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FLAVAN-3,4-DIOLS IN ANTHOCYANIN BIOSYNTHESIS, ENZYMATIC FORMATION WITH FLOWER EXTRACTS FROM *CALLISTEPHUS CHINENSIS*

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Abstract—Feeding of leucopelargonidin and leucocyanidin, but not of naringenin or dihydroquercetin, to petals of an acyanic mutant of *Callistephus chinensis*, blocked by recessive alleles of the gene F, led to formation of the respective anthocyanidin glucosides, whereas in a further acyanic mutant, blocked by recessive alleles of the gene A, none of these compounds initiated anthocyanin synthesis The results strongly support the role of flavan-3,4-diols in anthocyanin biosynthesis Moreover, in enzyme preparations from cyanic *Callistephus* flowers, dihydroflavonol 4-reductase activity was demonstrated catalysing the stereospecific reduction of (+)-dihydrokaempferol to (+)-3,4-*cis*-leucopelargonidin with NADPH as co-factor With NADH the reaction rate was only about 35% of that observed with NADPH A pH-optimum around 6.7 was determined. In agreement with the anthocyanidin pattern of *Callistephus* flowers, (+)-dihydroquercetin and (+)-dihydromyricetin were also reduced by the enzyme preparations to the respective flavan-3,4-*cis*-diols They are even better substrates than dihydrokaempferol. As expected from the feeding experiments, the gene F but not the gene A controls dihydroflavonol 4-reductase activity; enzyme activity is in addition controlled by gene G.

INTRODUCTION

It has been shown by supplementation experiments on genetically defined acyanic flowers of Matthiola incana that flavan-3,4-diols (leucoanthocyanidins) are intermediates in anthocyanin biosynthesis [1]. Moreover, in cell-free extracts from *Matthiola* flowers a soluble enzyme (dihydroflavonol 4-reductase) could be demonstrated which catalyses an NADPH-dependent stereospecific reduction of (+)-dihydroflavonols to flavan-3,4-*cis*-diols [2] Feeding of flavan-3,4-diols, but not of dihydroflavonols, initiated anthocyanin synthesis in one of the white flowering mutants of *Matthiola* indicating a biosynthetic block between dihydroflavonol and flavan-3,4-diol The fact that this mutant lacks dihydroflavonol 4-reductase activity provided strong evidence for this enzyme being specifically involved in anthocyanin biosynthesis (Fig. 1) [1, 2]

For a further evaluation of the role of this enzyme in the anthocyanidin pathway we have now investigated genetically defined lines of *Callistephus chinensis*. In the flowers of this plant four genes (Ch, A, F and G) are involved in the biosynthesis of the anthocyanidin molecule. The gene Ch is known to control chalcone isomerase activity [3]. The genes A, F and presumably also G interfere with the anthocyanin pathway after dihydroflavonol formation but the stage of their action was not known [4]

In this paper we report on supplementation experiments with acyanic mutants of *Callistephus* using different precursors and on the enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-diols with enzyme prepar-

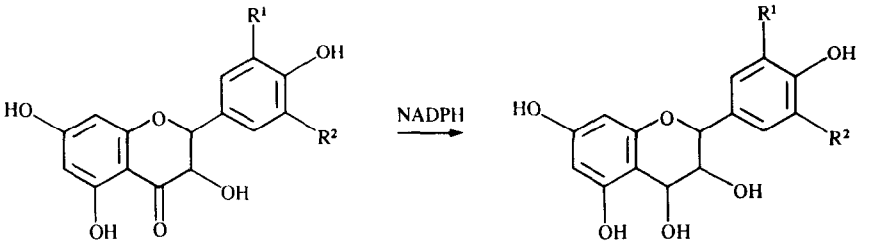

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Fig 1 Dihydroflavonols and flavan-3,4-diols used at the studies and the reaction of dihydroflavonol 4-reductase. $R_1 = R_2 = H$ 1 = dihydrokaempferol, 2 = leucopelargonidin $R_1 = OH$, $R_2 = H$ 1 = dihydroquercetin, 2 = leucocyanidin $R_1 = R_2 = OH$ 1 = dihydromyricetin; 2 = leucodelphinidin

ations from *Callistephus* flowers The studies confirm the important role of dihydroflavonol 4-reductase in anthocyanin biosynthesis Moreover, the genes F and G can be correlated with reductase activity, whereas the gene A concerns one of the still unknown steps between flavan-3,4-diols and anthocyanidins

RESULTS

Supplementation experiments

These experiments were performed on a range of acyanic mutants with recessive alleles of the genes A and F, respectively. A mutant with only recessive alleles for the gene G was not available The petals were placed in aqueous solutions of three types of precursors the flavanone naringenin, the dihydroflavonol dihydroquercetin and the flavan-3,4-diols leucopelargonidin and leucocyanidin (Fig 1) [5] Neither the administration of naringenin nor that of dihydroquercetin to the acyanic petals initiated anthocyanin synthesis (Table 1), indicating that recessive alleles of both the gene F and A cause a block after dihydroflavonol formation. Supplementation with flavan-3,4-diols, however, allowed a differentiation between the two genes Thus, in the petals of line 10a and of other lines with recessive alleles of the gene F, but dominant alleles of the genes A and G, anthocyanin synthesis could be observed from both leucopelargonidin and leucocyanidin, whereas the petals of lines with recessive alleles of the gene A (lines 07,08) remained again acyanic (Table 1)

In *Callistephus* flowers, lines with the dominant allele R possess flavonoid 3',5'-hydroxylase activity, whereas recessive genotypes (rr) lack this activity [7] As expected, feeding of leucopelargonidin to petals of a recessive genotype (line 10e) initiated the formation of pelargonidin derivatives and form leucocyanidin cyanidin derivatives were formed In the presence of flavonoid 3',5'-hydroxylase activity (line 10a), however, the same result was found and delphinidin derivatives were not formed (Table 1)

Enzymatic results

The supplementation experiments furnished strong evidence for the gene F being involved in the reduction of dihydroflavonols to flavan-3,4-diols This reaction is catalysed by dihydroflavonol 4-reductase (Fig 1). Corresponding enzymatic studies on cyanic and acyanic *Callistephus* lines should definitely prove the action of the gene F

When enzyme preparations from flowers of line 06 were incubated with (+)-(^{14}C)dihydrokaempferol in the presence of NADPH, one radioactive product was detected on TLC plates It comigrated with (+)-leucopelargonidin (Fig 1) in the solvent systems 1-3 which, however, do not separate the 3,4-*cis* and the 3,4-*trans* isomers of leucoanthocyanidins (Table 2) The isomer formed during enzymatic reduction could be decided by TLC in solvent system 4 (Table 2) and by two-dimensional chromatography with solvent system 4 in the first and solvent system 1 in the second dimension As expected [2, 10, 11], the radioactive product comigrated with the synthetic 3,4-*cis* isomer of leucopelargonidin

Dihydroflavonol 4-reductase activity could also be demonstrated in other cyanic *Callistephus* lines Moreover, reductase activity was present in enzyme preparations from flowers of the acyanic line 07 with the dominant allele F (Table 1) It was completely absent, however, in flower extracts from lines with recessive F alleles (line 10a, Table 1) Surprisingly, line 08 with dominant alleles of the gene F but recessive alleles of the gene G also lacked reductase activity (Table 1) Other enzyme activities involved in anthocyanin biosynthesis are present in both line 10a and line 08 In mixtures of enzyme extracts from line 06 and lines without reductase activity, no inhibition in the rate of conversion of dihydrokaempferol to leucopelargonidin was observed The absence of reductase activity in the latter lines is therefore not caused by an inhibitor

The further characterisation of the reductase reaction was performed with enzyme extracts from line 06 and (+)-dihydrokaempferol as substrate Leucopelargonidin formation was linear with protein concentration up to 20 μg protein per assay Linearity with time was observed for about 30 min The reaction had a pH-optimum between 6 5 and 7 0 with halfmaximal activities at pH 6 1 and 7 5 Under standard conditions a specific enzyme activity of about 0 4 μkat/kg protein was measured

Dihydroflavonol 4-reductase activity is strictly dependent on NADPH Saturation with NADPH was achieved with 1 mM per assay, even a ten-fold increase in NADPH concentration did not affect the reaction Substitution of NADPH by NADH (5 mM) reduced the reaction rate to 35% of the rate observed with NADPH

Incubation at different temperatures revealed a linear

Table 1 Genotype, phenotype, anthocyanin formation from precursors and dihydroflavonol 4-reductase activity of some lines of *Callistephus chinensis*

Line	Genotype	Flower colour	Main flower flavonoids	Anthocyanins formed from the precursors NAR	DHK	LPg	LCy	Dihydroflavonol 4-reductase activity (μkat/kg Protein)
06	ChChAAFFGGrr	red	Anthocyanins	n.s.	n.s.	n.s.	n.s.	0 4
07	chchaaFFGGrr	yellow	Isosalipurposide		-			0 13
08	ChChaaFFggrr	white	Flavonols			-		0 0
10a	ChChAAffGGRR	ivory	Flavonols	-		Pg	Cy	0 0
10h	chchAAffGGrr	yellow	Isosalipurposide	-	-	Pg	Cy	0 0

n s = Not studied, - = no reaction, NAR = naringenin, DHK = dihydrokaempferol, LPg = leucopelargonidin, LCy = leucocyanidin, Pg = pelargonidin derivatives, Cy = cyanidin derivatives

Table 2 R_f-values (× 100) of dihydroflavonols and flavan 3,4-diols on cellulose plates

Compounds	Solvent system 1	2	3	4	
Dihydrokaempferol	65	37	90	93	
Dihydroquercetin	37	34	84	86	
Dihydromyricetin	19	30	70	73	
Leucopelargonidin	27	52	79	80	(*trans*)
				64	(*cis*)
Leucocyanidin	12	50	61	56	(*trans*)
				38	(*cis*)
Leucodelphinidin	3	46	50	39	(*trans*)
				19	(*cis*)

increase of enzyme activity from 0 up to 30° and thereafter a drastic decrease to about 20% of the maximal activity in incubations at 46° Surprisingly, at O° the enzyme already exhibited a relatively high activity of 40% of the activity found at 30° Moreover, in incubations at low temperature, enzyme activity could be observed for at least 15 hr. No by-products were found to be formed during these long time incubations Flower extracts containing 10% (v/v) glycerol could be frozen in liquid nitrogen and stored at −70° for several months wihtout loss of reductase activity No enzyme activity remained, however, when petals were frozen and stored under the same conditions Addition of KCN (5 mM), EDTA (2 mM) and diethylpyrocarbonate (0.5 mM) did not influence enzyme activity, whereas *p*-chloromercuribenzoate (0 1 mM) reduced product formation to 75% and diethyldithiocarbamate (2 mM) even to about 50% in comparison to the standard assay without additions.

When the enzyme extracts were prepared from a line with flavonoid 3′,5′-hydroxylase activity, leucopelargonidin, leucocyanidin and leucodelphinidin were found to be formed from (+)-dihydrokaempferol as substrate Correspondingly, (+)-dihydroquercetin and (+)-dihydromyricetin also served as substrates for dihydroflavonol 4-reductase The 3,4-*cis* isomers of (+)-leucocyanidin and (+)-leucodelphinidin (Fig 1, Table 2) were identified as products of the respective enzyme reactions by the chromatographic methods described above. Leucodelphinidin could barely be extracted from the reaction mixture with ethyl acetate Therefore, the enzyme assays with dihydromyricetin as substrate were directly spotted on the TLC plate and separated in solvent systems 1 or 4. In contrast to *Matthiola*, where further reaction products of unknown structure were observed in the aqueous phase [2], with *Callistephus* flower extracts leucodelphinidin was the only reaction product from dihydromyricetin as substrate

Surprisingly, dihydroquercetin and dihydromyricetin (0.04 nmol each) were found to be completely reduced to the respective leucoanthocyanidins under standard conditions, whereas dihydrokaempferol (0.04 nmol) was only converted to 50% (at best) to leucopelargoidin Moreover, in the presence of dihydrokaempferol (0.02 nmol) and dihydroquercetin (0.02 nmol) as substrates in the same enzyme assay, only 3% of the available dihydrokaempferol but 70% of the dihydroquercetin were reduced to the respective leucoanthocyanidin. Similar results were obtained with mixtures of dihydromyricetin and dihydrokaempferol (60% and 3 4% conversion, respectively), whereas in incubations with 0 02 nmol of each dihydroquercetin and dihydromyricetin both substrates were completely converted to the respective leucoanthocyanidins

DISCUSSION

Supplementation of acyanic mutants with potential precursors again proved to be a valuable method for the elucidation of blocks in the biosynthetic pathway of anthocyanins Feeding of leucopelargonidin and leucocyanidin but not of dihydroquercetin or naringenin led to anthocyanin synthesis in line 10a (genotype AAffGG). In contrast, line 07 (genotype: aaFFGG) remained acyanic with all precursors fed The latter result is of importance, since it proves that chemical conversion of flavan-3,4-diols to anthocyanins does not occur during supplementation in the acyanic *Callistephus* flowers. Thus, flavan-3,4-diols are true intermediates in anthocyanin biosynthesis Moreover, the supplementation experiments suggest that recessive allels of the gene F block the reduction of dihydroflavonols to flavan-3,4-diols, whereas the gene A interferes with the anthocyanin pathway after flavan-3,4-diol formation In agreement with this assumption, in chemogenetic studies appreciable amounts of dihydroflavonols were found to accumulate in the flowers of lines with recessive alleles of the gene F (Teusch, M. and Forkmann, G , unpublished results)

During feeding experiments dihydrokaempferol (4′-OH) is converted to dihydromyricetin (3′,4′,5′-OH) in the presence of flavonoid 3′,5′-hydroxylase activity (data not shown). But from leucopelargonidin (4′-OH) and leucocyanidin (3′,4′-OH) only the respective anthocyanidins, and not delphinidin (3′,4′,5′-OH), were formed. Thus, the dihydroflavonol stage is obviously the latest point for the action of flavonoid 3′,5′-hydroxylase [6, 8] Similar results were found during supplementation experiments on *Matthiola* flowers for the action of flavonoid 3′-hydroxylase [1].

The demonstration of dihydroflavonol 4-reductase activity in *Callistephus* flower extracts confirms the supplementation experiments and furnishes strong support for the rule of this enzyme in anthocyanin biosynthesis. All *Callistephus* lines with cyanic flowers contain this enzyme activity and it is also present in lines with recessive alleles of the gene A. In contrast, flower extracts from line 10a with recessive alleles of the gene F lack reductase activity Surprisingly, however, line 08 with the dominant F allele and recessive G alleles did also not contain reductase activity Thus, obviously both the gene F and the gene G control this enzyme A similar situation was found in *Matthiola* flowers, where the genes e and g control reductase activity [1, 2] Here, there is some evidence for the gene e being the structural gene for dihydroflavonol 4-reductase, whereas the gene g rather exerts a regulatory function [2, 12, 13]. Up to now, however, in the case of the genes F and G of *Callistephus* flowers, such evidence is not available.

The properties of dihydroflavonol 4-reductase from *Callistephus* flowers are similar to those of the respective enzymes from *Matthiola* [2], *Dianthus*, *Petunia* and tomato (Ruhnau, B, Spörlein and Forkmann, G., unpublished results) and to the dihydroflavonol 4-reductase which are involved in proanthocyanidin synthesis rather than anthocyanidin formation [10, 11, 14, 15]. In all

cases a stereospecific, NADPH-dependent reduction of (+)-dihydroflavonol to the respective flavan-3,4-*cis*-diols was observed

In agreement with the presence of pelargonidin, cyanidin and delphinidin derivatives in *Callistephus* flowers (9, 16) and with the finding that dihydroflavonols but not flavan-3,4-diols can be hydroxylated in the 3′,5′-position, the respective hydroxylated dihydroflavonols dihydrokaempferol, dihydroquercetin and dihydromyricetin were found to be reduced by the enzyme to the corresponding flavan-3,4-diols

The reductase from *Matthiola* flowers also uses the three dihydroflavonols as substrate [2] In the latter plant, the reaction rate with the three substrates was not measured and no competition experiments with two dihydroflavonols in one enzyme assay were performed The results of such studies with *Callistephus* flowers suggest that dihydroquercetin and dihydromyricetin are considerably better substrates for reduction than dihydrokaempferol This result corresponds to chemogenetic studies on *Callistephus* flowers, where appreciable amounts of dihydrokaempferol were found in both acyanic and cyanic flowers In contrast, dihydromyricetin was never observed in cyanic flowers and dihydroquercetin occurred only in traces (Teusch, M and Forkmann, G., unpublished results) In this context the substrate specificity of dihydroflavonol 4-reductase from *Petunia* flowers and tomato seedling is worth mentioning The enzymes from both plants not at all reduce dihydrokaempferol to leucopelargonidin, but they use mainly dihydromyricetin as substrate for the reduction reaction (Ruhnau, B, Spörlein and Forkmann, G, unpublished results)

EXPERIMENTAL

Plant material The investigations included besides the anthocyanin producing line 06 (genotype ChChAAFFGGrrmm) a range of other cyanic lines and the acyanic flowering lines 07, 08, 10a and 10e [3] of *Callistephus chinensis* (Table 1) Gen Ch controls chalcone isomerase activity [3] Genes A, F and G concern anthocyanin synthesis after dihydroflavonol formation The multiple alleles of the R-locus (R, r′, r) govern the B-ring hydroxylation pattern of anthocyanins [7, 9, 16] Gene M controls the 5-*O*-glucosylation of anthocyanins [9, 16, 17] The plants were cultivated during the summer months in the experimental garden of our institute

Chemicals and synthesis of labelled substrates (+)-Dihydroflavonols and 4-coumaroyl-CoA were from our laboratory collection (+)-Flavan-3,4-*trans*-diols were kind gifts from W Heller (Neuherberg, F R G) and L Britsch (Freiburg, F R G) The respective 3,4-*cis*-isomers were prepared according to ref (14) (2-^{14}C)Malonyl-CoA (2 22 GBq/mmol) was purchased from Amersham-Buchler (Braunschweig, F R G) and diluted to 1 03 GBq/mmol with unlabelled material from Sigma Labelled (+)-dihydrokaempferol, (+)-dihydroquercetin and (+)-dihydromyricetin (3 09 GBq/mmol each) were prepared enzymatically from (^{14}C)malonyl-CoA and 4-coumaroyl-CoA as described [2, 8]

Enzyme preparation and enzyme assay The preparation of the crude extracts from buds and young flowers and gel filtration of the extracts was performed according to ref [2] The standard enzyme assay contained in a total volume of 50 μl 0 04 nmol radioactive substrate (116 Bq), 250 nmol NADPH in 10 μl H_2O and 20 μg protein in 0 1 M McIlvaine buffer, pH 6 8, with 2 8 mmol 2-mercaptoethanol and 10% (v/v) glycerol Incubation was carried out for 30 min at 25°. The mixture was immediately extracted twice (50 and 30 μl) with EtOAc and the extract chromatographed on a cellulose plate with solvent system 1 In case of dihydromyricetin as substrate, 25 μl of the reaction mixture were also spotted directly on a cellulose plate and separated in solvent system 1 or 4 Radioactivity was localized by scanning the plates For the quantitative determination of the reaction, radioactive zones were stripped off [18] and counted in Unisolve 1 in a scintillation counter

Dependence of reaction on pH and on temperature Crude extract was subjected to buffer exchange by gel filtration on Sephadex G-50 using 0 1 M McIlvaine buffer with 2 8 mmol 2-mercaptoethanol and 10% (v/v) glycerol between pH 5 5 and 7 5 Incubations were carried out with 40 μl enzyme solution of the respective pH and 10 μl NADPH solution in water The temperature dependence of the reaction was measured in standard enzyme assays incubated at 0, 7, 14, 25, 30, 37 and 45

Analytical methods Protein was determined according to ref [19] with bovine serum albumin as standard Supplementation experiments were performed according to ref [5] using method 2 for the administration of the precursors Standard procedures were used for the analysis of the anthocyanidin derivatives formed [20] TLC was carried out on precoated cellulose plates (Schleicher & Schull, Dassel, F R G) in (i) $CHCl_3$–HOAc H_2O (10 9 1, by vol), (ii) *n*-BuOH–HOAc–H_2O (6 1 2), (iii) 6% HOAc, and (iv) *n*-BuOH satd with 0 01 M Pi buffer, pH 6 8 Dihydroflavonols and flavan-3,4-diols (leucoanthocyanidins) were detected by spraying the plates with 0 1% aqueous fast blue B salt solution and subsequent exposure to NH_3 vapours Dihydroflavonols were also detected by the Zn–HCl test [21] The flavan-3,4-diols formed by enzymatic reduction of labelled dihydroflavonols were identified by co-chromatography with the synthetic compounds Radioactivity was localized by scanning the plates and in case of 2D chromatography using the Betacamera (Berthold, Wildbad, F R G)

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REFERENCES

1 Heller, W, Forkmann, G, Britsch, L and Grisebach, H (1985) *Planta* **165**, 284
2 Heller, W, Britsch, L, Forkmann, G and Grisebach, H (1985) *Planta* **163**, 191
3 Kuhn, B, Forkmann, G and Seyffert, W (1978) *Planta* **138**, 199
4 Seyffert, W (1982) *Biol Zbl* **101**, 465
5 Forkmann, G (1977) *Planta* **137**, 159
6 Tabak, A I H, Schram, A W and Bennink, G I H (1981) *Planta* **153**, 462
7 Stotz, G (1983) Thesis, Tubingen
8 Stotz, G and Forkmann, G (1982) *Z Naturforsch* **37c**, 19
9 Forkmann, G (1977) *Phytochemistry* **16**, 299
10 Stafford, H A, Lester, H H (1984) *Plant Physiol* **76**, 184
11 Kristiansen, K N (1986) *Carlsberg Res Commun* **51**, 51
12 Teusch, M, Forkmann, G and Seyffert W (1986) *Z Naturforsch* **41c**, 699
13 Kappert, H (1957) *Zuchter* **19**, 140
14 Stafford, H A, Lester, H H and Porter, L J (1985) *Phytochemistry* **24**, 333
15 Stafford, H A and Lester, H H (1985) *Plant Physiol* **78**, 791
16 Wit, F (1937) *Genetica* **19**, 1
17 Teusch, M (1986) Thesis, Tubingen

18 Redgwell, R J, Turner, N A and Bieleski, R L (1974) *J Chromatogr.* **88**, 25
19 Bradford, M M (1976) *Anal Biochem* **72**, 248
20 Harborne, J B (1967) *Comparative Biochemistry of the Flavonoids* Academic Press, New York
21 Barton, G M (1968) *J Chromatogr.* **34**, 562