Is the *in vivo* Photosystem I Function Resistant to Photoinhibition? An Answer from Photoacoustic and Far-Red Absorbance Measurements in Intact Leaves

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Preillumination of intact pea leaves with a strong blue-green light of 400 W m^{-2} markedly inhibited both photoacoustically monitored O_2-evolution activity and PS II photochemistry as estimated from chlorophyll fluorescence measurements. The aim of the present work was to examine, with the help of the photoacoustic technique, whether this high-light treatment deteriorated the *in vivo* PS I function too. High-frequency photoacoustic measurements indicated that photochemical conversion of far-red light energy in PS I was preserved (and even transiently stimulated) whereas photochemical energy storage monitored in light exciting both PS I and PS II was markedly diminished. Low-frequency photoacoustic measurements of the Emerson enhancement showed a spectacular change in the PS II/PS I activity balance in favor of PS I. It was also observed that the linear portion of the saturation curve of the far-red light effect in the Emerson enhancement was not changed by the light treatment. Those results lead to the conclusion that, in contrast to PS II, the *in vivo* PS I photofunctioning was resistant to strong light stress, thus confirming previous suggestions derived from *in vitro* studies. Estimation of the redox state of the PS I reaction center by leaf absorbance measurements at *ca.* 820 nm suggested that, under steady illumination, a considerably larger fraction of PS I centers were in the closed state in high-light pretreated leaves as compared to control leaves, presumably allowing passive adjustment of the macroscopic quantum yield of PS I photochemistry to the strongly reduced photochemical efficiency of photoinhibited PS II.

Introduction

Exposure of plant leaves to an incident light fluence rate exceeding that required for the saturation of the photosynthetic electron transport causes a gradual decline in photosynthetic activity – a phenomenon known as photoinhibition [1, 2]. Although the exact molecular bases of this process are still debated, it is believed that the primary site of action of intense light is located in photosystem (PS) II [2]. Two possible mechanisms underlying photoinhibition damage have emerged, one based on the central role played by damage and repair of D_1, the 32 kDa herbicide-binding protein [3] and the other based on damage and repair of components of the primary charge separation [4]. Alternatively, even though it is related to a transient inhibition of photosynthesis, reversible photoinhibition has been interpreted as an adaptative mechanism which reduces excitation energy delivery to the sensitive PS II reaction center by increasing non-photochemical energy dissipation in the PS II-chlorophyll antennae [5, 6].

The response of PS I to bright light has received considerably much less attention. This photosystem is generally supposed to be less susceptible to photoinhibition than PS II. This conclusion is based on *in vitro* studies of partial electron transfer reactions in the presence of artificial electron donors and acceptors in thylakoid membranes isolated from photoinhibited leaves [7–11]. Unfortunately, this suggestion has not been confirmed by *in situ* analyses of the PS I behavior in whole plant leaves exposed to photoinhibitory treatments. To the best of our knowledge, there is so far only one work [12] which provides data on the *in vivo* PS I function, showing that PS I may be still operative in the unicellular alga *Chlamydomonas reinhardtii* preilluminated with a strong light. The main rea-

Abbreviations: B_1, fraction of closed PS I reaction centers; E, Emerson enhancement; P_{700}, reaction center of PS I; V, relative variable chlorophyll fluorescence; PS, photosystem; ϕ_p, quantum yield for photochemistry in PS II; PES, photochemical energy storage.

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son for this lack of information probably lies in the fact that, in contrast to PS II activity which can be readily monitored through *in vivo* measurements of the characteristics of the fluorescence emission from PS II-chlorophyll antennae, the photofunctioning of PS I is much less easily accessible by *in vivo* measurements.

In this paper, we used the photoacoustic technique, aided by modulated fluorometry and absorbance spectroscopy, to study the *in vivo* PS I response to high-light stress. Although the photoacoustic method consists primarily in the microphone detection of the pulsed heat emission in samples illuminated with intensity-modulated light [13], it can also provide indirect information on various aspects of photosynthesis using adequate methodologies (recently reviewed in ref. [14]). Examples of application are given in the present paper where photoacoustic spectroscopy was used to study the relative activities of PS I and PS II in intact pea leaves pre-exposed to strong light stress.

Materials and Methods

Pea plants (*Pisum sativum* L.) were grown as described elsewhere [15]. Light produced by a Hansatech LS2 light source (Hansatech, Kings Lynn, U.K.) was passed through a CS 9782 filter (300–600 nm, Corning, New York, U.S.A.) combined with a Calfex C heat-reflecting filter (Balzers, Liechtenstein) and was transmitted onto attached pea leaflets using a 1.3-cm-diameter fiberoptic light guide. The fluence rate of this blue-green light, measured with a YSI-Kettering 65A radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.), was 400 W m^{-2} at the leaf surface. In order to avoid leaf dehydration which has been shown to affect the characteristics of the photoacoustic signals [16], the (attached) leaves were placed on wet filter paper. Leaf temperature, continuously monitored with a thermocouple thermometer (Digi-Sense 8528-20, Cole-Parmer Instrument Co., Chicago, U.S.A.), was maintained at a constant temperature of 25 °C throughout the light treatment, as described elsewhere [15].

After the photoinhibitory treatment, small leaf discs (diameter, 1 cm) were placed in a photoacoustic cell that has been described [17]. A complete description of the experimental setup utilized in the present work and the methodology used to analyze *in vivo* photoacoustic signals generated by leaves can be found elsewhere [18, 19]. The modulated O_2-evolution component of the photoacoustic signal was measured with a low-intensity broadband light (360–600 nm, 8 W m^{-2}) modulated at a low frequency of 17.5 Hz and was separated from the photothermal component by light saturation and phase adjustment using the vectorial method developed in ref. [20]. The Emerson enhancement (E) was measured by adding a strong far-red background light (>700 nm, *ca.* 30 W m^{-2}) to the modulated blue-green measuring beam: E is defined as the ratio of the amplitude of the O_2-evolution signal measured in presence of this additional far-red light to the signal amplitude measured in its absence. Leaves were illuminated for *ca.* 10 min with the far-red continuous light before measuring E. Photochemical energy storage (PES) was measured according to ref. [21], with either a broadband light (360–600 nm, 8 W m^{-2}) or a far-red light (>700 nm, 12 W m^{-2}). PES measurements were performed at a high frequency of modulation (367 Hz), where modulated O_2-evolution is damped out and only the photothermal signal exists: PES was calculated as the difference between the maximal photothermal signal measured in presence of a photosynthetically saturating background light (*ca.* 500 W m^{-2}) and the actual photothermal signal (without the background light) expressed in % of the maximal signal. PES is a measure of the efficiency of photosynthetic photochemistry comparable to the quantum yield.

A Hansatech twin channel fluorometer (MFMS-2) was used to measure simultaneously modulated 685 nm chlorophyll fluorescence and leaf absorbance at around 820 nm in pea leaves adapted to an actinic light (300–600 nm) of 10 W m^{-2}. The technical characteristics of the fluorometer and the experimental protocol used to measure the initial (F_o), steady-state (F_s) and maximal (F_m) levels of chlorophyll fluorescence in leaves photosynthesizing under steady-state conditions has been previously explained [22]. The actual quantum yield (ϕ_p) for photochemistry through PS II was estimated in light-adapted leaves from the F_s and F_m fluorescence levels [22, 23]:

$$\phi_p = 1 - (F_s/F_m) \quad (1)$$

The relative variable chlorophyll fluorescence

$$V = (F_s - F_o)/(F_m - F_o) \quad (2)$$

was used as a qualitative indicator of the fraction B_2 of closed PS II reaction centers. In reality, V is an hyperbolic function of B_2 [24]. It should be noticed that V is equal to $1-q_p$ where q_p is the so-called photochemical quenching coefficient [25].

Light-induced changes in leaf absorbance at around 820 nm, presumably reflecting changes in the redox state of the reaction center P_{700} of PS I [26], were monitored using a Hansatech P_{700}-measuring system employed in the reflection mode. The upper surface of the leaf was irradiated with a pulsed 820 nm light (4.8 kHz) emitted by an infrared light emitting diode combined with an interference filter (820 ± 20 nm). Reflected light was detected by a photodiode screened with a 820 nm interference filter. The lower surface of the leaf was covered with aluminium foil in order to eliminate transmitted light. The percentage of oxidized P_{700}^+ was evaluated following the procedure described in [27]: steady-state illumination (300–600 nm, 10 W m^{-2}) was interrupted and the fast absorbance decrease (ΔS) occurring in the dark was taken as representative of the spontaneous re-reduction of oxidized P_{700}^+ accumulated in the light. The maximal changes in absorbance $(\Delta S)_{max}$, which supposedly corresponds to a complete oxidation of P_{700}, was obtained by applying far-red light (730 nm) of saturating intensity of *ca.* 25 W m^{-2}. The fraction of P_{700} in the oxidized state (B_1) was then calculated as follows:

$$B_1 = (\Delta S)/(\Delta S)_{max} \quad (3)$$

Results and Discussion

Exposure of attached pea leaves to a strong blue-green light of 400 W m^{-2} altered the photosynthetic electron transport system, as indicated by the drastic reduction of the modulated O_2-evolution component of the *in vivo* photoacoustic signal (Fig. 1). After 30 min, the O_2-signal amplitude fell to less than 50% of its value determined before the light treatment. A slight increase in O_2-evolution was however noticed after *ca.* 40 min exposure to 400 W m^{-2}, suggesting that pea leaves had a certain, though limited, capacity to adapt to high-light stress. A similar recovery was observed for other photosynthetic parameters (*cf.* below, Fig. 3 and 4).

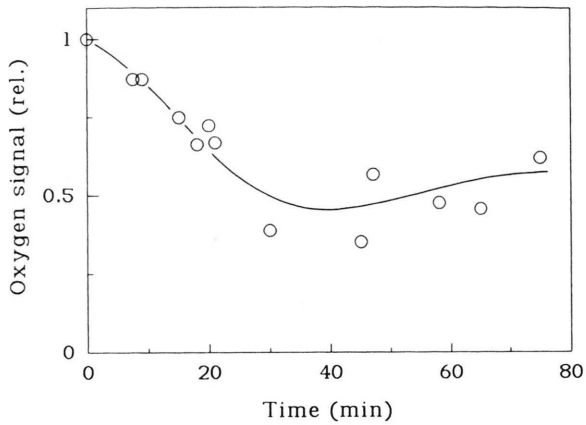

Fig. 1. O_2-evolution-related component of the *in vivo* photoacoustic signal generated by pea leaves pre-exposed for various times to a strong blue-green light of 400 W m^{-2}. Modulated measuring light: 360–600 nm, 17.5 Hz, 8 W m^{-2}.

As mentioned in the Introduction, high-light-induced inhibition of photosynthesis has been related to a loss of PS II function. Fig. 2 shows that, indeed, strong light stress was accompanied by a marked reduction of the actual quantum yield ϕ_p for photochemistry through PS II, as determined

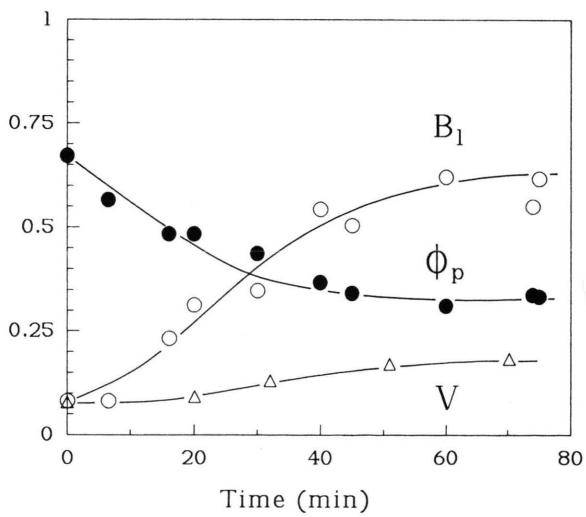

Fig. 2. Time course of the changes in the relative variable chlorophyll fluorescence V ($= (F_s - F_o)/(F_m - F_o)$), the quantum yield for photochemistry in PS II (ϕ_P) and the fraction B_1 of closed PS I centers in pea leaves pre-exposed to a strong light of 400 W m^{-2}. Absorbance and chlorophyll fluorescence measurements were performed in leaves adapted to a broadband light (300–600 nm) of 10 W m^{-2}.

from *in vivo* modulated chlorophyll fluorescence measurements. The problem we wanted to address in this paper was to determine whether the *in vivo* PS I activity was affected too. To this end, we took advantage of some features of the photoacoustic method which provide information on the relative activities of PS I and PS II.

Photochemical energy storage

The photoacoustic technique offers the unique possibility of measuring indirectly the energy of the photochemical processes that compete with radiationless pigment deexcitation. This can be achieved by saturating the photochemical activity with a strong background light (added to the modulated measuring light) which causes an almost complete conversion of the absorbed modulated light to heat. Thus, neglecting the low-yield chlorophyll fluorescence, the comparison of the heat emission level measured with and without the photosynthetically saturating background light gives an estimation of the energy which was stored in relatively long-lived intermediates of the photochemical processes [21]. When those measurements are performed at a modulation frequency of 367 Hz (as it is the case here), the observed photochemical products have a lifetime of at least 0.4 ms (reciprocal of the angular frequency).

In Fig. 3 is compared the photochemical energy storage (PES) monitored in modulated far-red light, absorbed almost exclusively in PS I, with that monitored in broadband light (360–600 nm) exciting both PS I and PS II. When measured with the 360-to-600-nm light, energy storage was observed to markedly decrease in response to strong light stress. The PES value was around 16% before light stress and fell to *ca.* 7% after 30 min exposure to 400 W m^{-2}. As shown in the inset of Fig. 3, a good correlation was observed between the changes in PES and the inhibition of O_2 evolution. Although the exact nature of the component(s) responsible for the energy storage monitored during photoacoustic experiments is unknown, it has been shown that PES reflects photochemical events involving both PS I and PS II [28] and it is believed that, under conditions where the light distribution between the two photosystems is well balanced, PS I and PS II contribute equally to the energy storage (S. Malkin, pers. comm.). In contrast

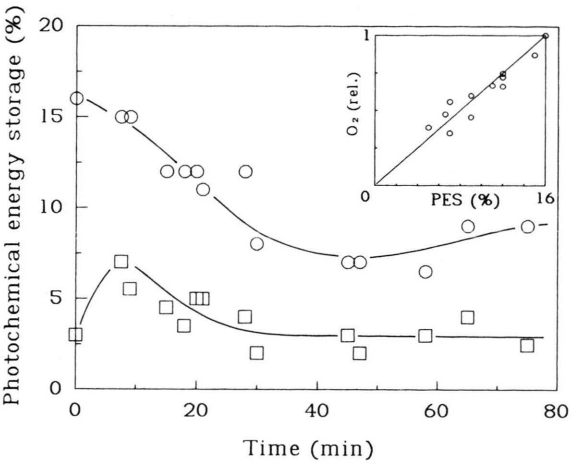

Fig. 3. Time course of the changes in photochemical energy storage monitored in far-red light (□, 12 W m^{-2}, >700 nm) or in broadband light (○, 8 W m^{-2}, 360–600 nm) in pea leaves pre-exposed to a strong light of 400 W m^{-2}. Inset: correlation between the O_2-evolution signal and photochemical energy storage (PES) monitored with the 360-to-600-nm light.

when leaves were irradiated with PS I-exciting far-red light (which did not induce any O_2 production, data not shown), the measured PES is specifically related to the PS I photofunctioning, most probably reflecting the production of photochemical products by means of cyclic electron flow around this photosystem [12, 29]. It has been demonstrated that PES monitored in algal cells with a pulsed 710 nm light is insensitive to the herbicide diuron but is greatly reduced by an inhibitor of the plastoquinol oxidation [12], thus suggesting that the operation of this PS I-mediated cyclic electron flow involves this latter electron carrier. In control pea leaves illuminated with the pulsed far-red light, PES was relatively small (*ca.* 3%). As previously reported [29], such a low energy storage by cyclic electron flow through PS I seems to be characteristic of C_3-type higher plants. Clearly, high-light stress did not reduce the extent of this energy storage; in fact, rather than an inhibition, a noticeable increase (× 2.5) in PES was observed during the first 20 min of the treatment, suggesting a transitory stimulation of the *in vivo* PS I function. Although the reasons of this transitory stimulation of PS I function remain to be determined, enhanced cyclic transport in PS I might be an adap-

tive response providing energy required for recovery processes and survival [12].

Emerson enhancement

One of the most interesting feature of the photoacoustic method is the possibility of measuring the Emerson enhancement (*i.e.* the enhancement of O_2 evolution by addition of far-red background light) which indicates the ratio β/α where β and α are, respectively, the maximum photochemical potentials (quantum yield × light distribution) of PS II and PS I [30]. Fig. 4 shows that E spectacularly decreased from *ca.* 17% (in control leaves) to less than 5% (in photoinhibited leaves). Such an effect shows that, during photoinhibition stress, PS I became less and less limiting for the non-cyclic electron transfer rate (*i.e.* α increased relative to β), thus pointing to a selective effect on PS II. It was also noticed that E decreased more rapidly than the other measured parameters (ϕ_p, PES, O_2 signal). This can be easily explained if we consider that the inhibition of the photochemical activity was specifically due to photodamage of PS II (β) whereas the pronounced change in $E = \beta/\alpha$ reflected both the decrease in β and the concomitant increase in α observed during the first phase of the light treatment (Fig. 3).

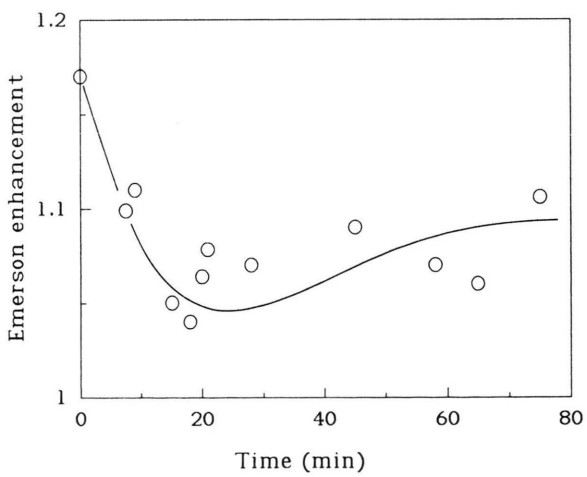

Fig. 4. Changes in the Emerson enhancement measured in pea leaves pre-exposed for various times to a strong light (400 W m^{-2}, 300–600 nm). Modulated measuring light: 360–600 nm, 8 W m^{-2}, 17.5 Hz. Far-red background light: >700 nm, 30 W m^{-2}.

The Emerson enhancement provides another opportunity to check the effects of strong light stress on PS I *via* the analysis of the saturation curve of the enhancement effect of the far-red background light. Fig. 5 shows the E *vs.* far-red light intensity curve, the slope of the linear portion providing a relative measure of the quantum yield of PS I for the enhancement effect [30]. It can be seen that exposure of pea leaves to high-light stress did not induce any substantial change in the slope of the linear plot. This observation provides an independent proof of the robustness of PS I regarding photoinhibition stress.

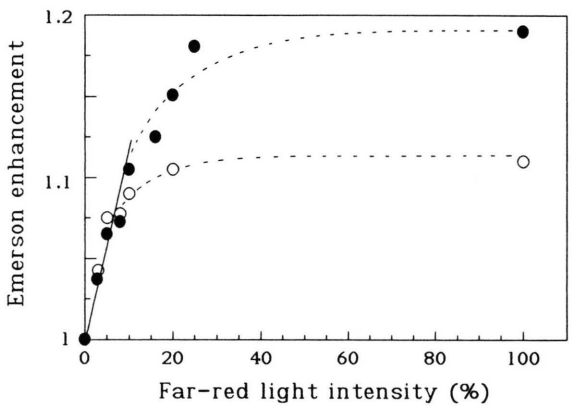

Fig. 5. Dependence of the Emerson enhancement values on the intensity of the far-red background light in pea leaves before (●) and after (○) exposure to a strong light stress (400 W m^{-2} for 40 min).

Leaf absorbance at 820 nm

Light-induced changes in the leaf absorbance at around 820 nm, which are indicative of the photooxidation of the reaction center P_{700} of PS I [26], were monitored concomitantly with PS II-chlorophyll fluorescence in pea leaves which had been experiencing strong light stress (Fig. 2). Before the photoinhibitory treatment, the P_{700} pool was almost completely reduced ($B_1 < 0.1$) in pea leaves under steady illumination with a relatively weak actinic light (10 W m^{-2}). Light pretreatment was observed to cause a progressive (steady-state) closure of the PS I centers (*i.e.* B_1 rose). After 60 min at 400 W m^{-2}, nearly 70% of the PS I traps were closed in the low actinic light. The steady-state level of P_{700}^+ is determined by the rate of P_{700} oxidation in PS I and the rate of its concomitant

re-reduction by electrons derived from PS II *via* plastocyanin. Then, accumulation of oxidized P_{700}^+ indicates the establishment of an imbalance between those two reactions in favor of the former one. In contrast, the relative stability of the variable chlorophyll function V suggests that the PS II reaction centers remained largely open throughout the light treatment. As excitation energy transferred to oxidized P_{700}^+ is dissipated as heat [31], the actual quantum yield $\phi_P^{PS\,I}$ of photochemistry in PS I is directly dependent on the proportion of photochemically active open centers as follows:

$$\phi_P^{PS\,I} = (1 - B_1)E_{1a}/J_1 \quad (4)$$

where E_{1a} is the excitation energy flux from the absorbing pigments and the reaction center and J_1 is the light absorption in PS I. Thus, the steady-state accumulation of oxidized P_{700}^+ in photoinhibited leaves irradiated with an actinic light of 10 W m^{-2} is in agreement with the idea of a selective photoinactivation of PS II, as it can be interpreted as a passive response of PS I, adjusting its macroscopic photochemical yield to the decreased photochemical efficiency of photoinhibited PS II.

Conclusion

In vivo photosynthetic parameters reflecting properties of PS II alone (ϕ_P) or of the tandem PS II + PS I (O_2 signal, PES) were markedly affected in high-light-treated pea leaves whereas parameters indicative of the PS I activity relative to that of PS II (E, B_1) point to a preferential photodestruction of PS II. More, the photoacoustic monitoring of PES in far-red light indicated a full preservation, if not a stimulation, of the capacity of PS I to mediate electron transport. Thus, from those results as well as the invariability of the linear portion of the saturation curve of E (Fig. 5), it seems clear that a light treatment which was very harmful to PS II did not inactivate the *in vivo* PS I function. Consequently, the conclusion, previously drawn from studies of chloroplastic membranes isolated from photoinhibited leaves, that PS I can tolerate high incident light intensities seems to be also valid *in vivo*. This tolerance to strong light stress can be an intrinsic property of PS I or can be the result of control processes which efficiently protect PS I *in vivo*, as suggested by *in vitro* experiments showing substantial inactivation of PS I during illumination of isolated chloroplasts [32].

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