Elzen PJM van den, Veltkamp E, Mol JNM (1986) Floral tissue of *Petunia hybrida* (V30) expresses only one member of the chalcone synthase multigene family. Nucleic Acid Res 14:5229–5239

Koes RE, Spelt CE, Mol JNM, Gerats GM (1987) The chalcone synthase multigene family of *Petunia hybrida* (V30): sequence homology, chromosomal localization and evolutionary aspects. Plant Mol Biol 10:159–169

Koes RE, Spelt CE, Mol JNM (1989a) The chalcone synthase multigene family of *Petunia hybrida* (V30): differential, light-regulated expression during flower development and UV light induction. Plant Mol Biol 12:213–225

Koes RE, Spelt CE, Elzen PJM van den, Mol JNM (1989b) Cloning and molecular characterization of the chalcone synthase multigene family of *Petunia hybrida*. Gene 81:245–257

Koes RE, Blockland R van, Quattrocchio F, Tunen AJ van, Mol JNM (1990) Chalcone synthase promoters in petunia are active in pigmented and unpigmented cell types. Plant Cell 2:379–392

Kreuzaler F, Ragg H, Fautz E, Kuhn DN, Hahlbrock K (1983) UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselium hortense*. Proc Natl Acad Sci USA 80:2591–2593

Kristiansen KN (1984) Biosynthesis of proanthocyanidins in barley: genetic control of the conversion of dihydroquercetin to catechin and procyanidins. Carlsberg Res Commun 49:503–524

Kristiansen KN (1986) Conversion of (+)-dihydroquercetin to (+)-2,3-*trans*-3,4-*cis*-leucocyanidin and (+)-catechin with an enzyme extract from maturing grains of barley. Carlsberg Res Commun 51:51–60

Lawton MA, Dixon RA, Hahlbrock K, Lamb CJ (1983) Elicitor induction of mRNA activity. Eur J Biochem 130:131–139

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor/NY

Marocco A, Wissenbach M, Becker D, Paz-Ares J, Saedler H, Salamini F, Rohde W (1989) Multiple genes are transcribed in *Hordeum vulgare* and *Zea mays* that carry the DNA binding domain of the *myb* oncoproteins. Mol Gen Genet 216:183–187

Martin C, Carpenter R, Sommer H, Saedler H, Coen ES (1985) Molecular analysis of instability in flower pigmentation of *A. majus*, following isolation of the *pallida* locus by transposon tagging. EMBO J 4:1625–1630

Martin C, Prescott A, MacKay S, Bartlett J, Vrijlandt E (1991) Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant J 1:37–49

Mehdy MC, Lamb CJ (1987) Chalcone isomerase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. EMBO J 6:1527–1533

Niesbach-Klösgen U, Barzen E, Bernhardt J, Rohde W, Schwarz-Sommer Z, Reif HJ, Wienand U, Saedler H (1987) Chalcone synthase genes in plants: a tool to study evolutionary relationships. J Mol Evol 26:213–225

O'Reilly C, Shepherd NS, Pereira A, Schwarz-Sommer Z, Bertram I, Robertson I, Robertson DS, Peterson PA, Saedler H (1985) Molecular cloning of the *A1* locus of *Z. mays* using the transportable elements *En* and *Mu1*. EMBO J 4: 877–882

Paz-Ares J, Wienand U, Peterson PA, Saedler H (1986) Molecular cloning of the *c* locus of *Zea mays:* a locus regulating the anthocyanin pathway: EMBO J 5:824–833

Paz-Ares J, Ghosal D, Wienand U, Peterson PA, Saedler H (1987) The regulatory *C1* locus of *Z. mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. EMBO J 6:3553–3558

Reddy AR, Britsch L, Salamini F, Saedler H, Rohde W (1987) The *A1* locus in *Zea mays* encodes dihydroquercetin reductase. Plant Sci 52:7–13

Reif HJ, Niesbach U, Deumling B, Saedler H (1985) Cloning and analysis of two genes for chalcone synthase from *Petunia hybrida*. Mol Gen Genet 199:208–215

Reimold U, Kröger M, Kreuzaler F, Hahlbrock K (1983) Coding and 3'non-coding nucleotide sequence of chalcone synthase mRNA and assignment of amino acid sequence of the enzyme. EMBO J2:1801–1805

Rohde W, Barzen E, Marocco A, Schwarz-Sommer Z, Saedler H, Salamini F (1987) Isolation of genes that could serve as traps for transposable elements in *H. vulgare*. Barley Genet V: 533–541

Rohde W, Marocco A, Wissenbach M, Barzen E, Kristiansen KN, Salamini F (1988) Anthocyanin biosynthesis in barley: characterization of structural and putative regulatory genes. In: Styles DE, Gavazzi GA, Racchi ML (eds) The genetics of flavonoids. Edizioni unicopli Milan, pp 79–95

Ryder TB, Cramer CL, Bell JN, Robbins MP, Dixon RA, Lamb CJ (1984) Elicitor rapidly induces chalcone synthase mRNA in *Phaseolus vulgaris* cells at the onset of the phytoalexin defense response. Proc Natl Acad Sci USA 81:5724–5728

Ryder TB, Hedrick SA, Bell JN, Liang X, Clouse SD, Lamb CJ (1987) Oranisation and differential activation of a gene family encoding the plant defense enzyme chalcone synthase in *Phaseolus vulgaris*. Mol Gen Genet 210:219–233

Schröder J, Kreuzaler F, Schäfer E, Hahlbrock K (1979) Concomitant induction of phenylalanine ammonia-lyase and flavanone synthase mRNAs in irradiated plant cells. J Biol Chem 254:57–65

Schwarz-Sommer Z, Shepherd N, Tache E, Gierl A, Rohde W, Leclercq L, Mattes M, Berndigen R, Peterson PA, Saedler H (1987) Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays*. EMBO J 6:287–294

Scott-Moncrieff R (1939) The genetics and biochemistry of flower colour variation. Ergeb Enzymforsch 8:277–306

Siggaard-Andersen M, Kauppinen S, Wettstein-Knowles P van (1991) Primary structure of a cerulenin binding β-ketoacyl-(acyl carrier protein) synthase from barley chloroplasts. Proc Natl Acad Sci USA (in press)

Sommer H, Saedler H (1986) Structure of the chalcone synthase gene of *A. majus*. Mol Gen Genet 202:429–434

Stafford HA (1989) The enzymology of proanthocyanidin biosynthesis. In: Hemingway RW, Karchesy JJ (eds) Chemistry and significance of condensed tannins. Plenum Publishing, New York, pp 47–70

Staiger D, Kaulen H, Schell J (1990) A nuclear factor recognizing a positive regulatory upstream element of the *A. majus* chalcone synthase promoter. Plant Physiol 93:1347–1353

Tunen AJ van, Koes RE, Spelt CE, Krol AR van der, Stuitje AR, Mol JNM (1988) Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida:* coordinate, light-regulated and differential expression of flavonoid genes. EMBO J 7:1257–1263

Tunen AJ van, Hartman SA, Mur LA, Mol JNM (1989) Regulation of chalcone flavanone isomerase (CHI) gene expression in *Petunia hybrida*: the use of alternative promoter in corolla, anthers and pollen. Plant Mol Biol 12:539–551

Wettstein D von, Jende-Strid B, Ahrenst-Larsen B, Sørensen JA (1977) Biochemical mutant in barley renders chemical stabilization of beer superfluous. Carlsberg Res Commun 42:341–351

Wettstein D von, Nilan RA, Ahrenst-Larsen B, Erdal K, Ingversen J, Jende-Strid B, Kristiansen KN, Larsen J, Outtrup H, Ullrich SE (1985) Proanthocyanidin-free barley for brewing: progress in breeding for high yield and research tool in polyphenol chemistry. MBAA Techn Quart 22:41–52

Wienand U, Sommer H, Schwarz-Sommer Z, Shepherd N, Saedelr H, Kreuzaler F, Ragg H, Fautz E, Hahlbroock K, Harrison B, Peterson PA (1982) A general method to identify plant structural genes among genomic DNA clones using transposable element induced mutations. Mol Gen Genet 187:195–201

Wienand U, Weidemann U, Niesbach-Klösgen U, Peterson PA, Saedler H (1986) Molecular cloning of the *C2* locus of *Zea mays*, the gene coding for chalcone synthase. Mol Gen Genet 203:202–207