MUTATIONS AFFECTING FLAVONOID SYNTHESIS IN BARLEY

by

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The anthocyanin, anthocyanidin and proanthocyanidin content of 52 induced barley mutants with altered anthocyanin pigmentation in different organs of the plant has been investigated. Comparing these mutants no correlation between the amount of anthocyanin in the plant and the amount of proanthocyanidin in the dry grains is found. The proanthocyanidins of the lemma, palea, pericarp, testa and aleurone of Bonus, Foma, Kristina, Herta, Balder and 37 *ant* mutants yielded upon hydrolysis delphinidin and cyanidin. In 50 of the mutants belonging to at least 16 gene loci only anthocyanin synthesis is impaired. In mutant *ant* 13-13 a step in the biosynthetic pathway leading to anthocyanins, catechins and proanthocyanidins is blocked.

1. INTRODUCTION

The flavonoids comprise numerous and widespread natural plant products which are biosynthetically derived from the aromatic amino acids phenylalanine and tyrosine via the corresponding cinnamic acids. In plants the basic C₁₅ skeleton probably results from a coupling of a cinnamate unit produced by the shikimic acid pathway with three acetate units (4). Once the C₁₅ intermediate has formed it can be modified in a limited number of steps to

yield the different flavonoid classes which differ in the oxidation state of the central ring.

Most of the brightly coloured blue and red plant pigments are anthocyanins, i.e., anthocyanidin glucosides containing one or more sugar residues and occasionally various other organic acid residues.

The proanthocyanidins are dimers or higher oligomers of flavan-3-ols. They yield anthocyanidins and catechins upon hydrolysis. Fruits have proved to be particularly rich in proan-

Table 1

Data on anthocyanin (*ant*) mutants in barley
(Information in columns 1-6 courtesy U. LUNDQVIST)

Mutant	Year of isolation	From variety	Mutagen (1)	Dose	Treatment (2)	Stem	Auricles	Anthocyanin in (3) Awn	Lemma	Special remarks
<i>ant</i> 1-1	1952	Bonus	α -rays (6)	500 rad	Resting	-	-	-	-	
• 1-2	1955	Bonus	n (5)	400 rad	Resting	-	-	-	-	
• -3	1955	Bonus	n (4)	40 rad	Resting	-	-	+	+	Late
• 1-4	1954	Bonus	n (4)	40 rad	Soaked	-	-	-	-	
• 3-5	1956	Bonus	n (4)	634 rad	Resting	-	-	-	+	Late, short
• 3-8	1957	Bonus	α -rays	100 days x 24 rad	Chronic	-	-	-	+	Late, short
• 5-9	1958	Bonus	El	0.04% x 2 hrs	Resting	-	-	-	+	
• -10	1958	Foma	El	0.1% x 2 hrs	Resting	-	+	+	-	
• 4-11	1959	Foma	El	0.07% x 2 hrs	Soaked	-	-	-	+	Late
• 7-12	1960	Foma	El	0.05% x 4 hrs	Soaked	-	+	++	++	Late
• 13-13	1960	Foma	EMS	0.25% x 24 hrs	Resting	-	-	-	-	Late
• -14	1961	Foma	PMS	1.0% x 24 hrs	Resting	-	-	++	-	Late
• 2-15	1962	Foma	EMS	0.17-0.33% x 24 hrs	Resting, recurrent	-	-	-	-	
• 4-16	1962	Foma	EMS + n (5)	0.08-0.5% x 5-24 hrs + 100 rad	Resting recurrent	-	-	-	+	Extremely late, very small side florets
• 4-17	1962	Foma	EMS + n (5)	0.08-0.5% x 5-24 hrs + 100 rad	Resting, recurrent	-	-	-	+	Extremely late, very small side florets
• 5-18	1963	Foma	n (5)	650 rad	Resting	-	-	-	+	
• 15-19	1963	Foma	BMS	0.7% x 5 hrs	Resting	-	++	++	++	
• 2-20	1963	Foma	EMS	0.08-0.5% x 5-24 hrs	Resting, recurrent	-	-	-	-	
• 8-21	1963	Foma	EMS	0.08-0.5% x 5-24 hrs	Resting, recurrent	-	+	+	+	Late
• 16-22	1963	Foma	EMS + n (5)	0.17-0.5% x 5-24 hrs + 100 rad	Resting, recurrent	-	+	+	+	Late
• 2-23	1963	Foma	EMS	0.08-0.5% x 5-24 hrs	Resting, recurrent	-	-	-	-	
• 5-24	1963	Foma	EMS + n (5)	0.08-0.5% x 5-24 hrs + 300-500 rad	Resting, recurrent	-	-	-	+	
• 2-25	1963	Foma	EMS	0.17-0.5% x 5-24 hrs	Resting, recurrent	-	-	-	-	
• 2-26	1963	Foma	EMS	0.17-0.5% x 5-24 hrs	Resting, recurrent	-	-	-	-	

• 2-27	1963	Foma	EMS	0.08-0.5% x 5-24 hrs	Resting, recurrent	-	-	-	Late
• 4-28	1964	Foma	El	0.01% x 5 hrs	Resting	-	+	+	Late
• 9-29	1963	Foma	EMS	0.17-0.5% x 5-24 hrs	Resting, recurrent	+	+	+	
• 10-30	1965	Foma	3-OH-EMS	1.5% x 24 hrs	Resting	-	+	+	Late
• 4-31	1965	Foma	EMS	0.25% x 24 hrs	Resting	-	-	+	
• 4-32	1966	Foma	El	0.01% x 5 hrs	Resting	-	-	+	
• 6-33	1966	Foma	ENU	0.015% x 24 hrs	Resting	-	+	+	
• 11-34	1967	Foma	n (4)	400 rad	Resting	+	+	+	
• 12-35	1967	Foma	EMS	0.17% x 24 hrs	Resting	-	+	+	
• 14-36	1967	Foma	n (4)	300 rad	Resting	-	+	+	
• 4-37	1967	Foma	EMS	0.25% x 24 hrs	Resting	-	-	+	Late
• 6-38	1968	Kristina	γ-rays	8000 rad	Resting	-	+	+	
• 3-39	1970	Kristina	γ-rays	27000 rad	Resting	-	-	+	Late, short
• 4-40	1954	Bonus	x-rays	4500 rad	Resting	-	-	+	
• 4-1	1973	Mari	γ-rays	chronic	Chronic	-	-	-	
• 4-2	1974	Kristina	γ-rays	15000 rad	Resting	-	-	+	Extremely late
• 4-3	1974	Sd 71120	Iso-PMS	0.1%	Resting	-	-	-	
• 1101	1952	Bonus	n (4)	30 rad	Soaked	++	++	+++	+++
• 1102	1962	Foma	EMS	0.08-0.5% x 5-24 hrs	Resting	++	++	+++	+++
• 1103	1963	Foma	n (5)	100-500 rad	Resting, recurrent	++	++	+++	Late
• 1104	1963	Foma	n (5)	100-500 rad	Resting recurrent	+++	++	+++	++
• 1105	1964	Foma	El	0.01% x 5 hrs	Resting	++	++	+++	++
• 1106	1962	Foma	EMS	0.08-0.5% x 5-24 hrs	Resting	+++	++	+++	++
• 1201	1963	Foma	EMS + n (4)	0.17-0.33% x 24 hrs + 70-420 rad	Resting, recurrent	++	+++	+++	Late
• 1202	1965	Foma	MNU	0.25% x 24 hrs	Resting	++	++	++	++
• 1203	1965	Foma	γ-rays	13000 rad	Resting	++	++	+++	++
• 1204	1967	Foma	n (4)	300 rad	Resting	++	++	++	++
• 1301	1953	Bonus	n (4)	326 rad	Resting	+++	++	+++	+++

(1) EMS = ethyl methanesulfonate; PMS = propyl methanesulfonate; BMS = butyl methanesulfonate; El = ethyleneimine; ENU = N-ethyl-N-nitroso-urethane; MNU = N-methyl-N-nitroso-urethane; 3-OH-EMS = 3-hydroxy-ethyl-methanesulfonate.

(2) Refers to the conditions of the treated plant material; *resting*: resting seeds with a water content of 11%; *soaked*: seeds which have been soaked in water for 12 or 24 hrs; *recurrent*: treatment of resting seeds in two or three successive years with the same or different mutagens; *chronic*: growing plants irradiated chronically from a ^{60}Co or ^{137}Cs source during the whole vegetation period.

(3) Anthocyanin in the different organs of the plant was visually determined as follows: +++ = more than in the wild type; ++ = as in the wild type; + = less than in the wild type; - = no anthocyanin.

(4) Fast neutrons from cyclotron (3)

(5) Neutrons in reactor center (1)

(6) α-rays from radon

thocyanidins and unripe fruit and seeds contain more proanthocyanidins than ripe ones. The catechins (flavan-3-ols) occur together with the proanthocyanidins in the plant tissues (16). Both the catechins and the proanthocyanidins are unglucosylated.

The flavonoid content of a series of induced mutants in barley with altered anthocyanin pigmentation in various parts of the plant has been investigated. The anthocyanin content of the different organs of the plant was estimated visually and spectrophotometrically and the anthocyanidin and proanthocyanidin content of the dry grains analysed. Diallelic crosses among the mutants have so far identified 16 genes (*ant*) affecting flavonoid synthesis in barley (7). The two anthocyanin-free varieties Balder and Alva were found to be allelic with mutants in the *ant* 2 gene.

2. MATERIALS AND METHODS

2.1. Plant material

From the collection of artificially induced mutants kept at the Swedish Seed Association in Svalöv and the Institute of Genetics, University of Lund, 52 recessive gene mutants with altered anthocyanin pigmentation were kindly supplied by Professor ÅKE GUSTAFSSON and Mrs. UDDA LUNDQVIST together with detailed information on their origin. The gene symbol *ant* is used for genes in barley affecting the anthocyanin, anthocyanidin and proanthocyanidin content. The first figure following the symbol *ant* designates the locus and the second figure the allele (7). Data on the origin and characteristics of the 52 *ant* mutants are given in Table I. Of these mutants 41 are deficient in anthocyanin and 11 mutants develop more anthocyanin than their mother varieties. The majority of the mutants namely 37 were isolated from the variety Foma, 10 from Bonus, 3 from Kristina, one from Mari and one from Svalöv line 71120. The mutants as well as their mother varieties were grown in observation plots. The amount of anthocyanin in the stems, auricles, awns and lemmas was visually estimated every week until harvest. Grains of the varieties Jet, Black African variety, Bonanza, Blue Indian variety, Gopal and Black Hulless, Balder and Herta were analysed for comparison.

2.2. Anthocyanin determination

Samples of auricles and awns ranging in fresh weight from 0.2-1.0 g were extracted with 1% HCl in methanol at +4°C (17). The amount of anthocyanin was determined by measuring the difference in absorption at 530 nm of the extract before and after the addition of H₂O₂, which oxidizes the anthocyanin (15). A Zeiss PMQ III spectrophotometer was used.

2.3. Anthocyanidin identification

The procedure used for soaking and peeling samples of barley grains was based on the method of MULLICK and BRINCK (11). About 10 g of barley grains were soaked in water of about 0°C for four days. Then the grains were peeled manually and each was divided into three parts: (1) palea + lemma, (2) pericarp + testa, and (3) the aleurone layer with some adhering endosperm. Each sample was placed in 10 ml of 1% HCl in methanol and extracted at about 0°C for 48 hours. The solutions were decanted and washed with petroleum ether. Concentrated HCl was added to the extracts which were then placed on a boiling water bath for 30 minutes. The resulting anthocyanidins were extracted with iso-amylalcohol and spotted together with pure delphinidin, cyanidin, and pelargonidin (Calbiochem, Los Angeles, USA), on 0.25 mm Avicel SF cellulose (Serva Entwicklungslaboratorium, Heidelberg, FRG) thin layer chromatography plates. Two solvent systems were used for development: (1) propionic: propionic acid – formic acid – concentrated HCl – water, 2.5:1:6 v/v/v/v (10) and (2) formic: formic acid – concentrated HCl – water, 10:1:3, v/v/v (13). The chromatograms were examined under visible and UV-light as well as after treatment with ammonia fumes.

2.4. Proanthocyanidin determination

The proanthocyanidin content of the grains of *ant* mutants and their mother varieties was determined according to a method modified from HARRIS and RICKETTS (5) and described in V. WETTSTEIN et al. (18). The method of DADIC and MORRISON for simultaneous determination of proanthocyanidins and catechins in barley

(2, 18) was used with four *ant* mutants and the varieties Black Hulless and Alva. DADIC's method is of advantage because it considers both the proanthocyanidin and the catechin content of the barley grains.

3. RESULTS

3.1. Field observations

The results of the field observations on anthocyanin pigmentation are summarized in Table I. The amount of anthocyanin in the stems, auricles, lemmas and awns varied with the developmental stage of the plant and the genotype. Maximal pigment concentration occurred first in the auricles, then in the awns and finally in the lemmas. When the plants turned yellow the amount of anthocyanin

decreased, and by the time the plants were ripe anthocyanin was no longer detectable in most of the genotypes. Exceptional is mutant *ant* - 1301, which has a strong red stem at harvest time.

Seven of the mutants contained more anthocyanin than the wild types in at least two of the investigated organs and four contained slightly more pigment at least in some years. A group of 41 mutants had reduced amounts of anthocyanin. Twelve of the *ant* mutants were anthocyanin-free throughout the growing season. Sixteen of the mutants were slower in development than the wild types and three others headed extremely late. Weather conditions, especially temperature had a strong influence on the amount and time of pigment formation.

Table II

Anthocyanidins and anthocyanin in the grain of different barley lines (Amounts are indicated as follows: ++ large, + small, - none).

Lines	Part of the grain	Colour	Anthocyanidins			
			Delphinidin	Cyanidin	Pelargonidin	Anthocyanin
Bonus, Foma, Kristina	lemma + palea	yellow	+	+	-	+
	pericarp + testa	yellow	+	+	-	-
	aleurone	yellow	++	+	-	-
Herta	lemma + palea	yellow	+	+	-	+
	pericarp + testa	yellow	+	+	-	-
	aleurone	yellow	++	+	-	+
Balder	lemma + palea	yellow	+	+	-	+
	pericarp + testa	yellow	+	+	-	-
	aleurone	yellow	++	+	-	-
37 <i>ant</i> -mutants	lemma + palea	yellow	+	+	-	-+
	pericarp + testa	yellow	+	+	-	-
	aleurone	yellow	++	+	-	-
Jet, Black African variety	lemma + palea	black	+	+	-	-
	pericarp + testa	black	+	+	-	-
	aleurone	yellow	+	+	-	-
Bonanza, Blue Indian variety	lemma + palea	yellow	+	+	-	+
	pericarp + testa	yellow	+	+	-	-
	aleurone	blue	+	+	-	++
Gopal	-					
Black Hulless	pericarp + testa	purple	++	++	+	++
	aleurone	blue	+	++	+	++

Table III

R_f values of anthocyanidins isolated from barley.

Anthocyanidin	Solvent system	
	Propionic	Formic
Cyanidin	0.26	0.44
Cyanidin (standard)	0.26	0.44
Delphinidin	0.13	0.28
Delphinidin (standard)	0.14	0.29
Pelargonidin	0.51	—
Pelargonidin (standard)	0.53	—

3.2. Anthocyanidins

In Tables II and III are listed the anthocyanidins identified in the various parts of the grain with the aid of thin layer chromatography. The investigated barley varieties belong on the basis of the colour of the ripe grain to 4 groups: (A) yellow, (B) black, (C) blue and (D) purple. The results are in agreement with those of MULLICK *et al.* (12) who investigated 8 other barley varieties which include varieties with yellow (called white), black, blue, and purple grains.

(A) Foma, Bonus, Kristina and 37 analysed *ant* mutants all have yellow coloured lemma, palea, pericarp, testa, and aleurone. Balder, considered as an anthocyanin-free variety, and Herta, considered as very anthocyanin-rich also fall in this category. Before hydrolysis the extracts contained no anthocyanin and were light yellow in colour, except some extracts prepared from lemmas + paleas early in the autumn, which contained a very small amount of anthocyanin and were pale pink. In Herta the aleurone layer of some grains contained a small amount of anthocyanin. The distribution and concentration of the anthocyanidins released from the proanthocyanidins and the small amounts originating from the anthocyanins were undistinguishable in all these varieties. Extracts from lemma + palea, pericarp + testa and aleurone contained the anthocyanidins cyanidin and delphinidin. Most of the anthocyanidin was present in the aleurone layer with a little in the pericarp + testa and the lemma + palea. In both lemma + palea and pericarp + testa the concentrations of cyanidin

and delphinidin were about the same whereas extracts from the aleurone layer contained approximately twice as much delphinidin as cyanidin.

(B) In the black varieties Jet and an unnamed African variety, delphinidin and cyanidin occurred in similar amounts as in the yellow lines. Since no anthocyanin was present in the black varieties all measured anthocyanidin in the extracts from lemma + palea, pericarp + testa and aleurone must have been released from proanthocyanidins. The black colour of the lemma and pericarp of these varieties is not attributable to anthocyanidin.

(C) In the blue varieties, Bonanza and an unnamed Indian variety, delphinidin and cyanidin occurred in the lemma + palea, pericarp + testa and aleurone layer. In both pericarp + testa and aleurone the concentrations of delphinidin and cyanidin were about the same. The blue varieties contain a small amount of anthocyanin in the lemma + palea, none in the pericarp + testa and much anthocyanin in the aleurone layer.

(D) The pericarp + testa and aleurone layer of the purple varieties Gopal and Black Hulless contained delphinidin and cyanidin and a third anthocyanidin, pelargonidin. In addition they differed from the other studied varieties by having more cyanidin. The grains of Gopal and Black Hulless were very purple. The pericarp + testa of Gopal was richer in anthocyanin than the aleurone layer. Black Hulless, on the other hand, had a blue very anthocyanin-rich aleurone layer and less pigment in the pericarp + testa. However, the amount of anthocyanin in the aleurone varied among the grains. Thus, in both varieties the anthocyanins contributed significantly to the amount of anthocyanidins observed.

3.3. Proanthocyanidins

Proanthocyanidins were determined with the modified HARRIS and RICKETTS method as well as the DADIC and MORRISON procedure in grains from lines distinct by their content of anthocyanins in the vegetative parts and from the variety Black Hulless with its high content of anthocyanin in the grain. (Tables IV and V).

Table IV

Comparison of anthocyanin and proanthocyanidin content in different lines of barley.

Genotype	Anthocyanin OD/g fresh weight		Proanthocyanidin in grain mg/100 g dry weight (HARRIS and RICKETTS)	
	Awns	Auricles	1973	1974
Foma	0.9	0.5	161	150
Bonus	1.0	0.5	202	187
Kristina	0.9	0.4	217	–
<i>ant</i> -1106	13.5	0.5	150	149
<i>ant</i> -1201	4.4	3.9	175	170

The amount of anthocyanin in the auricles and awns of Foma, Bonus and Kristina is very similar (Table IV). The proanthocyanidin content varied between 161 and 217 mg per 100 g dry weight which is not considered significantly different. Mutant *ant*-1106 with its drastically increased anthocyanin content in the awns did not reveal a parallel increase in the proanthocyanidin content of the grain. Mutant *ant*-1201 which has an elevated content of anthocyanin in the awns and auricles likewise contained a standard amount of proanthocyanidin.

In Table V both proanthocyanidins and catechins are considered. The anthocyanin-rich grains of the variety Black Hulless show the highest total polyphenol content. If this dif-

ference is significant it could be entirely due to the anthocyanin content of the grain. The mutants with reduced or non-detectable amounts of anthocyanin in the vegetative plant parts had in the grains a similar content of proanthocyanidin and catechin with the notable exception of *ant* 13-13 (18), which does not form significant amounts of proanthocyanidin and catechin.

It can be concluded from these observations that the content of anthocyanin can vary independently from that of proanthocyanidin.

For a more detailed analysis of the variation in proanthocyanidin content a large number of *ant* mutants has been studied as listed in Table VI. Of the mutants with an increased amount of

Table V

Proanthocyanidin and catechin content in the grain of 1976 determined according to the DADIC and MORRISON method.

Category	Genotype	Mg proanthocyanidin per 100 g dry weight	Mg catechin per 100 g dry weight	Mg total polyphenols per 100 g dry weight
Anthocyanin in grain	Black Hulless	228	206	434
Mutants with reduced amount of anthocyanin in plant	<i>ant</i> 3-39	218	125	343
	<i>ant</i> -42	207	158	365
Mutants without anthocyanin in plant	<i>ant</i> 2-Alva	174	220	394
	<i>ant</i> -41	205	143	348
	<i>ant</i> -43	172	133	305
	<i>ant</i> 13-13	1	4	5

Table VI

Proanthocyanidin content of the grains in different *ant* mutants and their mother varieties in 1973.

Category	Genotypes	Proanthocyanidin mg/100 g dry weight (HARRIS and RICKETTS)
Wild types	Foma, Bonus, Kristina	161, 202, 217
Mutants with increased amount of anthocyanin	<i>ant</i> -1101,-1103,-1102,-1101,-1201,-1104,-1106	248, 245, 228, 223, 175, 174, 150
Mutants with slightly increased amount of anthocyanin	<i>ant</i> -1105,-1204,-1203,-1202	181, 181, 169, 154
Mutants with reduced amount of anthocyanin	<i>ant</i> 3-5, 3-8 4-11, 4-16, 4-17, 4-28 » 4-31, 4-32, 4-37, 4-40 » 5-9, 5-18, 5-24 » 6-33, 6-38 » 7-12 » 8-21 » 9-29 » 10-30 » 11-34 » 12-35 » 14-36 » 15-19 » 16-22 » -3, -14	269, 217 178, 144, 150, 190, 144, 153, 207, 173 202, 185, 182 168, 209 197 165 169 202 176 162 171 168 179 218, 169
Mutants without anthocyanin	<i>ant</i> 1-1, 1-2, 1-4 » 2-15, 2-20, 2-23, 2-25, » 2-26, 2-27, 2-Alva, 2-Balder	195, 180, 208 191, 146, 171, 161, 156, 155, - , -
Mutant without anthocyanin	<i>ant</i> 13-13	3

anthocyanin in the plant 4 had a slightly elevated amount of proanthocyanidin, whereas 3 contained as much as the wild types. Disregarding mutant *ant* 13-13 no clear difference can be discovered among the mutants with slightly increased or reduced amounts of anthocyanin or those without anthocyanin. The mutants also do not differ from the wild types. A remarkable constant amount of proanthocyanidin in the grain emerges from the survey of these mutants.

4. DISCUSSION

Most higher plants possess the potential to form anthocyanidin under appropriate metabolic conditions. The anthocyanidin pigmen-

tation may only become visible at a certain stage of development for example during ripening of the fruit. In barley the anthocyanin pigmentation in the stem, auricles, awns and lemmas culminates just before the plant turns yellow. In ripe barley there is usually no visible anthocyanin in the grains except for varieties with blue or purple grains. Comparing different genotypes there is no correlation between the amount of anthocyanin in the barley plant or grain and the amount of proanthocyanidin in the ripe grains of the same plant.

The variation in proanthocyanidin content among the different *ant* mutants was with the exception of *ant* 13-13 small, not exceeding a factor of 2. Other surveys for the content of proanthocyanidins and catechins in different

barley varieties including anthocyanin-free ones have revealed likewise little variation in proanthocyanidin and catechin content (9, 14).

It is generally considered that proanthocyanidin containing plants can form anthocyanidin and the anthocyanidin formation may occur when the supply of biological reductant is cut off and the intermediate flav-3-en-3-ol is diverted by oxidation to form anthocyanidins (6). Frequently an inverse relationship between the synthesis of anthocyanin and the synthesis of proanthocyanidin has been reported (8). Genetic blocking of anthocyanin synthesis in barley does not lead to an automatic proanthocyanidin elevation. One mutant has been identified, which is blocked in both proanthocyanidin and anthocyanidin synthesis. It will be of interest if a mutant blocked in proanthocyanidin synthesis but not in anthocyanin synthesis can be isolated.

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