

Protein dynamics in thylakoids of the desiccation-tolerant plant *Boea hygropica* during dehydration and rehydration.

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Protein Dynamics in Thylakoids of the Desiccation-Tolerant Plant *Boea hygroskopica* during Dehydration and Rehydration¹

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Plants of *Boea hygroskopica* F. Muell were dehydrated to 9% relative water content (RWC) by withholding water for 26 d, and afterward the plants were rehydrated. Leaves were taken from control plants after 7, 12, and 26 d from the beginning of dehydration, and after 6 and 48 h from rehydration. The RWC decreased by 80% during dehydration, but the leaves regained RWC with rehydration. Dehydrated plants showed lesser amounts of proteins, lipids, and chlorophyll, all of which increased following rewatering. The lipid-to-protein ratio, which decreased during dehydration, returned to control level after 48 h of rehydration. Thylakoid lipids were more unsaturated when RWC reached the value of 9%. EPR measurements of spin-labeled proteins showed the presence of three different groups of proteins with different mobility in thylakoid membranes. The rotational correlation time of groups 1 and 2 increased with dehydration and decreased upon rehydration, whereas group 3 showed little changes. Desiccation did not cause thylakoid swelling or breakage, but the membrane system assemblage showed changes in thylakoid stacking. After 48 h of rehydration the membrane system recovered completely the organization of the fully hydrated state, showing several well-defined and regularly distributed grana.

Desiccation-tolerant (resurrection) plants are able to withstand drastic water losses approaching 0.2% relative water content (RWC; Sgherri et al., 1994a, 1994b; Navari-Izzo et al., 1995; Oliver et al., 1998) and show normal physiological characteristics upon rehydration. The remarkable tolerance to prolonged anhydrobiosis in desiccation-tolerant plants suggests that they are able to maintain essential structure and physiological integrity in the dry state and/or are able to repair injury caused by dehydration as soon as they are rehydrated. Many studies have been undertaken to unravel the mechanisms involved in desiccation tolerance, but little is known about changes in membrane structure and function during dehydrating and rehydrating phases. Of crucial importance in desiccation-tolerant plants are the physical properties of the photosynthetic apparatus, which is very sensitive and liable to injury, and needs to be maintained or quickly repaired as soon as water enters again into the cells. Membrane proteins are particularly important for the functionality of the photosynthetic apparatus. The functions of membrane proteins are influenced by the lipid matrix in which they are embedded, and changes in the physical

properties of bulk membrane lipids can alter the behavior of integral membrane proteins. Some membrane enzymes are active only when associated with specific lipids, indicating that the boundary lipids immediately surrounding the proteins are important for their function (Vogg et al., 1998). It is therefore reasonable to postulate for resurrection plants that changes in the lipid-protein interactions within the membrane may occur during dehydration and rehydration phases. The physiological relevance of possible changes in membrane dynamics has received considerable interest, since alteration in membrane lipid composition has been implicated as an important factor in several environmental stresses (Lynch and Steponkus, 1987; Navari-Izzo et al., 1993; Sgherri et al., 1993; Carlsson et al., 1994; Quartacci et al., 1995). Spin-labeled proteins have been found to be particularly useful in the study of lipid-protein interactions (Quinn and Williams, 1990; Quartacci et al., 1995). In response to drought, the adaptation shown by a tolerant cultivar of wheat in comparison with a sensitive one was probably due to the presence of a more fluid bilayer, as evidenced by electron paramagnetic resonance (EPR) analysis, because in the former cultivar non-bilayer forming lipids and free fatty acids did not accumulate (Quartacci et al., 1995).

Boea hygroskopica is a homoiochlorophyllous plant that during dehydration of its detached leaves retains amounts of chlorophyll comparable with those of its

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fresh detached tissues (Navari-Izzo et al., 1995). Furthermore, spin-labeled thylakoid proteins of dried detached leaves of *B. hygroscopica* showed lower sulfhydryl (SH) group level and increased spin motion than control leaves (Navari-Izzo et al., 1994).

In this study our aim was to investigate the relation between changes in the composition of the thylakoid membrane of the resurrection plant *B. hygroscopica* induced during a dehydration-rehydration cycle, and changes in the physical properties of thylakoid membrane proteins spin-labeled with 3-maleimido proxyl. We also carried out ultrastructural analyses to evaluate the dehydration-rehydration effects on thylakoid integrity, and to display possible correlations between changes in the chemical-physical characteristics of membranes and their organization in chloroplasts.

RESULTS

Dehydration for 26 d (D3) decreased the RWC by 80% in comparison with fully hydrated plants (Fig. 1). The rate of water loss during dehydration was quite low in the first 7 d (D1); the RWC decreasing only by 1.58% each day; afterward, the rate of water loss increased so that after a further 5 d of withholding water (D2), the RWC lowered from 79% to 48% and in the following 14 d (D3) it reached 9%. After 6 h of rehydration (R1) the leaves rapidly regained RWC, which recovered to control values after 48 h (R2). A reduction in total chlorophyll (-76%) and chloro-

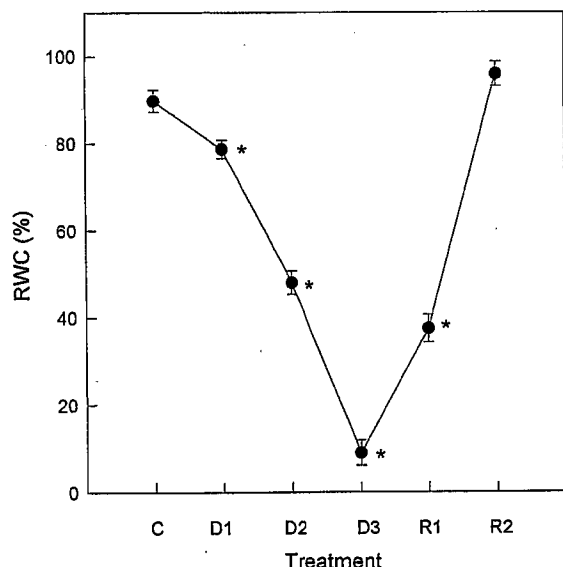


Figure 1. RWC of leaves from *B. hygroscopica* plants subjected to dehydration and rehydration. C, Control; 7 (D1), 12 (D2), and 26 (D3) d after dehydration; 6 (R1) and 48 (R2) h after rehydration. Results are the means of three repetitions of two independent experiments \pm SE ($n = 6$). One-way ANOVA was used for comparisons between the means. The index, being a percentage, was subjected to arcsin transformation for analysis. Means followed by an asterisk are significantly different from control at $P \leq 0.01$.

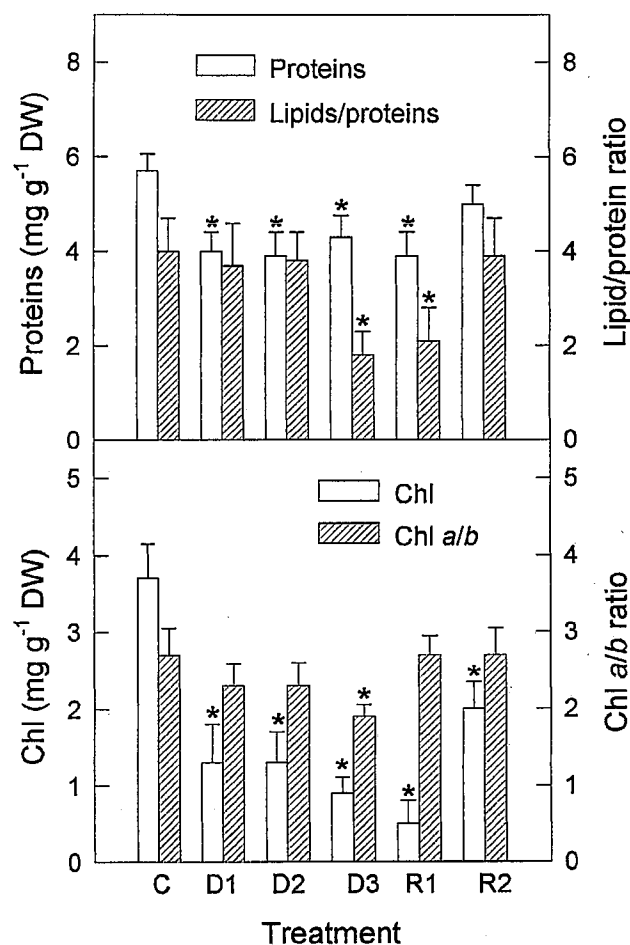


Figure 2. Proteins, lipid-to-protein ratio, chlorophyll, and chlorophyll *a/b* ratio of thylakoids from *B. hygroscopica* plants subjected to dehydration and rehydration. C, Control; 7 (D1), 12 (D2), and 26 (D3) d after dehydration; 6 (R1) and 48 (R2) h after rehydration. Results are the means of three repetitions of two independent experiments \pm SE ($n = 6$). One-way ANOVA was used for comparisons between the means. The index, when a ratio, was subjected to arcsin transformation for analysis. Means followed by an asterisk are significantly different from control at $P \leq 0.01$.

phyll *a/b* ratio (-30%) occurred during drying, but upon rehydration both values approached control amounts (Fig. 2). Proteins of thylakoid membranes on a dry weight basis decreased in the first 7 d of dehydration (-30%) and remained unchanged until 48 h after rehydration, when they increased again to control values (Fig. 2). The lipid-to-protein ratio remained unchanged in the first 12 d of dehydration indicating that lipids also were reduced on a dry weight basis (-35%). Lipid-to-protein ratio decreased sharply at the end of the dehydration (lipids reduced by -66% compared with controls), but after rehydration the ratio returned to the control value (Fig. 2). Unsaturation remained remarkably stable during dehydration and rehydration (Fig. 3); a shift toward more unsaturated membranes ($+10\%$) was only observed at 9% RWC when linoleic and linolenic

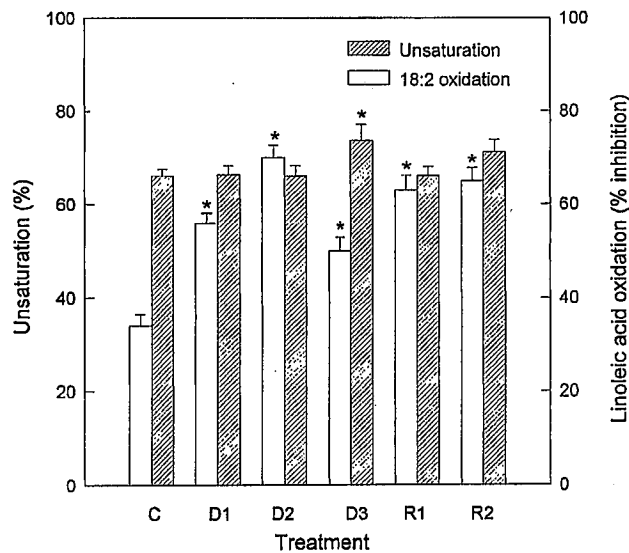


Figure 3. Unsaturation and antioxidant activity of thylakoids from *B. hygrosopica* plants subjected to dehydration and rehydration. C, Control; 7 (D1), 12 (D2), and 26 (D3) d after dehydration; 6 (R1) and 48 (R2) h after rehydration. Results are the means of three repetitions of two independent experiments \pm SE ($n = 6$). One-way ANOVA was used for comparisons between the means. The index, being a percentage, was subjected to arcsin transformation for analysis. Means followed by an asterisk are significantly different from control at $P \leq 0.01$.

acid increased (Table I). The antioxidant capacity of *B. hygrosopica* was always maintained above the control value, even when the RWC reached its lowest value in D3 (Fig. 3).

An experimental EPR spectrum for labeled thylakoid membranes is shown in Figure 4a where a strongly immobilized component (S) and a weakly immobilized (W) component are plain. A change in the relative proportions of the two components in covalently spin-labeled proteins has been used to demonstrate decreased membrane protein mobility as a consequence of protein lateral aggregation or

changes in protein conformation (Andersson and Anderson, 1980; Lynch et al., 1987).

We started the data processing by fitting the two components of experimental EPR spectra at the same time. Initial attempts indicated that spectra calculated by assuming a "two-site" model cannot fit the line shape of the strongly immobilized component, even when adopting various models, e.g. Brownian diffusion or strong jump diffusion for the dynamics of the spin-labeled proteins (Meirovitch et al., 1984). On the contrary, a good agreement was obtained by choosing a "three-site" model, which combined three groups of spin-labeled proteins with different mobility within the lipid bilayer (Fig. 5). Simulated spectra were obtained as a sum of unequal contributions (normalized with respect to values obtained after double integration) calculated for the different groups of spin-labeled proteins. The simulation of the dehydrated sample D2 at 298 K is shown in Figure 4b. The concentrations of the spin-labeled SH of thylakoid proteins and the rotational correlation times (τ) obtained at 298 K for all dehydrated and rehydrated samples are reported in Figure 5. Group 3 resulted to be the most, and group 2 the least representative of thylakoid membrane proteins. At physiological temperature the rotational correlation times of groups 1 and 2 showed almost the same behavior, increasing with dehydration and decreasing upon rehydration. In contrast, rotational correlation times for group 3 were virtually constant (Fig. 5).

Experimental EPR spectra of all dehydrated and rehydrated samples in the temperature range 268 to 303 K were simulated using the model described above. The temperature dependence of τ and the mobile portion (MP) concentration of membrane proteins (group 2) are shown in Figures 6 and 7, respectively. The Arrhenius plots of τ show broad changes in the protein motions and, at low temperature the curves tend to meet because of the rigid environment in which the proteins are immersed. Alterations in membrane protein conformation can produce non-linear Arrhenius plots of spin-label mobility. More-

Table I. Fatty acid composition (mol %) of thylakoid membranes of *B. hygrosopica* during dehydration and rehydration

Results are the means of three independent experiments each analyzed twice \pm SE ($n = 6$). One-way ANOVA was used for comparisons between the means. The index, being a percentage, was subjected to arcsin transformation for analysis. Means followed by an asterisk are significantly different from control at $P \leq 0.01$.

Fatty Acid	C	D1	D2	D3	R1	R2
14:0	0.7 \pm 0.2	0.9 \pm 0.2	0.6 \pm 0.1	0.7 \pm 0.1	0.9 \pm 0.2	0.6 \pm 0.2
16:0	28.3 \pm 2.6	31.0 \pm 1.5	29.5 \pm 1.3	22.0 \pm 0.9*	27.5 \pm 2.0	24.4 \pm 1.0
16:1 c	1.1 \pm 0.2	1.7 \pm 0.3	1.6 \pm 0.3	1.1 \pm 0.2	1.4 \pm 0.2	1.4 \pm 0.3
16:1 t	0.8 \pm 0.2	1.4 \pm 0.4	0.4 \pm 0.1	0.8 \pm 0.1	0.5 \pm 0.2	0.8 \pm 0.2
18:0	4.7 \pm 0.5	3.9 \pm 0.2*	3.8 \pm 0.3*	3.4 \pm 0.3*	5.2 \pm 0.6	3.8 \pm 0.4*
18:1	6.8 \pm 0.5	7.9 \pm 1.0	8.4 \pm 0.7	7.6 \pm 1.2	7.6 \pm 0.8	8.2 \pm 1.1
18:2	43.5 \pm 2.4	43.7 \pm 1.8	43.5 \pm 1.5	47.2 \pm 1.3*	43.7 \pm 0.8	47.3 \pm 1.5*
18:3	13.7 \pm 0.8	11.0 \pm 1.2*	11.0 \pm 1.5*	16.1 \pm 1.2	12.4 \pm 0.9*	12.3 \pm 1.5*
Others ^a	Trace	1.1 \pm 0.3*	1.1 \pm 0.3*	0.9 \pm 0.2*	0.5 \pm 0.2*	1.2 \pm 0.3*

^a Others, 20:0 + 20:1.

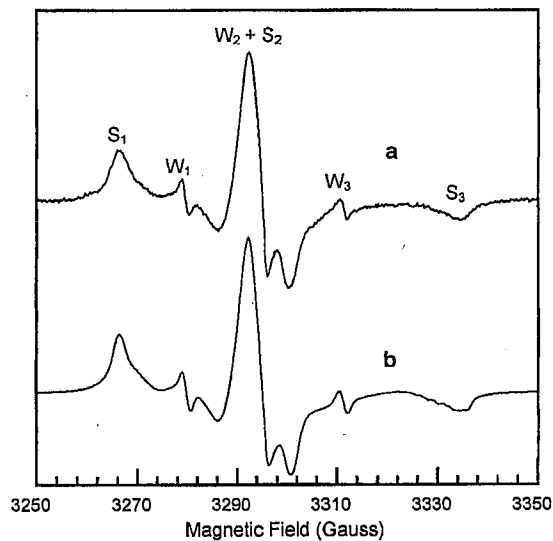


Figure 4. Experimental (a, at 298 K) and fitted (b) EPR spectra of 3-maleimido proxyl-labeled membrane proteins of *B. hygropscopica* after 12 d (D2) of withholding water.

over, the triphasic shapes displayed by the MP of membrane proteins (group 2) support the indication of changes in protein conformation (Fig. 7). Thus the theoretical model (Silvius and McElhaney, 1981) of the temperature-dependent conformational changes of proteins has been used to fit, by non-linear least-squares, the Arrhenius plot and the MP data. The best fits (solid lines), superimposed on the experimental data, are shown in Figures 6 and 7, respectively. The agreement between experimental and calculated data is very good. The midpoint temperature of conformational change (T_C) was 282 K in all samples, whereas the triphasic peculiarity of plots was progressively lost with the increase of dehydration. Plants dehydrated for 26 d (D3) showed nearly bi-phasic plots.

At the ultrastructural level the thylakoid system of hydrated chloroplasts was organized in several well-defined and regularly distributed grana connected by parallel stroma lamellae (Fig. 8A). The inner membranes were maintained in dried organelles, where no thylakoid swelling or breakage had taken place, but an apparent change in the membrane system assemblage could be noticed (Fig. 8B). Most thylakoids were appressed to form large stacks in which, however, the regular flattened profile of the single thylakoids was still distinguishable. No damage to chloroplast membranes occurred during rehydration, although the intergranal lamellae appeared to spread apart during drying and rehydration (6 h). Thylakoid interactions were little changed after 6 h of watering (Fig. 9A), whereas after 48 h (Fig. 9B) the membrane system recovered the organization found in the fully hydrated state, with the several smaller grana and stromal lamellae continuous again.

DISCUSSION

Dehydration involves many physical and chemical changes, resulting in disrupted membrane integrity, decreased activities of enzymes, lipid peroxidation, and de-esterification (Loggini et al., 1999; Navari-Izzo and Rascio, 1999; Leprince et al., 2000). The question of whether or not endomembranes of poiki-

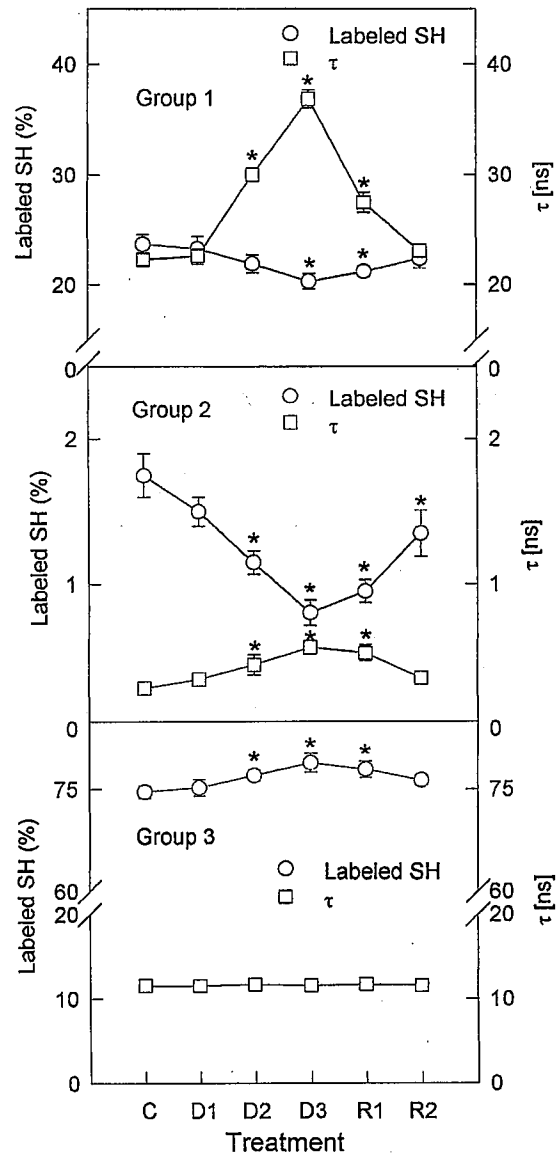


Figure 5. Labeled SH of proteins and rotational correlation time (τ) in thylakoids from *B. hygropscopica* plants subjected to dehydration and rehydration. C, Control; 7 (D1), 12 (D2), and 26 (D3) d after dehydration; 6 (R1) and 48 (R2) h after rehydration. Groups 1 to 3 are referred to proteins with different mobility within the lipid bilayer. Results are the means of three repetitions of two independent experiments \pm SE ($n = 6$). One-way ANOVA was used for comparisons between the means. The index, when a percentage, was subjected to arcsin transformation for analysis. Means followed by an asterisk are significantly different from control at $P \leq 0.01$.

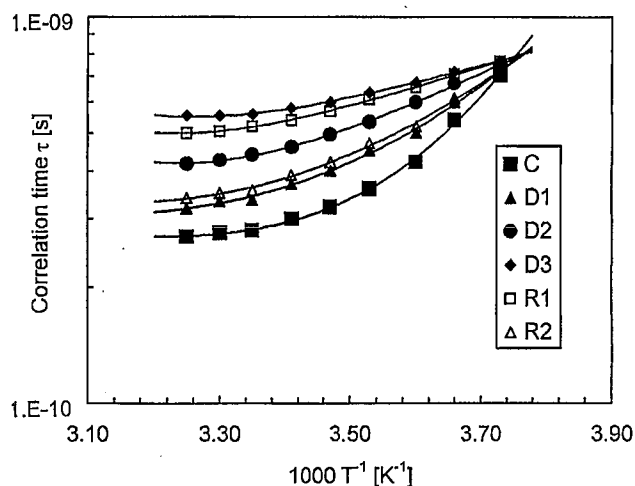


Figure 6. Arrhenius plots of the rotational correlation time as a function of the reciprocal of the absolute temperature for 3-maleimido proxyl-labeled membrane proteins (Group 2) of *B. hygrosopica*. C, Fully hydrated; 7 (D1), 12 (D2), and 26 (D3) d after dehydration; 6 (R1) and 48 (R2) h after rehydration.

lohydric plants can maintain their structure during dehydration or regain it upon rehydration is of considerable interest, since the organization of membranes is required for cell survival.

Under the conditions used in the present study, 3-maleimido proxyl has been shown to label only protein SH groups and has no effect on the fatty acid composition or fluidity of the membranes, nor does the procedure alter the thylakoid protein composition (Lynch et al., 1987). Our spectra, analyzed according to Budil et al. (1996), indicate that in thylakoids of *B. hygrosopica* there are contributions from three dominant groups of spin-labeled proteins (Fig. 5). Since changes in lipid bilayers are able to alter protein conformation, the EPR spectrum reflects microscopic properties that may be related to other macroscopic properties. In groups 1 and 2 the minimum in protein concentrations matched the maximum of stiffening of maleimido proxyl-labeled sulfhydryl groups during dehydration and rehydration processes. In contrast, group 3 showed a maximum of concentration and represents a group in which during dehydration and rehydration the mobility of membrane proteins of *B. hygrosopica* remained unaltered. Taking this together, the changes detectable by protein spin labeling reflect conformational changes in membrane proteins that have been induced, at least in part, by the different alterations in lipid bilayer composition previously observed in thylakoids of detached leaves of *B. hygrosopica* (Navari-Izzo et al., 1995). Moreover, alterations in the conformation and molecular dynamics of membrane proteins of group 2 can reasonably be suggested also by analyzing the trends of temperature plots for τ (Fig. 6) and MP (Fig. 7). It has been proposed that inflections and break points in temperature plots of motion param-

eters represent a critical temperature at which phase transitions in lipid membranes occur. Nevertheless, they may reflect more subtle and less dramatic changes in molecular ordering of lipids (Dickens et al., 1980). Gel-to-liquid-crystalline phase transition, steric effects of lipids on proteins due to fluidity change, lateral and/or vertical displacement of proteins within the lipid bilayer could lead to conformational alterations and, possibly, to changes in rotational motion of membrane proteins. Taking all EPR data into account, it is also reasonable to propose that changes in lipid fluidity and phase properties modulate the physical conformation of membrane proteins during dehydration and rehydration.

A lower spin label mobility during drying of *B. hygrosopica* has to be expected (Fig. 5) as a 2-fold increase in τ has been previously seen in a drought-sensitive cultivar of wheat when the RWC decreased to 78% (Quartacci et al., 1995). In *B. hygrosopica* dehydrated to a 9% RWC the τ of groups 1 and 2 increased by twice also, indicating that in spite of the severe dehydration, deleterious reactions and changes in structure and chemical composition of the membranes were slowed down and protein cross-linking was limited during dehydration.

The main ultrastructural change in the thylakoid system related to the water status variations was the extent of membrane overlapping that greatly increased during dehydration, (Fig. 8A) and was regained afterward to the initial degree with recovery of hydration (Fig. 8B).

The increased thylakoid stacking in dried chloroplasts could be a consequence of membrane and/or environmental changes leading to a weakening of the repulsive force between the membrane surfaces (Barber, 1982). The rise in the protein-to-lipid ratio may play a role in thylakoid stacking (Fig. 2) assisted by a

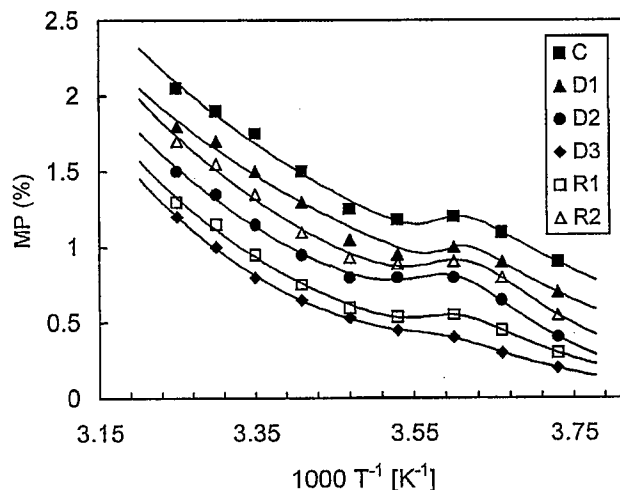


Figure 7. Effects of dehydration and rehydration on the MP of 3-maleimido proxyl-labeled membrane proteins (Group 2) of *B. hygrosopica*. C, Fully hydrated; 7 (D1), 12 (D2), and 26 (D3) d after dehydration; 6 (R1) and 48 (R2) h after rehydration.

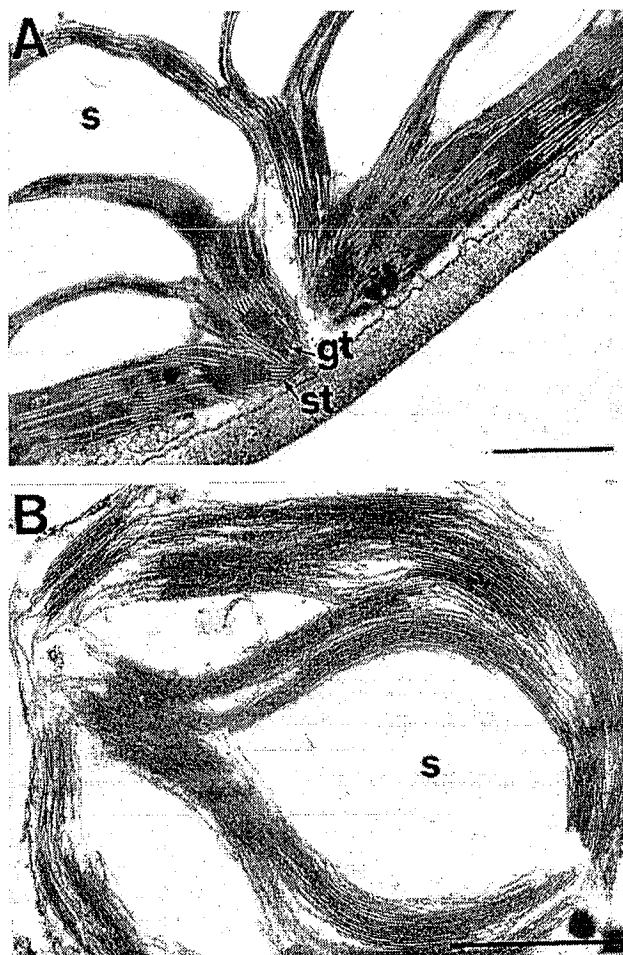


Figure 8. A, Inner membrane organization in fully hydrated chloroplasts. Note the numerous grana (gt) and stroma (st) thylakoids (bar = 1 μm). B, Dried chloroplast with huge thylakoid stacks (bar = 1 μm). s, Starch.

relative enrichment in proteins with a low surface charge, like the light harvesting complex II of the photosystem II distal antenna, which is responsible for thylakoid overlapping also in normally hydrated chloroplasts (Andersson and Anderson, 1980; Barber, 1986). This would be closely related to the decrease in chlorophyll *a/b* ratio during dehydration (Fig. 2), since most of the chlorophyll *b* is bound to these proteins (Green and Durnford, 1996). Another influential factor might be the rise due to water loss in the stroma ionic charge screening the repulsive force between thylakoids (Barber, 1982).

Apart from their modified surface interactions, the chloroplast membranes always maintain a well-preserved structure. Neither dehydration or rehydration caused thylakoid damage, such as fracture and swelling, which are usually suffered by chloroplast membranes of drought-sensitive, but also by some drought-adapted species (Poljakoff-Mayber, 1981; Maroti et al., 1984).

One of the deleterious effects of desiccation is the formation of activated oxygen species during water

loss (Navari-Izzo et al., 1996; Kranner and Grill, 1997; Sherwin and Farrant, 1998; Navari-Izzo and Rascio, 1999). In *B. hygroscopica* during dehydration and rehydration a tight control of superoxide production (Navari-Izzo et al., 1995), as well as an induction of antioxidant mechanisms (Sgherri et al., 1994a, 1994b), and an increase in antioxidative defenses in thylakoid membranes (Fig. 3) may have limited the concentrations of hydroxyl radicals and hydrogen peroxide to non-harmful levels. The decrease in thylakoid proteins observed during dehydration (Fig. 2) may be associated with degradation of lipoprotein thylakoid structure (Navari-Izzo et al., 1995) and may, at least in part, explain the increase in soluble proteins observed in resurrection plants (Daniel and Gaff, 1980; Navari-Izzo et al., 1997a, 1997b; Sgherri et al., 1994a). Depletion of SH readily accessible for labeling (Fig. 5) should be correlated with increased aggregation of membrane proteins as a significant fraction of oxidized groups can be involved in the formation of disulfide bridges. How-

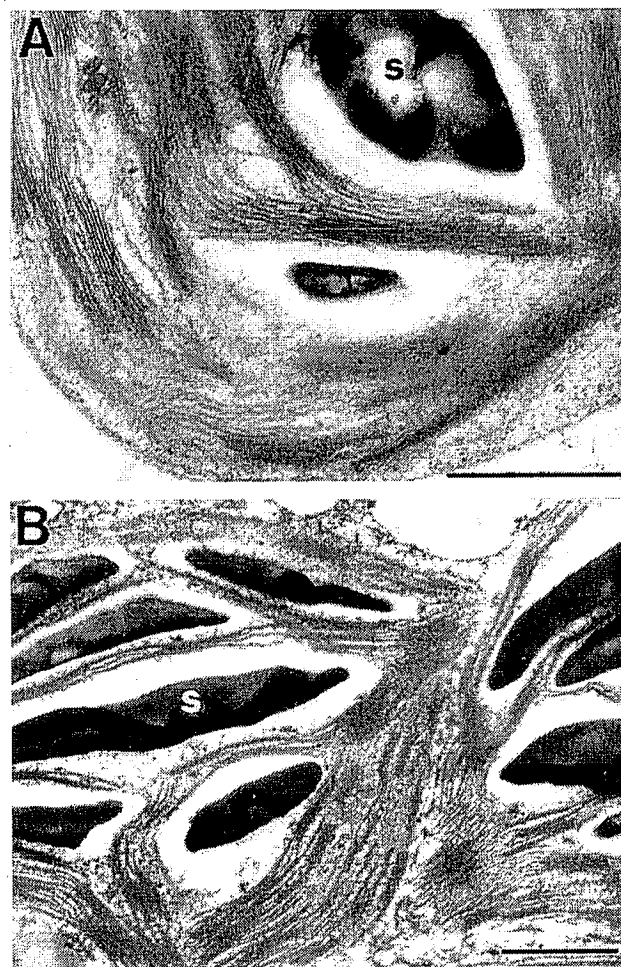


Figure 9. A, Chloroplast after 6 h of watering. Large thylakoid stacks are visible in the stroma (bar = 1 μm). B, Thylakoid organization 48 h after rehydration. Several small grana and stroma thylakoids can be seen in the chloroplasts (bar = 1 μm). s, Starch.

ever, protein thiols may be also oxidized to further products (Radi et al., 1991), and thiol conjugates may be formed. Neither of the latter cases has to be connected with protein aggregation. Moreover, membrane protein dynamics may be expected to result in rapid exchange between accessible and inaccessible SH groups so that the ratio of more mobile to less mobile proteins decreased during drying and increased upon rehydration (Fig. 5). Gross lipid analysis of membranes yields data insufficient to predict membrane fluidity accurately. A progressive increase in rotational correlation time (Fig. 5) and a higher protein-to-lipid ratio (Fig. 2) during drying of *B. hygroskopica* is followed by an opposite pattern upon rehydration confirming that the main effect of the lipid-protein interaction is upon the chain mobility of the membrane, fluidity appearing to be inversely correlated to the protein-to-lipid ratio of the membrane itself (Shinitzky, 1984). Many of the differences found in the lipid moieties between well-hydrated and desiccated tissues of *B. hygroskopica* (Navari-Izzo et al., 1995) might be explained as a form of adaptation, but the differences do not necessarily produce equivalent changes in physical properties.

The increase in unsaturation of thylakoid lipids (Fig. 3) in dehydrated plants may be the result of a decrease in lipid oxidation reactions involving superoxide radicals. Consistent with this during rehydration, when superoxide formation increased again (Navari-Izzo et al., 1994), the unsaturation level decreased. Furthermore, there are some indications of fluidity-mediated control of fatty acid desaturases, decreased fluidity being almost invariably accompanied by increased desaturase activity and vice versa (Martin et al., 1976). According to their finding, decreasing fluidity would have a stimulatory effect on one or more of the membrane-associated fatty acid desaturases, possibly by inducing conformational changes in the desaturase enzymes. In accordance with this, when membrane fluidity decreased during drying of *B. hygroskopica* the unsaturation level increased and when upon rehydration the fluidity increased again the unsaturation level decreased (Figs. 3 and 5).

In flowering plants the fluidity of thylakoid membranes during drought has to be maintained within fairly narrow limits for efficient photosynthetic functions. In *B. hygroskopica* membrane function is maintained within acceptable limits by compensating for perturbations induced by changes in the environment, but there is no reason to believe that maintenance of a constant fluidity or its increase is necessary for compensation of all membrane functions. There would be little need for *B. hygroskopica* to increase membrane fluidity to sustain activity in the dry state, since metabolic activity in dry resurrection plants is low and energy is stored for renewed metabolism when water is available again.

MATERIALS AND METHODS

Plant Material

Plants of *Boea hygroskopica* F. Muell were grown in well-watered pots containing leaf mold in a controlled environment at 27°C day and night, a 16-h photoperiod, 80% to 90% relative humidity, and a photon flux density of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by fluorescent (Osram L39W/20) cool-white light (20 W m^{-2}). Dehydration was imposed by withholding water. Samples were harvested at the beginning of dehydration from fully hydrated plants (C), and then after 7 (D1), 12 (D2), and 26 (D3) d from the beginning of stress imposition. Afterward, plants were rehydrated and the harvests were carried out after 6 (R1) and 48 (R2) h from rehydration. The experiment was run in duplicate, and for each experiment, at each sampling date, three samples were collected. Samples were selected from youngest fully expanded leaves, comparable in size for control and dehydrated plants. RWC values were determined as previously reported (Sgherri et al., 1994a, 1994b).

Isolation of Thylakoid Membranes

Thylakoids were isolated by homogenizing the leaves with a blender four times for 20 s each in an ice-cold medium (1:6, w/v), consisting of 50 mM Tricine [N-[2-hydroxy-1,1-Bis(hydroxymethyl)ethyl]glycine]-KOH (pH 7.4) containing 0.33 M Suc, 10 mM diethyldithiocarbamic acid, 5 mM ascorbic acid, and 5 mM Na_2EDTA , essentially following the procedure of Navari-Izzo et al. (1995). The homogenate was filtered through six layers of Miracloth and then centrifuged at 200g for 5 min and at 3,000g for 10 min. The pellets were resuspended for 30 min in a hypotonic buffer consisting of 10 mM Tris-HCl (pH 7.4) and 150 mM KCl, and then centrifuged at 12,000g for 20 min. The pelleted thylakoid membranes were again washed by resuspension with the same buffer and centrifuged as above. To avoid oxidation, all the solutions were depleted of oxygen under vacuum and equilibrated with nitrogen. All steps were carried out at 4°C. The purity of thylakoid membranes was checked by the analysis of chlorophyll and marker enzymes for mitochondria, peroxisomes, and plasma membrane (Navari-Izzo et al., 1995).

Lipid Analysis

The isolated thylakoids were first boiled in isopropanol for 5 min and lipids were then extracted for 2 h at 4°C with chloroform:methanol (2:1, v/v), containing butylhydroxytoluol as antioxidant. The total lipids were quantified as previously reported (Navari-Izzo et al., 1989). The fatty acid methyl ester derivatives were obtained, after heating at 70°C for 1 h, by transmethylation (Douce et al., 1990) with a mixture containing methanol:benzene:sulfuric acid (100:5:5, v/v). Qualitative and quantitative analyses of fatty acid methyl esters were carried out by gas liquid chromatography using heptadecanoic acid as the internal standard, as previously described (Navari-Izzo et al., 1995).

Chlorophyll and Protein Determination in Thylakoids

The thylakoid pellet was resuspended with 10 mM Tris-HCl (pH 7.4), 150 mM KCl, and 1 mM EDTA. Aliquots of thylakoid membrane were diluted with a solution containing 80% (v/v) acetone and 2.5 mM sodium phosphate buffer (pH 7.8), kept in an ice bath for 10 min, and centrifuged at 12,100g for 15 min at 4°C. The supernatant's absorbance was read at 646.6 and 663.6 nm (Porra et al., 1989). The pellet, dried under N₂, was delipidized with chloroform and dried again under N₂. Thylakoid proteins were solubilized by incubating the pellet at 35°C for 30 min with Tris-Gly buffer, consisting of 34 mM Tris and 46 mM Gly (pH 7.9), containing 2% (w/v) SDS. Protein determination was performed according to Bensadoun and Weinstein (1976).

Chlorophyll Determination in the Leaves

Leaves were ground in a solution containing 80% (v/v) acetone and 2.5 mM sodium phosphate buffer (pH 7.8) and the pellets were extracted four times. The supernatants from the subsequent extractions were pooled and their absorbance read at 646.6 and 663.6 nm (Porra et al., 1989).

Antioxidant Assay

The antioxidant activity of thylakoid membranes was determined in the lipid extracts by monitoring their ability to inhibit linoleic acid oxidation as described previously (Navari-Izzo et al., 1992).

Protein Spin Labeling

Isolated thylakoid membranes were labeled with the paramagnetic probe 3-maleimido proxyl, essentially as described by Quartacci et al. (1995). In brief, thylakoid membrane suspensions (4.0 mg protein mL⁻¹ in 10 mM morpholinoethane sulfonic acid, pH 6.6) were added to 100 μL of 4 mM 3-maleimido proxyl. The tubes were capped under nitrogen and incubated in the dark for 16 h at 4°C. Thylakoid membranes were then washed in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 10,000g for 20 min until the free spin label could not be detected in the supernatant.

EPR Spectra Measurements and Analyses

The concentrated membrane suspension was taken up in a capillary that was sealed at one end, inserted into a quartz holder, and then placed in the microwave cavity of the spectrometer. Spectra were recorded in a temperature range of 268 to 303 K using a spectrometer (model E-112, Varian, Palo Alto, CA) equipped with a Varian variable-temperature control accessory. The spectrometer was interfaced to an AST Premium 486/25-MHz EISA computer by means of a homemade data acquisition system (Ambrosetti and Ricci, 1991; Pinzino and Forte, 1992). At each temperature, spectra were recorded at a field setting of 3,307 G, a microwave power setting of 5 mW, a frequency of 9.2 GHz, a time constant of 0.125 s, and a modulation amplitude of

1.25 G. 2,2-Diphenyl-1-picrylhydrazyl powder was used as a g value standard (g = 2.0037).

The mobility of membrane proteins can be characterized by a motion parameter, the rotational correlation time (τ). This parameter was determined by the best fit of the line shape of the experimental EPR spectrum using the Normalized Recursive Least-Square Lattice implementation of the Slow-Motional EPR Line Shape Calculation Programs EPRL PC Version for Windows 95/NT (Budil et al., 1996) with a Brownian motion model of isotropic rotational diffusion.

The first step in the best-fit procedure was the proper choice of all elements of the nitrogen hyperfine (A) and electron Zeeman (g) tensors that are required as input for computing rotational correlation times. The principal values of the tensors for the 3-maleimido-labeled proteins could not be determined from the low-temperature (150 K) EPR spectrum because of the very broad lines. We adopted the principal values of the g magnetic parameter determined from the simulation of rigid-limit spectrum in 3-maleimido-labeled human serum albumin $g_{xx} = 2.0084$, $g_{yy} = 2.0061$, and $g_{zz} = 2.0025$ (Marzola et al., 1991). The components of the A tensor were selected using the "scaling procedure," which has been used in the past with reasonable results (Meirovitch and Freed, 1984; Meirovitch et al., 1984). The values determined from the rigid-limit spectrum in 3-maleimido proxyl-human serum albumin ($A_{xx} = A_{yy} = 6.325$ G and $A_{zz} = 36.1$ G; Marzola et al., 1991) were scaled to fit the weakly immobilized component observed in the experimental spectra with isotropic nitrogen hyperfine splitting constant. The tensor components $A_{xx} = A_{yy} = 6.0$ G and $A_{zz} = 35.2$ G were obtained from this procedure and were used for the best fitting of all EPR spectra.

Transmission Electron Microscopy

Samples from leaves of *B. hygrosopica* were fixed for 2 h in 6% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9), post-fixed in 1% (w/v) osmium tetroxide in the same buffer, and then processed for electron microscopy as described previously (Quartacci et al., 1997). Thin sections, cut with an ultramicrotome (Ultracut, Reichert-Jung, Tokyo), were examined with a transmission electron microscope (TEM 300, Hitachi, Wien, Austria) operating at 75 kV.

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