Plant Physiol. (1996) 111: 403–412

Characterization of Membrane Properties in Desiccation-Tolerant and -Intolerant Carrot Somatic Embryos

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In previous studies, we have shown that carrot (*Daucus carota* L.) somatic embryos acquire complete desiccation tolerance when they are treated with abscisic acid during culture and subsequently dried slowly. With this manipulable system at hand, we have assessed damage associated with desiccation intolerance. Fast drying caused loss of viability, and all K^+ and carbohydrates leached from the somatic embryos within 5 min of imbibition. The phospholipid content decreased by about 20%, and the free fatty acid content increased, which was not observed after slow drying. However, the extent of acyl chain unsaturation was unaltered, irrespective of the drying rate. These results indicate that, during rapid drying, irreversible changes occur in the membranes that are associated with extensive leakage and loss of germinability. The status of membranes after 2 h of imbibition was analyzed in a freeze-fracture study and by Fourier transform infrared spectroscopy. Rapidly dried somatic embryos had clusters of intramembraneous particles in their plasma membranes, and the transition temperature of isolated membranes was above room temperature. Membrane proteins were irreversibly aggregated in an extended β-sheet conformation and had a reduced proportion of α-helical structures. In contrast, the slowly dried somatic embryos had irregularly distributed, but nonclustered, intramembraneous particles, the transition temperature was below room temperature, and the membrane proteins were not aggregated in a β-sheet conformation. We suggest that desiccation sensitivity of rapidly dried carrot somatic embryos is indirectly caused by an irreversible phase separation in the membranes due to de-esterification of phospholipids and accumulation of free fatty acids.

Anhydrobiosis is a general phenomenon in nature. Animals, like nematodes or *Artemia* cysts and also plant organs, such as pollen and seeds, are able to survive almost complete dehydration (reviewed by Crowe and Crowe, 1992). Many studies have been undertaken to unravel the mechanisms involved in desiccation tolerance. It has been postulated that phase changes of membranes are involved in the desiccation sensitivity of dry organisms (Simon, 1974; Senaratna and McKersie, 1983a, 1983b; Crowe and Crowe, 1992).

Carrot (*Daucus carota* L.) somatic embryos survive desiccation to low moisture contents (5% of dry weight) when they are treated with ABA (38 μM) at the proper stage of development (Tetteroo et al., 1995). Furthermore, the drying time has to be sufficiently slow (at least 4 d) to secure survival, and prehydration of the dry somatic embryos in moist air before imbibition is necessary to prevent imbibitional injury. The mechanism of imbibitional injury has been characterized as the transient loss of permeability of the plasma membrane during its transition from the gel to the liquid-crystalline phase (Crowe et al., 1989a; Hoekstra et al., 1989). Imbibitional damage can be avoided by prehydration over water vapor or by heating above T_m. The finding that dry carrot somatic embryos also must be prehydrated before imbibition (Tetteroo et al., 1995) points to a similar type of phase change in the membranes of these embryos.

The conformational status of membranes is predominantly determined by the prevailing PLs and can be excellently analyzed by FTIR, both in vitro and in situ (Crowe et al., 1989b). In hydrated conditions, the membranes of living organisms generally are in the liquid crystalline phase. During water removal (drying), the distance between the PL headgroups decreases, leading to increased van der Waals' interactions between the acyl chains (reviewed by Crowe et al., 1992). The PL bilayer will then pass into the gel phase. Because of defects in the bilayer at the interphases of gel and liquid crystalline domains during the phase transition, the membranes become permeable to solutes (Chapman et al., 1967; Hammoudah et al., 1981; Cameron and Dluhy, 1987). This often results in extensive damage and even cell death.

Avoidance of phase transitions in membranes is considered to be important for survival under dry conditions (Crowe et al., 1992). Phase transitions due to dehydration can be prevented by replacing the water molecules around the polar headgroup by di- and oligosaccharides (Crowe et al., 1984; Crowe et al., 1987). Another way of avoiding phase transitions is by formation of more unsaturated acyl

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Abbreviations: FFA, free fatty acid(s); FTIR, Fourier transform IR spectroscopy; IMP, intramembraneous particles; PEMs, proembryogenic masses; PF, plasmatic face; PL, phospholipid; TEM, transmission electron microscope; T_m, gel-to-liquid crystalline phase transition temperature; X:Y, fatty acid with X carbons and Y *cis* double bonds.

chains, which decrease T_m of membrane lipids (Lynch and Steponkus, 1989). This occurs in pollen, as has been demonstrated by in vivo T_m measurements of membranes (Hoekstra et al., 1992b). During dehydration, T_m will rise but may not exceed ambient temperatures because of the high content of 18:3 acyl chains. Thus, organisms can become desiccation-tolerant by enhancing their unsaturation level. However, double bonds are very sensitive to free radical attack, and the resulting lipid peroxides may cause disruption of the membrane structure, leading to extensive leakage.

Extensive leakage of desiccation-sensitive tissues may be caused not only by a phase change of the PLs but also by problems with membrane proteins. Proteins may become irreversibly aggregated because of phase separation of the PLs (Hemminga et al., 1992). Protein secondary structures can be exquisitely studied in vivo with FTIR (Wolkers and Hoekstra, 1995).

In the present paper, we used carrot somatic embryos as the experimental system to study desiccation tolerance mechanisms. Using slow and fast drying techniques, which provide viable and dead somatic embryos, respectively, we investigated whether phase changes or phase separation in the membranes are the main cause of desiccation sensitivity. We measured leakage, PL content, and acyl chain composition. TEM studies of freeze-fracture replicas of plasma membranes were compared with FTIR studies on membrane phase behavior and protein secondary structure. Several causes of membrane leakage in carrot somatic embryos are discussed.

MATERIALS AND METHODS

Plant Material

Carrot (Daucus carota L.) somatic embryos, genotype Trophy, were produced according to described methods (Tetteroo et al., 1995). Survival of desiccation was varied by the use of different drying regimes and prehydration treatments before imbibition. Desiccation tolerance was measured as germination percentage after 10 d of imbibition on B_5 medium (Gamborg et al., 1968). Somatic embryos were scored as having survived desiccation when they showed elongated roots and green cotyledons.

K^+ Leakage

Approximately 10 mg of dry somatic embryos were homogenized with a mortar and pestle in 250 μL of 0.1 N HCl to extract total K^+. After centrifugation, the supernatant was diluted 50 times, and subsequently the K^+ content was measured with a flame photometer (PFP 7, Jenway, Felsted, UK).

To assess K^+ leakage, approximately 10 mg of dry somatic embryos were prehydrated in moist air for 4 h and then allowed to imbibe in 5 mL of K^+-free B_5 medium in a 9-cm Petri dish. During imbibition, the somatic embryos were continuously agitated on a rotary shaker. In the course of imbibition, 40-μL samples of the medium were regularly taken, diluted to 4 mL with Milli-Q (Millipore) water, and immediately analyzed with the flame photometer. K^+ leakage was expressed as percentage of the total K^+ content.

Carbohydrate Leakage

Total carbohydrates from approximately 20 mg of dry somatic embryos were extracted for 15 min at 76°C in 1 mL of 80% methanol. Raffinose was used as the internal standard. The methanol was evaporated with a Speed Vac (Savant Instruments, Farmingdale, NY). The dry samples were suspended in water and centrifuged with an Eppendorf centrifuge. After dilution of the supernatant in Milli-Q water, the samples were analyzed on a Dionex HPLC (Dionex, Sunnyvale, CA) equipped with a Carbopac-1 column and a pulsed electrochemical detector (for details, see Tetteroo et al., 1994). One hundred millimolar NaOH was used as the eluent. The data were captured with a Spectra Physics (San Jose, CA) integrator model SP4400 and Spectra Physics software (Labnet, Chromnet).

To assess carbohydrate leakage, approximately 20 mg of dry somatic embryos were prehydrated in moist air for 4 h and then allowed to imbibe in 4 mL of Suc-free B_5 medium in a 9-cm Petri dish, to which raffinose (1 mg mL^{-1}) was added as the internal standard. During the imbibition, the somatic embryos were continuously agitated on a rotary shaker. In the course of imbibition, 20-μL samples of the medium were regularly taken. Carbohydrate extraction and analysis were performed as indicated above. Leakage was expressed as percentage of the total carbohydrate content.

Phospholipid and FFA Analyses

Lipid extraction and analysis were performed according to methods previously described by Hoekstra and van Roekel (1988). In brief, somatic embryos were homogenized with a mortar and pestle in $CHCl_3$:methanol (2:1, v/v) with diheptadecanoyl-phosphatidylcholine and heptadecanoic acid as the internal standards. After 5 min of mild ultrasonic treatment, the homogenate was centrifuged, and the supernatant was washed with 0.2 volume of 0.9% NaCl solution. After centrifugation, the $CHCl_3$ layer was recovered and passed over an anhydrous Na_2SO_4 column. The dried $CHCl_3$ was evaporated, and the residue was dissolved in 1 mL of $CHCl_3$. Phospholipids were separated from neutral lipids and FFA with a SEP-PAK silica cartridge (Waters, catalog no. 51900) according to Juaneda and Rocquelin (1985). The PL fraction was transmethylated with 3 mL of 0.3 N KOH in methanol for 15 min at 70°C under vigorous shaking. The samples were cooled, and subsequently 1 mL of a saturated NaCl solution and 1.5 mL of hexane were added. After mixing and phase separation (centrifugation), the hexane fraction containing the methylated fatty acids was collected and passed over an anhydrous Na_2SO_4 column. The samples were then injected in a Perkin-Elmer 8320 Capillary Gas Chromatograph, equipped with a flame ionization detector and a CPsil 88 column (50 m long, 0.50 mm in diameter). The oven temperature during an analysis was 60°C for 2 min, then further heating at 30°C min^{-1} to 150°C, and then 3°C

min^{-1} to 240°C. The amounts of the fatty acids were calculated on the basis of peak areas after identification on the basis of retention times of known standards.

The FFA were separated from di- and triacyl glycerides by TLC, using a mixture of hexane:ether:acetic acid (80:20:1, v/v) as the developing solvent. After they were scraped off, the FFA were directly methylated with freshly prepared diazomethane. GC analysis was performed as described for PL.

Electron Microscopy

Somatic embryos, enveloped with B_5 medium, were mounted in golden specimen carriers (3 mm, Balzers, Liechtenstein). Directly afterward, the carriers were plunged into liquid propane (at −180°C) and stored in liquid N_2 (−196°C). Freeze-fracture replicas were prepared in a BAF 400 freeze-fracture apparatus (Balzers) at −120°C and 10^{-7} torr. Platina was evaporated at an angle of 40°, and carbon was evaporated to support the replicas. To digest all somatic embryo material, the replicas were treated with 3% cellulase Y/C (Seishin Pharmaceuticals, Tokyo, Japan) and 0.05% macerozym R-10 (Serva, Heidelberg, Germany) in 0.1% Mes buffer (pH 5.5) containing 8% mannitol. Replicas were cleaned with 50% CrO_3 solution for 2 h and overnight soaking in 9% NaOCl solution. After the replicas were mounted on electron microscope grids, electron micrographs were made with a transmission electron microscope (JEM-1200 EX II, JEOL) at 80 kV. IMP analyses were performed with an image analysis program (TIM version 3.3, Difa Measuring System, Breda, The Netherlands). Only plasma membranes were used for this quantitative analysis. We considered IMP distribution to be clustered when more than 5 to 10 IMPs are grouped together with distinct spaces between the clusters.

Fresh torpedo-shaped somatic embryos were directly freeze-fixed in liquid propane after the culture period in vitro. Slowly and rapidly dried somatic embryos were first prehydrated in moist air for 4 h (100% RH) at 25°C and were further allowed to imbibe in B_5 medium for 2 h before the freeze-fixation. The membrane fracture face nomenclature of Branton et al. (1975) was used for the description of the freeze-fracture images.

Microsomal Membrane Isolation

Somatic embryos were prehydrated in moist air for 4 h at 25°C and subsequently incubated in B_5 medium for 2 h. The embryos (200 mg dry weight) were then homogenized with a mortar and pestle using 1 mM EDTA, 1 mM EGTA, 1 mM diethylenetriamine-pentaacetic acid, 5 mM ascorbic acid, and 1 mM DTT in 10 mM Tes buffer, pH 7.4. The homogenate was centrifuged two times at 10,000*g*. The supernatant was then subjected to high-speed centrifugation (35 min at 100,000*g*), and after resuspension of the pellet in either H_2O or D_2O, the high-speed centrifugation was repeated. The isolated membrane pellet was directly used for FTIR analysis.

IR Spectroscopy

FTIR spectra were recorded on a Perkin-Elmer 1725 FTIR spectrometer equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and a Perkin-Elmer microscope interfaced to a personal computer. Membrane pellets were sandwiched between two CaF_2 windows and loaded in a temperature-controlled cell. For membrane fluidity studies, 50 to 60 FTIR spectra were recorded in the range between −60 and 80°C. For protein studies, spectra were recorded at room temperature. The optical bench was purged with dry, CO_2-free air (Balston, Maidstone, Kent, UK) at a flow rate of 25 L min^{-1}. The acquisition parameters were 4 cm^{-1} resolution, 512 co-added interferograms (32 in the membrane fluidity studies), 2 cm s^{-1} moving mirror speed, 3500 to 900 cm^{-1} wave number range, and triangle apodization function. The time needed for acquisition and processing of a spectrum (512) was 4.5 min.

Spectral analysis and display were carried out using the IR Data Manager Analytical software, versions 2.5 and 3.5 (Perkin-Elmer). The spectral region between 3000 and 2800 cm^{-1} was selected, and second derivative spectra were calculated. The second derivative spectrum was normalized, and the band position was calculated as the average of the spectral positions at 80% of the total peak height. Membrane fluidity was monitored by observing the band position of the CH_2 symmetric stretching band at approximately 2850 cm^{-1}. T_m was estimated from the discrete shifts in these band positions with temperature, i.e. the temperature at which half of the hydrocarbon-containing compounds have melted. The spectral region between 1800 and 1500 cm^{-1} was selected for protein studies. This region contains the amide-I and the amide-II absorption bands of the protein backbones. Procedures to generate second derivative spectra and deconvolved spectra were carried out according to Wolkers and Hoekstra (1995).

RESULTS

Leakage

For maximum germination, dry somatic embryos must be prehydrated for at least 4 h in moisture-saturated air

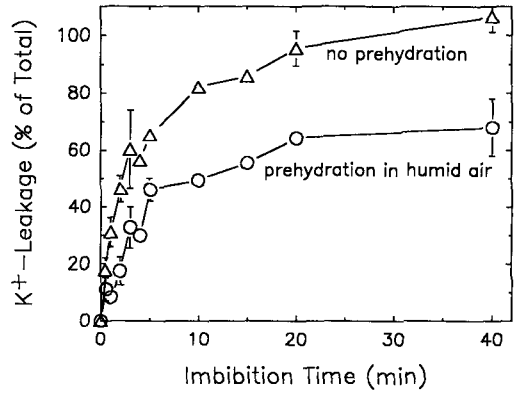

Figure 1. K^+ leakage during imbibition of slowly dried viable carrot somatic embryos with (○) and without (△) prehydration in humid air for 4 h. Data are means of triplicate leakage experiments. Error bars (±SD) are indicated when they exceed symbol size.

before imbibition in B_5 medium (Tetteroo et al., 1995). Without prehydration, somatic embryos leaked more K^+ and at a faster rate over the first 5 min than with prehydration (Fig. 1). These results clearly indicate that dry somatic embryos are sensitive to imbibitional stress and that leakage of endogenous solutes is one of the causes for their nonviability. However, the prehydrated embryos, which germinated approximately 100%, still leaked over 50% of the total K^+ present, which makes the relationship of solute loss with viability less clear-cut.

The drying rate also determines whether somatic embryos retain their viability after dehydration to 5% moisture content (Tetteroo et al., 1995). Fast drying was detrimental, whereas slowly dried somatic embryos survived completely. Figure 2 demonstrates the difference in K^+ leakage between slowly and rapidly dried somatic embryos, both of which had been subjected to the 4-h prehydration treatment. Despite prehydration, which reduces imbibitional damage (see Fig. 1), the intolerant somatic embryos leaked more K^+ and at a higher rate. The drying rate had the same effect on leakage of umbelliferose and Suc from the somatic embryos (Fig. 3).

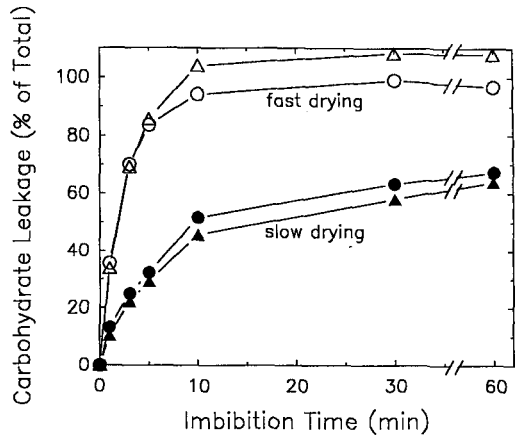

Figure 3. Leakage of umbelliferose (○, ●) and Suc (△, ▲) during imbibition of desiccation-tolerant (slow drying, ● and ▲) and intolerant (fast drying, ○ and △) carrot somatic embryos. Prehydration conditions were as in Figure 2. A single extraction was performed.

Phospholipid Analysis

The extensive leakage of K^+ and sugar from the intolerant somatic embryos (rapidly dried) cannot be simply explained by a phase change of membranes associated with rehydration; the underlying chemical changes should also be considered. Therefore, we have analyzed the PL content and acyl chain composition of fresh, nondried somatic embryos, of dried tolerant and intolerant somatic embryos, and of the latter two after imbibition for 2 h (Table I). Because data on acyl chain composition are the averages of measurements of five independent somatic embryo cultures at different seasons of the year, fairly large variations were observed. No substantial differences in acyl chain composition were found. This implies that lipid peroxidation could not have played an important role in the observed leakage of solutes from the desiccation-sensitive somatic embryos that had imbibed.

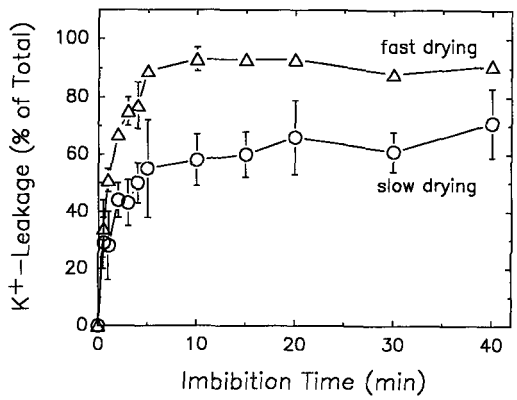

Figure 2. K^+ leakage during imbibition of desiccation-tolerant (after slow drying, ○) and -intolerant (after fast drying, △) carrot somatic embryos. Average of triplicate imbibition experiments. All embryos were prehydrated in saturated air for 4 h. Error bars (±SD) are indicated when they exceed symbol size.

However, the PL content of the dried, intolerant somatic embryos decreased by about 20% during rapid drying in all five replicates (Table I). Such a decrease was not observed when the somatic embryos were slowly dried. This means that lipid breakdown must have taken place during the 4 h of fast drying. Prehydration in humid air for 4 h followed by 2 h of incubation in B_5 medium further decreased the PL content, much more in the desiccation-sensitive specimens than in the tolerant ones.

Analysis of the content of FFA in the dried, desiccation-sensitive and -tolerant somatic embryos revealed elevated de-esterification in the sensitive embryos, which is in agreement with the loss of PLs (Table I). The sensitive specimens that had imbibed for 2 h, particularly, had increased FFA contents.

Freeze-Fracture

To visualize a possibly altered ultrastructure of membranes, freeze-fracture replicas were studied with TEM. An electron micrograph of the PF side of a plasma membrane in a fresh somatic embryo is shown in Figure 4. The somatic embryos were treated with 38 μM ABA, which rendered them potentially desiccation tolerant when they were dehydrated slowly. The membranes had a random IMP distribution, and plasmodesmata were also observed (see arrows in Fig. 4). These ultrastructural features corresponded to the results of Emons et al. (1992) with carrot PEMs and were typical of a physiologically functional membrane (Leshem, 1992). Quantitative data of the IMP distribution and other features of these membranes are indicated in Table II. The extraplasmatic face and PF sides were initially scored separately, but no differences in distribution were found between them, so we have combined the data in Table II. Of the 102 membranes from fresh, nondried somatic embryos that were scored, 40% showed microfibril imprints and 97% had a flat surface, which indicated that

Table I. *Content and acyl chain composition of PLs in fresh, desiccation-tolerant (after slow drying) and -intolerant (after fast drying) carrot somatic embryos*

The latter two specimens were also rehydrated in vapor-saturated air for 4 h followed by 2 h of imbibition in B_5 medium. The FFA contents are also given. Data are averages of five independent experiments, except the FFA content data, which are the average of two experiments.

Treatment	FFA Content	PL Content	Mole Percent Fatty Acids in PL				
			16:0	18:0	18:1	18:2	18:3
	$mg\ g^{-1}$	$mg\ g^{-1}$					
Fresh, no drying	2.0	35.35	23.5	2.2	18.7	52.8	2.5
Dry after slow drying	1.9	33.96	21.9	2.2	17.9	55.3	2.3
Dry after rapid drying	4.7	27.43	24.9	2.5	20.4	49.8	2.3
2 h imbibed (slow drying)	1.5	20.54	24.0	1.6	14.0	57.1	3.1
2 h imbibed (rapid drying)	8.1	9.60	27.1	1.9	16.2	48.9	5.5
LSD (P=0.05)	2.6	7.05	11.3	3.0	12.3	8.6	2.9

the fresh embryo cells were fully turgid (Pearce, 1985). Only 10% of the membranes had exocytose configurations (not shown on micrographs), which have earlier been described in carrot PEMs by Emons et al. (1992).

Plasma membranes in tolerant somatic embryos that had imbibed for 2 h looked similar to those in fresh somatic embryos, except that they had fewer microfibril imprints (not shown on micrographs) and were more undulated (Fig. 5; Table II). This can be explained by the fact that the 2-h imbibition period is not sufficiently long to allow the cells to become fully turgid. These membranes also contained 3 times more exocytose configurations (Table II) compared with those in fresh somatic embryos. These exocytose configurations might function as storage organs for PLs during the water stress because the decrease in cell volume during dehydration necessitates a reduction of membrane area. The IMPs in the plasma membranes of the desiccation-tolerant somatic embryos were somewhat irregularly distributed (Fig. 5), but clustering was rare (Table II).

Membranes in intolerant somatic embryos (rapid drying) that had imbibed clearly showed different features (Fig. 6). Almost all replicas (98%; see Table II) displayed a distinct clustering of IMPs, which was indicative of domain formation of PLs and membrane proteins. Moreover, plasmodesmata, microfibril imprints, and exocytose configurations were lacking, which was an indication that these membranes had lost their physiological functions.

We also analyzed freeze-fracture images of plasma membranes in somatic embryos developed on a medium with the suboptimal ABA concentration of 3.8 μM (Table II). These embryos acquired only partial desiccation tolerance (25%) when they are dried slowly (Tetteroo et al., 1995). This partial survival was also reflected in their membrane features. As much as 93% of the plasma membranes in the slowly dried somatic embryos had clustered IMPs, whereas

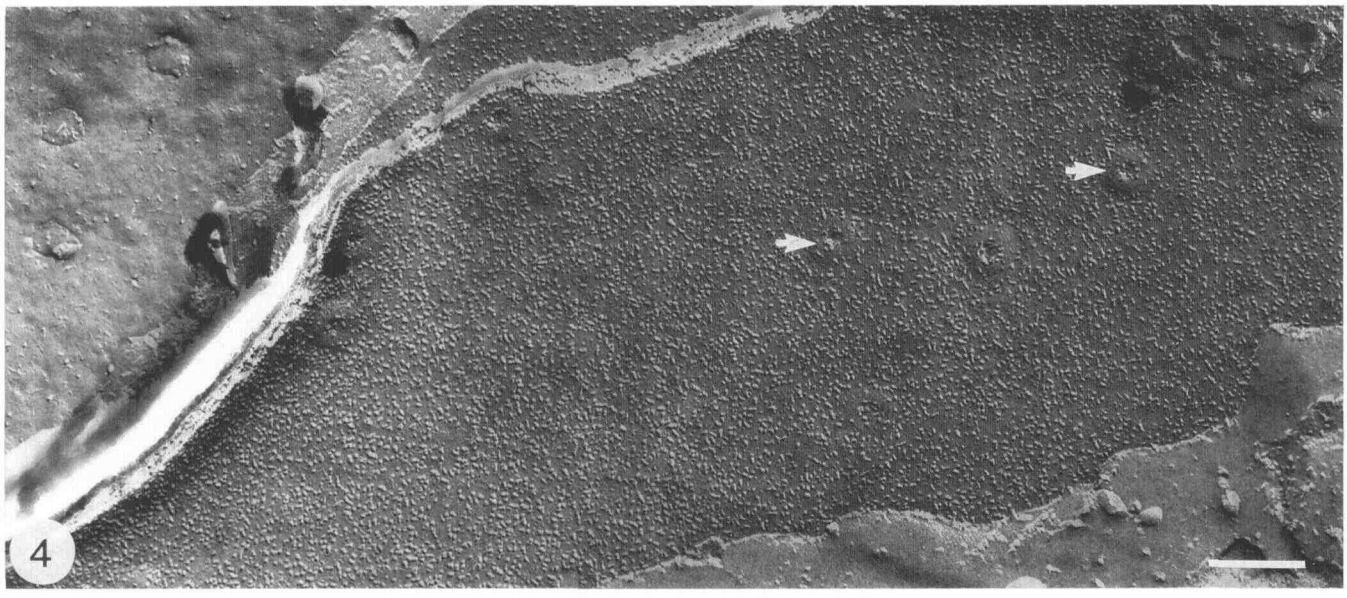

Figure 4. TEM micrograph of a freeze-fractured fresh somatic embryo cultured in liquid medium containing 38 μm ABA. The IMPs in the PF of the plasma membrane are randomly distributed, and plasmodesmata also are visible (arrow). Bar = 200 nm.

Table II. *Quantative data of the visual properties of freeze-fracture replicas of plasma membranes in fresh, nondried carrot somatic embryos and in rehydrated tolerant (after slow drying) and intolerant (after fast drying) somatic embryos*

Rehydration was for 4 h in water vapor-saturated air, followed by 2 h in B_5 medium. Somatic embryos were treated with either 38 μM ABA or the marginal concentration of 3.8 μM 1 week before the drying procedures.

Treatment and Status of Somatic Embryos	Viability	Number of Membranes Observed	IMP Distribution		Membrane Surface		Other Features		
			Random	Clustered	Flat	Undulated	Plasmodesmata	Microfibril imprints	Exocytose configurations
	% germination					%			
38 μM ABA									
Fresh	100	102	100	0	97	3	28	40	10
Slowly dried	100	154	92	8	51	49	6	8	31
Rapidly dried	0	54	2	98	22	78	0	2	0
3.8 μM ABA									
Fresh	100	106	99	1	85	15	24	26	16
Slowly dried	25	42	7	93	55	45	0	0	0
Rapidly dried	0	20	0	100	60	40	0	0	0

no plasmodesmata, microfibril imprints, or exocytose configurations were found.

Membrane Fluidity

At a certain point during the rapid drying or the subsequent rehydration procedure, membranes in the desiccation-sensitive somatic embryos had undergone a phase separation (Fig. 6). The phase-separated intramembraneous proteins might have irreversibly aggregated, from which it could be erroneously concluded that the membrane lipids were still in gel phase after 2 h of imbibition. This question was resolved by FTIR analysis of the CH_2 vibrational freedom (wave number range from 3000-2800 cm^{-1}). A typical plot of the position of the CH_2 symmetric stretching band (at approximately 2850 cm^{-1}) against the temperature is shown in Figure 7. The shape of the curve suggests the existence of two lipid populations in each membrane pellet, one with a T_m between −20°C and −10°C and one with a T_m above 0°C. Of the many explanations that could be generated, the most likely is that the lowest T_m is due to neutral lipid (oil) that was entrapped during the membrane isolation procedure and that the higher T_m is from the membrane phospholipids. Nonetheless, there is a considerable difference between the phase-transition curves of the isolated membrane preparations. Membranes from the rapidly dried somatic embryos had a higher average T_m (22°C) than those from the slowly dried ones (8°C). The measurements had to be conducted on isolated microsomal membranes because in situ FTIR measurements of the CH_2 absorbance in the intact somatic embryos are difficult to interpret because of oil interference (Hoekstra et al., 1993). At room temperature, membranes isolated from the rehydrated viable somatic embryos were mainly in the liquid crystalline phase, whereas at least part

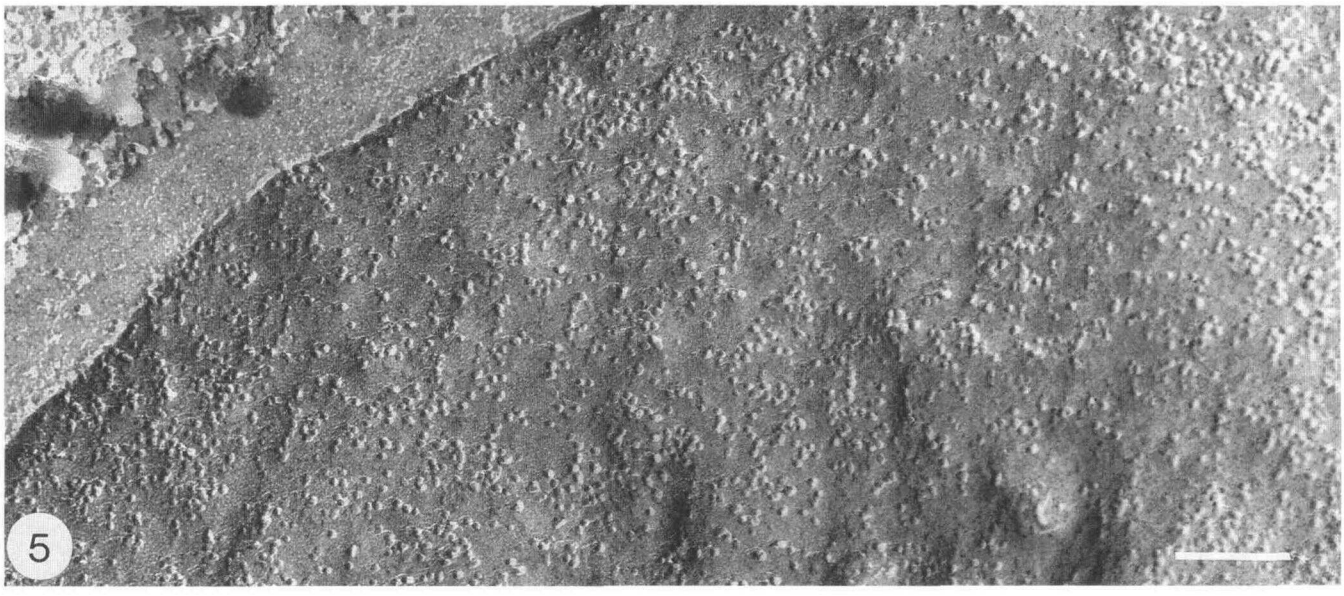

Figure 5. TEM micrograph of a freeze-fractured desiccation-tolerant somatic embryo, cultured as in Figure 4. After slow drying to 0.05 g H_2O g^{-1} dry weight, the embryos were rehydrated in humid air for 4 h and allowed to imbibe in B_5 medium for 2 h. The IMPs in PF are irregularly distributed in an undulated plasma membrane. Bar = 100 nm.

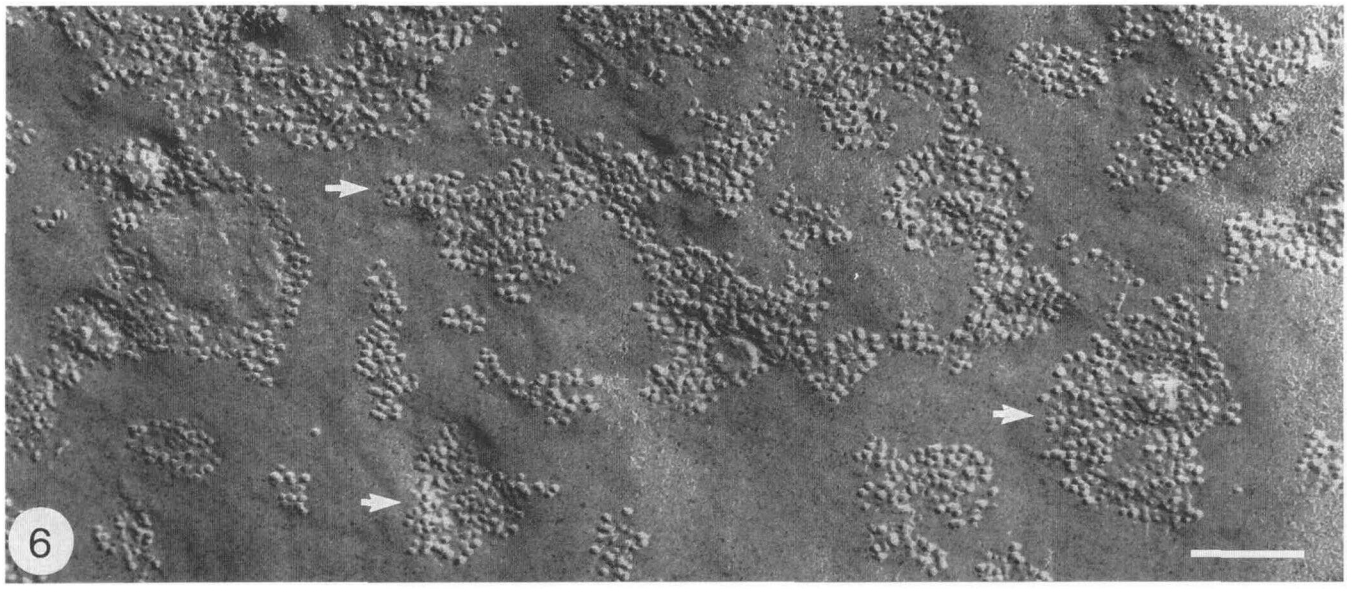

Figure 6. TEM micrograph of a freeze-fractured desiccation-intolerant somatic embryo, cultured as in Figure 4. After fast drying, somatic embryos were rehydrated in humid air for 4 h and allowed to imbibe in B_5 medium for 2 h. An undulated PF of the plasma membrane shows a distinct clustering of IMPs (arrows). Bar = 100 nm.

of the membrane fraction from the nonviable embryos was in the gel phase.

Membrane Protein Secondary Structure

To analyze whether the clustering of IMPs is associated with a possible irreversible aggregation of intramembraneous proteins in the desiccation-sensitive somatic embryos, FTIR absorbance spectra were made of the isolated membranes in D_2O over the region between 1800 and 1500 cm^{-1} (Fig. 8A). Three major absorption bands could be observed. The absorption band at approximately 1744 cm^{-1} could be assigned to ester bonds of the lipids. Those at approximately 1655 cm^{-1} and 1548 cm^{-1} represent the amide-I and amide-II bands of proteins, respectively (Susi et al., 1967). From the areas of the bands at approximately 1744 and 1655 cm^{-1}, it was calculated that the lipid-to-protein ratio decreased by a factor of 1.7 in the membranes from rapidly dried somatic embryos, which is in agreement with the considerable loss of PLs, as shown in Table I.

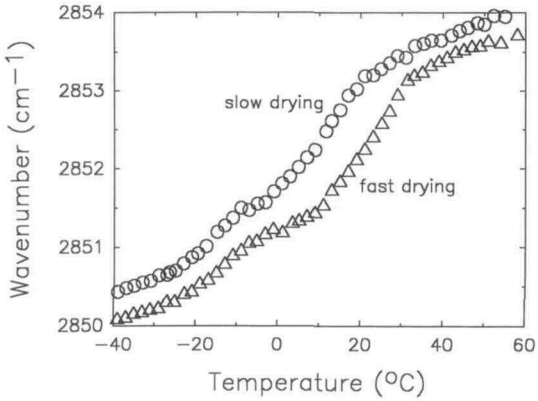

Figure 7. Wave number versus temperature plot (FTIR) of microsomal membranes isolated from slowly and rapidly dried carrot somatic embryos (representative plots). The data points represent the symmetric CH_2 stretching vibration of slowly dried embryos (○) and rapidly dried embryos (△).

To resolve the protein secondary structures in the isolated membranes, the spectra were deconvolved (Fig. 8B). Three bands could be distinguished in the amide-I region of IR spectra of the isolated membranes from slowly dried somatic embryos, namely at 1640, 1655, and 1680 cm^{-1}. In the isolated membranes from rapidly dried somatic embryos, two of the amide-I bands shifted partly to lower wave numbers, namely 1636 and 1653 cm^{-1}. The band at approximately 1655 cm^{-1} is dominated by α-helical structure (Surewicz and Mantsch, 1988; Haris et al., 1989; Bandekar, 1992). The band at approximately 1640 contains contributions from random coil and turn structures (Surewicz and Mantsch, 1988). The band at 1680 most likely represents turn-like structures. Rapid drying decreased the amount of α-helical structure compared with slow drying, which was deduced from the line-height ratios of the amide-I bands. The band at 1636 cm^{-1} indicates the presence of β-sheet structures (Susi et al., 1967) in rapidly dried somatic embryo membranes. Our results indicate that rapid drying of the somatic embryos is correlated with a decoiling of the α-helical structure in favor of β-sheet structures, characterized by peptide C=O groups having hydrogen bonds more colinearly oriented with the NH groups.

DISCUSSION

Preservation of membrane integrity in the dry state has been proposed to be a key factor in the survival of anhydrobiotic organisms (Crowe and Crowe, 1992). Membrane integrity is generally determined by measuring leakage of cytoplasmic solutes like K^+ or sugars into the imbibition medium (McKersie and Stinson, 1980; Senaratna and Mc-

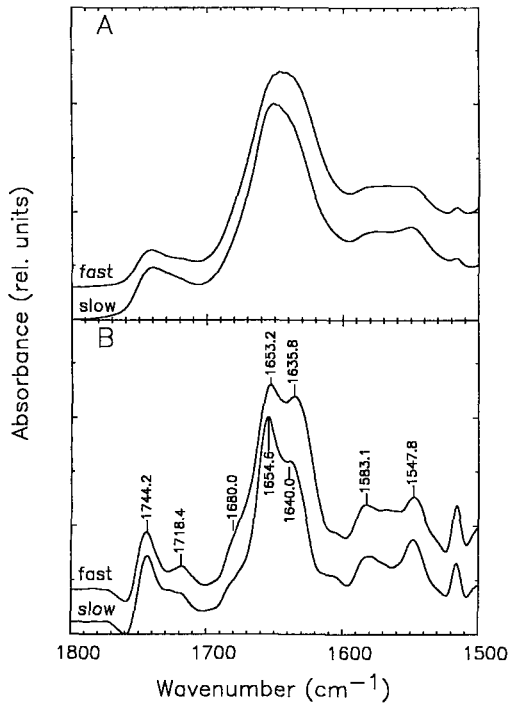

Figure 8. Absorbance (A) and deconvolved absorbance (B) FTIR spectra of isolated membranes from somatic embryos that had imbibed and were either slowly or rapidly dried (representative spectra). Membranes were prepared in D_2O. The prehydration was for 4 h in humid air, followed by 2 h of incubation in B_5 medium.

Kersie, 1983b; Hoekstra et al., 1992a). Our leakage experiments also demonstrated that membranes play a crucial role in the acquisition of desiccation tolerance by carrot somatic embryos. Within 5 min, all cytoplasmic solutes had leached from intolerant somatic embryos, which indicated a complete loss of membrane integrity during dehydration and rehydration, similar to that in desiccation-intolerant soybean embryos (Senaratna and McKersie, 1983a, 1983b).

However, tolerant somatic embryos leached as much as 50% of their endogenous K^+ and sugars but at a lower rate. This considerable loss from viable somatic embryos might be explained by the location of these solutes. K^+ will also be located in intracellular spaces because the somatic embryos are developed in B_5 medium, which is high in K^+. However, the losses of umbelliferose and Suc are not related to their original presence in B_5 medium. Umbelliferose was never added to the medium, and the added Suc was rapidly converted to Glc and Fru. These sugars might be actively transported to the outside of the plasma membrane during drying to enhance protection. The absolute requirement for such sugar transport was demonstrated in yeast mutants lacking the trehalose transporter in the membrane (Crowe et al., 1996). Thus, the K^+ and sugars in the imbibition medium may in part originate intercellularly and not from leakage through the plasma membrane. Another explanation for the considerable leakage from tolerant somatic embryos might be that not all embryo cells are viable and that the solutes originate from dead cells in the nevertheless 100% viable somatic embryos. However, the lack of browning reactions in the rehydrated viable embryos does not favor this explanation. Alternatively, a transient leakage independent of phase changes may occur upon imbibition regardless of tolerance state.

Our data indicate the occurrence of two types of membrane injuries (Figs. 1 and 2). The two types seemed similar because they showed similar leakage kinetics. However, the first type of injury (imbibitional damage) can be circumvented by prehydration (Fig. 1). Imbibitional damage has been previously described in pollen (Hoekstra and van der Wal, 1988). Zygotic embryos of different plant species also demonstrate this kind of damage but usually only when the testa is ruptured (Duke and Kakefuda, 1981). This again indicates that somatic embryos resemble zygotic embryos and that the seed coat has an important function in desiccation tolerance. The second type of damage, arising during the rapid drying, is irreversible (Figs. 2 and 3).

We analyzed freeze-fracture replicas of membranes in rapidly dried somatic embryos to explain the increased permeability. The electron micrographs clearly showed the occurrence of lateral phase separations of PLs and membrane proteins. Such phase separations were also detected in rehydrated microsomes of lobster muscles, which had lost their physiological function during lyophilization (Crowe and Crowe, 1992), and in membranes of drought-stressed wheat leaves, which showed increased leakage (Pearce, 1985).

Phase transitions can be caused by dehydration of membranes and may lead to domain formation. After rehydration, the components may be fully mixed again. However, the membranes in rapidly dried, desiccation-intolerant somatic embryos remain phase separated, which might be explained in two ways. On the one hand, the PLs in the membranes might remain in gel phase after rehydration because of the accumulation of FFA and lipid peroxides, both originating from free radical activity (Senaratna et al., 1984, 1985a, 1985b). On the other hand, the intramembraneous proteins might have irreversibly aggregated in extended β-sheet structures (Sanders et al., 1993), which prevents them from redistributing in the liquid crystalline membrane. The aggregated proteins might thus form the disturbances in the membrane through which the leakage occurs.

Our PL and FFA analyses support the first hypothesis. A decrease of the PL content and an increase of the FFA content were found in the rapidly dried somatic embryos and were typically the result of de-esterification of acyl chains from the glycerol backbone (Table I). As reported by Senaratna et al. (1985b), we also did not detect changes in composition or saturation level of the acyl chains. Furthermore, the FTIR data on the fluidity of the membranes support the occurrence of gel phase in desiccation-intolerant somatic embryos but not in the tolerant ones (Fig. 7).

However, we also gathered evidence for the second hypothesis in relation to protein structure. FTIR spectroscopy is one of the most suitable techniques to study protein secondary structure in situ and also in isolated membranes (Surewicz et al., 1993). Both theoretical and experimental studies with model polypeptides and proteins have shown

that there is a good correlation between the amide-I band frequency and the type of secondary structure (Surewicz and Mantsch, 1988; Haris et al., 1989; Bandekar, 1992). The spectra of the amide-I region of membranes isolated from *Daucus* somatic embryos are typical of membrane proteins (Haris et al., 1989; Garcia-Quintana et al., 1993), where a major contribution from α-helical structures has been reported. We interpret the shift to lower wave numbers of the amide-I band and the decrease in the amount of α-helical structure (Fig. 8) as a decoiling of α-helical proteins into intermolecular-extended β-sheet conformation in the isolated membranes from the rapidly dried somatic embryos.

We have demonstrated with TEM studies in combination with FTIR analysis that both gel phase lipid and membrane protein aggregation occur in membranes during rapid drying of carrot somatic embryos. However, it could also be that these are secondary effects because membranes from intolerant embryos that had imbibed for 2 h were used, which were already leaky directly after imbibition. Because it is almost impossible to get good quality freeze-fracture replicas of plasma membranes of dry materials, we chose to analyze rehydrated specimens. As is shown in Table I, breakdown reactions may have taken place during the 2-h imbibition period.

An important remaining question is which processes occurring during slow drying are involved in the prevention of phase transitions/separations and irreversible protein aggregation? Rapid drying almost certainly prevents gene expression and extensive protein synthesis, which may be required for desiccation tolerance.

We conclude that the integrity of the plasma membrane has to be preserved for carrot somatic embryos to survive dehydration to low moisture contents. The occurrence of lateral phase separations of PLs and membrane proteins during removal of the water could be the main cause of the loss of membrane function. The resulting extensive leakage of cytoplasmic solutes causes the somatic embryos to lose their viability.

ACKNOWLEDGMENTS

We thank Elena Golovina and Lekha Sreedhar for critically reading the manuscript and Tineke van Roekel for excellent technical assistance.

Received November 3, 1995; accepted February 15, 1996.

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