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Phase behaviour and molecular species composition of oat root plasma membrane lipids. Influence of induced dehydration tolerance

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Tolerance to dehydration induced by repeated water-deficit stress is well correlated to changes in the lipid composition of oat root cell plasma membranes. The molecular species of the two dominant phospholipids phosphatidylcholine and phosphatidylethanolamine were determined. The four major species were 16:0/18:2, 16:0/18:3, 18:2/18:2 and 18:2/18:3. In contrast to the large changes in plasma membrane lipid composition in other respects, induced tolerance resulted in very weak alterations concerning the phospholipid molecular species pattern. Only minor alterations, appearing as a decrease in the 18:3-containing lipids, occurred. Total lipids of microsomes and isolated plasma membranes of root cells were analysed by X-ray crystallography at different degrees of hydration. The lipid phase behaviour at different degrees of hydration was further confirmed by polarization microscopy. In the presence of excess water all membrane lipids adopted a reversed micellar configuration. The plasma membrane lipids from root cells with induced dehydration tolerance formed upon dehydration two coexisting lamellar structures. The importance of the phase behaviour at different degrees of hydration for the membrane properties and the relation to membrane composition is discussed.

Introduction

In many ecological situations temporary or long periods of water deficit is one of the major environmental factors influencing growth. This means a stress situation where the root system plays an important role for survival. Being often exposed to drastic changes in water potential of the surrounding medium, the root system must be able to resist and acclimate to water deficits. The mechanism behind this phenomenon is far from fully understood but there is evidence in the literature that root cell membranes and above all the plasma membrane changes its composition to acclimate to new conditions [1]. Analysis of the membrane composition of root cells exposed to water deficit stress shows some major changes that implies differences in the membrane permeability [2,3]. As shown by Nyström and Liljenberg [4] oat seedlings with induced water deficit tolerance have a lower ion leakage of the roots as well as a lower relative water content compared to controls during a long term dehydration stress. Further, they maintain a positive turgor pressure much longer during stress exposure than the non-acclimated control plants. A recent study of rape seedlings exposed to repeated water-deficit stress showed changes in total amount of acyl lipids, phospholipid composition and the ratio free sterol to phospholipid [5,6] as well as physical behaviour of the membrane lipids [7].

The aim of the present investigation was to further characterize the plasma membrane of oat seedling roots from control plants and from plants acclimated to water-deficit stress by a molecular species determination of the major phospholipids. The physical behaviour of microsomal and plasma membrane lipids was characterized by studying the mesomorphic phase transitions with low-angle X-ray crystallography and polarization microscopy during hydration.

Materials and Methods

Plant cultivation and dehydration stress induction

Oats (*Avena sativa* L. cv. Seger) were sown in gravel in plastic pots perforated in the bottom. The pots filled with gravel to a height of 9 cm were placed in plastic

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bins containing nutrient solution [5]. The plants were cultivated and exposed to stress in growth chambers with 16 h day (18°C) and 8 h night (12°C). Moderate water-deficit stress was applied on day five by placing the pots on a coarse stainless steel net permitting the gravel to dry effectively. The stress acclimation program consisted of three cycles of 24 h stress + 24 h rewatering. For further details on cultivation and stress treatment see Norberg and Liljenberg [1] and Svenningsson and Liljenberg [5].

Harvest, microsomal and plasma membrane isolation

Total root systems were cut into one cm pieces and were homogenized with a Waring blender in an isolation medium: 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 1.0 mM EGTA. The homogenate was filtered through double layers of nylon cloth and then centrifuged at $10000 \times g$ for 10 min. The supernatant was then centrifuged at $60000 \times g$ for 30 min. The microsomal membranes were resuspended in a 5.0 mM phosphate buffer (pH 7.8) with 0.25 M sucrose and 6.0 mM KCl and added to an aqueous polymer two-phase system containing 6.5% Dextran T500 (Pharmacia, Uppsala, Sweden) and 6.5% polyethylene glycol 3350 (Carbowax Union Carbide, CT, USA) for preparation of a pure plasma membrane fraction [1,8,9].

Extraction of lipids and identification of molecular species

Lipids were extracted from the resuspended membranes by immediately adding 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 aggitation and phase separation the chloroform phase was removed and the aqueous phase washed once with twice the volume of chloroform. Total plasma membrane lipids were evaporated under a stream of nitrogen, dissolved in a small volume of chloroform and applied on a silicic acid column (Silicar CC-4; Mallinckrodt). The column was then eluted in three fractions with chloroform, acetone and methanol in proportions; 1:1.5:1 [3].

The methanol fraction, containing the phosholipids, was then evaporated under a stream of nitrogen and the residue was dissolved in a small volume chloroform/methanol (9:1, v/v), and applied on a HPTLC-plate (Silica 60; Merck, Germany). Mobile phase in the TLC system was chloroform/methanol/acetic acid/0.6 M aqueous ammonium chloride (80:20:10:3, v/v). The bands corresponding to PC and PE were scraped off the plate and lipids were extracted from the gel using chloroform/methanol (1:2, v/v). The solvent was evaporated under a stream of nitrogen and 0.5 ml 10 mM phosphate buffer (pH 7.4) containing 250 μM calcium chloride, 100 μM zinc chloride and 40 units/mg lipid of phospholipase C (*Bacillus cereus*, grade I; Boehringer Mannheim, Germany) was added to the tube. The test tube was then flushed with nitrogen and put in an ultrasonic bath for 10 min at 37°C. The diacylglycerols formed by enzymatic degradation were extracted from the buffer by 2×1 ml chloroform.

The chloroform was then evaporated under a stream of nitrogen and the diacylglycerols were derivatized to tBDMS ethers by addition of equal volumes of 2 M *t*-butyldimethylsilyl chloride in dimethyl formic amide and 5 M imidazole in dimethyl formic amide [10]. The stoppered test tube was then heated to 80°C for 20 min, cooled, and the tBDMS ethers extracted by addition of one volume of diisopropyl ether/water (25:2, v/v). The two phases were separated and the upper phase containing tBDMS ethers were evaporated under a stream of nitrogen, dissolved in a small volume of diisopropyl ether and injected on the gas chromatograph. For gas chromatography a Hewlett-Packard 5890 gas chromatograph equipped with flame ionisation detector and a 5970 HP mass selective detector was used. Injection was in split mode, 1:3, injector temp. 305°C, detector temp. 300°C and oven temp. 275°C. Hydrogen was used as carrier gas for the flame ionisation detector and helium for the mass selective detector. In both cases a 60 m capillary column RTx 2330 (Restek; USA) i.d. 0.25 mm and 0.20 μm film thickness was used.

Preparation for low-angle X-ray crystallography

After evaporation of the solvent, until constant weight, the lipid samples were transferred to steel cuvettes with mica windows. Equilibrium with water took place in the sample cuvettes. The X-ray camera used was built according to Stenhagen [11] with point focus and flat film. Collimator to sample distance was 160 mm.

Polarization microscopy

After X-ray crystallography analysis the samples were immediately studied with polarization microscopy. In addition, dry samples were transfered to microscope slides and covered with cover slips. Water was then cautiously added at the rim of the cover slip. As the water moved in to the lipid sample the changes in birefringence and texture were followed and photographed in the microscope (Vanox, Olympus; Germany).

Results

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were identified by mass spectrometry as tBDMS derivatives of their diacylglycerols. Diagnostic peaks were pseudomolecular ion $(M-57)^+$ and $(RCO+74)^+$ ions [10]. Four prominent molecular species were positively identified, 16:0/18:2, 16:0/18:3,

TABLE I

Results from X-ray diffraction and polarization microscopy analysis of oat root plasma membrane lipids from acclimated and control plants

Samples	Polarization microscopy aspects	X-ray diffraction long spacing lines	Structure
Control			
dehydrated	anisotropic	one sharp band at 49 Å	lamellar
excess water	isotropic, liquid, grainy appearance	one diffuse band at 57 Å	reversed micellar (L2 phase)
Acclimated			
dehydrated	anisotropic	two sharp bands at 53 and 42 Å	coexcisting lamellar phases
excess water	isotropic, liquid, grainy appearance	one diffuse band at 57 Å	reversed micellar (L2 phase)

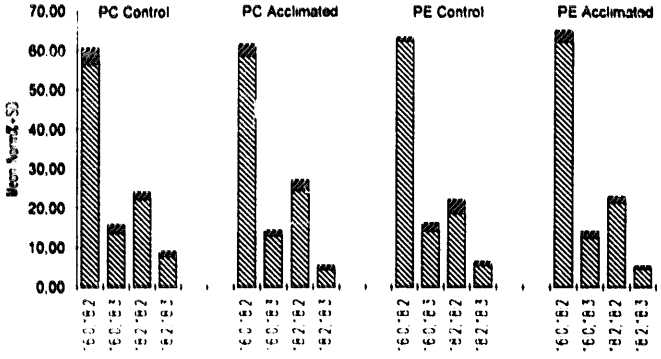

Fig. 1. Molecular species composition of phosphatidylcholine and phosphatidylethanolamine from oat root plasma membranes. Mean normalized percent ± standard deviation.

18:2/18:2 and 18:2/18:3. The peaks were quantified by flame ionisation detection and relative amounts are displayed in Fig. 1. No significant difference in molecular species composition were found between acclimated and control plants except for a small difference in the level of PC 18:2/18:3, which was lower in acclimated plants. The main difference was between PC and PE where PE had a higher level of 16:0/18:2 and a lower level of 18:2/18:2.

A complete phase diagram determination was not obtained due to small amounts of membrane lipid material. Phases were characterized in the dry state and in excess water both with low-angle X-ray diffraction and with polarization microscopy (see Tables I and II).The phase sequences were continuously studied in the microscope during rehydration of the dry lipid samples. The physical behaviour of the microsomal lipids and the plasma membrane lipids was different in the dry state. A one dimensional lamellar structure was found in the dry state of both microsomal and plasma membrane lipids of oat root controls, whereas coexisting lamellar structures were found in plasma membrane lipids from acclimated plants (Table II). The latter phase was anisotropic and showed X-ray long spacing index as a segregated lamellar structure indicating a segregation into two coexisting lamellar phases. In the dry state the microsomal lipids of the acclimated plants were associated into a lamellar structure similar to the control plants. On hydration (excess water) both microsomal and plasma membrane lipids from acclimated as well as control plants were converted to an isotropic liquid phase with grainy apperance as observed in the microscope and with an X-ray pattern with a diffuse single diffraction line at 57Å. The isotropic liquid phase has tentatively been regarded as reversed micellar (L2 phase). These observations were made several times with different samples and the same behaviour was repeated.

Discussion

A repeated moderate water-deficit stress results in an acclimation of oat seedlings to the stress and in-

TABLE II

Results from X-ray diffraction and polarization microscopy analysis of oat root microsomal lipids from acclimated and control plants

Samples	Polarization microscopy aspects	X-ray diffraction long spacing lines	Structure
Control			
dehydrated	anisotropic	one sharp band at 40 Å	lamellar
excess water	isotropic, liquid, grainy appearance	one diffuse band at 57 Å	reversed micellar (L2 phase)
Acclimated			
dehydrated	anisotropic	one sharp band at 40 Å	lamellar
excess water	isotropic, liquid, grainy appearance	one diffuse band at 57 Å	reversed micellar (L2 phase)

TABLE III

Lipid composition of oat root plasma membranes of acclimated and control plants

Lipid class	Control	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
PC/PE	1.1 ±0.1	0.69±0.16

Mean ± S.E. of three isolations [1].

duces an increased tolerance [4]. The plasma membranes of the oat seedling roots has a complex lipid composition and repeated moderate water-deficit stress (acclimation) implies numerous changes of the membrane [1]. The principal changes are shown in Table III. As the free sterols (campesterol 15%, stigmasterol 68% and sitosterol 17%) increase the sterol/phospholipid ratio increases during acclimation [1]. The relatively high level of cerebrosides, a distinct feature of plant plasma membranes, decreases to half the original level. In addition, a drastic change in the ratio of the major phosphlipids PC/PE occurs during acclimation. The total phospholipid level, however, is more or less constant upon acclimation.

The major molecular species of PC as well as PE were 16 : 0/18 : 2, 16 : 0/18 : 3, 18 : 2/18 : 2 and 18:2/18:3. Apart from a significant difference in the molecular species pattern between PC and PE no significant changes occurred in the different proportions of molecular species during acclimation. The fact that PC/PE-ratio showed drastic changes during acclimation although species composition did not, suggests that regulatory enzymes during acclimation are not acyltransferases. The regulation might be on the level of synthesis, degradation or by headgroup transferases [12]. This still remains to be investigated. Similarities in species composition between PE and PC also suggests that acyltransferases are not lipid class specific.

The increased relative proportion of PE, a potent non-lamellar forming lipid, might increase the tension of the plasma membrane.The plasma membrane of oat roots contains a cerebroside with one dominant 24 : 1-OH acyl chain [13]. This long chained lipid would, in a system of otherwise uniform chain composition, favour and lower the lamellar to reversed hexagonal phase transition temperature [14,15]. The decrease in the level of cerebrosides could then be viewed as counteracting the change in PC/PE ratio.The increased level of free sterols following acclimation is conceived to prevent demixing of acyl lipid components. This may otherwise lead to local enrichment of non-lamellar forming components causing phase transitions [16].

To further study the consequences of the changes in the plasma membrane lipid composition following acclimation to dehydration, the physical behaviour of the lipid mesomorphic phases were studied by low-angle X-ray crystallography and polarization microscopy. Total lipids of microsomes and purified plasma membranes from acclimated and control oat seedlings were analysed dry (water residue < 10%) and with excess water present. All samples, control as well as acclimated adopted a reversed micellar configuration (L2 phase) in excess water. The L2 phase has a one-dimensional swelling behaviour as the liquid crystal phase and it may occur in excess water [17]. The dry lipids showed a single lamellar phase with one exception: the lipids of acclimated plasma membranes where the X-ray diffraction pattern revealed two sharp lines at 42Å and 53Å. The X-ray difraction together with the characteristic pattern seen in the polarization microscope is indicating the occurrence of two coexisting lamellar phases. Such segregation might arise of different reasons during dehydration and a likely explanation would be an enrichment and aggregation of different lipids eventually resulting in two separate lamellar phases [18]. The acclimation-induced compositional alteration of the plasma membrane lipids together with different charge densities would then cause segregation of the phases. When the lamellar phases are hydrated the increased disarrangement and increased mobility of the acyl chains implies a more wedgeshaped form of the molecules leading to a transition to a reversed micellar phase. The phase behaviour of the oat plasma membrane and microsomal lipids are not in agreement with that of microsomal lipids from rape seedlings exposed to a similar stress program. Even though some of the changes found for oat plasma membrane lipids also occurred in rape microsomes [5,7].

To put forward a mechanistic explanation for and possible advantage of the altered plasma membrane composition and changed mesomorphic phase behaviour of the membrane to water-deficit stress is difficult at this stage of knowledge. Natural membranes are extraordinary heterogenous, assymetric bilayers with distinct inside and outside. Isolated membrane lipids on the other hand, behave as liquid crystals and could be characterized as relatively homogenous bulk aggregates with well defined water concentrations [19]. One explanation for different lipid behaviour in model systems as compared to the situation in intact membranes is most probably absence of surface active integral proteins. There is no generally accepted model for determining the impact of changes in protein/lipid ratios on membrane physical behaviour. However, to increase the knowledge of structure-function relations of natural membranes it is important to further study

the physico-chemical properties of isolated purified membrane components individually and as complex in simplified model systems.

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