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Recovery from Photoinhibition in Peas (*Pisum sativum* L.) Acclimated to Varying Growth Irradiances[1]

Role of D1 Protein Turnover

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D1 protein turnover and restoration of the photochemical efficiency of photosystem II (PSII) after photoinhibition of pea leaves (*Pisum sativum* L. cv Greenfeast) acclimated to different light intensities were investigated. All peas acclimated to different light intensities were able to recover from photoinhibition, at least partially, at light intensities far above their growth light irradiance. However, the capacity of pea leaves to recover from photoinhibition under increasing high irradiances was strictly dependent on the light acclimation of the leaves; i.e. the higher the irradiance during growth, the better the capacity of pea leaves to recover from photoinhibition at moderate and high light. In our experimental conditions, mainly D1 protein turnover-dependent recovery was monitored, since in the presence of an inhibitor of chloroplast-encoded protein synthesis, lincomycin, only negligible recovery took place. In darkness, neither the restoration of PSII photochemical efficiency nor any notable degradation of damaged D1 protein took place. In low light, however, good recovery of PSII occurred in all peas acclimated to different light intensities and was accompanied by fast degradation of the D1 protein. The rate of degradation of the D1 protein was estimated to be 3 to 4 times faster in photoinhibited leaves than in nonphotoinhibited leaves under the recovery conditions of 50 μmol of photons m^{-2} s^{-1}. In moderate light of 400 μmol of photons m^{-2} s^{-1}, the photoinhibited low-light peas were not able to increase further the rate of D1 protein degradation above that observed in nonphotoinhibited leaves, nor was the restoration of PSII function possible. On the other hand, photoinhibited high-light leaves were able to increase the rate of D1 protein degradation above that of nonphotoinhibited leaves even in moderate and high light, ensuring at least partial restoration of PSII function. We conclude that the capacity of photoinhibited leaves to restore PSII function at different irradiances was directly related to the capacity of the leaves to degrade damaged D1 protein under the recovery conditions.

Photoinhibition of PSII, which occurs under sustained high irradiance, is a phenomenon currently under intense study (Prasil et al., 1992; Aro et al., 1993b). Although several different mechanisms have been proposed for photoinhibition on the basis of in vitro experiments, the exact mechanism of in vivo photoinhibition of PSII electron transport is still unknown. After initial light-induced inactivation of PSII electron transport, a subsequent event in photoinhibition is the irreversible damage to the D1 protein, one of the heterodimeric polypeptides of the PSII reaction center complex (Aro et al., 1993b). Restoration of PSII electron transport activity after such photoinhibitory damage can only occur via degradation and de novo synthesis of the D1 protein and reactivation of the PSII complex. Additionally, reversible photoinhibition or "down-regulation" of PSII occurs at high photon flux densities, but this inhibition state relaxes without D1 protein synthesis and should be regarded as a regulatory mechanism for the dissipation of excess excitation energy. These regulatory mechanisms generally require minutes rather than hours to relax (Krause and Weiss, 1991), although some slowly relaxing protective mechanisms have also been reported (Demmig-Adams, 1990).

PSII of high-light-grown or sunlight-grown plants appears to withstand high irradiances without incurring photoinhibition. This is achieved by a rapid degradation and synthesis of the D1 protein and reassembly of the PSII complex (Öquist et al., 1992a, 1992b; Tyystjärvi et al., 1992; Aro et al., 1993a). However, the role of rapid light-dependent turnover of the D1 protein under high irradiance in vivo remains controversial; some authors (Demmig-Adams and Adams, 1992) suggest that D1 protein turnover plays only a minor role in protection against photoinhibition, especially in sunlight-acclimated plants. Low-light-grown or shade plants, on the other hand, do not seem to rely on fast turnover of the D1 protein (Öquist et al., 1992a; Tyystjärvi et al., 1992; Aro et al., 1993a); therefore, they become photoinhibited at lower irradiances than high-light leaves.

Since D1 protein synthesis is strictly light dependent (Prasil et al. 1992), it is not surprising that light is required for the recovery of PSII function. It is puzzling, however, that the fastest rates observed for restoration of PSII function generally need only low light intensities, usually less than 50 μmol of photons m^{-2} s^{-1} (Greer et al., 1986; Skogen et al., 1986;

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Abbreviations: D1, D1 reaction center protein of PSII; ELIP, early light-inducible protein; F_V and F_M, variable and maximum fluorescence, respectively; LHCII, light-harvesting Chl *a*/*b* proteins.

Greer and Laing, 1988). There is also some evidence that even moderate light, as low as 200 μmol of photons m^{-2} s^{-1}, may restrict recovery from photoinhibition (Greer and Laing, 1988; Le Gouallec et al., 1991). These studies have raised an important question: Does some D1 protein turnover indeed function at high irradiances at which photoinhibition becomes manifest? Certainly photoinhibition of PSII is significantly enhanced in the presence of inhibitors of chloroplast-encoded protein synthesis (Greer et al., 1986; Lidholm et al., 1987; Schuster et al., 1988; Tyystjärvi et al., 1992). Although these results suggest a crucial role for the repair cycle of PSII via D1 protein turnover during high illumination, the relationship between D1 protein turnover and the capacity of recovery from photoinhibition with respect to incident irradiance is still poorly understood.

To test our hypothesis that the restoration of PSII function during recovery from photoinhibition is directly linked to the capacity of leaves to degrade D1 protein, we have investigated the capacity of peas (*Pisum sativum* L.) acclimated to different light intensities to recover from photoinhibition under various photon flux densities. Our results emphasize that light acclimation of pea leaves directly determines the capacity of PSII to recover from photoinhibition under increasing photon flux densities; in turn, this is directly related to the capacity of light-acclimated peas to degrade D1 protein under recovery conditions.

MATERIALS AND METHODS

Plant Material

Peas (*Pisum sativum* L. cv Greenfeast) were grown in a compost:perlite mixture in growth chambers under photon flux densities of 65 (low-light peas), 250 (medium-light peas), and 700 μmol m^{-2} s^{-1} (high-light peas) during a 12-h photoperiod at 23°C. Seedlings (2–5 weeks old, depending on the photon flux density during growth) were used for the experiments. To ensure the uniformity of leaves, the fourth leaf pair representing the youngest, fully developed pair of leaves was always used.

Light Treatment of Leaf Discs

Leaf discs, floating adaxial side up on water, were illuminated with an ARC HMI studio lamp. Light was passed through a heat filter, and different photon flux densities to induce photoinhibition were obtained by changing the distance between the lamp and the leaf discs. To induce 40 to 50% inhibition in F_V/F_M, low-light peas were illuminated at a PPFD of 1600, medium-light peas were illuminated at 2300, and high-light peas were illuminated at 2800 μmol m^{-2} s^{-1} for 2.5 to 3 h. Different recovery light regimes were obtained by using neutral density filters. Temperature was controlled at 23°C during both photoinhibition and recovery experiments.

Lincomycin Treatment of Pea Leaves

When the role of chloroplast-encoded protein synthesis was studied in relation to the susceptibility of pea leaves to photoinhibition and subsequent recovery, the petioles were first immersed in a 0.6 mM lincomycin solution and the leaves were illuminated in a fume hood at a photon flux density of 20 μmol m^{-2} s^{-1} for 3 to 4 h. The volume of lincomycin solution taken up by the leaves corresponded to 100 to 150% of the leaf water content. No incorporation of [^{35}S]Met (see below) into the D1 protein of lincomycin-treated leaves was observed, indicating that the chloroplast-encoded protein synthesis was uniformly inhibited in the leaves. Photoinhibition of lincomycin-treated leaf discs was induced by illumination at 1600 μmol of photons m^{-2} s^{-1} for 1 h.

Pulse and Chase Experiments with L-[^{35}S]Met

Radioactive labeling of the leaf discs with [^{35}S]Met and chasing of radioactivity in the D1 protein were performed as described earlier (Aro et al., 1993a). By chasing the radioactivity in prelabeled D1 protein, we specifically measured the degradation of the D1 protein. When the rate of D1 protein degradation was studied in photoinhibited leaves under various recovery conditions, the labeling was performed for 2 h under photoinhibitory light, the intensity of which was chosen according to individual growth irradiance of the light-acclimated peas. Subsequently, the leaf discs were washed twice with 10 mM cold Met and further incubated in cold Met under the same photoinhibitory light for 1 h to eliminate radioactive nonprotein metabolic pools before the initial chase sample (chase, 0 min) was taken. The leaf discs were then transferred to various light intensities for recovery from photoinhibition.

Thylakoid membranes were rapidly isolated and stored in liquid nitrogen as described earlier (Aro et al., 1993a). Thylakoid polypeptides were solubilized (65°C, 5 min) and electrophoretically separated essentially according to the method of Ljungberg et al. (1986); 4 M urea was used in both the solubilizing and gel buffers. After SDS-PAGE, the gels were stained, treated with amplifier, and dried. Dry gels were exposed to Molecular Dynamics (Sunnyvale, CA) storage phospho screens for approximately 82 h. The screens were scanned on a Molecular Dynamics 400S PhosphorImager, and quantification of the radioactivity in D1 protein and LHCII polypeptides was made using the ImageQuant volume integration software. Radioactivity in LHCII polypeptides was generally used as an internal standard when the rate of D1 protein degradation was calculated, since the light treatments did not affect the radioactivity level of LHCII polypeptides during the chase period.

Chl Fluorescence Measurements

Measurements of the F_V/F_M of leaf discs were made with a Hansatech plant efficiency analyzer. Before the measurements, the leaf discs were always dark adapted for 30 min to allow relaxation of fast fluorescence-quenching components. We found that this long dark adaptation was required especially for photoinhibited material.

Chl Determinations

Chl was extracted in 80% acetone (25 mM Hepes, pH 7.5) and quantified using the extinction coefficients and wavelengths determined by Porra et al. (1989).

Curve Fitting

The decrease in radioactivity of the D1 protein during the chase period was fitted to a first-order equation, weighting the data points individually according to their SD values. The Fig. P software (Biosoft, Cambridge, UK) was used for curve fitting.

Only apparent rate constants could be obtained for degradation of photodamaged D1 protein in photoinhibited leaves, since the proportion of photodamaged D1 protein out of the radiolabeled pool of the D1 protein in the beginning of the chase is hard to assess with great accuracy.

RESULTS

Restoration of the F_V/F_M Ratios in Photoinhibited Leaves under Various Light Conditions

Our aim was to induce an equal level of photoinhibition of PSII in peas acclimated to different light intensities to have uniformly photoinhibited leaves for studies of the capacity of PSII to recover from photoinhibition. The decrease in F_V/F_M under high irradiance approaches with time a steady-state level (Fig. 1), which depends not only on the photon flux density but also on the light acclimation of the leaves (Tyystjärvi et al., 1992; Aro et al., 1993a). Thus, we found that it was more reliable to induce photoinhibition that approaches the same steady-state level in all the differently light-acclimated peas. This was done by choosing the photon flux density for the treatment of pea leaves according to their growth irradiance; low-light peas were treated at a PPFD of 1600 μmol m^{-2} s^{-1} for 3 h, medium-light peas were treated at 2300 μmol m^{-2} s^{-1} for 2.5 h, and high-light peas were treated at 2800 μmol m^{-2} s^{-1} for 2.5 h (Fig. 1). These photoinhibition treatments induced similar amounts of functional versus nonfunctional PSIIs in peas acclimated to different light intensities.

The decline in photochemical efficiency of PSII during photoinhibition was determined by the decline in the Chl F_V/F_M ratio after 30 min of dark adaptation. F_V/F_M ratios have been shown to be linearly correlated with both the quantum yields of light-limited O_2 evolution (Demmig and Björkman, 1987) and the number of functional PSII centers (Öquist et al., 1992a, 1992b).

As shown in Figure 1A, low-light peas recovered from photoinhibition nearly as well, whether the PPFD was 50 or 200 μmol m^{-2} s^{-1}. At 400 μmol m^{-2} s^{-1}, only partial restoration of F_V/F_M took place, whereas at 1000 μmol m^{-2} s^{-1} no recovery was possible; however, no further inhibition was evident either. Medium-light peas showed better restoration of the photochemical efficiency of PSII at PPFDs of 400 and 1000 μmol m^{-2} s^{-1} than low-light peas (Fig. 1B). High-light peas recovered from photoinhibition with almost equally fast rates at 50, 200, and 400 μmol of photons m^{-2} s^{-1}; even at 1000 μmol m^{-2} s^{-1} notable restoration of F_V/F_M took place (Fig. 1C). Irrespective of the light acclimation of the pea leaves, maximum recovery was obtained at 50 μmol of photons m^{-2} s^{-1}.

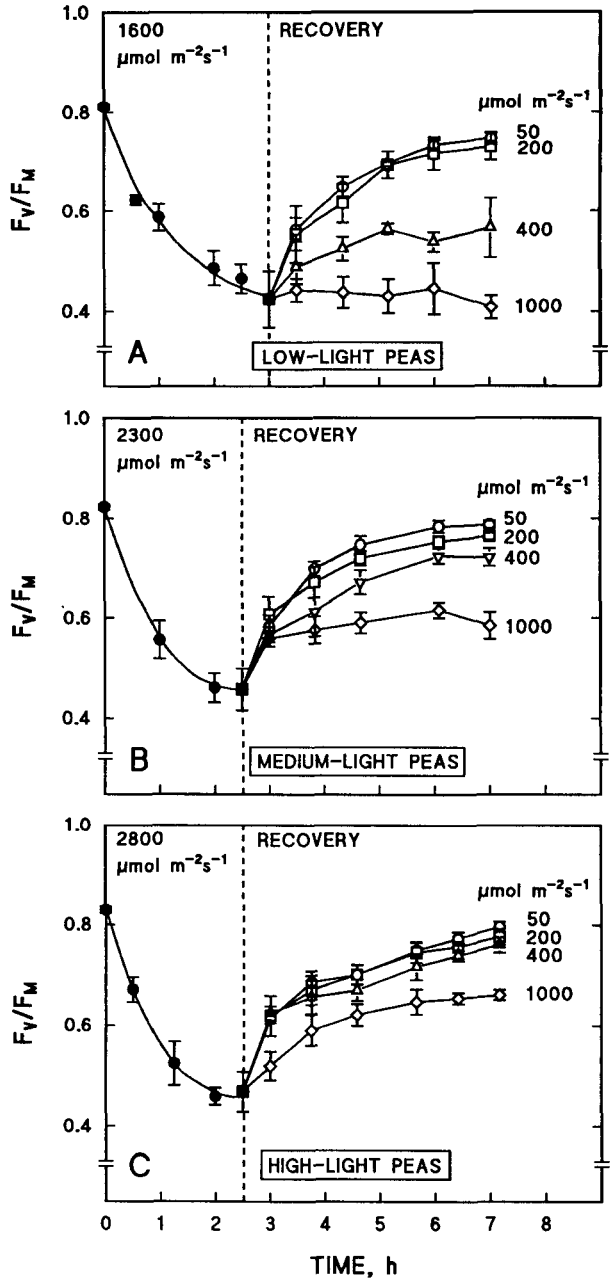

Figure 1. Restoration of F_V/F_M at various photon flux densities after photoinhibition of pea leaves acclimated at different light intensities. Peas were grown at 65 (A), 250 (B), and 700 μmol of photons m^{-2} s^{-1} (C). Leaf discs were illuminated to induce 40 to 50% photoinhibition of PSII, and restoration of the F_V/F_M ratios was subsequently followed at irradiances of 50 (○), 200 (□), 400 (△), and 1000 (◇) μmol of photons m^{-2} s^{-1}. Results are means ± SD; n = 5 to 6.

Restoration of F_V/F_M Is Dependent on Chloroplast-Encoded Protein Synthesis

To analyze the role of chloroplast-encoded protein synthesis in recovery from photoinhibition in high- and low-light-acclimated peas, we used the inhibitor lincomycin. Uptake of lincomycin by leaves does not affect photosynthetic function

(Tyystjärvi et al., 1992). Both the high- and low-light leaves treated with lincomycin were severely photoinhibited at 1600 μmol of photons m^{-2} s^{-1} (Fig. 2; 48 and 57% photoinhibition during 1 h, respectively) compared with untreated leaves under the same conditions (7 and 35% photoinhibition in high- and low-light peas, respectively). Significantly, lincomycin markedly reduced the difference in susceptibility to photoinhibition between high- and low-light leaves, confirming our earlier results with pumpkin (Tyystjärvi et al., 1992) and pea leaves (Aro et al., 1993a). When chloroplast-encoded protein synthesis was inhibited with lincomycin, there was a dramatic decrease in recovery at low light (Fig. 2), with only minor restoration of F_V/F_M ratios in both high- and low-light pea leaves. Moreover, in the presence of lincomycin, total darkness prevented even this slight restoration in F_V/F_M (results not shown); this indicates that some very low level of light-dependent, but protein synthesis-independent, restoration of PSII activity also existed. The molecular background of this restoration of PSII is unknown.

Restoration of the F_V/F_M ratios after photoinhibition has often been reported to be biphasic (Krause and Weis, 1991; Schnettger et al., 1992b; Van Wijk and van Hasselt, 1992). It has been postulated that the initial fast recovery phase, which lasts 10 to 50 min, represents reactivation of PSII centers without D1 protein turnover, whereas the slow phase, which lasts several hours, would represent actual repair of PSII centers via D1 protein turnover (Schnettger et al., 1992b). In our experiments with peas, no distinct separation of the restoration of F_V/F_M values into fast and slow phases could be made (Fig. 1). Peas showed only minor D1 protein turnover-independent restoration of F_V/F_M, which is clearly demonstrated by the lincomycin experiments (Fig. 2). The apparent absence of the fast protein turnover-independent recovery phase is likely to be due to our long dark adaptation (30 min) of pea leaves prior to measuring the F_V/F_M ratios. Dark relaxation of the fluorescence components in photoinhibited leaves is probably a complex process, including

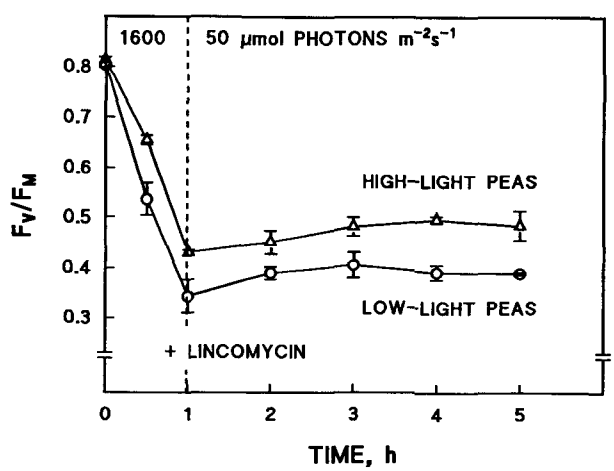

Figure 2. Effect of lincomycin on the restoration of F_V/F_M ratios at 50 μmol of photons m^{-2} s^{-1} after photoinhibition of leaf discs at 1600 μmol of photons m^{-2} s^{-1}, which also occurred in the presence of lincomycin. Peas were grown at 65 (○) and 700 (△) μmol of photons m^{-2} s^{-1}. Results are means ± SD; $n = 5$.

more than the energy-dependent quenching component, since a relatively long time, up to 30 min, was required for relaxation (data not shown). It is likely that a prominent fast recovery phase often seen after photoinhibition (Schnettger et al., 1992b) can be regarded as the reversible down-regulation of PSII, which mainly relaxes in darkness. The long dark-adaptation period of pea leaves before the F_V/F_M ratios were recorded simplified the kinetics of our recovery experiments and also allowed us to follow the restoration of F_V/F_M ratios related mainly to the D1 protein turnover-dependent recovery of PSII function. Results of recovery experiments in the presence of lincomycin strongly support this conclusion (Fig. 2).

Degradation of the D1 Protein in Nonphotoinhibited and Photoinhibited Leaf Discs under Recovery Conditions

During illumination, both damage to PSII and repair of damaged PSII centers occur simultaneously. When leaves are photoinhibited during sustained high irradiance, the rate of damage to PSII exceeds the rate of repair (Greer et al., 1986). Since no net loss of the D1 protein from thylakoid membranes can be detected, in spite of severe photoinhibition of PSII (Kettunen et al., 1991; Schnettger et al., 1992a), accumulation of PSII centers with damaged but nondegraded D1 protein in the grana membranes can be envisaged under photoinhibitory conditions. The restoration of PSII electron transport is possible only after the damaged D1 protein has been degraded and replaced with a newly synthesized one. Therefore, if PSII centers with damaged D1 protein indeed accumulate during photoinhibition of leaves, there should be a fast degradation of the D1 protein when photoinhibited leaves are transferred to low light for recovery, much faster than in nonphotoinhibited leaves under the same conditions.

To test this hypothesis, we next compared the rate of degradation of the D1 protein in both control (nonphotoinhibited) and photoinhibited light-acclimated pea leaves under conditions similar to those used in the recovery experiments in which F_V/F_M ratios were monitored (Fig. 1). D1 protein was radiolabeled by incubating leaf discs with [^{35}S]Met for 2 h at nonphotoinhibitory light (control leaves) or at photoinhibitory light (photoinhibited leaves). After labeling, the leaf discs were further illuminated at the same photon flux density for 1 h before the initial chase sample was taken (chase 0 min). This ensured that a significant portion of the radiolabeled D1 protein in photoinhibited leaves was damaged and targeted for degradation, whereas in nonphotoinhibited control leaves, the number of damaged PSII centers was minimal. About 50% inhibition of PSII was induced under photoinhibitory light in pea leaves acclimated to different light intensities (see "Materials and Methods" for exact PPFDs used). After the incorporation period, D1 protein and LHCII polypeptides were the most heavily labeled thylakoid polypeptides in both control (Fig. 3A, lane 0) and photoinhibited leaf discs (Fig. 3, B and C, lanes 0). During the subsequent chase at an irradiance of 50 μmol of photons m^{-2} s^{-1}, very little loss of radioactivity occurred from the D1 protein in nonphotoinhibited leaves (Fig. 3A). This is in marked contrast to the photoinhibited leaves, which showed a faster rate of degradation of the radiolabeled D1 protein

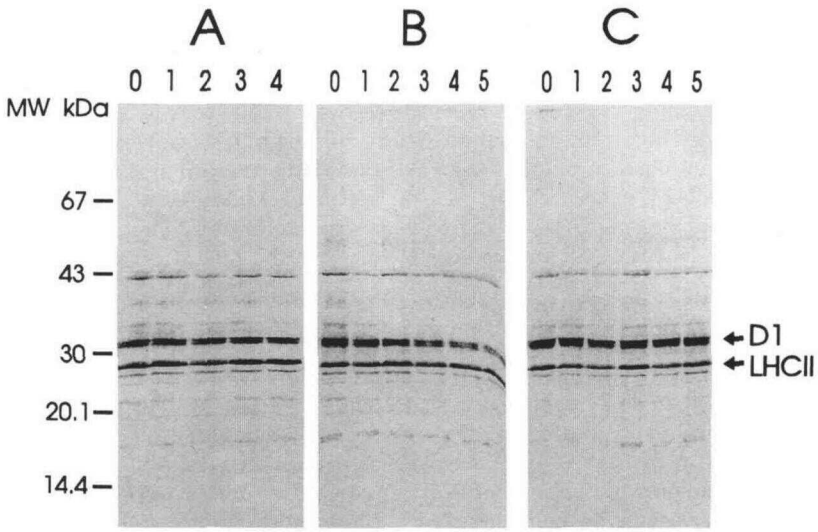

Figure 3. PhosphorImager printout of thylakoid membrane polypeptides after [^{35}S]Met labeling of leaf discs and separation of the polypeptides with SDS-PAGE. Leaf discs from peas grown at 65 μmol of photons m^{-2} s^{-1} were pulse labeled at 200 μmol of photons m^{-2} s^{-1}, which did not induce photoinhibition of PSII (A), and at photoinhibitory light of 1600 μmol of photons m^{-2} s^{-1} (B and C) for 2 h. Subsequently, the leaf discs were washed and incubated in cold Met under the same conditions for 1 h more, before the chase of the radioactivity in the D1 protein was started at 50 μmol of photons m^{-2} s^{-1} (A and B) or in darkness (C). D1 protein was present as a double band representing nonphosphorylated and phosphorylated forms of the D1 protein (Aro et al., 1993a), which in quantitative calculations, however, were taken as one entity. Radioactivity in the D1 protein was chased for 0 (lane 0), 45 (lane 1), 90 (lane 2), 135 (lane 3), 180 (lane 4), and 225 min (lane 5).

upon transfer to recovery conditions of 50 μmol of photons m^{-2} s^{-1} (Fig. 3B). However, if photoinhibited leaves were transferred to absolute darkness, only minor loss of radioactivity from the D1 protein was observed during the chase period (Fig. 3C).

The first-order fits of the degradation of the D1 protein under recovery irradiances of 50 and 400 μmol of photons m^{-2} s^{-1} in control and photoinhibited low-light and high-light peas are shown in Figure 4. In the nonphotoinhibited leaves of both the low- and high-light peas, D1 protein was degraded only slowly at 50 μmol of photons m^{-2} s^{-1}, with an apparent half-life of 6 to 7 h (Fig. 4, A and C). In contrast, the degradation rate of the D1 protein was significantly accelerated if the leaves had been photoinhibited prior to the

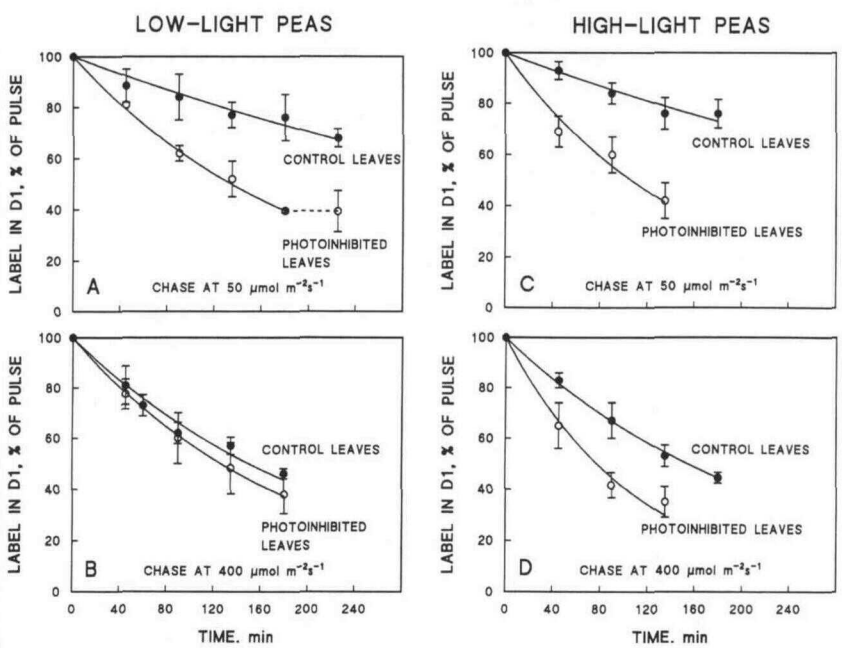

Figure 4. Degradation of the D1 protein at an irradiance of 50 μmol of photons m^{-2} s^{-1} (A and C) and at 400 μmol of photons m^{-2} s^{-1} (B and D) in control, nonphotoinhibited leaves (solid symbols) and in photoinhibited leaves (open symbols) of low-light (A and B) and high-light peas (C and D). Approximately 50% photoinhibition of PSII was induced in low-light peas by illumination at a PPFD of 1600 μmol m^{-2} s^{-1} for 3 h and in high-light peas by illumination at a PPFD of 2800 μmol m^{-2} s^{-1} for 2.5 h. Pulse labeling of thylakoid proteins was as described in Figure 3. Solid lines represent fits to a first-order equation. Data points were weighted according to their SD. Results are means ± SD; n = 2 to 4.

low-light chase, with apparent half-lives of about 2 h (134 and 106 min for low-light and high-light peas, respectively; Fig. 4, A and C). A similar enhancement in the rate of D1 protein degradation after photoinhibition was measured in medium-light peas (data not shown). Thus, good restoration of F_V/F_M in photoinhibited peas at 50 μmol of photons m^{-2} s^{-1} (Fig. 1) was indeed accompanied by a fast rate of D1 protein degradation ($t_{1/2}$ = 2 h), which is much faster than that measured at 50 μmol m^{-2} s^{-1} in leaves without previous photoinhibitory treatment ($t_{1/2}$ = 6–7 h) (Fig. 4, A and C). It is also of interest that the rates of restoration of the F_V/F_M ratios at low light (50 μmol of photons m^{-2} s^{-1}) were not significantly different in pea leaves acclimated to different light intensities (Fig. 1).

In contrast, however, when the irradiance during the recovery phase of low-light peas was increased to 400 μmol m^{-2} s^{-1}, the rate of D1 protein degradation in the photoinhibited leaves (apparent $t_{1/2}$ = 151 min) was not much enhanced from that measured at the same light without previous photoinhibition treatment ($t_{1/2}$ = 165 min) (Fig. 4B). Note also that the restoration of F_V/F_M in low-light peas under 400 μmol of photons m^{-2} s^{-1}, an irradiance about 6-fold higher than their growth irradiance, was very limited (Fig. 1A).

High-light peas, however, behaved quite differently. When the irradiance during the recovery period was increased to 400 μmol of photons m^{-2} s^{-1} (60% of their growth irradiance), the photoinhibited high-light leaves were able to further increase the rate of D1 protein degradation (apparent $t_{1/2}$ = 77 min) relative to that measured at 400 μmol m^{-2} s^{-1} in leaves without previous photoinhibitory treatment ($t_{1/2}$ = 154 min; Fig. 4D). High-light leaves also readily recovered from photoinhibition, even at a PPFD as high as 400 μmol m^{-2} s^{-1} (Fig. 1).

Restoration of the F_V/F_M after photoinhibitory illumination of pea leaves was very limited in darkness: clearly light is required for the recovery of PSII function (Fig. 5A). This is consistent with the fact that the degradation of the D1 protein in photoinhibited leaves also was extremely slow in darkness (Figs. 3C and 5A) and only slightly faster than in nonphotoinhibited pea leaves in the dark (not shown). This contrasts markedly with the situation when recovery was followed at low light (Fig. 5B); fast recovery was always accompanied by fast degradation of the D1 protein.

Finally, we paid special attention to the synthesis of ELIPs, which have been suggested to be induced at high irradiances and may be involved in the protection of leaves against photoinhibition (Adamska et al., 1992). The molecular masses of ELIPs vary; however, in peas a prominent high-light-inducible ELIP of about 17 to 19 kD has been reported (Adamska et al., 1992, 1993). Although we induced nearly 50% photoinhibition of PSII in the pea leaves acclimated to different light intensities (Fig. 1), we could detect no distinct radioactive bands corresponding to ELIP in our experiments (Fig. 3, B and C). Therefore, we wish to emphasize that ELIP synthesis is probably not directly involved in protection against photoinhibition but may be observed only in extremely severe conditions that lead to a net loss of the D1 protein from the thylakoid membranes and to photo-oxidative damage to the whole thylakoid structure. This is consistent with the considerations that the expression of ELIP starts only when the first signs of Chl bleaching become evident (Adamska et al., 1993): in our photoinhibition treatments, no Chl bleaching in pea leaves occurred.

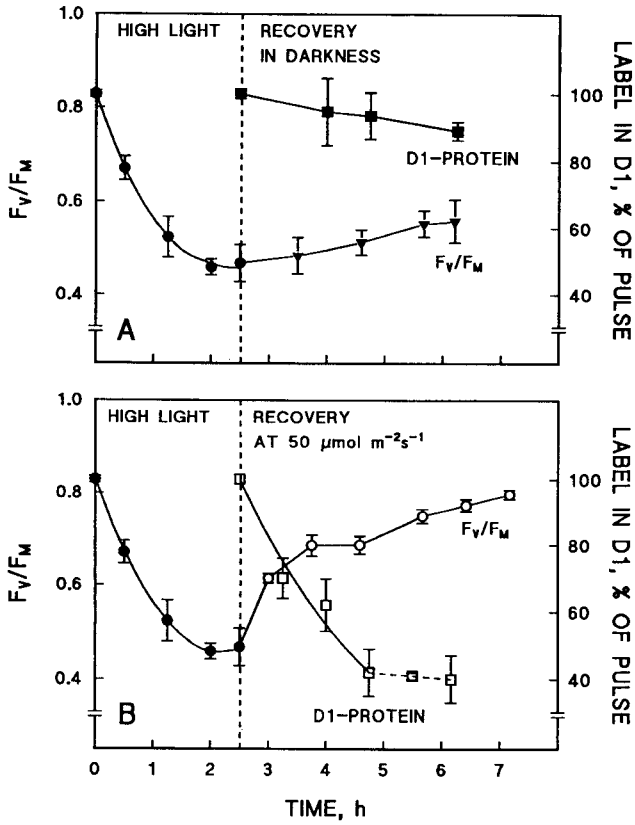

Figure 5. Restoration of the F_V/F_M ratios and degradation of the D1 protein in darkness (A) and at low light (B) after photoinhibitory treatment (2800 μmol of photons m^{-2} s^{-1} for 2.5 h) of leaf discs (●). Experiments were done with high-light peas grown at 700 μmol of photons m^{-2} s^{-1}. Pulse labeling of the thylakoid membranes with [^{35}S]Met was carried out during the high-light treatment (see legends for Figs. 3 and 4). The first part of the loss of radioactivity in the D1 protein at 50 μmol of photons m^{-2} s^{-1} (solid line) is a fit to a first-order equation. D1 protein degradation values are the means ± SD from three independent experiments. F_V/F_M values are the means ± SD of six leaf discs.

DISCUSSION

After photoinhibition, the restoration of the photochemical efficiency of PSII is generally most rapid at low irradiance (Greer et al., 1986; Skogen et al., 1986). Only very poor recovery of PSII photochemistry takes place in darkness, and photon flux densities as low as 200 μmol m^{-2} s^{-1} have been reported to severely inhibit the recovery process (Greer and Laing, 1988; Le Gouallec et al., 1991), at least in the shade-tolerant species used to make this generalization. Our recent findings that high rates of the PSII repair cycle, via rapid turnover of the D1 protein, constitute a survival strategy for plants at high light (Öquist et al., 1992a; Tyystjärvi et al., 1992; Aro et al., 1993a) seem at odds with these earlier results. We have recently shown that, when the PSII repair

cycle is active, photoinhibition of PSII becomes manifest with increasing irradiances only when the rate of D1 protein degradation cannot be further enhanced to rapidly remove all of the damaged D1 protein from PSII centers (Aro et al., 1993a). The capacity of leaves to degrade D1 protein under photoinhibitory light levels was strictly dependent on the type of light acclimation of the leaves, with low-light leaves having a slower rate of D1 protein degradation than high-light leaves (Tyystjärvi et al., 1992; Aro et al., 1993a). Consequently, we wondered whether the light acclimation of leaves and, therefore, their capacity to degrade D1 protein would also determine the light conditions under which recovery from photoinhibition is possible.

In all the pea leaves acclimated to different light intensities, the fastest recovery occurred at the lowest irradiance, 50 μmol of photons m^{-2} s^{-1}, used in our experiments (Fig. 1). However, 200 μmol of photons m^{-2} s^{-1} was almost as good for recovery, even in peas grown at very low irradiance (65 μmol of photons m^{-2} s^{-1}). When the recovery irradiance was further increased, clear differences in the recovery capacity became evident between the differently light-acclimated pea leaves. At 400 μmol m^{-2} s^{-1}, low-light leaves (growth irradiance of 65 μmol of photons m^{-2} s^{-1}) recovered only poorly, whereas high- and medium-light leaves recovered nearly as fast as at 50 μmol m^{-2} s^{-1}. When the recovery irradiance was increased to 1000 μmol m^{-2} s^{-1}, recovery was totally inhibited in low-light leaves but only partially in medium-light and high-light leaves (Fig. 1).

Our results clearly demonstrate that the capacity of leaves to recover from photoinhibition at different light intensities depends on the light acclimation of the leaves; the higher the incident light intensity during growth, the better the capacity of the leaves to recover from photoinhibition at increasing irradiances. According to our hypothesis (Tyystjärvi et al., 1992; Aro et al., 1993a), this behavior should be directly related to the capacity of the leaves to degrade damaged D1 protein, which we have postulated to be an important regulatory factor in the repair cycle of PSII. To test this hypothesis further, we measured the rates of D1 protein degradation in both control and previously photoinhibited leaves.

During the recovery period in low light, the rate of D1 protein degradation in photoinhibited low-light leaf discs (apparent $t_{1/2}$ = 134 min) far exceeded that in nonphotoinhibited leaves ($t_{1/2}$ = 372 min) (Fig. 4A); similar results were obtained with medium-light peas (data not shown) and high-light peas (Fig. 4C). Apparently, the concentration of the substrate of the degradation reaction (i.e. the amount of damaged D1 protein) was always greater in photoinhibited leaves than in control leaves. When photoinhibited leaves with damaged but nondegraded D1 protein were transferred to low light for recovery, fast degradation (Figs. 4A and 5B) and resynthesis (Huse and Nilsen, 1989) of the D1 protein ensured rapid restoration of PSII function in all cases (Fig. 1). In nonphotoinhibited low-light leaves, the D1 protein was degraded with nearly the maximum rate already at 400 μmol of photons m^{-2} s^{-1} ($t_{1/2}$ = 165 min) (Aro et al., 1993a), and only a slightly faster rate was measured in leaves that were photoinhibited prior to the chase experiments (apparent $t_{1/2}$ = 151 min; Fig. 4B). Thus, as expected, these low-light leaves possessed only a poor capacity for restoration of the F_V/F_M ratios at 400 μmol of photons m^{-2} s^{-1}. In contrast, with high-light peas, the rate of D1 protein degradation at 400 μmol of photons m^{-2} s^{-1} was significantly enhanced in photoinhibited (apparent $t_{1/2}$ = 77 min) relative to nonphotoinhibited leaves ($t_{1/2}$ = 154 min; Fig. 4D), thus allowing for fast recovery from photoinhibition (Fig. 1).

Our results demonstrate that, just as the light acclimation of leaves affects the susceptibility of pea leaves to photoinhibition (Aro et al., 1993a), light acclimation also modifies the capacity of PSII to recover from photoinhibition under moderate and high irradiances. Photoinhibition of PSII in vivo is a balance between damage to PSII and concurrent recovery (Greer et al., 1986). We have recently shown that the degradation of the damaged D1 protein regulates the rate of the repair cycle in higher plants (Aro et al., 1993a). Although the rate of D1 protein degradation increases with increasing irradiance, the rate becomes saturated when photoinhibitory light levels are reached (Aro et al., 1993a). A further increase in the photon flux density significantly enhances photoinhibition. Under photoinhibitory conditions, when the rate of damage to PSII exceeds the rate of repair, PSII centers with damaged D1 protein accumulate in the thylakoid membrane. Moreover, the maximum capacity for D1 protein degradation strictly depends on light acclimation of pea leaves. Therefore, the lower the growth light irradiance, the more susceptible the pea leaves are to photoinhibition (Aro et al., 1993a). Our present experiments further support these conclusions; they also show that photoinhibited leaves are able to recover from photoinhibition only under those irradiances that are not high enough to saturate the rate of D1 protein degradation of similarly light-acclimated but nonphotoinhibited leaves. High-light-acclimated leaves recover from photoinhibition at higher photon flux densities than low-light leaves because of their better capacity for D1 protein degradation and thus for the whole repair cycle of PSII.

We have hypothesized previously that the degree of thylakoid stacking probably plays a key role in the regulation of the rate of D1 protein degradation (Tyystjärvi et al., 1992; Aro et al., 1993a); low-light leaves with extensive thylakoid stacking have a lower capacity for D1 protein degradation than high-light leaves, which have a relatively smaller proportion of appressed membranes (Anderson et al., 1988). At high irradiances the rate of D1 protein degradation in pea leaves grown at different light intensities is linearly related to the Chl *a/b* ratio (Aro et al., 1993a), which is an index of thylakoid membrane stacking. In general, pea leaves were capable of at least partial recovery from photoinhibition at light intensities far higher than those experienced during growth. This occurs because peas are not photoinhibited until the irradiance is at least double their growth irradiance (Aro et al., 1993a), unlike *Brassica* leaves, in which the maximum rate of D1 protein degradation occurs at irradiances comparable to their growth irradiance (Sundby et al., 1993).

It is generally accepted that only poor recovery from photoinhibition can occur in darkness (Fig. 5A; Skogen et al., 1986; Greer and Laing, 1988). Synthesis of the D1 protein is regulated at the level of translation in higher plants, and the accumulation or stabilization of full-length D1 protein requires light (Eichacker et al., 1990; Danon and Mayfield,

1991; Kuroda et al., 1992; Taniguchi et al., 1993), although the exact mode of regulation still remains to be solved. Defective synthesis or stabilization of the D1 protein has been thought to be an underlying reason for the failure to repair damaged PSII centers in darkness. However, in photoinhibited leaves that contain PSII centers with damaged but nondegraded D1 protein (Tyystjärvi et al., 1992; Aro et al., 1993a; Kim et al., 1993; Schnettger et al., 1992a), only minor degradation of the D1 protein took place when leaves were transferred to darkness. These results suggest that the inability of pea leaves to recover from photoinhibition in darkness may also involve the failure to degrade the damaged D1 protein. This raises an important question: Is the D1 protein-specific protease activated by light in vivo and, conversely, inactivated in darkness? This possibility would not contradict D1 protein degradation in numerous in vitro experiments with isolated thylakoids and PSII preparations, since leaves for these experiments are usually collected during the light period. Another explanation could involve a light-regulated dephosphorylation of the D1 protein as a prerequisite for degradation in higher plants (Aro et al., 1993b). Whatever the mechanism for the retardation of the degradation of damaged D1 protein in darkness, its physiological significance is possibly to prevent the accumulation of D1 protein-depleted PSII centers and to ensure the coordinated degradation and resynthesis of the D1 protein (Aro et al., 1993b).

Although we demonstrated here that recovery from photoinhibition is not necessarily hindered at high light and depends on the prior acclimation of leaves, it is noteworthy that the recovery from photoinhibition in variously light-acclimated leaves was maximal at 50 μmol of photons m^{-2} s^{-1} (Fig. 1). Chow (1994) postulated that the low-irradiance requirement for optimal recovery from photoinhibitory damage is due to the need to maintain an optimal stromal pH for protein synthesis. The establishment of a maximum stromal pH of about 7.8 saturates at 50 μmol of photons m^{-2} s^{-1}, and this pH is also optimal for protein synthesis.

In the present study we showed that the damaged D1 protein that accumulates in the thylakoid membrane during photoinhibition is degraded under recovery conditions. At low irradiance, the recovery from photoinhibition occurred with nearly the same rate in all peas acclimated to different light intensities and was accompanied by rapid degradation of the damaged D1 protein. At increasing irradiances, the capacity for restoration of PSII function was strictly dependent on growth-light irradiance of peas and was related to the capacity of photoinhibited leaves to enhance the rate of D1 protein degradation relative to that of nonphotoinhibited leaves under the same conditions. Damaged D1 protein was not degraded in darkness, and no marked recovery from photoinhibition took place. The capacity to restore PSII function following photoinhibition is directly related to the capacity of leaves to degrade damaged D1 protein under recovery conditions.

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