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D1 protein degradation during photoinhibition of intact leaves

A modification of the D1 protein precedes degradation

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Illumination of intact pumpkin leaves with high light led to severe photoinhibition of photosystem II with no net degradation of the D1 protein. Instead, however, a modified form of D1 protein with slightly slower electrophoretic mobility was induced with corresponding loss in the original form of the D1 protein. When the leaves were illuminated in the presence of chloramphenicol the modified form was degraded, which led to a decrease in the total amount of the D1 protein. Subfractionation of the thylakoid membranes further supported the conclusion that the novel form of the D1 protein was not a precursor but a high-light modified form that was subsequently degraded.

D1 protein; Photoinhibition; Photosystem II

1. INTRODUCTION

Photoinhibition of PSII occurs when intact plants, chloroplasts or PSII preparations are exposed to strong light [1,2]. The site of photoinhibition in PSII may vary depending on the functional status of PSII [3]. Under aerobic conditions in vitro, photoinhibition is followed by degradation of the D1 protein [4–5]. D1 is the thylakoid protein with the fastest turn over [6,7], and photoinhibition further enhances its turnover in the green alga *Chlamydomonas reinhardtii* [8–10]. The exact mechanism and the site of D1 protein degradation in the thylakoid membrane are still obscure. There is evidence that before the final proteolytic cut, the D1 protein is modified [11], and only the irreversibly modified protein is degraded [12–13]. Experiments with *C. reinhardtii* suggest that after a light-induced modification, the core complex of PSII migrates from the appressed to the non-appressed thylakoid membrane region and functions there as an acceptor for the precursor of the D1 protein [14].

Although D1 degradation has clearly been demonstrated in photoinhibition of isolated systems and in unicellular algae, it has been more difficult to prove that D1 degradation is enhanced also during photoinhibition of higher plants in vivo [15–16]. In the present study we show that although turnover of the D1 protein is accelerated during photoinhibition, net decrease in the amount of the protein does not normally occur.

Abbreviations: CAP, chloramphenicol; D1', modified form of the D1 protein; PPFD, photosynthetic photon flux density; PSII, photosystem II

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2. MATERIALS AND METHODS

Pumpkin (*Cucurbita pepo* L.) plants were grown at a PPFD of 1000–1300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. For some experiments, specifically indicated in the text, the plants were grown at an extremely low PPFD, 50 to 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Fully expanded leaves of 3- to 5-week-old plants were used in the experiments.

Leaves were detached and the petioles were immersed in water or in an aqueous CAP solution (1 g/l) and incubated in the dark for 3 h. The leaves were illuminated at the PPFDs of 1500–3000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 20°C in saturating humidity. A 1200 W HMI arc lamp was used as a light source. For recovery from photoinhibition, the leaves were transferred to a growth chamber at 20°C and a PPFD of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 24 h with their petioles in the same solution as during the high light treatment.

Thylakoids were isolated [17] from control and treated leaves, and further fractionated into appressed and non-appressed membrane regions essentially according to [18]. The light-saturated rate of PSII oxygen evolution was measured with an oxygen electrode using phenyl-*p*-benzoquinone (1 mM) as an electron acceptor. Chlorophyll was determined in 80% acetone according to Arnon [19]. The thylakoids and thylakoid subfractions were solubilized and separated by denaturating SDS electrophoresis on a 12–22.5% (w/v) polyacrylamide gel including 4 M urea [20]. Polypeptides were transferred to Immun-Lite TM membrane (BioRad) and the immunodetection of the D1 protein was carried out using a BioRad chemiluminescence kit. An LKB laser densitometer was used to scan the immunoblots.

3. RESULTS

Figure 1 shows the photoinhibition of PSII oxygen evolution when pumpkin leaves were exposed to a PPFD of 3000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Leaves with 70% inhibition in PSII activity readily restored the activity during subsequent incubation in low light for 24 h (arrowheads in Fig. 1). As long as PSII photoinhibition could be

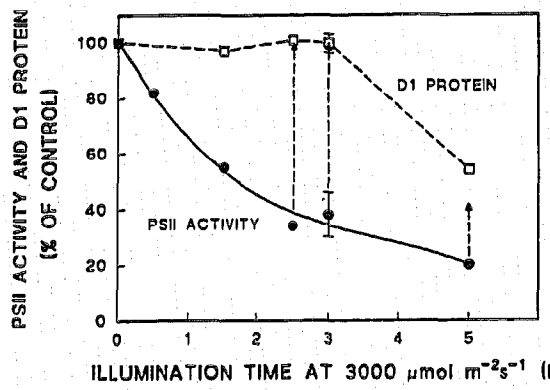

Fig. 1. Photoinhibition of PSII activity (■), and D1 protein degradation (□) during illumination (3000 μmol photons·m^{-2}·s^{-1}, 20°C) of intact pumpkin leaves. The arrowheads indicate the capacity of PSII to recover within 24 h incubation at low light after the photoinhibitory treatment.

reversed by subsequent incubation in low light, no decrease in the D1 protein content could be detected during illumination (Fig. 1). Very long treatments led to incomplete recovery and decrease in the D1 protein. After these long treatments, chlorophyll bleaching became evident during the recovery period. Bleaching-associated D1 degradation will not be further considered here.

Although D1 did not decrease during the photoinhibitory treatment, highly resolving electrophoresis revealed that after illumination the D1 protein was present in two distinct forms. The new, modified form of the D1 protein, designated D1*, migrated in the gel slightly more slowly than the original form (Fig. 2). During recovery from photoinhibition in low light, the modified form nearly disappeared and the original one became dominating again (Fig. 2) through de novo synthesis (see below).

Illumination of the leaves for 3 h at a PPFD of 2000 μmol·m^{-2}·s^{-1} did not induce photoinhibition of PSII activity in the absence of CAP, but when chloroplast protein synthesis was blocked with CAP, 45% inhibition was induced. D1 hardly degraded, even in the presence of CAP (Fig. 3A). However, when the leaves were subsequently transferred to low light, degradation of the high light modified D1 took place. The original D1 form of the non-inhibited PSII centers persisted during illumination and the 24 h incubation of leaves in recovery conditions. Increasing the PPFD to 3000 μmol·m^{-2}·s^{-1} induced more severe photoinhibition (60–75% inhibition in PSII activity), but the effect of CAP was less pronounced. In the presence of CAP, most of the D1 protein had already degraded during the high light treatment (Fig. 3B).

To test if the D1* really belongs to the photoinhibited PSII, the same plant species was grown under extremely low light conditions, which resulted in higher susceptibility to photoinhibition and, more importantly, to a much slower rate of recovery (E. Tyystjärvi, R. Kettu-

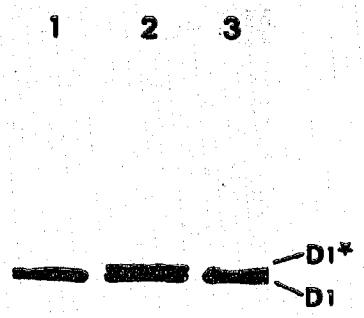

Fig. 2. Immunological demonstration of the appearance of the modified form of the D1 protein (D1*) during photoinhibitory illumination of pumpkin leaves and its disappearance during the recovery period at low light. Lane 1, non-treated leaves; lane 2, leaves photoinhibited at 3000 μmol photons·m^{-2}·s^{-1} at 20°C for 3 h; lane 3, subsequently fully recovered at low light for 24 h. 1.5 μg of chlorophyll was applied into each well.

nen and E-M. Aro, in preparation). Even in these plants, no net loss of the D1 protein occurred during illumination; only the modification of the D1 protein was evident. When the low light-grown plants were illuminated in the presence of CAP, accumulation of D1* was indeed correlated with increase in photoinhibition of PSII (Fig. 4). D1* started to degrade only slowly

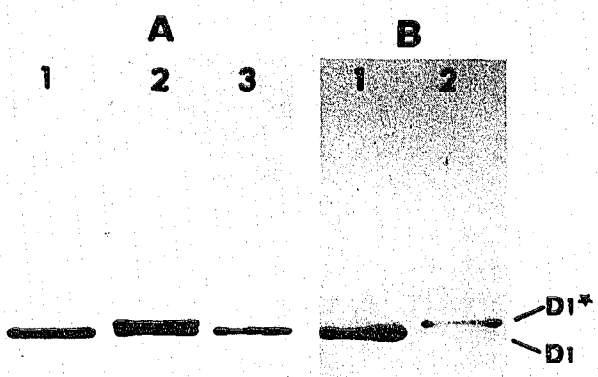

Fig. 3. An immunoblot demonstrating changes in the D1 protein of pumpkin leaves during illumination and subsequent incubation at low light in the presence of CAP. (A) Leaves illuminated at a PPFD of 2000 μmol·m^{-2}·s^{-1} for 3 h with 45% inhibition and (B) at 3000 μmol·m^{-2}·s^{-1} for 3 h with 75% inhibition in PSII activity. Lane 1, control leaves; lane 2, illuminated leaves; lane 3, subsequently incubated at low light for 24 h. 1.5 μg of chlorophyll was applied into each well.

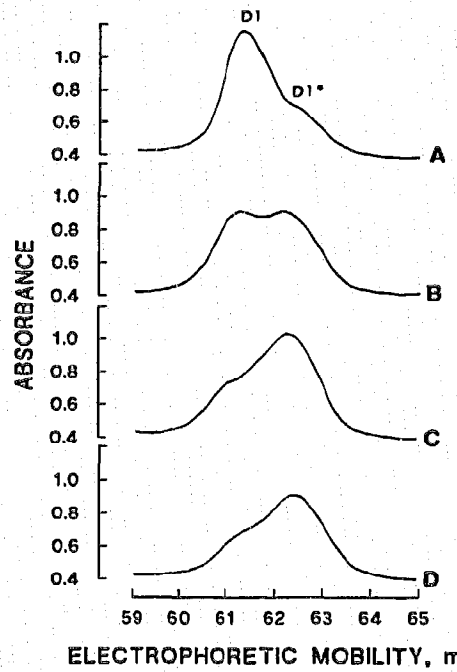

Fig. 4. Laser densitograms of the immunoblots of the D1 protein indicating modifications and degradation during illumination (1500 μmol·m^{-2}·s^{-1}) of low-light-grown pumpkin leaves in the presence of CAP. (A) Control leaves; (B) leaves illuminated for 1 h (40% inhibition of PSII activity), (C) for 2 h (69% inhibition) and (D) for 3 h (72% inhibition). 3 μg of chlorophyll was applied into each well.

during illumination (Fig. 4, curve D), in spite of severe photoinhibition of PSII.

Fractionation of thylakoid membranes into appressed and non-appressed regions revealed that D1* was present after illumination only in the appressed membranes (Fig. 5). We could not detect the precursor form of the D1 protein in non-appressed membranes [11,21].

4. DISCUSSION

No net degradation of the D1 protein occurs during high light treatment, if the leaves were not irreversibly damaged and were capable of recovering from photoinhibition. However, a photoinhibition-enhanced degradation of the D1 protein was clearly demonstrated when the high light treatments were performed in the presence of CAP, an inhibitor of chloroplast protein synthesis. In very high light (3000 μmol·m^{-2}·s^{-1}) in the presence of CAP, severe photoinhibition of PSII activity was accompanied by degradation of the D1 protein already during the treatment. However, illumination with CAP at 2000 μmol·m^{-2}·s^{-1} for 3 h induced less photoinhibition and less D1 protein degradation that was completed only during the subsequent incubation in low light.

D1 protein did not degrade directly after photoinhibition of PSII but degradation occurred via a modified form which migrated in the gel slightly more slowly than the original D1 protein (Fig. 3A). Photoinhibition-induced irreversible modification of the D1 protein has already been postulated [12,13] but the two forms have not been directly separated from each other. D1 turnover experiments with *Spirodela* first revealed a modified form of D1 (32*) [11], which was suggested to have a role in light-dependent degradation. Here we report for the first time the photoinhibition-induced modification of the D1 protein. Accumulation of D1* was proportional to photoinhibition of PSII in low light grown pumpkin leaves, which strongly suggests that photoinhibited PSII have their D1 in the D1* form. Similar electrophoretic mobilities of 32* [11] and D1* in relation to original D1 support the view that photoinhibition-induced D1 degradation is just an enhancement of normal turnover.

D1* could be detected only in the appressed thylakoid membrane regions (Fig. 5, [11]) which are the main sites for photoinhibition of PSII [22–24]. However, this does not necessarily mean that D1 also degrades in appressed membranes in vivo although the protease is probably an integral part of the PSII core complex itself [25]. The lack of net loss of the D1 protein during photoinhibition in vivo suggests that D1 degradation and insertion of the newly synthesized D1 to the reaction centre are tightly synchronized. The insertion of newly synthetized D1 protein to the reaction center has conclusively been shown to take place in the stroma thylakoids [14,21]. Translocation of the PSII reaction center from the site of damage in appressed membranes to non-appressed regions has been indicated in *Chlamydomonas* cells [14], and it is plausible that the D1 protein degrades in vivo only after this translocation, concomitantly with the insertion of new precursor D1 into the reaction center complex. If this is the case, D1* must degrade rapidly in the stroma thylakoids since it did not accumulate there (Fig. 5).

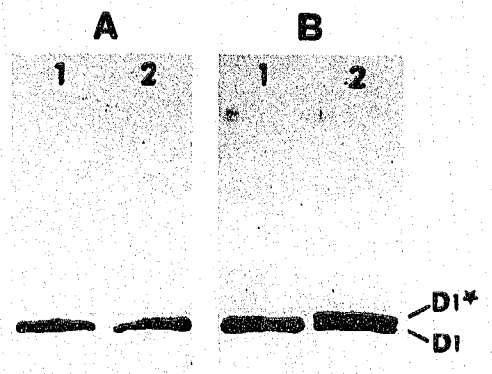

Fig. 5. An immunoblot of the D1 protein from (A) non-appressed and (B) appressed membranes. Lane 1, control membranes; lane 2, membranes from leaves illuminated at a PPFD of 3000 μmol·m^{-2}·s^{-1} for 3 h. 3 μg of chlorophyll was applied into each well in (A), and 0.8 μg in (B).

Moreover, in low light grown pumpkin plants with extensive thylakoid appression (not shown) but only a limited capacity to recover from photoinhibition (Tyystjärvi et al., in preparation), D1*, instead of degrading immediately in the appressed membranes, becomes long-living during illumination (Fig. 4). This indicates that D1* is not readily degraded in the appressed membranes in vivo. We suggest that the modification of the D1 protein in appressed membranes is a signal for a photoinhibited PSII core to migrate to non-appressed membranes for a concerted degradation and replacement of the D1 protein. Scarcity of stroma thylakoids may limit the recovery from photoinhibition in shade plants, because the abundance of stroma thylakoids sets a limit to the number of PSII centers that can cycle between appressed and non-appressed regions.

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