Lipid Composition Determines the Effects of Arbutin on the Stability of Membranes

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ABSTRACT Arbutin (hydroquinone-β-D-glucopyranoside) is an abundant solute in the leaves of many freezing- or desiccation-tolerant plants. Its physiological role in plants, however, is not known. Here we show that arbutin protects isolated spinach (*Spinacia oleracea* L.) thylakoid membranes from freeze-thaw damage. During freezing of liposomes, the presence of only 20 mM arbutin led to complete leakage of a soluble marker from egg PC (EPC) liposomes. When the nonbilayer-forming chloroplast lipid monogalactosyldiacylglycerol (MGDG) was included in the membranes, this leakage was prevented. Inclusion of more than 15% MGDG into the membranes led to a strong destabilization of liposomes during freezing. Under these conditions arbutin became a cryoprotectant, as only 5 mM arbutin reduced leakage from 75% to 20%. The nonbilayer lipid egg phosphatidylethanolamine (EPE) had an effect similar to that of MGDG, but was much less effective, even at concentrations up to 80% in EPC membranes. Arbutin-induced leakage during freezing was accompanied by massive bilayer fusion in EPC and EPC/EPE membranes. Twenty percent MGDG in EPC bilayers completely inhibited the fusogenic effect of arbutin. The membrane surface probes merocyanine 540 and 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-C_6-HPC) revealed that arbutin reduced the ability of both probes to partition into the membranes. Steady-state anisotropy measurements with probes that localize at different positions in the membranes showed that headgroup mobility was increased in the presence of arbutin, whereas the mobility of the fatty acyl chains close to the glycerol backbone was reduced. This reduction, however, was not seen in membranes containing 20% MGDG. The effect of arbutin on lipid order was limited to the interfacial region of the membranes and was not evident in the hydrophobic core region. From these data we were able to derive a physical model of the perturbing or nonperturbing interactions of arbutin with lipid bilayers.

INTRODUCTION

Arbutin (4-hydroxyphenyl-β-D-glucopyranoside) is a glycosylated hydroquinone (Fig. 1) that has been found at extraordinarily high concentrations in the leaves of several plant species, such as *Vaccinium* spp. (Suau et al., 1991). It has been used pharmaceutically in humans for centuries, either as plant extracts or, in more recent decades, in purified form, because of its diuretic and urinary antiinfective properties. There is nothing known about the physiological role of arbutin in the plants that synthesize it. The tolerance of many of these plants against environmental stresses such as frost and drought, however, could be related to the presence of arbutin. This is especially striking in the resurrection plant *Myrothamnus flabellifolia*, where arbutin constitutes as much as 25% of the dry weight of the leaves (Bianchi et al., 1993; Suau et al., 1991), which, assuming a uniform distribution in the cells, translates into a concentration of ~100 mM. This concentration would of course be higher if arbutin were restricted to specific cellular compartments. Resurrection plants are able to survive complete dehydration for extended periods of time. Although the physiological mechanisms underlying desiccation tolerance have not been completely understood, the accumulation of soluble sugars and other solutes, such as arbutin, is widely recognized as an important part of the cellular stress protection in plants (Crowe et al., 1992; Ingram and Bartels, 1996; Steponkus, 1984). This is true for desiccation and freezing, as plants employ similar biochemical adaptations to cope with the two stresses (Hughes and Dunn, 1996; Steponkus, 1984). This can be rationalized from the fact that during freezing, ice crystallization leads to an effective removal of liquid water and consequently to freeze-induced dehydration. Therefore, over a wide range of temperatures/water contents, freezing and desiccation challenge cellular structures with the same physical stresses. Only at the extremes of desiccation, when more cellular water is removed than would crystallize during freezing under physiologically relevant conditions, would the two treatments result in physically different stresses (Crowe et al., 1990).

A possible function of arbutin in plant stress tolerance could be the inhibition of membrane degradation in partly or completely desiccated or frozen leaves. Ioku et al. (1992) showed that arbutin has antioxidative properties for membrane lipids, and Oliver et al. (1996) reported that it can inhibit the enzyme phospholipase A_2 (PLA_2) in partially dehydrated liposomes. This inhibitory activity is most likely mediated by a direct interaction of arbutin with the lipid bilayer (Oliver et al., 1998), which is already seen with completely hydrated membranes and is probably enhanced when water is removed. In both hydrated and dry bilayers, made from different, pure species of phosphatidylcholine,

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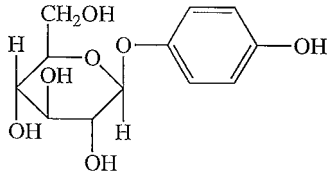

FIGURE 1 Line drawing of the chemical structure of arbutin (4-hydroxyphenyl-β-D-glucopyranoside).

the phase transition temperature between the gel and liquid-crystalline phases (T_m) is significantly reduced in the presence of arbutin (Oliver et al., 1996; 1998), indicating that its interaction with membranes has an influence on the physical properties of the membrane lipids.

Surprisingly, this interaction leads to a destabilization of large unilamellar PC vesicles during drying (Oliver et al., 1998). This finding is obviously at odds with the proposed role of arbutin in plant stress tolerance. It should be recognized, however, that pure phospholipid vesicles might not be an ideal model system for the study of the stress tolerance of plant membranes. Because the intracellular localization of arbutin has not been determined in any plant and consequently its natural target membranes are unknown, we decided to use isolated chloroplast thylakoid membranes from spinach as a well-defined experimental target membrane for arbutin. Because freeze-thaw damage to thylakoids (Hincha et al., 1996) and to liposomes containing different thylakoid lipids (Hincha et al., 1998) has been extensively studied, we have used the resulting knowledge to investigate the effects of arbutin on the freeze-thaw stability of membranes. Differences from results obtained with pure PC membranes could be expected, because thylakoids contain mostly glycolipids. The nonbilayer lipid monogalactosyldiacylglycerol (MGDG) accounts for ~50% of the thylakoid lipid content, while the bilayer lipids digalactosyldiacylglycerol (DGDG) (~25%), phosphatidylglycerol (PG) (~15%), and sulfoquinovosyldiacylglycerol (SQDG) (~10%) make up the other half (Webb and Green, 1991).

In the present paper we show that arbutin is a cryoprotectant for thylakoid membranes and that the cryoprotective effect is dependent on the presence of MGDG in large unilamellar liposomes. Results from experiments with several membrane probes indicate that arbutin influences the physical state of the membrane lipids in the headgroup and interfacial regions and that there are characteristic differences between membranes that contain MGDG and those that do not.

MATERIALS AND METHODS

Lipids and membrane probes

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) or from Sigma. Galactolipids were purified as described in detail in recent publications (Hincha and Crowe, 1996; Hincha et al., 1998), from fresh spinach (*Spinacia oleracea* L.) leaves obtained from a local market in Davis, CA. Alternatively, MGDG and DGDG from soybean leaves were purchased from Lipid Products (Redhill, UK). There were no detectable differences in our experiments in the effects of arbutin on liposomes made with the lipids from different sources. Carboxyfluorescein (CF) was obtained from Molecular Probes (Eugene, OR) and was purified according to the procedure described by Weinstein et al. (1984). *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE), *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (Rh-PE), 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-C_6-HPC), and trimethylammoniumpropyl-1,6-diphenyl-1,3,5-hexatriene (TMAP-DPH) were purchased from Molecular Probes. Merocyanine 540 (MC540), 1,6-diphenyl-1,3,5-hexatriene (DPH), and trimethylammonium-DPH (TMA-DPH) were obtained from Sigma.

Preparation of liposomes

The different lipids were mixed in chloroform, dried under a stream of N_2, and stored under vacuum overnight to remove traces of solvent. Mixtures of different lipids were made by weight and are expressed as a percentage (w/w). All liposomes were prepared from hydrated lipids, using a hand-held extruder (MacDonald et al., 1991; Avestin, Ottawa, Canada) with two layers of polycarbonate membranes (Poretics, Livermore, CA) with 100-nm pores.

Liposome freezing experiments

Liposomes (20 μl) were mixed with an equal volume of concentrated solutions of arbutin made in 10 mM TES, 0.1 mM EDTA, 50 mM NaCl (TEN buffer, pH 7.4) (final lipid concentration 5 mg ml^{-1}). The tubes were placed in a bath containing ethylene glycol cooled to −18°C. After 5 min the samples were crystallized by touching the outside of the tubes with a spatula cooled in liquid nitrogen. The samples were kept frozen for 3 h and thawed in a water bath at room temperature. Control samples were incubated at 0°C for 3 h. Freeze-thaw damage was determined either as leakage of the soluble marker CF or as membrane fusion. The figures show the means ± SD from three parallel samples. Where no error bars are visible, they were smaller than the symbols.

Leakage and fusion measurements

For leakage experiments, 10 mg of lipid was hydrated in 0.5 ml of 100 mM CF, 10 mM TES, 0.1 mM EDTA (pH 7.4). After extrusion, the vesicles were passed through a column (0.5 × 10 cm) of Sephadex G-50 (Pharmacia) equilibrated in TEN to remove the CF not entrapped by the vesicles. The eluted samples had a lipid concentration of ~10 mg ml^{-1}. For leakage measurements, 5 μl of sample was diluted in a cuvette in 3 ml of TEN. Measurements were made in a Perkin-Elmer LS-5 fluorometer at an excitation wavelength of 460 nm and an emission wavelenght of 550 nm. Fluorescence of CF is strongly quenched at the high concentration inside the vesicles and is increased when CF is released into the medium. The total CF content of the vesicles (100% leakage value) was determined after lysis of the membranes with 50 μl of 1% Triton X-100. For resonance energy transfer measurements (Struck et al., 1981), two liposome samples were prepared in TEN, one of which contained 0.5 mol% each of NBD-PE and Rh-PE, and the other contained only unlabeled lipids. After extrusion, liposomes were combined at a ratio of 1:9 (labeled:unlabeled), resulting in a lipid concentration of 10 mg ml^{-1}. Membrane fusion was measured by resonance energy transfer (Struck et al., 1981) as described in detail in recent publications (Hincha et al., 1998; Oliver et al., 1998).

Determination of freeze-thaw damage to thylakoids

Thylakoids were isolated from spinach (*Spinacia oleracea* L. cv. Monnopa) leaves as described previously (Hincha and Schmitt, 1992). The

membranes were washed three times in 10 mM $MgCl_2$, 20 mM K_2SO_4. Samples (0.2 ml) containing ~0.5 mg chlorophyll ml^{-1}, 5 mM $MgCl_2$, 10 mM K_2SO_4, 150 mM K-glutamate, 50 mM sucrose (artificial stroma medium; Hincha and Schmitt, 1988), and additional arbutin were placed in a freezer at −20°C for 3 h and were rapidly (within 2–3 min) thawed in a water bath at room temperature. Control samples were kept for the same time at 0°C. After thawing, the membranes were sedimented by centrifugation (15 min at 16,000 × *g*), and the supernatants were mixed with an equal volume of electrophoresis sample buffer (Laemmli, 1970). Proteins were fractionated on 15% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels under reducing conditions and were then transferred to nitrocellulose membranes by electroblotting (Towbin et al., 1979). Unoccupied binding sites on the membranes were blocked by incubation in 5% (w/v) milk powder, 0.1% (v/v) Tween 20 in 25 mM Tris, and 150 mM NaCl (pH 7.5; Johnson et al., 1984). Filters were probed with rabbit anti-spinach plastocyanin antiserum (Hincha et al., 1985). Bound IgG on the filters was visualized with a goat anti-rabbit IgG serum labeled with horseradish peroxidase (BioRad) as described by Sieg et al. (1996). The stained bands were quantified with a laser densitometer. For comparison with the frozen-thawed samples, plastocyanin was completely liberated from thylakoids by sonication with a tip sonicator for 5 min at 80 W. The membranes were removed by centrifugation (30 min at 20,000 × *g*), and the supernatant was treated as described above.

Partitioning of merocyanine 540 and NBD-C_6-HPC into liposome membranes

To assess the effects of arbutin on the surface properties of membranes, we used the dye MC540 as described by Bakaltcheva et al. (1994) and the fluorescent probe NBD-C_6-HPC as described by Lee and Lentz (1997). For MC540 measurements, liposomes (0.3 mg ml^{-1}) were suspended in TEN containing up to 200 mM arbutin. Samples were incubated at 0°C for 30 min, and then MC540 was added to a final concentration of 10^{-5} M. After 15 min, the absorbance was measured at 570 nm and 530 nm on a Uvikon 922 double-beam spectrophotometer (Kontron Instruments, Neufahrn, Germany) at room temperature. The reference cuvette contained liposomes and arbutin without MC540. The data were corrected for the effect of arbutin on the absorbance of MC540 in the absence of liposomes.

The increase in fluorescence emission from NBD-C_6-HPC resulting from the partitioning of the fluorescently labeled lipid into preformed liposomal membranes was measured with a Kontron SFM 25 fluorometer at 25°C. Liposomes (0.1 mg ml^{-1}) were suspended in a cuvette in TEN containing different concentrations of arbutin. NBD-C_6-HPC was added as a concentrated solution in methanol to a final lipid/probe ratio of 200:1 and a final methanol concentration of 0.1% (v/v). The resulting fluorescence emission was measured at 530 nm, with excitation at 470 nm. The data at all arbutin concentrations were corrected for the fluorescence of NBD-C_6-HPC in the absence of liposomes (under 1%). Arbutin had no measurable influence on the fluorescence emission of the probe in the absence of membranes.

Steady-state anisotropy of membrane lipids

The dynamics of lipids in liposome membranes in the presence of different concentrations of arbutin was determined by measuring the degree of depolarization of the fluorescence emitted from the probes DPH, TMAP-DPH, TMA-DPH, and NBD-PE (Lentz, 1993). DPH is a hydrophobic molecule and is widely used for measuring the order of the lipid fatty acyl chains in the core region of the bilayer, whereas TMAP-DPH and TMA-DPH are anchored at the water/lipid interface, because of their additional charged trimethylammonium group (Engel and Prendergast, 1981; Prendergast et al., 1981). NBD-PE is an indicator of the mobility of the lipid headgroup region of the membranes (Lentz et al., 1996). DPH, TMAP-DPH, or TMA-DPH in dimethyl formamide was added to a liposome suspension (0.1 mg ml^{-1}) in TEN containing up to 200 mM arbutin in a stirred cuvette at 25°C. The lipid/probe ratio was 200:1, and the final dimethyl formamide concentration was 0.1% (v/v). Measurements were carried out on a Kontron SFM 25 spectrofluorimeter with polarization filters. Fluorescence was excited at 360 nm, and emission was recorded at 450 nm. NBD-PE in chloroform was mixed with the other lipids at a lipid/probe ratio of 200:1, and liposomes were prepared in TEN as described above. The liposomes were suspended in TEN and arbutin as above, and fluorescence depolarization was measured at an excitation wavelength of 470 nm and an emission wavelength of 530 nm.

RESULTS

To investigate the effects of arbutin on the stress tolerance of plant membranes, we have frozen isolated spinach thylakoids in a simplified artificial stroma medium, which elicits freeze-thaw damage similar to that in the in vivo situation (Hincha and Schmitt, 1988). As a molecular marker for membrane damage we used plastocyanin, a soluble electron transport protein that is localized in the lumen of thylakoid vesicles. Its appearance in the supernatant of membrane samples after centrifugation is closely related to the inactivation of photosynthetic electron transport, in both leaves and isolated thylakoids, and indicates transient membrane rupture (see Hincha et al., 1996 for a comprehensive review).

Fig. 2 shows that a large part (71%) of the total plastocyanin content of thylakoids was released from the membrane vesicles after thawing. Increasing concentrations of arbutin up to 150 mM reduced the amount of plastocyanin in the supernatants to 32%, close to the level of the unfrozen control samples (23%). At the highest concentration em-

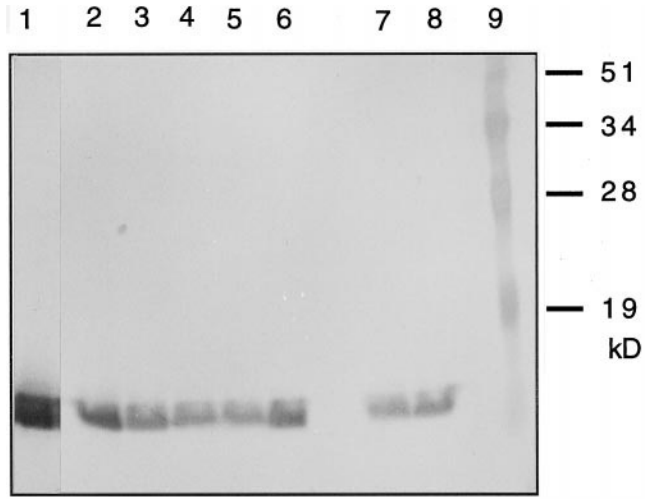

FIGURE 2 Western blot analysis of plastocyanin released from isolated spinach thylakoid membranes. An equal volume of membrane supernatant was applied to all lanes. As a control for complete loss of plastocyanin, thylakoids were treated with ultrasonication (*lane 1*). Loss of plastocyanin during a freeze-thaw cycle to −20°C is shown in lanes 2–6 as a function of the arbutin concentration (*lane 2*: 0 mM; *lane 3*: 50 mM; *lane 4*: 100 mM; *lane 5*: 150 mM; *lane 6*: 200 mM). Lanes 7 and 8 show the plastocyanin released during storage of thylakoids at 0°C in the absence and presence of 200 mM arbutin, respectively. Lane 9 shows standard proteins. The molecular mass of these proteins is indicated in kilodaltons on the right.

ployed in these experiments (200 mM) freeze-thaw damage increased again, to 56% plastocyanin leakage. It should be noted that this increase was specific for the freeze-thaw treatment, as the control samples stored at 0°C for 3 h in the presence of 200 mM arbutin showed no significant increase in plastocyanin release (Fig. 2, *lanes 7* (23%) and *8* (30%)).

To elucidate whether the cryoprotective behavior of arbutin for thylakoids depended on any specific membrane component, liposomes were prepared from isolated thylakoid lipids. To make the results directly comparable to those reported in Fig. 2, liposomes were prepared in buffer, and arbutin was added to the samples in different concentrations. Therefore, in all experiments reported here, arbutin was only present on the outside of the vesicles. Freeze-thaw damage was then assessed as leakage of a soluble fluorescent marker, carboxyfluorescein (CF). Parallel samples were always incubated for 3 h at 0°C in the absence and presence of 200 mM arbutin. For the fluorimetric measurements, all samples were diluted in TEN buffer at a ratio of 1:600 (v/v). In no case was the leakage in the 0°C controls containing 200 mM arbutin increased compared to controls containing liposomes in TEN buffer alone (data not shown). Therefore, the leakage reported in the figures can be attributed to the stresses during a freeze-thaw cycle and was not caused by the osmotic stresses associated with the incubation in the presence of high arbutin concentrations under nonfreezing conditions or the subsequent dilution during measurements.

As a first step in the analysis, the nonbilayer lipid MGDG was chromatographically separated from the bilayer lipids DGDG, PG, and SQDG, constituting the DG+ fraction (Hincha et al., 1998). The lipids were then reconstituted into liposomes containing 80% DG+ and either 20% egg phosphatidylcholine (EPC) or MGDG. A freeze-thaw cycle induced CF leakage that depended on both the lipid composition and the concentration of arbutin (Fig. 3). As described in detail before (Hincha et al., 1998), MGDG destabilizes membranes and leads to increased leakage during freezing. Concentrations higher than 20% MGDG in the membranes result in increased leakage, even in the absence of an additional stress.

Arbutin strongly increased leakage in liposomes without MGDG, but at low concentrations it protected liposomes that contained 20% MGDG in their membranes. At arbutin concentrations above 50 mM, however, leakage increased again. To see whether the observed cryoprotection by arbutin was due exclusively to the presence of MGDG in the membranes, or whether interactions with other lipids also played a role, liposomes were prepared from EPC containing different fractions of MGDG (Fig. 4). The results indicate that the presence of MGDG is indeed sufficient to modify the response of EPC vesicles to arbutin during freezing. Leakage at all arbutin concentrations was reduced by as little as 10% MGDG, compared to pure EPC membranes. With 15% MGDG no increase in leakage occurred over the whole concentration range. With 20% MGDG leakage increased already in the absence of arbutin, and here

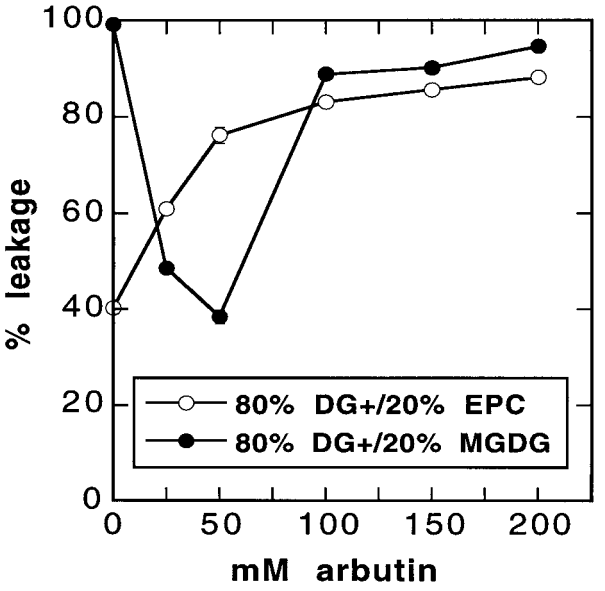

FIGURE 3 Freeze-thaw damage to large unilamellar liposomes in the presence of arbutin. Liposomes were prepared from mixtures of 80% MGDG-depleted chloroplast lipids (DG+) and 20% EPC or 20% MGDG. The samples were frozen at −18°C for 3 h. Freeze-thaw damage was determined as leakage of the soluble marker carboxyfluorescein (CF).

the cryoprotective effect was evident again (compare Fig. 3). Leakage increased somewhat at higher arbutin concentrations, but even at 200 mM arbutin it was still well below the leakage from EPC vesicles. Fig. 5 shows that both the cryotoxic effect for EPC membranes and the cryoprotective effect for membranes containing 20% MGDG only required very low concentrations of arbutin. The cryotoxic effect was

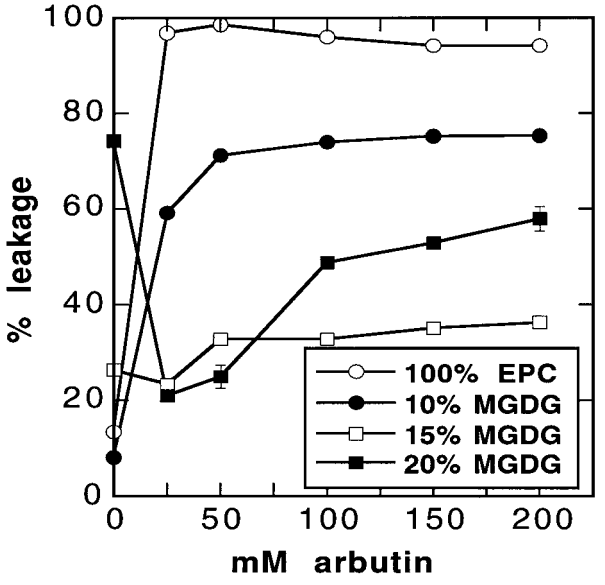

FIGURE 4 CF leakage from liposomes in the presence of different concentrations of arbutin during freezing. The membranes were composed of EPC and different fractions of MGDG.

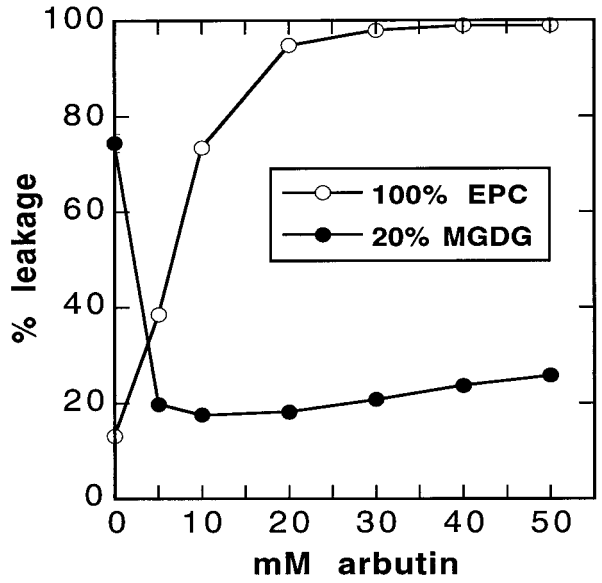

FIGURE 5 Freeze-thaw damage to liposomes in the presence of arbutin. The membranes were composed of either 100% EPC or 80% EPC and 20% MGDG.

saturated at 20 mM and the cryoprotective effect already at 5 mM.

That this dramatic reversal of the effect of arbutin was not due to the presence of galactose in the lipid headgroups, but specifically required MGDG, is demonstrated in Fig. 6. Membranes containing 50% DGDG and 50% EPC showed the same leakage as pure EPC membranes, and liposomes made from 50% DGDG, 20% MGDG, and 30% EPC showed the same leakage as those made from 20% MGDG and 80% EPC (compare Fig. 4).

The main physical difference between MGDG and DGDG is that they are nonbilayer and bilayer lipids, respectively. MGDG forms a hexagonal II (H_{II}) phase in excess water at temperatures above −15°C (Sanderson and Williams, 1992). This raised the question of whether the effect of MGDG on the cryobehavior of arbutin was due to its nature as a nonbilayer lipid. To test this possibility, liposomes were prepared from EPC and different fractions of egg phosphatidylethanolamine (EPE), another nonbilayer lipid (see McIntosh, 1996 for a review). As noted before (Hincha et al., 1998), EPE can be incorporated into vesicles at a higher percentage than MGDG. Only at concentrations above 80% is bilayer integrity compromised in the absence of freezing. Freeze-thaw damage was significantly increased at EPE concentrations of 40% or more already in the absence of arbutin (Fig. 7). Nevertheless, the cryotoxic effect of arbutin was ameliorated when EPE was present in the membranes. This became clearly visible at EPE concentrations above 20% (Fig. 7). Optimal results were obtained at 60% EPE, where a low concentration of arbutin was slightly protective. However, arbutin did not show a clear cryoprotective effect when CF leakage increased during freezing due to the presence of a high fraction of EPE in the membranes (Fig. 7, 80% EPE). This was in marked contrast to the situation with MGDG (Fig. 4).

We have shown previously that the destabilization of liposomes during freezing is based on different mechanisms in the presence of MGDG or EPE (Hincha et al., 1998). Whereas leakage in EPE-containing vesicles was related to increased bilayer fusion, MGDG induced no fusion, even under conditions of complete leakage. These differences in fusion behavior during freezing also persisted in the presence of arbutin (Fig. 8). Arbutin itself induced massive

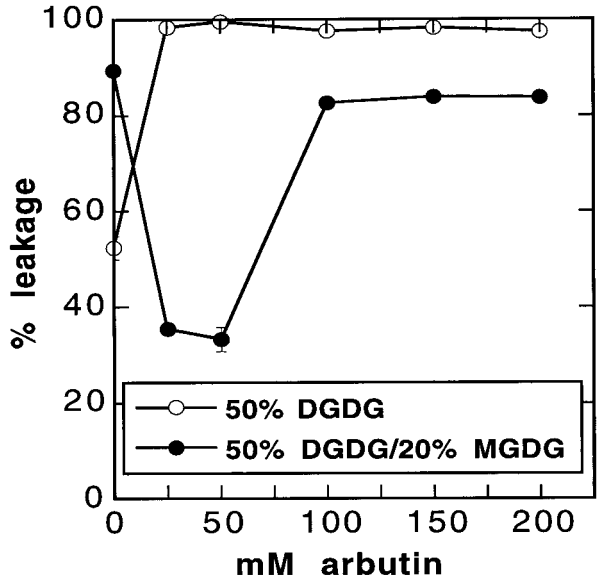

FIGURE 6 Freeze-thaw damage to liposomes in the presence of arbutin. The membranes were composed of either 50% EPC and 50% DGDG, or 30% EPC, 50% DGDG, and 20% MGDG.

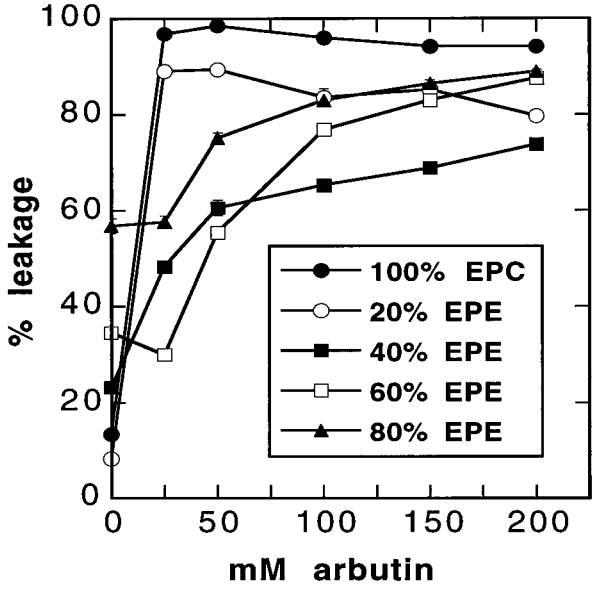

FIGURE 7 CF leakage from liposomes in the presence of different concentrations of arbutin during freezing. The membranes were composed of EPC and different fractions of EPE.

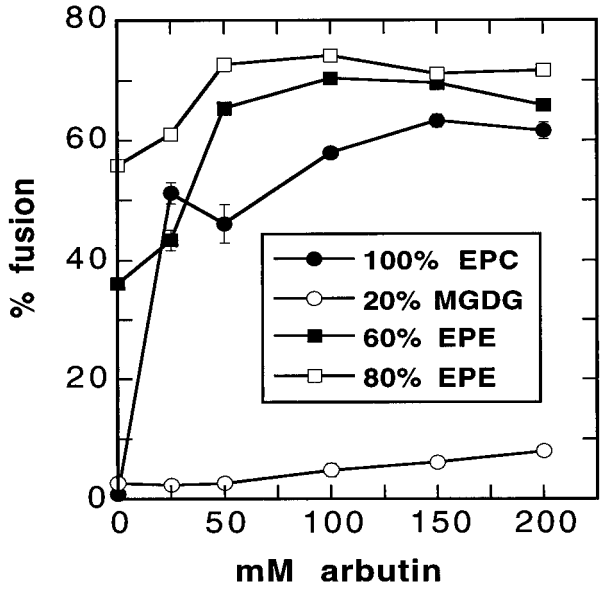

FIGURE 8 Freeze-thaw damage to liposomes in the presence of different concentrations of arbutin during freezing, measured as bilayer fusion. The membranes were composed of EPC and different fractions of either MGDG or EPE.

bilayer fusion between liposomes made from pure EPC, and this fusion was almost completely inhibited by the presence of 20% MGDG in the membranes (61.6% fusion in 100% EPC liposomes; 8.0% in 80% EPC/20% MGDG liposomes after freezing in the presence of 200 mM arbutin). Bilayers containing 60% or 80% EPE, on the other hand, showed increased fusion already in the absence of arbutin, and fusion increased further with increasing arbutin concentrations (Fig. 8). This was probably the reason why EPE was not as effective in reducing the cryotoxicity of arbutin or actually transforming it into cryoprotection.

Evidence from previous experiments (Oliver et al., 1998) indicates that arbutin interacts directly with lipids by inserting into membranes with its phenol moiety. Therefore, it was of interest to see how far the different effects of arbutin on the freeze-thaw stability of liposomes of different composition could be related to changes in the physical behavior of the membrane lipids. Membranes of three different lipid compositions (100% EPC; 80% EPC/20% MGDG; 50% EPC/50% EPE) were used throughout these experiments. Unfortunately, spectroscopic measurements of the kind reported next cannot be performed on frozen samples, and because of the limited solubility of arbutin the concentrations obtained during freezing cannot be achieved in unfrozen solutions. Because the effects described in the following figures are all concentration dependent, they are probably greatly magnified under freezing conditions. Assuming that only the rather hydrophobic phenol part of arbutin (Fig. 1) would partition between the fatty acyl chains and that the glucose moiety would remain in a more hydrophilic environment, the properties of the interfacial layer of the membranes are most likely to be perturbed by arbutin.

Measurements with two different probes gave independent evidence that the surface properties of liposomes were modified in the presence of arbutin (Figs. 9 and 10). MC540 is a dye that partitions into the membranes and orients parallel to the glycerol backbones of the lipids (Lelkes and Miller, 1980). Its absorbance at 570 nm increases greatly in a hydrophobic environment such as the hydrophobic interior of a membrane, whereas the second absorbance maximum at 530 nm is slightly reduced (Verkman and Frosch, 1985). Therefore, the degree of partitioning of MC540 into membranes can be estimated from the absorbance ratio A_{570}/A_{530} (Bakaltcheva et al., 1994). The observed reduction in this absorbance ratio as a function of the arbutin concentration in the suspending medium indicated that less MC540 could partition into the membranes (Fig. 9). This effect was most pronounced with membranes containing 50% EPE, and it was smallest in pure EPC membranes.

Almost identical results were obtained with the fluorescent lipid probe NBD-C_6-HPC (Fig. 10). These short-chain PC molecules were solubilized in an organic solvent, and a small volume was added to a stirred cuvette containing liposomes in different concentrations of arbutin. In the aqueous environment the NBD-C_6-HPC forms micelles in which NBD fluorescence is strongly quenched. Upon collision with liposomes, labeled lipids partition into the membranes and are released from the quenching inside the micelles. Therefore, partitioning can be quantitated from the fluorescence emission of the membrane-associated NBD. Fig. 10 shows that the partitioning of NBD-C_6-HPC was strongly reduced in the presence of arbutin and that the effect was most pronounced in membranes containing 50%

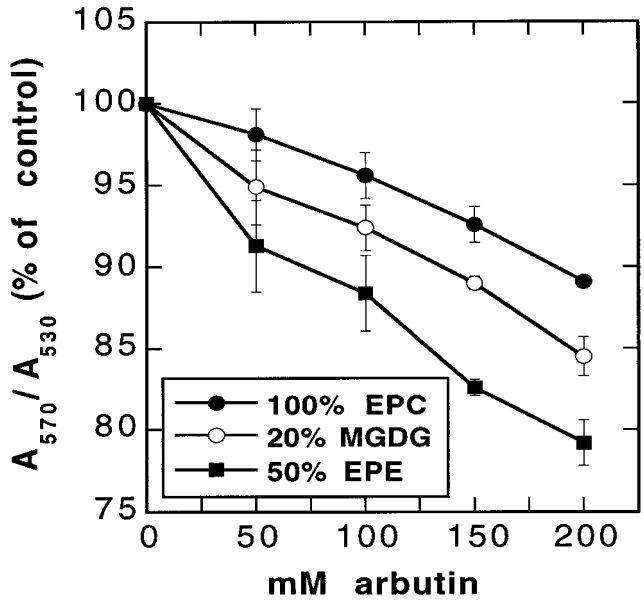

FIGURE 9 Absorbance ratio A_{570}/A_{530} of MC540 in the presence of liposomes of different composition and different concentrations of arbutin. A reduction in the absorbance ratio indicates reduced partitioning of the dye into the lipid headgroup region of the membranes. The means ± SD of three parallel samples are shown.

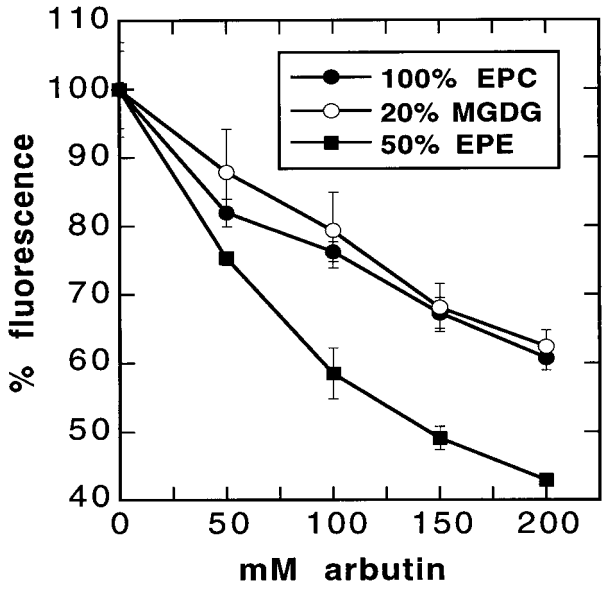

FIGURE 10 Fluorescence emission of the lipid probe NBD-C_6-HPC in the presence of liposomes of different composition as a function of the concentration of arbutin. Reduced fluorescence emission indicates a reduced ability of the probe to partition into the membranes. The means ± SD of three parallel samples are shown.

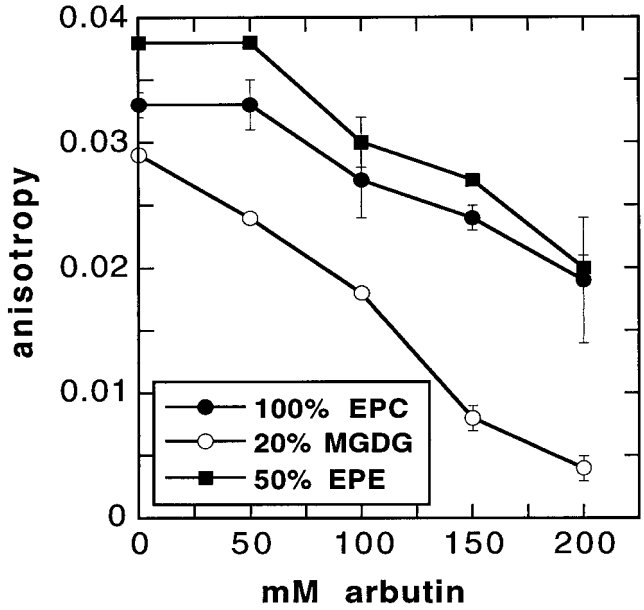

FIGURE 11 Steady-state fluorescence anisotropy of NBD-PE in liposomes of different composition as a function of the concentration of arbutin. NBD-PE reports on the fluidity in the lipid headgroup region of the membranes. The means ± SD of three parallel samples are shown.

EPE. Membranes formed of pure EPC or of EPC containing 20% MGDG were equally affected, but significantly less than membranes with EPE.

In the next set of experiments, the mobility of the lipids in different regions of the membranes was probed as a function of the arbutin concentration in the suspending medium. The steady-state anisotropy of the lipid headgroup region was measured with the probe NBD-PE. It can be seen (Fig. 11) that the mobility of the headgroups increased with increasing arbutin concentration in liposomes of all three lipid compositions. Membranes containing 100% EPC or 50% EPC/50% EPE were almost equally affected (a difference between 0 and 200 mM arbutin of 0.014 and 0.018 for EPC and EPC/EPE, respectively), whereas the membranes containing 20% MGDG showed a stronger decrease in headgroup anisotropy (a difference of 0.025 between 0 and 200 mM arbutin).

The ordering of the lipid acyl chains was differently affected by arbutin than were the headgroups. The probe that reports on the anisotropy closest to the glycerol backbone region was TMA-DPH. It showed a decrease in chain mobility with increased arbutin concentration, both for pure EPC membranes and for membranes containing 50% EPE (Fig. 12). There was no significant difference in anisotropy between samples containing 0 or 200 mM arbutin when 20% MGDG was present in the membranes. When the DPH moiety was located three carbon atoms lower down the fatty acyl chains (TMAP-DPH), the increase in chain order with increasing arbutin concentration was smaller for all lipid compositions but was still statistically significant in all cases (Fig. 12). The membranes containing 20% MGDG, however, were again the least affected. Consistent with the expected position of arbutin in the interfacial region of the membranes, there was no influence on the dynamics of the fatty acyl chains in the hydrophobic core region (Fig. 12; DPH) of the bilayers, independent of the lipid composition. With all four membrane probes, anisotropy measurements were also performed on the different liposomes in the presence of 200 mM NaCl. There were no significant differences between samples with or without additional NaCl (data not shown), indicating that the effects of arbutin were the results of specific interactions with the membranes and not nonspecific osmotic effects.

DISCUSSION

Although arbutin is a product of secondary metabolism that is found in several different plant species, its intracellular localization, its biosynthetic pathway, and its physiological role are all unknown. Based on its presence in high concentrations (up to 100 mM) in a resurrection plant and some frost-hardy species (Bianchi et al., 1993; Suau et al., 1991), as well as its antioxidative activity (Ioku et al., 1992) and the ability to inhibit PLA_2 (Oliver et al., 1996), it has been hypothesized that arbutin may play a role in plant stress tolerance. However, the fact that arbutin induced leakage in PC vesicles during drying (Oliver et al., 1998) and freezing (Fig. 4) cast serious doubts on this hypothesis, as the potentially beneficial effects would be rendered meaningless, if it severely destabilized cellular membranes at the same time. We have now shown that arbutin was cryoprotective for plant chloroplast thylakoid membranes (Fig. 2). This indicates that in addition to the indirectly stabilizing effects on membranes that have been reported before, arbutin also

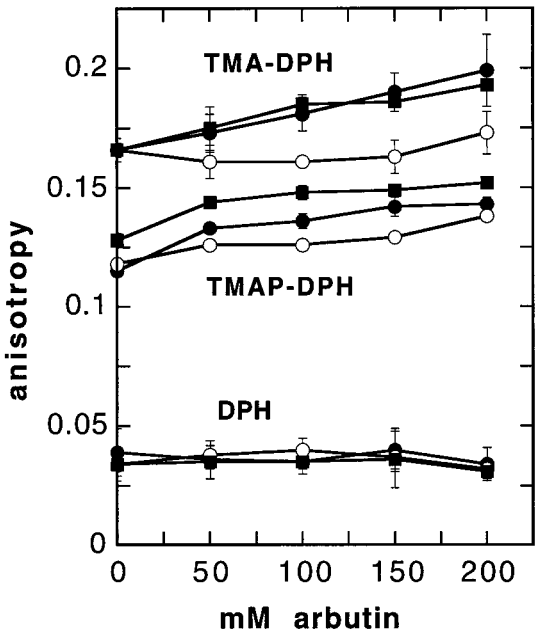

FIGURE 12 Steady-state fluorescence anisotropy of TMA-DPH, TMAP-DPH, and DPH in liposomes of different composition as a function of the concentration of arbutin. The symbols are the same as in Fig. 11 and indicate the means ± SD of three to six parallel samples. The probes report on the dynamics of the lipids in the hydrophobic core region of the membranes (DPH) or progressively closer to the membrane-solution interface (TMAP-DPH, TMA-DPH). For TMAP-DPH the differences for each lipid composition between samples in the absence of arbutin and samples containing 200 mM arbutin were significantly different at $p = 0.0005$ in a *t*-test. For TMA-DPH significant differences were found for EPE ($p = 0.0005$) and EPC ($p = 0.01$). No significant difference was found for liposomes containing MGDG ($p > 0.375$).

has directly stabilizing effects under stress conditions, which strongly argue for the proposed role of arbutin in plant stress tolerance.

This poses the problem that arbutin was cryoprotective to thylakoids under conditions similar to those under which it was cryotoxic to EPC vesicles. This is an interesting situation, because phospholipid vesicles have been used by numerous researchers as a model system for cellular membranes, and the results have come to be accepted as valid for membranes in general. The results presented here indicate that phospholipid vesicles are not a generally applicable model system, as even a small fraction of a plant galactolipid was sufficient to completely reverse the cryotoxic effects of a solute. Up to 15% MGDG ameliorated the cryotoxicity of arbutin (Fig. 4), whereas 20% resulted in a cryoprotective effect (Figs. 3–6). Cryoprotection in this case was complete at only 5 mM arbutin (Fig. 5). This reversal was not a general property of galactolipids, as DGDG was completely ineffective (Fig. 6). The other thylakoid lipids (PG, SQDG) had only a weak influence on the interplay between MGDG and arbutin (Fig. 3).

We have used thylakoids as a model to determine the effects of arbutin on a plant membrane. The natural target membrane(s) of arbutin, however, are unknown. The results obtained with liposomes containing EPE (Fig. 7) indicate that other cellular membranes, such as the plasma membrane, which contain no MGDG but PE (Lynch and Steponkus, 1987; Uemura et al., 1995), may also be less susceptible to destabilization by arbutin than pure PC membranes. But even at high concentrations, EPE was much less effective than MGDG in reversing the cryotoxic effects of arbutin. Of course, other components of plant membranes, such as cerebrosides and phytosterols, could also influence the response to arbutin. A comparison of the cryoprotection of thylakoids (Fig. 2) and liposomes containing 20% MGDG (Figs. 3 and 4) shows that the protective concentration range of arbutin was different. In liposomes, leakage was reduced only at concentrations up to ~50 mM, while higher concentrations increased leakage again. Thylakoids, on the other hand, were protected by up to 150 mM arbutin, and even in the presence of 200 mM freeze-thaw damage was only moderately increased, from 32% to 56%. This difference could be due to lipid-protein or arbutin-protein interactions, which are absent in liposomes. Alternatively, the high fraction of MGDG in thylakoids (50% of the total lipids) could further stabilize the membranes against the deleterious effects of high arbutin concentrations. Unfortunately, this cannot be investigated with liposomes, as such concentrations of MGDG would lead to the formation of H_{II} phase (Sprague and Staehelin, 1984), which disrupts the bilayers.

Although MGDG is a nonbilayer lipid, it does not induce bilayer fusion during freezing, in contrast to EPE (Fig. 8; Hincha et al., 1998). Arbutin-induced fusion between EPC bilayers during freezing was prevented by the presence of MGDG in the membranes, but not by EPE. This difference may explain why MGDG has a much stronger effect on freeze-thaw-induced leakage in the presence of arbutin than EPE. At high arbutin concentrations, however, leakage from liposomes containing 20% MGDG increased again, without a corresponding increase in fusion (compare Figs. 4 and 8). Obviously, arbutin can induce leakage during freezing both through fusion and through a nonfusogenic mechanism. During drying of PC liposomes only nonfusogenic leakage was observed in the presence of arbutin (Oliver et al., 1998), because in these experiments the sugars (sucrose and trehalose) that were also present formed a carbohydrate glass, which is known to prevent fusion (Sun et al., 1996).

The facts discussed above indicate that arbutin has complex effects on membranes, which become apparent under stress conditions and, depending on the lipid composition, may result in leakage and/or fusion. These effects are exerted via a direct interaction of arbutin with the lipids through the insertion of the phenol moiety (Fig. 1) into the membranes (Oliver et al., 1998). The reduced partitioning of MC540 and NBD-C_6-HPC into the membranes indicated that the glycerol backbone region was more tightly packed in the presence of arbutin (Figs. 9 and 10). The packing of the phenol rings of arbutin between the lipids also resulted in a decreased mobility of the fatty acyl chains close to the glycerol backbone (Fig. 12; TMA-DPH, TMAP-DPH). The

higher mobility of the lipid headgroups in the presence of arbutin (Fig. 11) indicates that the insertion of arbutin increased the spacing between the lipid molecules and reduced interactions between the headgroups (Fig. 13).

In accordance with the hypothesis that only the phenol, but not the glucose part of arbutin partitions between the lipids, the anisotropy of the core region of the membranes was not influenced by arbutin (Fig. 12; DPH). On the other hand, if the spacing between the lipids were increased because of the insertion of arbutin at the membrane-solution interface, the mobility of the fatty acyl chains in the core region might be expected to increase (Fig. 13). That this is indeed the case is indicated by the decrease in T_m determined with PC membranes by differential scanning calorimetry (Oliver et al., 1996, 1998), which is expected to be more sensitive than the depolarization measurements with DPH. Steady-state anisotropy values depend on both the rotational dynamics and the order parameter of DPH. Changes in the two parameters can partly compensate, reducing the apparent changes in anisotropy and thus making this method less sensitive than DSC.

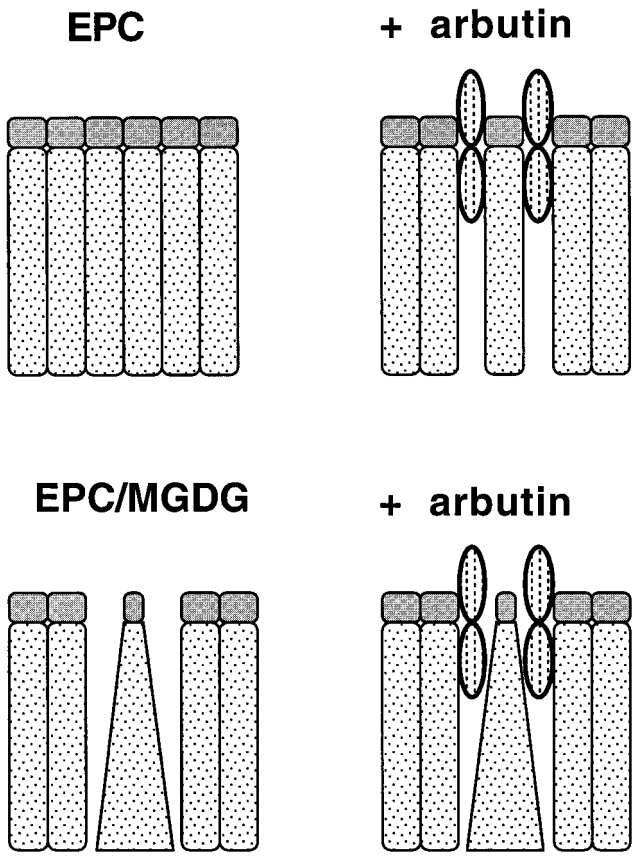

FIGURE 13 Schematic representation of cross sections of the outer monolayer of liposome membranes containing either only EPC (*cylindrically shaped molecules*) or a mixture of EPC and MGDG (*cone-shaped molecules*) in the absence and presence of arbutin. Darkly shaded parts of the lipids represent the headgroups, lightly shaded parts the acyl chain regions. Arbutin is depicted as two elipses, with the lower part representing the phenol ring and the upper part the glucose moiety (compare Fig. 1).

The increased fusion between EPC vesicles during freezing in the presence of arbutin (Fig. 8) might also be related to the increased spacing between the lipid hydrocarbon chains. According to the "shape concept" of lipid polymorphism (de Kruijff, 1987), the phase preference of a membrane lipid can be understood as the result of the relative volumes occupied by hydrophobic acyl chain regions and hydrophilic headgroup regions, as depicted schematically for EPC and MGDG in Fig. 13 (see Janes, 1996; Webb and Green, 1991 for reviews). Cylindrically shaped lipids such as EPC form bilayers in aqueous dispersions, whereas cone-shaped lipids such as MGDG or EPE arrange in H_{II} phase. During freeze-induced dehydration, PC and PE demix and the PE in closely approaching bilayers forms H_{II} (Rand, 1981; Webb et al., 1993), resulting in vesicle fusion. Because the intercalating arbutin molecules in EPC membranes increase the spacing of the lipids (Fig. 13), this could effectively change the overall shape of the PC molecules as their acyl chains have more space to move in. This would explain the fusogenic effect of arbutin in EPC bilayers (Fig. 8).

In contrast to EPE, MGDG does not induce fusion during freezing (Fig. 8). Apparently, during close approach of the membranes, interactions between the galactose headgroups stabilize the bilayers (see Hincha et al., 1998, for a more detailed discussion). Nevertheless have we observed increased leakage from MGDG-containing liposomes during freezing, which might be related to the curvature stress induced in the membranes by the presence of the cone-shaped lipid molecules. This could be ameliorated by the partitioning of arbutin into the "empty spaces" on the membrane surface (Gawrisch and Holte, 1996) created by MGDG, which would reduce the curvature stress (Fig. 13) and, at low arbutin concentrations, nonfusogenic leakage. The same argument can also be applied to the reduced leakage from EPE-containing liposomes that was observed (Fig. 7), which was also not related to fusion (Fig. 8). A similar stabilizing effect in the presence of PE has been observed for short-chain alkanols (reviewed in Gawrisch and Holte, 1996), which also locate at the lipid-water interface and increase the repulsion between lipid headgroups (compare Fig. 11). Apparently, at high arbutin concentrations, the capacity of the membranes to accommodate the solute is reached and the disruptive effects dominate again.

The hypothesis outlined above is also in agreement with the data on lipid dynamics (Fig. 12). The anisotropy measured with TMA-DPH showed that arbutin interacted with MGDG-containing membranes without interfering with acyl chain mobility, indicating that "empty spaces" on the membrane surface were filled (Fig. 12). The reduced partitioning of MC540 and NBD-C_6-HPC (Figs. 9 and 10) indicated, in addition, that the packing of the interfacial region had nevertheless become tighter. In EPC membranes, on the other hand, arbutin inserts between the acyl chains and constrains their mobility. Apparently, in EPE-containing membranes, no "open spaces" of sufficient size are created on the surface, and arbutin leads to hindered motion

of the upper parts of the acyl chains (Fig. 12). These differences between the effects of EPE and MGDG may also explain why much more EPE than MGDG can be incorporated into EPC membranes without compromising the bilayer structure.

In conclusion, the data presented in this paper are strong evidence that arbutin plays an important role in plant stress tolerance by directly and indirectly stabilizing cellular membranes. This protective role, however, is strictly dependent on the presence of a nonbilayer lipid, such as MGDG, in the membranes. The dramatically different effects of arbutin on membranes of different lipid composition can be understood in light of the spectroscopic data, on the basis of the "shape concept" of lipid polymorphism.

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