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Alterations of wheat root plasma membrane lipid composition induced by copper stress result in changed physicochemical properties of plasma membrane lipid vesicles

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Abstract

A response when wheat is grown in excess copper is an altered lipid composition of the root plasma membrane (PM). With detailed characterisation of the root PM lipid composition of the copper-treated plants as a basis, in the present study, model systems were used to gain a wider understanding about membrane behaviour, and the impact of a changed lipid composition.

PMs from root cells of plants grown in excess copper (50 μM Cu^{2+}) and control (0.3 μM Cu^{2+}) were isolated using the two-phase partitioning method. Membrane vesicles were prepared of total lipids extracts from the isolated PMs, and also reference vesicles of phosphatidylcholine (PC). In a series of tests, the vesicle permeability for glucose and for protons was analysed. The vesicles show that copper stress reduced the permeability for glucose of the lipid bilayer barrier. When vesicles from stressed plants were modified by addition of lipids to resemble vesicles from control plants, the permeability for glucose was very similar to that of vesicles from control plants. The permeability for protons did not change upon stress.

Electron paramagnetic resonance (EPR) of the lipid vesicles spin probed with *n*-doxylstearic acid (nDSA) was used to explore the lipid rotational freedom at different depth of the bilayer. The EPR measurements supported the permeability data, indicating that the copper stress resulted in more tightly packed bilayers of the PMs with reduced acyl chain motion.

Keywords: Wheat root; Copper stress; Plasma membrane; EPR; Vesicle permeability

1. Introduction

Membranes are the most important structures in plants regulating ion content in cells, and the plasma membrane (PM) of root cells is the first functional barrier that may come in contact with toxic metals. Changes in the lipid composition of PM may contribute to the control of membrane packing properties and also be of importance concerning lipid–protein interactions due to induced changes in the lipid microenvironment surrounding proteins [12]. Subsequently, this influences membrane functions and its efficiency as a selectively permeable barrier [18,21]. Plant cell membranes are dynamic in behaviour, with a lipid composition changing with variations in the environment. The maintenance of the structural integrity of the PM and, as a consequence, of permeability and protein–lipid interaction properties, is a prerequisite for survival during adverse environmental conditions [3,7].

Redox active metals such as copper, when present in excess, play an important role in the onset of peroxidative damage to plasma membrane lipids (PMLs) catalysing the production of free radicals [20,21] and converting hydroperoxides into reactive alkoxy and peroxy radicals [8]. In a previous study, it was evidenced that growth in copper excess (50 μM Cu^{2+}) strongly affected the lipid composi-

Abbreviations: EPR, electron paramagnetic resonance; nDSA, *n*-doxylstearic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PM, plasma membrane; PML, plasma membrane lipid

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tion of wheat root PM, and in particular caused a remarkable decrease in the saturation degree and in the phosphatidylcholine to phosphatidylethanolamine (PC/PE) molar ratio [21]. The observed changes were also a higher K^+ ion efflux and an increased NADPH-dependent superoxide radical formation.

Plant PML vesicles represent useful models for studying physical properties of membrane constituents, and their changes following external perturbations. Moreover, reconstitution experiments can be employed to get information on the contribution of different molecular interactions to the physical behaviour of the membranes when the composition is changed [2,3].

With a detailed characterisation of the Cu^{2+}-treated wheat root PML composition as a basis [21], we intended in the present study to gain a better understanding of the physicochemical properties of PML vesicles. For this purpose, the glucose and proton permeability of vesicles made of lipids extracted from PM of wheat roots grown in the presence of 50 μM Cu^{2+} was studied in comparison with that of vesicles made of total lipids extracted from control wheat root PMs. Vesicles to which exogenous phospholipids (PC and PE) were added, was investigated to establish importance of the PC/PE molar ratio for the permeability properties.

In a study correlational to the permeability measurements, electron paramagnetic resonance (EPR) spectroscopy was used, as a physical method to measure lipid mobility within the lipid bilayer of vesicles. Since PMLs are not paramagnetic, the samples were doped with stearic acid molecules containing a stable nitroxide radical (doxylstearic acid or DSA). Such DSAs have been widely used as spin probes in natural and model membranes to obtain information about the dynamics of the lipid bilayers through their degree of mobility [4,5,11,25]. In the present work, two spin probes, 5DSA and 16DSA, with the nitroxide group located at carbon atoms 5 and 16, respectively, were used to explore lipid rotational freedom at different depths in the bilayer.

2. Materials and methods

2.1. Chemicals

Soybean PC and soybean PE, oleoyl-palmitoyl PC and oleoyl-palmitoyl PE, β-sitosterol, pyranine, valinomycin and DSA spin probes were purchased from Sigma-Aldrich (Steinhem, Germany). Carboxyfluorescein was from Calbiochem (La Jolla, CA, USA). Solvents of analytical grade were from Merck (Darmstadt, Germany).

2.2. Plant cultivation and stress treatment

Wheat seedlings (*Triticum durum* Desf. cv. Creso) were grown in hydroponic culture with continuous aeration in a growth chamber with day/night temperatures of 21/16 °C, a 16-h photoperiod, a photon flux density of 400 μmol m^{-2} s^{-1} and 70–75% RH. For further details on plant cultivation, see Ref. [21]. In the preparation of the nutrient solution, precautions were taken to avoid the use of salts containing Cu^{2+} as a contaminant. Plants were continuously grown with either nutrient solution containing 0.3 (control, C) or 50 μM Cu^{2+} as $CuSO_4$ (stress, S) for 11 days. Roots were then harvested, rinsed with cold water and used immediately for PM preparation.

2.3. Harvest and membrane preparation

At harvest, roots were cut into 2-cm pieces and immediately homogenised using a Waring blender in two volumes of an extraction medium consisting of 50 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 3.0 mM Na_2EDTA, 10.0 mM ascorbic acid and 5.0 mM diethyldithiocarbamic acid. The homogenate was filtered through four layers of a nylon cloth and centrifuged at 10,000 × *g* for 10 min. The supernatant was further centrifuged at 65,000 × g for 30 min to yield a microsomal pellet, which was resuspended in 2 ml of a buffer containing 5 mM K-phosphate, pH 7.8, 0.25 M sucrose and 3.0 mM KCl. PMs were isolated using an aqueous two-phase system originally developed for wheat leaves [9], but optimized for wheat roots. The microsomal suspension (1.00 g) was loaded onto the aqueous two-phase polymer system to give a final composition of 6.5% (w/w) Dextran T500, 6.5% (w/w) polyethylene glycol, 5.0 mM K-phosphate (pH 7.8), 0.25 M sucrose and 3.0 mM KCl. The PM was further purified using a two-step batch procedure. The resulting upper phase was diluted 4-fold with 50 mM Tris–HCl (pH 7.5) containing 0.25 M sucrose, and centrifuged for 30 min at 100,000 × g. The resultant PM pellet was resuspended in the same buffer containing 30% ethylene glycol and stored at − 80 °C. All steps of the isolation procedure were carried out at 4 °C. To check the purity of the PM, the activity of the vanadate-sensitive ATPase as a marker enzyme was determined. Cytochrome *c* oxidase, NADH cytochrome *c* reductase and NO_3^--sensitive ATPase activities were used as markers of mitochondria, endoplasmic reticulum and tonoplast, respectively [21].

2.4. Lipid extraction

The PM suspension was thawed on ice and the lipids were extracted by addition of two volumes of boiling 2-propanol followed by two volumes of chloroform/methanol (2:1, v/v) containing butylhydroxytoluol (50 μg ml^{-1}), two volumes of chloroform, and one volume of distilled water. After gentle agitation and phase separation by centrifugation, the chloroform phase was collected. The aqueous phase was washed once more with two volumes of chloroform. The two chloroform phases were pooled and stored at − 18 °C under N_2.

2.5. Experimental design

Stock solutions of lipid mixtures in chloroform were prepared. The different mixtures used for the model experiments are described in Table 1. For vesicle preparation, the samples of each mixture were evaporated to dryness at 30 °C under N_2.

2.6. Vesicle preparation

The vesicles were prepared according to the procedure of Berglund et al. [2,3], however modified and scaled to require only one fifth of lipid material. The proton and glucose permeability measurements were performed on the same vesicles and therefore the vesicles were loaded with both carboxyfluorescein and pyranine. In a test tube, 100 μl of 10.0 mM HEPES–NaOH buffer (pH 7.7) containing 1.0 mM EDTA, 50.0 mM KCl, 1.0 mM carboxyfluorescein and 2.0 mM pyranine were added to 1.0 mg of lipids. The test tube was heated in a water bath (60 °C) for 2 s and then shaken. The procedure was repeated five times. The vesicles were then frozen in liquid nitrogen and immediately thawed in the water bath, again for five times. The multilamellar vesicles were then incubated at room temperature (22 °C) for 15 min before the extrusion step. The unilamellar vesicles were produced by a high-pressure extrusion technique [15,16]. The vesicles were extruded seven times through a double polycarbonate filter, pore size 200 nm, using a Liposofast Basic Equipment (Avestin, Ottawa, Canada). Valinomycin was added after the extrusion step and the vesicles were incubated overnight at 4 °C [2,6]. Unilamellar vesicles were separated from untrapped fluorescence dye by gel exclusion chromatography on a G-75 Sephadex column (0.5 $cm^2 \times 15$ cm). A UV lamp was used for detection of the vesicles on the column.

2.7. Permeability measurements

Due to the limited amount of PML, both glucose and proton permeability measurements were performed on the same vesicles. Preliminary experiments did not show any interference between pyranine and carboxyfluorescein (data not shown). The fluorescence of carboxyfluorescein is not pH dependent above pH 6.5, and pyranine is not concentration dependent.

The proton permeability was measured by adding HCl to the vesicle suspension. To 1.5 ml vesicle suspension, 50 μl of 0.1 M HCl was added causing a drop in the pH of the external solution from 7.7 to 7.1 ($\pm$ 0.05). The permeation of protons across the membrane was recorded as a decrease in fluorescence. The pH of the vesicle suspension was measured before and after each permeability measurement. The pyranine fluorescence was monitored in a quartz cuvette at 508 nm (excitation 460 nm) [2,6].

The glucose permeability was then measured by adding 1.5 ml of 1.6 M glucose solution to 1.5 ml vesicle suspension causing an immediate drop in the fluorescence. The subsequent increase in fluorescence was then monitored at 515 nm (excitation 494 nm) [2,3]. Fluorescence was recorded on an Aminco, SLM 8000 C fluorescence spectrophotometer (SLM Instruments, Urbana, IL). Both permeability measurements were performed at 22 °C.

2.8. Data analysis

The obtained fluorescence data follow a 'saturation in time' type of curve. However, for the first 200 and 100 s of the glucose and proton permeability measurements, respectively, a linear response (r^2>0.95) was observed which allowed the initial slope of the fluorescence function to be calculated. The obtained values were normalised and compared to the data of soy-PC, for which the initial slope of fluorescence vs. time was set to 1.0 [2,3].

2.9. Spin labeling and EPR measurements

Stock solutions of the fatty acid spin probes, 5- and 16DSA, were made up in ethanol (1 mg ml^{-1}). The probes were added to vesicle lipids dissolved in chloroform at a label to lipid ratio of 1:100 (w/w). This ratio was chosen to obtain a good quality of the signal avoiding spin–spin interactions. The solvent of the lipid preparations was removed under a stream of N_2. Fifty microliters of an aqueous buffer consisting of 10 mM HEPES–NaOH (pH 7.6), 1 mM Na_2EDTA and 50 mM KCl was added to the dry residues and vesicles were prepared as described above. For EPR analysis, the vesicle suspension was transferred to a 100-μl glass capillary tube, which was sealed and inserted into a quartz sample holder and put in the microwave cavity of the spectrometer. Spectra were recorded at 25 °C using a Varian (Palo Alto, CA) E-112 spectrometer equipped with a Varian E257 temperature control unity. The spectrometer was interfaced to a 100-MHz personal computer by means of an acquisition board [1] and a software package especially designed for EPR and ENDOR experiments [19]. For each spectrum, a field setting of 3265 G, a microwave power setting of 10 mW and a modulation amplitude of 1.25 G were employed.

Table 1
Phosphatidylcholine to phosphatidylethanolamine (PC/PE) molar ratio and double bond index (DBI) of vesicles used in the experiments

Vesicle	PC/PE	DBI
Control	0.7	2.1
Cu-stressed	0.3	0.9
Control + PE	0.4	1.7
Cu-stressed + PC	0.5	2.0
PC/PE/sitosterol	0.3	1.6
PC/PE/sitosterol	0.7	2.1
Soy-PC	–	12.2

Table 2

(a) Results from the one-way ANOVA on the effects of different lipid composition for the PML vesicles on permeability behaviour

	DF	Mean square	*F*	P-value
Glucose permeability	4	0.00006632	24.86	< 0.0004

(b) Results from Tukey's multiple comparisons test on the effects of different lipid composition for the PML vesicles on glucose permeability

	C	S	S + PC	C + PE	Soy-PC
C	–	**	ns	*	ns
S	**	–	**	***	**
S + PC	ns	**	–	ns	ns
C + PE	*	***	ns	–	ns
Soy-PC	ns	**	ns	ns	–

ns: Not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

2.10. Statistical treatment

All data from the permeability measurements were statistically treated by one-way ANOVA analysis, Tukey's post hoc, by the software SAS-system for Windows version 8, and the statistical analysis is displayed in Table 2.

3. Results

3.1. Effects of Cu^{2+} excess on root PMs

Growth of wheat in a hydroponical medium containing 50 μM Cu^{2+} resulted in effects at different organisation levels. As reported in a previous paper [21], growth in Cu excess resulted in an altered PML composition, a decreased PC/PE ratio (from 0.7 for the control to 0.3 for the Cu-treated sample), an increased proportion of saturated fatty acids and a reduction in the lipid/protein ratio. In addition, the PM pellet fraction isolated from the copper-stressed plants was somewhat darker in colour compared to the off-white pellet from the control plants.

3.2. Vesicle preparation and composition

Large unilamellar vesicles (∅ 200 nm) used for permeability and EPR measurements were prepared from the total lipid extract of PM fractions isolated from Cu-stressed and control wheat roots. To avoid the lack of lipid material usually occurring in this kind of investigations, the membrane vesicle preparation procedure was modified to a reduced scale. By using this new experimental approach, it was possible to prepare each sample from 1 mg of PML. Moreover, it was possible to have two fluorescent markers, carboxyfluorescein and pyranine, present in the vesicles at the same time so that permeability to glucose and protons could be analysed on the same sample. To better understand the permeability and EPR results of PML vesicles from stressed (S) and control (C) plants, vesicles artificially enriched with specific lipids were also prepared. In particular, PML of stressed plants were enriched with soy-PC and 16:0/18:1 PC (sample S + PC) to increase the PC/PE ratio and their double bond index (DBI) to values similar to those of control PML (Table 1). On the other hand, control vesicles were modified in an attempt to mimic the vesicles of PML from stressed plants (sample C + PE). For this purpose, they were enriched with 16:0/18:1 PE which resulted in an increase in the proportion of PE and a slight reduction of the DBI (Table 1). However, the lowest PC/PE ratio that could be reached was 0.4 due to the difficulty in obtaining stable vesicles with increasing proportion of PE.

Model vesicles were also prepared with soy-PC and soy-PE, and also 16:0/18:1 PC and 16:0/18:1 PE, added in the same proportions found in control and stressed root PMs, and their permeabilities were measured and used as reference (Table 1). The soy-PC and PE used had a molecular species composition very similar to PC and PE of wheat root cell PMs (control roots). Furthermore, similar PC + PE vesicles containing β-sitosterol (10 mol%), which is the predominant free sterol of wheat root PMs [21] were made (Table 1).

3.3. Permeability measurements

A comparison of the permeabilities to glucose of the different vesicles investigated is shown in Fig. 1. Vesicles prepared from soy-PC with little variation in permeability data are used as a reference. On the other hand, a large variation in permeability was observed for the PC + PE + sitosterol model vesicles (data not shown), reflecting the difficulties in preparing vesicles with low mass ratios of these unsaturated lipids.

Control vesicles showed a high permeability to glucose. On the contrary, a significantly reduced permeability to glucose was found for the S vesicles as compared to the control (Fig. 1). The S + PC modified vesicles showed a permeability to glucose very close to that of the control,

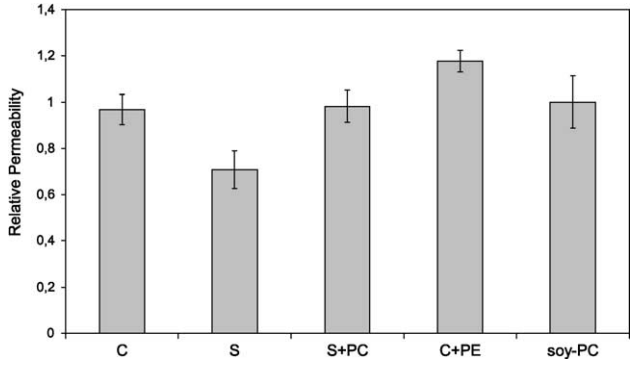

Fig. 1. The permeability of the plasma membrane lipid vesicles for glucose given as the relative permeability. Control (C), copper stressed (S), phosphatidylethanolamine (PE), phosphatidylcholine (PC). $n = 3$ (C,S,S+PC, C+PE), $n = 4$ (soy-PC). The data represent the means ± S.D.

whereas the PE-enriched vesicles showed the highest permeability to glucose. No differences were observed in the proton permeability of any of the PML vesicles (data not shown). The statistical significance of the pemeability data is displayed in Table 2.

3.4. EPR measurements

Lipid chain mobility was investigated at two depths in the bilayers by stearic acid analogues, 5DSA and 16DSA, spin labeled at two different positions along the hydrocarbon chain. In the case of 5DSA, the nitroxide moiety is five carbons away from the carboxyl headgroup. This spin probe therefore reports on the mobility of the double bilayer regions close to the polar lipid headgroups. On the other hand, 16DSA has its nitroxide moiety 16 carbons away from the headgroup and reports on the lipid mobility close to the core of the bilayer. EPR spectra of vesicle-incorporated 5DSA and 16DSA recorded at 25 °C are shown in Figs. 2 and 3, respectively. Line shapes indicate that as the nitroxide probe was placed further down the fatty acyl chain toward the center of the bilayer, the spectra displayed a rotational mobility gradient typical of lipid bilayers [17], from a relatively restricted polar head region to a more mobile terminal methyl region. A minor second component typical of spin probes undergoing fast isotropic motion appeared in all the recorded spectra. This sub-spectrum can be attributed to probes that were not incorporated into the vesicle bilayer, and was considered insignificant for vesicle mobility. Besides this minor component, spectra of 5DSA showed high anisotropy having resolved extrema both in the wings and in the central region, as typical for limited motions of the chain segments close to polar headgroups. Such spectra

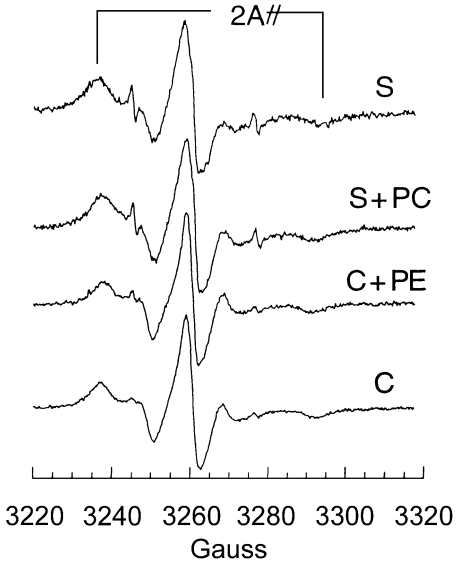

Fig. 2. EPR spectra of 5DSA incorporated in PML vesicles at 25 °C. Control (C), copper stressed (S), phosphatidylethanolamine (PE), phosphatidylcholine (PC).

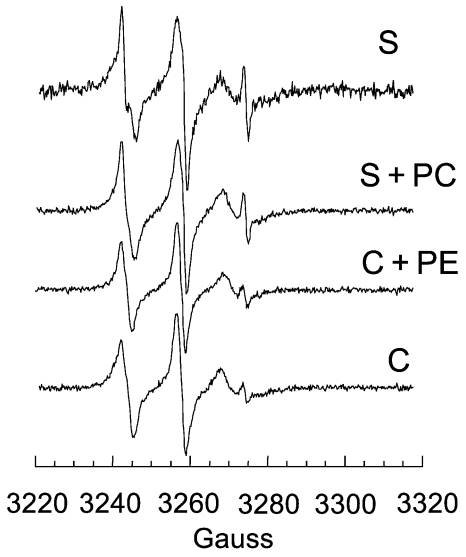

Fig. 3. EPR spectra of 16DSA incorporated in PML vesicles at 25 °C. Control (C), copper stressed (S), phosphatidylethanolamine (PE), phosphatidylcholine (PC).

could be further analysed by determining the outer (or parallel) splitting $2A_{//}$, which is inversely related to lipid mobility [4,5]. On the basis of this spectral parameter, the lowest mobility was shown by the S sample ($2A_{//} = 57.6 \pm 0.5$ G). A higher mobility was found for lipids from control roots ($2A_{//} = 55.9 \pm 0.5$ G), whereas S + PC and C + PE vesicles showed a higher mobility than C vesicles ($2A_{//} = 54.4 \pm 0.5$ and 53.7 ± 0.5 G for S + PC and C + PE, respectively).

Spectra of 16DSA incorporated into the bilayer did not show anisotropy at 25 °C. In fact, three-line spectra appeared (Fig. 3) with line widths sensitive to motions. Such spectra can be characterised by an effective correlation time (τ_R) for the spin probe motion [4,5] by assuming that the motion is in the fast regime and isotropic [14,22].

The highest τ_R value was found for the S vesicles ($\tau_R = 2.2 \pm 0.1$ ns), indicating a more hindered dynamics of the spin probe in the lipid bilayer of the stressed sample. 16DSA in the control vesicles showed a correlation time ($\tau_R = 2.0 \pm 0.1$ ns) shorter than in the S ones, but longer than in the S + PC and C + PE samples ($\tau_R = 1.7. \pm 0.1$ ns and $\tau_R = 1.8 \pm 0.1$ ns, respectively).

4. Discussion

Data from the present work show that it is possible to isolate PMs using the two-phase-partitioning method, extract total lipids from these membranes and get a sufficiently large amount of lipids, for vesicle preparation. Results of glucose permeability studies on model vesicles prepared from soy-PC + PE in proportions similar to those of the corresponding phospholipids of wheat PMs showed large variability, and did not result in treatable data. How-

ever, the tendency was that an increase in the proportion of PE resulted in increased permeability to glucose of the vesicles (data not shown). The large variability may be associated with the destabilizing effect of PE which is a non-lamellar forming lipid. It also reflected the difficulties in preparing vesicles with low mass ratios of unsaturated lipids. Results from preliminary experiments have shown that vesicles with a simple composition cannot be loaded with high proportions of PE without impairing vesicle stability (data not shown). Above a certain threshold concentration, PE might cause a high tension of the monolayers resulting in reduced stability of the bilayers [10] causing an increased permeability for low molecular substances such as glucose. The addition of β-sitosterol to vesicles made only of PC and PE caused a stabilisation of the vesicles, with a lower variation in permeability data (data not shown).

Stable vesicles were obtained from PM total lipids of control as well as of copper-stressed plants. When compared to model vesicles, PML vesicles from control plants showed a higher permeability to glucose. The lipid composition of these vesicles was characterized by a relatively high DBI (2.1), where PE itself had an even higher DBI (2.8). The higher permeability could be explained by the high content of unsaturated PE, due to its packing characteristics.

Copper-treated PMs were characterised by a much lower PC/PE ratio in comparison with the control plants, and phospholipids changed their fatty acid composition toward a higher saturation degree [21]. As far as PML vesicles are concerned, these changes implied that the relatively larger proportion of the non-lamellar forming lipid PE, which would have caused large tension in the individual lipid layers and destabilisation of the vesicle membrane, in this case was to a large part counteracted by the more saturated fatty acids. As a result, the PML vesicles were stable and with a significant low permeability to glucose in comparison with the control. It is, however, difficult to explain the low permeability exclusively referring to the higher saturation degree of the phospholipids. Following copper treatment, no changes in the levels of the minor phospholipids as well as the cerebrosides and free sterols of the PMs were observed. Thus, these components do not seem to be responsible for the reduced permeability. On the other hand, the sterols might interact preferably with the more saturated phospholipids favouring a reduced permeability [24]. Furthermore, we cannot exclude the presence of a thus far unknown component of the PML vesicles from stressed plants with the effect of increasing the packing order of the lipids resulting in lowered glucose permeability.

To provide more evidence that the different permeability behaviour of the PML vesicles made from copper-stressed and control plants was the result of a modified lipid composition, a method was used where PML vesicles were enriched with specific lipids. In the first case, PC molecular species were added to PML vesicles from stressed plants resulting in an increased PC/PE ratio and also an increased DBI, resembling the composition of the PML vesicles made from control plants (Table 1). The permeability properties of these modified PML vesicles from copper-stressed plants were very similar to those from control plants (Fig. 1).

In another test, an attempt was made to mimic the composition of the PML vesicles from stressed plants by addition of PE species to vesicles from control plants. However, it was not possible to reduce DBI below the value of 1.7 although 16:0/18:1 PE was used. Following the addition of PE, the permeability to glucose increased above the values for PML vesicles from the control plants. This might partially be explained by the high DBI, with a high content of unsaturated PE, causing destabilisation and increased permeability.

Regarding the constant proton permeability of the PML vesicles, these results show that the dramatic changes in lipid composition during Cu stress did not affect bilayer permeability to protons. Sustained proton gradients at the PMs of the root cells are prerequisites for a selective ion uptake and in the longer perspective for survival.

EPR spectroscopy has been widely used for the quantitative assessment of the rotational mobility of lipid molecules and as a measure of molecular order within membranes [13,20,23]. The EPR measurements indicate that lipids extracted from copper-stressed wheat root PMs form more tightly packed vesicles in which the molecular motions of the acyl chains are reduced. Moreover, investigations on nitroxide radicals probing the bilayer at different depth suggest that changes in mobility associated with stress involve different parts of the bilayer in a similar way (Figs. 2 and 3). To summarise, the results from the EPR measurements support the permeability results, and the copper stress resulted in more tightly packed bilayers of the PMs with reduced acyl chain motion.

When confronted with a change in the physical environment, cells carefully balance the content of unsaturated fatty acids and lamellar phase-forming and non-lamellar phase-forming lipids in their membranes. Changes in the PML composition of the root cells as a response to copper stress is a consequence of copper toxicity with effects on lipid metabolism and lipid transport to PM but might also be a reaction of the cells to sustain proton gradients and maintain the membrane permeability barrier.

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