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The rate constant of photoinhibition, measured in lincomycin-treated leaves, is directly proportional to light intensity

(photosynthesis/photosystem II/D1 protein/chlorophyll fluorescence)

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ABSTRACT **Pumpkin leaves grown under high light (500–700 μmol of photons $m^{-2} \cdot s^{-1}$) were illuminated under photon flux densities ranging from 6.5 to 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the presence of lincomycin, an inhibitor of chloroplast protein synthesis. The illumination at all light intensities caused photoinhibition, measured as a decrease in the ratio of variable to maximum fluorescence. Loss of photosystem II (PSII) electron transfer activity correlated with the decrease in the fluorescence ratio. The rate constant of photoinhibition, determined from first-order fits, was directly proportional to photon flux density at all light intensities studied. The fluorescence ratio did not decrease if the leaves were illuminated in low light in the absence of lincomycin or incubated in darkness in the presence of lincomycin. The constancy of the quantum yield of photoinhibition under different photon flux densities strongly suggests that photoinhibition *in vivo* occurs by one dominant mechanism under all light intensities. This mechanism probably is not the acceptor side mechanism characterized in the anaerobic case *in vitro*. Furthermore, there was an excellent correlation between the loss of PSII activity and the loss of the D1 protein from thylakoid membranes under low light. At low light, photoinhibition occurs so slowly that inactive PSII centers with the D1 protein waiting to be degraded do not accumulate. The kinetic agreement between D1 protein degradation and the inactivation of PSII indicates that the turnover of the D1 protein depends on photoinhibition under both low and high light.**

Photoinhibition of photosynthesis has been characterized as a high-light-induced stress reaction in plants (for review, see refs. 1 and 2). Several definitions of photoinhibition have been presented, and many authors include in the concept all light-induced phenomena that lower the efficiency of photosynthesis. We define photoinhibition as the light-dependent irreversible inactivation of photosystem II (PSII) reaction center activity, which can be restored only via the degradation and synthesis of the D1 protein. This strict definition allows for comparison of the kinetics of photoinhibition in different conditions but excludes several high-light-specific, reversible phenomena often referred to as photoinhibition.

We have shown that photoinhibition is a first-order reaction (3) and that its rate constant, k_{PI}, is directly proportional to photon flux density (4). However, these experiments were done under high, photoinhibitory light, and it therefore remained unclear if photoinhibition is somehow related to the photon flux exceeding the flux that can be safely dissipated through photosynthesis (5–7). On the contrary, the first-order nature of photoinhibition suggests that each photon absorbed by PSII causes photoinhibition with the same probability. In the present paper, we describe results that prove the latter.

Reciprocity between the amount of light and the duration of illumination was demonstrated for photoinhibition of isolated chloroplasts by Jones and Kok (8). Very recently, Park *et al.* (9) published data demonstrating that the law of reciprocity holds for photoinhibition of intact leaves too. The present study confirms most of their results, but we conclude that photoinhibition is a one-photon phenomenon instead of a photon counter-type poisoning process (9).

The degradation and synthesis of the D1 protein are rapid under both high and low light if compared to other thylakoid proteins (10), and several hypotheses have been put forward to explain the reasons for the fast turnover. The high-light-dependent and normal turnover of the D1 protein have often been treated separately because photoinhibition has been considered to be limited to light levels above the saturation of photosynthesis and because it has been assumed that photoinhibitory damage does not occur at low light. It was even suggested that the D1 protein turns over for reasons not at all related to light-induced damage to PSII (11). The rapid resynthesis of the D1 protein usually makes it impossible to detect the light-dependent loss of the D1 protein if the synthesis is not blocked during the experiment. Furthermore, since the D1 protein is degraded after but not simultaneously with photoinhibition of the reaction center, the dependence of the rate of degradation on light is far from linear (3). It must also be noted that because neither photoinhibition nor degradation of the D1 protein occurs with zero-order kinetics, fixed-time assays have no relevance with respect to the kinetics. The complexity of the kinetics has promoted the suggestions that exposure of plants to high light does not necessarily induce degradation of the D1 protein (12) or that the relationship between photoinhibition and D1 protein degradation is obscure (13). It has also been suggested that the degradation is a specific feature of laboratory-grown plants while adaptation of field-grown plants to full sunlight does not involve enhanced turnover of the D1 protein (14). The results of the present study strongly suggest that turnover of the D1 protein *in vivo* is always induced by the light-induced irreversible inactivation, or photoinhibition, of PSII.

MATERIALS AND METHODS

Plant Material. Pumpkin (*Cucurbita pepo* L.) plants were grown in a 16-h light/8-h dark rhythm in a phytotron under the photosynthetic photon flux density (PPFD) of 500–700 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during the light phase. The relative humidity was 70% and temperature was 22°C. The leaves were harvested at the end of the dark period. One set of experiments was done with pumpkin plants grown in the field.

Photoinhibition Treatments of Leaves. The leaves were first kept in darkness for 4–6 h with the petioles in lincomycin

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Abbreviations: DCIP, 2,6-dichloroindophenol; PPFD, photosynthetic photon flux density; PSII, photosystem II.

solution (1 g/liter). In some cases, water was used instead of lincomycin as indicated. The illumination was done in a phytotron and the PPFD values were adjusted with neutral density filters (Lee Filters, Andover, England). The temperature of the chamber was 22°C, and the temperature of the illuminated leaves was around 27°C. The petioles were in the lincomycin solution during the whole illumination period. During the illumination, samples were taken from the leaves for measurements of fluorescence, PSII electron transport activity, and D1 protein and chlorophyll content.

Fluorescence Measurements. The initial (F_0) and maximum (F_{max}) fluorescence levels were measured from leaf discs with a pulse amplitude modulated fluorometer (PAM 101; Heinz Walz, Effeltrich, Germany), using a saturating flash (7000 μmol of photons $m^{-2} \cdot s^{-1}$; duration, 1 s) for F_{max}. The leaf discs were dark adapted for 1–2 h before each measurement to allow most of the reversible light-induced quenching phenomena to relax. The FIP fluorescence software (Q_A-Data, Turku, Finland) was used to drive the fluorometer and to analyze the results.

Measurements of PSII Electron Transport Activity. Thylakoids were isolated and light-saturated PSII electron transport activity was measured with 2,6-dichloroindophenol (DCIP) as electron acceptor as described (15). PSII oxygen evolution was measured in one set of experiments with a Hansatech oxygen electrode, with 2,6-dichlorobenzoquinone (1 mM) as electron acceptor.

D1 Protein Content of Thylakoids. The D1 protein content of thylakoids isolated from treated leaves was measured by quantitative immunoblotting as described (16). Thylakoid proteins were solubilized at 65°C (17), separated by SDS/PAGE (12% acrylamide/6 M urea), and electrotransferred to a poly(vinylidene difluoride) membrane (18). The antibody (Research Genetics, Huntsville, AL) used for immunodetection of the D1 protein is directed against amino acids 232–242 of the D1 protein, and BioRad's chemiluminescence kit was used to visualize the D1 protein.

Chlorophyll. Chlorophyll was determined from leaf discs and thylakoid samples as described (19).

RESULTS

The Rate Constant of Photoinhibition, Measured in Lincomycin-Treated Leaves, Is Directly Proportional to Photosynthetic Photon Flux Density. The results of the present study confirm the finding (3) that photoinhibition is a first-order reaction: the decrease in F_V/F_{max} in the presence of lincomycin fitted well to first-order kinetics under all photon flux densities (Fig. 1*A*); first-order behavior under low light is confirmed by the random error residuals at PPFD of 9 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 1*B*). We carefully checked, by incubating leaves in darkness in the presence of lincomycin and by illuminating the leaves without lincomycin, that light-induced irreversible inactivation of PSII was the main factor contributing to the inhibition of PSII even under low light (Fig. 1*A*). Experiments done under high light also confirmed the importance of D1 protein turnover in field-grown pumpkin plants (Fig. 1*C*).

The rate constant of photoinhibition was directly proportional to photon flux density under all PPFD values ranging from 6.5 to 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 2). The proportionality constant p between k_{PI} and PPFD was the same in leaves grown in the field and in a growth chamber, and it was the same as earlier obtained (4) for pumpkin leaves grown under high light (Fig. 2). The leaves contained 480 μmol of chlorophyll per m^2, and by assuming one PSII center per 400 chlorophylls we get 1.2 μmol of PSII per m^2. The proportionality constant p is 0.00022 $h^{-1} \cdot \mu mol^{-1} \cdot m^2 \cdot s$ (Fig. 2*B*), or 6.11×10^{-8} $\mu mol^{-1} \cdot m^2$. The apparent quantum yield of photoinhibition, Φ_{PI}, is now calculated from the initial slope of the decrease in active PSII as follows:

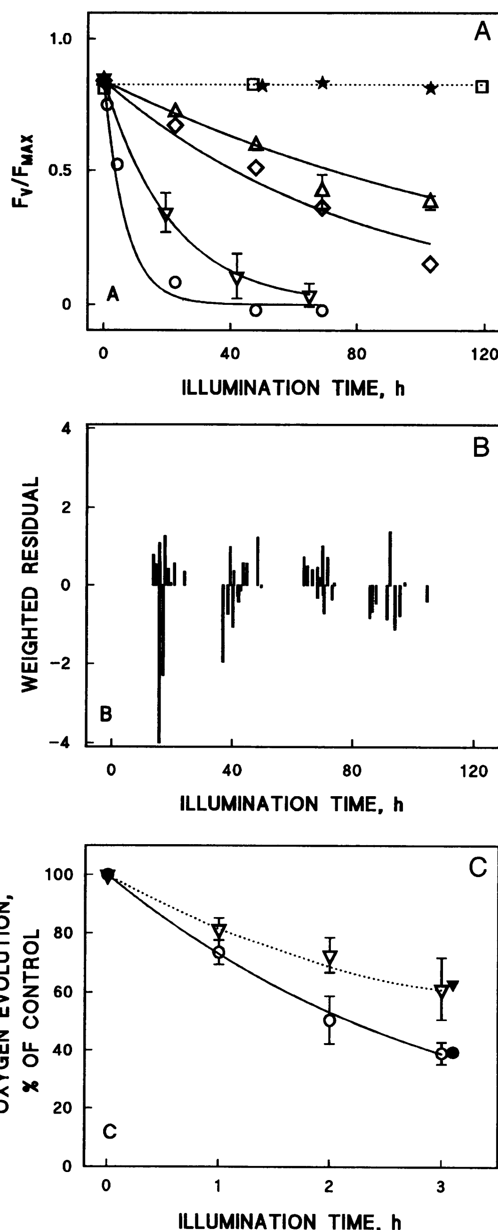

FIG. 1. (*A*) Changes in the F_V/F_{max} ratio of growth chamber-grown pumpkin leaves during incubation at the PPFD values of 548 (○), 307 (▽), 32 (◇), 9 (△), and 0 (★) $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the presence of lincomycin and at 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the absence of lincomycin (□). (*B*) Weighted error residuals from experiments done at a PPFD of 9 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the presence of lincomycin. (*C*) Field-grown pumpkin leaves were illuminated at 1500 μmol of photons $m^{-2} \cdot s^{-1}$ in the presence (circles) and absence (triangles) of lincomycin. F_V/F_{max} (open symbols) was measured from leaf discs and PSII oxygen evolution activity (H_2O to a quinone acceptor) (solid symbols) was measured from thylakoids isolated from the treated leaves. In both *A* and *C*, each point represents the mean of three to four leaves, and the SD is indicated if larger than the symbol. All solid lines are first-order fits. The F_V/F_{max} ratio was 0.83 ± 0.01 before illumination in lincomycin-treated leaves and 0.83 ± 0.02 in leaves not treated with lincomycin.

$$\Phi_{PI} = \frac{1}{\text{PPFD}} \times \frac{d}{dt}\left(1.2\frac{\mu\text{mol}}{\text{m}^2} \times e^{-p \times \text{PPFD} \times t}\right)_{(t=0)}, \quad [1]$$

which has a numerical value of 7×10^{-8} damaged PSII per photon. Φ_{PI} calculated in this way describes the stationary quantum yield of photoinhibition in the absence of lincomycin.

The quantum yield based on light absorbed by PSII pigments instead of light incident on the leaf would be higher by a factor

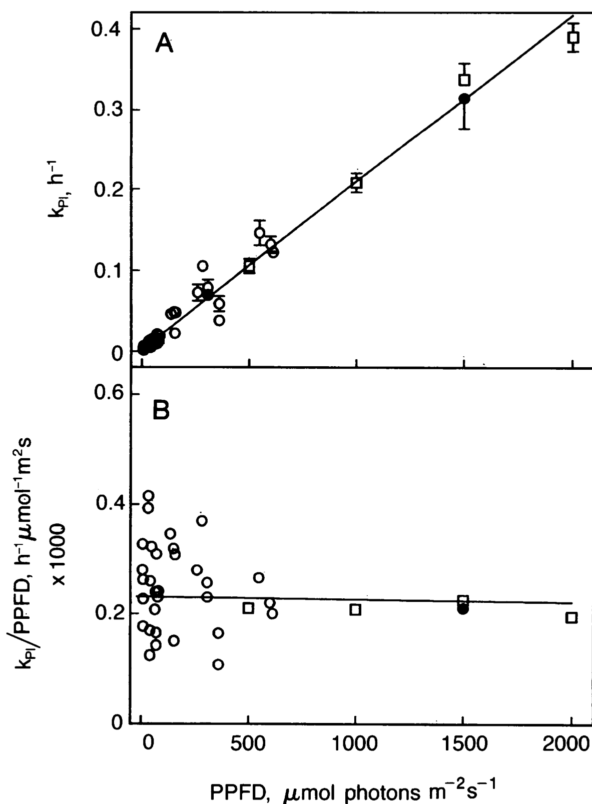

FIG. 2. Photon flux density dependence of the rate constant of photoinhibition, k_{PI}, measured in the presence of lincomycin (*A*) and photon flux density dependence of the proportionality constant *p* between k_{PI} and PPFD, which is a measure of the quantum yield of photoinhibition (*B*). Pumpkin leaves were illuminated in the presence of lincomycin, and k_{PI} values were obtained from F_V/F_{max} ratios measured from leaf discs. The first-order rate constant of photoinhibition was obtained by curve fitting. ○ and □, Plants grown in a growth chamber; ●, plants grown in the field. Each point refers to an experiment with three to four leaves, and bars showing SD are drawn if larger than the symbol. Four data points (□; each point representing three or four independent experiments) were taken from our earlier similar experiments (4) with pumpkin leaves grown in a growth chamber at 1000 μmol of photons $m^{-2} \cdot s^{-1}$.

of ≈2.5, since PSI pigments absorb ≈50%, and ≈20% are reflected or transmitted by the leaf.

PSII Activity and D1 Protein Decrease with F_V/F_{max}. The decrease in PSII electron transfer activity, measured as the light-saturated rate of DCIP reduction by thylakoids isolated from illuminated leaves, correlated linearly with the decrease in F_V/F_{max}, except for very low values of inhibition where the PSII activity decreased more rapidly than the fluorescence ratio (Fig. 3 *A* and *C*). We speculate that this slight deviation from first-order kinetics in the PSII activity data is related to the optical properties of leaves. A rapid initial decrease in the oxygen flash yield of lincomycin-treated pea leaves (9) may have the same origin; this decrease was attributed to an unstable PSII subpopulation, which is photoinhibited with a higher quantum yield than bulk PSII (9). Such an unstable subpopulation has, however, not been detected in isolated thylakoids, which are optically more homogenous than leaves (3).

The degradation rate of the D1 protein was PPFD dependent even under low-light conditions (Fig. 3*B*), the D1 protein content of thylakoids decreasing with the same kinetics as the PSII activity (Fig. 3 *A* and *D*).

Low-Light Photoinhibition Occurred on Top of Leaf Senescence. Senescence is a highly controlled process in which certain cell components are degraded, and it has been shown that senescence can proceed in the absence of chloroplast protein synthesis (20). Senescence-related decrease in the chlorophyll a/b ratio and loss of chlorophyll occurred during the light treatments of the excised leaves with an apparent half-time of 4.6 days without correlation with light intensity. The photosynthetic characteristics of the remaining functional PSII centers are unchanged during early phases of senescence, as evidenced by unchanged quantum yield and light-saturated oxygen evolution activity and an unchanged F_V/F_{max} ratio in partially senescent leaves (ref. 21; see also Fig. 2*A*). Both PSII activity and the D1 protein content of excised pumpkin leaves remained stable in darkness in the presence of lincomycin when measured on a chlorophyll basis (data not shown). However, senescence prevents the use of leaf area as a normalization parameter in long photoinhibition experiments done with excised leaves.

Both F_{max} and F_0 May Increase During Photoinhibition. The F_{max} yield of the leaves decreased if the photoinhibition treatment was done in high light. However, the decrease in F_{max} during photoinhibition gradually changed to an increase in lower photoinhibitory light (Fig. 4 *A* and *C*). In moderate light, F_{max} first decreased and then began to increase (Fig. 4*C*). The F_0 level was relatively constant during photoinhibition under high light, but it increased with decreasing F_V/F_{max} in medium and low light (Fig. 4*B*).

DISCUSSION

Photoinhibition Is Not Only a Stress Reaction. The results of this study show that photoinhibition *per se* is not restricted to high light but occurs *in vivo* under all light intensities. Without the chloroplast protein synthesis-dependent mechanism that repairs the light-induced damage to PSII, plants cannot survive even under low light (Fig. 1*A*). This repair mechanism is normally rapid enough to prevent the symptoms of photoinhibition from appearing under optimal growth conditions.

The Molecular Mechanism of Photoinhibition Must Have a Constant Quantum Yield. The ratio of k_{PI} to PPFD is the same between 6.5 and 2000 μmol of photons $m^{-2} \cdot s^{-1}$ in pumpkin leaves. The constancy of the quantum yield of photoinhibition of lincomycin-treated leaves in the whole range of physiologically relevant photon flux densities suggests that only one reaction governs the kinetics of photoinhibition *in vivo*. Thus, an essential criterion when considering the mechanism of photoinhibition *in vivo* is that the quantum yield of the reaction is independent of light intensity.

Treatment with an inhibitor of chloroplast protein synthesis is necessary for measurement of the rate of photoinhibition, but there is reason to believe that the same reaction occurs even if protein synthesis is allowed.

Two molecular mechanisms of photoinhibition have been shown to function *in vitro*: donor-side (22–24) and anaerobic acceptor-side photoinhibition (25). The acceptor-side mechanism is generally thought to function when oxygen-evolving PSII preparations or thylakoids are illuminated *in vitro* in the absence of added electron acceptors. In addition to the donor and acceptor side mechanisms, a specific low-light mechanism of D1 protein turnover was recently proposed (26). This low-light-syndrome is here considered as a third mechanism of photoinhibition since the rapid low-light-induced degradation of the D1 protein would lead to the irreversible loss of PSII activity even if the degradation occurred without preceding inhibition. Elucidation of the degradation pattern of the D1 protein has so far been the only method available for resolving the mechanism of photoinhibition *in vivo*, and the published results point to the acceptor-side mechanism (27).

The acceptor-side mechanism (25) requires at least two quanta: a priming charge separation must first singly reduce the first stable electron acceptor (Q_A) to make it possible for

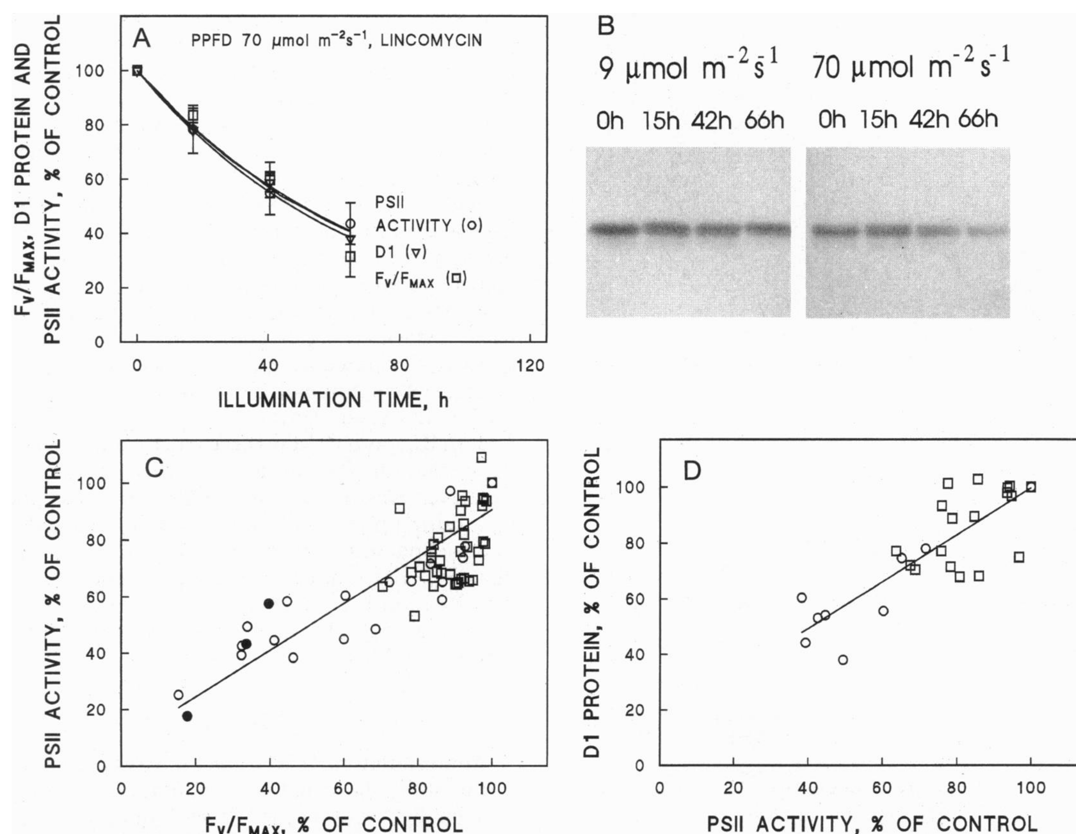

FIG. 3. Light-saturated PSII activity, D1 protein content of thylakoids, and ratio of variable/maximum fluorescence during illumination of pumpkin leaves in the presence of lincomycin. Fluorescence was measured from discs punched from the leaves, and the PSII electron transfer activity and D1 protein content were measured from thylakoids isolated from the treated leaves. A representative experiment with four leaves is shown in *A* and an immunoblot of the D1 protein from thylakoids isolated from pumpkin leaves after illumination at 9 and 70 μmol of photons $m^{-2} \cdot s^{-1}$ for 0, 15, 42, and 66 h, as indicated, is shown in *B*. Correlation between PSII activity and F_V/F_{max} is shown in *C* for pumpkin leaves incubated at 9 (□), 70 (○), and 1500 (●) μmol of photons $m^{-2} \cdot s^{-1}$ for different times. PSII activity was measured as reduction of DCIP (□ and ○) or as oxygen evolution (H_2O to dichlorobenzoquinone; ●). The experiments at 1500 μmol of photons $m^{-2} \cdot s^{-1}$ were done with field-grown pumpkin plants and the others were done with plants grown in a growth chamber. (*D*) Correlation between the loss of PSII activity (H_2O to DCIP) and decrease of the D1 protein content of thylakoids is shown for pumpkin leaves illuminated at 9 (□) and 70 (○) μmol of photons $m^{-2} \cdot s^{-1}$. (*C* and *D*) Each point represents an experiment with one leaf. Control rate of DCIP reduction was 435 ± 60 μmol of DCIP per mg of chlorophyll per h and the control rates of dichlorobenzoquinone-dependent oxygen evolution were 238 ± 50 μmol of O_2 per mg of chlorophyll per h in the thylakoids isolated from lincomycin-treated leaves and 232 ± 9 μmol of O_2 per mg of chlorophyll per h in thylakoids isolated from leaves not treated with lincomycin.

another charge separation to yield a doubly reduced Q_A. At PPFD of 6.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the average time between two quanta absorbed by the same PSII reaction center in pumpkin leaves is ≈0.2 s, long enough for virtually all Q_A to become oxidized. A significant contribution of acceptor-side photoinhibition or any other multiquantum reaction is much less probable at low light than at high photon flux densities. Since it is likely that the same mechanism is responsible for photoinhibition *in vivo* under both high and low light, we have to conclude that the anaerobically characterized acceptor-side mechanism does not as such contribute to photoinhibition *in vivo*. Secondly, model calculations show that the quantum yield of the specific low-light mechanism (26) increases enormously with decreasing photon flux density (28), and therefore a significant contribution from this mechanism does not comply with our observed constancy of the quantum yield. The contradiction between our results and the data on light dependence of the amount of D1 protein degraded during a 4-h treatment of *Chlamydomonas* (28) may be only apparent, as our data show photoinactivation while the data in ref. 28 show D1 protein degradation.

Donor-side photoinhibition consists of several reactions, which sum into a non-first-order reaction (24). The kinetic pattern indicates that the donor-side mechanism does not function in intact leaves in the same way as it functions when the oxygen-evolving complex has been deliberately inactivated. However, it is possible that the primary reaction of photoinhibition *in vivo* is the oxidation of nearby pigment or protein components by one of the highly oxidizing electron donors of PSII. Such oxidation resembles donor-side photoinhibition.

Based on the reciprocity between the intensity and duration of illumination, Park *et al.* (9) suggest that PSII cumulatively registers absorbed photons and becomes damaged, on the average, only after a large number of photons. We find this explanation kinetically problematic, since the observed first-order kinetics of photoinhibition strongly suggest a one-photon reaction.

The quantum yield of photoinhibition *in vivo*, from 7×10^{-8} (based on incident light) in the present study to 3×10^{-7} (calculated for the bulk PSII in ref. 9 based on light absorbed by PSII pigments) is the same order of magnitude as that of the photoinhibition of oxygen-evolving PSII membrane fragments (29) or as can be estimated by recalculating from photoinhibition of isolated thylakoids illuminated in the absence of added electron acceptors (30, 31). This similarity suggests that the aerobic acceptor-side photoinhibition *in vitro* may occur with the same mechanism as photoinhibition *in vivo*. However, the occurrence of photoinhibition in low light suggests that this mechanism is not the acceptor-side photoinhibition mechanism characterized in the anaerobic case (25). It is also possible that photoinhibition *in vivo* follows a mechanism not yet

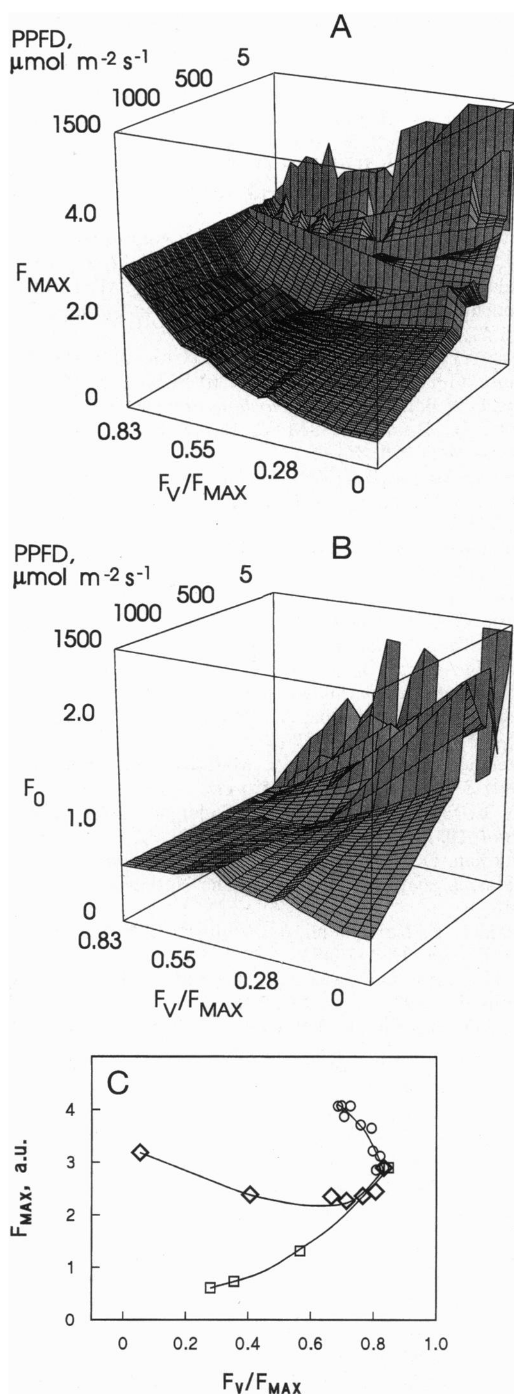

FIG. 4. Behavior of F_{max} (*A* and *C*) and F_0 (*B*) fluorescence levels as a function of the F_V/F_{max} ratio during illumination of pumpkin leaves in the presence of lincomycin under different PPFD values. Surface graphs were obtained by unweighted linear interpolation of F_{max} and F_0 values first along the F_V/F_{max} axis and then along the PPFD axis. Three representative graphs are shown in *C*, where each experimental point represents the mean of three to four leaves illuminated at 9 (○), 135 (◇), or 1500 (□) μmol of photons $m^{-2} \cdot s^{-1}$. a.u., Arbitrary units.

characterized *in vitro*, or that some known mechanism is only partly operational.

Photoinhibition Senses All Light but Down-Regulation of PSII May Sense Excess Light Only. The purpose of using lincomycin was to specifically inhibit D1 protein synthesis, which would have masked the kinetics of photoinhibition. Down-regulatory reactions of PSII (32–36) are usually considered to be less dependent on chloroplast protein synthesis. However, inhibiting chloroplast protein synthesis may also partly or totally block the down-regulation of PSII, as protein turnover may be involved in the photoprotective mechanisms (37). Inhibition of down-regulation would explain why the k_{PI} versus PPFD curve does not bend at high light (Fig. 2*A*).

The rate of photoinhibition depends on total irradiance and not on the amount of excess light that cannot be dissipated by photosynthesis (Fig. 2). In this respect, photoinhibition differs from reversible down-regulation, which lowers the quantum efficiency of active, open PSII reaction centers (Φ_{PSII}). The light response curve of Φ_{PSII} is sigmoidal, with the most gentle slope below the light saturation of photosynthesis (38). Such behavior is expected if down-regulation senses excess energy instead of all light. Correlation with excess light is also inferred in the down-regulation-related synthesis of zeaxanthin from other xanthophylls (14, 39). Unambiguous quantification of the effect of down-regulation on the rate constant of photoinhibition remains a challenge for further study.

D1 Protein Turnover Depends on Photoinhibition Even in Low Light. The relationship between the normal rapid turnover of the D1 protein (at low light) (10, 40, 41) to the photoinhibitory rapid turnover under high light (see, e.g., ref. 2) has been a matter of debate since it has been assumed that photoinhibitory damage does not occur at low light. Low light is especially suitable for examining the reason for the degradation of the D1 protein since the lag between the loss of PSII activity and the degradation of the D1 protein is negligibly short if photoinhibition is very slow. The finding (Fig. 3) that the degradation of the D1 protein under low light depends on photon flux density in exactly the same way as photoinhibition depends on PPFD under all light intensities strongly suggests that there is only one dominant turnover mechanism of the D1 protein and that this mechanism is degradation of the D1 protein after photoinhibitory damage. The kinetics of photoinhibition should therefore always be taken into account when considering the kinetics of the degradation of the D1 protein (see ref. 3).

The finding that pumpkin plants grown under field conditions behaved in the same way as those grown under high light in a growth chamber (4) indicates that the rapid turnover of the D1 protein occurs in field-grown plants as well. This result supports our previous finding (4, 42) that the fast turnover of the D1 protein is an essential feature of plants growing under high light.

Is Increase in F_0 Masked by a Change in the Overall Fluorescence Yield During Photoinhibition? A decrease in F_{max} usually accompanies the decrease in the F_V/F_{max} ratio during photoinhibition treatments under high light, while the behavior of F_0 is more variable (43–46). The light-dependent behavior of F_{max} and F_0 in the presence of lincomycin (Fig. 4) lend support to the hypothesis originally presented by Björkman (43): the decrease in PSII activity results in an increase in F_0, but a simultaneous change in the overall level of fluorescence (both F_0 and F_{max}) can mask this increase. The underlying reasons for the changes in F_0 and F_{max} remain to be evaluated; in light of earlier results (21), the increase in the overall fluorescence level under low light probably is not related to senescence.

Photoinhibition Is as Common as Light. The occurrence of photoinhibition even under extremely low light reveals the ecological importance of this phenomenon. Contrary to what was thought in the past, photoinhibition is not confined to stress conditions. Thus, any defects in the delicate machinery functioning in the repair of the photoinhibitory damage (1, 2) are potentially hazardous to plants irrespective of their growth conditions.

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