

# Differential Regulation of Two Genes Controlling the Biosynthesis of Isovitexin 7-O-Galactoside in *Silene* Plants

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

*Silene*, Isovitexin 7-O-Galactosylation Genes, *O7g* and *Xgal*, Differential Regulation

The expression of the allelic isovitexin 7-O-glycosylation genes *gG* (transfer of glucose) and *gX* (transfer of xylose) was studied in cotyledons, rosette leaves, stem leaves and petals of *Silene* plants. These studies revealed that *gG* is expressed in all ontogenetic stages, whereas its allele *gX* is only expressed in the petals. In the vegetative parts of *gX* individuals 7-O-xylosylation is replaced by 7-O-galactosylation. The possibility that *gX* encodes an enzyme activity that catalyzes different reactions in the petals and the vegetative parts resulting in the accumulation of the 7-O-xyloside and the 7-O-galactoside respectively, has been disproved. It is shown that there are two different enzymes catalyzing the biosynthesis of isovitexin 7-O-galactoside. These 7-O-galactosyltransferase activities differ with respect to heat inactivation, pH optimum, flavone acceptor specificity and Michaelis-Menten enzyme kinetic parameters. The genes controlling these enzyme activities are regulated differentially, with gene *O7g* (described previously by Steyns *et al.* [11]), expressed in the cotyledons and the rosette leaves and *Xgal* in the stem leaves and petals.

## Introduction

The flavonoids present in the white campion *Silene pratensis* belong to the class of the C-glycosylflavones. The basic flavone skeletons found are isovitexin (6-C-glucosylapigenin), isoorientin (6-C-glucosylluteolin), isoscoparin (6-C-glucosylchrysophenol) and its 8-C-glucosyl isomers vitexin, orientin and scoparin. The 7-OH of the A-ring and the 2''-OH of the C-C bound glucose may be glycosylated (Mastenbroek *et al.* [1]). Acylation of the sugar bound to the 7-OH group has also been demonstrated (Niemann [2]).

In the petals of *Silene* plants the biosynthesis of the isovitexin glycosides is controlled by the loci *g*, *f* and *gl*, which segregate independently. The 2''-O-glycosylation is controlled by the loci *gl* and *f* with each one recessive and two dominant alleles. The dominant alleles code for allozymes which differ with respect to the sugar to be transferred. Thus the allozymes encoded by *glA* and *glR* transfer arabinose and rhamnose respectively (Besson *et al.*; Heinsbroek *et al.* [3, 4]), whereas those encoded by *fG* and *fX* transfer glucose and xylose (Brederode and Nigtevecht [5]; Nigtevecht, unpublished).

The 7-O-glycosylation is controlled by the *g* locus with glucose or xylose transferred. The transfer of xylose to isovitexin or its 2''-O-glycosides is accomplished by the allozymes encoded by *gX* and *gX'*, which differ in maximal reaction velocity (Brederode and Nigtevecht; Brederode *et al.* [6, 7]). Two alleles have been identified for the 7-O-glucosylation. The allozyme encoded by *gGm* transfers glucose to isovitexin but not to isovitexin 2''-O-rhamnoside, whereas the enzyme encoded by *gGd* preferentially recognizes the 2''-O-rhamnoside and not isovitexin (Steyns and Brederode [8]).

Plants homozygous for the recessive alleles of the loci *gl*, *f* and *g* accumulate isovitexin in their petals (Brederode and Nigtevecht [9]). Yet, in the cotyledons and the rosette leaves of these plants glycosides of isovitexin are present (Steyns *et al.* [10]). This is due to the expression of the genes *O7g* and *D6a*, which control the 7-O-galactosylation and the 2''-O-arabinosylation respectively (Steyns *et al.* [11]).

Thus the study of the 7-O- and 2''-O-glycosylation of isovitexin has revealed the existence of allozymes differing in substrate specificity for the sugar donor as well as the flavone acceptor, the existence of genes which are only expressed early in the ontogeny of the plant and the existence of differentially regulated genes encoding the same product (*glA*, *D6a*). The flavone glycosylation genes identified so far in *Silene* plants have been summarized in Fig. 1.

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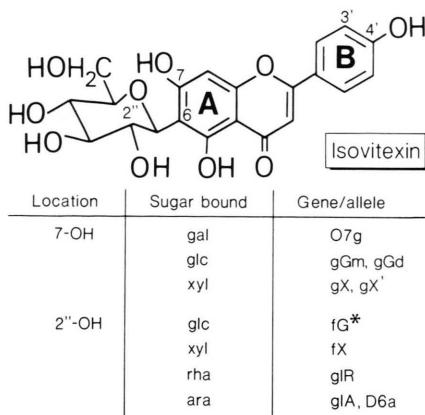


Fig. 1. Genetic control of isovitexin glycosylation. The denotation *fG* has replaced the denotation *Fg* described previously (Brederode and Nigtevecht [5]). This is due to the finding of a third allele, *fX*, in addition to *fG* and the recessive *f* (Nigtevecht, unpublished results).

In most of the flavone glycosylations isovitexin is the flavone acceptor. If at a particular stage of development several flavone glycosylation genes are expressed, this means that the enzymes encoded by these genes must compete for the same substrate. The relative accumulation of the flavone glycosides *in vivo* may be the result of the enzyme kinetic properties of the various glycosylation enzymes. This has been demonstrated for the dominancy of *gG* over *gX* in the petals of *gG/gX* heterozygotes (Brederode *et al.*; Brederode and Nigtevecht [7, 12]). In the vegetative parts competition for a particular enzyme substrate may be expected as well, *e.g.* we have demonstrated the presence of the 7-O-glycosylation enzymes encoded by *gG* and *O7g* or the 2''-O-glycosylation enzymes encoded by *fG* and *D6a* in cotyledons of *gG/gG* and *fG/fG* seedlings respectively (Steyns *et al.* [11]). The *in vivo* flavone glycoside patterns during ontogeny therefore not only are the result of allozyme specificity and differential gene regulation but probably also reflect the enzyme kinetic properties of the various glycosyltransferases.

In the present paper we have studied the expression of the genes *gG* and *gX* during ontogeny and their interrelation with the genes *O7g* and *D6a*, which are known to be only expressed in the cotyledons and the rosette leaves (Steyns *et al.* [11]).

## Experimental

### Plant material

Seedlings resulting from crosses between *gG/gX gG/gf f/f* and *g/g gG/gf f/f* individuals were grown in a climate chamber (daytemp. 24 °C; nighttemp. 15 °C; light-dark regime 16–8 h) at about 26000 lux (Metal halide lamps, Philips HPI/T 400 W). Cotyledons of 50 seedlings were harvested 14 days after sowing. Another thirty individuals were pricked out 14 days after sowing. The first and the second formed leaf pairs of 20 individuals were harvested 24 and 28 days after pricking out with the plantlets having developed 4–6 and 5–7 leaf pairs respectively. One half of each leaf pair was stored at -20 °C for later enzyme preparations, the other half being used for flavone extraction. Most plants flowered about 40 days after pricking off, having developed 8–9 leaf pairs.

### Flavone extraction and chromatography

Flavones were extracted with 70% aqueous methanol. Extracts were chromatographed on Schleicher and Schull paper (46 × 56 cm, 2043a) with the developing solvents BAW (n-butanol/acetic acid/water, 4:1:5, upper phase) or 1% HCl. In this way the 7-O-glucoside and the 7-O-xyloside of isovitexin can be discriminated. Discrimination between the 7-O-glucoside and the 7-O-galactoside is achieved by thin layer chromatography on a silica-60 layer with the developing solvent EPWM (ethyl-acetate/pyridine/water/methanol, 80:20:10:5) (Steyns *et al.* [11]).

### Enzyme preparation

Cotyledons, rosette leaves, stem leaves or petals were homogenized in a Potter-Elvehjem homogenizer at 0 °C in 50 mM Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.0, 20 mM 2-mercaptoethanol, 0.01% triton X-100, 5% soluble polyvinylpyrrolidone (*Mr* approx. 44000). The homogenate was centrifuged at 38000 × g for 20 minutes. The supernatant is the "crude homogenate", which can be used to test for the presence of various flavone-O-glycosyltransferase activities. The flavone-O-glycosyltransferases in the crude homogenate were precipitated with ammonium sulphate in two steps. The first step, resulting in 35% saturation, removes polyvinylpyrrolidone and most of the phenolic compounds upon centrifu-

gation (20 min,  $38000 \times g$ ). The supernatant is then brought to 60% saturation. The precipitate, obtained by centrifugation (20 min,  $38000 \times g$ ), is resolved in 50 mM  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  buffer, pH 7.0, 20 mM 2-mercaptoethanol and applied onto Sephadex G25, equilibrated and eluted with the same buffer, to desalt and remove residual phenolics and other low molecular weight material. The now "partly purified flavone-O-glycosyltransferases" are present in the void volume and can be stored at  $-20^\circ\text{C}$ .

#### Enzyme assays

The standard enzyme assay was performed in a final volume of 30  $\mu\text{l}$  at  $30^\circ\text{C}$  in a shaking water-bath. The flavone acceptor was dissolved in 2  $\mu\text{l}$  ethyleneglycol monomethylether (EGME). Unless indicated otherwise the flavone acceptor was used at saturating or nearly saturating concentrations. The  $^{14}\text{C}$ -labeled UDP-glycoside sugar donors, dissolved in 2  $\mu\text{l}$  2% *aq.* ethanol, were used at low specific activity, obtained by dilution with the unlabeled UDP-glycoside. The final concentrations in the reaction mixtures were, unless stated otherwise, 0.7 mM for UDP-xylose (1 Ci/mol) and between 0.5 and 2 mM for UDP-galactose (specific activities between 0.63 and 1 Ci/mol).

The enzyme reaction was stopped by addition of 10  $\mu\text{l}$  20% trichloroacetic acid in methanol. The denatured protein was pelleted. The supernatant was subjected to two-dimensional paper chromatography together with the appropriate flavone carrier(s) according to Steyns *et al.* [11]. The amount of radioactivity incorporated and the protein content of the enzyme preparation were determined as described (Steyns *et al.* [11]).

#### Determination of pH optimum

The "partly purified enzyme preparation" was diluted five times with  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  buffer of desired pH (final phosphate concentration 50 mM). Enzyme activity was monitored by the standard enzyme assay.

#### Heat-inactivation

Samples of the "partly purified enzyme preparation" were heated in a water-bath at  $45^\circ\text{C}$  in separate vessels for the indicated periods of time and then placed in ice. The residual enzyme activity, express-

ed as percentage of the  $T = 0$  minutes sample, was tested for by the standard enzyme assay during 120 min.

#### Immunological inactivation

Partly purified enzyme preparations were used to test the effect on enzyme activity of increasing amounts of anti-serum raised against the 7-O-glucosyltransferase encoded by gene *gG*. The immunoglobulins were coupled to protein A-sepharose. After washing, the enzymes in the petal extracts were allowed to bind to the immunoglobulins. The protein A-sepharose was then precipitated and the supernatant tested for the remaining enzyme activity, expressed as percentage of the activity scored with control serum, by the standard enzyme assay. This procedure will be described in detail elsewhere.

#### Molecular weight determination

Partly purified enzyme preparations were applied onto Sephadex G100 (40  $\times$  1.5 cm), equilibrated and eluted with 50 mM  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  buffer, pH 7.0, 20 mM 2-mercaptoethanol. Fractions collected were tested for enzyme activity by the standard enzyme assay. The G100 column was calibrated with ferritin, bovine serum albumin, chicken egg albumin, chymotrypsinogen A and cytochrome *c* (Boehringer calibration proteins).

## Results

#### i. Flavone-O-glycoside patterns during ontogeny

The *gG* and *gX* individuals used in the present study resulted from two crosses between heterozygous *gG/gX* and homozygous *g/g* individuals (footnotes \* and \*\*). The segregation between *gG/g* and *gX/g* individuals was 42/48 in the one and 26/21 in the other cross.

To investigate which flavones were present in the cotyledons, rosette and stem leaves of either *gG/g* or *gX/g* plants, methanolic extracts of these organs were screened by paper and thin layer chromatography (PC and TLC respectively). PC analysis of the cotyledons of 50 seedlings revealed that the flavone pattern was similar for all individuals: an isovitexin

\* The *gG* plants are of the *gGm* type.

\*\* The plants with genotype *gG/g*, *gX/g*, *gG/gX* or *g/g* are homozygous for the recessive alleles of the loci *gl* and *f*.

7-O-glycoside, co-chromatographing with isovitexin 7-O-glucoside (7G), and an isovitexin 7,2''-O-diglycoside. The presence of a sugar bound to the 2''-OH group of isovitexin can be explained by the expression of gene D6a and therefore the diglycoside is probably an isovitexin 7-O-glycoside 2''-O-arabinoside. The sugar bound to the 7-OH group cannot be xylose, however, because authentic isovitexin 7-O-xylose 2''-O-arabinoside did not co-migrate with the diglycoside present in the cotyledons. The genotype of about half of the tested individuals must be *gX/g*, however, and it appears therefore that in these individuals 7-O-xylosides are absent from the cotyledons.

In the rosette and stem leaves of *gG/g* plants 7G was the main flavone. In the first and the second formed rosette leaves small amounts of isovitexin 7-O-galactoside (7Gal) were present as well; in addition some plants accumulated minute amounts of the afore mentioned diglycoside. In the stem leaves B-ring modification had taken place: in addition to 7G the 7-O-glucosides of the 3'-OH/3'-OCH<sub>3</sub> substituted isovitexin derivatives isoorientin and isoscoparin accumulated.

The rosette and stem leaves of *gX/g* individuals did not accumulate isovitexin 7-O-xyloside (7X). Instead 7Gal was the main flavone present. In some plants minute amounts of the afore mentioned diglycoside were present in the first and second formed rosette leaves. B-ring modification also occurred in the stem leaves of the *gX/g* plants. However, isoorientin and isoscoparin were not 7-O-glycosylated.

From these results we may conclude that in the vegetative parts of *gX/g* plants 7-O-xylosides are absent, *i.e.* gene *gX* is not expressed at the product level.

#### *ii. Flavone O-glycosyltransferase activities during ontogeny*

The absence of the 7-O-xyloside in the vegetative parts of *gX/g* plants does not exclude that *gX* may be expressed at the enzyme level, *e.g.* it has previously been demonstrated that the amount of 7X accumulating in the petals of *gX/gG* individuals is low as compared to the amount of 7G as the consequence of differences in enzyme kinetic parameters (Brederode and Nigtevecht [12]).

The presence of isovitexin 7-O-xylosyltransferase activity was tested for in the cotyledons, rosette and

stem leaves, and petals of *gX/g* individuals using crude homogenates (see Experimental). The 7-O-xylosyltransferase activity could only be demonstrated in the petals. In the vegetative parts this enzyme activity was replaced by a 7-O-galactosyltransferase activity. These results correlate with the accumulation of 7X in the petals and of 7Gal in the vegetative parts. It was therefore surprising to note that the 7-O-galactosyltransferase activity was also present in the petals.

In the *gG/g* individuals 7-O-galactosyltransferase activity was restricted to those parts in which accumulation of 7Gal had been shown, *i.e.* this enzyme activity was absent beyond the second formed pair of rosette leaves. The 7-O-glucosyltransferase activity, however, was present throughout ontogeny.

We may thus conclude that in the vegetative parts of *gX/g* individuals neither isovitexin 7-O-xylosides nor the 7-O-xylosyltransferase activity are present. In the vegetative parts of these plants the 7-O-xylosylation of isovitexin has been replaced by the 7-O-galactosylation.

In *g/g* individuals the 7-O-galactosylation of isovitexin is restricted to the cotyledons and the rosette leaves (Steyns *et al.*; Brederode and Steyns [10, 13]). The transfer of galactose in these parts is controlled by locus *O7g* (Steyns *et al.* [11]). In individuals with a dominant 2''-O-glycosylation gene but recessive for the *g* locus the presence of isovitexin 7-O-galactose 2''-O-glycosides is restricted to these parts as well (unpublished results). The 7-O-galactosylation early in the ontogeny of *gG/g* individuals can also be explained by the expression of *O7g* (the diglycoside found is therefore probably isovitexin 7-O-galactose 2''-O-arabinoside). The presence of 7Gal in the stem leaves of *gX/g* individuals is therefore surprising. Even more surprising is the presence of 7-O-galactosyltransferase activity in the petals of these individuals. Three possible explanations are conceivable:

1) gene *gX* encodes an enzyme activity that is regulated differently in the petals and the vegetative parts, *i.e.*, depending on the metabolic environment either 7X or 7Gal is synthesized; the presence of the 7-O-galactosyltransferase activity, but not its product, in the petals of *gX/g* plants may support this suggestion

2) the presence of *gX* implies a different regulation of *O7g* resulting in its expression beyond the rosette leaf stage

3) the 7-O-galactosyltransferase activity present in the stem leaves and petals of *gX/g* plants is controlled by a locus other than *O7g*.

*iii. Is the 7-O-galactoside biosynthesis beyond the rosette leaf stage controlled by the **g** locus?*

To refute the first possibility one has to demonstrate that the biosynthesis of 7Gal and 7X is accomplished by different enzymes. The 7-O-galactosyl and the 7-O-xylosyltransferase activity present in partly purified petal homogenates were compared with respect to responses to heat-inactivation, responses to anti-serum raised against the 7-O-glucosyltransferase encoded by *gG* and incorporation kinetics. Fig. 2a shows the heat-inactivation at 45 °C. After 30 minutes the 7-O-xylosyltransferase activity has disappeared, whereas about 30% of the original activity of the 7-O-galactosyltransferase is left. Fig. 2b demonstrates that the 7-O-xylosyltransferase is inactivated by the anti-serum raised against the *gG* encoded 7-O-glucosyltransferase, whereas the 7-O-galactosyltransferase is not. Finally Fig. 2c shows that both enzyme activities also differ with respect to incorporation kinetics. From these results we may conclude that the biosynthesis of 7Gal and 7X in *gX/g* individuals is accomplished by two different enzymes, *i.e.* the 7Gal synthesis in these plants is not controlled by the *g* locus.

*iv. Is the 7-O-galactoside biosynthesis beyond the rosette leaf stage controlled by the **O7g** locus?*

The second and the third possibility mentioned to explain the presence of 7Gal and the enzyme catalyzing its biosynthesis beyond the rosette leaf stage in *gX/g* plants can be tested by comparing the biochemical properties of the 7-O-galactosyltransferase activities present in the cotyledons of *g/g* plants (*O7g* enzyme) and in *gX/g* petals. These enzyme activities will have the same properties if the presence of *gX* results in an altered regulation of the expression of *O7g* and will differ if encoded by different loci.

The 7-O-galactosyltransferase activities were partly purified by ammonium sulphate precipitation and Sephadex G25 gel chromatography. The responses to anti-serum against the *gG* glucosyltransferase, the apparent molecular weight, the responses to heat-inactivation at 45 °C, pH optimum, flavone acceptor specificity and Michaelis-Menten enzyme kinetic parameters were determined.

Both enzyme activities were not inhibited by the glucosyltransferase anti-serum (see also Fig. 2b).

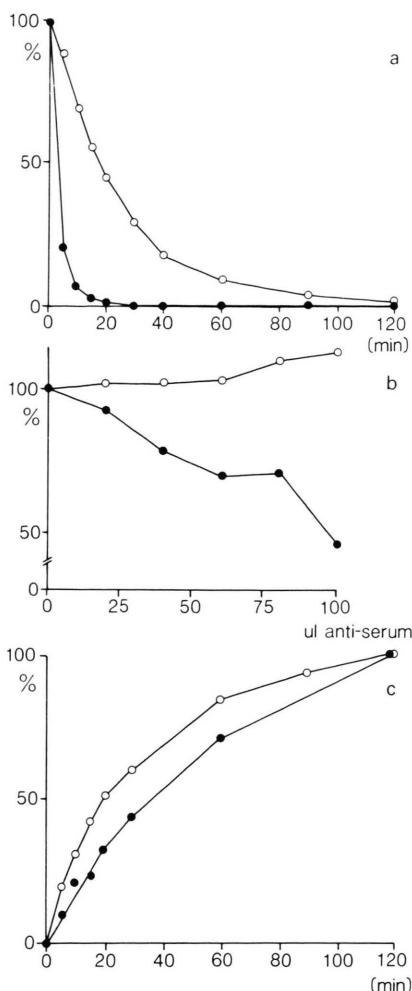


Fig. 2. Heat-inactivation (a), immunological inactivation (b) and incorporation kinetics (c) of the 7-O-xylosyl- and 7-O-galactosyltransferase activities from *gX/g* petals. The partly purified petal enzyme preparation was incubated with isovitexin and UDP-[<sup>14</sup>C]xylose to monitor the 7-O-xylosyltransferase activity (●) and with isovitexin 2'-O-arabinoside and UDP-[<sup>14</sup>C]galactose to monitor the 7-O-galactosyltransferase activity (○).

(a) Heat-inactivation at 45 °C. The residual enzyme activity is expressed as percentage of the activity at  $T = 0$  min, amounting to 535 cpm for the xylosyl- and 1330 cpm for the galactosyltransferase.

(b) The activities at  $T = 0$  min were 330 cpm for the xylosyl- and 4490 cpm for the galactosyltransferase.

(c) The incorporation is expressed as percentage of the incorporation at 120 min, amounting to 1035 cpm for the xylosyl- and 680 cpm for the galactosyltransferase activity.

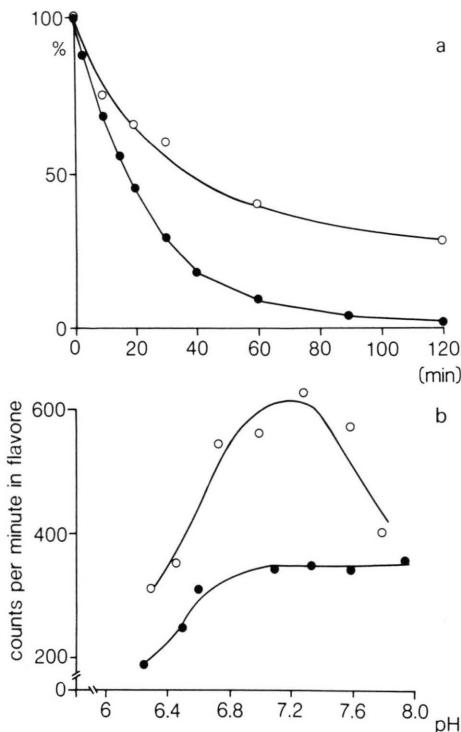


Fig. 3. Heat-inactivation (a) and pH dependence (b) of the 7-O-galactosyltransferase activities from *g/g* cotyledons and *gX/g* petals. The 7-O-galactosyltransferase activities from *g/g* cotyledons (○) or *gX/g* petals (●) were tested for by the standard enzyme assay with isovitexin 2''-O-arabinoside as the flavone acceptor.

(a) Heat-inactivation at 45 °C. At  $T = 0$  min the enzyme activities were 1400 cpm for the cotyledon and 1330 cpm for the petal preparation; the protein contents at  $T = 0$  min were 0.34 and 1.3 mg/ml respectively.

(b) pH dependence in phosphate buffer.

The apparent molecular weight was estimated by Sephadex G100 gel chromatography. Both enzyme

activities eluted at the same volume, corresponding to an apparent molecular weight of about 40000 Dalton. Under similar conditions the *gG* controlled glucosyltransferase eluted at a volume corresponding to about 45000 Dalton.

The heat-inactivation curves at 45 °C are shown in Fig. 3a. Half-maximal activity is attained after 18 minutes for the petal enzyme preparation and after 40 minutes for the cotyledon preparation. The pH curves for the two enzyme activities in phosphate buffer also differed (Fig. 3b).

The *O7g* controlled 7-O-galactosyltransferase activity preferentially transfers galactose to the 2''-O-glycosides of isovitexin rather than to isovitexin itself (Steyns *et al.* [11]). This is also true for the 7-O-galactosyltransferase activity present in the *gX/g* petals as shown in Table I. This table reveals, however, slight differences in acceptor specificities with re-

Table I. Flavone acceptor specificities of the 7-O-galactosyltransferase activities present in petals of *gX/g* plants and cotyledons of *g/g* plants respectively.

Flavone acceptor	Relative enzyme activity <i>gX/g</i> petals	Relative enzyme activity <i>g/g</i> cotyledons
Isovitexin	13	20
6R	56	57
6G	100	91
6A	76	100
6X	43	64

Enzyme activities were tested for by the standard enzyme assay during 15 minutes with saturating concentrations of the flavone acceptor and a fixed amount of UDP-[<sup>14</sup>C]galactose. The isovitexin 2''-O-glycosides 6R, 6G, 6A and 6X stand for the rhamnoside, glucoside, arabinoside and xyloside respectively. On prolonged incubations (30 and 60 minutes respectively) the relative enzyme activity did not change.

Enzyme source	Flavone acceptor	Product formed	True $K_m$ flavone [mM]	True $K_m$ sugar donor [mM]	True $V_{max}$ [pkat/mg protein]
<i>g/g</i> cotyledons	isovitexin	7Gal	0.025	8.2	1.8**
<i>g/g</i> cotyledons	6A	7Gal6A	0.015	0.56	9.3
<i>gX/g</i> petals	6G	7Gal6G	0.46	11.0	65.3
<i>gG/g</i> petals	isovitexin	7G	0.043	1.15	41.8

The sugar donor for the 7-O-galactosyltransferases is UDP-galactose, whereas UDP-glucose is the donor for the 7-O-glucosyltransferase. The isovitexin O-glycosides 6A, 6G, 7Gal and 7G are the 2''-O-arabinoside, 2''-O-glucoside, 7-O-galactoside and 7-O-glucoside respectively. 7Gal6A and 7Gal6G are the corresponding diglycosides.

Table II. Michaelis-Menten enzyme kinetic parameters for the 7-O-galactosyltransferases present in *g/g* cotyledons and *gX/g* petals, and the 7-O-glucosyltransferase present in *gG/g* petals.

\*\* Data derived from Brederode and Steyns [13].

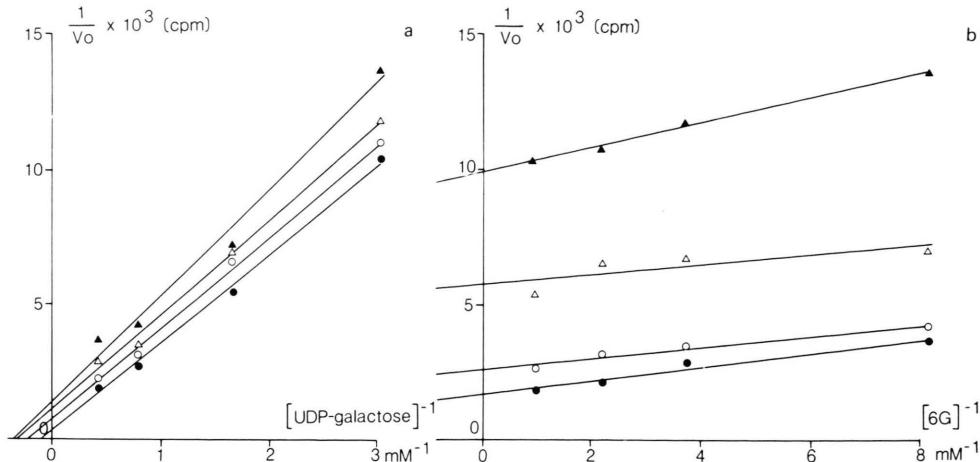


Fig. 4. Lineweaver-Burk plots of the reciprocal of the initial reaction velocity versus the reciprocal of the mmolar concentration of UDP-galactose (a) or isovitexin 2''-O-glucoside (6G) (b). The fixed and the variable concentrations of UDP-galactose and 6G are same in (a) and (b). The UDP-galactose (0.36 Ci/mol) concentrations in the reaction mixture were 0.33 (▲), 0.61 (△), 1.25 (○) and 2.36 (●) mM respectively. The 6G concentrations were 0.12 (▲), 0.27 (△), 0.45 (○) and 1.04 (●) mM respectively.

The reaction velocity was determined at 30 °C in a final volume of 30 µl during 20 minutes. The reaction mixture contained 25 µl enzyme preparation (partly purified from *gX/g* petals), 3 µl UDP-[<sup>14</sup>C]galactose and 2 µl 6G (in EGME). The pH of the reaction mixture was 7.1 (42 mM phosphate, 16.7 mM 2-mercaptoethanol); the protein content was 72.5 µg.

spect to the 2''-O-glucoside (6G), 2''-O-arabinoside (6A) and 2''-O-xyloside (6X) of isovitexin respectively.

The Michaelis-Menten enzyme kinetic parameters were determined with 6A as the flavone acceptor for the *O7g* enzyme and 6G as the acceptor for the enzyme from *gX/g* petals to obtain maximal activity. At fixed amounts of the one substrate the initial reaction velocity depended on the concentration of the other substrate. This is shown in Fig. 4 for the enzyme from *gX/g* petals. The "true  $V_{max}$ " and the "true  $K_m$ " values for the flavone acceptor and the sugar donor were obtained by the method of Florini and Vestling [14]. This is shown in Fig. 5 for the enzyme from *gX/g* petals. The "true  $K_m$ " values for the flavone acceptor and the sugar donor as well as the "true  $V_{max}$ " differ markedly for the 7-O-galactosyltransferases present in the cotyledons of *g/g* and the petals of *gX/g* individuals, the ratios being 30, 20 and 0.14 respectively (Table II).

From the differences in response to heat inactivation, pH optimum, flavone acceptor specificity and Michaelis enzyme kinetic parameters we may conclude that the 7-O-galactosyltransferase present in the cotyledons of *g/g* individuals is different from the

7-O-galactosyltransferase present in the petals of *gX/g* plants.

The binding of galactose to the 7-OH group of isovitexin or an isovitexin 2''-O-glycoside is therefore controlled by two genes that are differentially regulated. Gene *O7g* is expressed in the cotyledons

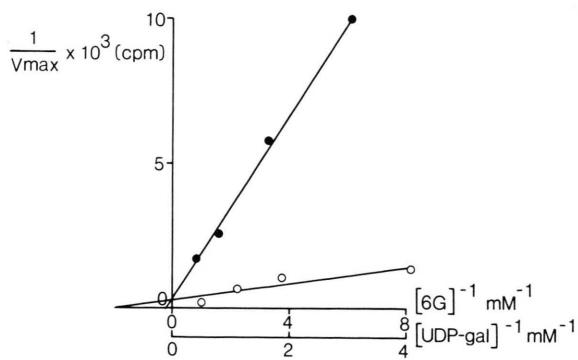


Fig. 5. Secondary plots of the reciprocal of the initial reaction velocity at infinite concentration of one of the two enzyme substrates (obtained by extrapolation to  $1/s = 0$  in Fig. 4a/b) against the reciprocal of the isovitexin 2''-O-glucoside (6G) concentration (○) or the reciprocal of the UDP-galactose concentration (●).

and the rosette leaves (Steyns *et al.* [10, 11]), whereas gene *Xgal* controls the 7-O-galactosylation in the stem leaves and the petals.

## Discussion

The study of the 7-O- and 2''-O-glycosylation of the flavone isovitexin in *Silene* plants has revealed some interesting features with regard to the genetic regulation and the biosynthesis of flavones. First, genetical and biochemical investigations on the biosynthesis of the flavone glycosides present in the petals have shown the existence of alleles encoding allozymes differing in substrate specificity, not only for the sugar to be transferred but also for the flavone acceptor to which a specific sugar is transferred (Fig. 1). Second, two loci (*O7g*, *D6a*) have been identified, which are only expressed in early ontogenetic stages (Steyns *et al.* [11]). Third, the biosynthesis of a particular flavone glycoside can be controlled by different genes which are differentially regulated. The latter has been shown for the biosynthesis of isovitexin 2''-O-arabinoside. Gene *D6a* directs the biosynthesis of this compound in the cotyledons and the rosette leaves, whereas gene *gla* controls its synthesis in stem leaves and petals. In the present paper it is shown that an analogous situation exists for the 7-O-galactosylation of isovitexin. Gene *O7g*, which has been described previously (Steyns *et al.* [11]), is only expressed in the cotyledons and the rosette leaves. This gene was identified in plants, which are unable to glycosylate isovitexin in the petals due to the absence of dominant 7-OH and 2''-OH glycosylation genes (genotype *g/g gl/gl f/f*). Gene *Xgal* is expressed in the stem leaves and the petals. We cannot be certain whether this gene is also expressed in the cotyledons and the rosette leaves as gene *O7g* seems to be always present in these organs and the biochemical properties of the enzymes encoded by *Xgal* and *O7g* do not allow implicit discrimination in a mixture. Gene *Xgal* was identified in *gX/g* individuals, which synthesize isovitexin 7-O-xyloside in their petals. The 7-O-xyloside and the enzyme catalyzing its biosynthesis could only be demonstrated in the petals of these individuals. On the other hand, in *gG/g* individuals the 7-O-glucoside and the enzyme catalyzing its biosynthesis could be demonstrated in the cotyledons, rosette leaves, stem leaves and the petals. The possibility that gene *gX* encodes an enzyme

catalyzing two different reactions during ontogeny, *i.e.* the biosynthesis of the 7-O-xyloside in the petals and that of the 7-O-galactoside in the vegetative parts, is refuted in this paper. The alleles *gG* and *gX* therefore seem to be regulated quite differently during ontogeny.

Until now 7-O-galactosylation of isovitexin or its 2''-O-glycosides beyond the rosette leaf stage has only been demonstrated in individuals harboring gene *gX*. This suggests that the genes *Xgal* and *gX* are linked. Alternatively, it is conceivable that *Xgal* and *gX* are not linked, but that the *gX* chromosome carries another gene controlling the expression of *Xgal*.

As the *Xgal* enzyme activity is only present in *gX/g* plants the presence of 7Gal in the rosette leaves of *gG/g* individuals is the result of the activity of *O7g*. In these leaves *gG* is expressed as well. The amount of 7G produced exceeds that of 7Gal. This may be explained by differences in maximal reaction velocities of the respective enzymes (Table II), if we assume that *in vivo* both enzymes are saturated with isovitexin.

In the petals of *gX/g* individuals both *gX* and *Xgal* are expressed at the enzyme level, yet 7Gal does not accumulate. This cannot be explained, however, by differences in maximal reaction velocities of the respective enzymes, which both amount to about 10 pkat/mg protein. The affinity ( $K_m$ ) of both enzymes for the common substrate isovitexin, however, differs by at least a factor 12. The  $K_m$  for the *gX* enzyme is very low ( $\ll 0.04$  mm: ref. [6]), whereas that for the *Xgal* enzyme is estimated to be at least 0.5 mm (by analogy with the  $K_m$  values determined for the *O7g* enzyme: refer to Table II). This may indicate that *in vivo* the available isovitexin is predominantly captured by the *gX* enzyme leaving the *Xgal* enzyme unsaturated. At comparable maximal reaction velocities of both enzymes this may result in a very low amount of 7Gal produced, an amount too low to be detected by paper chromatography. In addition it is also conceivable that 7X inhibits the *Xgal* enzyme activity. We did not test this possibility, however.

The close resemblance in biochemical properties of the 7-O-galactosyltransferases encoded by *O7g* and *Xgal* may indicate that these genes represent duplicated genes. The altered regulation of these genes may have arisen from either the duplication event or the accumulation of mutations.

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