

## Irreversible Structural Changes in Thylakoid Membranes at High Temperatures. Detection by Luminescence and EPR

D. MARKOVIĆ<sup>1</sup>, M. JEREMIĆ<sup>2</sup>, Č. RADENOVIĆ<sup>3</sup> and M. SCHARA<sup>4</sup>

1 Faculty of Technology, University of Niš,  
16000 Leskovac, Yugoslavia

2 Department of Physical Chemistry, Faculty of Science, University of Belgrade,  
POB 550, 11001 Belgrade, Yugoslavia

3 Maize Research Institute, Zemun Polje,  
POB 89, 11081 Belgrade, Yugoslavia

4 Institute Josef Stefan,  
61000 Ljubljana, Slovenia

**Abstract.** The character of structural changes in thylakoid membranes caused by temperature variation was investigated. Experiments were performed on maize leaf segments *in vivo* and in a closed temperature cycle 24–50–24°C. Two biophysical methods were used for detection: luminescence and EPR. Arrhenius plots of delayed fluorescence (DF) versus reciprocal temperature revealed two break points,  $T_1$  and  $T_2$ . The MeFASL(10.3) spin probe monitoring properties close to membrane surface detected only  $T_2$  transition temperature. The results were interpreted in terms of a fluidity change which starts in a membrane centre at  $T_1$  and gradually displaces toward the surface at  $T_2$ . The  $T_1$  and  $T_2$  transition temperatures are sensitive to pretreatment history of plants indicating that high temperature and drought – induced membrane alterations are irreversible. Activation energies  $E_1$ ,  $E_2$  and  $E_3$  were determined for temperature regions below  $T_1$  between  $T_1$  and  $T_2$ , and above  $T_2$ , respectively. The  $E_1$  and  $E_3$  activation energies showed greater sensitivity to stress than did  $E_2$ . There are some indications that the DF method could be used to screen temperature sensitive and temperature resistant genotypes.

**Key words:** Thylakoid membrane — Temperature — Leaf — *Zea mays* L. — Luminescence

### Introduction

The temperature as an environmental factor affects practically all physiological and biochemical processes in plants and definitely determines the development

and expansion (Vallejos 1979). A primary consequence of temperature action on biomembranes is change in molecular ordering of membrane lipids (Raison et al. 1979; Kawada and Nosoh 1981). Membrane lipids undergo phase transition from fluid state at high temperatures to an ordered gel state at low temperatures (Fork 1979). Phase transitions are of vital importance for biomembrane functioning because enzymes within membranes form activated complex only in a fluid environment (Wolfe 1979). A good example for the relation between the ordering of membrane lipids and membrane enzymes activity is the report by Raison and Chapman (1976). These authors simultaneously monitored respiratory enzymes activity and membranes fluidity (by incorporating EPR probes inside them) to detect temperatures at which discontinuities at linear Arrhenius plot occur.

Some consequences of temperature action are specific for thylakoid membranes only as they differ from other membrane types by their functions connected with the presence of the photosynthetic apparatus.

Many processes connected with the photosynthetic apparatus are temperature dependent. Temperature affects electron transport; it is temperature independent at lower and temperature dependent at high temperatures (Itoh 1977; Metee et al. 1984). This is different from temperature dependence of energy transfer in photosynthetic apparatus (Sarai and Yomosa 1981; Kee and Ort 1982). Other temperature - induced changes in thylakoid membranes include: loss of membrane proteins (Mollenhauer et al. 1983), quantitative changes in pigment content (Musser et al. 1984), changes in energy transfer between PSII and PSI (Weis 1984) as well as in PG&PC content (Lynch and Thompson Jr. 1984). Similar and even various types of biomembranes, connected with significantly different processes, have been shown to behave similarly under temperature influence. Murata et al. (1975) compared phase transition temperatures in lipid constituents of *Anacystis nidulans* membrane fragments with temperatures at which dramatic changes of some processes connected with the photosynthetic apparatus occur (Chl fluorescence, oxidoreduction of P-700 reaction trap, O<sub>2</sub> evolution, transition between the two photoactive states of chloroplasts. Murata et al. (1979) also renewed methods and procedures for the detection of phase transitions in thylakoid membrane lipids; these included luminescence and EPR.

In our previous paper (Marković et al. 1987) we described DF phenomenon and showed that DF may serve as a "natural probe" to study structural changes in thylakoid membranes at high temperatures. In the present work the influence of high temperatures on the structure of thylakoid membranes was studied using DF. The emphasis is now on the character of structural changes induced in thylakoid membranes by high temperatures; two different maize genotypes were used which are variously rated based on their physiological resistance toward temperature and drought. The DF investigations are supplemented by EPR measurements.

## Experimental techniques

### *Luminescence*

Two maize genotypes which differ in their resistance to temperature and drought were used in these experiments: ZPL 1304, rated as resistant and ZPLB 389, rated as sensitive. We used 8–11 weeks old plants grown in the field from where they were collected in late spring and early summer (May to July). The experimental plants were subjected to different pretreatments before delayed fluorescence (DF) measurements. The control plants (plants *A*) were used immediately after being collected. The second set of experiments was carried out with plants which, after having been collected, were kept in darkness and dry laboratory air at 25°C for 24 hours (plants *B*).

Details of the experimental setup are explained elsewhere (Vučinić 1983). Leaf segments cut under water were placed on a temperature controlled plate inside a Becquerel phosphoroscope where they were adapted to the temperature of the plate and darkness. DF intensity was measured in the dark period of intermittently illuminated leaves using a 150 W halogen lamp. One cycle consisted of 2 ms light and 8 ms dark. DF was registered from ms 3 to 7 of darkness using a cooled photomultiplier.

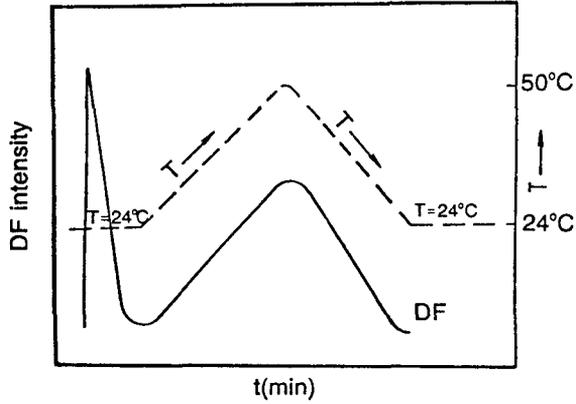
Measurements of the temperature dependence of DP were performed as follows. After the induction period and as soon as a steady state level was reached, the temperature of the leaf was gradually increased (Fig. 1) in the 24–50°C range. Then, from about 50°C, the temperature was gradually decreased to 24°C at the same rate. The rate of the temperature change was 2.2°C/min. The DF intensity and the temperature of the leaf surface were registered simultaneously with a two-channel strip chart recorder.

### *EPR*

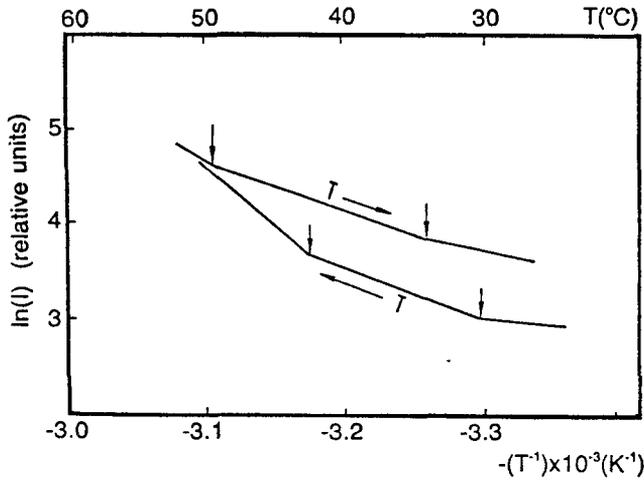
The spin probe used was palmitic acid methylester labeled with doxyl group on the 5<sup>th</sup> atom of the acyl chain (MeFASL (10.3)). The structural formula is shown in Fig. 3. Fresh leaf segments were suspended in phosphate buffer supplemented with  $2 \cdot 10^{-4}$  mol/l of the spin probe. The sample was placed into a glass capillary for EPR measurements. EPR spectra were taken on a Varian E-9-X-band spectrometer in a temperature cycle 24–50–24°C. The temperature was controlled by a Varian variable temperature controller and measured by a Cu-constantan thermocouple.

## Results and Discussion

The temperature dependence of DF intensity, which shall be called thermal DF curve, is shown in Fig. 1. DF emission grows gradually with the temperature increase from 24°C till about 50°C, and it drops with the decreasing temperature,



**Figure 1.** Temperature induced changes of steady state of DF emission (thermal DF curve) in the temperature cycle 24-50-24 °C. The broken line shows temperature variation.



**Figure 2.** Arrhenius plot of thermal DF curve for control plant, genotype ZPL 1304. Transition temperatures are marked by the arrows.

but never reaches the original level. Arrhenius plots of thermal DF curve could be fitted with three straight line segments defining break points, which we call transition temperatures  $T_1$  and  $T_2$ . As it can be seen from Fig. 2, this applies for the positive ( $\overline{T}$ ) as well as the negative temperature gradient ( $\overleftarrow{T}$ ). The straight line segments were fitted to experimental data by computer program using the

**Table 1.** Transition temperatures and activation energies from Arrhenius plots of thermal DF curves for both genotypes and both pretreatments.  $\bar{T}$  - temperature increase,  $\overleftarrow{T}$  - temperature decrease. Figures in the brackets are standard deviations.

| Genotype ZPL 1304 |                                   |               |                     |                   |                   |                   |                         |
|-------------------|-----------------------------------|---------------|---------------------|-------------------|-------------------|-------------------|-------------------------|
|                   | $T_1$<br>(°C)                     | $T_2$<br>(°C) | $T_2 - T_1$<br>(°C) | $E_1$<br>(kJ/mol) | $E_2$<br>(kJ/mol) | $E_3$<br>(kJ/mol) | $E_3 - E_1$<br>(kJ/mol) |
| Plant A           | $\bar{T}$ 29.8<br>(1.3)           | 40.6<br>(1.1) | 10.8                | 3.6<br>(2.0)      | 30.2<br>(6.5)     | 74.2<br>(7.8)     | 70.6                    |
|                   | $\overleftarrow{T}$ 33.4<br>(1.5) | 42.8<br>(2.0) | 9.4                 | 13.2<br>(4.5)     | 29.4<br>(6.5)     | 46.2<br>(6.8)     | 33.0                    |
| Plant B           | $\bar{T}$ 27.8<br>(1.3)           | 33.0<br>(1.3) | 5.2                 | 11.8<br>(4.6)     | 30.0<br>(5.5)     | 50.6<br>(5.6)     | 34.2                    |
|                   | $\overleftarrow{T}$ 31.0<br>(1.3) | 40.7<br>(2.0) | 9.7                 | 27.7<br>(5.5)     | 36.7<br>(3.8)     | 42.5<br>(5.5)     | 14.7                    |
| Genotype ZPLB 389 |                                   |               |                     |                   |                   |                   |                         |
| Plant A           | $\bar{T}$ 30.0<br>(1.3)           | 39.0<br>(1.5) | 9.0                 | 15.7<br>(3.0)     | 34.0<br>(5.0)     | 66.2<br>(7.2)     | 50.5                    |
|                   | $\overleftarrow{T}$ 31.5<br>(1.3) | 38.2<br>(1.2) | 6.7                 | 24.7<br>(2.2)     | 35.2<br>(4.0)     | 37.2<br>(5.0)     | 12.5                    |
| Plant B           | $\bar{T}$ 27.2<br>(1.2)           | 32.2<br>(1.5) | 5.0                 | 17.0<br>(3.0)     | 37.5<br>(3.2)     | 51.7<br>(7.2)     | 41.5                    |
|                   | $\overleftarrow{T}$ 33.5<br>(1.5) | 41.2<br>(2.0) | 7.7                 | 28.0<br>(3.2)     | 40.0<br>(5.0)     | 49.5<br>(4.8)     | 21.5                    |

least squares method. From the slopes of the straight lines, activation energies were calculated. They were noted  $E_1$ ,  $E_2$  and  $E_3$  for the temperature regions below  $T_1$ , between  $T_1$  and  $T_2$  and above  $T_2$ , respectively. Transition temperatures and activation energies are summarized in the Table 1. The values were averaged over 8-10 independent experiments.

The Arrhenius plot of the thermal DF curve shows a clear hysteresis (Fig. 2) suggesting that the structural changes of the thylakoid membrane caused by high temperatures are of irreversible nature. To the same conclusion came Laine-

**Table 2.** The break temperature obtained from linear plots of EPR parameters (+) and (-) indicate positive and negative gradient of EPR parameter slope at given temperature  $\overline{T}$  and  $\overleftarrow{T}$  mean temperature increase and decrease, respectively

|                          | $I_0$        | $I_1$        | $2T_{  }$    |
|--------------------------|--------------|--------------|--------------|
| Genotype ZPL 1304        |              |              |              |
| $\overline{T}$ (°C)      | 37(-)        | 37(-)        | 35(+)        |
| $\overleftarrow{T}$ (°C) | 39(+)        | 39(+)        | 34(+)        |
| Genotype ZPBL 389        |              |              |              |
| $\overline{T}$ (°C)      | 35(-), 47(+) | 35(-), 46(+) | 36(+), 39(-) |
| $\overleftarrow{T}$ (°C) | 40(+)        | -            | 36(+)        |

Boszormenyi et al (1972), who studied DF temperature dependence using isolated spinach chloroplast in a closed temperature cycle

The variation of parameters (Table 2) can be discussed in terms of i) an influence of the pretreatment on the same genotype exposed to increasing temperature (24–50°C), ii) an influence of the pretreatment on the same genotype exposed to decreasing temperature (from 50 to 24°C), iii) a comparison of the parameters of the positive with that of the negative temperature gradient for the same genotype and the same pretreatment, iv) a comparison of parameters of two different genotypes for the same temperature gradient and the same pretreatment

i) Drought lowered  $T_1$  and  $T_2$  compared to the control plants, 27.8 versus 29.8°C, and 33.0 versus 40.6°C for  $T_1$  and  $T_2$ , respectively for genotype ZPL 1304, and 27.2 versus 30.0°C and 32.2 versus 39.0°C for  $T_1$  and  $T_2$ , respectively for genotype ZPBL 389. This indicates that the natural protective mechanism of a plant is affected by drought. It should be noted that  $T_2$  is lowered more significantly than  $T_1$ . For both genotypes, the difference  $T_2 - T_1$  appears to be an indicator for a smaller heterogeneity of those thylakoid membrane lipid mixtures that are influenced by drought. Their values of about 10 for control plants are lowered almost 50% for plants subjected to drought.

The  $E_1$  activation energies are slightly increased in plants subjected to drought. From the kinetic point of view, the increase of activation energies is in accord with the observation (Havaux and Lannoye 1985) that backward reactions in electron transport chain become slower during dehydration, the higher the activation energy the slower the reaction,  $E_2$  activation energies seem to be unaffected by drought. The  $E_2$  values (30–37 kJ/mol) are in a good agreement with the activation energy of 34.7 kJ/mol obtained for DF emission from *Chlorella* (Jursinic and Govindjee 1972). However, for both genotypes,  $E_3$  energies, as well as the  $E_3 - E_1$  difference are smaller for plants subjected to drought. This is opposite of what is found for

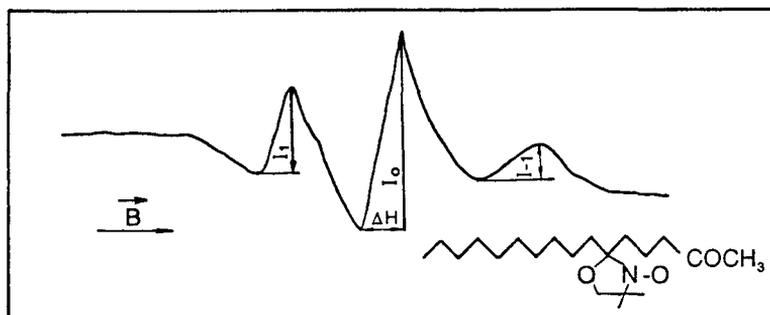
$E_1$  activation energies. The explanation could be significant irreversible change occurring in the high temperature region (40–50°C). This picture which generally reflects a fall in a plant resistance is further supported by the fact that Hill reaction becomes inactivated between 41–46°C (Nolac and Smillie 1977).

ii) For the reversed part of the temperature cycle, plant pretreatment shifts the transition temperatures in opposite directions. However, differences  $T_2 - T_1$  are not affected by pretreatment, remaining the same for control and treated plants contrary to the behavior under the positive temperature gradient. This is another piece of evidence for irreversible changes in fluidity of the thylakoid membrane occurring at higher temperatures. From this point of view, prolonged drought at room temperature and short heat stress over 40°C have the same effect on  $T_2 - T_1$  difference, and hence on membrane fluidity.

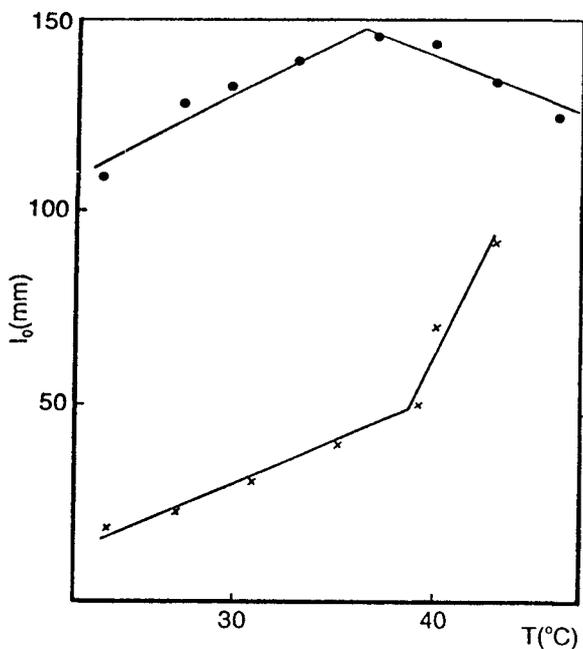
iii) A comparison of data obtained with the same genotype under positive and negative temperature gradient shows unchanged transition temperatures for control plants (within experimental error). The same is true for  $E_2$  activation energies. However,  $E_1$  and  $E_3$  activation energies differ significantly between the two parts of the temperature cycle. These results suggest that temperature variation in the 24–50–24°C cycle affects the rate of reverse reaction in Photosystem II but has no effect on microviscosity variation in the membranes of control plants, as defined by transition temperatures  $T_1$  and  $T_2$ . It will be shown below using EPR experiments that  $T_1$  and  $T_2$  define gradual change of fluidity starting from the membrane centre and spreading towards the surface. Plants subjected to drought stress show significant differences between thermal parameters obtained for positive and those for negative temperature gradient. For example, transition temperature, in particular for the temperature sensitive genotype, differ more than three standard deviations. This shows clearly that drought causes significant change in membrane fluidity.

iv) The parameters in the Table 1 provide some indication for the use of luminescence to distinguish between temperature sensitive and temperature resistant genotypes. Transition temperatures and their differences seem to be more useful for this purpose than activation energies, since the former better reflect the state of microviscosity inside of thylakoid membrane; activation energies mirror reverse reaction kinetics in Photosystem II. However, it must be said that the differences between the two genotypes are mostly within the experimental error. This, however, does not preclude using luminescence for this purpose. It is reasonable to suppose that standardized growth conditions in a strictly controlled environment would decrease individual variation and allow favored differences between the two genotypes to be detected by the luminescence method.

In Fig. 3, a typical EPR spectrum of MeFASL(10,3) and its structural formula are presented. MeFASL(10,3) is a spin probe monitoring the properties of the hydrocarbon core close to the polar head region. It is typically hydrophobic and therefore in water most of its spectra are strongly broadened due to aggregation of



**Figure 3.** EPR spectrum of spin probe MeFASL (10,3) incorporated in leaf tissue. Genotype ZPL 1304.



**Figure 4.** The temperature dependence of  $I_0$ , obtained for increasing (●) and decreasing temperature (×), respectively. Genotype ZPL 1304.

the spin probe molecules. The appearance of the resolved spectra means dilution usually accomplished by the penetration of these molecules into the hydrophobic region of the membrane.

The intensity of EPR spectra, as well as maximal hyperfine splitting  $2T_{II}$  measured from the outermost extrema, which are related to the ordering and dynamics of the hydrocarbon chains of the more ordered regions close to the surface of the hydrocarbon core of the membrane, show temperature dependences. As an example, the temperature dependence of  $I_0$  peak is shown in Fig. 4. It has a break as about 37°C which we defined as transition temperature  $T_2$ . Transition temperatures observed for all EPR parameters lay within 34–40°C (Table 2).

In order to measure fluidity of the thylakoid membrane, we chose MeFASL (10,3) for EPR and chlorophyll of the reaction center of Photosystem II as an internal probe for DF measurements. The results obtained indicate a relationship between changes in microviscosity of the lipid bilayer and the functional state of the thylakoid membrane. The monitoring properties of spin probe MeFASL(10,3) close to the membrane polar region revealed only transition temperature  $T_2$ . The values are in good agreement with those obtained by DF measurements. The fluidity transition observed by DF at lower temperatures ( $T_1$ ) probably take place in the lipid bilayer centre where they can not be detected by MeFASL(10,3). It seems that the temperature, at which distinct fluidity change occurs (the transition temperature) within the hydrocarbon core of the membrane, gradually grows from the centre to the membrane surface. This "transition temperature gradient" resembles the well established fluidity gradient in the membrane. This is also in agreement with activation energies (Table 1) which increase significantly after each transition temperature. This means that less energy is required to increase the mobility for an already disordered and less viscous region than for an ordered and more viscous state.

The Raison-Chapman criterion of temperature resistance (Raison and Chapman 1976) says that abrupt changes in biomembranes fluidity concern only temperature sensitive and not temperature resistant plants. According to this criterion neither of the genotype used in the present work can be rated as temperature resistant; genotype ZPLB 389 may be rated as less resistant according to break temperatures. The high degree of consistency found for DF and EPR data in our experiments definitely supports the conclusion that it was the same processes of membrane fluidity change observed in both experiments.

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