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Effects of Chilling Injury in the Light on Chlorophyll Fluorescence and D1 Protein Turnover in Cucumber and Pea Leaves

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Abstract: Light-chilling effects were investigated in chilling-sensitive cucumber (*Cucumis sativus* L. cv. Ilmichungjang) and chilling-resistant pea (*Pisum sativum* L. cv. Giant) leaf discs in relation to possible damage in D1 protein. In both plants, dark-chilling did not cause any noticeable changes in (Fv)m/Fm, and lincomycin did not affect the decrease in (Fv)m/Fm caused by light-chilling. This result suggests that the *de novo* synthesis of D1 protein did not occur actively during light-chilling. In pea light-chilled for 6 h, the decreased (Fv)m/Fm was partly recovered in the dark, and almost complete recovery was observed in the light. In cucumber light-chilled for 3 h, the reduced (Fv)m/Fm decreased further for the initial 2 h recovery process in the light regardless of the treatment of lincomycin and recovered very slowly. In both plant species, the treatment of lincomycin inhibited the recovery process in the light, but did not significantly inhibit the process in the dark. In cucumber leaves pulse-labeled with [^{35}S]Met, the labeled band intensities of isolated pigment-protein complexes were almost the same during the 6 h light-chilling, but significant decreases in band intensities were observed during the 3 h recovery period. This result suggests that the irreversibly damaged D1 protein was degraded during the recovery period. However, no noticeable changes were observed in the pea leaves during the 12 h chilling and 3 h recovery period. The polyacrylamide gel electrophoresis of the pigment-protein complexes showed that the principal lesion sites of light-chilling were different from those of room temperature photoinhibition.
Key words: chlorophyll fluorescence, D1 protein, light chilling, photoinhibition, pigment-protein complex.

Photoinhibition is usually considered to result principally from the inactivation of reactions associated with PSII (Prasil *et al.*, 1992; Aro *et al.*, 1993b). Low temperature is an important environmental stress which makes photosynthesis more sensitive to photoinhibition, so that even low photon flux density (PFD) may cause photoinhibition (Öquist *et al.*, 1987; Baker *et al.*, 1988; Öquist and Huner, 1991). Chilling-sensitive plants are injured more severely or quickly than chilling-resistant plants by chilling under a moderate PFD for only a few hours (Taylor and Rowley, 1971; Wright and Simon, 1973; Garber, 1977; Ha *et al.*, 1996).

When photoinhibition is induced by a high PFD, the electron transport through PSII is inhibited. Acceptor and/or donor sides of D1 protein are thought to be the first target(s) of high light (Andersson *et al.*, 1992). In acceptor-side photoinhibition, there is a sequential array of events leading to overreduction of the primary quinone acceptor Q_A, which leads to the formation of a P680 Chl triplet and singlet oxygen. The donor-side photoinhibition is thought to involve the accumulation of the highly oxidizing species $P680^+$ and $Tyr\text{-}Z^+$. These species will lead to oxidative damage of D1 protein, triggering it for degradation.

The damaged D1 protein is removed and replaced with new one (Aro *et al.*, 1993b). During this process, the reaction center is disassembled and the remainders of the reaction center components migrate laterally to stroma lamellae where reassembly with new D1 protein takes place. Under chilling conditions, degradation and synthesis of D1 protein of the reaction center appear to be slowed down (Chow *et al.*, 1989; Gong and Nilsen 1989; Aro *et al.*, 1990). The rates of migration, protein assembly and ligation of cofactors are also slowed down at low temperatures (Kyle, 1987).

However, there are several reports suggesting that the target sites of low temperature photoinhibition are not primarily PSII (Havaux and Davaud, 1994; Terashima *et al.*, 1994). However, Ogren and Öquist (1984a) reported that the electron transport *via* PSI was unaffected by low temperature photoinhibition. Kok *et al.* (1965), Satoh (1970), Satoh and Fork (1982) and Og-

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ren and Öquist (1984b) reported inhibitions in PSI as well as in PSII under rate-limiting light conditions.

The chilling injury specific to chilling-sensitive plants can give clues for the understanding of the plant responses to chilling stress and the mechanism of chilling resistance. Ha *et al.* (1996) reported that the photosynthetic apparatus of pea leaves was reversibly injured by light-chilling, but the damage in cucumber leaves seemed to be irreversible. The irreversible damage occurred within 6 h of light-chilling according to the changes in several parameters of Chl fluorescence induction kinetics.

The aim of this work was to understand the target sites of light-chilling specific to chilling-sensitive cucumber in comparison with chilling-resistant pea leaves. Focuses were given to the D1 protein turnover and the change of pigment-protein complexes of thylakoid membranes, because the damage and disassembly of D1 protein will affect the organization of pigment-protein complexes.

Materials and Methods

Plant materials, growth conditions and chilling treatment

Pea (*Pisum sativum* L. cv. Giant) and cucumber (*Cucumis sativus* L. cv. Ilmichungjang) plants were grown in a growth chamber under continuous light, with PAR of 50 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ provided by white fluorescence lamps. Leaf discs excised from fully expanded young leaves were floated on water with the abaxial side down. For dark-chilling, the leaf discs were chilled at 4℃ in the dark. For light-chilling, the leaf discs were illuminated on their adaxial side at 4℃ under the same light conditions used for their growth. For recovery after chilling, leaf discs floated on water were transferred back to the chamber where they were grown.

Measurements of Chl fluorescence

Chl fluorescence was measured using a portable plant efficiency analyzer (Hansatech Instruments Ltd, England). The leaf discs were dark-adapted for 20 min before Chl fluorescence was monitored. The Chl fluorescence signals were detected using a PIN photodiode after passing through a long-pass filter (50% transmission at 720 nm). Chl fluorescence was recorded in a time span from 10 μsec to 5 sec with 200 readings at 10 μsec intervals for the first 2 msec, 998 readings at 1 msec intervals until the recording period was 1 sec, and finally 40 readings at 0.1 sec intervals. A line of best fit through the initial data points (8~24) was extrapolated to time zero (the start of illumination) to determine the initial fluorescence (Fo). The maximum yield of fluorescence (Fm) was obtained by a saturation beam from an array of 6 light emitting diodes (peak of emission, 650 nm; PAR of 1200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) focused on to the sample surface to provide a homogeneously illuminated light spot of about 4 mm in diameter. The maximum variable fluorescence ((Fv)m) was obtained by subtraction of Fo from Fm. The ratio of (Fv)m/Fm was used to show the potential quantum yield of photochemical reactions in photosynthesis (Horton and Bowyer, 1990) to reduce the variation of data measured from different locations on a leaf and those from different leaves.

Pulse and chase labeling with [^{35}S]Met

For the radioactive labeling of thylakoid proteins, leaf discs floating on 10 mL of solution containing 1 mCi of [^{35}S]Met in 0.4% Tween 20 were illuminated (PAR of 50 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) for 1.5 h (Aro *et al.*, 1993a). The abaxial sides of leaf discs (diameter of 0.5 cm) were brushed gently to facilitate infiltration of [^{35}S]Met. Immediately after the pulse period, leaf discs were washed twice in a solution with 10 mM unlabeled Met, and 0.4% Tween 20, and further illuminated in the presence of cold Met for 1.5 h before taking the first chase sample.

Thylakoid membrane preparation

Thylakoid membrane was prepared according to Aro *et al.* (1993a) with some modifications. Leaf discs were ground with a glass homogenizer in an ice-cold grinding buffer (50 mM HEPES, pH 7.6, 0.3 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$). The homogenate was filtered through a layer of cheese cloth and centrifuged at 4,500×*g* for 5 min. Pellets were then washed twice with washing buffer (similar to grinding buffer, except for 0.1 M sorbitol). Thylakoid pellets were resuspended in a small aliquot of washing buffer, and immediately used for experiments. The Chl content was determined according to Arnon (1949).

Electrophoretic analysis of photosynthetic pigment-protein complex

Electrophoretic separation of the pigment-protein complexes in isolated thylakoids was performed using the low-ionic native green gel system of Allen and Staehelin (1991). The resolving gel contained 25 mM Tris HCl (pH 8.3), 50 mM glycine, and 10% glycerol. The stacking gel contained 25 mM Tris HCl (pH 6.3), 50 mM glycine, and 10% glycerol. The electrode buffer contained 25 mM Tris, 192 mM glycine (pH 8.3), and 0.1% SDS. A uniform 8% polyacrylamide gel with an acrylamide to bisacrylamide ratio of 100 : 1 was used. Polymerization was initiated at room temperature by the addition of 0.1% ammonium persulfate and 0.05%

NNN'N'-tetramethylethylenediamine. Membrane pellets were washed twice in 2 mM Tris maleate (pH 7.0) and resuspended in a solubilization buffer (0.45% octyl glucoside, 0.45% decyl maltoside, 0.1% lithium dodecyl sulfate, 10% glycerol, and 2 mM Tris maleate (pH 7.0)). The ratio of total nonionic detergent to Chl was adjusted to 20:1 (w/w). Samples were incubated on ice for 30 min, and insoluble material was pelleted at 15,000 rpm (Micro17R, Hanil, Korea) for 7 min. A 30 μL aliquot of the sample was loaded per lane, and run on 1.5 mm thick, 6 cm gel using a precooled apparatus (Multi-Protein II Cell, Bio-Rad, USA) in the cold refrigerator for 2~3 h with a 6 mA constant current. The native green gel containing samples labeled with [^{35}S] Met was exposed to X-ray film for visualization.

Two-dimensional SDS-PAGE

From the green gel lanes, 1.5 mm thick strips were excised and incubated for 30 min at room temperature in solubilization buffer containing 1× stacking gel buffer, 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, and 10% glycerol. The treated gel strips were loaded directly onto the stacking gel of 1.5 mm thickness using the buffer system of Laemmli (1970). Electrophoresis was performed using an LKB 2001 Vertical Electrophoresis Unit (LKB, Sweden) with a 30 mA constant current per gel, and the resulting gel was silver-stained. The gel with [^{35}S]Met-labelled samples was embedded in scintillant (20% 2,5-diphenyloxazole), dried, and exposed to X-ray film.

Results and Discussion

The effects of chilling on the Chl fluorescence

The effects of both light- and dark-chilling on the Chl fluorescence from chilling-sensitive cucumber and chilling-resistant pea leaf discs were examined for 12 h (Fig. 1). In the absence of light, a slow decrease in (Fv)m/Fo was observed in both plants, but no difference was observed between the two plants. However, a 56% reduction in (Fv)m/Fm was observed in cucumber leaves by light-chilling for 12 h, although only a 17% decrease in this ratio was measured in pea leaves.

During light-chilling, the decrease in (Fv)m/Fm, especially in cucumber, was caused by both an increase in Fo and a decrease in Fm (Ha *et al.*, 1996). The decrease in Fm may be due to an increase in the rate of thermal energy dissipation (Somersalo and Krause, 1988, 1989) and/or damage in PSII centers. Kirilovsky *et al.* (1990) interpreted the increase of Fo as a reversible modification of PSII reaction centers by light-chilling in *Chlamydomonas*. The modified closed center has a shift in the redox potential of the Q_A/Q_B^- couple

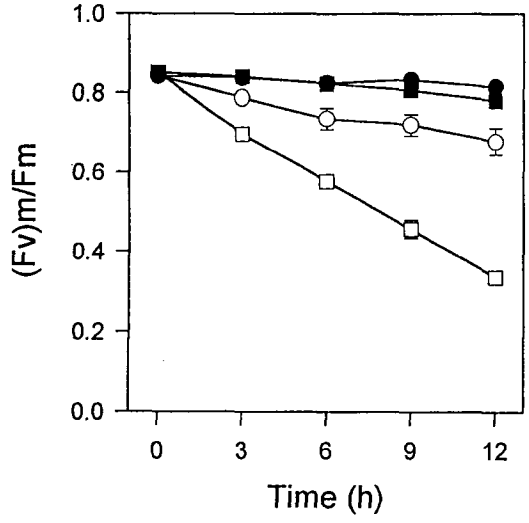

Fig. 1. The effect of light and chilling on the (Fv)m/Fm in cucumber and pea leaf discs. Cucumber (□,■) and pea (○,●) leaf discs were chilled at 4℃, in the presence (□,○) or in the absence (■,●) of light (50 μmol·m^{-2}·s^{-1}).

which was induced by the conformational change of the D1 protein. The conformational change leads to an increase of the activation energy of recombination and electron transport reactions, which results in an increase in Fo. Greer *et al.* (1988) explained the increase of Fo in kiwifruit at a low temperature as the reduction of the rate constant for photochemistry due to irreversible damage at or near the PSII reaction centers.

The effects of lincomycin on the changes in Chl fluorescence

To see if the difference in light-chilling injury between pea and cucumber leaves was due to the different turnover rate of D1 protein, the chloroplast encoded protein synthesis was blocked by the addition of 250 mg/L lincomycin. Leaf discs were treated with lincomycin for 4 h in the weak light (PAR of 0.5 μmol·m^{-2}·sec^{-1}) and washed with distilled water before the start of chilling and (Fv)m/Fm was monitored during chilling (Fig. 2) and during subsequent recovery periods (Fig. 3).

During light-chilling, lincomycin did not cause any significant effects on the decrease of (Fv)m/Fm in both pea and cucumber leaf discs (Fig. 2). A similar result was reported by Ottander *et al.* (1993) in barley leaves under light-chilling. This result suggests that there is almost no *de novo* synthesis of D1 protein for the repair of damaged reaction centers during chilling in the light in both plants, and that the D1 turnover rate is not the reason for the differences in the chilling sensitivities between pea and cucumber leaves.

In pea leaves chilled for 6 h in the light, a gradual

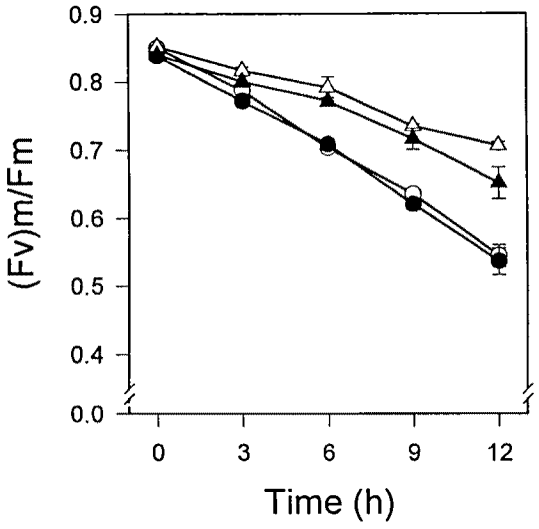

Fig. 2. The lincomycin independency of the light-chilling effects on the (Fv)m/Fm in cucumber and pea leaf discs. Cucumber (○,●) and pea (△,▲) leaf discs were chilled at 4℃ in the light (50 μmol·m^{-2}·s^{-1}). Control samples are indicated as open symbols and 250 mg/L lincomycin-treated ones as closed symbols.

increase of (Fv)m/Fm was observed during the subsequent recovery period at 25℃ for 6 h both in the light and in the dark (Fig. 3(A)). The extent of increase in (Fv)m/Fm in the light was a little higher than that in the dark, indicating the possible requirement of photosynthetically driven energy for the complete recovery. Similar results were reported by Gombos *et al.* (1994) in *Synechocystis* PCC6803 and by Leitsch *et al.* (1994) in spinach.

The treatment of lincomycin inhibited the recovery process in the light, but did not significantly inhibit the process in the dark (Fig. 3(A)). This result indicated that the recovery process in the light is supported by active protein synthesis. The recovery process shown in the light could be thought of as a process additional to the process in the dark, but this was not true according to this result. At present, it can not be answered why the process in the dark was not involved in the process in the light. Gombos *et al.* (1994) in *Synechocystis* PCC6803 obtained similar results using chloramphenicol. Similar results as those shown in Figs. 2 and 3 were also obtained using 1 mg/mL chloramphenicol (data not shown). Leitsch *et al.* (1994) reported that a fast and a slow process were involved in the recovery process in the light and they suggested that the treatment of streptomycin blocked only the slow recovery process. However, they did not show experimental data for the effect of streptomycin on the dark and light recovery processes in light-chilled spinach.

In the recovery experiments using cucumbers, leaf discs were light-chilled for 3 h when the extent of reduction in (Fv)m/Fm was comparable with that of pea

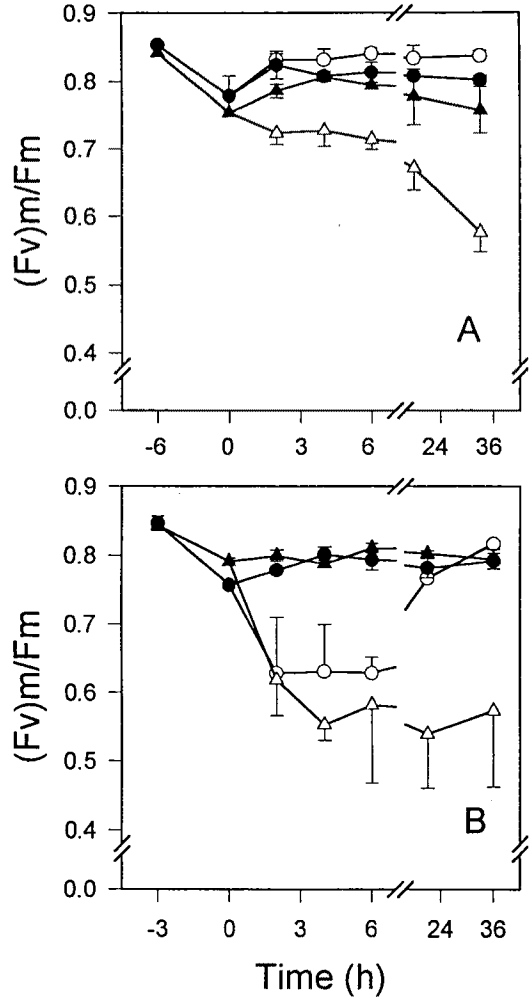

Fig. 3. The effect of light and lincomycin on the restoration of (Fv)m/Fm in light-chilled pea (A) and cucumber (B) leaf discs. Pea leaf discs were chilled at 4℃ for 6 h (from −6 h to 0 h) in the light (50 μmol· m^{-2}·s^{-1}), and cucumber leaf discs were chilled at 4℃ for 3 h (from −3 h to 0 h) in the light (50 μmol· m^{-2}·s^{-1}). After chilling, they were recovered at 25℃ in the presence (○,△) or in the absense (●,▲) of light (50 μmol·m^{-2}·s^{-1}). Control samples are indicated as circular symbols and 250 mg/L lincomycin-treated ones as triangular symbols.

leaf discs light-chilled for 6 h. The reduced (Fv)m/Fm by light-chilling decreased further for the initial 2 h recovery process in the light regardless of lincomycin treatment (Fig. 3(B)). After that, (Fv)m/Fm in lincomycin-untreated cucumber leaves increased slowly, but this slow recovery process was blocked in the lincomycin-treated leaf discs. The initial decrease in (Fv)m/Fm shown in the light-recovery process was not observed in the dark, and no significant effect of lincomycin was observed. The recovery process in the dark was very slow or insignificant (Fig. 3(B)). The effect of dark-induced senescence was negligible because there was no significant difference between the experiments performed in the presence or in the absence of 0.3 M

sucrose, a known inhibitor of dark-induced senescence (data not shown).

The dark recovery of the reduced (Fv)m/Fm shown in pea leaves (Fig. 3(A)) suggests that there is no significant irreversible damage in D1 protein during the light-chilling period in pea leaves. If D1 protein is irreversibly damaged by light-chilling, the increase in (Fv)m/Fm should not be observed during the dark recovery period because light is necessary to repair the damaged D1 protein (Bedbrook *et al.*, 1978; Mohamed and Jansson, 1989). The reduction in (Fv)m/Fm during light-chilling in pea leaves was mainly due to the decrease in Fm (Ha *et al.*, 1996), which can result from the increase in thermal energy dissipation, a protective mechanism of chilling-resistant plants (Somersalo and Krause, 1988, 1989). Because the extent of the decrease in (Fv)m/Fm in cucumber leaves light-chilled for 3 h was similar to that of pea leaves light-chilled for 6 h, additional explanations are necessary for the initial decrease in (Fv)m/Fm during the light-recovery period in the cucumber leaves shown in Fig. 3(B).

As an explanation, D1 protein was irreversibly damaged by light-chilling, but its disassembly and degradation process occurred only at room temperature in the light. The rate of *de novo* synthesis of D1 protein might be very slow in cucumber leaves during the recovery process. However, the disassembly and degradation process might occur at room temperature in the dark, but (Fv)m/Fm was not decreased at all. As an additional explanation, the possible damage in PSI during light-chilling might not be repaired easily in a short period of time, which resulted in slow recovery with about a 6 h delay (Fig. 3(B)).

Chasing [^{35}S]Met labeled D1 protein

To check the first possibility of irreversible damage in D1 protein, newly synthesized proteins in cucumber and pea leaf discs were labeled with [^{35}S]Met for 90 min, and the decay of labeled D1 protein was traced during chilling and the subsequent recovery period (Fig. 4). In light-chilled pea leaf discs, there were no changes in the band patterns of the native green gel and in the band patterns in the autoradiogram of the gel (Fig. 4(A) and 4(C)). In light-chilled cucumber leaf discs, there were no significant changes in the band patterns of the native green gel except for some changes in the intensities of minor bands (Fig. 4(B)). The autoradiogram of the gel showed only minor changes in band intensities after a 6 h light-chilling, but significant decreases in band intensities during the subsequent 3 h recovery period (Fig. 4(D)). Two-dimensional SDS-PAGE analyses of bands of the native green gel showed that the spots of the autoradiogram were mostly from D1 protein

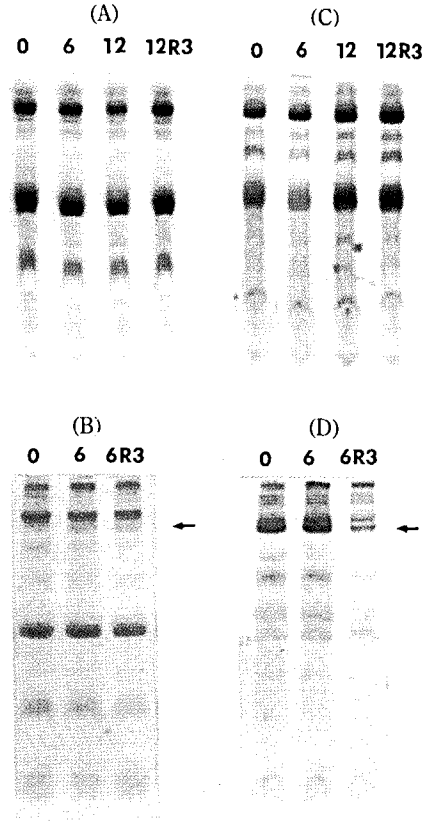

Fig. 4. Pigment-protein complexes and corresponding autoradiogram of cucumber and pea leaf discs during light chilling or during the subsequent recovery period. Pea leaf discs (A) and cucumber leaf discs (B) were pulse-labeled with [^{35}S]Met for 90 min, and chilled in the light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 0, 6, or 12 h at 4℃. Pea and cucumber leaf discs were transferred to 25℃ in the light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and recovered for 3 h after 6 h (6R3) and 12 h (12R3) light-chilling, respectively. (C) and (D) are corresponding autoradiograms of (A) and (B), respectively.

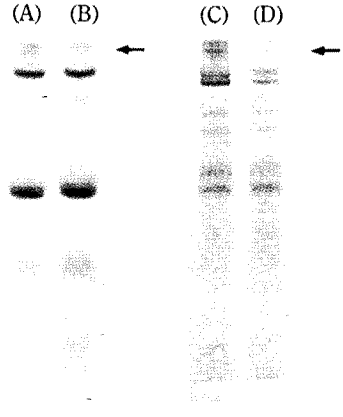

Fig. 5. Pigment-protein complexes and corresponding autoradiogram of cucumber leaf discs during room temperature photoinhibition or during the subsequent recovery period. Cucumber leaf discs were pulse-labeled with [^{35}S]Met for 90 min (A), and photoinhibited with PFD of 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 3 h (B) at 25℃. (C) and (D) are corresponding autoradiograms of (A) and (B), respectively.

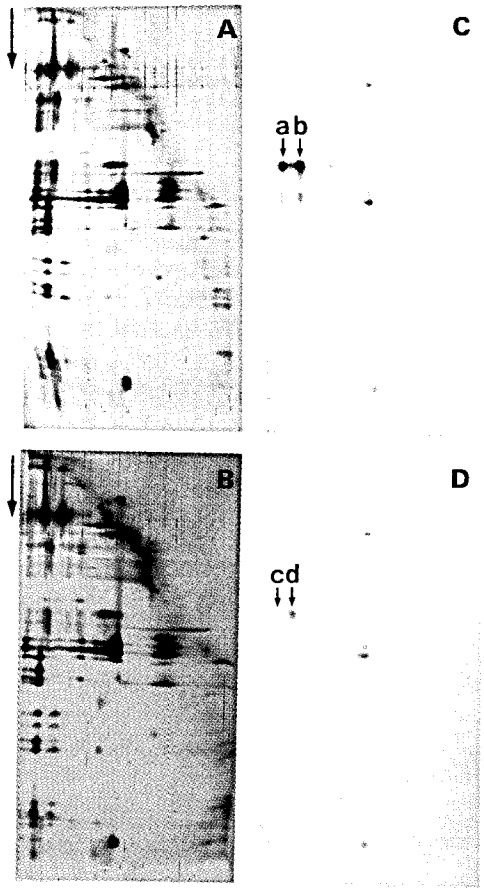

Fig. 6. Two-dimensional SDS-PAGE analysis of polypeptide composition of pigment-protein complexes and corresponding fluorogram in room temperature photoinhibited cucumber leaf discs. Cucumber leaf discs were pulse-labeled with [^{35}S]Met for 90 min (A), and photoinhibited with PFD of 1200 μmol·m^{-2}·s^{-1} for 3 h (B) at 25℃. (C) and (D) are corresponding autoradiograms of (A) and (B), respectively.

and light-harvesting complex II (LHCII) (see Fig. 6). In cucumber leaf discs light-chilled for 12 h, intensities of some bands in the autoradiogram of the native green gel were reduced in a similar manner to those observed in leaves light-chilled for 6 h and recovered for 3 h (data not shown).

These results suggest that the pigment-protein complexes were not disassembled during a 6 h light-chilling in the cucumber leaf discs, but the irreversibly damaged D1 protein was rapidly degraded during the subsequent recovery period. Similarly, Aro *et al.* (1990) reported that the loss of D1 protein was observed only during the recovery period after light-chilling, but not during light-chilling.

Comparison of effects of light-chilling with room temperature photoinhibition

To compare the effects of light-chilling with room temperature photoinhibition, cucumber leaf discs were photoinhibited for 3 h in the high light (1200 μmol·m^{-2}·sec^{-1} PAR), resulting in a decrease in (Fv)m/Fm down to 0.4. As shown in Fig. 5, photoinhibition caused a decrease in the intensity of a slowly migrating band of the native green gel and in the intensity of a corresponding band in the autoradiogram, as indicated by arrows in lanes (B) and (D), respectively. In the two-dimensional SDS-PAGE analysis of bands of the native green gel shown in Fig. 6, the slow migrating bands seemed to contain oligomeric forms of PSII (possibly a dimeric form (Bassi *et al.*, 1990; Santini *et al.*, 1994)). The labeled D1 protein (arrow (a) in Fig. 6(C)) was thought to be degraded first by photoinhibition, resulting in a faint spot (arrow (c) in Fig. 6(D)). Instead of its decrease, the intensity of an another spot was increased during photoinhibition (see the spot indicated by an arrow (d) compared with a spot indicated by an arrow (b) in Fig. 6). This second spot is assumed to be the D1 protein of PSII without LHCII.

A comparison between the effect of light-chilling shown in Fig. 4 and the effect of room temperature photoinhibition shown in Fig. 5 leads us to suggest that the principal damage caused by light-chilling is different from that caused by room temperature photoinhibition.

In addition to the damage in PSII caused by light-chilling, there are many reports on its inhibitory effect on PSI (Havaux and Davaud, 1994; Terashima *et al.*, 1994). In our preliminary experiments, we also observed an appearance of a PSI form without some small extrinsic peptides located in the stromal side of PSI by light-chilling in cucumber leaves, which is located in a position indicated by an arrow in Fig. 4(B) with the same mobility as a PSII form with labeled D1 protein indicated by an arrow in Fig. 4(D). The damage in PSI might be the additional cause for the initial drop in (Fv)m/Fm for 2 h in cucumber leaf discs light-chilled for 3 h (Fig. 3(B)).

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