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Serological and genetical studies on the evolution of substrate specificity of flavone glycosyltransferase genes in *Silene*

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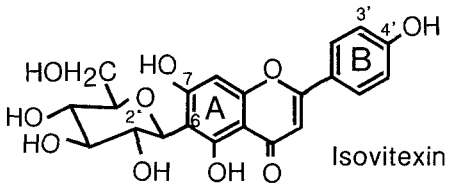
Summary. The variation in flavone glycosylation patterns in *Silene* is the result of the expression of six genetic loci, which control either the presence of allozymes differing in substrate specificity or isozymes regulated differently during development. Serological studies showed that at least three of these six loci are evolutionarily related. The genetic mechanisms leading to these complicated variation patterns and the role of this polymorphism for the plant in its interaction with the environment are discussed.
Key words. Evolution; substrate specificity; serological homologies; flavone biosynthesis; *Silene;* glycosyltransferases.

The flavones present in *Silene pratensis* and *S. dioica,* the white and red campion respectively, belong to the class of the C-glycosylflavones. The main flavone skeleton found is isovitexin (fig. 1). The 7-OH of the A ring and the 2″-OH of the C–C bound glucose may be glycosylated[1].

Until now 11 dominant genes, spread over 6 loci, which control the glycosylation of isovitexin have been identified in *Silene*. The 2″-O-glycosylation is controlled by the loci gl, f and D6a; the 7-O-glycosylation by the loci g, 07g and Xgal. Three alleles have been identified both for the locus gl (gl, glA, glR) and f (f, fG, fX). The allozymes encoded by glA and glR transfer arabinose and rhamnose respectively[2,3], whereas those encoded by fG and fX transfer glucose and xylose[4]. Five alleles have been identified for the g locus (g, gX, gX′, gGm and gGd). The transfer of xylose to the 7-OH group of isovitexin or its 2″-O-glycosides is accomplished by the allozymes encoded by gX and gX′, which differ in maximal reaction velocity[5,6]. 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[7]. The loci gl, f and g segregate independently. Plants homozygous for the recessive alleles of these loci (genotype g/g, gl/gl, f/f) are unable to glycosylate isovitexin in the petals[8]. Yet, in the cotyledons and the rosette leaves of these plants glycosides of isovitexin are present[9]. This is due to the expression of the genes 07g and D6a, which control the 7-O-galactosylation and the 2″-O-arabinosylation respectively[10]. Biosynthesis of isovitexin 7-O-galactoside beyond the rosette leaf stage, controlled by Xgal, was found to be dependent on the presence of gene gX[11]. Hitherto no recessives or other alleles have been identified for the loci D6a, 07g and Xgal. The dominant flavone glycosylation genes identified until now in *Silene* plants have been summarized in figure 1.

So far, 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 for the flavone acceptor, and the existence of differently regulated genes encoding the same product (glA/D6a and Xgal/07g). Variation in expression of isovitexin glycosylation genes is found not only within the plant, but also between geographical races and between the various *Silene* species within section Elisanthe[12]. The complicated pattern of variation in the expression of isovitexin glycosylation genes suggests an adaptive component in this variation, which can easily be tailored by the plant. This leaves us with the question of how this is accomplished by the plant, i.e. what are the evolutionary relationships between these various alleles and loci.



Location	Sugar bound	Gene/allele
7-OH	gal	O7g, Xgal
	glc	gGm, gGd
	xyl	gX, gX'
2"-OH	glc	fG
	xyl	fX
	rha	glR
	ara	glA, D6a

Figure 1. Genetics of isovitexin glycosylation in *Silene*.

Materials and methods. 1. *Purification of gGm controlled isovitexin 7-O-glucosyltransferase.* 20 g petals were homogenized with an Ultra Turrax homogenizer in 60 ml 20 mM 2-mercaptoethanol, 5% polyvinylpyrrolidone w/v (MW 40,000), 0.01% Triton X-100, 50 mM Pi* buffer pH 7.0. The homogenate was pressed through 4 layers of cheesecloth and centrifuged for 15 min at 38,000× g. Proteins were precipitated by ammonium sulphate in 2 steps (0–35%; 35–60%). The second ammonium sulphate precipitate was washed twice with and resuspended in 5 mM 2-mercaptoethanol, 25 mM Pi buffer pH 7.0, and applied onto a 2.5 × 60 cm Sephacryl S-200 (superfine) column preequilibrated with the same buffer. Fractions containing isovitexin 7-O-glucosyltransferase activity were pooled and applied to a DEAE Sephadex column (1.0 × 10 cm) equilibrated with 25 mM Pi buffer. After two column volumes elution with this buffer a linear KCl gradient (0–150 mM) was applied. The fractions with glucosyltransferase activity eluted at about 125 mM KCl. These fractions were desalted over Sephadex G-25 equilibrated with 25 mM Pi buffer pH 7.0 and then used for the induction of antiserum.

2. *Preparation of antisera.* An equal volume of the partly purified denaturated glucosyltransferase fraction (in the case of the antiserum against the recessive an analogous purified protein fraction isolated from plants homozygously recessive for the flavone glycosylation loci g, gl and f) was mixed with complete or incomplete Freund's adjuvant; 1 mg of protein was injected s.c. into rabbits. Boosters were given every fortnight for a period of 9 months, after which the rabbits were bled and the sera prepared by centrifugation after coagulation at room temperature.

3. *Enzyme inhibition tests.* 100 µl protein A-Sepharose (Pharmacia, Sweden) was suspended in 100 µl 1:10 diluted PBS-buffer (= 80 g NaCl, 2 g KCl, 11.2 g K2HPO4, 1.5 g NaH2PO4 dissolved in 1 l, pH 7.4) and added to varying amounts of antiserum, supplemented to 100 µl with either normal rabbit serum, or with serum raised against the protein fraction of homozygous recessive plants (the ratios 1:0, 4:1, 3:2, 1:1, 2:3, 1:4 and 0:1, were used). After addition of 3 µl Triton X-100, the mixture was incubated by shaking at room temperature for 60 min. The protein-A Sepharose, with bound antibodies, was pelleted and washed three times with equal volumes of 10-fold diluted PBS-buffer. The washed protein-A-antibody complex was then incubated for 60 min at room temperature with partly purified enzyme preparations[10] from plants differing in flavonoid glycosylation genotype. After precipitation of the protein-A, antibody, antigen complex, the remaining glycosyltransferase activity in the supernatant was tested as described before[10].

Results and discussion. a) Serological cross-reactivity. To demonstrate relationships between the various genes, we raised antiserum against a specific glycosyltransferase, the 7-O-glucosyltransferase controlled by gGm (partly purified from petals of plants with genotype gGm/., gl/gl, f/f; in these petals the other glycosyltransferases are not demonstrable), and tested the cross reactivity of this antiserum with the glycosyltransferases controlled by the various other genes.

The fact that the enzyme activity to which antibodies were elicited was only partially purified restricts the type of immunoassay with which cross-reactivity with other enzyme activities can be tested, to monitoring the inhibition of enzyme activity as the result of antigen-antibody interaction. Techniques such as immunodiffusion or immunoelectrophoresis rely on monospecific antisera and therefore cannot be applied. Enzyme inhibition methods assume that the binding between the antibody and the enzyme results in inactivation and that it is unlikely that antibodies directed against contaminating proteins affect this enzyme-antibody interaction. That this is a valid assumption has been shown by Ohnishi[13], who obtained essentially the same results when determining evolutionary relations between various species with antisera raised against pure or impure immunogens. On the other hand, structural homology between various proteins may be overlooked if antigenic determinants to which antibodies were produced are not involved with active sites. This problem

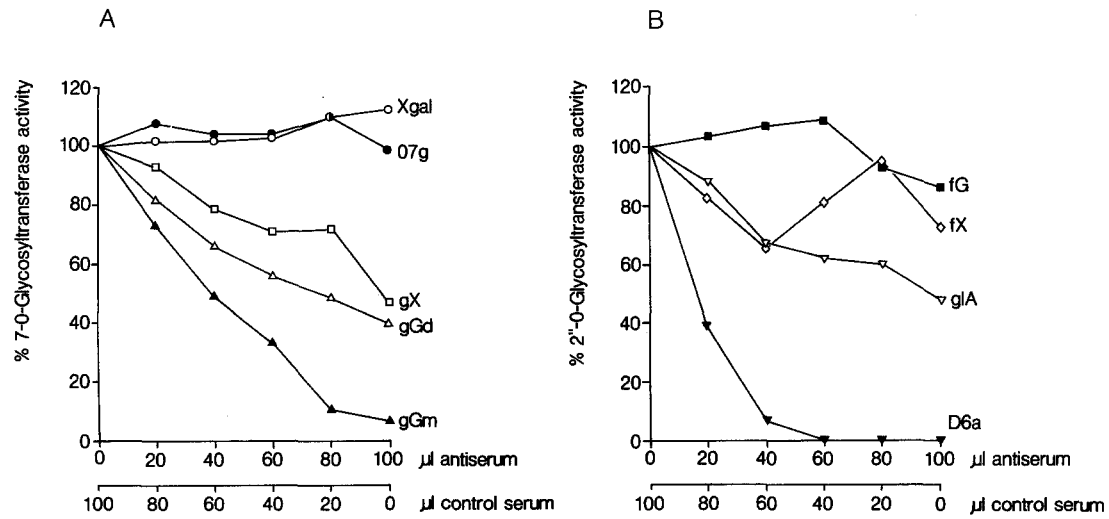


Figure 2. Cross reactivity of antibody raised against protein controlled by gGm, with proteins controlled by other genes involved with isovitexin glycosylation. *A* Proteins involved in 7-O-glycosylation; *B* 2"-O-glycosylation.

may be circumvented by precipitating the enzyme-antibody complexes.

Cross-reactivity between the various isovitexin glycosylation proteins was tested by such an adapted inhibition method. Antiserum was first incubated with protein A (coupled to Sepharose), which binds the Fc portion of a variety of mammalian immunoglobulins. The protein A-antibody complexes were then precipitated, washed and incubated with enzyme preparations from plants with a known glycosylation genotype. Cross-reactivity was indicated by diminished enzyme activity in the supernatant after precipitation of the protein A-antibody-antigen complexes.

The results obtained with the 7-O-glycosyltransferases encoded by the genes gGd, gX, Xgal and 07g are shown in figure 2A. The antiserum directed against the gGm controlled isovitexion 7-O-glucosyltransferase decreases the activity of the isovitexin 2″-O-rhamnoside 7-O-glucosyltransferase and isovitexin 7-O-xylosyltransferase respectively. This result is not unexpected as the genes controlling these enzyme activities are allelic to gGm. The 7-O-galactosyltransferase activities, controlled by the genes 07g and Xgal, do not cross-react. This is remarkable in view of the close resemblance in substrate specificity of these enzymes (showing preference for 2″-O-glycosides) with the gGd enzyme, the difference being the transfer of galactose instead of its stereoisomer glucose.

The cross-reactivity tests with the 2″-O-glycosyltransferases are shown in figure 2B. The isovitexin 2″-O-glucosyltransferase, encoded by gene fG, is only slightly inhibited at high antiserum concentrations, whereas the results obtained with the isovitexin 2″-O-xylosyltransferase, encoded by the allelic variant fX, are somewhat ambiguous but also tend to show a slight homology. The isovitexin 2″-O-arabinosyl-transferase activities, encoded by gene glA or gene D6a, clearly are inhibited by the antiserum. The D6a controlled enzyme activity seems to be always present in all genotypes[19]; the enzyme is, however, not demonstrable beyond the rosette leaf stage[20]. It could be argued that an inactivated D6a protein is present in stem leaves and petals and as a consequence antibodies against this inactivated protein are present, which would explain the inactivation. However, this argument must then be applied also to the 07g controlled enzyme activity, yet this enzyme is not inactivated. It appears therefore that the inactivation of the D6a controlled enzyme is the result of the presence of epitopes common to the protein to which the antiserum was raised and not to the presence of an inactivated protein. The stronger binding with the D6a protein may be explained by a relatively low concentration of the protein. Alternatively, it is possible that on this protein homologous epitopes are more exposed than on the gGm controlled protein. A possible explanation for the latter phenomenon may be that the antiserum was raised against a denaturated protein fraction, resulting in the exposure of epitopes which may be more hidden in the native protein.

The glR controlled isovitexin 2″-O-rhamnosyltransferase activity can only be tested indirectly (the substrate UDP-rhamnose is not commercially available) by making use of the NADPH dependent conversion of UDP-glucose into UDP-rhamnose[9] which is demonstrable in the crude extract. The UDP-rhamnose is then further used as a substrate for the glR controlled incorporation into isovitexin 2″-O-rhamnoside. This cumbersome test system results in a rather poor incorporation into the product, which made it impossible to draw reliable conclusions with regard to the inhibition caused by the antiserum.

From the above it can be concluded that the loci g, gl and D6a control isovitexin glycosylation enzymes that share serologically recognizable epitopes, i.e. the enzymes have common structural elements. Given this homology between 7-O-glucosyl, 7-O-xylosyl- and 2″-O-arabinosyltransferase genes, it is hard to imagine that the other genes, controlling 2″-O-glucosyl-, 2″-O-xylosyl- or 7-O-galactosyltransferases, evolved independently. Probability calculations show that such convergent evolution of proteins with approximately the same function is a highly unlikely event[16]. Instead it is more probable that the failure to detect cross-reactivity is due to the insensitivity of the method applied. Examples of the relative insensitivity of immune inhibition or precipitation test are given by investigations on rat hexokinase isoenzymes[17,18] and rye triosephosphate isomerase isoenzymes[19]. It is also conceivable that the antibodies were directed against epitopes which were lost by the non-cross-reacting proteins during evolution. As the antibodies were apparently not directed against the sugar donor or acceptor sites the difference in estimated molecular weights between the 7-O-glucosyl- and 7-O-galactosyltransferases respectively (45,000 versus 40,000 daltons[11]) may support the latter suggestion. The absence of cross-reactivity of some of the glycosyltransferases does not exclude a priori that these enzymes are not related to the other gylocosyltransferases between which serological relations are demonstrated. Further and more detailed studies with more sensitive techniques are required, e.g. the use of cDNA of a specific mRNA which can be used as a probe for detecting homology at the DNA level between the various loci. Attempts in this direction are being pursued at present.

b) *Genetic mechanisms leading to variation in isovitexin glycosylation genes.* At present one can only speculate on the genetic mechanisms which have led to the variation in glycosylation genes. The allelic variants of the g locus can easily be explained by the accumulation of mutations in duplicated (possibly silenced) genes, or by gene conversion.

The serological interaction between the unlinked loci g, gl and D6a (which comes to expression in g/g, gl/gl as well as gG/gG, gl/gl and g/g glR/glR individuals) requires the introduction of some form of translocation with a concomitant change in regulation. This may have occurred for the genes D6a and glA, both controlling the presence of isovitexin 2″-O-arabinoside, with glA expressed throughout ontogeny and D6a only in the cotyledons and the rosette leaves. An analogous situation may exist for the differentially regulated isovitexin 7-O-galactosylation genes Xgal and 07g.

c) *Role of polymorphism.* Throughout evolution the development of proteins with new functions must have played an important role. On the other hand, the development of a functional protein is an unlikely event. Once a successful enzyme is found, it is far more efficient to modify it than to seek an entirely new solution. Contrary to the rather static environment in the cells of higher organisms, which appears to preclude strong changes in the interaction within and between primary biochemical pathways, the pathways acting at the interface with the environment are more prone to selection. Especially in higher plants the latter pathways are of importance. Plants are restricted to a single place and are – apart from morphological adaptations – more dependent upon the synthesis of chemicals, i.e. the so-called secondary compounds, for the interaction with the environment. It is therefore not surprising that countless variations in secondary compounds occur in higher plants[20].

Roth[21] argues that glycosyltransferases may be the evolutionary antecedents of the immunoglobulins, a class of proteins involved in the chemical discrimination between self and non-self in mammals. The variation in glycosyltransferase genes found in *Silene* may support the assumption of the general role of glycosyltransferases being involved in chemical defence mechanisms. In higher plants, however, the evolution of these mechanisms has resulted in less variable and efficient mechanisms when compared to those in mammals. Contrary to that in mammals, in which each individual has to build up its own defensive complex and is unable to transmit it to the next generation, the defence mechanisms in higher plants are heritable. It is of profit to the offspring, which tend to grow close to the stand of the parents, to possess the genetical potency which enabled the parents to survive.

The elucidation of the mechanisms leading to this variation may provide insight into the mechanisms plants use to cope with a

changing environment and may thus be of importance for plant improvement.

*Abbreviations: Pi = Na2HP04/KH2P04.

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Acetaldehyde induces mature endoreduplicated *Allium cepa* root cells to divide

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Summary. In *Allium cepa* root tips treated with acetaldehyde, metaphase cells showing diplochromosomes are occasionally observed. The short treatment time excludes the possibility that the endoreduplication has been induced by the chemical. Instead, it seems that acetaldehyde is able to stimulate, directly or indirectly, the division of mature cells previously endoreduplicated.
Key words. Acetaldehyde; endopolyploid cells; diplochromosomes.

In most higher plants, both monocotyledons and dicotyledons, the cells of mature root tissues, in their process of development, undergo chromosome doubling by endomitotic reduplication without subsequent mitosis[1-3]. This chromosome doubling in differentiated tissues was first reported after stimulation of these mature cells to divide by wounding[4] or by treatment with plant hormones, namely auxins[5] or kinetin[6]. In all these cases, it has been well established that the endomitotic reduplication had occurred prior to these treatments rather than being induced by the experimental procedure itself.

On the other hand, cells showing diplochromosomes, i.e., chromosomes made up of four chromatids, are frequently observed among the polyploid mitoses which are characteristic of the nodular tissues of leguminosae after inoculation with *Rhizobium leguminosarum*[7]. Nevertheless, in spite of the extensive studies carried out, the internal molecular mechanisms which control these cells, preventing them from dividing under normal conditions, are poorly understood to date.

Acetaldehyde, the first metabolite of ethanol, has been reported to be clastogenic (able to break chromosomes) and to induce sister chromatid exchange[8]. In a study performed by us in order to evaluate the effectiveness of this chemical with regard to its cytotoxicity and genotoxicity in *Allium cepa* root tip cells[9], metaphase cells showing diplochromosomes were occasionally observed. In this plant material, Hervas[10] has reported the mitotic activity of endopolyploid cells after a pulse treatment with excess thymidine.

The aim of the present study was to try to determine, for a short-term experimental schedule, the effectiveness of both acetaldehyde and thymidine in inducing endoreduplicated cells to divide. The short treatment time in acetaldehyde was chosen because of the high toxicity of this chemical.

Materials and methods. The material consisted of root meristems of *A. cepa*. The onion bulbs, 15–30 g in weight, were grown in the dark at constant temperature (20 °C) with tap water renewed every 24 h. All the treatments began when the roots were 15–20 mm in length.

Roots, still attached to the bulbs, were treated with acetaldehyde (Merck) dissolved in distilled water at different concentrations (0.05; 0.1 and 0.2%) for 2 h or with a solution of thymidine (Fluka) in tap water at a concentration of 5 mg/ml for 10 min followed by either a treatment with 0.05% colchicine in distilled water for 2 h, or with 0.05% acetaldehyde for the same period. After treatments, root tips were fixed in mixture of methanol: acetic acid (3:1) at 5 °C overnight and prepared as Feulgen squashes.

Results and discussion. No metaphase cells showing diplochromosomes were observed after a 2-h-treatment with 0.05% colchicine. Both for 0.05 and 0.1% acetaldehyde, a strong C-

Effectiveness of short-term treatments with acetaldehyde and thymidine in the induction of endopolyploid mitosis in *A. cepa* root tips

Agent	Dose	Post-treatment	Normal mitosis per meristem (mean ± SEM)[a]	Endopolyploid mitosis (mean ± SEM)
Colchicine	0.05%	–	1.157.6 ± 12.2	0
Acetaldehyde	0.05%	–	356.0 ± 6.7	0
	0.1%	–	446.0 ± 7.5	0.3 ± 0.2
	0.2%	–	n.m.	n.m.
Thymidine	5 mg/ml	0.05% Colch.	934.3 ± 10.9	0
	5 mg/ml	0.05% Acetald.	526.2 ± 8.2	0.06 ± 0.1

[a] 30 root tips, 6 from each of 5 bulbs were analyzed in all cases. n.m., no mitosis.