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GENETIC CONTROL OF FLAVONE SYNTHASE II ACTIVITY IN FLOWERS OF *GERBERA* HYBRIDS

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Key Word Index—*Gerbera* hybrids; Compositae; flavone biosynthesis; flavone synthase II (FNS II); genetic control.

Abstract—In flower extracts of defined genotypes of *Gerbera* hybrids an enzyme activity was demonstrated which catalyses the introduction of a double bound between the C atoms 2 and 3 of the flavanones naringenin and eriodictyol. The products formed were the corresponding flavones, apigenin and luteolin. Similar to flavone synthases II from other plant species, the enzyme activity of this cytochrome P450-dependent monooxygenase was found to be localized in the microsomal fraction. The reaction required NADPH as cofactor and had a pH-optimum of about 7.5. Flavone synthase II activity was detectable only in flower extracts of genotypes with dominant wild-type alleles at the locus *Fns*, but not in lines with recessive alleles (*fns fns*). The results establish for the first time a correlation between a gene and the enzyme activity of flavone synthase II.

INTRODUCTION

Flower of *Gerbera* hybrids (Compositae) are known to contain several glycosides from the two flavones, apigenin and luteolin [1]. These compounds belong to one of the most abundant groups of naturally occurring flavonoid classes. Besides their occurrence in flowers, where they contribute to the flower colour together with other flavonoids, they were also found in many other parts of higher plants [2].

A soluble flavone-forming enzyme (flavone synthase I, FNS I) has been described from parsley (*Petroselinum hortense*) cell suspension cultures. This enzyme requires molecular oxygen, 2-oxoglutarate, Fe^{2+} and ascorbate as cofactors. Based on this cofactor requirement it was classified as a 2-oxoglutarate-dependent dioxygenase [3]. In contrast, flavone formation in flowers of a wide range of plant species [4–7] is catalyzed by an NADPH-dependent microsomal enzyme, called flavone synthase II (FNS II). Enzyme activity is affected by typical cytochrome P450 inhibitors, such as tetcyclacis, ketokonazole and ancymidol [8], indicating that this enzyme is a cytochrome P450-dependent monooxygenase [9]. The observation that a secondary compound is formed by two distinctly different enzymatic reaction systems [10, 11] is quite rare in nature and may be of significance in the consideration of the evolution of flavonoid biosynthesis in plants [3].

Many genes are known to control single steps in flavonoid biosynthesis in several plant species [12]. Analytical work on *Gerbera* hybrids has indicated that the formation of flavones is controlled by one gene called *fns* (Fig. 1) [13]. In genotypes with the dominant allele (*fns*$^+$) flavones were present, whereas in flowers with the recessive alleles (*fns fns*) no flavones were detected.

We now report on the enzymatic conversion of flavanones to flavones with enzyme preparations from flowers of chemogenetically defined genotypes of *Gerbera* hybrids, on the characterization of FNS II, and demonstrate for the first time a correlation between genotype and enzyme activity for flavone formation.

RESULTS

Naringenin has variously been demonstrated to be substrate for both FNS II and flavonoid 3′-hydroxylase (F3′H), which co-occur in membrane preparations and use NADPH as a cofactor (Fig. 1) [4, 8]. Separation of the two enzyme activities was achieved in *Gerbera* by the use of line "Th 58" with the dominant allele *fns*$^+$ but recessive alleles (*f3′hf3′h*) for F3′H activity as an enzyme source.

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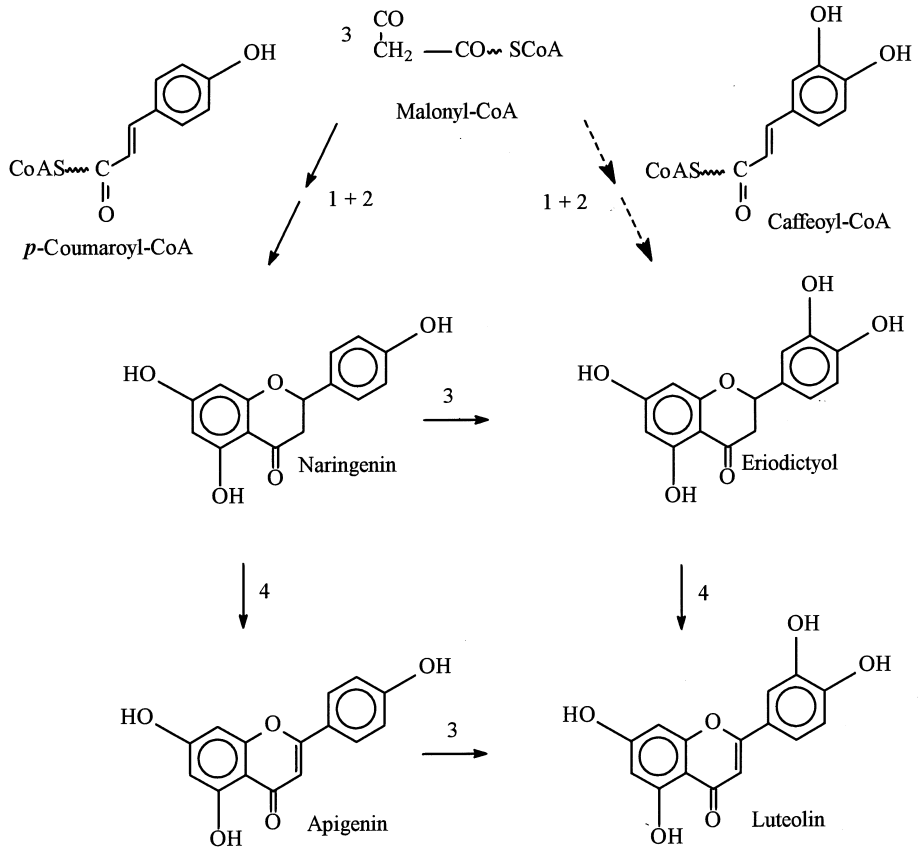


Fig. 1. Biosynthetic pathway of flavones. Structural formulas of substrates and products. Chalcone synthase (1); chalcone isomerase (2); flavonoid 3′-hydroxylase, gene *F3′*h (3); flavone synthase II, gene *Fns II* (4).

The incubation of crude extract from line "Th58" with labeled naringenin in the presence of the cofactor NADPH gave one radioactive product only, which comigrated with the authentic flavone apigenin on TLC performed with solvent systems 1–4 (Table 1).

Microsomal fractions prepared by Mg^{2+}-precipitation contained the bulk of FNS II activity. The reaction was strictly dependent on the cofactor NADPH. The substitution of NADPH by NADH considerably reduced the formation of apigenin. Exclusion of oxygen from the enzyme assay by addition of glucose and glucose oxidase led to complete loss of enzyme activity (Table 2).

Table 1. R_f-values (×100) of substrates and products on cellulose TLC plates

Compound	Solvent system			
	1	2	3	4
Naringenin	87	59	89	92
Eriodictyol	68	47	81	84
Apigenin	80	20	82	88
Luteolin	54	11	65	75

The reaction was linear with protein concentration up to about 30 μg protein per assay. Linearity with time was observed up to 25 min. The

Table 2. Subcellular localization, cofactor and oxygen requirement of the FNS II activity in flowers of *Gerbera* hybrids

Enzyme source	Additions	dpm in apigenin*
Crude extract	none	0
	NADPH	925
Microsomal pellet	none	0
	NADPH	3305
	NADH	90
Supernatant of microsomal pellet	NAPDH	270
Crude extract or microsomal pellet	none	895/3.215
	glucose + glucose oxidase	0

*Apigenin formed with 10 μg protein.

Table 3. Effect of several additions to the FNS II assay on enzyme activity

Additions	FNS II (%)
None	100
2 mM EDTA	93
5 mM KCN	58
0.5 mM DPC	67
0.1 mM Chloromercuribenzoate	78
2 mM DDC	93
0.1 mM *o*-Phenanthroline	110
2 mM Fe^{2+}	9
2 mM Fe^{3+}	33
50 μM Tetcyclacis	110
50 μM Ketoconazol	28
50 μM Ancymidol	40

DDC, Diethyl dithiocarbamate; DPC, Diethyl pyrocarbonate; EDTA, ethylene diamine tetra-acetate.

highest conversion of naringenin to apigenin occurred when the incubation was carried out at pH values around 7.5 and at a temperature of 25°C. Storage of crude extract for 40 min on ice or preincubation up to 40 min at 25°C had no effect on enzyme activity. This indicated that FNS II activity was sufficiently stable in the crude extract for enzyme characterization. When flowers were frozen in liquid nitrogen and stored at −70°C, no loss of extractable enzyme activity was found. Protein extracts containing 10% (v/v) glycerol could also be frozen in liquid nitrogen and stored for several weeks at −70°C with nearly no loss of enzyme activity. Without glycerol, loss of about 50% within 7 weeks was observed.

The incubation of labeled eriodictyol with enzyme preparations from line "Th58" and NADPH led to the formation of luteolin (Fig. 1), which was identified by co-chromatography with the authentic compound in the four solvent systems mentioned above (Table 1). Under standard conditions, the conversion rate for eriodictyol to luteolin was 45% compared to that of naringenin to apigenin.

Strong inhibition was found with ketokonazole and ancymidol, but not with tetcyclacis. Suprisingly, addition of Fe^{2+} iron to the assay strongly reduced the FNS II activity, too. Substantial inhibition was also observed upon addition of diethyl pyrocarbonate (DPC), chloromercuribenzoate, Fe^{3+} iron and KCN to the enzyme assays, whereas addition of EDTA, *o*-phenanthrolin or diethyl dithiocarbamate (DDC) had no appreciable effect (Table 3).

The course of enzyme activity for FNS II as well the flavone accumulation were studied during the development of buds and flowers. FNS II activity is present already in the very small buds of stage 1 and increased to a maximum in flowers of stage 5. In the following stages, the activity decreased slowly to nearly zero at stages 10 and 11. Accumulation of flavones started at stage 2 and increased concomitantly with FNS II activity. The amount of apigenin derivatives remained constant with aging of the flowers (Fig. 2).

Incubation of enzyme preparations from other cyanic as well as acyanic lines with the dominant allele *fns*$^+$ led to essentially the same results described for line "Th58". In lines, where additional F3′H activity was present, eriodictyol and luteolin where formed from naringenin in addition to apigenin. However, FNS II activity was found neither in crude extracts nor in microsomal preparations from flowers of lines with recessive alleles (*fns fns,* line Clivia 18-1), but in lines with F3′H activity, eriodictyol was still formed. In mixed enzyme assays containing FNS II activity and enzyme preparation from flowers of lines with recessive *fns* alleles, flavone formation was not inhibited.

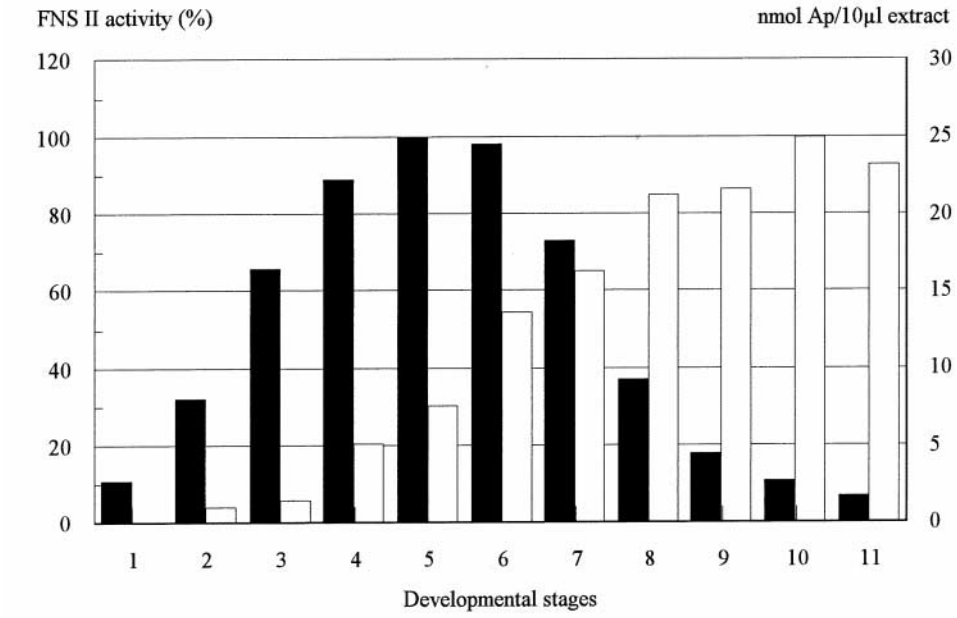
DISCUSSION

A great number of genes are known which control the activity of definite enzymes on the biosynthesis of flavonoids in various plant species. Among these, a gene controlling enzyme activity for flavone formation has not yet been characterized. In *Gerbera* hybrids the gene *Fns*, responsible for this biosynthetic step, has now been documentated chemogenetically [13].

The enzyme catalysing this reaction, FNS II, had been shown in flower extracts of several plant species [4, 7, 8, 14] and in osmotically stressed *Glycine max* cell suspension cultures [9]. We have now been able to demonstrate enzyme activity for the oxidation of flavanones to the respective flavones with flower extracts of chemogenetically defined *Gerbera* hybrids and to investigate the oxidation reaction in different genotypes affecting flavone synthesis in the flowers.

Gerbera buds and flowers earlier proved to be a valuable source for enzymes of the flavonoid pathway including F3′H [15]. The most elegant way to investigate FNS II activity separately from F3′H was the use of chemogenetically defined mutants which lack this hydroxylating enzyme activity [13].

As found for several other plant species, flavone formation in *Gerbera* is catalysed by a microsomal-bound enzyme which has an absolute requirement for NADPH and molecular oxygen and clearly not by the soluble 2-oxoglutarate-dependent dioxygenase found in parsley [3]. Incubation with the respective cofactors led to the formation of dihydroflavonols instead of flavones (data not shown). Furthermore, the pH at which maximal enzyme activity is exhibited is similar in all investigated plant species [4, 7]. Inhibition by typical cytochrome P450 inhibitors identify FNS II as a cytochrome-P450-dependent monooxygenase like flavonoid 3′-hydroxylase, flavonoid 3′,5′-hydroxylase and cinnamate 4-hydroxylase [11]. The strong inhibition of FNS II enzyme activity with Fe^{2+}- and



Length of ray floret corolla (mm)	<5	5 - 10	10 - 15	15 - 23	23 - 26	26 - 35	35 - 40	40 - 50	50 - 55	55 - 60	55 - 60
Description	closed buds	ray floret visible		start of pigmentation	pigmented ligule	ray floret	inflorescence half-opened	fully opened inflorescence			senescent ray florets

Fig. 2. The courses of apigenin (Ap) content □ and enzyme activity for flavone formation ■ during flower development in *Gerbera* line "Th58" with description of developmental stages [18]. Stage 5 was set as 100.

Fe^{3+}-ions may due to their ability to form radicals in the presence of ascorbate. Resulting hydroxyl radicals are known to react with many biological molecules [16, 17]. Other metal ions, such as copper and zinc, had similar effects on enzyme activity. In addition, EDTA, which forms complexes with the cofactor for dioxygenases Fe^{2+} and strongly inhibited FNS I [3], shows no effect on FNS II enzyme activity from *Gerbera*.

In agreement with the chemogenetic results and the genetic data described earlier [13], enzyme activity for FNS II was only present in flower extracts prepared from genotypes with the dominant allele *fns*$^+$. In enzyme preparations from recessive genotypes (*fns fns*) no conversion of flavanones to flavones was found. These results establish for the first time a correlation between a gene and enzymatic formation of flavones. This correlation proves that the gene *Fns* of *Gerbera* actually controls the step flavanone to flavone and that the enzyme activity measured in the *in vitro* assay is definitely responsible for the oxidation of flavanones to flavones *in vivo*.

EXPERIMENTAL

Plant material

Defined genotypes, flavone-producing (genotype *fns*$^+$) and non-flavone-producing mutants (genotype *fns fns*) of *Gerbera* hybrids were obtained from chemogenetic investigations [13] and cultivated in a greenhouse under standard conditions. The flavone-producing line "Th58" and the flavone-free line "Clivia 18-1" are distinguished from each other by the flavonoid composition and carotenoid content of the flowers. Eleven different stages were already defined to divide the flower development [18].

Chemicals

Naringenin, eriodictyol, apigenin and luteolin were obtained from Roth (Karlsruhe, Germany). (2-^{14}C)-Malonyl-CoA (55 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, U.S.A.) and diluted to 0.7 nmol and 110000 dpm contained in 5 µl destillated water. (4a, 6, 8-^{14}C)-Naringenin and (4a, 6, 8-^{14}C)-eriodictyol were prepared as decribed [3, 19] using partially purified

chalcone synthase (CHS) and chalcone isomerase (CHI) from parsley suspension cell cultures. 4-Coumaroyl-CoA and caffeoyl-CoA for naringenin and eriodictyol synthesis, as well as the specific cytochrome P450 inhibitors ketoconazole and tetcyclacis, were kind gifts from Dr W. Heller (Neuherberg, Germany). Ancymidol was obtained from Sigma (Deisenhofen, Germany).

Buffer solutions

The following buffer were used: (1) 0.1 mol/l Tris–HCl (containing 28 mmol/l 2-mercaptoethanol and 10 mmol/l Na-ascorbate), pH = 7.5; (2) 0.1 mol/l Tris–HCl, pH = 7.5; (3) as for (1) but with 0.05 mol/l Tris; (4) Britton–Robinson-buffer II (between pH 5.0 and 10.0).

Enzyme preparation and protein determination

All steps were performed using buffer 1 at 4°C. Preparation of crude extract and microsomal fractions by precipitation with $MgCl_2$ from fresh flower tissue was performed as described [20]. Protein was determined by the method of Bradford [21] using bovine serum albumin (Fluka, Neu-Ulm, Germany) as a standard.

Standard enzyme assay

The incubation mixture (final volume 200 μl) contained: 175 μl buffer 2, 0.3 nmol radioactive substrate (67 Bq; naringenin or eriodictyol, respectively), 2.0 nmol unlabelled substrate, 10 μl 20 mmol/l NADPH and 15 μl crude extract or microsomal prepn. After incubation for 20 min at 25°C the reaction was stopped by adding 20 μl MeOH containing a mixture of the respective flavonoids. The reaction mixture was extracted twice with EtOAc (100 + 50 μl). The pooled upper phase was chromatographed on a cellulose plate with solvent system 1. The radioactivity was localized and determined with the Fuji BAS 1000 Bio-Imaging Analyzer.

Determination of pH optimum

For enzyme preparation buffer 3 was used. The enzyme assays were carried out in mixtures of 175 μl buffer 4, 15 μl crude extract and 10 μl 20 mmol/l NADPH in water.

Oxygen dependence

Exclusion of O_2 from enzyme assay was carried out as described by Kochs and Grisebach [9] using glucose oxidase.

Analytical methods

For TLC precoated cellulose plates (Schleicher and Schüll G 1440, Dassel, Germany) were used with the following solvent systems: (1) $CHCl_3$–HOAc–H_2O (10:9:1); (2) 30% HOAc; (3) HOAc–HCl–H_2O (30:3:10) and (4) *t*-BuOH–HOAc–H_2O (3:1:1). Standard methods were used for extraction and identification of flavonoids [22, 23]. Flavones were detected under UV-light (243 nm) before and after fuming with ammonia. Flavanones were detected by reduction with sodium borohydride and subsequent exposure to HCl fumes [24].

The flavone content of buds and flowers during development was estimated by extraction of the pigments from petals of different stages with EtOAc in a tissue to solvent ratio of 1:40 (g/ml) during 48 h at 4°C in the dark. Characterization and quantification was performed by HPLC [25]. 10 μl of 75% MeOH extract was injected to a Spherisorb ODS II column (particle size 5 μm, 250 × 4.6 mm, Bischoff, Leonberg, Germany). Detection was performed with a diode array detector model 168 (Beckmann, München, Germany).

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