The Expression of the Isovitexin 7-O-Xylosylating Gene *gX* in *Silene pratensis* and *S. dioica* is Restricted to the Petals

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The isovitexin-O-glycosylation patterns of rosette leaves, stem leaves and petals of *Silene dioica* plants were investigated. The 7-O-xylosylation of isovitexin, controlled by gene *gX,* only occurs in the petals. In the leaves isovitexin is 7-O-galactosylated, which is controlled by gene *Xgal.* Acyl groups may be linked to either the 7-O- or the 2″-O-substituted sugar; the former only occurs in the leaves, whereas the latter takes place both in leaves and petals. The restriction of the *gX* controlled enzyme activity and its product to the petals of *Silene dioica* plants is in complete agreement with previously obtained results (reference [2]) on the expression of *gX* in the genetic background of the closely related *Silene pratensis,* into which *gX* can be introduced by introgressive hybridization.

On the basis of serological enzyme inhibition studies, it is argued that the absence of 7-O-xylosylation in the leaves is not due to post-translational inactivation of the *gX* controlled enzyme. The regulation of the expression of gene *gX* throughout ontogeny therefore differs markedly from that of two allelic *g* locus variants, controlling the 7-O-glucosylation of isovitexin.

Introduction

The variation in the glycosylation pattern of isovitexin (6-C-glucosylapigenin) in the two closely related species *Silene pratensis* and *S. dioica,* is the result of variation in the expression of a set of 6 loci, which control the presence of 11 different isovitexin-O-glycosyltransferases (Table I). The phenolic 7-OH group of isovitexin may be substituted with glucose, xylose and galactose, whereas glucose, xylose, arabinose and rhamnose may be bound to the 2″-OH group of the C-C bound glucose. The concerted action of the 7-O- and 2″-O-glycosylation genes results in the synthesis of isovitexin 7,2″-O-diglycosides.

The 7-O-xylosylation is governed by gene *gX.* This gene is most commonly found in the red flowering *S. dioica* [1], but may also be encountered in the white flowering *S. pratensis* as the result of introgressive hybridization. The allelic variant of *gX, gGm,* controls the 7-O-glucosylation and is present both in *S. dioica* and *S. pratensis.*

In isogenic lines with a *S. pratensis* background the expression of the genes *gGm* and *gX* differs throughout ontogeny with *gGm* expressed in all ontogenetic stages and *gX* only in the petals [2]. In the vegetative parts of *gX* plants 7-O-xylosylation is replaced by 7-O-galactosylation; two different enzyme activities catalyze the biosynthesis of the respective 7-O-glycosides. The genetic control of the 7-O-galactosyltransferase is assigned to gene *Xgal.* Preliminary evidence suggests that the genes *gX* and *Xgal* are linked. Steyns and Brederode [2] showed that the *Xgal* controlled enzyme activity is also present in the petals; yet, only trace amounts of the 7-O-galactoside are produced.

Table I. Genetics of the isovitexin 7-O- and 2″-O-glycosylation in *Silene pratensis* and *S. dioica.*

Locus	Gene/allele	Transferred sugar	Reference	
	g	none	[10]	7-O-glycosylation
g	*gGm, gGd*	glucose	[8, 11]	
	gX, gX′	xylose	[12, 13]	
O7g	*O7g*	galactose	[4]	
Xgal	*Xgal*	galactose	[2]	
	gl	none	[10]	2″-O-glycosylation
gl	*glA*	arabinose	[14, 15]	
	glR	rhamnose	[14, 16]	
	f	none	[17]	
f	*fG*	glucose	[17]	
	fX	xylose	[18]	
D6a	*D6a*	arabinose	[2]	

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The differential regulation of the *g* locus controlled enzyme variants throughout ontogeny may be due to the genetic background in which *gX* and *gGm* are present. We wondered whether the expression of *gX* throughout ontogeny is different in *S. dioica,* in which this gene is commonly encountered. In the present paper we therefore report on the flavones and flavone-O-glycosyltransferases present throughout ontogeny of *S. dioica* plants.

Experimental

a. Seeds of a cross (code number 36F) between two *Silene dioica* individuals, originating from a population in the Vosges mountains (France), were sown on earth in a climate chamber. The climate chamber conditions were: light-dark regime of 16–8 h, 26,000 lux, day temperature 24 °C, night temperature 15 °C. After two months the conditions were changed (12 h light/12 h dark, night temperature 8 °C) to induce flowering, which occurred about one month later.

b. Flavone extraction, purification and structure elucidation as well as glycosyltransferase assays have been described previously [3, 4].

c. Thin layer chromatography of flavones was performed with the following developing solvents:

I. BAW (*n*-butanol/acetic acid/water, 4/1/5, upper phase).

II. 1% HCl (1% hydrogen chloride in water).

III. 15% HOAc (15% acetic acid in water).

IV. EPWM (ethylacetate/pyridin/water/methanol, 80/20/10/5).

The developing solvents I–III were run on cellulose (Merck Fertigplatten), whereas silica 60 (Merck Fertigplatten) was used for IV.

Results and Discussion

i. Flavonoids in petals of S. dioica plants

Two-dimensional paper chromatography of methanolic petal extracts revealed the presence of three UV-absorbing flavonoid spots: A, B and C.

Compound C was identified as isovitexin 7-O-xyloside (7X) by co-chromatography with authentic 7X on thin layer chromatograms developed with the solvents I–IV and by the presence of an isovitexin 7-O-xylosyltransferase activity in a partly purified petal protein extract.

Thin layer chromatography of spot A in developing solvent IV revealed a mixture of two compounds, A1 and A2 (Table II), with A2 as the major component. UV spectral shifts with several diagnostic reagents [5] indicated the presence of free 4′-OH and 5-OH groups in A1/A2, whereas the 7-OH group was substituted. Total hydrolysis with trifluor acetic acid (TFA) yielded isovitexin, arabinose and xylose. Partial acid hydrolysis resulted in a mixture of two 7-O-glycoside intermediates. The most prominent of these 7-O-glycosides was identified as 7X by co-chromatography. The other 7-O-glycoside surprisingly co-chromatographed with isovitexin 7-O-galactoside (7Gal); this compound was detected because 7X is more acid labile than 7Gal so that the amount of the latter relatively increases in a partial hydrolysate. The presence of 7Gal was confirmed by demonstrating a 7-O-galactosyltransferase activity in a partly purified petal protein extract. In this enzyme preparation 2″-O-arabinosyl- and 2″-O-xylosyltransferase activities could be demonstrated as well; on the other hand, no 7-O-arabinosyltransferase activity was detected. Taken together these results suggest that A may be a mixture of four isovitexin 7,2″-O-diglycosides: isovitexin 7-O-xylose 2″-O-arabinoside (7X6A), isovitexin 7-O-xylose 2″-O-xyloside (7X6X), isovitexin 7-O-galactose 2″-O-arabinoside (7Gal6A) and isovitexin 7-O-galactose 2″-O-xyloside

Table II. Thin layer R_f values (× 100) of the flavones present in the petals (A–C) and the vegetative parts (D–G) of *S. dioica* plants.

Developing solvent	A1	A2	B	C	D1	D2	E	F	G
I: BAW	43	43	55.5	46	32	34.5	46	50	47
II: 1% HCl	55	55	56	14.5	53	53	54	25	35
III: 15% HOAc	73	73	76	44	72	72	74	57	65
IV: EPWM	25	28.5	34	33	16.5	21	25.5	35	29

A1 = isovitexin 7-O-xylose 2″-O-xyloside (7X6X).
A2 = isovitexin 7-O-xylose 2″-O-arabinoside (7X6A).
B = isovitexin 7-O-xylose 2″-O-arabinoacyl.
C = isovitexin 7-O-xyloside (7X).
D1 = isovitexin 7-O-galactose 2″-O-xyloside (7Gal6X).
D2 = isovitexin 7-O-galactose 2″-O-arabinoside (7Gal6A).
E = isovitexin 7-O-galactose 2″-O-arabinoacyl.
F = isovitexin 7-O-galactoacyl 2″-O-arabinoacyl.
G = isovitexin 7-O-galactoacyl 2″-O-arabinoside.
The acylgroup attached to the 7-O-bound galactose is a cinnamic acid derivative, whereas that linked to the 2″-O-bound arabinose is probably a small aliphatic group.

(7Gal6X). The concentrations of 7Gal6A and/or 7Gal6X must be very low as no galactose was detected in the sugar analysis of A.

Spectral analysis and acid hydrolysis of compound B revealed that B is also an isovitexin derivative with free 4′-OH and 5-OH groups and a substituted 7-OH group. Two sugars are released from B: arabinose and xylose. The relative high R_f values in the aqueous solvents II and III suggest that B is an isovitexin 7,2″-O-diglycoside; the unusual high mobilities in the developing solvents I and IV, however, point to a less hydrophilic character (Table II). Alkaline hydrolysis of B gave A2. The moiety esterified to A2 is linked to the 2″-OH bound sugar. This is inferred from the occurrence of a small amount of an unstable TFA hydrolysis intermediate, which further decomposed into the 2″-O-glycoside. This 2″-O-glycoside co-chromatographed with isovitexin 2″-O-arabinoside (6A), which indicates that A2 is 7X6A and A1 is 7X6X.

In summary, the petals of the *S. dioica* plants contain 7X, 7X6X, 7X6A, an unknown acylated 7X6A derivative, 7Gal6A and/or 7Gal6X. The relative contributions to the total flavone content are 35% for 7X, 55% for 7X6A/7X6X, 10% for the acylated 7X6A derivative B (the amounts of 7Gal6A and/or 7Gal6X are negligible). The accumulation of these compounds indicates that the glycosylation genes expressed in the petals are: *gX, fX, glA* and *Xgal* (see also Table I).

ii. Flavonoids in the vegetative parts of S. dioica plants

Paper and thin layer chromatography (PC and TLC respectively) of methanolic extracts of rosette and stem leaves indicated that the flavone patterns in both types of leaves were identical, but clearly different from the petals (Table II). In the following these leaf types are therefore referred to as the vegetative parts.

Two-dimensional PC revealed the presence of four UV-absorbing flavonoid spots: D, E, F and G. These compounds were purified and then subjected to TLC. The TLC analysis revealed that D was a mixture of two compounds: D1/D2 (Table II).

The structure elucidation of the compounds D1, D2 and E was analogous to that undertaken for the petal flavonoids. The compounds D1 and D2 were identified as isovitexin 7-O-galactose 2″-O-xyloside and isovitexin 7-O-galactose 2″-O-arabinoside respectively. Compound E was shown to be a derivative of D2 by alkaline hydrolysis. It is likely that the esterified moiety is the same as that esterified to compound B. The difference between E and B therefore is the 7-O-bound sugar, which is galactose in the vegetative parts and xylose in the petals.

The compounds F and G were also shown to be isovitexin derivatives. Both compounds converted into D2 upon alkaline hydrolysis. The presence of a cinnamic acid derivative bound to F and G was indicated by the appearance of a blue fluorescing compound with high relative mobility in developing solvent I (R_f = 0.85) upon alkaline hydrolysis, the greenish fluorescence of F and G upon spraying with Na_2CO_3 and the elevated B-ring absorbance between 325–330 nm in the methanol spectrum [6]. The structure of the cinnamic acid derivative was not further investigated. Partial TFA hydrolysis indicated that this acyl group is esterified to the 7-O-bound galactose. Prolonged storage of F and G in methanol revealed instability. Decay products of F were identified as G, E and D2, whereas G converted into D2. These results indicate that F and G are the 7-O-acylated derivatives of E and D2 respectively.

From the analyses performed on the flavones from the vegetative parts and the petals, we may conclude that 7-O-xylosylation of isovitexin, controlled by *gX*, does not take place in the vegetative parts.

The structure elucidation of the C-glycoflavones present in the red campion, *S. dioica*, is complicated by the fact that all isovitexin glycosylation genes identified so far in *Silene* section Elisanthe, may be present in *S. dioica* [1]. Here it is shown that the flavone pattern in *S. dioica* is also influenced by acylation. The binding of a cinnamic acid derivative to the 7-O-substituted sugar seems restricted to the leaves. This kind of acylation has also been demonstrated in the leaves of *S. pratensis* with ferulic acid bound to the glucose moiety of isovitexin 7-O-glucoside [6]. Independent of the 7-O-glycoside acylation another kind of acylation occurs at the 2″-O-substituted sugar. This acyl moiety may be aliphatic in nature, explaining the increased R_f value in developing solvent I as compared to the unacylated compounds (Table II). On the other hand, the relative mobilities in the aqueous solvents II and III suggests that this hypothetic aliphatic group is small. Available literature suggests that the unknown acyl

moiety may be acetate (for references refer to Chopin *et al.*, [7]).

iii. Differential regulation of g locus controlled allozymes

The flavonoid pattern during ontogeny clearly demonstrates that the 7-O-xylosylation of isovitexin is also restricted to the petals in its common genetic background, *S. dioica*. The results obtained are completely comparable with those for *gX* hybridization plants with a *S. pratensis* genetic background [2]. This means that the expression of the *gX* controlled 7-O-glycosylation is quite different from the 7-O-glycosylation controlled by its allelic variants *gGm* and *gGd* [8]. This different regulation of the flavone 7-O-glycosylation during ontogeny suggests that the *g* locus may be a regulatory rather than a structural gene locus. The existence of four *g* locus controlled enzyme variants, differing in substrate specificity *(gGm, gGd, gX)* or maximal reaction velocity *(gX')*, and showing serological cross-reactivity [9], however, strongly argues against this possibility.

The absence of the 7-O-xyloside in the vegetative parts may be explained in several ways: the expression of gene *gX* may be impaired at the transcriptional or translational level or, alternatively, translation takes place, but the protein formed is subject to rapid turnover or is rendered inactive by post-translational modification. The presence as well as the amount of the *gX* controlled protein may be studied by use of a specific anti-serum or the anti-serum raised against the allelic enzyme variant controlled by *gGm*, which also recognizes the *gX* controlled protein. The possibility of post-translational modification of isovitexin glycosylating proteins, resulting in enzymatic inactivation, can be extended to question the differential regulation of the genes *D6a* and *O7g* as well. These genes are expressed in the cotyledons and the rosette leaves, but not in the stem leaves and petals: enzymes as well as enzyme products are not detectable beyond the rosette leaf stage [4]. If, however, inactivated *D6a* and *O7g* controlled proteins are present in the petals, the polyclonal anti-serum raised against the *gGm* controlled protein is expected to contain antibodies against these proteins as well. Enzyme inhibition tests of this polyclonal anti-serum with active *D6a* and *O7g* proteins, however, revealed that the *D6a* enzyme is inactivated, whereas the *O7g* enzyme activity remains unaffected [9]. This result argues against the presence of inactive forms of isovitexin glycosylating proteins in organs where the flavone product and the enzyme catalyzing its biosynthesis are not demonstrable, and may therefore be taken as indirect evidence that the expression of *gX* in the vegetative parts is blocked at the transcriptional or translational level.

In the vegetative parts 7-O-galactosylation replaces 7-O-xylosylation. The biosynthesis of the 7-O-galactoside beyond the rosette leaf stage, assigned to the expression of gene *Xgal*, is dependent on the presence of gene *gX* [2]. This suggests that *gX* and *Xgal* are genetically linked. This linkage, combined with the differential and sequential expression of these genes during ontogeny, may in turn suggest that *gX* and *Xgal* represent duplicated genes. Enzyme inhibition tests with the *gGm* anti-serum, which have shown that the *gX* enzyme is inactivated, whereas the *Xgal* enzyme is not [2], however, argue against this possibility.

Further and more detailed studies of the variation of the isovitexin glycosylating genes, their genetic regulation and interrelation, require more sensitive techniques, *e.g.* the use of cDNA of a specific mRNA. Attempts in this direction are presently pursued.

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