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SULPHATIDES AND POLLINATION IN *OENOTHERA MISSOURIENSIS*

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Key Word Index—*Oenothera missouriensis*; Onagraceae; lipids; pollen; style; sulphatides; self-incompatibility.

Abstract—Analyses of the sulphatides in the pollen and style of *Oenothera missouriensis* show that these membranous lipids are comparatively less important in the styles than in the pollen. Incompatible pollination is followed by a large increase in sulphatides, whereas cross-pollination also causes an increase in sulphatide but to a much lesser extent. This mobilization of sulphatides in the membrane is discussed in term of permeability.

INTRODUCTION

During the fertilization process, a sequence of events occurs that is known to be initiated by a self–nonself discrimination. This mutual recognition between a pollen grain and a pistil is controlled by mechanisms strictly defined by genetic controls from both partners. Gametophytic incompatibility provides definitive evidence for cell recognition as a pollen carrying an S-incompatibility allele is rejected by a style bearing the same allele [1–3]. Soon after pollination, the pollen tube of *Oenothera* begins its growth and interacts with neighbouring stylar tissues [4–6]. Only the compatible pollen tube can reach the ovary, while the incompatible one is rejected.

It is apparent from our preliminary work [7–9] that it is of interest to consider the relationship between pollination and membrane functions and more precisely between pollination and permeability. Evidence linking SL (cerebroside sulphates) with ion permeability is mainly indirect [10, 11]. This property has been attributed to: (a) their galactose sulphate groups which can bind cations; (b) and their ceramide structure which can adjust their fluidity characteristics to fit specific requirements and, hence, regulate permeability, as well as the activity of membrane bound enzymes; and (c) moreover SL have been claimed to be selective cofactors for Na^+, K^+ and Ca^{2+} ATPases [12, 13]. Thus, selective permeability is dependent on the SL content, and also on the fluid character of their ceramide moiety, and is influenced in a predictable way by factors, such as SL content, length and degree of unsaturation of their aliphatic chains [14–16].

The present study was undertaken to investigate sulphatide structures and their relevance to changes in membrane permeability following pollination.

RESULTS

Analysis of SL

Our present study was conducted on both pollen and unpollinated pistils (styles and stigmas, the ovary being discarded) of *Oenothera missouriensis* derived from three genetic clones corresponding to the S-incompatibility alleles S_1 ($S_1 \cdot S_1$), B_b ($S_1 \cdot S_2$) and B_g ($S_2 \cdot S_4$). We also examined styles which underwent compatible cross pollination ($S_1 \times B_g$ and $B_g \times S_1$), incompatible self pollination and semicompatible pollination ($B_b \times B_g$). In the latter case, only the pollen grains bearing the S_4 allele gave normal pollen tubes.

We purified SL and our results listed in Table 1 and expressed in nmol GSL/g fr. wt represent the average of five assays with s.d.s.

Comparison of pollen and unpollinated styles. When compared with the unpollinated styles, pollen proved to be mainly characterized by higher amounts of SL. The analysis of each clone shows that pollen SL can represent up to 26 times the amount of stylar SL. There thus appears to be a general pattern that pollen is significantly richer in SL than styles.

Contribution of pollination. We have estimated the average weight of pollen grains used to ensure pollination. It represents less than 0.02% of the weight of fresh styles and stigmas. So the error due to pollen sulphatides is statistically negligible. As expected, some significant differences are recorded between self and cross pollination, SL amounts being greatly increased by self pollination. Cross pollination also increases SL amounts but to a much lesser extent.

Analysis of FA distribution among SL

The FA compositions of SL from *Oenothera missouriensis* have been characterized and are presented in Table 2.

Comparison of pollen and unpollinated styles. The three pollen samples (B_g, B_b, S_1) are very similar in FA composition with almost half of the total FA content being palmitic acid (C16:0) and stearic acid (G18:0), the other major acid is oleic (C18:1). The unpollinated styles are similar to pollen in that their major FA are also palmitic, stearic and oleic acids. Most often lipids from *Oenothera* oils or pollen are characterized by a high content of homo-γ-linolenic acid (C18:3ω6). This fatty

Abbreviations: LCB, long-chained bases; GSL, glycosphingolipids; SL, sulphatide; FA, fatty acid.

Table 1. Evolution of SL with pollination in *Oenothera missouriensis*

	Expressed in nmol/g tissue			Compared with virgin style			Compared with cross pollination		
Genotypes	$S_1 \cdot S_1$	$S_2 \cdot S_2$	$S_2 \cdot S_4$	$S_1 \cdot S_1$	$S_1 \cdot S_2$	$S_2 \cdot S_4$	$S_1 \cdot S_1$	$S_1 \cdot S_2$	$S_2 \cdot S_4$
Unpollinated styles	1.3±0.1	3±0.7	1.5±0.2	1	1	1	—	—	—
Pollen	34 ±0.8	27±3.1	32 ±2.1	26	9	21	—	—	—
Self pollination	2.6±0.20	5±1.5	6 ±1	2	1.67	4	1.3	2.5	3
Cross pollination	2.0±0.50	2±0.50	2 ±0.70	1.53	0.66	1.33	1	1	1

Table 2. FA composition of SL from *Oenothera missouriensis*

	Pollen			Styles							
	B_g	B_b	S_1	B_g virgin	$B_g \cdot B_1$	$B_g \cdot B_g$	S_1 virgin	$S_1 \cdot B_g$	$S_1 \cdot S_1$	B_b virgin	$B_b \cdot B_g$
C14:0	6.09	4.55	3.16	4.30	1.98	2.54	11.06	3.40	4.57	9.35	6.50
C15:0	4.79	3.58	3.60	4.62	0.80	2.57	7.65	4.34	2.36	8.74	3.88
C16:0	26.21	31.52	31.46	34.66	33.67	38.27	47.35	43.04	32.40	36.17	40.57
C16:1	4.91	4.10	3.54	5.70	0.78	5.19	3.04	3.54	—	5.88	3.91
C17:0	1.54	1.47	1.40	3.37	1.13	2.13	2.36	1.50	—	1.85	1.77
C18:0	17.43	22.94	20.70	17.03	31.78	19.84	14.40	31.07	17.27	15.91	20.80
C18:1	15.02	11.32	15.17	8.80	8.27	16.12	4.95	5.72	6.40	11.96	6.01
C18:2	5.22	3.24	3.57	4.47	7.12	3.13	1.33	4.68	12.54	3.88	4.88
C18:3	1.09	1.34	4.21	1.40	5.32	0.64	0.66	—	9.27	1.94	—
C20:0	5.60	7.42	6.10	5.90	4.08	3.58	1.48	2.70	5.04	1.24	2.98
C20:3	—	1.33	—	—	—	—	1.96	—	—	1.37	2.14
C22:0	6.97	4.24	2.27	2.93	2.34	2.89	1.95	—	3.84	1.70	4.90
C24:0	5.12	2.94	4.81	6.81	2.72	3.09	1.80	—	6.30	—	1.65
Unsaturated FA	26.24	21.33	26.49	20.57	25.57	28.66	11.94	13.94	28.21	25.03	16.94
Odd FA	6.33	5.05	5.00	7.99	1.93	4.70	10.01	5.84	2.36	16.43	5.65
C18:2/C18:1	0.35	0.29	0.24	0.51	0.86	0.19	0.27	0.82	1.96	0.32	0.81

acid was the major FA (40%) of pollen ceramides and mono-and diglycosylceramides. It was not encountered in stylar glycolipids, or in our sulphatides.

The high degree of saturation can be explained by the fact that most often SL are characterized by extra long unsaturated fatty acids and also that lipids from plasma membrane of plant origin are generally more saturated and richer in palmitic acid than lipids of animal origin [17]. However, when compared to pollen, styles proved to be mainly characterized by a higher degree of saturation.

Odd FA content is always lower in pollen, when compared to virgin styles. It seems to be a general pattern that styles are characterized by high content of odd FA. At least four divisions may be made on the basis of chain length. FA can be subdivided according to their short (C14–C15), medium (C16–C17), C18 and long (C20–C24) aliphatic chains. Pistils are characterized by short and medium FA (*ca* 62% in styles and 44% in pollen). Pollen, on the other hand is richer in long FA (16% in pollen and 9% in styles). As expected, FA composition of SL is very different from that of phospholipids [18, 19]. SL are richer in stearic acid and long chain FA (C22–C24). Such long FA have been observed in the SL fraction of plants [13, 17]. This degree of variability in FA composition among pollen and stylar SL led us to examine the behaviour of SL after pollination.

The contribution of pollination. The main result of compatible cross pollination is an increase of stearic (C18:0) and linoleic acids (C18:2), while short and medium FA (C14–C17) decrease. The pattern of evolution of the long FA is not homogenous and some variations are recorded with both the degree of saturation and the analysed clones. For example long saturated FA decrease. The ratio C18:2/C18:1 is enhanced by compatible pollination up to two or three times the values encountered in unpollinated styles. With incompatible self pollination, it is difficult to summarize the contribution of self pollination; however, the C14, C15 and C17 FA content is reduced and the degree of unsaturation is slightly increased. The above ratio C18:2/C18:1 is only slightly influenced by incompatible pollination. No other typical developments can be recorded and the results seemed to be clone-dependent. In the semicompatible mating $B_b \cdot B_g$ ($S_1 \cdot S_2 \times S_2 \cdot S_4$), there is a characteristic increase of C16, C22 and C24 FA which is not recorded elsewhere.

To summarize, it is apparent that the cross pollination process induces organized responses according to a general pattern. By contrast the influence of self pollination is less regular and can vary with the analysed clones. This correlates well with previous observations (neutral GSL) which show a radical difference between cross and self pollination. Changes in FA distribution are probably related to the incompatibility reaction.

Table 3. Sphingosine base composition of SL from *Oenothera missouriensis*

		Pollen			Styles								
Aldehydes	LCB	B_g	S_1	B_b	B_b virgin	$B_b \cdot B_g$	$B_b \cdot B_b$	S_1 virgin	$S_1 \cdot B_g$	$S_1 \cdot S_1$	B_g virgin	$B_g \cdot S_1$	$B_g \cdot B_g$
C14:0	d16:0–t17:0	1.07	6.12	3.43	8.24	4.90	6.28	3.33	10.11	10.53	3.57	13.20	5.55
C14:1	d16:1–t17:1	9.98	13.25	4.63	5.06	10.40	7.24	5.33	11.50	19.08	1.60	1.43	12.24
C15:0	d17:0–t18:0	7.12	3.28	4.37	2.78	3.50	3.02	6.14	5.80	16.83	16.74	14.60	4.88
C15:1	d17:1–t18:1	6.60	5.97	0.59	2.59	3.38	5.52	2.66	3.87	9.74	1.78	25.50	5.04
C16:0	d18:0–t19:0	19.00	20.03	11.17	5.55	6.57	5.32	11.80	16.57	5.20	7.27	2.38	8.11
C16:1	d18:1–t19:1	9.06	4.12	49.12	39.38	23.45	28.80	30.98	13.40	5.86	36.54	6.35	16.86
C17:0	d19:0–t20:0	15.30	8.61	12.07	15.93	14.38	15.45	9.78	10.40	11.35	6.98	6.65	16.62
C17:1	d19:1–t20:1	6.51	3.12	6.06	8.73	8.20	14.43	6.44	4.00	7.45	6.11	5.10	7.68
C18:0	d20:0 t21:0	15.75	23.36	5.32	6.34	15.28	8.36	12.19	8.20	7.63	12.13	10.08	13.21
C18:1	d20:1–t21:1	9.60	12.13	3.23	5.39	9.93	5.57	11.34	16.14	6.32	7.27	14.70	9.80
	Unsaturated LCB	41.74	38.59	63.63	61.15	55.36	61.56	56.75	48.91	48.45	53.30	53.08	51.62

Analysis of LCB distribution among SL

Whilst exhibiting a mixed FA composition, SL may also vary, albeit much less extensively, in their sphingosine content. The LCB composition: chain length, degree and position of unsaturation, has been characterized and is presented in Table 3.

Comparison of pollen and unpollinated styles. Some differences in major LCB are recorded according to the clone. LCB profile of pollen SL displays some general features. The SL of pollen from *Oenothera missouriensis* are relatively rich in sphinganine (d18:0), eicosa-sphinganine (d20:0), eicosa-sphingenine (d20:1) and in the corresponding trihydroxylated sphingosine bases, t19:0, t21:0 and t21:1, which can produce the same aldehydes. By contrast, the SL of unpollinated styles have appreciable amounts of d18:1–t19:1 and d19:1–t20:1 sphingosine bases. When compared to pollen, styles proved to be mainly characterized by a lower degree of saturation.

The contribution of pollination. The analysis of the LCB distribution with pollination is extremely complex; however, no attempt to make subdivisions according to chain length was undertaken, the analysed LCB being relatively homogenous. Pollination induces variations among LCB distributions, which can be considered as general features. Some rare distortions occur in certain clones. As expected some significant differences are registered between cross and self pollination. This concerns the d18:0–t19:0 and d19:1–t20:1 sphingosine bases.

The first group (d18:0–t19:0) considered as pollen major LCB is enhanced by cross pollination. By contrast it decreases with self pollination. The other (d19:1–t20:1), considered as typically stylar LCB, is increased by self pollination only. Self pollination increases the degree of saturation to a larger extent than cross pollination. Thus, there appears to be a general pattern which correlates the LCB composition of SL to a definite part of the flower.

DISCUSSION

The data presented here show that SL metabolism changes after pollination (Table 4). Some appreciable variations in amounts are recorded after pollination. For example, self pollination significantly enhances amounts of SL when compared to cross pollination. FA and LCB profiles also vary during the fertilization process. Cross pollination for 15 hr induces a rapid decrease of short and medium chain FA (for example palmitic acid), correlated with an increase of stearic and oleic acid and a slight enhancement in the degree of saturation. In addition an abrupt decrease in stylar LCB compensated by increased amounts of pollen LCB is characteristic of cross pollination. A radical contrast is recorded for self pollination which promotes less organized and less typical variations.

As SL are claimed to act on ion permeability [11, 13], our data suggest that after self pollination, the number of ion transport sites (amount of SL) as well as fluidity character of SL are modified. Such variations can change the osmotic balance and the electrochemical ion gradient across the pollen tube membrane. Energy is contained in a transmembrane ion gradient [20] and it is reasonable to assume that a dramatic effect could be exerted upon the pollen tube on disruption of the energized step. Changes in SL amounts might lead to the opening of ionic channels. As many metabolic processes depend on the ion-charge gradient across membranes [20], SL may be used to regulate a variety of metabolic processes through ionic mobilization.

EXPERIMENTAL

Material. The present analysis was performed with *Oenothera missouriensis* Sims (a self incompatible species) grown in the Botanical Garden, Strasbourg (France). The different clones have been characterized and isolated by Linder [21]. They were grown in identical environmental conditions to avoid modifications of incompatibility responses. The clones of *Oenothera missouriensis* used were S_1 ($S_1 \cdot S_1$ alleles), B_b ($S_1 \cdot S_2$) and B_g ($S_2 \cdot S_4$). When flowering (in June and July), the floral buds were harvested, the stamens either discarded or collected and the flowers then underwent *in vitro* pollination. Our present study was conducted on both pollinated and unpollinated styles of *Oenothera missouriensis* derived from three genetic clones corresponding to the S incompatibility alleles [21] and on styles which underwent compatible and incompatible pollination. The assessment of pollen tubes within styles was performed by cytolocalization of

Table 4. Evolution of SL with pollination

		Self pollination	Cross pollination
SL amounts		↑↑↑	↑
FA	Unsaturated FA	↑↑↑	↑
	C18:0/C16:0	↑	↑↑↑
	C18:2/C18:1	No common features	
LCB	Unsaturated LCB	↘	↘
	Stylar LCB	↗	↘
	Pollen LCB	↘	↗

Ascending arrows represent an increase of a given parameter, while descending ones represent a decrease.

callose plugs after staining with anillin blue [22]. After 15 hr pollination, which is the time necessary for a complete growth of compatible pollen tubes through stylar tissues [23], 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 [24] as modified by Karlsson *et al.* [25]; a 15 min extraction in a blender 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 [25]; 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 (8:4:3). After partition, the lower phase (GSLs) was evaporated.

Purification of GSLs. The FA 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 silicic acid, elution vol. 10 mg/g silicic acid. The GSLs thus purified were collected (pGSL).

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

Prep. TLC. Additional purification of SL was achieved by 20 × 20 cm prep. TLC with $CHCl_3$–MeOH–H_2O (65:25:4). The elution was performed with $CHCl_3$–MeOH (1:1).

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 acid–α-naphthol sprays. For identification of SL, additional TLC was performed in *n*-PrOH–NH_3–H_2O (12:1:2) where SL have R_f 0.82 [26].

Fluorimetric determination of GSLs [27]. Samples of GSLs (1–100 nmol) were heated in 0.5 ml 1 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 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.

GSL hydrolysis. The various GSL were subjected to acid hydrolysis (1 N HCl in MeOH with 10 mol H_2O) for 23 hr in N_2 [28].

Analysis of FA composition. The FA of all the GSL released by acid hydrolysis according to ref. [28] were extracted by *n*-hexane (3 × 3 ml), evaporated to dryness and transmethylated [29]. After extraction with heptane, the methyl esters of each extract were separated and identified by GC using two capillary columns, one packed with Carbowax 20 M (10%) and the other with DEGS (10%). The temp. of the columns were, respectively, 190° and 170°. For each methyl ester mixture, two analyses were performed on both columns. The peak areas were calculated by an integrator. The results were expressed as means (nmol/%) of three determinations for each extract.

Analysis of the LCB composition. After the removal of FA the LCB were selectively extracted from the MeOH phase with Et_2O under alkaline conditions [30]. They were then converted into stable DNP derivatives by 1-fluoro-2,4-dinotrobenzene [31] and finally into aldehydes by $Pb(OAc)_4$ oxidation [32]. The aldehydes were analysed by TLC [26] and GC (10% DEGS on Chromosorb W60–80 mesh, column temp. 150°, and a N_2 stream of 19–20 ml/min) [30, 31].

In order to confirm the aldehyde identifications, a complementary study was performed by oxidizing the aldehydes with $KMnO_4$ without prior catalytic hydrogenation [33]. The FA obtained were then transesterified by a MeOH–H_2SO_4 mixture [29], purified by TLC and chromatographed by GC (10% DEGS on chromosorb W60–80 mesh AW with a column temp. of 176° and a N_2 stream of 25 ml/min) [34].

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