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CHLOROPHYLL FLUORESCENCE IN CUCUMBER (*Cucumis sativus* L.) AND PEA (*Pisum sativum* L.) LEAVES UNDER CHILLING STRESS IN THE LIGHT AND DURING THE SUBSEQUENT RECOVERY PERIOD

SUK-BONG HA, YOUNG-JAE EU and CHOON-HWAN LEE*
Department of Molecular Biology, Pusan National University, Pusan 609-735, Korea

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Abstract — To investigate the chilling sensitivity related injuries in the photosynthetic apparatus of cucumber leaves, the light-chilling induced alterations of chlorophyll fluorescence transients in cucumber leaves were compared with those in pea leaves. As an early effect of light-chilling, an increase in Fp/Fm† was observed in both pea and cucumber leaves, which was saturated by about 6 h chilling. However, the saturated value of Fp/Fm was almost 1.0 in cucumber, in contrast to about 0.8 in pea. During the recovery period after 24 h chilling, the light-chilling induced changes in pea seemed to be reversed, but those in cucumber leaves were thought to be irreversible, because Fo was increased significantly. Light-chilling caused significant decreases in qQ and qE in cucumber leaves, but qR was increased until 6 h, and decreased thereafter. In both pea and cucumber leaves, Fm was increased by 2 h dark treatment. The Fm from the predarkened pea leaf discs was higher than the value from the preilluminated ones during the whole period of light-chilling (500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR). However, the predarkened cucumber leaf discs showed a reduction in Fm and an increase in Fo during the 2 h chilling in the light. These results indicate that the causes of chilling sensitivities in photosynthetic apparatus of cucumber leaves are possibly related with the damage in PSI reaction center and the ability of acidification of lumen by PSII.

INTRODUCTION

A chilling temperature is defined as a temperature low enough to cause plant tissue damage without causing freezing of tissue water.[1] For most chilling sensitive plants, chilling temperatures are between 0 and 10°C.[2] Chilling sensitive plants are injured more severely or quickly in the light than in the dark.[3,4]

Chilling sensitive plants under strong illumination show a fast and large inhibition of light- and CO_2-saturated photosynthesis.[5] The quantum efficiency of photosynthesis, therefore, is remarkably declined.[6] Photoinhibition of photosynthesis is not caused by a decrease of the leaf pigment concentration since light-induced leaf pigment degradation only starts after prolonged exposure to light during chilling.[7]

Initial events inducing photoinhibition of photosynthesis might be chilling-induced structural changes of lipid domains,[8] conformational changes of proteins in the chloroplast membrane[9] or conformational changes in some regulatory enzymes.[10]

Changes in membrane fluidity[11,12] or partial lipid phase transition[13] was observed under short-term chilling. Quinn[14] suggested that small areas of gelphase lipids is formed by the solidification of domains of highermeltingpoint lipids present in the chilling sensitive species. During cooling the highmelting-point lipids separate into the gel phase domain and exclude the membrane proteins together with the lowermeltingpoint lipids. These changes might disturb the distribution of the components of photosynthetic apparatus.

* To whom correspondence should be addressed.
† *Abbreviations* : D-P, Kautsky notation of the different points in the Chl fluorescence transients; Fm, maximal level of Chl fluorescence; Fo, constant or initial Chl fluorescence; Fp, fluorescence level at 'peak' (P) level; Fr, maximal rate of fluorescence increase in the D-P rise curve; Fv, variable Chl fluorescence; (Fv)m, maximum Fv (Fm minus Fo); Q_A, a primary quinone acceptor in PSII; Q_B, a secondary quinone acceptor in PSII; qE, energy-dependent quenching; qQ, photochemical quenching; qR, non-photochemical quenching remaining after the reversal of qE; RT, room temperature.

Light has deleterious effects on chilling sensitive plants by a prolonged exposure to chilling. Many of the long-term effects are due to the photooxidation of photosynthetic apparatus, which include the degradation of leaf pigments[7] and chloroplast unsaturated lipids,[3,15] structural damages to the chloroplast[16], and the inhibition of the electron transport activities.[17] The photooxidation is generally considered to be due to the activated superoxide ion (O_2^-) formed through Mehler reaction by blocking dark reaction, and/or singlet oxygen ($^1O_2$) formed by the excitation energy transfer of chlorophyll (Chl^1) in triplet state to triplet oxygen ($^3O_2$).[18,19,20]

Changes in photosynthetic apparatus can be sensitively monitored by measuring Chl fluorescence emitted from plant leaves.[21,22] Recently, Chl fluorescence technique has been improved by using a weak modulated light in conjunction with a system that selectively detects the fluorescence emitted at the same frequency and phase as the modulated source.[23] By using the modulated source, a rapid and sensitive detection of Chl fluorescence from intact leaves is possible, even in the fields, because this technique does not need isolation and purification of the various components of the photosynthetic apparatus and the equipments are handy and relatively low in cost.

By using this technique, the light-chilling induced changes in photosynthetic apparatus of a chilling sensitive cucumber were investigated.[24] Although many chilling-induced changes in the Chl fluorescence transients were observed, some of them were common to both chilling sensitive and chilling resistant plants, e.g. rise in Fr during the first 2 h light-chilling. To understand the mechanism of plant responses related with the chilling tolerance, comparisons among plant species showing different sensitivity to chilling stress are necessary. Chilling stress induced changes in some parameters were dependent on plant species under investigation: as an example, Fo was not a good indicator for chilling resistance screening among rice cultivars.[25]

In this study, the light-induced alterations of Chl fluorescence in cucumber leaves were investigated in details in comparison with a chilling resistant pea, as an extension of the work reported by Ha *et al.*[24]

MATERIALS AND METHODS

Plant materials, growth and chilling treatment conditions. Cucumber (*Cucumis sativus* L. cv. Ilmichungjang) and pea (*Pisum sativum* L. cv. Sparkle) seeds were germinated in moistened cloth at 25°C for 24 h in the dark, and grown at 25°C-28°C under continuous light from white fluorescence tubes giving PAR of 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$. For chilling, 15-20 day-old seedlings were placed at 4°C under the same light condition used for their growth. Plants were well-watered to reduce the effect of drought stress. For recovery after chilling, plants were transferred back to the chamber where they had grown.

Chl fluorescence measurements and quenching analysis. Chl fluorescence transients and quenching coefficients were measured in a pulse modulated (PAM) fluorometer (Walz, Germany) as described by Ha *et al.*[24] Leaf discs (10 mm in diameter) were excised, and placed immediately in petri dishes filled with distilled water. After dark-adaption for 20 min, Chl fluorescence from the adaxial side of the leaves was measured. The initial fluorescence (Fo) was measured with modulating beam (0.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) alone, and actinic light (110 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) was provided by a light emitting diode (H2000, Stanley, Japan). The maximum fluorescence (Fm) was induced by a

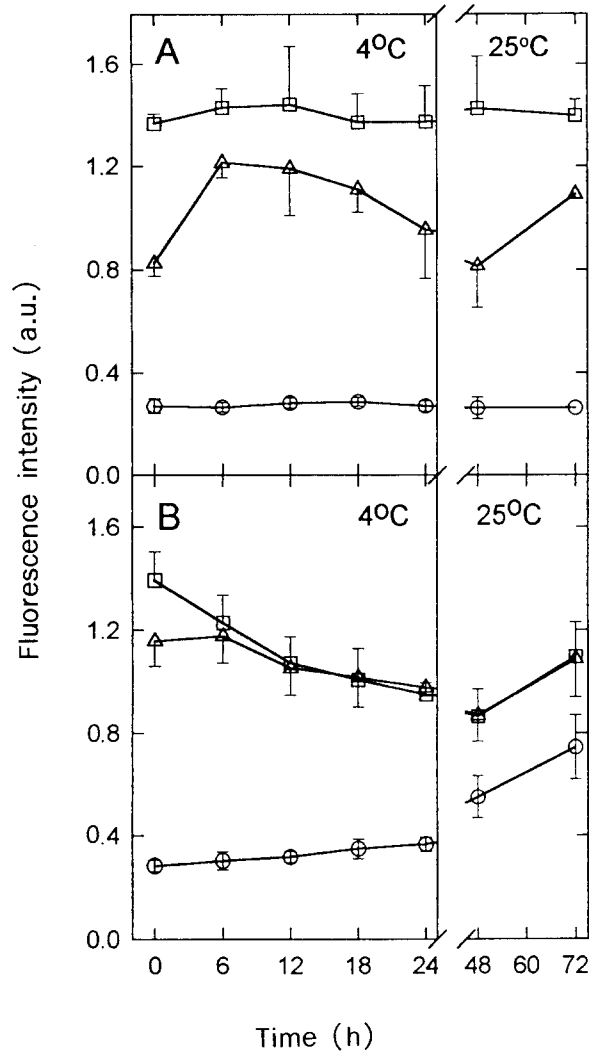

Figure 1. Changes in Fm, Fp and Fo in pea and cucumber leaves during light-chilling and the subsequent recovery period. (A) pea and (B) cucumber leaves were chilled at 4°C and recovered at 25°C in the light (20 $\mu mol \cdot m^{-2} \cdot s^{-1}$). (□) Fm, (△) Fp and (○) Fo.

saturated light pulse (2200 μmol · m^{-2} · s^{-1} PAR) provided by a halogen lamp (KL1500, Schott, Germany) for 0.8 s. Quenching coefficients were calculated as described by Oxoborough and Horton.[26]

RESULTS AND DISCUSSION

Light-chilling induced changes in parameters of Chl fluorescence transients. To investigate the effect of chilling in the light on photosynthesis of chilling sensitive plants, alterations of Chl fluorescence transients in cucumber were compared with those in pea. Figures 1 and 2 show the changes in Fm, Fp and Fo, and the changes in Fp/Fm and (Fv)m/Fm with the progress of the light-chilling and during the subsequent recovery period in the light, respectively.

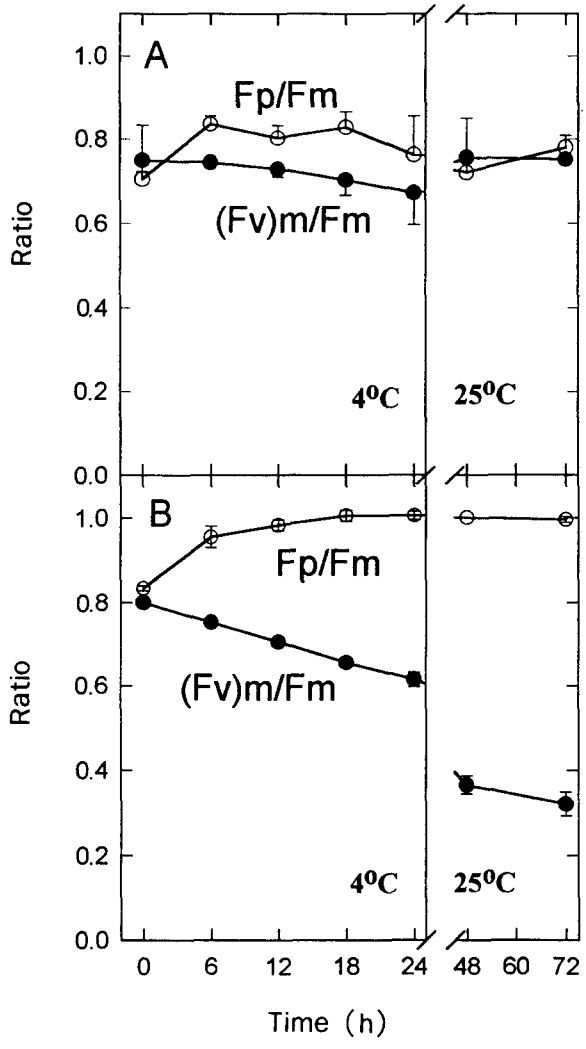

Figure 2. Changes in (Fv)m/Fm and Fp/Fm in pea and cucumber leaves during light-chilling and the subsequent recovery period. (A) pea and (B) cucumber leaves were chilled at 4°C and recovered at 25°C in the light (20 μmol · m^{-2} · s^{-1}). (●) (Fv)m/Fm and (○) Fp/Fm.

In Figure 1(A), the chilling resistant pea showed no significant changes in Fm and Fo with an increase in Fp during the initial 6 h chilling. In contrast, the chilling sensitive cucumber showed a decrease in Fm and a gradual increase in Fo, and a rather fast increase in Fo was observed during the subsequent recovery period (Fig. 1(B)).

As shown in Figure 2, the relative increase in Fp compared to Fm was almost saturated within 6 h in both plant species, suggesting that this phenomenon was not related to the differences in chilling sensitivity between the two species. The early increase in Fp/Fm is related to the increase in Fr during 2 h light-chilling and also to the increase in Fr during 15 min dark-chilling reported in Ha *et al.*[24] However, the saturated value of Fp/Fm was almost 1.0 in cucumber, in contrast to about 0.8 in pea (Fig. 2), suggesting that the decrease in membrane mobility may be the cause of the increase in Fp/Fm to 0.8, and the decrease in membrane mobility[11,12] may not be enough to block the Q_A^- reoxidation completely in pea. Photochemical reactions in cucumber were almost completely blocked by light-chilling, possibly due to the damage in PSI reaction centers.[27,28]

Although the reduced Fp and (Fv)m/Fm by light-chilling was restored during the recovery period in pea, the reduced (Fv)m/Fm was decreased more during the recovery period in cucumber leaves (Figs. 1 and 2). The light-chilling induced changes in pea seemed to be reversible. However, the photosynthetic machinery in cucumber was irreversibly damaged by the light-chilling. This idea is supported by the significant increase in Fo during the recovery period in cucumber (Fig. 1), such that the irreversibly damaged (presumably) PSII centers during light-chilling[29] seemed to be broken down further during the recovery period.

Light-chilling induced changes in overall Chl fluorescence transients. As reported in Ha *et al.*,[24] the noticeable changes in the overall Chl fluorescence transients were the increase in Fv after the P level and the decrease in the energy dependent quenching (qE). Because the increase in Fv may be a very early symptom of chilling, leaf discs were put in a dark aluminum chamber at 4°C for 15 min and Chl fluorescence was measured at 4°C. In both plants, both Fp and Fv in the transients after P level were observed after dark-chilling for 15 min (Fig. 3). One of the causes for both of these phenomena is the reduction of membrane lipid mobility by dark-chilling. We can assume that the decrease in lipid mobility resulted in the increases in Fp/Fm during the 6 h light-chilling in both plant species shown in Figure 2.

However, the decrease in Fv after P level was almost

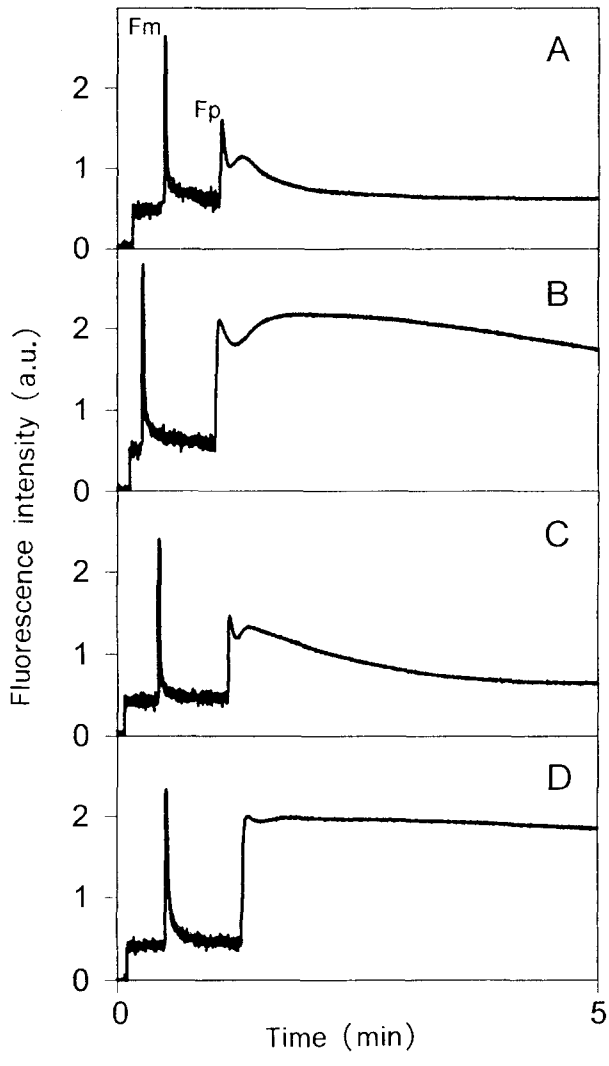

Figure 3. Early changes in the overall Chl fluorescence transients in pea and cucumber leaf discs during dark-chilling. (A) pea leaf discs measured at 25°C after 15 min dark-incubation at 25°C, (B) pea leaf discs measured at 4°C after 15 min dark-chilling at 4°C, (C) cucumber leaf discs measured at 25°C after 15 min dark-incubation at 25°C, and (D) cucumber leaf discs measured at 4°C after 15 min dark-chilling at 4°C. The initial saturation light pulses were given to measure Fm.

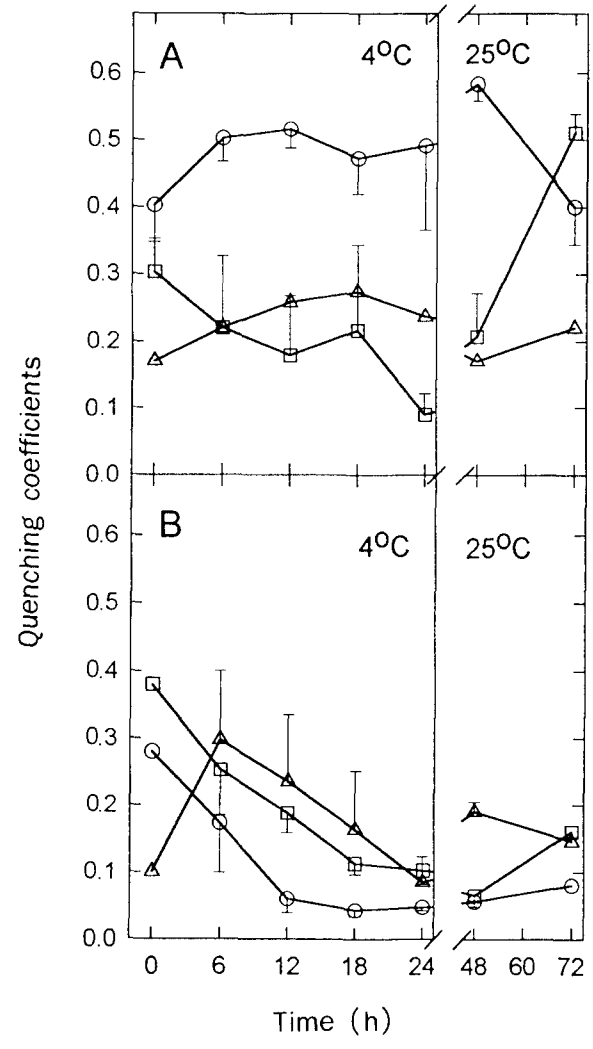

Figure 4. Changes in qQ, qE and qR in pea and cucumber leaves during light-chilling and the subsequent recovery period. (A) pea and (B) cucumber leaves were chilled at 4°C and recovered at 25°C in the light (20 μmol · m^{-2} · s^{-1}). (○) qQ, (□) qE and (△) qR.

completely blocked in cucumber leaf discs (Figs. 3(C) and 3(D)). Although this can be explained by the reduction of membrane lipid mobility by dark-chilling, the extent of the increase in Fr by dark-chilling was very similar in both plant species.[24] Therefore, the blockage of the decrease in Fv after P level shown in cucumber, but not in pea, was caused by some kinds of hindrance of Q_A reoxidation.[10]

Light-chilling effects on fluorescence quenching coefficients. Differences in the overall Chl fluorescence transients are closely related to Chl fluorescence quenching. The changes in quenching coefficients in pea leaves were rather slow and in a lesser degree when compared with those in cucumber leaves (Fig. 4). In Figure 4(B), light-chilling caused significant decreases in qQ and qE in cucumber leaves. By light-chilling, qR increased until 6 h, and decreased thereafter. Gradual changes in the quenching coefficients were observed during the 6 h light-chilling period[24] and were apparent just after 1 h chilling in the light. After about 6 h light-chilling, the Chl fluorescence from cucumber leaves could not recover at RT (data not shown). Possible causes of this relatively long-term damage is the irreversible damage in the D1 protein[31] and the inactivation of PSI by photooxidation.[18,19,20,27,28] The decrease in qR after 6 h light-chilling is probably a sign of unrecoverable damage, although this remained to be confirmed.

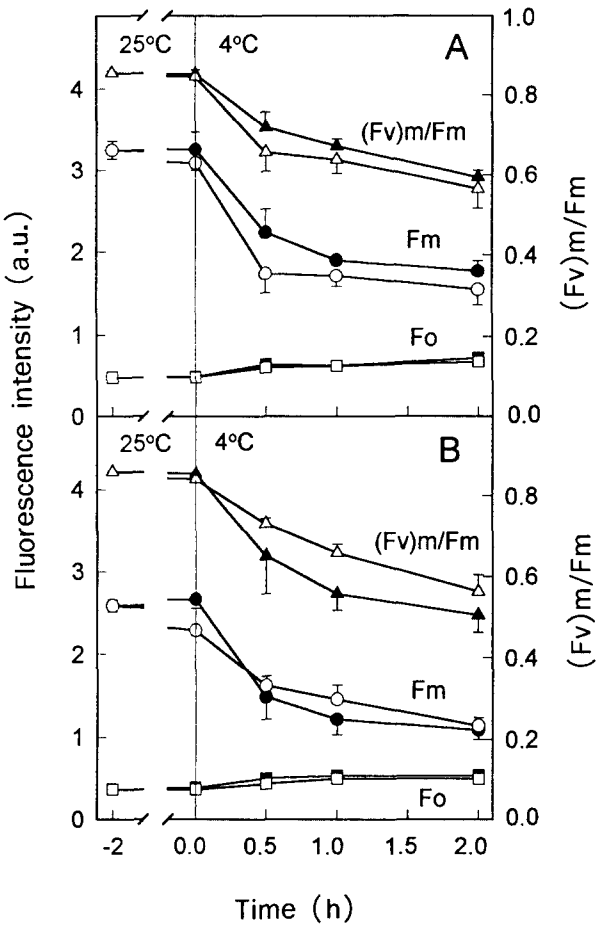

Figure 5. The effect of pre-dark treatment on the chilling-induced changes in (Fv)m/Fm, Fm and Fo in pea and cucumber leaf discs. (A) pea and (B) cucumber leaves were chilled at 4°C in the light (500 μmol · m^{-2} · s^{-1}). (Fv)m/Fm (△, ▲), Fm (○, ●) and Fo (□, ■) were measured in predarkened leaves (closed symbols) and in preilluminated leaves with PAR of 20 μmol · m^{-2} · s^{-1} at 25°C (open symbols).

The possible relationship of chilling resistance with the zeaxanthin cycle. The decrease in qE is due to the blockage of ΔpH formation across the thylakoid membrane[32] and/or the hindrance the role of xanthophyll cycle in the energy dissipation.[33] Demmig-Adams *et al.*[34] suggested that the formation of large amount of zeaxanthin was probably an important factor in the acclimation of plants to chilling temperatures. To examine the possible involvement of the zeaxanthin cycle in chilling resistance, pea and cucumber leaf discs were kept in the dark for 2 h. The 2 h dark incubation may be enough to epoxidate zeaxanthin to violaxanthin, because the qE relaxation was normally completed in about 10 min and Noctor *et al.*[35] detected no zeaxanthin after 50 min dark treatment of spinach leaves.

In both pea and cucumber leaves, Fm was increased by 2 h dark treatment, suggesting the zeaxanthin contents were decreased by the dark treatment. The Fm from the predarkened pea leaf discs was higher than the value from the preilluminated ones during the whole period of light-chilling (Fig. 5(A)), supporting the protective role of zeaxanthin by the energy dissipation.[34] However, the unprotected (predarkened) cucumber leaf discs showed a reduction in Fm and an increase in Fo compared with the preilluminated ones during 2 h chilling in the light (500 μmol · m^{-2} · s^{-1} PAR) (Fig. 5(B)). Similar changes were reported in mangrove (*Rhizophora mangle*) leaves during the recovery period in the light (100 μmol · m^{-2} · s^{-1}) by Demmig-Adams *et al.*[34]

The measurement of data shown in Figure 5 was performed at 4°C after 5 min dark period at 4°C. When the parameters were measured at RT after 5 min dark period at RT, similar results were obtained (data not shown), suggesting that no noticeable recovery process was involved during the 5 min dark period at RT.

While the unprotected pea leaf discs were not damaged more than the protected ones, the unprotected cucumber leaves suffered severer damages. Although our results cannot explain the protective mechanisms to light-chilling in chilling resistant pea leaves, one of the differences in chilling susceptibility in the light is probably related with the ability of acidification of lumen by PSII. We have observed a decrease in qE during 1 h light-chilling,[24] and Peeler and Naylor[36] reported uncoupling in cucumber thylakoids by light-chilling, even during the chloroplast isolation procedure on ice. Whether the two species show differences in the zeaxanthin contents and/or the uncoupling in cucumber is a primary cause for the most chilling injuries[36,37] remain to be elucidated.

CONCLUSION

In conclusion, the causes of chilling sensitivities in photosynthetic apparatus of cucumber leaves could be predicted by analyzing the chilling-induced alterations of Chl fluorescence transients in cucumber leaves compared with those in pea leaves. In cucumber leaf discs, a significant decrease in qE was observed by light-chilling and the predarkened leaf discs showed a reduction in Fm and an increase in Fo during the 2 h chilling in the light. These results suggest that the causes of chilling sensitivities in photosynthetic apparatus of cucumber leaves are possibly related with the ability of acidification of lumen by PSII and possibly with the ability of zeaxanthin photoprotection,

although we do not have conclusive data for the contents of zeaxanthin.

After the first 6 h light-chilling, the saturated value of Fp/Fm was almost 1.0 in cucumber, in contrast to about 0.8 in pea. The almost complete blockage of photochemical quenching could be explained by the reduction of thylakoid membrane mobility[11,12] and/or by the hindrance in the electron transport pathways starting from PSII. Because the extent of the increase in Fr by dark-chilling was very similar in both plant species,[24] the blockage of the decrease in Fv after P level and the saturation of Fp/Fm to 1.0 are due to some kinds of hindrance of Q_A^- reoxidation,[30] possibly due to the damage in PSI reaction centers.[27,28]

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