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 gg, 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 gIR, gIA and the recessive gl) is involved in the glycosylation of the hydroxyl group at position 2’ of the 6-C-bound glucose. The alleles gIR and gIA 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]).

The genes gG, gIR 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 accu-
mulation 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 glyco-
sylate 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 1 6 -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 redis-
solving 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 com-
bination 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 desic-
cator above silica gel for at least one hour.

Results

i. Identification of flavone glycosides in foliage leaves

The development of the flavone pattern in seed-
lings 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, where-
as 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/butanol/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].

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<th>PC: Rf × 10²</th>
<th>BAW</th>
<th>1% HCl</th>
<th>15% HOAc</th>
<th>Acid Hydrolysis</th>
<th>aglycone(s)</th>
<th>sugar(s)</th>
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<tr>
<td>A. 88D-glycosides: Rf values and products of acid hydrolysis</td>
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<td>IV-7-O-gluc &amp; 32 &amp; 29 &amp; 60 &amp; IV + V &amp; gal + ara</td>
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<td>B. Spectra of 88D-glycosides</td>
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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 out 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 DR. 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 ng/mg dry weight, 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 asterisk 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 and 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
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*, *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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