J. Membrane Biol. 166, 61–70 (1998)

The Journal of
Membrane
Biology

© Springer-Verlag New York Inc. 1998

Phenotypic Adaptation of Tonoplast Fluidity to Growth Temperature in the CAM Plant *Kalanchoë daigremontiana* Ham. et Per. is Accompanied by Changes in the Membrane Phospholipid and Protein Composition

M. Behzadipour[1], R. Ratajczak[1], K. Faist[1], P. Pawlitschek[1], A. Trémolières[2], M. Kluge[1]

[1]Institut für Botanik, Technische Universität Darmstadt, Schnittspahnstrasse 10, D-64287 Darmstadt, Germany
[2]Institut de Biotechnologie des Plantes, Université de Paris-Sud, F-91405 Orsay, France

Received: 4 March 1998/Revised: 28 July 1998

Abstract. The present study deals with the phenotypic adaptation of tonoplast fluidity in the CAM plant *Kalanchoë daigremontiana* to changes in growth temperature. Tonoplast fluidity was characterized by measuring fluorescence depolarization in membranes labeled with fluorescent fatty acid analogues and by following formation of eximeres in membranes labeled by eximere-forming fluorophores. With both techniques it was found that exposure of the plants to higher growth temperature compared with the control decreased the fluidity of the tonoplast while exposure to lower growth temperature caused the opposite. Three hours of high temperature treatment (raised from 25°C to 35°C; ''heat shock'') were sufficient to decrease the tonoplast fluidity to roughly the same extent as growth under high temperature for 30 days. The phenotypic response of tonoplast fluidity to changes in growth temperature was found only in the complete membrane, not however in the lipid matrix deprived of the membrane proteins. Heat treatments of the plants decreased the lipid/protein ratio while exposure to low temperature (for 30 days) increased it. Heat treatments led to a decrease in the percentage of linolenic acid (C18:3) and linoleic acid (C18:2), heat shock and low temperature treatments induced an increase in the percentage of linoleic acid (C18:3), with concomitant decrease in the percentage of linoleic acid (C18:2). However, in the case of heat shock, increase in linolenic acid concerned mainly monogalactosyldiacylglycerol, while with low temperature treatment linoleic acid increased in phosphatidylcholine. Both treatment of the plants with high and low temperature led to a slight decrease in the contribution of phosphatidylcholine and phosphoethanolamine to the total phospholipid content of the tonoplast. High-temperature treatment of the plants not only decreased the phospholipid/protein ratio in the tonoplast, but also led to the occurrence of a 35 kDa polypeptide in the tonoplast which cross-reacted with an antiserum against the tonoplast H^+-ATPase holoenzyme. The important role of membrane proteins in bringing about the phenotypic rigidization of the tonoplast was mimicked by reconstitution experiments showing that incorporation of the proteins isolated from the tonoplast into phosphatidylcholine vesicles decreased the fluidity of this membrane system. As to be expected from the analyses in the natural membrane, the degree of this effect depended on the phospholipid/protein ratio.

Key words: Crassulacean acid metabolism (CAM) — Excimer technique — Fluorescence polarization — Homeoviscous adaptation — *Kalanchoë daigremontiana* — Membrane fluidity

Introduction

One of the key processes of crassulacean acid metabolism (CAM) consists in the reversible storage of malic acid in the vacuole of photosynthetically active cells (Kluge & Ting, 1978; Osmond, 1978; Winter, 1985; Lüttge et al., 1995), with acid accumulation during the night and net export (''deacidification'') during the day. In many CAM plants the capability of the vacuoles to accumulate malic acid is seriously disturbed by high actual temperatures (*see* the mentioned reviews on CAM), presumably via alteration of membrane ''fluidity'' (Kluge et al., 1991; Kliemchen et al., 1993; Kluge & Schomburg, 1996; for discussion of the term ''membrane fluidity'' *see* e.g., Kluge & Galla, 1995). In this context the find-

Correspondence to: M. Kluge

ing is interesting that in the obligate CAM plant *Kalanchoë daigremontiana* increase of growth temperature or, in the case of the facultative CAM plant *Mesembryanthemum crystallinum*, the induction of CAM by increase of soil salinity decreases the fluidity of the tonoplast (Kliemchen et al., 1993). This phenotypic change in the tonoplast fluidity can be interpreted in terms of compensatory adaptation which guarantees undisturbed functioning of the membranes under stress (Kliemchen et al., 1993). In physiological experiments the latter authors also found that the changes in tonoplast fluidity could be related to changes in the behavior of reversible storage of malic acid in the vacuole.

Phenotypic adaptation of membrane fluidity occurs not only in plants but also in microorganisms and animals and is denoted in the literature as ''homeoviscous adaptation'' (HVA; for review *see* Hazel, 1988; Parola, 1993). In microorganisms and animals, HVA was found to be the result of changes in the composition of the membrane lipids (Hazel, 1988; Nozawa & Umeki, 1988; Parola, 1993). However, as far as the tonoplast of CAM plants is concerned, the mechanism behind HVA is not yet clear. Schomburg & Kluge (1994) provided evidence that in the latter case HVA depends largely on the proteins of the tonoplast, but the question remained open to which extent and in which manner the lipid fraction might be also involved.

The present study was carried out to improve the knowledge on the mechanism behind HVA in the tonoplast of CAM plants by addressing the following questions: (i) Does heat-induced HVA occur if the plants are exposed to elevated growth for much shorter times than the 30 days applied in the experiments previously reported? (ii) Does lowering as well as increasing of growth temperature bring about HVA of the tonoplast? (iii) Is the heat-induced HVA accompanied by changes in the tonoplast lipid and polypeptide composition? (iv) Is it possible to mimic the influence of tonoplast proteins on membrane fluidity by reconstitution experiments with an artificial membrane system? Finally, we wanted to find out whether HVA in the CAM plant tonoplast previously visualized solely by ESR spectroscopy (Kluge et al., 1991; Kliemchen et al., 1993) can also be observed if the membrane fluidity is measured by means of fluorescence labeling techniques. Such verification appeared to be important since it has been shown (Svetec et al., 1995) that in many cases application of a single biophysical method is not sufficient to obtain unequivocal information about the fluidity behavior of a given biomembrane.

Materials and Methods

Plant Material

The experiments were performed with the obligate CAM plant *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie derived from a clone kept at the Botanical Garden Darmstadt, Germany. Control plants were held in a phytotron under the following standard conditions: 25°C (day)/17°C (night); long-day photoperiod (14-hr day/10-hr night), with a photon flux density (wavelength 400–700 nm) of 250–300 μmol m^{-2} sec^{-1} at the level of the first fully expanded leaves; relative air humidity 50% (day)/80% (night). For the high temperature (HT) acclimation experiments, prior to tonoplast preparation the plants were transferred for 30 days from the standard conditions to 34°C (day)/25°C (night), with the other conditions remaining the same as mentioned above. For low temperature acclimation (LT), the plants were transferred for 30 days to 17°C (day)/7°C (night). For ''heat shock'' experiments the plants were transferred during the last 3 hr of the light period from 25°C (day) to 35°C (day) for 3 hr.

Preparation of Tonoplast Vesicles and Reconstitution of Liposomes

Tonoplast vesicles were isolated from fully expanded leaves of 5- to 7-month old plants. The leaves were harvested from the plants at the end of the light period. The tonoplast isolation was carried out by homogenization of the tissue in a blender (Starmix, Braun, Melsungen, Germany), followed by sucrose density centifugation (100,000 × *g* for 105 min, 55.2 Ti rotor, Beckman) according to the method of Bremberger et al. (1988) except that, in order to inhibit protease activity, all buffers used in our experiments for the extraction and purification of the tonoplast vesicles contained EDTA (5 mM), leupeptin (1 μM), pepstatin (1 μM) and PMSF (1 mM).

Membrane proteins were removed from the tonoplast fraction by acetone precipitation. One volume of membrane suspension was mixed with 5 volumes of acetone. The mixture was kept at −20°C for 1 hr and centrifuged at 5,000 × *g* for 15 min. The supernatant was collected, the acetone was evaporated under a nitrogen stream and the remaining lipid film was resuspended by sonification in 1 ml buffer (5 mM HEPES, 3 mM $MgSO_4$, pH 7.0).

Solubilization of tonoplast proteins and their incorporation into phosphatidylcholine liposomes (PC liposomes) were carried out according to Behre et al. (1992).

Lipid Analyses

Lipids were extracted from the membrane preparations by chloroform according to Bligh & Dyer (1959). An aliquot of total lipid extract was transmethylated for fatty acid analysis (Metcalfe et al., 1966), and another aliquot was used for determination of lipid classes by thin layer chromatography (DC) on silica gel plates using chloroform/acetone/methanol/acetic acid/water (50/20/10/10/5 by vol) for separation of polar lipid classes and petroleum ether/diethyl ether/acetic acid (70/30/0.4 by vol) for separation of neutral lipids. The lipid spots were visualized by exposure of the chromatogram for 2 min to iodine vapor. Afterwards, the spots were scraped from the DC plates and transmethylated according to Metcalfe et al. (1966) for quantitative determination of fatty acids and analysis of fatty acid composition of the fractions. Fatty acid methylesters were analyzed at 170°C by isothermic capillary gas-liquid chromatography on Carbowax columns (50-m length, 0.25-mm diameter). The data shown in the tables represent analysis of membrane fractions pooled from 3 to 5 isolations. The precision of the lipid analysis is in the range of 1%.

Fluorescence Depolarization Measurements

The tonoplast fluidity was estimated by measuring temperature profiles of fluorescence depolarization after labeling of the membranes with the

fluorescent lipid analogue 1,6-diphenyl-1,3,5-hexatriene (DPH) (Parola, 1993; Shinitzky & Barenholz, 1978). The tonoplast fractions in 3 mM $MgSO_4$, 5 mM HEPES, pH 7.0 were incubated with 1 mM DPH dissolved in tetrahydrofurane to give a final total lipid to probe molar ratio of 100 to 300:1. The suspension was incubated for 30 min in the dark at 37°C under a nitrogen stream just prior to fluorescence measurements using a Perkin-Elmer LS50B fluorometer. Excitation wave length was 360 nm; emission wave length 430 nm. The degree of polarization (*P*) was calculated from the equation:

$$P = (I_{\parallel} - I_{\perp} \times G)/(I_{\parallel} + I_{\perp} \times G)$$

with *G:* the correction factor inherent to the apparatus; $I_{\parallel}$ and $I_{\perp}$: fluorescence intensities recorded through a polarization filter orientated in parallel and perpendicularly to the vertically polarized excitation light. Temperature profiles of membrane fluidity were estimated by measuring fluorescence depolarization while the temperature of the membrane suspension (in the following denoted as ''actual temperature'') was gradually changed. Each data point shown in the figures represents the averaged degree of polarization that was measured during 10 sec.

Excimere Techniques

The formation of excimers (''excited dimers'') from monomer fluorophors integrated as probes in biomembranes requires interaction of the label molecules. Thus, excimer formation, which can be visualized by the excimer/monomer fluorescence intensity ratio (*I**/*I* ratio) depends on lateral diffusion of the probe in the membrane and is therefore a reliable indicator for membrane fluidity and phase separation (Galla & Hartmann, 1980; Galla, 1988).

In the present study pyrene-labeled phosphatidylcholin (β-py-C_{10}-HPC) was taken as a probe and integrated in tonoplasts as follows: membrane material equivalent to 200 μg phospholipid was suspended in 3 ml of buffer containing 3 mM $MgSO_4$, 5 mM HEPES, pH 7.0. The probe, dissolved in ethanol, was added to give a concentration of 1 mol% with respect to the membrane lipids. Afterwards, the samples were incubated in a water bath at 37°C for 30 min in the dark under a nitrogen stream. In pre-experiments it was made sure that 1 mol% label concentration in the membrane lipids was in the range where this parameter is linearly correlated to the *I**/*I* ratio. Fluorescence intensities were measured by a Perkin-Elmer LS50B fluorometer. Excitation wave length was 346 nm; emission wave length 381 nm for the monomer (*I*) and 487 nm for the dimer (*I**). Temperature profiles of the *I**/*I* ratio were measured, with an integration time of 5 sec for fluorescence measurement at each given temperature.

SDS-PAGE and Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on slab gels containing 10% (w/v) acrylamide monomer using the Laemmli buffer system (Laemmli, 1970). After electrophoresis, gels were either stained with silver (Oakley et al., 1980) or proteins were electrophoretically transferred to Immobilon P teflon membrane for 45 min at 25 V. Immunostaining was performed with alkaline phosphatase conjugated goat-anti-rabbit IgG after incubation of the blots with polyclonal rabbit antisera against the holoenzyme of the tonoplast H^+-ATPase of *K. daigremontiana* (Haschke et al., 1989) or against subunit A (67 kDa) of the tonoplast H^+-ATPase of *M. crystallinum* (Ratajczak et al., 1994*b*).

Other Assays

Immunoprecipitation of the tonoplast H^+-ATPase holoenzyme was performed with a polyclonal rabbit antiserum against subunit A of the tonoplast H^+-ATPase of *M. crystallinum* as described by Ratajczak et al. (1994*b*). Protein was determined with Amidoblack 10^B after Popov et al. (1975) using bovine serum albumin as standard. Rates of ATP hydrolysis were calculated from the determination of the amount of inorganic phosphate produced during incubation of tonoplast vesicle preparations for 60 min at 37°C (after Ames, 1966). As specific inhibitors of ATP-hydrolysis activity 50 mM nitrate (tonoplast H^+-ATPase), 1 mM azide (mitochondrial H^+-ATPase), and 0.1 mM vanadate (plasmalemma H^+-ATPase) were used.

The amount of tonoplast phospholipids was estimated by the determination of the phosphate content of tonoplast preparations after Ammon & Hinsberg (1935).

Statistical Analysis

Arithmetic means were analyzed by Student's *t*-test. Linear regression analysis using the Graph Pad Prism computer program was applied to test the membrane fluidity profiles for differences in the slopes and intercept. The significant term is used in the present paper strictly in its statistical meaning with $P < 0.05$.

Abbreviations

CAM, crassulacean acid metabolism; C16:0, palmitic acid; C18:0, stearic acid, C18:1, oleic acid (D 9 octadecamonoenic acid); C18.2, linoleic acid (D 9,12 octadecadienoic acid); C18:3, linolenic acid (D 9,12,15 octadecatrienoic acid); DGDG, digalactosyldiacylglycerol; EDTA, ethylenediamineteracetic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; FFA, free fatty acids; HS, heat shock; HT, high temperature (34°C day/25°C night); HVA, homeoviscous adaptation; LT, low temperature (17°C day/7°C night); MDGD: monogalactosyldiacylglycerol; PA, phosphatidic acid, PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipids; PMSF, phenylmethylsulfonyl fluoride; RH, relative air humidity; SQDG, sulfoquinovosyldiacylglycerol.

Results

Characterization of the Tonoplast Vesicles

The purity of the tonoplast material was evaluated from the ATP hydrolysis activity of the tonoplast H^+-ATPase in the presence of specific inhibitors (Jochem & Lüttge, 1987). The azide-resistant, nitrate-sensitive ATP hydrolysis activity at pH 8.0, as a marker for tonoplast H^+-ATPase, was as high as 65.2 ± 3.9%, 70.8 ± 7.8% and 45.5 ± 7.6% of the total ATP hydrolysis activity of control, HT and LT plants (Table 1). There were no differences in the azide-resistant, nitrate-sensitive ATP hydrolysis activity at pH 8.0, as compared to the total ATP hydrolysis activity in preparations between control and HT plants. In contrast, there was a decrease of ATP hydrolysis activity for LT plants and as well as after exposure to a heat shock. Contamination by mitochondrial ATPase (azide-sensitive activity) at pH 8.0 and by plasmalemma ATPase (vanadate sensitive activity) at pH 6.5 was low. Student's *t*-test did not reject the null hypothesis for the equality of means at a significance level of $\alpha = 0.05$.

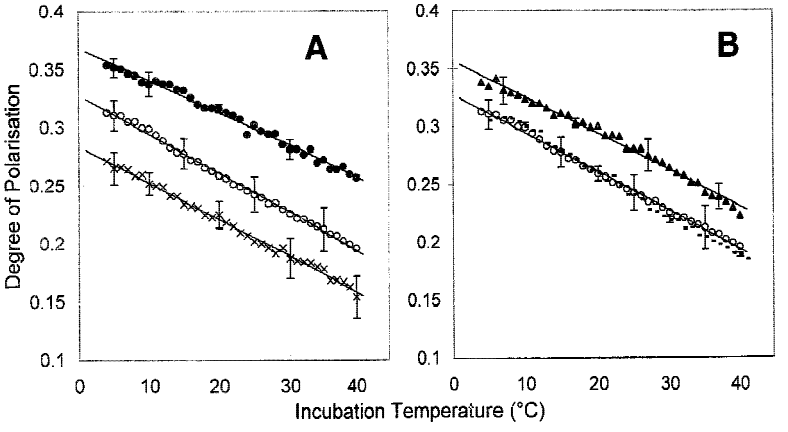

Fig. 1. Fluorescence polarization of DPH in tonoplasts of *K. daigremontiana* as function of incubation temperature. The diagram compares tonoplasts isolated from plants adapted to different growth temperatures prior the membrane isolation. (*A*) Effects of the acclimation (long term, 30d), to different growth temperatures. O: control (25°C(day)/17°C(night)); ●: high temperature treatment (HT, 34°C/25°C) and ×: low temperature treatment (LT, 17°C/7°C) (*B*) Effects of short term (3 hr) exposure to a heat shock from 25 to 35°C (▲) and to a cold treatment from 25 to 12°C (−); control (○). The values represent arithmetic means of $n = 5$ independent experiments. For sake of clarity the standard deviations (vertical bars) are indicated only for a few values.

Response of Tonoplast Fluidity to Growth Temperature

To avoid confusion, the following two types of temperature response have to be strictly distinguished, namely, (i) response of tonoplast fluidity to the environmental temperature the plants received prior the isolation and biophysical characterization of the membrane (i.e., response to ''growth temperature,'' which represents a kind of memory effect), and (ii) response of tonoplast fluidity to the temperature at which fluorescence polarization was measured (''incubation temperature''). As indicated by the decline of the degree of fluorescence polarization (Fig. 1*A* and *B;* Fig. 2), the gradual increase of incubation temperature between 5 and 40°C tested so far led in all cases to a steady fluidization of the tonoplast. Discontinuities in the temperature profiles of fluorescence polarization did not occur, suggesting that there was no sudden phase transition of the tonoplast lipids by increase of the actual temperature. This is in contrast to the result of previous EPR studies (Kluge et al., 1991).

For any given incubation temperature complete tonoplasts from plants grown for 30 days at high temperature (HT plants) always showed a significantly higher degree of fluorescence polarization, i.e., lower membrane fluidity, compared with the controls grown at normal temperature (NT) (Fig. 1*A*). In other words, a higher incubation temperature was required with the tonoplast of HT plants to reach a given degree of fluidity. However, the difference in tonoplast fluidity between NT and HT plants visible in the complete membrane disappeared if the proteins were removed from the tonoplast and only the lipid matrices were compared (Fig. 2). The protein-free tonoplast lipid matrix of the HT plants showed even slightly lower values of fluorescence polarization indicating somewhat higher fluidity of the lipids compared with the controls (Fig. 2).

Figure 1*B,* in comparison with Fig. 1*A,* shows that

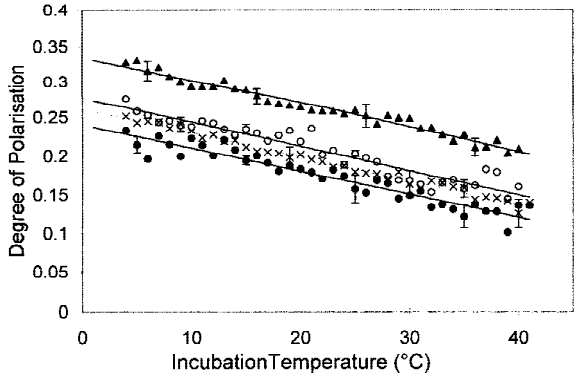

Fig. 2. Fluorescence polarization of DPH in tonoplasts of *K. daigremontiana* deprived of their proteins. As in Fig. 1 the diagram compares tonoplast fluidity of plants held at different growth temperatures prior membrane isolation. ○: control; ▲: heat shock (HS), ×: low temperature treatment (LT); ●: high temperature treatment (HT).

exposure for three hours to high temperature (''heat shock'') decreased the tonoplast fluidity to nearly the same extent as observed in the plants grown for 30 days under HT. In case of heat shock treatment removal of the proteins had no effects on the fluidity of lipid matrix (Fig. 2).

In contrast to the response to HT, growing the plants under low temperature (LT) for 30 days shifted the fluorescence polarization profile of the tonoplast to a significantly lower level (Fig. 1*A*) indicating that the membrane became more fluid with respect to the controls. In contrast to the heat shock, low temperature shock, i.e., shifting the plants for three hours from 25 to 12°C, had no effects on the fluidity of the tonoplast (Fig. 1*B*).

Figure 3*A* and *B* show temperature profiles of excimere/monomer fluorescence intensity (I^*/I) ratios in β-py-C_{10}-HPC labeled tonoplasts isolated from differently grown plants of *K. daigremontiana*. The I^*/I ratios increased in response to the incubation temperature sug-

gesting that due to thermotropic fluidization of the membrane the lateral diffusion of the probe increased leading to an increase in the frequency of excimere formation. In none of the curves obtained there was an abrupt change in the slope which provides further evidence that no sudden phase transitions occurred in the membrane. There were significant differences in the slopes of the temperature versus *I**/*I* correlation curves obtained with the entire membranes of differently treated plants (Fig. 3*A*). That is, in the tonoplasts of HT plants the increase of intensity ratios in response to incubation temperature was clearly less steep compared with the control (NT Plants). This suggests that adaptation to high temperature decreased the susceptibility of the tonoplast to thermotrope fluidization. The opposite holds true for the tonoplast of LT plants where increase of incubation temperature led to a steep increase in the *I**/*I* ratios. If the proteins were removed from the tonoplast (Fig. 3*B*), the slopes of the *I**/*I* temperature increased considerably in the case of the controls and even more so in the case of HT plants suggesting that lateral motion of the label molecules in the membranes was facilitated by this treatment. In contrast, with the tonoplast of LT plants, where high lateral motion of the label was already observed in the entire membrane, removal of proteins had practically no further effect on membrane fluidity as shown by the same slopes of the concerned temperature response curves of Fig. 3*A* and *B*, respectively.

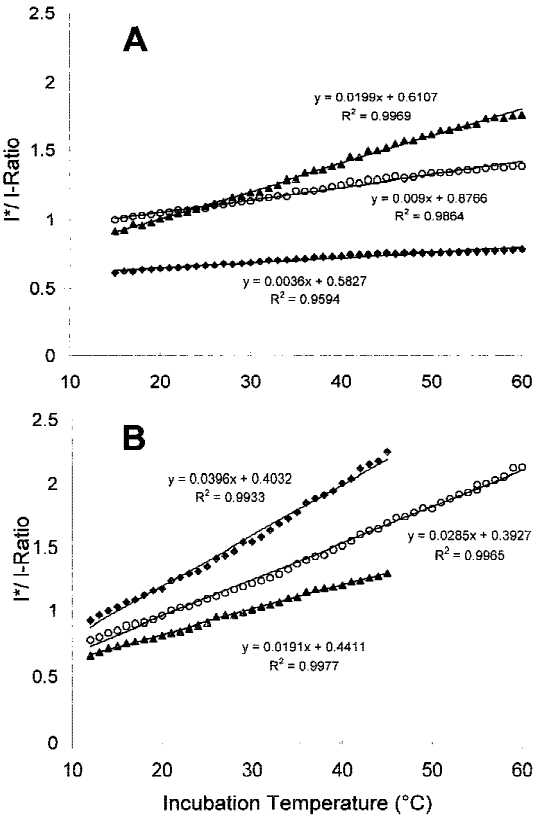

Fig. 3. Change of the excimere/monomere fluorescence intensity ratios (*I**/*I*-Ratio) in response to incubation temperature, exhibited by pyrene-PC-labeled tonoplast preparations of *K. daigremontiana*. The data show the results of a labelling experiment using tonoplast material pooled from three isolations. (*A*) Entire tonoplasts; (*B*) tonoplasts deprived of their proteins. The membrane material derived from the same preparations as shown in (*A*). Symbols: ○: control (plants grown under NT); ◆: plants grown for 30 days under HT conditions; ▲: plants grown for 30 days under LT conditions.

Response of Tonoplast Lipid Composition to Growth Temperature

The decrease in tonoplast fluidity after exposure of the plants to a heat shock for 3 hr (Fig. 1*B*) or after acclimation to high temperature for 30 days (Fig. 1*A*) was paralleled by changes in the lipid fraction of the tonoplast. Compared with the control, the total amount of lipids decreased in the case of HT plants by about 20% and the content of total fatty acids by about 19% and by 14% after a heat shock with respect to the protein content of the membrane. In contrast, the low-temperature treatment increased the content of total fatty acids by about 23% (*data not shown*).

As shown in Table 2, the acclimation to high temperature led to a change in the fatty acid composition of the tonoplast. The percentage of linoleic acid and linoleic acid decreased from 34 to 22% and from 23 to 18%, respectively. After HT treatment there was a slight increase in palmitic acid, whereas stearic acid increased from 5% to 11%. The analyses of lipid classes showed that the percentage of PC and PE in tonoplasts of HT plants were lower compared to control plants (Fig. 4).

Upon exposure of the plants to the heat shock (HS), the saturated fatty acid compositions of the tonoplast lipids were not markedly changed with respect to the controls. However, the percentage of linoleic acid decreased from 34 (controls) to 23%, whereas linolenic acid increased from 24 to 35%. The analyses of lipid classes revealed that upon heat shock PC and PE decreased, while galactolipids increased (Fig. 4). Moreover, it was found that in PC, the proportion of linoleic acid decreased from 37 to 27% and in PE from 36 to 26%. Simultaneously, the proportion of linolenic acid in the MGDG-bound fatty acids substantially increased from 23 to 41%.

Also in the case of LT treatment there was an increase in the contribution of linolenic acid to total fatty acids with concomitant decrease in the percentage of linoleic acid. However, in contrast to heat shock, with LT treatment the increase in linolenic acid (from 17 to 23%) occurred almost exclusively in PC while in the fatty acids of PE and the galactolipids the degree of saturation increased (Table 3).

Table 1. Characterization of tonoplast vesicle preparations from *K. daigremontiana*

	ATP hydrolysis activity (% of total activity)		
	Nitrate-sensitive, azide-resistant activity at pH 8.0	Azide-sensitive activity at pH 8.0	Vanadate-sensitive activity at pH 6.5
Control ($n = 8$)	65.20 ± 3.90	8.10 ± 3.40	8.50 ± 5.50
HT ($n = 10$)	70.80 ± 7.80	10.70 ± 4.60	7.90 ± 7.80
LT ($n = 7$)	45.54 ± 7.60	10.60 ± 3.60	8.60 ± 5.70
Heat shock ($n = 5$)	49.90 ± 8.10	10.40 ± 6.10	8.20 ± 4.20

The tonoplast vesicle preparations from *K. daigremontiana* were grown under normal conditions (control), high temperature (HT), low temperature (LT) or after a heat shock from 25 to 35° for 3 hr by determination of ATP-hydrolysis activity in presence of specific inhibitors. Inhibitor concentrations were 1 mM azide (mitochondrial H^+-ATPase; measured at pH 8.0), 50 mM nitrate (tonoplast H^+-ATPase; measured at pH 8.0), and 0.1 mM vanadate (plasmalemma H^+-ATPase; measured at pH 6.5). Total ATP-hydrolysis activities at pH 8.0 were 108.9 (control), 111.2 (HT), 91.09 (LT) and 78.24 (heat shock) μmol P_i mg protein^{-1} hr^{-1}. The values represent arithmetic means ± standard deviation.

Table 2. Fatty acid composition (mol%) of tonoplasts isolated from *K. daigremontiana*

Treatment	C16:0	C18:0	C18:1	C18:2	C18:3
Control	32.3	5.0	4.7	34.2	23.7
HT	35.9	10.9	10.9	22.4	18.4
HS	32.5	6.3	3.4	23.1	34.7
LT	28.7	9.8	8.3	22.1	29.0

The tonoplasts isolated from *K. daigremontiana* were grown at normal temperatures (control), at high temperature (HT), at low temperature (LT), and after a heat shock from 25 to 35°C for 3 hr (HS).

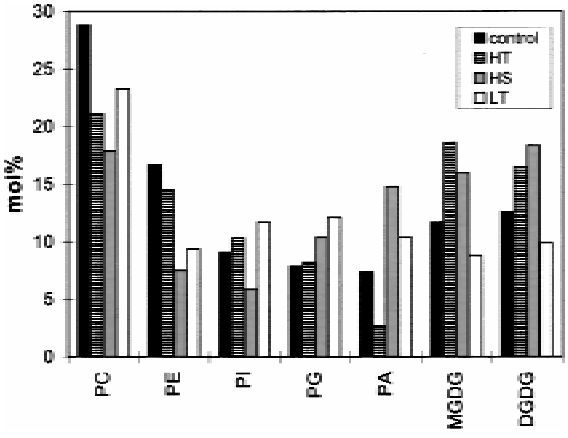

Fig. 4. Lipid class compositions (mol%) of tonoplasts isolated from plants adapted to different growth temperature regimes as indicated in the insert. The values represent arithmetic means of two analyses carried out with a tonoplast sample where membrane material of 5 independent isolations was pooled.

Responses of Tonoplast Proteins to Growth Temperature

The responses of tonoplast proteins were analyzed after HT treatment. Also in this experiment we found that parallel to the decrease of tonoplast fluidity (Fig. 1*A*) the lipid/protein ratio of the membrane decreased from 3.08 ± 0.45 ($n = 5$) in the controls to 2.20 ± 0.45 ($n = 5$) in the treated plants. The difference was significant on the level of $\alpha = 0.05$ (Student's *t*-test).

The polypeptide compositions of tonoplast vesicle preparations from control plants and heat treated plants were practically identical, with the exception that in preparations from heat treated plants an additional 35 kDa polypeptide was present (Fig. 5). Interestingly, Western blot analysis revealed that this polypeptide cross-reacted with an antiserum directed against the holoenzyme of the tonoplast H^+-ATPase of *K. daigremontiana* (Fig. 6, lane 2). Since there was no cross-reaction of the 35 kDa polypeptide with an antiserum raised against subunit A of the tonoplast H^+-ATPase (of *M. crystallinum*) it is unlikely that the 35 kDa polypeptide is a proteolytic fragment of subunit A (Fig. 6, lane 4). The tonoplast H^+-ATPase holoenzyme was immunoprecipitated from solubilized tonoplast proteins by an antiserum monospecific for subunit A of the enzyme. Western blot analysis of immunoprecipitated tonoplast H^+-ATPase with subsequent immunostaining using an antiserum against the tonoplast H^+-ATPase holoenzyme showed all known subunits in the precipitate, while the 35 kDa polypeptide is not a part of the tonoplast H^+-ATPase holoenzyme (Fig. 7).

Experiments with PC Liposomes and Proteoliposomes Reconstituted with Tonoplast Proteins

The experiments shown in Fig. 2 and previous experiments by Schomburg & Kluge (1994) demonstrated that the removal of the membrane proteins from the tonoplast of *K. daigremontiana* abolished the phenotypic rigidization of this membrane due to increase of growth tem-

Table 3. Fatty acid composition (mol%) of lipid classes of tonoplasts isolated from *K. daigremontiana*

Lipid class	Treatment	C16:0	C18:0	C18:3	C8:2	C18:3
PC	Control	37.5	5.8	6.9	37.4	12.5
	HT	47.6	13.9	8.0	21.9	8.7
	HS	46.6	7.5	4.5	24.8	16.7
	LT	35.7	9.0	6.9	24.5	22.8
PE	Control	49.1	5.7	3.3	36.3	5.5
	HT	46.0	17.1	12.0	16.4	4.2
	HS	55.7	8.0	3.6	25.9	7.3
	LT	36.4	32.9	6.9	13.4	10.4
PG	Control	67.0	8.8	8.1	11.5	2.6
	HT	52.6	19.9	11.1	8.8	2.8
	HS	65.5	8.9	6.3	12.1	3.8
	LT	38.3	7.3	34.6	12.1	5.8
PA	Control	41.0	8.9	6.4	38.0	11.0
	HT	45.8	3.7	19.8	21.7	9.0
	HS	44.9	7.3	4.6	30.1	12.0
	LT	31.2	14.8	32.0	12.6	5.8
MGDG	Control	43.8	6.6	4.3	20.7	23.3
	HT	48.3	15.3	20.4	11.9	4.4
	HS	35.6	8.5	3.4	12.7	40.9
	LT	39.6	19.7	7.9	17.4	15.5
DGDG	Control	25.5	11.6	3.9	10.1	49.3
	HT	26.1	27.9	6.2	3.4	4.8
	HS	25.0	12.5	2.9	9.2	50.0
	LT	40.9	13.5	12.2	10.3	10.7

The tonoplasts isolated from *K. daigremontiana* were grown at normal temperatures (control), at high temperature (HT), at low temperature (LT), and after a heat shock from 25 to 35°C for 3 hr (HS).

perature. Aiming to mimic rigidization of the membranes by tonoplast proteins, we have reconstituted proteins isolated from the tonoplast of *K. daigremontiana* into phosphatidylcholine (PC) vesicles. The incorporation of tonoplast proteins into the PC liposomes led to a marked increase in the degree of fluorescence polarization (Fig. 8) indicating decrease of membrane fluidity. This effect clearly depended on the phospholipid/protein ratio established in the reconstituted vesicles. That is, the fluidity of the membrane decreased when the lipid/protein ratio was shifted to lower values.

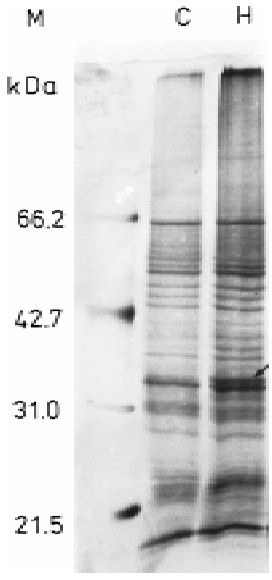

Fig. 5. Silver-stained polyacrylamide gel showing the polypeptide composition of tonoplast vesicles from control plants (lane C) and HT plants (lane H) of *K. daigremontiana.* The arrow indicates the position of a 35 kDa polypeptide which was exclusively present in heat treated plants (HT). Slots were loaded with about 5 μg protein. M: molecular mass standard; numbers on the left hand margin indicate molecular masses of standard proteins.

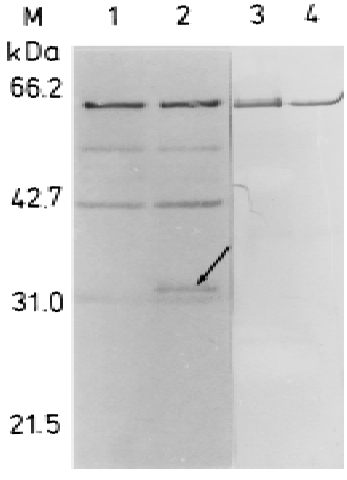

Fig. 6. Western blot analysis of tonoplast proteins from controls (lanes 1, 3) and HT plants (lanes 2, 4) of *K. daigremontiana.* Immunostaining was performed with an antiserum against the holoenzyme of the tonoplast H^+-ATPase of *K. daigremontiana* (lanes 1, 2) or against subunit A of the tonoplast H^+-ATPase of *M. crystallinum* (lanes 3, 4). The arrow indicates the 35 kDa heat related polypeptide. M: molecular mass standard; numbers on the left hand margin indicate molecular masses of standard proteins.

Discussion

In the present paper fluorescence labeling techniques were applied to visualize in the CAM plant *K. daigremontiana* the response of tonoplast fluidity to growth temperature. The observed effects of increase in growth temperature are fully consistent with previous findings obtained by EPR spectroscopy in the same experimental system (Kluge et al., 1991; Kliemchen et al., 1993; Schomburg & Kluge, 1994). Thus, there is now unequivocal evidence that at least in CAM plants increase of growth temperature leads adaptively to a decrease in the fluidity of the tonoplast. Moreover, our present results show for the first time that already three hours of treatment with high temperatures are sufficient to bring about rigidization of the tonoplast, and that lowering of

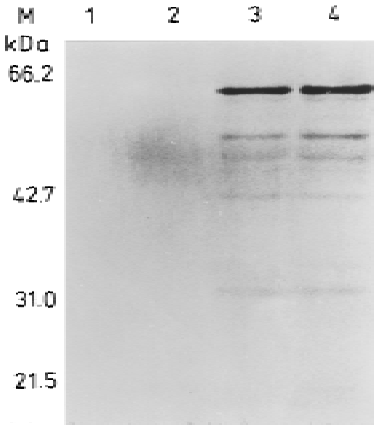

Fig. 7. Western blot analysis of tonoplast proteins from controls (lane 3) and HT plants (lane 4) of *K. daigremontiana* which were immunoprecipitated by an antiserum against subunit A of the tonoplast H^+-ATPase of *M. crystallinum.* Control experiments which were performed in the absence of antiserum against subunit A or in the absence of tonoplast proteins are shown in lanes 1 and 2, respectively. Immunostaining was performed using an antiserum against the tonoplast H^+-ATPase holoenzyme of *K. daigremontiana.* M: molecular mass standard; numbers on the left hand margin indicate molecular masses of standards.

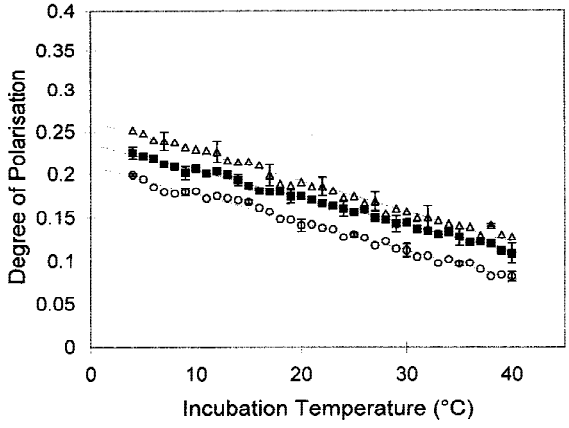

Fig. 8. Changes in membrane fluidity of PC vesicles containing *K. daigremontiana* tonoplast proteins from control plants at various lipid/protein (*I/p*, w/w) ratios (*I/p*). ○: PC vesicles without proteins ($n = 4$); ■: *I/p* ratio = l0 ($n = 3$); Δ: *I/p* ratio = 5 ($n = 3$).

growth temperature increased the tonoplast fluidity. This latter finding is consistent with the classical manifestation of homeoviscous adaptation known from biomembranes of microorganisms and animal cells (Hazel, 1988; Nozawa & Umeki, 1988; Parola, 1993).

As far as the rigidization of the tonoplast by increase of growth temperature is concerned, for the following reasons our findings clearly support the hypothesis of Schomburg & Kluge (1994) that this effect is mainly due to stabilization of the bulk lipid matrix by membrane proteins: First, as shown by our measurements of fluorescence polarization and excimere formation, the heat-induced decrease of tonoplast fluidity occurring in the complete tonoplast is abolished if the proteins are removed from the native membrane. Second, it is well known that the lipid constituents of biomembranes can be stabilized by interaction with membrane proteins (Parola, 1993). Indeed, we found that parallel to the phenotypic rigidization of the tonoplast there was a clear shift in the lipid/protein ratios in favor of the proteins. Finally, we were able to mimic the influence of variation of the lipid/protein ratio on membrane fluidity by reconstitution experiments with tonoplast proteins and artificial liposomes.

Whether or not that rigidization is solely a matter of changes in the lipid/protein ratios remains to be studied in detail. However, in this context our finding is interesting that the heat-induced decrease in tonoplast fluidity was accompanied by the appearance of a 35 kDa tonoplast polypeptide which was not detectable in the normally grown controls. As can be seen clearly in Fig. 5, the 35 kDa polypeptide has only a minor contribution to the total tonoplast protein amount suggesting that this protein did not lead to a drastic change of the lipid/protein ratio. However, the 35 kDa polypeptide might have a more specific role in membrane rigidization. It is too early to interpret this polypeptide in terms of heat shock proteins (Vierling, 1991), but it is conceivable that it belongs to that category. Recently, the heat-shock mediated induction of an hydrophobic 30 kDa membrane protein has been observed in yeast (Regnacq & Boucherie, 1993).

The identity of the 35 kDa tonoplast polypeptide has to be shown by future investigations, but it is worth mentioning in this context that stress-related appearance of tonoplast polypeptides with a molecular mass of about 30 kDa, which cross-react with antisera against a subunit of the tonoplast H^+-ATPase, has already been observed in other plants. In *M. crystallinum,* polypeptides with molecular masses of 28 and 32 kDa, which cross-reacted with an antiserum against the H^+-ATPase holoenzyme of *K. daigremontiana,* appeared in tonoplast vesicles after salinity induced C_3-CAM transition (Bremberger et al., 1988). In contrast to the 35 kDa heat-response polypeptide of *K. daigremontiana,* these polypeptides were shown to be closely attached to the H^+-ATPase holoenzyme (Ratajczak et al., 1994*b*; Zhigang et al., 1996) by immunoprecipitation experiments performed under identical conditions as used in the present study (Zhigang et al., 1996). In *Citrus sinensis,* salt stress led to the appearance of a 35 kDa tonoplast polypeptide (Bañuls et al., 1995) which cross reacted with an antiserum against the holoenzyme of the tonoplast H^+-ATPase of *K. daigremontiana* but was not found to be a constituent of the H^+-ATPase holoenzyme complex (Bañuls et al., 1995). Further studies are needed to find out whether these

polypeptides and the 35 kDa heat related polypeptide of *K. daigremontiana* described in the present study are of similar origin. Although several protease inhibitors such as PMSF, EDTA, leupeptin and pepstatin were present during isolation of tonoplast vesicles, proteolytic breakdown can not be completely excluded. It is also possible that all the mentioned polypeptides are products of *in vivo* degradation of high molecular mass subunits of the tonoplast H-ATPase occurring under certain stress conditions and still exhibiting distinct metabolic functions as it is the case for the 32 kDa polypeptide of *M. crystallinum*. Alternatively, it is conceivable that the 35 kDa heat response polypeptide has nothing to do with the ATPase complex and that the reaction with the antiserum derives from accidental structural similarities with the ATPase-subunits.

The obvious importance of membrane proteins in bringing about HVA of the tonoplast does not exclude the possibility that membrane lipids are also involved in this process. This becomes evident by our lipid analyzes. The basic lipid composition of the tonoplast we have found in normally grown *K. daigremontiana*, agrees well with the data reported by Haschke et al. (1989). The latter authors found that in *Mesembryanthemum crystallinum* induction of CAM had no effect on the tonoplast lipid composition, albeit in this species CAM induction is accompanied by a decrease in tonoplast fluidity (Kliemchen et al., 1993). In the case of *K. daigremontiana*, however, the present study revealed that alterations in tonoplast fluidity in response to growth temperature were paralleled by changes at the level of the membrane lipids. Surprisingly, and in contradiction to the generally admited opinion, we observed that heat shock treatment significantly increased the degree of unsaturation of the tonoplast lipids by increase in the percentage of linolenic acid, although the fluidity of the complete membrane (Fig. 1*B*) and that of the protein-free lipid matrix significantly decreased (Fig. 2). At first glance this contradictory result can reasonably be explained by the decrease in the level of PC and PE which occurred in parallel to the increase in linolenic acid. Moreover, PC and PE became more saturated due to a large decrease in the percentage of linoleic acid. On the other hand, an increase was observed in the level and degree of unsaturation of MGDG. MGDG and PE are typical ''nonbilayer-forming'' lipids, and it has been shown that incorporation of this type of lipid into a membrane affects its order and dynamics and with it the transport taking place across the concerned membrane (De Kruiff, 1997). Altogether, the tonoplast of *K. daigremontiana* responds to heat shock at the level of the lipid composition in a subtle manner. First, as in most other systems, the decrease in the degree of unsaturation of the main bilayer-forming lipids can be expected to increase the rigidity of the tonoplast. However, this rigidization is counterbalanced by the increase in the level and the degree of unsaturation of nonbilayer forming MGDG which is known to disturb the bilamellar structure and with it the degree of order in a membrane. Also in lowering of growth temperatures (LT Plants) the tonoplast lipids of *K. daigremontiana* respond in a complicated way. That is, PC which represents one of the main bilayer forming lipids in the membrane, became more desaturated which should lead to a fluidization of the membrane. However, this fluidization is obviously compensated by the increase in saturation in PE (which is also a nonbilayer forming lipid) and in the two galactolipids so that the protein-free lipid matrix of the tonoplast from LT plants shows the same fluidity as the control (Fig. 2).

In conclusion, our data suggest that HVA of the *K. daigremontiana* tonoplast is a multifaceted phenomenon concerning both the membrane proteins and lipids. We are aware that the gapless explanation of HVA in the tonoplast requires a more precise understanding of the lipidic microdomains around the membrane proteins. Studies dealing with this problem are now in progress in our laboratories.

The question still remains, whether growth temperature can also shift the tonoplast fluidity in others than in CAM performing cells, and if such shifts concern also the plasmalemma and other membranes. For thylakoid membranes the existence of homoeoviscous adaptation has been demonstrated and could be related to acclimation of photosynthesis to high environmental temperatures (Quinn, 1988). It is reasonable to propose that the temperature related phenotypic adaptability of tonoplast fluidity shown for *K. daigremontiana* is also ecophysiologically relevant. In nature, CAM succulents as represented by our experimental plant are exposed to drastic diurnal fluctuations of temperature ranging from about 10°C in the night to often more than 35°C in full sun during the day. Also seasonal changes in the environmental temperatures have to be put into account. It is conceivable that the shown phenotypic adaptation of tonoplast fluidity helps to avoid excessive thermotropic fluidization or rigidization of the tonoplast thus guaranteeing undisturbed functioning of the vacuole under environmental temperature stress. It is also conceivable that the adaptation of tonoplast fluidity is an important component in the mechanism behind the marked increase in cellular heat-resistance shown by desert CAM plants in response to short- and long-term exposure to exceptional high environmental temperatures (Nobel, 1988).

This work was supported by the Deutsche Forschungsgemeinschaft (Bonn, Germany) in the frame of the SFB 199 (TP B1 and B3). We would like to thank Mrs. Monika Medina-España and Mrs. Upmeyer for help in preparing the manuscript. We also thank Mrs. Annick Bernardo-Connan for her technical assistance in the analysis of lipids and fatty acids.

References

Ames, B.N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *In:* Methods of Enzymology. S.P. Colwick and N.O. Caplan, editors. Vol. 8. pp. 115–118. Academic Press, New York

Ammon, R., Hinsberg, K. 1935. Colorimetrische Phosphor-und Arsenbestimmung mit Ascorbinsäure. *Z. Physiol. Chemie* **239:**207

Bañuls, J., Ratajczak, R., Lüttge, U. 1995. NaCl-stress enhances proteolytic turnover of the tonoplast H^+-ATPase of *Citrus sinensis:* Appearance of a 35 kDa polypeptide still exhibiting ATP-hydrolysis activity? *Plant Cell Environ.* **18:**1341–1344

Behre, B., Ratajczak, R., Lüttge, U. 1992. Selective reconstitution of the tonoplast H^+-ATPase of the crassulacean-acid metabolism plant *Kalanchoë daigremontiana. Bot. Acta* **105:**260–265

Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *J. Biochem. Physiol.* **37:**911–917

Bremberger, C., Haschke, H.-P., Lüttge, U. 1988. Separation and purification of the tonoplast ATPase and pyrophosphatase from plants with constitutive and inducible crassulacean acid metabolism. *Planta* **175:**465–470

De Kruiff, B. 1997. Biomembranes: lipids beyond the bilayer. *Nature* **386:**129–130

Galla, H.-J. 1988. Spektroskopische Methoden in der Biochemie. Georg Thieme Verlag; Stuttgart

Galla, H.-J., Hartmann, W. 1980. Excimer-forming lipids in membrane research. *Chem. Phys. Lipids* **27:**199–219

Haschke, H.-P., Bremberger, C., Lüttge, U. 1989. Transport proteins in plants with crassulacean acid metabolism: Immunological characterization of ATPase subunits. *In:* Plant Membrane Transport. J Dainty et al., editors. pp. 149–54. Elsevier Science Publishers BV

Haschke, H.-P., Kaiser, G., Martinoia, E., Hammer, U., Teucher, T., Dorne, A.J., Heinz, E. 1990. Lipid profiles of leaf tonoplastes from plants with different CO_2-fixation mechanisms. *Botanica Acta* **103:**32–38

Hazel, J.R. 1988. Homeoviscous adaptation in animal cell membranes. *In:* Advances in Membrane Fluidity. R.C. Aloia, C.C. Curtain and L.M. Gordon, editors. Vol. 3. p. 149–189. Alan R. Liss, New York

Iwasaki, I., Arata, H., Kijima, H., Nishimura, M. 1992. Two types of ion channels involved in the malate ion transport across the tonoplast of a crassulacean acid metabolism plant. *Plant Physiol.* **98:**1494–1497

Jochem, P., Lüttge, U. 1987. Proton transporting enzymes at the tonoplast of the leaf cells of the CAM plant *Kalanchoe daigremontiana* I. *In:* The ATPase. *J. Plant. Physiol.* **129:**251–268

Kliemchen, A., Schomburg, M., Galla, H.-J., Lüttge, U., Kluge, M. 1993. Phenotypic changes in the fluidity of the tonoplast membrane of crassulacean-acid metabolism plants in response to temperature and salinity stress. *Planta* **189:**403–409

Kluge, M., Galla, H.-J. 1995. Thermotropic responses of biomembranes exemplified with the tonoplast of crassulacean acid metabolism plants. *Progress in Botany* **57:**81–92

Kluge, M., Kliemchen, A., Galla, H.-J. 1991. Temperature effects on crassulacean acid metabolism: EPR spectroscopic studies on the thermotropic phase behaviour of the tonoplast membranes of *Kalanchoë daigremontiana. Bot. Acta* **104:**355–360

Kluge, M., Schomburg, M. 1996. The tonoplast as a target of temperature effects on CAM. *In:* Crassulacean acid metabolism. Biochemistry, Ecophysiology and Evolution. K. Winter, A.P. Smith and J.A.C. Smith, editors. pp. 72–78. Springer Verlag, Heidelberg

Kluge, M., Ting, I.P. 1978. Crassulacean acid metabolism. Analysis of an ecophysiological adaptation. Ecological Studies. Vol. 30. Springer Verlag, Heidelberg

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685

Lüttge, U., Fischer-Schliebs, E., Ratajczak, R., Kramer, D., Berndt, E., Kluge, M. 1995. Functioning of the tonoplast in vacuolar C-storage and remobilization in crassulacean acid metabolism. *J. Exp. Bot.* **46:**1377–1388

Lüttge, U., Smith, J.A.C. 1984. Mechanism of passive malic-acid efflux from vacuoles of the CAM plant *Kalanchoë daigremontiana. J. Membrane Biol.* **81:**149–158

Metcalfe, L.D., Schmidtz, Pelka, J.R. 1966. A rapid preparation of fatty acid methylesters from lipids for gas chromatographic analysis. *Anal. Chem.* **38:**514–515

Nobel, P.S. 1988. Environmental Biology of Agaves and Cacti. Cambridge University Press. Cambridge, New York

Nozawa, Y., Umeki, S. 1988. Regulation of membrane fluidity in unicellular organisms. *In:* Advances in Membrane Fluidity. R.C. Aloia, C.C. Curtain and L.M. Gordon, editors. Vol. 3. pp. 239–257. Alan R. Liss, New York

Oakley, B.R., Kirsch, D.B., Morris, N.R. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105:**361–362

Osmond, C.B. 1978. Crassulacean acid metabolism: A curiosity in context. *Ann. Rev. Plant Physiol.* **29:**379–414

Parola, A.H. 1993. Membrane lipid-protein interaction. *In:* Biomembranes Physical aspects. M. Shinitzky, editor. pp. 159–277. VCH Weinheim

Popov, N., Schmitt, M., Schulezek, S., Mathies, H. 1975. Eine störungsfreie Mikromethode zur Bestimmung des Proteingehaltes in Gewebehomogenaten. *Acta Biol. Med. Ger.* **34:**1441–1446

Quinn, P.J., Li, L.J. 1987. Phase transition behavior of monogalactosyldiacylglycerol. *In:* The metabolism, structure and function of plant lipids. P.K. Stumpf, J.B. Mudd, and W.D. Nes, editors. pp. 181–183. Plenum Press, New York, London

Ratajczak, R., Kemna, I., Lüttge, U. 1994*a.* Characteristics, partial purification and reconstitution of the vacuolar malate transporter of the CAM plant *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie. *Planta* **195:**226–236

Ratajczak, R., Richter, J., Lüttge, U. 1994*b.* Adaptation of the tonoplast V-type H^+-ATPase of *Mesembryanthemum crystallinum* to salt stress, C_3-CAM transition and plant age. *Plant Cell Environ.* **17:**1101–1112

Regnacq, M., Boucherie, H. 1993. Isolation and sequence of HSP30, a heat-shock gene coding for a hydrophobic membrane protein. *Current Genetics* **23:**435–442

Schomburg, M., Kluge, M. 1994. Phenotypic adaptation to elevated temperatures of tonoplast fluidity in the CAM plant *Kalanchoë daigremontiana* is caused by membrane proteins. *Bot. Acta* **107:**328–332

Shinitzky, M., Barenholz, Y. 1978. Fluidity parameters of lipid regions determined by fluorescence polarization. *Biochim. Biophys. Acta* **515:**367–394

Svetec, J., Furtula, V., Nemec, M., Nothnagel, E.A., Scharam, M. 1995. Transport and dynamics of molecules dissolved in maize root cortex membranes. *J. Membrane Biol.* **143:**19–28

Vierling, E. 1991. The roles of heat shock proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol.* **42:**579–620

Winter, K. 1985. Crassulacean acid metabolism. *In:* Photosynthetic mechanisms and the environment. J. Barber and N.R. Baker, editors. pp. 329–387. Elsevier, Amsterdam

Zhigang, A., Löw, R., Rausch, T., Lüttge, U., Ratajczak, R. 1996. The 32 kDa tonoplast polypeptide D_i associated with the V-type H^+-ATPase of *Mesembryanthemum crystallinum* L. in the CAM state: A proteolytically processed subunit B? *FEBS Lett.* **389:**314–318