Differential Regulation of Flavone Glycosylation during Ontogeny of *Silene pratensis*

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Two isovitexin glycosides have been found in the cotyledons and foliage leaves of *Silene pratensis* plants that are unable to glycosylate isovitexin in their petals (genotype *gg glgl fgfg*). The glycosides (isovitexin 7-O-galactoside and isovitexin 7-O-galactose 2″-O-arabinoside) were present only in the lower leaves: leaves produced later in the development of the flower stem accumulated only the aglycon isovitexin. The transition in the flavone composition during the ontogeny of the plants could be influenced by light intensity. In plants grown at low light intensity, glycoside production continued until a higher leaf pair number than in plants grown at higher light intensities. However, the effect of light intensity is indirect: the transition in the flavone composition is correlated with the transition from rosette leaves to stem leaves. The presence of the 7-O-galactosides in cotyledons and rosette leaves suggests that in addition to the *g*, *gl* and *fg* loci, there are further glycosylating loci which are not expressed in stem leaves and petals.

Introduction

Genetical and biochemical analyses have shown that there are three independent loci involved in the glycosylation of the basic flavone isovitexin (6-C-glucosylapigenin) in petals of *Silene pratensis* and *S. dioica*. The locus *g* is responsible for the attachment of sugars to the hydroxyl group at position 7 in the A-ring and there are three alleles known for this locus. The alleles *gG* and *gX* code for glycosyltransferases that bind glucose and xylose respectively (van Brederode and van Nigtevecht [1, 2]). In the presence of the recessive allele *g* no sugars are attached to the 7-OH group. The locus *gl* (with the alleles *glR*, *glA* and the recessive *gl*) is involved in the glycosylation of the hydroxyl group at position 2″ of the 6-C-bound glucose. The alleles *glR* and *glA* control the binding of rhamnose and arabinose respectively (Besson *et al.*; Heinsbroek *et al.* [3, 4]). The dominant allele *Fg* of the third locus, *fg*, is responsible for the attachment of glucose to the 2″-OH group (van Brederode and van Nigtevecht [5]).

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The genes *gG*, *glR* and *fg* are characteristic of *S. pratensis*, the white campion, whereas all the alleles of the three loci have been found in the red campion, *S. dioica* (Mastenbroek *et al.* [6]).

More complicated flavonoid patterns can be found in the vegetative parts of the plants, mainly as a result of the action of the genes *P* and *Me* in these parts. The presence of gene *P* can lead to the formation of isoorientin (isovitexin with an additional 3′OH group in the B-ring) and the 3′OH of isoorientin can be methylated by the action of gene *Me* resulting in the formation of isoscoparin (van Brederode and Kamps-Heinsbroek; van Brederode *et al.* [7, 8]). The glycosyltransferases which attach sugars to isovitexin recognize isoorientin and isoscoparin as substrates as well (van Brederode *et al.* [9]).

S. pratensis plants with the recessive alleles only (genotype *gg glgl fgfg*), are unable to glycosylate isovitexin in the petals and consequently the only flavone found in the petals is isovitexin itself. In foliage leaves, however, glycosides can be detected in addition to the aglycones isovitexin, isoorientin and isoscoparin. In view of the genetics of the flavone glycosylation this is rather surprising. Does this mean that the expression of the flavone glycosylating loci is differentially regulated during the

ontogeny of the plant or are loci other than the three already described involved in glycosylating the aglycones?

Preliminary experiments showed that the accumulation of the glycosides during the ontogeny of the plants could be influenced by light intensity. In this paper we describe the identification of the leaf glycosides and the effect of light intensity on the accumulation of these glycosides.

Experimental

a. Plant material

Silene pratensis plants that were unable to glycosylate isovitexin in the petals (genotype *gg glgl fgfg*; code number 88D) were obtained by crossing and selection of plants originally found in populations near Nijmegen, The Netherlands. Seedlings were grown in a climate chamber (daytemp. 24 °C, nighttemp. 15 °C, light-dark regime 16–8 h) at 8000, 17000 and 30000 lux (Metal halide lamps, HPI/T 400 W).

b. Extraction and identification

Flavones of leaves and petals were extracted with 70% aqueous methanol. For quantitative purposes leaves were extracted four times with 10 µl/mg freshweight. Flavonoid identification was done according to standard techniques (Mabry *et al.* [10]), including cochromatography with known referents, acid hydrolysis and UV-spectrum analysis.

c. Quantification of flavonoids

Flavones were quantified *in situ* by densitometric scanning of thin layer chromatograms.

Extracts of leaves were concentrated stepwise by evaporating to dryness in a desiccator and redissolving in known volumes of methanol. 2 µl samples were then chromatographed on thin layer plates (cellulose, Merck Fertigplatten, 0.1 mm thick; solvent 15% acetic acid in water). Spots were located on the chromatogram and the amounts of flavones in them were estimated by measuring the amount of light remitted at 335 nm. The spots were scanned in the direction of the chromatographic run. The apparatus used was the Camag Z-scanner in combination with the Zeiss PMQII-amplifying unit and photodetector, a Zeiss standard light source (H30DS Hydrogen lamp) and the Zeiss M4QII monochromator. The *in situ* absorption spectra of the flavones coincided well with the spectra in methanol (Mabry *et al.* [10]). Recorded emission peaks were cut out and weighed. A calibration curve of isovitexin 7-O-glucoside was used to estimate the flavone content of leaf extracts by interpolation. Every thin layer plate contained at least three different concentrations of isovitexin 7-O-glucoside, labeled with 14C in the glucose-moiety (4300 cpm/µg) according to van Brederode and van Nigtevecht [1]). The labeling was done to check quantitative application of the samples. In the concentration range from 0.1 up to 1.0 µg linear curves were obtained both for the quantitative application of samples and the amount of light remitted. The deviation of the mean of duplicate samples is within 5%. The standard deviation of the reproducibility of a measurement is 4–5% ($n = 10$).

The flavone content was related to dryweight. After the last extraction the leaves were heated at 80 °C for 20–24 h, followed by cooling in a desiccator above silica gel for at least one hour.

Results

i. Identification of flavone glycosides in foliage leaves

The development of the flavone pattern in seedlings of 88D was followed from the stage of the cotyledons towards the stage of flowering. During the ontogeny of the plant there is a transition from compounds with the characteristics of an isovitexin diglycoside and an isovitexin monoglycoside to isovitexin itself.

These compounds were purified by paper chromatography and the structure was elucidated by UV-spectrum analysis and acid hydrolysis. These analyses revealed that the compounds were indeed flavone glycosides. Upon treatment with 1 N HCl at 100 °C the monoglycoside releases galactose, whereas the diglycoside contains galactose and arabinose. Isovitexin was the main component in the aglycone fraction for both glycosides. The small amounts of vitexin found as well, can be explained by the Wessely-Moser rearrangement (see Mabry *et al.* [10]). Analysis of the UV-spectrum in methanol and several diagnostic reagents indicated that no free 7-OH occurred in either. Partial hydrolysis of the diglycoside showed that arabinose detaches very

quickly: the monoglycoside thus formed releases galactose upon hydrolysis. Spectrum analysis revealed that the 7-OH of this monoglycoside was occupied. From these data it can be concluded that the monoglycoside is isovitexin 7-O-galactoside and that in the diglycoside galactose is bound to the 7-OH group. In view of our knowledge of the structure of the diglycosides found in other genotypes of *S. pratensis* (Besson *et al.* [3]), and the fact that the chromatographic behaviour of the diglycoside resembles that of isovitexin 7-O-glucose 2″-O-arabinoside (Table I), it can be concluded that arabinose, released from the diglycoside, is bound to the 2″OH of the 6-C-bound glucose. Consequently, the diglycoside found in 88D is isovitexin 7-O-galactose 2″-O-arabinoside. The results of the identification are summarized in Table I.

The finding of a 7-O-galactoside was rather surprising. Therefore, plants of the same genotype, but obtained from another cross (56D), were investigated as well. In this cross we found the same glycosides. In addition, in crude protein extracts of 56D cotyledons an enzyme activity could be detected that was able to synthesize the 7-O-galactoside with isovitexin and UDP-galactose as substrates (van Brederode and Steyns, accompanying paper).

In both 88D and in 56D the main flavone in the cotyledons is the diglycoside. In cross 56D the diglycoside is only present in the cotyledons, whereas in 88D the diglycoside can also be found in substantial amounts in the first formed leaves.

ii. Influence of light intensity on the flavone pattern during the ontogeny of the plant

Seedlings of 88D were grown in the climate chamber at 8000, 17000 and 30000 lux respectively. The flavones in the leaf pairs of plants grown at the three light levels were then investigated by paper chromatography.

At all light levels the glycosides were found in the first formed leaves and aglycones in later ones. However, with increasing light intensity the transition of glycosides towards aglycones takes place at earlier developmental stages. At the lowest light level the glycosides disappear in the sixth or seventh leaf pair, whereas at higher light levels the aglycone

Table I. A. For solvents of paperchromatography (PC) see van Brederode and Kamps-Heinsbroek [7]. IV and V stand for isovitexin and vitexin respectively. Glc, gal and ara stand for glucose, galactose and arabinose respectively. Sugar identification is based on cochromatography with known referents in BAW or in the following thin layer chromatograms: 1. silica gel 60 (Merck) – aceton/water 90/10, 2. silica gel 60 (Merck) – aceton/water/chloroform/methanol 75/5/10/10, 3. cellulose (Merck) – formic acid/butanon/t-butanol/water 15/30/40/15. Sugars were made visible by spraying with anilinebiphthalate in water-saturated butanol (2.5 g/100 ml) followed by heating at 110 °C for several minutes. B. UV-absorption peaks and -shoulders(sh) are given in nanometers. For reagents see Mabry *et al.* [10].

	PC: Rf × 10^2			Acid Hydrolysis	
	BAW	1% HCl	15% HOAc	aglycone(s)	sugar(s)
A. 88D-glycosides: Rf values and products of acid hydrolysis					
88D-monoglycoside	31	27	56	IV + V	gal
IV-7-O-glc	32	29	60		
88D-diglycoside	27	56	73	IV + V	gal + ara
IV-7-O-glc 2″-O-ara	30	60	76		
IV	63	16	42		
V	44	6	22		
B. Spectra of 88D-glycosides					
	88D monoglycoside			88D diglycoside	
Reagents	UV-spectrum			UV-spectrum	
MeOH	271-333			270-332	
+NaOMe	247-274-304-359sh-391			249-276-301-359sh-395	
+NaOAc	269-358sh-392			261sh-269-350-392	
+NaOAc/H_3BO_3	270-338			270-337	
+$AlCl_3$	233-248-278-300-348-374			233-250sh-278-300-348-375	
+$AlCl_3$/HCl	233-246-279-300-346-374			233-250sh-278-300-344-375	

is already the main flavone in the third or fourth leaf pair. Variation occurs between seedlings grown at the same light level: the transition in the flavone pattern cannot be assigned therefore to a specific leaf pair.

There was also within-population variation in the accumulation of the diglycoside and of isoorientin and isoscoparin. The diglycoside did not accumulate in all plants investigated (at 8000, 17000 and 30000 lux 5 out of 19, 8 out of 26 and 7 out of 25 plants respectively); however, the monoglycoside was encountered in all the plants. The products of the genes P and Me, isoorientin and isoscoparin, were not encountered in all plants (at 8000, 17000 and 30000 lux 2 out of 19, 8 our of 26 and 8 out of 20 plants respectively).

It is likely that the absence of the diglycoside and of isoorientin and isoscoparin in some plants is the result of genetic variation in 88D. In order to be able to follow the occurrence of the various flavones during the ontogeny of the plant, leaf pairs of single plants were harvested. The changes in the amounts of the flavones were quantified. Figure 1 shows the amounts of the various flavones, expressed in µg/mg dryweight, present in the successive leaf pairs of three plants grown at 8000, 17000 and 30000 lux. From this figure it can be seen that at 8000 lux the transition in the flavone pattern occurs in the sixth leaf. At 17000 lux this transition occurs in the fourth leaf, although traces of the monoglycoside can still be detected in the fifth and sixth leaves. At 30000 lux the glycosides are detected in leaves 1 to 4, whereas in later developed leaves only isovitexin is found.

Figure 1 also demonstrates that as soon as substantial amounts of isovitexin accumulate the amount of the diglycoside drops sharply. The monoglycoside is less sensitive in this respect. Neither glycoside is present in the sessile leaves on the stem (denoted by an asterix in Fig. 1). In these leaves the glycosides are replaced by the aglycones. Care has to be taken with the interpretation of the quantities of the flavones at the various light levels shown in the three histograms of Fig. 1. Leaves were picked in one single harvest and there may be a cumulative effect of light on the flavone content. Substantial variation in dryweight will influence the pattern of the histogram. Nevertheless, it can be concluded from Fig. 1 that high light levels enhance flavone accumulation (see also Niemann *et al.* [11]).

Discussion

Plants of *S. pratensis* that are unable to glycosylate isovitexin in the petals (genotype *gg glgl fgfg*), contain isovitexin glycosides in cotyledons and foliage leaves. These glycosides were identified as isovitexin 7-O-galactoside amd isovitexin 7-O-galactose 2″-O-arabinoside. The glycosides are present in leaves with a clear petiole, whereas the aglycones are predominantly detected in sessile

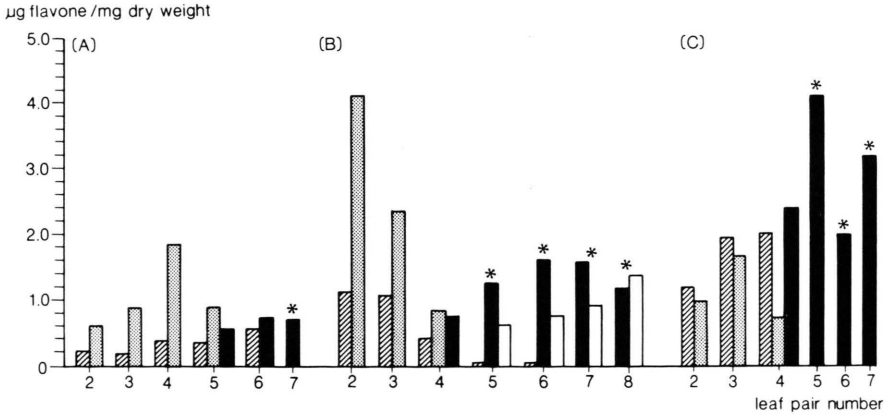

Fig. 1. Quantification of flavones in leaves of 88D grown at (A) 8000 lux (B) 17000 lux (C) 30000 lux. ▨ = isovitexin 7-O-galactoside; ▩ = isovitexin 7-O-galactose 2″-O-arabinoside; ■ = isovitexin; □ = isoorientin/isoscoparin. Asterisks (×) denote sessile leaves. The absence of isoorientin and isoscoparin in (A) and (C) is due to genetic variation. Both flavones can accumulate at these light intensities (see text)

leaves. The leaves with a clear petiole are characteristic of the rosette, whereas typical stem leaves are sessile. The transition from typical rosette leaf to typical stem leaf can be found in the one or two leaves located on the stem but with an intermediate petiole length. The transition in the flavone pattern is correlated with the transition in the leaf morphology. This means that the genetic regulation of the flavone glycosylation changes during the ontogeny of the plant and differs in rosette and stem leaves. This resembles results found with isoenzyme patterns during the ontogeny of *S. pratensis* by Mastenbroek *et al.* [12]. These authors demonstrated that isoenzyme variation within a plant was restricted to rosette leaves, whereas the pattern in stem leaves was constant.

At low light levels the ontogeny of the plants is retarded. This can be inferred from the observation that, at low light levels, the plants produce more rosette leaves (with a clear petiole) prior to stem formation. The effect of light intensity on the accumulation of the glycosides is therefore indirect. In this respect the disappearance of the glycosides may be correlated with the moment the plant starts stem formation and subsequently flowering.

The binding of galactose to the hydroxyl group at position 7 and of arabinose to the 2″ hydroxyl of isovitexin presented us with the problem of whether this binding is coded for by the loci *g* (7OH), *gl* and *fg* (2″OH) or whether other loci are involved in glycosylating isovitexin. We reasoned that in the case of involvement of the loci *g*, *gl* and *fg* it would be impossible to find 7-O-galactosides or 2″-O-arabinosides in the presence of two dominant alleles at the corresponding loci. This was not found to be the case (J. M. Steyns *et al.*, in preparation). Thus, it can be concluded that two hitherto unknown isovitexin glycosylating loci have been found. They have escaped earlier detection because their expression is limited to early ontogenetic stages.

The finding of the 7-O-galactosides in rosette leaves also explains the discrepancy between isovitexin glycosides found in Silene by our group and by Wagner *et al.* [13]. Wagner *et al.* [13] found a number of 7-O-galactosides in *S. pratensis*, whereas our population-genetic studies of the flavone glycosylation in petals of *S. pratensis* and *S. dioica* did not reveal any galactosides. It seems likely that Wagner *et al.* [13] investigated mainly rosette leaves of *S. pratensis* plants with the normally occurring genotypes and therefore found a number of 7-O-galactosides. It has been observed that in rosette leaves of plants of genotypes with one or more of the dominant alleles of the loci *g*, *gl* and *fg*, isovitexin 7-O-galactosides can be detected (J. M. Steyns, unpublished).

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