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Predicting the Extent of Photosystem II Photoinactivation Using Chlorophyll *a* Fluorescence Parameters Measured during Illumination

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The temperature dependence of the relationship between the decline in activity of photosystem II (PSII) and a chlorophyll *a* fluorescence parameter combining the excitation pressure ($1–q_P$) and efficiency of excitation energy capture by open PSII reaction centers in the light-acclimated state (F_v'/F_m') was investigated in cotton leaves. A formula for the prediction of PSII inactivation is proposed on the basis of the results obtained. By comparison of the predicted and actual levels of PSII photoinactivation, the rate of PSII recovery was estimated from chlorophyll *a* fluorescence parameters measured during the day for attached cotton leaves exposed to suboptimal morning temperatures in a greenhouse.

Keywords: Cotton — *Gossypium hirsutum* L. — Photoinhibition — Photosystem II — Repair.

Abbreviations: EF, excessive light flux, $F_{v,}$ variable chlorophyll fluorescence in dark-adapted state, F_v', variable chlorophyll fluorescence in light-adapted state, F_m, maximal chlorophyll fluorescence in dark-adapted state, F_m', maximal chlorophyll fluorescence in light-adapted state, NPQ, non-photochemical chlorophyll fluorescence quenching coefficient, q_P, photochemical chlorophyll fluorescence quenching coefficient, PFD, photon flux density.

Introduction

The extent of photosystem II (PSII) photoinactivation has been shown to depend directly on the redox state of Q_A (Öquist et al. 1992, Ottander et al. 1993, Melis 1999) and inversely on the level of thermal energy dissipation (Demmig-Adams and Adams 1992, Osmond et al. 1993, Niyogi 1999) in PSII complexes. The Q_A redox state and the level of thermal energy dissipation are traditionally estimated using the photochemical (q_P) and non-photochemical (NPQ) chlorophyll fluorescence quenching coefficients, respectively (reviewed by Maxwell and Johnson 2000, Rohácek 2002). Combination of these parameters can provide a quantitative expression of the susceptibility of PSII to photoinhibition. For instance, the ratio $(1–q_P)$/NPQ, has been introduced as an index of PSII susceptibility to light stress (Osmond 1994, Park et al. 1995a, Park et al. 1996). Ögren (1991) described an alternative means of using photochemical and non-photochemical coefficients of chlorophyll fluorescence quenching to predict the decline in PSII activity.

The principal disadvantage to the use of NPQ is that this parameter requires knowledge of dark-adapted maximal fluorescence (Fm; Maxwell and Johnson 2000). F_m can be impractical or impossible to measure in some experimental settings. A meaningful comparison of F_m' with F_m, as in the calculation of NPQ ($F_m/ F_m'–1$), requires that both are measured from precisely the same region of the leaf with the probe of the fluorometer at precisely the same distance and orientation relative to the leaf surface. These measuring conditions can be hard to achieve, particularly in the field. Furthermore, in response to environmental stress, particularly to cold temperatures, many plants exhibit sustained thermal energy dissipation that persists through the evening and results in quenched pre-dawn values for F_m (Adams et al. 1995). The use of such quenched values for F_m in the calculation of NPQ will lead to an underestimate.

The ratio of variable to maximal chlorophyll *a* fluorescence measured for light-adapted leaves (F_v'/F_m') is considered as an alternative measure of the level of non-photochemical energy dissipation in PSII complexes (Rohácek 2002). F_v'/F_m' is widely applied to estimate the efficiency of excitation energy capture by open PSII reaction centers for light-acclimated leaves (Harbinson et al. 1989, Genty et al. 1989). Development of non-photochemical energy dissipation in PSII complexes brings about a decrease in F_v'/F_m'. Therefore this parameter is a measure of the ability of the photosynthetic apparatus to dissipate light energy absorbed by PSII antennae (Oxborough and Baker 1997).

$(1–q_P)F_v'/F_m'$, an analog of the parameter $(1–q_P)$/NPQ, can be used to estimate the susceptibility of PSII to photoinhibition. Demmig-Adams et al. (1996) applied this parameter as an estimate for so called "excessive" light energy (E). The meaning of the parameter was also discussed by Weis and Lechtenberg (1989). According to Demmig-Adams et al. (1996) $E = (1–q_P)F_v'/F_m'$ represents the portion of light energy absorbed by PSII antennae that is not used in electron transport nor dissipated thermally. The parameter was alternatively referred to as "unaccounted for" (Adams and Barker 1998, Adams et al. 1999) light energy. The approach has been successfully employed in studies comparing the extent of PSII photoinactivation and $(1–q_P)F_v'/F_m'$ levels during exposure to stressful

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environmental conditions, such as drought, low temperature, and high photon flux density (PFD), especially for shade-acclimated plants transferred to high light (Martin et al. 1999, Fleck et al. 2000, Kornyeyev et al. 2002, Olivera and Penuelas 2001, Lima et al. 2002, Kato et al. 2003). Such conditions generally lead to an increase in the level of $(1–q_P)F_v'/F_m'$. In the present article, we applied this parameter to predict PSII inactivation under continuously changing environmental conditions in a greenhouse.

The goal of the present study was to investigate the relationship between PSII activity and the total amount of "excessive" light (excessive light exposure) at different temperatures in order to develop a formula for the prediction of the decline in PSII activity in cotton leaves treated with lincomycin, an inhibitor of PSII repair. Secondarily, comparison between predicted and actual levels of PSII photoinactivation (those observed in the absence of lincomycin) offers the opportunity to estimate the rate of PSII repair under fluctuating light and temperature regimes.

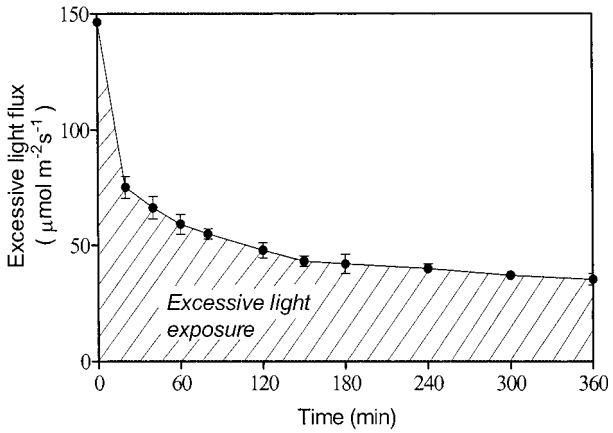

Fig. 1 Changes in the level of excessive light flux (EF = $(1–q_P)F_v'/F_m'\times PFD\times 0.75\times 0.5$) during dark-to-light transition at 10°C. Cotton leaves used for this experiment were pre-treated with lincomycin. Hatched area represents the total amount of photons captured by PSII complexes with Q_A in reduced state (excessive light exposure). PFD = 500 μmol m^{-2} s^{-1}, data are means ± SD, n = 3–6.

Results

In analogy to a widely used approach to estimate the rate of electron transport through PSII on the basis of the quantum yield of electron transport in PSII ($q_P\times F_v'/F_m'$), and taking into account the incident PFD, coefficients for leaf absorbance, and for the sharing of absorbed photons between PSI and PSII (Genty et al. 1989, Maxwell and Johnson 2000), we propose that excessive light flux (EF) can be estimated using the following formula:

$$EF = (1–q_P)F_v'/F_m'\times PFD\times 0.75\times 0.5 = E\times PFD\times 0.75\times 0.5 \quad (1)$$

The value of the last coefficient (0.5) assumes an equal distribution of excitation between PSI and PSII (Krall and Edwards 1992). The coefficient 0.75 is used to account for cotton leaf absorbance (see Björkman and Demmig 1987). A similar approach was used previously by Kato et al. (2003). Changes in EF during a dark-to-light transition (Fig. 1) reflect the decrease in the portion of "excessive" light (E) caused by the development of photochemical and non-photochemical energy quenching. Integration of EF over the time of illumination (see Fig. 1) gives an estimate for the total amount of photons trapped by PSII complexes in excess of that which could be used in electron transport or safely dissipated as heat (excessive light exposure):

$$\text{Excessive light exposure} = \sum_{i=2}^{n} t \times (EF_i + EF_{i-1})/2 \quad (2)$$

t is time between EF measurements; EF_i and $EF_{i–1}$ are the levels of EF measured at the current and previous time-points, respectively; n = total number of time-points. F_v/F_m was used as the level of E at t = 0, because at the beginning of the chilling treatment (i = 1) q_P was assumed to be 0. The average level of EF calculated as $(EF_i + EF_{i–1})/2$ for each time period was multiplied by the duration of this period and the results obtained for all periods were summed to obtain the estimation of total of excessive light absorbed during the period of the light treatment. In this paper, the term "excessive light exposure" is used instead of the previously introduced "Time-dependent averaged E" (Kornyeyev et al. 2001, Kornyeyev et al. 2002) because such integration represents cumulative "excessive" light energy. Excessive light exposure reflects the amount of photons captured by "closed" but potentially active PSII complexes with Q_A in reduced state. Light energy captured by PSII complexes with Q_A in reduced state (Q_A^-) results in double reduction of Q_A (Q_A^{2-}) thereby triggering processes leading to PSII inactivation (Noguchi 2002).

In order to study the relationship between excessive light exposure and the extent of PSII inactivation, we assessed the decline in dark-adapted F_v/F_m as a manifestation of PSII photoinactivation and a measure of the decrease in the amount of functional PSII reaction centers (Öquist et al. 1992, Flexas et al. 2001, Rohácek 2002). It should be stressed that while decreases in F_v/F_m and PSII photoinactivation are well correlated, the loss of D1 protein has been shown to lag behind the loss of PSII activity during chilling-induced photoinhibition (Aro et al. 1990, Schnettger et al. 1994). This indicates that the decline in D1 protein content and PSII inactivation are different processes (Salonen et al. 1998, Ottander et al. 1993). Therefore, the results we present do not address D1 protein dynamics, specifically. PSII photoinactivation can also be measured as a decrease in the rate of photosynthetic oxygen evolution under light and CO_2 saturation. However, it has been established previously that the combined action of illumination and low temperature brings about a significant decline in PSI activity as well as PSII activity, especially for chilling sensitive species,

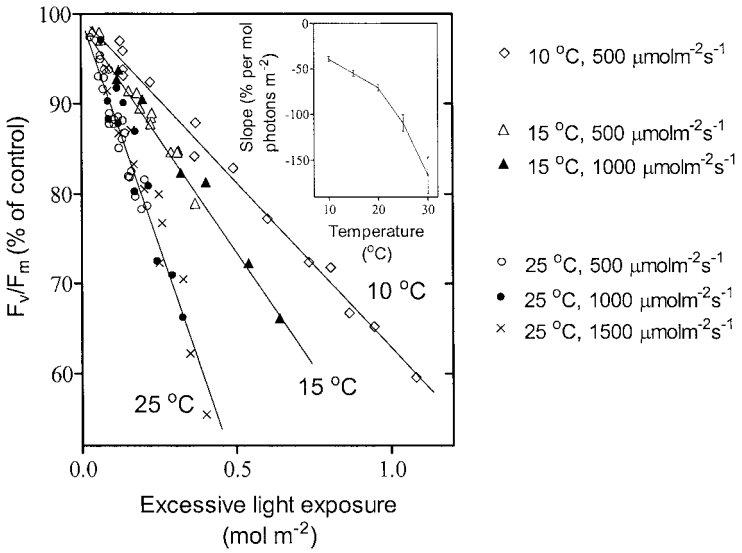

Fig. 2 Values of F_v/F_m expressed as the percent of initial, dark-acclimated values measured before the photoinhibitory treatment versus excessive light exposure for cotton leaf discs at different temperatures and PFDs in the oxygen electrode chamber. Leaf discs were pretreated with lincomycin to inhibit chloroplast repair processes. *Insertion.* Temperature dependence of the slope for the relationship between F_v/F_m and excessive light exposure. PFD = 500 μmol m^{-2} s^{-1}, data are means ± SD, n = 3–6.

such as cotton, the subject of the present study (Kornyeyev et al. 2003, see also Sonoike 1996, Terashima et al. 1998). PSI limitations on the rate of oxygen evolution may complicate the interpretation of this technique as a measure of PSII activity for samples previously exposed to illumination under chilling conditions.

The extent of PSII photoinactivation, measured as the decrease in F_v/F_m, was compared to the level of excessive light exposure at different temperatures for samples treated with lincomycin to inhibit repair processes (Fig. 2). At each temperature, an inverse linear relationship was observed, and the slope was insensitive to the PFD of the exposure. Interestingly, the steepness of the slope was positively correlated with temperature, i.e. the same level of excessive light exposure corresponded to a higher extent of PSII photoinactivation at warmer temperatures. It should be noted that, in contrast to the steepness of the slope for the relationship between F_v/F_m and excessive light exposure, the rate constant of PSII photoinactivation increases as the temperature declines below optimum. In our experiment, the level of the rate constant of PSII photoinactivation was 1.5 times higher at 10°C than at 25°C (PFD = 500 μmol m^{-2} s^{-1}). This difference can be explained by a faster increase in excessive light exposure at lower temperatures, which occurs because of the slow development of photochemical and non-photochemical energy quenching. Despite a lower sensitivity to excessive light exposure observed at low temperature, the levels of $(1–q_P)F_v'/F_m'$ detected under these conditions are noticeably higher, especially at the beginning of the illumination (Fig. 3).

The temperature dependency of the slope for the relationship between F_v/F_m and excessive light exposure can be described as follows:

$$\text{slope} = -18.6\, e^{0.071T}\ (R^2 = 0.991), \quad (3)$$

where T is temperature in °C. Equation 3 is based on the data obtained at 10–30°C (see inset in Fig. 2). In order to check for a possible hysteresis effect, leaf discs were illuminated for 1-h periods at 25, 10, and then 25°C. PFD was 1,000 μmol m^{-2} s^{-1} during the entire light treatment. Slopes calculated for data obtained before (88±3% mol^{-1} m^2) and after (83±5% mol^{-1} m^2) low temperature period were not significantly different (P = 0.273, values are means ± SD, n = 3). Therefore no evidence of a hysteresis effect was observed.

One may suggest that Equation 3 can be used to predict the value of F_v/F_m after light exposure:

$$F_v/F_m\ (\%\ \text{of control}) = 100 - 18.6\, e^{0.071T} \times \text{Excessive light exposure} \quad (4)$$

Equation 4 was obtained for lincomycin-treated leaves. Nevertheless, it can be used to estimate the overall percentage of PSII photoinactivation in untreated leaves during light treatment.

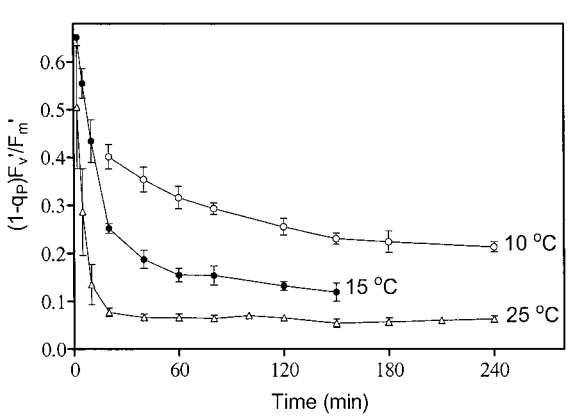

Fig. 3 Time course of $(1–q_P)F_v'/F_m'$ during light adaptation at different temperatures. PFD = 500 μmol m^{-2} s^{-1}, data are means ± SD, n = 3–6.

PSII recovery (reactivation) and PSII photoinactivation occur simultaneously in untreated leaves. As a result, the level of PSII activity manifested in untreated leaves is higher than that in lincomycin-treated leaves (Tyystjärvi and Aro 1996) where recovery is inhibited. The following equation can be applied to estimate the rate of PSII recovery for the period of time (t):

$$\text{Rate of PSII recovery} = (\text{Predicted loss of PSII activity} - \text{Actual loss of PSII activity})/t \quad (5)$$

The loss of PSII activity in the absence of recovery can be predicted on the basis of excessive light exposure (see Equation 6 derived from Equation 4), and the actual loss of PSII activity can be calculated as the difference between F_v/F_m levels measured at the beginning and at the end of the time period of analysis.

$$\text{Predicted loss of PSII activity (\% of control)} = 18.6\, e^{0.071\,T} \times \text{Excessive light exposure} \quad (6)$$

Other researchers have developed alternative means of estimating the rate of PSII repair from chlorophyll *a* fluorescence emission (see Wünschman and Brand 1992, Lee et al. 2001). According to the literature cited above, the rate of PSII recovery depends on the content of damaged PSII complexes:

$$dA/dt = k_{REC}\,(1-A), \quad (7)$$

where A represents the fraction of PSII complexes which are photosynthetically active and k_{REC} is the first order rate constant for recovery of PSII activity. dA/dt is the rate of recovery. Therefore,

$$k_{REC} = \text{rate of PSII recovery}/(1-A) \quad (8)$$

In order to verify the validity of our approach for the estimation of PSII recovery on the basis of excessive light exposure we calculated k_{REC} using the values of the rate of PSII recovery obtained from Equation 5 (Fig. 4). An increase in the leaf temperature led to higher levels of k_{REC}. Similar results were previously obtained by Lee et al. (2001) and Wünschman and Brand (1992). Lee et al. (2001) reported the rate of PSII recovery of 0.34 μmol PSII m^{-2} h^{-1} for leaves of *Capsicum annuum* at 25°C and PFD of 900 μmol m^{-2} s^{-1} (34% of initial activity per hour taking into account the initial level of PSII activity of 1 μmol PSII m^{-2}). Applying the same photoinhibitory treatment for cotton leaf discs, we found that 21±3% of the initial PSII activity was recovered during 1 h (mean ± SD, $n = 3$).

Previous methods of estimating PSII photoinactivation and recovery (Wünschman and Brand 1992, Lee et al. 2001) cannot be applied to fluctuating light and temperature regimes that would be encountered in field and greenhouse settings. We employed Equations 5 and 6 to estimate the rate of PSII repair in warm-acclimated cotton subjected to photoinhibitory conditions of cool morning temperatures and natural illumination for two consecutive days (Fig. 5A). On both days, the minimum F_v/F_m in the time-courses coincided with the middle of the solar day (Fig. 5B). This suggests that the greatest disequilibrium between PSII photoinactivation and repair occurred prior to midday. Interestingly, midday levels of F_v/F_m were higher on the second day of the experiment in comparison to the first. The application of the parameter $(1-q_P)F_v'/F_m'$ allowed us to compare the rates of PSII recovery on the first and second day of stress treatment.

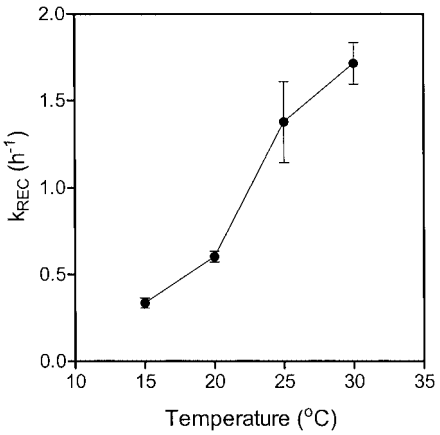

Fig. 4 Temperature dependence of the rate constant of PSII recovery (k_{REC}). PFD = 900 μmol m^{-2} s^{-1}, data are means ± SD, $n = 3$.

The rate of PSII recovery (expressed as % of initial activity per hour) for the period between two consecutive time points was calculated as the difference between predicted and actual losses of PSII activity divided by the duration of time (t) between these time points (Equation 5). Actual loss of PSII activity was calculated as the differences between F_v/F_m obtained for samples taken at the time points mentioned above. PSII activity was expressed as % of the control level determined for non-stressed leaves before sunrise. The calculation of the loss of PSII activity (% of initial activity) was based on excessive light exposure (Equation 6) assessed for the same period as for determination of actual loss of PSII activity with leaf temperature averaged between the values taken during current and previous measurements. Excessive light exposure was calculated separately for every leaf used in the experiment on the basis of periodic chlorophyll *a* fluorescence and PFD measurements. Leaf temperatures slightly above 30°C were recorded for a few time points (Fig. 5A). However, only temperatures between 10 and 30°C were used for calculation of the predicted loss of PSII activity, because T in Equation 6 is the average between the temperature values registered at current and previous measurements for the same leaf. The same time points for collection of samples and determination of the parameter $(1-q_P)F_v'/F_m'$ were used in the calculation of both actual and predicted PSII activities.

The maximal rate of PSII repair was detected in the early afternoon (Fig. 5C) when PFD was high and F_v/F_m was declin-

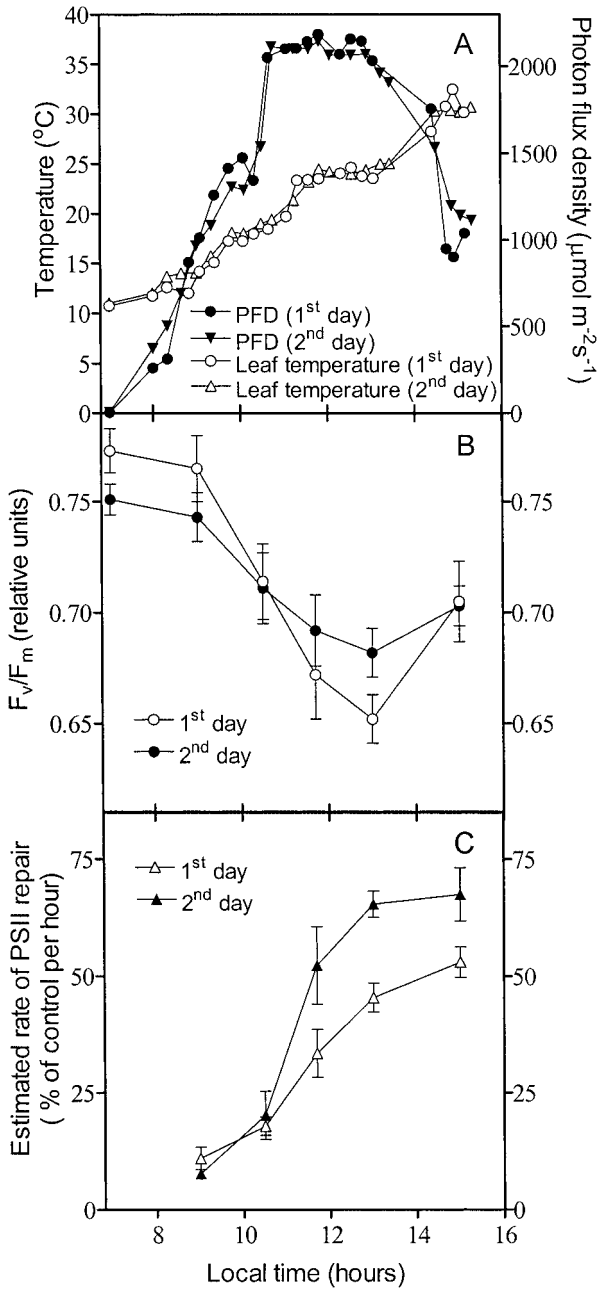

Fig. 5 (A) Diurnal changes in leaf temperature and photon flux density (PFD) during "cool mornings" experiment in the greenhouse for two consecutive days. Attached untreated with lincomycin leaves were used for experiment. (B) Diurnal changes in F_v/F_m reflecting the time-course of PSII photoinactivation. (C) The rate of PSII repair calculated as differences between the predicted and actual levels of PSII photoinactivation. The prediction of PSII inactivation was based on the measurements of chlorophyll fluorescence parameter $(1–q_P)F_v'/F_m'$ (see text for details). The measurements of the leaf temperature and PFD (A) were conducted the same day as measurements of F_v/F_m (B). The results were used for calculation of the rate of PSII repair (C). Data are means ± SD, $n = 4$.

ing. An increase in the rate of PSII repair was observed on the second day of the cool morning experiment, suggesting that PSII repair is subject to acclimatory increases in capacity in response to environmental conditions that increase the rate of PSII photoinactivation.

Discussion

In the present paper we continued the investigation of the linear relationship between the PSII activity and the total amount of the light energy absorbed in excess of what can be utilized in photosynthesis or dissipated as heat (Kornyeyev et al. 2001, Kornyeyev et al. 2002). The previously published data support the approach of using the parameter $(1–q_P)F_v'/F_m'$ to estimate the susceptibility of PSII to photoinhibition proposed by Demmig-Adams et al. (1996). It was recently demonstrated by Kato et al. 2003 that the rate constant of the PSII photoinactivation was proportional to the absolute level of the excess energy (the parameter similar to excessive light flux used in the present paper). The measurements of excessive light flux, however, can be used to predict the extent of PSII photodamage only under confined conditions. The magnitude of excessive light flux changes temporally even when illumination and temperature are held constant (Fig. 1, see also Kornyeyev et al. 2002). These changes reflect the increase in the level of regulatory thermal dissipation (down-regulation) and electron transport, which minimize the proportion of light that is excessive during the adaptation to illumination. A decrease in the leaf temperature brings about a noticeable delay in the development of both photochemical and non-photochemical energy quenching in PSII complexes. As a result of this delay, the share of excessive light ($(1–q_P)F_v'/F_m'$) declines more slowly at low temperatures, reaching the steady-state level only after several hours at 10°C (Fig. 3). It was shown earlier that the rate constant of photoinhibition calculated for the first 40 min of illumination at low temperature (10°C) was significantly higher in comparison to that calculated for the period between 300 and 360 min (Kornyeyev et al. 2002). On the one hand, these data support the relationship between the level of excessive light and the extent of photoinhibition (higher level of E ($E = (1–q_P)F_v'/F_m'$) at the beginning of the illumination is associated with higher rate constant of photoinhibition). On the other hand, the results described above suggest that it could be more effective to use the total amount of photons trapped by PSII complexes in excess (excessive light exposure) for the prediction of PSII photodamage instead of using the magnitude of excessive energy flux at a single time-point. The advantage of the application of excessive light exposure is self-evident in the case of the fluctuating light and temperature regimes.

According to the data described in the present paper, the prediction of PSII photoinactivation on the basis of excessive light requires taking into account the leaf temperature. It was determined that the same level of excessive light exposure is associated with higher decrease in PSII activity at warmer tem-

perature (Fig. 2). This non-intuitive temperature dependency of PSII photoinactivation has been observed previously for isolated thylakoids (Aro et al. 1990) and in vivo (Schnettger et al. 1994). Although low temperatures have been shown to inhibit proteolytic degradation of D1 (Ottander et al. 1993, Salonen et al. 1998), PSII photoinactivation can occur in the absence of D1 degradation, especially during exposure to chilling. Whatever the mechanism, lower vulnerability to PSII photoinactivation during exposure to chilling may be viewed as photoprotective and may be mediated by chilling-induced phosphorylation of D1 and possibly other PSII core proteins (Kruse et al. 1997, Salonen et al. 1998).

One of the potentially interesting applications of the approach for the calculation of PSII photodamage introduced in the present paper could be the estimation of the rate of the PSII recovery. An example of this application is shown in Fig. 5. An increase in the rate of PSII recovery was observed on the second day of exposure to stressful conditions (low morning temperature). Enhanced resistance to photodamage via increased rates of thermal dissipation or electron transport may also occur over this time-scale; however, these processes are accounted for during in the calculation of excessive light exposure.

In the middle of the solar day, the rate of PSII repair (calculated as the percentage of PSII activity on the basis of F_v/F_m measurements) is estimated as 65–70% of the control level per hour (Fig. 5C, second day of stress treatment). Taking into account the correlation between F_v/F_m and the amount of functional PSII complexes (Flexas et al. 2001, Park et al. 1995b), one may suppose that more than a half of the PSII complexes are renewed in cotton leaves during one hour of light exposure. This observation confirms the importance of the PSII repair cycle as a component of photoprotection in cotton plants.

Materials and Methods

Cotton plants, *Gossypium hirsutum* L. cv. Coker 312, were grown in 8-liter pots in a greenhouse at ~30/26°C (day/night) with a natural photoperiod. Plants were fertilized with Hoagland's solution twice a week. The youngest fully expanded leaves of 5- to 8-week-old plants were used for all analyses. The leaves were treated with lincomycin, an inhibitor of chloroplast protein synthesis and PSII repair processes, as described previously (Kornyeyev et al. 2002). The leaves were harvested at sunrise by cutting their petioles under water. They were immediately transferred to microcentrifuge tubes containing 1 mg ml^{-1} lincomycin (863 units mg^{-1}) and kept in the dark for 3 h at room temperature. At the end of this dark incubation period, the concentration of lincomycin in the bulk leaf tissue (C_L) was 1.22–2.47 mM as estimated from the formula: $C_L = C_S(W_S/W_L)$, where C_S is the inhibitor concentration in the solution, W_S is the weight of the solution taken up by a leaf, and W_L is the fresh weight of the leaf (Bilger and Björkman 1994). The discs from leaves treated with lincomycin and subjected to photoinhibitory conditions exhibited no increase in F_v/F_m when subsequently exposed to conditions that favored repair processes (3 h at 10 μmol photons m^{-2} s^{-1} and room temperature).

Chlorophyll *a* fluorescence emission was measured with a pulse amplitude-modulated fluorometer (PAM 101/103, Heinz Walz GmbH, Effeltrich, Germany). The experimental protocol of Schreiber et al. (1986) and the nomenclature of van Kooten and Snel (1990) were used. Measurements on discs from lincomycin-treated leaves were conducted through a port in the oxygen electrode chamber (Hansatech, King's Lynn, Norfolk, U.K.) at various times during the treatment. Leaf discs (10 cm^2), acclimated to darkness for 1.5 h, were exposed to the desired temperature for 20 min prior to illumination in the chamber. A flow of humidified air was used as the CO_2 supply. Attached, untreated leaves were used for diurnal experiments in a temperature-controlled greenhouse.

Leaf discs possessed values for F_v/F_m of 0.78±0.01 (mean ± SD, n = 20) prior to the period of experimental light exposure. For some of the 10-cm^2 leaf discs, smaller (1 cm^2) discs were removed during illumination to determine F_v/F_m following 3 h of incubation in darkness at 25°C. No significant differences were found in the levels of F_v/F_m when identical photoinhibitory treatments were followed by 3 h of dark acclimation at 10°C or 25°C (data not shown).

Acknowledgments

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