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Quality control of Photosystem II under light stress – turnover of aggregates of the D1 protein *in vivo*

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Abstract

When photodamaged under excessive light, the D1 protein is digested and removed from Photosystem (PS) II to facilitate turnover of the protein. *In vitro* studies have shown that part of the photodamaged D1 protein forms aggregates with surrounding polypeptides before being digested by a protease(s) in the stroma [Yamamoto Y (2001) Plant Cell Physiol 42: 121–128]. The aim of this study was to examine whether light-induced aggregation of the D1 protein also occurs *in vivo*. The following results were obtained: (1) PS II activity in spinach leaves was significantly inhibited by weak illumination (light intensity, 20–100 μE m^{-2} s^{-1}), as monitored by chlorophyll fluorescence Fv/Fm, when the leaves were kept at higher temperatures (35–40 °C); (2) aggregation of the D1 protein, as well as cleavage of the protein, was detected in thylakoids isolated from spinach leaves that had been subjected to heat/light stress; (3) aggregates of the D1 protein disappeared after incubation of the leaves at 25 °C in the dark or under illumination with weak light. Since it is dependent on the presence of oxygen, aggregation of the D1 protein is probably induced by reactive oxygen species produced in thylakoids upon illumination at elevated temperatures. Consistent with this notion, singlet oxygen production in thylakoid samples under illumination was shown to be stimulated significantly at higher temperatures.

Abbreviations: D1 – the reaction center-binding protein of Photosystem II; PS II – Photosystem II; ROS – reactive oxygen species; P680 – the primary electron donor of Photosystem II; Chl – chlorophyll

Introduction

The reaction center D1 protein of photosystem (PS) II is prone to photodamage under excessive light. Damage to PS II is closely related to decreased photosynthetic yield in plants during the daytime as a result of high irradiation with visible light; this phenomenon is called photoinhibition of PS II. The details of the photoinhibition process have been studied both *in vitro* and *in vivo*, but several important questions essential for fully understanding the mechanism of protein damage and degradation remain unanswered.

Degradation of the D1 protein is not the only major event that occurs after photodamage of the D1 protein. Recent investigations have demonstrated significant cross-linking of the D1 protein with surrounding polypeptides, namely D2, CP43 and PsbE protein, under photoinhibitory conditions *in vitro* (Yamamoto and Akasaka 1995; Ishikawa et al. 1999; Yamamoto 2001). Protein cross-linking or protein aggregation has been

shown to be a serious hazard to cells in general. For instance, prion disease and other amyloid-related human diseases are caused by accumulation of large protein aggregates in cells. A similar situation might also occur in plant cells, especially near the reaction center of PS II where reactive oxygen species (ROS) and endogenous radicals such as $P680^+$ and Chl^+ are easily generated by photochemical reactions under various stress conditions. In the present study, we show that light-induced aggregation of the D1 protein takes place *in vivo* as well as *in vitro*. The data obtained strongly suggest that the turnover of D1 aggregates is a general process that occurs under natural heat/light stress conditions *in vivo*.

Materials and methods

Fresh spinach leaves were purchased from a local market and intact chloroplasts were isolated according to a method reported previously (Mullet and Chua 1983) using buffer solution containing 0.1 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$ and 50 mM Tricine-KOH (pH 7.6) (solution A). Thylakoid membranes were prepared by osmolysis of the intact chloroplasts with hypotonic solution containing 5 mM $MgCl_2$ and 10 mM Hepes-KOH (pH 7.5). The thylakoid membranes were washed twice and suspended in solution A to a final chlorophyll concentration of 0.5 mg Chl ml^{-1}. PS II-enriched membranes were prepared by treating thylakoids with Triton X-100 (Yamamoto et al. 2004a). They were then suspended in solution A and stored at −80 °C until use. All preparation steps were carried out at 4 °C under green safe light. For preparation of spinach leaf disks, fresh leaves were cut with a cork-borer (diameter, 20 mm).

For heat treatment of thylakoids and PS II-enriched membrane, the samples were put in black plastic tubes (volume, 1 ml) and incubated in a circulating water bath for a given time at 40 °C. Heat treatment, if leaf disks was carried out by making them were float in a water bath at 40 °C. After incubation for a given period, the leaf disks were homogenized in 5 ml of solution containing 0.1 M sucrose, 0.2 M NaCl and 50 mM KH_2PO_4-NaOH (pH 6.9), and the homogenate was quickly filtered through two layers of tissue paper and centrifuged at 10,000 × *g* for 5 min at 4 °C. The pellet was then suspended in buffer A and used for SDS/urea-polyacrylamide gel electrophoresis (SDS/urea-PAGE) and Western blot analysis. Where indicated, the samples were heat-treated under anaerobic conditions created by addition of 10 mM glucose, 0.1 mg ml^{-1} glucose oxidase and 0.1 mg ml^{-1} catalase to the samples.

SDS/urea-PAGE and Western blot analysis were carried out as described previously (Yamamoto and Akasaka 1995; Yamamoto et al. 2004b). The primary antibodies used for Western blot analysis were against the D1 protein (225R-249V in the DE-loop, donated by Dr Mitsue Miyao of the National Institute of Agrobiological Resources, Japan). As the secondary antibody, a horseradish peroxidase-conjugated anti-rabbit antibody (BioRad, Japan) was used. The immunodecorated bands were detected by sensitive fluorography with enhanced chemiluminescence (ECL) (Amersham, Japan). Chlorophyll fluorescence was measured with a plant efficiency analyzer (PEA; Hansatech, UK). Fv/Fm was monitored as an index of PS II activity. Before the measurements, the spinach leaf disks were dark adapted for 30 min. Singlet oxygen was detected using a JEOL TE 200 EPR spectrometer as described previously (Suh et al. 2000).

Results and discussion

Spinach thylakoids and PS II-enriched membranes were illuminated both at 25 and 40 °C with either low (100 μE m^{-1} s^{-1}) or high light (1800 μE m^{-1} s^{-1}), and the behavior of the photodamaged D1 protein was assayed by SDS/urea-PAGE and Western blot analysis with an antibody against the D1 protein (Figure 1). Light-induced formation of D1 aggregates was stimulated significantly when the samples were incubated at 40 °C compared to at 25 °C. Heat (40 °C) treatment of the thylakoids *per se* appeared to induce cleavage of the D1 protein, producing a 23-kDa D1 fragment. It was also apparent that generation of D1 aggregates and formation of the 23-kDa fragment compete with each other under illumination at higher temperatures. When thylakoids were illuminated with either low or high light at 40 °C, a large fraction of the 23-kDa fragment observed in thylakoid samples kept in the dark at the same temperature was sacrificed for a significant elevation in the D1

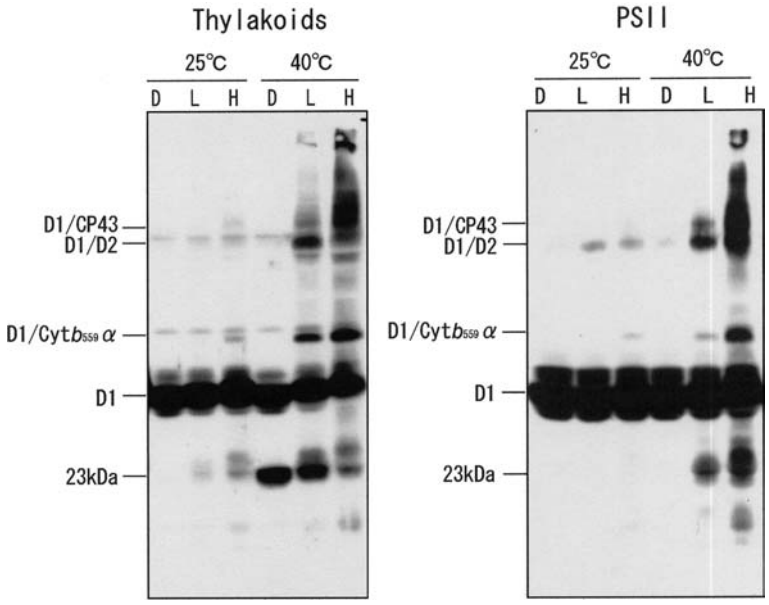

Figure 1. Effects of heat and light stress on the D1 protein in thylakoids and PS II-enriched membranes. D, L and H represent dark control, low light (150 μE m^{-2} s^{-1}) and high light (1800 μE m^{-2} s^{-1}) conditions, respectively. The D1 protein was assayed by SDS/urea-PAGE and Western blot analysis. The locations of the D1 protein, the cross-linked products and a 23 kDa fragment of the D1 protein are indicated on the left side of the gels.

aggregation level. These data might suggest that the sites of heat-induced cleavage and light-induced cross-linking of the D1 protein are close to each other, or possibly the same. It is known that oxygen-evolving system of PS II is more sensitive to heat stress than the reaction center itself is. However, when the thylakoids or PS II membranes were treated at 35–40 °C for 30 min, no PsbO, P, Q and Mn atoms were released from PS II (data not shown), and these results suggest that the oxygen evolving system was not impaired severely under these conditions. Thus, the donor-side photoinhibition of PS II probably does not take place by the illumination of the temperature stressed samples, and the aggregation of the D1 protein observed here is most likely due to the acceptor-side photoinhibition of PS II.

We examined the synergistic effect of heat and light stresses on PS II activity in intact leaves by monitoring leaf chlorophyll fluorescence, Fv/Fm (Figure 2). When spinach leaf disks were illuminated (light intensity, 20–1800 μE m^{-2} s^{-1}) at 25 °C, no significant change in PS II activity was seen. In contrast, PS II activity was inhibited significantly when the leaf disks preincubated at 40 °C were illuminated. Even illumination at an intensity as low as 20–100 μE m^{-2} s^{-1} was enough to induce inhibition. Recently, it was shown that ROS may inhibit repair of the photodamaged D1 protein (Nishiyama et al. 2004). It is possible that by illumination of the samples at higher temperatures, ROS may damage the repair system of the

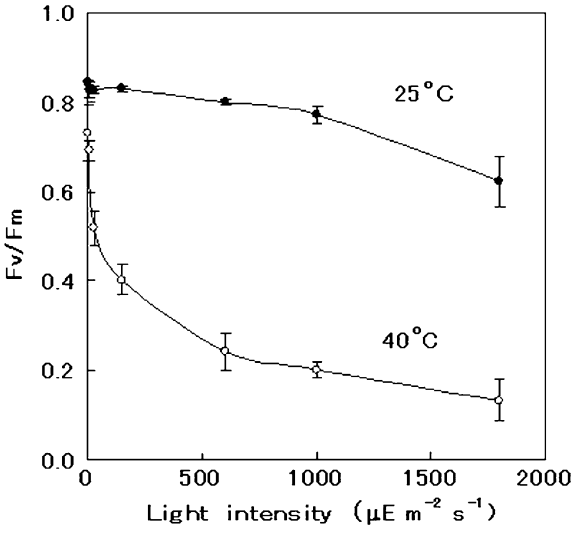

Figure 2. Synergistic effect of heat and light stress on PS II activity in spinach leaf disks. Chlorophyll fluorescence, Fv/Fm, was plotted against light intensity at 25 and 40 °C. Values are means ± SE ($n = 5$).

D1 protein, which stimulates inhibition of the PS II activity.

A question related to the inhibition of PS II activity shown in Figure 2 is, whether the same types of D1 deterioration seen in isolated thylakoids occur in leaves when exposed to high light at elevated temperature. To answer this question, D1 derivatives were assayed by SDS/urea-PAGE and Western blot analysis with thylakoid membranes isolated from spinach leaf disks immediately after exposure to heat/light stress. As a result, both aggregation and degradation of the D1 protein were observed in the treated leaf disks (Figure 3). In the control experiment, where leaf disks were illuminated at 25 °C, neither D1 aggregation nor D1 degradation was detected.

The D1 aggregates formed in isolated thylakoids or PS II membranes upon illumination with excessive light are digested by protease(s) in the stroma (Ishikawa et al. 1999; Ferjani et al. 2001; Yamamoto et al. 2004b). To see if the D1 aggregates produced in leaves are also removed by proteolytic activity, leaf disks exposed to heat/light stress were incubated at 25 °C in the dark or under dim light (7 μE m^{-2} s^{-1}) then subjected to an assay for D1 derivatives. The overall level of D1 derivatives, that is, aggregates and degradation products, decreased with dark or low light incubation (see the last two lanes in Figure 3). It is thus likely that the formation and degradation of D1 aggregates can be observed in leaves under natural heat/light stress conditions.

Heat-induced generation of the 23 kDa D1-cleavage product in thylakoids appeared independent of oxygen (data not shown), likely ruling out ROS as a causative agent of the cleavage process, while supporting the action of protease(s). On the other hand, D1 aggregation was detected in thylakoids when illuminated only in the presence of oxygen (data not shown), suggesting the participation of ROS in the aggregation process. Furthermore, stimulated aggregation of the D1 protein in samples illuminated at a high temperature (40 °C) might suggest that ROS generation in thylakoid membranes is enhanced as a combined effect of heat and light. Although the ROS responsible for stimulating D1 aggregation at elevated temperature is yet to be identified, it could

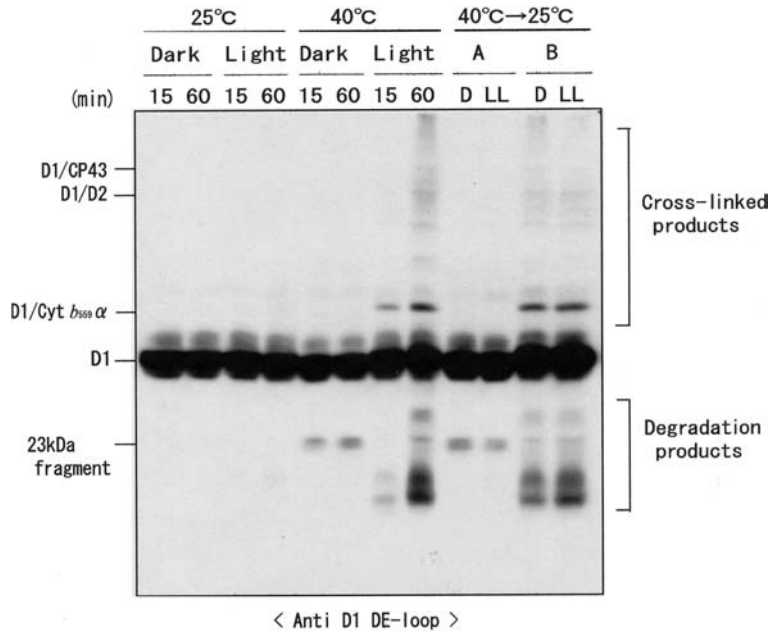

Figure 3. Effects of heat and light stress in spinach leaf disks. The leaf disks were illuminated (light intensity, 1800 μE m^{-2} s^{-1}) or incubated in the dark for the period specified on the top of the gel at either 25 or 40 °C. Thylakoids were then isolated from the leaf disks immediately after each treatment and the D1 protein was assayed by SDS/urea-PAGE and Western blot analysis with an antibody against the DE-loop of the D1 protein. The spinach leaf disks incubated at 40 °C for 60 min either in the dark (shown as A) or light (shown as B) were further incubated at 25 °C for 15 min in the dark (D) or under illumination of low light (L) (light intensity, 7 μE m^{-2} s^{-1}). The locations of the D1 protein, aggregates of the D1 protein and degradation products are shown on both sides of the gel.

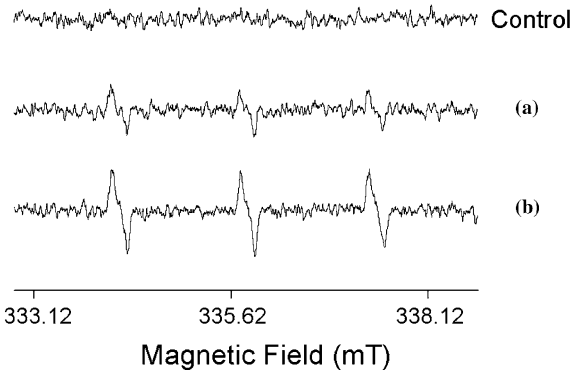

Figure 4. Singlet oxygen production in thylakoids under high light conditions. Thylakoid samples containing 2,2,6,6-tetramethyl-4-piperidone were illuminated (light intensity, 2000 μE m^{-2} s^{-1}) at either 25 (A) or 40 °C (B) for 10 min. The formation of 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl as a result of singlet oxygen reaction with the trap compound was then detected by ESR in a Jeol TE 200 spectrometer at an operating frequency of approximately 9.42 GHz. The control is for thylakoids kept in the dark at 25 °C.

be singlet oxygen because this active oxygen was produced at a remarkably increased rate in thylakoids illuminated at 40 °C compared to those illuminated at 25 °C (Figure 4). It has been proposed that the PS II reaction center is the generation site of singlet oxygen that damages the D1 protein. In connection with the heat/light effects in thylakoids, however, light-harvesting complex II (LHC II) might be assumed to be the site of singlet oxygen production. It seems reasonable to suppose that heat-induced weakening of the interaction between LHC II and the reaction center complex at higher temperature interrupts excitation energy transfer in PS II, resulting in an increased steady-state population of antenna Chl molecules in the triplet excited state that, in turn, interact with O_2 to produce singlet oxygen at an increased level. Alternatively, conformational changes in the reaction center protein of PS II, especially that of the binding sites of bound plastoquinone Q_A and Q_B at elevated temperature might induce superoxide anion production at these sites upon illumination, which should also induce aggregation of the D1 protein. The present results strongly suggest that the D1 protein of PS II is a common target of heat and light stresses and that formation of D1 aggregates becomes prominent when plants are exposed to high light in the day-time on hot days. Plants might avoid serious situations resulting from accumulation of aggregates in PS II by developing an efficient proteolytic system that removes the hazardous aggregates.

Acknowledgements

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