Stabilization of photosynthetic machinery against low-temperature photoinhibition by fatty acid unsaturation of membrane lipids in plants

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Abstract

Chilling tolerance of plants are closely correlated with the degree of fatty acid unsaturation of membrane lipids. We studied the effects of low-temperature photoinhibition on the photochemical efficiency of photosystem II in terms of fatty acid unsaturation of thylakoid membranes lipids isolated from chilling-sensitive plants and chilling-resistant ones. To directly test the chilling tolerance of photosynthetic machinery in relation to membrane lipids, we further compared wild type tobacco plants with that of transgenic tobacco plants, in which the sensitivity to chilling had been enhanced by genetic modification of fatty acid unsaturation of chloroplast membrane lipids. The transgenic tobacco plants were found to contain reduced levels of unsaturated membrane fatty acids after being transformed with cDNA for glycerol-3-phosphate acyltransferase from squash. The functional integrity of photosystem II during and recovery of photosynthesis from low-temperature photoinhibition will be discussed in connection with the degree of fatty acid unsaturation of chloroplast membranes lipids.

Introduction

Low temperature and high-intensity light are important examples of environmental stresses on plants, and they act synergistically to induce low-temperature photoinhibition (Öquist and Huner, 1993). The photoinhibition is caused by photo-induced damage to the D1 protein of the photosystem II protein (PS II) complex (Andersson *et al.*, 1992). Under light conditions that allow normal

growth of plants, the photo-induced damage to the D1 protein of PS II is reversed by repair system that includes degradation and *de novo* synthesis of the D1 protein (Aro *et al.*, 1993b; Kanervo *et al.*, 1995). By contrast, under strong illumination that causes photoinhibition of the PS II complex, the rate of the photodamage to the D1 protein is higher than that of the repair and thus, the amount of active D1 protein decreases, with resultant loss of PS II activity (Greer *et al.*, 1986; Miyao *et al.*, 1995).

In previous studies, it was demonstrated that sensitivity of tobacco plants to low-temperature photoinhibition could be modified by gene-technologically manipulating the degree of fatty acid unsaturation of membrane phosphatidyl-glycerols (PG). Such manipulation was achieved by transformation of tobacco plants with cDNA for glycerol-3-phosphate acyltransferase cloned from squash (Murata *et al.*, 1992).

The aim of the present study was to examine in further detail the sensitivity of PS II to chilling temperatures of plants having different chilling sensitivity. We tried to test directly the effect of fatty acid unsaturation of membrane PG on the sensitivity of PS II to low- temperature photoinhibition, using transgenic tobacco plants, in which the extent of fatty acid unsaturation of PG in chloroplast membranes had been genetically manipulated.

Materials and Methods

1. Plant materials

Leaves from 45-day-old plants of spinach (*Spinacia oleracea* var. Glabra) and 30 day-old-plants of squash (*Cucurbita moschata* Duch var. Shirakikuza) that had been cultivated hydroponically at 22℃ under continuous light (200 μ mol m^{-2} s^{-1}) and at 25℃ with 16h of light daily (200 μ mol m^{-2} s^{-1}), respectively, were used for experiments. Leaves of sweet potato and cabbage were obtained from plants that had been cultivated in a green house. Wild-type and transgenic (T-SQ) tobacco plants (*Nicotiana tabacum* var. Samsun) were obtained and grown as described previously (Murata *et al.*, 1992; Moon *et al.*, 1995). Leaves from 3-month-old tobacco plants were used for experiments.

2. Other Methods

Isolation of thylakoid membranes, extraction of lipids and analysis of fatty acids were conducted according to the methods previously described (Moon *et al.*, 1995; Murata *et al.*, 1982). Measurement of chlorophyll fluorescence from leaf disks were measured by using Plant Efficiency Analyser (Hansatech, Norfolk, U.K.) as described previously (Moon *et al.*, 1998a).

Results

1. The degree of fatty acid unsaturation of PG in chilling-sensitive plants and chilling-resistant ones

As shown in Figure 1, fatty acid analysis of the isolated thyalkoid membrane lipids revealed that the mole percentages of *cis*-unsaturated PG were 86% and 22% in thylakoid membranes of spinach and squash plants, respectively, indicating that spinach has a higher proportions of *cis*-unsaturated molecules of PG than squash by 64% (Moon *et al.*, 1998b). In parallel with the results, the mole percentages of *cis*-unsaturated molecules of PG extracted from leaves of pea and sweet potato, another chilling-sensitive species, were estimated to be 84% and 36%, respectively (Murata *et al.*, 1982).

PG (% of molecular species)
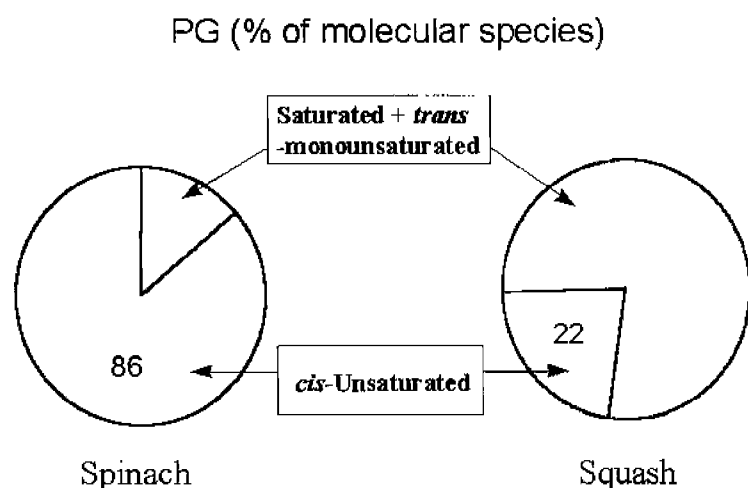
Figure 1. Estimated mole percentages of *cis*-unsaturated molecules of phosphatidylglycerol (PG) in thylakoid membranes isolated from leaves of spinach and squash plants.

## 2. Photoinhibition of chilling-sensitive plants and chilling-resistant ones

To compare the chilling sensitivity of plants having different adaptive capabilities to thermal environment, photo-induced inactivation of their PS II photochemical efficiency was examined according to the incubation temperatures in terms of chlorophyll fluorescence. Figure 2 A-B show the extent of photoinhibition of the PS II complex in leaf disks of spinach and squash, measured as the ratios of Fv to Fm (Fv/Fm). The results indicate that exposure of squash plants to strong light induced a marked inactivation of the PS II photochemical efficiency, relatively to spinach which showed a slight decrease, suggesting that the PS II complex of squash plants was more susceptible to chilling temperature than that of spinach.

Since the extent of photoinhibition in leaves is determined by the counteracting two processes, namely the light-induced inactivation of the PS II complex and its repair from the photoinhibited form (Greer et al., 1986), we seperated these two processes into the individual ones by inducing photoinhibition in the presence of lincomycin, which serves as an inhibitor of chloroplast-encoded protein synthesis (Figure 2 C-D). Although the extent of photoinhibition was accelerated by lincomycin in both types of plant, the differential sensitivity to strong light, that had been observed previously between the two types of plants, became no more apparent by treatment with lincomycin. This observation suggests that the rate of the photo-induced inactivation was almost identical between the two types of plants but that the rate of recovery from low-temperature photoinhibition was far greater in spinach than in squash.

3. Recovery from low-temperature photoinhibition in chilling-sensitive plants and chilling -resistant ones

Figure 3 shows the time course of restoration of PS II photochemical efficiency, measured in terms of Fv/Fm, in chilling-resistant species, spinach and cabbage, and in chilling-sensitive ones, sqaush and sweet potato. Leaf disks were first exposed to strong light at 1 ℃ for 3 or 4h to reduce the ratios to about 25% of the original values. Then, increases in each ratio were followed during incubation of the photoinhibited leaves at 17 ℃ in darkness or in the light. During incubation under

dim light following photoinhibition, there was a clear difference in the extent of recovery of the PS II complex between chilling-sensitive species and chilling-resistant ones. After recovery for 4h under irradiation by light of 0.07 mmol m^{-2} s^{-1}, ratios of Fv to Fm of cabbage and spinach were found to restore to 70% of the original levels, whereas those of squash and sweet potato restored only to 40%.
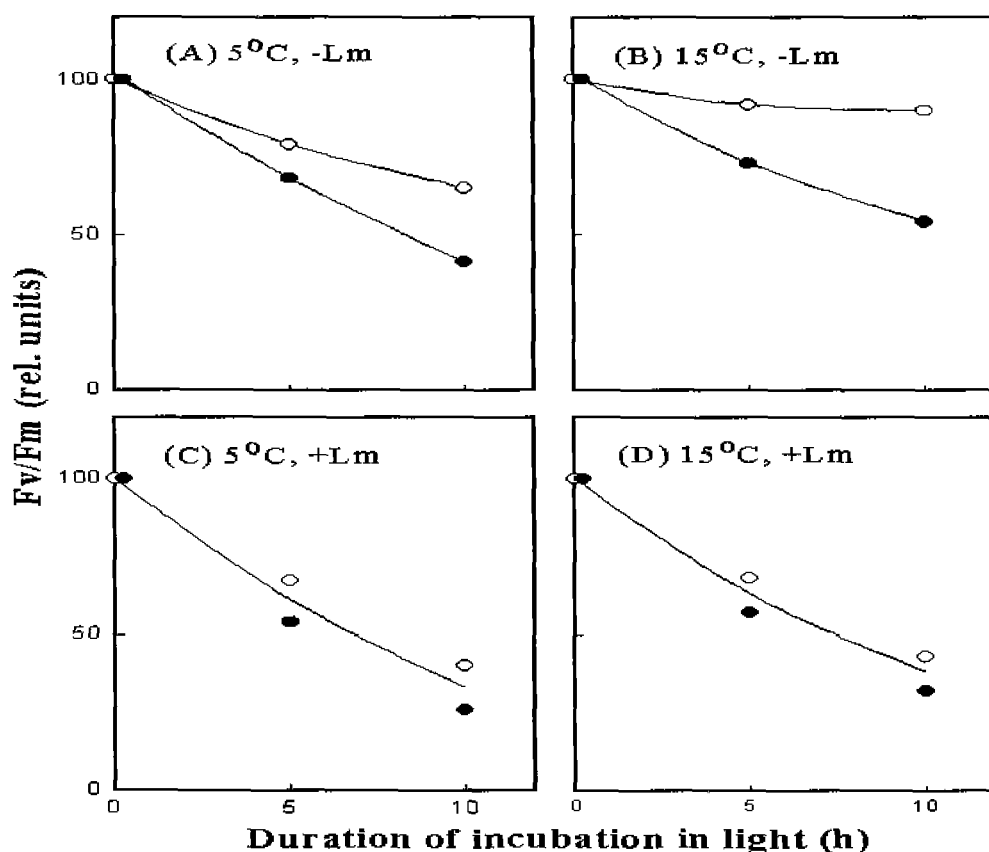
Figure 2. Photoinhibition of the PS II complex at 5℃ and 15℃, monitored in terms of ratios of Fv to Fm, in leaves of spinach and squash in the absence (A, B) or in the presence (C, D) of lincomycin. Lincomycin was administered by immersing petioles in 0.6mM lincomycin for 4h at 25℃ at a light intensity of 0.1mmol quanta m^{-2} s^{-1}. To induce photoinhibition, leaf disks were incubated at a light intensity of 0.6mmol quanta m^{-2} s^{-1} at the designated temperatures. The initial values of Fv/Fm measured at 25℃ 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 within ± 5%. Lm, lincomycin. (A) 5℃, without lincomycin; (B) 15℃, without lincomycin; (C) 5℃, with lincomycin; (D), 15℃, with lincomycin; (O) Spinach; (●) squash.
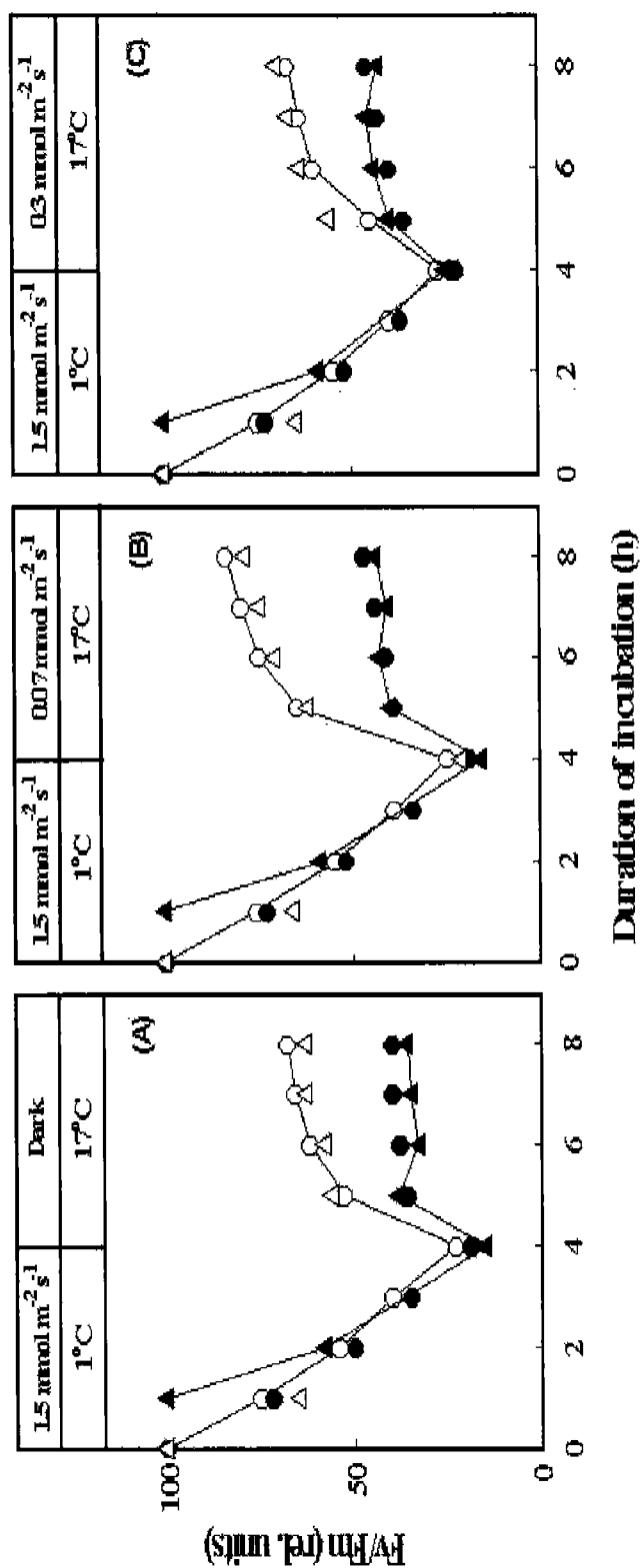
Figure 3. Restoration of PS II photochemical efficiency, monitored in terms of ratios of Fv to Fm, after photoinhibition in leaves of spinach, cabbage, squash and sweet potato. To induce photoinhibition, leaf disks were exposed to light at 1 ℃ at an intensity of 1.5mmol quanta m^{-2} s^{-1} for 3h with respect to squash and for 4h with respect to spinach. To allow recovery from low-temperature photoinhibition, then the leaf disks were incubated at 17℃ in darkness (A), in light of at an intensity of 0.07mmol quanta m^{-2} s^{-1} (B), or in light at an intensity of 0.3mmol quanta m^{-2} s^{-1} (C). The initial values of Fv/Fm measured at 25℃ were taken as 100% and corresponded to 0.80, 0.82, 0.81 and 0.79, in spinach, cabbage, squash and sweet potato, respectively. The values were obtained from the results of three independent experiments. The deviation of values was within ± 5%.

(○) Spinach; (△) cabbage; (●) squash; (▲) sweet potato.

4. The degree of fatty acid unsaturation of PG in wild-type and transgenic tobacco plants

To gain better insight into the mechanism of chilling tolerance of photosynthesis in terms of membrane lipids, we carried out genetic transformation of tobacco plants with cDNA for glycerol-3-phosphate acyltransferase from squash. The transgenic plants showed lowered extent of fatty acid unsaturation of membrane lipids, in particular, the extent of unsaturation of phosphatidylglycerol being most effectively modified (Murata *et al.*, 1992).

As shown in Figure 4, the fatty acid analysis of thylakoid membrane lipids revealed that the mole percentages of *cis*-unsaturated molecules of PG were 66% and 24% in wild-type and transgenic tobacco plants, respectively, indicating that transgenic plants had a depressed amount of *cis*-unsaturated PG molecules than wild-type plants by 42%.

PG (% of molecular species)
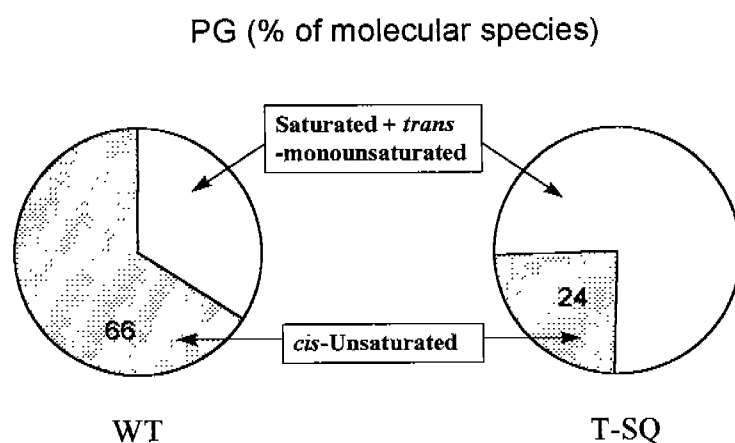
Figure 4. Estimated mole percentages of *cis*-unsaturated molecules of phosphatidylglycerol (PG) in thylakoid membranes isolated from wild-type (WT) and transgenic tobacco plants (T-SQ).

5. Photoinhibition of wild-type and transgenic tobacco plants

To further dissect the role of fatty acid unsaturation of membrane lipids in the sensitivity of photosynthesis to chilling, we directly tested the effects of modification of fatty acid unsaturation of membrane lipids on the susceptibility of the PS II complex to low temperature photoinhibition, using wild-type and transgenic tobacco

plants. The altered level of *cis*-unsaturated PG molecules in thylakoid membranes was shown to lead to the modification of the ability of plants to tolerate low temperature, i.e. enhanced sensitivity to chilling. Figure 5 A-B show the profiles of the decline in Fv/Fm during photoinhibition in leaves of wild-type and transgenic tobacco plants. When leaf disks were exposed to light at an intensity of 0.6 mmol m^{-2} s^{-1}, the extent of photoinhibition at 5℃ was markedly increased, as compared to that occurred at 15℃. Although little difference was recognized in the extent of photoinhibition at 15℃ between the two type of plants, photoinhbition at 5℃ marked a clear discrepancy in decline of Fv/Fm..

Figure 5 C-D show the time course of induction of photoinhibition at 5℃ and 15℃ in leaves of tobacco plants in the presence of lincomycin. Lincomycin accelerated the photoinhibition in the two types of tobacco plant. Moreover, the difference in the extent of photoinhibition between wild-type and transgenic plants that had been observed during exposure to 5℃, in the absence of lincomycin, became unrecognizable by treatment with lincomycin.

6. Recovery from low-temperature photoinhibition in wild-type and transgenic tobacco plants

Figure 6A shows the return to normal values of Fv/Fm during recovery of leaves of wild-type and transgenic tobacco plants from low-temperature photoinhibition. Leaf disks were exposed to strong light at low temperature until Fv/Fm had declined to about 20% of the original level. Then the ratios of Fv to Fm were monitored after shifting the leaf disks to 25℃ under dim light to allow them to recover from the photoinhibited state. It was found that after 4h-incubation for recovery, the ratios that had been restored from low-temperature photoinhibition was almost twice greater in wild-type plants than in transgenic ones.
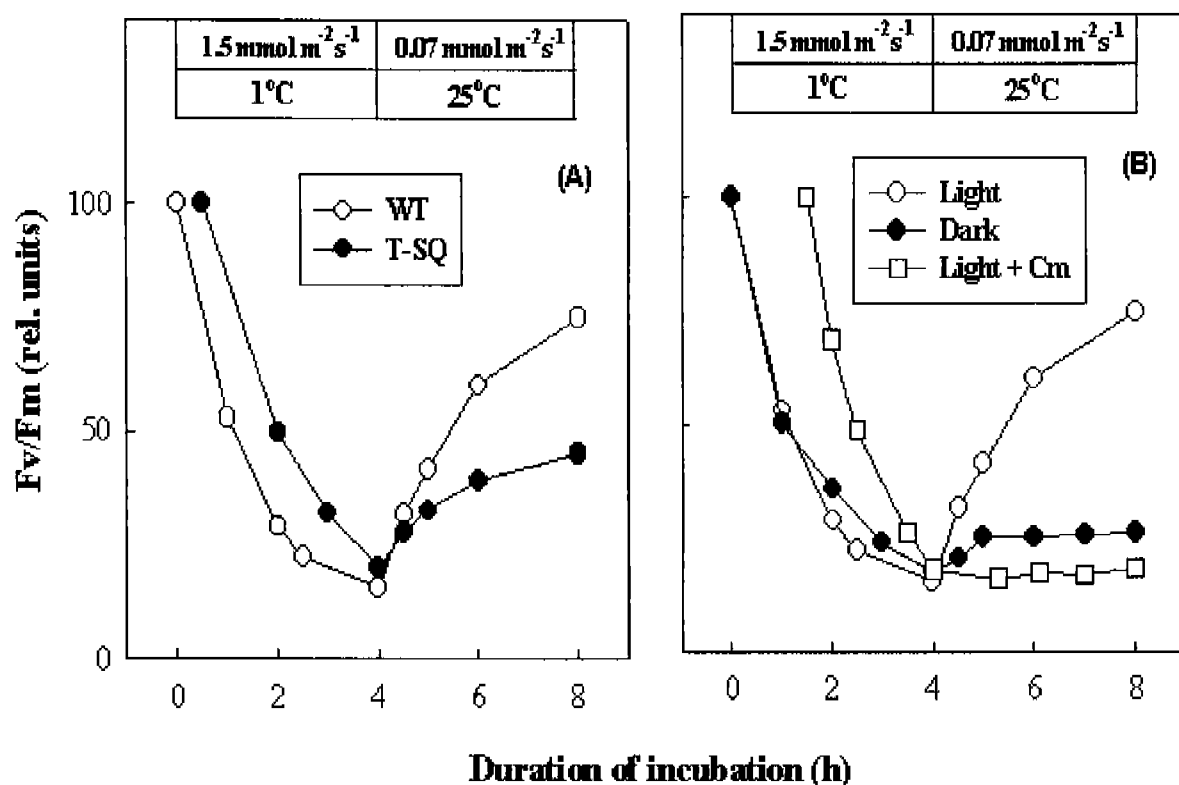
The effects of light and chloroamphenicol, which are modulators of repair of the phtosystem II complex during recovery from photoinhibition, were also studied in wild-type tobacco plants (Fig. 6B). For this purpose, photoinhibition was induced by strong light at 1℃ in the presence, or in the absence of chloramphenicol. Then the leaf disks were allowed to recover at 17℃ in darkness or under dim light. Increase

in Fv/Fm were markedly promoted during incubation under dim light. In contrast to this, recovery in darkness or in the presence of chloramphenicol was very slow or negligible, suggesting that the recovery of the PS II complex from low-temperature photoinhibition requires photosynthetically-supported protein synthesis in chloroplasts.
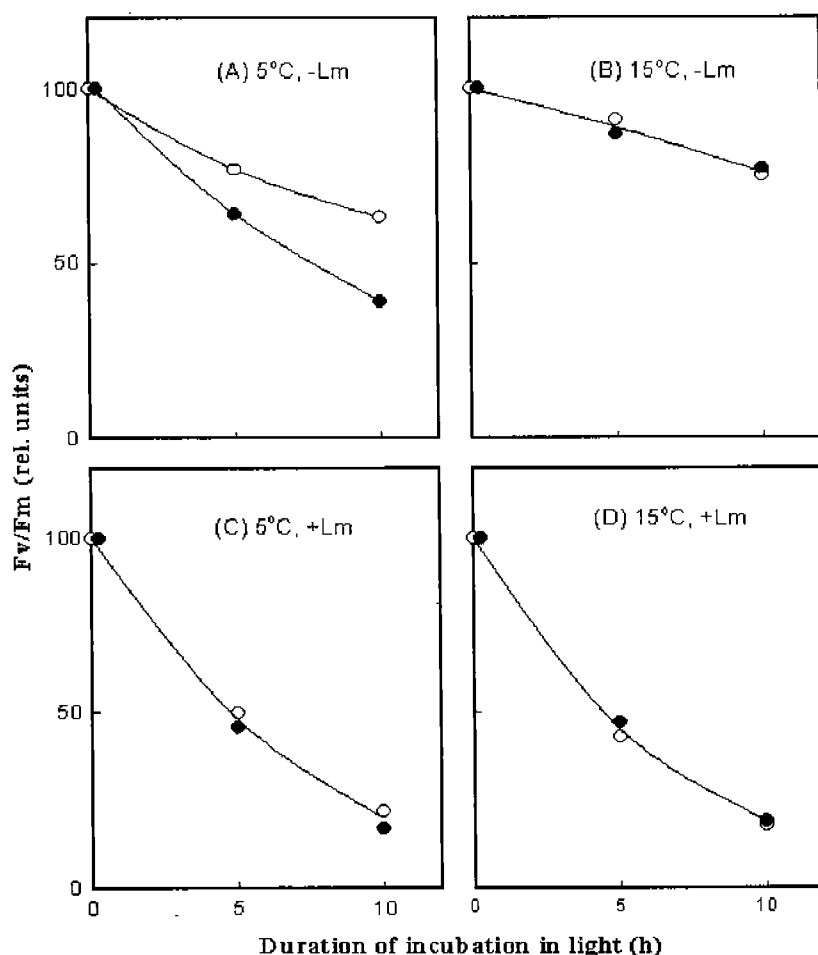
Figure 5. Photoinhibition of PS II photochemical efficiency, monitored in terms of ratios of Fv to Fm, in leaves of wild-type (WT) and transgenic tobacco plants (T-SQ) in the absence (A, B) or in the presence (C, D) of lincomycin. Experimental details were the same as described in the legend of Figure 1. The initial values of Fv/Fm measured at 25℃ were taken as 100% and corresponded to 0.79 and 0.80 for wild-type and transgenic tobacco plants, respectively. The values were obtained from the results of three independent experiments. The deviation of values was within ± 5%. Lm, lincomycin. (A) 5℃, without lincomycin; (B) 15℃, without lincomycin; (C) 5℃, with lincomycin; (D), 15℃, with lincomycin; (O) Wild type; (●) transgenic tobacco plants (T-SQ).

$1.5\,mmol\,m^{-2}s^{-1}$	$0.07\,mmol\,m^{-2}s^{-1}$
1°C	25°C

(A)

Fv/Fm (rel. units)

100

50

0

—O— WT
—●— T-SQ

0   2   4   6   8

$1.5\,mmol\,m^{-2}s^{-1}$	$0.07\,mmol\,m^{-2}s^{-1}$
1°C	25°C

(B)

—O— Light
—●— Dark
—□— Light + Cm

0   2   4   6   8

Duration of incubation (h)

Figure 6. (A) Restoration of PS II photochemical efficiency, monitored in terms of ratios of Fv to Fm, after photoinhibition in leaves of wild-type and T-SQ transgenic tobacco plants. Leaf disks were exposed at 1°C to light at an intensity of 1.5 mmol quanta $m^{-2}\,s^{-1}$ for 4h. To induce recovery from low-temperature photoinhibition, then the leaf disks were incubated at 25°C in dim light at an intensity of 0.07mmol quanta $m^{-2}\,s^{-1}$. (O) Wild type; (●) transgenic tobacco plants (T-SQ). (B) Effects of light and chloramphenicol (Cm) on restoration of PSII activity after photoinhibition in leaves of wild type tobacco plants. To induce photoinhibition at low temperature, leaves were exposed to strong light at an intensity of 2.0mmol quanta $m^{-2}\,s^{-1}$ at 1°C in the absence or presence of chloramphenicol (0.1mM). Then they were incubated for recovery from low-temperature photoinhibition at 25°C in darkness or in light (0.07mmol $m^{-2}\,s^{-1}$). (O) Light; (□) light plus chloramphenicol; (●) darkness. The values were obtained from the results of three independent experiments. The deviation of values was within ± 5%.

Discussion

The proportions of *cis*-unsaturated molecules of phosphatidylglycerol (PG) of membrane lipids is particularly important in the tolerance of plants to chilling (Murata, 1983; Roughan, 1985; Nishida and Murata, 1996). The relative levels of molecular species of PG in membrane lipids of cabbage and spinach were shown to be 98% and 86% of the total PG, respectively. By contrast, squash and sweet potato leaves had only 22% and 36% of the total PG, respectively. Both chilling-sensitive species and the transgenic tobacco plants were found to contain low amounts of *cis*-unsaturated PG in membrane lipids.

On the other hand, the photochemical efficiency of PS II of transgenic tobacco plants was more sensitive to low-temperature photoinhibition than wild-type plants. When the extent of low-temperature photoinhibition was compared, chilling-sensitive squash was found to be more sensitive to photoinhibition than chilling-resistant spinach, indicating that there are clear discrepancy in the photochemical efficiency of the PS II complex between them in tolerating low temperatures.

During photoinhibition *in vivo*, two processes are suggested to counteract against each other (Greer *et al.*, 1986). One is the inactivation process induced by high light, which includes the photodamage to the D1 protein of the PS II complex, and the other is recovery process, which includes degradation of the photodamaged D1 protein, synthesis of the D1 protein *de novo* and reintegration of the D1 protein into the PS II complex (Aro *et al.*, 1993b). We seperated the inactivation process from the recovery process of the repair cycle of PS II by applying lincomycin to leaves. By doing so, the discrepancy in the extent of Fv/Fm, that had been observed between spinach and squash, as well as between wild-type and transgenic tobacco plants, became almost abolished. Since photoinhibition in the presence of lincomycin blocks repair reactions of photodamaged PS II complex, the result implies that the lesser degree of fatty acid unsaturation of membrane PG little affects the rate of light-induced inactivation of the PS II complex during low-temperature photoinhibition.

To examine the role of repair reactions of the photodamaged PS II complex during photoinhibition, we tested whether the recovery of the PS II complex from low-temperature photoinhibition was responsible for the observed differences in chilling

sensitivity between wild-type and transgenic tobacco plants, and also between chilling-resistant species and chilling-sensitive ones (Fig. 6). The results showed that there were clear differences in the rate of recovery from photoinhibition between the two types of plants; chilling resistant groups, i.e. spinach, pea, and wild-type tobacco plants, showed much faster restoration of Fv/Fm than the chilling-sensitive groups, i.e. squash, sweet potato, and transgenic tobacco plants. The observation indicates that the higher extent of fatty acid unsaturation of membrane PG is responsible for the faster recovery of PS II complex from low-temperature photoinhibition, probably due to the acceleration of recovery process from low-temperature photoinhibition. Furthermore, the analysis of the process of recovery under various light conditions showed that the recovery under low-intensity light (0.07 mmol m^{-2} s^{-1}) was faster than that occurred in darkness, suggesting that photosynthetically produced energy is important in recovery process. Chloramphenicol completely blocked the recovery, a result suggesing that the recovery was supported by active protein synthesis.

It is widely accepted that repair of photodamaged PS II centers constitutes a complex cycle, including degradation of photodamaged D1 protein, *de novo* synthesis, and insertion of newly synthesized D1 protein into PS II complex, migration of PS II complexes between appressed and nonappressed thylakoid regions (Aro *et al.*, 1993a). Recent findings that fatty acid unsaturation of membrane lipids not only accelerates accumulation of mature, functional D1 protein (Kanervo *et al.*, 1997) but also promotes exchange of the D1 protein forms in cyanobacterial thylakoid membranes (Sippola *et al.*, 1998) hint a functional role of membrane fluidity in the turnover of D1 protein. Depressed level of membrane PG unsaturation could lower fluidity of the thylakoid membrane in transgenic tobacco plants, leading to impaired replacement of photodamaged D1 with photochemically efficient form of D1, which causes unstabilization of PS II function at lowered temperatures. Yet the exact role of PG in membrane lipids during turnover of D1 protein remains to be answered.

On the basis of the observations, we propose that fatty acid unsaturation of chloroplast membrane lipids is important in regulating membrane fluidity at low temperatures, thereby accelerating recovery of photosynthetic machinery from the photoinhibited state, including enhanced repair of photodamaged D1 protein, but

that it little affects the rate of photo-induced inactivation of photosynthetic machinery.

Acknowledgments

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