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CHANGES IN GLYCOSPHINGOLIPIDS DURING POLLINATION IN *OENOTHERA MISSOURIENSIS*

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Abstract—The results of an analysis of the glycosphingolipids in the pollen and in the style of *Oenothera missouriensis* show that these membranous lipids are comparatively less important in the styles than in the pollen. The pollen is especially rich in monoglycosylceramides and also in acid glycosphingolipids. Compatible pollination is followed by a large increase in monoglycosylceramides whereas incompatible self-pollination causes a decrease in the amounts of triglycosylceramides whatever the genotype of the flower.

INTRODUCTION

Glycosphingolipids (GSLs) are characterized by the presence of a long-chain base (LCB: an amine diol with a long carbon chain), a fatty acid (linked by an amide bond at position 2) and a carbohydrate chain (through a glycosidic linkage at position 1). Their structural complexity is due to the large variation of each class of constituent: bases (about 60), fatty acids (more than 200) and the nature and number of glycosidic residues (from 0 to more than 10) [1, 2]. Such diversity may be related to the specificity built into these cell-surface molecules. GSLs have been identified in plants [3] but their function has not yet been clearly defined. Nevertheless, some of their physiological properties have been established: their role in water permeability [4, 5]; ion transport [6, 7]; their immunological properties [8]; and their roles in cell interactions, cellular recognition and also contact inhibition processes [9, 10].

We have examined changes in these membranous lipids in the styles and the pollen of *Oenothera missouriensis* and their distribution among the various subgroups in connection with gametophytic self incompatibility (GSI). This GSI phenomenon inhibits pollen tube growth in a style which bears the same incompatibility S allele. The GSI reaction is induced by a signal following contact between pollen and stylar cellular surfaces. Other cellular events implying a previous recognition stage induced by intercellular contact (as contact inhibition, tumoral and transformation processes) are known to evince changes in GSL pattern [11, 12].

Moreover, glycolipids are thought to be relatively more specific to particular tissues than phospholipids and are subject to variations according to the species [2] or genetic clone [13]. Until recently, it was thought that membranous glycoproteins were only involved in the process of recognition but recent data show GSLs as receptors for a wide variety of molecules which can influence intracellular events [14]. GSLs are not only receptors for lectins but they can also bind toxins; they are also blood group antigens (ABO) [14]. Similar investigations have been conducted in *Petunia hybrida* which also possesses a GSI system [15, 16]. No one has suggested so far what the precise role of GSLs in the recognition process and in the SI systems is. We thought it would be interesting to see whether GSL distribution changes during growth following a recognition interaction.

RESULTS

Our present study was conducted on both pollen and unpollinated styles of *Oenothera missouriensis* derived from three genetic clones corresponding to the S incompatibility alleles [20] S_1 (S_1S_1), Bb (S_1S_2) and Bg (S_2S_4). We also examined styles which underwent compatible cross-pollination ($S_1S_1 \times S_2S_4$ and $S_2S_4 \times S_1S_1$), incompatible self-pollination and semi-compatible pollination ($S_1S_2 \times S_2S_4$); in the latter case, only the pollen grains bearing the S_4 allele gave normal pollen tubes.

We divided the lipids into neutral or acidic components and finally obtained five fractions: F_1, the ceramides (Cer); F_2, monoglycosylceramides (MGCer); F_3, di- and triglycosylceramides (DGCer and TGCer); F_4, tetraglycosylceramides (TrGCer); and finally F_5, polar lipids. Our results (Table 1) are expressed in nanomol of GSL/g fr. wt and represent the average of five assays with standard deviations.

Comparison of pollen and style

When compared with the unpollinated styles, pollen proved to be mainly characterized by higher amounts of GSLs. The analysis of each GSL class shows that this is a general feature, except in the case of the ceramides. One may also note the relatively high amounts of MGCer (F_2) and the acidic lipids in pollen (Table 2). Together, fractions F_2 and E represent more than 60% of total pollen GSLs. Moreover, if we determine the relative percentage of acid GSLs versus total GSLs, we obtain the results shown in Table 3. The ceramides represent the most important group of GSLs in unpollinated styles, except in the case of the S_1 clone which possesses a larger amount of monoglycosylceramides.

Table 1. Average values of glycosphingolipid assays in different clones of *Oenothera missouriensis*

		S_1 plant				Bb plant				Bg plant			
		Pollen	S_1S_1 unpoll.	$S_1S_1 \times S_1S_1$ self-poll.	$S_1S_1 \times S_2S_4$ cross-poll.	Pollen	S_1S_2 unpoll.	$S_1S_2 \times S_1S_2$ self-poll.	$S_1S_2 \times S_2S_4$ cross-poll.	Pollen	S_2S_4 unpoll.	$S_2S_4 \times S_2S_4$ self-poll.	$S_2S_4 \times S_1S_1$ cross-poll.
A		94.4 ± 1.7	20.6 ± 1.1	40.6 ± 0.5	70.5 ± 2.1	91.3 ± 0.9	23.3 ± 2.4	60.0 ± 3.2	48.5 ± 2.7	87.7 ± 2.7	25.1 ± 1.6	44.0 ± 3.7	61.5 ± 1.9
F_1		5.4 ± 0.7	7.0 ± 1.2	13.0 ± 1.1	16.0 ± 1.8	6.3 ± 0.5	10.0 ± 2	23.0 ± 2	7.0 ± 1.4	6.7 ± 0.5	10.0 ± 1.6	13.0 ± 1.6	13.0 ± 1.5
F_2		31.0 ± 0.7	8.0 ± 0.6	18.0 ± 1.7	40.0 ± 3.1	32.0 ± 1.9	5.0 ± 1.5	20.0 ± 1.9	30.0 ± 3	28.0 ± 2.5	8.0 ± 1.3	18.0 ± 1.9	35.0 ± 1.8
F_3		15.0 ± 1.6	1.8 ± 0.2	4.0 ± 0.7	8.0 ± 0.8	16.0 ± 2.7	2.0 ± 0.6	8.0 ± 1.6	5.0 ± 1.8	13.0 ± 2.7	3.0 ± 0.9	5.0 ± 0.8	6.0 ± 1.3
F_4		6.0 ± 1	1.5 ± 0.3	1.2 ± 0.2	2.5 ± 0.4	5.0 ± 1.2	2.5 ± 0.3	2.0 ± 0.4	1.8 ± 0.5	5.0 ± 1.5	1.5 ± 0.4	0.7 ± 0.2	2.5 ± 0.5
F_5		3.0 ± 0.4	1.0 ± 0.3	1.8 ± 0.2	2.0 ± 0.4	5.0 ± 1.7	1.8 ± 0.4	2.0 ± 0.5	3.0 ± 1.2	3.0 ± 0.7	1.1 ± 0.3	1.3 ± 0.4	3.0 ± 0.6
E		34.0 ± 0.8	1.3 ± 0.1	2.6 ± 0.2	2.0 ± 0.5	27.0 ± 3.1	3.0 ± 0.7	5.0 ± 1.5	2.0 ± 0.5	32.0 ± 2.1	1.5 ± 0.2	6.0 ± 1	2.0 ± 0.7
(nanomol/g)													
F_1	B	2.5	33	31	26	7	36.8	27	15	18	50	13	23
F_2	B	35	39	44	55	35	20	46	61	47	15	70	58
F_3	B	15	9	9.8	12	17	8	9	10	10	7	7	10
F_4	B	6.5	6	3	4	5	10	6	4	6	8	2.6	4
F_5	B	3.5	5	4	3	5	8	6	6	6	7	5.1	4
E	B	37.5	6	6	7	30	16	6	5	14	12	2.6	7
F_1	C	0.8	1	1.9	2.3	0.7	1	2.3	0.7	0.7	1	1.3	1.3
F_2	C	3.9	1	2.3	5	6.4	1	4	6	3.5	1	2.3	4.4
F_3	C	8.3	1	2.2	4.4	8	1	4	2.5	4.3	1	1.7	2
F_4	C	4	1	0.8	1.7	2	1	0.8	0.7	3.3	1	0.5	1.7
F_5	C	3	1	1.5	2	2.8	1	1.1	1.7	2.7	1	1.1	2.8
E	C	26	1	2	1.5	9	1	1.7	0.7	21	1	4	1.3

Key: A = Total GSLs, B = percentage in each class, C = relative amounts compared with unpollinated styles, E = acid GSLs.

Table 2. Amounts of acidic GSLs (fraction E) in nmol/g

	Pollen	Styles
S_1S_1 (S_1)	34	1.3
S_1S_2 (Bb)	27	3
S_2S_4 (Bg)	32	1.5

Table 3. Relative percentage of acidic GSLs vs total GSLs

	Pollen	Unpollinated styles
S_1S_1 (S_1)	36	6.3
S_1S_2 (Bb)	29.6	13
S_2S_4 (Bg)	36.4	5.9

The contribution of pollination

Generally speaking, pollination increases the amounts of GSLs in the styles, since pollen is relatively richer in GSLs than the unpollinated style. There are, however, differences between cross- and self-pollination. Thus self-pollination increases both Cer and MGCer while the main result of cross-pollination is an increase in Cer and a much larger increase in MGCer (Table 1). These differences are the same whatever the genotype of the style.

In the particular case of semi-compatible mating (Bb × Bg), there is a characteristic increase in MGCer which is very similar to that in compatible mating. Comparison of lipid constituents in the three genotypes of *Oenothera missouriensis* (Table 1) shows that there are relative increases in Cer, MGCer and DGCer after cross- and self-pollination. Remarkably enough, the levels of triglycosylceramides decrease after self-pollination while the pollen and stylar contributions should actually show a large increase; there must, therefore, be some metabolic upset induced by an incompatible mating. In addition, self-pollination leads to an increase in the relative amounts of acidic GSLs.

Statistical analysis. Variance analysis based on genotype, lipid fraction and mating type has been performed on the stylar results (Table 4). The following statistical facts emerge. When comparing genotypes, the average amounts for all six lipid fractions do not vary significantly. On the other hand, the averages for the GSL distribution among the various fractions present the highest variance value. The averages between the three stylar groups (unfertilized, cross- and self-pollinated) are highly significant. The GSL distribution depends mainly on the state of the style. The genotype vs fraction interaction is significant but has a weaker coefficient. This implies that the GSL distribution also depends on the genotype. In comparison, the genotype vs style interaction is more important, which means that if GSL distribution fluctuates with stylar condition, this event is under genotypic control. The biggest interaction is that between style and lipid fraction. The importance of a particular fraction is mainly related to the stylar condition. The second-order interaction is also significant. If we consider the genotype vs style interaction, it varies with a given F fraction and the genotype only has a slight influence.

To summarize, it is apparent that the pollination process is the most important parameter for determining the distribution of the glycosphingolipids among the different plant tissues. By contrast, the influence of the genotype is much less pronounced.

DISCUSSION

The present study fails to show any inter-relation between a given glycosphingolipid and a given S allele because, whatever the flower genotype, the distribution of and variation in GSLs are very similar. Nevertheless, pollination induces changes in the GSL pattern. Pollination results in a reduced level of the more complex tetraglycosylceramides and polar lipids with a corresponding accumulation of ceramides and monoglycosylceramides. In other physiological processes involving recognition, a simplification of the glycolipid pattern (shorter carbohydrate chains) and Cer–MGCer accumulation has been noted [17–19]. Under the same conditions, this change in pattern has also been observed in *Petunia hybrida*, a GSI species [15, 16].

Table 4. Variance analysis of GSL amounts in unpollinated, self- and cross-pollinated styles (S_1S_1, S_1S_2 and S_2S_4 genotypes)

Source of variance	Degree of freedom	S_2	F
Genotype (A)	2	0.60	0.42 NS
Fraction (B)	5	2518.64	1749.06^{xxx}
Style (C)	2	886.73	615.78^{xxx}
Genotype × fraction (A × B)	10	14.54	10.10^{xxx}
Genotype × style (A × C)	4	96.06	66.71^{xxx}
Style × fraction (B × C)	10	479.13	332.73^{xxx}
A × B × C	20	23.05	16.10^{xxx}
Error	216	1.44	

NS = non-significantly different than 1.
xxx = significant at the level 0.01.

Changes in GSL distribution and structure are probably related to the process of fertilization and more especially to the stage of contact and recognition which initiates it. One may note that self- and cross-pollination do not induce exactly the same changes in the various lipid classes. For instance, self-pollination affects principally the F_4 and acidic fractions. Our data strongly suggest that, after pollination, glycolipid metabolism changes, such a change being initiated by the recognition reaction. Whether the existing membrane GSLs of pollen and stylar tissues are involved in recognition processes, as cell surface glycoproteins are, is still open to question. It also seems necessary to determine the mechanism causing these modifications after cellular contact and it remains to be seen whether these alterations in metabolism are the causes or the results of the self-incompatibility system.

EXPERIMENTAL

Biological material. The clones of *Oenothera missouriensis* Sims used were S_1 (S_1S_1 alleles), Bb (S_1S_2) and Bg (S_2S_4). When flowering (in June and July), the floral buds were harvested, the stamens discarded or collected, and then the flowers underwent *in vitro* pollination. 15 Hr after pollination, which is the time necessary for a complete growth of compatible pollen tubes through stylar tissues [21], the styles were collected and kept at −20° before use.

Isolation of total lipids. Total lipids were extracted from frozen material (pollen 3 g, styles 50 g) according to the Folch procedure [22] as modified by Karlsson [23]; a 15 min extraction in a blendor in $CHCl_3$–MeOH (2:1), 20 ml/g of frozen tissue. After 2 hr at room temp. and filtration, the residue was re-extracted twice with the same solvent and the remaining residue was refluxed in boiling $CHCl_3$–MeOH (1:1). The combined extracts were partitioned overnight with 9% NaCl and the upper phase was discarded.

Mild alkaline hydrolysis and partition. The removal of glycerol ester lipids was achieved by mild alkaline hydrolysis [5]; 1 g total lipid was suspended in 100 ml 0.1 M KOH in MeOH–H_2O (9:1). The vessel was flushed with N_2, closed and left, with stirring, in the dark at room temp. for 18 hr. The hydrolysis was stopped by slow addition of 2 M HCl with stirring until pH 2–3 was reached. $CHCl_3$ and H_2O were added to obtain $CHCl_3$–MeOH–H_2O proportions of 8:4:3 (by vol.). After partition the lower phase was evapd.

Purification of GSLs. The fatty acids and cholesterol were eluted in pure $CHCl_3$ from a column of silicic acid and GSLs were eluted in 75% (by vol.) MeOH in $CHCl_3$ followed by pure MeOH. The load was 100 mg lipid/g of silicic acid, the elution volume 10 ml/g silicic acid.

DEAE-cellulose column chromatography. The DEAE-cellulose converted into the acetate form was equilibrated in $CHCl_3$–MeOH (2:1) for a few hr. Lipid (50 mg or less) was applied for each g of cellulose. Neutral lipids were eluted in $CHCl_3$–MeOH (52:1), 100 ml for each g of cellulose. Acidic lipids were eluted in 5% (w/v) LiCl in MeOH (25 ml/g of cellulose). H_2O and $CHCl_3$ were added to this eluate in order to emulsify the lipids. LiCl was then removed by dialysis.

Silicic acid column chromatography. For further separation of neutral lipids, silicic acid chromatography was used. The load was 10–25 mg lipid/g silicic acid. The GSLs were gradually eluted with increasing concns of MeOH in $CHCl_3$, i.e. F_1 (Cer) by $CHCl_3$–MeOH 98:2, F_2 (MGCer) 92:8, F_3 (DGCer) 85:15, F_4 (TGCer + TrGCer) 45:55, and F_5 (polar lipids) by pure MeOH.

Analytical TLC. All preparative steps were monitored by TLC in $CHCl_3$–MeOH–H_2O (65:25:4). The chromatograms were visualized by brief I_2 exposure or by Rhodamine or sulphuric/α-naphthol sprays.

Fluorimetric determination of GSLs [24]. Samples of GSLs (1–100 nmol) were heated in 0.5 ml M HCl in aq. MeOH (MeOH–H_2O, 41:9) at 70° for 18 hr in screw-capped tubes. After cooling in ice, the soln was neutralized with 0.25 ml 2 N NaOH, and mixed with 0.75 ml 0.2 M sodium borate buffer, pH 8.0. Et_2O (1.5 ml) was added to the hydrolysis tube, followed by 0.5 ml freshly prepared Et_2O containing 0.015% fluorescamine. After capping the tube, the mixture was vigorously stirred, and following phase separation, the Et_2O soln transferred to a fluorometer tube for quantitative determination of the fluorescence intensity, with excitation and emission wavelengths of 385 and 480 nm respectively. The fluorescence intensity was directly proportional to the GSL amounts. A standard assay was provided.

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