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**Rapid Report**

# Heat injury of barley leaves detected by the chlorophyll fluorescence temperature curve

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The ratio of fluorescence intensity at the first maximum of the chlorophyll fluorescence temperature curve at 52°C (M1) to the intensity at 30°C (F(T30)) is shown to be a sensitive indicator of time-dependent heat stress (49°C) in barley leaves changing in correlation with other fluorescence parameters.

A complex of chlorophyll *a* fluorescence methods is increasingly applied in plant physiology to detect plant stresses [1–3]. High temperatures belong to the most investigated effects. Leaves and isolated chloroplasts show a marked reduction in their photosynthetic activity after exposure to temperatures above 40–45°C. It has been documented that PS II is more susceptible to thermal damage than PS I [4,5]. The temperature induced changes in chlorophyll fluorescence intensity or quantum yield upon linear heating regime in the region from 20 to 60°C or higher temperatures were called the fluorescence temperature curves (FTC) [6,7]. The FTC of leaves and chloroplasts depends on the excitation and emission wavelengths, on the intensity of excitation light, on pH and ionic strength of the medium and on the heating rate [6–10]. The essential points of FTC were lately used for characterization of heat and drought adaptation of plants [11–13]. Two critical temperatures ($T_{C1} \approx 45$–$48$°C, $T_{C2} \approx 53$–$55$°C) triggering successive irreversible changes in the photosynthetic apparatus have been postulated using a special linear-constant heating regime [14, 15].

In the present investigation, the changes in the FTC upon the time-dependent pre-incubation of barley leaf at 49°C are compared to changes in chlorophyll fluorescence induction at room temperature and fluorescence spectra at 77 K.

Seedlings of spring barley (*Hordeum vulgare* L., cv. Zenit) were grown in a cultivation chamber under light intensity of 20 W $m^{-2}$ at the regime – light 16 h/22°C, dark 8 h/18°C. In the growth phase of the second leaf a central segment of the primary leaf blade was excised and immersed in the dark in distilled water of constant temperature 49°C for a time interval from 1 s to 1 h. This temperature is chosen slightly higher than the first critical temperature $T_{C1}$ of irreversible changes [14]. The dark treatment was chosen to avoid the protection of the PS II photochemical activity by light [16].

A laboratory-made spectrofluorimeter with a computer driven system of the linear heating and fluorescence detection was used [14]. Weak actinic light (~ 2 W $m^{-2}$) of 436 nm with 15 nm spectral half-width was used for the chlorophyll fluorescence excitation. The FTC was detected at 685 nm (emission of PS II) with the spectral resolution of 6 nm. A leaf segment was immersed in distilled water and heated at a rate of 4 C°/min. To measure the chlorophyll fluorescence spectrum at 77 K (spectral half-width of emission monochromator was from 3 to 6 nm) in a given moment of the incubation treatment, the segment surface was briefly dried with filter paper and immediately

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Abbreviations: Chl, chlorophyll; F685 (F695, F735), fluorescence bands with maxima at about 685 (695, 735) nm; $F_0$, fluorescence at open reaction centers of Photosystem II; $F_m$, maximal fluorescence at closed reaction centers of Photosystem II; $F_v$, variable fluorescence equals $F_m - F_0$; FTC, chlorophyll fluorescence temperature curve; F(T30), fluorescence intensity at 30°C; M1, fluorescence intensity at first FTC maximum; $Q_A$ ($Q_B$), primary (secondary) plastoquinone electron acceptor of Photosystem II; RC, reaction centre.

(max. 5 s) immersed in a liquid nitrogen bath (77 K) in a glass Dewar cryostat.

The ratio $F_v/F_m$ of chlorophyll fluorescence induction at room temperature was detected by using a Plant Stress Meter manufactured by Biomonitor AB S.C.I. (Umea, Sweden) [17]. Recording time of 1 s and photon flux density of 400 $\mu$mol $m^{-2}$ $s^{-1}$ were used. Before the measurements, the leaves were predarkened for at least 15 min. The fluorescence induction curves were transferred from the memory of the Plant Stress Meter to a personal computer (PC-AT) for further evaluation. A special program for determining the $F_{pl}$ value as the first inflection point of fluorescence induction curve has been developed.

Fig. 1 shows the FTCs of an unheated leaf and of incubated leaves at 49°C for 90 s and 1 h, respectively. The curves are normalized at the M1 value. It can be clearly seen that a relative enhancement of the F(T30) value occurred with the increasing incubation time together with a shift of the maximum to lower temperatures. Only a decreasing trend without any maximum was obtained in the FTC of a leaf incubated for 1 h.

The M1/F(T30) parameter of FTC is defined as a ratio of fluorescence intensity at the first FTC maximum (M1) to the intensity at 30°C (F(T30)). The dependence of this parameter on the incubation time is presented in Fig. 2a. The standard M1/F(T30) value (2.02 ± 0.36) for a non-incubated leaf was obtained as an average of four FTC measurements. This ratio was very sensitive to the heat treatment and gradually decreased with the time of leaf incubation. The first

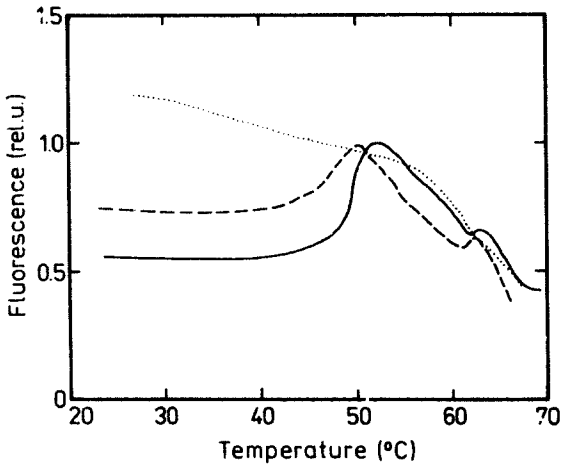


Fig. 1. Fluorescence temperature curves of barley leaf without incubation (solid line) and of leaves incubated for 90 s (dashed line) and 60 min (dotted line) in distilled water at 49°C. The curves were normalized at M1 value. Segment of primary barley leaf (Chl $a$/Chl $b$ = 2.95) was heated in distilled water at a rate of 4°C/min. Low actinic light excitation ( ~ 2 W $m^{-2}$) of 436 nm was applied, emission wavelength was 685 nm.

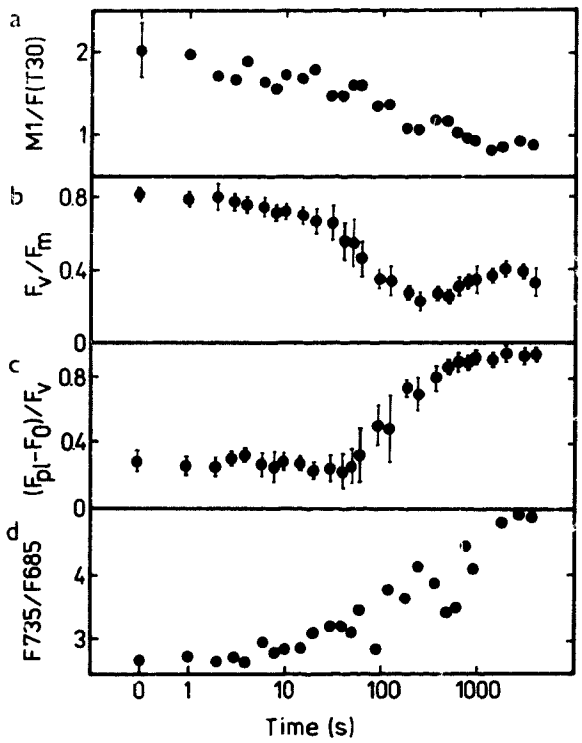


Fig. 2. Time courses of the FTC parameter M1/F(T30) (a), of the $F_v/F_m$ (b) and $(F_{pl} - F_0)/F_v$ (c) obtained from the chlorophyll fluorescence induction curve and of the uncorrected fluorescence band ratio F735/F685 (d) at 77 K during leaf incubation treatment at 49°C from 1 s to 60 min. The time axis representing heat treatment of the leaf are given in logarithmic scale. The M1/F(T30) value of non-incubated leaf is taken as average of 4 FTCs with standard deviation (S.D.). In the cases of the chlorophyll fluorescence induction parameters (b, c), each point represents the average of four values with S.D.

maximum was present in the FTCs up to incubation of 22.5 min and its position shifted from 52°C for the standard leaf to 47°C (data not shown). The positions of the first maximum for the time of incubation greater than 22.5 min were determined approximately with the help of linear regression method.

The meaning of the chlorophyll fluorescence induction curve was described in detail in [1,18]. The points I and D are usually not discernible and may be designated as a plateau ($F_{pl}$) as was suggested by Forbush and Kok [19] and quite frequently used [20–22].

The dependence of the $F_v/F_m$ value on the time of incubation at 49°C is shown in Fig. 2b. The $F_v/F_m$ ratio decreased from the beginning of the incubation treatment. The lowest value ($F_v/F_m = 0.226$) was reached after 6 min of incubation. For an unknown reason, the following $F_v/F_m$ ratios increased to the value of 0.413 after 30 min incubation and then decreased mildly to the final value 0.337.

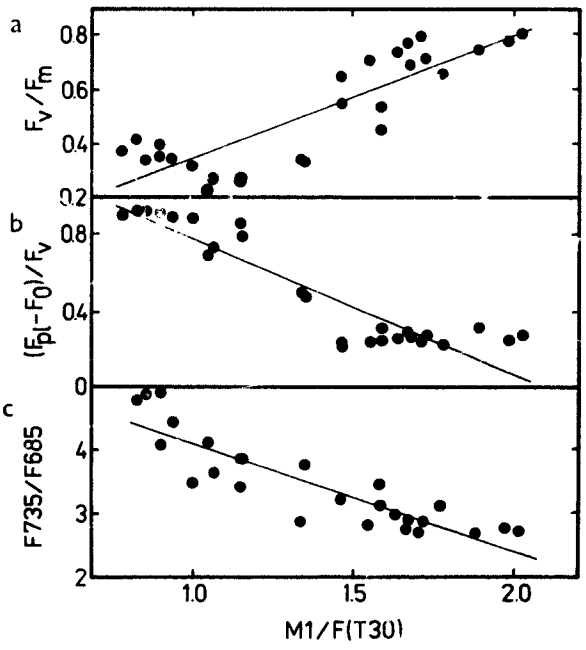


Fig. 3. (a) Correlation between M1/F(T30) and $F_v/F_m$ values. Correlation coefficient $r = 0.857$. (b) Correlation between M1/F(T30) and $(F_{pl} - F_0)/F_v$ values. Correlation coefficient $r = -0.923$. (c) Correlation between M1/F(T30) and F735/F685 values. Correlation coefficient $r = -0.881$.

Another parameter obtained from the fluorescence induction, the $(F_{pl} - F_0)/F_v$ value, was investigated during the leaf incubation treatment (Fig. 2c). The $F_{pl}$ value was detected according to Melis [23]. The $(F_{pl} - F_0)/F_v$ ratio was suggested to represent a relative amount of the PS II $O_B$-nonreducing centres in the total number of PS II centres [21]. The measured $(F_{pl} - F_0)/F_v$ ratio remained relatively constant up to the incubation time of 50 s (Fig. 2c), sharply increased in the incubation times from 1 min to 30 min and finally reached a constant maximal level ($\sim 0.92$).

The dependence of the F735/F685 band ratio of the chlorophyll fluorescence spectra at 77 K on the leaf incubation time at 49°C is shown in Fig. 2d. The F685 and F695 bands are usually ascribed to the inner antennae of PS II whereas the F735 band belongs to LHC I [1,18]. The increase of the F735/F685 value upon the incubation with no change in the band position was observed. Only a mild decrease of F685/F695 was detected (data not shown).

To analyze a suitability of the FTC for a detection of heat injury, the correlations of M1/F(T30) value with the other detected fluorescence parameters were evaluated. The results of these analyses are expressed in Fig. 3a for $F_v/F_m$ (correlation coefficient $r = 0.857$), in Fig. 3b for $(F_{pl}-F_0)/F_v$ ($r = -0.923$) and in Fig. 3c for F735/F685 ($r = -0.881$). The lowest absolute value of the correlation coefficient between M1/F(T30) and $F_v/F_m$ is probably caused by an unexpected increase of the $F_v/F_m$ parameters at incubation times above 6 min (see Fig. 2b).

The FTC has been already measured both under $F_0$ and $F_m$ conditions [7]. Under $F_0$ conditions, all RC II are open and $Q_A$ is in its oxidized state. The main fluorescence rise in the FTC course was suggested to reflect a blocking of PS II reaction centers [7, 10]. A shape of FTC similar to that of $F_0$ conditions was obtained by us for an unaffected leaf at steady state conditions (light excitation of 2 W $m^{-2}$) (see Fig. 1).

At the $F_m$ level, all the RCs II are closed by high light or DCMU presence. At this situation, only a decreasing curve was obtained [7,24]. This type of FTC corresponds with the FTC of the leaf incubated for 1 h (Fig. 1).

The inner antenna of PS II is usually thought to emit the 685 nm fluorescence [1]. The quantum yield of chlorophyll fluorescence is increased when the process of photosynthetic electron transport through PS II is reduced due to the heat stress. Based on this assumption, the heat exposure of leaf at 49°C inactivated a part of PS II centres causing a relative enhancement of F(T30) value and consequently a decrease of the M1/F(T30) value. (Figs. 1, 2a). From this, the M1/F(T30) value may be suggested as a factor giving information about the activity of Photosystem II.

The $F_v/F_m$ ratio reflecting the potential photochemical efficiency of PS II [17,25] has become a widely used measure of plant stress as it is rather constant among healthy leaves of many different species and decreases upon specific stress effects [25].

In our case, the use of $F_v/F_m$ was suitable for the heat injury detection in the leaves incubated within first 5 min (see Fig. 2b). However, an unexpected increase of the $F_v/F_m$ ratio at longer incubation times does not apparently mean a partial recovery of the situation in PS II and remains to be explained.

On the other hand, the behavior of $(F_{pl} - F_0)/F_v$ value (Fig. 2c) displayed a continuously increasing accumulation of the PS II $Q_B$-nonreducing centres upon incubation [23]. For this reason, the application of $(F_{pl} - F_0)/F_v$ seems to be more acceptable than the $F_v/F_m$ value for the determination of the long-termed heat stress in barley leaves [26].

The increase of F735/F685 at 49°C (Fig. 2d) should document the functional separation of LHC II complexes from the PS II systems (Fig. 2d) and/or a preferential delivery of excitations to PS I. This notion is in agreement with the suggestion of Schreiber and Armond [7] and Berry and Björkman [4]. This effect seems to proceed in parallel way to the FTC changes.

Based on the favourable results of correlation between the M1/F(T30) and other fluorescence parameters (Fig. 3), the M1/F(T30) ratio of the FTC may be

suggested as a sensitive indicator of the heat stress injury in plant tissues.

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