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RAPID RECOVERY OF PHOTOSYNTHESIS FROM PHOTOINHIBITION IS RELATED TO FATTY ACID UNSATURATION OF CHLOROPLAST MEMBRANE LIPIDS IN CHILLING-RESISTANT PLANTS

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Abstract – The susceptibility of chilling-resistant spinach plants, and of chilling-sensitive squash plants to photoinhibition was compared in terms of the activity of photosystem II, in relation to the degree of fatty acid unsaturation of chloroplast membrane lipids. From thylakoid membranes of the plants, monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol, and phosphatidylglycerol were seperated as major lipid classes. It was found that the content of *cis*-unsaturated fatty acids of phosphatidylglycerol was greater by 32% in spinach than that in squash. When leaf disks were exposed to light at 5°C, 15°C and 25°C, photochemical efficiency of photosystem II, measured as the ratio of the variable to the maximum fluorescence of chlorophyll, declined markedly in squash plants, as compared to spinach plants. When leaf disks were exposed to strong light in the presence of lincomycin, an inhibitor of protein synthesis in chloroplasts, photoinhibition was accelerated in the two types of plants. Moreover, lincomycin treatment abolished the differences in the degree of susceptibility to strong light, which had been observed between the two types of plants. When the extent of photoinhibition of photosystem II-mediated electron transport was compared in thylakoid membranes isolated from the two types of plants, there were no differences in the degree of inactivation of photosystem II activity. However, when intact leaf disks were exposed to strong light either at 10°C or at 25°C, and then were allowed to recover either at 17°C or at 25°C in dim light, chilling-resistant plants such as spinach and pea showed marked recovery from photoinhibition, in contrast to chilling-sensitive plants, such as squash and sweet potato, whose recovery was strongly dependent on the temperature. These findings are discussed in relation to the unsaturation of fatty acids in membrane phosphatidylglycerol. It appears that fatty acid unsaturation of membrane lipids accelerates the recovery of photosystem II from photoinhibition, without affecting the photo-induced inactivation process of photosystem II associated with photoinhibition.

INTRODUCTION

Chilling-sensitive plants exhibit the inhibition of growth and development, in contrast to chilling-resistant plants, when exposed to non-freezing, low temperatures.[1,2] The symptoms of chilling stress, accompanied by the physical or physiological changes, as shown by chilling-sensitive plants at low temperatures, are termed chilling injury. Many economically important crops such as cotton, soybean, maize, cucumber, squash and rice, that are originated from tropical or subtropical areas, are chilling-sensitive. Contrastingly, plants such as spinach, rye and *Arabidopsis thaliana*, that are originated from the temperate zone, are chilling-resistant.

According to the proposal on the mechanism of chilling sensitivity, made by Lyons (1973)[1] and Raison (1973),[3] the formation of a lipid gel phase in cell membranes at low temperature is the primary event in chilling injury, which is accompanied by a series of processes leading to the death of the cells. When a model membrane enters the phase-seperated state in which mixtures of gel phase and liquid crystalline phase exists, the membrane becomes leaky to small molecules and electrolytes. This phase-seperated state destroys the formation of ion gradient across the membranes, that are essential to the function of plant cells. This hypothesis has been supported by Murata and his coworkers,[4,5] in the blue-green alga *Anacysitis nidulans*. However, it is not clear if this mechanism of chilling sensitivity is also operative in cell membranes of higher plants.

In higher plants, the amount of saturated fatty acids (16:0, 18:0 or 16:1- *trans* fatty acids) in phosphatidylglycerol

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† *Abbreviations:* Chl, chlorophyll; DGDG, digalactosyl diacylglycerol; FAME, fatty acid methyl esters; Fo, yield of instantaneous fluorescence; Fm, maximum yield of fluorescence induced by pulses of saturating light; Fv, variable yield of fluorescence; MGDG, monogalactosyl diacylglycerol; PBQ, phenyl-*p*-benzoquinone; PG, phosphatidylglycerol; PS, photosystem; Q_A, primary electron acceptor of PS II; SQDG, sulfoquinovosyl diacylglycerol.

(PG) has been shown to be closely correlated with chilling sensitivity.[6,7] Chilling-resistant plants contain saturated PGs less than 20% of the total PGs in chloroplast membranes, while chilling-sensitive ones have more than 40%.[8,9] In relation to chilling sensitivity of plants, we have shown that the elevated levels of saturated PGs in the transgenic plants, into which the gene for glycerol-3-phosphate acyl transferase from squash had been introduced, depressed the recovery of photosynthetic apparatus from low-temperature photoinhbition (Moon *et al.*, 1995).[10] However, the molecular mechanism of low-temperature photo-inhibition has not been fully analyzed in chilling-sensitive plants and chilling-resistant ones, especially in terms of fatty acid unsaturation of their membrane lipids.

In this paper we report that chilling-resistant plants show much more efficient recovery of photosystem (PS) II complex from the photoinhibited state than chilling-sensitive plants, and that recovery process from low-temperature photoinhibition is accelerated by fatty acid unsaturation of chloroplast membrane lipids.

MATERIALS AND METHODS

Cultivation of plant materials. Spinach (*Spinacia oleracea*), a chilling-resistant plant, and squash (*Cucurbita moschata*), a chilling-sensitive plant were employed as plant materials, together with, when necessary, other speceis such as pea (*Pisum sativum*, chilling-resistant) and sweet potato (*Ipomoea batatas*, chilling-sensitive). Germinated spinach plants in vermiculite medium, were supplied with 1 : 1,000 diluted fertilizer solution (Hyponex, Hyponex Co., Marysville, OH, U.S.A.) and were grown at 22°C in white fluorescent light at 100 μmol quanta m^{-2} s^{-1}, with photoperiod of 12 h. Squash and pea plants were grown in the same condition as that of spinach other than the temperature of 25°C and continuous light. To obtain leaves of sweet potato, its tubers were grown hydroponically in the same condition as that of squash.

Preparation of thylakoid membranes. Thylakoid membranes were isolated from mature leaves, following the procedure of Moon *et al.* (1995),[10] after homogenization of tissues in 50 m*M* Na/K-phosphate buffer (pH 7.4) containing 1.0 *M* glycinebetaine, 20 m*M* NaCl, 5 mM $MgCl_2$ and 100 m*M* sucrose (medium A). Thylakoid membranes were resuspended in 1.0 ml of 25 m*M* Mes-NaOH buffer (pH 6.5) containing 1.0 *M* glycinebetaine, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 300 m*M* sucrose and adjusted to a final chlorophyll (Chl) concentration of 1 mg/ml.

Extraction of lipids and analysis of fatty acids. Lipids were extracted from the isolated thylakoid membranes by the method of Bligh and Dyer (1959)[11] with trivial modifications. Classes of polar lipids were fractionated by ion-exchange column chromatography on DEAE-Toyopearl 650 C (Sigma, St. Louis, MO, U.S.A.), column chromatography on silicic acid, and thin-layer chromatography with precoated silica-gel plates (No. 5721, Merck, Rahway, NJ, U.S.A.), essentially as described in Murata *et al.* (1982).[6] The fractionated lipids were subjected to methanolysis in 2.5 ml of 5% HCl/CH_3OH at 85°C for 2.5 h. To each of the samples, 50 nmole pentadecanoic acid (15:0) were added as an internal standard for quantification of the fatty acid methyl esters (FAME). The resultant methyl esters were analyzed by GLC with an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.), equipped with a split-splitless injector and a hydrogen flame-ionization detector. Seperation of FAME was realized with a DB-23 capillary column (0.25mm i.d. × 30m, J&W Scientific, Folsom, CA, U.S.A.) coated with (50%-cyanopropyl) methylsiloxane at a thickness of 0.25μm. The flow rate of hydrogen was 2ml/min, and samples were injected in the splitless mode. The oven temperature was initially held at 160°C for 2 min, then increased at a rate of 4°C/min up to 230°C. The injector and detector were heated at 250°C and 300°C, respectively. FAME were identified from their retention times by comparison with those of authentic samples. The amounts of FAME were determined from areas under chromatographic peaks by calculation with a computer software, in which a correction was made for differences with respect to the relative sensitivity of FAME that were due to their molecular mass.

Exposure of thylakoid membranes to light. Thylakoid membranes corresponding to 10μg of Chl were suspended in 1 ml of medium A. The suspension was illuminated at 5°C, 15°C and 25°C in a temperature-controlled reaction vessel with actinic light of various intensities provided by an incandescent lamp in combination with a yellow optical filter (Y-46, Hoya, Tokyo, Japan) and a heat absorbing filter (HA-50, Hoya).

Measurement of PS II-mediated electron transport activity. The PS II-mediated electron transport from H_2O to phenyl-*p*-benzoquinone (PBQ) from the isolated thylakoid membranes were measured with Clark-type oxygen electrode at 25°C, based on the procedure of Moon *et al.* (1995).[10]

Photoinhibition and recovery from the photoinhibited state. Leaf disks of 1 cm^2 were placed in water layer at a depth of about 1 cm in a temperature-controlled water bath. To induce photoinhibition, the leaf disks were illuminated by light from a high-intensity incandescent lamp. After photoinhibitory treatment, to allow recovery from photoinhibition, leaf disks were transferred to dim light at an intensity of 0.07 mmol m^{-2} s^{-1}.

Measurement of chlorophyll fluorescence. Chlorophyll fluorescence from leaf disks were measured by using Plant Efficiency Analyser (Hansatech, Norfolk, U.K.). Before measurement of chlorophyll fluorescence, the disks were held in complete darkness for 10 min at 25°C, followed by far-red light illumination for 30s from PAM 102 (Walz, Effeltrich, Germany), to ensure complete reoxidation of the primary stable PS II electron acceptor (Q_A). Then, fluorescence was excited by illumination with an array of 6 high intensity light-emitting diodes (LEDs) which were focused onto the leaf surface to provide even illumination over the exposed area of leaf (4 mm dia.). The LEDs provided red light of a peak wavelength of 650 nm, which is readily absorbed by the chloroplasts of the leaf. The fluorescence signal received by

the sensor unit during recording was digitised by using a fast analog/digital converter. The initial fast rise in the fluorescence signal was digitized to give resolution of the Fo value, which designates the fluorescence level when all reaction centers of PS II are open - namely when the primary electron acceptors of PS II are fully oxidized. After 2 milliseconds, a slower acquisition rate of data points was adopted (1,000 readings per second) until the recording period is 1 second. Then the key fluorescence parameters of Fm, Fv, and the ratio Fv/Fm were automatically calculated for the light level used during measurement. Fm represents the maximal yield of fluorescence emitted from dark-adapted leaves. The variable fluorescence, Fv, was given by the difference between Fm and Fo.[12]

RESULTS

Lipids of thylakoid membranes

Lipids were extracted from isolated thylakoid membranes and seperated into several lipid classes - namely, monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG) and PG (Table 1). Except for DGDG, the proportion of the individual lipid classes was not very different between spinach and squash. PG accounted for small proportion - namely, less than 10% of the total polar lipids in the two types of plant, in consistence with the other report.[22]

As shown in Table 2, fatty acid analysis of the isolated thyalkoid membrane lipids revealed that spinach plant contained 16:3 in MGDG, a characteristics of 16:3 plants, in contrast to squash plant in which 16:3 is absent in MGDG, a characteristics of 18:3 plants.[13] More importantly, the sum of *cis*-unsaturated fatty acids of PG were 43% and 11% in thylakoid membranes of spinach and squash plants, respectively, indicating that spinach has a higher degree of fatty acid unsaturation in PG than squash by 32% (Table 2). The sum of *cis*-unsaturated fatty acids of PG extracted from leaves of pea and sweet potato was reported to be 42% and 18%, respectively.[6]

Photoinhibition of photosynthesis in leaves

Table 1. Composition of lipid classes in thylakoid membranes isolated from spinach and squash plants.

Origin of thyalkoid membranes	Lipid class, mol %			
	MGDG	DGDG	SQDG	PG
Spinach	64	23	4	8
Squash	59	32	3	6

The values are the means of results from two independent experiments. The deviation of values was < 4% in every case.

Table 2. Fatty acids in polar lipids from thylakoid membranes isolated from spinach and squash plants.

	Fatty acid, mol %									
						18:1				Σ*cis*-
Lipid class	16:0	16:1c	16:1t	16:3	18:0	9-*cis*	11-*cis*	18:2	18:3	unsat. FA, mol%
Spinach										
MGDG	1	1	tr	19	1	tr	tr	2	74	96
DGDG	9	tr	tr	3	tr	tr	1	3	82	89
SQDG	48	0	2	2	1	0	0	10	37	49
PG	20	0	36	tr	1	tr	1	7	35	43
Squash										
MGDG	2	0	1	0	tr	tr	tr	2	95	97
DGDG	11	0	1	0	1	tr	tr	2	85	87
SQDG	42	0	1	0	3	2	0	4	48	54
PG	69	0	14	0	6	2	1	1	7	11

16:0, Hexadecanoic acid (palmitic acid); 16:1c, 9-*cis*-hexadecenoic acid (palmitoleic acid); 16:1*t*, 3-*trans*-hexadecenoic acid; 18:0, octadecanoic acid (stearic acid); 18:1, 9-octadecenoic acid (oleic acid) and 11-octadecenoic acid (vaccenic acid); 18:2, 9,12-octadecadienoic acid (linoleic acid); 18:3, 9,12,15-octadecatrienoic acid (α-linolenic acid); tr, trace (less than 0.5%). Σ*cis*-unsat. FA is the sum of the *cis*-unsaturated fatty acids. The values are the means of results obtained from two independent experiments.

Fig. 1 shows time courses of photoinhibition at 5°C, 15°C and 25°C of the PS II complex in spinach and squash leaves, as determined in terms of ratios of Fv to Fm. In the absence of lincomycin (Fig. 1 A-C), the decline in the ratios of Fv to Fm was greater at lower temperatures in leaves of both species of plants. However, squash was more susceptible than spinach to photoinhibtion at all temperatures tested. The extent of photoinhibition in leaves represents the outcome of the competition between the light-induced inactivation of the PS II complex and the subsequent recovery from the photoinhibited state.[14] In view of this perspective, we seperated the two processes into the individual ones by inducing photoinhibition in the presence of lincomycin, an inhibitor of protein synthesis in chloroplasts (Fig. 1 D - F). Lincomycin accelerated photoinhibition in both spinach and squash. Moreover, the difference in the susceptibility to photoinhibition between the two species of plants was indistinguishable in the presence of lincomycin, suggesting that rate of the photo-induced inactivation was about the same in the two species.

Photoinhibition in isolated thylakoid membranes

Fig. 2 shows the dependence on time of the photo-inhibition at 5°C, 15°C and 25°C of oxygen evolution by thylakoid membranes from spinach and squash. The extent of photoinhibition was identical in the thylakoid

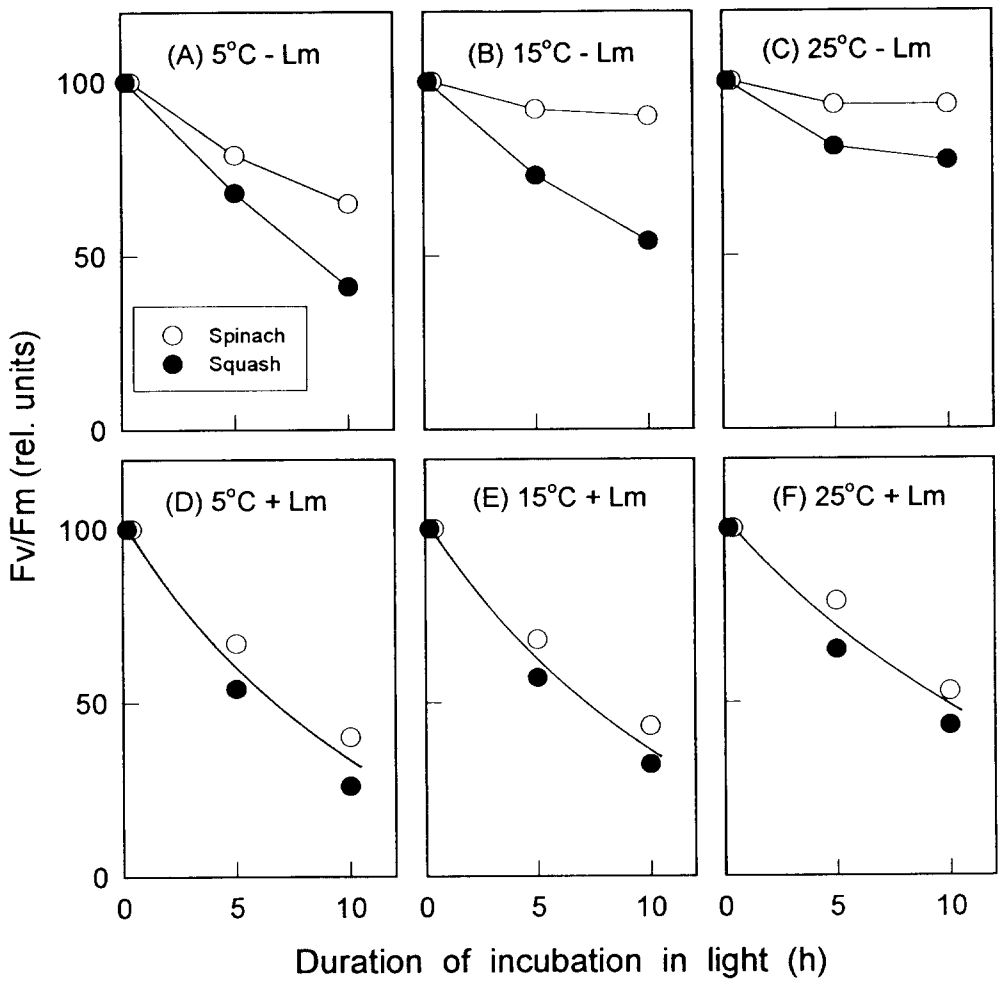

Figure 1. Photoinhibition of PS II activity, monitored in terms of the ratio of Fv to Fm, in spinach and squash leaves in the presence of lincomycin and in the absence of lincomycin. Lincomycin was administered before the photoinhibitory incubation by immersing petioles in 0.6 m*M* lincomycin for 4 h at 25°C at a light intensity of 0.1 mmol m^{-2} s^{-1}. Leaf disks were incubated at a light intensity of 0.6 mmol m^{-2} s^{-1} at designated temperatures. The initial values of Fv/Fm measured at 25°C were taken as 100% and corresponded to 0.81 and 0.80 for squash and spinach, respectively. The values were obtained from the results of three independent experiments. The deviation of values was < 5% in each case. (A) 5°C, without lincomycin; (B) 1°C, without lincomycin; (C) 25°C, without lincomycin; (D) 5°C, with lincomycin; (E) 15°C, with lincomycin; (F) 25°C, with lincomycin. Lm, lincomycin; (○) spinach; (●) squash.

membranes from the two plant sources. Fig. 3 shows the dependence on light intensity of photoinhibition at at 5° C, 15°C and 25°C of oxygen evolution by thylakoid membranes from spinach and squash. With increase in the intensity of light, the extent of photoinhibition increased. However, there was no difference in the extent of photoinhibition between the thylakoid membranes from spinach and squash.

Recovery of photosynthesis from photoinhibition

The extent of photoinhibition *in vivo* results from the outcome of competition between the photo-induced process of inactivation and the counteracting recovery process.[14] Therefore, we compared the extent of recovery from photoinhibition in a leaf system, in which both the inactivation process and the recovery process were operative. Figs. 4A and B show the return to normal values of ratios of Fv to Fm during recovery of leaves of spinach and squash, respectively, from various duration of photoinhibition at 25°C. Leaf disks were exposed to strong light (2,800 μmol m^{-2} s^{-1}) for various periods at 25 °C. Then, the change of Fv/Fm ratios was monitored at 25 °C under dim light. Photoinhibition of leaf disks caused a sharp decline in the ratio of Fv to Fm. However, following 30-min photoinhibition, the depressed ratio was rapidly restored to the original level within an hour. With longer durations of photoinhibition, Fv/Fm ratios dropped more sharply. When the photo-inhibited leaf disks were transferred to weak light to induce recovery from the photoinhibited state, unrecovered portion of Fv/Fm was shown apparently in a dosage-dependent manner, which might be called irrecoverable part of photoinhibition. Furthermore, the extent of irrecoverable portion of photoinhibition was

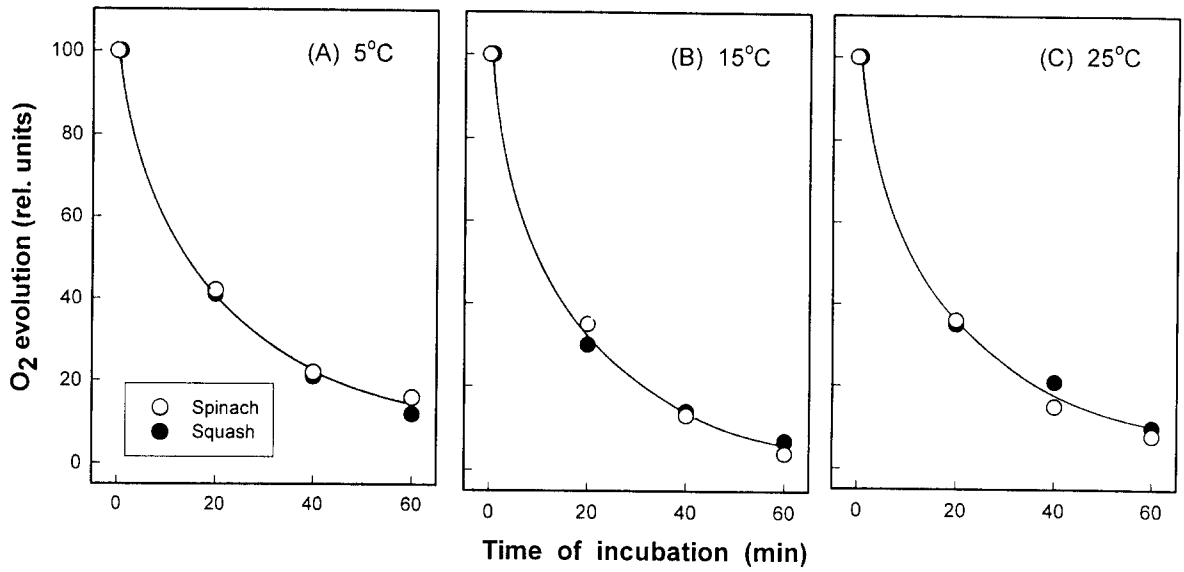

Figure 2. Time-dependent photoinhibition of the PS II-mediated transport of electrons in thyakoid membranes isolated from spinach (○) and squash (●) plants. Thylakoid membranes (10 μg of Chl per ml) were incubated at 5°C (A), 15°C (B), or 25°C (C) with irradiation at 1.5 mmol m^{-2} s^{-1} in 1.0 ml of 25 m*M* Mes buffer, adjusted with NaOH to pH 6.5, that contained 10 m*M* NaCl, 300 m*M* sucrose and 1.0 M betaine. Then the transport of electrons from H_2O to PBQ were monitored with a Clark-type oxygen electrode. The absolute values for evolution of oxygen before incubation were taken as 100 arbitrary units, which corresponded to 252 ± 20 and 173 ± 15 μmol of O_2 · (mg Chl)$^{-1}$ · h^{-1} in thylakoid membranes from spinach and squash, respectively. The values were calculated from results of three independent experiments. The deviation of values was < 5% in each case.

more greater in squash than in spinach, again indicating that squash is more susceptible to strong light even at room temperature, when the plants are exposed to strong light at an intensity of 2,800 μmol m^{-2} s^{-1}.

Fig. 5 shows temperature-dependent recovery of PS II from photoinhibition in leaves of spinach and squash. Leaf disks were exposed to strong light for designated periods of photoinhibition at 10°C (Figs. 5C and D) and 25°C (Figs. 5A and B). Then, ratios of Fv to Fm was monitored in dim light at 17°C (Figs. 5B and D) and 25°C (Figs. 5A and C). As shown in Figs. 5A and B, spinach and squash, when recovered at 25°C, exhibited about the same rate of PS II recovery from photoinhibition, regardless of the temperature of

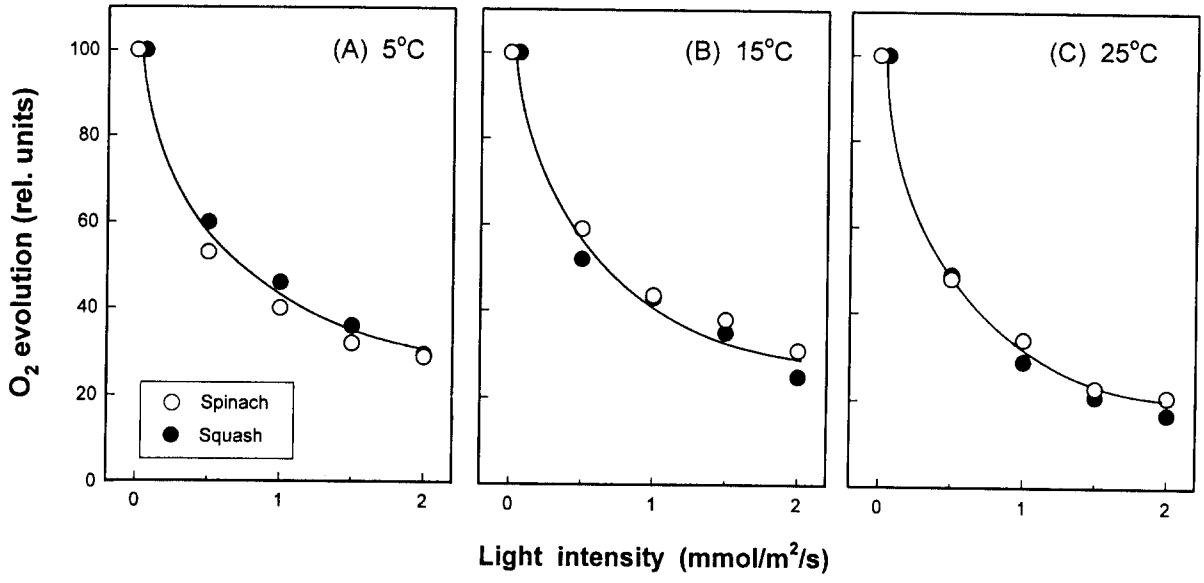

Figure 3. Effects of the intensity of light on the photoinhibition of the PS II-mediated transport of electrons in thyakoid membranes isolated from spinach (○) and squash (●) plants. Thylakoid membranes (10 μg of Chl per ml) were incubated for 20 min at 5°C (A), 15°C (B), or 25°C (C), with irradiation at 1.5 mmol m^{-2} s^{-1} in the same buffer as in Fig. 2. Oxygen evolution was measured as in Fig. 2.

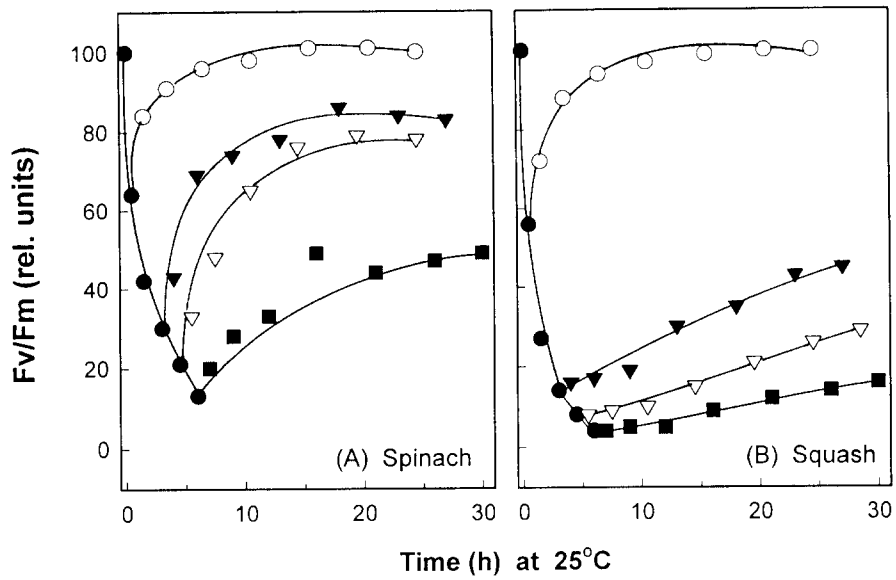

Figure 4. Restoration of Fv/Fm ratios from the photoinhibited state in leaf disks of spinach (A) and squash (B) plants. Leaf disks were exposed to light at an intensity of 2.8 mmol m^{-2} s^{-1} for different periods at 25°C to induce photoinhibition. The leaf disks were then incubated at 25°C in dim light at an intensity of 0.07 mmol m^{-2} s^{-1} for recovery from photoinhibition. The initial values of Fv/Fm measured at 25°C was taken as 100% that corresonded to 0.84 and 0.82 in spinach and squash, respectively. The values were obtained form the results of three independent experiments. The deviation of values was $<5\%$ in each case. (●) photoinhibitory light treatment; (○) recovery from 0.5h-photoinhibition; (▼) recovery from 3h-photoinhibition; (▽) recovery from 4.5 h-photoinhibition; (■) recovery from 6h-photoinhibition.

photoinhibitory treatment. However, when recovered at 17°C, spinach showed more rapid recovery of PS II from photoinhbition (Figs. 5C and D), suggesting that recovery of spinach photosynthesis from photoinhibition is not as much dependent on the temperature as that of squash.

Fig. 6 shows temperature-dependent recovery of PS II from photoinhibition in leaves of pea and sweet potato. Leaf disks were exposed to strong light for designated periods of photoinhibition at 10°C (Figs. 5C and D) and 25°C (Figs. 5A and B). Then, changes in the ratios of Fv to Fm was monitored in dim light at 17°C (Figs. 5B and D) and 25°C (Figs. 5A and C). Pea plants were markedly efficient in recovering PS II from photoinhibition, in contrast to sweet potato plants, which showed severe decline in Fv/Fm after 10°C-photoinhibition, and showed very minor recovery of PS II even at 25°C

DISCUSSION

In the present study, we compared the extent of low-temperature photoinhibition in chilling-resistant and chilling-sensitive plants, in relation to the extent of fatty acid unsaturation of thylakoid membrane lipids. When the susceptibility to strong light was compared between spinach and squash, squash was found to be more sensitive to photoinhibition than spinach (Fig. 1) Reductions in PS II photochemical efficiency, measured as the ratio of Fv to Fm (Fv/Fm) do not necessarily reflect damage to PS II involving, for example, the degradation of thyalkoid proteins. These reductions may also indicate a controlled, sustained increase in the thermal dissipation of excess energy occurring, for example, in the antenna chlorophyll, which is closely correlated with a protectiv role of zeaxanthin.[15]

However, PS II photochemical efficiency is reduced under strong light *via* the photo-induced damage to the reaction center, involving, for example, the breakdown of D1 protein in thylakoid membranes.[16] We attempted to examine this possibility by applying lincomycin, an inhibitor of chloroplast-encoded protein synthesis, to leaves under photoinhibitory treatment. When the leaves were treated with lincomycin before photoinhibitory treatment, the differential susceptibility to strong light, which had been previously observed between the two types of plants, as in Figure 1A-C, became almost indistinguishable, as shown in Figure 1D-F. Based on these observations, we attempted to further examine PS II photoinhibition in terms of light-induced degradation and repair of D1 protein.

Two competing processes are operative, during photoinhibtion *in vivo*:[14] light-induced inactivation, which includes damage to the D1 protein of the PS II complex; and the recovery processes, which includes degradation of the damaged D1 protein, synthesis of the D1 protein *de novo* and reintegration of the D1 protein into the PS II complex. To determine which of the two processes is responsible for the difference in sensitivity to photoinhibition between chilling-sensitive and

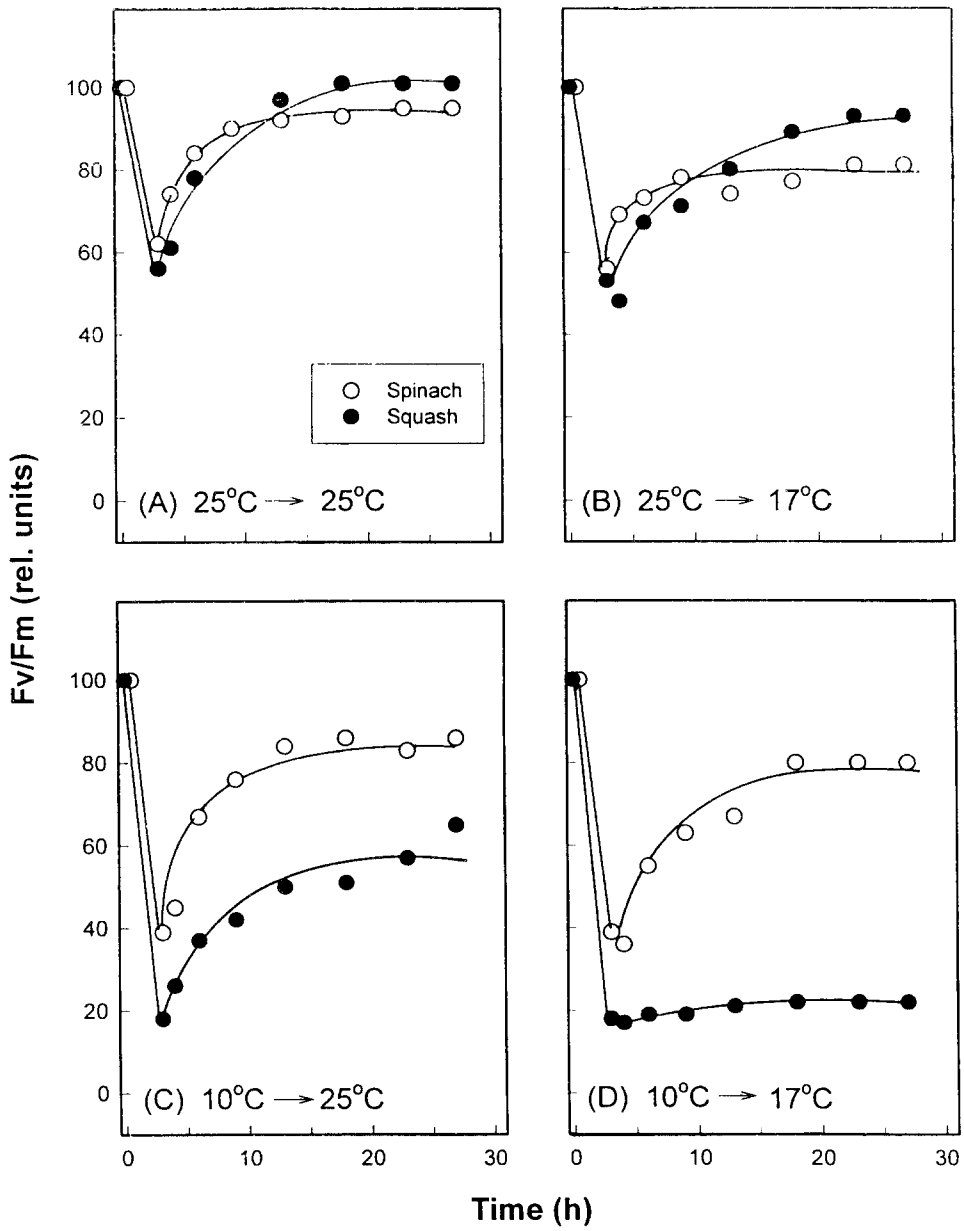

Figure 5. Temperature-dependent restoration of Fv/Fm ratios from photoinhibition in leaf disks of spinach and squash. Leaf disks were exposed to light at an intensity of 1.5 mmol m^{-2} s^{-1} for 3 h at 25°C (A, B) and 10°C (C, D) to induce photoinhibition. The leaf disks were then incubated at 25°C (A, C) and 17°C (B, D) in dim light at an intensity of 0.07 mmol m^{-2} s^{-1} for recovery from photoinhibition. The initial values of Fv/Fm measured at 25°C was taken as 100% and corresonded to 0.84 and 0.82 in spinach and squash, respectively. The values were obtained form the results of two independent experiments. The deviation of values was $< 5\%$ in each case. (○) spinach; (●) squash.

chilling-resistant plants, we tried to seperate the inactivation process from the recovery process associated with photoinhibition. This seperation was carried out by using the isolated thylakoid membrane systems, in which the recovery process is not active. In consistence with the results in Fig. 1, when the PS II-mediated electron transport activity was monitored during exposure of thylakoid membranes to strong light, the obvious differences in sensitivity to photoinhibition, which had been observed between the intact leaves of spinach squash, were almost abolished (Figs. 1, 2 and 3). The results indicate that the light-induced inactivation of the PS II complex occurs at the same rate in chilling-sensitive plants as in chilling-resistant plants. This observation suggests that the different level of fatty acid unsaturation in PG, observed between spinach and squash (Table 2), does not affect the inactivation process of photoinhibtion of PS II.

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Cultivation of plant materials. Spinach (*Spinacia oleracea*), a chilling-resistant plant, and squash (*Cucurbita moschata*), a chilling-sensitive plant were employed as plant materials, together with, when necessary, other speceis such as pea (*Pisum sativum*, chilling-resistant) and sweet potato (*Ipomoea batatas*, chilling-sensitive). Germinated spinach plants in vermiculite medium, were supplied with 1 : 1,000 diluted fertilizer solution (Hyponex, Hyponex Co., Marysville, OH, U.S.A.) and were grown at 22°C in white fluorescent light at 100 μmol quanta m^{-2} s^{-1}, with photoperiod of 12 h. Squash and pea plants were grown in the same condition as that of spinach other than the temperature of 25°C and continuous light. To obtain leaves of sweet potato, its tubers were grown hydroponically in the same condition as that of squash.

Preparation of thylakoid membranes. Thylakoid membranes were isolated from mature leaves, following the procedure of Moon *et al.* (1995),[10] after homogenization of tissues in 50 m*M* Na/K-phosphate buffer (pH 7.4) containing 1.0 *M* glycinebetaine, 20 m*M* NaCl, 5 mM $MgCl_2$ and 100 m*M* sucrose (medium A). Thylakoid membranes were resuspended in 1.0 ml of 25 m*M* Mes-NaOH buffer (pH 6.5) containing 1.0 *M* glycinebetaine, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 300 m*M* sucrose and adjusted to a final chlorophyll (Chl) concentration of 1 mg/ml.

Extraction of lipids and analysis of fatty acids. Lipids were extracted from the isolated thylakoid membranes by the method of Bligh and Dyer (1959)[11] with trivial modifications. Classes of polar lipids were fractionated by ion-exchange column chromatography on DEAE-Toyopearl 650 C (Sigma, St. Louis, MO, U.S.A.), column chromatography on silicic acid, and thin-layer chromatography with precoated silica-gel plates (No. 5721, Merck, Rahway, NJ, U.S.A.), essentially as described in Murata *et al.* (1982).[6] The fractionated lipids were subjected to methanolysis in 2.5 ml of 5% HCl/CH_3OH at 85°C for 2.5 h. To each of the samples, 50 nmole pentadecanoic acid (15:0) were added as an internal standard for quantification of the fatty acid methyl esters (FAME). The resultant methyl esters were analyzed by GLC with an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.), equipped with a split-splitless injector and a hydrogen flame-ionization detector. Seperation of FAME was realized with a DB-23 capillary column (0.25mm i.d. × 30m, J&W Scientific, Folsom, CA, U.S.A.) coated with (50%-cyanopropyl) methylsiloxane at a thickness of 0.25μm. The flow rate of hydrogen was 2ml/min, and samples were injected in the splitless mode. The oven temperature was initially held at 160°C for 2 min, then increased at a rate of 4°C/min up to 230°C. The injector and detector were heated at 250°C and 300°C, respectively. FAME were identified from their retention times by comparison with those of authentic samples. The amounts of FAME were determined from areas under chromatographic peaks by calculation with a computer software, in which a correction was made for differences with respect to the relative sensitivity of FAME that were due to their molecular mass.

Exposure of thylakoid membranes to light. Thylakoid membranes corresponding to 10μg of Chl were suspended in 1 ml of medium A. The suspension was illuminated at 5°C, 15°C and 25°C in a temperature-controlled reaction vessel with actinic light of various intensities provided by an incandescent lamp in combination with a yellow optical filter (Y-46, Hoya, Tokyo, Japan) and a heat absorbing filter (HA-50, Hoya).

Measurement of PS II-mediated electron transport activity. The PS II-mediated electron transport from H_2O to phenyl-*p*-benzoquinone (PBQ) from the isolated thylakoid membranes were measured with Clark-type oxygen electrode at 25°C, based on the procedure of Moon *et al.* (1995).[10]

Photoinhibition and recovery from the photoinhibited state. Leaf disks of 1 cm^2 were placed in water layer at a depth of about 1 cm in a temperature-controlled water bath. To induce photoinhibition, the leaf disks were illuminated by light from a high-intensity incandescent lamp. After photoinhibitory treatment, to allow recovery from photoinhibition, leaf disks were transferred to dim light at an intensity of 0.07 mmol m^{-2} s^{-1}.

Measurement of chlorophyll fluorescence. Chlorophyll fluorescence from leaf disks were measured by using Plant Efficiency Analyser (Hansatech, Norfolk, U.K.). Before measurement of chlorophyll fluorescence, the disks were held in complete darkness for 10 min at 25°C, followed by far-red light illumination for 30s from PAM 102 (Walz, Effeltrich, Germany), to ensure complete reoxidation of the primary stable PS II electron acceptor (Q_A). Then, fluorescence was excited by illumination with an array of 6 high intensity light-emitting diodes (LEDs) which were focused onto the leaf surface to provide even illumination over the exposed area of leaf (4 mm dia.). The LEDs provided red light of a peak wavelength of 650 nm, which is readily absorbed by the chloroplasts of the leaf. The fluorescence signal received by

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