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Flow Cytometric Fluorescence Anisotropy of Lipophilic Probes in Epidermal and Mesophyll Protoplasts from Water-Stressed *Lupinus albus* L.

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ABSTRACT

The blue emission anisotropy, *r*, of two lipophilic probes, diphenylhexatriene (DPH) and its trimethyl-ammonium derivative (TMA-DPH), has been measured in foliar *Lupinus albus* L. protoplasts for the first time by flow cytometry. Distinctive values have been obtained for protoplasts of epidermal and mesophyll origin, identified by their intensities of chlorophyll fluorescence. Fluorescence microscopy confirmed that TMA-DPH remained in the plasma membrane while DPH penetrated into intracellular lipid domains. Typical emission anisotropy values at 22°C for mesophyll and epidermal protoplasts, respectively, were 0.225 and 0.312 with TMA-DPH, and 0.083 and 0.104 with DPH. This indicates that epidermal cells—and notably their plasma membranes (TMA-DPH)—have higher lipid microviscosity and/or more ordered lipid structure. Two lupin genotypes characterized as resistant or susceptible to drought were analyzed with or without 9 days of water stress shown to increase ion leakage from foliar discs. Water stress greatly increased the apparent fluidity, and more so in the susceptible genotype; the effect was more pronounced in the chlorophyll-containing mesophyll cells than in the epidermal cells.

The permeability of proteolipidic cell membranes arises from the interaction of their biochemical and biophysical properties (24). The lipid domain of a membrane is more or less fluid and the lipids are ordered with more or less structure (1, 28); such properties appear to affect the hydration control of the membrane (24), lateral heterogeneity and protein conformation (*e.g.* in microsomal membranes from bean cotyledons) (19).

Leaf protoplasts have here been studied with two lipophilic fluorescent probes whose emission anisotropy, *r*, is related in a nonlinear manner to the dynamic property microviscosity (28) and to structural order of the lipid matrix. We use the term 'lipid fluidity' to englobe the two aspects (30). In fact, lipid order dominates the anisotropy coefficient in steady-state measurements, r_s, through the limiting fluorescence anisotropy, r_∞ (16). Of the two probes used (below), this is less so for TMA-DPH[1] (25).

Protoplasts have been used for polarization studies previously (3, 18, 31), but the present work is unique in (a) using TMA-DPH for plasma membrane localization, (b) obtaining single cell values, notably to characterize Chl-containing and Chl-less cells, and (c) using the gating function of flow cytometry to eliminate debris, broken cells, or blue autofluorescent cells from the final calculations and to minimize any contribution of diffused light. Cytometric fluorescence polarisation studies are common with mammalian material (8, 11, 13, 26), but there are only two elementary measures concerning plant cells (5, 6).

Among the pleiotropic effects of drought upon plants, this report concentrates upon the notion of homeoviscosity in the cellular processes of regulation of membrane fluidity in response to external factors: this has been demonstrated in some cases *e.g.* upon variation of external pressure (28). Typically, a drought-resistant plant maintains its membrane integrity for a longer period during a water stress (23). Low temperatures provoke rigidification of cell membranes: cold resistant plants tend to maintain membranes relatively more fluid at cold temperatures (28). Of four wheat cultivars tested, only cold-resistant varieties increased membrane fluidity during the 'hardening process' (31). On the other hand, marked decreases in membrane fluidity appear to be associated with extreme processes such as cut flower senescence (3) or ripening (18), linked to lipoxygenase activity leading to selective depletion of unsaturated fatty acids (10) and superoxide production (20).

Several genotypes of white lupin (*Lupinus albus* L.) have been ranked for their resistance to water stress according to recovery from a period without watering (15). There is a correlation between drought resistance and ion leakage in this collection: the efflux of solutes from leaf discs, assessed by conductivity, is 40% less in a resistant genotype (15). A similar association has been found for cotton plants rendered more or less drought tolerant by the photoperiod regime (9). During the onset of dehydration in lupin, the level of characteristic membrane lipids falls with respect to total leaf lipids, especially in drought susceptible genotypes (15). The ratio of free sterols to phospholipids increases with water stress in *Brassica napus* (29). Lipids of the plastidial compartment are also clearly modified by this stress (15).

The current study was motivated by the following questions with respect to membrane fluidity and order. (a) Do epidermal and mesophyll cells have similar values? (b) Is there an intrinsic difference in the watered state between two characteristic lupin genotypes, one resistant and the other susceptible to drought? (c) Are similar membrane changes produced in these genotypes by plant water stress?

[1] Abbreviations: TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5hexatriene *p*-toluenesulfonate; DPH, 1,6-diphenyl-1,3,5hexatriene; CV, coefficient of variation; S, Siemens.

MATERIALS AND METHODS

Plant Culture

Two genotypes of *Lupinus albus* L. produced by the 'Groupement d'Interet Economique LUPSEM' (Lusignan, France) were used: the drought-resistant G_1 and drought-susceptible G_9. Plants were cultivated for 1 month with nutrient medium and then studied watered or stressed by an abrupt cessation of watering during 9 d or less (15).

Protoplast Isolation

Protoplasts were obtained by enzymatic incubation of leaves 17 h at 20°C in the dark in a medium containing 400 mM sorbitol, 10 mM Mes (pH5.6), macerozyme R10, cellulase R10, and driselase. They were filtered through 150 μm mesh nylon and rinsed with the same medium by three centrifugations at 65*g* for 5 min, then stored at room temperature in the dark.

Cytology

Leaf fragments were fixed in glutaraldehyde, poststained with osmium tetroxide and included in araldite. Semithin transversal sections (Reichert OM41 microtome) were mounted, cleared, and stained with toluidine blue 0.1% (pH 8.9).

Protoplasts were observed on a Reichert Polyvar epifluorescence microscope using the U1 filter combination (excitation 330–380 nm, emission 418 nm longpass).

Fluorescent probes DPH and TMA-DPH (Molecular Probes, Eugene, OR) were prepared as 2 mM stock solutions in, respectively, acetonitrile and dimethylformamide and stored at 4°C. An intermediary dilution, 50 μM, of TMA-DPH was prepared daily with an aliquot from the 2 mM stock added to protoplast medium and agitated vigorously for 10 min to eliminate the dimethylformamide; this was then added to give 1 μM TMA-DPH in the protoplast suspension, and analysis was started after 4 min. Such a procedure with an intermediary dilution was not feasible with DPH due to its low solubility in protoplast medium: an aliquot of the stock was added directly to protoplast suspensions to obtain 5 μM DPH. Collins and Grogan (8) stress the importance of stringent staining protocols when using DPH, as it does not form stable micelles. All experiments were conducted at 22°C under dim incandescent light and using glassware rather than plastic.

Bulk Spectrofluorometry

Uncorrected excitation and emission spectra were obtained on suspensions of 20,000 protoplasts mL^{-1} using a Kontron SFM25 spectrofluorometer. The same instrument, fitted with polarizers on the excitation and emission axes, was used with labeled liposome suspensions to assess steady-state Emission Anisotropy at 357 nm excitation and 430 nm emission, applying a grating correction factor.

Flow Cytometry

The EPICS V flow cytometer (Coulter, Hialeah, FL) had a 100 μm nozzle and the standard optic such that each cell was exposed and measured for about 1.5 μs, *i.e.* two orders above the fluorescence lifetime of these probes. The argon laser (Spectra-Physics 2025-05) set at 351 + 364 nm, 50 mW, had vertical polarization. Forward angle light scatter was used to gate the analysis upon intact cells. The fluorescent emissions were filtered (Fig. 1) through a 408 nm long-pass, split with a 590 nm dichroic sending red Chl fluorescence through a 630 nm long-pass filter to a first photomultiplier; the blue fluorescence continued through a 465 nm short-pass filter, was split with a 50% mirror at 45° so that the vertically polarized component (I_{vv}) passed through a vertical polarizer to a photomultiplier, and the horizontally polarized component (I_{vh}) was reflected through a horizontal polarizer to another photomultiplier. The signals were processed on an analog card linked to a 1024 channel ADC to derive the blue emission anisotropy (r),

$$r = \frac{I_{vv} - I_{vh}}{I_{vv} + 2I_{vh}}.$$

Incidentally, r is related to the oft-used coefficient of polarization P as

$$r = \frac{2P}{3 - P}.$$

A prerequisite was that the two 'blue' photomultipliers be balanced: once each had been set roughly appropriate for the fluorescence intensities of the cells, they were balanced by inserting a 357 nm half-wave retardation plate in the laser beam. When this is turned at 45° the excitation is horizontally polarised and the emission on the horizontal plane is isotropic such that $I_{hv} = I_{hh}$. The photomultiplier voltages were then set so that their signals on the oscilloscope had the same amplitude and, more accurately, so that a cytogram of I_{hv} versus I_{hh} showed $x = y$. The half-wave retardation plate was then removed, restoring the vertical polarisation of the incident light. Insertion of the 590 nm dichroic did not modify the measured blue Emission Anisotropy.

Statistics

The value r was assessed object by object: the mean and the CV were calculated on 100,000 liposomes or on 2,000 to 10,000 protoplasts. Student's t-test was used to compare the means of several treatments.

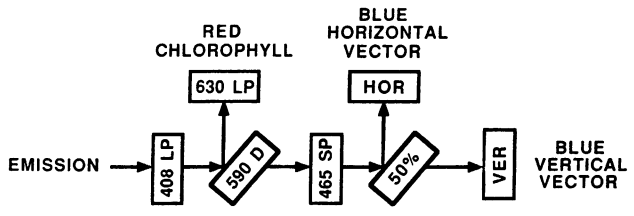

Figure 1. The filter arrangement used to analyse the emission collected through a collimator at 90° to the laser and stream axes of the EPICS V flow cytometer. Three composants—red emission due to Chl and vertical and horizontal polarization vectors of the blue emission—are directed to separate photomultipliers. See "Materials and Methods."

RESULTS AND DISCUSSION

Physiology of the Leaf

The resistant genotype had a leaf water content (LWC) and a leaf water potential (assessed with a Scholander xylem pressure chamber) initially lower than the susceptible genotype (15). But the resistant plants lost water more slowly during water stress, confirming a higher resistance. After 8 d of water stress, where

$$LWC = \frac{(FW - DW)\cdot 100\%}{DW}$$

the resistant genotype had a leaf water content of 441% and a leaf water potential of −1.2 MPa, while the susceptible genotype had only 249% water content and a water potential of −1.6 MPa (DW and FW, dry and fresh weight, respectively).

In a study of ion leakage rates from leaf discs, conductivity (15) of the bathing medium with leaves in the watered state was significantly higher for the susceptible (33.8 ± 1.9 μS) relative to the resistant genotype (21.8 ± 1.8 μS). During the water stress, this leakage (conductivity) increased for leaves from both genotypes and this increase was always much larger for the susceptible genotype. After 8 d of water stress, ion leakage in the susceptible genotype was 1.4-fold that of the resistant (52.8 *versus* 38.2 μS).

Establishing the Cytometric Methods

Probes

Protoplast preparations were only retained if stable. Elementary tests substantiated their viability: they appeared intact and TMA-DPH labeled only the plasma membrane; they became fluorescent with fluorescein diacetate; they were able to synthesize different classes of neutral and polar lipids from 1-[^{14}C]acetate (TLC data not presented); more than 85% excluded Evans blue. By fluorescence microscopy (Fig. 2), TMA-DPH was clearly localized in the plasmalemma of intact protoplasts, as expected from the work of Kuhry *et al.* (17) and Schroeder *et al.* (27), these latter reporting that TMA-DPH localized preferentially in the inner leaflet of synaptic plasma membranes, anchored at the lipid-water interface (25). DPH, although difficult to observe by microscopy due to rapid quenching, appeared to penetrate into the cell, marking both the plasmalemma and intracellular membranes, in accordance with Bouchy *et al.* (4) and Collard and De Wildt (7) and in opposition to Borochov *et al.* (3). Lipid domains of broken cells were labeled by both probes. Protoplasts were free of cellulose inasmuch as Calcofluor White did not label them.

By flow cytometry, a time study of fluorescence intensity and *r* upon addition of the probes to intact lupin protoplasts showed that TMA-DPH labeling reached a plateau before the first reliable data point (10 s) and *r* was stable thereafter. In "stabilized" protoplasts (below), DPH labeling intensity increased throughout 40 min and although mean *r* was stable, the CV of *r* increased after 20 min. Such kinetics are coherent with the microscopic observations above. The measured *r* was

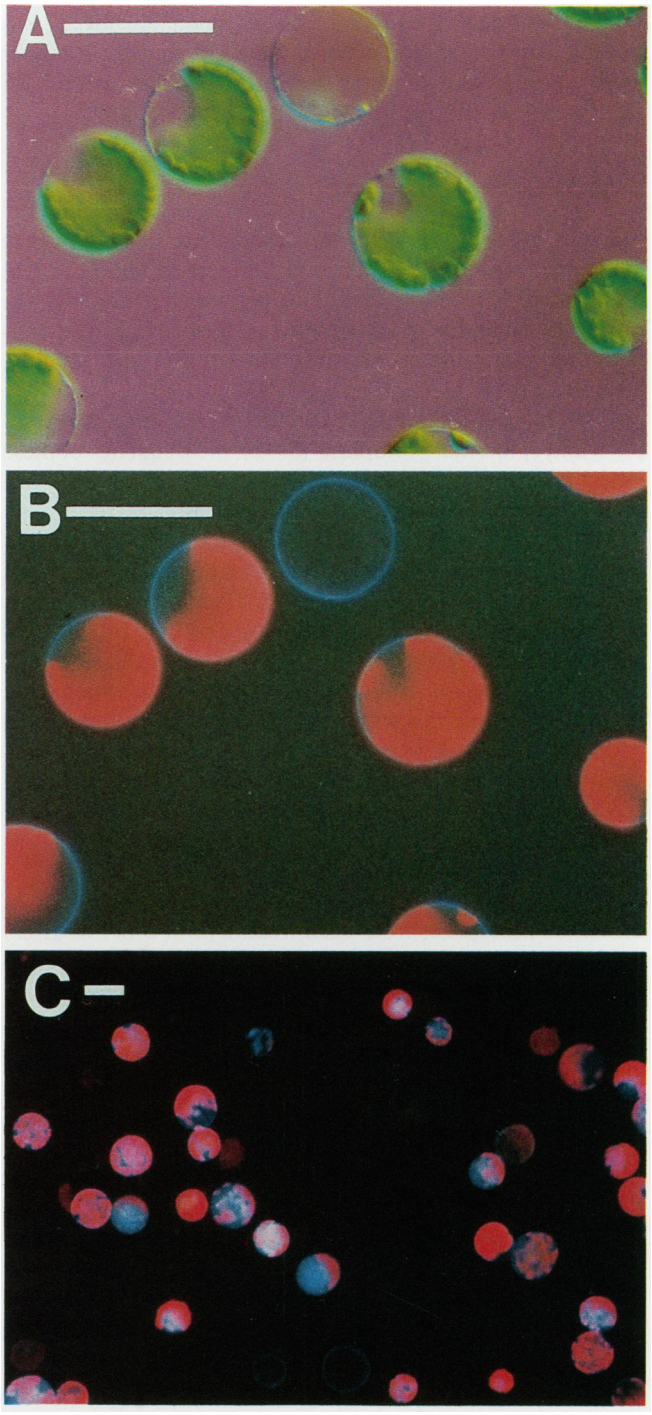

Figure 2. Lupin leaf protoplasts labelled with 1 μM TMA-DPH: (A) by differential interference contrast microscopy; (B and C) by epifluorescence. Note the red emission of Chl and the blue TMA-DPH labeling of the plasmalemma in both Chl^+ and Chl^- protoplasts (membranes were negative in controls). The heterogeneity of such suspensions is more evident in C, some protoplasts having intense blue autofluorescence (photographed as white) while cellular debris of broken protoplasts has labeled with TMA-DPH. Bar = 50 μm.

independent of [DPH] between 0.5 and 10 μM (Fig. 3). Labeled cells are apparently damaged above 100 mW excitation, and the gain of emission intensity upon labeling, F, was optimal between 30 and 60 mW (data not shown). Analyses were routinely started 4 min after adding probes, using 5 μM DPH and 1 μM TMA-DPH with 50 mW excitation.

Natural Cellular Fluorescence

Lupin protoplasts have two characteristic fluorescence emissions (Fig. 2), a peak at 690 nm corresponding to Chl and a broad blue emission peaking between 435 and 460 nm under UV excitation (peak 365 nm) and probably corresponding to several constituents such as NADPH and, mainly, alkaloids.

The Chl emission serves in cytometry to distinguish mesophyll and epidermal protoplasts (5, 12), the frequency of these latter in our preparations being about 0.28, concording with counts in transversal leaf photomicrographs.

The intensity of blue fluorescence, characterized using bulk fluorometry, was similar for the two genotypes but increased with water stress. But flow cytometry revealed the dispersion of individual cellular levels (Fig. 4) and the presence of subtypes differing about 15-fold in intensity: in this example, 5.4% of protoplasts from watered plants had strong blue fluorescence while this was 29.8% after 3 d without watering and 35% after 8 d. It was essential to optimize the probe signal relative to this spectrally similar cellular autofluorescence which we were unable to quench. Increasing the probe concentration did not usefully increase the signal, and could possibly perturb membrane properties.

We measured the fluorescence intensity and anisotropy in unstained protoplasts from each treatment in order to assess the error due to this (variable) background. Letting F be the gain in mean intensity, I, upon adding the probe, namely,

$$F = \frac{I \text{ with probe}}{I \text{ autofluorescence}}$$

we found that for DPH $5 < F < 16$ while for TMA-DPH $15 < F < 35$. The less favorable factors corresponded to preparations with high initial autofluorescence (8 d stressed plants). The error introduced by this natural cellular background will

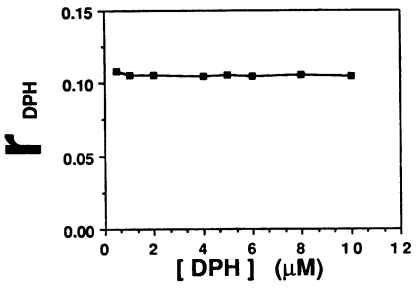

Figure 3. Mean emission anisotropy of DPH as a function of probe concentration added to lupin protoplasts, measured by flow cytometry after 4 min. The anisotropy of the blue cellular autofluorescence was 0.164 (before addition of DPH).

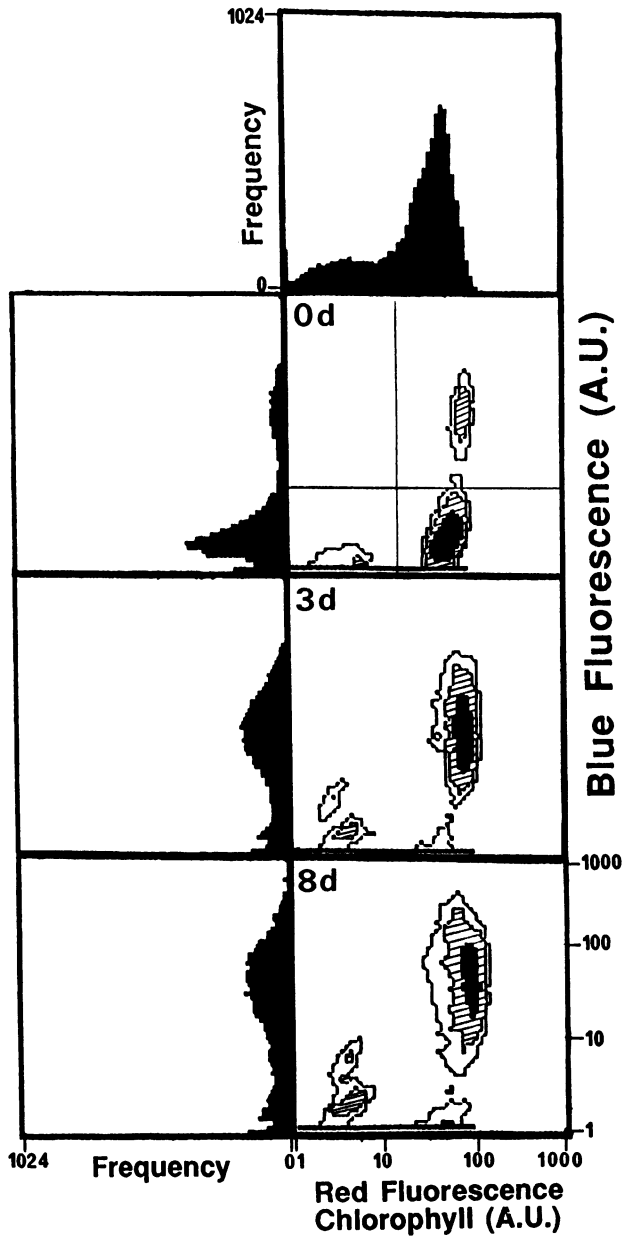

Figure 4. Cytograms corresponding to 20,000 lupin foliar protoplasts each analysed for red Chl and natural blue fluorescence. The axes (64 × 64 channel resolution) are logarithmic. Three classes of frequency are indicated by the contours which delimit coordinates having >30 protoplasts (*solid black*), 10 to 29 protoplasts (*hatched*), 5 to 9 protoplasts (*white*). Statistics for each class were obtained by a typical quadrant analysis (x, y : 25, 20) demonstrated on the first cytogram. Corresponding histograms are shown as projections for the blue fluorescence, and for the red fluorescence only on day 0. The protoplasts were from plants of the resistant genotype, after 0, 3, or 8 d without watering. Stress increased the frequency of strong blue fluorescing protoplasts (the highest contour level is reached) but had little incidence upon their modal fluorescence intensity (its Y coordinate was stable).

depend upon its intensity and its anisotropy relative to that of the membrane probe. It can be shown that

$$r_c = \frac{F \cdot r_a - r_n}{F - 1}$$

where r_a (apparent) and r_n (natural) are values of r obtained in presence and absence of added probe, F as above, and r_c is the true (corrected) emission anisotropy of the membrane probe. Obviously, the error increases as F decreases and as r_n differs from r_c. Fortunately, natural cellular fluorescence of lupin protoplasts has an anisotropy close to that of the membrane-inserted probes: in the worst sample actually encountered ($F = 4.5$) true r_c was overestimated 11% by using the apparent r_a.

The above calculations were made with mean population values: it was not possible in the flow cytometer to obtain values for the same cell before and after labeling! In practice, we introduced an analysis gate to eliminate from the calculation any exceptionally blue protoplasts (the upper few percent of the population), and then used the apparent value, r_a.

Validation

By comparison of the two techniques spectrofluorometry and cytofluorometry, the optical configuration and electronics of a similar cytometer have already been validated (11). Similarly, no significant difference between bulk fluorometry measurements and those from flow cytometry was observed with HeLa cells (8). We abandoned an initial objective of using liposomes as reference material for each experiment, as such reference preparations were not stable and their fluorescence intensities were highly dispersed. In our case, flow cytometry with protoplasts was simpler and more reproducible than with liposomes, but we chose not to make bulk fluorometric measurements with protoplasts due to the importance of sedimentation, broken cells and light scattering. Moreover, distinctive cell subpopulations cannot be detected.

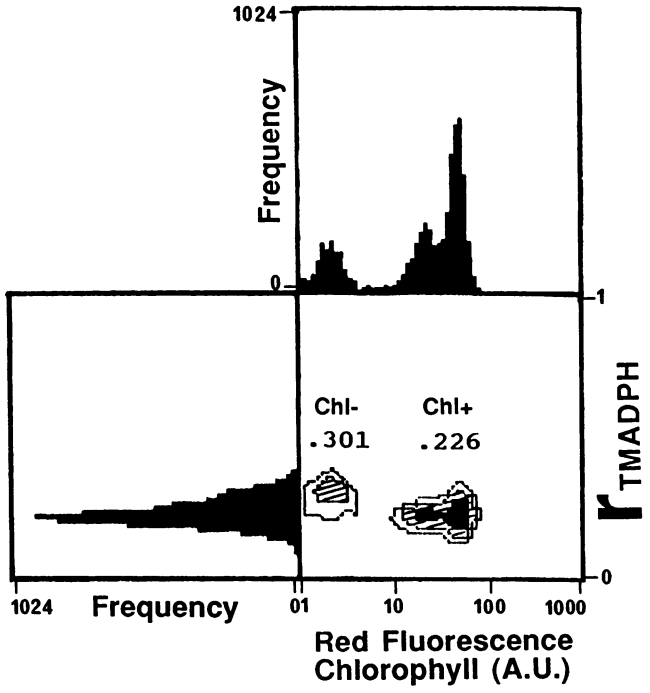

Figure 5. Cytogram corresponding to 6,000 protoplasts of the resistant lupin genotype each analysed for red chl (log scale) and TMA-DPH emission anisotropy (linear). Three classes of frequency are indicated by the contours which delimit coordinates having >35 protoplasts (*solid black*), 12 to 34 protoplasts (*hatched*), 4 to 11 protoplasts (*white*). Corresponding histograms are shown as projections. The monomodal anisotropy histogram in fact confounds distinctive anisotropy values for Chl^- and Chl^+ protoplasts; the $r_{TMA\text{-}DPH}$ values shown are the means of each class as delimited in the bivariate analysis. The two contiguous Chl^+ subclasses have similar modal anisotropy, and other results (not shown) indicate that mesophyll protoplasts with even much less Chl still have this same anisotropy.

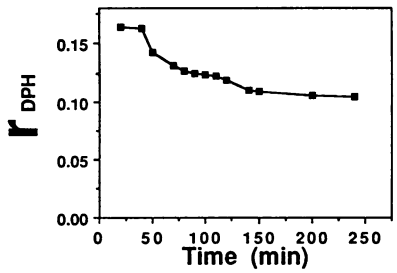

Figure 6. Effect of storage at 4°C upon lupin protoplasts which were subsequently labeled with DPH and assessed for emission anisotropy.

Application to Lupin Genotypes

Mesophyll versus Epidermal Protoplasts

Using the red fluorescence of chl in a multiparametric study, we could associate each anisotropy reading with the type of protoplast, namely of mesophyll or epidermal origin (Fig. 5). The two classes of protoplasts have markedly different membrane properties: indeed, a change of their frequency in a suspension can significantly modify the mean of the whole population, without there necessarily being any change in the membrane structure of either subpopulation! This potential artifact of bulk measurements (in cuvettes) is avoided by flow cytometry.

The following results include both situations, where the mesophyll and epidermal cells respond to treatments in similar or in opposite ways. But whatever the genotype or the physiological state of the original plant or of the protoplasts, mesophyll protoplasts have a far more fluid lipid domain, whether assessed with DPH (averaging cellular lipids) or TMA-DPH (addressing the plasma membrane). This cannot simply be an artefact of energy transfer from the probes to Chl: TMA-DPH is not in the chloroplasts, and DPH values in mesophyll protoplasts were independent of Chl 'content' (red fluorescence).

Modulation in Vitro: *Time, Temperature, Chemicals*

When the cellulase incubation was shortened from 17 h to 2.5 h, the resulting protoplasts yielded lower $r_{TMA\text{-}DPH}$ values; although the global r_{DPH} did not change, in fact the anisotropy fell significantly in mesophyll protoplasts but rose in the epidermal subpopulation.

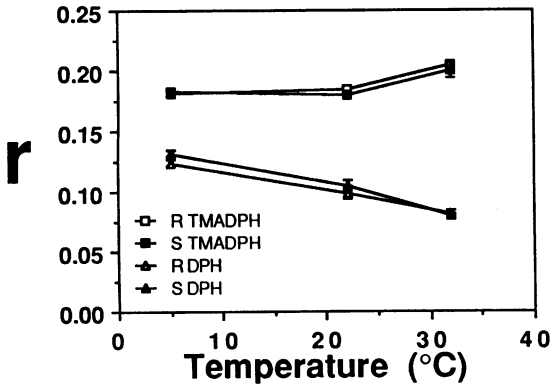

Figure 7. Emission anisotropy measured at three temperatures in lupin protoplasts from resistant (R) or susceptible (S) genotypes after labeling with TMA-DPH or DPH. The 95% confidence intervals do not exceed the symbols except were shown.

Protoplasts were stored in the dark at 4 or 22°C, labeled and *r* was measured at 22°C. Storage for 4h increased the apparent membrane fluidity (Fig. 6) (except in the case of the 8d stressed susceptible genotype where the decline was not significant, r_{DPH} being already low at zero time). The decline was significantly stronger in protoplasts stored at 22°C. The formation of triglycerides in such protoplasts shown by [^{14}C] acetate incorporation (data not presented) would favour such a decline in r_{DPH}. Before studies, protoplasts were routinely stored at 4°C for 120 min after purification, and treatment comparisons were made at similar times thereafter. Apparently this storage only affected the plasma membrane of mesophyll protoplasts, as no significant change occurred in $r_{TMA\text{-}DPH}$ of epidermal protoplasts.

As the temperature during anisotropy measurement was increased, r_{DPH} decreased (Fig. 7). This increase in fluidity with temperature is normal (13, 21) (and with protoplasts) (18). The $r_{TMA\text{-}DPH}$ in protoplasts did not change between 5 and 22°C, except for a slight rise for only the resistant genotype, and then increased strongly at 32°C (Fig. 7). Given that the rate of rotational reorientation within lipids will certainly not decrease with temperature (r_0 should decrease), this result can only be interpreted as an increased structural order of the probes's domain within the plasma membrane at 32°C. We propose that this ordering of the probe's domain is due to protein redistribution at higher temperatures, which has been shown to contribute positively to fluorescence anisotropy (16, 30). With each fluorescent probe, mesophyll and epidermal protoplasts showed similar responses. The temperature losses on the flow cytometer were excessive, and precluded experimentation to establish Arrhenius plots.

We tested the effect of several compounds expected to modify the plasma membrane (calcium 10 mM, spermine 10 mM, DMSO 2%, KCl 20 mM) or to have physiological action (ethanol 0.4% v/v, naphthyl acetic acid 10 μM). Following a 20 min preincubation, spermine and KCl increased $r_{TMA\text{-}DPH}$; a small increase (as expected) with Ca^{2+} was not significant, and DMSO decreased $r_{TMA\text{-}DPH}$ (Table Ia). Naphthyl acetic acid, DMSO, spermine, and ethanol each increased r_{DPH} significantly (Table Ib).

Table I. *Emission Anisotropy (Mean ± SD·10^4) of (a) TMA-DPH or (b) DPH Assessed Either on the Whole Population (Global) or Specifically on Mesophyll (Chl$^+$) and Epidermal (Chl$^-$) Lupin Protoplasts after 20 min Treatments as Shown*

Lipophilic Probe Treatments[a]	Emission Anisotropy					
	Global		Chl$^+$		Chl$^-$	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
(a) TMA-DPH						
Control	0.162	18	0.143	34	0.165	20
Spermine 10 mM	0.165	16	0.146	25	0.170	13
Ca^{2+} 10 mM	0.163	14	0.143	22	0.167	06
DMSO 2%	0.156	30	0.136	98	0.157	09
KCl 20 mM	0.167	18	0.149	23	0.173	09
(b) DPH						
Control	0.073	01				
Spermine 10 mM	0.077	04				
DMSO 2%	0.090	13				
FA 25 $\mu g \cdot mL^{-1}$	0.065	01				
Ethanol 0.4%	0.078	01				
NAA 10 μM	0.081	17				

[a] FA, farnesylacetone; NAA, naphthyl acetic acid.

Genotypic Differences

When results were pooled from five experiments, an analysis of variance showed that 80 to 90% of the variability was due to the experiment number, namely day-to-day, plant-to-plant, or systematic cytometric differences. (The CV for the mean of 40 independent measurements on the same day was only 1.5% for r_{DPH} and 2.2% for $r_{TMA\text{-}DPH}$, but when grouping experiments from 4 d this rose to 7.4% and 4.3%, respectively.)

For global $r_{TMA\text{-}DPH}$ there was no significant (P = 0.05) genotypic difference in plasma membrane fluidity: average emission anisotropies were 0.197 and 0.198 for resistant and susceptible genotypes respectively. Only the Chl-less protoplasts differed significantly with respect to $r_{TMA\text{-}DPH}$, and here the susceptible genotype had the lesser lipid fluidity. Mesophyll protoplasts did not show a significant difference for $r_{TMA\text{-}DPH}$. However, genotypic differences were observed for global r_{DPH}; the average r_{DPH} values over five experiments were 0.092 and 0.090 for resistant and susceptible genotypes

Table II. *Emission Anisotropy of (a) TMA-DPH or (b) DPH in Protoplasts from Resistant or Susceptible Lupin Plants with or without Watering for 9 d*

Lipophilic Probe	Emission Anisotropy			
	Resistant		Susceptible	
	Watered	Unwatered	Watered	Unwatered
(a) TMA-DPH				
Mean *r*	0.228	0.184	0.222	0.179
Conf int 5%[a]	0.0060	0.0033	0.0055	0.0073
(b) DPH				
Mean *r*	0.081	0.050	0.073	0.036
Conf int 5%	0.0017	0.0020	0.0029	0.0073

[a] The 5% confidence interval.

Table III. *Emission Anisotropy of (a) TMA-DPH or (b) DPH in Mesophyll (Chl$^+$) and Epidermal (Chl$^-$) Protoplasts from Resistant or Susceptible Lupin Cultivars*

Lipophilic Probe	Emission Anisotropy			
	Resistant		Susceptible	
	Chl$^+$	Chl$^-$	Chl$^+$	Chl$^-$
(a) TMA-DPH				
Mean *r*	0.225	0.312	0.222	0.325
SD[a]	0.0034	0.0014	0.0030	0.0013
(b) DPH				
Mean *r*	0.083	0.104	0.077	0.098
SD[a]	0.0012	0.0017	0.0010	0.0014

[a] Calculated from five measures.

respectively. Protoplasts of the susceptible genotype have greater lipid fluidity: *e.g.* in one experiment (Table II) r_{DPH} was 0.081 ± 0.002 for the resistant and 0.073 ± 0.003 for the susceptible genotype. Both protoplast classes showed the same significant difference (Table III).

Taking TMA-DPH as a plasma membrane probe, we therefore cannot affirm that there is any intrinsic difference between normally watered plants of these genotypes with respect to the fluidity of the plasma membrane.

For protoplasts of either genotype, r_{DPH} decreased as temperature increased from 5 to 32°C (Fig. 7). The slope of the regression, temperature *versus* r_{DPH}, was −0.0016 for the resistant genotype and −0.0019 for the susceptible.

Effect of Water Stress

For both genotypes and both protoplast classes, a water stress applied for 8 d or more produced a significant increase in membrane fluidity (Fig. 8). Exceptionally, the fluidity increase assessed with DPH was not significant for Chl-less protoplasts of the resistant genotype. The fall in anisotropy was always greater for mesophyll protoplasts, *e.g.* over 9 d r_{DPH} fell 0.032 for mesophyll and 0.007 for epidermal protoplasts of the susceptible genotype. In short, stress amplified the initial difference observed between protoplast classes.

With DPH, the anisotropy following water stress was significantly lower in the susceptible variety; with TMA-DPH, the change was not genotype-dependent.

CONCLUSION

For the first time, separate anisotropy values have been obtained for mesophyll and epidermal protoplasts. It is the mesophyll cells which apparently have greater lipid fluidity and they are more particularly affected by stress.

Clearly, water stress produces a marked increase in the apparent lipid fluidity, both in the plasma membrane (from TMA-DPH) and in the sum of lipid domains (DPH). The effect of stress upon the plasma membrane, as an increase in membrane fluidity observed at the protoplast level with TMA-DPH, was independent of genotype; but the increase in membrane permeability on leaf discs was genotype-dependent (15). Therefore these two plasma membrane phenomena are probably not directly related.

On the other hand, interpretation of data from DPH on protoplasts is difficult. The value corresponds to an amalgam of the various anisotropies of different pools of DPH in a given cell—in membranes, in organelles, and in lipid droplets. This global value indicates slightly greater lipid fluidity in the susceptible genotype.

The strong decline in r_{DPH} with stress, particularly in the susceptible genotype, is no doubt dominated by changes in intracellular lipids. Insertion of DPH in the nascent triglyceride droplets would lower the anisotropy observed in protoplasts. For these results are consistent with biochemical studies of the same material (15): triglyceride content increased sixfold during 11 d without watering plants of the susceptible genotype. Watered plants of the two genotypes had similar lipid profiles. Total lipids per g dry weight declined upon stress (almost halved for the susceptible genotype), especially the plastidial galactolipids but also the polar lipids of phosphatidylcholine and phosphatidylethanolamine characteristic of the plasma membrane. The unsaturation index only decreased later, upon senescence, as in microsomal membranes from senescing carnation (10). If the observed increase in fluidity over 8 or 9 d cannot be attributed to any decrease in the lipid unsaturation index, it is probably due to weakening of the protein-lipid interactions in membranes, including the plasmalemma (TMA-DPH results). Physiologically, the genotypes differ greatly under stress (15); in the present biophysical analysis, the differences were only slight.

Legge *et al.* (18) reported much higher anisotropy values for DPH on tomato pericarp protoplasts, steady-state aniso-

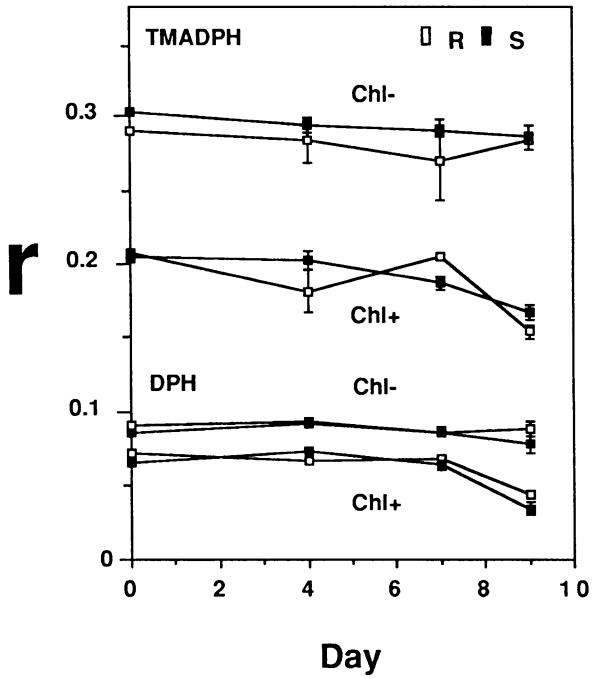

Figure 8. Emission anisotropy of mesophyll (Chl$^+$) or Chl-less (Chl$^-$) subpopulations after TMA-DPH (*upper curves*) or DPH (*lower*) labeling of lupin protoplasts of the resistant (R, *open symbols*) or susceptible (S, *solid*) genotypes. The plants had gone 0, 4, 7, or 9 d without watering. The 95% confidence intervals do not exceed the symbols except were shown. One of six such experiments.

tropy increasing to 0.25 upon fruit ripening. That report interpreted DPH data as if the probe were in the plasma membrane, whereas we have always observed that DPH can penetrate to intracellular lipids. Intermediary values, $r \simeq 0.13$, have been reported (3).

Senescence is commonly accompanied by rigidification of membranes, in contrast to the present effects of water-stress. The anisotropy values observed in the basal state (watered plants or freshly isolated protoplasts) may result from an organisation imposed upon the lipid domain by membrane proteins and the increased fluidity may reflect a greater segregation of, respectively, lipid- and protein-rich domains, without gel phase formation. This idea has not been tested. (The reported treatment with 20 mM KCl presumably removed some extrinsic membrane proteins, but would not have affected the intrinsic proteolipidic structure.) Furthermore, one could quite simply use Nile Red, a metachromatic dye with yellow emission in neutral lipid domains and red emission in polar domains, to follow the formation of lipid droplets in protoplast classes.

This is the first study of plant protoplasts with TMA-DPH, a probe more appropriate than DPH due to its specific localization. The use of protoplasts in such studies (*e.g.* refs. 18 and 31) causes constraints during the measure (*e.g.* light scattering, background fluorescence) which can largely be overcome with flow cytometry. A more general problem is the biophysical interpretation of data from such complex material (reflecting various membranes, lipid droplets, with or without proteins), having neither the simplicity of purified membranes nor the integrity of the initial leaf material. The partitioning of probes between various cellular lipids is not exactly known; time-resolved fluorescence is excluded. But in fact, the protoplast material was surprisingly convenient to handle, and we propose that such an approach will be useful in revealing cell responses *in vitro*.

Flow cytometric studies have been initiated with phytotoxins and polluants on protoplasts (2). Given the current interest in transmembrane signal transduction, development of biophysical methods capable of following rapid changes in the lipid domains of whole cells is warranted. There is an increase in membrane fluidity in B lymphocytes in <1 s following induction with anti-immunoglobulin M; this is protein kinase C dependent and more rapid than the increase in cytoplasmic calcium (22). Rigidification of soybean plasma membranes may be induced by an auxin (14). Unfortunately the amplitude of any shift in 'lipid fluidity' following a physiological response will probably be low (*cf.* Table I). In this context, plant cell flow cytometry (reviewed in ref. 5) offers the advantage that one may distinguish (and sort) different cell types, enabling a study of reactive subpopulations. A responding cell type may be followed despite the inertia of other cells which will dampen any global assessment.

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