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Effects of sterol-binding agent nystatin on wheat roots: The changes in membrane permeability, sterols and glycoceramides

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ABSTRACT

Plant sterols are important multifunctional lipids, which are involved in determining membrane properties. Biophysical characteristics of model lipid and isolated animal membranes with altered sterol component have been intensively studied. In plants however, the precise mechanisms of involvement of sterols in membrane functioning remain unclear. In present work the possible interactions between sterols and other membrane lipids in plant cells were studied. A useful experimental approach for elucidating the roles of sterols in membrane activity is to use agents that specifically bind with endogenous sterols, for example the antibiotic nystatin. Membrane characteristics and the composition of membrane lipids in the roots of wheat (*Triticum aestivum* L.) seedlings treated with nystatin were analyzed. The application of nystatin greatly increased the permeability of the plasma membrane for ions and SH-containing molecules and decreased the total sterol level mainly as a consequence of a reduction in the amount of β-sitosterol and campesterol. Dynamic light-scattering was used to confirm the *in vitro* formation of stable complexes between nystatin and β-sitosterol or cholesterol. Sterol depletion was accompanied by a significant rise in total glycoceramide (GlCer) content after 2 h treatment with nystatin. Analysis of the GlCer composition using mass spectrometry with electrospray ionization demonstrated that nystatin induced changes in the ratio of molecular species of GlCer. Our results suggest that changes in the sphingolipid composition can contribute to the changes in plasma membrane functioning induced by sterol depletion.

1. Introduction

Among the factors determining the structure and functioning of plant membranes, sterols play essential roles. In contrast to fungal or mammalian cells where ergosterol and cholesterol, respectively, are the predominant sterol forms, the plasma membranes (PM) of higher plant cells contain a complex mixture of sterols (Benveniste, 2004). The predominant sterols in plants are β-sitosterol, campesterol, stigmasterol, and the presence of cholesterol has also been described (Schaller, 2003). Phytosterols can be conjugated with sugars, usually glucose, which in turn can be acylated by fatty acids (FA) to form steryl glycosides and acyl-steryl glycosides, respectively. As integral components of the membrane lipid bilayer, sterols not only regulate membrane fluidity and permeability but also modulate the activity and distribution of membrane-bound proteins such as receptors, enzymes, and components of signaling pathways (Hartmann, 1998; Kim et al., 2010). Therefore, sterols are involved in the regulation of almost every aspect of plant metabolism, including growth, development and stress responses (Beck et al., 2007; Quartacci et al., 2002).

An important property of sterols is their ability to form van der Waals interactions with the saturated alkyl residues of sphingolipids; this enables them to achieve a high packing density and promotes the formation of lipid microdomains (Wang and Silvius, 2000). Specific microdomains enriched with typical plant sterols, steryl glycosides and glycosylceramides, have been detected in PM and Golgi bodies isolated from different plants, e.g. arabidopsis, tobacco, alfalfa (Laloi et al., 2007; Mongrand et al., 2004). Such lipid microdomains can form platforms for the aggregation of signalling

Abbreviations: FA, fatty acid; GlCer, glycoceramides; GPI, glycosylphosphatidylinositol; LCFA, long-chain fatty acid; +ESI-MS, mass spectrometry with electrospray ionization; PM, plasma membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; 2D-TLC, two-dimensional thin-layer chromatography.

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complexes, and consist of various signal and defence proteins, including glycosylphosphatidylinositol (GPI)-anchored proteins. For example, in detergent-resistant membranes isolated from roots of *Medicago truncatula*, 270 proteins have been identified, including those involved in signalling, secretion, programmed cell death, cell wall functioning, and also redox enzymes (peroxidase, l-ascorbate oxidase, NADP ubiquinone oxidoreductase, and cytochrome b_{561}) (Lefebvre et al., 2007).

The dependence of membrane functioning on its sterol component has been intensively studied using model lipid and isolated animal membranes (Hac-Wydro, 2010; Makky et al., 2010). In plants however, the precise mechanisms of involvement of sterols in membrane functioning remains unclear. A useful approach for elucidating the roles of sterols in membrane activity is to deplete sterols using agents such as nystatin that specifically bind with endogenous sterols (Akaike and Harata, 1994). The polyene antibiotic nystatin is widely used in medicine to suppress the growth of pathogenic fungi (Gale et al., 1972; Semis et al., 2010). Using model lipid membranes it has been shown that antibiotics of this group (nystatin, levorin, amphotericin B) interact with membrane sterols via the polyene bonds. This results in the formation of polyene pores consisting of an equal number of molecules of antibiotics and sterols with hydrophilic hydroxyl groups along the pore surface (Coutinho and Prieto, 2003; Finkelstein and Holz, 1973). In microorganisms, nystatin induces the loss of potassium ions, phosphates, sugars and other compounds, leading to cell death (Marini et al., 1961; Sharma et al., 2010). In animal membranes, nystatin binds with cholesterol, but the ability of this antibiotic to bind with plant sterols has not yet been shown. Interestingly, treating the roots of wheat seedlings with nystatin caused dramatic changes in various physiological parameters, such as membrane potential, rate of respiration and heat production (Gordon et al., 2005). However, the exact mechanism by which nystatin influences plant membrane lipids is currently unknown. Therefore, the main goal of the present investigation was to study the nystatin-induced changes in cellular membranes in plant roots. Specifically, we aimed to examine the changes in the sterol content and ratio of sterol molecular species *in vivo* after nystatin treatment of wheat roots. We also studied if nystatin induces alterations in other membrane lipids, in particular phospholipids and sphingolipids. Changes in these lipids can contribute not only to the changes in membrane permeability but also to the functioning of lipid microdomains, which are enriched in sterols and sphingolipids (Mongrand et al., 2004). We also tested the ability of nystatin to bind with phytosterols *in vitro*.

2. Results

2.1. Nystatin-induced membrane permeability of ions and free SH-groups

Treating excised wheat roots with nystatin greatly increased the permeability of the PM for ions and SH-containing molecules. Roots incubated with 20 μM nystatin displayed a rapid (within 15 min) and sustained leakage of K^+ ions that exceeded K^+ release in the control by at least four times (Fig. 1). Nystatin also caused the alkalinization of the root incubation medium by 0.5 pH units compared with the control (data not shown). Moreover the concentration of free SH-groups in root incubation medium significantly increased following treatment with nystatin, progressively rising over 6 h (Fig. 2).

2.2. Sterol content and composition following nystatin treatment

Nystatin significantly affected the changes in sterol levels and species composition that occurred in roots during 6 h after their excision from seedlings. In excised roots incubated in $CaCl_2$ solution without nystatin (the control), the total sterol content changed only slightly during the first 4 h, but doubled after the next 2 h (Fig. 3). When excised roots were incubated in the presence of nystatin, the total sterol content was similar to that in the control during first 2 h, but increased after 4 h. However, during the following 2 h nystatin reduced the concentration of sterols, and

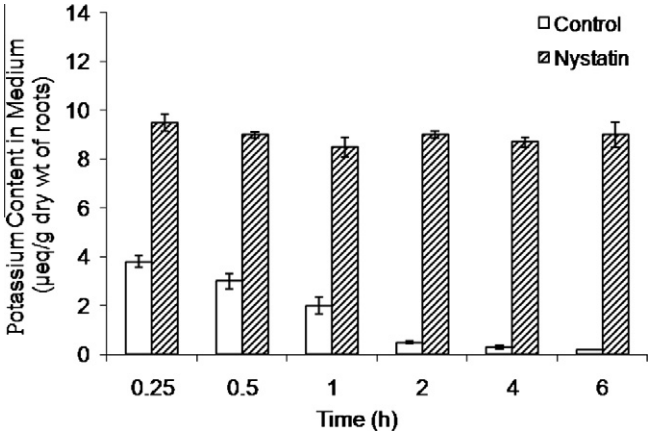

Fig. 1. Potassium leakage from wheat roots estimated as a content of potassium ions in the root incubation medium following 20 μM nystatin treatment. Values represent means ± SD, $n = 5$.

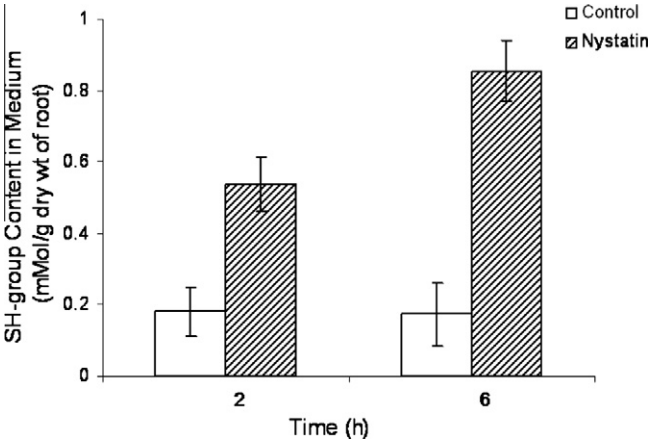

Fig. 2. The content of SH-groups in the incubation medium of wheat roots after 20 μM nystatin exposure. Values represent means ± SD, $n = 5$.

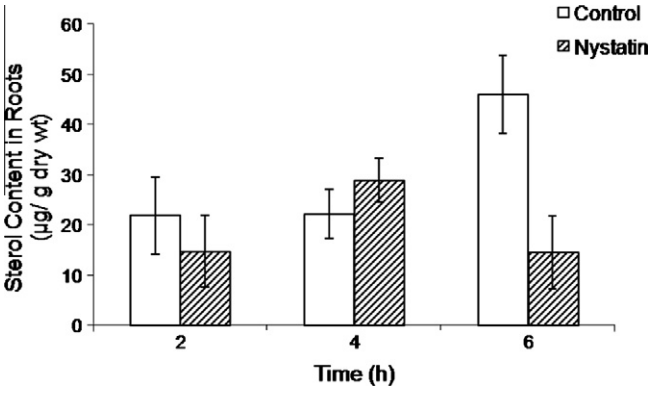

Fig. 3. The total content of sterols in wheat roots following 20 μM nystatin treatment. Values represent means ± SD, $n = 3$.