Protection of Photosystem II by Light in Heat-Stressed Pea Leaves

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Modulated 685-nm chlorophyll fluorescence was measured under steady-state conditions in pea leaves adapted to moderate actinic light. When the measurements were performed after a short exposure to heat (42 °C) in darkness, the maximal \( F_m \) and steady-state \( F_s \) fluorescence levels were dramatically and irreversibly quenched whereas the basic fluorescence \( F_0 \) remained unchanged. Concomitantly, photosynthetic \( O_2 \) evolution was irreversibly inhibited. Analysis of the fluorescence data suggested that the heat treatment affected primarily the PS II reaction center, with the main effect being presumably an increased thermal dissipation of the excitation energy transferred to the reaction centers. In contrast, when heat stress was imposed in the presence of light, the loss of variable fluorescence \( (F_m - F_s) \) was much less marked and was fully reversible. In addition, no inhibition of \textit{in vivo} \( O_2 \) evolution was observed when the samples heated in the light were recooled at 25 °C. The results indicate that light acted as an efficient protector of PS II against heat injury.

Introduction

In general, when plant leaves are exposed to environmental constraints such as drought [1, 2], chilling [3, 4] or freezing [4, 5], damage to photosynthesis is strongly enhanced in the presence of light. For example, Taylor and Rowley [6] showed that exposing chilling-sensitive plants to a temperature of 10 °C and an irradiance of 170 W m\(^{-2}\) for a few days caused a drastic inhibition of photosynthesis, with the degree of inhibition being directly proportional to the level of irradiance. This phenomenon is attributed to the reduced rate of photosynthesis which leads to a situation where absorption of light energy by the pigment antennae is in excess to what can be dissipated by photosynthesis, creating conditions for photoinhibition to occur [2, 4, 7]. Thus, when placed in stressful environments, plants are sensitized to photoinhibition stress. Although there is still controversy as to the exact molecular mechanism leading to photoinhibition, the primary site of action seems to be located in PS II [7]. Two possible mechanisms underlying photoinhibitory damage have emerged, one based on the central role played by damage and repair of \( D_1 \), the 32 kDa herbicide-binding protein [8] and the other based on damage and repair of the photochemical reaction center itself [9]. Decreased rates of repair could be in part responsible for increased photoinhibition under environmental stress conditions such as low temperatures [4]. Typically, photoinhibition is accompanied by severe modifications of the characteristics of the PS II-chlorophyll fluorescence emission which are often used to diagnose a photoinhibitory stress [10, 11].

This paper demonstrates that, rather than causing injury, light can also give high protection during exposure to stress, as reported in a few previous reports [12, 13]. Using a modulated chlorophyll fluorescence technique combined with separate measurements of \textit{in vivo} \( O_2 \) evolution, the PS II function was probed in pea leaves subjected to heat treatments in the light or in the dark. It was shown that heat stress in the dark caused irreversible alteration of PS II, with the main effect being presumably located in the reaction center. In contrast, light at high temperature had protecting effect since the heat-induced changes observed in the characteristics of \textit{in vivo} PS II-chlorophyll fluorescence and \( O_2 \) evolution after heat stress in the dark were greatly reduced or even absent in the light.
Materials and Methods

The experiments were performed on mature leaves of pea (Pisum sativum L.). Plants were grown in a glasshouse under natural sunlight (average intensity at midday, around 60 W m\(^{-2}\)) and controlled temperature (25 °C) and air humidity (60%) conditions.

In vivo PS II-chlorophyll fluorescence emission from attached leaves was measured using an Hansatech modulated fluorescence instrument, as previously described in detail [14]. In brief, the initial level \(F_0\) of modulated chlorophyll fluorescence was excited by a low-intensity 585-nm light (\(<0.025\) W m\(^{-2}\)) pulsed at a frequency of 870 Hz. Fluorescence was detected at 685 nm with a photodiode. The maximal fluorescence level \(F_m\) was determined by applying a 1-s pulse of intense light (photosynthetically active radiation, 320–640 nm, 500 W m\(^{-2}\)). Most of the chlorophyll fluorescence measurements were made with leaves photosynthesizing under steady-state conditions after prolonged adaptation to an actinic light (320 to 640 nm) of moderate intensity (usually, 45 W m\(^{-2}\)). All light intensities were measured with a YSI-Kettering 65A radiometer. The chlorophyll fluorescence signals were analyzed using a theoretical model, presented in detail elsewhere [14], which is based on the analysis of the energy fluxes in the photochemical apparatus of photosynthesis as in [15, 16]. Leaf temperature was adjusted by circulation of water (from a Colora WK 3D thermostated water bath) through a block of plexiglas which was placed in firm contact with the leaf sample. Temperature of control leaves was maintained at 25 °C whereas that of stressed leaves was 42 °C (unless specified otherwise).

Oxygen exchanges by leaf discs of 8-mm diameter were measured with a Yellow Spring Clark-type oxygen electrode, as described elsewhere [17]. Photosynthetic \(O_2\) production was monitored in red light, the intensity of which was adjusted using neutral density filters.

Results

Fig. 1A shows modulated 685-nm chlorophyll fluorescence signals measured at 25 °C (trace a) or 42 °C (trace b) in dark-adapted pea leaves. The transitory closure of all the PS II traps by a short pulse of intense, photosynthetically saturating, light resulted in a rapid rise of the modulated fluorescence intensity from the initial level (\(F_0\)), elicited by a dim modulated yellow light, to the maximal level (\(F_m\)). This flash-induced fluorescence rise is caused by the transfer of the excitation energy from the closed PS II reaction centers back to the chlorophyll antennae, increasing the energy flux through the PS II dissipative pathways (including fluorescence). Under the assumption that the probability for this energy recycling (\(p_{b2}\)) is close to 1, the ratio \((F_m - F_0)/F_m\) can be used as a direct estimate of the maximal quantum yield \(\Phi_{\text{open}}\) for photochemistry in PS II with all reaction centers in the open configuration [14, 18, 19]. In control pea leaves at 25 °C, this ratio was close to 0.8. A short exposure (15 min) of the dark-adapted leaves to a high temperature of 42 °C caused some changes in the fluorescence signals (measured at 42 °C): the \(F_0\) level was slightly increased (+10%) whereas the height of the \(F_m\) peaks was reduced by around 28%. As a consequence, heated leaves had a slightly decreased \((F_m - F_0)/F_m\) ratio of around 0.75. The inset of Fig. 1A shows that \((F_m - F_0)/F_m\) decreased linearly with time of exposure to heat stress, although the extent of this decrease was rather limited.

When the leaves heated in the dark were adapted (at 42 °C) to an actinic light of moderate intensity (45 W m\(^{-2}\)), the maximal (\(F_m\)) and steady-state (\(F_s\)) levels of modulated chlorophyll fluorescence were dramatically quenched (Fig. 1B, trace c) as compared to the \(F_m\) and \(F_s\) levels measured in leaves kept at 25 °C (trace b in Fig. 1B). In contrast, there was no significative difference between the \(F_0\) levels measured at the two temperatures. When the actinic light was switched off, there was a fast and marked rise in the fluorescence yield of the heat stressed leaves. This overshoot appeared to be a complex phenomenon; it was suppressed by far-red light (data not shown) indicating that it does not reflect changes in the fluorescence emission of open PS II reaction centers (\(F_o\)) but probably the reduction of QA by a built-up internal electron donor pool. This fluorescence overshoot will be examined in detail in further work. Table I gives the relative values of the different fluorescence levels calculated from a large number of leaves, indicating that the above changes were statistically sig-
A consequence of the differential effects of heat stress on the $F_0$, $F_s$, and $F_m$ levels was a spectacular decrease in the amplitude of both the variable fluorescence $F_v = F_0$ (around $-70\%$) and the maximal variable fluorescence $F_m - F_0$ ($-80\%$) in the steady state. Thus, when pea leaves, previously heated in the dark, were exposed to light, a considerable part of the variable part of the chlorophyll fluorescence emission from PS II was lost.

Trace b in Fig. 1 B is the in vivo fluorescence signal emitted by pea leaves which were treated at $42 \degree C$ in the presence of light ($45 \text{ W m}^{-2}$). It can be seen that the combination of heat and light during pretreatment had much less effects on the characteristics of the modulated fluorescence signals (at steady state) than heat stress in the dark. As compared to control leaves (trace a in Fig. 1 B), there was a $25\%$-reduction of the $F_m$ amplitude and almost no change in the $F_s$ level ($-6\%$) (cf. Table I).

In Fig. 2 are shown the time courses of the changes in $F_0$, $F_s$, and $F_m$ during heat stress in the absence (Fig. 2 A) and in the presence of light (Fig. 2 B). During heat stress in the dark, both $F_v$ and $F_m$ were rapidly quenched (within around $10\min$) to a very low level close to the basic fluorescence level (i.e. variable chlorophyll fluorescence disappeared almost completely). When leaves were irradiated during the heat treatment, the $F_m$ changes were much less marked, following in biphasic kinetics: during the first $10\min$, the maximal level $F_m$ was decreased by around $50\%$ and, after this, it slowly increased, suggesting a progressive adaptation of the photosynthetic apparatus to high temperature.

A small, but significant, decrease in $F_v$ was also observed by applying a short pulse of intense light (\(\uparrow\)). Trace a: leaves at $25 \degree C$; trace b: signals obtained at $42 \degree C$ in leaves kept at this high temperature for $15\min$ in the dark. The inset shows the changes in the $(F_m - F_v) / F_m$ ratio measured in leaves incubated in the dark at $42 \degree C$ for various times.

B: Leaves were adapted to an actinic light of $45 \text{ W m}^{-2}$ until the modulated chlorophyll fluorescence intensity reached a steady-state level $F_v$. The maximal fluorescence level $(F_m)$ was induced by applying a pulse of intense light (\(\uparrow\)) and the initial level $(F_0)$ was obtained by simultaneously switching off the actinic light and applying a short pulse of far-red light (\(\downarrow\)). Trace a: leaves at $25 \degree C$; traces b and c were measured at $42 \degree C$ in leaves pretreated at this temperature for $15\min$ in the light ($45 \text{ W m}^{-2}$, trace b) or in the dark (trace c).
Table I. Characteristics of modulated 685-nm chlorophyll fluorescence signals monitored at 25 °C in control leaves and at 42 °C in leaves heated at this high temperature for 15 min in the dark or in the light. Measurements were performed under steady-state conditions in leaves adapted to a light of 45 W m⁻². The parameter 1 - V = q_p
is the fluorescence ratio (F_m-F_s)/(F_m-F_o). Data are mean values (arbitrary units) calculated from 7 to 12 separate measurements ± standard deviations. In brackets: values expressed in % of those measured at 25 °C.

<table>
<thead>
<tr>
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<th>25 °C (n = 7)</th>
<th>Treated at 42°C in the dark (n = 12)</th>
<th>Treated at 42°C in the light (n = 7)</th>
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<tr>
<td>F_o</td>
<td>13.9 ± 0.8</td>
<td>13.5 ± 2.8 (97%)</td>
<td>14.3 ± 1.9 (103%)</td>
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<tr>
<td>F_s</td>
<td>19.6 ± 1.6</td>
<td>15.2 ± 2.6 (77%)</td>
<td>18.5 ± 1.6 (94%)</td>
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<tr>
<td>F_m</td>
<td>55.0 ± 6.6</td>
<td>21.3 ± 4.3 (39%)</td>
<td>41.1 ± 7.9 (75%)</td>
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<tr>
<td>F_m-F_o</td>
<td>41.1</td>
<td>7.8 (19%)</td>
<td>26.8 (65%)</td>
</tr>
<tr>
<td>F_s-F_o</td>
<td>5.7</td>
<td>1.7 (29%)</td>
<td>4.2 (74%)</td>
</tr>
<tr>
<td>1-V</td>
<td>0.86</td>
<td>0.78 (91%)</td>
<td>0.84 (98%)</td>
</tr>
</tbody>
</table>

The relative variable fluorescence V = (F_s-F_o)/(F_m-F_o)*, which is non-linearly related to the fraction of closed PS II reaction centers [14, 20].

* V is equal to 1-q_p, where q_p is the so-called photochemical fluorescence quenching coefficient [21].

Figure 2. Effects of a heat treatment (42 °C) in the dark (A) or in the presence of a light of 45 W m⁻² (B) on the initial (F_o, ◦), steady-state (F_s, △) and maximal (F_m, ◦) levels of PS II-chlorophyll fluorescence measured in light-adapted pea leaves. Measurements were performed at 42 °C under steady-state conditions in leaves adapted to a light of 45 W m⁻², as in Fig. 1B. Inset: changes in the maximal variable fluorescence (F_m-F_o) in the steady state during the heat-treatments (◊, in the light (L); ◦, in the dark (D)).

was not much affected by the various stress treatments: from the data of Fig. 1, 1-V was calculated to be close to 0.85 in control leaves and in leaves heated in the light and around 0.8 in leaves heated in the dark (Table I). This relative stability of V suggests that the marked chlorophyll fluorescence changes reported above were not accompanied by appreciable changes in the redox state of PS II.

As shown in Fig. 3, the F_m quenching observed after heat stress in the light was almost completely reversed upon transfer of the leaves to a temperature of 25 °C. Recovery was rapid, with a measured half-time t½ in the order of 5 min. In contrast, the marked reduction of the variable fluorescence...
This fast reversal of the $F_{m}$-quenching suggests the involvement of the rapidly relaxing “energy”-dependent quenching of chlorophyll fluorescence related to the proton gradient.
Photoprotection was also observed at high light intensities (>45 W m⁻²) although the \((F_m - F_o)\) value was decreased as compared to that measured in leaves exposed to lower intensities (10–45 W m⁻²) during heat stress. This effect could possibly result from the dynamic photoregulation of PS II deactivation which strongly favors non-photochemical deactivation over photochemistry in high-lights [11, 22–24]. This “reversible photoinhibition” is known to affect preferentially the height of the \(F_m\) peaks, to occur with a relatively long time constant (>30 min) [11, 23, 24] and to be markedly enhanced at high temperature [24]. This adjustment of the energy dissipation pathways in PS II probably interfered with the heat-induced loss of variable PS II-fluorescence examined here. However, the (partial) reversibility of the fluorescence changes monitored after transfer of the leaves at room temperature (25 °C) for 20 min indicates that high-lights had also protecting effects against heat-induced alteration of PS II. The relatively long relaxation time of “reversible photoinhibition” could be responsible for the incomplete recovery after heat stress at the highest light intensities tested (ca. 150 W m⁻²).

Fig. 6 shows the temperature dependence of the chlorophyll fluorescence changes reported above. When the leaves were exposed to the various temperatures (from 22 to 48 °C) in the dark, maximal variable fluorescence \(F_m - F_o\) started to decrease already at a temperature as “low” as 30 °C whereas, in the presence of light, no significant change in \(F_m\) was observed at temperatures lower than around 40 °C. At leaf temperatures higher than 47 °C, there was no difference between leaves heated in the dark and those heated in the light. The insert of Fig. 6 shows that the fluorescence parameter \(1-V\) remained largely unchanged during the various heat treatments except, however, for extreme temperature conditions (>44 °C).

**Discussion**

This study shows that the response of PS II to high temperature stress *in vivo* strongly depends on the light environment. The heat-induced changes in the characteristics of PS II-chlorophyll fluorescence (measured in light-adapted leaves photosynthesizing under steady-state conditions) were indeed much more marked in leaves which were kept in darkness during heating than in leaves illuminated during the treatment (Fig. 1 B). After a short exposure at 42 °C in the dark, illumination of the leaves led to a drastic and irreversible quenching of *in vivo* chlorophyll fluorescence (above \(F_o\)) whereas, in leaves heated in the presence of light, the extent of this fluorescence quenching was limited and the fluorescence changes were fully reversible (Fig. 3). It was also observed that heat stress under dark conditions caused a pronounced and irreversible decrease in both the quantum yield and the light-saturated rate of \(O_2\) evolution whereas these two parameters remained unchanged after heat stress in the light (Fig. 4). Consequently, light can be considered as a protective factor limiting the alteration of PS II brought about by heat. This photoprotection was observed even with light of very low intensity (Fig. 5).

The effects of heat stress in the dark on PS II seemed to be rather complex. When the PS II functioning was tested in dark-adapted samples by examining flash-induced changes in the chlorophyll fluorescence yield (Fig. 1 A), there was only a small decrease in the \(((F_m - F_o)/F_m)_{dark}\) ratio, suggesting that the maximal photochemical efficiency of PS II was largely preserved. In fact, the most striking effect of heat stress in the dark was observed when leaves were readapted to light after the pretreatment. The response of PS II to continuous actinic
light was indeed strongly perturbed, with the main effect being a drastic quenching of chlorophyll fluorescence above the $F_0$ level. The relative stability of the $F_0$ fluorescence suggests that the main heat effect lies in the reaction center and not in the chlorophyll antennae since otherwise the amplitudes of $F_0$ and $F_m - F_0$ would have been simultaneously modified. As previously demonstrated [14], the theoretical expressions of $F_0$ and $F_m$ in terms of rate constants are:

$$F_0 = J_2 k_{2F}/(k_{2b} + k_{N'})$$

$$F_m = J_2 k_{2F}/(k_{2b} + k_{N'} - k_{2b} p_{b2})$$

where $J_2$ is the light absorption flux in PS II and $k_{2F}$, $k_{2b}$ and $k_{N'}$ are, respectively, the rate constant for fluorescence, the rate constant for photochemistry and the sum of the rate constants of all the nonphotochemical energy dissipation processes in PS II excluding PS II – PS I energy transfer (i.e. heat losses, PS II – PS I energy transfer by “spillover” and fluorescence). Consequently, the observed constancy of $F_0$ (Fig. 2) indicates that the terms $J_2 k_{2F}$ and $k_{2b} + k_{N'}$ were unaffected by the stress treatments used in this study. Then, if we exclude the improbable case where changes in $k_{2b}$ were exactly compensated by inverse changes in $k_{N'}$, the values of the rate constants of deexcitation of the PS II antenna chlorophylls were stable. This latter idea was also confirmed by the finding that the relative variable fluorescence $V$ did not change significantly in the heated leaves (see Fig. 1 B and 6). The parameter $V$ is a complex function of all the rate constants of exciton-consuming reactions in PS II (as well as in PS I) [14] and hence it can be expected that changes in the rate constant of photochemistry ($k_{2b}$) and/or the nonphotochemical rate constant ($k_{N'}$) would have modified the amplitude of $V$. Thus, comparison of Eqns. (1) and (2) indicated that the spectacular decrease in the height of the $F_m$ peaks associated with unchanged $F_0$ levels resulted from a reduction of the probability $p_{b2}$. In the limit (for example, for prolonged exposure (>30 min) to 42 °C in the dark, see Fig. 2 A), $F_0 \equiv F_m$ and $p_{b2} \equiv 0$. In other words, open and closed PS II centers had the same apparent behavior as regards their fluorescence emission. This latter characteristic makes PS II resembling PS I, which does not generate a fluorescence of variable yield, and indicates that, after heat stress in the dark, in vivo 685-nm chlorophyll fluorescence was no longer able to provide useful information on the photochemical activity of PS II in the steady state.

By definition, the probability $p_{b2}$ is equal to the ratio between the energy flux $E_{b2}$ from the closed PS II reaction centers (denoted by the subscript b) to the chlorophyll pool (subscript 2) and the sum of all the energy fluxes leaving the PS II traps (i.e. $E_{b2} + E_{b2}$) where $E_{b2}$ is the energy flux by radiative ($E_{b2}$) and nonradiative ($E_{n2}$) energy dissipation, with $E_{b2} \gg E_{b2}$. Thus, in closed PS II centers,

$$p_{b2} = E_{b2}/(E_{b2} + E_{b2})$$

As $E_{b1} = k_{b1} P_b *$ where $P_b *$ is the concentration of excited reaction center pigment $P_{880}$. Eqn. (3) can be expressed in terms of rate constants as follows:

$$p_{b2} = k_{b2}/(k_{b2} + k_{b2})$$

It is usually assumed (see, for example, refs. [14, 18, 19]) that the rate constant $k_{b2}$ for the energy recycling between the closed PS II traps and the chlorophyll antennae is much higher than that of thermal energy dissipation $k_{nh}$, so that $p_{b2}$ is high ($p_{b2} \equiv 1$). On the contrary, in open reaction centers, most of the transferred energy is believed to be used for photochemistry and, consequently, $p_{b2}$ is assumed to be very low ($p_{b2} \equiv 0$). Our results indicated however that the assumption $p_{b2} = 1$ in closed centers is not valid for heat stressed leaves in which the marked lowering of the $F_m$ peaks pointed to a reduction of the $p_{b2}$ value and to an increased radiative and/or nonradiative deactivation ($k_{b2}$) of the reaction center (cf. Eqn. (4)) as the rate of energy transfer from a donor (b) to an acceptor (chlorophyll pool 2) is a function of the distance separating them [25], major changes in $k_{b2}$ can be excluded since otherwise the rate constant of the inverse transfer ($k_{b2}$) would have been affected too.

As the heat-induced quenching of $F_m$ manifested mainly when the leaf samples were illuminated with continuous background light (Fig. 1), it is tempting to explain the above phenomena on the basis of the fluorescence quenching associated with establishing a “high energy state” in the thylakoids [26–28]. This type of fluorescence quenching (related to the light-induced proton gradient) has also been assumed to reflect a change in the rate constant $k_{b1}$ of nonradiative (thermal) deexcitation of
the PS II reaction center [26–29]. Ultrastructural alterations of the thylakoid membrane [27, 29] or synthesis of the carotenoid zeaxanthin [30] are usually evoked to explain the modulation of the thermal decay of $P_{b}^{*}$ by the pH gradient.

Let us consider the initial photochemical events in PS II. The simplest reaction scheme can be written as:

(a) $P_{b}$ Pheo $\rightarrow P_{b}^{*}$ Pheo
(b) $P_{b}^{*}$ Pheo $\rightarrow P_{b}^{+}$ Pheo$^{-}$
(c) $P_{b}^{+}$ Pheo$^{-}$ + $Q_{A}$ $\rightarrow P_{b}^{+}$ Pheo + $Q_{A}^{+}$
(d) $P_{b}^{+}$ Pheo + D $\rightarrow P_{b}$ Pheo + D$^{+}$

where Pheo is pheophytin, $Q_{A}$ is the primary stable (quinone) electron acceptor of PS II and D is the reduced donor to $P_{b}^{+}$. In closed centers, reaction (c) cannot occur and part of the excitation energy is transferred back to the antenna chlorophylls ($E_{b}$). However, the following, heat producing, reduction-oxidation reaction can also occur:

(c') $P_{b}^{+}$ Pheo$^{-}$ $\rightarrow P_{b}$ Pheo + heat.

This reaction, as any oxido-reduction reaction, is dependent on the proton concentration [31]. Major structural changes are known to take place in heat-treated thylakoid membranes [32]. It is then conceivable that heat stress (in the dark) brought about conformational changes in the thylakoid membrane increasing the accessibility of protons to the complex $P_{b}^{+}$ Pheo$^{-}$ and hence favoring reaction (c'). In this context, it is interesting to note that 9-aminoacridine measurements have shown a stimulation of the light-induced proton uptake into the thylakoid space under heat conditions [33]. If reaction (c') becomes predominant, excitation energy transferred to the PS II centers is chiefly dissipated as heat (i.e. $E_{b}$ rises) and closed centers behave as open centers as regards chlorophyll fluorescence emission (i.e. $F_{m}$ is close to $F_{0}$), although no stable photochemical work is performed. In contrast, when heat stress was imposed in the presence of light, the reaction centers passed in a cyclic way through the $P_{b}^{+}$ Pheo$^{-}$ state. A closer proximity of the reaction center molecule and the Pheophytin molecule in that ionized state could possibly stabilize the complex and hinder heat-induced changes in the reaction center conformation, thus preventing (or limiting) reaction (c'). It is clear that this hypothesized mechanism of fluorescence quenching which is presented here as a working hypothesis needs to be substantiated by further studies. Irrespective of the exact causes of the heat-induced loss of variable PS II-fluorescence, our results demonstrate the importance of light as an environmental factor avoiding irreversible damage of PS II in leaves exposed to high temperature. This observed photoprotection could have important ecological implications since it is usually in the daytime, when solar irradiation is relatively high, that plants encounter high temperature in the field.