Analysis of photoinhibition of photosynthesis at chilling temperature

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低温処理による光合成障害の解析

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Table of Contents

List of Figures

Abstract

Plants of tropical or subtropical origins tend to suffer damage to photosynthesis at chilling temperature. In order to clarify the mechanism of chilling sensitivity in molecular level, we have to establish a model plant sensitive to chilling stress. In this report, chilling-induced inhibition in photosynthesis was studied in the miniature dwarf tomato cultivar (*Solanum lycopersicon* cv. Micro-Tom) that could be a new model plant for the study of chilling sensitivity. Chlorophyll fluorescence and photo-oxidizable P-700 were measured using a pulse amplitude modulated (PAM) system and a spectrophotometer. The stronger the photon flux density during the light chilling treatment becomes, the more the decrease of the maximum quantum yield of photosystem II (PSII) photochemistry (Fv/Fm) is. On the other hand, the inhibition of PSI is observed under low light condition, and the extent of the inhibition is not much increased by the increase of photon flux density during the treatment. The recovery of PSI after the chilled leaves were returned to the normal growth condition was a slow process and rate limiting for the recovery of total electron transfer. When decrease of Fv/Fm and increase of non-photochemical quenching (NPQ) was induced by the pretreatment of high light before the chilling treatment, the photoinhibition of PSI was suppressed. Even when chilling tolerant spinach was used, the photoinhibition of PSI could be induced *in vitro* in broken chloroplasts but not in intact chloroplast, irrespective of the temperature during the treatment. These results indicate that a mechanism exists to protect PSI in chloroplast in chilling tolerant plants.

Introduction

Plants that have been introduced from tropics and subtropics area often suffer a distinct physiological damage when they are exposed to low but not freezing temperatures (Hetherington et al. 1989). This phenomenon is referred to as chilling injury. Because the phenomenon is observed at temperatures above 0°C, chilling injury is quite distinct from freezing injury, which is mainly associated with physical disorders caused by freezing of water. The chilling-sensitive plants develop chilling injury at temperatures below a specific threshold temperature of around 10°C, and the chilling stress causes lowered growth and productivity in these plants. Although the chilling injury could be induced in complete darkness, it is more marked under light condition (Terashima et al. 1989). Thus, chilling injury in the light is regarded as a kind of photoinhibition (Sonoike 1998). The combination of low temperature and low light is typical of conditions experienced in the temperate climates in the early morning when temperature is lowest in a day. Therefore it is highly desirable to improve the chilling tolerance of horticultural crops such as common bean (*Phaseolus vulgaris* L.), tomato (*Lycopersicon esculentum*) and cucumber (*Cucumis sativus* L.) that have tropical or sub-tropical origin. To establish such improvement, it is necessary to clarify the physiological and molecular mechanisms of chilling response in horticultural crops.

Photosystem II (PSII) is generally the most sensitive to environmental stress, especially in the case of high light stress (Barber and Andersson 1992; Aro et al. 1993) whereas photosystem I (PSI) is relatively stable. However, when low photon flux density is combined with chilling temperature, the damage to PSII is often negligible whereas PSI is severely damaged. The extent of inhibition of PSI is highly dependent on

temperature (Sonoike 1999). Selective photoinhibition of PSI in cucumber (Terashima et al. 1994) and potato (Havaux and Davaud 1994) was reported in 1994 (Sonoike 1996; Sonoike 1998). Generally, low temperature is coming up in the morning when the light is weak. In cucumber leaves, several hours of weak illumination (100–200 μmol m^{-2} s^{-1}) at chilling temperature caused a decrease in PSI activity with almost no inhibition of PSII activity. A study with cucumber has indicated that inactivation of PSI occurs by chilling treatment under light and the inactivation remains even after the temperature recovers to the normal level. Therefore, the damage to PSI must be more serious than that to PSII for the plant survival. In the case of PSII, the turnover rate of the PSII protein is relatively high, and the activity of PSII rapidly recovers after the cessation of the stress treatments. It must be noted that the inhibition of PSI is completely suppressed *in vitro* as well as *in vivo* by the addition of DCMU or DBMIB, which blocks electron transfer from PSII to PSI (Havaux and Davoud 1994; Sonoike 1995). This observation implies that any kind of inhibition in PSII activity might protect PSI from photoinhibition. However it is not known whether the protection of PSI through the inhibition of PSII takes place *in vivo.*

Whereas the photoinhibition of PSI was observed in chilling-sensitive cucumber leaves (Terashima et al. 1994), the PSI activity in chilling-tolerant spinach leaves has been reported to be unaffected at chilling temperature (Somersalo and Krause 1990). Interestingly, the photoinhibition was observed in both plants when isolated thylakoid membranes were illuminated (Sonoike 1995). The results suggest that P-700 itself is not a chilling-sensitive component and some component(s) of the protective mechanism exist *in vivo.* However, the identity of this component is totally unknown, and we do not know whether the component exists in chloroplast or in cytosol.

To elucidate the molecular mechanism of photoinhibition at chilling temperature in future, analysis of a model plant, with which researchers can use technique of molecular biology and genomic information, is inevitable. In this study, a new tomato cultivar (*Lycopersicon esculentum* cv. Micro-Tom) was used. Micro-Tom is a miniature dwarf tomato cultivar that was originally bred for home gardening (Scott and Harbaugh 1989). The size of tomato plant is relatively large with a relatively long life cycle. However Micro-Tom has been used in the studies of plant science to overcome these problems (Meissner et al. 1997). This variety of plant grows to a similar size as *Arabidopsis* and has a shorter life cycle than other tomato varieties, routinely producing seeds within 12 weeks after being planted. Recently a large, publicly available Micro-Tom EST database, MiBASE, (http://www.kazusa.or.jp/microtom/) has been generated with support from Kazusa DNA Research Institute (Yamamoto et al. 2005) and tomato genome sequencing programs have been launched as part of the internationally coordinated International Solanaceae Genome Project (SOL) consortium (http://www.sgn.cornell.edu/solanaceae-project/). In addition, it is possible to use technique of molecular biology such as recombinant DNA experiment in Micro-Tom (Sun et al. 2006). These features are similar to those of *Arabidopsis*; and this tomato is considered to be a new model plant. Hence, I determined to use Micro-Tom as a model plant for photoinhibition experiments.

The aims of this study is (a) to estimate the chilling sensitivity of Micro-Tom, (b) to verify whether the inhibition of PSII that is observed by light chilling treatment protect PSI from further photoinhibition *in vivo*, and (c) to determine the localization of protective mechanism in chilling tolerant plants. I investigated the process of PSI photoinhibition and repair in Micro-Tom. I showed that electron flow of down stream of

PSII measured by photochemical quenching recovered quickly, although the recovery of photo-oxidizable P-700 and the effective quantum yield of electron transfer (ϕII) was a slower process. I also demonstrated that the photoinhibition of PSI was observed in the thylakoid membrane but not in the intact chloroplast. I believe that the overall results would become the basis for the molecular analysis of the chilling sensitivity in plants.

Materials and Methods

Plant materials

Cucumber (*Cucumis sativus* L. cv. Nanshin; from Takii, Kyoto, Japan) were germinated in vermiculite and grown hydroponically in a growth chamber at 30°C under continuous light at 100 μmol m^{-2} s^{-1}. Humidity was not controlled. Hydroponic solution (Hewitt's solution that contained 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 100 μM NaCl, 50 μM H_3BO_3, 50 μM Fe(III)-EDTA, 10 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM Na_2MoO_4, and 0.2 μM $CoSO_4$) was continuously aerated. Fully developed, second main leaves from approximately 3 week-old plants were used for experiments. Tomato (*Solanum lycopersicon* cv. Micro-Tom; from Kazusa DNA Research Institute, Chiba, Japan) plants were grown 23°C, under continuous light at 100 μmol m^{-2} s^{-1} and 65% relative humidity. Seed were sowed on soil-pot (Jiffy-seven; Sakata Seed, Yokohama, Japan). After germination, they were transplanted in vermiculite and fertilized soil. The plants were fed with commercial nutrient solution (Hyponex; N:P:K=6:6:6, Hyponex Japan, Osaka, Japan) once a week. Fully developed, third main leaves from approximately 3- to 4-week-old plants were used for experiments. Spinach leaves (*Spinacia oleracea* L.) were purchased from a local market.

Photoinhibitory treatments of leaves

Plants leaves were placed on the surface of temperature-controlled water with dorsal side down. Light from fluorescent lamps (FPL27EX-N; TOSHIBA, Tokyo, Japan) was used for inhibitory treatments. The photon flux densities of inhibitory light were measured at leaf level using photometer (Li-250; Li-Cor, Lincoln, Neb., USA).

Attached leaves were used for recovery experiments, while detached leaves were used for simple inhibitory experiments.

Determination of chlorophyll fluorescence and absorption chage due to P-700 in vivo by a pulse-modulated system

Fluorescence was measured using a pulse-modulated fluorometer (PAM 101/103; Walz, Effeltrich, Germany). Minimum fluorescence (F_0) was recorded after dark adaptation for 10 min. Maximum fluorescence (Fm) was obtained by applying a 0.8-s saturating light pulse (Cucumber; 5,600 μmol m^{-2} s^{-1}, Tomato; 2000 μmol m^{-2} s^{-1}) from a light source (KL 1500; Schott, Wiesbaden, Germany). The maximal quantum yield of PSII photochemistry was calculated as $Fv/Fm=(Fm-F_0)/Fm$. The light-adapted state fluorescence yield (F) was monitored continuously and a saturating light pulse was supplied at intervals of 20 s to determine maximum variable fluorescence (Fm'). The effective quantum yield of electron transfer (ϕII) and non-photochemical quenching of chlorophyll fluorescence (NPQ) at the light-adapted state were defined as (Fm'-F)/Fm' and (Fm-Fm')/Fm', respectively. F_0' represents the minimum fluorescence in the light-adapted state. The effective quantum yield of PSII photochemistry (Fv'/Fm') and the photochemical quenching of chlorophyll fluorescence (qP) were calculated as ($Fm'-F_0'$)/Fm' and ($Fm'-F$)/($Fm'-F_0'$), respectively.

The absorption change around 830 nm due to P-700 oxidation in vivo was measured using a pulse-modulated system (PAM 101/102; Walz, Effeltrich, Germany; Schreiber et al. 1988). P-700 was oxidized by far-red light from a photodiode (FR-102). The irradiance of the far-red light was 13.5 W m^{-2} unless otherwise stated.

Isolation of thylakoid membranes and determination of P-700 in vitro

For isolation of thylakoid membranes, cucumber leaves were homogenized for 10 s with a Polytron homogeniser (Kinematica, Luzern, Switzerland) in an ice-cold buffer containing 50 mM Tris/HCl (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M Sucrose. The homogenate was filtered through a layer of 20-μm nylon mesh and the filtrate was centrifuged at 2000 g for 2 min. The pellet was suspended in the same buffer. To determine the concentration of photooxidizable P-700, light minus dark difference absorption changes of P-700 at 701 nm were measured using a spectrophotometer (model 356; Hitachi, Tokyo, Japan). Actinic light was provided by a tungsten lamp passing through two layers of blue filters (CS 4-96; Corning Glass, Coming, N.Y., USA) and a 7-cm layer of water. The photomultiplier was protected with a cut-off filter (R-69; Toshiba) and an interference filter (Toshiba; transmittance peak at 701 nm). The reaction mixture contained thylakoid membranes equivalent to 15 μM Chl, 50 mM Tris-HCl (pH 7.5), 10 mM sodium ascorbate, 80 μM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), 10 μM DCMU, 0.01% (w/v) DM and 3 μM methyl viologen (Terashima et al. 1994; Sonoike1995). The difference absorption coefficient of P-700 was assumed to be 63 mM^{-1} cm^{-1} (Sonoike and Katoh 1990). Chlorophyll concentrations were determined after extraction with 80% acetone according to Porra et al. (1989).

Preparation of intact chloroplast and photoinhibition

For preparation of intact chloroplast, spinach leaves were homogenized in an ice-cold GR buffer that contained 10 mM HEPES-KOH (pH 7.5), 66 mM D-Sorbitol, 5 mM Sodium ascorbate, 0.2 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.4 mM EDTA (pH 8.0) for less than 3 s with a Polytron homogeniser. The homogenate was filtered through a layer of 20-μm

nylon mesh and the filtrate was centrifuged at 4000 g for 20 s. The pellet was suspended in the same buffer and then the suspension was centrifuged at 8000 g for 10 min on Percoll step gradient (30%/70% Percoll). Intact chloroplasts precipitated were re-suspended in the same buffer. The intactness of the chloroplast was assessed by determining the rate of electron transport through PSII with externally added potassium ferricyanide as an electron acceptor using pulse-modulated fluorometer as described above. For isolation of thylakoid membranes, intact chloroplast were osmotically shocked in a hypotonic buffer that contained 5 mM Tricine-NaOH (pH7.8), 5 mM NaCl, 1mM $MgCl_2$ for 1min at room temperature and then centrifuged at 2000 g for 2 min. The pellet was suspended in the GR buffer. Photoinhibitory treatment of chloroplasts and thylakoid membranes were carried out at 25 ^{o}C or 4 ^{o}C for 90 min by illumination at 100 μmol m^{-2} s^{-1} in the GR buffer containing 50 μM Chl.

Results

Physiological analysis of photoinhibition at chilling temperature in Micro-Tom

First, I examined the effects of various photoinhibitory treatments of Micro-Tom leaves at different temperatures. The changes in the functional PSI content were determined *in vivo* as an absorption change upon the photo-oxidation of P-700, the reaction-center chlorophyll of PSI. The quantum yield of PSII was estimated as a fluorescence parameter, Fv/Fm, through the pulse amplitude modulation (PAM) measurement of chlorophyll fluorescence. The effect of low temperature treatment for 5 h on the photosynthetic characteristics of Micro-Tom leaves under growth light (100 μmol m^{-2} s^{-1}) is shown in Fig. 3. Both PSI and PSII did not show any change by the treatment above 10°C, whereas inhibition was observed when the leaves were treated below 10°C. This sudden decrease in photosynthesis activities demonstrated the existence of a specific threshold temperature for the photoinhibition, which is commonly observed in chilling-sensitive plants. The apparent functional PSI content decreased by 30%, while the decrease in Fv/Fm was 10%. These results indicate that not only tomato but also Micro-Tom is a chilling-sensitive plant.

The effects of various PFD (0, 50, 100, 200, 300 μmol m^{-2} s^{-1}) during the chilling treatment at 0°C for 5 h on PSI and PSII are shown in Fig. 4. When the chilling treatment at 0°C was applied in complete darkness, decrease of Fv/Fm and photo-oxidizable P-700 *in vivo* were not observed at all. The result indicates that chilling injury in Micro-Tom is regarded as a kind of photoinhibition and PFD of normal growth condition is necessary for the inhibition. The stronger the PFD during the light chilling

treatment becomes, the more the decrease of Fv/Fm and photo-oxidizable P-700 determined *in vivo*. When P-700 was photochemically determined *in vitro* after the isolation of thylakoid membranes from the treated leaves, the situation becomes different. If the photon flux density during the treatment exceeds that of the growth environment, the inhibition did not increase any more, and may be even mitigated in some extent. Thus, the difference of the decrease of photo-oxidizable P-700 between *in vivo* and *in vitro* is observed at high photon flux density than normal growth condition (100 μmol m^{-2} s^{-1}). The cause of the difference could be ascribed to underestimation of the amount of photo-oxidizable P-700 due to unsaturated actinic light for the *in vivo* determination of photo-oxidizable P-700.

Cyclic electron transfer around PSI is induced by chilling treatment in cucumber leaves (Sonoike 1999). The euction of P-700 by cyclic electron transfer may compete with the photo-oxidation of P-700 by far-red actinic light, resulting in underestimation of the apparent absorption change. In order to check this possibility, the effect of the irradiance of far-red excitation light on the apparent P-700 content was compared between the untreated leaves and the light chilling treated leaves. Although oxidation of P-700 in the untreated leaves *in vivo* was fully saturated by far-red light below 10 W m^{-2}, far-red light at 13.5 W m^{-2} was not enough to oxidize P-700 completely in the leaves just after the chilling treatment (Fig. 5A). The difference between the contents of P-700 determined *in vitro* and *in vivo* can be ascribed to the enhanced reduction pressure on PSI by cyclic electron transfer or the increased antenna size in chilling leaves. In order to distinguish these two possibilities, the light-saturation curve for P-700 oxidation was determined *in vitro* where the effect of cyclic electron transfer could be neglected.

Apparently, light saturation of P-700 oxidation in chilled leaves occurs around 50 μmol m^{-2} s^{-1}, which is even lower than the light saturation point observed in the untreated leaves (Fig. 5B). The result indicates that the effect of cyclic electron transfer induced by the chilling treatment caused underestimation of P-700 content after the chilling treatment due to the enhancement of cyclic electron transfer. We henceforth determined the extent of the photo-oxidation of P-700 in thylakoid membranes that is isolated from the leaves just after the chilling treatment, to eliminate the effect of cyclic electron on the photoinhibition of PSI reaction center itself.

Protection of PSI through the inactivation of PSII

The electron flow from PSII is a prerequisite for the photoinhibition of PSI. Addition of DCMU, an inhibitor of PSII, was reported to suppress the inactivation of PSI both *in vivo* and *in vitro*. The protection of PSI might take place not only during the chilling treatment but also after the treatment when temperature recovers to the normal level. To verify this hypothesis, the changes of photosynthetic parameter after chilling treatment under light condition (300 μmol m^{-2} s^{-1}, 0°C, 5h) were determined periodically (Fig. 6). Overall photosynthetic yield decreased just after the chilling treatment. Although Fv/Fm, effective quantum yield of PSII (Fv'/Fm'), and effective quantum yield of electron transfer (ϕII) recovered to 90% in the first 24 h after the treatment, these parameters was not fully recovered until 72 h after the treatment. The extent of the decrease of P-700 was much smaller than these parameters, but the recovery was very slow. All the parameter values finally reached the untreated level at 72 h after the treatment. Interestingly, the decrease of photochemical quenching (qP), an indicator of

the redox level of plastoquinone pool between PSI and PSII recovered to the original level within 24 h after the treatment. These results, especially the constant qP value after 24 h, suggest that 1) the inhibition of PSI by chilling treatment is not critical in Micro-Tom just after the treatment, 2) the recovery of PSI from photoinhibition under normal growth condition is very slow, and 3) the rate-limiting step of the recovery process would be PSI after 24 h subsequent to the chilling treatment.

PSI is readily photoinhibited in leaves of cucumber (*Cucumis sativus*) at chilling temperatures by weak illumination with almost no damage to PSII and its inactivation remain for 6 days under normal growth condition (Kudoh and Sonoike 2002a). In order to determine whether the situation observed above is also true for the chilling treatment of cucumber, I investigated that the periodic changes of photosynthetic parameters after the chilling treatment (200 μmol m^{-2} s^{-1}, 4°C, 5h) of cucumber leaves (Fig. 7). The amount of functional P-700 decreased by 70% and the level of ϕII decreased by 80% just after the chilling treatment. The functional P-700 and ϕII was still 50% of the untreated level even at 72 h after the chilling treatment. The qP decreased 50% of the original level just after the treatment, and recovered to 70% of untreated level at 72 h after the treatment. Fv/Fm was also decreased by the chilling treatment, but the extent of the decrease was much smaller than that of functional PSI, and the recovery is also slow. In contrast to Micro-Tom, the photoinhibition of PSI by the chilling treatment was very severe in cucumber. However, the recovery of yield of electron transfer, as well as the yield of PSII, proceeded also in cooperation with that of PSI.

In order to check whether the photoinhibition of PSI is suppressed by the photoinhibition of PSII, I tried to induce selective photoinhibition of PSII by the

pretreatment of Micro-Tom by high light (6000 μmol m^{-2} s^{-1}) for 5 min. When a leaf was treated for 5 min under high light (6000 μmol m^{-2} s^{-1}), Fv'/Fm' was decreased to 55% of control level (Fig. 8A), while the decrease was not observed in a leaf treated under 100 μmol m^{-2} s^{-1} for 5 min. The amount of photooxidizable P-700 determined in isolated thylakoid membrane was also reduced by the pretreatment of high light (Fig. 8B). In this case, however, the further decrease of photooxidizable P-700 by subsequent chilling treatment was not observed. This protective effect of high light pretreatment may be brought about the decrease of the yield of PSII (Fv'/Fm'). Decrease of Fv'/Fm' could be due to either the decrease of maximum quantum yield of PSII (Fv/Fm) or the increase of non-photochemical quenching of chlorophyll fluorescence (NPQ). NPQ is a mechanism that harmlessly dissipates excess excitation energy as heat. Since the decrease of Fv/Fm (Fig. 8C) and the increase of NPQ (Fig. 8D) were observed upon the high light pretreatment, both factors are involved in the protection of PSI.

Protection of PSI by the factor only present in vivo

Although the photoinhibition of PSI in leaves is not observed in chilling sensitive plants such as spinach, inhibition of PSI is induced in isolated thylakoid membranes from spinach leaves (Sonoike 1995). The observation suggests that photoinhibition of PSI is a universal phenomenon and a mechanism exists to protect PSI *in vivo*. That protective mechanism should be the chilling sensitive component in chilling sensitive plants. To determine the localization of the protective mechanism, intact chloroplasts and thylakoid membranes isolated from spinach leaves were illuminated at 4°C for 90 min. After the treatment, the amount of P-700 was determined by the absorption change

at 701 nm. Both at 4°C (Fig. 9A) and at 25°C (Fig. 9B), the amount of P-700 in thylakoid membranes decreased upon photoinhibitory treatment, while that in intact chloroplasts was much smaller. The decrease of the amount of P-700 was less than 10% in complete darkness both in intact chloroplasts and in thylakoid membranes (Fig. 9A, B).

Discussion

The recent studies of the post-damage reactions in cucumber (Kudoh and Sonoike 2002a) and in *Arabidopsis* (Zhang and Scheller 2004) showed that a partial recovery was observed after photoinhibition of PSI. When PSI is inhibited, an imbalance in electron transport between PSI and PSII would be induced. In some cases, PSII may also be damaged but it would be quickly recovered resulting in more reduced conditions in plastoquinone pool between PSI and PSII. The imbalance in electron transport capacity is expected to lead to an adjustment of the PSI/PSII ratio in the same way as that during adaptation to changes in light quality (Pfannschmidt et al. 1999). In Micro-Tom, ϕII slowly recovered as well as the change of the functional PSI during the recovery period, whereas qP completely recovered in 24 h after the leaves were returned to the normal growth conditions (Fig. 6). The observation supports the idea that the adjustment of the PSI/PSII ratio takes place to prevent the excess electron flow into PSI. Dissociation of peripheral antenna system from PSII core might be an early structural change in the PSII complex that serves to limit electron donation to PSI. In cucumber, ϕII, photo-oxidizable P-700, and qP showed a similr change during the recovery period (Fig. 7). The inhibition of PSI in cucumber by chilling treatment was much larger than that in Micro-Tom. However, the recovery of photosynthetic capacities occurred in cooperation with that of PSI either in Micro-Tom or in cucumber. I conclude that the recovery of PSI from photoinhibition under normal growth condition was rate-limiting step in the recovery process in both plants to prevent further damage to PSI.

The recovery of PSII activity was completely recovered by 72 h after the leaves were returned to the normal growth conditions (Fig. 6). This is perhaps not surprising

because the turnover rate of D1 protein, one of the targets of PSII photoinhibition, is known to be fast, with reported half-times of 1-2 h (Sundby et al. 1993; Neidhardt et al. 1998). However, the result in Micro-Tom is very different from the observation in cucumber leaves exposed to light at chilling temperatures, and no full recovery was observed even in 72 h after the chilling treatment (Kudoh and Sonoike 2002a). Perhaps, the severity of the damage in cucumber may delay the normal PSII repair process, while turnover of D1 protein is not so much interfered by the relatively small inhibition of PSI in Micro-Tom.

The photoinhibition of PSI was suppressed when Fv/Fm decreased and NPQ was induced (Fig. 8). Thus, inactivation of PSII and the induction of thermal dissipation processes *in vivo* induced by the high light pre-treatment may actually protect PSI from photoinhibition. It is well known that D1 protein, one of the targets of PSII photoinhibition, is a protein with rapid turnover rate and that the photoinhibition of PSII can be rapidly recovered by the replacement of D1. On the other hand, because of the slow recovery of PSI from photoinhibition, the inhibition of PSI must be more harmful for plant survival than that of PSII. It is reasonable to assume that the inactivation of PSII under various stresses can be regarded as a protective mechanism of PSI from photoinhibition.

The photoinhibition of PSI was only observed in chilling-sensitive plants at chilling temperatures (Terashima et al. 1994), and the PSI activity in chilling tolerant plants has been reported to be unaffected at chilling temperature (Somersalo and Krause 1990). On the other hand, the photoinhibition of PSI in isolated thylakoid membranes from spinach was observed not only at 4°C but also at 25°C (Fig. 9). It seems that the photoinhibition of PSI is not a phenomenon unique to chilling-sensitive plants at least in

the case of inhibition *in vitro*. The results, together with the earlier observation in cucumber, demonstrate that the chilling-sensitive component in plant is not PSI itself but some mechanism to protect PSI *in vivo*. The components of such protective machinery might exist in chloroplast since PSI was susceptible to photoinactivation even at room temperature in isolated thylakoid membrane but not in intact chloroplasts (Fig. 9).

One of the candidates for such a protective mechanism in chloroplasts is scavenging system of reactive oxygen species. The major site of superoxide production in chloroplast is the reducing side of PSI, and enzymes that scavenge reactive oxygen species, such as superoxide dismutase (SOD) and ascorbate peroxidase, are localized at or near the PSI reaction center (Miyake and Asada 1992; Ogawa et al. 1995). The primary target of reactive oxygen species in photoinhibition of PSI is iron-sulfur centers, which serves as the terminal electron acceptors in PSI (Sonoike and Terashima 1994; Sonoike et al. 1995). Subsequently, PsaA and PsaB proteins, the heterodimer subunits of the PSI reaction center, are degraded (Sonoike 1996). SOD itself also may be a primary target of the chilling stress in the light (Choi et al. 2002). The amount of P-700 in thylakoid membranes decreased upon photoinhibitory treatment, while that in chloroplasts was much smaller (Fig. 9). This observation supports the idea that when SOD in chloroplast becomes inactivated, it can no longer protect PSI against inactivation by reactive oxygen species. The activity of SOD in chloroplast may correlate with chilling-sensitivity.

An alternative candidate for the protective mechanism is cyclic electron flow around PSI (CEF-PSI). CEF-PSI has an important function in the protection of photosynthetic apparatus from photodamage (Endo et al. 1999; Munekage et al. 2002; Munekage and Shikanai 2005). CEF-PSI is composed of two main pathway, one

ferredoxin (Fd) dependent and the other NAD(P)H dependent (Asada et al. 1993; Mi et al. 1995). Although Fd is significant component in both pathways, that is lost during the isolation of thylakoid membrane because of water-soluble nature of the protein. This may be one of the reasons why the photoinhibition of PSI take place in isolated thylakoid membrane but not in chloroplast (Fig. 9).

CEF-PSI is induced under chilling stress in the light (Kudoh and Sonoike 2002a; Bukhov et al. 2004) and this is also true for Micro-Tom, in which I observed apparent absorbance changes of P-700 decreased by the reducing pressure from cyclic electron flow (Fig. 4B). Zhang and Scheller (2004) reported that changes in cyclic electron flow in chilling-tolerant plant, *Arabidopsis*, seemed to have little influence on the P-700 measurements, judging from the fact that the rate of P-700 reduction after far-red light illumination did not show any significant changes during stress and also in recovery phase. This result is very different from that of Micro-Tom (Fig. 5) and of cucumber (Kudoh and Sonoike 2002a). The effect of CEF-PSI may have a role to prevent the photoinhibition at chilling temperatures, thus observed only in chilling-sensitive plants.

The existence of a specific threshold temperature for the photoinhibition (Fig. 3), as well as the induction of CEF-PSI (Fig. 5), demonstrates the chilling-sensitivity in Micro-Tom. In Micro-Tom, the extent of photoinhibition was smaller in comparison with a cucumber (Fig. 6 and 7). Since PSI is readily photoinhibited in leaves of cucumber, previous study could not induce selective photoinhibition of PSII and determine if photoinhibition of PSII protects PSI from photoinhibition (Kudoh and Sonoike 2002b). However, I can induce selective photoinhibition of PSII by the pretreatment by high light by means of relative stability of PSI in Micro-Tom (Fig. 8).

Using various advantages as experimental material including the relative stability of PSI, techniques of molecular biology or genome sequencing information that are available in Micro-Tom, it may be possible to elucidate the mechanism of chilling sensitivity in chilling sensitive plants in future.

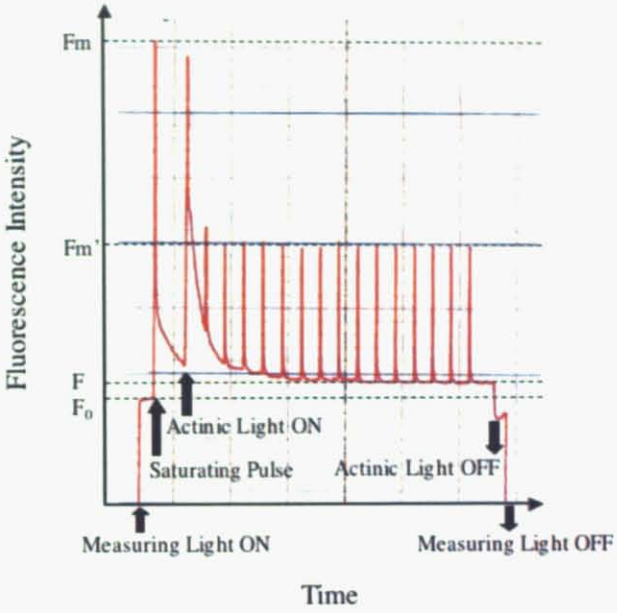

Fig. 1 Measurement of chlorophyll fluorescence using PAM spectrometer

Minimum original fluorescence (F_0) is obtained upon the irradiation of the very weak, pulse-modulated measuring light to dark-adapted leaf. Maximum fluorescence (Fm) is obtained upon the irradiation of saturating light pulse. Subsequently, leaf is illuminated with the continuous actinic light. F is the steady-state fluorescence level that results from the actinic light. Fm' is the maximum yield of fluorescence in actinic light-acclimated leaf. F_0' is minimum fluorescence just after the actinic light off.

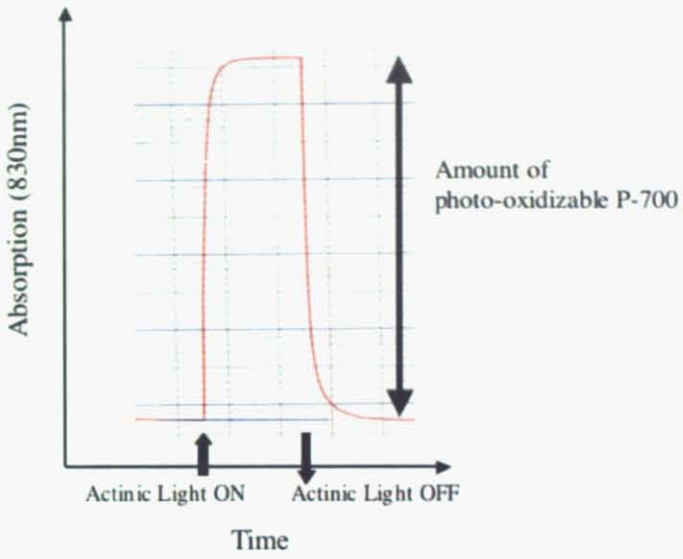

Fig. 2 Mesurement of photo-oxidizable P-700 using PAM spectrometer

P-700, the PSI reaction center chlorophyll dimer, is oxidized to P-700^{+} upon the onset of far-red (FR: peak at 735 nm) actinic light. The characteristic absorption of P-700^{+} around 830 nm appeared upon the application of FR. Photo-oxidized P-700 is re-reduced after turning off FR light.

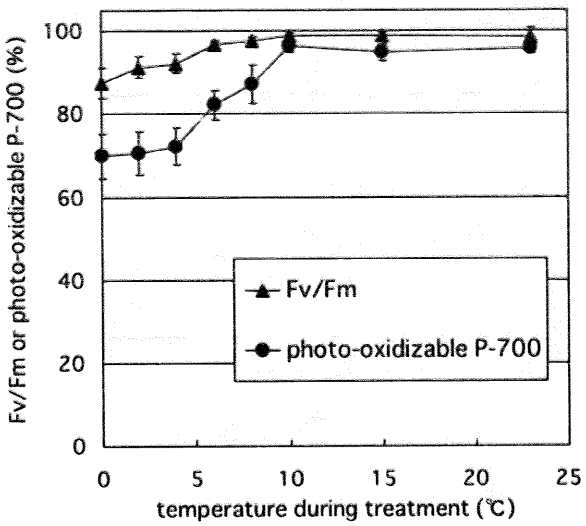

Fig. 3 Effect of temperature on photosynthesis of Micro-Tom leaves

The dorsal side of Micro-Tom leaves was placed down on the surface of temperature-controlled water. Chlorophyll fluorescence and absorption change due to the oxidation of P-700, the reaction center chlorophyll of PSI was measured using PAM *in vivo*. The relative amounts are presented as a percentage of the activity before the treatment (300 μmol m^{-2} s^{-1} for 5 h). Each bar represents the SD of independent experiments on four different leaves. The maximum quantum yield of PSII photochemistry (Fv/Fm) was estimated (closed triangles). The changes in the functional PSI content per unit leaf area were determined (closed circles).

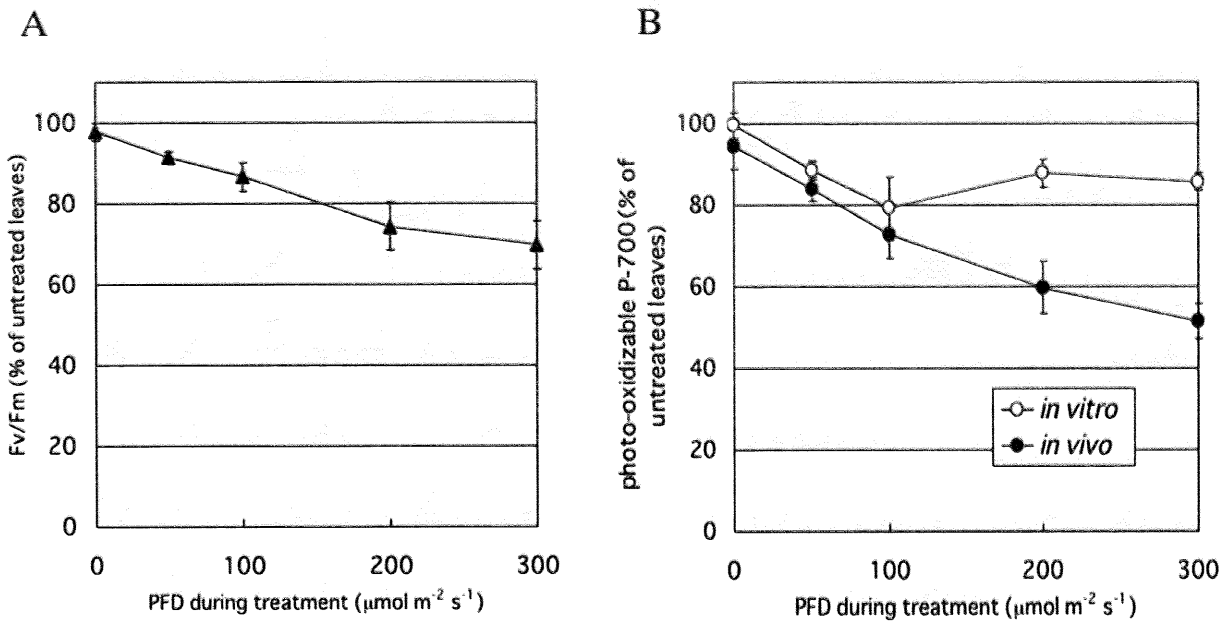

Fig. 4 Effect of photon flux density (PFD) on photosynthetic activity during the chilling treatment of Micro-Tom leaves at 0℃ for 5 h

(A) Fv/Fm was measured with leaves treated at the indicated PFD. (B) The P-700 content was determined with intact leaves (closed circles; *in vivo*) or with thylakoid membranes isolated just after the chilling treatment (open circles; *in vitro*). The relative amounts are presented as a percentage of the activity before the treatment. Each bar represents the SD of independent experiments on four different leaves.

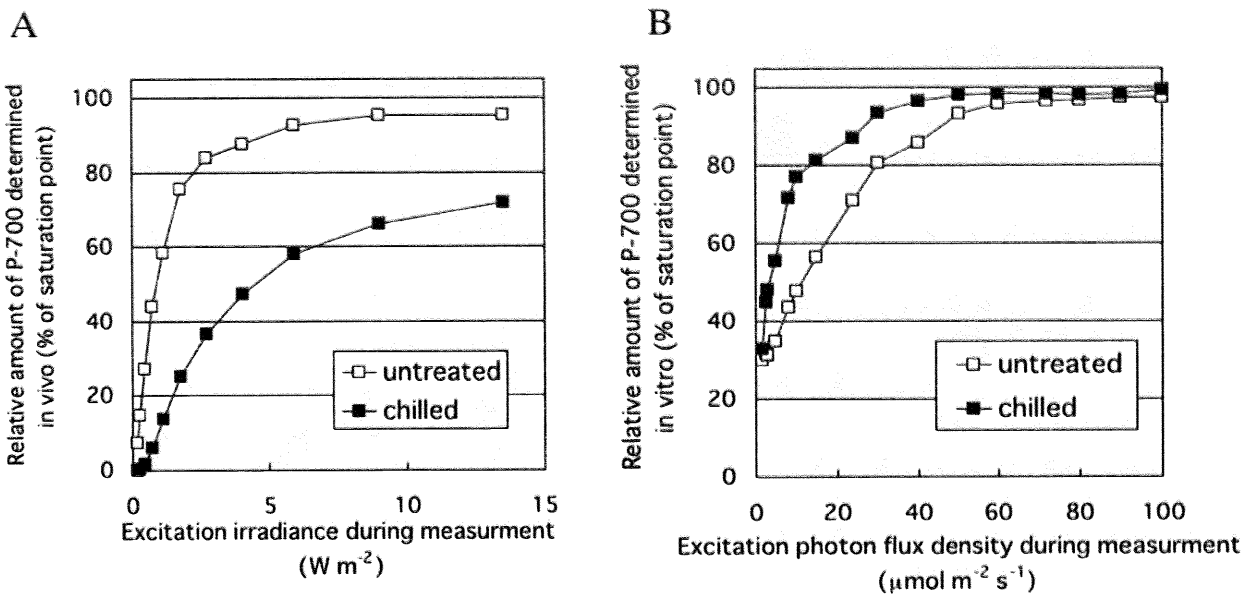

Fig. 5 Effect of chilling on the excitation light-saturation curve of P-700 photooxidation determined *in vivo* (A), and *in vitro* (B)

The chilling treatment of Micro-Tom leaves was performed at 0℃ in the light at 300 μmol m^{-2} s^{-1} for 5 h. Apparent extent of the absorption change due to photooxidation of P-700 was determined with untreated leaves (open squares) or chilled leaves (closed squares). (A) and (B) represents the change in photo-oxidizable P-700 content determined *in vivo* and *in vitro*, respectively. The relative amounts of photo-oxidizable P-700 were presented as percentages of the saturation point. Saturation point was estimated using the equation of Michaelis-Menten kinetics, assuming photosynthetic reaction to be an enzymatic reaction.

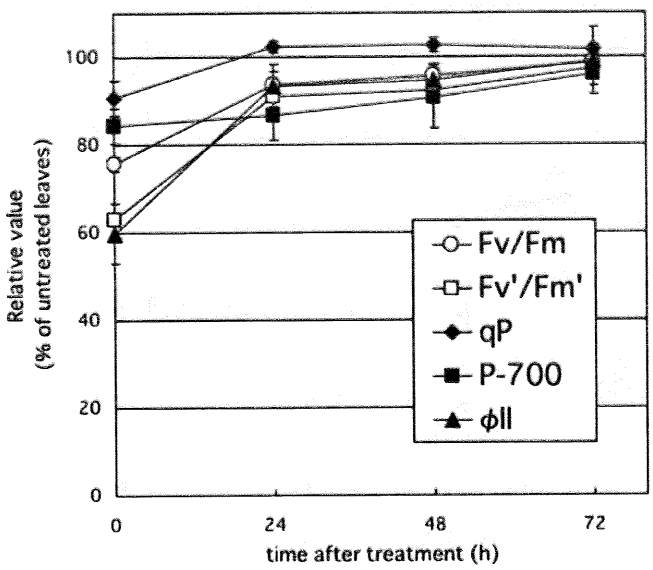

Fig. 6 Recovery of photosynthesis in Micro-Tom

Chilling treatment of Micro-Tom leaves was performed in the light at 300 μmol m^{-2} s^{-1} at 0℃ for 5 h. After the chilling treatment, the plants were returned to the normal growth condition. The relative amounts were presented as a percentage of the activity before treatment. The effective quantum yield of PSII photochemistry (Fv'/Fm'; open squares), the photochemical quenching of chlorophyll fluorescence (qP; closed diamonds), the maximum quantum yield of PSII photochemistry (Fv/Fm; open circles), and the effective quantum yield of electron transfer (ϕII; closed triangles) were estimated from chlorophyll fluorescence. The P-700 content was determined with thylakoid membranes isolated at the time indicated (closed squares). Each bar represents the SD of independent experiments on four different leaves. The actinic photon flux density was 100 μmol m^{-2} s^{-1}.

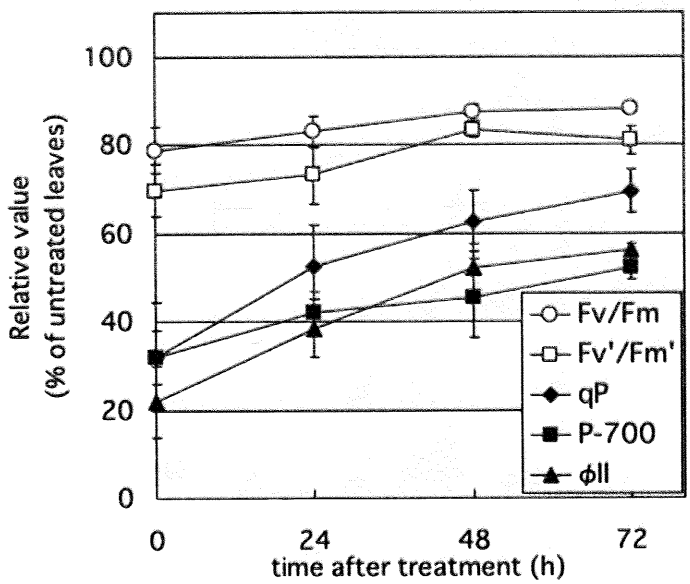

Fig. 7 Recovery of photosynthesis in cucumber leaves

Chilling treatment was performed by placing the dorsal side of cucumber leaves on the surface of temperature-controlled water at 4°C for 5h. Photon flux density during the chilling treatment was 200 μmol m^{-2} s^{-1}. After the chilling treatment, the plants were returned to the normal growth condition. Respectively, symbols have the same identity as in Fig. 6. Each bar represents the SD of independent experiments on three different leaves. The actinic photon flux density was 200 μmol m^{-2} s^{-1}.

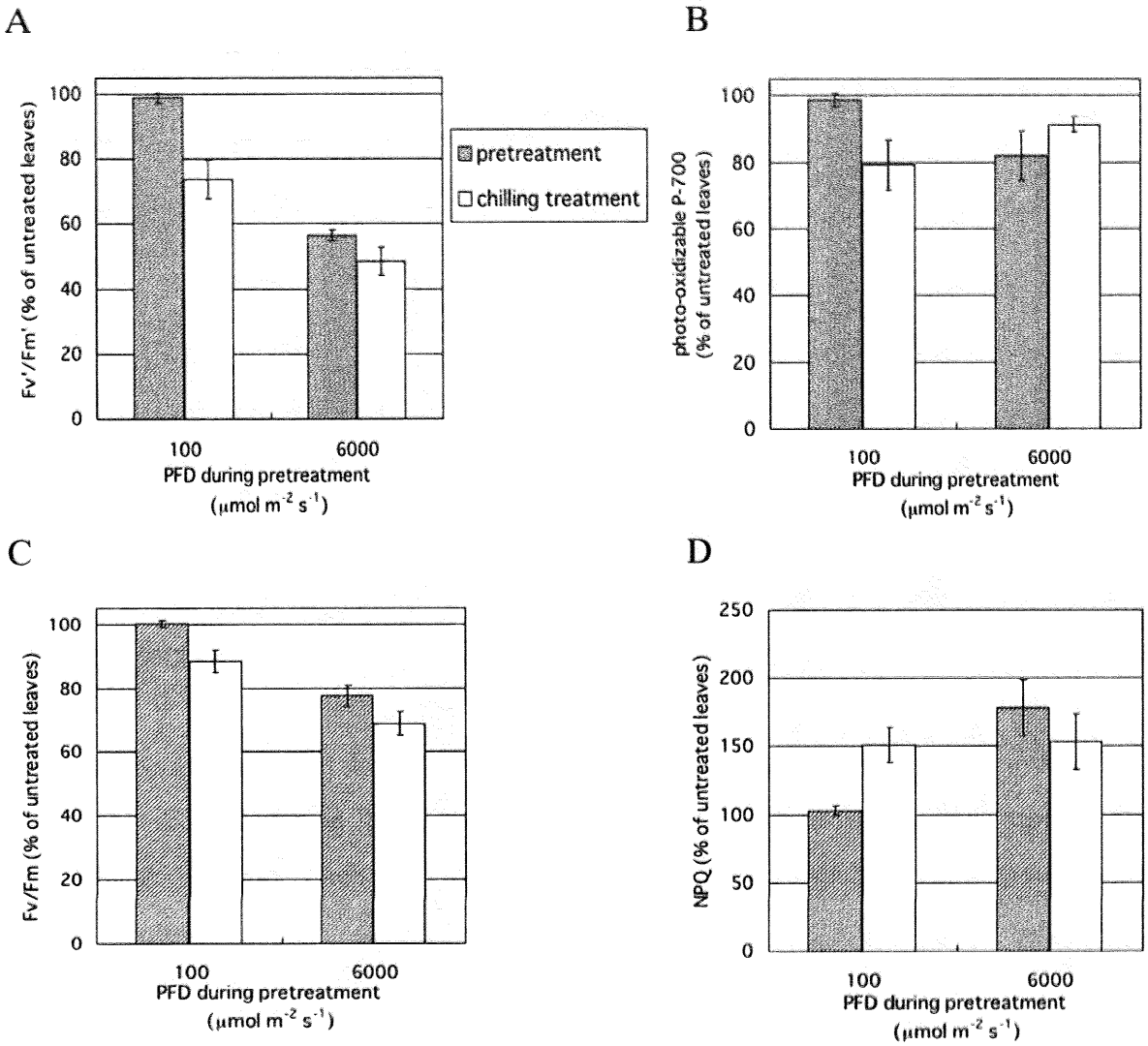

Fig. 8 Effect of pretreatment by high photon flux density (PFD) on photosynthetic activity in the light at 300 μmol m^{-2} s^{-1} at 0°C for 5 h after the illumination for 5 min at indicated PFD

The pretreatment was performed in growth chamber and the subsequent chilling treatment was performed by the same method as in Fig. 3. The actinic PFD was 100 μmol m^{-2} s^{-1} when non-photochemical quenching of chlorophyll fluorescence (NPQ) was estimated using PAM. The effective quantum yield of PSII photochemistry (Fv'/Fm') and the maximum quantum yield of PSII photochemistry (Fv/Fm) were estimated from chlorophyll fluorescence. The P-700 content was determined with thylakoid membranes isolated just after treatment. Each bar represents the SD of independent experiments on four different leaves.

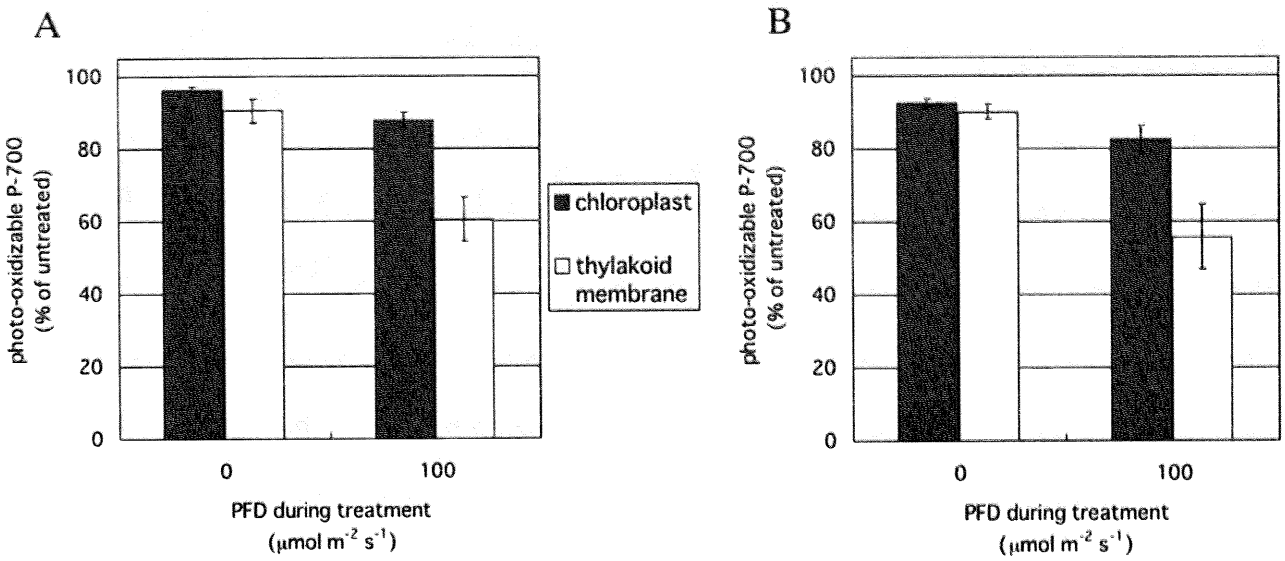

Fig. 9 Photoinhibition of PSI in chloroplast or in thylakoid membrane from spinach at 4℃ (A) and at 25℃ (B)

Intact chloroplast and thylakoid membrane from spinach were treated at 4°C and at 25°C for 90 min at indicated photon flux density (PFD). The values were presented as a percentage of the untreated sample. Each bar represents the SD of independent experiments on three different samples.

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