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 s⁻¹) were illuminated under photon flux densities ranging from 6.5 to 1500 μmol m⁻² 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.

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 μmol m⁻² 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.

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.

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

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solution (1 g/liter). In some cases, water was used instead of lincomycin as indicated. The illumination was done in a phytootron 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 (F₀) and maximum (Fₘₐₓ) 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⁻²s⁻¹; duration, 1 s) for Fₘₐₓ. 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 Fₐ photoprotection software (Qₐ-D ata, Turku, Finland) was used to drive the fluorometer and to analyze the results.

**Measurements of PSI Electron Transport Activity.** Thylakoids were isolated and light-saturated PSI electron transport activity was measured with 2,6-dichloroindophenol (DCIP) as electron acceptor as described (15). PSI 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 electroblotted 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 F₉/Fₘₐₓ in the presence of lincomycin fitted well to first-order kinetics under all photon flux densities (Fig. 1A); first-order behavior under low light is confirmed by the random error residuals at PPFD of 9 μmol m⁻²s⁻¹ (Fig. 1B). 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. 1A). Experiments done under high light also confirmed the importance of D1 protein turnover in field-grown pumpkin plants (Fig. 1C).

The rate constant of photoinhibition was directly proportional to photon flux density under all PPFD values ranging from 6.5 to 1500 μmol m⁻²s⁻¹ (Fig. 2). The proportionality constant ρ between ρ 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 m², and by assuming one PSI center per 1000 chlorophylls we get 1.2 μmol of PSII per m². The proportionality constant ρ is 0.00022 h⁻¹μmol⁻¹m⁻²s⁻¹ (Fig. 2B), or 6.11 × 10⁻⁸ μmol ml⁻¹m²⁻¹s⁻¹. The apparent quantum yield of photoinhibition, Φₚₚ, is now calculated from the initial slope of the decrease in active PSII as follows:

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\Phi_p = \frac{1}{\text{PPFD}} \times \frac{d}{dt} (1.2 \mu \text{mol m}^{-2} \times e^{-\rho \times \text{PPFD} \times t}) \quad (t = 0),
\]

which has a numerical value of 7 × 10⁻⁸ damaged PSII per photon. Φₚₚ calculated in this way describes the stationary quantum yield of photoinhibition in the absence of lincomycin.

The quantum yield based on light absorbed by PSI pigments instead of light incident on the leaf would be higher by a factor.
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 Fv/Fm ratio in partially senescent leaves (ref. 21; see also Fig. 2A). 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 Fv/Fm and F0 May Increase During Photoinhibition.** The Fmax yield of the leaves decreased if the photoinhibition treatment was done in high light. However, the decrease in Fmax during photoinhibition gradually changed to an increase in lower photoinhibitory light (Fig. 4A and C). In moderate light, Fmax first decreased and then began to increase (Fig. 4C). The F0 level was relatively constant during photoinhibition under high light, but it increased with decreasing Fv/Fm in medium and low light (Fig. 4B).

**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-dependence mechanism that repairs the light-induced damage to PSII, plants cannot survive even under low light (Fig. 1A). 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 kph to PPFD is the same between 6.5 and 2000 μmol of photons m⁻²s⁻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 vivo: 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 quantum absorptions, a single electron transfer, a single priming charge separation. The single electron transfer decreases the quantum yield of the reaction (QA) to make it possible for...
which function indicates that the donor-side mechanism does not function in intact leaves in the same way 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 photooxidation.

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, after only a large number of photons. We find this explanation kinetically problematic, since the observed first-order kinetics of photooxidation strongly suggest a one-photon reaction.

The quantum yield of photooxidation in vivo, from $7 \times 10^{-8}$ (based on incident light) in the present study to $3 \times 10^{-7}$ (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 photooxidation of oxygen-evolving PSII membrane fragments (29) or as can be estimated by recalculating from photooxidation of isolated thylakoids illuminated in the absence of added electron acceptors (30, 31). This similarity suggests that the aerobic acceptor-side photooxidation in vitro may occur with the same mechanism as photooxidation in vivo. However, the occurrence of photooxidation in low light suggests that this mechanism is not the acceptor-side photooxidation mechanism characterized in the anaerobic case (25). It is also possible that photooxidation in vivo follows a mechanism not yet understood.
characterized in vitro, or that some known mechanism is only partly operational.

**Photo inhibition 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 photo inhibition. 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 $k_T$ versus PPFD curve does not bend at high light (Fig. 2A).

The rate of photo inhibition 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 irreversible down-regulation, which lowers the quantum efficiency of active, open PSII reaction centers ($\Phi_{PSII}$). The light response curve of $\Phi_{PSII}$ 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 photo inhibition remains a challenge for further study.

**D1 Protein Turnover Depends on Photo inhibition Even in Low Light.** The relationship between the normal rapid turnover of the D1 protein (at low light) (10, 40, 41) to the photo inhibitor rapid turnover under high light (see, e.g., ref. 2) has been a matter of debate since it has been assumed that photo inhibition 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 photo inhibition 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 photo inhibition 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 photo inhibition damage. The kinetics of photo inhibition 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 F0 Masked by a Change in the Overall Fluorescence Yield During Photo inhibition?** A decrease in $F_{max}$ usually accompanies the decrease in the $F_v/F_{max}$ ratio during photo inhibition 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.

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

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