The Flavones of the European Species of *Silene* Section Elisanthe

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Z. Naturforsch. **38 c,** 894–898 (1983); received July 18, 1983

Flavones, Flavone Glycosylating Genes, *Silene* Species

The flavones present in three European species of *Silene* section Elisanthe (*Silene diclinis*, *S. heuffelii* and *S. marizii*) have been identified. The flavones of these species were compared with the flavones present in the three remaining species of section Elisanthe in Europe (*S. dioica*, *S. noctiflora* and *S. pratensis*). It was found that isovitexine-7-O-glucoside is present in all these species. The other flavones, notably the 2″-O-glycosylated ones, show a distribution over the species. The presence of flavone glycosylating genes in *S. diclinis*, *S. heuffelii* and *S. marizii* was tested by biochemical means. The results of this experiment show that the gene *g-G*, controlling the formation of isovitexin-7-O-glucoside, is the basic flavone glycosylating gene in European species of section Elisanthe. The other flavone glycosylation genes, *g-X*, *gl*, *gl-A*, *gl-R*, *fg* and *Fg* are not present in all species.

Introduction

There are six species of *Silene* section Elisanthe in Europe. Three of the species, *S. pratensis* (£), *S. dioica* and *S. noctiflora*, are widespread [1–3], while the three others are endemics. *S. diclinis* [4, 5] and *S. marizii* [6] occur only in few localities in the Iberian peninsula, and *S. heuffelii* is restricted to the Balkans [3]. The flavones present in the petals of the two very closely related species, *S. pratensis* and *S. dioica*, have been investigated thoroughly [7, 8]; Mastenbroek and Knorr, unpublished) and up to 29 flavones have been identified. All flavones identified in *S. pratensis* and *S. dioica* are derived from isovitexin (6-C-glucosyl-apigenin) [8–10].

Three unlinked independent loci are involved in the glycosylation of isovitexin in the mature plant and its flowers [8, 11]. For these three loci, *g*, *gl* and *fg*, three, three and two alleles respectively have been found. The two dominant alleles of the *g*-locus, *g-G* and *g-X*, code for proteins that bind glucose and xylose respectively to the free 7-OH group of isovitexin; *g* is the recessive allele. At the *gl*-locus *gl* is the recessive allele, while *gl-R* and *gl-A* control the binding of rhamnose and arabinose to the 2″-OH group. *Fg* is the dominant allele at the *fg*-locus and controls the binding of glucose to the 2″-OH, *fg* is the recessive allele [7].

The dioecious species of section Elisanthe in Europe are closely related [2] and seem suitable to investigate the evolutionary pathways leading to the various species. To do this we must obtain many different data for the various species such as on isozymes, morphology, flavones etc. In this paper we present the results of the identification of the flavones present in individuals of the endemic European species from the section Elisanthe of the genus Silene.

(£) *S. pratensis* is the correct name in *Silene* for *S. alba* [12] and is also known as *Melandrium album.*

Materials and Methods

Chemicals used

UDP-[^{14}C]glucose 292 Ci/mol, UDP-[^{14}C]xylose 267 Ci/mol and UDP-[^{14}C]arabinose 183 Ci/mol were obtained from New England Nuclear.

Plant material

Seed of the Spanish endemics, *S. diclinis* and *S. marizii*, was provided by Dr. H. C. Prentice. Seed of *S. heuffelii* was provided by the botanical garden of Cluj, Romania. Individuals of *S. diclinis* and *S. marizii* were grown in the greenhouse. Individuals of *S. heuffelii* were, after germination, stored in the cold for two months and then transferred to the greenhouse. All individuals treated in this way flowered six months after germination. 135 plants were investigated for *S. diclinis*, and 62 and 75 were screened for *S. marizii* and *S. heuffelii* respectively. The petals of five flowers per plant were collected and stored at 4 °C in 0.2 ml 1% HCl in 70% methanol.

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0341-0382/83/1100-0894 $ 01.30/0

Separation and isolation of the flavones

The identification of the flavones of the individual plants was carried out by means of one-dimensional paper chromatography on Schleicher and Schuell 1 paper in the developing solvents *n*-butanol-acetic acid-water (4:1:5 v/v/v, upper phase (BAW)) and 1% HCl in water (v/v); reference samples were co-chromatographed. The flavones are visible under UV (366 nm) as dark spots and after spraying with 5% sodium carbonate (w/v) the spots fluoresced yellow (isovitexin-7-glycosides and diglycosides) or dull brown (isovitexin-2″-glycosides). Larger quantities of flavone for the UV spectra and sugar and aglycone identification were obtained by chromatography on Whatmann no. III paper, using BAW and water as developing solvents. After elution with methanol the eluate was concentrated at reduced pressure in a rotation evaporator.

Sugar and aglycone identification

The isolated flavones were hydrolysed in 1 ml 50% methanol/1 N HCl for one hour in a fused tube in a boiling waterbath. The aglycone formed was extracted with *n*-butanol. The extract was concentrated at reduced pressure and co-chromatographed with the appropriate references on cellulose (Merck DC fertigplatten) coated thin layer plates in the developing solvents:

1) *n*-butanol-acetic acid-water (4:1:5 v/v/v, upper phase);
2) 1% HCl in water (v/v);
3) 15% acetic acid in water (v/v).

The sugar-containing water phase was passed through an ion exchanger (Merck Ionenaustauscher V), concentrated under reduced pressure and chromatographed on a kiesel-gel coated thin layer plate with 90% Acetone in water (v/v) together with reference samples. The sugars were made visible by spraying the plate with a solution of 2.58 g aniline hydrogenphtalate in 100 ml butanol saturated with water followed by heating at 100 °C for 10 min [13].

Enzymatic assay of petal preparations

Crude protein extracts of petals were made according to van Brederode and van Nigtevecht [14, 15]. The presence of the genes *g-G*, *g-X*, *gl-A*, *gl-R* and *Fg* was tested using isovitexin and the appropriate ^{14}C labeled UDP-glycoside as substrates. The standard reaction mixture consisted of 20 µl extract, 2 µl 1% isovitexin (w/v) in ethylene glycol mono-methyl ether and 2 µl UDP-[^{14}C]glycoside with the specific activities as mentioned under chemicals used. Incubation was for 20 min at 30 °C, after which the reaction was stopped by addition of an equal volume of 1% trichloroacetic acid in methanol w/v. The reaction mixture was applied to Whatman no. III paper and chromatographed 2-dimensionally in the BAW and water together with the appropriate references. The flavone spots were detected under UV, cut out and placed in a scintillation vial. After addition of 20 ml scintillation solvent (4 g 2,5-diphenyloxazole, 50 mg 1,4-bis-2-(5-phenyloxazoyl)-benzene in 1 litre toluene) the vial was counted in a Packard liquid scintillation spectrometer (counting yield approximately 75%).

Results and Discussion

The upper half of Table I presents the flavones found in *S. diclinis*, *S. marizii* and *S. heuffelii*. The lower half of the same table presents the flavones that can be found in the other three species [8, 11]; (Kamps-Heinsbroek and Maas, unpublished). All the glycosides can be found in *S. dioica* with the exception of those of vitexin. On the other end of the scale is the species with only a few isovitexin-glycosides: *S. noctiflora.* Only two glycosides are present in *S. noctiflora:* isovitexin-7-O-glucoside (7G) and an unknown compound referred to as 7G+. Hydrolysis of this compound yields glucose as sugar and isovitexin as aglycone. Its UV-spectrum in methanol and different diagnostic reagents [16] is exactly like that of isovitexin-7-O-glucoside. The only difference between the two compounds is their mobility in the chromatography-systems used, presumably because of the presence of some extra, unidentified, moiety in the molecule. This flavone, 7G+, is present in all species investigated.

S. marizii has three extra flavones in addition to those found in *S. noctiflora:* isovitexin-2″-O-glucoside (6G), isovitexin-7-O-glucose-2″-O-glucoside (7G6G) and isovitexin-7-O-glucose-2″-O-rhamnoside (7G6R). The first two of these are also present in the three remaining species: *S. diclinis*, *S. heuffelii* and *S. pratensis.* Isovitexin-7-O-glucose-2″-arabinoside (7G6A) and isovitexin-2″-O-arabinoside (6A) are also present in *S. diclinis.* *S. pratensis* and

Table I. Flavones found in the six species of *Silene*-section Elisanthe.

Species	7G	7X	6R	6A	6G	7G+	8X	8G	7G6R	7G6A	7G6G	7X6R	7X6A	7X6G
S. marizii	+	−	−	−	+	+	−	−	+	−	+	−	−	−
S. diclinis	+	−	−	+	+	+	−	−	−	+	+	−	−	−
S. heuffelii	+	−	−	−	+	+	−	−	+	−	+	−	−	−
S. pratensis	+	−	+	−	+	+	+	+	+	−	+	−	−	−
S. dioica	+	+	+	+	+	+	?	?	+	+	+	+	+	+
S. noctiflora	+	−	−	−	−	+	−	−	−	−	−	−	−	−

abbreviations used:
7G = isovitexin-7-O-glucoside;
7X = isovitexin-7-O-xyloside;
6R = isovitexin-2″-O-rhamnoside;
6A = isovitexin-2″-O-arabinoside;
6G = isovitexin-2″-O-glucoside;
7 G+ = isovitexin-7-O-glucoside containing an unknown extra group;
8G = vitexin-2″-O-glucoside;
8X = vitexin-2″-O-xyloside;
7G6R = isovitexin-7-O-glucose-2″-O-rhamnoside;
7G6A = isovitexin-7-O-glucose-2″-arabinoside;
7G6G = isovitexin-7-O-glucose-2″-O-glucoside;
7X6R = isovitexin-7-O-xylose-2″-O-rhamnoside;
7X6A = isovitexin-7-O-xylose-2″-O-arabinoside;
7X6G = isovitexin-7-O-xylose-2″-O-glucoside.

symbols used: + = present; − = absent and ? presence unknown.

S. heuffelii have the same flavone contents. As well as 7G, 7G+, 6G and 7G6G there are two more compounds present: isovitexin-2″-O-rhamnoside (6R) and isovitexin-7-O-glucose-2″-O-rhamnoside (7G6R). In some populations of *S. pratensis* from the USSR there are also two glycosides of the isomere of isovitexin vitexin, vitexin-2″-O-glucoside (8G) and vitexin-2″-O-xyloside (8X) [17]. The Russian botanical gardens, which send us the seed, ascribe these populations to *Melandrium boissieri*. In the Flora Europaea *M. boissieri* is classified under *S. pratensis* (1) but the flavones as well as its morphological appearance seem to indicate that it might be a distinct species.

Some conclusions can be drawn from our results:

1) The flavone 7G is always present in the species of Silene section Elisanthe; 7X is restricted to *S. dioica.*
2) *S. noctiflora* is the only species in which 6G and 7G6G are absent.
3) The diclycoside 7G6R is found in four of the six species investigated.
4) *S. dioica* and *S. diclinis* are the only species in which 6A and 7G6A are present. It is notably that these also are the only species with red petals, all others having white petals.

The glycosylation of the flavones in the petals of *S. dioica* and *S. pratensis* is controlled by three unlinked independent loci: *g*, *gl* and *fg* [8, 11]. The rather close relationship between these two species and the other species of section Elisanthe makes it likely that the same flavone glycosylation loci are present in all species. Assuming this, it can be inferred from Table I which glycosylation genes are present in *S. diclinis*, *S. marizii* and *S. heuffelii*. In *S. marizii* the presence of 7 G6R and 7G then indicates the action of the allele *g-G* of the *g*-locus, 6G and 7G6G indicate the presence of the gene *Fg* and 7G6R points at the presence of *gl-R*. The same genes are found in *S. heuffelii*. The genes present in *S. diclinis* on the basis of Table I should be: *g-G*, *gl-A* and *Fg*. An elaborate crossing program with known genotypes of *S. pratensis* and *S. dioica* needs to be carried out to prove that these genes indeed are present in *S. marizii*, *S. diclinis* and *S. heuffelii*.

The presence in the flower of the isovitexin-O-glycosyl transferases can be tested by incubating a crude homogenate of the petals with an UDP-[^{14}C]glycoside and isovitexin as substrates. The results of these tests are presented in Table II. In *S. marizii* the presence of *g-G* and *Fg* was inferred from Table I, so in the test incubation with UDP-[^{14}C]glucose as sugar donor activity was

expected at the 7G-spot, the 6G-spot and possibly the diglycoside spot of the chromatogram. Table II shows the presence of high activity at the 7G and the diglycoside spots, but only a very low activity at the 6G spot. The high activity at the 7G spot indicates very clearly the presence of *g-G*. The activity at the 6G spot is higher than the back ground level, but is not high enough to be used as evidene for the presence of *Fg*. The high activity at the diglycoside spot can be explained in various ways. One possibility is that the labeled 6G that is formed by the action of *Fg* immediately is converted into 7G6G by the action of *g-G*. Another possibility is that the crude petal homogenate contained a rather large amount of 2″-O-glycosides (6G and 6R) which were converted into 7G6G by the action of *g-G*. A third explanation could be that the action of *Fg* turns over the 7G present in the homogenate to the diglycoside. As 7G, 6G (and 6R) were present in the chromatograms of the individuals plants used in this biochemical test none of these possibilities can be singled out. Therefore, this experiment cannot be used to determine the presence of *Fg* in *S. marizii*. The third gene that, according to Table I, is present in *S. marizii* is *gl-R*. It is not possible to obtain UDP-[^{14}C]rhamnose from commercial sources, so the presence of this gene must be tested indirectly. In an incubation of UDP-[^{14}C]glucose in the presence of NADPH some UDP-[^{14}C]rhamnose is formed out of the UDP-[^{14}C]glucose [18]. *gl-R* activity can be shown with this UDP-[^{14}C]rhamnose. Table II shows that a rather high activity is recovered from the 6R spot, indicating the presence of *gl-R* in *S. marizii*. Activity is also found at the 7G and diglycoside spots, due to the presence of UDP-[^{14}C]glucose in the reaction mixture. Table I does not indicate the presence of either *gl-A* or *g-X*. The tests for these genes with UDP-[^{14}C]arabinose and UDP-[^{14}C]xylose show no activity, confirming the findings of Table I.

The presence of *gl-A* and *g-G* in *S. diclinis* is supported by the results presented in Table II. Again nothing can be said about the presence of *Fg*. The high activity found in the test with UDP-[^{14}C]xylose at the 6A spot is rather puzzling. Table I does not show the presence of a flavone xyloside in *S. diclinis*. Van Brederode and Kamps-Heinsbroek (unpublished), however, demonstrated considerable epimerase activity in crude homogenates of petals of *S. pratensis*, causing the transformation of UDP-xylose into UDP-arabinose. As no xylosides were found in *S. diclinis*, it is likely that the epi-

Table II. Results of biochemical tests.

Species	sugar (1)	cpm recovered from the position of (2, 3)						activity like
		7G	7X	6G	6R	6A/X	diglyc.	
S. marizii	glc	4550	–	40	80	–	1300	*g-G* (4)
	rha	5500	–	100	500	–	800	*g-G*, *gl-R* (4)
	xyl	–	0	–	–	0	0	
	ara	–	0	–	–	0	0	
S. diclinis	glc	200	–	30	200	–	2500	*g-G* (4)
	rha	450	–	0	120	–	650	*g-G* (4)
	xyl	–	0	–	–	1900	100	*gl-A* (5)
	ara	–	0	–	–	1500	100	*gl-A*
S. heuffelii	glc	140	–	30	0	–	300	*g-G* (4)
	rha	140	–	30	60	–	1000	*g-G* (4)
	xyl	–	0	–	–	0	0	
	ara	–	0	–	–	50	0	
S. noctiflora	glc	1850	–	–	–	–	–	*g-G*

1) sugars: glc = glucose; rha = rhamnose; xyl = xylose and ara = arabinose.
2) cpm = counts per minute. All tests were at least done in duplicate. The control value (incubation stopped at t = 0 with TCA) was 30 cpm. This value has been substracted from all values in the table. In all tests performed no activity was found at the 7G+ spot.
3) Symbol used: – = not determined.
4) The activity at the diglycoside spot could be the result of monoglycosides present in the crude homogenate; see text for details.
5) An epimerase activity is likely to be present which turns UDP-(^{14}C)-xylose over into UDP-[^{14}C]arabinose.

Table III. The distribution of the dominant flavone glycosylating genes over the six Silene species.

Species	*g-G*	*g-X*	*gl-A*	*gl-R*	*Fg*
S. dioica	+	+	+	+	+
S. pratensis	+	−	−	+	+
S. diclinis	(+)	−	(+)	−	(+)
S. marizii	(+)	−	−	(+)	(+)
S. heuffelii	(+)	−	−	(+)	(+)
S. noctiflora	(+)	−	−	−	−

Symbols used:
+ = present; − = absent; (+) = presence likely, but not formally proven by crossing experiments.

merase is also active in this species. Table II shows that only *g-G* could be demonstrated in *S. heuffelii*. Concerning *Fg* the same problems occur as in *S. marizii*. Table I indicates that *gl-R* should be present as well, but only a very low activity is found at the 6R spot. Here similar explanations could be offered for the absence of a clear *gl-R* activity as have been presented for the lack of a clear *Fg* activity. *S. noctiflora* was only tested for a *g-G* like activity as no other spot, with the exception of 7G+, has ever been found in this species (Maas, unpublished). The result of this test is very straightforward: as expected a very high activity was recovered from the 7G spot.

The results from the Tables I and II can be taken together to give Table III: the likely distribution of the six European species of *Silene* section Elisanthe. The presence of the genes in *S. pratensis* and *S. dioica* is confirmed by crossing experiments [11]. The presence of the genes of the other species is based on the results presented in this paper. It is evident from Table III that:

1) *g-G* is always present in the species of *Silene* section Elisanthe, *g-X* is only present in *S. dioica*.
2) The alleles of the *gl*-locus show a clear distribution among the species. *gl-A* is only present in the red flowering species *S. dioica* and *S. diclinis*, while *gl-R* is found in four of the six species investigated.
3) *Fg* is only missing from *S. noctiflora*.

These data will be useful in reconstructing the evolutionary pathways leading to these species [19], although other data are needed for this as well. Crossing experiments already have been performed [2], but more work needs to be done on morphological and biochemical characters of the various species. A very promising way of looking at the evolutionary pathways will be to isolate and compare the DNA of the flavone glycosylating genes of the *Silene* species [19].

Acknowledgements

We wish to thank Dr. H. C. Prentice for providing the seed of *S. marizii* and *S. diclinis*. We also wish to express our thanks to her for her discussion and comments on the manuscript.

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