Plant Physiol. (1991) 96, 1136–1141
0032-0889/91/96/1136/06/$01.00/0

Received for publication December 13, 1990
Accepted March 25, 1991

Lipids of Plasma Membranes Prepared from Oat Root Cells[1]

Effects of Induced Water-Deficit Tolerance

Peter Norberg and Conny Liljenberg*

Department of Plant Physiology, University of Göteborg, Carl Skottsbergs G. 22, S-413 19 Göteborg, Sweden

ABSTRACT

Plasma membranes were isolated from oat (*Avena sativa*) roots by the phase-partitioning method. The membranes were exposed to repeated periods of moderate water-deficit stress, and a water-deficit tolerance was induced (acclimated plants). The plasma membranes of the controls (nonacclimated plants) were characterized by a high phospholipid content, 79% of total lipids, cerebrosides (9%) containing hydroxy fatty acids (>90% 24:1-OH) and free sterols, acylated sterylglucosides, sterylglucosides, and steryl esters, together amounting to 12%. Major phospholipids were phosphatidylcholine and phosphatidylethanolamine with lesser amounts of phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid. After the membranes were acclimated to dehydration, the lipid to protein ratio decreased from 1.3 to 0.7 micromoles per milligram. Furthermore, the cerebrosides decreased to 5% and free sterols increased from 9% (nonacclimated plants) to 14%. Because the total phospholipids did not change significantly, the free sterol to phospholipid ratio increased from 0.12 to 0.19. There was no change in the relative distribution of sterols after acclimation. The ratio of phosphatidylcholine to phosphatidylethanolamine changed from 1.1 in the nonacclimated plants to 0.69 in the acclimated plants. The results show that acclimation to dehydration implies substantial alterations in the lipid composition of the plasma membrane.

Lipids play a fundamental role in determining the physical and chemical properties of biomembranes. The total cellular metabolic activity is affected by permeability, transfer activity, enzyme reactions, and lipid phase transition characteristics of the membranes. Plant cell membranes are dynamic with composition changing with the developmental stage (11, 30) and with variation in environment (12, 14, 31). Of the cellular membranes the plasma membrane, by its localization limiting the cytoplasm, is the initial critical target when the cells are exposed to abiotic environmental stress. However, information concerning lipid composition of plant cell plasma membranes is quite limited because of difficulties in isolating plasma membranes with a high degree of purity and in quantities needed for detailed studies.

Water-deficit stress or drought causes a changed membrane structure and function (*e.g.* 18, 26, see also review in ref. 1). There are several reports concerning correlation between water stress and changed membrane lipid composition (12, 13, 19, 28). Oat (*Avena sativa*) plants repeatedly exposed to a moderate water-deficit stress followed by a recovery period of rewatering will induce tolerance against subsequent exposure to a prolonged water stress period (P. Norberg and C. Liljenberg, unpublished data). The repeated stress cycle which is regarded as an acclimation process to dehydration is characterized by prolonged survival and prolonged continued growth of the roots as compared with the nonacclimated plants during exposure to a long period of severe stress. Furthermore, the root cell membranes had reduced permeability as measured by ion leakage. The roots of the acclimated plants had a lower relative water content as well as absolute water content compared with the controls (nonacclimated plants). As the long-term stress advances and the water potential of the environment as well as in the root cells decreased, the latter had the capacity to maintain a difference between the osmotic potential and water potential of the root cells much longer than those of the nonacclimated plants (P. Norberg and C. Liljenberg, unpublished data). Apart from changes in membrane lipid composition in general (12, 28), the above-related repeated water stress treatment, inducing dehydration tolerance, could, in addition, cause alterations in the lipid phase behavior, which was shown with microsomal lipids from rape root cells (20). To formulate an experimentally based mechanistic explanation for the changed plasma membrane behavior of root cells in response to water-deficit stress, a much more detailed knowledge of the chemical structure and physical properties of the membranes is needed. To more profoundly investigate the influence of dehydration acclimation on the lipid composition of root cell plasma membranes with likely implications for the lipid phase behavior leading to altered membrane properties, the objective of the present investigation was to characterize, in detail, the lipid composition of oat root cell plasma membranes from acclimated and nonacclimated plants.

MATERIALS AND METHODS

Plant Cultivation and Stress Treatment

Oats (*Avena sativa* L., cv Seger) were sown in gravel in plastic pots with holes in the bottom. The grains were evenly distributed over the surface and then covered with a thin layer of stones. The pots filled with gravel up to a height of 9 cm were placed in plastic bins containing nutrient solution (7) to a depth of 6 cm. The plants were grown in growth chambers

[1] This work was supported by grants from the Swedish Natural Science Research Council.

for 11 d (16-h day at 18°C and 8-h night at 12°C, 60% RH, 500 μmol/s/m photon flux density at the level of the leaves). After 5 d the plants were exposed to a water-deficit stress program. Excess nutrient solution was drained off and gravel and roots were dried effectively by placing the pots on a coarse stainless steel net. The stress program consisted of three 48-h cycles with 24 h of stress and 24 h of rewatering. After the final rewatering period, the plants were harvested.

Harvest and Membrane Preparation

The root system was washed in cold distilled water, cut in 1-cm pieces and homogenized in a Waring blender in the following isolation medium: 10 mM Tris-HCl buffer (pH 7.5) with 250 mM sucrose and 1.0 mM EGTA. The homogenate was filtered through two layers of nylon cloth and centrifuged for 15 min at 10,000*g*. The supernatant containing the membranes was then centrifuged at 60,000*g* for 30 min. The microsomal membrane pellet was resuspended in 5.0 mM potassium phosphate buffer (pH 7.8) containing 250 mM sucrose and 6.0 mM KCl.

Plasma membranes were prepared by partitioning the microsomal suspension in a 24-g aqueous polymer two-phase system containing 6.5% dextran T 500 (Pharmacia Fine Chemicals, Uppsala, Sweden) and 6.5% PEG 3350 (Carbowax Union Carbide Corp.) in 5.0 mM potassium phosphate buffer (pH 7.8) with 250 mM sucrose and 6.0 mM KCl. For further detailed information about the aqueous polymer two-phase system, see the paper by Sommarin *et al.* (27) and the references therein.

The upper phase containing the plasma membranes was diluted 5 to 10 times in 10 mM Tris-HCl buffer (pH 7.5) and 250 mM sucrose, centrifuged for 1 h at 100,000*g*, and resuspended in a small volume of 10 mM Tris-HCl buffer (as above). A defined portion of the different phases was frozen in liquid nitrogen and stored in a freezer (−18°C) until needed, *e.g.* protein and enzymatic activity assays.

Lipid Extraction and Separation

The lipids were immediately extracted from the membrane suspension by addition of two volumes of boiling 2-propanol followed by two volumes of chloroform:methanol (2:1, v/v), two volumes of chloroform, and one volume of distilled water. After agitation and phase separation, the chloroform phase was removed and the aqueous phase was washed once more with two volumes of chloroform. The two chloroform phases were pooled and dried, dissolved in a defined volume of chloroform, and stored at −18°C.

The total lipid extract was run on a silicic acid column (Silicar CC-4; Mallinckrodt, Inc.). The lipids were eluted with chloroform (neutral lipids), acetone (glycolipids), and methanol (phospholipids and polyglycolipids) in that order (12). The composition of each fraction from the column chromatography was examined by TLC on silica gel plates (Merck 60, Darmstadt, Germany) using the following solvents: petroleum ether (boiling point, 40–60°C):diethyl ether:formic acid (85:15:1, by volume) for neutral lipids; chloroform:methanol:acetic acid:water (85:15:15:3.5, by volume) for polar lipids. The spots were visualized with iodine vapor and identified by their R_F values relative to authentic standards. The separated lipids were scraped off the plates and extracted from the gel with chloroform:methanol (1:2, v/v) followed by methanol. The acyl lipids, except for steryl esters and cerebrosides, were converted to methyl esters by methanolysis in 2.5% HCl (gaseous) in dry methanol at 70°C for 1 h after a known amount of heptadecanoic acid was added as an internal standard. Total fatty acids were prepared by the same methanolysis procedure using a portion of the total lipid extract, to which was added the internal standard.

Analysis of Lipids

Sterol Analysis

The steryl esters were subjected to alkaline hydrolysis in 1 M NaOH in 90% ethanol at 65°C overnight. Cholesterylheptadecanoate was used as an internal standard. The sterols were then extracted with petroleum ether. The sterylglycosides and acylated sterylglycosides were hydrolyzed in 2.5% HCl (gaseous) in dry methanol at 70°C for 1 h. β-Cholestanol was used as an internal standard. The sterols were then extracted with petroleum ether. Free sterols and sterols from esters, glycosides, and acylated glycosides were further analyzed by GC.

Cerebroside Analysis

The acetone fraction containing the ceramides was dried and dissolved in a small defined volume of acetone. The ceramides were then further separated from the glycolipids of the acetone fraction on a silicic acid column (Merck LiCroprep Si-60, 15–25 μm) by sequential elution with 10 mL chloroform followed by 15 × 1-mL fractions of chloroform:methanol (90:10, v/v), then 10 mL chloroform: methanol (3:1, v/v), and finally 10 mL chloroform:methanol (1:3, v/v), all given per g silicic acid. The composition of the 1-mL fractions was examined by chromatography on high performance thin layer chromatography plates (Merck, high performance silica gel 60) with chloroform:methanol:water (80:20:2, by volume). The ceramides were detected on the high performance thin layer chromatography plates by exposure to iodine vapor or spraying with orcinol reagent (9) and developed at 140°C for 4 min. Fractions containing ceramides were combined and dried for further examination. For fatty acid determination the cerebrosides were hydrolyzed under reflux in 2 M aqueous methanolic HCl for 18 h (9). Methyl esters were extracted using petroleum ether and further reacted with silylating reagents to form *O*-trimethylsilyl fatty acid methyl esters before analysis by GC.

GC-MS

Sterols were analyzed by GC-MS after they were converted to trimethylsilyl derivatives. Methyl esters of fatty acids and trimethylsilyl ethers of sterols were run on a gas chromatograph (Hewlett Packard 5890) equipped with two detectors, a flame ionization detector and a mass selective detector (Hewlett Packard 5970, quadrupole instrument with electron impact ionization at an electron energy of 70 eV). The fatty acid methyl esters were separated on a 30-m × 0.32-mm i.d. DB225 (J and W Scientific) fused silica column (0.25-μm film

thickness) which was temperature programmed from 170°C (held for 20 min) to 195°C at 5°C/min (held until termination of run). Injector and detector temperatures were maintained at 210°C. The flow was maintained at 1.8 mL/min. Split injection was used. The trimethylsilyl ethers of the sterols were separated on a 30-m × 0.32-mm i.d. DB1701 (J and W Scientific) (0.25-μm film thickness) fused silica column operated at 250°C. Injector and detector temperatures were maintained at 270°C. The flow was 1.7 mL/min.

The cerebroside fractions were permethylated according to the method of Månsson *et al.* (16). Positive fast atom bombardment MS of the permethylated fractions and GC-MS of the partially methylated alditol acetates were performed as described previously (15, 16).

Analysis of Protein and Enzymatic Activity

Protein was analyzed by a modification of the Lowry procedure (17). The marker enzyme for the plasma membrane was glucan synthetase II (4). Marker enzymes for endoplasmic reticulum and mitochondria were NADPH-Cyt *c* reductase and Cyt *c* oxidase, respectively (6). Tests with the markers showed that these organelles were present in negligible amounts.

RESULTS

The lipids of plasma membranes from oat root cells are above all dominated by the phospholipids constituting 79% of the total lipids (control plants) (Table I). Two other lipid classes are major components of the membranes, the cerebrosides and the different forms of sterols. Acclimation to dehydration brought about by a repeated moderate water-deficit stress program resulted in numerous changes in the lipid composition of the plasma membrane (Table I). Although the level of phospholipids was unchanged, the lipid to protein ratio decreased from 1.3 to 0.7 μmol/mg. There was also a drastic decrease in the level of cerebrosides, from 9 to 5 mol % of total lipids. Sterols are present in the membrane as free, esterified glycosides and acylated glycosides. The steryl esters and the sterylglycosides are minor components. Free sterols and acylated sterylglycosides constituted 9 and 3 mol %, respectively, of total lipids from the nonacclimated root cells (Table I). After the stress program, there was a significant relative increase in the free sterols to 14 mol % of total lipids. The species composition of major free sterols is shown in Table II. Following acclimation to dehydration the mol % acylated sterylglycosides, sterylglycosides, and steryl esters decreased in total to a level equivalent to the increase in free sterols (Table III). Plasma membranes usually contain high levels of cerebrosides rich in hydroxy fatty acids. MS analysis of the cerebrosides present in oat root cell plasma membranes revealed a glycosyl ceramide of which >90% contained 24:1-OH in nonacclimated as well as in acclimated plants. Furthermore, the spingosine base was shown to be only one, 4,8-sphingadienine (21). The predominant phospholipids were PC and PE with lesser amounts of PG, phosphatidylinositol, and PA. The level of total phospholipids did not change after acclimation. However, substantial alterations of the relative proportions of the individual phospholipids occurred (Table IV). The ratio PC/PE was 1.1 in nonacclimated and 0.69 in acclimated membranes. The content of PA and PG decreased from 11 to 9 mol % (NS) and from 9 to 4 mol %, respectively, of total phospholipids after acclimation. The major fatty acids of total phospholipids were 16:0, 18:2, and 18:3. Small increases in 18:2 and decrease in 16:0 and 18:3 after acclimation were not significant (Table V). The differences between the fatty acid patterns of PC and PE were small. However, a higher proportion of 16:0 and a lower proportion of unsaturated 18C acids together with a higher proportion of long-chain fatty acids (20:0, 20:1, 20:2, 22:0, 22:1, and 24:0) of PE compared with PC characterized these lipids and persisted after acclimation. In addition to a polyglycolipid (13), trace amounts of MGDG[2] and DGDG were observed (not shown here). These minor components showed higher proportions of saturated fatty acids than the phospholipids . The polyglycolipid, which was the largest glycolipid component, decreased after acclimation and so did MGDG and DGDG but to a lesser degree.

Table I. *Lipid Composition of Oat Root Plasma Membranes of Acclimated and Nonacclimated Plants*

Mean ± SE of two to three isolations.

Lipid Class	Nonacclimated	Acclimated
Free sterols (mol %)	8.9 ± 0.2	13.5 ± 0.7
Steryl ester + steryl glycoside (mol %)	<3	<3
ASG (mol %)	2.7 ± 0.1	3.4 ± 0.4
Cerebroside (mol %)	9.4 ± 1.7	4.7 ± 0.7
Phospholipids (mol %)	79.5 ± 1.5	75.7 ± 4.4
Free sterol/phospholipid	0.12 ± 0.01	0.19 ± 0.01
Lipid/protein (μmol/mg)	1.32 ± 0.43	0.69 ± 0.17

Table II. *Composition of Free Sterols in Acclimated and Nonacclimated Plants*

Mean ± SE of two to three isolations.

Sterol Class	Nonacclimated	Acclimated
Campesterol	14.6 ± 1.9	15.0 ± 0.9
Stigmasterol	68.3 ± 3.7	68.7 ± 2.5
Sitosterol	17.0 ± 2.5	16.4 ± 3.1

[2] Abbreviations: MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

Table III. *Sterol Composition of Acclimated and Nonacclimated Oat Root Plasma Membranes*

Mean ± SE of two to three isolations.

Sterol Class	Nonacclimated	Acclimated
	mol %	
Free sterol	73.5 ± 2.1	80.5 ± 0.4
ASG	26.6 ± 2.4	17.7 ± 2.3
Steryl glycosid	1.0 ± 0.3	0.2 ± 0.02
Steryl ester	1.5 ± 0.4	0.8 ± 0.01
Total sterol (of total lipids)	14.6 ± 0.3	19.9 ± 1.1

Table IV. *Phospholipid Composition of Oat Root Plasma Membranes of Acclimated and Nonacclimated Plants*

Mean ± SE of two to three isolations.

Phospholipid	Nonacclimated	Acclimated
	mol %	
Phosphatidylserine + phosphatidylinositol	3.2 ± 2.2	2.7 ± 2.3
PG	9.3 ± 1.9	4.1 ± 2.8
PC	22.6 ± 1.6	16.4 ± 2.7
PE	20.9 ± 0.7	25.7 ± 8.3
PA	10.9 ± 1.4	9.5 ± 1.0
PC/PE	1.1 ± 0.1	0.69 ± 0.16

DISCUSSION

There are a few detailed descriptions of the lipid composition of highly purified plasma membranes now available. So far there seem to be large differences between the plasma membrane lipid composition of the same plant organ from different species (14, 23, 25) and also between different organs within one species (23, 25). Two lipid analyses of oat root plasma membranes have been reported (10, 25). Of these the data from the analyses of Sandstrom and Cleland (25) show striking similarities with the lipid composition of plasma membranes from the nonacclimated oat of the present investigation. Oat root plasma membranes are characterized by a high content of phospholipids, 79 mol % of total lipids, compared with plasma membranes from other investigated plant organs (14, 23). The major phospholipids are PC and PE in a ratio of 1.1 in nonacclimated plants. The characteristic major lipids are the free sterols and the cerebrosides. MS analysis of the cerebrosides present in oat root cell plasma membranes revealed a glucosylceramide of which >90% contained 24:1-OH as an acyl component in nonacclimated as well as acclimated plants. There was only one sphingosine base, 4,8-sphingadienine (21). Recently long-chain hydroxy fatty acids were found to be the dominant acyl chains of cerebrosides in rye leaf plasma membranes (14).

Of the total sterol content, 74% were free sterols, and of the sterol derivatives, acylated glycosides dominated, a situation that is supposed to be regulated by plasma membrane localized enzymes catalyzing the interconversion between free sterol and sterol derivatives (5, 8). Total sterols amounted to 12 mol % of total membrane lipids, which is a lower content than was found by Sandstrom and Cleland for oat roots (25). Different varieties, different growing conditions, and above all different developmental stages (ages) might play a role. The relative distribution of sterol molecular species was remarkably stable in the membranes of nonacclimated and acclimated plants and was also the case for the fatty acid pattern of the phospholipids with only small nonsignificant changes.

When oat as well as rape plants were exposed to repeated cycles of moderate stress (acclimation), the effect on the membrane lipid level was a marked general decrease. This is in accordance with what Ouedraogo *et al.* (22) found for cotton during water-deficit stress and for field-grown maize during conditions of water deficit (19). A general decrease in membrane lipids was found for whole root lipid analyses as well as for analyses of isolated membrane fractions, independently of which base was used for the data, per plant, fresh weight, dry weight, or mg protein. The membrane lipid decrease is interpreted as causing a reduction of the total membrane area of the cells. It is interesting that total phospholipids measured as mol % of total lipids was not significantly reduced after acclimation, whereas free sterols increased from 9 to 14 mol% which led to an increase of the ratio of free sterols to phospholipids from 0.12 to 0.19. The relative distribution within phospholipids was changed during acclimation. The most notable change was between PC and PE, for which the PC/PE ratio of plasma membranes from nonacclimated plants was 1.1 and from acclimated was 0.69; also, PG, one of the minor components, decreased significantly. Whether PA is a natural component of the plasma membrane is an open question. However, despite precautions to reduce phospholipase D activity (high pH and addition of EGTA), a certain level of PA was present. MGDG and DGDG, known as membrane-building units of plastids, were also found together with the plasma membrane lipids. Whether these trace amounts of MGDG and DGDG found here are true components of the root cell plasma membranes or the result of

Table V. *Fatty Acid Composition of PC, PE, and Total Phospholipids of Acclimated and Nonacclimated Plants*

Mean ± SE of two to three isolations.

Lipid Class	Acyl group						
	16:0	16:3	18:0	18:1	18:2	18:3	Others[a]
				mol %			
Phospholipids							
Nonacclimated	27.9 ± 1.0	1.1 ± 0.3	0.4 ± 0.1	1.6 ± 0.5	50.8 ± 1.5	17.2 ± 0.6	3.0 ± 0.6
Acclimated	26.9 ± 0.8	1.5 ± 0.0	0.6 ± 0.05	2.1 ± 0.5	51.5 ± 1.4	15.3 ± 0.6	3.0 ± 0.9
PC							
Nonacclimated	27.2 ± 0.8	1.6 ± 0.6	0.3 ± 0.05	1.7 ± 0.5	54.1 ± 1.7	15.9 ± 0.4	1.8 ± 0.4
Acclimated	28.4 ± 2.3	1.6 ± 0.2	1.3 ± 1.1	3.8 ± 1.7	49.7 ± 3.8	12.7 ± 1.7	3.2 ± 0.9
PE							
Nonacclimated	32.3 ± 0.9	1.3 ± 0.2	0.4 ± 0.01	1.3 ± 0.1	50.4 ± 0.9	12.9 ± 0.2	3.0 ± 0.5
Acclimated	31.8 ± 1.1	1.9 ± 0.1	0.4 ± 0.01	1.8 ± 0.2	52.0 ± 1.8	12.0 ± 1.8	3.9 ± 1.4

[a] 16:0, 20:0, 20:1, 20:2, 22:0, 22:1, 24:0.

contamination by proplastid envelopes is a difficult question to answer.

The property of mesomorphism of membrane lipids within the liquid crystalline state implies a possibility of lipid phase transitions occurring, at least in certain regions, of biomembranes. In addition, it should be pointed out that the plasma membrane proteins probably play an important role in the mesomorphic phase behavior of the membrane lipids. In view of the dynamic membrane lipid phase behavior, the cerebrosides and PC of our system could be regarded as candidates favoring an L_α phase (3) and as being present in the studied system in proportions preventing phase separations (2). If the proteins are disregarded, a marked increased proportion of PE, and especially with a high degree of unsaturation, might lead to an increased tension in the bilayer favoring phase transitions toward more curved structures. Within the natural bilayer membrane, the increased tension would lead to formation of reversed micellar aggregates. This tendency is further reinforced by the drastic decrease in cerebrosides. However, as studies by Tate and Gruner (29) have shown, to fulfill the requirements for the lipids to fill the reversed hexagonal lattice, especially at larger radii of the water cylinder, the lipid composition should contain a certain level of long-chain hydrocarbons. If the lipid composition is not provided with the distribution of hydrocarbon lengths necessary for the H_{II} phase or reversed micellar aggregates, this could be a factor that favors an L_α phase because fewer geometrical packing constraints exist in that situation.

The phase behavior of microsomal lipids from rape exposed to dehydration acclimation was studied in model systems (20). During the stress program (acclimation), a change of the lipid composition (28) and of the lipid phase behavior were reported. There are now indications that dehydration acclimation also affects the phase behavior of oat root plasma membrane lipids (P. Norberg and C. Liljenberg, unpublished data).

Another factor affecting membrane behavior is the sterol level. During acclimation to dehydration, the relative proportions of sterols was increased. The sterols, regarded as modulators of bilayer lipids, might counteract tendencies of phospholipid demixing and thereby prevent phase transition.

Dehydration of the root cells with reduction of the hydrostatic pressure probably implies temporary shrinkage of the membrane area facing the wall. This could be fulfilled by a reduction of plasma membrane components or by frequent invaginations of the plasma membrane. During dehydration acclimation, the plasma membrane levels of sterols and PE increased. This possibility of changing the membrane lipid composition might be regarded as a system with a capacity to increase its curvature and thereby facilitate repeated invaginations of the membrane.

ACKNOWLEDGMENT

We wish to express our gratitude to Dr. A. S. Sandelius for valuable discussions.

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