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Binding of Sterols Affects Membrane Functioning and Sphingolipid Composition in Wheat Roots

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Abstract—The present work was devoted to the exploration of the role of sterols in the functioning of membranes in root cells. Membrane characteristics and composition of the membrane lipids in the roots of wheat (*Triticum aestivum* L.) seedlings treated with exogenous cholesterol and antibiotic nystatin, which specifically binds with endogenous sterols, were analyzed. Cholesterol caused a fall of membrane potential, acidification of the incubation medium, decrease in potassium leakage of roots, and increase in the level of exogenous superoxide radical. Similarly to cholesterol, the application of nystatin also induced the depolarization of the plasma membrane, but in contrast with cholesterol it was accompanied by alkalinization of the incubation medium and decrease in the level of exogenous superoxide radical. Analysis of membrane lipids showed that following nystatin treatment the total sterol content in roots did not change, while the level of complex sphingolipids represented mainly by glycoceramides became higher. Using mass spectrometry with electrospray ionization ($^+$ESI-MS) for the analysis of the glycoceramide composition, we showed that nystatin induced changes in the ratios of molecular species of glycoceramides. It was suggested that the modification of the sterol component of plasma membrane could influence membrane functioning by changing the sphingolipid composition of lipid rafts.

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Biological membranes are heterogeneous, constantly changing structures, certain parts of which are enriched in sphingolipids and sterols and organized into specific lipid domains – rafts [1]. These structures are supposed to regulate the transduction of various signals into the cell, including those initiating cell division, differentiation, and various stress responses [2]. In addition, it was found that the detergent-resistant membranes of plant cells are particularly rich in biologically important hydrocarbon synthases, in particular callose and cellulose synthases, involved in the synthesis of cell wall. Specifically, 73% of plasma membrane glucan synthesis is detected in the detergent-resistant membranes [3]. Physicochemical properties of lipid rafts are defined, among other conditions, by the qualitative composition of contained sterols, the depletion of which can lead to the destruction of these domains and release of their contents from the membranes [4]. There is no doubt that the change of sterol component entails significant changes in the membrane functioning in general. It was shown that the addition of sterols to model membranes composed of phospho- and sphingolipids promotes ordering of their saturated acyl chains, but it retains the lateral mobility of lipids [4]. Most studies on the mechanism of regulation by sterols of membrane physicochemical properties were carried out by using cholesterol. It was found that cholesterol facilitates denser packing of liquid-organized domains of artificial membranes by reducing the spaces between lipid

Abbreviations: FA, fatty acids; O_2^-, superoxide anion-radical; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ROS, reactive oxygen species; TLC, thin-layer chromatography.

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molecules [5]. This sterol has a condensing effect on phospholipids, forming complexes of fixed stoichiometry with phospholipids. In addition, cholesterol can play an important role in relieving tension between the liquid-organized and liquid-unorganized domains [6].

Most studies on the role of sterols in membrane functioning are carried out on model lipid membranes and isolated membranes of animal origin. However, despite the fact that cholesterol is the main membrane sterol of animals, it has also been found in plant membranes, particularly in detergent-resistant membranes of *Arabidopsis thaliana*, where its content can reach 19% of total sterol amount [7]. So it can be supposed that membrane sterols are key links in the ion permeability regulation of plant membranes as well. This was confirmed in experiments with the polyene antibiotic nystatin specifically binding with sterols [8]. It was shown that under the action of nystatin there is a significant increase in membrane conductivity of plant cells for water molecules [9], potassium ions, as well as increased oxygen consumption of cells and the increase in the rate of heat release [10]. However, the biochemical mechanisms of the involvement of sterols in the plant membrane functioning remain insufficiently studied. In particular, it remains unclear how binding or enrichment of the sterol component of plant membranes can influence the composition of membrane lipids, including phospho- and sphingolipids. How can changes in the composition of these lipids affect the functioning of the membranes?

The goal of this work was to study the membrane characteristics and composition of membrane lipids in the wheat roots following their treatment with of exogenous cholesterol and binding of endogenous sterols by nystatin.

MATERIALS AND METHODS

Experiments were performed on the roots of 4-day-old wheat seedlings (*Triticum aestivum* L.), Kazan jubilee variety. Seedlings were grown in H_2O (control) or 50 μM cholesterol at 20°C. Immediately after cutting off the roots, they were incubated in 0.25 mM $CaCl_2$ with 50 μM cholesterol or 20 μM nystatin for 2 h. Cholesterol and nystatin were dissolved in 0.2% dimethyl sulfoxide (DMSO). Roots incubated in 0.25 mM $CaCl_2$ solution with the addition of an appropriate quantity of DMSO served as a control. In all variants, the original incubation solutions did not contain potassium ions and had pH of 6.0.

The potassium ion content in the root incubation medium was determined using a Phlapho-41 flame photometer (Carl Zeiss, Germany). The pH was measured using a Mettler Toledo pH instrument (USA). The membrane potential of wheat root cells was measured using a standard microelectrode technique [11]. Superoxide radical content ($O_2^{\cdot-}$) was determined by the conversion of epinephrine to adrenochrome [12], which was estimated spectrophotometrically (480 nm; $\varepsilon = 4020\ M^{-1}\cdot cm^{-1}$). Specificity of $O_2^{\cdot-}$ formation was confirmed with the use of superoxide dismutase.

Lipids were extracted with a mixture of isopropanol and chloroform using the method of Nichols [13] with modifications [14]. Individual phospho- and sphingolipids were analyzed with two-dimensional thin-layer chromatography (TLC) on 6 × 6 cm plates with 5-20 μ silica gel according to the method of Vaskovsky and Terekhova [15] with modifications in the solvent systems chloroform–methanol–toluene–28% ammonia (65 : 30 : 10 : 6) (first direction) and chloroform–methanol–toluene–acetone–acetic acid–water (70 : 30 : 10 : 5 : 4 : 1) (second direction). Chromatographic zones corresponding to the lipid compounds were visualized with 5% solution of H_2SO_4 in ethanol or iodine vapor. Glycerolipids were identified with standard markers and specific reagents for individual functional groups [16]. Individual classes of phospholipids were eluted with chloroform–methanol (1 : 1) to investigate their fatty acid composition. Glycoceramides were eluted according to a similar scheme.

Fatty acids from glycerolipids were analyzed as methyl esters, which were prepared by methanolysis of individual lipids with 2.5% H_2SO_4 in methanol at 70°C for 2 h. The fatty acid methyl esters were separated on a gas–liquid chromatograph (Kristall 5000.1; Russia) using a Hewlett Packard HP-23 (30 m × 0.32 mm × 0.25-μm) capillary column (USA). The initial thermostatted temperature of 170°C was maintained for 3 min with a further increase to 220°C at rate of 4°C/min. The evaporator temperature was 230°C, and the detector temperature was 250°C. The carrier gas was nitrogen. Fatty acids were identified by comparing the values of their retention times with standard markers (Sigma, USA).

The molecular type composition of glycoceramides (GlCer) isolated from control and nystatin-treated roots was determined by the value of m/z using an MX-5310 high resolution time-of-flight mass spectrometer with orthogonal input and electrospray ion source (ESI-TOF) developed in the Institute of Analytical Instrument Engineering, Russian Academy of Sciences. All spectra were obtained in the positive ion mode in the range of 150-2000 m/z. The capillary spray voltage was 3.3 kV, and the temperature of the dissolving gas was 50°C. Samples were dissolved in methanol–acetonitrile–water–formic acid (44 : 44 : 10 : 2). The sample volume was 10-50 μl, and sample flow rate was 1.5 μl/min.

The structure of GlCer molecular types was analyzed in detail on control samples with a Thermo 7-T LTQ FT mass spectrometer (Germany) with electrospray ionization (ESI) equipped with a Proxeon Biosystems electrospray ion source (Denmark). Instrument readings were collected in positive ions mode. Capillary voltage was 1.6 kV. Temperature of the converting capillary was 225°C. Mass spectra were recorded in the range from 200

to 1500 m/z. Individual ions were fragmented in the collision cell of the mass spectrometer at 30 eV in the instrument ion trap. Fragment ions were measured both in the ion trap and in the FT part of the mass spectrometer. Mass spectra were processed with the Xcalibur program and interpreted manually.

Amount of total glycoceramides and individual phospholipids was determined with a DenSkan densitometer (Russia). Standard solutions of phosphatidylcholine and galactosylceramide (Sigma) were used to construct calibration curves.

The experimental data were processed by the method of variation statistics. Biological experiments were repeated in triplicate with triplicate analytical tests. The tables and figures present mean values and standard errors. Statistical significance of differences between the control and the experiment samples was assessed with Student's criterion with confidence level $P_1 = 0.95$.

RESULTS

Cholesterol and sterol-binding nystatin induced significant changes in membrane characteristics of wheat roots. Treatment of roots with 50 μM cholesterol or 20 μM nystatin caused significant plasma membrane depolarization (Table 1), but these compounds had the opposite effect on the membrane permeability to ions. Growing wheat seedlings and subsequent 2-h incubation of excised roots in a solution of 50 μM cholesterol led to acidification of the incubation medium by 0.6 pH unit compared with the control sample (Table 1). As seen in Table 1, both in control sample and in the presence of exogenous cholesterol the incubation of excised roots was accompanied by the release of potassium ions from the cells. However, under cholesterol treatment, the plasma membrane permeability for K^+ was significantly less than in the control sample, which was reflected by a reduced content of K^+ in the incubation medium. A 2-h incubation of roots with 20 μM nystatin promoted apoplast alkalinization as well as a significant K^+ release from cells (Table 1).

Cultivation of seedlings and incubation of roots in the presence of cholesterol caused a significant increase in extracellular $O_2^{\cdot-}$ (Table 1). In contrast, treatment of roots with nystatin resulted in significant decrease in content of extracellular $O_2^{\cdot-}$ (Table 1).

Study of the composition of membrane lipids showed that the binding of endogenous sterols after 2 h treatment of

Table 1. Effects of cholesterol and of nystatin on membrane potential, pH, K^+ content in the incubation medium, and the formation of $O_2^{\cdot-}$ in wheat roots (2 h incubation)

Treatment	Membrane potential, mV	pH of incubation medium	K^+ content in incubation medium, μeq/h per g fresh weight	$O_2^{\cdot-}$ content, μmol/min per g fresh weight
Control	100.3 ± 0.2	5.3 ± 0	0.8 ± 0.3	8.2 ± 0.3
Cholesterol (50 μM)	77.8 ± 1.4	4.7 ± 0	0.2 ± 0.1	12.1 ± 0.8
Nystatin (20 μM)	72.5 ± 1.2	5.8 ± 0.1	10.6 ± 0.3	4.8 ± 0.3

Table 2. Content of the main fatty acids in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) after 2-h nystatin treatment of roots

Fatty acids	Content in PC, %		Content in PE, %	
	control	nystatin (20 μM)	control	nystatin (20 μM)
14:0	1.7 ± 0.2	0.9 ± 0.4	2.0 ± 0.5	1.9 ± 0.5
16:0	34.6 ± 8.7	34.9 ± 3.1	31.8 ± 1.8	32.3 ± 4.9
$16:1^{\Delta9}$	2.3 ± 0.9	2.5 ± 1.6	4.4 ± 0.5	4.2 ± 0.1
18:0	4.9 ± 0.1	5.3 ± 0.7	5.5 ± 0.5	8.7 ± 4.1
$18:1^{\Delta9}$ + $18:1^{\Delta11}$	12.9 ± 0.1	8.7 ± 1.1*	13.7 ± 2.4	13.9 ± 2.4
$18:2^{\Delta9,12}$	27.6 ± 2.8	34.3 ± 0.7**	28.2 ± 2.7	27.1 ± 0.2
$18:3^{\Delta9,12,15}$	11.4 ± 4.1	11.3 ± 0.2	9.1 ± 0.8	8.7 ± 0.4

* Here and in Table 3, differences significant at $P < 0.01$.
** Here and in Table 3, differences significant at $P < 0.05$.

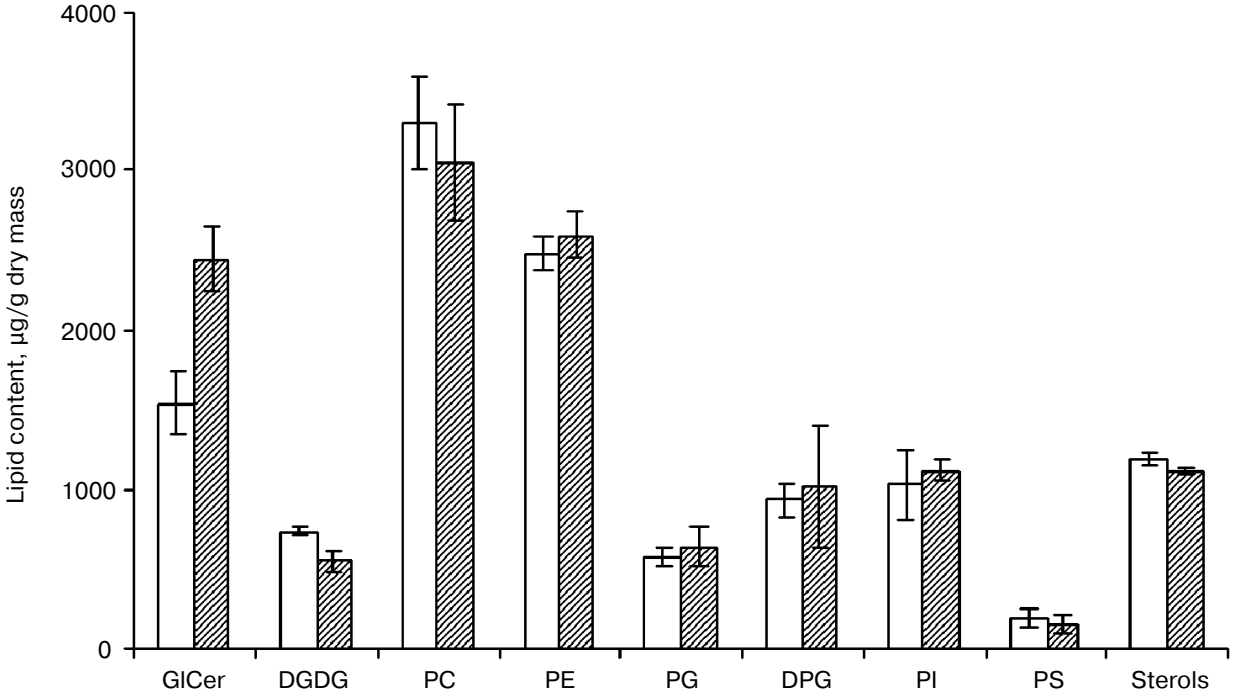

Fig. 1. Composition and content of membrane lipids in roots of wheat treated with 20 μM nystatin. Control, open columns; nystatin, shaded columns. GlCer, glycoceramides; DGDG, digalactosyldiacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

roots with nystatin does not change the content of glycerolipids including digalactosyldiacylglycerols and individual classes of phospholipids compared with the control level. Analysis of fatty acids (FA) of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) also did not show any significant changes induced by nistatin. Only a slight decrease in the relative content of $C_{18:1}$ ($18:1^{\Delta 9}$ + $18:1^{\Delta 11}$) acid was registered, which was compensated by an increased level of $C_{18:2}$ in the PC fraction (Table 2). Interestingly, the sterol content also remained unchanged (Fig. 1).

Nystatin treatment had the greatest influence on sphingolipids, namely the qualitative and quantitative composition of glycoceramides (GlCer). Total content of GlCer in the roots under treatment with the channel former increased approximately 1.5-fold (Fig. 1). Furthermore, as revealed by mass spectrometry (ESI-TOF), nystatin treatment altered the ratio of GlCer molecular species (Table 3). The mass spectra of total GlCer of wheat roots mostly recorded ions of $[M+Na]^+$ with m/z 736, 738, 764, 822, and 864. There were also trace amounts of ions with m/z 792, 794, 850, and 866. The mass spectra of control samples were dominated by molecular ions with m/z 764 and 822, whereas in nystatin-treated samples the spectra were dominated by ions with m/z 736 and 738, and the relative abundance of ions with m/z 764 and 822 substantially decreased.

The GlCer molecular species were identified by analysis of their fragments obtained with tandem mass spectrometry ($^+$ESI-MS/MS). As illustrated by the three molecular species of GlCer with m/z 736, 764, and 866, the fragmentation of compounds of this class was accompanied by a loss of 162 mass units, indicating the presence of a glycosidic bond in the molecule and the formation of daughter ions with m/z 574, 602, and 704, respectively (Fig. 2). In addition, there were fragments formed after the simultaneous separation of hexose and water (daughter ions with m/z 556, 584, and 686). As is generally known, $^+$ESI-MS/MS fragmentation of complex GlCer is accompanied by rupture of the amide bond and the cleavage of the fatty acid molecule [17, 18]. The newly formed glycosylated sphingoid base is regarded as one of the most important characteristic fragments defining the structure of GlCer. Analysis of individual GlCer molecular species revealed that their characteristic fragments were subsidiary ions with m/z 482, 484, and 500, indicating the presence of sphingadienine, sphingenine, and hydroxysphingenine, respectively.

Based on this analysis, it was found that ions with m/z 736, 764, 792, and 820 are monohexosylceramides containing 4,8-sphingodienine ($d18:2^{\Delta 4,8}$) as sphingoid base and $C_{16:0(OH)}$, $C_{18:0(OH)}$, $C_{20:0(OH)}$, and $C_{22:0(OH)}$ fatty acids, respectively. The structure of GlCer with m/z 738, 794, 822, and 850 with $C_{16:0(OH)}$-$C_{24:0(OH)}$ fatty acid residues includes 8-sphingenine ($d18:1^{\Delta 8}$). Another group of GlCer (m/z 864 and 866) are compounds with 4-

Table 3. Content of glycoceramide molecular species after 2-h nystatin treatment of roots

Sphingoid base	Fatty acid	$[M + Na^+]$ (*m/z*)	Content, %	
			control	nystatin (20 μM)
$d18:2^{\Delta4,8}$	16:0(OH)	736.5	15.1 ± 2.2	29.4 ± 1.6*
$d18:1^{\Delta8}$	16:0(OH)	738.5	10.9 ± 2.4	20.3 ± 3.3**
$d18:2^{\Delta4,8}$	18:0(OH)	764.5	36.4 ± 7.6	22.1 ± 0.8**
$d18:2^{\Delta4,8}$	20:0(OH)	792.6	trace	trace
$d18:1^{\Delta8}$	20:0(OH)	794.6	–"–	–
$d18:2^{\Delta4,8}$	22:0(OH)	820.6	–"–	–
$d18:1^{\Delta8}$	22:0(OH)	822.6	25.9 ± 2.2	13.5 ± 0.4*
$d18:1^{\Delta8}$	24:0(OH)	850.6	trace	–
$t18:1^{\Delta8}$	24:1(OH)	864.7	12.0 ± 1.6	14.7 ± 2.1
$t18:1^{\Delta8}$	24:0(OH)	866.7	trace	trace

hydroxy-8-sphingenine ($t18:1^{\Delta8}$) as sphingoid base and $C_{24:1(OH)}$ and $C_{24:0(OH)}$ fatty acids.

DISCUSSION

Our research has shown that the modulation of the sterol component of membranes leads to changes in the structure of membrane lipids and, consequently, affects the permeability and the functioning of membranes. Sterols are important structural component of membranes of any origin. The presence of sterols has a stabilizing effect on membranes and facilitates ordering of the structural components of membranes. Artificial modification of sterol component of membranes can be achieved through stimulation or suppression of sterol synthesis, as well as by the addition of exogenous sterols or binding of endogenous sterols. In the present work we used cholesterol treatment to increase the sterol content of cells, and addition of the polyene antibiotic nystatin to bind endogenous sterols. Both compounds induced significant changes in the characteristics of root cells membranes, in particular, a significant plasma membrane depolarization (Table 1). However, one can assume that the nature of this decrease in the membrane potential varies. Under the action of cholesterol, depolarization can be due to an increase in microviscosity of membranes, its "condensing" effect, and the redistribution of electric charges [19]. These properties of cholesterol probably contributed to a significant decrease in membrane permeability for K^+ and H^+ and led to the observed apoplast acidification (Table 1). Reduced plasma membrane ion permeability under the influence of excess cholesterol is consistent with the data on increased permeability of lysosomal membranes to K^+ and H^+ in cholesterol deficiency [20].

In contrast to the cholesterol effect, plasma membrane depolarization induced by nystatin is possibly caused by formation of polyene channels due to specific binding of nystatin with membrane sterols [21]. It is known that nystatin is capable of forming specific pores (channels) consisting of two subunits and containing hydrophilic hydroxyl groups along the surface of the pores and hydrophobic heptane segment, which interacts with sterols and the hydrophobic environment of the bilayer. The formation of these pores leads to the acidification of intracellular contents through K^+/H^+ or K^+/carbohydrate metabolism and the loss of esters of phosphorus, organic acids, nucleotides, and proteins [22]. Thus, the dissipation of the transmembrane gradient observed in the present study, which was accompanied by a decrease in membrane potential and a significant increase in membrane permeability for K^+ and H^+, confirms the formation of polyene pores in the membranes of root cells under nystatin treatment.

Changing the physicochemical properties of the plasma membrane in cells saturated with cholesterol or binding of endogenous sterols with nystatin has an impact not only on the transport of ions, but also on the activity of redox enzymes involved in the formation of reactive oxygen species (ROS). ROS formation and detoxification is one of the key mechanisms of membrane permeability regulation, in particular through the oxidation of SH-groups of proteins and membrane lipids. A significant increase in extracellular levels of O_2^- under cholesterol treatment of roots compared with the level of O_2^- in the control cells may indicate the activation of the plasma membrane redox system (Table 1). Studies on mammalian macrophages showed that 7-ketocholesterol treatment induced activation of NAD(P)H-oxidase, the main redox system of plasma membrane, which was accompanied by enhanced formation of O_2^- [23], and an excess of

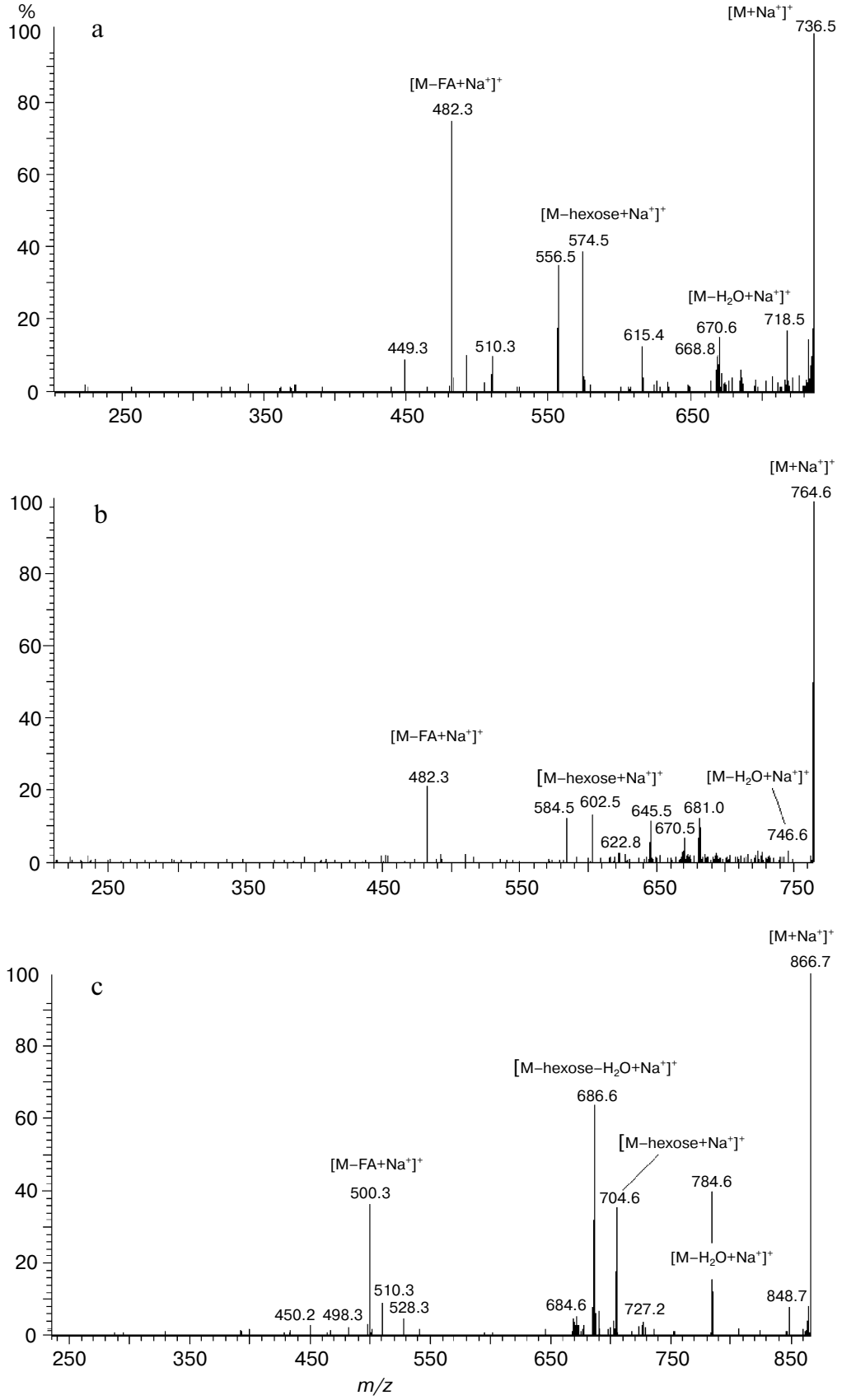

Fig. 2. $^{+}$ESI-MS/MS spectra of fragments of cationized molecular ions $[M+Na^{+}]^{+}$ with m/z 736 (glucose/d18:$2^{\Delta4,8}$/$C_{16:0(OH)}$) (a), 764 (glucose/d18:$2^{\Delta4,8}$/$C_{18:0(OH)}$) (b), and 866 (glucose/t18:$1^{\Delta8}$/$C_{24:0(OH)}$) (c).

sterols induced programmed cell death [24]. In contrast, the binding of endogenous sterols by nystatin in the root cells resulted in a significant decrease in content of extracellular O_2^- (Table 1). A possible reason for nystatin-induced reduction of O_2^- can be a violation of the sterol-enriched lipid domains structure (rafts), upon which membrane ROS-generating systems are anchored. In [25] the presence of the redox enzymes such as peroxidase, L-ascorbate-oxidase, NADP-ubiquinone-oxidoreductase, and cytochrome b_{561}, which are involved in the formation and metabolism of ROS, was demonstrated in lipid rafts isolated from the roots of alfalfa and *Medicago truncatula*. It is believed that modification of the composition and/or intermolecular interactions of sterols with the lipid and protein environment leads to changes in the structure of lipid rafts and, consequently, the activity of related proteins, such as redox enzymes.

The question arises as to what changes in membrane lipid composition can cause changes in membrane characteristics and redox reactions in root cells following the binding of endogenous sterols? It was found that the binding of endogenous sterols by nystatin did not result in a reduction of their contents (Fig. 1). Chromatographic analysis of other lipid compounds showed that the treatment of roots with nystatin did not change the content of the basic membrane glycerolipids, but the amount of sphingolipids increased significantly (Fig. 1). Accumulation of sphingolipids, namely GlCer, was accompanied by a change in the ratio of their individual molecular species. In particular, under nystatin treatment the relative content of GlCer N-acylated with $C_{16:0(OH)}$ acid doubled, which was accompanied by decreasing levels of molecular species containing the FA with a greater chain length. As it is known, plant GlCer containing long chain ($>C_{20}$) hydroxy-FA are one of the most important factors in stabilizing the membrane and reducing its permeability [26]. However, GlCer acylated with shorter FA cannot show such activity and even, on the contrary, increase the permeability of the bilayer for different ions. At the same time, aglycone GlCer, formed after the enzymatic breakage of a glycosidic bond or in the reactions of synthesis *de novo*, such as C_{16}-ceramides, can form stable channels that are permeable not only for low molecular weight substances, but also for some high-molecular-weights compounds, in particular for cytochrome *c* [27, 28]. It is possible that the membrane depolarization accompanied by intense release of potassium observed during nystatin treatment was caused by changes in the GlCer composition, namely increased levels of molecular species containing short-chain hydroxy-FA, and a decrease in molecular species containing long-chain hydroxy-FA.

Sphingolipids and their metabolites are not only structural components of membranes, but also bioactive molecules involved in signaling and cell regulation. Functions of sphingolipids have been well studied in animal cells, but information on their biological activity in plants is very limited. Recent studies have shown that sphingosine-1-phosphate is involved in the mobilization of calcium ions into cells [29]. A similar effect for ceramide was demonstrated on callus culture of *Arabidopsis*. Interestingly, the effect of these compounds was mediated by increased oscillations of intracellular calcium, but was not associated with the generation of ROS [30]. In contrast, the mechanism of signal transduction involving free sphingoid bases often includes the formation of active oxygen metabolites [31]. Concerning complex GlCer, their involvement in cell signaling has long been disputed. However, recently it was shown that surface GlCer of one cell can interact with neighboring GlCer with the formation of a new GlCer–GlCer complex, significantly modulating the transmission of cellular signals [32, 33]. Change in membrane sphingolipid profile is supposed to affect the structuring and functional activity of rafts.

Despite the abundance of biophysical evidence for interference of sterols and sphingolipids in simple model systems, there is only sporadic evidence of their interaction in cells. It was found that the animal cells with a vector mutation on sterol metabolism differed mainly in the composition of membrane sphingolipids, which affected the structure of lipid rafts, physiological activity, and phenotype of the cells [34]. A comparative study of antibiotic effects on the synthesis of ergosterol in cells of the yeast *Candida albicans*, including in the wild type and mutants, showed that the major factors that heighten the sensitivity of mutants to antibiotics is a destabilization of the interactions of sphingolipids in rafts, but not the increase in fluidity and permeability of the plasma membrane caused by a reduction in ergosterol [35].

The data obtained in this study suggest that modification of sterol status is a key component in the regulation of functional activity of plant membranes. The binding of sterols leads to a change in the ratio of glycoceramide molecular types that can affect the structuring of lipid rafts, the functional activity of their component enzymes and, consequently, the modulation of reception and transduction of cellular signals.

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